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Cyclic AMP Stimulates SF-1-Dependent CYP11A1 Expression through Homeodomain-Interacting Protein Kinase 3-Mediated Jun N-Terminal Kinase and c-Jun Phosphorylation▽

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Steroids are synthesized in adrenal glands and gonads under the control of pituitary peptides. These peptides bind to cell surface receptors to activate the cyclic AMP (cAMP) signaling pathway leading to an increase of steroidogenic gene expression. Exactly how cAMP activates steroidogenic gene expression is not clear, except for the knowledge that transcription factor SF-1 plays a key role. Investigating the factors participating in SF-1 action, we found that c-Jun and homeodomain-interacting protein kinase 3 (HIPK3) were required for basal and cAMP-stimulated expression of one major steroidogenic gene, *CYP11A1*. HIPK3 enhanced SF-1 activity, and c-Jun was required for the functional interaction of HIPK3 with SF-1. Furthermore, after cAMP stimulation, both c-Jun and Jun N-terminal kinase (JNK) were phosphorylated through HIPK3. These phosphorylations were important for SF-1 activity and *CYP11A1* expression. Thus, we have defined HIPK3-mediated JNK activity and c-Jun phosphorylation as important events that increase SF-1 activity for *CYP11A1* transcription in response to cAMP. This finding has linked three common factors, HIPK3, JNK, and c-Jun, to the cAMP signaling pathway leading to increased steroidogenic gene expression.

Steroidogenic factor 1 (SF-1) (also called Ad4BP or NR5A1) is a member of the nuclear receptor superfamily, which consists of DNA binding transcription factors (39). In contrast to other nuclear receptors, which are activated by ligands (51), nuclear receptors 5A are orphan receptors because the existence of their ligands is still under debate (31, 53). One characteristic of the nuclear receptor 5A family is the presence in all family members of a conserved region called the Ftz-F1 box that functions in the recognition of a DNA sequence (52). In addition to this domain and the activation domain 2 present in all nuclear receptors (51), SF-1 also contains a Pro-rich domain located at amino acids (aa) 110 to 172 that functions as an activation domain (11, 32).

SF-1 is a key regulator for steroid biosynthesis. Steroid hormones are synthesized in steroidogenic tissues such as adrenal gland, gonad, placenta, and brain. SF-1 is involved in the regulation of adrenal and testicular steroidogenic genes such as *StAR*, hydroxysteroid dehydrogenase genes (*HSD3B* and *HSD11B*), *MC2R*, and those in the *CYP* family encoding cytochrome P450 enzymes (*CYP11A1*, *CYP11B1*, *CYP17*, and *CYP21*) (41). SF-1 does not, however, control the regulation of steroidogenic genes in brain, placenta, and gut (26). In placenta, LBP-1b and LBP-9 can control the expression of *CYP11A1* (23, 24), and TreP-132 has been shown to regulate steroidogenesis in placenta and brain (13, 16).

In the adrenal gland, steroids are synthesized from three cortical zones. Zona glomerulosa is involved in the synthesis of mineralocorticoids under the regulation of salt and angiotensin II through the calcium/calmodulin/protein kinase C pathway and by interleukin 6 (15). Zona fasciculata and zona reticularis are involved in the synthesis of glucocorticoids and precursors for sex hormone under the control of the pituitary peptide hormone adrenocorticotropic hormone. Adrenocorticotropic hormone binds to cell surface receptors, causing the accumulation of intracellular cyclic AMP (cAMP) (46). cAMP activates protein kinase A (PKA), which triggers a series of signaling cascades leading to an ultimate increase in SF-1 activity and expression of steroidogenic genes. The precise mechanism connecting cAMP elevation to increased SF-1 activity and stimulation of steroidogenic gene transcription is still not clear.

The *cis* regulatory elements in the promoters of steroidogenic genes that participate in cAMP-dependent gene expression have been extensively studied. In addition to SF-1, proteins such as CREB, c-Jun, ATF, and C/EBP that bind to cAMP-responsive sequences can regulate steroidogenic gene expression (5, 34, 35, 43, 54). Among them, c-Jun interacts with SF-1 physically and functionally to activate the *CYP11A1* promoter (32).

Usually c-Jun is activated by growth factors through the mitogen-activated protein kinase (MAPK) signal transduction pathway (10). Upon stimulation, Jun N-terminal kinase (JNK), a MAPK, phosphorylates N-terminal Ser-58, -63, and -73 and Thr-89, -91, and -93 of c-Jun, resulting in its activation (2, 10, 51). Replacement of these Ser/Thr by Ala ablates N-terminal phosphorylation, forming dominant negative Ala-c-Jun. On the other hand, replacement of these Ser/Thr residues with Asp results in the formation of constitutively active Asp-c-Jun (49). Although regulated mainly by the MAPK pathway, c-Jun

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can also be regulated by PKA (8). However, the mechanism connecting PKA to c-Jun activation has not been characterized.

Another protein regulated by JNK is homeodomain-interacting protein kinase (HIPK), which is a nuclear Ser/Thr protein kinase functioning in transcriptional corepression, cell differentiation, growth suppression, and apoptosis (9, 42, 56). Three family members, HIPK1, -2, and -3, have been identified (28), and HIPK2 and -3 have been shown to increase c-Jun phosphorylation (20, 36).

Through our interest in steroidogenic gene expression, we have previously examined the activation of steroidogenic gene *CYP11A1* by SF-1 (17, 18). In this report we demonstrate that HIPK3 and c-Jun interact with and activate SF-1 in basal and cAMP-dependent *CYP11A1* transcription. Activation of the cAMP pathway leads to increased HIPK3-mediated JNK and c-Jun phosphorylation, resulting in increased *CYP11A1* expression. Our finding points to a novel regulatory mechanism by which JNK, c-Jun, and HIPK3 participate in SF-1 action to increase cAMP-dependent CYP11A1 expression.

MATERIALS AND METHODS

Cell culture and reagents. Y1 mouse adrenocortical tumor cells (44) and human placental JEG3 cells (29) were maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal calf serum. The human lung adenocarcinoma cell line H1299 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. 8-Br-cAMP (1 mM) was added to Y1 cells for 24 h for stimulation experiments.

Plasmids and small interfering RNA (siRNA). Plasmids for pCDNA3.1-SF-1-HA (37), phscc2.3kb (21), pVP16-HIPK3, HIPK3, K226R, full-length HIPK3, N-HIPK3 (36), pCDNA3-Flag-JNK1, pCDNA3-Flag-JNK1 (APF) (48), JNK1-HA (57), and c-Jun derivatives wild-type (WT) c-Jun, Ala-c-Jun, and Asp-c-Jun (50) have been described previously. The mammalian two-hybrid expression vector Gal4-SF-1 was created by fusing the SF-1 fragments aa 80 to 461 and 120 to 461 with a Gal4 DNA binding domain (DBD). Double-stranded RNA oligonucleotides against exon 1 of mouse HIPK3 (7) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Ambion, Austin, TX.

Transient transfection and reporter assay. JEG3 cells and H1299 cells were transfected using Lipofectamine 2000 (Invitrogen, San Diego, California). Y1 cells were transfected using Lipofectamine Plus reagent for DNA and Oligofectamine for siRNA (Invitrogen). Cells were harvested at 48 h after transfection and subjected to luciferase or β-galactosidase assays. Reporter activities were normalized with internal control RSV-β-gal. At least three independent experiments were performed, and the standard deviations from the means are represented as error bars.

Immunoblotting and antibodies. Cells were harvested and lysed in lysis buffer (100 mM potassium phosphate [pH 7.8], 0.2% Triton X-100, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) or sodium dodecyl sulfate gel loading buffer 48 h after DNA transfection or 3 days after siRNA transfection. Equal amounts of total proteins were separated by gel electrophoresis and then transferred to Immobilon P membranes (Millipore, Billerica). Membranes were incubated with anti-c-Jun (sc-45; Santa Cruz Biotechnology Inc., Santa Cruz, California), anti-phospho-c-Jun (sc-822; Santa Cruz Biotechnology Inc.), anti-JNK, anti-phospho-JNK (Cell Signaling Technology, Inc.), anti-SF-1 (32), anti-hemagglutinin (anti-HA), anti-CYP11A1 (22), anti-HIPK3 (Abgent Co., San Diego, California), anti-Flag, or anti-Hsp70 antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 45 min. Signals were detected by chemiluminescence assays. SF-1 antibody (32) was used for coimmunoprecipitation in Y1 cells and then with light-chain-specific secondary antibody.

Protein binding assay. In glutathione *S*-transferase (GST) pull-down assays, c-Jun was in vitro transcribed and translated using the TNT reticulocyte lysate system (Promega, Madison, Wisconsin) and simultaneously labeled with [^{35}S]Met. These proteins were incubated with recombinant GST-SF-1-FP and glutathione beads in NENT buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 0.5% NP-40) for 2 h at 4°C, precipitated, and analyzed by gel electrophoresis and autoradiography. In coimmunoprecipitation experiments, after transfection with SF-1-HA, Flag-sHIPK3 (aa 159 to 1191), or c-Jun-HA, H1299 lysate in IPH buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% NP-40) plus 1× complete protease inhibitor mixture (Roche, Basel, Switzerland) was incubated with 1 μg of mouse anti-HA antibody or normal mouse immunoglobulin G and 30 μl of 50% protein A-Sepharose beads for 2 h. Proteins bound to Sepharose beads were precipitated, eluted with HA or Flag peptides, and then examined by immunoblotting.

RESULTS

c-Jun and HIPK3 interact with SF-1 physically and functionally. We previously showed that SF-1 interacts with c-Jun in vitro (25, 32). To further investigate the functional significance of this interaction, we confirmed this interaction in cells by carrying out a coimmunoprecipitation experiment. SF-1-HA and c-Jun were cotransfected into H1299 cells, which were chosen because of their high transfection efficiency. The SF-1-HA complex was immunoprecipitated with anti-HA antibodies, and c-Jun was found to be present in this complex by immunoblotting (Fig. 1A, upper panel). This indicates that SF-1 and c-Jun interact with each other in vivo. We also detected the interaction of SF-1 and c-Jun by a GST pull-down experiment. Full-length c-Jun was synthesized in vitro using reticulocyte lysate. Both phosphorylated (p-c-Jun) and unphosphorylated forms of c-Jun, with sizes of 45 kDa and 40 kDa, respectively, were obtained (Fig. 1A, lower panel) (38). ^{35}S-labeled c-Jun was directly associated with SF-1-FP (Ftz-F1 box and Pro-rich region of SF-1, aa 78 to 172) linked to GST. These results prove that c-Jun interacts with SF-1 in vivo and in vitro.

In a search for SF-1-interacting proteins by yeast two-hybrid analysis, we identified HIPK3 as one of the proteins that interact with SF-1-FP (data not shown). To confirm this interaction, we performed mammalian two-hybrid analysis in H1299 cells. The Gal4 DBD was fused with SF-1 fragments of various lengths, which were then coexpressed with a reporter gene and HIPK3 (aa 159 to 1191) fused with the transactivation domain VP16. The amount of SF-1/HIPK3 interaction was reflected by the level of reporter activity. When VP16-HIPK3 was cotransfected with the Gal4 DBD fused with aa 80 to 461 of SF-1 (Δ80), but not Gal4-DBD fused to a shorter (Δ120, aa 120 to 461) or a control vector, the reporter activity was increased (Fig. 1B). This result indicates that the region in aa 80 to 120 of SF-1 is required for interaction with HIPK3. Moreover, the coimmunoprecipitation experiment also detected interaction between Flag-sHIPK3 (aa 159 to 1191) and SF-1-HA that were overexpressed in H1299 cells (Fig. 1C). Furthermore, endogenous SF-1-associated proteins were coimmunoprecipitated with anti-SF-1 antibody, and HIPK3 was detected in the precipitated pellet (Fig. 1D). These data indicate that HIPK3 and SF-1 form a complex in Y1 cells.

Having established physical interactions of SF-1 with HIPK3 as well as with c-Jun, we examined the function of these interactions by testing their effects on the promoter activity of *CYP11A1*, a well-known SF-1 target gene. We confirmed our previous data that c-Jun potentiates SF-1 activity by using a β-galactosidase reporter gene in JEG-3 cells (Fig. 1E) (32). The ability of HIPK3 to synergize with SF-1 function was also tested in a reporter assay. *CYP11A1-Luc* reporters were used to monitor the transcriptional activity of cotransfected SF-1 and HIPK3 in H1299 cells. The results

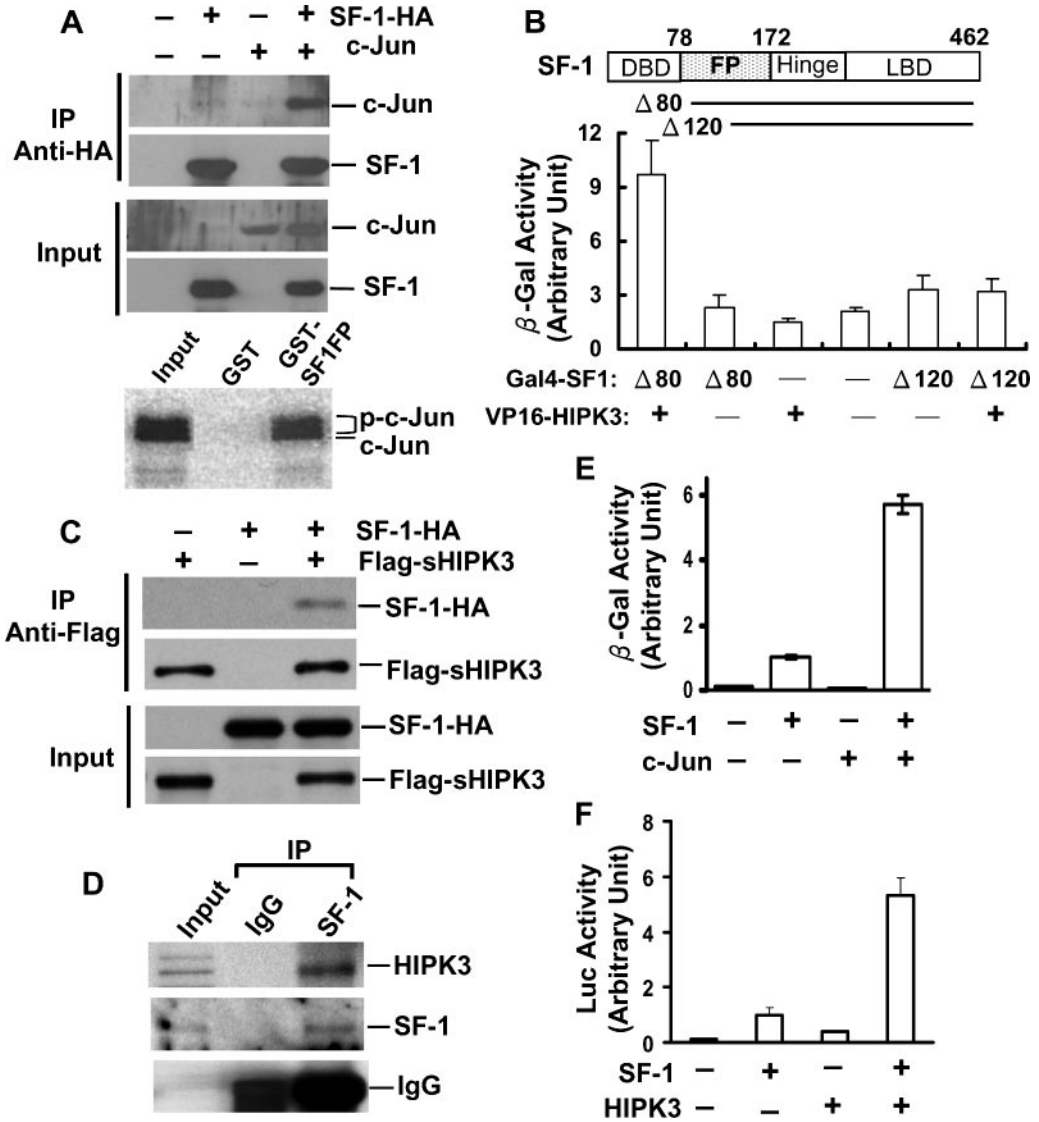

FIG. 1. HIPK3 and c-Jun interact with SF-1 and enhance its transcriptional activity. A. Detection of c-Jun and SF-1 interaction by coimmunoprecipitation (upper panel) and GST pull-down assay (lower panel). After expression of SF-1-HA and c-Jun in H1299 cells, the SF-1-HA complex was immunoprecipitated (IP) by anti-HA antibody, and SF-1 and c-Jun were detected by Western blotting (upper panel). Ten percent of input proteins were also directly immunoblotted. Full-length ^{35}S-labeled c-Jun was in vitro translated, pulled down with beads containing GST or GST fused with the FP domain of SF-1 (GST-SF1-FP), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lower panel). B. Interaction of SF-1 and HIPK3 by mammalian two-hybrid analysis. The top diagram shows the structure of SF-1 and its deletion mutants. The Gal4 DBD fused with SF-1 aa 80 to 461 (Δ80) or aa 120 to 461 (Δ120) was coexpressed with the activation domain of VP16 fused with HIPK3 (aa 159 to 1191) (VP16-HIPK3) into H1299 cells. The level of β-galactosidase activity was detected and normalized with control luciferase activity of pGL2. Control experiments included lysates from transfections with vectors only (−). C. Detection of interaction between HIPK3 and SF-1 by coimmunoprecipitation. After expression of SF-1-HA and Flag-sHIPK3 (aa 159 to 1191) in H1299 cells, the HIPK3 protein complex was immunoprecipitated with anti-Flag antibody or by direct loading to the gel (input). Western blotting was then performed to detect SF-1-HA and Flag-sHIPK3. D. Endogenous HIPK3 and SF-1 form a complex in Y1 cells. Total Y1 cell lysate was immunoprecipitated with anti-SF-1 antibody or an immunoglobulin G (IgG) control. The proteins in the immunoprecipitate were then detected with antibodies against HIPK3 or SF-1 by Western blotting. Input represents 1/40 of the lysate before immunoprecipitation. E. c-Jun promotes the transcriptional activity of SF-1. The expression vectors of SF-1-HA, c-Jun, or both were transfected with *CYP11A1*-β-*gal* reporter into JEG3 cells. Relative β-galactosidase activities are shown. F. HIPK3 facilitates the transcriptional ability of SF-1. HIPK3 expression plasmids were transfected into H1299 cells with *CYP11A1*-β-*gal* reporter in the presence or absence of SF-1-HA. Relative β-galactosidase activities are shown. The results were obtained from at least three independent experiments. Error bars indicate standard deviations.

showed that HIPK3 and SF-1 synergistically up-regulated reporter activity (Fig. 1F).

The domain of HIPK3 that is required for SF-1 coactivation was tested. S-HIPK3 (aa 159 to 1191) enhanced the activity of SF-1 to activate *CYP11A1-Luc* in H1299 cells, but a kinase-deficient HIPK3 mutant (K226R) and a mutant HIPK3 with a C-terminal truncation (N-HIPK3) had no effect (Fig. 2A). Full-length HIPK3 (aa 2 to 1191) appeared to have activity

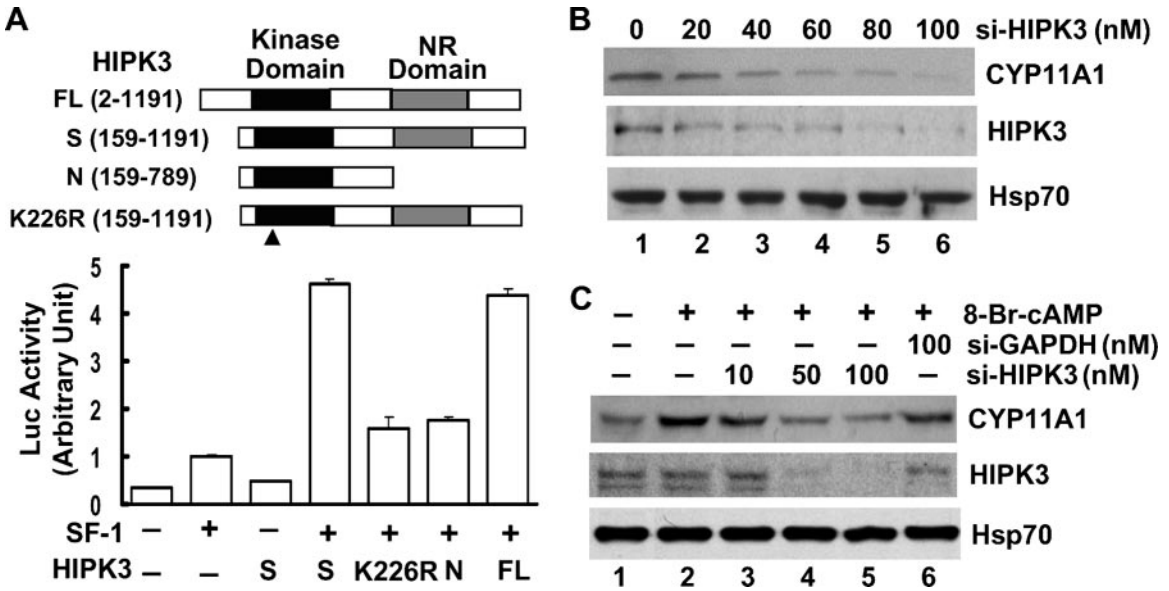

FIG. 2. HIPK3 is essential for basal and cAMP-stimulated CYP11A1 expression, and its kinase activity is important. A. Kinase activity and the C-terminal interaction domain are essential for HIPK3 coactivation function. The top diagram shows full-length (FL) HIPK3 (aa 2 to 1191) and its derivatives S-HIPK3 (aa 159 to 1191), K226R (aa 159 to 1191) with a point mutation (depicted by a triangle) and deficient in kinase activity, and the N-terminal part of HIPK3 (aa 159 to 789) lacking the nuclear receptor (NR) interaction domain. The relative activities of the *CYP11A1-Luc* reporter in H1299 cells are shown in the lower panel. Error bars indicate standard deviations. B. Reduction of CYP11A1 expression upon HIPK3 blockage by siRNA. After double-stranded siRNA against mouse HIPK3 (si-HIPK3) was transfected into Y1 cells, HIPK3 and CYP11A1 were detected by immunoblotting. C. HIPK3 is required for cAMP-stimulated CYP11A1 expression. Different amounts of si-HIPK3 or negative control si-GAPDH were transfected into Y1 cells, and 1 mM of 8-Br-cAMP was added and left for 24 h. Amounts of HIPK3 and CYP11A1 were detected by immunoblotting. Hsp70 was used as an internal control.

similar to that of S-HIPK3 (aa 159 to 1191). These results show that HIPK3 enhanced SF-1 activity and that both the kinase and the C-terminal nuclear receptor interaction domains of HIPK3 were required for this interaction.

HIPK3 siRNA reduces the amount of CYP11A1. Besides reporter gene assays, we also checked the expression of the SF-1 target CYP11A1 after blocking HIPK3 with a double-stranded siRNA (si-HIPK3) in Y1 cells. The CYP11A1 level was decreased in a dose-dependent manner after transfection of si-HIPK3 (Fig. 2B). Furthermore, si-HIPK3 also reduced 8-Br-cAMP-stimulated CYP11A1 levels (Fig. 2C). The siRNA against a housekeeping gene, the GAPDH gene, used as a negative control, did not interfere with CYP11A1 expression. From these results, we conclude that HIPK3 participates in basal and cAMP-stimulated CYP11A1 expression.

Phosphorylation of c-Jun is important for CYP11A1 expression. N-terminal phosphorylation of c-Jun is known to activate c-Jun (10). Whether this phosphorylation is involved in cAMP stimulation and *CYP11A1* transcription was further investigated in this study. The effect of cAMP on c-Jun phosphorylation was first examined. 8-Br-cAMP treatment dramatically enhanced the level of phosphorylated c-Jun in a delayed fashion, with an initial observation of phosphorylation at 3 h, and later peaked at 12 h, while the amount of total c-Jun was unaffected (Fig. 3A). This pattern of delayed c-Jun phosphorylation parallels the previously published time course of cAMP-stimulated CYP11A1 expression (22), indicating a possible role of c-Jun phosphorylation in cAMP-stimulated CYP11A1 expression.

N-terminal phosphorylation is important for the transactivation function of c-Jun. To test whether this N-terminal phosphorylation is also involved in *CYP11A1* activation, mutant plasmids of c-Jun encoding unphosphorylatable Ala-c-Jun or constitutively active Asp-c-Jun were transfected into Y1 cells (Fig. 3B). Ala-c-Jun repressed both basal and cAMP-stimulated *CYP11A1-Luc* activities. In contrast, Asp-c-Jun resulted in a higher level of *CYP11A1-Luc* expression, which was further enhanced by 8-Br-cAMP (Fig. 3B, lower panel).

In addition to checking promoter activity, we also examined the effect of c-Jun on cAMP-stimulated CYP11A1 protein expression in Y1 cells by immunoblotting followed by quantitation of the results after densitometric scanning. Ala-c-Jun decreased the amount of cAMP-stimulated CYP11A1 in a dose-dependent manner, while the amounts of internal controls (SF-1 and Hsp70) were not affected (Fig. 3C).

To further test the effects of c-Jun modifications on CYP11A1 expression, we transfected different c-Jun derivatives into Y1 cells (Fig. 3D). Ala-c-Jun transfection resulted in a 50% reduction of CYP11A1 levels compared to WT c-Jun transfection (compare lane 1 with lane 3). Asp-c-Jun-transfected cells exhibited higher basal CYP11A1 levels, which were further increased after cAMP stimulation (Fig. 3D). A similar pattern of *CYP11A1* mRNA levels induced by c-Jun derivatives was also detected using quantitative PCR (data not shown). This indicates the participation of c-Jun N-terminal phosphorylation in the stimulation of CYP11A1 expression, resulting in increased promoter activity and accumulation of its RNA and protein in Y1 cells.

HIPK3 and cAMP influence c-Jun phosphorylation. Since HIPK3 kinase activity and c-Jun phosphorylation are both im-

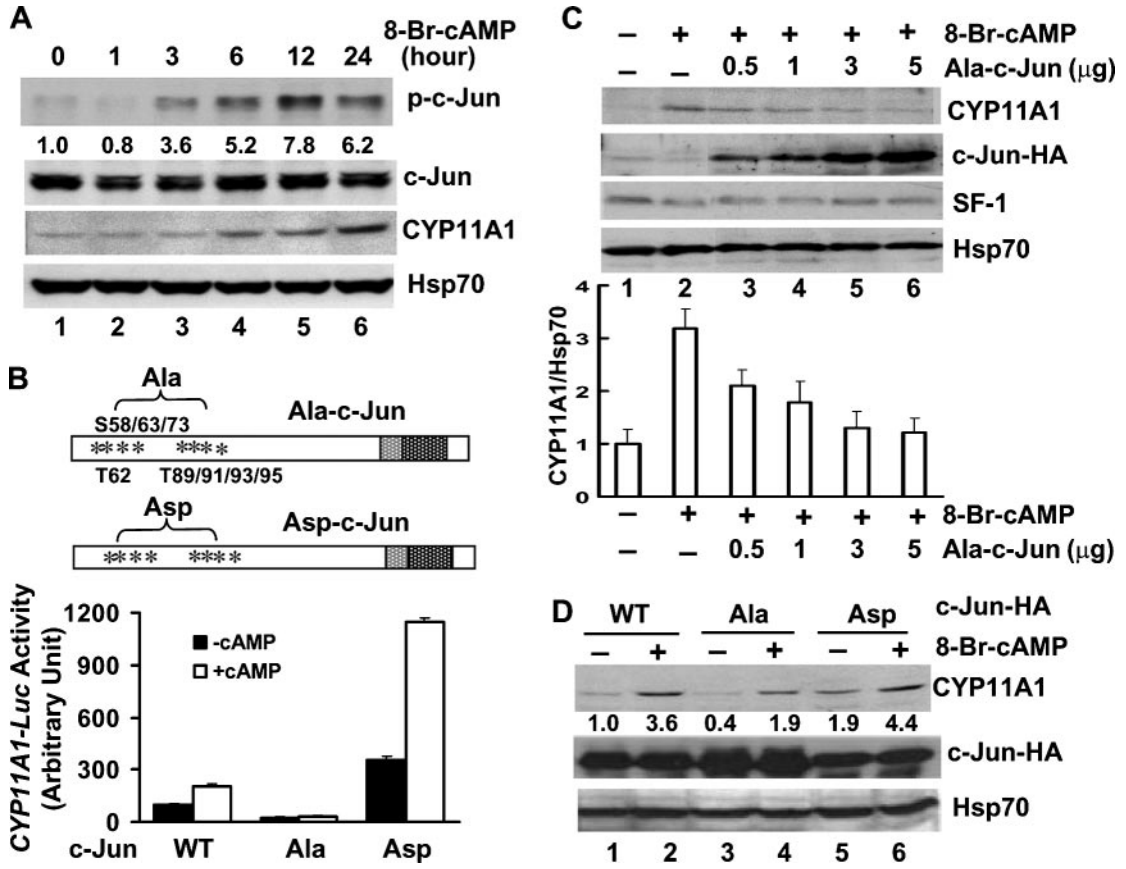

FIG. 3. CYP11A1 expression is regulated by c-Jun through its N-terminal phosphorylation. A. The c-Jun phosphorylation level was increased after cAMP treatment. The amounts of phospho-c-Jun (p-c-Jun), c-Jun, and Hsp70 were detected by immunoblotting and are shown after treatment with 1 mM 8-Br-cAMP in Y1 cells for the time indicated above each lane. The numbers below the top gel represent the quantitative amounts of p-c-Jun after densitometric scanning. B. Effect of c-Jun phosphorylation on SF-1 mediated transcription. The diagram at the top shows the modification of c-Jun. The transactivation domain of c-Jun is located at the N terminus, which contains Ser/Thr residues at amino acids 58, 63, 73, 89, 91, and 93 that were mutated to Ala or Asp to form dominant negative Ala-c-Jun and constitutively active Asp-c-Jun, respectively. An HA epitope tag was also included. The effects of WT c-Jun-HA, Ala-c-Jun-HA, and Asp-c-Jun-HA on CYP11A1-Luc expression in Y1 cells in the presence (□) or absence (■) of 8-Br-cAMP are shown. Error bars indicate standard deviations. C. Ala-c-Jun reduces the expression of c-AMP stimulated CYP11A1. Increasing doses (0.5, 1, 3, 5 and μg) of expression plasmids for Ala-c-Jun-HA were transfected into Y1 cells, followed by incubation with 8-Br-cAMP for 24 h. The levels of endogenous CYP11A1, SF-1, Hsp70, and exogenous Ala-c-Jun-HA as detected by immunoblotting are shown. The quantitation of the amounts of CYP11A1 versus Hsp70 is shown in the lower panel. D. Effects of c-Jun phosphorylation mutants on CYP11A1 expression. Plasmids (2 μg) encoding c-Jun derivatives were transfected into Y1 cells, and treated with or without 8-Br-cAMP. The expression levels of CYP11A1, Hsp70, and HA-c-Jun are shown. The numbers below the top gel represent amounts of CYP11A1 quantitated from three independent immunoblotting experiments.

portant for CYP11A1 expression, the relationship and signal transduction of c-Jun and HIPK3 was further investigated. The physical interaction between Flag-sHIPK3 and c-Jun-HA was detected using coimmunoprecipitation after transfection into H1299 cells (Fig. 4A). The HIPK3 protein complex was precipitated by anti-Flag antibody, and the c-Jun-HA protein was also detected by immunoblotting only after cotransfection with Flag-sHIPK3 (Fig. 4A, lane 3). This indicates that HIPK3 and c-Jun could form a complex.

Since HIPK3 physically interacted with c-Jun, its ability to activate c-Jun was measured by supplying exogenous HIPK3 (Fig. 4B). Overexpression of Flag-sHIPK3 increased the level of p-c-Jun in H1299 cells, while the kinase-deficient K226R mutant failed to enhance c-Jun phosphorylation (Fig. 4B, lane 5). Our quantification revealed more than fivefold induction of phosphorylated c-Jun by HIPK3 transfection, but K226R had no effect (Fig. 4B). This indicates that the kinase activity of HIPK3 is important for c-Jun phosphorylation.

We further examined the role of HIPK3 in c-Jun phosphorylation in the cAMP stimulation pathway by blocking HIPK3 activity in Y1 cells with si-HIPK3. Phosphorylation of c-Jun was decreased upon increasing the concentration of si-HIPK3 (Fig. 4C). CYP11A1 expression was also decreased. This loss-of-function result demonstrates that HIPK3 participates in cAMP-stimulated c-Jun phosphorylation and CYP11A1 expression.

c-Jun mediates HIPK3 function to activate CYP11A1 expression. Since c-Jun and HIPK3 both interact with SF-1, functional correlation of HIPK3 and c-Jun in CYP11A1 expression was examined using a reporter assay with H1299 cells. Flag-sHIPK3 potentiated SF-1 transcriptional activity, indicating synergistic effects. While this activity was not affected by

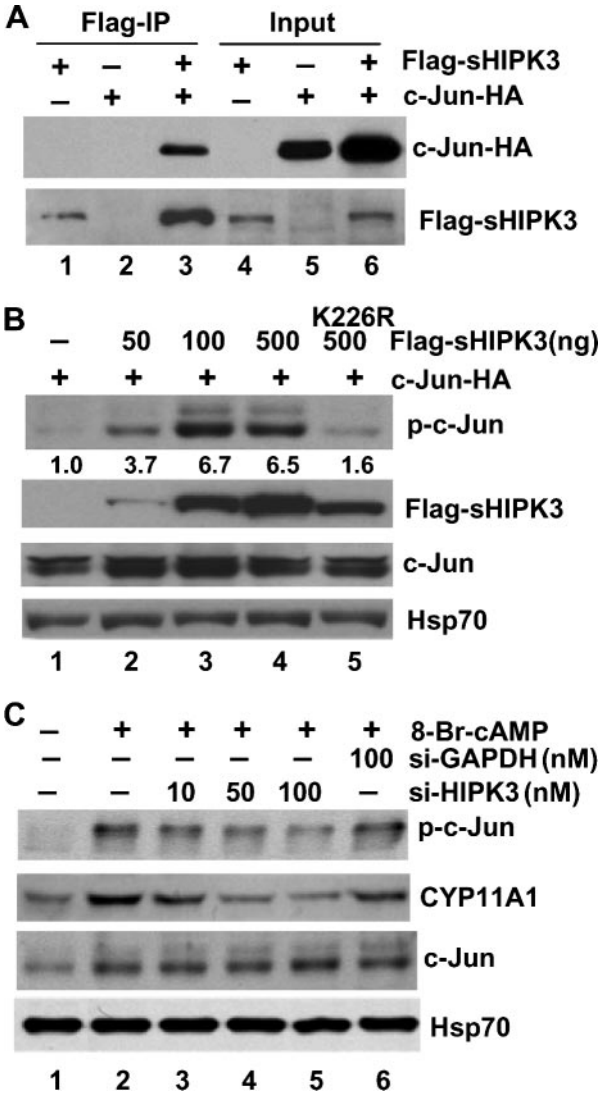

FIG. 4. HIPK3 interacts with c-Jun and contributes to its N-terminal phosphorylation. A. Detection of interaction between c-Jun and HIPK3 by coimmunoprecipitation. After expression of Flag-sHIPK3 and/or c-Jun-HA in H1299 cells, the HIPK3 complex was immunoprecipitated (IP) with anti-Flag antibody. Western blotting was then performed using anti-Flag and anti-HA antibodies to detect HIPK3 and c-Jun. B. HIPK3 results in increased c-Jun phosphorylation. Expression vectors of c-Jun and Flag-sHIPK3 (aa 159 to 1191) or its kinase-deficient mutant K226R (aa 159 to 1191) were transfected into H1299 cells at the dosage shown above each lane. Proteins were detected with specific antibodies. The numbers below the top gel are the quantities of intensity of p-c-Jun after densitometric scanning. C. HIPK3 siRNA causes reduction of cAMP-stimulated c-Jun phosphorylation. si-HIPK3 or negative control si-GAPDH at the concentrations (nM) shown above each lane was transfected into Y1 cells, followed by incubation with 1 mM 8-Br-cAMP for 24 h. Levels of p-c-Jun and CYP11A1 were detected by immunoblotting. Hsp70 was the internal control. All the data were confirmed in at lease three independent experiments.

WT c-Jun, it was repressed by increasing amounts of dominant negative Ala-c-Jun (Fig. 5A). This indicates that c-Jun participates in the synergistic effects of HIPK3 and SF-1. Moreover, the K226R mutant HIPK3 and Ala c-Jun failed to activate the *CYP11A1* reporter gene, indicating that the kinase activity of HIPK3 and the N-terminal phosphorylation of c-Jun were both required (Fig. 5B).

Not only promoter activity but also CYP11A1 protein levels in Y1 cells were reduced after blocking HIPK3 with si-HIPK3 (Fig. 5C, lane 2). Cotransfection of Asp-c-Jun reversed this inhibitory effect (Fig. 5C, lane 4). These results indicate that c-Jun mediates HIPK3 activity to activate CYP11A1 expression.

The requirement of HIPK3 to influence c-Jun function in *CYP11A1* expression was also investigated by use of a reporter assay with Y1 cells (Fig. 5D). In the absence of HIPK3 siRNA, Asp-c-Jun and WT c-Jun enhanced reporter activity three- and twofold, respectively. The luciferase activity of the *CYP11A1* reporter was reduced by si-HIPK3 transfection to less than half compared to that with control siRNA transfection. Overexpression of Asp-c-Jun in the presence of siRNA against HIPK3 could rescue reporter activity to the control level without gene knockdown. This rescue effect of Asp-c-Jun was exactly like the previous immunoblotting result (Fig. 5C). In contrast, WT c-Jun failed to rescue the reporter activity when HIPK3 activity was silenced by siRNA. This showed that only Asp-c-Jun, which mimics the phosphorylated c-Jun, could overcome the HIPK3 deficiency for CYP11A1 regulation. Thus, both Western blot and reporter activity results indicate that HIPK3 function is important for CYP11A1 expression and that the c-Jun effect is downstream from HIPK3 for SF-1-dependent *CYP11A1* expression.

HIPK3 regulates JNK and c-Jun phosphorylation for CYP11A1 expression. We detected increased N-terminal phosphorylation of c-Jun after HIPK3 transfection (Fig. 4B), and these phosphorylated residues are well-known targets of JNK (10). Therefore, the role of JNK in HIPK3-dependent *CYP11A1* regulation was investigated. We detected decreased phospho-JNK (p-JNK) and p-c-Jun upon increasing the concentration of si-HIPK3 in Y1 cells, although the total amounts of JNK, c-Jun, and Hsp70 were not affected (Fig. 6A). This indicates the participation of HIPK3 in JNK phosphorylation. To further investigate the role of HIPK3 in JNK phosphorylation, we transfected increasing amounts of Flag-sHIPK3 into H1299 cells and found that ectopic expression of HIPK3 enhanced the level of p-JNK by twofold and that K226R mutant transfection could not increase JNK phosphorylation (Fig. 6B).

We further studied the requirement of JNK for *CYP11A1* expression by using a dominant negative mutant of JNK1, called Flag-APF, in which the phosphorylation motif Thr-Pro-Tyr was replaced by Ala-Pro-Phe (48). After transfection of increasing amounts of Flag-APF in Y1 cells, the level of p-c-Jun was repressed in a dose-dependent manner (Fig. 6C, third panel). In addition, CYP11A1 expression was also decreased (Fig. 6B, top panel). These results further confirm that the JNK signaling pathway including JNK and c-Jun is required for cAMP-dependent CYP11A1 expression.

DISCUSSION

In this study, we examined the mechanism underlying SF-1-regulated CYP11A1 expression. Increased cAMP levels in steroidogenic cells trigger a series of signaling events, resulting in increased phosphorylation of JNK and c-Jun. HIPK3 plays a key role in JNK and c-Jun phosphorylation. Phosphorylated

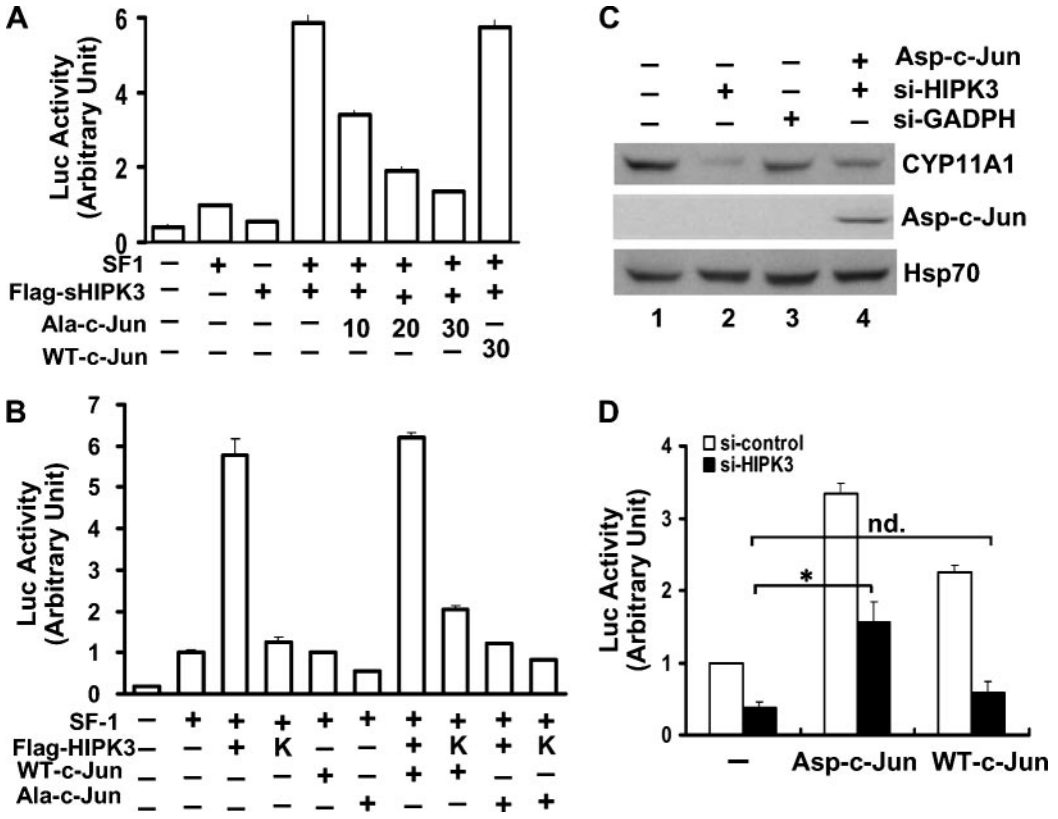

FIG. 5. c-Jun is involved in the action of HIPK3 to enhance SF-1 activity. A. Ala-c-Jun impairs the ability of HIPK3 to activate SF-1. Shown are luciferase (Luc) reporter activities after cotransfection of H1299 cells with ph*SCC-Luc*, *CYP11A1-Luc*, and expression vectors for SF-1, Flag-sHIPK3 (aa 159 to 1191), and/or Ala-c-Jun or WT c-Jun. Error bars indicate standard deviations. B. Kinase activity of HIPK3 and N-terminal phosphorylation of c-Jun are fundamental for CYP11A1 activation. Shown are Luciferase (Luc) reporter activities after cotransfection into H1299 cells on 24-well plates with *CYP11A1-Luc* and different combinations of expression vectors for SF-1, Flag-sHIPK3 (aa159 to 1191) or K226R, and WT c-Jun or Ala-c-Jun. At least three independent experiments were performed. C. Asp-c-Jun rescues CYP11A1 expression that is impaired by HIPK3 siRNA. The amounts of CYP11A1, c-Jun-HA, and Hsp70 after Y1 cells were transfected with double-stranded siRNA against mouse HIPK3 (si-HIPK3) or GAPDH (si-GAPDH) in the presence (+) or absence (−) of Asp-c-Jun expression vector are shown. D. Functional requirement of HIPK3 in c-Jun action to regulate the CYP11A1 promoter. The *CYP11A1-Luc* reporter was transfected with vectors encoding WT c-Jun or Asp-c-Jun in the presence of 70 μM of HIPK3 siRNA into Y1 cells, and the luciferase (Luc) reporter activities are shown. The transfection of scrambled RNA oligonucleotides (si-control) was used as a negative control to reveal basal reporter activity. All the data were confirmed in at lease three independent experiments. nd, no significant difference; ∗, $P < 0.001$.

c-Jun can enhance SF-1 activity for *CYP11A1* transcription. Thus, we show that HIPK3, JNK, and c-Jun participate in a cAMP-dependent pathway to enhance SF-1 activity.

We showed that N-terminal phosphorylation of c-Jun was important for enhancing SF-1 activity and *CYP11A1* expression. The evidence comes from parallel patterns of induction by cAMP, as well as the decreased expression of CYP11A1 when c-Jun function was blocked by a dominant negative mutant. In addition, constitutively active Asp-c-Jun can increase CYP11A1 expression. Thus, N-terminal phosphorylation of c-Jun is an important step in cAMP-stimulated CYP11A1 expression. Yet c-Jun is probably not the only downstream effector that regulates *CYP11A1* under cAMP control. Our data show that although Asp-c-Jun has higher basal activity to activate *CYP11A1*, its activity can be further stimulated by cAMP (Fig. 3). Other partner molecules probably also participate in gene activation by cAMP. Proteins such as CREB/ATF that bind to the cAMP-responsive sequence can be likely candidates (55).

CREB and ATF-1 can bind to similar cAMP-responsive sequences (6, 54). Thus, CREB and ATF-1 may also be able to potentiate SF-1 function like c-Jun, although this possibility has not been tested. Whether c-Jun, ATF, CREB, and the like play redundant or distinct roles in steroidogenic gene regulation is not clear. One possibility is that they contribute to the regulation of all steroidogenic genes; the other possibility is that they select different steroidogenic promoters for function. In brief, cAMP stimulation and HIPK3 activity can cause multiple effects to regulate CYP11A1 expression, and the identities of other effector molecules should be further studied.

The involvement of HIPK3 and c-Jun in the cAMP signaling pathway has not been carefully investigated before, since our knowledge of c-Jun and HIPK3 is mostly about their participation in the regulation of cell proliferation and apoptosis (2, 42). However, the cAMP-PKA pathway does cross talk to the growth factor signaling pathway (3). PKA is known to activate MAPK signaling and c-Jun in the regulation of cell proliferation (8, 47). Here we provide evidence showing that HIPK3 is involved in the cAMP signaling pathway for c-Jun phosphorylation and activation.

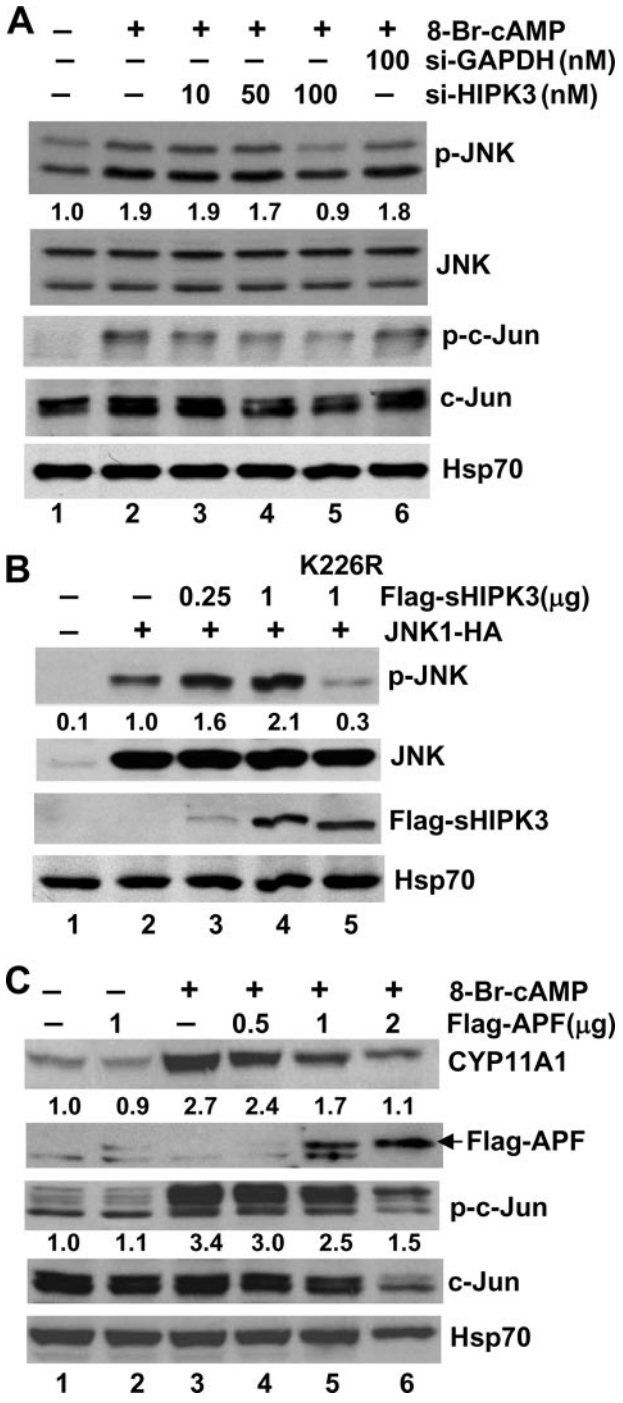

FIG. 6. HIPK3 regulates JNK activity which participates in CYP11A1 expression. A. HIPK3 siRNA causes reduction of cAMP-stimulated JNK phosphorylation. si-HIPK3 or negative control si-GAPDH at the concentrations (nM) shown above each lane was transfected into Y1 cells, followed by incubation with 1 mM 8-Br-cAMP for 24 h. Levels of p-JNK and p-c-Jun were detected by immunoblotting. The numbers below the top gel represent the quantitative amounts of p-JNK after densitometric scanning. The amounts of total c-Jun and JNK were controls. B. HIPK3 causes increased JNK1 phosphorylation. Expression vectors of JNK1-HA and Flag-sHIPK3 (aa 159 to 1191) or its kinase deficient mutant K226R (aa 159 to 1191) were transfected into H1299 cells at the dosage shown above each lane. Proteins were detected with specific antibodies. The numbers below the top gel are the intensities of p-JNK after densitometric scanning. C. Overexpression of dominant negative JNK reduces the expression of cAMP-stimulated CYP11A1. Increasing doses (0.5, 1, 2 μg) of expression plasmids for dominant negative JNK (Flag-APF) were transfected into Y1 cells on six-well plates, followed by incubation with 8-Br-cAMP for 24 h. The levels of endogenous CYP11A1, p-c-Jun, total c-Jun, and exogenous Flag-APF as detected by immunoblotting are shown. Hsp70 was the internal control. The amounts of CYP11A1 and p-c-Jun versus Hsp70 after quantitation are shown below the panel. At least three independent experiments were performed.

JNK and c-Jun phosphorylation can be regulated by HIPKs, but they are probably not the direct substrates of HIPKs. The direct targets of HIPKs include FADD (42), p53 (12), Daxx (14, 20), Groucho (4), AML-1 (1), and Pax6 (27). HIPK1 and HIPK2 activate the JNK pathway through ASK1 (33) and Daxx (20), respectively. Likewise HIPK3 can probably also activate the JNK pathway through proteins like ASK1 and Daxx.

Increased cAMP and PKA activity is the first step in the stimulation of steroidogenic gene expression (46). Two kinase proteins to date have been shown to mediate a cAMP effect on the regulation of steroidogenic gene transcription. One is mitogen-activated protein kinase phophatase 1, which mediates PKA activity to enhance *CYP17* transcription (45). The other is salt-inducible kinase, which regulates *CYP11A1*, *CYP11B*, and *StAR* expression in response to cAMP (40). We have now shown HIPK3 as another kinase which functions in cAMP-induced *CYP11A1* expression. The mechanism of the action of all these kinases in steroidogenic gene expression is unclear. However, both mitogen-activated protein kinase phophatase-1 and salt-inducible kinase are induced within 1 h after cAMP stimulation, while HIPK3 activity as assessed by c-Jun phosphorylation was enhanced only many hours after cAMP treatment. Therefore, these proteins appear to play different roles in early and late phases of gene activation, respectively.

HIPK proteins were originally identified for their roles in apoptosis (19, 30, 42). Previous data and our current data show that all three HIPKs participate in JNK and c-Jun phosphorylation (20, 33). The pathways that control the activities of HIPKs, however, are not completely understood. HIPK1 and HIPK2 activities can be induced by tumor necrosis factor alpha and transforming growth factor β, respectively (20, 33). Here we show that HIPK3 activity is under the control of cAMP signaling in adrenal Y1 cells. Thus, HIPK3 can function in JNK and c-Jun phosphorylation not only for the control of apoptosis but also for steroidogenesis. Since HIPK1 and -2 are very similar in structure and functions to HIPK3, it will be interesting to test whether HIPK1 and -2 also play a role in steroidogenesis. Although HIPK1 and HIPK2 are generally considered to be present in every cell type, their existence in steroidogenic cells has not been examined.

HIPK3 interacts with another nuclear receptor, androgen receptor (AR), in addition to SF-1 (36). Similar to the case for SF-1, the kinase activity of HIPK3 is important for AR function. It will be interesting to find out whether HIPK3 enhances AR function through c-Jun phosphorylation just like SF-1 and whether this HIPK3-enhanced activity can be generalized to other nuclear receptors.

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REFERENCES

1. **Aikawa, Y., L. A. Nguyen, K. Isono, N. Takakura, Y. Tagata, M. L. Schmitz, H. Koseki, and I. Kitabayashi.** 2006. Roles of HIPK1 and HIPK2 in AML1- and p300-dependent transcription, hematopoiesis and blood vessel formation. EMBO J. **25:**3955–3965.
2. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta **1072:**129–157.
3. **Bornfeldt, K. E., and E. G. Krebs.** 1999. Crosstalk between protein kinase A and growth factor receptor signaling pathways in arterial smooth muscle. Cell Signal **11:**465–477.
4. **Choi, C. Y., Y. H. Kim, Y. O. Kim, S. J. Park, E. A. Kim, W. Riemenschneider, K. Gajewski, R. A. Schulz, and Y. Kim.** 2005. Phosphorylation by the DHIPK2 protein kinase modulates the corepressor activity of Groucho. J. Biol. Chem. **280:**21427–21436.
5. **Christenson, L. K., P. F. Johnson, J. M. McAllister, and J. F. Strauss, 3rd.** 1999. CCAAT/enhancer-binding proteins regulate expression of the human steroidogenic acute regulatory protein (StAR) gene. J. Biol. Chem. **274:** 26591–26598.
6. **Clem, B. F., E. A. Hudson, and B. J. Clark.** 2005. Cyclic adenosine 3′,5′-monophosphate (cAMP) enhances cAMP-responsive element binding (CREB) protein phosphorylation and phospho-CREB interaction with the mouse steroidogenic acute regulatory protein gene promoter. Endocrinology **146:**1348–1356.
7. **Curtin, J. F., and T. G. Cotter.** 2004. JNK regulates HIPK3 expression and promotes resistance to Fas-mediated apoptosis in DU 145 prostate carcinoma cells. J. Biol. Chem. **279:**17090–17100.
8. **de Groot, R. P., and P. Sassone-Corsi.** 1992. Activation of Jun/AP-1 by protein kinase A. Oncogene **7:**2281–2286.
9. **Deng, X., D. Z. Ewton, B. Pawlikowski, M. Maimone, and E. Friedman.** 2003. Mirk/dyrk1B is a Rho-induced kinase active in skeletal muscle differentiation. J. Biol. Chem. **278:**41347–41354.
10. **Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
11. **Desclozeaux, M., I. N. Krylova, F. Horn, R. J. Fletterick, and H. A. Ingraham.** 2002. Phosphorylation and intramolecular stabilization of the ligand binding domain in the nuclear receptor steroidogenic factor 1. Mol. Cell. Biol. **22:**7193–7203.
12. **D'Orazi, G., B. Cecchinelli, T. Bruno, I. Manni, Y. Higashimoto, S. Saito, M. Gostissa, S. Coen, A. Marchetti, G. Del Sal, G. Piaggio, M. Fanciulli, E. Appella, and S. Soddu.** 2002. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat. Cell Biol. **4:**11–19.
13. **Duguay, Y., A. Lapointe, B. Lavallee, D. W. Hum, and S. Rivest.** 2003. Cloning of murine TReP-132, a novel transcription factor expressed in brain regions involved in behavioral and psychiatric disorders. Mol. Psychiatry **8:**39–49.
14. **Ecsedy, J. A., J. S. Michaelson, and P. Leder.** 2003. Homeodomain-interacting protein kinase 1 modulates Daxx localization, phosphorylation, and transcriptional activity. Mol. Cell. Biol. **23:**950–960.
15. **Foster, R. H.** 2004. Reciprocal influences between the signalling pathways regulating proliferation and steroidogenesis in adrenal glomerulosa cells. J. Mol. Endocrinol. **32:**893–902.
16. **Gizard, F., B. Lavallee, F. DeWitte, and D. W. Hum.** 2001. A novel zinc finger protein TReP-132 interacts with CBP/p300 to regulate human CYP11A1 gene expression. J. Biol. Chem. **276:**33881–33892.
17. **Guo, I. C., M. C. Hu, and B. C. Chung.** 2003. Transcriptional regulation of CYP11A1. J. Biomed. Sci. **10:**593–598.
18. **Guo, I. C., H. M. Tsai, and B. C. Chung.** 1994. Actions of two different cAMP-responsive sequences and an enhancer of the human CYP11A1 (P450scc) gene in adrenal Y1 and placental JEG-3 cells. J. Biol. Chem. **269:**6362–6369.
19. **Hofmann, T. G., A. Moller, H. Sirma, H. Zentgraf, Y. Taya, W. Droge, H. Will, and M. L. Schmitz.** 2002. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat. Cell Biol. **4:**1–10.
20. **Hofmann, T. G., N. Stollberg, M. L. Schmitz, and H. Will.** 2003. HIPK2 regulates transforming growth factor-beta-induced c-Jun NH(2)-terminal kinase activation and apoptosis in human hepatoma cells. Cancer Res. **63:** 8271–8277.
21. **Hu, M. C., S. J. Chou, Y. Y. Huang, N. C. Hsu, H. Li, and B. C. Chung.** 1999. Tissue-specific, hormonal, and developmental regulation of SCC-LacZ expression in transgenic mice leads to adrenocortical zone characterization. Endocrinology **140:**5609–5618.
22. **Hu, M. C., I. C. Guo, J. H. Lin, and B. C. Chung.** 1991. Regulated expression of cytochrome P-450scc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein. Biochem. J. **274:**813–817.
23. **Huang, N., and W. L. Miller.** 2000. Cloning of factors related to HIV-inducible LBP proteins that regulate steroidogenic factor-1-independent human placental transcription of the cholesterol side-chain cleavage enzyme, P450scc. J. Biol. Chem. **275:**2852–2858.
24. **Huang, N., and W. L. Miller.** 2005. LBP proteins modulate SF1-independent expression of P450scc in human placental JEG-3 cells. Mol. Endocrinol. **19:**409–420.
25. **Huang, Y., M. Hu, N. Hsu, C. L. Wang, and B. Chung.** 2001. Action of hormone responsive sequence in 2.3 kb promoter of CYP11A1. Mol. Cell Endocrinol. **175:**205–210.
26. **Keeney, D. S., Y. Ikeda, M. R. Waterman, and K. L. Parker.** 1995. Cholesterol side-chain cleavage cytochrome P450 gene expression in the primitive gut of the mouse embryo does not require steroidogenic factor 1. Mol. Endocrinol. **9:**1091–1098.
27. **Kim, E. A., Y. T. Noh, M. J. Ryu, H. T. Kim, S. E. Lee, C. H. Kim, C. Lee, Y. H. Kim, and C. Y. Choi.** 2006. Phosphorylation and transactivation of Pax6 by homeodomain-interacting protein kinase 2. J. Biol. Chem. **281:**7489–7497.
28. **Kim, Y. H., C. Y. Choi, S. J. Lee, M. A. Conti, and Y. Kim.** 1998. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J. Biol. Chem. **273:**25875–25879.
29. **Kohler, P. O., and W. E. Bridson.** 1971. Isolation of hormone-producing clonal lines of human choriocarcinoma. J. Clin. Endocrinol. Metab. **32:**683–687.
30. **Kondo, S., Y. Lu, M. Debbas, A. W. Lin, I. Sarosi, A. Itie, A. Wakeham, J. Tuan, C. Saris, G. Elliott, W. Ma, S. Benchimol, S. W. Lowe, T. W. Mak, and S. K. Thukral.** 2003. Characterization of cells and gene-targeted mice deficient for the p53-binding kinase homeodomain-interacting protein kinase 1 (HIPK1). Proc. Natl. Acad. Sci. USA **100:**5431–5436.
31. **Krylova, I. N., E. P. Sablin, J. Moore, R. X. Xu, G. M. Waitt, J. A. MacKay, D. Juzumiene, J. M. Bynum, K. Madauss, V. Montana, L. Lebedeva, M. Suzawa, J. D. Williams, S. P. Williams, R. K. Guy, J. W. Thornton, R. J. Fletterick, T. M. Willson, and H. A. Ingraham.** 2005. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. Cell **120:**343–355.
32. **Li, L. A., E. F. Chiang, J. C. Chen, N. C. Hsu, Y. J. Chen, and B. C. Chung.** 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.
33. **Li, X., R. Zhang, D. Luo, S. J. Park, Q. Wang, Y. Kim, and W. Min.** 2005. Tumor necrosis factor alpha-induced desumoylation and cytoplasmic translocation of homeodomain-interacting protein kinase 1 are critical for apoptosis signal-regulating kinase 1-JNK/p38 activation. J. Biol. Chem. **280:**15061–15070.
34. **Manna, P. R., D. W. Eubank, E. Lalli, P. Sassone-Corsi, and D. M. Stocco.** 2003. Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1. J. Mol. Endocrinol. **30:**381–397.
35. **Manna, P. R., D. W. Eubank, and D. M. Stocco.** 2004. Assessment of the role of activator protein-1 on transcription of the mouse steroidogenic acute regulatory protein gene. Mol. Endocrinol. **18:**558–573.
36. **Moilanen, A. M., U. Karvonen, H. Poukka, O. A. Janne, and J. J. Palvimo.** 1998. Activation of androgen receptor function by a novel nuclear protein kinase. Mol. Biol. Cell **9:**2527–2543.
37. **Monte, D., F. DeWitte, and D. W. Hum.** 1998. Regulation of the human P450scc gene by steroidogenic factor 1 is mediated by CBP/p300. J. Biol. Chem. **273:**4585–4591.
38. **Nateri, A. S., L. Riera-Sans, C. Da Costa, and A. Behrens.** 2004. The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. Science **303:** 1374–1378.
39. **Nuclear Receptors Nomenclature Committee.** 1999. A unified nomenclature system for the nuclear receptor superfamily. Cell **97:**161–163.
40. **Okamoto, M., H. Takemori, and Y. Katoh.** 2004. Salt-inducible kinase in steroidogenesis and adipogenesis. Trends Endocrinol. Metab. **15:**21–26.
41. **Parker, K. L., and B. P. Schimmer.** 1997. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocrinol. Rev. **18:** 361–377.
42. **Rochat-Steiner, V., K. Becker, O. Micheau, P. Schneider, K. Burns, and J. Tschopp.** 2000. FIST/HIPK3: a Fas/FADD-interacting serine/threonine kinase that induces FADD phosphorylation and inhibits fas-mediated Jun NH(2)-terminal kinase activation. J. Exp. Med. **192:**1165–1174.
43. **Sarkar, D., F. Kambe, Y. Hayashi, S. Ohmori, H. Funahashi, and H. Seo.** 2000. Involvement of AP-1 and steroidogenic factor (SF)-1 in the cAMP-dependent induction of human adrenocorticotropic hormone receptor (ACTHR) promoter. Endocrinol. J. **47:**63–75.
44. **Schimmer, B. P.** 1985. Isolation of ACTH-resistant Y1 adrenal tumor cells. Methods Enzymol. **109:**350–356.

45. **Sewer, M. B., and M. R. Waterman.** 2003. CAMP-dependent protein kinase enhances CYP17 transcription via MKP-1 activation in H295R human adrenocortical cells. J. Biol. Chem. **278:**8106–8111.
46. **Simpson, E. R., and M. R. Waterman.** 1988. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu. Rev. Physiol. **50:**427–440.
47. **Stork, P. J., and J. M. Schmitt.** 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. **12:**258–266.
48. **Tournier, C., A. J. Whitmarsh, J. Cavanagh, T. Barrett, and R. J. Davis.** 1997. Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proc. Natl. Acad. Sci. USA **94:**7337–7342.
49. **Treier, M., D. Bohmann, and M. Mlodzik.** 1995. JUN cooperates with the ETS domain protein pointed to induce photoreceptor R7 fate in the Drosophila eye. Cell **83:**753–760.
50. **Treier, M., L. M. Staszewski, and D. Bohmann.** 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell **78:**787–798.
51. **Tsai, M. J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63:** 451–486.
52. **Ueda, H., G. C. Sun, T. Murata, and S. Hirose.** 1992. A novel DNA-binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein. Mol. Cell. Biol. **12:**5667–5672.
53. **Wang, W., C. Zhang, A. Marimuthu, H. I. Krupka, M. Tabrizizad, R. Shelloe, U. Mehra, K. Eng, H. Nguyen, C. Settachatgul, B. Powell, M. V. Milburn, and B. L. West.** 2005. The crystal structures of human steroidogenic factor-1 and liver receptor homologue-1. Proc. Natl. Acad. Sci. USA **102:**7505–7510.
54. **Wang, X. L., M. Bassett, Y. Zhang, S. Yin, C. Clyne, P. C. White, and W. E. Rainey.** 2000. Transcriptional regulation of human 11beta-hydroxylase (hCYP11B1). Endocrinology **141:**3587–3594.
55. **Watanabe, N., H. Inoue, and Y. Fujii-Kuriyama.** 1994. Regulatory mechanisms of cAMP-dependent and cell-specific expression of human steroidogenic cytochrome P450scc (CYP11A1) gene. Eur. J. Biochem. **222:**825–834.
56. **Zhang, Q., Y. Yoshimatsu, J. Hildebrand, S. M. Frisch, and R. H. Goodman.** 2003. Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP. Cell **115:**177–186.
57. **Zhou, G., K. A. Mihindukulasuriya, R. A. MacCorkle-Chosnek, A. Van Hooser, M. C. Hu, B. R. Brinkley, and T. H. Tan.** 2002. Protein phosphatase 4 is involved in tumor necrosis factor-alpha-induced activation of c-Jun N-terminal kinase. J. Biol. Chem. **277:**6391–6398.