The AP-1 family member FOS blocks transcriptional activity of the nuclear receptor steroidogenic factor 1

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Summary
Steroid production in the adrenal zona glomerulosa is under the control of angiotensin II (Ang II), which, upon binding to its receptor, activates protein kinase C (PKC) within these cells. PKC is a potent inhibitor of the steroidogenic enzyme CYP17. We have demonstrated that, in the ovary, PKC activates expression of FOS, a member of the AP-1 family, and increased expression of this gene is linked to CYP17 downregulation. However, the pathway and the molecular mechanism responsible for the inhibitory effect of PKC on CYP17 expression are not defined. Herein, we demonstrated that Ang II inhibited CYP17 through PKC and ERK1/2-activated FOS and that blocking FOS expression decreased PKC-mediated inhibition. Although CYP17 transcription was activated by the nuclear receptor SF-1, expression of FOS resulted in a decrease in SF-1-mediated gene transcription. FOS physically interacted with the hinge region of SF-1 and modulated its transactivity, thus preventing binding of cofactors such as SRC1 and CBP, which were necessary to fully activate CYP17 transcription. Collectively, these results indicate a new regulatory mechanism for SF-1 transcriptional activity that might influence adrenal zone-specific expression of CYP17, a mechanism that can potentially be applied to other steroidogenic tissues.

Key words: SF-1, AP-1, Adrenal, CYP17

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Introduction
The production of individual steroids within the ovary, testis or adrenal gland relies on the selective expression of specific steroid-metabolizing enzymes. In the adrenal gland, each zone of the cortex is characterized by the production of unique steroids based on a distinct pattern of expression of specific steroid-metabolizing enzymes (Rainey, 1999). In particular, the zona glomerulosa produces mineralocorticoids, mainly aldosterone, whose levels are regulated primarily by angiotensin II (Ang II) and potassium (Bird et al., 1993). The zona glomerulosa is characterized by high expression of aldosterone synthase (CYP11B2) (Ogishima et al., 1992; LeHoux and Ducharme, 1995) and low expression levels of 17α-hydroxylase-17,20-lyase (CYP17) (Suzuki et al., 2000). In this distinct expression pattern of CYP11B2 and CYP17, Ang II appears to play a central role. Presumably, the zone-specific expression of CYP17 depends on trans-acting factors that are differentially expressed or regulated in the glomerulosa and fasciculata. These factors, which remain poorly defined, would interact with specific elements in the promoter of this gene, either activating or repressing transcription.

Ang II binds to its receptor, a member of the G-protein-coupled receptor family, and activates protein kinase C (Mazzocchi et al., 1997; Tian et al., 1998). Activation of PKC exerts a potent inhibitory effect on CYP17 mRNA levels (McAllister and Hornsby, 1988; Naseeruddin and Hornsby, 1990; Brentano et al., 1990; McGee et al., 1996; Bird et al., 1998) and enzymatic activity (Rainey et al., 1991a). PKC is capable of directly activating a cascade of phosphorylation events that results in the activation of ERKs (extracellular-regulated kinases) (Cacace et al., 1996; Cai et al., 1997; Tian et al., 1998). In particular, it has been shown that Ang II activates ERKs in primary cultures of rat adrenal glomerulosa cells (Otis et al., 2005), bovine adrenal cells (Cherradi et al., 2003) and H295R adrenal cells (Watanabe et al., 1996). It has been shown that silencing of ERK1/2, using selective inhibitors, increases CYP17 expression in the H295R adrenal cell model (Sewer and Waterman, 2003). Once activated, ERK1 and ERK2 phosphorylate numerous substrates in all cellular compartments including various membrane proteins, cytoskeletal proteins and nuclear substrates such as SRC1, ELK1, MEF2, c-Myc, STAT3 and FOS (Roux and Blenis, 2004).

Ang II also stimulates expression of the activator protein 1 (AP-1) family members (Nogueira et al., 2007). FOS belongs to the AP-1 family of transcription factors (Curran and Franza, 1988), which include FOS members (FOS, FOSB, Fra-1 and Fra-2) and JUN members (JUN, JUNB and JUND). FOS forms heterodimers with JUN family members in order to bind, with high affinity, to an asymmetric recognition sequence (AP-1 site) in the promoter of numerous mammalian genes (Curran and Franza, 1988). Most AP-1 transcription factors are present at low levels in cells but are rapidly induced and activated in response to specific stimuli. Ang II has been shown to increase FOS expression in primary cultures of rat, bovine and ovine adrenal cells (Viard et al., 1992; Clark et al., 1992), as well as in human adrenocortical tumor cell line H295R (Nogueira et al., 2007). A role for FOS in controlling steroidogenesis in the adrenal gland has been investigated, analyzing its ability to control transcription of steroidogenic acute regulatory (StAR) protein in mouse (Shea-Eaton et al., 2002) and bovine adrenal cells (Rincon Garriz et al., 2009). More recently, a

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study demonstrated the ability of FOS to increase expression of CYP11B2 in H295R cells (Romero et al., 2007). We have shown that in ovarian theca cells there is an inverse correlation between FOS and CYP17 expression (Beshay et al., 2007), and that silencing FOS can reverse PKC-dependent inhibition of CYP17. We believe that the ability of FOS to repress CYP17 expression will probably have broader application to other steroidogenic tissues such as the adrenal. However, to date there are no studies showing a correlation between FOS and CYP17 expression in the human adrenal gland. Herein, we tested the hypothesis that Ang II repression of CYP17 results from activation of PKC and ERK1/2, leading to increased expression of AP-1 members. Our observations explain, at least in part, the molecular mechanism for repressed CYP17 expression in the adrenal zona glomerulosa.

Results

Opposing effects of PKC and Ca^{2+} signaling on CYP17 expression

The human H295R adrenal cell line is a pluripotent model that can be manipulated to produce steroids characteristic of each adrenocortical zone, thus making it an appropriate model to define the molecular mechanisms that inhibit CYP17 expression (Rainey et al., 1994). Treatment of H295R cells with Ang II alone did not change CYP17 levels, whereas treatment with TPA, a selective activator of the PKC pathway, reduced *CYP17* mRNA expression by 80% (Fig. 1A). These data support a role for the PKC pathway in blocking CYP17 expression and causing cells to resemble a glomerulosa-like cell line. Ang II, in addition to activating PKC, increases intracellular Ca^{2+}. K^+, another key regulator of glomerulosa cell aldosterone production, also acts by intracellular Ca^{2+}. Fig. 1B shows that treatment of H295R cells with K^+ increased *CYP17* gene expression by 4-fold. Since both Ca^{2+} signaling and PKC were activated, we hypothesized that Ang II alone does not act as a repressor of CYP17. Because our previous studies established that Ang II acts as a repressor of agonist stimulation of CYP17, we wanted to determine whether Ang II can block K^+ stimulation of CYP17. Both Ang II and TPA were able to completely block the K^+ induction of *CYP17* mRNA (Fig. 1B). Ang II treatment (24 hours) was combined with KN93 (to inhibit CaM kinases) or GF109203X (to inhibit PKC). Treatment with Ang II in the presence of KN93 resulted in a decrease in *CYP17* mRNA levels by 75% (Fig. 1C). By contrast, treatments with Ang II plus the PKC inhibitor GF109203X resulted in a significant increase in *CYP17* gene expression (Fig. 1C). These data suggest that glomerulosa cell activation of PKC might be the physiological mechanism that leads to an inhibition of CYP17 expression.

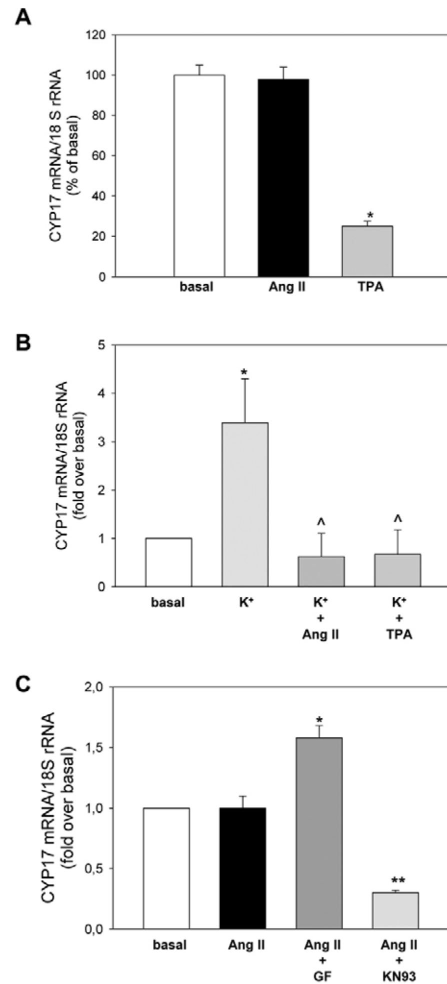

Fig. 1. Opposing actions of Ca^{2+} and PKC signaling on *CYP17* expression. Real-time RT-PCR was used to quantify *CYP17* transcript levels. Basal (untreated) cells were used as control. (**A**) Cells were incubated for 24 hours with Ang II (100 nM) and TPA (10 nM). (**B**) Cells were incubated for 24 hours with K^+ (20 mM) alone or combined with Ang II (100 nM) or TPA (10 nM). (**C**) Cells were incubated for 24 hours with Ang II (100 nM), alone or in combination with the PKC inhibitor GF109203X (GF; 5 μM) or with the Cam kinase inhibitor KN93 (5 μM). Real-time RT-PCR data represent the mean + s.e.m. of three independent RNA samples from H295R cultures and are expressed as relative difference from basal (calibrator) as indicated in the Materials and Methods section. *P<0.05 compared with basal; ^P<0.001 compared with K^+; **P<0.001 compared with basal.

Activation of PKC induces FOS expression in H295R cells

Treatment of H295R cells with TPA resulted in a time-dependent inhibition of *CYP17* mRNA expression that was completely abolished by the presence of the protein synthesis inhibitor cycloheximide (Fig. 2A). These data indicated that the synthesis of new proteins is required for TPA-mediated inhibition of *CYP17*. We have previously shown that Ang II mediates FOS induction in adrenal cells (Nogueira et al., 2007). Because PKC is activated by Ang II after binding its receptor, we proposed that FOS could also be a factor regulated by TPA. Microarray analyses of mRNA from untreated (basal) H295R cells or cells treated with either Ang II or TPA, are shown in Fig. 1B,C. Most of the AP-1 family members examined were upregulated by Ang II and TPA (Fig. 2B,C). The greatest increases, following treatment with Ang II or TPA, were observed for *FOS* (300- and 194-fold, respectively) and *FOSB* (500- and 65-fold, respectively) followed by *JUNB* (14- and 9-fold, respectively), *JUN* (4.2- and 2-fold, respectively) and *JUND* (2-fold each). Although both *Fra-1* and *Fra-2* increased 2-fold following treatment with Ang II, only *Fra-1* was increased by TPA treatment (2-fold). Interestingly, under basal conditions, *FOS* and *FOSB* are present at very low levels compared with *JUN* or *JUND*. However, cells treated with Ang II or TPA increased *FOS* and *FOSB* expression to levels similar to those seen for *JUN* mRNA (Fig. 2B,C). A time-course study of *FOS* mRNA levels indicated that the presence of TPA increased *FOS* expression and this increase was also maintained after prolonged treatment (Fig. 3A), with an

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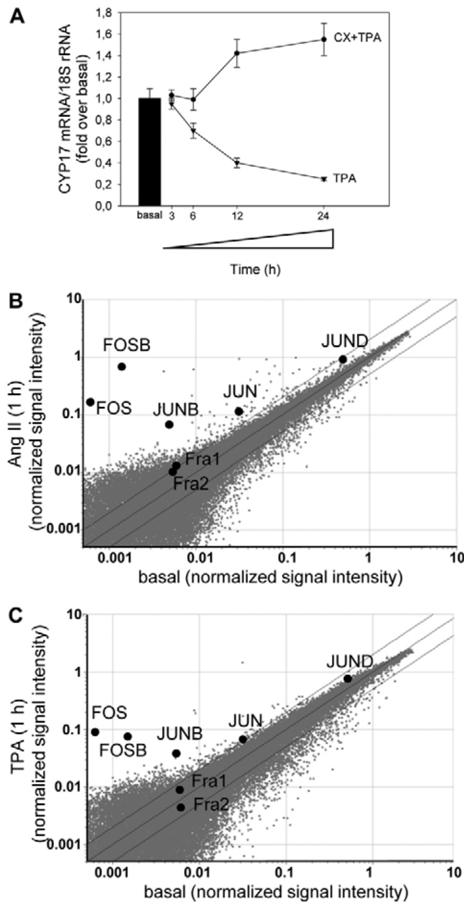

Fig. 2. TPA-induced inhibition of *CYP17* mRNA is dependent on protein synthesis. (**A**) H295R cells were incubated for the indicated times with TPA (10 nM) alone or in the presence of cycloheximide (CX; 10 μM). Real-time RT-PCR analysis was used to quantify *CYP17* transcript levels. Real-time RT-PCR data represent the mean + s.e.m. of three independent RNA samples from H295R cultures and are expressed as relative difference from basal (calibrator). *P<0.001 compared with basal. (**B**) Microarray analysis of H295R cells treated for 1 hour with 100 nM Ang II versus basal (untreated) cells and (**C**) of cells treated for 1 hour with 10 nM TPA versus basal cells. Each spot represents a unique sequence with a total of approximately 38,500 transcripts examined per array. Pure signal was normalized to a list of 100 normalization control probe sets provided by Affymetrix. Spots outside of the parallel lines represent mRNAs with greater than 2-fold differences in expression. AP-1 family member genes are indicated.

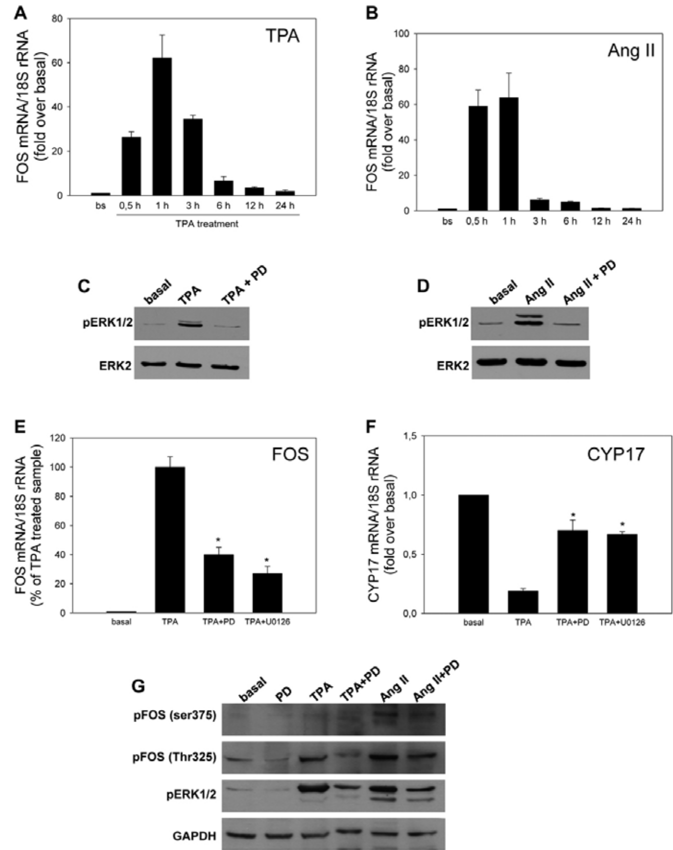

Fig. 3. PKC-dependent effects on FOS and CYP17 require ERK1/2 activation. (**A**,**B**) H295R cells were incubated for the indicated times with TPA (10 nM; A) or with Ang II (100 nM; B). Real-time RT-PCR analysis was used to quantify *FOS* transcript levels. (**C**,**D**) Cells were incubated for 5 minutes with TPA (10 nM; C) or with Ang II (100 nM; D) alone or in the presence of PD98059 (PD; 10 μM) added 30 minutes before stimulation. Phosphorylation of ERK1/2 was examined by immunoblot analysis using equivalent amounts of proteins. ERK2 was used as a loading control. (**E**) Cells were incubated for 1 hour with TPA (10 nM) alone or in the presence of PD (10 μM) or U0126 (10 μM) added 30 minutes before stimulation. Real-time RT-PCR analysis was used to quantify *FOS* transcript levels. (**F**) Cells were incubated for 24 hours with TPA (10 nM) alone or in the presence of PD (10 μM) or U0126 (10 μM) added 30 minutes before stimulation. Real-time RT-PCR analysis was used to quantify *CYP17* transcript levels. Real-time RT-PCR data represent the mean + s.e.m. of three independent RNA samples from H295R cultures and are expressed as relative difference from basal (calibrator). *P<0.001 compared with TPA. (**G**) Cells were incubated for 1 hour with TPA (10 nM) and Ang II (100 nM) alone or in the presence of PD (10 μM) added 30 minutes before stimulation. Phosphorylation of FOS on Ser375 and Thr325 and of ERK1/2 were examined by immunoblot analysis using equivalent amounts of proteins. GAPDH was used as a loading control.

expression pattern similar to the one seen under Ang II treatment (Fig. 3B).

MAPK family members ERK1/2 have been previously shown to be part of the Ang-II-activated pathway in H295R cells (Watanabe et al., 1996). We confirmed these data by showing the ability of both Ang II and TPA to induce phosphorylation of ERK1/2 after 5 minutes treatment (Fig. 3C,D). Activation of these kinases was blocked by the presence of the MEK inhibitor PD98059 (PD; Fig. 3C,D). To confirm the importance of ERK1/2 in the activation of the AP-1 genes, cells were pre-incubated with two different ERK1/2 inhibitors, PD and U0126, prior to TPA treatment followed by measurement of *FOS* mRNA (Fig. 3E). Induction of *FOS* by TPA was reduced by 60% in the presence of the ERK1/2 inhibitor PD and by 70% in the presence of U0126 (Fig. 3E). TPA inhibition of *CYP17* was also blocked by the addition of both inhibitors (Fig. 3F). As ERK1 and ERK2 are also able to directly phosphorylate FOS (Chen et al., 1993; Monje et al., 2003), we examined the effects of Ang II and TPA on FOS phosphorylation (Fig. 3G). Western blot analysis using antibodies against phospho-serine 374 and phospho-threonine 325 indicated that both treatments increased FOS phosphorylation, an effect that was prevented by the presence of PD. These data confirm a role for ERK1/2 in controlling FOS at both transcriptional and post-translational levels.

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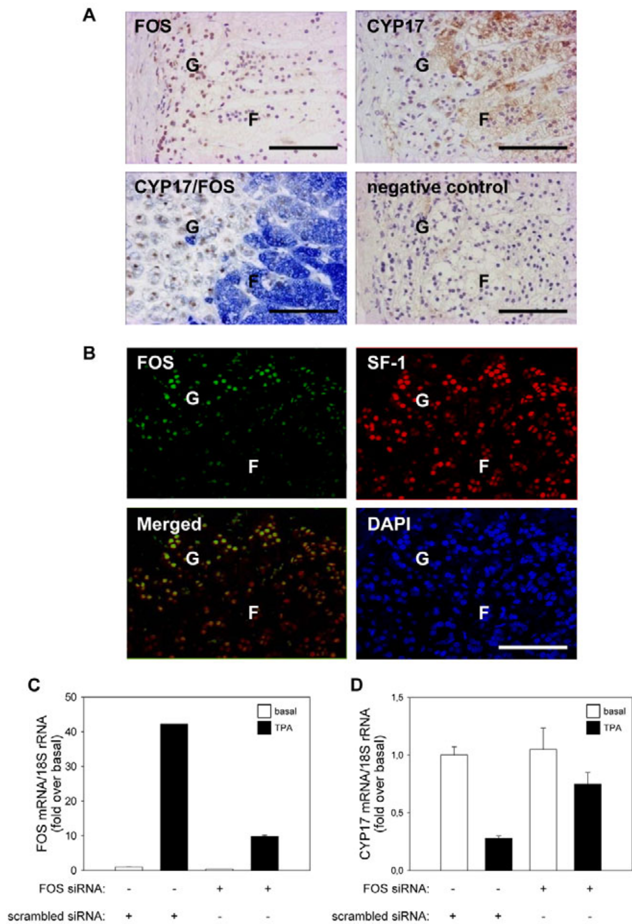

Fig. 4. An inverse correlation exists between FOS and CYP17 expression in human adrenal. (**A**) Immunohistochemical analysis of FOS (left upper photo) and CYP17 (right upper photo) in human adult adrenal glands. Double immunostaining for FOS and CYP17 (left lower photo). The right lower panel is the negative control for FOS expression. Immunoreactivity is indicated with brown staining; Hematoxylin was used for counterstaining, which gave the blue coloring. For double immunostaining for FOS and CYP17, the first antibody used was FOS and it was visualized by DAB (brown). Then a CYP17 antibody was used, which was stained by alkaline phosphatase substrate kit III (blue). Each panel is of a serial section of the same adrenal gland. G, glomerulosa; F, fasciculata. Scale bars: 100 μm. (**B**) Double immunofluorescence for SF-1 and c-FOS in the human adrenal gland. The majority of adrenocortical cells in the zona glomerulosa were positive for both SF-1 and c-FOS. SF-1 immunoreactivity was represented as red (Alexa Fluor 647), whereas c-FOS immunoreactivity was shown as green (Alexa Fluor 488). Colocalization of SF-1 and c-FOS are indicated as yellow color in the merged photo. Nuclei of H295R cells were visualized by DAPI staining. Scale bars: 100 μm. (**C**,**D**) H295R cells were grown for 2 days in the presence of siRNA for FOS and then treated for 1 hour (C) or 24 hours (D) with TPA (10 nM). Real-time PCR for *FOS* (C) and *CYP17* (D) in H295R cells following siRNA gene silencing was performed. Data represent the mean + s.e.m. of two independent RNA samples from H295R cultures and are expressed as relative difference from cells untreated and transfected with scrambled siRNA (basal calibrator).

FOS mediates CYP17 repression and is expressed in the zona glomerulosa

FOS and CYP17 expression was examined in human adrenal glands using immunohistochemistry. Immunoreactive FOS was localized primarily to the nucleus of cells in the zona glomerulosa (Fig. 4A, left upper photo), where CYP17 expression was absent (Fig. 4A, left lower photo). Double immunostaining for FOS and CYP17 was performed, indicating the differential expression of the two proteins (Fig. 4A, right upper photo).

FOS and SF-1 localization was examined in human adrenal glands by confocal microscopy using specific antibodies as indicated in the Materials and Methods. Immunoreactive FOS was localized primarily in the nucleus of cells in the zona glomerulosa (Fig. 4B, FOS), where SF-1 expression was abundant (Fig. 4B, SF-1). We were also able to determine that FOS and SF-1 colocalized in cells of the zona glomerulosa (Fig. 4B, merged).

Transfection of H295R cells with siRNA for *FOS* decreased its expression in H295R cells (Fig. 4C) and showed that the presence of this transcription factor is necessary for PKC-mediated inhibition of *CYP17* (Fig. 4D). TPA inhibited *CYP17* mRNA expression by 65%, but when FOS expression was knocked down (with siRNA), this inhibition was only 25% (Fig. 4D).

FOS containing AP-1 dimers regulates *CYP17* gene transcription

AP-1 proteins regulate gene transcription by binding to specific consensus sequences (AP-1 sites) in the promoter of target genes. Sequence analysis of the *CYP17* promoter identified several potential AP-1 sites. We used ChIP analysis to show endogenous binding of AP-1 proteins to the *CYP17* promoter. Not only FOS, but also JUN, binding was induced by treatment with Ang II and TPA (Fig. 5A,B). However, binding of FOS, but not JUN, was reduced by blocking ERK1/2 activation using the inhibitor PD (Fig. 5A,B).

Dimers of JUN and FOS were able to decrease *CYP17* transcription by 40% (Fig. 5C). Because human *CYP17* transcription is enhanced by SF-1 (Hanley et al., 2001), and potential AP-1 sites are in close proximity to the SF-1 sites, we tested the effect of dimers formed between FOS and JUN on the SF-1-induced transcription of *CYP17* (Fig. 5D). Cells were transfected with constant amounts of SF-1 and JUN in combination with increasing doses of FOS (Fig. 5D). SF-1 induced *CYP17* transcription by 10-fold, whereas FOS inhibited SF-1 activation of *CYP17* transcription in a concentration-dependent manner, with a significant inhibition observed with as little as 1 ng of expression vector, a 50% inhibition with 10 ng and an 80% inhibition with 100 ng (Fig. 5D).

FOS represses CYP17 through direct interaction with SF-1

FOS-mediated repression of CYP17 was abolished when the three *SF-1* sites were mutated (Fig. 5E), indicating that the inhibitory effect is specific for SF-1. To determine which of the potential *AP-1* sites was responsible for the inhibition, we performed a mutational analysis (Fig. 5F,G). A cDNA construct was mutated at the putative *AP-1* site, at –164 bp, and used in co-transfection experiments. JUN–FOS dimers completely lost their ability to interfere with SF-1-activated transcription of *CYP17* (Fig. 5F). However, the mutated construct showed a lower basal transcriptional activity when compared with wild-type *CYP17* construct (Fig. 5G). This was owing to the ability of JUN alone, in the absence of FOS, to induce *CYP17* basal transcription (Fig. 5H).

FOS-mediated repression of CYP17 was overcome by co-transfection with increasing amounts of SF-1 (Fig. 6A). Addition of the highest dose of SF-1 reversed AP-1 inhibition by 80%. Using a promoter construct containing AP-1 sites and co-transfecting with JUN–FOS, we were able to induce transcription

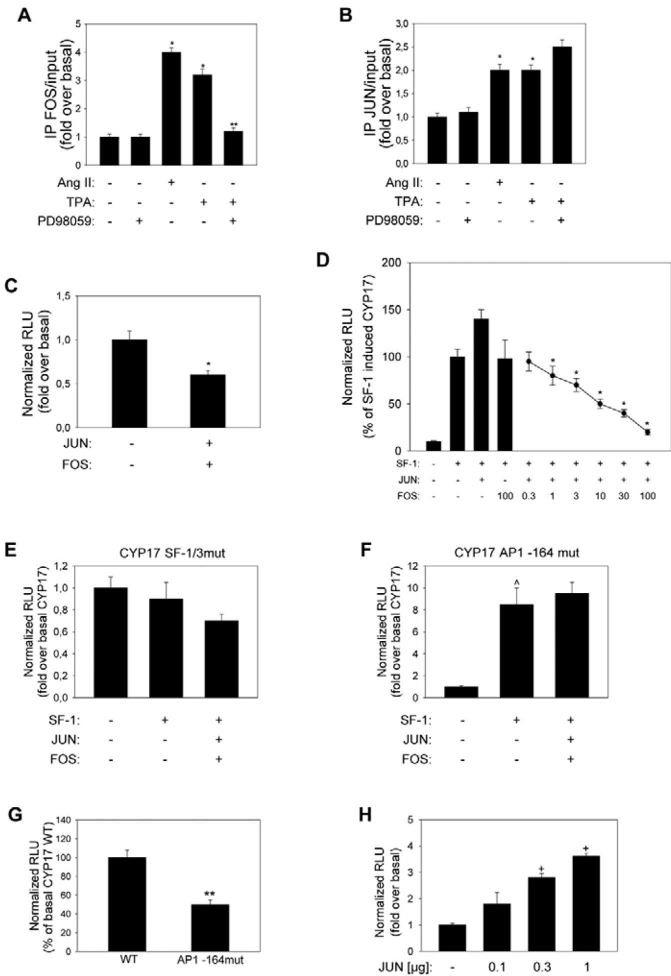

Fig. 5. JUN and FOS proteins are recruited to the *CYP17* promoter and repress its transcription. (**A**,**B**) H295R cells were untreated (basal) or incubated for 1 hour with Ang II (100 nM) or TPA (10 nM) alone or in the presence of PD98059 (10 μM). In vivo binding of JUN and FOS to the *CYP17* promoter was examined using ChIP assay. Immunoprecipitated (JUN, FOS) and total (10% input) DNA were subject to real time RT-PCR using specific primers. Ct values from immunoprecipitated samples were normalized to the input Ct values (**P*<0.001 compared with basal and ***P*<0.001 compared with TPA). (**C**) H295R cells were transfected with a *CYP17* promoter construct (1 μg) and 0.1 μg of expression vector for JUN and FOS. (**D**) H295R cells were co-transfected with 1 μg of a luciferase promoter construct containing 381 bp of the *CYP17* promoter, increasing doses of *FOS* (0.3, 1, 3, 10, 30, 100 ng), *SF-1* (0.3 μg) and *JUN* (0.1 μg) expression plasmids. (**E**,**F**) H295R cells were co-transfected with 1 μg of *CYP17* reporter construct mutated at the *SF-1* sites (CYP17 SF-1/3mut; E) or of CYP17 AP1 –164 mut (F) together with SF-1 (0.3 μg), JUN (0.1 μg) and FOS (0.1 μg). (**G**) Cells were transfected with CYP17 reporter construct (WT) or a construct containing 381 bp of the promoter region mutated at an *AP-1* site (CYP17 AP1 –164 mut). (**H**) Cells were transfected with the *CYP17* reporter and increasing doses of *JUN*. Data were normalized to co-expressed β-gal. Results represent the mean + s.e.m. of data from two to three independent experiments, each performed in triplicate. **P*<0.001 compared with SF-1; ^*P*<0.001 compared with basal (without expression vectors) mut CYP17; ***P*<0.05 compared with CYP17 WT; $^{+}$*P*<0.001 compared with basal (without expression vectors) WT CYP17.

by 5.5-fold (Fig. 6B). Addition of increasing amounts of SF-1 resulted in the reduction of AP-1-mediated induction of transcription in a concentration-dependent manner (Fig. 6B). These results suggested an interaction between FOS and SF-1. Therefore, we examined the potential for a direct interaction between FOS and SF-1. To this purpose, we used SF-1 antibodies to precipitate H295R nuclear protein followed by western analysis using a FOS antibody. As shown in Fig. 6C, western analysis for FOS revealed its presence in SF-1 immunoprecipitates, and this interaction increased if cellular FOS levels were increased after treatment with Ang II or TPA (Fig. 6C,D). We also investigated which region of SF-1 interacted with FOS by using deletion constructs of the *SF-1* coding region in GST pull-down experiments (Fig. 6E). The full-length construct confirmed a direct interaction between SF-1 and FOS. However, interaction between the two proteins was lost when the hinge region was removed (Fig. 6F). This region was shown to be important for co-factors binding to nuclear receptors (Bain et al., 2007); in particular, SF-1 was shown to interact with CREB binding protein (CBP) and SRC1. Importantly, interaction of SRC1 and FOS with SF-1 was lost when the hinge region was deleted (Fig. 6G).

FOS competes with co-factors binding to SF-1 on the *CYP17* promoter

Because SF-1 transcriptional activity depends on binding of co-activators, we tested the hypothesis that AP-1 factors inhibit SF-1-dependent *CYP17* transcription through competition for common co-activators including CBP and/or SRC1. To test this hypothesis, we used ChIP and re-ChIP analysis to show eventual changes in SF-1, SRC1 and CBP binding to the *CYP17* promoter (Fig. 7A–C). Although promoter binding of SF-1 did not change (Fig. 7A), treatment with Ang II or TPA reduced CBP and SRC binding to SF-1 (Fig. 7B,C). By blocking ERK1/2 activation, the reduction in SRC and CBP binding to SF-1 was lost (Fig. 7B,C). Co-transfection experiments with a *CYP17* reporter construct and increasing amounts of expression vectors for CBP and/or SRC1 (Fig. 7D) supported our hypothesis. Increasing expression of both factors was able to completely overcome FOS-mediated inhibition of *CYP17* promoter activity and, when CBP and SRC1 were added simultaneously at the concentration of 0.3 μg, the final effect was a significant induction of gene transcription of 2-fold over SF-1 alone.

Discussion

CYP17 has a specific pattern of expression in the adrenal cortex, being selectively expressed in the zonae fasciculata and reticularis and absent in the zona glomerulosa. The molecular mechanisms causing the zonal pattern of CYP17 expression within the adrenal cortex are not known.

Multiple studies support the ability of Ang II to block induction of CYP17 protein levels in several models (Rainey et al., 1991a; Rainey et al., 1991b; Rainey et al., 1992; Bird et al., 1992). It has long been our hypothesis that the expression of CYP17 can be inhibited by PKC (Mason et al., 1986; Rainey et al., 1991a; Bird et al., 1992; Bird et al., 1996; Bird et al., 1998). The inhibitory effect of PKC activation on CYP17 expression has been confirmed by several laboratories (McAllister and Hornsby, 1988; Naseeruddin and Hornsby, 1990; Brentano et al., 1990). Herein, we further determine the downstream signaling events that inhibit CYP17. Based on previous observations indicating *FOS* as a rapid-response gene activated by Ang II (Nogueira et al., 2007), and on the observation that protein synthesis was needed for TPA inhibition of CYP17, we wanted to determine if FOS induction could be dependent on PKC activation. Our microarray analysis and time-

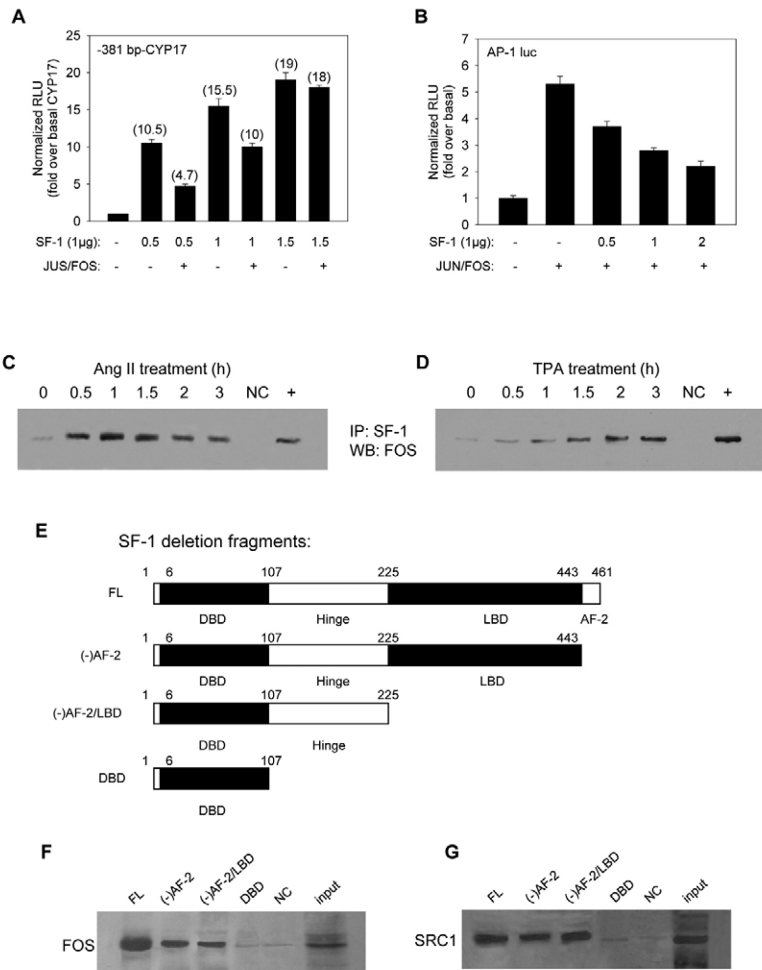

Fig. 6. Direct interaction between SF-1 and FOS. (**A**) H295R cells were transfected with the *CYP17* reporter plasmid and increasing doses of SF-1 expression vector and 0.1 µg of JUN and FOS expression vectors. (**B**) H295R cells were transfected with a luciferase promoter construct containing *AP-1* sites. Cells were cotransfected with increasing doses of SF-1 expression vector and 0.1 µg of expression vector for JUN and FOS. (**C**,**D**) H295R cells were treated for the indicated times with Ang II (100 nM; C) or TPA (10 nM; D). Nuclear extracts were immunoprecipitated (IP) with an anti-SF-1 antibody and then used for immunoblot analysis (WB) using an anti-FOS monoclonal antibody. (**E**) Schematic diagram depicting deletion fragments of the *SF-1* coding region. DNA fragments were cloned into pGEX4T1 and used in GST pulldown experiments. (**F**,**G**) One hundred micrograms of purified GST-tagged SF-1 proteins were incubated with 5 µl of in-vitro-synthesized FOS (F) or SRC1 (G) protein. Immunocomplexes (IP) were formed using an anti-SF-1 antibody. Precipitated samples were used for western blot analysis using an anti-FOS monoclonal antibody or an anti-SRC-1 antibody. The GST protein was immunoprecipitated like the samples and used as negative control (NC). One microliter of in-vitro-synthesized FOS or SRC-1 was loaded on the gel and used as positive control (input). GST pulldown results shown represent data from three independent experiments.

course study revealed the ability of TPA, as well as of Ang II, to induce elevated *FOS* expression levels. We have previously shown that activation of ERK1/2 is part of the Ang-II-activated pathway in H295R cells (Watanabe et al., 1996). Here, we demonstrate that ERK1/2 is downstream of PKC in the pathway leading to FOS induction. In fact, increased *FOS* levels after treatment with TPA are lost in the presence of a specific ERK1/2 inhibitor PD or U0126. It has been shown that ERK phosphorylates multiple residues within the carboxylterminal transactivation domain of FOS, thus resulting in increased FOS transcriptional activity (Monje et al., 2003). In particular, phosphorylation of FOS at serine 375 (Ser375) by ERK potentiates AP-1 transactivation capabilities and primes FOS for phosphorylation at threonine 325 (Thr325). Phosphorylation of Thr325 stabilizes FOS heterodimers and enhances promoter transactivation by AP-1 complexes (Chen et al., 1993; Chen et al., 1996). We found that phoshorylation on both residues is increased after Ang II and TPA treatment and is prevented by the presence of ERK inhibitor. These events were well-coordinated with the inhibitory effect on *CYP17* mRNA levels in that the inhibition of ERK1/2 after treatment with PD or U0126 results in a decreased ability of PKC to inhibit *CYP17* expression. This is in agreement with previous reports showing that silencing of ERK1/2 increases CYP17 expression in the H295R adrenal cells (Sewer and Waterman, 2003). Furthermore, ACTH, which is a known positive regulator of CYP17, decreases ERK activity in the adrenal cortex in vivo and in the mouse adrenal Y1 cell line (Watanabe et al., 1997). These previous data, together with ours, support the hypothesis that ERK activation is involved in the downregulation of CYP17.

Our immunohistochemical analysis shows that FOS expression is higher in the zona glomerulosa, where CYP17 is absent, and that FOS is low in the fasciculata and reticularis, where CYP17 is highly expressed. This inverse correlation between FOS and CYP17 made us hypothesize that FOS was the factor responsible for CYP17 downregulation in the zona glomerulosa. To confirm our hypothesis, we used siRNA to downregulate *FOS* expression in the H295R adrenal cell line. The knockdown of this gene was able to significantly block the ability of TPA to inhibit *CYP17* expression, demonstrating that inhibition of *CYP17* through PKC activation depends on the increased expression of *FOS* and on its binding to the *CYP17* promoter.

Transcriptional regulation of *CYP17* is mediated by several transcription factors (Sewer and Jagarlapudi, 2009), including the nuclear receptor SF-1 (Hanley et al., 2001; Sewer et al., 2002; Dammer et al., 2007). An analysis of the human *CYP17* promoter revealed the presence of AP-1 sites located in between the SF-1 sites, leading us to hypothesize an interference of AP-1 factors with SF-1-mediated transcription of *CYP17*. Our transfection experiments demonstrated that AP-1 dimers can result in an inhibition of SF-1-mediated activation of *CYP17*. Mutation of a putative AP-1 site present on the *CYP17* promoter at nucleotide –164 blocked AP-1 dimer ability to block SF-1-mediated transcription of the gene.

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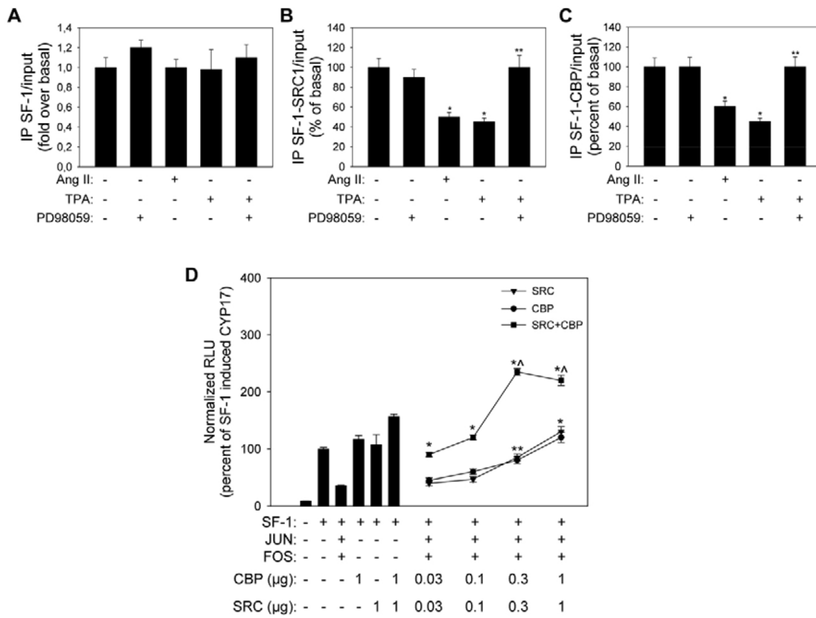

Fig. 7. FOS competes with co-factors binding to SF-1 on the *CYP17* promoter. (**A–C**) In vivo binding of SF-1 (A) to the *CYP17* promoter was examined using a ChIP assay. Interaction of SRC1 (B) and CBP (C) to SF-1 on the *CYP17* promoter was tested with a re-ChIP assay. All samples were immunoprecipitated with an anti-SF-1 antibody. For re-ChIP, samples were re-precipitated with an anti-SRC1 or anti-CBP antibody. Immunoprecipitated (SF-1, SF-1/SRC1, SF-1/CBP) and total (10% input) DNA were subject to real-time RT-PCR using specific primers. Ct values from immunoprecipitated samples were normalized to the input Ct values. Results represent the mean + s.e.m. of data from 3 independent experiments (*P<0.001 compared with basal and **P<0.001 compared with TPA). (**D**) H295R cells were transfected with the luciferase promoter construct containing 381 bp of the promoter region of the human *CYP17* gene in the presence of the expression vector for SF-1 (0.3 μg), JUN (0.1 μg), FOS (0.1 μg), CBP (1 μg), SRC1 (1 μg) or increasing doses of SRC-1, CBP or SRC1 plus CBP as indicated by the table under the graph. Results represent the mean + s.e.m. of data from three independent experiments, each performed in triplicate. *P<0.001 and **P<0.05 compared with SF-1+JUN/FOS; ^P<0.05 compared with SF-1.

Interestingly, mutation at the AP-1 site resulted in a decrease in basal *CYP17* transcription (about 50%), whereas a similar event was also observed in a study on the role of AP-1 proteins in the regulation of the StAR gene (Shea-Eaton et al., 2002), leading authors to suggest a more complex role for AP-1 members in StAR transactivation. We demonstrated that this is owing to the ability of JUN alone to increase basal *CYP17* transcription, suggesting that in the absence of FOS as seen in the inner adrenal zones, the AP-1 site is bound by JUN, which activates *CYP17* transcription. Importantly, ACTH, a positive regulator of CYP17, activates SAPK (also called JNK kinase), which is responsible for phosphorylation and activation of JUN (Watanabe et al., 1997). The activation of the PKC pathway inducing FOS possibly causes the formation of JUN–FOS dimers. Studies on the capacity of the JUN or FOS protein family members to bind DNA have revealed that the different dimers have different binding affinities. Specifically for homodimers, JUN has the strongest affinity, followed by JUND and finally JUNB (Ryseck and Bravo, 1991). Importantly, heterodimers of any of the JUN proteins with any of the FOS family members have a greatly enhanced binding activity compared with homodimers (Ryseck and Bravo, 1991).

Based on the position of the AP-1 and SF-1 sites on the CYP17 promoter, we examined the interaction between SF-1 and AP-1 factors. It has been shown by in vitro studies that SF-1 and FOS can interact (Manna et al., 2004) and it is a likely possibility that a similar interaction occurs in our model. An immunoprecipitation assay confirmed an interaction that increased with increasing amounts of FOS, as is seen following stimulation of H295R cells with TPA or Ang II. Using GST pull-down, we investigated the SF-1 region involved in this interaction. As expected, interaction occurred between FOS and the full-length SF-1 and this interaction was lost when the hinge region was removed. These data clearly show that the presence of the hinge is required for the interaction. However, a participation of the AF-2 domain cannot be excluded as we observed decreased FOS binding following AF-2 deletion. Indeed, the deletion might have changed the conformation of other domains and thus modify potential interaction. The absence of interaction with the DNA-binding domain (DBD) alone could also reflect the nature of the severely truncated protein and severe conformational change. Within the context of the longer protein, the interaction could be in the DBD, which without the rest of the protein would exist in a different non-interacting conformation when truncated to 107 amino acids. The hinge region, particularly between residues 187–245, was shown to be necessary for the activation capacity of SF-1-mediating interaction with the co-activator SRC1 (Crawford et al., 1997). SRC1 and CBP have been described as cofactors for various nuclear receptor proteins, including SF-1 (Liu and Simpson, 1997; Ito et al., 1998; Lund et al., 2002). Importantly, in GST pull-down experiments, the deletion of the AF-2 domain in SF-1 did not change the amount of SF-1 and SRC1 interaction, indicating a possible difference between FOS and SRC1 in the interacting region of SF-1 protein. Importantly, in GST pull-down experiments, the deletion of the AF-2 domain in the full-length SF-1 did not change the amount of SF-1 or SRC-1 required for interaction, indicating a possible difference between FOS and SRC1 in the interacting region of SF-1 protein. We proposed CBP and/or SRC1 as common limiting cofactors that could account for inhibition of SF-1-dependent gene expression by AP-1. It has been demonstrated that CBP interacts with SF-1 in the H295R cell model and that it potentiates SF-1-activated transcription of CYP11A1 (Monte et al., 1998). In the above-mentioned study the authors proposed that the interaction of SF-1 with CBP would trigger RNA-polymerase-II-dependent transcription and suggested that SF-1-mediated *CYP17* gene expression was also upregulated by the presence of CBP in the NCI-H295 model. Therefore, we investigated the possibility that FOS might prevent cofactors from binding to SF-1, which should lead to a decrease in its transcriptional activity. This is a valid possibility as it has been demonstrated that the composition of the AP-1 dimers plays an important role in determining the final effect on gene transcription as a consequence of a conformational change of the DNA after binding of AP-1 dimers (Kerppola and Curran, 1993; Kerppola and Curran, 1997). As a consequence, the JUN–FOS dimer might modify the conformation of the *CYP17* promoter in such a way that SF-1 bound to the three SF-1 sites cannot share cofactors or that an RNA polymerase complex cannot be formed.

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ChIP assays demonstrated unchanged levels of SF-1 to the *CYP17* promoter in the presence of both Ang II and TPA, whereas the same treatments decreased the amount of SRC1 and CBP that associated with SF-1, confirming a displacement of cofactors from SF-1 after increasing FOS levels in the cell. In co-transfection experiments, increasing the amounts of CBP and/or SRC1 was able to rescue SF-1-induced activation of *CYP17* transcription from the inhibition caused by the presence of AP-1 dimers.

In conclusion, we show for the first time the cellular pathway that is activated by Ang II and is responsible for the downregulation of CYP17 in the human adrenal cells. In this pathway, Ang II activates PKC, resulting in phosphorylation of ERK1/2, which leads to an increase in FOS levels. FOS interactions with SF-1 act to partially inhibit its induction of CYP17. Importantly, to support the hypothesis that a similar mechanism can be applied to other steroidogenic tissues, we recently published that FOS is involved in the inhibition of CYP17 expression in ovarian theca cells (Beshay et al., 2007). Taken together, these findings enhance our understanding of the mechanisms allowing steroidogenic tissues to tightly control the expression of steroid-metabolizing enzymes, thus allowing them to produce specific steroid hormones. Potentially, this novel mechanism for AP-1 transcription factors in the regulation of SF-1 can be applied to other SF-1 activated genes containing both AP-1 and SF-1 sites.

Materials and Methods

Cell culture

NCI-H295R (H295R) cells were cultured as previously reported (Nogueira et al., 2007). Cell monolayers were subcultured onto 100 mm culture dishes for ChIP assay and nuclear extracts (10×10^6 cells/plate), 30 mm dishes for protein or RNA extraction and siRNA experiments (4×10^6 cells/plate), and 12-well culture dishes for transfection experiments (4×10^5 cells/well). The plates were used for experiments 48 hours later.

Expression vectors

Expression vectors containing the full-length sequence for human *v-JUN* (GenBank acc. no. BC006175), *JUNB* (GenBank acc. no. BC004250) and *v-FOS* (GenBank acc. no. BC004490) were originally purchased from ATCC (Manassas, VA), and *JUND* (GenBank acc. no. NM_005354) plasmid was a gift from Dr Marcello Maggiolini (University of Calabria, Italy) (Vinciguerra et al., 2004). Coding sequences for the genes were excised from their original vectors and subcloned into pcDNA3.1 zeo (+) (Invitrogen, Carlsbad, CA). The coding sequence for *FOSB* (GenBank acc. no. NM_006732) was amplified in a PCR reaction using reverse-transcribed mRNA from H295R cells. Primers for the amplification were based on the published sequence of the *FOSB* gene: forward, 5′-TGTGCCCAGGGAAATGTTTCAGGC-3′ and reverse, 5′-AATCCTCCTGTCCGATGGCAGTGGC-3′, which produced a 1202 bp fragment. The PCR product was cloned into TOPO II vector (Invitrogen), digested with *Eco*RI and subcloned into pcDNA3.1 zeo (+). Human *SF-1* was provided by Dr Meera Ramayya (University of Washington, Seattle, WA) (Wong et al., 1996) and *SRC1* plasmid was provided by Dr Bert O'Malley (Baylor College of Medicine, Houston, TX) (Onate et al., 1995). Coding sequences for the *SF-1* and *SRC1* plasmids were excised from each vector and subcloned into the pcDNA3.1 zeo (+) expression plasmid.

Transfection assay

The preparation of promoter constructs containing the human *CYP17* wild type and with mutation of the three *SF-1* sites has been described previously (Hanley et al., 2001). The *AP-1* site was mutated using the Site-Directed Mutagenesis Kit (Stratagene) with the following set of primers: forward, 5′-GAGGTTTGCCC-TGGAGTTGAGC***TG***GCCCT-3′ and reverse, 5′-AGGGC***CA***GCTCAACTCC-AGGGCAAACCTC-3′, where the underlined bases indicate the *AP-1* site and bases in bold italic indicate the mutation (bases 1716–1744 of GenBank number M63871). *AP-1*-luciferase reporter plasmid was a gift from Dr Marcello Maggiolini (Vinciguerra et al., 2004). Transfections were carried out for 6 hours using the transfection reagent Transfast (Promega, Madison, WI). For co-transfection experiments, indicated amounts of expression plasmids were included in the transfection reaction, and the total amount of DNA was kept constant by addition of carrier DNA [empty expression vector, pcDNA3.1 zeo (+)]. To normalize luciferase activity, cells were co-transfected with 50 ng of pSV β-galactosidase control vector (Promega). Following transfection, cells were incubated with 2.0 ml low-serum medium for 18–24 hours to allow for recovery and expression of foreign DNA. Cells were then lysed and assayed for enzyme activity using the Luciferase Assay System (Promega) and β-Galatosidase Assay System (Tropix, Applied Biosystems, Foster City, CA).

Protein assay and western blot analysis

Cells were lysed in passive lysis buffer (Promega). Protein content and polyacrylamide gel electrophoresis were carried out on samples as previously reported (Beshay et al., 2005). Proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes by wet transfer for 1 hour at 25 V. Following transfer, membranes were incubated overnight at 4°C using antibody against phospho-c-FOS serine 375 (pSer375; Abcam, Cambridge, UK), phospho-c-FOS threonine 325 (pThr325; Abcam, Cambridge, UK), pERK (Cell Signaling Technology) or ERK (Cell Signaling Technology). To assure equal loading of nuclear proteins, membranes were stripped and incubated overnight at 4°C with antibody against GAPDH (Santa Cruz Biotechnology) at a 1:1000 dilution. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and immunoreactive bands were visualized with the ECL Western Blotting Detection System (Amersham Pharmacia Biotech).

Quantitative real-time PCR (qPCR)

RNA extraction and qPCR were performed as previous reported (Sirianni et al., 2005) Primers for the amplification were based on published sequences for the human *AP-1* and *CYP17* genes. The nucleotide sequences of the primers for *CYP17* have been previously published (Sirianni et al., 2005). PCR reactions and quantifications were performed as previously published (Sirianni et al., 2005). *AP-1* factor amplification was accomplished using the following primers: *JUN*, forward 5′-AGCTGGAGCGCCTGATAATC-3′ and reverse 5′-CTCCTGCTCATCTG-TCACGTTCT-3′ and *FOS*, forward 5′-AGGAGGGAGCTGACTGATACACT-3′ and reverse 5′-TTTCCTTCTCCTTCAGCAGGTT-3′.

Microarray analysis

RNA from H295R cells untreated (basal) or treated for 1 hour with TPA (10 nM) or Ang II (100 nM) were hybridized to an Affymetrix human HG-U133plus oligonucleotide two-microarray set containing more than 54,000 probe sets representing over 38,500 independent human genes (Affymetrix, Santa Clara, CA). The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000. Results were analyzed using GeneSpring version 6.1 software (Silicon Genetics, Redwood City, CA). Pure signal values were normalized using a list of 100 normalization control probe sets published by Affymetrix and used to identify genotypic differences between untreated and treated cells. Hierarchical clustering algorithms were used to determine steroidogenic gene expression patterns in the two treated samples.

Chromatin immunoprecipitation (ChIP)

This assay was performed using the ChIP assay kit from Upstate Technology (Lake Placid, NY) according to manufacturer's instructions. On day 1, confluent cultures were treated for 1 hour with 10 nM TPA, 100 nM Ang II or with PD98059 for 30 minutes before treatment with or without 10 nM TPA. Following treatment, DNA and protein complexes were crosslinked with 1% formaldehyde at 37°C for 10 minutes. Next, cells were collected and re-suspended in 400 μl of SDS lysis buffer and left on ice for 10 minutes before sonication. Of the supernatants, 10 μl were kept as input (starting material, to normalize results), while 100 μl were diluted 1:10 in 900 μl of ChIP dilution buffer and immunocleared with 80 μl of sonicated salmon sperm DNA protein A agarose for 6 hours at 4°C. Immunocomplex was formed using 2 μg of the specific antibodies anti-FOS (sc-253) and anti-JUN (sc-44) (Santa Cruz Biotechnology) and anti-SF-1 antisera (a gift from Ken-ichirou Morohashi, National Institute for Basic Biology, Okazaki, Japan) overnight at 4°C (day 1). Immunoprecipitation with salmon sperm DNA and protein A agarose was carried out at 4°C for 2 hours. DNA and protein complexes were reverse-crosslinked overnight at 65°C (day 2). Extracted DNA was resuspended in 20 μl of TE buffer (day 3). Samples immunoprecipitated with SF-1 were used for re-ChIP experiments. In this case, on day 2, instead of reverse crosslinking, samples were immunoprecipitated for a second time using anti-SRC1 or anti-CBP antibodies overnight at 4°C. On day 3, samples were reverse-crosslinked and on day 4 DNA was extracted. A 5 μl volume of each sample and input was used for qPCR using *CYP17* promoter primers: forward, 5′-CCTTTAAC-AGTCCCTGCTACTTG-3′ and reverse, 5′-GGGCACAAGGAGGCCTTTA-3′. PCR reactions were performed in the iCycler iQ Detection System (Biorad, Hercules, CA), using 0.1 μM of each primer in a total volume of 50 μL. SYBR Green Universal PCR Master Mix (Biorad) was used for gene amplification. Negative controls contained water instead of cDNA. Final results were calculated using the ΔΔCt method as explained in the real-time session, using the input Ct value to normalize the data and with the basal sample used as the calibrator.

Immunohistochemistry and immunofluorescence

Non-pathologic human adult adrenal tissues were retrieved from autopsy files of Tohoku University Hospital, Sendai, Japan. These tissues were fixed in 10% formalin and embedded in paraffin wax. Histological examinations revealed no significant pathologic abnormalities, including nodules or neoplasms. Review of the clinical history revealed that those patients had not received any form of adrenocortical steroids prior to their demise. A Histofine Kit (Nichirei, Tokyo, Japan) was used for

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immunohistochemistry. Antigen retrieval for FOS immunostaining was performed by heating the slides in an autoclave at 120°C for 5 minutes in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The rabbit polyclonal antibody for c-FOS (Ab-2) was purchased from Calbiochem (San Diego, CA), the rabbit polyclonal antibody for CYP17 has been described in detail previously (Sasano et al., 1989) and SF-1 antibody was purchased from Perseus Proteomics (Tokyo, Japan). For immunohistochemistry, tissues were blocked with normal goat serum followed by incubation overnight with rabbit anti-CYP17 antibody (1/2000) and rabbit anti-FOS antibody (Calbiochem, 1/500). Visualization of immunoreactivity employed the streptavidin-biotin amplification method. Briefly, the antigen-antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer pH 7.6 and 0.006% H_2O_2) and counterstained with hematoxylin. For a negative control, the sections were incubated with normal rabbit IgG instead of the primary antibodies and no specific immunoreactivity was detected in these sections. Double immunofluorescence was done after the use of a goat serum blocking step. Tissue sections were then incubated overnight with mouse anti-SF-1 antibody (1/200) and rabbit anti-FOS antibody (1/200). After the incubation with goat anti-rabbit IgG-Alexa Fluor 488 (Invitrogen) and goat anti-mouse IgG-Alexa Fluor 647 (Invitrogen), counterstain was performed by 4′-6-diamidino-2-phenylindole (DAPI) included in ProLong Gold antifade reagent (Invitrogen). The cellular expression and localization of SF-1 and FOS was observed with fluorescence microscope (DMRXA, Leica).

RNA interference

FOS SMARTpool siRNA and scrambled siRNA were purchased from Upstate Technology. Twenty-four hours after plating cells into 30 mm dishes at 4×10^6 cells, siRNAs were transfected to a final concentration of 100 nM using the RNAiFect Transfection Reagent (Qiagen Inc., Valencia, CA). *FOS*-specific knockdown was checked using real-time PCR on RNA extracted from cells transfected for 48 hours and then treated with TPA (10 nM) for 1 hour. *CYP17* mRNA levels were measured in samples transfected for 24 hours and then treated with TPA (10 nM) in low-serum medium for 24 hours.

Immunoprecipitation

Nuclear extracts were isolated as described above. For immunoprecipitation assays, nuclear proteins were incubated overnight at 4°C, with rotation, with anti-SF-1 antisera (a gift from Dr K. Morohashi, National Institute for Basic Biology, Okazaki, Japan) and protein-A-sepharose beads. The mixture was then centrifuged, and the supernatant discarded. Beads were washed three times and then used for western analysis as described above. Membranes were incubated overnight at 4°C with a monoclonal FOS antibody (sc-447; Santa Cruz).

GST pulldown assay

SF-1 full-length and deleted fragments (1515 bp, 858 bp, 507 bp) were obtained with PCR from the full-length gene (1570 bp) and subcloned into *Xba*I-*Eco*RI cloning sites of pGEX4T1 (Amersham). GST-SF-1 fusion proteins were transformed in *Escherichia coli* strain BL21(DE3) pLysS (Promega). Following induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 6 hours, cells were lysed in NENT buffer containing lysozyme, DNase and RNase. The soluble protein fraction was incubated overnight with glutathione-sepharose 4B resin at 4°C. GST fusion proteins bound to the resin were washed four times with ice-cold PBS. The concentration of purified proteins was determined spectroscopically and 100 μg were incubated overnight with 5 μl of in-vitro-synthesized FOS and SRC1 protein in 500 μl of NENT buffer. The resin was centrifuged to remove supernatant, washed three times with PBS and boiled for 5 minutes in 1×Laemmli sample buffer (Sigma). The released proteins were used for western analysis. Membranes were incubated overnight at 4°C with FOS antibody (sc-447; Santa Cruz) or with anti SRC1 antibody (sc-8995; Santa Cruz).

Data analysis and statistical methods

Results from a minimum of three independent experiments were analyzed using one-way ANOVA with Student-Newman-Keuls multiple-comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

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