The role of JUN in the regulation of PRKCC-mediated STAR expression and steroidogenesis in mouse Leydig cells

Pulak R Manna and Douglas M Stocco

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA

(Correspondence should be addressed to D M Stocco; Email: doug.stocco@ttuhsc.edu)

Abstract

Activator protein 1 (JUN) transcription factors (JUN and FOS) play critical roles in a wide variety of signaling processes including those in the protein kinase C (PRKCC) pathway, a pathway that is instrumental in the expression of the steroidogenic acute regulatory (STAR) protein. In the present study, we determined the functional involvement of one of the key JUN family members, JUN, in the regulation of PRKCC-dependent STAR expression and steroidogenesis. MA-10 mouse Leydig tumor cells treated with an activator of PRKCC, phorbol 12-myristate 13-acetate (PMA), demonstrated increases in the expression of the STAR and CYP11A1 proteins and progesterone synthesis, which coincided with the expression and phosphorylation of JUN (P-JUN). PMA was also capable of enhancing the cAMP analog, (Bu)₂cAMP, which stimulated JUN, STAR, P-STAR and progesterone levels. The induction of *Jun* mRNA expression and steroid synthesis by PMA requires *de novo* protein synthesis. Chromatin immunoprecipitation studies revealed the association of P-JUN with the STAR proximal promoter and that PMA specifically enhanced *in vivo* P-JUN–DNA interaction. Electrophoretic mobility shift assays and reporter gene analyses demonstrated that JUN binds to the JUN motif (–81/–75 bp) in the STAR promoter, and that JUN–DNA-binding activity was highly correlated with the induction of JUN by PRKCC signaling. Overexpression of JUN increased the PMA-mediated transcription of the *Star* gene, an event markedly decreased by TAM-67, a dominant negative mutant of JUN. Targeted silencing of endogenous JUN, by small interfering RNA, was correlated with the repression of basal- and PMA-mediated STAR expression and progesterone synthesis. These findings describe the mechanisms by which JUN influences PRKCC signaling and provide additional and novel insight into the regulation of the steroidogenic machinery in mouse Leydig cells.

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Introduction

The biosynthesis of steroid hormones is initiated upon mobilization of cholesterol from cellular stores to the mitochondrial inner membrane that is the site of cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) and is the rate-limiting and regulated step in steroidogenesis. The 37/30 kDa steroidogenic acute regulatory (STAR) protein mediates this crucial step and has been demonstrated to be an acute regulator of steroidogenesis in steroidogenic tissues (Clark *et al.* 1994, Lin *et al.* 1995, Stocco & Clark 1996, Christenson & Strauss 2000, Manna & Stocco 2005, Miller 2007). Transcriptional and/or translational inhibition of STAR expression has been shown to markedly decrease steroid biosynthesis, whereas ~10–15% of steroid synthesis is mediated by STAR-independent mechanisms (Lin *et al.* 1995, Clark *et al.* 1997, Manna *et al.* 2001, Manna & Stocco 2005). In the adrenals and gonads, while the cAMP/protein kinase A (PRKACA) pathway is the predominant signaling cascade involved in tropic hormone-stimulated STAR expression and steroid biosynthesis, PRKCC has also

been demonstrated to play a role in these processes (Stocco & Clark 1996, Jo *et al.* 2005, Manna & Stocco 2005, Manna *et al.* 2007). By itself, the effect of PRKCC on steroid synthesis is considerably less than that of PRKACA signaling; however, PRKCC can potentiate gonadotropin and/or cAMP-mediated steroidogenic responsiveness, and by doing so, undoubtedly functions in gonadal/adrenal steroidogenic processes reviewed in Refs Manna & Stocco (2005) and Stocco *et al.* (2005).

While the PRKCC signaling cascade has been demonstrated to be involved in influencing the steroidogenic response, there are conflicting reports in the literature that demonstrate either positive, negative, or no effects of PRKCC on steroidogenesis in different cell models reviewed in Ref. Stocco *et al.* (2005). We have demonstrated that activation of the PRKCC pathway dramatically increases STAR expression but only moderately elevates progesterone synthesis, and that the latter process is due to the inability of PRKCC to phosphorylate STAR (Jo *et al.* 2005, Stocco *et al.* 2005, Manna *et al.* 2006a,b), a requirement indispensable for optimal steroid synthesis (Arakane *et al.* 1997, LeHoux *et al.* 2004). Notably, while PRKCC, by itself, had no effect on

STAR phosphorylation, it greatly enhanced cAMP-stimulated phosphorylation of STAR at Ser194 and concurrently steroid synthesis in mouse Leydig cells (Jo *et al.* 2005, Manna *et al.* 2006b, 2007). However, those studies did not provide information on the regulatory mechanisms by which PRKCC signaling is evolved in STAR expression and steroidogenesis.

Mapping of STAR promoter sequences in mouse demonstrated the importance of the first 150 nucleotides (highly conserved in other species) in the transcriptional regulation of the *Star* gene (Manna *et al.* 2002a, 2003b, Hiroi *et al.* 2004, Clem *et al.* 2005). Within this region, we have identified a motif (5'-TGACTGA-3') that recognizes both the cAMP response element (CRE) and JUN (JUN and FOS) family proteins (Manna *et al.* 2002a, 2004). JUN (JUN, JUNB, and JUND) and FOS (FOS, FOSB, FOSL1, and FOSL2) belong to the basic leucine zipper (bZIP) group of transcription factors that bind to DNA as dimers. In particular, they bind to the sequence TGA (C/G)TCA, referred to as the tetradecanoylphorbol acetate response element (JUN/TRE), with distinct affinity and specificity, but exhibit varying degrees of transcriptional activity (O'Shea *et al.* 1992, Rutberg *et al.* 1997, Bakiri *et al.* 2002). The closely related CRE (TGACGTCA) is also a target for selective JUN/FOS dimers (Hai & Curran 1991, Rutberg *et al.* 1999, Manna & Stocco 2007). JUN and FOS are immediately response genes and are thought to function in coupling extracellular signals to phenotypic responses by influencing the expression of target genes. These factors are induced by a wide range of extracellular stimuli, including PRKCC and also by processes associated with mitogenesis, differentiation, and neuronal excitation (Abate *et al.* 1991, Karin *et al.* 1997, Li *et al.* 1997, Manna *et al.* 2002b, 2004). Furthermore, phosphorylation of JUN/FOS on Ser and Thr residues has been reported to be involved in the transcription of a number of genes (Abate *et al.* 1993, Chen *et al.* 2001, Manna *et al.* 2006b, Manna & Stocco 2007). Utilizing MA-10 mouse Leydig tumor cells, a cell line possessing many characteristics of its normal counterparts, as an experimental model, the present findings provide for the first time evidence that JUN binds, both *in vivo* and *in vitro*, to the JUN motif in the STAR promoter where it is effective in regulating PRKCC-mediated STAR expression and steroidogenesis.

Material and methods

Plasmids, transfections, and luciferase assays

The -151/-1 (JUN Wt) and -151/-1 JUN mutant (JUN Mut) STAR promoters were constructed and

subcloned into the pGL3 basic vector (Promega) that contains the firefly luciferase gene as a reporter (Manna *et al.* 2002a, 2004). The wild type JUN, dominant negative JUN (TAM-67), and pRL-SV40 expression plasmids have been previously described (Manna *et al.* 2004, 2006b). MA-10 cells (Ascoli 1981) were transfected using FuGENE HD-transfection reagent (Roche Diagnostics Corp.), under optimized conditions (Manna *et al.* 2004, Manna & Stocco 2007). Briefly, the -151/-1 STAR (wild type and mutant) reporters were transfected with different expression plasmids (1:1). Transfection with small interfering RNAs (siRNAs) was performed using X-tremeGENE siRNA transfection reagent (Roche Diagnostics Corp.), as described previously (Dyson *et al.* 2008). Silencer negative control siRNA and the JUN siRNAs (#1, 5'-CAGUAAACCCUAAGAUCUA-3', and #2, 5'-CCAA-GUGCCGAAAAGGAA-3') were obtained as annealed oligos from Ambion Inc., (Austin, TX, USA).

Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay system (Promega), using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA), as described previously (Manna *et al.* 2002a, 2003a, Manna & Stocco 2007).

Immunoblotting

Western blot analyses were carried out with total cellular protein (Manna *et al.* 2006a, 2007). Briefly, equal amounts of protein (20–30 µg) were loaded on either 10 or 12% SDS-PAGE (Mini Protean II System, Bio-Rad Laboratories Inc). The proteins were electrophoretically transferred onto Immuno-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad), which were probed with the specific antibodies (Abs) that recognize JUN (sc-45, 1:2000), JUNB (sc-8051, 1:1000), JUND (sc-74, 1:1000), FOS (sc-8047, 1:2000), FOSB (sc-48, 1:1000), FOSL1 (sc-183, 1:1000), FOSL2 (sc-171, 1:1000), actin (sc-7210, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), P-JUN (Ser⁶³, 1:1500; Cell Signaling Technology Inc., Beverly, MA, USA), STAR (1:20 000; Bose *et al.* 1999), P-STAR (Ser¹⁹⁴, 1:10 000; Manna *et al.* 2006b), and CYP11A1 (1:5000; Chemicon International Inc., Temecula, CA, USA). Horseradish peroxidase-conjugated secondary Abs against rabbit, mouse, or goat IgG were used to recognize relevant primary Abs. Immunodetection of different proteins was determined with the Chemiluminescence Imaging Western Lightning Kit (Perkin-Elmer, Boston, MA, USA). The membranes were exposed to X-ray films (Marsh Bio Products Inc., Rochester, NY, USA), and the intensity of immunospecific bands was quantified using a computer-assisted image analyzer (Visage 2000, BioImage, Ann Arbor, MI, USA). All western blotting experiments were repeated three to five times.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were carried out with MA-10 nuclear extracts (NE) and *in vitro* transcribed/translated JUN (Manna *et al.* 2002a, 2003a, 2004). The sense strands of the oligonucleotide sequences used were the following: -83/-67 wild type, 5'-GGAATGACTGATGACTTTT-3'; -83/-67 mutant (-83/-67 M), 5'-GGAATAGATCTTGACTTTT-3'; consensus JUN (Promega), 5'-CGCTTGATGAGTCAGCCGAA-3'. The double-stranded oligonucleotide was end-labeled with [α -³²P]-dCTP (Perkin-Elmer) using Klenow fill-in reaction, and DNA-protein-binding assays were carried out under optimized conditions (Manna *et al.* 2002a, 2004). Briefly, NE (12 μ g) and *in vitro* translated JUN (2–5 μ g) were incubated in 20 μ l reaction buffer (10 mM HEPES, 1 mM EDTA, 4% Ficoll, 10 mM dithiothreitol, 1 μ g poly dIdC, 40 mg/ml BSA, and 2 μ M ZnSO₄, pH 7.9) for 15 min at room temperature. The ³²P-labeled DNA probe was added alone or in the presence of 100-fold molar excess of unlabeled sequence, and incubation was continued for another 15 min. When JUN (sc-45) and FOS (sc-8047) Abs were used, binding reactions were incubated on ice for 45 min prior to the addition of ³²P-labeled DNA. Binding reaction was then subjected to electrophoresis on 5% polyacrylamide gels, which were dried, and the complexes were visualized using a phosphor imaging device (Molecular Dynamics, Sunnyvale, CA, USA). These experiments were repeated at least three times.

Quantitative reverse transcription and PCR (RT-PCR)

The isolation and amplification of mouse STAR (Clark *et al.* 1994) and JUN (GenBank accession number NM_010591) cDNAs were carried out using total RNA, as described previously (Manna *et al.* 1999, 2007). Briefly, the primer pairs used were: STAR sense, 5'-GACCTTGAAAGGCTCAGGAAGAAC-3', and STAR antisense, 5'-TAGCTGAAGATGGACAGACTTGC-3'; JUN sense, 5'-GCTAACCCCGTGAAGTGACGG-3' and JUN antisense, 5'-GTAGACCGGAGGCTCACTGTG-3'. The variation in RT-PCR efficiency was assessed with an L19 ribosomal protein gene using the following primer pairs: L19 sense 5'-GAAATCGCCAATGCCAACTC-3' and L19 antisense 5'-TCTTAGACCTGCGAGCCTCA-3' (Chan *et al.* 1987). RT and PCR were run sequentially in the same assay tube using 2 μ g total RNA. The cDNAs generated were further amplified by PCR using the primer pairs listed above. The molecular sizes of STAR, JUN, and L19 were determined on 1.2% agarose gels, which were then vacuum dried, exposed to X-ray film (Marsh Bio Products), and the levels of different signals were quantified using a computer-assisted image analyzer (Visage 2000).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using a kit (Upstate/Chemicon) following the manufacturer's instructions, under optimized conditions (Manna & Stocco 2007). Briefly, cells were incubated with formaldehyde to cross-link DNA and its associated proteins and sonicated in lysis buffer. Following centrifugation, the supernatant was immunoprecipitated with either P-JUN Ab (as above) or IgG for 14 h at 4 °C. The chromatin-antibody-protein A agarose complexes were washed subsequently with low salt, high salt, LiCl, and Tris/EDTA buffers. Protein-DNA complexes were eluted with freshly made elution buffer (1% SDS, 0.1 M NaHCO₃), and then the eluted materials were incubated at 65 °C for 4 h to reverse the formaldehyde cross-linking. The resulting samples were treated with 0.5 M EDTA, 1 M Tris-HCl (pH 6.5), and 10 mg/ml proteinase K (10 mg/ml) for 1 h at 45 °C, and the purified DNA samples were used for PCR using [α -³²P]-dCTP in the dNTP mixture (Manna & Stocco 2007). PCR was performed with ~100 ng of DNA and the proximal mouse STAR promoter primers: sense, 5'-CTGGTCTCCCTTTACACAGTC-3', and antisense, 5'-GGCGCAGATCCAGTGCCTGC-3' (Caron *et al.* 1997, Manna & Stocco 2007). PCR products were determined on 2% agarose gels. Gels were vacuum dried, exposed to X-ray film (Marsh Bio Products), and the resulting signals were visualized by autoradiography. These experiments were repeated four times.

JUN PathDetect transreporting assay

MA-10 cells were transfected with the pathway-specific pFA2-JUN transactivator plasmid (Stratagene, La Jolla, CA, USA), in the presence of a pFR-Luc reporter, using FuGENE HD-transfection reagent (Roche Diagnostics Corp.), as described previously (Manna *et al.* 2006a,b). The transactivator protein consists of the activation domain of JUN (aa1-223) fused with the LGALS4 DNA-binding domain (aa1-147). The pFR-Luc plasmid contains a synthetic promoter of the yeast LGALS4-binding sites that control the expression of the firefly luciferase gene. Cells were also co-transfected with JUN and TAM-67 to determine the functional specificity of JUN. Following 36 h of transfection, cells were pre-treated without or with GF-10923X (GFX) and then treated with phorbol 12-myristate 13-acetate (PMA) for an additional 6 h, and luciferase activity in the cell lysates was determined using a TD 20/20 Luminometer (Turner Designs).

Statistical analysis

All experiments were repeated three to seven times. Statistical analysis was performed by ANOVA using

Statview (Abacus Concepts Inc., Berkeley, CA, USA) followed by Fisher's protected least significant differences test. Data represent the mean $\pm$ S.E.M. and $P < 0.05$ was considered significant.

Results

Effects of PMA on JUN and FOS expression, steroidogenesis, and on the JUN PathDetect assay

The function of JUN on the PRKCC-mediated regulation of STAR expression and steroidogenesis was investigated using MA-10 cells. As illustrated in Fig. 1A, cells treated with PMA (10 nM) for 6 h demonstrated a 3.1 ± 0.5 -fold increase in the expression of JUN protein, when compared with untreated cells. Inhibition of PRKCC activity by GF-10923X (GFX; 20 μ M, (Jo *et al.* 2005, Manna *et al.* 2006b, 2007)) abolished PMA-induced JUN expression. Under similar experimental paradigms, while PMA induced a pronounced increase in JUN expression, it had varying effects, albeit lesser ones, on the protein levels of other JUN and FOS family members. In particular, PMA showed little to no effects on JUNB, FOSB, and FOSL2 levels but increased ($P < 0.05$) JUND, FOS, and FOSL1 expression, when compared with their respective controls. On the other hand, treatment with PMA showed a 5.6 ± 1.2 -fold increase in progesterone synthesis over basal levels (1.7 ± 0.3 ng/mg protein), a response that was attenuated $\sim 75\%$ by GFX (Fig. 1B). GFX alone had no apparent effects on either JUN and FOS expression or progesterone synthesis. The induction of JUN expression and steroid synthesis by PMA occurred maximally between 4 and 8 h, decreased thereafter (8–12 h), and was further decreased ($P < 0.01$) at 24 h, but was still higher than controls (data not shown). These findings suggest that among the different JUN and FOS members, JUN plays a predominant role in PRKCC-mediated steroid synthesis.

The involvement of PRKCC-responsive JUN expression was further verified using the pathway-specific transreporting assay. Cells transfected with the pFA2-JUN transactivator plasmid demonstrated 1.9 ± 0.3 - and 3.7 ± 0.8 -fold increases in luciferase activity over basal in response to 5 and 10 nM PMA respectively (Fig. 2). GFX in combination with PMA strongly decreased ($P < 0.01$) PMA-induced responsiveness. Co-expression of pFA2-JUN and TAM-67 (a dominant negative mutant of JUN) inhibited basal- and PMA-mediated luciferase activities by 55–70%, clearly demonstrating the importance of JUN in the PRKCC signaling pathway.

To ascertain the involvement of JUN in PRKCC signaling, expression of JUN and its post-translational

Figure 1 Effects of PMA on the expression of JUN and FOS proteins and steroid synthesis. (A) MA-10 cells were pretreated without or with GFX (20 μ M) for 30 min, and then treated in the absence or presence of PMA (10 nM) for an additional 6 h. Expression of different JUN and FOS proteins was determined by western blotting using 25 μ g of total protein. Immunoblots shown are representative of three to five independent experiments. Actin expression was assessed as a loading control. Accumulation of progesterone in the media of the same treatment groups was determined and expressed as ng/mg protein, which represent the mean $\pm$ S.E.M. of five independent experiments (B). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

Figure 2 Role of PMA on JUN PathDetect transreporting assay. MA-10 cells were transfected with the pathway-specific pFA2-JUN transactivator plasmid in the presence of pFR-Luc reporter, as described in Materials and methods. The pRL-SV40 was included in the transfection to normalize transfection efficiency. The specificity of JUN responsiveness was evaluated by co-expressing TAM-67 (a dominant negative mutant of JUN). Following 36 h of transfection, cells were pretreated for 30 min without or with GFX (20 μM) and then incubated with two different doses of PMA (5 and 10 nM) for an additional 6 h. Luciferase activity in the cell lysates was determined ($\pm$ S.E.M., $n=7$) and expressed as relative light units (RLU; luciferase/renilla). Letters above the bars indicate that these groups differ significantly from each other at least at $P<0.05$.

modification following phosphorylation were evaluated. Cells treated with PMA (10 nM) showed 2.9- and 3.2 ± 0.5 -fold increases in JUN expression and JUN phosphorylation (P-JUN, Ser⁶³) over untreated cells respectively, demonstrating that the increases in JUN expression and its phosphorylation in response to PMA were similar (Fig. 3A and B). While GFX abrogated PMA-induced JUN and P-JUN levels, a PRKACA inhibitor H-89 (20 μM, (Jo *et al.* 2005, Manna *et al.* 2006b, 2007)) had no effects on these parameters, suggesting the specificity of JUN in PRKCC signaling. Likewise, PMA demonstrated an 8.3 ± 0.9 -fold increase in STAR protein expression over basal. Notably, while H-89 had no effects on JUN and P-JUN levels, both GFX and H-89 attenuated ($P<0.01$) PMA-responsive STAR expression and progesterone (not illustrated) synthesis, suggesting an involvement of signaling crosstalk between PRKACA and PRKCC in the steroidogenic response (Fig. 3A and B). Consistent with our previous findings, phosphorylation of STAR (Ser¹⁹⁴, P-STAR) was undetectable in any of these treatments (data not shown; Jo *et al.* 2005, Manna *et al.* 2006b, 2007).

To better understand signaling events, the effects of PRKCC on JUN, STAR, CYP11A1, and steroid levels were determined. Cells treated with PMA (0–50 nM) demonstrated increases in the expression of JUN, STAR, CYP11A1, and progesterone levels in a concentration-dependent manner (Fig. 3C). The magnitude of responses at 10 nM PMA was ~ 2.7 -, 8.7 -, 1.9 -, and 5.3 -fold on JUN, STAR, CYP11A1 proteins, and progesterone synthesis, when compared with their respective controls. The lower than expected fold increase ($P<0.05$) in the level of CYP11A1 protein observed in response to PMA was in all likelihood a result of its higher basal expression (Ilvesmaki & Voutilainen 1991, Bird *et al.* 1995), which may be critical in producing the increased level of progesterone observed in PMA-treated cells. These findings also indicate that the unexpectedly small increase in progesterone production associated with the marked induction of STAR expression by PMA was due to the lack of STAR phosphorylation.

In additional studies, the influence of PMA on (Bu)₂cAMP-stimulated JUN expression and steroidogenesis was studied, since PRKCC has been demonstrated to enhance PRKACA responsiveness in different steroidogenic cells (Tilly & Johnson 1989, Jo *et al.* 2005, Manna *et al.* 2006a,b, 2007). MA-10 cells treated with PMA (10 nM) for 6 h demonstrated a 2.8-fold increase in JUN expression over untreated cells; however, its level was unaffected in response to a suboptimal dose (0.1 mM) of (Bu)₂cAMP (Fig. 4A). The concentration of (Bu)₂cAMP chosen was based on previous findings; however, it is noted that (Bu)₂cAMP (0.5/1.0 mM) was shown to have no effect on JUN expression but it attenuated P-JUN level below controls at 6 h (Clem *et al.* 2005, Jo *et al.* 2005, Manna *et al.* 2007, Manna & Stocco 2007). Addition of PMA to (Bu)₂cAMP-treated cells produced a 1.8 ± 0.3 increase in JUN expression over the response seen with PMA alone, suggesting the involvement of additional factor(s) in this process since (Bu)₂cAMP has no effect on JUN expression. On the other hand, both PMA and (Bu)₂cAMP alone increased STAR expression and progesterone synthesis over their respective basal levels. However, cells treated with PMA and (Bu)₂cAMP together dramatically augmented STAR, P-STAR, and progesterone levels when compared with their responses individually (Fig. 4A and B). The combined effects of PMA and (Bu)₂cAMP on JUN, STAR, P-STAR, and progesterone levels were significantly affected ($P<0.05$) both by GFX and H-89. Whereas these inhibitors markedly attenuated STAR expression and steroid synthesis when the cells were co-treated with PMA and (Bu)₂cAMP, the effect of H-89 was more pronounced than GFX, indicating that intracellular cAMP plays an important role in regulating PRKCC/PRKACA-dependent steroidogenesis.

Induction of PRKCC-mediated JUN expression and steroid synthesis requires *de novo* protein synthesis

The effect of PRKCC on JUN expression and its relevance to steroid synthesis was studied for its dependence on protein synthesis. As depicted in Fig. 5, MA-10 cells treated with PMA (10 nM) showed a 3.5 ± 1.1 -fold increase in *Jun* mRNA expression. Addition of the protein synthesis inhibitor, cycloheximide (Chx, 5 μ M), to PMA-treated cells significantly inhibited ($P < 0.01$) *Jun* gene expression (Fig. 5A and B). Chx alone had no effect on *Jun* mRNA expression. Progesterone production in the media increased 5.8-fold in response to PMA and this response was decreased $\sim 80\%$ by Chx (Fig. 5B). These results demonstrate a link between JUN and steroid synthesis and indicate that the induction of PMA-mediated JUN expression and steroid synthesis is dependent on translation in these cells.

Interaction of JUN with the JUN motif in the STAR promoter and its consequences on *Star* gene expression

To understand molecular events, the involvement of JUN in PRKCC-mediated regulation of the steroidogenic response was assessed using ChIP assays. It can be clearly seen from Fig. 6A that cells treated with varying doses of PMA (0–50 nM) demonstrated increases in P-JUN association with the STAR proximal promoter in a dose-responsive manner, reaching a maximum of 2.7 ± 0.4 -fold at 10 nM, decreasing slightly at 50 nM, but remaining elevated over control. Figure 6B demonstrated that the induction of PMA-mediated association of P-JUN with the STAR promoter was markedly decreased ($P < 0.01$) by GFX. No signals were observed using DNA recovered from IgG-treated groups. These findings document the physical interaction of

Figure 3 Effects of PMA on JUN, P-JUN, STAR, CYP11A1, and steroid levels. MA-10 cells were pretreated without or with GFX (20 μ M) and H-89 (20 μ M) for 30 min, and then treated in the absence or presence of PMA (10 nM) for a further 6 h, as indicated. Expression of JUN, P-JUN, and STAR in the cell lysates (22 μ g of protein) was determined by western blotting (A). Immunoblots shown are representative of three to four independent experiments. Integrated optical density (IOD) values of each band were quantified and compiled data ($\pm$ S.E.M., $n=3$) are presented (B). Cells were also treated with increasing doses of PMA (0–50 nM) for 6 h, and levels of JUN, STAR, and CYP11A1 were determined by immunoblotting using 25 μ g of total protein (C). Actin expression (A and C) was assessed as loading controls. Progesterone synthesis in the media of the same treatment groups was determined and expressed as ng/mg protein, which represent the mean $\pm$ S.E. of three independent experiments (C). Letters above the bars indicate that these groups differ significantly from each other at least at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus respective controls.

Figure 4 Effects of PMA and (Bu)₂cAMP on the expression of JUN, STAR, P-STAR, and progesterone levels in MA-10 cells. Cells were pretreated either with GFX (20 μ M) or H-89 (20 μ M) for 30 min, and then treated in the absence or presence of PMA (10 nM), (Bu)₂cAMP (0.1 mM), or a combination of both for a further 6 h, as indicated (A). These experiments were repeated four times, and representative immunoblots illustrate JUN, STAR, and P-STAR levels using 25 μ g total protein. Actin expression was assessed as a loading control. (B) Accumulation of progesterone in the media of the same treatment groups was determined and expressed as ng/mg protein ($\pm$ S.E.M., $n=4$). Letters above the bars indicate that these groups differ significantly from each other at least at $P<0.05$.

P-JUN-DNA with the STAR promoter following the activation of PRKCC signaling.

To obtain more insight into these mechanisms, EMSA DNA-protein binding was carried out using MA-10 NE and *in vitro* transcribed/translated JUN. As shown in Fig. 7A, a ³²P-labeled probe (–83/–67) encompassing the JUN region of the STAR promoter resulted in the formation of two DNA-protein complexes (I and II) with untreated MA-10 NE (lane 2). PMA (10 nM) treatment further increased DNA-protein complex formation (compare lanes 2 and 3). DNA-protein binding was strongly inhibited by its unlabeled sequence (lane 4). An Ab that recognizes

Figure 5 Induction of PMA-mediated *Jun* mRNA expression and progesterone production requires ongoing protein synthesis. MA-10 cells were treated (in duplicates) without (Con) or with PMA (10 nM), PMA plus cycloheximide (Chx; 5 μ M), and Chx alone for 6 h, and subjected to *Jun* mRNA expression by RT-PCR analysis (A). A representative autoradiogram illustrates the expression of *Jun* and L19 mRNAs in different treatment groups using 2 μ g total RNA. IOD values of each band were quantified and normalized to the corresponding L19 bands (B). Accumulation of progesterone in the media was determined and expressed as ng/mg protein (B). Results represent the mean $\pm$ S.E.M. of four independent experiments. Note the different scales on the graph. * $P<0.05$; ** $P<0.01$ versus respective controls.

JUN in immunoblots diminished DNA-protein complexes (lane 6), suggesting that MA-10 NE binding to the –83/–67 region is predominantly JUN protein. JUN proteins heterodimerize with FOS members, and FOS is reported to be the best-known partner of JUN (Angel & Karin 1991). FOS Ab showed a decrease ($P<0.05$) in DNA-protein binding (lane 5). In additional experiments (Fig. 7B), the –83/–67 probe illustrated the presence of a specific DNA-protein complex (I) with *in vitro* translated JUN (lanes 2–6) and that the binding was increased with a higher amount of JUN (compare lanes 2 and 3). Unlabeled consensus JUN (Con JUN, lane 4) and JUN Ab (lane 5) nearly abolished formation of the DNA-protein complex. Binding was not affected with an unlabeled sequence containing JUN mutant (–83/–67 M, lane 6), suggesting that JUN binds to the JUN region of the STAR promoter and plays an important role in regulating *Star* gene transcription.

Figure 6 Effect of PMA on the association of JUN with the STAR promoter. MA-10 cells were pretreated without or with GFX (20 μ M) for 30 min and then incubated with either increasing (0–50 nM; A) or a fixed dose (10 nM; B) of PMA for 6 h as indicated, and ChIP assays were carried out as described in Materials and methods. Cross-linked sheared chromatin obtained from control (Con) and PMA treatments were immunoprecipitated (IP) with anti-P-JUN or IgG. Recovered chromatin was subjected to PCR analysis using primers (–170/+1 bp) encompassing the JUN region of the STAR promoter. Autoradiograms shown are representative of three to five independent experiments.

The functional relevance of JUN in PRKCC-mediated STAR expression and steroid synthesis

The impact that JUN has on PRKCC-mediated STAR expression and steroidogenesis was further elucidated utilizing overexpression and silencing approaches. MA-10 cells transfected with the –151/+1 bp STAR reporter plasmid (JUN Wt) displayed a 1.9 ± 0.3 -fold increase in luciferase activity in response to PMA (Fig. 8A). This segment was chosen based on our previous studies (Manna *et al.* 2002a, 2004). Disruption of the JUN element (JUN Mut) resulted in an $\sim 50\%$ decrease in STAR reporter activity, suggesting the importance of the JUN motif in PRKCC-mediated STAR promoter responsiveness. Cells transfected with the JUN expression plasmid (JUN), within the context of the –151/+1 bp STAR fragment, demonstrated an additional 2.4 ± 0.5 -fold increase in luciferase activity over the response seen with PMA in mock-transfected cells (Fig. 8B). GFX markedly decreased PMA-induced STAR reporter activity. The transactivation potential of JUN in PMA-mediated STAR reporter activity was strongly attenuated by TAM-67, demonstrating that JUN plays a key role in PRKCC-dependent transcription of the *Star* gene.

The results obtained with JUN overexpression were further corroborated with endogenous JUN knock-down studies. As illustrated with western blot (Fig. 9A), and RT-PCR (Fig. 9B), analyses, MA-10 cells transfected with 100 nM of either of the two JUN-specific siRNAs (JUN siRNA #1 and #2) decreased basal JUN protein and *Jun* mRNA levels between 75 and 95%, and concomitantly inhibited PMA-induced responsiveness when compared with a silencer negative control siRNA (Con siRNA). *Star* mRNA expression (Fig. 9B) and progesterone synthesis (Fig. 9C) under basal and PMA-stimulated conditions followed similar patterns to those of JUN levels, and were decreased by 45–55%. Collectively, these findings demonstrate that JUN binds to the JUN motif in the STAR promoter and mediates PRKCC-mediated steroid synthesis by controlling the transcription of the *Star* gene in mouse Leydig cells.

Discussion

Regulation of steroid biosynthesis in Leydig cells is largely mediated through the interaction of LH/hCG to its specific receptor, which results in several intracellular modifications including the activation of cAMP-dependent PRKACA and the phosphorylation of proteins. Ligand–receptor interaction also stimulates phospholipase C and triggers the activation of the downstream PRKCC pathway that has been demonstrated to be involved in regulating STAR expression and steroid synthesis in Leydig cells (Jo *et al.* 2005, Manna & Stocco 2005, Stocco *et al.* 2005). The present studies extend these observations by elucidating the molecular events in which the JUN transcription factor JUN acts to drive PRKCC-dependent STAR expression and steroidogenesis in mouse Leydig cells.

Our findings demonstrate that the activation of PRKCC signaling by PMA increases the expression and phosphorylation of JUN, events that are in close coordination with the expression of STAR and CYP11A1 and the synthesis of progesterone. Induction of *Jun* gene expression and steroid synthesis in response to PRKCC was effectively inhibited by cycloheximide, indicating a requirement for *de novo* protein synthesis. Inhibition of PRKCC, but not PRKACA, essentially abolished JUN/P-JUN expression, indicating the specificity of JUN in the PRKCC signaling pathway. However, PRKCC is a weak inducer of progesterone synthesis in MA-10 cells, an observation in agreement with previous findings that demonstrated moderate effects of PRKCC on steroid synthesis and on the expression of several steroidogenic enzymes, including CYP11A1 in different steroidogenic cells (Chaudhary & Stocco 1988, Ilvesmaki & Voutilainen 1991, Bird *et al.* 1995, Jo *et al.* 2005, Manna *et al.* 2006b, 2007). We have previously reported

Figure 7 Binding of the JUN region in the STAR promoter to MA-10 NE and to *in vitro* translated JUN, using EMSAs. NE (12 μg) obtained from control (lane 2) and PMA (10 nM) treated (lanes 3–6) MA-10 cells were incubated with the ³²P-labeled probe specific to the JUN region (–83/–67) of the STAR promoter (A). DNA–protein complexes (labeled as I and II) were challenged with its unlabeled sequences (lane 4), FOS Ab (lane 5), and JUN Ab (lane 6). Binding of *in vitro* translated JUN (lanes 2–6) was also evaluated with the –83/–67 probe (B). DNA–protein complex (labeled as I) was challenged with unlabeled consensus JUN (Con JUN, lane 4), JUN Ab (lane 5), and mutant JUN (–83/–67 M, lane 6) sequences. Cold competitors were used at 100-fold molar excess. Migration of free probes is shown. These experiments were repeated three to four times, and representative phosphor images from each group are illustrated.

that steroid synthesis elicited by PRKCC is compromised by the obligatory need for PRKACA to phosphorylate and activate STAR in mouse Leydig cells (Jo *et al.* 2005, Manna *et al.* 2006b, 2007). In addition to stimulation of steroidogenesis by PRKCC, inhibitory effects of PRKCC on gonadal and adrenal steroid biosynthesis have also been demonstrated reviewed in Ref. Stocco *et al.* (2005). The differential effects of PRKCC in steroidogenesis thus appear to depend on tissue specificity, ligand concentrations, receptor–ligand interactions, and the short- or long-term experimental conditions.

Whereas the inhibition of PRKACA by H-89 was found to have no effects on PRKCC-mediated JUN and

P-JUN levels, it markedly affected STAR, P-STAR, and steroid synthesis. PMA does not elevate intracellular cAMP (Chaudhary & Stocco 1988, Muller *et al.* 1998); therefore, the repression of PRKCC-mediated STAR expression and steroid synthesis by a PRKACA inhibitor strongly indicates the involvement of crosstalk in these signaling pathways. We demonstrate that PMA is capable of potentiating (Bu)₂cAMP-induced JUN, STAR, P-STAR, and steroid levels, which reinforces the probability that PRKCC potentiates the activity of PRKACA that is involved in regulating steroidogenesis (Tilly & Johnson 1989, Jo *et al.* 2005, Manna *et al.* 2006a,b, 2007). The observation that inhibition of PRKACA activity decreased the steroidogenic response

Figure 8 Functional involvement of the JUN element on *Star* gene expression. (A) MA-10 cells were transfected with either the –151/+1 bp STAR reporter segment (JUN Wt) or the –151/+1 containing a mutation in the JUN Mut, in the presence of pRL-SV40 (renilla/luciferase for determining transfection efficiency). pGL3 basic (pGL3) was used as a control. Utilizing the –151/+1 STAR reporter segment, cells were also transfected with empty vector (pcDNA3), wild type JUN, or JUN plus TAM-67 as indicated, in the presence of pRL-SV40 (B). Following 36 h of transfection, cells were pretreated without or with GFX (20 μ M) for 30 min, and then incubated in the absence (basal) or presence of PMA (10 nM) for a further 6 h. Luciferase activity in the cell lysates was determined and expressed as RLU (luciferase/renilla). Results represent the mean $\pm$ S.E. of five independent experiments. Letters above the bars indicate that these groups differ significantly from each other at least at $P < 0.05$.

mediated by PMA could indicate that the synergistic effects of PMA and (Bu)₂cAMP on STAR, P-STAR, and steroid levels require the involvement of endogenous cAMP (Jo *et al.* 2005, Manna *et al.* 2006b, 2007). On the other hand, further increases in PMA-mediated JUN

Figure 9 Silencing of JUN and its relevance to STAR expression and progesterone synthesis. MA-10 cells were transfected with either a negative control siRNA (Con siRNA) or two JUN-specific siRNAs (JUN siRNA #1 & #2) at 100 nM concentrations, as described in Materials and methods. Following 40 h of transfection, cells were treated without (basal) or with PMA (10 nM) for an additional 6 h, and subjected to western blot (A) and RT-PCR (B) analyses. Representative immunoblots and autoradiogram illustrate the expression of JUN and actin (western blot) and JUN, STAR, and L19 (RT-PCR) in different treatment groups respectively. Actin and L19 were used as loading controls. Similar results were obtained from three independent experiments. (C) Accumulation of progesterone in the media of the same treatment groups was determined ($\pm$ S.E.M., $n=3$). Letters above the bars indicate that these groups differ significantly from each other at least at $P < 0.05$.

levels in the presence of (Bu)₂cAMP suggests that additional factor(s) may be involved in these signaling pathways, since the latter had no effect on JUN expression. Previously, we have demonstrated that cAMP-dependent factors, while unable to enhance intracellular cAMP by themselves, are capable of

enhancing hCG-mediated cAMP levels in mouse Leydig cells (Manna *et al.* 2006a,b). The role of PRKACA in ongoing steroid synthesis has been demonstrated by using different PRKACA inhibitors and speculated on a mechanism for the crosstalk between different signaling pathways in steroidogenic cells via guanine nucleotide exchange factors (Chin & Abayasekara 2004). Nonetheless, PMA has been demonstrated to decrease the expression of a negative regulatory factor DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1), and simultaneously increase extracellular signal-regulated kinase 1/2 and CREB1 signaling, situations that have positive effects on STAR and steroid synthesis (Jo *et al.* 2005, Manna *et al.* 2006a,b, 2007). Therefore, a balance between the repressor and inducer functions generated through PRKCC activity may allow for fine tuning the steroidogenic machinery in mouse Leydig cells.

The previous identification of a JUN element (Manna *et al.* 2004) in the cAMP-responsive region of the mouse STAR promoter provided the opportunity to delineate the relevance of JUN in PRKCC-mediated *Star* gene expression. We demonstrate that the disruption of the JUN-binding site resulted in the repression of basal and PMA-dependent *Star* gene expression, which reinforces the involvement of JUN and FOS in the transcription of the *Star* gene in previous findings (Manna *et al.* 2002b, 2004, Shea-Eaton *et al.* 2002, Jo *et al.* 2005, Manna & Stocco 2007). Nevertheless, an oligonucleotide probe encompassing the JUN motif can specifically bind MA-10 NE in a PRKCC-responsive manner as well as *in vitro* translated JUN, and that DNA-protein binding was affected by mutations in the JUN site, JUN Ab, and consensus JUN. It is noteworthy that the JUN motif in the STAR promoter is analogous to a CRE-like element that interacts with CRE-DNA-binding proteins (Manna *et al.* 2002a, 2003a). Similar overlapping sequences have been shown to target the CCAAT/enhancer-binding protein β (Silverman *et al.* 1999, 2006). Therefore, three families of proteins can bind to this region, a phenomenon that is perhaps not surprising since they share bZIP motifs and possess high degrees of homology in their DNA-binding domains. Members of these families can form selective dimers depending on the dimer composition and can alter their DNA-binding specificities in diverse physiological responses (Benbrook & Jones 1990, Hai & Curran 1991, Rutberg *et al.* 1999, Manna & Stocco 2007). Consistent with this, the binding of MA-10 NE to the JUN motif was inhibited by FOS Ab, indicating the prevalence of cross-family dimerization (Manna *et al.* 2004). Moreover, JUN-DNA-binding activity correlated with the induction of JUN/P-JUN in response to PRKCC. *In vivo* ChIP data revealed that PMA increased the association of P-JUN with the STAR proximal

promoter. We and others have shown that treatment with cAMP analog increases the binding of several transcription factors, including JUN/P-JUN, to the proximal but not the distal, STAR promoter, that occurs concurrently with the co-regulator CREB1-binding protein (CBP; Hiroi *et al.* 2004, Clem *et al.* 2005, Manna & Stocco 2007). CBP and its functional homolog, p300, are recognized as bridging proteins, involved in the communication between transcription factors and the basal transcriptional machinery, and have been shown to participate in the activities of many transcription factors including JUN/FOS (Bannister *et al.* 1995, Vo & Goodman 2001, Manna & Stocco 2007). Hence, it is plausible that the PRKCC-mediated induction of P-JUN results in the recruitment of CBP to function in regulating *Star* gene transcription.

In view of the present studies, it is apparent that the JUN transcription factor JUN plays an essential role in the PRKCC-mediated regulation of STAR expression and steroidogenesis. In support of this, the JUN proto-oncogene, but not other JUN factors, has been demonstrated to be involved in maintaining the integrity and function of the adrenal cortex (Lehoux *et al.* 1998). JUN and FOS have been demonstrated to have diverse effects in cellular functions and are able to interact with different proteins and mediate a variety of transcriptional responses (Karin *et al.* 1997, Sarkar *et al.* 2000, Shea-Eaton *et al.* 2002, Manna *et al.* 2004, Manna & Stocco 2007). Conceivably, the roles for JUN and FOS in PRKCC-responsive STAR expression and steroidogenesis are speculated to be different since they lack sequence similarity outside the DNA-binding and dimerization domains. Mice lacking JUN are embryonic lethal with embryos dying between mid- and late gestation due to several tissue defects, underscoring the importance of JUN during development (Hilberg *et al.* 1993, Johnson *et al.* 1993). Our findings show that overexpression of JUN increased basal and PMA-mediated *Star* gene expression and that these responses were markedly affected by TAM-67, demonstrating that JUN plays an integral role in the transcription of the *Star* gene. Consequently, targeted silencing of endogenous JUN was shown to coordinately suppress PRKCC-dependent STAR expression and steroid synthesis. JUN dimerizes with many bZIP members, and thus the repression of JUN in concurrence with PRKCC-mediated steroidogenesis may affect relevant transcription factor(s), a possibility that requires additional investigation.

In summary, these findings provide insight into the mechanisms by which JUN regulates the PRKCC signaling pathway involved in the steroidogenic response in mouse Leydig cells. Also, our present findings support the observation that the PRKCC-dependent induction of STAR expression and steroid synthesis involves signaling crosstalk with PRKACA.

JUN binds to the JUN motif in the STAR promoter and influences PMA-induced steroid synthesis by controlling transcription of the *Star* gene. Whether JUN acts similarly in other steroidogenic cells in response to PRKCC remains to be elucidated.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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