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Transcription factors SF1 and cJUN cooperate to activate the *Fdx1* promoter in MA-10 Leydig cells

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ABSTRACT

The Ferredoxin 1 (FDX1) protein supports steroid biosynthesis in steroidogenic cells through electron transfer to the rate-limiting steroidogenic enzyme, CYP11A1. The latter catalyzes the conversion of cholesterol to pregnenolone through side chain cleavage inside the mitochondria. Thus far, only several transcription factors have been implicated in the regulation of mouse *Fdx1* promoter activity in Leydig cells. These include the nuclear receptor SF1 and SP1. Since two conserved regulatory elements for AP1 transcription factors have been located at −764 and −617 bp of the *Fdx1* promoter, we hypothesized that cJUN may cooperate with other partners to regulate *Fdx1* in Leydig cells. Indeed, we report that SF1 and cJUN interact and cooperate to activate the *Fdx1* promoter in MA-10 and TM3 Leydig cells. Furthermore, we found that such activation requires different regulatory elements located between −124 and −306 bp of the *Fdx1* promoter and involves recruitment of SF1 to this region. Using RNA interference, the importance of SF1 in transcriptional regulation of *Fdx1* was confirmed, whereas cJUN was dispensable even though it cooperated with SF1 to upregulate *Fdx1* expression in MA-10 cells. Thus, our data provides new insights in the molecular mechanisms that control mouse *Fdx1* transcription, possibly leading to regulation of CYP11A1 enzyme activation, in Leydig cells.

1. Introduction

In Leydig cells, the main steroidogenic cells within the mammalian testis, *Cyp11a1* gene expression and steroidogenesis are mainly regulated by the pituitary hormone LH that binds to its G-protein coupled receptor, leading to production of cAMP, activation of cAMP-regulated pathways and resulting in transcriptional activation. As limiting factors of testicular testosterone production, the steroidogenic acute regulatory protein (STAR) is responsible for the transport of cholesterol into mitochondria, while ferredoxin 1 (FDX1) and cytochrome P450scc (CYP11A1) are involved in the conversion of cholesterol into pregnenolone. After being reduced by the flavoprotein ferredoxin reductase, FDX1 supplies electrons to CYP11A1.

Several transcription factors have been implicated in the regulation of *Fdx1* promoter activity within Leydig cells. These include the nuclear receptor steroidogenic factor 1 (SF1, Ad4BP, NR5A1) and SP1. Indeed, SF1 binds to the −794 and −614 regulatory elements, whereas SP1 is being recruited to the −110 and −70 DNA elements to activate human *Fdx1* expression in steroidogenic cells [1–3]. The nuclear receptor SF1 is a critical regulator of steroidogenic cell development and of many steroidogenic genes [4,5]. Since *Fdx1* is ubiquitously expressed and also plays an important role in the mitochondrial electron transport chain, its tissue- and cell-specific expression as well as hormonal regulation in steroidogenic cells may result from cooperation between transcription factors. Indeed, a multitude of such cooperations have been characterized for *Star* promoter regulation and involve SF1 and CEBPB [6], SP1 and SF1 [7], GATA4 and cJUN [8] as well as SF1 and cJUN [9].

Transcription factors belonging to the AP1 family are ubiquitously expressed and can be divided in two groups based on their amino acid similarity; the FOS (cFOS, FRA1, FRA2, and FOSB) and the JUN (cJUN, JUNB, and JUND) subfamilies [10]. AP1 family members have been shown to bind as dimers to specific DNA sequences located in the promoters of target genes [10]. The FOS subfamily members must heterodimerize with JUN proteins, whereas JUN members can form either homo- or heterodimers with any other AP1 members. Interestingly, AP1 transcription factors may interact with SP1 [11] to regulate *Fdx1* expression in Leydig cells.

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Other potent interacting partners for SF1, SP1 and/or AP1 contributing to *Fdx1* regulation are members of the SOX family of transcription factors. Indeed, SOX8 is known to physically interact with cJUN, SF1 and SP1 [12,13], in addition to being expressed in human testicular Leydig cells [14]. Thus, SOX transcription factors may participate in functional cooperations contributing to regulation of *Fdx1* expression in Leydig cells.

Since the DNA binding element for SOX transcription factors is located in the −665 bp region of the mouse *Fdx1* promoter and is surrounded by numerous conserved regulatory elements for other transcription factors such as cJUN at −764 and −617 bp, we hypothesized that SOX and/or cJUN transcription factors may cooperate with other partners to regulate *Fdx1* transcription in Leydig cells. Indeed, we report that the mouse *Fdx1* promoter is being regulated by a cooperation between cJUN and SF1 in MA-10 Leydig cells. In addition, using *Fdx1* promoter deletions, we found that the region between −306 and −124 bp is important for such activation of *Fdx1* expression and involves recruitment of SF1 to this region. Our data also suggest that SOX8 is dispensable and identify cJUN as a novel regulator of *Fdx1* gene expression in steroidogenic Leydig cells.

2. Materials and methods

2.1. Chemicals

8Br-cAMP (8-Bromoadenosine 3′,5′-cyclic monophosphate), FSK (forskolin), IBMX (3-Isobutyl-1-methylxanthine), CGS–9343B and IPZ (importazole) were purchased from Sigma-Aldrich Canada (Oakville, Canada).

2.2. Plasmids

The −1003 to +45 bp mouse *Fdx1*-luciferase promoter construct (−1 kb) has been described previously [15]. Deletions of the *Fdx1* promoter to −306 (−306 bp) and −124 bp (−124 bp) were obtained by PCR using the −1003 bp *Fdx1* promoter as template, along with the common reverse primer containing a KpnI (underlined) cloning site (5′-GGG GTA CCG CGG AGT CCT GCG CAG TGC TGA G-3′) and the following forward primers containing a BamHI cloning site: −306 bp, 5′-CGG GAT CCC TTT TGC TGT AAC TTG TAT TCT-3′; and −124 bp, 5′-CGG GAT CCC TGG AAC AGT GCT CG-3′. All promoter fragments were cloned into a modified pXP1 luciferase reporter plasmid [16] and subsequently verified by sequencing (Sequencing and Genomes Genotyping Platform, CHUL Research Centre, Quebec City, Canada). The mouse SF1 and LRH1 expression vectors have been described previously [17,18]. The cJUN expression vector [19] was obtained from Dr Dany Chalbos (Institut National de la Santé et de la Recherche Médicale, Endocrinologie Moléculaire et Cellulaire des Cancers, Montpellier, France). The cJUN deletion construct, lacking amino acids 1-168 (Δ1-168), was obtained by amplification of cDNA using the forward primer containing a BamHI cloning site, 5′-GCG GGA TCC ATG GTC TAC GCC AAC CTC AGC AA-3′, and reverse primer containing a XhoI cloning site, 5′-CCC TCG AGT CAA AAC GTT TGC AAC TGC TG-3′, followed by cloning into pCDNA3. The mouse SP1 expression vector was obtained from Dr Robert Viger (CHUQ Research Centre, Laval University, Quebec, Canada). The mouse SOX8 and SOX9 expression vectors have been described previously [20,21] and were generously provided by Dr. Michael Wegner (Institut für Biochemie, Universität Erlangen, Germany) and Dr. James Wells (Cincinnati Children's Hospital Research Foundation, Cincinnati, OH), respectively. The mouse SOX4 expression vector was purchased from OriGene (Cat #: MR207005, OriGene Technologies, Inc., Rockville, MD). Expression vector for CEBPB [22] was provided by Dr Steven McKnight (UT Southwestern Medical Center at Dallas, Dallas, TX, USA). The NFKB1 (p50) and RELA (p65) expression vectors [23] were provided by Dr Richard Pope (Northwestern University Feinberg School of Medicine, Chicago, IL, USA). The mouse CREB1 expression vector was obtained by amplification of the cDNA using the forward primer containing a BamHI cloning site, 5′-CGG GAT CCA AAT GAC CAT GGA ATC TGG AGC-3′, and reverse primer containing a XbaI cloning site, 5′-GCT CTA GAT TAA TCT GAT TTG TGG CAG TAA AGG-3′, followed by cloning into pCDNA3.

2.3. Cell culture and transfections

Mouse MA-10 Leydig cells [24], provided by Dr. Mario Ascoli (University of Iowa, Iowa City, Iowa), were grown as described previously [25] for mRNA and protein extractions and in Dulbecco modified Eagle medium (DMEM)/F12 medium supplemented with 15% horse serum for transfections. This well-studied tumor Leydig cell line originated spontaneously in a C57Bl/6J mouse [24] and retains many characteristics of their non-transformed counterparts. For instance, MA-10 cells are able to produce progesterone in response to both hormonal stimulations (LH and hCG) and cAMP analogues [24,26]. Non tumorigenic mouse TM3 Leydig cells [27] were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM/F12 medium supplemented with 2.5% fetal bovine serum and 5% horse serum. African Green monkey kidney fibroblast CV-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM medium supplemented with 10% newborn calf serum. Cells were cultured at 37 °C and 5% CO_2. MA-10 cells were transfected using the jetPRIME® reagent (Polyplus Transfection, Illkirch, France) according the manufacturer's instructions followed by treatments using medium without serum and analysis of lysates for luciferase activity as previously described [25,28]. TM3 and CV-1 cells were transfected using polyethylenimine at a ratio of 3:1 with DNA [29]. Treatments were done using activators of the cAMP/PKA pathway such as 0.5 mM 8Br-cAMP, 10 μM FSK (an activator of the adenylate cyclase) and 1 mM IBMX (a non-specific inhibitor of cAMP and cGMP phosphodiesterases) or selective inhibitors for importin (importazole at 40 μM) and calmodulin (CGS–9343B at 50 μM) for 6 h. Concentrations used for the inhibitors have been optimised previously [30].

2.4. siRNA transfection

RNA interference oligonucleotides (siRNAs) directed against SF1 (Cat.: s77130) or cJUN (Cat.: s68564), were purchased from Thermo Fisher Scientific (Waltham, MA USA) and transfected in MA-10 Leydig cells using jetPRIME® transfection reagent (Polyplus Transfection, Illkirch, France). As a negative control, Silencer® Select negative control No. 2 siRNAs were used (Thermo Fisher Scientific). After 48 h, MA-10 cells were incubated with vehicle or 0.5 mM 8Br-cAMP, followed by mRNA isolation, cDNA synthesis and quantitative real-time PCR assays.

2.5. Quantitative real-time PCR

RNA was isolated from cultured MA-10 Leydig cells using the E.Z.N.A. Total RNA kit (Omega Bio-Tek, Inc., Norcross, GA). The purity and concentration of the isolated RNA was assessed using a Nanodrop 1000 where 260/280 ratios were approximately 2.1. The qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) was used to synthesize cDNA. Quantitative real-time polymerase chain reaction (qPCR) was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers (Integrated DNA Technologies, Coralville, Iowa) used are

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