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# MAP kinase phosphatase-3 (MKP-3) is transcriptionally and post-translationally up-regulated by hCG and modulates cAMP-induced p21 expression in MA-10 Leydig cells

Mercedes Mori Sequeiros García [a], Natalia V. Gómez [a], Alejandra Gorostizaga [a], Andrea Acquier [a,b], Silvia I. González-Calvar [c,d], Carlos F. Mendez [a,b], Cristina Paz [a,*]

[a] Institute for Biomedical Research (INBIOMED), Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, (C1121ABG) Buenos Aires, Argentina
[b] Pharmacology Unit, School of Dentistry, University of Buenos Aires, Marcelo T. de Alvear 2142, (C1122AAH) Buenos Aires, Argentina
[c] Institute of Biology and Experimental Medicine, National Council for Scientific and Technical Research, Vuelta de Obligado 2490, (C1428DN) Buenos Aires, Argentina
[d] School of Medicine, University of Buenos Aires, Paraguay 2155, (C1121ABG) Buenos Aires, Argentina

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ABSTRACT

Luteinizing hormone (LH) activates ERK1/2, MAP kinases (MAPKs) necessary for its action on steroidogenesis and cell proliferation, and also induces MAPK phosphatase-1 (MKP-1), which rapidly dephosphorylates nuclear ERK1/2. MKP-3 is a cytoplasmic ERK-phosphatase up-regulated by proliferative stimuli. MKP-3 also dephosphorylates transcription factor FOXO1, promoting its transport to the nucleus. Here we analyzed MKP-3 expression in MA-10 Leydig cells and demonstrated that LH receptor (LHR) activation with human gonadotropin hormone (hCG) and an analog of its second messenger, 8Br-cAMP, up-regulates MKP-3 by transcriptional and post-translational mechanisms. It is known that FOXO1 drives the expression of the cell cycle inhibitor p21. Since the activation of this transcription factor by MKP-3 has been reported, we assessed the effect of shRNA against MKP-3 on p21mRNA levels. 8Br-cAMP increased these levels (2-fold at 2 h) and MKP-3 down-regulation reduced this effect. Our work demonstrates that LH/hCG tightly up-regulates MKP-3 which in turn, dephosphorylates ERK1/2 and drives p21 expression. These events could contribute to counteract hormonal action on cell proliferation.


## 1. Introduction

Mitogen-activated protein kinases (MAPKs: ERKs, JNKs and p38) exert profound effects on a variety of physiological processes such as proliferation, differentiation and apoptosis (Ashwell, 2006; Raman et al., 2007). MAPKs display maximal enzymatic activity upon phosphorylation on threonine and tyrosine, thus, the magnitude and duration of their activity are linked to the action of phosphatases able to dephosphorylate and inactivate them.

MAPK phosphatases (MKPs) are dual specificity (threonine and tyrosine) phosphatases (DUSPs) involved in MAPK regulation (Boutros et al., 2008; Huang and Tan, 2012; Keyse, 2008). MKP family members differ in their subcellular localization, tissue-specific expression, inducibility by various types of signals, induction kinetics and selectivity for dephosphorylating specific MAPKs. MKP-1 and -3 are archetypes of the MKP family. MKP1 (or DUSP1) is a nucleus-localized phosphatase induced by different growth factors and stress signals while MKP-3 (or DUSP6) is a cytoplasmic enzyme induced by different proliferative stimuli – but not by environmental stress – and is characterized as a highly specific phosphatase for attenuating ERK1/2 signaling (Zhao and Zhang, 2001). Accordingly, enhanced basal ERK1/2 phosphorylation has been described in the heart of MKP-3 deficient mice (Maillet et al., 2008). Therefore, MKPs exhibiting different subcellular localization and induction kinetics lead to strict spatio-temporal control of MAPKs.

It is accepted that MKP substrates are confined specifically to MAPKs. However, at least for a few members of the MKP

*Abbreviations:* DAPI, 4′,6-diamidino-2-phenylindole; DUSPs, dual specificity phosphatases; FGF, fibroblast growth factor; FOXO1, Forkhead box protein O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GnRH, gonadotropin releasing hormone; L19, ribosomal L19 protein; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MAPK, mitogen-activated protein kinase; MEK1/2, kinases upstream ERK1/2; MKP, MAPK phosphatase; NES, leucine-rich nuclear export signal; P-ERK1/2, phospho-ERK1/2; RT, reverse transcription; shRNA, short hairpin RNA; StAR protein, Steroidogenic Acute Regulatory protein; STARD1, StAR gene.

* Corresponding author. Address: Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 5th Floor, (C1121ABG) Buenos Aires, Argentina. Tel.: +54 1145083672.
*E-mail address:* crispaz@fmed.uba.ar (C. Paz).

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family, growing evidence supports the notion that MKP substrates constitute a broader group of phosphoproteins (Huang and Tan, 2012). Indeed, it has been reported that MKP-3 interacts with and dephosphorylates Forkhead box protein O1 (FOXO1), an event that promotes its nuclear translocation and subsequent activation of key gluconeogenic genes (Jiao et al., 2012; Wu et al., 2010). FOXO1 participates in the regulation of genes related not only to metabolism but also to other processes, like cell cycle arrest, through the induction of the cell cycle inhibitor p21 (Roy et al., 2010). Moreover, FOXO1 negatively regulates ovarian granulose cell proliferation and differentiation induced by FSH (follicle-stimulating hormone) (Park et al., 2005). In addition, in gonadotrope cells, FOXO1 represses basal and GnRH-induced (gonadotropin releasing hormone) transcription of the LH (luteinizing hormone) beta-subunit (Arriola et al., 2012). Therefore, all these processes are potentially modulated by MKP-3.

In steroidogenic cells, the corresponding trophic hormones up-regulate MKPs (Bey et al., 2003; Brion et al., 2011; Casal et al., 2007; Sewer and Waterman, 2003), which play a role in the regulation of steroidogenesis (Bey et al., 2003; Brion et al., 2011; Casal et al., 2007; Sewer and Waterman, 2003). In Leydig cells, LH regulates cell function through a mechanism involving protein kinase A (PKA) activation (Podesta et al., 1976) and PKA-dependent ERK1/2 activation (Hirakawa and Ascoli, 2003). Moreover, Evaul and Hammes demonstrated that, in both mouse tumor Leydig cell lines as well as primary mouse Leydig cells, LH-induced cAMP/PKA leads to epidermal growth factor receptor (EGFR) transactivation, which then activates ERK1/2 (Evaul and Hammes, 2008). LH up-regulates Steroidogenic Acute Regulatory (StAR) protein, which facilitates the access of cholesterol to the inner mitochondrial membrane (Stocco and Clark, 1996), the rate-limiting step of steroid synthesis (Crivello and Jefcoate, 1980). Because ERK activity participates in the expression of the gene encoding for StAR protein (STARD1) (Gyles et al., 2001) and also in StAR protein activation (Poderoso et al., 2008), the down-regulation of steroidogenesis by MKPs is expected. We have demonstrated that the activation of the LH receptor with human Chorionic Gonadotropin (hCG) rapidly induces MKP-1, and that the down-regulation of this enzyme by a specific *short hairpin* RNA (shRNA) increases the hormonal effects on ERK1/2 activity, StAR gene expression and steroidogenesis (Brion et al., 2011). Our results highlight the participation of ERK1/2 in steroidogenesis and are consistent with the conclusions reached by Evaul and Hammes, who postulated that the activation of GFR/ERK1/2 pathway is necessary for early LH-induced steroidogenesis (Evaul and Hammes, 2008).

In addition to the regulation of steroid synthesis, LHR activation increases Leydig cell proliferation. In primary cultures of rat Leydig cells, LHR stimulation induces cell proliferation through an ERK-dependent pathway (Shiraishi and Ascoli, 2007). The same conclusion was drawn using a more physiological model consisting of adult mice carrying a testicular-specific deletion of MEK (kinase upstream ERK1/2), which show Leydig cell hypoplasia and hypergonadotropic hypogonadism, among other abnormalities (Yamashita et al., 2011). Moreover, primary cultures of Leydig cells from these mice exhibit low phospho-ERK1/2 (P-ERK1/2) levels after hCG or cAMP stimulation (Yamashita et al., 2011). Therefore, the regulation of cell proliferation by LH could be an additional target of MKPs.

As LH is a proliferative stimulus for Leydig cells, we hypothesized that LHR activation regulates MKP-3 expression. This phosphatase could promote p21 expression, through dephosphorylation and activation of FOXO1. To test this hypothesis, we examined the effect of hCG on MKP-3 and the impact of MKP-3 down-regulation on p21 expression.

## 2. Materials and methods

### 2.1. Materials

Monoclonal antibodies against MKP-3, β-tubulin and FLAG M2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Millipore Corporation (Billerica, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Polyclonal antibodies against P-ERK1/2 and total ERK1/2 were from Cell Signaling Technology, Inc. (Boston, MA, USA). 2-(2-Amino-3-methoxyphenyl) 4H-1-benzopyran-4-one (PD98059), actinomycin D and 8-Bromo-cAMP (8Br-cAMP) were from Sigma (St. Louis, MO, USA) and human chorionic gonadotropin (purified hCG, batch CR-125 of biological potency 11900 IU/mg) was a gift from NIDDK, NIH (Bethesda, MA, USA). All other reagents were of highest quality available.

### 2.2. Cell cultures

The MA-10 cell line, a clonal strain of mouse Leydig tumor cells generously provided by Dr. Mario Ascoli (University of Iowa, College of Medicine, Iowa, IO, USA), was handled as originally described (Ascoli, 1981). After 24 h of serum starvation, the cells were incubated with hCG, 8Br-cAMP or other agents.

### 2.3. Plasmid constructs

FLAG-tagged MKP-3 construct (pFLAG-MKP-3) was generated using the p3xFLAG-CMV™-7.1 expression vector. Mouse total RNA was used to obtain total cDNA by reverse transcription. From this sample, a fragment of 1188 kb corresponding to the full-length coding region of MKP-3 (NM_026268.3) was amplified using the following primers: forward, TTGCGGCCGCTATGATAGATACGCTCA-GA (which contains the cleavage site for NotI) and reverse, ACGCGAATGAAGGAATGGGGACAACTC (which contains the cleavage site for EcoRI). This fragment was purified and fused into p3xFLAG-CMV™-7.1 using NotI/EcoRI restriction sites. p3xFLAG-CMV-7.1 containing ERK2 (pFLAG-ERK2) was kindly provided by Melanie Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX).

A vector for the expression of shRNA against MKP-3 under the control of the polymerase-III H1-RNA promoter (pSUPER.retro-MKP-3) was obtained using the pSUPER.retro vector (OligoEngine, Seatle, WA, USA). A pair of 60-nt annealed DNA oligonucleotides (containing a 17-nucleotide target sequence derived from murine MKP-3 mRNA) was inserted between the *Bgl*II/*Hind*III restriction sites of the pSUPER.retro vector. The set of 60-nt oligos containing this sequence for MKP-3 shRNA is described below: sense, 5′-GATCCCCACGACATTGTTAAGATGAATTCAAGAGATTCATCTTAACAAT GTCGTTTTTTA-3′ and antisense, 5′-AGCTTAAAAAACGACATTGT-TAAGATGAATCTCTTGAATTCATCTTA ACAATGTCGTGGG-3′. Correct in-frame insertions were verified by sequencing.

### 2.4. Transfection assays

Cells were seeded the day before transfection, grown up to 80% confluence and transfected during 6 h using Lipofectamine 2000 reagent in Opti-MEM medium according to manufacturer's instructions (Invitrogen, Life Technologies, Inc.-BRL, Grand Island, NY, USA).

### 2.5. RNA extraction and Real-time PCR

Total RNA was extracted using Tri Reagent following the manufacturer's instructions (Molecular Research Center, Inc. Cincinnati, OH, USA). Reverse transcription was done using 2 μg of total RNA as previously described (Castilla et al., 2008) and Real-time PCR