MAPK Phosphatase-1 (MKP-1) Expression Is Up-Regulated by hCG/cAMP and Modulates Steroidogenesis in MA-10 Leydig Cells

Laura Brion, Paula M. Maloberti, Natalia V. Gomez, Cecilia Poderoso, Alejandra B. Gorostizaga, Maria M. Mori Sequeiros Garcia, Andrea B. Acquier, Mariana Cooke, Carlos F. Mendez, Ernesto J. Podesta, and Cristina Paz

Institute of Molecular Research in Hormonal, Neurodegenerative and Oncological Diseases, Department of Biochemistry, School of Medicine, University of Buenos Aires, C1121ABG Buenos Aires, Argentina

MAP kinases (MAPKs), such as ERK1/2, exert profound effects on a variety of physiological processes. In steroidogenic cells, ERK1/2 are involved in the expression and activation of steroidogenic acute regulatory protein, which plays a central role in the regulation of steroidogenesis. In MA-10 Leydig cells, LH and chorionic gonadotropin (CG) trigger transient ERK1/2 activation via protein kinase A, although the events that lead to ERK1/2 inactivation are not fully described. Here, we describe the hormonal regulation of MAPK phosphatase-1 (MKP-1), an enzyme that inactivates MAPKs, in MA-10 cells. In our experiments, human CG (hCG)/cAMP stimulation rapidly and transiently increased MKP-1 mRNA levels by a transcriptional action. This effect was accompanied by an increase in protein levels in both nuclear and mitochondrial compartments. In cells transiently expressing flag-MKP-1 protein, hCG/cAMP promoted the accumulation of the recombinant protein in a time-dependent manner (10-fold at 1 h). Moreover, hCG/cAMP triggered ERK1/2-dependent MKP-1 phosphorylation. The blockade of cAMP-induced MAPK kinase/ERK activation abated MKP-1 phosphorylation but only partially reduced flag-MKP-1 protein accumulation. Together, these results suggest that hCG regulates MKP-1 at transcriptional and posttranslational level, protein phosphorylation being one of the mechanisms involved in this regulation. Our study also demonstrates that MKP-1 overexpression reduces the effects of cAMP on ERK1/2 phosphorylation, steroidogenic acute regulatory gene promoter activity, mRNA levels, and steroidogenesis, whereas MKP-1 down-regulation by small interfering RNA produces opposite effects. In summary, our data demonstrate that hCG regulates MKP-1 expression at multiple stages as a negative feedback regulatory mechanism to modulate the hormonal action on ERK1/2 activity and steroidogenesis. **(*Endocrinology* 152: 2665–2677, 2011)**

The MAPK phosphatase (MKP) family comprises dual-specificity enzymes that dephosphorylate phosphothreonine and phosphotyrosine residues within MAPK (1, 2). A common feature of MAPK (ERK, c-Jun NH_2-terminal protein kinases, or JNK1/2, and p38 MAPK) is the requirement of dual threonine and tyrosine phosphorylation to display maximal activity (3). Consequently, MKPs down-regulate MAPKs activity. MAPK regulation has profound consequences for cell biology through their action on several processes, such as proliferation, development, apoptosis, differentiation, and inflammation (3–5). Therefore, the role of MKPs as regulatory components of such processes is now well recognized.

One member of the MKP family is MKP-1, a nucleus-localized phosphatase involved in the regulation of ERK1/2, JNK1/2, and p38 MAPK. Due to its cellular

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-0021 Received January 10, 2011. Accepted April 19, 2011.
First Published Online May 10, 2011

Abbreviations: AngII, Angiotensin II; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H-89, N-[2-(p-bromocynnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; hCG, human chorionic gonadotropin; L19, ribosomal L19 protein; LHR, LH receptor; MEK, MAPK kinase; MG-132, Z-Leu-Leu-Leu-al; MKP, MAPK phosphatase; nt, nucleotide; P4, progesterone; PD98059, 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one; pERK1/2, phospho-ERK1/2; PKA, protein kinase A; Scr, scrambled; SF1, steroidogenic factor 1; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory; WT, wild type.

localization, MKP-1 is responsible for nuclear MAPKs inactivation. Consequently, this phosphatase controls the activity and/or expression of MAPK-dependent transcription factors and, ultimately, gene transcription (6, 7). MKP-1 expression is tightly regulated both at transcriptional and posttranslational levels. Indeed, MKP-1 mRNA is rapidly induced by a broad array of extracellular stimuli (8–10), and MKP-1 protein is subjected to posttranslational modifications, such as phosphorylation and acetylation (11, 12).

In steroidogenic cells, the corresponding trophic hormones trigger MAPKs signaling pathways (13, 14). Accordingly, we and others have shown that ACTH (8, 15) and angiotensin II (AngII) (16) increase MKP-1 mRNA and protein levels in adrenocortical cells. Moreover, we have previously demonstrated that MKP-1 mRNA is accumulated in MA-10 Leydig cells in response to cAMP (17). However, information available on posttranslational regulation of MKP-1 by steroidogenic hormones is still scarce.

The LH regulates Leydig cell function through a mechanism that involves the interaction with its specific receptor [LH receptor (LHR)], protein kinase A (PKA) activation (18), and PKA-dependent ERK1/2 activation (14). The rate-limiting step in steroid biosynthesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane, a step that is facilitated by the steroidogenic acute regulatory (StAR) protein (19, 20). Although other regulatory proteins are known to contribute to this process (21, 22), LH regulates steroidogenesis through the induction of StAR gene (19, 20) and the activation of StAR protein (23, 24). Several reports demonstrate that StAR gene induction and StAR protein activation are dependent on PKA (19, 23) and also on ERK1/2 activity (24–26). In view of these results, the regulation of steroid production by MKP seems possible.

The present study was undertaken to analyze the effect of LHR activation by human chorionic gonadotropin (hCG) hormone on MKP-1 expression and to determine the role played by this enzyme in steroidogenesis in MA-10 Leydig cells. Our results demonstrate that hCG promotes both MKP-1 gene induction and protein stabilization by PKA-dependent mechanisms. Also, we report that MKP-1 overexpression reduces the hormonal effect on ERK1/2 activity and, consequently, on StAR expression and steroidogenesis, whereas its down-regulation by siRNA produces opposite effects. MKP-1 induction acts, therefore, as a negative feedback regulatory mechanism in the signaling pathway, by which hCG and, hence, LH modulate steroidogenesis.

Materials and Methods

Reagents

N-[2-(p-bromocynnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), Z-Leu-Leu-Leu-al (MG-132), 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one (PD98059), 8Br-cAMP, actinomycin D, and monoclonal antibody against FLAG M2 were purchased from Sigma (St. Louis, MO). Polyclonal antibodies against MKP-1 and against phospho-ERK1/2 (pERK1/2) and total ERK1/2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and New England Biolabs, Inc. (Beverly, MA), respectively. Monoclonal mouse antibody against β-tubulin was provided from Upstate Group, Inc. (Temecula, CA), whereas horseradish peroxidase-conjugated goat-antirabbit, goat-antimouse secondary antibodies as well as Immun-Blot polyvinylidene fluoride membrane were purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were of highest quality available.

Plasmid constructs and site-directed mutagenesis

The 1-kb murine StAR promoter fused to the *Firefly* luciferase reporter gene construct (pGL3-StAR) was generously provided by Colin Jefcoate (University of Wisconsin-Madison Medical School, Madison, WI). 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). The pRc/CMVi expression vector containing MKP-1 (pRc/CMV-MKP-1) was constructed as follows: the coding cDNA of MKP-1 was amplified by PCR using the 5′-CGCTCTAGAATGCCCTGTATCCAAGCTC-3′ and 5′-GGGGTACCTCAGAAAGAC AAGGTG-3 primers. The amplified fragment was cloned in the *Eco*RI site of pRc/CMVi vector. FLAG-tagged MKP-1 expression vector (pFLAG-MKP-1) was generated by digestion of pRc/CMV-MKP-1 vector with *Eco*RI/*Xba*I enzymes. The 1.4-kb fragment obtained corresponding to MKP-1 cDNA was purified and cloned in *Eco*RI/*Xba*I sites of p3xFLAG-CMV-7.1 vector.

siRNA plasmid vector was constructed as follows: a pair of 64-nucleotide (nt)-annealed DNA oligonucleotides was inserted between the *Bgl*II and *Hind*III restriction sites of the pSUPER.retro vector (OligoEngine) to express short hairpin small interfering RNA (siRNA) under the control of the polymerase-III H1-RNA promoter. A 19-nt target sequence derived from murine MKP-1 mRNA (accession NM_013642, 489–507 bp, siRNA1) was used. The set of 64-nt oligos containing this sequence is described below: sense, 5′- GATCCCCGCTCCACTCAAGTCTTCTTTT CAAGAGAAAGAAGACTTGAGTGGAGCTTTTTA-3′ and antisense, 5′-AGCTTAAA AAGCTCCACTCAAGTCTTCTTTCTCTTGAAAAGAAGACTTGAGTGGAGCGGG-3′. A second siRNA was used (targeting 764–783 bp of MKP-1 cDNA, siRNA2). Control siRNA were also designed as scrambled (Scr) sequences of siRNA1 and siRNA2: 5′-TCATTCATTCGCTATCCGC-3′ and 5′-CATTCCCTTCGGTAGACTC-3′, Scr1 and Scr2, respectively. Correct in-frame insertions were verified by sequencing.

The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to replace serine 359 and 364 for alanine in the pFLAG-MKP-1 sequence to generate the S359/364A flag-MKP-1 double mutant. Employed oligonucleotides (mutated nucleotides are underlined) were: sense, 5′-CTACCTTAAAGCCC CCATCACCACCGCTCCA AGCTG-3′ and an-

tisense, 5′-CAGCTTGGAGCGGTGGTGATGGG GGCTTTA-AGGTAG-3′. All constructs were verified by DNA sequencing.

Cell culture

The MA-10 cell line, a clonal strain of mouse Leydig tumor cells generously provided by Mario Ascoli (University of Iowa College of Medicine, Iowa City, IA) was handled as described elsewhere (27, 28). Cells were growth arrested by serum starvation for 24 h. After replacing the medium by fresh serum-free medium, the cells were incubated with or without hCG, 8Br-cAMP, MG-132, actinomycin D, PD98059, or H-89 as stated in the legend of the corresponding figures.

Transfection and luciferase assay

MA-10 cells were seeded the day before transfection and grown up to 80% confluence, then transfected during 16 h using Lipofectamine 2000 reagent in Opti-MEM medium according to manufacturer's instructions and before serum starvation. For luciferase assays, transfections were carried out in 24-well plates using 0.6 μg pRc/CMV-MKP-1 or pSUPER.retro.MKP-1 vectors, 0.2 μg pGL3-StAR, and 10 ng pRL *Renilla* luciferase expression vector as an internal control for transfection efficiency. StAR promoter activity was evaluated in cells treated with 8Br-cAMP 0.5 mM for 6 h, to achieve submaximal stimulation. Luciferase activity was measured using the Dual Luciferase Assay System (Promega Corp., Madison, WI) and the Synergy HT luminometer (BioTek Instruments, Inc., Winooski, VT).

RNA extraction, semiquantitative RT-PCR, and quantitative real-time PCR

Total RNA was extracted using TriZol reagent following manufacturer's instructions (Life Technologies, Carlsbad, CA). The RT and PCR analyses were made as previously described (29). Primers used for the amplification of MKP-1 cDNA were: forward, 5′-GAGCTGTGCAGCAAACAGTCC-3′ and reverse, 5′-CCGGTGGC-AAGTGAAACTCC-3′. Ribosomal protein L19 (L19) cDNA was used as an internal standard as previously described (29, 30). The reaction conditions were: one 5-min cycle at 95 C, followed by 23 cycles for MKP-1 or 20 for L19 at 95 C for 30 sec, 56 C for 30 sec, and 72 C for 60 sec.

For quantitative real-time PCR assay, isolated RNA was deoxyribonuclease-treated using DNase-Free kit (Ambion, Inc., Austin, TX). The RT and PCR analyses were made using 1 μg of total RNA. Reactions were performed using the SYBR Green Master Mix reagent kit (Applied Biosystems, Carlsbad, CA) using the following specific primers: StAR cDNA forward, 5′-TTGGGCATACTCAACAACCA-3′ and reverse, 5′-CCTTGA-CATTT-GGGTTCCAC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA forward, 5′-TGCACCAC-CAACTGCTTAGC-3′ and reverse, 5′-GGCATGGACTGTG-GTCATGAG-3′. The reaction conditions were: one 10-min cycle at 95 C, followed by 40 cycles at 95 C for 15 sec, 55 C for 30 sec, and 60 C for 30 sec. Data from the reactions were collected and analyzed by the Sequence Detection Software (version 1.3; Applied Biosystems). Relative quantification of gene expression was calculated using standard curves and normalized to GADPH in each sample. For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (31) was applied. An expression ratio was determined for each sample by calculating (Etarget).Ct(target)/(EGAPDH).Ct-(GAPDH), where E is the efficiency of the primer set and the cycle threshold, which is calculated as Ct = Ct(normalization cDNA) − Ct(experimental cDNA). The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction *vs.* Ct value [E = 10 − (1/slope)]. Efficiencies of 2 ± 0.1 were considered optimal.

Subcellular fractionation

Subcellular fractions were obtained by differential centrifugation as previously described (32, 33). Briefly, cells were washed in PBS and harvested in isotonic homogenization buffer A [20 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EDTA, 10 mM KCl, and 1.5 mM $MgCl_2$] supplemented with protease and phosphatase inhibitors. Samples were then homogenized by mechanical disruption with a pellet pestle motor (Kontes) and passed through a 75-μm pore filter to remove unbroken cells. The homogenates were centrifuged at 1000 × *g* for 10 min. The pellet containing nuclei was resuspended in buffer A followed by sonication. The resulting pellet was resuspended in buffer A and nuclei disrupted by sonication, whereas the supernatant was centrifuged for 10 min at 15,000 × *g* to obtain the mitochondrial fraction. All steps were performed on ice at 4 C. Purity of obtained subcellular fractions was assessed by enzymatic analysis, and fractions were considered of appropriate purity when, at least, 90% of the respective total enzymatic activity was recovered.

Western blot analysis

Proteins were separated by SDS-PAGE and electrotransferred to polyvinylidine fluoride membranes as previously described (8). Immunodetection was performed using the following antibody dilutions: rabbit polyclonal anti-MKP-1 (1:1000), mouse monoclonal anti-FLAG (1:10,000), rabbit polyclonal anti-pERK1/2 (1:5000), mouse monoclonal anti-β-tubulin (1:5000), rabbit polyclonal antitotal ERK1/2 (1:20,000), or mouse monoclonal antimitochondrial 48-kDa subunit of complex III (1:20,000). Bound antibodies were developed by incubation with secondary antibody (goat antirabbit or goat antimouse horseradish peroxidase conjugated) and detected by chemiluminescence using the enhanced chemiluminescence detection reagent (GE Life Sciences, Princeton, NJ).

Immunofluorescence and microscopy

MA-10 cells were grown on poly-L-lysine-coated glass coverslips as previously described (21) and treated as indicated. Glass coverslips were then incubated with rabbit polyclonal antibody anti-MKP-1 or mouse monoclonal antibody anti-FLAG, in a humidified chamber for 24 h at 4 C. Primary antibodies were detected by cy-2 conjugated goat anti-(rabbit IgG) Ig or cy3-conjugated goat anti-(mouse IgG) Ig (Molecular Probes, Eugene, OR). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The glass coverslips were mounted in FluorSave reagent (Calbiochem, San Diego, CA) and examined in an Olympus BX 50 epifluorescence microscope (Olympus, Tokyo, Japan).

Metabolic labeling and immunoprecipitation

The culture media of MA-10 cells were changed to phosphate-free DMEM containing 0.5 mCi/ml of [^{32}P]orthophosphate (PerkinElmer Life Sciences, Waltham, MA). Cells were metabolically labeled for 4 h followed by incubation with MG-132 for 1 h before a 15-min stimulation with 8Br-cAMP. Ra-

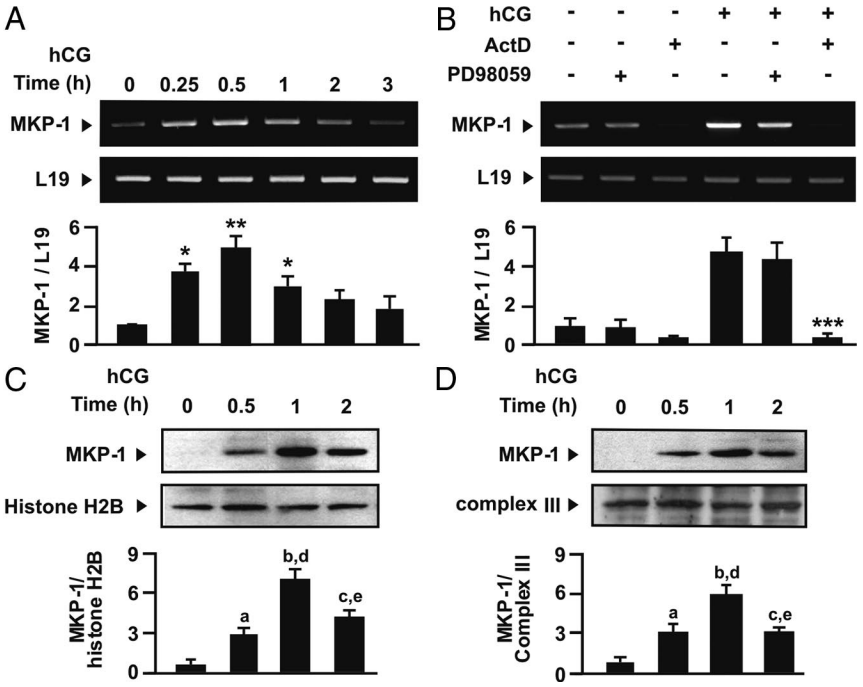

FIG. 1. hCG increases MKP-1 expression in MA-10 cells. MA-10 cells were serum starved and then stimulated with 20 ng/ml hCG for the indicated times (A) or incubated for 30 min in the absence or presence of 50 μM PD98059 or 5 μg/ml actinomycin D (ActD) and further incubated for 30 min with 20 ng/ml hCG (B). Total RNA was isolated, reversed transcribed, and subjected to semiquantitative PCR using specific primers for MKP-1 and L19 cDNA as loading control. PCR products were resolved in ethidium bromide-stained agarose gels. Figure shows representative gels (*upper panels*). Integrated optical density of each band was quantitated, and the values of MKP-1 were normalized against L19 abundance. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments (*lower panels*). *, $P < 0.05$; **, $P < 0.01$ *vs.* nonstimulated cells; ***, $P < 0.001$ *vs.* stimulated cells without ActD. MA-10 cells were serum starved and then stimulated with 20 ng/ml hCG for the indicated times (C and D). Cells were then homogenized and subjected to subcellular fractionation. Nuclear (C) or mitochondrial (D) proteins were analyzed by Western blotting using an anti-MKP-1 antibody. Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped, and histone H2B or complex III was detected using specific antibodies as loading controls for nuclear or mitochondrial proteins, respectively. Figure shows representative immunoblots (*upper panels*). Integrated optical density of each band was quantitated and MKP-1 values normalized against the corresponding loading controls and expressed in arbitrary units. Data represent the mean ± SEM of three independent experiments (*lower panels*). a and c, $P < 0.01$; b, $P < 0.001$ *vs.* nonstimulated cells; d and e, $P < 0.05$ *vs.* previous time point.

diolabeled cells were harvested, and total cell lysates were subjected to immunoprecipitation.

For immunoprecipitation assays, 400 μg of proteins were incubated with 2 μg of anti-FLAG antibody in lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5% Triton X-100, and 2 mM EDTA] supplemented with protease and phosphatase inhibitors for 1 h at 4 C. Then, 30 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) were added and incubated overnight at 4 C. Immune complexes were centrifuged and the supernatant removed. Beads were washed with 1 ml buffer [1 M NaCl and 20 mM Tris-HCl (pH 7.4)], then resuspended in Laemmli sample buffer, boiled, and resolved by SDS-PAGE. Gels were stained with Coomassie blue, dried, and used for autoradiography.

Determination of steroid production

Steroid production was determined by RIA as previously described (29).

Statistical analysis

Results are shown as the mean ± SEM. Statistical significance was evaluated using ANOVA followed by Tukey test. Differences were deemed significant when $P < 0.05$.

Results

hCG increases MKP-1 expression in MA-10 Leydig cells

The first experiments were performed to evaluate the effect of hCG (20 ng/ml) on MKP-1 mRNA levels in MA-10 Leydig cells. LHR stimulation by hCG produced a transient increase of MKP-1 mRNA levels, evaluated by semiquantitative RT-PCR. The effect of hCG was statistically significant already after 15 min, reached a maximum 5-fold increase at 30 min, and returned to basal levels after 2 h (Fig. 1A). Actinomycin D abolished the effect of hCG on MKP-1 mRNA (Fig. 1B), indicating that hCG/cAMP increases MKP-1 messenger levels through gene transcription activation. PD98059, an ERK1/2 upstream kinase [MAPK kinase (MEK)] inhibitor, had no effect on hCG-induced accumulation of MKP-1 mRNA, which suggests that this is an ERK1/2-independent event (Fig. 1B).

Next, we evaluated the effect of hCG on MKP-1 protein levels. Even when MKP-1 is described as a nuclear protein, its presence in mitochondria has also been reported (34). Therefore, proteins from both nuclear and mitochondrial compartments were analyzed by Western blotting. hCG increased the abundance of the protein in both nuclei and mitochondria in a time-dependent manner and with similar kinetics (Fig. 1, C and D). In both subcellular fractions, MKP-1 was barely detected in nonstimulated cells, whereas hCG stimulation of the cells resulted in an increase in protein levels that was already evident at 30 min, became maximal after 1 h (~7-fold), and declined thereafter (Fig. 1, C and D).

MKP-1 subcellular localization was confirmed by immunocytochemistry. MKP-1 was barely detected in control cells, but its nuclear accumulation was markedly evident in hCG-stimulated cells (Fig. 2A). An hCG-mediated increase in fluorescence was also detected in extranuclear structures, which was consistent with the detection of MKP-1 in the mitochondrial fraction (Figs. 1D and 2A).

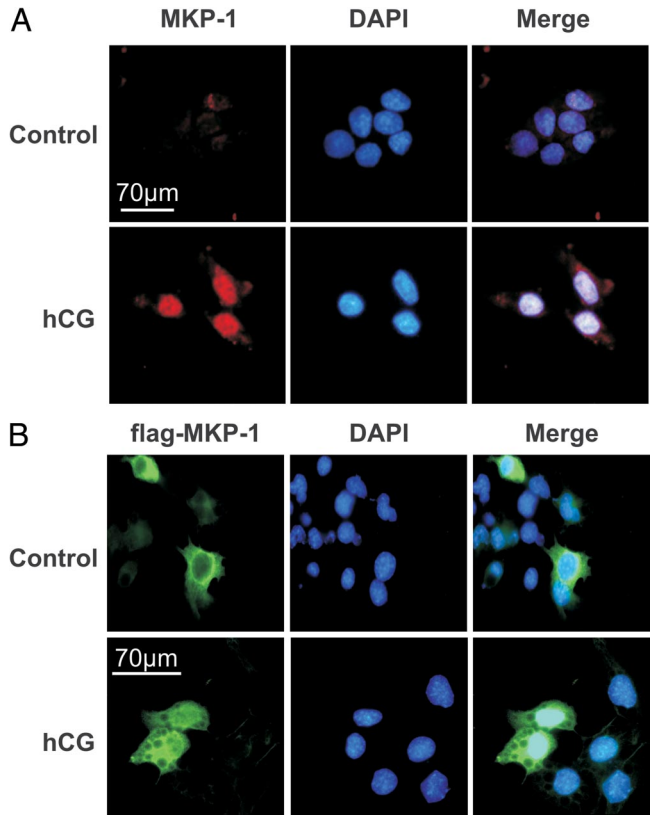

FIG. 2. hCG increases MKP-1 protein accumulation in the nucleus of MA-10 cells. A, MA-10 cells were grown on coverslips, serum starved, stimulated for 1 h with 20 ng/ml hCG, fixed, and stained with an antibody against MKP-1 (*red*) and DAPI (*blue*) and subjected to immunofluorescence microscopy. B, Cells were grown on coverslips, transfected with pFLAG-MKP-1, serum starved, and finally stimulated for 1 h with 20 ng/ml hCG. Cells were then fixed and stained with an anti-FLAG M2 antibody (*green*) and DAPI (*blue*) and subjected to immunofluorescence microscopy.

hCG and cAMP increase MKP-1 protein levels by a posttranslational mechanism

To test whether hCG regulates MKP-1 protein levels by posttranslational modifications, cells were transiently transfected for the overexpression of recombinant flag-MKP-1 protein. Because the expression of flag-MKP-1 protein is driven by a constitutively active promoter, monitoring recombinant protein levels is a useful approach to assess posttranslational effects of hCG on protein stability, regardless of its action on MKP-1 gene expression.

To determine whether the recombinant protein flag-MKP-1 displays a subcellular distribution similar to that of the endogenous protein, we also analyzed flag-MKP-1-overexpressing cells under immunofluorescence microscopy. In control cells, flag-MKP-1 was excluded from the nucleus, whereas in hCG-stimulated cells, it was equally distributed between the cytosol and the nucleus (Fig. 2B), which suggests that the localization in the nucleus is a hormone-dependent event. Western blot analysis of whole cell extracts showed an hCG/cAMP-promoted increase of flag-MKP-1 protein levels that was evident at 30 min, peaked at 1 h (9-fold), and declined thereafter (Fig. 3A, *upper and middle*). Endogenous MKP-1 protein levels in whole cell extracts from 8Br-cAMP-stimulated cells for the same time period (0–6 h) exhibited a similar and sustained time course (Fig. 3A, *lower*). A transient increase of flag-MKP-1 was also observed in nuclear and mitochondrial extracts obtained from 8Br-cAMP-stimulated cells (Fig. 3B). In control cells, flag-MKP-1 was not detected in the nuclear fraction (Fig. 3B, *left*), in agreement with the immunocytochemical observation, although it was detected in the mitochondrial fraction (Fig. 3B, *right*). These results suggest that flag-MKP-1 localization in the nucleus and in the mitochondria involves different mechanisms.

MKP-1 is a labile protein that is targeted for degradation by the ubiquitin-proteasome pathway (11). Accordingly, the proteasome inhibitor MG-132 mimicked the effect of 8Br-cAMP (with no additive effect) on MKP-1 protein levels (Fig. 3C). Given that MG-132 exposure leads to flag-MKP-1 accumulation in the absence of hCG/cAMP, we analyzed the effect of 8Br-cAMP on the subcellular distribution of flag-MKP-1 accumulated by MG-132 treatment in cells under protein synthesis inhibition by cycloheximide. As observed in Fig. 3D, 8Br-cAMP stimulation of MG-132-treated cells resulted in a decrease in flag-MKP-1 protein levels in the cytosolic fraction and in a significant increase of this protein in the nuclear fraction. This result and the fact that the effect is observed in cells exposed to a protein synthesis inhibitor added before 8Br-cAMP suggest that this stimulus promotes posttranslational modification(s) that leads to MKP-1 localization in the nucleus.

hCG/cAMP trigger MKP-1 protein accumulation and phosphorylation through ERK1/2 activity

A series of experiments were conducted to determine the role of ERK1/2 in hCG/cAMP-mediated flag-MKP-1 protein accumulation. First, we evaluated the effect of H-89 and PD98059 (PKA and MEK inhibitors, respectively) on flag-MKP-1 protein accumulation in control and stimulated cells. As observed in Fig. 4A, H-89 abated the effect of both hCG and 8Br-cAMP on flag-MKP-1, whereas PD98059 only partially reduced the effect of these stimuli (Fig. 4A). This demonstrates that hCG-mediated MKP-1 protein stabilization requires the action of PKA through both ERK1/2-dependent and ERK1/2-independent mechanisms. To further assess the role of ERK1/2 in MKP-1 posttranslational regulation by hCG, we studied the effect of ERK2 overexpression on flag-MKP-1 accumulation. The effect of 8Br-cAMP on flag-MKP-1 levels was higher in flag-ERK2-overexpressing cells, an effect that was markedly reduced in the presence of

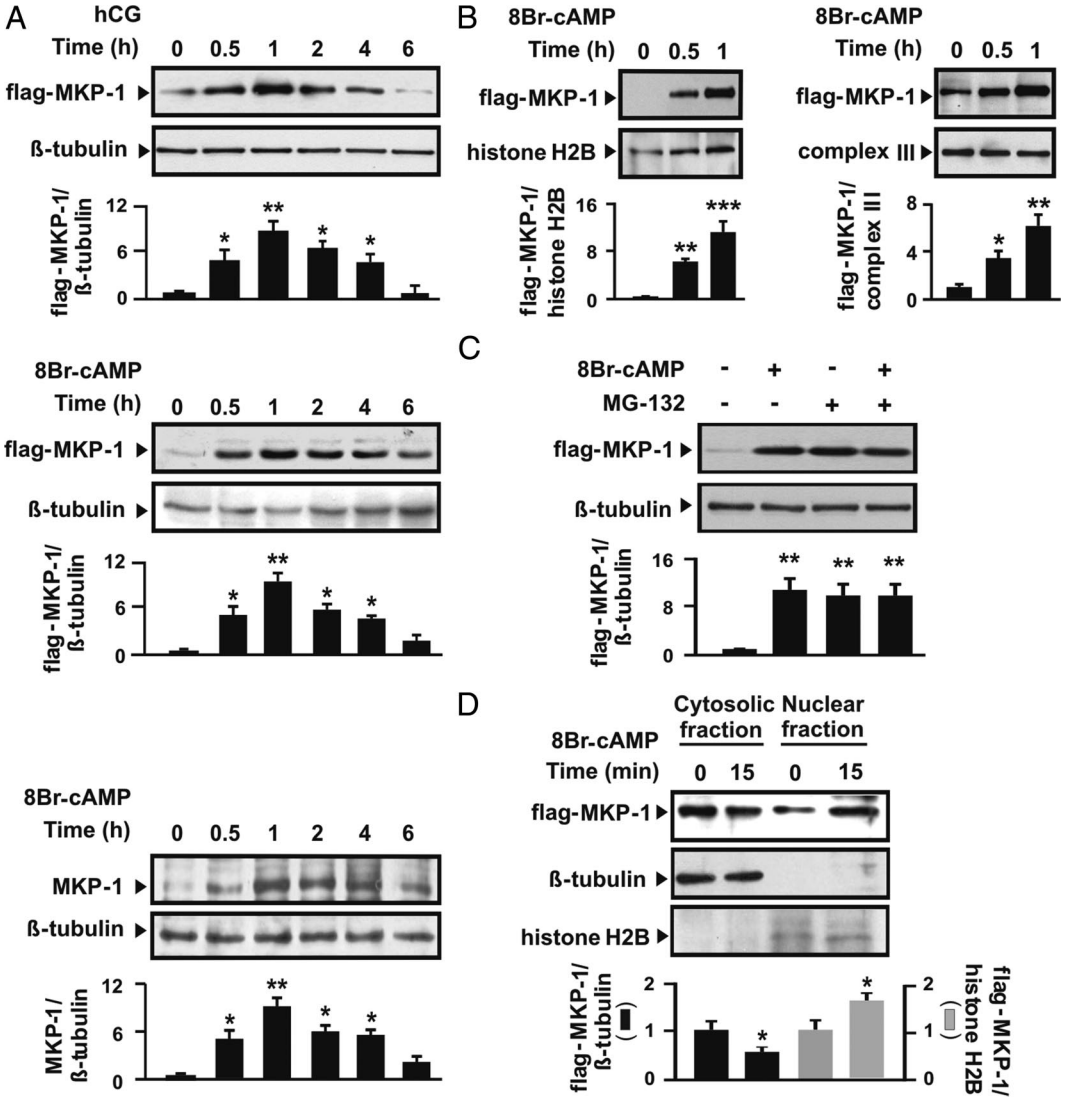

FIG. 3. hCG increases flag-MKP-1 protein levels by a posttranslational mechanism in MA-10 cells. MA-10 cells were transfected with pFLAG-MKP-1 (A, *upper and middle*, and B–D) or nontransfected (A, *lower*), serum starved, and stimulated for the indicated times with 20 ng/ml hCG or 0.5 mM 8Br-cAMP (A and B), incubated for 1 h in the presence or absence of 10 μM MG-132, and further incubated for 1 h with 8Br-cAMP (C), or preincubated with 10 μM MG-132 for 90 min and further incubated with 8Br-cAMP for 15 min in the presence of cycloheximide added 30 min before 8Br-cAMP (D). Cells were then homogenized and subjected to subcellular fractionation and total cell lysates (A and C) or nuclear (B, *left*, and D, *right*), mitochondrial (B, *right*), or cytosolic (D, *left*) proteins analyzed by Western blotting using an anti-FLAG M2 or anti-MKP-1 antibodies as indicated. Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped, and β-tubulin, histone H2B, or complex III was detected using specific antibodies as loading controls for total cell lysates, nuclear, or mitochondrial proteins, respectively. Figures show representative immunoblots (*upper panels*). Integrated optical density of each band was quantitated and flag-MKP-1 or MKP-1 values normalized against the corresponding loading control and expressed in arbitrary units. Data represent the mean ± SEM of three independent experiments (*lower panels*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* nonstimulated cells.

PD98059. In addition, flag-ERK2 overexpression *per se* produced a modest increase in flag-MKP-1 in nonstimulated cells (Fig. 4B).

Next, we tested whether hCG promotes MKP-1 protein phosphorylation and whether PKA and/or ERK1/2 are involved in this process. hCG and 8Br-cAMP induced ^{32}P labeling of flag-MKP-1 (Fig. 5A), whereas PD98059 prevented 8Br-cAMP-stimulated flag-MKP-1 phosphorylation (Fig. 5B). In summary, the results shown in Figs. 4 and 5 indicate that ERK1/2 activities are involved in MKP-1 phosphorylation and stabilization.

S359 and S364 within MKP-1 sequence are described as the major consensus sites for ERK1/2 phosphorylation, and phosphorylation of MKP-1 in these residues is known to impair its proteolysis (11). We thus compared the amounts of wild-type (WT) flag-MKP-1 and its doubly mutated form, S359A/S364A flag-MKP-1, accumulated after 1 h of 8Br-cAMP stimulation. The effect of the double mutation was evidenced as a significantly reduced accumulation of MKP-1 compared with WT flag-MKP-1-transfected cells (Fig. 5C). No difference was observed for the effect of the proteasome pathway inhibitor MG-132

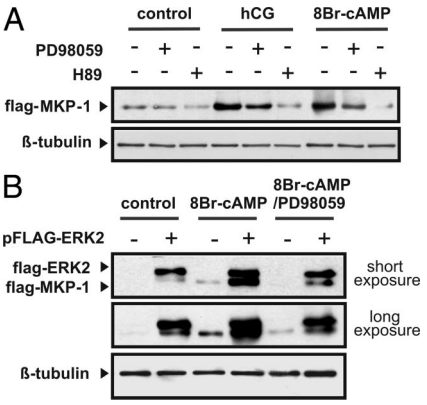

FIG. 4. hCG and cAMP promote MKP-1 protein accumulation through ERK1/2 activity. MA-10 cells were transfected with pFLAG-MKP-1 alone (A) or with pFLAG-MKP-1 and either pFLAG-ERK2 or the empty expression vector as indicated (B), serum starved, preincubated for 30 min in the presence or the absence of 50 μM PD98059 or 10 μM H-89, and further incubated for 1 h with hCG or 8Br-cAMP as indicated. Cells were then homogenized, and total cell lysate proteins were analyzed by Western blotting using an anti-FLAG M2 antibody. Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped and β-tubulin detected as loading control. Figure shows representative immunoblots from three independent experiments.

between S359A/S364A flag-MKP-1- and WT flag-MKP-1-transfected cells, which suggests that even when the mutated form cannot be phosphorylated and stabilized, it can be accumulated at similar levels to those of the WT form under proteolysis inhibition (Fig. 5C). Finally, we measured WT and S359A/S364A flag-MKP-1 levels in serum-starved cells upon 8Br-cAMP stimulation followed by protein synthesis inhibition. Again, we detected a significantly lower accumulation of S359A/S364A flag-MKP-1 compared with WT flag-MKP-1. Moreover, S359A/S364A flag-MKP-1 exhibited a faster degradation kinetics when compared with the wild-type form, because in stimulated cells, the levels of S359/S364 flag-MKP-1 decreased significantly after 30 min of chase, whereas the levels of WT flag-MKP-1 remained unchanged after this period (Fig. 5D).

MKP-1 expression affects cAMP-stimulated steroid production by regulating ERK1/2 activity

ERK1/2 activities play a relevant role in LH/cAMP-stimulated steroid synthesis (24–26). To determine the relationship between MKP-1 induction and the regulation of steroid production, we performed experiments in cells exhibiting up- or down-regulated MKP-1 expression by transiently transfecting cells with vectors carrying MKP-1 cDNA (pRc/CMV-MKP-1) or sequences encoding siRNA against MKP-1 (siRNA1 or siRNA2), respectively. MKP-1 overexpression resulted in a significant reduction of cAMP-stimulated steroid synthesis in MA-10 cells, measured as progesterone (P4) in the culture media by RIA (Fig. 6A). MKP-1 down-regulation by the siRNA showed, however, variable effects on 8Br-cAMP-mediated stimulation of MA-10 cells. Although siRNA1 and siRNA2 had no effect on short-term 8Br-cAMP stimulation (30 min), they significantly increased P4 production after 2 or 6 h of stimulation, as compared both with the empty vector pRc/CMV (mock transfection) or to vectors carrying scrambled siRNA1 or 2 sequences (Scr1 or Scr2, respectively) (Fig. 6A). We have also verified the effectiveness of the different constructs on MKP-1 protein levels. Western blot analysis of MKP-1, performed in whole cell extracts from stimulated cells (8Br-cAMP, 1 h) after transfection, showed that the protein levels reached after 8Br-cAMP stimulation were increased in cells transfected with pRc/CMV-MKP-1 and decreased in cells transfected with siRNA1 or siRNA2, compared with cells transfected with the empty vector (Mock) or Scr1 or Scr2 (Fig. 6B). Because Mock-, Scr1-, and Scr2-transfected cells exhibited similar MKP-1 protein levels after stimulation (Fig. 6B) and P4 production both in control and stimulated conditions (Fig. 6A), only mock transfection was used as control in the following experiments.

The effect of up- or down-regulation of MKP-1 on cAMP-dependent ERK1/2 phosphorylation was evaluated by Western blotting at times corresponding to maximal ERK1/2 activation and maximal increase of MKP-1 mRNA, respectively. As shown in Fig. 6C, 8Br-cAMP increased pERK1/2 levels in a time-dependent manner and consistent with MKP-1-mediated ERK1/2 dephosphorylation (Fig. 1C). Indeed, pERK1/2 levels reached its maximum after 15 min of 8B-cAMP stimulation, declined thereafter, and returned to basal level after 120 min (Fig. 6C). Hence, we evaluated the effect of up- or down-regulation of MKP-1 on pERK1/2 levels after 15 min (maximal ERK1/2 activation) and 30 min (maximal increase of MKP-1 mRNA) (Fig. 1A) of 8Br-cAMP stimulation, respectively. MKP-1 overexpression reduced ERK1/2 phosphorylation induced by 15 min of 8Br-cAMP stimulation (Fig. 6D), whereas MKP-1 down-regulation by siRNA promoted an increase in the extent of ERK1/2 phosphorylation evaluated at 30 min of stimulation (Fig. 6D). These results suggest that MKP-1 might contribute to the regulation of steroid production through the modification of ERK1/2 activity.

MKP-1 expression reduces the effect of cAMP on both StAR promoter activity and mRNA levels

To test whether the inhibitory effect of MKP-1 on P4 production involves an action on StAR gene expression, we evaluated StAR promoter activity and mRNA levels in cells transiently transfected with pRc/CMV-MKP-1 or

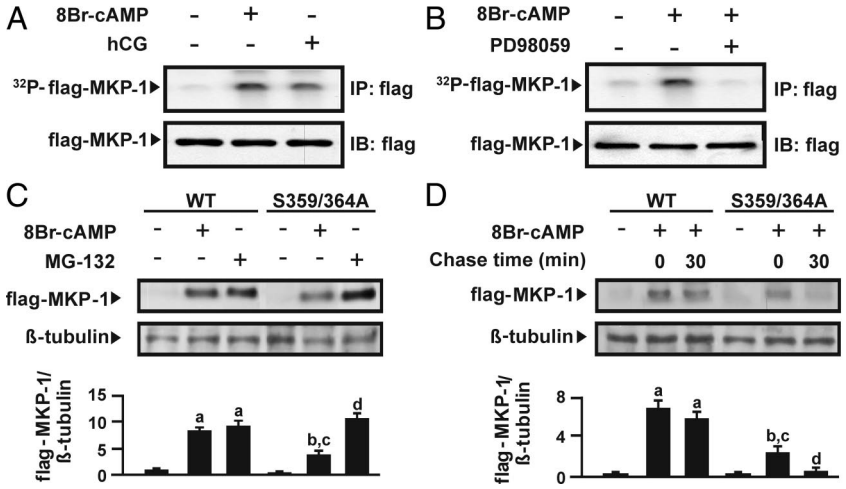

FIG. 5. hCG/cAMP promote MKP-1 phosphorylation by an ERK1/2-dependent mechanism in MA-10 cells. MA-10 cells were transfected with pFLAG-MKP-1, serum starved, and incubated with ^{32}P (500 μCi/ml) in phosphate-free medium. Cells were preincubated for 1 h with 10 μM MG-132 and stimulated with 0.5 mM 8Br-cAMP or 20 ng/ml hCG (A) or incubated for 30 min in the presence or the absence of 50 μM PD98059 and further incubated for 15 min with 8Br-cAMP (B). Cells were then homogenized and subjected to immunoprecipitation (IP) using an anti-FLAG M2 antibody. Immunoprecipitated flag-MKP-1 was resolved on SDS-PAGE and detected by autoradiography (*upper panels*) or protein immunoblotting (*lower panels*) with the anti-FLAG M2 antibody (IB). Figure shows representative results from three independent experiments. MA-10 Leydig cells were transfected with pFLAG-MKP-1 or with a double mutated form for overexpression of WT flag-MKP-1or its derivative S359A/S364A. After serum deprivation, cells were incubated in the absence or the presence of 0.5 mM 8Br-cAMP or 10 μM MG-132 for 1 h (C) or stimulated for 60 (0 chase time) or 90 min (30-min chase time) with 0.5 mM 8Br-cAMP plus 2 μg/ml cycloheximide, added 30 min after 8Br-cAMP (D). Cells were then harvested after 60 (0 chase time) or 90 min (30-min chase time) treatment, whereas control cells were incubated in the absence of 8Br-cAMP and harvested only after 60 min. Total cell lysates were subjected to electrophoresis and proteins analyzed by Western blotting using an anti-FLAG M2 antibody. Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped, and β-tubulin was detected using a specific antibody as loading control. Figure shows representative immunoblots (*upper panels*). Integrated optical density of each band was quantitated and WT or S359A/S364A flag-MKP-1 normalized against the loading control and expressed in arbitrary units. Data represent the mean ± SEM of three independent experiments (*lower panels*). C: a, $P < 0.001$ *vs.* nonstimulated WT flag-MKP-1; b, $P < 0.01$, *vs.* nonstimulated mutant flag-MKP1; c, $P < 0.001$ *vs.* stimulated WT flag-MKP-1; d, $P < 0.01$ *vs.* stimulated mutant flag-MKP-1. D: a, $P < 0.001$ *vs.* nonstimulated WT flag-MKP-1; b, $P < 0.05$ *vs.* nonstimulated mutant flag-MKP1; c, $P < 0.001$ *vs.* WT flag-MKP-1 at 0 chase time; d, $P < 0.01$, *vs.* mutant flag-MKP-1 at 0 chase time.

siRNA. As expected, 8Br-cAMP increased StAR promoter activity (pGL3-StAR) in mock-transfected cells, an effect that was reduced by PD98059 (Fig. 7A, *left panel*). MKP-1 overexpression, in turn, significantly reduced the effect of 8Br-cAMP on StAR promoter activity, whereas a significant increase in promoter activity was observed in cells transfected with siRNA1 (Fig. 7A, *right panel*) or siRNA2 (data not shown). Accordingly, variations in MKP-1 protein levels affected also 8Br-cAMP-induced StAR mRNA accumulation. As shown in Fig. 7B, MKP-1 overexpression significantly inhibited 8Br-cAMP-stimulated StAR mRNA induction, whereas siRNA down-regulation of the protein produced the opposite effect, as evidenced by real-time PCR (Fig. 7B).

Collectively, these results suggest that hCG-mediated MKP-1 induction plays an important role in controlling ERK1/2 activity and, consequently, StAR mRNA levels and steroid production in MA-10 Leydig cells.

Discussion

The present study confirms and extends our previous report showing a rapid and transient increase of MKP-1 mRNA in MA-10 Leydig cells exposed to 8Br-cAMP (17). In fact, here, we demonstrate, for the first time, that LHR stimulation by hCG tightly regulates MKP-1 protein expression, not only by promoting gene transcription but also by inducing posttranslational modifications, which increase MPK-1 protein accumulation. Also, we present evidence supporting a functional role of MKP-1 in steroidogenesis on the basis of its ability to reduce the effect of cAMP on StAR gene induction, a crucial ERK-dependent event in the hormonal regulation of steroidogenesis (26). Collectively, our data allow us to propose a model for the regulation of MKP-1 by hCG in MA-10 Leydig cells and for the role of this phosphatase in hCG/cAMP-stimulated steroidogenesis (Fig. 8).

Several reports, including ours, show that steroidogenic hormones, such as ACTH and AngII, increase MKP-1 mRNA and protein levels (8, 15, 16). Moreover, ACTH- and AngII-induced MKP-1 protein phosphorylation is also documented (15, 16). Here, we demonstrate that hCG-stimulated MKP-1 protein accumulation is linked to PKA- and ERK1/2-dependent posttranslational modifications and that hCG promotes the phosphorylation of MKP-1. Our results suggest that hCG-mediated phosphorylation is mainly an ERK1/2-dependent event and are in agreement with those of Casal *et al.* (16), who demonstrated that ERK1/2 inhibition blunted AngII-induced MKP-1 phosphorylation.

MKP-1 is a labile protein that is targeted for degradation by the ubiquitin-proteasome pathway (11). Accordingly, MKP-1 protein was not detected unless cells were stimulated with 8Br-cAMP. Even when overexpressed, the recombinant protein was barely detected unless cells were stimulated or treated with a proteasome inhibitor. Moreover, no additive effects were registered for the combina-

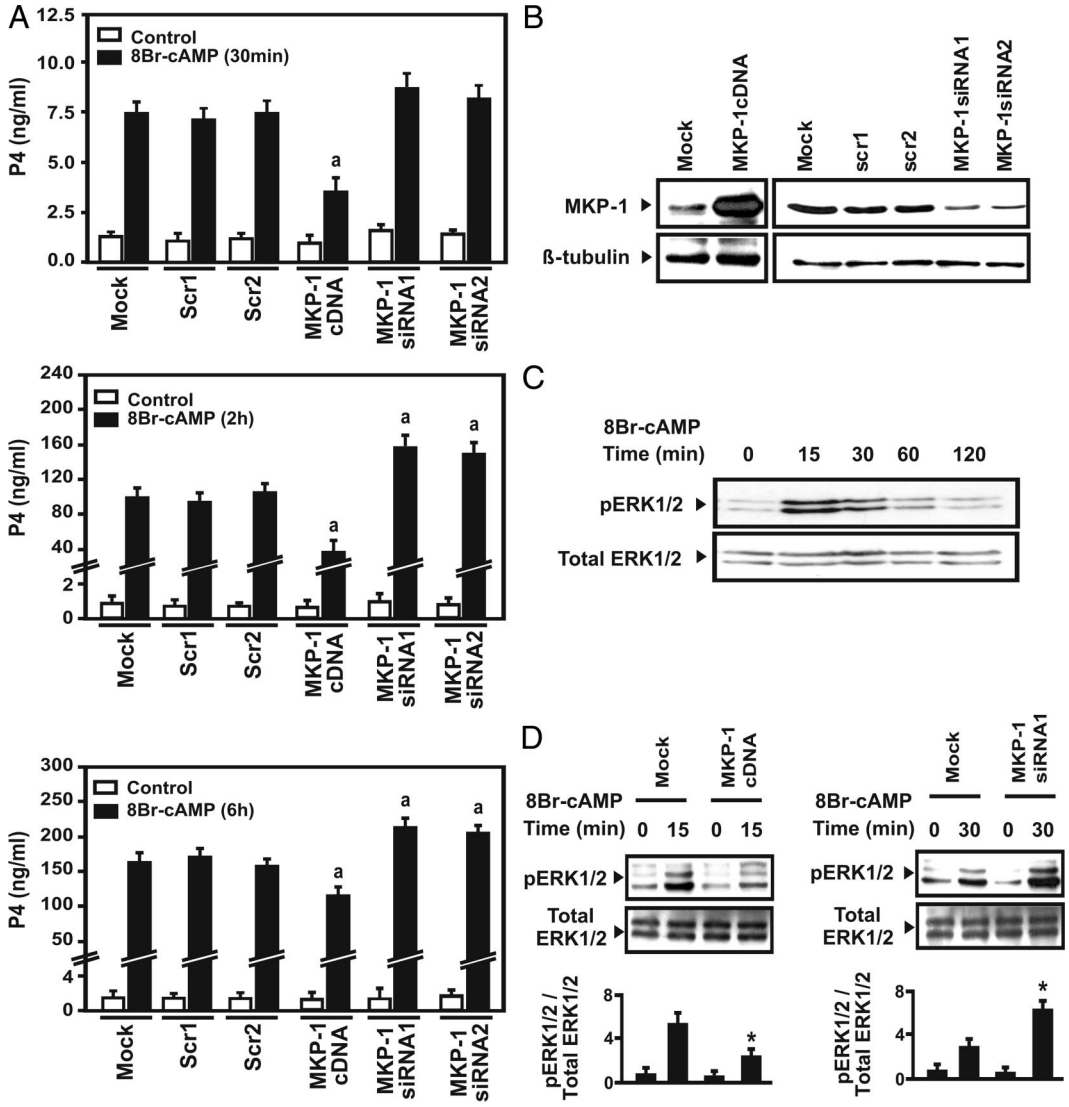

FIG. 6. MKP-1 reduces steroid production and ERK1/2 phosphorylation in cAMP-stimulated MA-10 cells. MA-10 cells were transfected (A, B, and D) or nontransfected (C), serum starved, and stimulated with 0.5 mM 8Br-cAMP for the indicated times (A and D) or for 1 h (B). Transfections included pRc/CMVi-MKP-1 (MKP-1 cDNA), pSUPER.retro-MKP-1 (MKP-1 siRNA1 or siRNA2), pSUPER.retro-scramble (Scr1 or Scr2), or the empty expression vector (Mock) as indicated. A, P4 levels were determined by RIA in the incubation media. Data represent the mean ± SEM of three independent experiments and are expressed as ng/ml. a, $P < 0.001$ *vs.* mock-transfected cells. B, C, and D, Cells were homogenized and total cell lysate proteins analyzed by Western blotting using an anti-MKP-1 antibody (B) or an anti-pERK1/2 antibody (C and D). Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped and β-tubulin (B) or ERK1/2 (C and D) were detected using specific antibodies as loading controls. Figure shows representative immunoblots (*upper panels*). Integrated optical density of each band was quantitated and MKP-1 or pERK1/2 values normalized against the corresponding loading controls and expressed in arbitrary units. Data represent the mean ± SEM of three independent experiments (*lower panels*). *, $P < 0.05$ *vs.* nonstimulated cells.

tion of 8Br-cAMP and the proteasome inhibitor. These observations raise the possibility that hCG and cAMP increase MKP-1 protein levels by reducing protein degradation through the proteasome pathway.

We show that ERK1/2 activation leads to MKP-1 phosphorylation and stabilization, whereas MKP-1 expression reduces the phosphorylation status of ERK1/2 promoted by 8Br-cAMP. These results support a reciprocal regulation between MKP-1 and ERK1/2 in MA-10 Leydig cells under hCG stimulation, as observed in other systems (2). It is possible that the intracellular concentrations, substrate affinity and binding of the enzymes involved in this reciprocal regulation will initially favor MKP-1 phosphorylation by ERK1/2, leading to MKP-1 accumulation. This event will then result in the dephosphorylation and consequent inactivation of ERK1/2 by the phosphatase (Fig. 8).

S359 and S364 are the major sites for ERK1/2 phosphorylation and responsible for a decrease in MKP-1 proteolysis (11). Indeed, S359 and S364 are involved in 8Br-cAMP-induced, ERK-mediated MKP-1 stabilization in MA-10 Leydig cells. However, sequence analysis of MKP-1

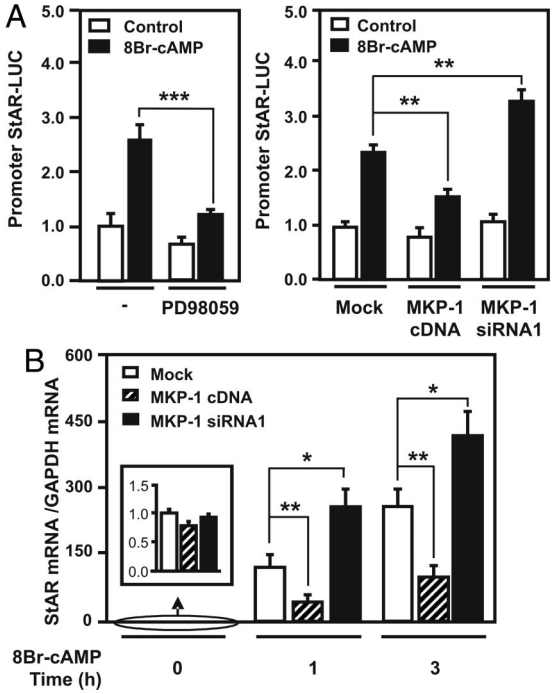

FIG. 7. MKP-1 decreases StAR promoter activity and mRNA expression in cAMP-stimulated MA-10 cells. A, MA-10 cells were transfected with pGL3-StAR and pRL *Renilla* luciferase vectors (*left panel*) or with pRc/CMVi-MKP-1 (MKP-1 cDNA), pSUPER.retro-MKP-1 (MKP-1 siRNA1), or the corresponding empty expression vector (Mock) as indicated (*right panel*). Cells were serum starved, stimulated for 6 h with 0.5 mM 8Br-cAMP in the presence or the absence of 50 μM PD98059, and cell extracts assayed for luciferase activity. Data represent the mean ± SEM of three independent experiments and are expressed in arbitrary units. B, MA-10 cells were transfected with MKP-1 cDNA, MKP-1 siRNA1, or the corresponding empty expression vector (Mock) as indicated. Cells were serum starved, stimulated for 1–3 h with 0.5 mM 8Br-cAMP, and total RNA isolated, reverse transcribed, and subjected to quantitative real-time PCR with specific primers for StAR and GAPDH cDNA as loading control. MKP-1 levels were normalized against the corresponding GAPDH mRNA levels. Data represent the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* mock-transfected cells; ***, $P < 0.001$ *vs.* nonstimulated cells.

reveals the existence of several consensus phosphorylation sites for ERK1/2 and *in vivo* phosphorylation in these different sites seems to affect MKP-1 stability in different ways (11, 35). Based on the results presented here, our current efforts are aimed to determine the role played by the different ERK1/2 phosphorylation consensus sites in hCG-mediated phosphorylation, stabilization, and subcellular localization of MKP-1.

We also provide evidence for ERK1/2-independent posttranslational modifications leading to hCG- and cAMP-mediated MKP-1 protein accumulation. Even when MKP-1 is known to be acetylated (12, 36), a link between MKP-1 acetylation and enhanced stabilization has not been established yet. Further studies are needed to determine the nature of ERK1/2-independent modifications that contribute to hCG-induced MKP-1 stabilization.

MKP-1 is described as a nuclear protein, although it lacks a classical nuclear targeting sequence (2). However, Wu *et al.* (37) identified, within MKP-1 amino terminus, a motif that mediates protein-protein interactions with nuclear-targeted hormone receptors and that could direct MKP-1 to the nucleus. Here, we demonstrate that flag-MKP-1 is entirely localized in the cytoplasm of nonstimulated cells, whereas it is equally distributed between the cytoplasm and the nucleus after hCG/cAMP stimulation. Moreover, flag-MKP-1 protein accumulated by inhibition of the proteasome reached the nuclear compartment mainly after 8Br-cAMP stimulation and by means of posttranslation modification(s). Whether hCG/cAMP trigger a posttranslational modification in MKP-1, which regulates its interaction with nuclear proteins and, thus, its nuclear localization is merely speculative. Therefore, elucidation of the true mechanism involved in the hormonal action on MKP-1 subcellular localization in MA-10 cells deserves further studies.

We have previously demonstrated the presence of MEK1/2 and ERK1/2 in the mitochondria of Leydig cells, which leads to ERK1/2-dependent StAR phosphorylation in this compartment after LH/cAMP stimulation (24). Because mitochondrial ERK1/2 activation is a transient event, the presence of MKP in this compartment was expected and confirmed by our work. Moreover, our finding is in agreement with that of Rosini *et al.* (34), who detected MKP-1 in the mitochondria of neurons upon nerve growth factor stimulation.

It is well documented that hCG-stimulated ERK1/2 phosphorylation is a rapid event that can be detected minutes after stimulation (24, 38). Although hCG-stimulated MKP-1 protein induction requires a longer stimulation time, our results show that the increase in MKP-1 protein levels coincides with ERK1/2 dephosphorylation. Because this event is completed after 60 min of stimulation, it follows that all ERK-dependent events necessary for full steroidogenesis occur during the first 60 min of stimulation. Taken together, these observations indicate that ERK1/2 activities play a crucial role in the early phase of steroid synthesis, even when evidenced in steroid production in a later phase. In agreement with this, we show here that down- or up-regulation of MKP-1 increases or decreases steroid production, respectively, through changes in pERK1/2 levels. Noteworthy, MKP-1 overexpression reduces P4 accumulation even after 6 h of stimulation, when MKP-1 protein levels have already decreased to basal levels. However, it should be noted that at 6 h or even 2 h after stimulation, the action of MKP-1 on pERK has already occurred.

Blocking ERK1/2 activation by PD98059 inhibits steroid synthesis (80–90% inhibition after 2 h of stimula-

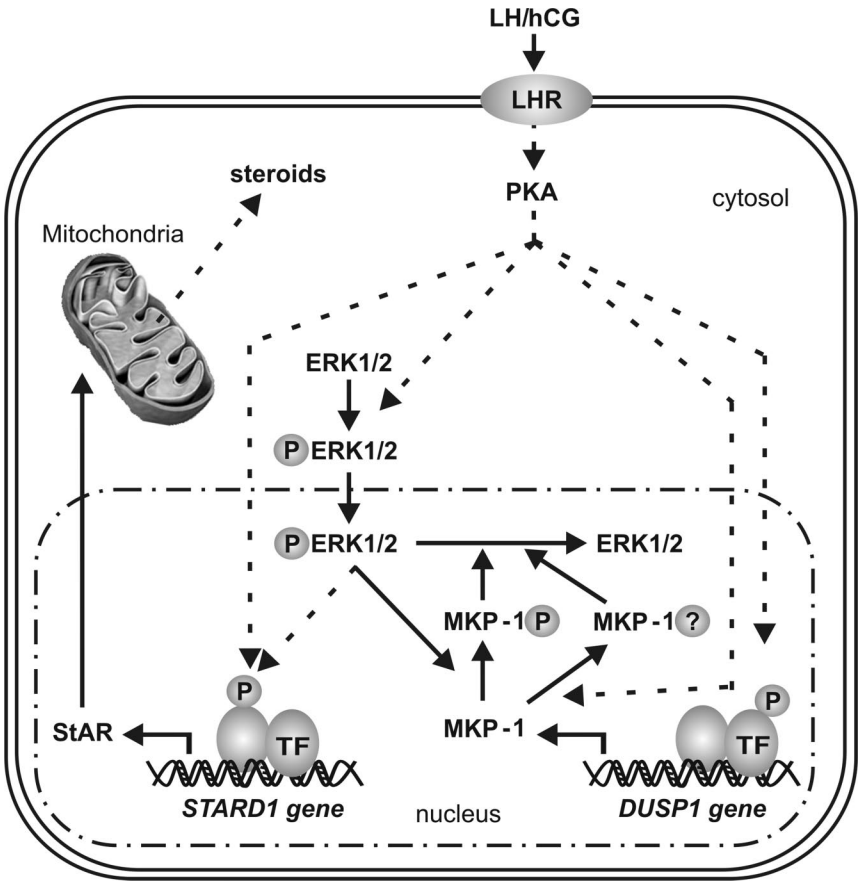

FIG. 8. Proposed model for the role of MKP-1 in steroidogenesis. Through its action on LHR, steroidogenic hormones trigger the activation of PKA, which leads to the rapid phosphorylation of MAPK ERK1/2. Activated ERK (P-ERK) translocates to the nucleus, where together with PKA, it phosphorylates and activates transcription factors (TF), leading to STARD1 induction. STARD1 product, StAR protein, acts in the mitochondria facilitating steroid synthesis. Simultaneously, activated PKA also induces MKP-1 gene (DUSP1) transcription. Activated PKA promotes also MKP-1 protein stabilization through a still unknown posttranslational mechanism. MKP-1 is also stabilized by ERK-mediated phosphorylation. Posttranslational modifications of MKP-1 lead to its accumulation in the cell. In turn, MKP-1 protein dephosphorylates ERK1/2, generating a negative feedback mechanism on its activity, thus terminating MAPK-regulated events involved in steroidogenesis and, as a consequence, decreasing steroid production. Direct effects are indicated by *solid lines*, whereas indirect effects are indicated as *dotted lines*. A *question mark* indicates an unknown posttranslational modification.

tion) (data not shown). Compared with this effect, MKP-1 overexpression produced a smaller reduction in cAMP-stimulated steroid synthesis (50%), which is in accordance with the effectiveness of the transfection system used in the present work.

The effect of MKP-1 expression on steroidogenesis could involve the down-regulation of mitochondrial ERK1/2 activity and, consequently, a decrease in StAR activity (24), a possibility that we have not analyzed in this work. On the other hand, the fact that ERK1/2 are involved in StAR transcription (25, 26) suggests that MKP-1 could modify steroid biosynthesis by acting on StAR gene induction. Our results showing a reduction of StAR promoter activity and mRNA levels are consistent with such hypothesis. Even when it was already demonstrated that MKP-1 expression modulates the effect of AngII on ERK activity and steroidogenesis in bovine glomerulosa cells (16), our findings demonstrate for the first time that MKP-1 down-regulates hCG-mediated StAR gene activation.

Several reports have shown controversial results on the involvement of ERK1/2 in StAR expression and steroid biosynthesis. Indeed, both inhibition and stimulation of steroidogenesis after inhibition of ERK1/2 pathway are reported (25, 26, 38, 39). The discrepancies on the role of ERK1/2 in StAR transcription might be due to different experimental conditions, which generate a different availability of factors, such as transcription factors, required for StAR expression. One such experimental condition is serum deprivation, a procedure used in this work. The results presented here are in agreement with those supporting a relevant role for ERK1/2 in StAR transcription and steroidogenesis.

The mechanism involved in MKP-1 regulation of StAR promoter activity awaits further characterization. It is known that StAR gene induction requires the hormone-dependent regulation of several transcription factors, such as steroidogenic factor 1 (SF1) and NUR77 (40), which are targets of ERK1/2. For example, LH stimulation of Leydig cells leads to the phosphorylation and activation of SF1 and increased StAR gene transcription (26). As for NUR77, its expression and phosphorylation by ERK1/2 has also been documented (41, 42). Interestingly, LH/cAMP rapidly and strongly induce this factor in Leydig cells (43, 44). Whether MKP-1 modulates those transcription factors acting on ERK1/2 activity in MA-10 Leydig cells deserves further investigation. Finally, it should be mentioned that the involvement of MKP-1 in the regulation of SF1-mediated gene transcription has been already documented (15, 45). Indeed, ACTH-mediated MKP-1 induction promotes SF1 dephosphorylation, which, in turn, leads to the increase of SF1-mediated CYP17 transcription in adrenocortical cells (46). Moreover, the regulation of ACTH receptor gene expression by ACTH involves a cyclic pattern of SF1-dependent transcriptional activation, a dynamic that could be modulated by MKP-1 expression (45).

In summary, we report here that hCG/cAMP regulate MKP-1 expression at several stages and that this regulation serves as a mechanism to modulate StAR gene induction and, consequently, steroid synthesis. However, additional work is required to fully elucidate the regulatory mechanism by which MKP-1 modulates hCG/cAMP-mediated StAR expression.

Acknowledgments

We thank Dr. Mario Ascoli (University of Iowa College of Medicine, Iowa City, IA) for kindly providing MA-10 cells, Dr. Colin Jefcoate (University of Wisconsin-Madison Medical School, Madison, WI) and Dr. Melanie Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX) for providing valuable plasmids, and Dr. Silvia González-Calvar and Dr. Candela González (Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina) for advice on real-time PCR analysis.

Address all correspondence and requests for reprints to: Cristina Paz, Ph.D., Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 5th Floor, C1121ABG Buenos Aires, Argentina. E-mail: crispaz@fmed.uba.ar.

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica Grants PICT 00909 and 00819, Universidad de Buenos Aires Grants M052 and M059, and by Consejo Nacional de Investigaciones Científicas y Técnicas Grants PIP 112-200801-00209 and 112-200801-01976 (to C.Pa. and E.J.P.).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Dickinson RJ, Keyse SM** 2006 Diverse physiological functions for dual-specificity MAP kinase phosphatases. J Cell Sci 119:4607–4615
2. **Boutros T, Chevet E, Metrakos P** 2008 Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. Pharmacol Rev 60:261–310
3. **Raman M, Chen W, Cobb MH** 2007 Differential regulation and properties of MAPKs. Oncogene 26:3100–3112
4. **Cagnol S, Chambard JC** 2010 ERK and cell death: mechanisms of ERK-induced cell death—apoptosis, autophagy and senescence. FEBS J 277:2–21
5. **Ashwell JD** 2006 The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol 6:532–540
6. **Fuller SJ, Davies EL, Gillespie-Brown J, Sun H, Tonks NK** 1997 Mitogen-activated protein kinase phosphatase 1 inhibits the stimulation of gene expression by hypertrophic agonists in cardiac myocytes. Biochem J 323(Pt 2):313–319
7. **Franklin CC, Kraft AS** 1997 Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. J Biol Chem 272:16917–16923
8. **Bey P, Gorostizaga AB, Maloberti PM, Lozano RC, Poderoso C, Maciel FC, Podestá EJ, Paz C** 2003 Adrenocorticotropin induces mitogen-activated protein kinase phosphatase 1 in Y1 mouse adrenocortical tumor cells. Endocrinology 144:1399–1406
9. **Gorostizaga A, Brion L, Maloberti P, Maciel FC, Podestá EJ, Paz C** 2005 Heat shock triggers MAPK activation and MKP-1 induction in Leydig testicular cells. Biochem Biophys Res Commun 327:23–28
10. **Tomasi ML, Ramani K, Lopitz-Otsoa F, Rodríguez MS, Li TW, Ko K, Yang H, Bardag-Gorce F, Iglesias-Ara A, Feo F, Pascale MR, Mato JM, Lu SC** 2010 S-adenosylmethionine regulates dual-specificity mitogen-activated protein kinase phosphatase expression in mouse and human hepatocytes. Hepatology 51:2152–2161
11. **Brondello JM, Pouysségur J, McKenzie FR** 1999 Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286:2514–2517
12. **Cao W, Bao C, Padalko E, Lowenstein CJ** 2008 Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. J Exp Med 205:1491–1503
13. **Le T, Schimmer BP** 2001 The regulation of MAPKs in Y1 mouse adrenocortical tumor cells. Endocrinology 142:4282–4287
14. **Hirakawa T, Ascoli M** 2003 The lutropin/choriogonadotropin receptor-induced phosphorylation of the extracellular signal-regulated kinases in leydig cells is mediated by a protein kinase a-dependent activation of ras. Mol Endocrinol 17:2189–2200
15. **Sewer MB, Waterman MR** 2003 CAMP-dependent protein kinase enhances CYP17 transcription via MKP-1 activation in H295R human adrenocortical cells. J Biol Chem 278:8106–8111
16. **Casal AJ, Ryser S, Capponi AM, Wang-Buholzer CF** 2007 Angiotensin II-induced mitogen-activated protein kinase phosphatase-1 expression in bovine adrenal glomerulosa cells: implications in mineralocorticoid biosynthesis. Endocrinology 148:5573–5581
17. **Gorostizaga A, Cornejo Maciel F, Brion L, Maloberti P, Podesta EJ, Paz C** 2007 Tyrosine phosphatases in steroidogenic cells: regulation and function. Mol Cell Endocrinol 265- 266:131–137
18. **Podesta EJ, Dufau ML, Catt KJ** 1976 Adenosine 3′,5′-monophosphate-dependent protein kinase of Leydig cells: in vitro activation and relationship to gonadotropin action upon cyclic AMP and steroidogenesis. FEBS Lett 70:212–216
19. **Clark BJ, Wells J, King SR, Stocco DM** 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J Biol Chem 269:28314–28322
20. **Stocco DM, Clark BJ** 1996 Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. Biochem Pharmacol 51:197–205
21. **Maloberti P, Castilla R, Castillo F, Maciel FC, Mendez CF, Paz C, Podestá EJ** 2005 Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis. FEBS J 272:1804–1814
22. **Hauet T, Yao ZX, Bose HS, Wall CT, Han Z, Li W, Hales DB, Miller WL, Culty M, Papadopoulos V** 2005 Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. Mol Endocrinol 19:540–554
23. **Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, Strauss 3rd JF** 1997 Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem 272:32656–32662
24. **Poderoso C, Converso DP, Maloberti P, Duarte A, Neuman I, Galli S, Maciel FC, Paz C, Carreras MC, Poderoso JJ, Podestá EJ** 2008 A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis. PLoS One 3:e1443
25. **Martinelle N, Holst M, Söder O, Svechnikov K** 2004 Extracellular signal-regulated kinases are involved in the acute activation of steroidogenesis in immature rat Leydig cells by human chorionic gonadotropin. Endocrinology 145:4629–4634
26. **Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud**

SJ, Jones PM 2001 ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. J Biol Chem 276:34888–34895
27. **Ascoli M** 1981 Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. Endocrinology 108:88–95
28. **Castillo AF, Maciel FC, Castilla R, Duarte A, Maloberti P, Paz C, Podestá EJ** 2006 cAMP increases mitochondrial cholesterol transport through the induction of arachidonic acid release inside this organelle in Leydig cells. FEBS J 273:5011–5021
29. **Castilla R, Gadaleta M, Castillo AF, Duarte A, Neuman I, Paz C, Cornejo Maciel F, Podestá EJ** 2008 New enzymes involved in the mechanism of action of epidermal growth factor in a clonal strain of Leydig tumor cells. Endocrinology 149:3743–3752
30. **Eisenberg E, Levanon EY** 2003 Human housekeeping genes are compact. Trends Genet 19:362–365
31. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
32. **Alonso M, Melani M, Converso D, Jaitovich A, Paz C, Carreras MC, Medina JH, Poderoso JJ** 2004 Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development. J Neurochem 89:248–256
33. **Stocco DM** 1983 Rapid, quantitative isolation of mitochondria from rat liver using Ficoll gradients in vertical rotors. Anal Biochem 131:453–457
34. **Rosini P, De Chiara G, Bonini P, Lucibello M, Marcocci ME, Garaci E, Cozzolino F, Torcia M** 2004 Nerve growth factor-dependent survival of CESS B cell line is mediated by increased expression and decreased degradation of MAPK phosphatase 1. J Biol Chem 279: 14016–14023
35. **Lin YW, Yang JL** 2006 Cooperation of ERK and SCFSkp2 for MKP-1 destruction provides a positive feedback regulation of proliferating signaling. J Biol Chem 281:915–926
36. **Chi H, Flavell RA** 2008 Acetylation of MKP-1 and the control of inflammation. Sci Signal 1:pe44
37. **Wu JJ, Zhang L, Bennett AM** 2005 The noncatalytic amino terminus of mitogen-activated protein kinase phosphatase 1 directs nuclear targeting and serum response element transcriptional regulation. Mol Cell Biol 25:4792–4803
38. **Manna PR, Jo Y, Stocco DM** 2007 Regulation of Leydig cell steroidogenesis by extracellular signal-regulated kinase 1/2: role of protein kinase A and protein kinase C signaling. J Endocrinol 193: 53–63
39. **Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss 3rd JF, Amsterdam A** 2001 The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. J Biol Chem 276:13957–13964
40. **Martin LJ, Tremblay JJ** 2009 The nuclear receptors NUR77 and SF1 play additive roles with c-JUN through distinct elements on the mouse Star promoter. J Mol Endocrinol 42:119–129
41. **Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E** 2002 Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638–1651
42. **Wingate AD, Arthur JS** 2006 Post-translational control of Nur77. Biochem Soc Trans 34:1107–1109
43. **Martin LJ, Boucher N, El-Asmar B, Tremblay JJ** 2009 cAMP-induced expression of the orphan nuclear receptor Nur77 in MA-10 Leydig cells involves a CaMKI pathway. J Androl 30:134–145
44. **Martin LJ, Boucher N, Brousseau C, Tremblay JJ** 2008 The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca2+/calmodulin-dependent protein kinase I. Mol Endocrinol 22:2021–2037
45. **Winnay JN, Hammer GD** 2006 Adrenocorticotropic hormone-mediated signaling cascades coordinate a cyclic pattern of steroidogenic factor 1-dependent transcriptional activation. Mol Endocrinol 20:147–166
46. **Sewer MB, Waterman MR** 2003 ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc Res Tech 61:300–307