

MAPK Phosphatase-2 (MKP-2) Is Induced by hCG and Plays a Role in the Regulation of CYP11A1 Expression in MA-10 Leydig Cells

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MAPKs such as ERK1/2 are dephosphorylated, and consequently inactivated, by dual specificity phosphatases (MKPs). In Leydig cells, LH triggers ERK1/2 phosphorylation through the action of protein kinase A. We demonstrate that, in MA-10 Leydig cells, LH receptor activation by human chorionic gonadotropin (hCG) up-regulates MKP-2, a phosphatase that dephosphorylates ERK1/2, among other MAPKs. After 2 hours, hCG and 8-bromo-cAMP (8Br-cAMP) significantly increased MKP-2 mRNA levels (3-fold), which declined to basal levels after 6 hours. MKP-2 protein accumulation exhibited a similar kinetic profile. In cells transiently expressing flag-MKP-2 protein, hCG/8Br-cAMP stimulation promoted the accumulation of the chimera (2.5-fold after 3 h of stimulation). Pharmacologic and biochemical approaches showed that the accumulation of flag-MKP-2 involves a posttranslational modification that increases MKP-2 half-life. MKP-2 down-regulation by a short hairpin RNA (MKP-2 shRNA) raised the levels of phosphorylated ERK1/2 reached by 8Br-cAMP stimulation. This effect was evident after 180 min of stimulation, which suggests that MKP-2 down-regulates the late phase of cAMP-induced ERK1/2 activity. Also, MKP-2 down-regulation by MKP-2 shRNA increased the stimulatory effect of 8Br-cAMP on both promoter activity and messenger levels of CYP11A1, which encodes for the steroidogenic enzyme P450_{scc} and is induced by LH/hCG through protein kinase A and ERK1/2 activities. Our findings demonstrate, for the first time, that LH/hCG tightly regulates MKP-2 expression, which modulates the induction of CYP11A1 by 8Br-cAMP. MKP-2 up-regulation might control ERK1/2 activity in a specific temporal frame to modulate the expression of a finite repertoire of ERK-dependent genes. (*Endocrinology* 154: 1488–1500, 2013)

The MAPK phosphatases (MKPs) constitute a family of dual specificity phosphatases that down-regulate the activity of MAPK family members, such as ERK1/2, c-Jun NH₂-terminal protein kinases (JNK1/2) and p38, through tyrosine and threonine dephosphorylation (1–3). MAPKs

are key regulators of several biological processes like proliferation, development, apoptosis, differentiation, and inflammation (4–6). Therefore, the role of MKPs as regulatory components of such processes is now well recognized.

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Abbreviations: 8Br-cAMP, 8-bromo-cAMP; CHX, cycloheximide; cytochrome; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; hCG, human chorionic gonadotropin; JNK1/2, c-Jun NH₂-terminal protein kinase; LHR, LH receptor; MG 132, Z-Leu-Leu-Leu-al; MKP, MAPK phosphatase; NLS, nuclear localization sequence; PD98059, 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one; P-ERK1/2: phospho-ERK1/2; PKA, protein kinase A; P450_{scc}, cholesterol side-chain cleavage enzyme; Scr, scrambled; SF1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein.

MKP family members can be subdivided into different groups according to their catalytic properties and subcellular localization (2). One group includes nuclear phosphatases such as MKP-1 and MKP-2. These enzymes display a rather broad specificity for inactivation of ERK1/2, JNK1/2, and p38 and are induced by several stimuli albeit with different kinetics. Indeed, MKP-1 mRNA levels are increased after 15–20 minutes of stimulation whereas the increase in MKP-2 mRNA levels requires a longer period of time (2, 3, 7).

Due to their nuclear localization, MKP-1 and MKP-2 play an important role in the regulation of gene transcription. Indeed, both enzymes are responsible for nuclear MAPK inactivation and, ultimately, for regulating the activity and/or expression of MAPK-dependent transcription factors (2, 3, 8).

In steroidogenic cells, the corresponding trophic hormones trigger MAPK signaling pathways (9, 10). Accordingly, we and others have shown that ACTH (11, 12) and angiotensin II (13) increase MKP-1 mRNA and protein levels in adrenocortical cells. Moreover, we have recently demonstrated that, in MA-10 Leydig cells, the stimulation of the LH receptor (LHR) with human chorionic gonadotropin (hCG) promotes both MKP-1 gene transcription and posttranslational modifications leading to transient MKP-1 protein accumulation (14). In addition, we demonstrated that MKP-1 induction contributes to the down-regulation of steroid production (14). Although there is certain evidence of MKP-1 regulation and function in steroidogenic cells, to our knowledge, information about MKP-2 in these cells is not yet available.

LH regulates Leydig cell function through a mechanism that involves the activation of both protein kinase A (PKA) and ERK1/2 in a PKA-dependent fashion (10, 15). 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 (16, 17). LH regulates steroidogenesis through the induction of StAR gene (STARD1) (16, 17) and the activation of StAR protein (18, 19). LH also regulates the expression of other steroidogenic genes (20, 21) such as CYP11A1, which encodes for the enzyme involved in the first and common step of steroid synthesis in all tissues: the cholesterol side-chain cleavage enzyme cytochrome P450_{scc} (20, 21). In Leydig cells, CYP11A1 expression is high in basal conditions and increases in response to cAMP, hCG, and LH, although it does so later than STARD1 (21, 22). Although it is well documented that ERK1/2 participate in STARD1 expression (14, 22) and StAR protein activation (19), the role of these kinases in CYP11A1 expression in Leydig cells has been scarcely analyzed. However, a recent study demon-

strates the participation of ERK1/2 in the expression of CYP11A1. Indeed, in Leydig cells derived from mice carrying a Leydig cell-specific deletion of the MAPK kinase upstream ERK1/2, hCG fails to induce ERK1/2 phosphorylation and CYP11A1 exhibits a lower expression (23). Therefore, in addition to PKA activity, the expression of CYP11A1 involves ERK1/2 signaling and might, as a consequence, be modulated by MKPs.

The aim of the present study was to determine whether hCG regulates MKP-2 expression and the impact of this regulation on hCG-induced ERK phosphorylation and CYP11A1 expression. We report that hCG increases MKP-2 protein levels through transcriptional and post-translational mechanisms and that this effect contributes to down-regulating ERK1/2 activity. Also, we report that MKP-2 overexpression reduces the effects of cAMP on CYP11A1 promoter activity and expression, whereas its down-regulation by a specific short hairpin RNA (shRNA) produces opposite effects. Because MKP-2 is expressed later than MKP-1, we conclude that these enzymes act within different temporal frames to “turn off” ERK1/2 activity and, consequently, to modulate the action of hCG—and hence, LH—on the expression of key steroidogenic genes and steroidogenesis.

Materials and Methods

Reagents

N-[2-(p-bromocynnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), Z-Leu-Leu-Leu-al (MG132), 4H-1-benzopyran-4-one (PD98059), actinomycin D, cycloheximide (CHX), 8-bromo-cAMP (8Br-cAMP), and monoclonal antibody against FLAG M2 were purchased from Sigma Chemical Co. (St Louis, Missouri). Antibody against MKP-2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Antibodies against phospho-ERK1/2 (P-ERK1/2), ERK1/2, and Histone H2B were from New England Biolabs, Inc. (Beverly, Massachusetts). Monoclonal mouse against β -tubulin was purchased from Upstate Group, Inc (Temecula, California). Horseradish peroxidase-conjugated goat-antirabbit and goat-antimouse secondary antibodies and Immun-Blot polyvinylidene difluoride membrane were purchased Bio-Rad Laboratories (Hercules, California). All other reagents were of the highest quality available.

Plasmid constructs

The murine CYP11A1 promoter fused to the firefly luciferase reporter gene construct (pGL3-CYP11A1) was generously provided by Dr. Bon-Chu Chung (Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan).

FLAG-tagged MKP-2 expression vector (pFLAG-MKP-2) was constructed using the p3xFLAG-CMV7.1 vector. Mouse total RNA was used to obtain total cDNA by reverse transcription. From this sample, a fragment of 1294 kb corresponding to the full coding region of MKP-2 (NM_176933.4)

was amplified using the following primers: forward, TTGCG-GCCGCTATGGTGACGATGGAG (which contains the cleavage site for *NotI*) and reverse, AAGGATCCGACTCCTGCA-CACACATGCTC (which contains the cleavage site for *Bam*HI). This fragment was purified and fused in p3xFLAG-CMVTM-7.1 *NotI/Bam*HI site.

shRNA plasmid vector was constructed as follows: a pair of 60-nucleotide annealed DNA oligonucleotides (containing a 17-nucleotide target sequence derived from murine MKP-2 mRNA) was inserted between the *Bgl*III/*Hind*III restriction sites of the pSUPER.retro vector (OligoEngine, Seattle, Washington) in order to express short hairpin small interfering RNA (shRNA) under the control of the polymerase-III H1-RNA promoter. The set of 60-nucleotide oligos containing this sequence for MKP-2 shRNA1 is described below: sense, 5'-GATCCCCAGACTGTCCCAATCACTTTTCAAGAGAA AAGTGATTGGGA CAGTCTT TTTTA-3'; and antisense, 5'-AGCTTAAAAAAGACTGTCCCAATCACTTTTCTCTTG AAAAAGTGATTGGGACAG TCTGGG-3'. A second shRNA was used (targeting 821-839 bp of MKP-2 cDNA, MKP-2 shRNA2). Control shRNA was also designed as scrambled (Scr) sequences of MKP-2 shRNA1 and MKP-2 shRNA2: 5'-ATCACAAGTTTGCTCCC-3' and 5'-AAGTTCTCCCACTGATT-3', Scr1 and Scr2, respectively. Correct in-frame insertions were verified by sequencing.

Cell culture

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 City, Iowa), was handled as originally described (24, 25).

Cells were growth arrested by serum starvation for 24 hours. After replacing the medium with fresh serum-free medium, the cells were incubated with or without hCG or 8Br-cAMP or MG 132 or Actinomycin D or 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, and then transiently transfected for 6 hours with Lipofectamine 2000 reagent (Invitrogen, San Diego, California) in Opti-MEM medium, according manufacturer's instructions. After 24 hours in culture in serum-free medium, cells were used for experiments. For luciferase assays, transfections were carried out in 24-well plates using 0.6 μ g of pFLAG-MKP-2, MKP-2 shRNA1 or MKP-2 shRNA2, or Scr vectors; 0.2 μ g of pGL3-CYP11A1 vector and 10 ng of pRL *Renilla* luciferase expression vector as an internal control for transfection efficiency.

CYP11A1 promoter activity was evaluated in cells treated with 0.5 mM 8Br-cAMP for 12 hours to achieve submaximal stimulation. Luciferase activity was measured using the Dual Luciferase Assay System (Promega Corp, Madison, Wisconsin) and the Synergy HT luminometer (BioTek Instruments, Inc, Winooski, Vermont).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TriZol reagent following manufacturer's instructions (Life Technologies, Gaithersburg, Maryland). The reverse transcription (RT) and PCR were conducted as previously described (26). The RT and quantitative real-time PCR

analyses were done using 2 μ g of total RNA. cDNA was amplified in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems [Foster City, California], Life Technologies). Reactions were performed using the SYBR Green Master Mix reagent kit (Applied Biosystems) and the following specific primers at a concentration of 0.3 μ M in each reaction: MKP-2 cDNA forward, 5'-GCCTACCTGATGAAGAAGCG-3' and reverse, 5'-TGGT-GAGCACCTGAGACTCA-3'; CYP11A1 cDNA forward, 5'-GTGCCTCCAGACTTCTTTTCG-3' and reverse, 5'-TTCTT-GAAGGGCAGCTTGTT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA forward, 5'-TGCACCAC-CAACTGCTTAGC-3' and reverse, 5'-GGCATGGAC TGTTGTCATGAG-3'.

Cycling conditions were as follows: step 1, 10 minutes at 95°C; step 2, 15 seconds at 95°C; step 3, 60 seconds at 60°C for CYP11A1 and GAPDH or 51°C for MKP2, repeated 40 times from step 2 to step 3. Data from the reactions were collected and analyzed by the Sequence Detection Software (version 1.3; Applied Biosystems). Melting curves were run to confirm specificity of the signal. Relative quantification of gene expression was calculated using standard curves and normalized to GAPDH in each sample. For the assessment of quantitative differences between samples in the cDNA target, the mathematical model of Pfaffl (27) was applied. An expression ratio was determined for each sample by calculating $(E_{\text{target}})^{\Delta C_t(\text{target})} / (E_{\text{GAPDH}})^{\Delta C_t(\text{GAPDH})}$, where E is the efficiency of the primer set and $\Delta C_t = C_t(\text{normalization cDNA}) - C_t(\text{experimental cDNA})$. The amplification efficiency of each set of primers was calculated from the slope of a standard amplification curve of $\log(\text{ng cDNA})$ per reaction vs C_t value ($E = 10 - [1/\text{slope}]$). Efficiency values of 2.0 ± 0.1 were considered optimal.

Subcellular fractioning

Subcellular fractions were obtained by differential centrifugation. Cells were washed in PBS and harvested in isotonic homogenization buffer, A Buffer (20 mM HEPES [pH 7.4], 250 mM sucrose, 1 mM EDTA, 10 mM KCl, 1.5 mM MgCl_2) supplemented with protease and phosphatase inhibitors. Samples were then homogenized by mechanical disruption with a pellet pestle motor (Kontes, Vineland, New Jersey) and passed through a 75 μ m-pore filter to remove unbroken cells. The homogenates were centrifuged at $1000 \times g$ for 10 minutes. The pellet containing nuclei was resuspended in A Buffer and later submitted to sonication. The supernatant was centrifuged at $105,000 \times g$ for 60 minutes in order to obtain cytosolic fractions as the supernatant. All steps were performed on ice at 4°C. Purity of subcellular fractions obtained 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 polyvinylidene difluoride membranes as previously described (11).

Immunodetection was performed using the following antibody dilutions: mouse monoclonal anti-FLAG (1:10,000), rabbit polyclonal anti-phospho-ERK1/2 (1:5000), rabbit polyclonal anti-MKP-2 (1:1000), mouse monoclonal anti- β -tubulin (1:5000), rabbit polyclonal anti-ERK1/2 (1:20,000), rabbit poly-

clonal anti-Histone H2B (1:5000). Bound antibodies were developed by incubation with secondary antibody (goat antirabbit and goat antimouse horseradish peroxidase conjugated) and detected by chemiluminescence using enhanced chemiluminescence detection reagent (GE Life Sciences, Princeton, New Jersey and Bio-Lumina reagent from Productos Bio-Lógicos (Buenos Aires, Argentina).

Pulse and chase assays

MA-10 Leydig cells were seeded into a 12-well plate. After overnight culture, cells were transfected with pFLAG-MKP-2 and serum starved. After 24 hours, they were stimulated or not with 0.5 mM 8Br-cAMP for 150 min and then incubated with 2 $\mu\text{g}/\mu\text{L}$ of CHX for different times. Cells were processed at different times after CHX treatment, and total protein was subjected to immunoblotting using FLAG antibody and β -tubulin antibody after the stripping. The relative levels of flag-MKP-2 were expressed as a percentage, taking the value registered after 30 min of CHX treatment (time 0) as 100%.

Immunofluorescence and microscopy

MA-10 cells were grown on poly-L-lysine glass coverslips and treated as previously described (28).

The glass coverslips were incubated with rabbit polyclonal antibody anti-MKP-2 or mouse monoclonal antibody anti-FLAG in a humidified chamber for 24 hours at 4°C. Primary antibodies were detected by cy-2-conjugated goat antirabbit IgG or cy-2-conjugated goat antimouse IgG, respectively (Molecular Probes, Eugene, Oregon). Glass coverslips were mounted in FluorSave reagent (Calbiochem, La Jolla, California) and examined in an Olympus FV300 laser scanning confocal microscope.

Statistical analysis

Results are shown as the mean \pm SEM. Statistical significance was evaluated using ANOVA followed by Tukey test. Differences were deemed significant when $P < .05$.

Results

hCG and 8Br-cAMP increase MKP-2 gene transcription

MKP-2 mRNA levels from stimulated cells were evaluated by real-time PCR. LHR stimulation with hCG (20 ng/mL) produced a transient increase in MKP-2 mRNA levels. The effect was statistically significant and maximum after 2 hours (\sim 3-fold) and declined to basal levels after 6 hours (Figure 1A). The kinetic profile of mRNA accumulation caused by 8Br-cAMP (0.5 mM) resembled the profile obtained under hCG stimulation (Figure 1B). In addition, the increase in MKP-2 mRNA levels was dependent on hCG concentration. As shown in Figure 1C, the minimal concentration that was effective in increasing messenger levels was 5 ng/mL. Actinomycin D abolished the effect of hCG, which indicates that LHR stimulation promotes MKP-2 gene transcription (Figure 1D). H-89, a PKA inhibitor, as well as PD98059, a MAPK kinase up-

stream ERK1/2 inhibitor, blunted MKP-2 gene induction by hCG, which suggests that the hormonal effect is a PKA- and ERK1/2-dependent event (Figure 1D).

MKP-2 protein levels were also increased after hCG stimulation, which caused a significant increase in MKP-2 protein levels after 3 hours (\sim 10-fold). This increase remained fairly constant for the next hour (Figure 1E).

Posttranslational modifications triggered by hCG/cAMP increase MKP-2 protein stability

In order to test whether hCG regulates the levels of MKP-2 by posttranslational modifications, cells were transiently transfected for the overexpression of recombinant flag-MKP-2 protein, the expression of which is driven by a constitutively active promoter. Thus, monitoring recombinant protein levels is a useful approach to assessing posttranslational effects of hCG, regardless of its action on MKP-2 gene expression.

Western blot analysis of whole-cell extracts showed an hCG- and 8Br-cAMP-promoted increase of flag-MKP-2 protein levels that was evident at 2 hours, peaked between 3 and 5 hours (\sim 2.5-fold), and declined to basal levels at 7 hours (Figure 2, A and B). In addition, 8Br-cAMP stimulation had no effect on the accumulation of other flag-tagged proteins such as flag-ERK2, which indicates that the effect of hCG/cAMP on flag-MKP-2 is specific for the MKP-2 moiety of the chimera (Figure 2C). These results and the fact that the chimera is expressed under a constitutive promoter suggest that the accumulation of flag-MKP-2 by hCG or 8Br-cAMP is due to an increase in MKP-2 half-life mediated by these stimuli. The preincubation of the cells with H-89, but not with PD98059, blocked the accumulation of flag-MKP-2 triggered by 3 hours of hCG (Figure 2D) or 8Br-cAMP stimulation (Figure 2E). As expected, the addition of H-89 with or without PD98059 had the same effect (Figure 2, D and E). Then, we tested whether the hormonal regulation of MKP-2 includes a posttranslational modification that increases the protein half-life. Pulse and chase experiments, performed according to the incubation scheme shown at the top of the figure, demonstrated that 8Br-cAMP increases flag-MKP-2 half-life (Figure 3A). Moreover, hCG treatment produced the same effect (data not shown). These results are in accordance with the accumulation of recombinant protein by these stimuli shown in Figure 2.

The levels of flag-MKP-2 were evaluated in cells incubated with MG132, a well-recognized proteasome inhibitor. The results showed that MG 132 is able to accumulate flag-MKP-2 protein (Figure 3B). This effect was significant after 1 hour and progressed in a time-dependent way. Even after 3 hours of treatment, serine-protease

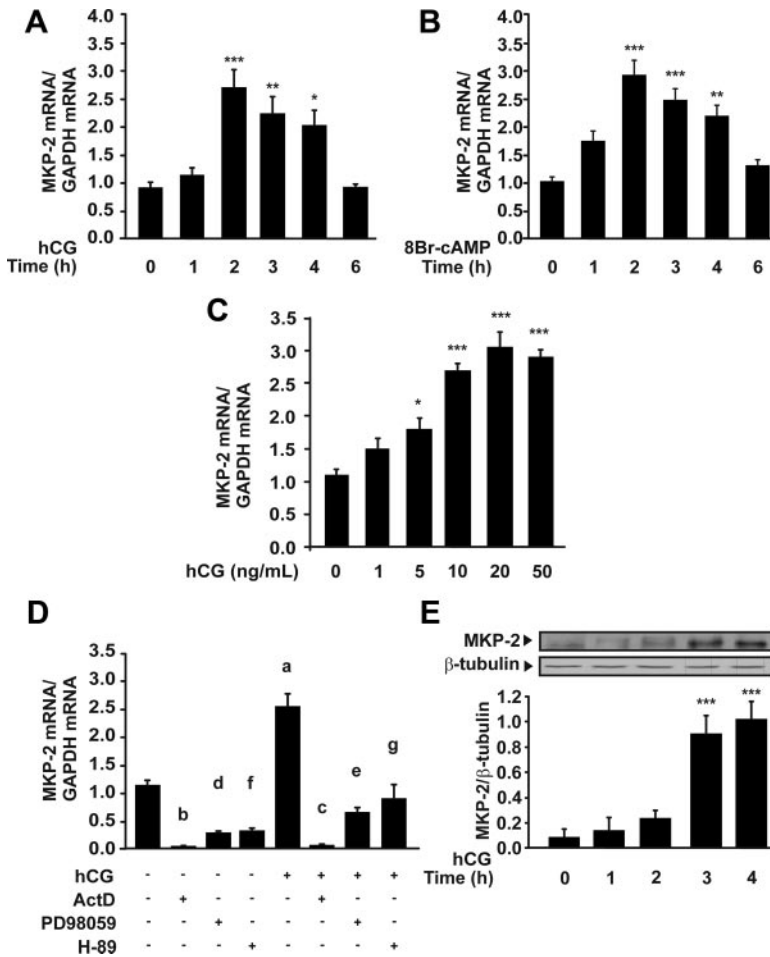


Figure 1. hCG and 8Br-cAMP Increase MKP-2 Expression in MA-10 Leydig Cells. MA-10 Leydig cells were serum starved and then stimulated with 20 ng/mL hCG (A) or 0.5 mM 8Br-cAMP (B) for the indicated times or incubated for 120 minutes with different concentrations of hCG (C), or for 30 minutes in the absence or presence of 5 μ g/mL actinomycin D (ActD), 50 μ M PD98059, or 10 μ M H-89 and further incubated for 120 minutes in presence or absence of 20 ng/mL hCG (D). Total RNA was isolated and subjected to reverse transcription and real-time PCR with specific primers for MKP-2 and GAPDH cDNA as loading control. MKP-2 mRNA levels were normalized against the corresponding GAPDH mRNA levels. Data represent the mean \pm SEM of three independent experiments. A, B, and C, * P < .05; ** P < .01; *** P < .001 vs nonstimulated cells. D, a, P < .001 vs nonstimulated cells; b, P < .01, d and f, P < .05 vs nonstimulated cells; c, e, and g, P < .001 vs hCG-stimulated cells. MA-10 Leydig cells were stimulated with 20 ng/mL hCG for the indicated times. Total cell lysates were analyzed by Western blotting using an anti-MKP-2 antibody (E). Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped, and β -tubulin was detected as loading control, using a specific antibody. Figure shows representative immunoblots (upper panels). Integrated optical density of each band was quantitated and MKP-2 values normalized against the loading control and expressed in arbitrary units. Data represent the mean \pm SEM of three independent experiments (lower panels). ***, P < .001 vs nonstimulated cells.

inhibitor Tosyllysine Chloromethyl Ketone (TLCK) did not increase the recombinant protein levels (Figure 3B). Flag-MKP-2 levels accumulated after 3 hours of 8Br-cAMP and MG132 treatment were similar and were not increased when the cells were treated with both reagents simultaneously (Figure 3C).

Flag-MKP-2 is accumulated in the nucleus after cell stimulation

We also analyzed MKP-2 in MA-10 cells by immunofluorescence and confocal microscopy. We found that, in basal conditions, MKP-2 is barely detected (Figure 4A, left), in concordance with the low levels of this protein detected by Western blot in nonstimulated cells (Figure 1E). The stimulation with 8Br-cAMP (Figure 4A, left) intensified MKP-2 signal and a predominantly nuclear localization was observed. Similar results were obtained in hCG-stimulated cells (data not shown). Because we intended to use the recombinant protein flag-MKP-2 in order to test the biological role of MKP-2, we analyzed the subcellular localization of the tagged protein in stimulated or control cells (Figure 4A, right), in order to confirm that this chimera and the endogenous protein behave in the same way. Flag-MKP-2 was even detected in basal conditions, which coincides with the detection of the recombinant protein in nonstimulated cells by Western blot (Figure 2B). Noteworthy, in basal conditions the protein was detected in the extranuclear compartment. 8Br-cAMP increased flag-MKP-2 signal, predominantly in the nucleus.

The fact that the nuclear localization is observed mainly in stimulated cell leads us to hypothesize that the hormonal stimulation could direct MKP-2 to the nucleus. To investigate this possibility, cells were treated to accumulate flag-MKP-2 in the absence of hormonal stimulation and then analyzed by immunofluorescence and confocal microscopy. The accumulation of the recombinant protein was performed by the incubation with MG132 for 1 hour. Then, protein synthesis was stopped by the addition of CHX to the culture media for 30 minutes. After that, 8Br-cAMP was added or not to the media and the incubation was prolonged for 15 minutes. Immunostaining of control cells showed a strong signal mainly in the extranuclear space (Figure 4B). However, this distribution changed after stimulation, because a short period of 8Br-cAMP treatment resulted in a weak signal in the extranuclear compartment and a strong signal in the nucleus (Fig 4B). Given that the stimulation was performed for a short period and in the presence of a protein synthesis inhibitor, the protein accumulated in the nucleus was not synthesized during this period of stimulation. Rather, accumu-

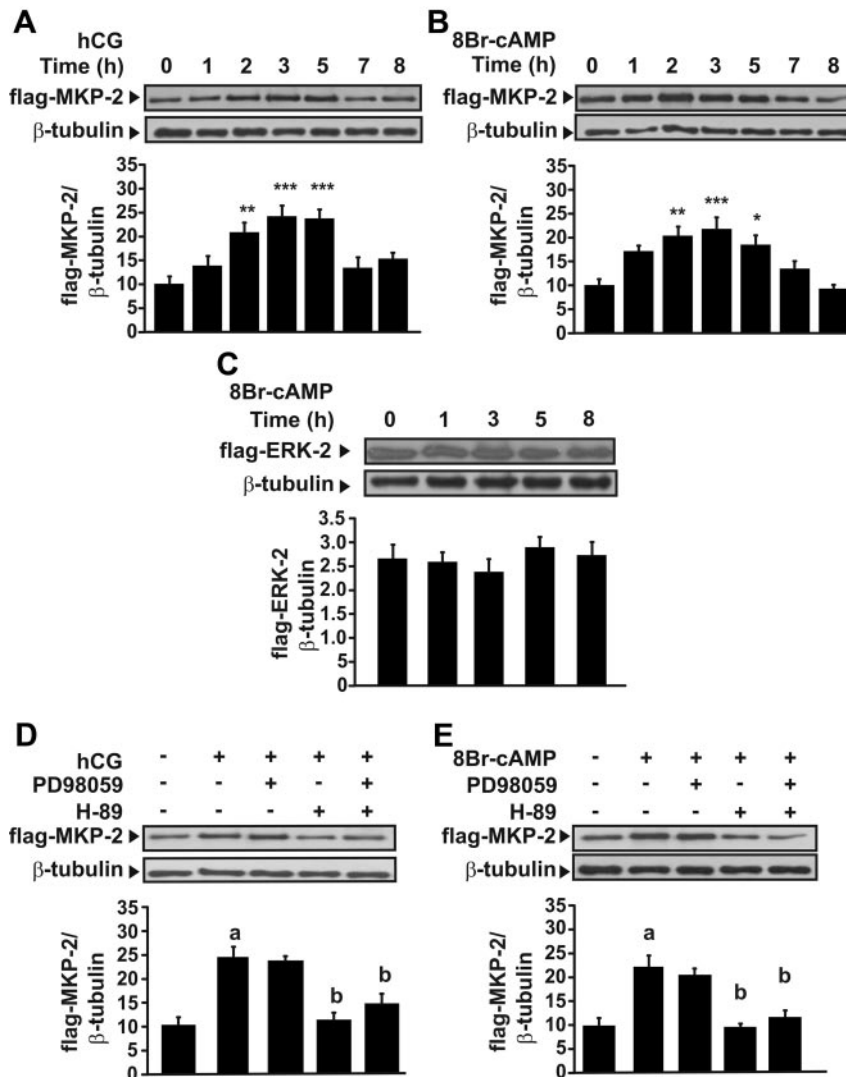


Figure 2. hCG Increases flag-MKP-2 Protein Levels by a Posttranslational Mechanism in MA-10 Leydig Cells. Cells were transfected with pFLAG-MKP-2 (A, B, D, and E) or pFLAG-ERK2 (C), serum starved, and stimulated for the indicated times with 20 ng/mL hCG (A) or 0.5 mM 8Br-cAMP (B and C) or incubated for 3 hours with 20 ng/mL hCG (D) or 0.5 mM 8Br-cAMP (E) after preincubation for 30 minutes with 50 μ M PD98059 or 10 μ M H-89. 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 was detected as loading control using a specific antibody. Figure shows representative immunoblots (upper panels). Integrated optical density of each band was quantitated, and flag-MKP-2 values were normalized against the loading control and expressed in arbitrary units. Data represent the mean \pm SEM of three independent experiments. A, and B, * P < .05; *** P < .01; **** P < .001 vs nonstimulated cells. D and E, a, P < .001 vs nonstimulated cells; b, P < .001 vs hCG- (D) or 8Br-cAMP- (E) stimulated cells.

lated protein could come from the extranuclear space. Together, these results suggest that 8Br-cAMP stimulation promotes the nuclear localization through a posttranslational mechanism. In accordance with these observations, experiments of subcellular fractionation and subsequent Western blot showed that 8Br-cAMP stimulation increases flag-MKP-2 in the nuclear fraction whereas it decreases in the cytoplasmic fraction (Figure 5A). Moreover, flag-MKP2 protein level accumulated by MG132

displays a similar pattern when cells are stimulated under protein synthesis inhibition (Figure 5B).

Down-regulation of MKP-2 by a specific shRNA alters the pattern of 8Br-cAMP-stimulated ERK1/2 phosphorylation

Further experiments were conducted to determine whether the down-regulation of MKP-2 could impact the kinetics or magnitude of ERK1/2 phosphorylation levels reached by cAMP stimulation. For this, we designed and cloned two specific short hairpin RNA against MKP-2 (MKP-2 shRNA1 and MKP2 shRNA2), which were effective in reducing flag-MKP-2 protein levels in control and 8Br-cAMP-stimulated cells for 2.5 hours, but not in reducing Flag-MKP-1 protein levels (Figure 6, A and B). These conclusions were obtained using the corresponding empty vector as control (mock transfections). However, it is important to mention that plasmids carrying the corresponding scrambled sequences (Scr) and empty plasmids produced similar effects (data not shown).

After testing the effectiveness of shRNA1 and 2 in down-regulating MKP-2 expression, we analyzed the levels of P-ERK1/2 reached after 8Br-cAMP stimulation in cells expressing MKP-2 shRNA or not. When the levels of P-ERK1/2 were analyzed in nontransfected cells stimulated with 8Br-cAMP (0–180 min), a rapid and transient increase in P-ERK1/2 was observed (Figure 6C). Even when P-ERK1/2 signal seemed to be extinguished after 120 min of stimulation, long exposure of immunoblots showed higher P-ERK1/2 levels in cells stimulated for long periods (120–180 min), as compared with unstimulated cells (Figure 6C). Next, we focused on these times of stimulation to assess the effect of MKP-2 shRNA or Scr expression on P-ERK levels. Next, we focused on these times of stimulation to assess the effect of MKP-2 shRNA or Scr expression on P-ERK levels. As shown in Figure 6, the early phase of ERK phosphorylation was not

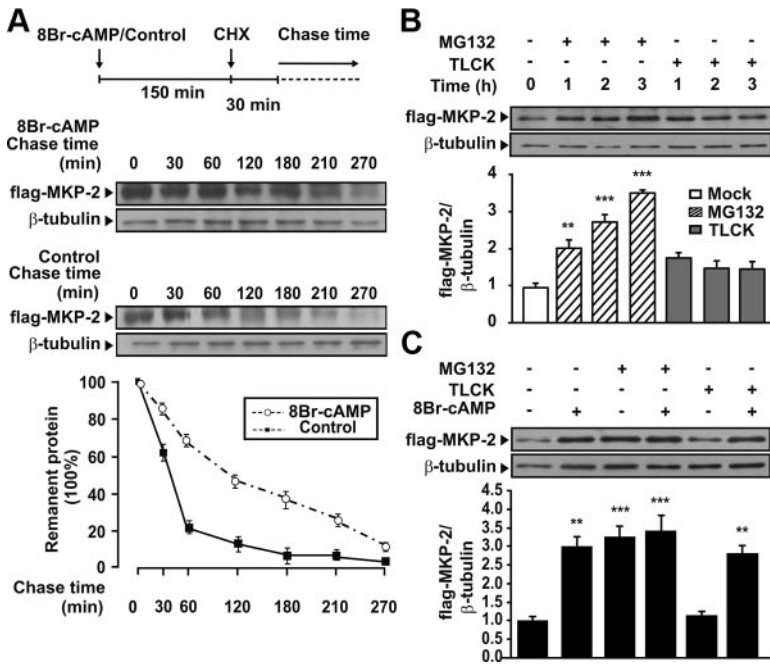


Figure 3. flag-MKP-2 Is Degraded through the Proteasome Pathway and Its Half-Life Is Increased by 8Br-cAMP. MA-10 Leydig cells were transfected with pFLAG-MKP-2, serum-starved, incubated with or without 0.5 mM 8Br-cAMP for 30 min (upper and lower panel, respectively) and further incubated with 2 μg/μl CHX for the indicated times (A) or incubated in the absence or presence of 10 μM MG-132 or 10 μM TLCK for the indicated times or left untreated (B) or incubated with 8Br-cAMP for 3 hours, alone or plus MG132 or plus TLCK (C). 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. Data represent the mean ± SEM of three independent experiments. B and C, *, *P* < .05; **, *P* < .01; ***, *P* < .001 vs nonstimulated cells.

modified by MKP-2 shRNA expression (Figure 6D, left). Moreover, we found that even up to 140 minutes of stimulation, P-ERK1/2 levels were not significantly different in MA-10 cells transfected with either MKP-2 shRNA or Scr (Figure 6D, right panel). However, at 180 and 200 min of stimulation, the levels of P-ERK1/2 in cells carrying shRNA were significantly higher than the levels detected in Scr-transfected cells stimulated for the same times (Figure 6D, right panel). Results shown in Figure 6D were obtained with MKP-2 shRNA1, which is indicated in this and subsequent figures as MKP-2 shRNA. However, similar results were obtained with MKP-2 shRNA2 (data not shown).

Down-regulation of MKP-2 increases the effect of 8Br-cAMP stimulation on both CYP11A1 promoter activity and mRNA levels

CYP11A1 gene expression induced by LH/cAMP occurs after a long period of stimulation and it is dependent on ERK1/2 activity (21, 29). Thus, we hypothesized that MKP-2 expression, by its action on ERK1/2 activity, could contribute to regulating CYP11A1 expression much like MKP-1 regulates the expression of STARD1 (14). To test

this possibility we assessed the effect of MKP-2 level variations, reached by transient transfections, on CYP11A1 promoter activity increased by 8Br-cAMP. As indicated in Figure 7A, CYP11A1 promoter activity was blunted by ERK1/2 inhibition (Figure 7A). Accordingly, MKP-2 overexpression reduced 8Br-cAMP-stimulated CYP11A1 promoter activity, whereas the down-regulation of MKP-2 by MKP-2 shRNA displayed the opposite effect (Figure 7B). Because we found no differences in promoter activity in cells carrying the empty vector and those carrying the Scr sequence, in the following experiments only Scr-transfected cells were used as control.

As expected, the effect of MKP-2 protein on CYP11A1 promoter activity was also observed in the synthesis of the corresponding mRNA. Indeed, the levels of CYP11A1 mRNA obtained after 12 hours of 8Br-cAMP stimulation were reduced by MKP-2 overexpression and increased by the down-regulation of MKP-2 by MKP-2 shRNA (Figure 7C). In Figure 6D we already demonstrated that the down-regulation of MKP-2 promotes an increase in 8Br-cAMP-mediated ERK phosphorylation in the period between 180 and 200 minutes of stimulation. Because the down-regulation of MKP-2 also increases CYP11A1 expression, it is concluded that CYP11A1 induction requires the action of ERK1/2 in this period.

Discussion

In Leydig cells, LHR activation promotes a signal transduction pathway that includes PKA activation, as well as the transient activation of ERK1/2 dependent on PKA (10). MKP-1 and MKP-2 are nuclear enzymes involved in the inactivation of the three major classes of MAPKs: ERKs, JNKs, and p38. The present study demonstrates that hCG and 8Br-cAMP regulate MKP-2 expression in MA-10 Leydig cells. To our knowledge, this is the first study describing the hormonal regulation of MKP-2 in steroidogenic cells. Our conclusion is supported by the following findings in cells under hCG or 8Br-cAMP stimulation: 1) increased MKP-2 mRNA and protein levels, 2) increased MKP-2 protein stability, and 3) increased MKP-2 accumulation in the nucleus. Moreover, our findings also support a functional role of MKP-2 in steroid synthesis. We found that MKP-2 shRNA increases the effect of 8Br-cAMP on: 1) P-ERK1/2 levels, mainly after

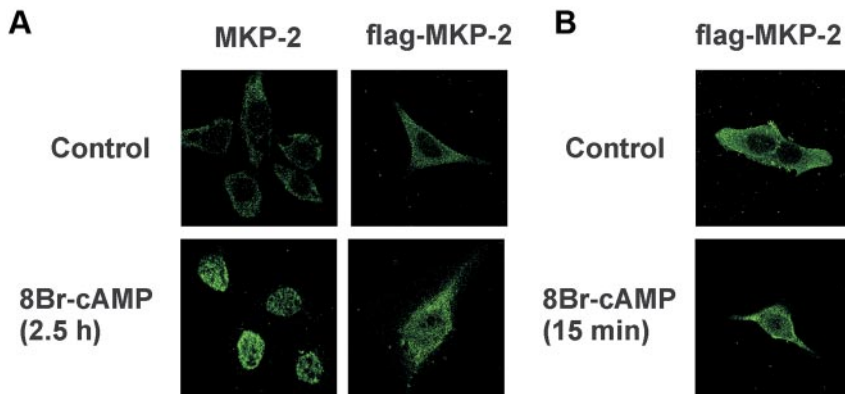


Figure 4. 8Br-cAMP Promotes the Nuclear Accumulation of flag-MKP-2. MA-10 Leydig cells were grown on coverslips, serum starved, stimulated or not (Control) with 0.5 mM 8Br-cAMP for 2.5 hours, fixed, subjected to immunofluorescence staining using an antibody against MKP-2, and analyzed by confocal microscopy (A, left). Cells were grown on coverslips, transfected with pFLAG-MKP-2, serum starved, and finally stimulated or not (Control) with 0.5 mM 8Br-cAMP for 2.5 hours (A, right) or incubated for 1 hour in the presence of 10 μ M MG132, and further incubated with 8Br-cAMP for 15 minutes in the presence of CHX added 30 minutes before 8Br-cAMP (B). Cells were then fixed, stained with an anti-FLAG M2 antibody, and subjected to immunofluorescence and confocal microscopy (A, right, and B).

180-200 minutes of stimulation, which indicates that MKP-2 expression specifically down-regulates the delayed phase of ERK1/2 activity and 2) CYP11A1 promoter activity and messenger levels. We have previously reported that, in MA-10 Leydig cells, hCG and cAMP rapidly induce MKP-1 expression, which contributes to both early P-ERK1/2 dephosphorylation after hCG/cAMP stimulation, and the regulation of a key protein in the acute regulation of steroidogenesis, ie, StAR protein (14). Therefore, these facts, together with the present data showing the regulation and function of MKP-2, demonstrate that hCG not only promotes ERK1/2 activation but also exerts a strict temporal control on the inactivation of ERK1/2 to differentially modulate gene expression.

The increase in MKP-2 mRNA is detected after 2 hours of hCG or 8Br-cAMP stimulation, which indicates that the kinetics of MKP-2 mRNA accumulation is slower than the kinetics of MKP-1 mRNA accumulation. Indeed, 15 minutes of stimulation with hCG or 8Br-cAMP are sufficient to produce a significant increase in MKP-1 mRNA in MA-10 Leydig cells (14). Moreover, hCG treatment causes MKP-2 protein accumulation after approximately 2 hours of treatment, whereas a robust amount of MKP-1 protein is detected after 15 minutes of stimulation (14). In accordance with our results, Zhang and Roberson (30) demonstrated that GnRH, which promotes MAPK activation in gonadotropes, induces both MKP-1 and MKP-2 with different temporal profiles. Therefore, these and our results suggest differential roles for these MKPs in the regulation of MAPK activity.

The increase in MKP-2 mRNA levels by hCG mainly occurs through de novo gene transcription and is depen-

dent on PKA and ERK1/2 activities. In line with our results, MKP-2 expression by GnRH also requires MAPK activation. Indeed, the inhibition of ERK1/2 or JNK1/2 is sufficient to block GnRH-induced MKP-2 expression (31). In MA-10 Leydig cells the activation of ERK1/2 by hCG is a PKA downstream event (10). This characteristic, together with the fact that a MEK inhibitor blunted the action of hCG on MKP-2 mRNA levels (Figure 1C), suggests that hCG promotes MKP-2 gene activation mainly through ERK1/2 activity triggered by PKA action. Thus, it is suggested that MKP-2 gene induction by hCG only involves events downstream of PKA that are dependent on ERK1/2.

The increase in flag-MKP-2 levels by hCG/8Br-cAMP could be linked to a decrease in the rate of degradation, because the chimera is expressed under a constitutive promoter and, consequently, displays a constant rate of production. Indeed, 8Br-cAMP stimulation resulted in an increase in flag-MKP-2 half-life. This result, together with the fact that PKA activation and the inhibition of proteasome have no accumulative effect on the amount of flag-MKP-2, supports the notion that a posttranslational modification triggered by hCG or cAMP prevents MKP-2 degradation, thus leading to its stabilization.

Peng et al. (32) have demonstrated that MKP-2 is phosphorylated by ERK1/2 on Serine 386 and Serine 391 in serum-exposed human breast cancer cells, which leads to protein stabilization. In our experiments, MA-10 Leydig cells were serum depleted for 24 h and further stimulated by hCG or 8Br-cAMP for different periods. Under these experimental conditions, which resulted in transient ERK1/2 activation, we showed a null effect of a MEK inhibitor on flag-MKP-2 accumulation. This result excludes the possibility of ERK1/2 participation in the stabilization of the chimera mediated by a posttranslational modification.

It is important to mention that MKP-2 sequence analysis reveals the presence of several PKA phosphorylation consensus sites. Therefore, MKP-2 could be a direct target of PKA in MA-10 cells under hCG stimulation. However, the posttranslational modification of MKP-2 induced by hCG and 8Br-cAMP in MA-10 Leydig cells remains undetermined.

MKP-2 lacks the classical motif of nuclear localization but it has two nuclear localization sequences (NLSs), and

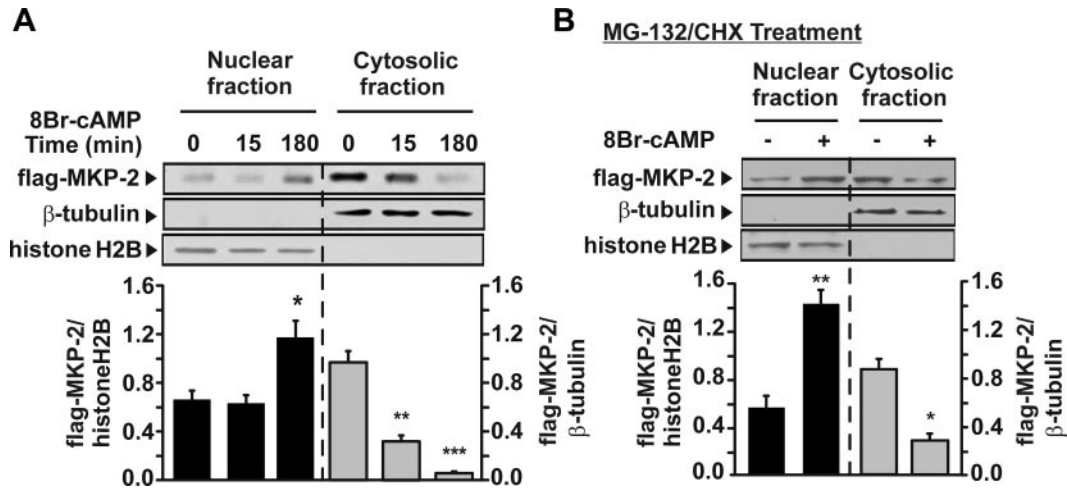


Figure 5. 8Br-cAMP Promotes the Nuclear Accumulation of flag-MKP-2. MA-10 Leydig cells were transfected with pFLAG-MKP-2, serum starved, and stimulated with 0.5 mM 8Br-cAMP (A) or incubated for 1 hour in the presence of 10 μ M MG132, and further incubated with 8Br-cAMP for 15 minutes in the presence of CHX added 30 minutes before 8Br-cAMP (B). Cells were then homogenized and subjected to subcellular fractioning, and nuclear and cytosolic 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 and histone H2B were detected as loading controls for total cytosolic or nuclear proteins, respectively. Figures show representative immunoblots (upper panels). Integrated optical density of each band was quantitated and flag-MKP-2 or MKP-2 values were normalized against the corresponding loading control and expressed in arbitrary units. Data represent the mean \pm SEM of three independent experiments (lower panels). * $P < .05$; ** $P < .01$; *** $P < .001$ vs the corresponding fraction from nonstimulated cells.

both participate in the nuclear localization of MKP-2 (NLS-1 and NLS-2) (33). We showed that, in cells exposed to a proteasome inhibitor, flag-MKP-2 is accumulated in the cytoplasm, and a short period of 8Br-cAMP stimulation causes its localization in the nucleus. Given that the stimulation was performed in the presence of a protein synthesis inhibitor, this study suggests that 8Br-cAMP promotes an MKP-2 posttranslational modification leading to its nuclear localization. Our current efforts aim to characterize this modification.

In gonadotropes, GnRH triggers both ERK1/2 and JNK1/2 phosphorylation and MKP-2 induction, after which JNK1/2 are dephosphorylated mainly by MKP-2 (30). In MA-10 Leydig cells, ERK phosphorylation by hCG stimulation is well documented (10). In contrast, to our knowledge, JNK1/2 phosphorylation under this condition has not been described. In our experiments, hCG stimulation resulted in strong phosphorylation of ERK1/2 but no apparent phosphorylation of JNK1/2 (data not shown). Therefore, JNK1/2 is unlikely to be a physiological substrate of MKP-2 in MA-10 cells under hCG stimulation. Our studies demonstrate that MKP-2 expression is linked to the late stage of ERK1/2 phosphorylation. MKP-2 may thus complete the dephosphorylation of ERK1/2, which, as we have demonstrated in previous work (14), is initiated by MKP-1.

In the search for the functional role of MKP-2 in MA-10 Leydig cells, we focused on the regulation of CYP11A1. The expression of this gene fulfills two essential requirements: 1) it is dependent on hCG/cAMP through an

ERK1/2-dependent pathway (23); and 2) it occurs in a temporal frame compatible with the regulation exerted by MKP-2, in the sense that 8Br-cAMP increases MKP-2 protein levels before significantly raising the level of P450_{scc} mRNA. Our studies demonstrate that MKP-2 down-regulation increases the effect of 8Br-cAMP on P-ERK1/2 levels and also on CYP11A1 promoter activity and mRNA levels, which suggests that hCG- and cAMP-induced MKP-2 modulates CYP11A1 gene expression. This modulation occurs through the dephosphorylation of P-ERK1/2, which, in turn, leads to the inactivation of at least one ERK-dependent protein involved in CYP11A1 expression.

It is well recognized that the regulation of CYP11A1 promoter by gonadotropins and cAMP involves transcription factors such as steroidogenic factor 1 (SF1), GATA4, cAMP response element-binding protein-1, and AP-1 family members (cFos and c-Jun), although the profile of transcription factors involved in this regulation exhibits tissue-specific differences (21, 34). Regarding the mechanism through which MKP-2 regulates the induction of CYP11A1 by cAMP, one possibility points at SF1 regulation. It is known that ERK1/2 promotes the phosphorylation of SF1 and its activity on STARD1 expression (22). Therefore, through its action on ERK1/2, MKP-2 could reduce the phosphorylation level of SF1, which would result in lower SF1 transcriptional activity. However, the phosphorylation of SF1 seems to have a different effect on the regulation of steroidogenic genes such as STARD1 and CYP17 (which encodes for P450_{c17}). Indeed, it has been

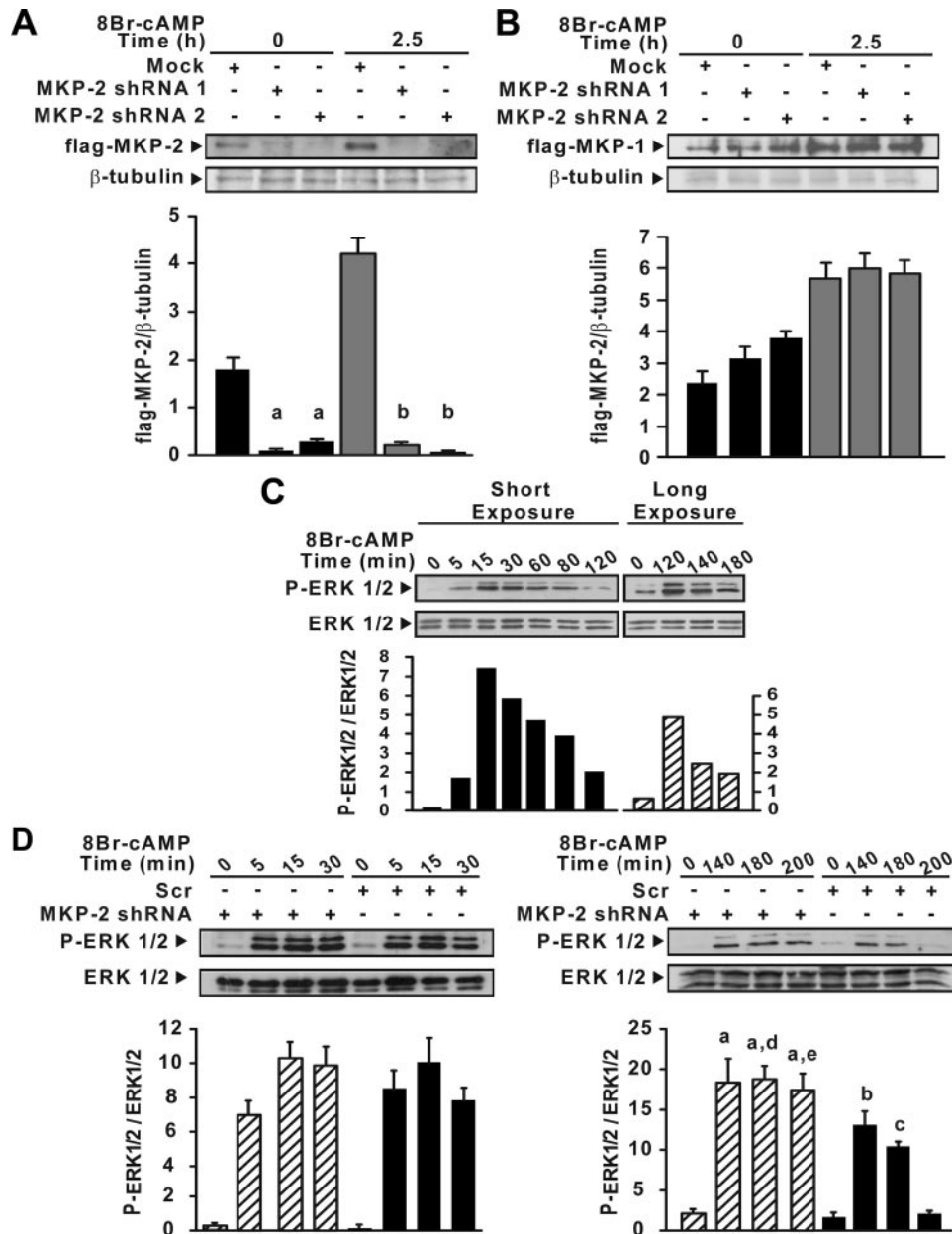


Figure 6. Effect of the Down-Regulation of MKP-2 by shRNA on ERK1/2 Phosphorylation. MA-10 Leydig cells were cotransfected with the empty vector pSUPER.retro (Mock) or pSUPER.retro-MKP-2 (MKP-2 shRNA) from two different clones (clone 1 and clone 2) together with the expression vectors pFLAG-MKP-2 (A) and pFLAG-MKP-1 (B). In C and D, cells were either not transfected or transfected with pSUPER.retro-scramble (Scr) or pSUPER.retro-MKP-2 (MKP-2 shRNA), respectively. Next day they were serum starved and then stimulated with 8Br-cAMP (0.5 mM) for the times indicated. Cells were then homogenized and total cell lysate proteins were analyzed by Western blotting using an anti-FLAG M2 antibody (A and B) and anti-P-ERK 1/2 antibody (C and D). Specific bands were detected by enhanced chemiluminescence. Membranes were then stripped, and β-tubulin (A and B) or total ERK1/2 (C and D) were detected as loading control. Figure shows representative immunoblots (upper panels). Integrated optical density of each band was quantified and flag-MKP-2 (A), flag-MKP-1 (B), and P-ERK (C and D) values were normalized against the corresponding loading control and expressed in arbitrary units. A, B, and D, Data represent the mean ± SEM of three independent experiments. A: a and b, $P < .001$ vs mock transfected cells. B: a, $P < .001$ vs the respective nonstimulated transfected cells. D: a, $P < .001$ vs nonstimulated cells expressing pSUPER.retro-MKP-2 (MKP-2 shRNA); b, $P < .01$, and c, $P < .05$ vs nonstimulated cells expressing pSUPER.retro-scramble (Scr); d, $P < .05$; e, $P < .001$ vs cells expressing pSUPER.retro-scramble (Scr) and stimulated with 8Br-cAMP for the same period. C, Data corresponding to a representative experiment.

documented that ACTH-mediated MKP-1 induction promotes SF1 dephosphorylation, which, in turn, leads to an increase in SF1-mediated CYP17 transcription in adrenocortical cells (12).

Winnay et al. (35) have demonstrated that ACTH-receptor gene expression by ACTH involves a cyclic pattern of SF1-dependent transcriptional activation, which, they postulate, could be modulated by MKP-1 expression.

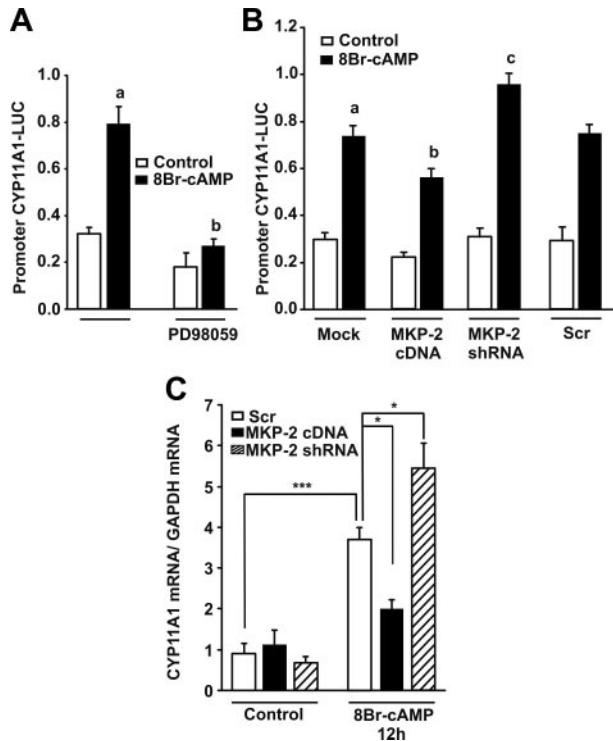


Figure 7. Effect of Overexpression and Silencing of MKP-2 on both CYP11A1 Promoter Activity and mRNA Levels. MA-10 cells were cotransfected with the reporter vector pGL3-CYP11A1 and the control pRL *Renilla* luciferase vector, together (B) or not (A) with the expression vectors pFLAG-MKP-2 (MKP-2 cDNA), pSUPER.retro-MKP-2 (MKP-2 shRNA), or pSUPER.retro-scramble (Scr). After 48 hours, cells were stimulated with 0.5 mM 8Br-cAMP for 12 hours in the presence or absence of 50 μ M PD98059 (A), and luciferase activity (RLU) was detected in cell lysates. Each value was normalized against the *Renilla* luciferase activity. The results are expressed as the mean \pm SEM of three independent experiments. A: a, $P < .001$ vs unstimulated cells incubated in absence of PD98059; b, $P < .001$ vs 8Br-cAMP-stimulated cells incubated in absence of PD98059. B: a, $P < .001$ vs nonstimulated mock-transfected cells; b, $P < .001$ vs 8Br-cAMP-stimulated mock-transfected cells and c, $P < .05$ vs 8Br-cAMP-stimulated mock-transfected cells. C, MA-10 cells were transfected with pFLAG-MKP-2 (MKP-2 cDNA), pSUPER.retro-MKP-2 (MKP-2 shRNA), or pSUPER.retro-scramble (Scr) as indicated. Cells were serum starved, stimulated for 12 hours with 0.5 mM 8Br-cAMP, and total RNA was isolated and subjected to reverse transcription and quantitative real-time PCR with specific primers for CYP11A1 and GAPDH cDNA as loading control. CYP11A1 levels were normalized against the corresponding GAPDH mRNA levels. Data represent the mean \pm SEM of three independent experiments. * $P < .05$; *** $P < .001$.

Whether MKP-2 coordinates a similar cyclic pattern of regulation of CYP11A1 is still to be elucidated.

In Y1 adrenocortical cells, basal and cAMP-stimulated expression of CYP11A1 involves the homeodomain-interacting protein kinase 3, which triggers both JNK1/2 and c-Jun phosphorylation (36). It is demonstrated that the stimulation with 8Br-cAMP raises the levels of phosphorylated c-Jun in a delayed fashion, starting after 3 hours, which promotes SF1 activation and CYP11A1 ex-

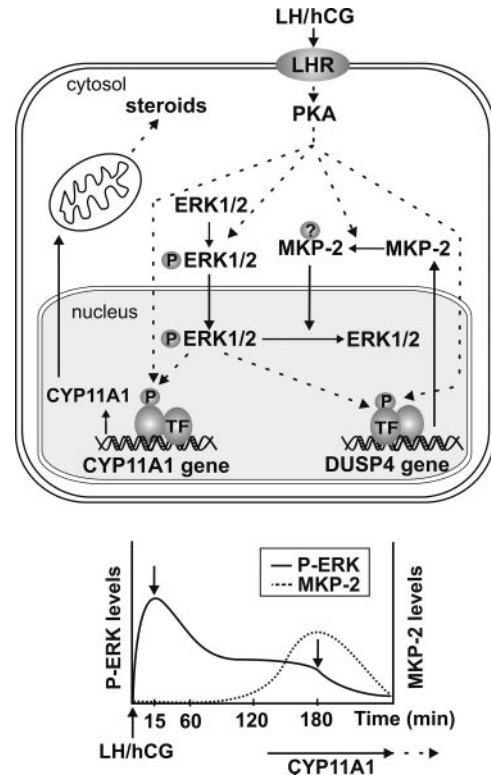


Figure 8. Proposed Model for the Role of MKP-2 in Steroidogenesis. LHR activation by LH/hCG triggers PKA activation, which leads to the rapid phosphorylation and activation of ERK1/2. Activated ERK (P-ERK) translocates to the nucleus where it phosphorylates and activates transcription factors (TF). TF activated by P-ERK, together with TF activated by PKA, lead to CYP11A1 induction. CYP11A1 encoded product, P450_{scc} protein, acts in the mitochondria in steroid synthesis. Simultaneously, activated PKA and P-ERK also induce DUSP4 gene transcription (DUSP4 or dual-specificity phosphatase-4: name of gene encoding MKP-2 protein). PKA also promotes MKP-2 protein stabilization through a still unknown posttranslational mechanism that leads to MKP-2 accumulation in the cell. In turn, MKP-2 protein induces ERK1/2 dephosphorylation after approximately 180 minutes of LH stimulation, generating a late negative feedback mechanism on its own expression, which contributes to “turning off” the ERK1/2-dependent events involved in steroidogenesis. Direct effects are indicated by solid lines, whereas indirect effects are indicated as dotted lines. A question mark indicates an unknown posttranslational modification. Levels of P-ERK decline markedly in the period between 15 and 90 minutes of hormone stimulation. Between 90 and 180 minutes, P-ERK levels remain unchanged. After 180 minutes a second step of dephosphorylation, due to MKP-2 expression, occurs and P-ERK levels reach the control levels.

pression (36). JNK1/2 phosphorylation by hCG was not detected in our system. However, even if hCG activated JNK1/2 with kinetics similar to c-Jun phosphorylation, MKP-2 would seem unlikely to have a role in the inactivation of JNK1/2, because this would imply a simultaneous increase in both phosphorylated JNK1/2 and MKP-2. Nevertheless, it is worth pointing out that our experiments were carried out in different experimental conditions, and that, when arrested by serum depletion, as we have proceeded, MA-10 Leydig cells could display different requirements for full stimulation.

The present work demonstrates the regulation of MKP-2 by hCG in MA-10 Leydig cells. Although it could be argued that this regulation does not operate in untransformed cells or in the whole animal, studies using an in vivo mouse model strongly suggest that the hormonal regulation of MKP-2 is a common mechanism in cell biology to counteract MAPK activity (30).

In summary, we have reported, for the first time, the up-regulation of MKP-2 in MA-10 Leydig cells elicited by hCG and cAMP. This regulation involves the ERK-dependent MKP-2 gene induction and a posttranslational modification that increases MKP-2 half-life. We also provide evidence that MKP-2 induction might contribute to the down-regulation of both ERK activity and CYP11A1 expression after LHR stimulation. Figure 8 shows a cartoon depicting the regulation of MKP-2 by hCG and its role in CYP11A1 expression.

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