

# Modulation of F-actin Rearrangement by the Cyclic AMP/cAMP-dependent Protein Kinase (PKA) Pathway Is Mediated by MAPK-activated Protein Kinase 5 and Requires PKA-induced Nuclear Export of MK5<sup>\*[5]</sup>

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The MAPK-activated protein kinases belong to the Ca<sup>2+</sup>/calmodulin-dependent protein kinases. Within this group, MK2, MK3, and MK5 constitute three structurally related enzymes with distinct functions. Few genuine substrates for MK5 have been identified, and the only known biological role is in *ras*-induced senescence and in tumor suppression. Here we demonstrate that activation of cAMP-dependent protein kinase (PKA) or ectopic expression of the catalytic subunit C $\alpha$  in PC12 cells results in transient nuclear export of MK5, which requires the kinase activity of both C $\alpha$  and MK5 and the ability of C $\alpha$  to enter the nucleus. C $\alpha$  and MK5, but not MK2, interact *in vivo*, and C $\alpha$  increases the kinase activity of MK5. Moreover, C $\alpha$  augments MK5 phosphorylation, but not MK2, whereas MK5 does not seem to phosphorylate C $\alpha$ . Activation of PKA can induce actin filament accumulation at the plasma membrane and formation of actin-based filopodia. We demonstrate that small interfering RNA-triggered depletion of MK5 interferes with PKA-induced F-actin rearrangement. Moreover, cytoplasmic expression of an activated MK5 variant is sufficient to mimic PKA-provoked F-actin remodeling. Our results describe a novel interaction between the PKA pathway and MAPK signaling cascades and suggest that MK5, but not MK2, is implicated in PKA-induced microfilament rearrangement.

The mitogen-activated protein (MAP)<sup>3</sup> kinase signaling pathways play important roles in several cellular processes,

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<sup>3</sup> The abbreviations used are: MAP, mitogen-activated protein; PKA, cAMP-dependent protein kinase; C $\alpha$ , catalytic subunit PKA isoform  $\alpha$ ; CRE, cAMP-response element; Me<sub>2</sub>SO, dimethyl sulfoxide; EGFP, enhanced green fluorescence protein; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MBP, myelin basic protein; MK, MAPKAPK; NES, nuclear export signal; NLS, nuclear localization signal; PTB, polypyrimidine tract-binding protein; RI, regulatory subunit PKA type I; siRNA, small interfering RNA; CREB, cAMP-response element-binding protein; PKI, protein kinase inhibitor; BSA, bovine serum albumin; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBS, phosphate-buffered saline.

including proliferation, differentiation, apoptosis, development, gene transcription, and motility. A typical MAP kinase module acts through subsequent phosphorylation and activation of a MAP kinase kinase kinase, a MAP kinase kinase, and a MAP kinase. The MAP kinase may phosphorylate non-protein kinase substrates or yet other protein kinases, referred to as MAP-activating protein kinases (MAPKAPK) (1–5). Based on the sequence homology of the kinase domain, the MAPKAPK belong to the superfamily of Ca<sup>2+</sup>/calmodulin-dependent protein kinases (6). The mammalian MAPKAPK include the ribosomal S6-kinases (RSK1–4 or MAPKAPK1a–d), the mitogen- and stress-activated kinases (MSK1 and -2), the MAPK-interacting kinases (MNK1 and -2), and the MAPKAPK MK2, MK3, and MK5. MK2 was isolated 15 years ago in an attempt to identify protein kinases involved in adrenergic control of glycogen synthase activity, whereas MK3 was first described as a protein encoded by a gene located in the small cell lung cancer tumor suppressor gene region 3p21.3 (7, 8). The murine MAPKAPK5 (MK5) or its human homologue PRAK was originally described as a downstream target of p38 MAPK (9, 10). MK5 shares ~45% sequence homology with MK2 and MK3, whereas MK2 and MK3 display 75% sequence identity. All three kinases contain the same conserved regulatory phosphorylation site (Thr-182, Thr-222, and Thr-201, respectively). Despite their high similarity, gene deletion experiments reveal that these three kinases possess separate functions (11, 12). MK2 is involved in many cellular processes like cell cycle regulation, inflammation, and cell migration (13–18), whereas the biological roles of MK3 and MK5 remain unclear. MK3 may be involved in transcription regulation through modulating the activity of the transcription factor E47 and the transcriptional repressor Polycomb group Bmi-1 protein. Moreover, MK3 may function as a tumor suppressor (19–21). The biological function of MK5 is poorly understood, as MK5 knock-out mice bred onto different backgrounds displayed either no obvious phenotype or embryonic lethality. The reason for this lethality, however, remains unknown. Furthermore, the atypical MAP kinases ERK3 and ERK4 are the only identified *in vivo* MK5-interacting partners, but the physiological relevance of the interaction remains elusive (11, 22–26). However, a recent study demonstrated that MK5 could phosphorylate p53 at serine 37 *in vivo*, that MK5 deficiency enhanced mutagen-induced skin carcinogenesis in mice, and that MK5<sup>-/-</sup> primary cells were more susceptible to oncogenic transformation. These findings point to a role for MK5 in tumor suppression (27). Our group, in collaboration

with others, found that increased levels of ERK3 during nerve growth factor-induced differentiation of PC12 cells was accompanied by increased MK5 activity, hence suggesting a role for MK5 in neuronal differentiation (24).

Cyclic AMP functions as an intracellular second messenger to transmit extracellular signals, such as hormones and neurotransmitters, to the intracellular environment. The major target of cAMP is the cAMP-dependent protein kinase (PKA), a tetrameric holoenzyme that consists of two regulatory (R) and two catalytic (C) subunits. After cAMP binds the R subunits, the C subunits dissociate and phosphorylate their substrates (28). The cAMP/PKA signaling pathway regulates a variety of cellular processes such as cell proliferation, differentiation, actin cytoskeletal rearrangements, and gene transcription (reviewed in Refs. 29–31). To optimize the responsiveness to a wide variety of signals, the PKA pathway interacts with other signaling pathways, including the MAPK pathways. In fact, PKA has been shown to modulate the activity of several MAPK cascades (32–35). Recently, the MAPKAP kinase, RSK1, was found to either bind the C or R subunit of PKA, depending on the phosphorylation status of RSK1, but the physiological relevance of this interaction is not completely understood (36).

A role for PKA in actin cytoskeletal changes is well illustrated, and it has been suggested that MK2/3/5 may be implicated in actin remodeling (reviewed in Refs. 11 and 30). However, whether there is a specific role for a certain MK in actin redistribution and whether PKA may recruit MKs to mediate actin rearrangement remains unsolved. This prompted us to examine whether MK5 might contribute in cAMP/PKA-provoked modification of the microfilament architecture in the rat pheochromocytoma cell line PC12. Our results demonstrate a new interaction between the cAMP/PKA and MK5 and recognize a novel role of MK5 as a mediator of cAMP-induced F-actin rearrangement.

## EXPERIMENTAL PROCEDURES

**Materials**—Forskolin, PKA-C, and PKA-R were purchased from Sigma. [ $\gamma$ - $^{32}$ P]ATP was obtained from GE Healthcare, and recombinant active and inactive His-MK5/PRAKs were from Invitrogen. The PRAKtide MK5 substrate peptide was purchased from Upstate (Charlottesville, VA). The monoclonal antibody against the regulatory RI $\alpha$  of PKA was a kind gift from Dr. Skålhegg (University of Oslo). Anti-PRAK (A-7), anti-ERK2 (C-14), and anti-PKA<sub>cat</sub> (C-20) were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-CREB (catalog number 9190) and anti-MK2 (catalog number 3042) were obtained from Cell Signaling (Beverly, MA), and anti-green fluorescent protein (ab290) was purchased from AbCam (Cambridge, UK). Anti-ERK3 antibodies have been described before (24). The monoclonal anti-Ras clone RAS10 antibody was from Upstate (catalog number 05-516). Alexa Fluor 488 antibody was from Invitrogen. The alkaline phosphatase-conjugated secondary antibodies sheep anti-mouse IgG and anti-rabbit IgG were from Sigma. Oligonucleotides were purchased from Sigma or Eurogentec (Seraing, Belgium).

**Plasmids**—The expression vectors for p38 $\beta$  and the activated MKK6E/E mutant, as well as for the EGFP fusion proteins with wild-type MK5, and the MK5 mutants K51E, T182A, and

L337A, and truncated MK5 residues 1–423 have been described previously (24, 37). Dr. Maurer generously provided the expression plasmid of the heat-stable inhibitor of PKA (pCMVPKI) (38). The C $\alpha$ -NLS plasmid was a kind gift of Dr. Thiel (39). The kinase-dead C $\alpha$  L72H was generated by site-directed mutagenesis using the primer (only one strand is shown): 5'-GAA-CAA-CTA-CGC-CAT-GCA-CAT-CTT-AGA-CAA-G-3'. The expression plasmids for cytoplasmically located MK5 variants were generated by cloning the NES signal of the Rev protein of human immunodeficiency virus into pEGFP-MK5. Tagging a protein with this sequence will direct it to the cytoplasm (40). Therefore, the complementary oligonucleotides 5'-CCGGAGACGCTCTACCACCGCTT-GAGAGACTTACTCTTGACCGAGCT-3' and 5'-CGGT-CAAGAGTAAGTCTCTCAAGCGGTGGTAGAGCGTCT-3' were ligated into the BspEI/Sac I sites of pEGFP-MK5, pEGFP-MK5 T182A, and pEGFP-MK5 L337A, respectively. To construct the RFP-C $\alpha$ -NLS and RFP-C $\alpha$ -NES expression plasmids, the BspEI and BamHI fragment of GPKAnls and GPKAnes, respectively, was ligated into the corresponding sites of pAsRed2-C1 (Clontech). The GPKAnls and GPKAnes plasmids were generously provided by Dr. S. H. Green (41). The plasmid pCRE-LUC was from Clontech. All newly generated plasmids and mutations were confirmed by sequencing.

**In Vitro Kinase Assay**—Phosphorylation of purified MK2 (Upstate/Millipore, Billerica, MA), MK5 (Upstate/Millipore), and ERK3 (24) by 2.5 units of PKA-C was performed in 25 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.05 mg/ml BSA, 2.5 mM dithiothreitol, 0.15 mM cold ATP, and 0.3  $\mu$ l of [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol; GE Healthcare) in a total volume of 40  $\mu$ l at 30 °C for 1 h. The reaction was stopped in 4 $\times$  LDS Sample buffer, and proteins were denatured at 70 °C for 10 min. The phosphorylation was analyzed on NuPAGE 4–12% BisTris SDS-PAGE (Invitrogen) for 50 min at 200 V and then subjected to autoradiography.

**Cell Culture and Transfection**—PC12 cells, a kind gift from Dr. Jaakko Saraste (University of Bergen, Norway), and PKA-deficient PC12<sup>A123.7</sup> cells (generously provide by Dr. J. Yao) were maintained in RPMI 1640 medium, supplemented with 10% horse serum (Invitrogen) and 5% fetal bovine serum, 2 mM L-glutamine, penicillin (110 units/ml), and streptomycin (100  $\mu$ g/ml). HeLa cells were maintained in Eagle's minimum essential medium supplemented with 1 $\times$  nonessential amino acids, 10% fetal calf serum, 2 mM L-glutamine, penicillin (110 units/ml), and streptomycin (100  $\mu$ g/ml). HEK293 cells were purchased from the European Collection of Cell Cultures (catalog number 85120602; Salisbury, Wiltshire, UK) and kept in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (110 units/ml), and streptomycin (100  $\mu$ g/ml). Cells were transfected with Lipofectamine 2000 (Invitrogen) or using the Nucleofection kit (Amaxa) according to the manufacturers' instructions. We routinely obtained >75% transfection efficiency in the different cell lines with these transfection agents as judged by green fluorescent-positive cells when using the pEGFP plasmid as a marker.

**Preparation of Nuclear and Cytoplasmic Extracts**—The NE-PER<sup>®</sup> nuclear and cytoplasmic extraction reagents (Pierce, cat-

## PKA-induced F-actin Rearrangement by MK5

alog number 78833) were used according to the manufacturer's instructions.

**Western Blotting**—For detection of co-immunoprecipitates and MK5, samples were analyzed by SDS-PAGE NuPAGE 4–12% BisTris SDS-PAGE (Invitrogen) according to the manufacturer's protocol and blotted onto a 0.45- $\mu$ m polyvinylidene difluoride membrane (Millipore, Billerica, MA). Immunoblotting was performed by first blocking the membrane with PBS-T (PBS with 0.1% Tween 20 (Sigma) containing 10% (w/v) dried skimmed milk for 1 h and probed either by 4D7, anti-PRAK, anti-MK2, anti-ERK2, anti-PKA cat. After four washes, the membrane was incubated with the appropriate secondary antibody for 1 h. Visualization of proteins was achieved by using CDP Star substrate (Tropix, Bedford, MA) and Lumi-Imager F1 from Roche Applied Science.

**Immunoprecipitation**—HeLa extracts were harvested and lysed in buffer containing 20 mM Tris·HCl, pH 7.5, 1% Triton X-100, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.27 M sucrose, 10 mM  $\beta$ -glycerophosphate, and Complete protease inhibitor mixture (Roche Applied Science). Lysates were cleared by centrifugation at 4 °C for 10 min at 15,000  $\times$  g. Lysates were incubated with the appropriate antibody for at least 1 h at 4 °C, before addition of 60  $\mu$ l of slurry (*i.e.* 30  $\mu$ l of protein G-agarose (GE Healthcare) equilibrated with 30  $\mu$ l lysis buffer) and incubated for an additional hour. The immunoprecipitates were then washed three times in lysis buffer and twice in 50 mM Tris·Cl, pH 8.0. Twenty  $\mu$ l of 2 $\times$  LDS sample buffer was added to the beads before denaturation at 70 °C for 10 min. The immunoprecipitates were either analyzed on Western blots or by means of kinase assays.

**Cell Staining and Microscopy**—Cells were rinsed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 4% formaldehyde. Next, the cells were washed twice with PBS and then permeabilized for 10 min with 0.1% Triton X-100. The cells were preincubated with PBS containing 1% BSA for 20–30 min and stained with Alexa Fluor 594 phalloidin (A12381; Molecular Probes, Eugene, OR) for 20 min. The cells were then washed with PBS and examined using confocal laser-scanning Zeiss LSM 510 META and Leica SP5 microscopes. Cell nuclei were visualized by DRAQ5 staining (Biostatus Ltd., Leicester-shire, UK). We routinely examined 50 cells or more, and representative pictures are shown under the "Results."

**Transient Transfection and Luciferase Assay**—HEK293 cells were plated 3  $\times$  10<sup>5</sup> cells per well in a 6-well plate. Cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. Luciferase assays were as described previously (42).

**Immune Complex Kinase Assay**—For the immune complex kinase assays, HeLa cells were transfected with expression plasmids encoding EGFP fusion proteins with wild-type MK5 or mutants and C $\alpha$ . Corresponding empty vector control plasmids (pEGFP and pRcCMV, respectively) were used as control. As a positive control for the assay, cells were either transfected with pEGFP-MK5 L337A, encoding an activated MK5 variant, or pEGFP-MK5 plus expression plasmids for the activated MKK6 E/E mutant and p38 $\beta$  MAPK. MK5 fusion proteins were immunoprecipitated using anti-green fluorescent protein antibody,

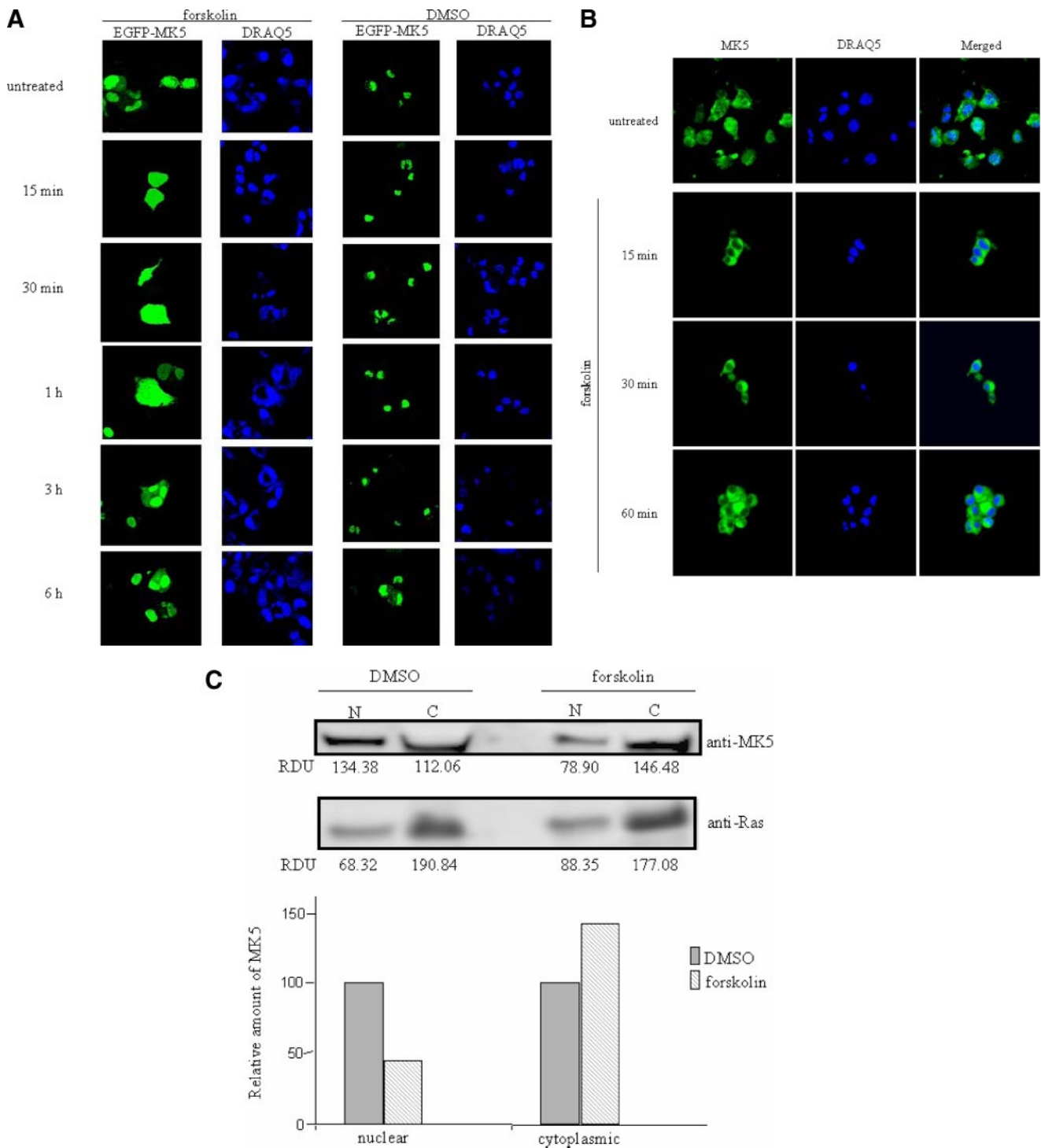
and the kinase activity was monitored against 30  $\mu$ M PRAKtide substrate peptide at 30 °C for 20 min in 50  $\mu$ l of buffer containing 40 mM Tris·Cl, pH 7.5, 0.4 mg/ml BSA, 15 mM MgCl<sub>2</sub>, 0.1 mM cold ATP, and 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol). Following incubation, 20- $\mu$ l aliquots were spotted onto phosphocellulose disks (Upstate) and washed extensively with 1% phosphoric acid before measurement of radioactive incorporation by scintillation counting.

**Quantitation of F-actin**—The amount of globular (G-actin) and filamentous actin (F-actin) was determined using the G-actin/F-actin *in vivo* assay kit from Cytoskeleton (Denver, CO) according to the instructions of the manufacturer. Briefly, cells were lysed, and the lysate was centrifuged at 100,000  $\times$  g for 1 h at 37 °C. The supernatants contained G-actin, whereas the pellet contained F-actin. As a control, cell lysates are either treated with F-actin enhancing solution (phalloidin) or F-actin depolymerization solution (cytochalasin) before ultracentrifugation. The amount of G- and F-actin was determined by Western blotting using an anti-actin-specific antibody.

**Small Interfering RNA (siRNA)**—Validated MK5-directed siRNA, purchased from Ambion Inc. (Austin, TX), was transfected by Nucleofection (Amamax) into PC12 cells according to the manufacturer's instructions using 100  $\mu$ M siRNA/10<sup>6</sup> cells. The levels of MK5 protein in untreated and siRNA-treated cells were monitored 48 h after transfection by Western blotting using anti-PRAK antibody (Santa Cruz Biotechnology) to verify the efficiency of reduction in MK5 expression.

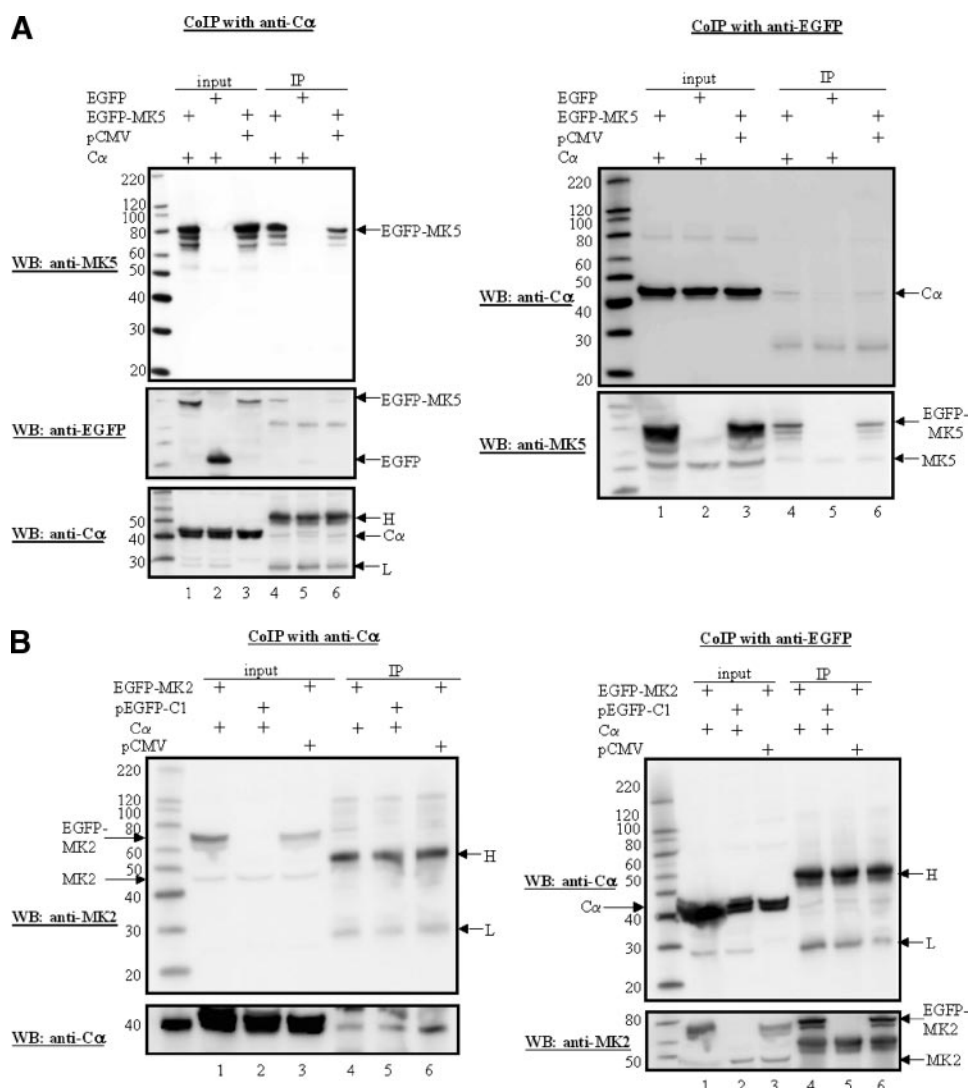
## RESULTS

**The cAMP/PKA Pathway Induces Nuclear Export of MK5**—Both endogenous and ectopically expressed MK5 as green fluorescent protein (EGFP)-, cyan fluorescent protein-, and hemagglutinin-tagged proteins reside predominantly in the nucleus in different cells (23, 37, 43). Studies have proven that the cAMP/PKA signaling pathway participates in actin remodeling and that MKs may regulate the cytoskeletal architecture through modulating the activity of cytoplasmic proteins involved in F-actin organization (reviewed in Refs. 11 and 30). Because the putative involvement of MK5 in stimulus-induced microfilament rearrangement raises the likelihood of cytoplasmic distribution of MK5, we tested whether activation of PKA triggered subcellular distribution of MK5 in PC12 cells. Thereto cells transfected with an expression plasmid for EGFP-MK5 fusion protein were treated with the cAMP-elevating agent forskolin. Consistent with previous findings of both ectopic and endogenous MK5, we observed MK5 mainly in the nucleus of unstimulated cells (23, 37, 43). A transient nuclear export of MK5 was observed in cells treated with forskolin, but not in control cells treated with the vehicle Me<sub>2</sub>SO. Increased cytoplasmic MK5 residence was observed 15 min after forskolin exposure and remained elevated for at least 1 h, after which EGFP-MK5 relocalized mainly to the nucleus. After 30 min of forskolin stimulation, the amount of cells displaying both nuclear and cytoplasmic EGFP-MK5 *versus* nuclear EGFP-MK5 alone increased from 6 to 84% (50 cells were counted). After 6 h, the subcellular distribution of MK5 was comparable with untreated cells (Fig. 1A). In 95% ( $\pm$ 2%; 50 cells were counted in several independent experiments) of the Me<sub>2</sub>SO-



**FIGURE 1. Activation of the cAMP/PKA signaling pathway induces transient nuclear exclusion of MK5.** *A*, PC12 cells were transfected with an expression plasmid for EGFP-MK5 and then stimulated with either 10  $\mu\text{M}$  forskolin (FSK) or vehicle Me<sub>2</sub>SO (DMSO) for the time points indicated. The subcellular localization of the EGFP-MK5 fusion protein over time was visualized by confocal microscopy. Cell nuclei were visualized by DRAQ5 staining. The subcellular distribution of EGFP-MK5 was monitored by confocal microscopy. Fifty cells were counted, and more than 80% displayed both nuclear and cytoplasmic presence of EGFP-MK5 after forskolin exposure. *B*, PC12 cells were treated with 10  $\mu\text{M}$  forskolin for the indicated time points, and endogenous MK5 was visualized with anti-PRAK antibody (Santa Cruz Biotechnology) as primary antibody and Alexa Fluor 488 as secondary antibody. Nuclei were stained with DRAQ5. *C*, PC12 cells were transfected with EGFP-MK5. Nuclear (N) and cytoplasmic (C) extracts were prepared from forskolin-treated cells (30 min) or vehicle Me<sub>2</sub>SO (DMSO)-treated cells. The presence of EGFP-MK5 was assayed by Western blotting. To ensure equal loading and to test for contamination between cytoplasmic and nuclear fractions, the membrane was re-probed with anti-Ras antibodies. The intensity of the signals was determined by densitometry, and the values are shown as relative densitometric units (RDU). *Bottom panel*, the ratio of RDU<sub>MK5</sub>:RDU<sub>RAS</sub> in nuclear (respectively cytoplasmic) fraction of Me<sub>2</sub>SO-treated cells was arbitrary set as 100%, and the ratios of RDU<sub>MK5</sub>:RDU<sub>RAS</sub> in the fractions of forskolin-treated cells were related to this.

## PKA-induced F-actin Rearrangement by MK5



**FIGURE 2. MK5, but not MK2, interacts with C $\alpha$  *in vivo*.** *A*, HeLa cells were co-transfected with expression plasmids for C $\alpha$  and EGFP-MK5 or corresponding empty vectors (pCMV and pEGFP, respectively). After 24 h, cells were lysed, and C $\alpha$  was immunoprecipitated (IP) with anti-C $\alpha$  antibody (left panel) or EGFP-MK5 was immunoprecipitated with anti-EGFP antibodies (right panel). The immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting (WB) was performed using either anti-MK5 antibody (left panel) or anti-C $\alpha$  antibody (right panel). The co-immunoprecipitates with anti-C $\alpha$  were also assayed with anti-EGFP antibodies (middle part of left panel). Lanes 1–3, input control of the transfected cells; lanes 4–6, immunoprecipitates with expression vectors for EGFP-MK5 and C $\alpha$ ; lanes 2 and 5, cells transfected with pEGFP vector and C $\alpha$  expression vector; lanes 3 and 6, cells transfected with EGFP-MK5 and empty vector for C $\alpha$  (pCMV). The positions of C $\alpha$ , MK5, the EGFP-MK5 fusion proteins, and the heavy (H) and light (L) IgG chains are indicated. To ascertain equal protein loading, the membranes were re-probed with an antibody against the immunoprecipitated protein (bottom panel). *B*, HeLa cells were co-transfected with expression plasmids for EGFP-MK2 and C $\alpha$  or with the corresponding empty vectors (EGFP and pCMV, respectively). After 24 h, cells were lysed, and C $\alpha$  was immunoprecipitated with anti-C $\alpha$  antibody (left panel) or EGFP-MK2 was immunoprecipitated with anti-EGFP antibodies (right panel). Lanes 1–3, input control of the transfected cells; lanes 4–6, Western blotting of the immunoprecipitates with anti-C $\alpha$  antibody. Lanes 1 and 4, cells transfected with expression vectors for EGFP-MK2 and C $\alpha$ ; lanes 2 and 5, cells transfected with empty vector for MK2 (EGFP) and C $\alpha$  expression vector; lanes 3 and 6, cells transfected with MK2 expression plasmid and empty vector for C $\alpha$  (pCMV). The molecular mass marker and the positions of MK2, MK2-EGFP fusion protein, and the heavy (H) and light (L) IgG chains are indicated. To ascertain equal protein loading, the membranes were re-probed with an antibody against the immunoprecipitated protein (bottom panel in the figure).

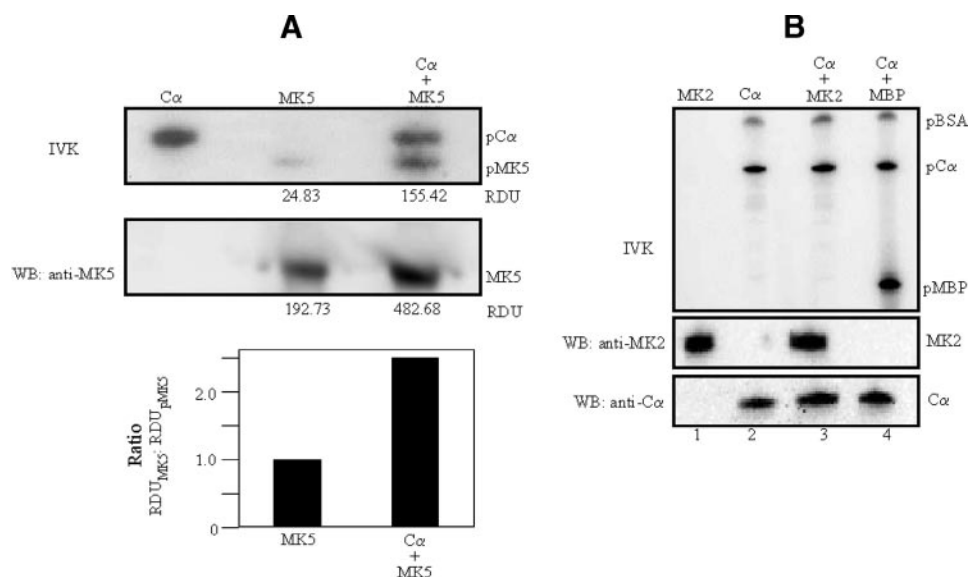
treated cells, MK5 was predominantly nuclear. Forskolin treatment had no effect on the subcellular localization of the EGFP protein (results not shown). Endogenous MK5 was also exported from the nucleus in cells with activated PKA signaling pathway with the same kinetics as EGFP-MK5 (Fig. 1B). These findings confirm that MK5 and EGFP-MK5 fusion protein

behave identically in their subcellular redistribution in response to forskolin. Monitoring the amount of MK5 in the cytoplasmic and nuclear fraction of untreated and forskolin-treated cells confirmed an increased nuclear export of MK5 after forskolin exposure. A 55% decrease of nuclear MK5 was observed after 30 min of forskolin treatment, whereas a 41% increase in cytoplasmic MK5 was monitored in forskolin-exposed cells (Fig. 1C).

Next, we tested whether overexpression of the catalytic subunit of PKA tagged with a nuclear localization signal (C $\alpha$ -NLS) was sufficient to induce nuclear export of MK5. Similar to forskolin-exposed cells, MK5 was located in the cytoplasm of cells that ectopically expressed C $\alpha$ -NLS (supplemental Fig. S1). To elaborate the contribution of PKA in forskolin-induced nuclear export of MK5, we used PC12<sup>A123.7</sup> cells, which are deficient in PKA (44). No subcellular redistribution of MK5 was monitored in these cells after forskolin treatment (supplemental Fig. S2). In conclusion, these results demonstrate that activation of the cAMP/PKA signaling pathway provokes nuclear export of MK5 in a time-dependent manner.

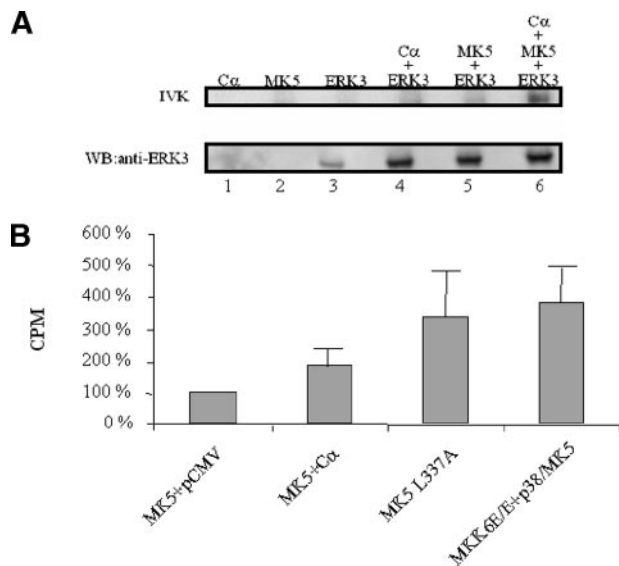
*The Catalytic C $\alpha$  Subunit of PKA Phosphorylates MK5 and Increases the Kinase Activity of MK5*—Previous studies by our group and others have shown that nuclear export of MK5 can be achieved by overexpression of the atypical MAP kinases ERK3 and ERK4. This nuclear exclusion requires the direct interaction between MK5 and ERK3 or ERK4 and leads to activation of MK5 (23–26). To find out whether PKA can interact with MK5, HeLa cells were transfected with EGFP-MK5 and C $\alpha$  expression plasmids, and reciprocal co-immunoprecipitation studies were performed with anti-C $\alpha$  antibody fol-

lowed by Western blotting with MK5 antibody or MK5 antibody, or co-immunoprecipitation with anti-EGFP antibody followed by Western blotting with anti-C $\alpha$  antibody. A specific interaction was monitored between these two proteins (Fig. 2A). The positive signal in Fig. 2A, lane 6 (lysates of cells ectopically expressing EGFP-MK5 but not C $\alpha$ ), is probably the result



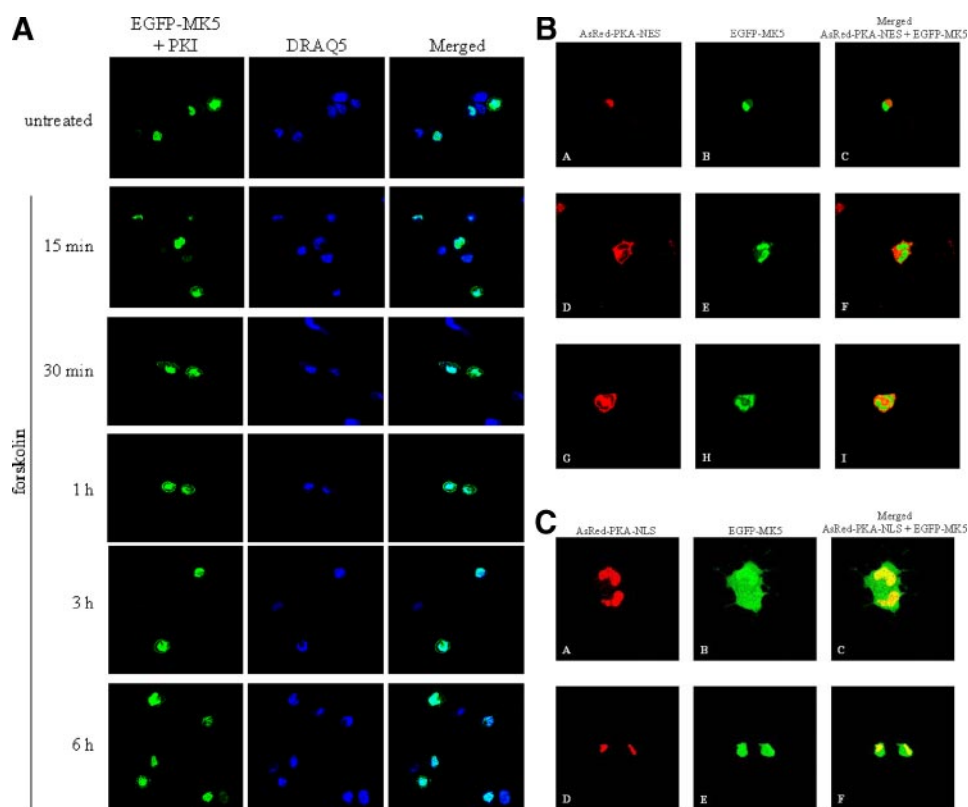
**FIGURE 3. The catalytic subunit C $\alpha$  of PKA increased the phosphorylation level of MK5, but not MK2, *in vitro*.** *A*, top panel (IVK) shows *in vitro* phosphorylation of MK5 by C $\alpha$ . Left lane, purified C $\alpha$ ; middle lane, purified MK5; right lane, C $\alpha$  plus MK5. The positions of phosphorylated C $\alpha$  (pC $\alpha$ ) and phosphorylated MK5 (pMK5) are indicated. Middle panel, the samples used for *in vitro* kinase assay were tested for equal amounts of MK5 protein by Western blot (WB) with anti-MK5 antibodies. The values below the top and middle panel represent relative densitometric units (RDU) of the scanned phospho-MK5 (pMK5) and MK5 signals, respectively. The ratio of relative densitometric units of total MK5 protein (Western blot signals) and of phosphorylated MK5 (*in vitro* kinase signals) is shown at the bottom panel. *B*, C $\alpha$  does not phosphorylate MK2 *in vitro*. Lane 1, MK2 protein; lane 2, purified C $\alpha$  protein; lane 3, MK2 protein plus C $\alpha$ ; lane 4, MBP plus C $\alpha$ . The lower panels represent Western blot with anti-MK2 and anti-C $\alpha$  antibodies, respectively. BSA, which is present in the kinase assay buffer, is also phosphorylated by C $\alpha$ . The positions of the phosphorylated proteins (pBSA, pC $\alpha$ , and pMBP) are shown. The amount of MK2 protein (middle panel) and C $\alpha$  (bottom panel) in each sample was assayed by Western blot.

of interaction between ectopically expressed MK5 and endogenous C $\alpha$ . No interactions were detected in the control pulldown assays using empty expression vectors. Because of the sequence similarity between MK2 and MK5, we investigated whether C $\alpha$  also associates with MK2. Cells were co-transfected with EGFP-MK2 and C $\alpha$  expression vectors or empty vectors as controls, and lysates were immunoprecipitated with anti-C $\alpha$  or anti-EGFP antibodies. The presence of the co-immunoprecipitated partner was detected by Western blot using anti-Mk2 or anti-C $\alpha$  antibodies, respectively. However, under these experimental conditions no interaction between MK2 and C $\alpha$  was detected (Fig. 2B). The MAPKAP kinase RSK1 was reported to interact with both the C $\alpha$  and the regulatory subunit of PKA (36). This prompted us to test whether such an interaction also occurred with MK5. No association between MK5 and the regulatory RI $\alpha$  subunit of PKA was



**FIGURE 4. The PKA catalytic subunit C $\alpha$  stimulates the kinase activity of MK5 *in vitro* and *in vivo*.** *A*, *in vitro* kinase (IVK) assay (top panel) was performed with purified C $\alpha$  and/or MK5 with recombinant ERK3 as substrate. Lane 1, purified C $\alpha$ ; lane 2, purified inactive MK5; lane 3, purified ERK3; lane 4, C $\alpha$  plus ERK3; lane 5, inactive MK5 plus ERK3; lane 6, C $\alpha$  plus inactive MK5 plus ERK3. The total amount of ERK3 protein in the samples was monitored by Western blot (WB, lower panel). *B*, cells were transfected with expression plasmids for the EGFP-MK5 fusion protein and C $\alpha$  or empty vector. As a positive control for the assay, cells were either transfected with plasmids encoding the constitutively active MK5 L337A mutant (EGFP-MK5 L337A) or with EGFP-MK5 and constitutively active MKK6/E plus p38 MAPK. Cell lysates were immunoprecipitated with anti-EGFP antibody, and the MK5 kinase activity was determined by an immune complex kinase assay with PRAKtide as a substrate. The relative kinase activity in of wild-type MK5 in the absence of C $\alpha$  was arbitrary set as 100%, and the activity of the other samples was related to this. The results represent the average of 5–6 independent experiments.

observed (results not shown). Next, we examined whether PKA, in agreement with the MK5-interacting protein kinases ERK3 and ERK4, possessed the ability to phosphorylate MK5 and activate its intrinsic kinase activity. An *in vitro* kinase assay demonstrated that both C $\alpha$  and MK5 possessed autophosphorylation activity, and that incubation of purified C $\alpha$  with purified MK5 increased the phosphorylation levels of MK5 ~2.5-fold but not of C $\alpha$  (Fig. 3A). C $\alpha$  did not phosphorylate MK2 but phosphorylated the myelin basic protein (MBP), which was used as a positive control (Fig. 3B). The inability of C $\alpha$  to phosphorylate MK2 is in agreement with our previous findings that MK2 and C $\alpha$  did not interact *in vivo* (Fig. 2B). To monitor the effect of PKA on the enzymatic activity of MK5, we performed an *in vitro* kinase assay. ERK3 was selected as a substrate for MK5. ERK3 displayed low levels of autophosphorylation activity. C $\alpha$  alone or inactive MK5 alone did not induce phosphorylation of ERK3. However, incubation of C $\alpha$  plus inactive MK5 strongly increased the phosphorylation levels of ERK3 (Fig. 3A, top panel). Western blotting with anti-ERK3 antibodies ensured that equal amounts of ERK3 substrate were used in the samples (Fig. 3A, bottom panel). These results suggest that C $\alpha$  enhanced the kinase activity of MK5 toward ERK3. Subsequently, we tested the effect of PKA on the *in vivo* kinase activity of MK5 using an immune complex kinase assays. Thereto, a series of co-transfections with EGFP-MK5 and C $\alpha$ -NLS expression plasmids or empty vectors (pEGFP-C1 and pRcCMV, respectively) were performed, and the protein kinase activity of MK5 toward the synthetic PRAKtide substrate was measured. Although EGFP alone or in the presence of C $\alpha$  had no kinase activity toward PRAKtide (results not shown), EGFP-



**FIGURE 5. Forskolin- or  $\text{Ca}$ -induced nuclear export of MK5 requires nuclear entrance of  $\text{Ca}$ .** *A*, PC12 cell that were co-transfected with an expression plasmid encoding PKI and EGFP-MK5 were treated with forskolin, and the subcellular localization of MK5 was monitored over time. The nuclei of the cells were visualized with DRAQ5 staining (blue channel). *B*, cytoplasmic retention of  $\text{Ca}$  prevents nuclear exclusion of MK5. PC12 cells were co-transfected with expression plasmids for EGFP-MK5 and Red-PKA-NES. The latter is a fusion protein of red fluorescent protein and  $\text{Ca}$  tagged with a functional NES. *Panels A, D, and G*, NES-tagged  $\text{Ca}$  was visualized directly (red channel); *panels B, E, and H*, EGFP-MK5 was visualized directly (green channel); *panels C, F, and I*, merged images of the red and green channels. *C*, nucleus-directed localization of  $\text{Ca}$  results in nuclear export of MK5. PC12 cells were co-transfected with expression vectors for EGFP-MK5 and for red fluorescent- $\text{Ca}$  fusion protein tagged with a functional NLS signal (As-Red-PKA-NLS). *Panels A and D*, NLS-tagged  $\text{Ca}$  was visualized directly (red channel); *panels B and E*, EGFP-MK5 was visualized directly (green channel); *panels C and F*, merged images of the red and green channels.

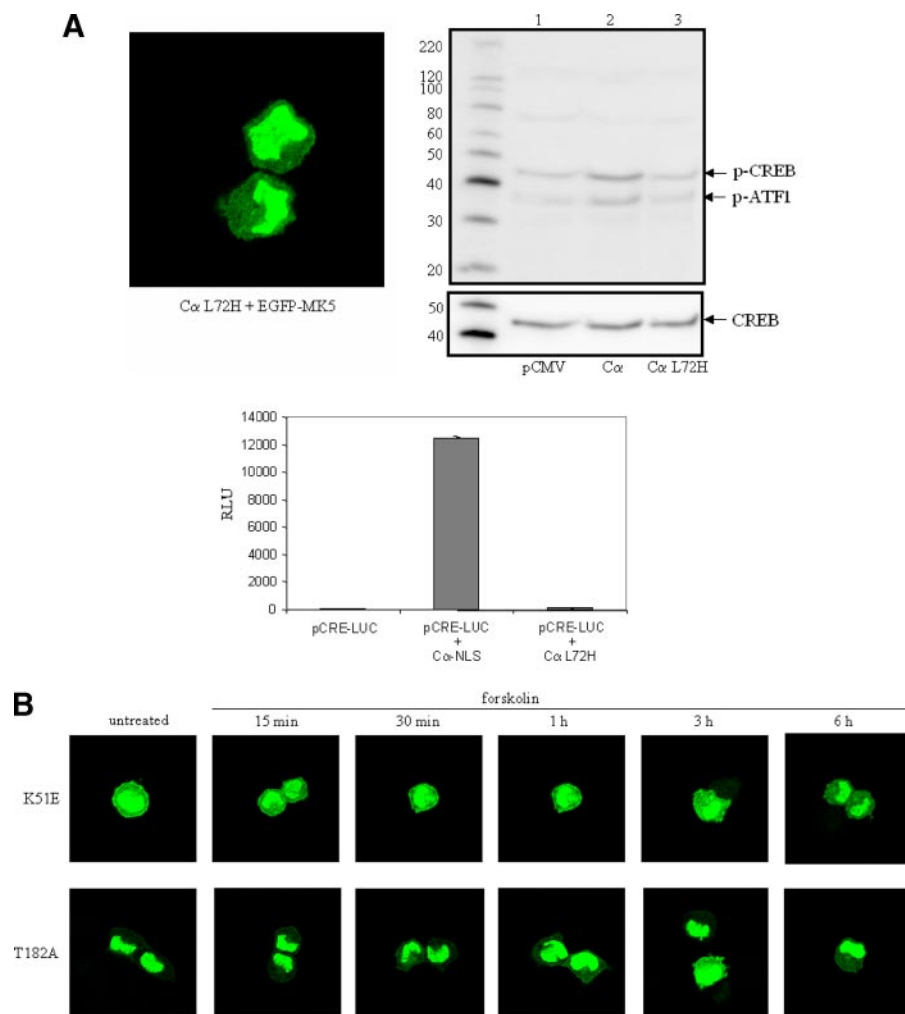
MK5 phosphorylated PRAKTide. Co-expression of  $\text{Ca}$  enhanced the kinase activity of MK5 ~2-fold in most experiments ( $n = 6$ ), although occasionally we did not observe an increase of MK5 activity in the presence of  $\text{Ca}$  ( $n = 2$ ). The constitutively active MK5 mutant L337A (37) and an activated MK5 by co-expression of active MKK6 E/E and p38 MAPK possessed a 3–5-fold increased kinase activity compared with wild-type MK5 (Fig. 4B). Forskolin stimulation alone also induced the kinase activity of MK5 (results not shown). In conclusion,  $\text{Ca}$  physically interacts with and stimulates the kinase activity of MK5 *in vivo*.

**PKA-induced Nuclear Export of MK5 Requires Nuclear Localization of  $\text{Ca}$** —The co-immunoprecipitation studies suggest an *in vivo* interaction between  $\text{Ca}$  and MK5, but they do not establish whether  $\text{Ca}$  is directly involved in the subcellular redistribution of MK5. We therefore wanted to determine whether nuclear entrance of  $\text{Ca}$  is required to induce nuclear export of MK5. We monitored the subcellular localization of MK5 in forskolin-treated cells that overexpress PKI, a peptide that contains a strong nuclear export signal and that can export  $\text{Ca}$  out of the nucleus (for a recent review see Ref. 45). Overexpression of PKI prevented forskolin-induced nuclear export of

MK5 in PC12 cells. Of the EGFP-MK5-expressing cells, in 98% of the cells ( $\pm 2\%$ ; 50 cells were counted in several independent experiments) MK5 was mainly in the nucleus (Fig. 5A). This is in contrast with the transient nuclear exclusion of MK5 in forskolin-treated PC12 cells (Fig. 1). To further ascertain the necessity of nuclear entry of  $\text{Ca}$  in MK5 cytoplasmic relocation, we compared the residence of EGFP-MK5 in cells co-transfected with an expression plasmid for EGFP-MK5 plus an expression vector for  $\text{Ca}$  tagged with either NLS or with NES. To visualize  $\text{Ca}$  and MK5 simultaneously, we used red fluorescence protein- $\text{Ca}$  fusion proteins (AsRed-PKA-NLS and AsRed-PKA-NES, respectively). The NES-tagged  $\text{Ca}$  subunit resided in the cytoplasm and was unable to induce nuclear export of MK5, whereas in cells expressing a  $\text{Ca}$ -NLS fusion protein both cytoplasmic and nuclear MK5 locations were observed (in  $90 \pm 2\%$  of the analyzed cells; Fig. 5, B and C). The levels of cytoplasmic MK5 in  $\text{Ca}$ -NLS expressing cells were increased compared with cells not expressing  $\text{Ca}$ -NLS (compare untreated cells in Figs. 1A and 5C).

**PKA-induced Nuclear Export of MK5 Requires Protein Kinase Activity of PKA as Well as MK5**—Next,

we investigated whether the kinase activity of  $\text{Ca}$  was required to provoke nuclear export of MK5. Thereto, expression plasmids for the kinase-dead  $\text{Ca}$  mutant ( $\text{Ca}$  L72H) and EGFP-MK5 were co-transfected in PC12 cells, and subcellular localization of MK5 was monitored. In contrast to an active  $\text{Ca}$  enzyme, no nuclear export was observed with the mutant catalytic PKA subunit. In fact, in 98% of the cells ( $\pm 2\%$ ; 50 cells were counted in several independent experiments), MK5 resided predominantly in the nucleus (Fig. 6A). Western blot analysis of cell extracts transfected with the kinase-dead  $\text{Ca}$  L72H mutant confirmed that this mutant had no enzymatic activity because phosphorylation of the transcription factor CREB, which is a genuine substrate for PKA, did not increase in the presence of this mutant, whereas increased phospho-CREB was detected in extracts prepared from cells transfected with the active  $\text{Ca}$  expression plasmid. Moreover, the kinase-dead  $\text{Ca}$  mutant was unable to stimulate CRE-dependent transcription (Fig. 6A, bottom panel). To decide whether MK5 enzyme activity is required for PKA-regulated nuclear export, we monitored the subcellular distribution of the kinase-dead MK5 K51E mutant and the T182A mutant in the phosphorylation loop. Both mutants also failed to relocate to the cytoplasm in the presence of activated



**FIGURE 6. The kinase activities of both the PKA catalytic subunit C $\alpha$  and MK5 are required to induce nuclear export of MK5.** *A*, PC12 cells were co-transfected with expression plasmids encoding EGFP-MK5 fusion protein and with nucleus-directed kinase-dead C $\alpha$ L72H mutant. The subcellular distribution of MK5 was monitored by confocal microscopy (*left panel on the top*). *Middle panel on the top*, phosphospecific CREB antibodies failed to detect increased phosphorylation of CREB at Ser-133 in cells expressing kinase-dead C $\alpha$  L72H, whereas phospho-CREB was monitored in cells expressing kinase active C $\alpha$ . *Lane 1*, extracts of cells transfected with empty vector pRCMV; *lane 2*, extracts of cells transfected with wild-type C $\alpha$  expression vector; *lane 3*, extracts of cells transfected with expression plasmid for C $\alpha$  L72H. The cells were serum-starved overnight after transfection before they were harvested for Western blotting analysis. The phospho-CREB antibodies cross-react with phospho-ATF1, a member of the CREB superfamily. The membrane was stripped and blotted with anti-CREB antibodies to ensure equal loading. The molecular mass (in kDa) of the protein marker is indicated. *Right panel on the top*, HEK293 cells were transfected using Lipofectamine 2000 with 100 ng of pCRE-LUC reporter plasmid and 100 ng of expression vector encoding wild-type C $\alpha$  or kinase-dead C $\alpha$  L72H. Four hours after transfection, the medium was replaced with fresh medium containing 0.2% serum. Luciferase activity (expressed as relative luciferase units; RLU) was measured 20 h later. The results represent the average  $\pm$  S.D. of three independent experiments. *B*, forskolin does not induce transient nuclear exclusion of the kinase death MK5 K51E and MK5 T182A mutants. Cells were transfected with expression plasmids for the EGFP-MK5 K51E (*top panel*) or EGFP-MK5 T182 (*bottom panel*) fusion proteins. Green fluorescence was monitored at different time points after stimulation with 10  $\mu$ M forskolin.

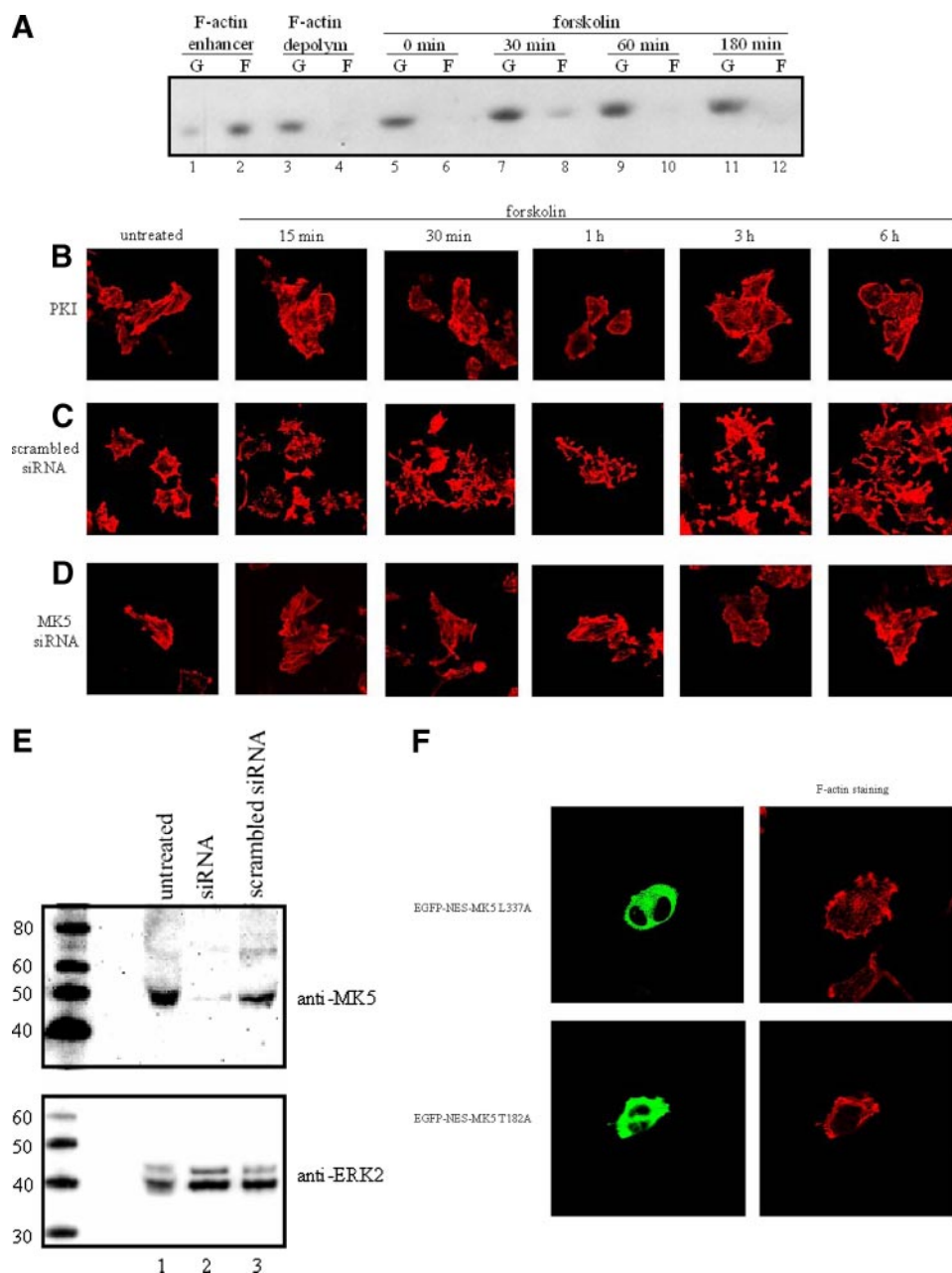
PKA (Fig. 6B). In agreement with our observations in PC12 cells, forskolin treatment of HeLa cells also induced transient nuclear export of EGFP-MK5 protein, but not of the kinase-dead T182A mutant, indicating a non-cell-specific mechanism (results not shown). These findings indicate that both an active C $\alpha$  and active MK5 are required for PKA-induced cytoplasmic translocation in PC12 cells.

**MK5 Mediates Forskolin-induced F-actin Rearrangement—**We and others noticed that forskolin treatment of PC12 cells resulted in altered cytoskeleton morphology (11, 30). There-

fore, we assessed the amount of F-actin in response to forskolin. A transient increase in F-actin was observed 30 min after forskolin treatment (Fig. 7A). Interestingly, the kinetics of F-actin rearrangement and increase coincided with the nuclear exclusion of MK5, presuming the involvement of the MK5 in PKA-induced F-actin remodeling. To address the possible implication of MK5 in cAMP/PKA-induced F-actin rearrangement, several different experimental approaches were conducted. We reasoned that the coupled event nuclear import of C $\alpha$ /cytoplasmic export of MK5 would be required for F-actin reorganization. Therefore, we compared the microfilament structure in untreated and forskolin-treated nontransfected cells or cells transfected with a PKI expression plasmid. F-actin remodeling was inhibited in cells overexpressing PKI (>75% of the PKI transfected cells did not display F-actin rearrangement after forskolin exposure; Fig. 7B). This underscores that nuclear import of PKA is necessary and suggests that nuclear export of MK5 may be required for cAMP/PKA-induced F-actin remodeling. Next, we examined forskolin-induced F-actin rearrangement in control cells and in cells in which the levels of MK5 protein had been depleted by validated siRNA against MK5. In agreement with previous reports, we observed that forskolin treatment of PC12 cells, which had been transfected with scrambled siRNA, resulted in F-actin remodeling (Fig. 7C). However, the majority of cells (>75%; 50 cells counted in two independent experiments) treated with MK5-directed siRNA did not show F-actin rear-

rangements after forskolin treatment (Fig. 7D). Although siRNA directed against MK5 transcripts strongly reduced MK5, but not ERK2 protein levels, scrambled siRNA did not reduce the levels of either of these proteins (Fig. 7E). Taken together, these results indicate that MK5 mediates forskolin-induced F-actin remodeling. To elaborate the role of activated, cytoplasmic MK5 in F-actin rearrangement, we generated an MK5 mutant that is constitutively active and that locates to the cytoplasm. Our previous study had shown that a single amino acid substitution in the functional NES motif of MK5 (L337A)

## PKA-induced F-actin Rearrangement by MK5



**FIGURE 7. Forskolin alters the amount of F-actin and PKA-induced F-actin remodeling is mediated by MK5.** *A*, PC12 cells were left untreated (*lanes 1–4*) or treated with forskolin for the time points indicated (*lanes 5–12*). After the cells were lysed, G-actin and F-actin were separated by ultracentrifugation, with G-actin found in the supernatant and F-actin present in the pellet. Both fractions were run on a denaturing polyacrylamide gel, and G-actin (G) and F-actin (F) were detected by Western blotting using an anti-actin antibody. F-actin enhancing solution was added to the lysate of cells, and the fractions after ultracentrifugation are shown in *lanes 1* and *2*. F-actin depolymerization solution was added to the lysate of cells, and the fractions after ultracentrifugation are shown in *lanes 3* and *4*. *B*, nuclear exclusion of activated PKA by PKI prevents forskolin-induced F-actin remodeling. Cells transfected with a PKI expression plasmid were stimulated with forskolin 24 h after transfection and were then monitored for microfilament structure by staining F-actin. *C*, PC12 cells, transfected with scrambled siRNA, were treated with forskolin ( $10 \mu\text{M}$ ) for the indicated time points, and the architecture of the microfilaments was examined by staining F-actin with Alexa Fluor 594 phalloidin. *D*, depletion of MK5 protein ablates forskolin-induced F-actin rearrangements in PC12 cells. Cells were transfected with  $100 \mu\text{M}$  siRNA/ $10^6$  cells and were stained for F-actin 48 h after transfection. *E*, treatment of PC12 cells with siRNA against MK5, but not with scrambled siRNA, reduced the MK5 protein levels, whereas the protein amounts of ERK2 remained unaffected. Cells were transfected with siRNA or left untreated, and lysates were prepared 24 h after transfection. The amount of MK5 was visualized by Western blotting. *F*, cytoplasmic expression of activated MK5 is sufficient to induce F-actin rearrangement. PC12 cells were transfected with either expression plasmids encoding EGFP fusion proteins with activated MK5 L337A or MK5 T182A mutants tagged with a functional NES. Twenty four hours after transfection, F-actin was stained, and the nuclear localization of MK5 was monitored.

converted MK5 in a constitutively active kinase. However, this mutant has lost its ability to shuttle to the cytoplasm (37). To conduct this active MK5 variant to the cytoplasm, we fused the NES sequence of the human immunodeficiency virus Rev protein to EGFP-MK5 to generate the fusion protein EGFP-NES-MK5 L337A. Studies by others had shown that this NES motif could direct nuclear exclusion of EGFP (40). Concordantly, the EGFP-NES-MK5 L337A protein resides exclusively in the cytoplasm (Fig. 7*F*, top panel). All cells expressing this cytoplasmic, activated MK5 mutant (50 cells counted in three independent experiments) could induce some F-actin rearrangement in the absence of forskolin (Fig. 7*F*, top panel). Overlay of the confocal microscopy images confirmed F-actin rearrangements in cells transfected with this MK5 mutant (results not shown), whereas no such changes were observed in nontransfected cells. Cytoplasmic expression of EGFP-NES-MK5 T182A (Fig. 7*D*, bottom panel) or EGFP-NES-MK5 (results not shown) did not affect the microfilament structure in these cells, despite the cytoplasmic location of MK5. These results prove that an activated, cytoplasmic MK5 can partially mimic forskolin-induced F-actin rearrangement, and suggest that PKA-dependent nuclear export and activation of MK5 is implicated in PKA-triggered reorganization of F-actin.

## DISCUSSION

The MAPKAPK MK5 was first described almost 10 years ago, but to date little is known about its activators, *in vivo* substrates, and biological functions. The atypical MAPKs ERK3 and ERK4, were the first identified genuine substrates for MK5, but the functional importance of these interactions remains unclear (23–26). The original study with *mk5*<sup>-/-</sup> mice revealed no obvious phenotypical defects (22), but a recent study with MK5-deficient mice led to the recognition of the

implication of MK5 in *ras*-induced senescence and tumor suppression in a p38 MAPK-dependent manner through activation of p53 (27). Results from our studies assign the following: (i) a novel mode of regulating the subcellular localization of MK5, (ii) PKA as a new activator for MK5, and (iii) a previously unrecognized biological function of MK5 in F-actin rearrangements.

MK5, which contains functional but overlapping NLS and NES motifs, accumulates in the nucleus of resting cells. The NLS signal seems to be dominant over NES, probably because of the conformation of the MK5 protein in which the NES is less accessible to exportin. However, in response to cellular stress, the NES motif may be unmasked, and MK5 can be exported to the cytoplasm (37, 43). In this study, we observed that activated PKA triggered nuclear export of MK5. Time studies revealed that at least 15 min were required for MK5 to exit the nucleus in response to forskolin (Fig. 1), whereas nuclear translocation of the catalytic subunit of PKA in response to forskolin was reported to plateau after 15 min (46). Similar kinetics may indicate that nuclear import of activated PKA is necessary for nuclear export of MK5. Cytoplasmic redistribution of MK5 is also observed when ERK3 and ERK4 are overexpressed. The mechanisms of ERK3/ERK4-mediated nuclear export of MK5 differ, however, from  $C\alpha$ -mediated export. Co-expression of a kinase-dead  $C\alpha$  did not lead to accumulation of MK5 in the cytoplasm, and similarly, the kinase-dead MK5 variants K51E and T182A were not excluded from the nucleus by an active  $C\alpha$  or forskolin. On the contrary, kinase-dead ERK3 and ERK4 mutants were able to induce nuclear export of wild-type MK5, but also of catalytically dead MK5 mutant proteins (23–26). Further studies that identify the interaction regions on MK5 will provide additional information on the mechanistic insight how  $C\alpha$  regulates the nucleocytoplasmic relocation of MK5. Our preliminary results show that a C-terminal truncated MK5 consisting of residues 1–423 is not transported out of the nucleus upon forskolin treatment. On the other hand, ERK3 still relocates this truncated MK5 mutant to the cytoplasm (24), indicating that nuclear exclusion of MK5 by PKA is not mediated by ERK3. ERK4, however, does not translocate this MK5 variant to the cytoplasm (26). Whether ERK4 is implicated in PKA-induced nuclear export of MK5 remains to be established. The C-terminal part of MK5, which is absent in MK2 and MK3, may be critical for PKA-induced MK5 nuclear export. Because the C-terminal part of MK5 is absent in MK2 and MK3, it may explain the unique role of MK5 in PKA-triggered F-actin remodeling.

Next, we have provided experimental evidence that, similar to ERK3 and ERK4, co-expression of  $C\alpha$  with MK5 stimulated the kinase activity of MK5 *in vivo*. We also found enhanced phosphorylation of MK5 by *in vitro* kinase assay. Whether this is due to increased phosphorylation of Thr-182 in the activation loop of MK5 remains unsolved. We have tried to inspect whether forskolin or  $C\alpha$  triggers phosphorylation of MK5 at Thr-182 by using two different commercially available antibodies (anti-phospho-PRAK (Thr-182) antibodies from Upstate, Charlottesville, VA, and anti-phospho-threonine-proline antibodies from Cell Signaling, Beverly, MA). Unfortunately, none of these antibodies worked in our hands, as they did not recognize *in vitro* phosphorylated MK5 or immunoprecipitated MK5

from forskolin-treated or  $C\alpha$ -transfected cells. Moreover, we were unable to detect PKA-induced phosphorylation of MK5 *in vitro* and in cell extracts with a phospho-PKA (Ser/Thr) PKA substrate antibody (catalog number 9624, Cell Signaling).

The  $C\alpha$ -MK5 interaction described here represents a new example of cross-talk between the cAMP/PKA and MAPK signaling pathways. Various branches of interaction between the cAMP/PKA pathway and the different MAPK cascades have been demonstrated, *e.g.* PKA-mediated regulation of Raf and p38 MAPK, and recently also of another MAPKAPK, RSK1 (32, 34, 36, 47). Unphosphorylated RSK1 interacted with the regulatory RI subunits, whereas activated RSK1 bound the catalytic subunits of PKA. The interaction with PKA also regulated the subcellular localization of RSK1. The authors demonstrated that disruption of the interaction of RI subunits with PKA-anchoring protein resulted in an increase in cytosolically active RSK1 levels. The mechanism by which PKA induces nuclear export of MK5 appears to be different from RSK1 because we did not detect an *in vivo* interaction between MK5 and RI. Chaturvedi *et al.* (36) did not investigate whether  $C\alpha$  phosphorylated RSK1, nor was the effect of this interaction on the intrinsic kinase activity of RSK1 tested. We found that  $C\alpha$  increased the phosphorylation levels of MK5, but not vice versa.

Finally, we solved the biological relevance for PKA-induced subcellular redistribution and activation of MK5 by demonstrating that MK5 acted as a mediator for PKA-induced F-actin remodeling. Cytoplasm-directed expression of a constitutively active MK5 variant mimicked forskolin-induced F-actin rearrangement, whereas ablation of MK5 expression by siRNA prevented forskolin-provoked F-actin rearrangements. MK5 is not the only protein that is involved in cytoskeletal architecture and whose subcellular location is regulated by PKA. The RNA-binding protein, polypyrimidine tract-binding protein (PTB), was exported from the nucleus during PKA-induced F-actin rearrangement/neurite outgrowth in PC12 cells. Nuclear export of PTB required PKA-mediated phosphorylation of Ser-16. PTB preferentially associated with  $\beta$ -actin mRNA and thwarting PTB expression by RNA interference disrupted PKA-induced but not nerve growth factor-induced neurite outgrowth in PC12 cells. Similarly to MK5, PTB represents a protein involved in cytoskeletal dynamics whose subcellular distribution is regulated by PKA (48).

Several experimental observations indicate that p38 MAPK, MK2, and Hsp27 play a role in mediating alternations in filamentous actin in response to stimuli. First, MK2, p38 MAPK, Hsp27, and Akt can form a multiprotein complex (49, 50). In addition, stress-inducing stimuli activated p38 MAPK, and this coincided with phosphorylation of MK2 and Hsp27 and changes in F-actin dynamics in different cell lines (51–55). Furthermore, a regulatory role for MK2 in F-actin dynamics during embryonic development was also proposed by the work of Paliga *et al.* (56). However, none of the above-mentioned studies directly addressed the role of MK5 in F-actin remodeling, nor was the participation of MK2 unequivocally proven by RNA interference studies or overexpression of dominant negative variants of these kinases, for example. Studies in rat pulmonary artery microvascular endothelial cells revealed a more direct causal involvement of MK2 in hypoxia-induced actin reorgani-

zation. Indeed, overexpression of a constitutively active MK2 resembled, whereas a dominant negative MK2 mutant prevented, the effect of hypoxia on F-actin structure (57). Stimuli-induced alterations in microfilament structure may require different pathways that signal through distinct MKs. For example, stimuli that activate p38 MAPK (e.g. oxidative stress) may engage MK2, whereas stimuli signaling through the cAMP/PKA (e.g. secretin) pathway may utilize MK5 to provoke changes in F-actin structures (51, 55, 58). The precise molecular mechanism by which MK5 participates in F-actin remodeling remains elusive, but may in concordance with MK2 imply phosphorylation of Hsp27. We could detect MK5-Hsp27 interaction by co-immunoprecipitation and found that MK5 phosphorylated Hsp27 *in vitro*.<sup>4</sup> On the other hand, studies by Gaestel and co-workers (22) with MK5-deficient mice jeopardize the role for MK5 in Hsp27 phosphorylation. The authors showed that Hsp27 did not interact with MK5 *in vivo*, at least in primary murine cells isolated from a strain with a mixed 129xC57/B6 genetic background (22). It cannot be ruled out that Hsp27 is a *bona fide* substrate for MK5 in rat PC12 cells or that PKA-provoked phosphorylation/activation or conformational changes of MK5 are required to enable *in vivo* interaction with Hsp27. Observations in *mk5*<sup>-/-</sup> mice further support a putative role for MK5 in regulation of the cytoskeleton structure and cell migration. Cell motility requires reorganization of the cytoskeleton, and directed cell migration is important for embryonic development. Depending on the genetic background, MK5-deficient animals, but not *mk2*<sup>-/-</sup> mice, displayed embryonic lethality with incomplete penetrance around day E11.5. This developmental failure may be the result of impaired cell migration (23).

Activation of the nuclear transcription factor CREB by phosphorylation at Ser-133 has been documented to be involved in cAMP-provoked cytoskeletal changes in PC12 cells (59). CREB can be phosphorylated by numerous protein kinases, including MAPKAPK (60). It is unlikely that MK5-mediated F-actin rearrangement in forskolin-treated PC12 cells requires the action of CREB because forskolin induced redistribution of MK5 to another subcellular compartment than CREB, and MK5 did not interact with CREB *in vivo*, nor did MK5 phosphorylate CREB or activate CREB-mediated transcription (61).

In conclusion, our studies have revealed a novel function of MK5 in microfilament rearrangement in response to increased cAMP levels and disclosed a new interaction between the cAMP/PKA and the MAPK signaling pathways. These findings may increase our molecular understanding of stimulus-induced changes in microfilament and cross-talk between signal transduction pathways. Whether MK5 is involved in other cytoskeletal rearrangements and/or cell motility events waits to be proven, but phosphorylation of the putative MK5 substrate, Hsp25/27, also contributes to cell migration. In addition, the F-actin interacting myosin light chain protein was shown to be an *in vitro* substrate for MK5 (10, 62–64). Where, when, and under which circumstances MK5 participates in cytoskeletal dynamics in an organism are questions that need to be

answered to increase our understanding of the role of MK5 in normal and possible pathological processes to apply therapeutic strategies in diseases associated with perturbed MK5 activity.

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REFERENCES

- Huang, C., Jacobson, K., and Schaller, M. D. (2004) *J. Cell Sci.* **117**, 4619–4628
- Roux, P. P., and Blenis, J. (2004) *Microbiol. Mol. Biol. Rev.* **68**, 320–344
- Imajo, M., Tsuchiya, Y., and Nishida, E. (2006) *IUBMB Life* **58**, 312–317
- Krens, S. F., Spaink, H. P., and Snaar-Jagalska B. E. (2006) *FEBS Lett.* **580**, 4984–4990
- Whitmarsh, A. J. (2007) *Biochim. Biophys. Acta* **1773**, 1285–1298
- Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298**, 1912–1934
- Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leever, S. J., Marshall, C., and Cohen, P. (1992) *EMBO J.* **11**, 3985–3994
- Sithanandam, G., Latif, F., Duh, F. M., Bernal, R., Smola, U., Li, H., Kuzmin, I., Wixler, V., Geil, L., and Shrestha, S. (1996) *Mol. Cell. Biol.* **16**, 868–876
- New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C. N., and Han, J. (1998) *EMBO J.* **17**, 3372–3384
- Ni, H., Wang, X. S., Diener, K., and Yao, Z. (1998) *Biochem. Biophys. Res. Commun.* **243**, 492–496
- Gaestel, M. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 120–130
- Gerits, N., Kostenko, S., and Moens, U. (2007) *Transgenic Res.* **16**, 281–314
- Han, J., and Ulevitch, R. J. (1999) *Nat. Cell Biol.* **1**, E39–E40
- Kotlyarov, A., Neiningner, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) *Nat. Cell Biol.* **1**, 94–97
- Kotlyarov, A., Yannoni, Y., Fritz, S., Laas, K., Telliez, J. B., Pitman, D., Lin, L. L., and Gaestel, M. (2002) *Mol. Cell. Biol.* **22**, 4827–4835
- Manke, I. A., Nguyen, A., Lim, D., Stewart, M. Q., Elia, A. E. H., and Yaffe, M. B. (2005) *Mol. Cell* **17**, 37–48
- Culbert, A. A., Skaper, S. D., Howlett, D. R., Evans, N. A., Facci, L., Soden, P. E., Seymour, Z. M., Guillot, F., Gaestel, M., and Richardson, J. C. (2006) *J. Biol. Chem.* **281**, 23658–23667
- Kobayashi, M., Nishita, M., Mishima, T., Ohashi, K., and Mizuno, K. (2006) *EMBO J.* **25**, 713–726
- Neufeld, B., Grosse-Wilde, A., Hoffmeyer, A., Jordan, B. W., Chen, P., Dinev, D., Ludwig, S., and Rapp, U. R. (2000) *J. Biol. Chem.* **275**, 20239–20242
- Voncken, J. W., Niessen, H., Neufeld, B., Rennfahrt, U., Dahlmans, V., Kubben, N., Holzer, B., Ludwig, S., and Rapp, U. R. (2005) *J. Biol. Chem.* **280**, 5178–5187
- Houben, R., Becker, J. C., and Rapp, U. R. (2005) *J. Carcinogen.* **20**, 23
- Shi, Y., Kotlyarov, A., Laas, K., Gruber, A. D., Butt, E., Marcus, K., Meyer, H. E., Friedrich, A., Volk, H. D., and Gaestel, M. (2003) *Mol. Cell. Biol.* **23**, 7732–7741
- Schumacher, S., Laas, K., Kant, S., Shi, Y., Visel, A., Gruber, A. D., Kotlyarov, A., and Gaestel, M. (2004) *EMBO J.* **23**, 4770–4779
- Seternes, O. M., Mikalsen, T., Johansen, B., Michaelsen, E., Armstrong, C. G., Morrice, N. A., Turgeon, B., Meloche, S., Moens, U., and Keyse, S. M. (2004) *EMBO J.* **23**, 4780–4791
- Kant, S., Schumacher, S., Singh, M. K., Kisper, A., Kotlyarov, A., and Gaestel, M. (2006) *J. Biol. Chem.* **281**, 35511–35519

<sup>4</sup>N. Gerits, T. Mikalsen, S. Kostenko, A. Shiryayev, M. Johannessen, and U. Moens, unpublished results.

26. Åberg, E., Perander, M., Johansen, B., Julien, C., Meloche, S., Keyse, S. M., and Seternes, O. M. (2006) *J. Biol. Chem.* **281**, 35499–35510
27. Sun, P., Yoshizuka, N., New, L., Moser, B. A., Li, Y., Liao, R., Xie, C., Chen, J., Deng, Q., Yamout, M., Dong, M. Q., Frangou, C. G., Yates, J. R., III, Wright, P. E., and Han, J. (2007) *Cell* **128**, 295–308
28. Skälhegg, B., and Taskén, K. (2000) *Front. Biosci.* **5**, D678–D693
29. Stork, P. J., and Schmitt, J. M. (2002) *Trends Cell Biol.* **12**, 258–266
30. Howe, A. K. (2004) *Biochim. Biophys. Acta* **1692**, 159–174
31. Johannessen, M., and Moens, U. (2005) in *Trends in Cellular Signalling* (Caplin, D. E., ed) pp. 41–78, Nova Science Publishers, Hauppauge, NY
32. Saxena, M., Williams, S., Taskén, K., and Mustelin, T. (1999) *Nat. Cell Biol.* **1**, 305–311
33. Delghandi, M. P., Johannessen, M., and Moens, U. (2005) *Cell. Signal.* **17**, 1343–1351
34. Houslay, M. D. (2006) *Sci. STKE* 2006, PE32
35. Pearson, G. W., Earnest, S., and Cobb, M. H. (2006) *Mol. Cell. Biol.* **26**, 3039–3047
36. Chaturvedi, D., Poppleton, H. M., Stringfield, T., Barbier, A., and Patel, T. B. (2006) *Mol. Cell. Biol.* **26**, 4586–4600
37. Seternes, O. M., Johansen, B., Hegge, B., Johannessen, M., Keyse, S. M., and Moens, U. (2002) *Mol. Cell. Biol.* **22**, 6931–6945
38. Day, R. N., Walder, J. A., and Maurer, R. A. (1989) *J. Biol. Chem.* **264**, 431–436
39. Thiel, G., Al Sarraj, V., Vinson, C., Stefano, L., and Bach, K. (2005) *J. Neurochem.* **92**, 321–336
40. Thyssen, G., Li, T. H., Lehmann, L., Zhuo, M., Sharma, M., and Sun, Z. (2006) *Mol. Cell. Biol.* **26**, 8857–8867
41. Bok, J., Zha, X. M., Cho, Y. S., and Green, S. H. (2003) *J. Neurosci.* **23**, 777–787
42. Johannessen, M., Delghandi, M. P., Seternes, O. M., Johansen, B., and Moens, U. (2004) *Cell. Signal.* **16**, 1187–1199
43. New, L., Jiang, Y., and Han, J. (2003) *Mol. Biol. Cell* **14**, 2603–2616
44. Yao, J., Erickson, J. D., and Hersch, L. B. (2004) *Traffic* **5**, 1006–1016
45. Dalton, G. D., and Dewey, W. L. (2006) *Neuropeptides* **40**, 23–34
46. Montminy, M. (1997) *Annu. Rev. Biochem.* **66**, 807–822
47. Dumaz, N., and Marais, R. (2005) *FEBS J.* **272**, 3491–3504
48. Ma, S., Liu, G., Sun, Y., and Xie, J. (2007) *Biochim. Biophys. Acta* **1773**, 912–923
49. Zheng, C., Lin, Z., Zhao, Z. J., Yang, Y., Niu, H., and Shen, X. (2006) *J. Biol. Chem.* **281**, 37215–37226
50. Wu, R., Kausar, H., Johnson, P., Montoya-Durango, D. E., Merchant, M., and Rane, M. J. (2007) *J. Biol. Chem.* **282**, 21598–21608
51. Huot, J., Houle, F., Marceau, F., and Landry, J. (1997) *Circ. Res.* **80**, 383–392
52. Larsen, J. K., Yamboliev, I. A., Weber, L. A., and Gerthoffer, W. T. (1997) *Am. J. Physiol.* **273**, L930–L940
53. Huot, J., Houle, F., Rousseau, S., Deschesnes, R. G., Shah, G. M., and Landry, J. (1998) *J. Cell Biol.* **143**, 1361–1373
54. Schäfer, C., Ross, S. E., Bragado, M. J., Groblewski, G. E., Ernst, S. A., and Williams, J. A. (1998) *J. Biol. Chem.* **273**, 24173–24180
55. Azuma, N., Akasaka, N., Kito, H., Ikeda, M., Gahtan, V., Sasajima, T., and Sumpio, B. E. (2001) *Am. J. Physiol.* **280**, H189–H197
56. Paliga, A. J. M., Natale, D. R., and Watson, A. J. (2005) *Biol. Cell* **97**, 629–640
57. Kayyali, U. S., Pennella, C. M., Trujillo, C., Villa, O., Gaestel, M., and Hassoun, P. M. (2002) *J. Biol. Chem.* **277**, 42596–42602
58. Kim, H. S., Yumkham, S., Kim, S. H., Yea, K., Shin, Y. C., Ryu, S. H., and Suh, P. G. (2006) *Exp. Mol. Med.* **38**, 85–93
59. Shimomura, A., Okamoto, Y., Hirata, Y., Kobayashi, M., Kawakami, K., Kiuchi, K., Wakabayashi, T., and Hagiwara, M. (1998) *J. Neurochem.* **70**, 1029–1034
60. Johannessen, M., and Moens, U. (2007) *Front. Biosci.* **12**, 1814–1832
61. Johannessen, M., Delghandi, M. P., Rykx, A., Dragset, M., Vandenheede, J. R., Van Lint, J., and Moens, U. (2007) *J. Biol. Chem.* **282**, 14777–14787
62. Hedges, J. C., Dechert, M. A., Yamboliev, I. A., Martin, J. L., Hickey, E., Weber, L. A., and Gerthoffer, W. T. (1999) *J. Biol. Chem.* **274**, 24211–24219
63. Pichon, S., Bryckaert, M., and Berrou, E. (2004) *J. Cell Sci.* **117**, 2569–2577
64. Rousseau, S., Dolado, I., Beardmore, V., Shpiro, N., Marquez, R., Nebrada, A. R., Arthur, J. S., Case, L. M., Tessier-Lavigne, M., Gaestel, M., Cuenda, A., and Cohen, P. (2006) *Cell. Signal.* **18**, 1897–1905