MAP Kinase Phosphatase 3 (MKP3) Interacts with and Is Phosphorylated by Protein Kinase CK2α*

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Mitogen-activated protein (MAP) kinases play a central role in controlling a wide range of cellular functions following their activation by a variety of extracellular stimuli. MAP kinase phosphatases (MKPs) represent a subfamily of dual specificity phosphatases, which negatively regulate MAP kinases. Although ERK2 activity is regulated by its phosphorylation state, MKP3 is regulated by physical interaction with ERKK2, independent of its enzymatic activity (Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkinstall, S., (1998) Science 280, 1262–1265; Farooq, A., Chaturvedi, G., Muttaba, S., Plotnikova, O., Zeng, L., Dhalluin, C., Ashton, R., and Zhou, M. M. (2001), Mol. Cell 7, 387–399; Zhou, B., and Zhang, Z. Y. (1999) J. Biol. Chem. 274, 35526–35534). The interaction of ERK2 and MKP3 allows the reciprocal cross-regulation of their catalytic activity. Indeed, MKP3 acts as a negative regulator on ERK2-MAP kinase signal transduction activity, representing thus a negative feedback for this MAPK pathway. To identify novel proteins able to complex MKP3, we used the yeast two-hybrid system. Here we report that MKP3 and protein kinase CK2 form a protein complex, which can include ERK2. The phosphatase activity of MKP3 is then slightly increased in vitro, whereas in transfected cells, ERK2 dephosphorylation is reduced. In addition, we demonstrated that CK2 selectively phosphorylates MKP3, suggesting cross-regulation between CK2α and MKP3, as well as a modulation of ERK2-MAPK signaling by CK2α via MKP3.

Dual specificity phosphatases (DSPs),1 capable of dephosphorylating serine/threonine and tyrosine residues, represent a subclass of the protein tyrosine phosphatase gene super family, which appears to play an important role in dephosphorylating and inactivating MAP kinases. Several distinct DSPs have now been reported, including CL100/MKP1, PAC1, hVH-2/MKP2/TYP1, hVH3/B23, hVH5/M3–6, MKP3/PYST1/rVH6, B59/PYST2/MKPX, MKP4, and MKP5 (1–5). Some DSPs are localized to different subcellular compartments, and moreover, several DSP genes undergo rapid induction following exposure to stress and/or growth factor extracellular stimulation (2, 6, 7). However, the control of their activity at the level of post-transcriptional regulation is less well understood.

A better understanding of the regulation of the activity of DSP will further strengthen our insight into the complex regulation of MAP kinase activity, which is crucial for many cellular events. The original concept of the linear model of signaling by MAP kinases is now modified and improved by the concept of signalosome (signaling complex), in which the interaction of signaling molecules, including kinases, phosphatases, and scaffold proteins, dictate the signaling response to extracellular stimuli, depending on the cellular context and determining a conditional cross-talk between different pathways. Long-term stimulation of the ERK2 MAP kinase pathway leads to differentiation, whereas short-term activation leads to proliferation in PC12 cells (8). The modulation of the signal strength and/or the duration of Ras/MAPK activity, for example, are important determinants for defining cell fate (4, 9, 10). Moreover, the strength of the mitogen stimulation can modulate the level of the cellular response (11). Another good example is given by the inhibitory effect of activated Akt on the ERK2 pathway in differentiated myoblasts. This inhibition does not occur in nondifferentiated myoblasts, suggesting a stage-dependent regulation of the cross-talk between these pathways (12), controlling either proliferation or differentiation of muscle cells, two mutually exclusive biological processes.

The four families of MAP kinase described so far (ERK1, ERK2/p38/JNK/ERK5) (3, 13) are interconnected with each other, as well as with other signaling pathways. Since the expression and regulation of MKP can be key elements for this cross-regulation, we were interested in finding binding partners that could modulate the enzymatic activity or substrate selectivity of MKP.

Many proteins of the MKP family are composed of similar domains. They share a high degree of similarity in their catalytic core located in the C terminus, whereas their regulatory domain is highly variable. It is believed that each member of the MKP family has relative specificity among the MAP kinase families mediated by their noncatalytic domain (14). MKP3 and MKP4 are highly selective for inactivating ERK1 and ERK2 (14, 15), MKP1 and M3/6 for p38 and JNK (16–18), and MKP2 for ERK1, ERK2, and p38 (19).

We focused our study on MKP3 and its target MAPK ERK1, ERK2. MKP3 is mainly expressed in lung and brain tissues but also in placenta, muscle, kidney and liver tissues, as shown by Northern blot analysis (15). It encodes a protein with a predicted mass of 42.3 kDa. MKP3 functions as an immediately

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1 The abbreviations used are: DSP, dual specificity phosphatase; MAP, mitogen-activated protein; MKP, MAP kinase phosphatase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GST, glutathione S-transferase; Bia/Pia, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CH2, Cdc25 homology; pNPP, p-nitrophenyl phosphate; GAL4BD, GAL4 DNA binding domain; GAL4AD, GAL4 activation domain.
ear early gene, and among other stimuli, it is transcriptionally up-regulated after ERK2 pathway activation (i.e. following nerve or epidermal growth factor (EGF) treatment of PC12 cells) (6). Its localization is restricted to the cytoplasm, where it is activated by the direct physical interaction of ERK2. MKP3 has a low basal activity, but in the presence of ERK2, its activity is 40-fold higher in vitro, independent of ERK2 kinase activity (1, 36). This reflects tight and specific binding between the ERK2 major C-terminal structural lobe and the MKP3 kinase interaction motif within the MKP3 N terminus Cdc25 homology (CH2) domains, which are highly conserved between members of the MKP gene family (20).

To identify proteins that influence MKP3 function, we performed a yeast two-hybrid screen with MKP3 as bait and a mouse brain cDNA library as a target. Protein kinase CK2 was identified by this genetic screen. Other proteins found in this screen are summarized in Fig. 2C. Protein kinase CK2 (formerly known as casein kinase 2), a ubiquitously expressed serine/threonine kinase, is one of the most highly conserved proteins among eukaryotic cells. It is composed of two catalytic α or α’ subunits and two regulatory β subunits that can form a holoenzyme with αβ2, αα’β2 or αβ2tetrameric structure. It has been suggested that this kinase plays a pivotal role in cell growth and proliferation. In addition, several findings support the role of CK2 in cell survival (21, 22). In this study, we demonstrated the direct interaction of CK2 with the MAP kinase phospatase MKP3. In addition, we showed that CK2 is able to phosphorylate MKP3 without affecting its catalytic activity in vitro. However, in a cellular context, CK2α seems to influence not only the phosphorylation but also the enzymatic activity of MKP3.

EXPERIMENTAL PROCEDURES

DNA Constructs and Yeast Two-hybrid Screen—pMTS-myc-MKP3, pMTS-myc-MKP4, pGEX-T3-MKP3, pGEX-T3-MKP4, pGEX-T3-MKP1, and pGEX-T3-MKP5 constructs have been described elsewhere (15,6,14–16,20). pSG5-C2α was kindly provided by Dr. Claude Cochet, (Biology Department, INSERM, Grenoble, France), pCDNA3-HA-JNK1/SAPKγ and pGEX-SAPKα/JNK2 were provided by Dr. B. Stockwell (Ontario Cancer Institute, Ontario, Canada). pGEX2T-ERK2-K52A was kindly provided by C. J. Marshall (Chester Beatty Labs, Imperial Cancer Research, London). Full-length MKP3 cDNA was fused to the GAL4 DNA binding domain in the pGBT9 vector as bait. Positive and negative cDNA controls were fused to the GAL4 activation domain in the pACT2 vector (Beatty Labs, Imperial Cancer Research, London). For co-immunoprecipitation assays, the cells were lysed in binding lysis buffer (Tris (50 mM), NaCl (150 mM), SDS (0.1%), deoxycholate (2.5%), Triton X-100 (1%), β-glycerophosphate (40 mM), NaF (10 mM), Na3VO4 (1 mM), okadaic acid (30 mM), microcin (25 mM), calciculin A (10 mM), and protease inhibitor tablet) (Roche Applied Science). For co-immunoprecipitation assays, the cells were lysed in binding lysis buffer (Tris (50 mM), NaCl (150 mM), Nonidet P-40 (1%), NaF (10 mM), sodium pyrophosphate (1 mM), Cali- ciferol (10 mM), Na3VO4 (1 mM), microcin (25 mM), calciculin A (10 mM), and protease inhibitor tablet), and the lysates were diluted 1.5 in binding lysis buffer and incubated 1 h at 4 °C before centrifugation at 14,000 revolutions/min. 50 μl of protein G-Sepharose beads containing 2 μg of antibody were added to the supernatant. After overnight incubation at 4 °C, the beads were extensively washed with binding lysis buffer and eluted with Laemmli sample buffer. All immunoblotting was performed using NuPAGE according to the manufacturer's instructions. To monitor the band shift of MKP3, we used 12% polyacrylamide hand-cast gels containing 1.4% bisacrylamide instead of 2.8%. The following antibodies were used for detection and immunoprecipitation: mouse monoclonal e-Myc 9E10 (Santa Cruz Biotechnology), mouse monoclonal CK2α (Calbiochem), mouse monoclonal hemagglutinin (Babeo), mouse monoclonal ERK1/2 (Upstate Biotechnology), rabbit polyclonal phos- pho-ERK1/2 Thr202/Tyr204 (Cell Signaling Technologies). Secondary antibodies were peroxidase-conjugated donkey anti-rabbit or anti-mouse (Jackson ImmunoResearch). Detection of the peroxidase was performed with ECL reagents (Amersham Biosciences) and revealed with Hyperfilm (Amersham Biosciences).

RESULTS

Identification of CK2α as a Binding Partner of MKP3 by Yeast Two-hybrid Screen—We have reported previously that MKP3 binds tightly to ERK2 but not to JNK or p38 and that regions localized within the C-terminal structural lobe of ERK are essential for binding and catalytic activation of MKP3 (1, 20). These subdomains include regions believed to be important on Glutathion-Sepharose-4B, were incubated in 600 μl of binding buffer containing Triton X-100 1%, sodium pyrophosphate (5 mM), NaF (50 mM), Tris acetate, pH 7.0 (20 mM), EDTA (1 mM), EGTA (1 mM), Na3VO4 (1 mM), sucrose (270 mM), β-glycerophosphate (10 mM), protease inhibitor tablet (Roche Applied Science), and β-mercaptoethanol (0.1%) with 0.05, 0.25, 0.5, and 2.5 μg of CK2α, as indicated in Fig. 3, in the absence or presence of 0.1, 0.5, 1, and 5 μg of CK2β for 4 h. The beads were washed twice with binding buffer and twice with Tris 10 mM, after which sample buffer was added to the dry beads, and the samples were boiled. Western blotting was then performed with an anti-CK2α antibody (Calbiochem) and developed with ECL reagent (Amersham Biosciences) following the manufacturer’s instructions.

Cell Culture, Transfection, Immunoblotting and Co-immunoprecipitation Experiments— COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). The cells were transfected with FuGENE 6 as described by the manu- facturer (Roche Applied Science) using 4 μg of total DNA/5wlm dish. (In the experiments, a combination of cDNAs was required, and wells containing only one of the constructs were transfected also with the empty vector to keep the total DNA amount constant.) Where indicated in the legend to Fig. 9, the cells were starved for 4 h in medium containing no serum and further stimulated with 10 nM of EGF (ORI- GIN) for 10 min. For direct immunoblotting, the cells were lysed in 200 μl of RIPA lysis buffer. Assay buffer (Tris, pH 8 (20 mM), NaCl (150 mM), SDS (0.1%), deoxycholate (2.5%), Triton X-100 (1%), β-glycerophosphate (40 mM), NaF (10 mM), Na3VO4 (1 mM), okadaic acid (30 mM), microcin (25 mM), calciculin A (10 mM), and protease inhibitor tablet) (Roche Applied Science). For co-immunoprecipitation assays, the cells were lysed in binding lysis buffer (Tris (50 mM), NaCl (150 mM), Nonidet P-40 (1%), NaF (10 mM), sodium pyrophosphate (1 mM), Calcium (10 mM), Na3VO4 (1 mM), microcin (25 mM), calciculin A (10 mM), and protease inhibitor tablet), and the lysates were diluted 1.5 in binding lysis buffer and incubated 1 h at 4 °C before centrifugation at 14,000 revolutions/min. 50 μl of protein G-Sepharose beads containing 2 μg of antibody were added to the supernatant. After overnight incubation at 4 °C, the beads were extensively washed with binding lysis buffer and eluted with Laemmli sample buffer. All immunoblotting was performed using NuPAGE according to the manufacturer's instructions. To monitor the band shift of MKP3, we used 12% polyacrylamide hand-cast gels containing 1.4% bisacrylamide instead of 2.8%. The following antibodies were used for detection and immunoprecipitation: mouse monoclonal e-Myc 9E10 (Santa Cruz Biotechnology), mouse monoclonal CK2α (Calbiochem), mouse monoclonal hemagglutinin (Babeo), mouse monoclonal ERK1/2 (Upstate Biotechnology), rabbit polyclonal phospho-ERK1/2 Thr202/Tyr204 (Cell Signaling Technologies). Secondary antibodies were peroxidase-conjugated donkey anti-rabbit or anti-mouse (Jackson ImmunoResearch). Detection of the peroxidase was performed with ECL reagents (Amersham Biosciences) and revealed with Hyperfilm (Amersham Biosciences).

MKP3 Phosphatase Activity—Phosphatase activity was measured in 96-well plates containing 20 μg of phosphatase buffer (imidazole (50 mM), MgCl2 (10 mM), and dithiothreitol (1 mM)); 2.5 μg of GST-MKP3 (previously pre-incubated with different concentrations of CK2α, as indicated), and 20 μg of the synthetic phosphate substrate p-nitrophenyl phosphate (pNPP), in the absence or presence of 3 μg of GST- ERK-K52A. After incubation for 120 min at room temperature, hydrolyzed pNPP was monitored at 405 nm.

In Vitro Phosphorylation—1 μg of GST-tagged MAPK phosphatases (MKP1, MKP3, MKP4, MKP5, MKP3αN, and MKP3αC), GST-tagged JNK2, or casein were incubated for 1 h at 30 °C in 100 μl of kinase buffer (Hepes (25 mM), pH 7.0, MgCl2 (10 mM), dithiothreitol (1 mM), NaVO4 (0.2 mM), and β-glycerophosphate (25 mM)) containing 10 μM0.06 Ci/ml mmole ATPγS (32P)ATP, and 0.1 μg of CK2α or ERK2. Some experiments were performed with 20 μg/ml heparin (Sigma). The reaction was stopped by the addition of sample buffer and further boiling of the samples at 95 °C. After loading on a 4–21% SDS-polyacrylamide gel (NOVEX), the gel was stained with Coomassie Blue, destained overnight, dried, and exposed to an autoradiography film (Amersham Biosciences).

RESULTS

Identification of CK2α as a Binding Partner of MKP3 by Yeast Two-hybrid Screen—We have reported previously that MKP3 binds tightly to ERK2 but not to JNK or p38 and that regions localized within the C-terminal structural lobe of ERK are essential for binding and catalytic activation of MKP3 (1, 20). These subdomains include regions believed to be important...
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MKP3-CK2α Interaction in Vitro and in Mammalian Cells—In an attempt to assess the specificity and molecular nature of this interaction, in vitro binding was performed by incubating increasing concentrations of recombinant CK2α or CK2β with a constant amount of GST-MKP3 immobilized on Sepharose beads. CK2α bound to MKP3 was visualized by Western blotting using specific antibodies against CK2α or CK2β (Fig. 3A, upper and middle panels, respectively). As a control for equal loading, a Coomassie Blue staining of GST-MKP3 is shown (Fig. 3A, lower panel). CK2α binding was observed only with MKP3 but not with Sepharose beads, GST, or MKP4 (except at the highest concentration), which is another MAP kinase phosphatase that also binds and inactivates ERK2 (Fig. 3B, lanes 9–14, respectively). These results indicate not only that the interaction is direct and does not require
the participation of other proteins but also is specific for MKP3. The starting material was also submitted to Western blot analysis to assess the proportion of CK2α retained by MKP3 (Fig. 3C). The result shows that almost all of CK2α is retained by MKP3. Although only the α subunit of CK2 was found to interact with MKP3 in the yeast two-hybrid system, Fig. 3, lanes 5–8, shows that CK2β does not compete for the binding of CK2α to MKP3 but rather can form a complex with MKP3 and CK2α, suggesting that, in vivo, MKP3 may bind to the holoenzyme.

MKP3 has been shown to directly interact with its physiological substrate ERK2. This interaction leads to the activation of MKP3 phosphatase activity, and it is independent on the phosphorylation state of ERK or MKP3 (1, 36). Since ERK2 and CK2α are both ubiquitously expressed protein kinases, and both bind and phosphorylate MKP3, we investigated whether their binding to MKP3 is mutually exclusive. For this, recombinant GST-MKP3-Sepharose was incubated with CK2α and increasing concentrations of eluted His-ERK2, as described under “Experimental Procedures.” Bound CK2α or ERK2 were detected by Western blot after SDS-PAGE separation of the washed MKP3-Sepharose beads. Recombinant CK2α consists of two bands of 45 kDa (corresponding to the full-length) and 39 kDa (corresponding to an autoproteolytic fragment). Although ERK2 was not capable of displacing the binding of the CK2α form with the lowest apparent molecular mass, binding of MKP3 to the full-length form of CK2α was clearly decreased by increasing concentrations of ERK2 (Fig. 4, upper panel, lanes 2–4), suggesting that the binding of both kinases to MKP3 is mutually exclusive. Moreover, these results may indicate that MKP3 binds to CK2α via two independent sites, one of them being functional to the binding of MKP3 and not competed by ERK2 in the N-terminal truncated form of CK2α, although possibly masked and therefore not functional in the full-length form. This is reinforced by the fact that the decrease on binding of CK2α to MKP3 correlates perfectly with the increase on ERK2 binding to MKP3. To ensure equal loading of MKP3, a Coomassie Blue staining was performed and is shown in the lower panel. To investigate whether MKP3 and CK2α could also interact in a mammalian cellular environment, we co-transfected myc-MKP3 and CK2α in COS-7 cells. Similar protein expression was controlled by Western blot analysis of the crude lysates (Fig. 5, lanes 1, 3, 5, and 7). After immunoprecipitation with α-Myc antibody, CK2α could be detected in the immunoprecipitates (Fig. 5A, panel WB: αCK2α, lane 6). MKP3 could be detected as well in CK2α immunoprecipitates but only upon co-transfection of both proteins (Fig. 5A, panel WB: α-Myc, lane 8). CK2α did not co-precipitate MKP4, a closely related MAPK phosphatase that also binds ERK2 (Fig. 5B, lanes 2 and 4). In addition, MKP3 did not co-precipitate with the MAPK JNK2 (Fig. 5C, lanes 2 and 4). It is of note that in crude cell lysates endogenous levels of CK2α could not be detected with the antibody used (Fig. 5, lanes 1 and 2). Taken together, these results suggest that CK2α can bind directly and specifically to MKP3 in vitro and in yeast and that this interaction may also occur in mammalian cells.

CK2α Specifically Phosphorylates MKP3 in Vitro—Because
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FIG. 3. CK2α binds to MKP3 in vitro. A, 2.5 µg of recombinant GST-MKP3, immobilized in Glutathion-Sepharose-4B beads, was preincubated with 0.05, 0.25, 0.5, and 2.5 µg of CK2α (lanes 1–4, respectively) or with 0.05, 0.25, 0.5, and 2.5 µg of CK2α and 0.1, 0.5, 1, and 5 µg of CK2β to reconstitute CK2 heterotetramers (lanes 5–8, respectively), as indicated under “Experimental Procedures.” After washing of the pelleted MKP3 beads, bound CK2α and CK2β were visualized by Western blot using specific antibodies against the CK2 subunits, as specified. Lower panel shows Coomassie Blue staining of the GST-MKP3 to monitor equal loading. B, to prove the specificity of MKP3 binding to CK2α, Glutathion-Sepharose beads alone (lane 9), bound to 2.5 µg of GST (lane 10), or 2.5 µg of GST-MKP4 (lanes 11–14) were incubated with 2.5 µg (lanes 9 and 10) or 0.05, 0.25, 0.5, and 2.5 µg of CK2α, as described under “Experimental Procedures.” Bound CK2α was detected as in A. Lower panel shows Coomassie Blue staining of the recombinant GST proteins to monitor equal loading. C, Western blot showing immunoreactivity of the same amounts of CK2α used in A and B loaded directly into a 4–21% SDS-polyacrylamide gel to provide an indication of the amounts of CK2α retained by MKP3 in A.

FIG. 4. In vitro binding of CK2α to MKP3 in the presence of ERK2. 2.5 µg of recombinant GST-MKP3, immobilized in Glutathion-Sepharose-4B beads, were incubated with 2.5 µg of CK2α in the absence (lane 1) or presence of 0.25, 0.5, 1, 2.5, and 5 µg of His-ERK2 (lanes 2–6, respectively), as indicated under “Experimental Procedures.” After washing of the pelleted MKP3 beads, bound CK2α was visualized by Western blot using specific antibodies against the CK2 subunits as specified (upper panel). Middle panel shows a Western blot with an anti-histidine antibody to monitor ERK2 retained in the complex. Lower panel shows a Coomassie Blue staining of the pelleted beads to monitor GST-MKP3 equal loading.

CK2α has been reported to have a large variety of substrates, its interaction with MKP3 prompted us to investigate whether MKP3 could behave as a substrate for CK2α, as we have described previously for ERK2 (1). MKP3 was effectively phosphorylated in vitro by CK2α. Fig. 6A shows the time course of the in vitro phosphorylation of recombinant MKP3 by CK2α. Quantification of the phosphate incorporation on MKP3 indicated 40% of phosphate incorporation into MKP3 after 2 h of incubation with CK2α (Fig. 6B). To investigate whether the in vitro phosphorylation of MKP3 by CK2α is specific and whether we could locate it to the catalytic C-terminal lobe of MKP3 or to the regulatory N-terminal lobe (14, 20), in vitro kinase assays, using as substrates other MAPK or MAPK phosphatases as well as the N- or C-terminal lobes of MKP3, were performed. In those assays, β-casein was used as a positive control for CK2α activity and heparin as an inhibitor of its activity. Fig. 7 shows that CK2α phosphorylates only MKP3 (both the N- and C-terminal lobes) but not MKP1, MKP4, MKP5, or JNK2. In addition, the experiment shown in Fig. 7 indicates that the activity of CK2α on MKP3 is even stronger than that of ERK2. GST itself was not phosphorylated by CK2α (data not shown). Interestingly, CK2α phosphorylates both the N- and C-terminal lobes of MKP3 with the same apparent potency, whereas phosphorylation of the N-terminal lobe of MKP3 by ERK2 is much stronger than that of the C-terminal lobe. The results shown in Fig. 7 not only suggest that MKP3 phosphorylation by CK2α is specific for MKP3 but also indicate that CK2α phosphorylates MKP3, at least at more than a single site.

The potential CK2α phosphorylation sites, identified by sequence homology with the consensus motif for CK2α, do not overlap with the ERK2 binding site or ERK2 phosphorylation sites, neither do they correspond to the catalytic domain of MKP3. Further investigations are needed to identify the specific CK2α phosphorylation sites on MKP3 and the role they play in interaction in vitro and in a cellular context. The results obtained so far suggest the role of CK2α in the abrogation of the binding and activation by ERK; however, they do not exclude an additional and independent effect of CK2α on MKP3. The fact that CK2α has strong selectivity for MKP3 compared with other MKPs is in accordance with this hypothesis.

CK2α Slightly Increases MKP3 Phosphatase Activity in Vitro in the Presence of ERK2—Recombinant MKP3 phosphatase activity can be measured by the hydrolysis of pNPP in vitro (29). We have demonstrated previously that binding of ERK2 to MKP3 strongly increases MKP3 phosphatase activity in vitro and in vivo (1). We have therefore investigated whether CK2α binding has an effect on MKP3 phosphatase activity. As shown
in Fig. 8 (inset), CK2α alone did not influence MKP3 in vitro phosphatase activity. However, when MKP3 was in complex with ERK2 K52A (which enhances MKP3 phosphatase activity), the addition of CK2α clearly induced a further concentration-dependent increase in MKP3 phosphatase activity (Fig. 8, closed circles). This effect is, as shown for ERK2 (1), independent of the phosphorylation of MKP3 by CK2α, because the experiments done in the presence of ATP to allow CK2α to phosphorylate MKP3 (not shown) and in the absence of ATP (Fig. 8) gave identical results; therefore this effect on MKP3
activity might be due to the physical interaction between both proteins, as we have demonstrated for ERK2 (1).

Expression of CK2α in Mammalian Cells Induces MKP3 Retarded Migration through SDS-Polyacrylamide Gels—COS-7 cells were transfected with pS53-CK2α and/or pMTSM-myc-MKP3, and the lysate was analyzed by immunoblot using either anti-CK2α or anti-Myc antibodies. A typical double band for Myc-MKP3 can be seen (15) with an apparent molecular weight of 42 and 43 kDa (Fig. 9, lane 3). When cells were co-transfected with both vectors, the analysis of the lysate showed the appearance of a third band for Myc-MKP3 with an apparent size of 44 kDa and the disappearance of the lower original band of 42 kDa (Fig. 9, lanes 5–7). This band shift is typical for protein phosphorylation, and MKP3, which was only slightly phosphorylated in vitro by CK2α, did not show the same shift in migration in the presence of CK2α (data not shown), whereas it has been published that, upon phosphorylation by ERK, MKP1 migration is modified (25). Although this experiment was not a final proof that the retarded migration of MKP3 in SDS-PAGE is due to the direct phosphorylation by CK2α, it suggests that MKP3 is a substrate for CK2α, not only in vitro but also in cells.

MKP3 Phosphatase Activity on ERK2 Is Reduced in the Presence of CK2α in Mammalian Cells—COS-7 cells co-transfected with Myc-MKP3 (2 μg) and the indicated amounts of CK2α-cDNAs, as described under “Experimental Procedures.” 48 h after transfection, the cells were starved for 4 h and activated with EGF (10 ng/ml) in a serum-free medium for 10 min. The lysates were submitted to immunoblot analysis with the indicated antibodies.

with MKP3 and CK2α were stimulated with EGF, and the lysates were submitted to immunoblot analysis with anti-Myc or anti-CK2α antibodies to monitor equal protein loading in each lane, as an internal control. EGF stimulation leads to a strong increase in the phosphorylation of ERK2 (not shown). As shown previously (16), overexpression of MKP3 blocked almost completely EGF-induced ERK2 phosphorylation (Fig. 9, lane 3), whereas CK2α expression did not interfere with EGF-induced ERK phosphorylation (Fig. 9, lane 2). Our results showed that, in addition, co-expression of CK2α slightly reverted the inhibitory effect of MKP3 on ERK2 phosphorylation in a concentration-dependent manner (Fig. 9, lanes 4–7). This might suggest that CK2α can influence MKP3 activity against ERK2 in a COS-7 cellular context, either by avoiding physical association of MKP3 and ERK2 or by another mechanism (structural change), not implying direct effect on its catalytic activity.

**DISCUSSION**

CK2α is a ubiquitously expressed and constitutively active serine/threonine kinase (21–23, 26). It forms a tetramer with two β regulatory subunits and two α catalytic subunits, although the α subunit also exists as a monomer. CK2 is involved in several cellular functions, including proliferation, cell cycle progression, and development. More than 160 potential substrates have been suggested for CK2, but the precise role and regulation of this protein kinase remains unclear. Because depletion of CK2α by antisense oligonucleotides impairs neuroblastoma cell neuritogenesis (12), one possible function of CK2 could be the regulation of neuronal differentiation. Moreover, it has been suggested that CK2 is necessary, but not sufficient, to induce proliferation and might promote cell survival (26, 27).

We first demonstrated MKP3 binding to CK2α by a yeast two-hybrid, and we confirmed this interaction in vitro and in mammalian cells by co-immunoprecipitation of co-expressed CK2α and MKP3. The in vitro experiments show a direct protein–protein interaction. The use of closely related MAPK phosphatases and MAPK proteins as controls suggests a rather high degree of specificity for the binding of MKP3 and CK2α. CK2β is also retained by MKP3 in the presence of CK2α.

We further investigated the enzymatic relationship between MKP3 and CK2α. Interestingly, we were able to show a strong and selective phosphorylation of MKP3 by CK2α in vitro. Indeed, CK2α favors phosphorylation of MKP3 over its prototyp-
MKP3 Interacts with and Is Phosphorylated by CK2α

CK2α phosphorylates MKP3 at least at two distinct domains, because mutants bearing deletions of the N or C terminus are both equally phosphorylated by CK2α. Phosphorylation of kinases at both regulatory and catalytic domains by a single kinase has already been reported. For example, multiple PKA phosphorylation sites have been identified in Raf-1 (28). The minimum consensus sequence for CK2 phosphorylation is (S/T)XX(X/E), where the Xs are preferentially acidic residues (29, 30). We have found eight corresponding consensus sites in MKP3, whereas we found only four in MPK1 and two in MPK4, correlating with the intensity of in vitro phosphorylation found in our experiments when we compared those MAPK phosphatases as substrates of CK2α. It is suggested that, because these sites in MKP3 neither overlap with those known to be important for ERK2 binding or phosphorylation nor locate into the catalytic domain of MKP3 (20, 29), MKP3 phosphorylation by CK2α does not necessarily imply the same molecular consequences as its phosphorylation by ERK2. This is in agreement with our finding that CK2α does not interfere with the activation of MKP3 by ERK but rather enhances it. To further investigate MKP3 phosphorylation by CK2α, we performed immunoprecipitation following co-expression of both proteins in COS-7 cells. Under these conditions, MKP3 is retarded in its migration on SDS-polyacrylamide gels. This modification in migration of MKP3 depends on the amount of CK2α expressed, suggesting that it is due to the phosphorylation by CK2α. In a similar experiment, MKP1, the migration of which was shown to be retarded following phosphorylation by MAPK (25), does not behave the same as MKP3 in the presence of CK2α (data not shown), confirming the specificity of CK2α for MKP3, observed also in vitro experiments.

Because MKP3 dephosphorylates ERK2 in cells (16), we used antibodies raised against phosphorylated ERK2 as a readout for MKP3 activity. Stimulation of COS-7 cells with EGF leads to a clear increase in phosphorylated ERK2, whereas in MKP3-expressing cells, phosphorylated ERK2 is reduced to background levels. CK2α transfection itself does not change the ERK2 phosphorylation state. However, when cells are co-transfected with MKP3 and CK2α, inhibition of ERK2 phosphorylation is slightly reversed, correlating with the expression level of CK2α and suggesting a loss of MKP3 function. These results suggest that CK2α might regulate MKP3 activity in a cellular context by a mechanism different from that which lowers its phosphatase activity. Different mechanisms can explain this down-regulation. Cellular localization is an important factor to take into account for the regulation of CK2 activity; the binding and phosphorylation of MKP3 could induce sequestration to defined cellular compartments and regulate CK2/MKP3 activity (23, 31). However, we were not able to show changes in subcellular localization of either CK2α or MKP3 by co-expression of both proteins and immunocytochemistry detection (data not shown). A functional link between CK2 and PP2A, another key protein phosphatase involved in the regulation of the MAPK pathway, has already been demonstrated (9, 27). In this case, CK2α was shown to interact with and improve the catalytic activity of PP2A, leading to MKP1 (physiological PP2A substrate) dephosphorylation and consequent down-regulation of ERK-dependent mitogenic signals. Here, we provide evidence for the complex formation between CK2α and MKP3, which affects MKP3 phosphatase activity towards ERK2 in vitro. Several hypotheses can be proposed to support such a consequence. Despite the inability of CK2α to inhibit MKP3 in vitro, the MKP3-dependent ERK2 dephosphorylation could be decreased by CK2α in an intracellular environment. Alternatively, we could speculate for a less direct regulation that would result from the competition between the two phosphatases (MKP3 and PP2A) for their binding to the monomeric form of CK2α. Indeed, MKP3 sequestered away from ERK2 by CK2α, could displace PP2A, which would no longer be activated to impair MEK1-mediated signaling, as proposed previously (32). Because the β subunit does not interfere with the binding of MKP3 to CK2α, the physiologically relevant entity binding MKP3 in vivo may be the holoenzyme tetrameric kinase. Indeed, this would confirm the growth-promoting role shown for CK2α in other studies.

MKP3 and CK2 are both enriched in the brain and appear to be up-regulated during neuronal differentiation (15, 24, 33), whereas expression is suppressed in mature neurons (28). MKP3 is strictly localized in the cytosol as well as CK2α, which is found in the cytosol of post-mitotic neurons (34, 35). Together with our results, this could indicate that in post-mitotic neurons, CK2α could interact with MKP3 in the cytosol, where it regulates MKP3 activity, attenuating its inhibitory effect on ERK activation. This could represent a novel mechanism for controlling the MAP kinase activation state and provides exciting insights into new potential mechanisms of regulatory crosstalk between kinase-based signaling systems, using specific phosphatases as intermolecular connectors for different pathways.

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