Discordance between the Binding Affinity of Mitogen-activated Protein Kinase Subfamily Members for MAP Kinase Phosphatase-2 and Their Ability to Activate the Phosphatase Catalytically*

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MKP-2 is a member of the mitogen-activated protein (MAP) kinase phosphatase family which has been suggested to play an important role in the feedback control of MAP kinase-mediated gene expression. Although MKP-2 preferentially inactivates extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) MAP kinase subfamilies, the mechanisms underlying its own regulation remain unclear. In this report, we have examined the MKP-2 interaction with and catalytic activity by distinct MAP kinase subfamilies. We found that the catalytic activity of MKP-2 was enhanced dramatically by ERK and JNK but was affected only minimally by p38. By contrast, p38 and ERK bound MKP-2 with comparably strong affinities, whereas JNK and MKP-2 interacted very weakly. Through site-directed mutagenesis, we defined the ERK/p38-binding site as a cluster of arginine residues in the NH2-terminal domain of MKP-2. Mutation of the basic motif abrogated its interaction with both ERK and p38 and severely compromised the catalytic activation of MKP-2 by these kinases. Unexpectedly, such mutations had little effect on JNK-triggered catalytic activation. Both in vitro and in vivo, wild type MKP-2 effectively inactivated ERK2 whereas MKP-2 mutants incapable of binding to ERK/p38 did not. Finally, in addition to its role as a docking site for ERK and p38, the MKP-2 basic motif plays a role in regulating its nuclear localization. Our studies provided a mechanistic explanation for the substrate preference of MKP-2 and suggest that catalytic activation of MKP-2 upon binding to its substrates is crucial for its function.

The mitogen-activated protein (MAP)1 kinases are critical components of the signal transduction pathways which mediate the cellular response to a variety of extracellular stimuli, ranging from growth factors to environmental stresses (1). So far, at least 10 MAP kinase family members have been identified in mammalian cells (1, 2). According to a tripeptide signature motif (Tyr-Xaa-Thr) in their kinase subdomain VIII, these protein kinases can be classified into three subfamilies including the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 (1–5). ERK1 and ERK2 are the archetypes of the ERK subfamily which are highly activated by growth factors. In contrast, the JNK and p38 subfamilies are activated preferentially by stress including ultraviolet light, heat shock, and lipopolysaccharide (3), and therefore they are also referred to as stress-activated protein kinases (2). Once activated, MAP kinases can translocate from the cytoplasm to the nucleus, leading to the phosphorylation of a multitude of transcription factors and altered gene expression (4, 5). The physiological functions of various MAP kinase subfamilies have been studied extensively in a large number of systems. In general, ERK activation is closely associated with cell proliferation, differentiation, and enhanced cell survival after cellular stress, although in certain situations, such as recently described for cisplatin treatment, ERK activation is required for the execution of apoptosis (6). On the other hand, activation of JNK and p38 is usually associated with enhanced apoptosis and production of inflammatory cytokines (7, 8), although there are notable exceptions in which JNK/p38 activation is necessary for cell proliferation and differentiation (9, 10). Because MAP kinase pathways play an important role in regulating many critical cellular processes, the precise regulation of these signaling proteins is crucial for the maintenance of cellular homeostasis.

The activities of all MAP kinases are regulated via reversible phosphorylation of the conserved threonine and tyrosine residues in their tripeptide signature motifs by specific MAP kinase kinases and protein phosphatases (1, 11–13). In mammalian cells, inactivation of MAP kinases is accomplished primarily by a family of dual specificity MAP kinase phosphatases that can act on both the phosphothreonine and phosphotyrosine residues. So far, nine distinct mammalian MAP kinase phosphatase family members have been characterized. According to their subcellular localization and patterns of transcriptional regulation, these phosphatases can be roughly divided into two groups (13). The first group includes MKP-3/Pyst-1, Pyst-2, MKP-4, MKP-5, and M3/6, which are localized predominantly in the cytosol and are therefore thought to control mainly the MAP kinase-regulated events that occur in the cytosol. The second group of enzymes includes MKP-1 (CL100/3CH134), MKP-2, PAC-1, and B23,
which are localized primarily in the nuclear compartment. Encoded by immediate early genes, these nuclear MAP kinase phosphatases are induced rapidly by many of the same stimuli that also activate MAP kinases (14–18). For this reason, it has been suggested that these MAP kinase phosphatases play an important role in the feedback control of MAP kinase signaling in the nucleus (12, 13).

Recently, it has been reported that several cytosolic MAP kinase phosphatases can interact with their substrate MAP kinases, and such an interaction increases their catalytic activities substantially (19, 20). These studies suggest that catalytic activation of MAP kinase phosphatases mediated through substrate binding may play an important role in determining their substrate preferences. However, not all cytosolic MAP kinase phosphatases undergo catalytic activation upon interaction with their substrates. For example, although MKP-5 interacts with its substrate MAP kinases through a basic motif in its NH2-terminal domain, this interaction has little effect on its catalytic activity (21, 22). Even though the substrate specificities for many nuclear MAP kinase phosphatases have been studied extensively (23, 24), relatively little is known about their abilities to interact with their substrates. Even less is known about the mechanisms underlying the interactions or the effects that these interactions have on their biochemical and physiological functions. In this report, we have characterized the interactions between a nuclear MAP kinase phosphatase MKP-2 (25, 26) and its substrate MAP kinases and the subsequent effects such interactions have on the catalytic activity of MKP-2. Our findings provide a mechanistic explanation for the substrate specificity of MKP-2 and strongly suggest that catalytic activation of MKP-2 upon binding to its substrates is crucial for its function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transient Transfection, and Treatment—**HeLa and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified atmosphere containing 5% CO2. 293T cells were transfected using FuGENE 6transfection reagent (Roche, Indianapolis) according to the manufacturer’s specifications. HeLa cells were transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (27). For the induction of apoptosis, HeLa cells were plated in six-well plates and transfected with pSR-based expression constructs for FLAG-tagged wild type or mutant MKP-2 proteins. 48 h after transfection, cell lysates were prepared and incubated with glutathione-Sepharose beads at 4 °C. The precipitates were then blocked with Tris-buffered saline (TBS) containing 10% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were fixed in freshly prepared 4% paraformaldehyde for 15 min at 4 °C and then blocked with Tris-buffered saline (TBS) containing 10% normal goat serum and 1% bovine serum albumin at room temperature for 1 h. After blocking, the cells were incubated at 4 °C overnight with the anti-FLAG antibody at a concentration of 20 μg/ml. The cells were washed twice with TBS containing 0.1% Triton X-100 (TBST) and then with TBS for 15 min, then incubated with an Alexa 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing five times with TBST, the cells were stained with 4,6-diamidino-2-phenylindole (DAP) to visualize the nuclei and examined under a Zeiss Axioskop microscope (Carl Zeiss, Inc., New York).

**Preparation of Recombinant Proteins—**Recombinant GST-MKP-2 protein and its derivatives were produced in 293T cells through transient transfections and isolated essentially as described previously (27). The proteinase K/SDS-glycine–Sepharose bead procedure was used to purify recombinant GST-MKP-2 protein for 15 min at 30 °C in 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM NaF, 1 mM Na3VO4, 20 mM microcystin-LR, 2 μM leupeptin, 2 μM aprotinin, and 1 mM phenylmethylsulfonfluoride.

**Expression Vectors—**Mammalian expression vector pSR-FLAG-MKP-2 and bacterial expression vector pET41a-FLAG-MKP-2 have been described previously (27). pEG-MKP-2 was generated by cloning the human MKP-2 cDNA into the mammalian expression vector pEG (20). All vectors expressing mutant MKP-2 proteins were generated with a site-directed mutagenesis kit (QuikChange, Stratagene). The human JNK1 cDNA (30) was cloned in the bacterial expression vector pGEX-2T-ERK2 and pGEX-2T-ERK2 sequencing. pGEX-ERK1 for producing human ERK1 (32) was a generous gift from Kun-Liang Guan. pGEX-2T-ERK2 and pGEX-2T-ERK2 were expressed in E. coli BL21 (DE3) and purified with glutathione-Sepharose affinity beads as described previously (27). His6-ERK2 was expressed in E. coli BL21 (DE3) and purified through batch binding to Ni2+-nitriloacetic acid-agarose (Qiagen, Valencia, CA) according to the manufacturer’s recommendation.

**GST Pull-down Assay—**To pull down MKP-2 with recombinant MAP kinases, HeLa cells were transfected with pSR-based constructs expressing FLAG-tagged wild type or mutant MKP-2 proteins, and lysates were prepared 48 h after transfection. Purified GST-MAP kinases (4 μg) that were bound to glutathione-Sepharose beads were incubated with these cell lysates for 16 h at 4 °C. The precipitates were then washed twice with lysis buffer containing 200 mM NaCl. The proteins were separated by electrophoresis and analyzed by Western blotting using the anti-FLAG antibody. To pull down endogenous MAP kinases using GST-MKP-2, 293T cells were transfected with pSR-based constructs expressing GST-tagged wild type or mutant MKP-2 proteins. 48 h after transfection, cell lysates were prepared and incubated with glutathione-Sepharose beads at 4 °C for 30 min. The proteins pulled down with the beads were washed similarly and then subjected to Western blot analysis using antibodies recognizing MAP kinases.

**p-Nitrophenyl Phosphate Hydrolysis Assay—**p-Nitrophenyl phosphate (p-NPP) hydrolysis by MKP-2 was carried out as described previously (27). Briefly, a series of 200-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 5 mM DTT, 20 μg p-NPP, and the indicated amounts of wild type or mutant GST-MKP-2 proteins were added to 96-well plates. The reactions were carried out at 25 °C in a microplate reader (Molecular Devices, Sunnyvale, CA) and monitored at 405 nm over a 5-h period. To assess the effect of various MAP kinases on MKP-2 activity, the indicated amounts of GST-MAPK fusion proteins were included in the reactions.

**Kinase Assays—**The protein phosphatase activity of MKP-2 toward ERK2 was examined indirectly through an in vitro phosphatase-coupled kinase assay. Briefly, 50 ng of activated recombinant rat ERK2 protein (Calbiochem) was incubated with the indicated amounts of purified recombinant GST-MKP-2 protein for 15 min at 30 °C in 50 μl of phosphate reaction buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM DTT, 20 μg p-NPP, and the indicated amounts of wild type or mutant GST-MKP-2 proteins were added to 96-well plates. The reactions were incubated at 30 °C. The reaction was stopped by the addition of Laemmli’s sample buffer. Phosphorylation of MBP was detected using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) after SDS-polyacrylamide gel electrophoresis.
To assess the phosphatase activity of MKP-2 in vivo, HeLa cells were transiently transfected with the indicated amounts of pSRα-based expression constructs for wild type or mutant MKP-2. 16 h after transfection, cells were first serum starved for 24 h and then stimulated with 20% fetal bovine serum for 5 min. Cells were harvested, and the activity of endogenous ERK2 was assessed by immunocomplex kinase assays as described previously (37).

RESULTS

MKP-2 Is Activated Preferentially by ERK and JNK—To examine whether different MAP kinases can stimulate the catalytic activity of MKP-2, recombinant MKP-2 was expressed in 293T cells as a GST fusion protein, purified using glutathione-Sepharose beads, and eluted. A mutant MKP-2 protein carrying a Cys-280Ser substitution in its catalytic site (MKP-2 C280S) was prepared similarly. Coomassie staining of the samples separated on NuPAGE gel revealed a major band of 65 kDa, a minor band of 50 kDa (tentatively identified as human translation elongation factor-1ε by mass spectrometry), and two faint bands of 32 and 26 kDa, respectively (Fig. 1A).

Western blot analysis of the proteins did not detect any contamination from the endogenous MAP kinase proteins (data not shown). Because truncation of 58 amino acids from the carboxyl terminus of MKP-2 reduced the size of GST-MKP-2 by about 6 kDa (data not shown), we concluded that this expression system had produced full-length MKP-2 proteins. Based on Coomassie staining of the gels, we estimated that the purity of the recombinant MKP-2 proteins was about 70%.

The basal activity of the recombinant MKP-2 proteins was measured through the hydrolysis of p-NPP (Fig. 1B). Recombinant MKP-2 exhibited low dose-dependent activity toward p-NPP, whereas MKP-2 C280S had no effect (Fig. 1B). The addition of ERK1 increased the catalytic activity of MKP-2 dramatically in a dose-dependent and saturable fashion (Fig. 1C). Interestingly, JNK1 also substantially enhanced the activity of MKP-2 in a dose-dependent manner (Fig. 1C), but compared with ERK1, it was a less effective stimulator of MKP-2. A plateau for MKP-2 activation by JNK1 was not achieved within the amount of JNK1 used. p38 only had a small effect on MKP-2 catalytic activity. The maximum activation of 3 μg of GST-MKP-2 achieved by p38 was less than 4-fold, and an activation plateau was reached with a relatively small amount of p38 (Fig. 1C). Because GST can form homodimers (38), interaction between the GST moieties of MKP-2 and MAP kinase fusion proteins may potentially complicate the interpretation of the p-NPP hydrolysis assays. To exclude such a possibility, we examined the effects of GST, GST-ERK2, and His6-ERK2 on the catalytic activity of MKP-2 (Fig. 1D).

MKP-2 Binds Preferentially to ERK and p38—To examine the ability of MKP-2 to bind to various MAP kinases, a GST

FIG. 1. Catalytic activation of MKP-2 by distinct MAP kinases. GST-MKP-2 and GST-MKP-2 C280S were expressed in 293T cells and purified under stringent washing conditions using glutathione-Sepharose beads (see “Experimental Procedures”). The phosphatase activity of GST-MKP-2 in the presence or absence of recombinant MAP kinases was measured as p-NPP hydrolysis at 25 °C by monitoring the absorbance at 405 nm (λmax). A, Coomassie staining of the purified recombinant MKP-2 or the C280S mutant proteins. Molecular masses of the protein markers are indicated on the left. B, dose response of the basal phosphatase activities for the wild type and the mutant MKP-2 proteins. p-NPP hydrolysis at the 3-h time point is presented. C, p-NPP hydrolysis by 3 μg of GST-MKP-2 after a 2-h incubation in the presence of the indicated amount of GST fusion proteins for ERK1, JNK1, and p38. D, p-NPP hydrolysis by 3 μg of GST-MKP-2 after a 2-h incubation in the presence of the indicated amount of GST, GST-ERK2, or His6-ERK2. Data presented are results from a representative experiment.
pull-down assay was performed. HeLa cells were transiently transfected with an expression construct for FLAG-tagged MKP-2 (pSRα-FLAG-MKP-2) and harvested 48 h after transfection. The lysate was incubated with immobilized GST-ERK1, GST-JNK1, GST-p38, or GST. An anti-FLAG antibody was used to detect the pulled-down FLAG-MKP-2 (Fig. 2A). Whereas MKP-2 was not brought down by GST, it was effectively pulled down by both GST-ERK1 and GST-p38, indicating that MKP-2 interacts specifically with both ERK1 and p38. Surprisingly, very little FLAG-MKP-2 was brought down by GST-JNK1 protein (Fig. 2A). To test whether this was caused by potential misfolding of the GST-JNK1 protein used in this assay, we performed GST pull-down assays with FLAG-tagged MKP-1 (27). As was reported previously (27, 39) and shown in Fig. 2B, MKP-1 was effectively pulled down by GST-ERK1, GST-p38, and GST-JNK1. This result, together with the fact that GST-JNK1 triggered a substantial catalytic activation of MKP-2 (Fig. 1C), argues that GST-JNK1 was properly folded and suggests that the affinity between JNK1 and MKP-2 is weak.

**A Basic Motif in the NH₂ Terminus of MKP-2 Is Responsible for Its Interaction with ERK and p38 and for Its Catalytic Activation by These MAP Kinases**—It has been proposed that the interaction between a MAP kinase phosphatase and a MAP kinase is mediated through a basic motif of the phosphatase and an acidic motif of the kinase (21). Because MKP-2 has a basic motif in its NH₂ terminus which consists of three arginine residues (Arg-74, Arg-75, and Arg-76), we tested whether this basic motif of MKP-2 is important for its interaction with different MAP kinases. A series of mutations in the MKP-2 basic motif was generated which either changed a single arginine residue or changed all three arginine residues (Arg-74, Arg-75, or Arg-76 alone, or all three residues together, almost completely eliminated the interaction between MKP-2 and p38, suggesting more prominent roles for these two residues in mediating the interaction with this kinase. When all three arginines were changed to alanines, the interaction between ERK1 and MKP-2 was virtually abolished (Fig. 3A, middle panel). Likewise, mutation of Arg-74, Arg-75, or Arg-76 alone, or all three residues together, almost completely eliminated the interaction between MKP-2 and p38 (Fig. 3A, bottom panel). The lack of difference among these mutant MKP-2 proteins in their abilities to bind to p38 is likely due to the weaker affinity of MKP-2 for p38 than for ERK1 (Fig. 2A).

To verify that the basic motif of MKP-2 mediates its interaction with endogenous ERK1/2 and p38 MAP kinases, wild type and mutant MKP-2 proteins were expressed in 293T cells as GST fusion proteins. These GST fusion proteins were enriched through glutathione-Sepharose beads (see “Experimental Procedures”) and analyzed by immunoblotting for co-purification of various endogenous MAP kinases (Fig. 3B). Although Coomassie staining of the enriched proteins indicated that similar amounts of wild type and mutant MKP-2 proteins were expressed and purified, only wild type MKP-2 brought down endogenous ERK1/2 and p38 MAP kinases (Fig. 3B, middle and bottom panel). Consistent with the notion that the
The wild type protein (Fig. 4), although the MKP-2 R74A/R75A/R76A mutation did not differ significantly from that of the wild type proteins purified under stringent conditions. The basal phosphatase activities of the single-residue substitution mutants of MKP-2 were produced as GST fusion proteins and were examined through ERK1/2 and p38. The role of the basic motif in the NH2-terminal domain of MKP-2 is responsible for its interaction with the acidic motif of ERK2 is necessary for maximal MKP-2 activation. It has been demonstrated that a sevenmaker mutation (D319N) at the COOH-terminal acidic motif of ERK2 abolishes the interaction between ERK2 with MKP-3 (19). To examine whether this domain of ERK2 was also responsible for MKP-2 activation, activation profiles of the wild type and the MKP-2 R74A/R75A/R76A mutant proteins were generated using either wild type ERK2 or the sevenmaker mutant (ERK2 D319N). As indicated in Fig. 6, like ERK1, ERK2 also triggered a dramatic catalytic activation of the wild type MKP-2. The sevenmaker mutant ERK2 (ERK2 D319N) only stimulated a small (about 3-fold) increase in MKP-2 activity, indicating that this acidic motif of ERK2 is necessary for maximal MKP-2 activation (Fig. 6A). Interestingly, a similar profile was observed for catalytic activation of the mutant MKP-2 (R74A/R75A/R76A) by wild type ERK2. However, combining the sevenmaker mutation in ERK2 and the R74A/R75A/R76A

**Fig. 4.** Role of the basic motif in ERK1-induced catalytic activation of MKP-2. GST fusion proteins for either wild type or mutant MKP-2 were produced in 293T cells and purified under stringent washing conditions (see "Experimental Procedures"). The kinetics of p-NPP hydrolysis by 3 μg of wild type or mutant GST-MKP-2 protein in the presence or absence of the indicated amount of GST-ERK1 or GST-p38 was monitored over a 5-h period of time. A, basal phosphatase activity of 3 μg of GST fusion proteins for either wild type or the indicated mutant MKP-2. p-NPP hydrolysis at the 2-h time point is shown. Data are presented as the mean ± S.D. from four independent experiments. The asterisk (*) indicates that the difference is statistically significant (t test, p < 0.05). B, effect of ERK1 on the catalytic activity of either wild type or mutant MKP-2 proteins. C, effect of p38 on the catalytic activation of wild type MKP-2 or the MKP-2 R74A/R75A/R76A mutant. Catalytic activation is expressed as ERK1- or p38-stimulated p-NPP hydrolysis at 2 h relative to the basal phosphatase activity. Data in B and C are from a representative experiment.

The affinity of MKP-2 for JNK1 was lower than that for either ERK or p38, the amount of JNK1 co-precipitated with GST-MKP-2 was barely detectable (data not shown). Taken together, these results clearly indicate that the basic motif in the NH2-terminal domain of MKP-2 is responsible for its interaction with ERK1/2 and p38.

The role of the basic motif in its catalytic activation by ERK1 was examined through p-NPP hydrolysis assays. Wild type and mutant proteins were produced as GST fusion proteins and purified under stringent conditions. The basal phosphatase activities of the single-residue substitution mutants of MKP-2 (R74A, R75A, or R76A) did not differ significantly from that of the wild type protein (Fig. 4A), although the MKP-2 R74A/R75A/R76A mutant consistently displayed a slight increase (about 2-fold) in basal phosphatase activity (Fig. 4A). Wild type MKP-2 underwent a very steep catalytic activation by ERK1, reaching a maximum level of about 25-fold above its basal activity (Fig. 4B). Similarly, upon incubation with ERK1, the R74A mutant also underwent substantial activation, although the magnitude of the increase was slightly lower. In contrast, the R75A mutation severely compromised its catalytic activation, with ERK1 only triggering a 6-fold increase above its basal activity. Likewise, the R76A mutation also substantially compromised its ability to undergo catalytic activation by ERK1. Predictably, the MKP-2 R74A/R75A/R76A mutant exhibited the most severe defect in its capacity to undergo activation (Fig. 4B). Similarly, the R74A/R75A/R76A mutation completely abolished the small increase in MKP-2 activity triggered by p38 (Fig. 4C). The fact that all mutants displayed a somewhat comparable basal activity suggests that all proteins were folded properly. These results indicate that the catalytic activation of MKP-2 by both ERK and p38 is mediated through the basic motif in the NH2 terminus of MKP-2.

The role of the basic motif of MKP-2 in JNK-induced catalytic activation was also examined through p-NPP hydrolysis assays. Surprisingly, both the wild type and the mutant MKP-2 proteins responded somewhat similarly to JNK1, reaching a comparable level of phosphatase activity (Fig. 5). This suggests that MKP-2 activation by JNK1 is mediated by a distinct domain.

**Sevenmaker Mutation Abolishes ERK-induced Catalytic Activation of MKP-2**—It has been demonstrated that a sevenmaker mutation (D319N) at the COOH-terminal acidic motif of ERK2 abolishes the interaction between ERK2 with MKP-3 and abrogates the stimulatory effect of ERK2 on MKP-3 (19). To examine whether this domain of ERK2 was also responsible for MKP-2 activation, activation profiles of the wild type and the MKP-2 R74A/R75A/R76A mutant proteins were generated using either wild type ERK2 or the sevenmaker mutant (ERK2 D319N). As indicated in Fig. 6, like ERK1, ERK2 also triggered a dramatic catalytic activation of the wild type MKP-2. The sevenmaker mutant ERK2 (ERK2 D319N) only stimulated a small (about 3-fold) increase in MKP-2 activity, indicating that this acidic motif of ERK2 is necessary for maximal MKP-2 activation (Fig. 6A). Interestingly, a similar profile was observed for catalytic activation of the mutant MKP-2 (R74A/R75A/R76A) by wild type ERK2. However, combining the sevenmaker mutation in ERK2 and the R74A/R75A/R76A

**Fig. 5.** Effect of JNK1 on the catalytic activity of either wild type MKP-2 or the indicated mutants. The kinetics of p-NPP hydrolysis by 3 μg of wild type or mutant GST-MKP-2 proteins in the presence or absence of the indicated amount of GST-JNK1 was monitored over a 5-h period. p-NPP hydrolysis at 2 h is shown. Data shown are from a representative experiment.

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substitution in MKP-2 did not compromise the catalytic activity further (Fig. 6A), thereby supporting the hypothesis that the acidic domain of ERK2 exerts its effect through the basic motif of MKP-2. Unlike the effect of the ERK2 sevenmaker mutation on ERK2-mediated MKP-2 activation, an equivalent mutation in JNK1 (E329N) had no influence on JNK1-mediated MKP-2 activation (Fig. 6B). This observation supports further the notion that JNK1 activates MKP-2 via a different mechanism.

Asp-249 of MKP-2 Is Critical for Both Basal Phosphatase Activity and ERK2-triggered Catalytic Activation of MKP-2—Upon ERK2 binding, closure of the acidic loop over the active site has been suggested as the underlying mechanism for the activation of Pyst-1, the human homolog of MKP-3 (40, 41). Asp262 at the acidic loop of Pyst-1 has been shown to be essential for the phosphatase to switch from its “low activity” form to its “high activity” form upon ERK2 binding. Because MKP-2 is highly homologous to Pyst-1 around its catalytic active site and also has an aspartate residue in the acidic loop (Asp-249), an Asp-249 → Asn mutation on MKP-2 was generated. The effect of this mutation on MKP-2 activity in the absence and the presence of ERK2 was examined by p-NPP hydrolysis assays. To our surprise, unlike Pyst-1/MKP-3, where mutating Asp-262 to Asn had little effect on its basal phosphatase activity, mutation of Asp-249 to Asn (D249N) on MKP-2 resulted in a 90% decrease in its basal phosphatase activity (Fig. 7A). Furthermore, the MKP-2 D249N mutant showed little response to stimulation by ERK2 (Fig. 7B), consistent with what was reported for Pyst-1. A GST pull-down assay indicated that the D249N mutant was able to bind to ERK with an efficiency comparable to that of wild type MKP-2 (Fig. 7C). Immunofluorescent studies indicated that both the wild type MKP-2 and the MKP-2 D249N mutant were localized in the cell nucleus (Fig. 7D). These results argue strongly that the defect exhibited by the D249N mutant was not caused by protein misfolding.

The Basic Motif of MKP-2 Is Critical for Inactivating ERK Signaling—The importance of the basic motif of MKP-2 for its function as a protein phosphatase was examined in vitro by a phosphatase-coupled kinase assay. Briefly, increasing amounts of purified GST-MKP-2 or its mutant derivatives were incubated with 50 ng of active recombinant ERK2 for 15 min. The tyrosine phosphatase inhibitor vanadate, together with \([\gamma-^{32}\text{P}]\text{ATP and MBP, was then added to the kinase/phosphatase reaction mixture and incubated for an additional 20 min.}\]

The remaining activity of ERK2 after treatment with MKP-2 was reflected in the phosphorylation of MBP (Fig. 8). Even at the lowest dose tested (25 ng), wild type MKP-2 inactivated ERK2 potently (Fig. 8, top panel, left side). Although the MKP-2 R74A mutant appeared to be somewhat less efficient than wild type MKP-2, it also effectively inactivated the kinase (Fig. 8, top panel, right side). Mutation at either Arg-75 or Arg-76 on MKP-2 resulted in a more significant defect in its ability to inactivate ERK2 (Fig. 8, middle panel). The R74A/R75A/R76A mutant displayed the most severe defect in its activity toward ERK2 (Fig. 8, bottom panel).

The role of the MKP-2 basic motif in its biochemical function as an ERK phosphatase in vivo was examined through transient transfection assays. HeLa cells were first transiently transfected with increasing amounts of expression vectors for either FLAG-tagged wild type MKP-2 or its mutants and then serum starved for 24 h. Finally, the cells were stimulated with 20% fetal bovine serum for 5 min and harvested. Given the high transfection efficiency achieved with this procedure (27) (see also Fig. 11), endogenous ERK2 could serve as a reporter of phosphatase activity. The activity of endogenous ERK2 was measured by immunocomplex kinase assays (Fig. 9). In cells transfected with an empty vector, serum stimulation resulted in a 15-fold increase in ERK2 activity within 5 min. In cells transfected with wild type MKP-2 vector, the inhibitory effect was already noticeable with 0.5 μg of expression vector, whereas 2 μg of the wild type MKP-2 plasmid resulted in a 79% reduction in ERK2 activity. Similarly, the R74A mutant was quite effective at inhibiting ERK2 kinase activity, although it was slightly less efficient than the wild type protein. The abilities of both the R75A and R76A mutants to inhibit ERK2 activity were compromised substantially, being noticeable only in cells transfected with the highest amount of expression vectors (8 μg). Remarkably, the R74A/R75A/R76A mutant showed no inhibitory activity toward ERK2 in our assays (Fig. 9). Western blot analysis of the cell lysates with FLAG antibody indicated that the wild type and all mutant MKP-2 proteins were expressed at comparable levels (Fig. 9, α-FLAG blot).

The Basic Motif of MKP-2 Is Required to Protect HeLa Cells from Cisplatin-induced Apoptosis Effectively—The physiological importance of the basic kinase binding motif in MKP-2 was demonstrated through inhibition of cisplatin-induced apoptosis of HeLa cells. It has been shown that ERK activity is required for the execution of apoptosis after cisplatin treatment of HeLa cells (6). This finding was confirmed by using two inhibitors of MEK1/2, U0126 and PD98059 (Fig. 10A). Treatment of HeLa cells with 20 μM cisplatin for 18 h induced chromatin fragmen-
The addition of U0126 into the medium blocked the apoptosis process completely (Fig. 10A, lower left panel). PD98059 also inhibited the cisplatin-induced apoptosis but to a less degree (Fig. 10A, lower right panel). In cells transfected with an empty vector (pSRa), cisplatin induced chromatin fragmentation in about 80% of the cells (Fig. 10B and C). By comparison, transfection of cells with the vector for wild type MKP-2 increased cell survival significantly (Fig. 10B). However, transfection with the vector for the MKP-2 D249N mutant proteins increased cell survival significantly (Fig. 10B). However, transfection with the vector for the MKP-2 D249N mutant proteins increased cell survival significantly (Fig. 10B). However, transfection with the vector for the MKP-2 D249N mutant proteins increased cell survival significantly (Fig. 10B). However, transfection with the vector for the MKP-2 D249N mutant proteins increased cell survival significantly (Fig. 10B). 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The Basic Motif of MKP-2 Is Involved in Nuclear Localization—Basic motifs formed by a cluster of arginine and/or lysine residues have been shown to serve as nuclear localization signals in a large number of proteins (42). Because MKP-2 is localized predominantly in the nucleus (Fig. 7), we investigated whether the basic motif also played a role in its subcellular localization. FLAG-tagged wild type and mutant MKP-2 proteins were transiently expressed in HeLa cells, and their subcellular localization was examined by immunofluorescence (Fig. 11). Consistent with the previous report (26), wild type MKP-2 was localized predominantly in the nucleus as confirmed by DAPI staining (Fig. 11, A and B). Similar to the wild type MKP-2, all three single-residue substitution mutants (R74A, R75A, and R76A) also displayed predominantly nuclear localization (Fig. 11, C–H). However, the MKP-2 R74A/R75A/R76A mutant presented a pattern of subcellular distribution that was significantly different from that of the other MKP-2 proteins (Fig. 11, I and J). Compared with wild type MKP-2 or the single-residue substitution mutants, the MKP-2 R74A/R75A/R76A mutant showed a greater presence in the cytoplasm. Reflecting this increase in the cytosolic fraction, the nuclear boundary was no longer clear, especially in the cells where the mutant was expressed at higher levels (Fig. 11, I and J). Nevertheless, when the R74A/R75A/R76A mutant was expressed at a lower level, the mutant still primarily localized in the nucleus. The expression level-dependent difference in subcellular localization in the R74A/R75A/R76A mutant suggests that the basic motif as a whole plays a role in the nuclear transport and/or retention of MKP-2.

DISCUSSION

In this report, we have investigated the interaction of MKP with its substrate MAP kinases and the subsequent effect of such interactions on its catalytic activity. We demonstrate that ERK1/2 and JNK1, but not p38, can trigger a potent catalytic activation of MKP-2 (Fig. 1), which provides an explanation for earlier observations that MKP-2 acts preferentially on ERK and JNK MAP kinases (23, 26). Unexpectedly, however, MKP-2 was found to interact preferentially with ERK and p38 (Figs. 2 and 3). This interaction involved three arginine residues within a basic motif in the NH₂ terminus of MKP-2 (Fig. 3). This basic...

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**Fig. 10.** Protective effects of wild type MKP-2 and the docking-deficient mutant against ERK-mediated apoptosis induced by 15 μM cisplatin (CDDP). A, effects of MEK1/2 inhibitors on cisplatin-induced apoptosis of HeLa cells. Shown is the DAPI staining of control cells (upper left panel), cisplatin-treated cells (upper right panel), cells treated with cisplatin plus either U0126 (lower left panel) or PD 098059 (lower right panel) for 18 h. The inhibitors remained in the medium until the cells were fixed for analysis. B, effects of overexpression of either wild type MKP-2 or the R74A/R75A/R76A mutant on cisplatin-induced apoptosis. HeLa cells in six-well plates were transfected with 2 μg of pSRa, pSRα-FLAG-MKP-2, or pSRα-FLAG-MKP-2 R74A/R75A/R76A. 24 h later, the cells were treated with cisplatin for 18 h. Cells were then fixed and stained by DAPI. Transfected plasmids are indicated above the panels. C, summary of cell survival after cisplatin treatment in cells transfected with different vectors. A and B are from representative experiments. Data presented in C are from two independent experiments and are expressed as the mean ± S.D. More than 400 cells were scored for each treatment.

**Fig. 11.** Subcellular localization patterns of the wild type or mutant MKP-2 proteins. FLAG-tagged wild type or mutant MKP-2 proteins were transiently expressed in HeLa cells, and subcellular localization was examined by immunofluorescence using anti-FLAG antibody (A, C, E, G, and I). Nuclei were visualized by DAPI staining (B, D, F, H, and J). Transfected vectors: A and B, FLAG-MKP-2; C and D, FLAG-MKP-2 R74A; E and F, FLAG-MKP-2 R75A; G and H, FLAG-MKP-2 R76A; I and J, FLAG-MKP-2 R74A/R75A/R76A. Images were acquired in the same field.
motif was also required for effective catalytic activation by ERK and p38 (Fig. 4). We further show that both in vitro and in vivo wild type MKP-2, but not the docking-deficient mutants, inactivated ERK2 efficiently (Figs. 8 and 9). Although the MKP-2 domain responsible for JNK1-induced catalytic activation remains unclear, the observations that JNK1 substantially activates MKP-2 in vitro (Fig. 1) and MKP-2 effectively inactivates JNK1 in vivo (23, 43) suggest that JNK1 interacts with MKP-2. This interaction was found to be very weak both in GST pull-down assays (Figs. 2 and 3) and in a yeast two-hybrid assay (data not shown), and we were thus unable to define the JNK interacting domain further. Because JNK1-triggered catalytic activation of MKP-2 is unaffected by mutation of the MKP-2 basic motif (Fig. 5), JNK1 is likely to interact with a different site on MKP-2. The observation that a mutation in JNK1 equivalent to the ERK2 sevenmaker mutation did not affect JNK1-mediated MKP-2 activation (Fig. 6B) further supports the notion that JNK1 may interact with MKP-2 through a different mechanism. Very recently, Slack et al. (39) have reported that a basic motif on MKP-1, which is highly homologous to that on MKP-2, does not participate in the interaction between JNK1 and MKP-1. Interestingly, mutation of an LXL motif in a cytosolic MAP kinase phosphatase, hVH5/M3/6, blocks JNK-mediated phosphorylation of hVH5 and inhibits the phosphatase activity of hVH5 toward JNK (44). Because this LXL motif is highly conserved in MKP-2, it is possible that the MKP-2 LXL motif is involved in MKP-2 catalytic activation by JNK1.

Several characteristics of catalytic activation of MKP-2 by ERK1/2 resemble those of MKP-3. The 25-fold increase in MKP-2 activity stimulated by ERK1/2 (Figs. 1 and 4) is comparable to the ERK2-induced activation of MKP-3 (19, 41). Maximal activation of MKP-2 by ERK2 also requires the ERK2 acidic motif (Fig. 6A). Like MKP-3 (45), the stimulatory effect of ERK1/2 on MKP-2 is mediated primarily through the basic motif of the phosphatase (Figs. 4 and 6A). The small but still significant activation (2–3-fold) of the MKP-2 R74A/R75A/R76A mutant by both the wild type and the sevenmaker mutant ERK2 proteins (Fig. 6A) suggests that at least an additional domain of ERK2 is involved in the maximal activation. A similar stimulatory effect was also observed in MKP-1 catalytic activation by the ERK2 sevenmaker mutant (39). However, it is unclear whether, in addition to serving as a docking site for MAP kinases, the basic motif of MKP-2 has another function in the catalytic activation. Compared with wild type MKP-2, the MKP-2 Arg-74/Arg-75/Arg-76 mutant exhibited a small increase in basal phosphatase activity (Fig. 4A). Mutation of the three arginines to glutamates or deletion of the region spanning the two Cdc25 homolog domains and the basic motif also resulted in a similar increase in basal activity (data not shown). It is possible that these small increases in basal activity were nonspecific effects caused by the severe mutations. Alternatively, in the absence of specific substrates, the NH2-terminal domain may have an inhibitory effect on the catalytic site of the phosphatase. Potentially, such an inhibition could prevent nonspecific actions toward other phosphorylated proteins. The small increases in the basal activities of these severe mutants may represent a relief of the inhibitory effect.

It was interesting to find that the Asp-249 → Asn mutation at the general acid loop of MKP-2 had different effects than did an equivalent mutation on Pyst-1/MKP-3 (Asp-262 → Asn). Based on the structures of VHR, Pyst-1 and other tyrosine phosphatases (41, 46, 47), both Asp-249 of MKP-2 and Asp-262 of Pyst-1 are predicted to act as the general acid responsible for protonating the tyrosine leaving group. Although mutating Asp-262 on Pyst-1 did not change the basal phosphatase activity of Pyst-1 significantly, it did abolish the ERK2-stimulated increase in Pyst-1 activity, indicating that Asp-262 is only required for adapting a high activity form (41). By contrast, the Asp-249 → Asn mutation on MKP-2 not only prevented its catalytic activation but also decreased its basal phosphatase activity by 90%, indicating that Asp-249 is necessary for MKP-2 function in both the unactivated and the activated state. As demonstrated in Fig. 7, this unique behavior of the MKP-2 D249N mutant was unlikely to result from improper folding. However, we cannot rule out the possibility that in addition to functioning as a general acid, Asp-249 may play a role in catalysis. In this regard, the equivalent residue in cdc25A (Asp-383) has been shown to play a structural role and form a salt bridge with an arginine side chain. Mutation of Asp-383 to Asn resulted in a 150-fold reduction in phosphatase activity (48).

Perhaps the most intriguing finding from this study is the cleardiscordance between the ability of a native MAP kinase to bind to an unactivated MAP kinase phosphatase and its ability to trigger phosphatase activation. Specifically, p38 effectively binds to MKP-2 (Figs. 2 and 3) but has only a marginal effect on its catalytic activity (Fig. 1C). On the other hand, JNK1 displays a lower affinity for MKP-2 (Fig. 2), but it induces a substantial catalytic activation (Fig. 1). If we assume that maximal activation of a MAP kinase phosphatase involves two distinct kinase domains, one for binding to the phosphatase and the other for forcing the phosphatase to adopt the active conformation (referred to as the activation domain), we can explain our results as follows. Although p38 has a favorable site for interaction with MKP-2, it may lack an effective activation domain for MKP-2. JNK1, on the other hand, lacks a high affinity binding site for MKP-2 but may have a very effective activation domain. This model is also consistent with the observation that a lower MKP-2 activation plateau was achieved at a much lower p38/MKP-2 ratio, whereas the higher activation plateau with JNK1 will require a substantially larger JNK1/MKP-2 ratio (Fig. 1). In this regard, investigation of MKP-2 activation by the p38/ERK chimeras (45) could be an ideal approach for defining the two domains.

The differential binding abilities of a given dual specificity phosphatase or a tyrosine phosphatase (49) to various MAP kinases present an intriguing channel for cross-talks between distinct MAP kinase cascades. For example, if MKP-2 is responsible for maintaining a low basal JNK activity in unstimulated cells, ERK1/2 and to a less degree p38 after stimulation may increase JNK activity by forming complexes with MKP-2 and sequestering its function. Such a mechanism mediated by MKP-2 and potentially also by other MAP kinase phosphatases may provide a reasonable explanation for JNK activation induced by the tumor promoter arsenite. Previously, Cavigelli et al. (50) have suggested that JNK activation by arsenite is mediated by inhibiting a constitutive dual specificity protein phosphatase. We and others have demonstrated that arsenite substantially activates both ERK1/2 and p38 (35, 51–53). Most importantly, preliminary results in our laboratory indicate that MKP-2 can be detected easily in both unstimulated HeLa cells and in resting WI-38 normal human diploid fibroblasts (data not shown). Given the fact that MAP kinase phosphatases and MAP kinase phosphatases compete for the same site on MAP kinases for interaction (21), even a slight shift in this balance could yield a staggering effect on MAP kinase activity.

Finally, several MAP kinase phosphatases have been shown to localize predominantly in the nucleus, including MKP-1, MKP-2, PAC-1, and B23 (13). The nuclear localization signals on these phosphatases are still unclear. We found that when all three arginine residues in the basic motif of MKP-2 were mu-
tated to alanines, the subcellular localization pattern was af-
fected significantly (Fig. 11). Although basic amino acid clus-
ters have been shown to serve as nuclear localization signals for many proteins (42), this was an unexpected finding here because all MAP kinase phosphatases have a basic motif, but they do not all localize to the nucleus (13). In this regard, it is interesting to note that all members of the nuclear MAP kinase phosphatase subgroup share an RRA/RK consensus sequence (13, 21). This basic motif of MKP-2 is clearly not the only (or entire) nuclear localization signal because a large fraction of the R74A/R75A/R76A mutant still localizes in the nucleus (Fig. 11). Considering the fact that proper subcellular localization is a prerequisite for its function, the compromised localization of the mutant MKP-2, as shown in the immunofluorescent studies (Fig. 11), could have a devastating effect on its function, especially in situations where de novo protein synthesis and nuclear transport are required.

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Discordance between the Binding Affinity of Mitogen-activated Protein Kinase Subfamily Members for MAP Kinase Phosphatase-2 and Their Ability to Activate the Phosphatase Catalytically

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