Distinct Binding Determinants for ERK2/p38α and JNK MAP Kinases Mediate Catalytic Activation and Substrate Selectivity of MAP Kinase Phosphatase-1*

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Mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1/CL100) is an inducible nuclear dual specificity protein phosphatase that can dephosphorylate and inactivate both mitogen- and stress-activated protein kinases in vitro and in vivo. However, the molecular mechanism responsible for the substrate selectivity of MKP-1 is unknown. In addition, it has been suggested that the signal transducers and activators of transcription 1 (STAT1) transcription factor is a physiological non-MAP kinase substrate for MKP-1. We have used the yeast two-hybrid assay to demonstrate that MKP-1 is able to interact selectively with the extracellular signal-regulated kinase 1/2 (ERK1/2), p38α, and c-Jun NH2-terminal kinase (JNK) MAP kinase isoforms. Furthermore, this binding is accompanied by catalytic activation of recombinant MKP-1 protein in vitro, and these end points show an absolute correlation with MKP-1 substrate selectivity in vivo. In contrast, MKP-1 does not interact with STAT1. Recombinant STAT1 does not cause catalytic activation of MKP-1; nor does MKP-1 block tyrosine phosphorylation of STAT1 in vivo. Both binding and catalytic activation of MKP-1 are abrogated by mutation of a conserved docking site in ERK2, p38α, and JNK1 MAP kinases. Within MKP-1, MAP kinase binding is mediated by the amino-terminal noncatalytic domain of the protein. However, mutation of a conserved cluster of positively charged residues within this domain abolishes the binding and activation of MKP-1 by ERK2 and p38α but not JNK1, indicating that there are distinct binding determinants for these MAP kinase isoforms. We conclude that the substrate selectivity of MKP-1 is determined by specific protein-protein interactions coupled with catalytic activation of the phosphatase and that these interactions are restricted to members of the MAP kinase family of enzymes.

The mitogen-activated protein (MAP) kinase isoforms are key components of cellular signal transduction pathways, which become activated in response to a wide variety of external stimuli. They can be subdivided into at least three classes based on sequence homology and differential activation by agonists (1–3); these include the growth factor-activated MAP kinases, extracellular signal-regulated kinase 1 (ERK1) and ERK2, and the stress-activated MAP kinases c-Jun amino-terminal kinase (JNK, SAPK1) and p38 (SAPK2) MAP kinases. In addition, a number of less well characterized members of the MAP kinase family have been identified such as BMK1/ERK5, ERK7, and ERK3 (4–6).

MAP kinase pathways relay, amplify, and integrate complex signals in order to elicit appropriate biological responses. In mammalian cells, these include cellular proliferation, differentiation, inflammatory responses, and apoptosis. These responses are associated with significant alterations in the pattern of cellular gene expression, and transcription factors are a major target of MAP kinase signaling in vivo (7). To phosphorylate these proteins, activated MAP kinases translocate to the cell nucleus, a process that is generally associated with prolonged activation of the MAP kinase (8). Therefore, the magnitude and duration of MAP kinase activation are critical determinants of biological outcome, and regulatory mechanisms governing the activation of MAP kinase are of key importance in both physiological and pathological cell functions.

The duration and magnitude of MAP kinase activation can be regulated at many points within the signal transduction pathway. However, it is now clear that the MAP kinase itself is a major target for regulation through the action of specific protein phosphatases. MAP kinase activation is dependent on the phosphorylation of both the threonine and tyrosine residues of the TXY motif found within the “activation loop” of the kinase. Since phosphorylation of both residues is required for activity, dephosphorylation of either residue is sufficient for inactivation. This can be achieved by protein-tyrosine phosphatases, serine/threonine-specific protein phosphatases, or dual specificity (threonine/tyrosine) protein phosphatases. It is now clear that dual specificity MAP kinase phosphatases (MKPs) play an important role in regulating the activity of MAP kinases (9). Thus far, nine distinct MKPs have been identified and characterized. These are MKP-1 (CL100) (10–13), PAC1 (14), MKP-2 (hVH2, TYP1) (15–17), hVH3 (B23) (18, 19), hVH5 (M3–6) (20, 21), MKP-3 (Pyst1, rVH6) (22–24), MKP-X (Pyst2, B59) (22, 25, 26), MKP-4 (27), and MKP-5 (28). MKPs can be divided into two broad classes. The first group, typified by MKP-1 and PAC1, are nuclear enzymes that are

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§ The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; MKP, MAP kinase phosphatase; pNPP, para-nitrophenyl phosphate; ATF2, activating transcription factor 2; HA, hemagglutinin; IFN, interferon; STAT, signal transducers and activators of transcription; GAS, γ-interferon activated sequence.
encoded by growth factor and stress-inducible genes (10, 14). The second group, typified by MKP-3, are predominantly cytosolic enzymes and are not encoded by immediate early genes (22, 25). Recent work has shown that certain dual specificity MKPs display marked substrate selectivity for different MAP kinase isoforms in vitro and in vivo. MKP-3 is ~100-fold more active toward ERK2 than p38α (SAP2a) in vitro (22). Furthermore, this substrate selectivity is also observed in vivo (22, 29). hVH-5 (M3/6), another cytosolic MKP, is highly specific for JNK and p38 MAP kinases (29).

The specific dephosphorylation of ERK2 by MKP-3 is accompanied by the formation of a tight physical complex between the two proteins (22). This binding is mediated by the amino-terminal noncatalytic domain of MKP-3, and removal of this domain abrogates substrate selectivity in vivo (30). Remarkably, this binding event also results in the catalytic activation of MKP-3 in vitro as revealed by a 30-fold increase in the ability of the enzyme to hydrolyze the chromogenic substrate paranitrophenyl phosphate (pNPP) (31). Catalytic activity mirrors substrate selectivity in vivo, since stress activated MAP kinase isoforms were unable either to bind to or increase the catalytic activity of MKP-3. The closely related enzymes MKP-X (Pytx2) and MKP-4 also undergo catalytic activation upon binding to ERK2 (25, 31), indicating that this may be a general mechanism by which all members of this family of MKPs are regulated. However, MKP-5, a recently characterized MKP, both binds to and inactivates p38 (SAPK2), but recombinant p38 protein does not catalytically activate MKP-5 in vitro (28).

The inducible nuclear phosphatase MKP-1 (CL100) was originally characterized as an ERK2 phosphatase in vitro and in vivo (11–13). However, it was subsequently demonstrated to have activity against JNK (SAPK1) and p38 (SAPK2) both in vitro and in vivo (22, 32, 33), and careful titration of expression levels in mammalian cells revealed it to be equally effective in inactivating these three MAP kinase isoforms in vivo (34). Despite this, nothing is known about the molecular basis for the substrate selectivity of MKP-1. Wild-type MKP-1 does not appear to be able to form a physical complex with ERK2 in vivo (12, 22), and it is not known if MKP-1 is subject to catalytic activation by mitogen- and stress-activated MAP kinases in vitro. In addition, the results of experiments using antisense mRNA to suppress the expression of MKP-1 indicate that a regulatory tyrosine residue within the signal transducers and activators of transcription 1 (STAT1) protein is dephosphorylated by MKP-1 in vivo, thus raising the possibility that there may be physiological non-MAP kinase substrates for this enzyme (35).

Here we demonstrate that the substrate selectivity of MKP-1 is governed by its ability to form physical complexes with ERK2, JNK1, and p38α MAP kinase isoforms in vitro and in vivo and that complex formation is accompanied by catalytic activation of MKP-1. In contrast, STAT1 is unable to bind to MKP-1 or to catalytically activate the enzyme; nor does over-expression of MKP-1 cause tyrosine dephosphorylation of STAT1 in vivo. Mutation of a conserved docking site within MAP kinases that is used by activators, regulators, and substrates of these enzymes abrogates both binding and catalytic activation of MKP-1. Within MKP-1, MAP kinase binding is mediated by the amino-terminal noncatalytic domain of the phosphatase. However, mutation of a conserved cluster of positively charged residues within this domain abrogates binding and catalytic activation only by ERK2 and p38α and not JNK1, indicating that MAP kinase interactions may be mediated by distinct binding sites. We conclude that the substrate selectivity of MKP-1 is governed by specific protein–protein interactions coupled with catalytic activation of the phosphatase, and our results strongly suggest that these interactions are restricted to the MAP kinase family of proteins.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The plasmids pSG5.MKP-1, pSG5.MKP-1CS, pSG5.p38a, pSG5.JNK1, and pSG5.ERK2 containing a single copy of either HA (12CA5) or Flag tag or the HA (12CA5) epitope tag have been previously described (22). The IFN-γ-responsive reporter, p(IF-1.GAS),tktλ−[39]lucter, and the ptkλ−[39]lucter reporter were kindly provided by Dr. S. Goodbourn. The plasmids (MLVGALELK, which encodes the C-terminal activation domain of Elk-1 fused to the GAL4 DNA binding domain; G5EAue, a GAL4-dependent luciferase reporter plasmid; pAG−1−147), which encodes the DNA binding domain of either PARG or p38, which encodes β-galactosidase) were kindly provided by Dr N. Jones. All other plasmid constructs are described in the Supplemental Material.

**Two-hybrid Analysis**—GAL4 DNA binding domain and activation domain fusion plasmids were transformed into the yeast strains J398−2A and Y187, respectively, according to the manufacturer’s instructions (CLONTECH). Semiquantitative analysis of two-hybrid interactions was performed using a β-galactosidase assay according to the manufacturer’s instructions (CLONTECH). To ensure that all protein–kinase interactions were detected, binding capability was assessed using each kinase isoform expressed as GAL4 DNA-binding domain and activation domain fusions where possible.

**Protein Expression and Purification**—Wild-type MKP-1 and MKP-1ΔL were expressed in Escherichia coli (BL21DE3[pLyS]SR), purified, and refolded as described previously for wild-type MKP-1 (10). His-tagged STAT1α and ERK2D319N were expressed in E. coli and purified on Ni2⁺-nitrilotriacetic acid resin according to the manufacturer’s instructions (Qiagen). Protein-containing fractions were pooled and dialyzed overnight at 4 °C against 1 x 2 liters DB2 (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM dithiothreitol, and 50% glycerol).

**Western Analysis**—For detection of Myc epitope-tagged proteins in cells, lysates were separated by SDS-polyacrylamide gel electrophoresis (10%), transferred to a 0.45-µm polyvinylidene difluoride membrane (Millipore Corp.), and immunoblotted with the anti-Myc monoclonal antibody 9E10 as previously described (22). For detection of endogenous phosphorylated STAT1α, immunoblotting was performed with an anti-phospho-STAT1 (Tyr701) polyclonal antibody according to the manufacturer’s instructions (New England Biolabs).

**Protein Phosphatase Assays**—Phosphatase activities and catalytic activation of MKP-1 was measured using pNPP hydrolysis at 25 °C as previously described (25). Initial rates of pNPP hydrolysis were determined using Microplate Manager III, version 1.57 (Bio-Rad).

**Gene Activators**—The activities of Myc epitope-tagged or HA (12CA5) epitope-tagged ERK2, JNK1, p38α, p38γ, and p38β immunoprecipitated from COS-1 cells using either 9E10 or 12CA5 monoclonal antibodies, respectively, were assayed as described previously (25).

**Cell Culture, Transfection, and Reporter Assays**—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected using a standard calcium phosphate method as previously described (22). Reporter gene assays for the detection of luciferase and β-galactosidase activities were performed using the Dual-Light system according to the manufacturer’s instruction (Tropix Inc.).

**RESULTS**

**MKP-1 Binds to both Mitogen- and Stress-activated MAP Kinases in Vivo**—A yeast two-hybrid assay was used to determine the specificity and selectivity of interactions between MKP-1 and a comprehensive panel of nine distinct mitogen- and stress-activated MAP kinase isoforms in vivo. Binding was determined by the activation of GAL4-dependent ADE2/HIS3/ lacZ reporters. Activation of HIS3 and ADE2 was assessed by selection on synthetic dropout medium deficient for histidine and adenine. The strength of these interactions was quantified by β-galactosidase assay. Full-length wild-type MKP-1 was shown to specifically interact with ERK1, ERK2, JNK1, and a kinase-negative form of p38α (Fig. 1A). A very weak interaction was detected with ERK5, while no interaction was seen with ERK3, ERK7, p38γ, or p38δ. MKP-1 interacted with ERK1, ERK2, JNK1, and p38α with a rank order of binding strength as follows: JNK1 ≫ ERK2 ≫ p38α > ERK1 (Fig. 1B). We
consistently find that ERK1 has a lower binding affinity for MKP-1 when compared with ERK2, and this is also the case when the ERK-specific phosphatase MKP-3 is used as bait in these assays (data not shown). The specificity of these protein-protein interactions was unaltered using a catalytically inactive mutant (Cys258 to Ser) form of MKP-1 (MKP-1CS); however, the strength of binding was somewhat increased (−2-fold) (Fig. 1B). Western blot analysis using GALA-specific antibodies (CLONTECH) verified expression of each of the MAP kinase fusion proteins used in the screen (data not shown).

**Binding to Mitogen- and Stress-activated MAP Kinases Is Associated with a Stimulation of MKP-1 Catalytic Activity**—It has been demonstrated previously for a subfamily of dual specificity MAP kinase phosphatases, which includes MKP-3 (Pyst1), Pyst2 (MKP-X), and MKP-4, that binding to ERK2 results in a dramatic increase in the catalytic activity of these enzymes in vitro (25, 31). Phosphatase activity of bacterially expressed MKP-1 (2.5 μg) toward pNPP was investigated to determine whether the selective binding of MKP-1 in vivo to ERK2, JNK1, and p38α was mirrored by catalytic activation in vitro. The phosphatase activity of MKP-1 was specifically increased in the presence of 10 μg of recombinant ERK2, JNK1, and p38α (Fig. 2). ERK5, which interacted only weakly with MKP-1 in our two-hybrid screen, caused a small but reproducible increase in activity (−1.6-fold). In contrast, no significant catalytic activation was observed upon the addition of recombinant ERK3, p38γ, or p38δ. The strength of the interactions between MKP-1 and these kinase isoforms in vivo directly parallels the level of catalytic activation observed in this in vitro assay. The phosphatase activity of MKP-1 was only moderately stimulated by p38α and ERK2 (−2-fold), whereas JNK1, which was shown to interact most strongly with MKP-1 in vivo, enhanced activity by up to 5-fold. Incubation of MKP-1 (2.5 μg) with increasing amounts of these MAP kinases (up to 20 μg) showed that the activation of MKP-1 was dose-dependent (Fig. 3, A–C) and at least in the case of p38α was saturable (Fig. 3C). Interestingly, Myc (9E10) epitope-tagged MKP-1 expressed in mammalian cells and immunoprecipitated using an anti-9E10 monoclonal antibody also exhibited phosphatase activity toward pNPP. Furthermore, an identical pattern of catalytic activation in the rank order JNK1 ≫ ERK2 = p38α was observed (Fig. 4). To ensure that this activity was not mediated by the presence of associated proteins co-immunoprecipitated with MKP-1, the catalytically inactive MKP-1CS form was also expressed in COS-1 cells. In contrast to the wild-type protein, immunoprecipitated MKP-1CS exhibited no measurable phosphatase activity (Fig. 4).

**MAP-1 Specifically Inactivates ERK2, JNK1, and p38α in Vivo**—Since MKP-1 exhibited a restricted ability to both bind to and be catalytically activated by different mitogen- and stress-activated MAP kinase isoforms, we set out to determine if these properties reflect the substrate selectivity of MKP-1 in vivo. COS-1 cells were cotransfected with plasmids encoding Myc (9E10) epitope-tagged MKP-1 and HA (12CA5) epitope-tagged ERK2, JNK1, p38α, p38γ, or p38δ. Transfected cells were then exposed to an appropriate stimulus to induce kinase activation, either serum (ERK2) or anisomycin (JNK1, p38α, p38γ, and p38δ). MAP kinases were then immunoprecipitated using an anti-HA (12CA5) monoclonal antibody and assayed...
MAP Kinase Interaction Domains on MKP-1

MAP kinases ERK2, JNK1, and p38α are associated with significant stimulation of MKP-1 phosphatase activity. Furthermore, only those MAP kinase isoforms that are able to bind and cause catalytic activation of MKP-1 are in vitro substrates for this dual specificity phosphatase.

STAT1α Is Not a Substrate for MKP-1—We have demonstrated previously that MKP-1 is unable to catalyze the dephosphorylation of a variety of non-MAP kinase proteins in vitro (11). However, a recent report thatSTAT1 tyrosine phosphorylation was specifically prolonged in the presence of an MKP-1 antisense oligonucleotide suggested that this transcription factor was also regulated by MKP-1 (35). The nuclear localization and subsequent transcriptional activity of STAT1 is regulated primarily by tyrosine phosphorylation; thus, dephosphorylation by a nuclear phosphatase would result in its inactivation. However, using two-hybrid analysis, we have shown that MKP-1 is incapable of interacting with STAT1α in vivo (Fig. 6A). This finding was confirmed by coimmunoprecipitation studies in vitro (data not shown). Furthermore, recombinant STAT1α (up to 20 μg) was incapable of increasing the phosphatase activity of MKP-1 toward pNPP (Fig. 6B), and overexpression of Myc epitope-tagged MKP-1 in COS-1 cells failed to prevent tyrosine phosphorylation of STAT1α in response to IFN-γ (Fig. 6C). Finally, overexpression of increasing amounts of MKP-1 (up to 2 μg) in COS-1 cells significantly reduced the transcriptional activity of the MAP kinase-regulated transcription factor Elk-1 (Fig. 6D) but did not modulate the transactivation potential of a STAT1α-dependent reporter in response to IFN-γ (Fig. 6E). In light of these results, we believe that it is most unlikely that MKP-1 fulfills a role as a nuclear phosphatase responsible for the dephosphorylation and subsequent inactivation of STAT1.

Sevenmaker Mutations in ERK2, JNK1, and p38α Abrogate Binding to MKP-1 in Vivo—The mutant sevenmaker form of ERK2, ERK2D319N, has been shown to exhibit enhanced kinase activity as a result of reduced sensitivity to inactivation by several phosphatases (31, 34, 36). This “docking domain” within the C terminus of ERK2 is common to all members of the mitogen- and stress-activated MAP kinase family (37). To determine whether this site was important for the observed interactions between MKP-1 and MAP kinase isoforms, sevenmaker mutations were introduced into ERK2, JNK1, and p38α. For p38α, aspartic acid residue 316 was mutated to asparagine (p38D316N). In the case of JNK1, two docking domain mutants were created: one in which aspartic acid 326 was replaced by asparagine (JNKD326N) and another in which three acidic residues within this domain, aspartic acid 326 and glutamic acid residues 329 and 331, were mutated to asparagine (JNK326N). Two-hybrid analysis showed that in vivo binding of both wild-type and C258S forms of MKP-1 to ERK2, p38α, and JNK1 was abrogated by mutation of these conserved acidic residues. For JNK1, mutation of aspartic acid residue 326 alone was sufficient to block interaction (Fig. 7A).

ERK2-sevenmaker Does Not Stimulate MKP-1 Phosphatase Activity in Vitro—Sevenmaker-like mutations within ERK2, JNK1, and p38α abrogated binding of MKP-1 to these kinase isoforms in vivo. Previous experiments have shown that this mutation within ERK2 abrogates its ability to bind to and catalytically activate the dual specificity phosphatase MKP-3 (31), and our experiments demonstrate that MAP kinase binding is essential for catalytic activation of MKP-1. To examine the effect of this mutation on the enzymatic activity of MKP-1 toward pNPP, recombinant MKP-1 (2.5 μg) was incubated with increasing amounts of either recombinant wild-type or D319N ERK2. The results clearly show that ERK2-dependent catalytic activation of MKP-1 was significantly reduced by the presence directly for kinase activity using an appropriate substrate, either myelin basic protein (ERK2 and p38 isoforms) or ATPβS (JNK1). The data clearly show that expression of MKP-1 specifically inhibits the kinase activity of ERK2, JNK1, and p38α but has no effect upon the activities of p38γ or p38δ (Fig. 5). Furthermore, the resistance of both p38γ and p38δ to inactivation by MKP-1 was maintained in titration experiments where even higher amounts (up to 20 μg) of MKP-1 were transfected and expressed (data not shown).

Taken together, these in vivo and in vitro findings demonstrate that highly specific interactions between MKP-1 and the
were transfected with 5 μg of a plasmid expression vector encoding either Myc-tagged wild-type MKP-1 or a catalytically inactive mutant (MKP-1CS). Following transfection, cells were lysed, and MKP-1 protein was immunoprecipitated using anti-Myc (9E10) monoclonal antibody. Immunoprecipitated MKP-1 was then assayed for pNPP hydrolysis either in the absence or presence of ERK2, JNK1, or p38δ as indicated. Assays were performed in triplicate, and the results of representative experiments are shown.

MAP Kinase Interaction Domains on MKP-1

The NH₂ Terminus of MKP-1 Is Required for Interaction with MAP Kinases—It has been demonstrated that the noncatalytic amino-terminal region of MKP-3 is required for interaction with ERK2 (30). To determine whether the corresponding region of MKP-1 is important for binding to ERK2, JNK1, and p38δ, a deletion mutant of MKP-1, MKP-1188 (lacking amino acid residues 1–188), was screened in a two-hybrid assay for its ability to interact with the kinase panel described previously. The β-galactosidase assay results show that loss of the noncatalytic NH₂ terminus of MKP-1 abrogates binding to ERK1, ERK2, ERK5, JNK1, and p38δ in vivo (Fig. 8).

Identification of a Selective MAP Kinase Interaction Motif within MKP-1—Recent work has identified a common docking site within the activators, substrates, and regulators of mitogen- and stress-activated MAP kinases (37). For the dual specificity MAP kinase phosphatase MKP-3, this motif is composed of a positively charged amino acid cluster within the noncatalytic amino-terminal domain known to be critical for ERK2 binding. This is immediately adjacent to a region found in all dual specificity MKPs, which has significant homology with the cell cycle regulatory phosphatase Cdc25 (38, 39). A similar motif was independently identified within the amino-terminal noncatalytic domain of the tyrosine-specific phosphatase Ptp3p in S. cerevisiae and is responsible for mediating the binding and inactivation of the yeast Fus3p MAP kinase by this enzyme (40).

Based on sequence homology with Ptp3p and MKP-3, we would predict that arginine residues at positions 53–55 would be essential for kinase recognition by MKP-1. To determine whether this motif is important for binding to ERK, JNK1, and p38δ, a mutant form of MKP-1 was constructed (MKP-1M) in which these three residues were mutated to alanine, serine, and alanine, respectively (Fig. 9A). Substrate binding was then determined by two-hybrid analysis. We find that mutation of these arginine residues completely abolishes the ability of MKP-1 to recognize ERK1, ERK2, and p38δ (Fig. 9, B and C). However, to our surprise, the binding of MKP-1 to JNK1 was completely unaffected, suggesting that the binding determinant for this MAP kinase isomorph is distinct from that used by ERKs and p38δ.

Only JNK1 Is Able to Increase the Phosphatase Activity of MKP-1M—For wild-type MKP-1, substrate binding was associated with marked stimulation of phosphatase activity in vitro. To investigate whether the MKP-1M mutant retains phosphatase activity and to determine if this activity could be stimulated specifically by JNK1 binding, recombinant MKP-1M was expressed in bacteria and assayed for enzymatic activity toward pNPP. MKP-1M exhibited an equivalent level of phosphatase activity to the wild-type protein, and the ability of JNK1 to cause catalytic activation of the phosphatase was completely unimpaired (Fig. 10). In contrast and as predicted by our binding data, MKP-1M did not show any increase in activity toward pNPP.

The NH₂ Terminus of MKP-1 Is Active and Undergoes Catalytic Activation. COS-1 cells go catalytic activation.

of this mutation, with at least 5 times more mutant ERK2 than wild-type protein required to achieve the same increase in MKP-1 phosphatase activity (Fig. 7B).

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catalytic activity when incubated with either recombinant ERK2 or p38α.

**MKP-1M Specifically Inactivates JNK1 Kinase Activity in Vivo**—Our in vitro and in vivo binding and activation data strongly suggest that MKP-1M will behave as a JNK1-specific phosphatase in vitro. To determine whether this is indeed the case, COS-1 cells were cotransfected with plasmids encoding Myc (9E10) epitope-tagged MKP-1M and either HA (12CA5) epitope-tagged ERK2, JNK1, or p38α. The kinases were then immunoprecipitated with an anti-HA (12CA5) monoclonal antibody from cells treated with either serum (to activate ERK2) or anisomycin (to activate JNK1 and p38α) and assayed directly for kinase activity against myelin basic protein (ERK2 and p38α) or ATF2 (JNK1). The results clearly show that only JNK1-dependent kinase activity could be abolished by MKP-1M expression; the kinase activities of ERK2 and p38α are completely unaffected (Fig. 11).

**DISCUSSION**

The prototypic dual specificity MAP kinase phosphatase MKP-1 is able to dephosphorylate and inactivate both mitogen- and stress-activated isoforms of MAP kinase in vitro and in vivo (11–13, 22, 32, 33). However, the full extent of MKP-1 substrate selectivity and its molecular basis have not been

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**Fig. 6.** STAT1 is not a substrate for MKP-1. **A,** semiquantitative analysis of yeast two-hybrid assays based on the level of induction of the β-galactosidase gene was performed to measure interactions between MKP-1 and either STAT1 or JNK1. **B,** phosphatase activity was measured as pNPP hydrolysis at 25 °C monitored by change in optical density at 405 nm. Catalytic activation is expressed as fold increase in the initial rate of pNPP hydrolysis by 2.5 μg of recombinant MKP-1 in the absence or presence of either 10 μg of recombinant STAT1 or JNK1. Assays were performed in triplicate, and the results of a representative experiment are shown. **C,** COS-1 cells were transfected with 5 μg of pSG5 expression vector encoding Myc-tagged MKP-1. Cells were then treated for various times with 1000 IU/ml interferon-γ, and cell lysates were analyzed by Western blotting using antibodies specific for STAT1 phosphorylated on tyrosine 701 and anti-Myc monoclonal antibody (9E10). **D,** COS-1 cells were transiently transfected with increasing amounts of pSG5.MKP-1 (0.25–2 μg) or pSG5 alone, along with 2.5 μg of the GAL4-dependent reporter G5E4Luc and either 2.5 μg of MLVGAELK or 2.5 μg of pAG(1–147). 1.25 μg of pJATLac was included in each transfection as a control. The transfected cells were either untreated or treated for 4 h with 0.1 μg/ml epidermal growth factor (EGF). GAL4-dependent luciferase activity was monitored using a luminometer. Elk-1-mediated expression levels were determined by correcting the luciferase activity to the β-galactosidase levels (mediated by pJATLac expression) and to the activity of the GAL4 binding domain plasmid, pAG(1–147). **E,** COS-1 cells were transiently transfected with increasing amounts of pSG5.MKP-1 (0.25–2 μg) or pSG5 alone, along with either 2.5 μg of an IFN-γ-responsive reporter p(IRF-1.GAS)6tkΔ[−39]luc or 2.5 μg of ptkΔ[−39]luc. 1.25 μg of pJATLac was included in each transfection as a control. The transfected cells were either untreated or treated for 5 h with 1000 IU/ml IFN-γ. GAS reporter-specific expression levels were determined by correcting the luciferase activity to the β-galactosidase levels and to the nonspecific activity of the ptkΔ[−39]luc reporter plasmid.
In an extensive yeast two-hybrid screen, we have characterized protein-protein interactions between MKP-1 and a comprehensive panel of mitogen- and stress-activated MAP kinase isoforms. We find that MKP-1 is only able to interact with a subset of these enzymes, specifically ERK1, ERK2, JNK1, and p38α. Our finding that MKP-1 is able to bind most tightly to JNK1 is supported by a recent study in which wild-type MKP-1 was shown to form a physical complex with JNK when expressed in the 293T human embryonic kidney cell line (41). A substrate-trapping mutant of MKP-1 (Cys258→Ser) was also used in this screen and found to interact with this same subset of MAP kinases, albeit with somewhat higher affinity. This indicates that such substrate traps do not promote inappropriate binding of dual specificity MKPs to other MAP kinase isoforms and underlines the utility of such mutants in defining MKP-substrate interactions.

For a distinct subfamily of ERK-specific MKPs that includes MKP-3 (Pyst1), MKP-X (Pyst2), and MKP-4 (Pyst3), substrate binding is associated with an increase in catalytic activity in vitro (25, 31). In contrast, the recently characterized dual specificity phosphatase MKP-5 can interact with and dephosphorylate p38α but is not catalytically activated by this kinase (28). Thus, it is unclear whether catalytic activation is a general mechanism that determines substrate selectivity.

Our data demonstrate for the first time that catalytic activation of MKP-1 is mediated by specific protein-protein interactions with ERK2, JNK1, and p38α. Significantly, the level of catalytic activation by these kinase isoforms is also consistent with their strength of binding in vivo. Although catalytic activation of MKP-1 by MAP kinases is dose-dependent, we find that activation by p38α MAP kinase saturates at lower concen-
This is despite the fact that JNK1 binds to MKP-1 more tightly than for JNK1. Recent studies of MKP-3 activation by ERK2 have revealed that multiple regions of the phosphatase are involved in its recognition by ERK2 (42). Furthermore, certain sites contribute to ERK2 binding but are not essential for MKP-3 activation. As yet, it is unclear exactly how JNK1 and MKP-1 dock in comparison with complexes.

FIG. 9. Mutations in the NH₂-terminal region of MKP-1 abrogate ERK and p38α but not JNK1 binding. A, the NH₂-terminal region of MKP-1 contains conserved amino acid residues (shown in boldface type) present in yeast tyrosine phosphatases and mammalian MKPs. The Cdc25 homology domain (A box) is overlined, and the amino acid substitutions introduced into MKP-1 are indicated. B, pGBKT.MKP-1M was transformed into PJ69-2A and mated with Y187 expressing the GAL4 AD kinase fusion panel described in the legend to Fig. 1. p38α was expressed in PJ69-2A as a GAL4 BD fusion and mated with pGADT7.MKP-1M transformed into Y187. Yeast diploids were selected onto SD-leu/trp and then restreaked onto SD-leu/trp/his/ade as before. C, semiquantitative analysis of the two-hybrid interactions based on the level of induction of the β-galactosidase gene was performed for full-length wild-type MKP-1 (hatched bars) and the mutant form MKP-1M (closed bars) in the presence of the indicated MAP kinase. Assays were performed in triplicate, and the results of a representative experiment are shown.

FIG. 10. Catalytic activation of MKP-1M is mediated specifically by JNK1. Phosphatase activity of wild-type MKP-1 and MKP-1M was measured as pNPP hydrolysis at 25 °C monitored by change in optical density at 405 nm. Catalytic activation is expressed as fold increase in the initial rate of pNPP hydrolysis by 2.5 μg of recombinant MKP-1 (hatched bars) and MKP-1M (closed bars) in the absence or presence of 10 μg of the indicated MAP kinase. Assays were performed in triplicate, and the results of a representative experiment are shown.

FIG. 11. MKP-1M specifically inactivates JNK1 in vivo. COS-1 cells were co-transfected with HA-tagged ERK2, JNK1, or p38α MAP kinases (2.5 μg) together with either empty pSG5 vector (2.5 μg) or pSG5 expression vector encoding Myc-tagged MKP-1M (2.5 μg). Following 12 h of growth, cells were either serum-starved overnight and restimulated with 15% fetal calf serum (ERK2) or treated with 10 μg/ml anisomycin for 30 min (JNK1 and p38α). MAP kinases were then immunoprecipitated using an anti-HA (12CA5) monoclonal antibody, and immunocomplex assays were performed using myelin basic protein (ERK2 and p38α) or ATF2 (JNK1) as substrates. Radiolabeled proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Equivalent expression of all MAP kinases and expression of MKP-1M was verified by Western blotting using the appropriate monoclonal antibodies (data not shown).
formed between MKP-1 and p38α. However, the results of our mutagenesis experiments indicate that different binding sites on MKP-1 are involved. Determination of the stoichiometry of binding between MKP-1 and different MAP kinases and quantitative measurements of binding affinity are currently under way, and these, together with studies of the mechanism by which MKP-1 undergoes catalytic activation (see below), may provide an explanation for our observations.

The levels of MKP-1 catalytic activation seen here are not comparable with the 20–30-fold increase in the activity of MKP-3 (Pyst1) and Pyst2 that results from binding to ERK2 (25, 31). However, we have observed that the basal activity of recombinant MKP-1 in the absence of MAP kinase is significantly higher when compared with MKP-3. This may reflect a fundamental difference in the position of the general acid loop of MKP-1 in the absence of bound substrate. The crystal structure of the catalytic domain of MKP-3 (Pyst1) suggests that activation mediated by ERK2 binding results from the displacement of the general acid (Asp267) to a more favorable position for catalysis (43). For MKP-1, the equivalent acidic residue (Asp271) may already be in a position to contribute significantly to catalytic activity in the absence of any interaction with MAP kinase. The role of this aspartate residue in the catalytic activation of MKP-1 is currently under investigation.

With respect to the substrate selectivity of MKP-1 in vivo, we find an absolute correlation between substrate binding, catalytic activation, and MAP kinase inactivation. Thus, MKP-1 is able to inhibit the activities of ERK2, JNK1, and p38α but not p38γ and p38δ. The ability of MKP-1 to discriminate between these p38 MAP kinase isoforms is particularly striking given that the sequence homology between p38α, p38γ, and p38δ is ∼60% (44, 45). In our two-hybrid assays, we did detect a very weak interaction between MKP-1 and ERK5 (Fig. 1B). Furthermore, recombinant ERK5 caused a small but reproducible increase in the catalytic activity of MKP-1 (∼1.6-fold). Our results are consistent with a recent report that overexpression of MKP-1 is able to reduce the kinase activity of ERK5 (46).

The sevenmaker mutation within ERK2 (D319N) exhibits reduced sensitivity to inactivation by a number of phosphatases including MKP-1 and MKP-3, both in vitro and in vivo (31, 34, 36). Both JNK1 and p38α have conserved aspartic acid residues at equivalent positions to Asp319 of ERK2, at Asp326 and Asp316, respectively, and Tanoue et al. recently demonstrated that mutation of Asp326 in JNK1 reduced its ability to interact with MKP-5 (37). We have found that mutation of these acidic residues in ERK2, JNK1, and p38α abrogated binding to MKP-1 in vitro, suggesting that they represent a conserved docking motif that is crucial for kinase-phosphatase interactions and thus critical for the regulation of kinase activity in vivo. Consistent with this, recombinant ERK2D319N was unable to induce catalytic activation of MKP-1 in vitro to the same level as wild-type ERK2. Similar results have also been reported for the catalytic activation of MKP-3 by ERK2D319N (31, 42).

The fact that we can still detect catalytic activation by ERK2D319N, albeit markedly reduced, suggests that other residues within ERK2 may be important for interaction with MKP-1. This is consistent with the finding that in p38 MAP kinase other acidic residues close to Asp313 (Asp311 and Asp315) are important for interactions with MKK6, MKP-5, and MNK1 (37). Furthermore, a recent report has identified regions of MKP-3 other than the basic motif as being important for ERK2 binding and catalytic activation (42). We have demonstrated that substrate binding is critical for catalytic activation; thus, it would be predicted that both JNKD326N and p38D316N will also be impaired in this assay. This is indeed the case, since it has recently been reported that a sevenmaker mutant of p38 MAP kinase also has a reduced ability to activate MKP-1 in vitro (47).

It is clear that the noncatalytic amino-terminal region of both yeast protein-tyrosine phosphatases and mammalian dual specificity MAP kinase phosphatases is critical for the binding of MAP kinases (37, 40). We identified three arginine residues within MKP-1 that would be predicted to mediate this and found that mutation of these residues abolishes the ability of MKP-1M to bind to and be activated by ERK1, ERK2, and p38α. Furthermore, overexpression of this mutant in COS-1 cells did not inhibit the activation of either ERK2 or p38α, thus establishing a critical role for this cluster of basic residues in mediating the activity of MKP-1 toward both ERK2 and p38α. However, to our surprise, MKP-1M was still able to bind to and be activated by JNK-1, and expression of MKP-1M in COS-1 inhibited the kinase activity of JNK1. To our knowledge MKP-1M is the only dual specificity phosphatase that is absolutely specific for JNK, since the M3/6 (hVHI-5) enzyme also inactivates p38α (29). Since there are currently no specific JNK inhibitors available, MKP-1M may be of considerable value in dissecting the specific role of JNK signaling in mediating a variety of biological end points.

Our results strongly suggest that the binding of MKP-1 to JNK1 is determined by sequences that are distinct from those that mediate binding of ERK1/2 and p38α. A motif has been identified within the transcription factors c-Jun and ATF2 and within the JNK scaffold protein JIP-1, which acts as a docking site for JNK (48). This 6-domain contains an LXXL motif located 3–5 amino acids downstream from a region containing a number of basic residues. Furthermore Net, a MAP kinase-regulated transcription factor, contains distinct binding domains for ERK/p38 and JNK, and the latter motif (designated the J box) also contains an LXXL element (49). Mutations within the J box abrogated binding of JNK to Net but had no effect upon interaction with either ERK2 or p38. Finally, it has been reported recently that the mutation of an LXXL element within the amino terminus of the JNK-specific phosphatase hVHI5 (M3/6) reduced the ability of this enzyme to dephosphorylate SAPK/JNK in vivo (50). MKP-1 also contains this LXXL motif, and we are currently examining the effects of mutating this and other residues close to the ERK/p38 binding site in an attempt to identify the sequences required for interaction with JNK1.

In conclusion, we have demonstrated that MKP-1 is able to form physical complexes with a restricted subset of MAP kinase isoforms and that MAP kinase binding results in catalytic activation of MKP-1. Both of these end points show an absolute correlation with the substrate selectivity of MKP-1, and our quantitative binding and catalytic activation data would indicate that JNK may be the preferred substrate for MKP-1 in vitro. This is in agreement with previous studies demonstrating that conditional expression of MKP-1 preferentially inhibits JNK in U937 cells (51) and more recent data showing that expression of MKP-1 is able to protect cells against apoptosis mediated by JNK activation (41, 52). In contrast, both the results of our previous studies (11) and experiments presented here do not support the idea that there are physiological non-MAP kinase substrates for MKP-1. In particular, we can find no evidence to support the recent proposal that MKP-1 is able to act on the STAT1 transcription factor. Finally, our identification of distinct determinants within the amino terminus of MKP-1 responsible for binding ERK2/p38α and JNK imply considerable complexity in the binding and catalytic activation

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2 D. N. Slack, O.-M. Seternes, M. Gabrielsen, and S. M. Keyse, unpublished observation.
of MKP-1 by MAP kinases. Future studies will be directed at dissecting these binding determinants within MKP-1 and determining their role in the regulation of MAP kinase signaling by this enzyme.

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