Mitogen-activated Protein Kinase (MAPK) Phosphatase 3-mediated Cross-talk between MAPKs ERK2 and p38α*

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MAPK phosphatase 3 (MKP3) is highly specific for ERK1/2 inactivation via dephosphorylation of both phosphorytrosine and phosphothreonine critical for enzymatic activation. Here, we show that MKP3 is able to effectively dephosphorylate the phosphostrictroline, but not phosphothreonine, in the activation loop of p38α in vitro and in intact cells. The catalytic constant of the MKP3 reaction for p38α is comparable with that for ERK2. Remarkably, MKP3, ERK2, and phosphorylated p38α can form a stable ternary complex in solution, and the phosphatase activity of MKP3 toward p38α substrate is allosterically regulated by ERK2-MKP3 interaction. This suggests that MKP3 not only controls the activities of ERK2 and p38α but also mediates cross-talk between these two MAPK pathways. The crystal structure of bisphosphorylated p38α has been determined at 2.1 Å resolution. Comparisons between the phosphorylated MAPK structures reveal the molecular basis of MKP3 substrate specificity.

MAPK pathways converge different extracellular stimuli into specific cellular responses and mediate various physiological processes, including cellular proliferation, apoptosis, differentiation, and stress responses. There are three major mammalian MAPK subfamilies: ERK, JNK and p38 (1–6). The ERKs are typically activated by growth factors and phorbol ester, whereas JNK and p38 MAPKs are primarily activated by cytokines and environmental stress. MAPK activity is tightly controlled by phosphorylation and dephosphorylation. Full activation of the MAPKs requires phosphorylation on both threonine and tyrosine residues in the TXY motif by their specific upstream dual specificity kinases (MAPK kinases). After activation, each MAPK phosphorylates a distinct spectrum of substrates, which include key regulatory enzymes, cytoskeletal proteins, nuclear receptors, regulators of apoptosis, and many transcription factors.

The regulated dephosphorylation of MAPKs plays a key role in determining the magnitude and duration of kinase activation and hence the physiological outcome of signaling. MAPK phosphatases (MKPs)² are important negative regulators of MAPK signaling. MKPs belong to a family of dual specificity phosphatases and specifically dephosphorylate both threonine and tyrosine residues in the activation loop of MAPKs. In mammalian cells, there are 10 distinct catalytically active MKPs. Several of these MKPs display distinct in vivo substrate preferences for the various MAPKs. For example, it has been shown that MKP3 selectively targets ERK, whereas MKP5 shows a preference for JNK and p38 (7–9). Therefore, there must be a fine regulatory mechanism for MAPK-MKP recognition.

MKP3 is localized predominantly in the cytoplasm and is highly specific for ERK1/2 inactivation (10–13). MKP3 contains a conserved catalytic domain in its C terminus and a less conserved MAPK binding domain at its N terminus (supplemental Fig. S1). The inactive ERK1/2, but not p38 and JNK MAPKs, can form a tight complex with MKP3 via the interaction between the N-terminal domain of MKP3 and the C-lobe of ERK2. Unlike other PTPases and constitutively active MKPs, MKP3 exhibits very low activity toward small phosphoesters, such as para-nitrophenyl phosphate (pNPP), and binding of ERK to the N-terminal domain of MKP3 causes its catalytic activation in vitro. Neither JNK nor p38 MAPKs stimulated significant MKP3 catalytic activity over basal levels (14–17). Thus, it has been suggested that the noncatalytic N-terminal domain of MKP3 plays an important role in controlling its substrate specificity and that the ERK-induced MKP3 activation may be responsible for the selective inactivation of ERK MAPKs by MKP3 (8). Although studies with small molecule substrates have yielded useful information about MKP3 catalysis, our knowledge of its physiological functions is somewhat less complete. Two important questions concerning the dephosphorylation mechanism of its authentic substrate remain unresolved. How does MKP3 differentially recognize and bind to MAPK isoforms? How does complex formation lead to conformational change within the catalytic domain of MKP3?

In this study, we challenge the notion that MKP3, a prototypical ERK-specific MKP, displays little or no activity toward the p38 MAPK isoform. Contrary to a large body of literature, our investigations first show that MKP3 is able to effectively

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The atomic coordinates and structure factors (code 3PY3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S8.

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§ The abbreviations used are: MKP, mitogen-activated protein kinase phosphatase; HePTP, hematopoietic protein-tyrosine phosphatase; STEP, striatal enriched phosphatase; PTP-SD, striatal enriched phosphatase-like protein-tyrosine phosphatase; MEG3, 7-methyl-6-thioguanosine-5′-pentaphosphate (pNPP), para-nitrophenyl phosphate; p38α/pY, p38α phosphorylated on both Thr180 and Tyr182; p38α/pT, p38α phosphorylated on Tyr182; p38α/pY, p38α phosphorylated on Thr180; ERK2/pTy, ERK2 phosphorylated on both Thr180 and Tyr182.

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dephosphorylate the phosphotyrosine, but not phosphothreonine, in the activation loop of p38α MAPK in vitro and in intact cells. The catalytic efficiency of MKP3 reaction for p38α substrate is comparable with that for ERK2 as well as other tyrosine-specific phosphatases, including HePTP, PTP-SE, and STEP. Remarkably, binding of ERK2 to the noncatalytic domain of MKP3 increases the catalytic activity of MKP3 toward phosphorylated p38α. These results suggest that phosphorylated p38α is a physiological substrate for MKP3. To gain further insight into the phosphorylation-dependent binding of p38α to MKP3, we determined the crystal structure of bisphosphorylated active p38α at 2.1 Å resolution. Comparisons between the phosphorylated kinase structures reveal that although p38α and p38γ have greater sequence similarity, the structure of phosphorylated p38γ is more similar to ERK2 than to p38α. The conformation of the activation lip of active p38α is distinct from the phosphorylated ERK2 and p38γ. This structural information reveals new insights into the mechanism of p38α activation by dual phosphorylation and the molecular basis of MKP3 substrate specificity.

EXPERIMENTAL PROCEDURES

Plasmid, Protein Expression, and Purification—The N-terminal His6-tagged human MKP5, human HePTP, rat PTP-SE (147–549), mouse STEP, human PP2Ca, and a C-terminal His6-tagged rat MKP3 and its mutants (MKP3C293S, MKP3/ΔN151, and MKP3C293S/ΔN151) were expressed in Escherichia coli BL21(DE3) and purified by a nickel-NTA column (Qiagen), followed by an anion exchange Source 15Q column (Amersham Biosciences). The mouse N-terminally His6-tagged unphosphorylated ERK2 and p38α were expressed in E. coli BL21(DE3) and purified by a nickel-NTA column, followed by Source 15Q column. Bisphosphorylated MAPK (ERK2/pTγY or p38α/pTγY) was obtained by coexpressing MAPK (ERK2 or p38α) and its cognate constitutively active MKK (MEK1EE or MKK6EE) in vivo. Both MAPK and its cognate MKK were subcloned into the same plasmid pETDuet-1 (Novagen). The bisphosphorylated p38α/pTγY and ERK2/pTγY proteins were expressed in E. coli BL21(DE3) and purified by nickel-NTA, followed by Source 15Q and a size exclusion Superdex 200 column. The purified proteins for enzyme assay were made to 20% glycerol and stored at −80 °C. The protein for crystallization was stored in buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM DTT) at −80 °C.

Crystallization of Phosphorylated p38α and Data Collection—Crystals of active p38α were obtained at 20 °C in 5 days using the hanging drop technique containing equal volumes of protein solution and reservoir buffer (20–25% (w/v) polyethylene glycol 3350, 0.2 M sodium citrate, 100 mM HEPES, pH 7.5). Crystals were transferred into the cryoprotectant containing reservoir buffer with 20% glycerol before being “flash-cooled” at 100 K in a stream of nitrogen gas. X-ray data were collected on a home laboratory x-ray system (Rigaku FR-E rotating anode x-ray generator with an R-AXIS IV++ image plate area detector). Data were integrated and scaled using HKL2000 (18). The crystals belonged to the orthorhombic space group P212121 with unit cell dimensions of a = 47.3 Å, b = 52.3 Å, c = 168.1 Å, and α = β = γ = 90°. There was one molecule in the asymmetric unit.

Structure Determination and Refinement—The structure was solved by molecular replacement using Phaser (19) with the search model of inactive p38α (Protein Data Bank code 1p38). Multiple rounds of the model building and the refinement were carried out with Coot (20) and PHENIX (21). The final Rfree was 18.2%, and the Rfactor was 23.9%. The final model spans nearly the entire molecule, comprising residues 1–354 and two covalently bound phosphate ions, with residues 355–360 missing because of disordered loops that are not visible in any electron density map. PROCHECK (22) was used to analyze the model stereochemistry. All of the residues were in the most favored and additional allowed regions of the Ramachandran plot. Data collection and structure refinement statistics are summarized in supplemental Table S1.

Enzyme Assays for Protein Phosphatases—Kinetic parameters for the dephosphorylation of the phosphorylated p38α and ERK2 were determined using a continuous spectrophotometric assay (23–25). This assay incorporates a coupled enzyme system, which uses purine nucleoside phosphorylase and its chromogenic substrate 7-methyl-6-thioguanosine (MESG) for the quantification of inorganic phosphate produced in the phosphatase reaction (26). All experiments were carried out at 25 °C in 1.8 ml of reaction mixture (pH 7.0 buffer containing 50 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, 100 μM MESG, 0.1 mg/ml purine nucleoside phosphorylase). The reaction was initiated by adding a catalytic amount of phosphates to a reaction mixture, and the dephosphorylation reaction of bisphosphorylated ERK2 and p38α was detected by monitoring the real-time phosphate release at 360 nm. The time courses of absorbance change at 360 nm resulting from the MESG/phosphorylase coupling reaction were recorded on a Lambda 14 PerkinElmer spectrophotometer equipped with a magnetic stirrer in the cuvette holder. Initial rates were determined from the linear slope of progress curves obtained, and the experimental data were analyzed using a nonlinear regression analysis program. The change in absorbance was due to the conversion of MESG to 7-methyl-6-thioguanine in the presence of inorganic phosphate released from the dephosphorylation of ERK2 and p38α by MKP3. Quantitation of phosphate release was determined using an extinction coefficient of 11,200 M−1 cm−1 for the phosphate-dependent reaction at 360 nm at pH 7.0 (27). The stoichiometry of MAPK phosphorylation was then calculated by dividing the relative concentration of the phosphate by that of ERK2 or p38α. The concentration of MESG was determined at 331 nm, using a molar extinction coefficient of 32,000 M−1 cm−1.

Gel Filtration Analysis—Size exclusion chromatography was performed to determine the apparent molecular weight of proteins. 0.1 ml of each sample was loaded onto a Superdex 200 column, preequilibrated in the buffer (50 mM MOPS buffer, pH 7.0, 100 mM NaCl, 0.1 mM EDTA), and run at 4 °C using a flow rate of 0.5 ml/min. The column was calibrated using the molecular weight markers.

Plasmids, Cell Culture, and Transfections—For expression in HEK293T cells, cDNAs coding the sequence of wild-type MKP3, mutant MKP3C293S, and HePTP were subcloned into the pCMV-Myc (Clontech) vector. Plasmid pCDL-SRα 296-
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FIGURE 1. Dephosphorylation of phosphorylated MAPK by MKP3. A, time courses of p38α/pTpY and ERK2/pTpY dephosphorylation by MKP3. The assay system contained 175 nM MKP3 and 4 μM ERK2/pTpY (curve 1) or 4 μM p38α/pTpY (curve 2). B, time-dependent dephosphorylation of p38α/pTpY upon treatment with different phosphatases. The assay system contained 0.75 μM p38α/pTpY. The reaction was initiated by the addition of 400 nM MKP3 and then followed by the addition of 177 nM HePTP and 200 nM MKP5 (as indicated) to the reaction mixture. Western blot analysis of dephosphorylation of p38α/pTpY (C) and p38γ/pTpY (D) by different phosphatases in vitro. p38α/pTpY or p38γ/pTpY were treated with MKP5, MKP3, HePTP, and PP2Cα individually in buffer (50 mM MOPS, pH 7.0, 100 mM NaCl, 10 mM Mg2+). C and D correspond to the upper and lower panels, respectively. p38α and p38γ proteins were detected by specific antibodies. p38α and p38γ were not separated on SDS-PAGE because they have the same molecular weight.

RESULTS
Recombinant Active p38α Is Rapidly and Specifically Dephosphorylated by MKP3—To understand the molecular basis of the substrate specificity of MKP3, we prepared milligram quantities of bisphosphorylated ERK2 and p38α (ERK2/pTpY and p38α/pTpY) (25, 28). This p38α/pTpY preparation corresponds to the highest reported activity against ATF2Δ109 for this enzyme (29). The values of $k_{\text{cat}}$ and $K_m$ were determined to be $4.14 \pm 0.06$ s$^{-1}$ and $1.81 \pm 0.09$ μM, respectively (supplementary Fig. S2). We then examined the dephosphorylation kinetics and substrate specificity of MKP3 for ERK2/pTpY and p38α/pTpY using a spectrophotometric method for the quantitation of inorganic phosphate (25). Fig. 1A shows typical progress curves of ERK2/pTpY and p38α/pTpY dephosphorylation by MKP3. Both MAPKs can be dephosphorylated rapidly by MKP3. When all the substrate has been converted to product, the phosphorylation stoichiometry of ERK2 was determined to be close to 2 mol of phosphate/mol of ERK2. In comparison, the dephosphorylation rate of p38α/pTpY was about 3-fold slower than that of ERK2/pTpY, and only one equivalent of inorganic phosphates was released upon treatment of p38α/pTpY with MKP3. The phosphorylation state of p38α was further determined by the amount of inorganic phosphate released from p38α upon treatment by various phosphatases (Fig. 1B). The addition of MKP3 (400 nM) to a reaction mixture contain-
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Effects of N-terminal domain on substrate specificity and ERK2-induced activation of MKP3. A, time-dependent dephosphorylation of p38α/pTpY upon treatment with different phosphatases. The initial mixture contains 0.9 μM bisphosphorylated p38α/pTpY. The reaction was initiated by the addition of 1.1 μM MKP3/ΔN151 and then followed by 177 nm HePTP and 200 nm MKP5. B, titration of the binding of MKP3 to ERK2. The assay system contained 10 mM pNPP and 5 μM MKP3. Increasing amounts of ERK2 were added to a solution in which the concentration of MKP3 was at least 10 times higher than the dissociation constant, and the intersection of the slope of the increase in the absorbance at 410 nm with the maximum value gives the stoichiometry. The dissociation constant, and the intersection of the slope of the increase in the absorbance at 410 nm with the maximum value gives the stoichiometry. The time courses of p38α/pTpY dephosphorylation by MKP3 in the absence and presence of ERK2. The reaction mixture contained 3 μM p38α/pTpY and 177 nm MKP3 plus 177 nm ERK2 (curve 1) or 177 nm MKP3 only (curve 2). D, Western blot analysis of dephosphorylation of p38α/pTpY by different phosphatases in vitro. p38α/pTpY were treated with MKP3, MKP3 plus ERK2, MKP3/ΔN151, HePTP, MKP5, and PP2Cα individually in buffer (50 mM MOPS, pH 7.0, 100 mM NaCl, 10 mM Mg2+) at 25 °C for 30 min, followed by Western blot using the indicated antibodies. The lower panel of the SDS-polyacrylamide gel (Coomassie-stained) shows the amounts of purified p38α/pTpY and other proteins used in the experiment.

A second intriguing possibility could be due to different origins of p38α proteins because the Xenopus p38α homologue was used by Groom et al. (10), and ours is of a mammalian origin. MKP3 might exhibit different substrate specificity for the recombinant p38 proteins. For example, we have looked at the ability of MKP3 to dephosphorylate bisphosphorylated p38α and found that MKP3 is unable to dephosphorylate this isomorph of p38 MAPK under identical experimental conditions (Fig. 1D). The N-terminal Region Is Not a Major Determinant for Substrate Specificity of MKP3—MKP3 is a rather inefficient enzyme toward small phosphoesters, such as pNPP. However, in the presence of ERK, the catalytic activity of MKP3 increases >35-fold toward pNPP (14, 31). The activation of MKP3 is thought to be achieved via the interaction between the N-terminal domain of MKP3 and the non-catalytic regions of ERK2. Thus, MKP3 substrate specificity is linked to the ability of substrate to induce productive orientation in the active site. To evaluate the role of the N-terminal domain of MKP3 in conferring tyrosine specificity to p38α, we examined the dephosphorylation of p38α/pTpY by MKP3/ΔN151 (an N-terminally truncated MKP3, lacking the first 1–151 amino acids) (32). Fig. 2A shows the time dependence of Pi release from p38α/pTpY for sequen-
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tional additions of different phosphatase into the coupled reaction system. After completion of MKP3/ΔN151-catalyzed p38α reaction, the addition of HePTP to the reaction mixture did not cause detectable Pi release, whereas the further addition of MKP5 produced another equivalent of inorganic phosphate. This result indicates that MKP3/ΔN151 still specifically dephosphorylated the phosphotyrosine of p38α/pTpY, and the N-terminal domain of MKP3 is not a major determinant for the tyrosine specificity toward p38α.

Next, we tested whether the ERK2-induced MKP3 activation is unique for small molecule aryl phosphates. In the initial experiments, the amount of ERK2 required to activate MKP3 maximally was determined by measuring the initial velocities at increasing concentrations of ERK2 and a fixed concentration of phosphatase substrate pNPP. Increasing amounts of ERK2 were added to a solution in which the concentration of MKP3 was at least 10 times higher than the dissociation constant, and the results were plotted to give the stoichiometry. Consistent with previous observations (33, 34), we found that the activation of MKP3 by ERK2 is saturable, and maximal activation resulted from an MKP3/ERK2 ratio of 1:1, suggesting a 1:1 binding stoichiometry between MKP3 and ERK2 (Fig. 2B). We then examined the effects of ERK2 on the MKP3-catalyzed dephosphorylation of p38α. Fig. 2C shows typical progress curves of the MKP3-catalyzed reaction in the absence and presence of ERK2. It can be seen from this figure that binding of ERK2 to MKP3 significantly increases the rate of MKP3-catalyzed p38α/pTpY dephosphorylation, whereas at the same time it still renders MKP3 incapable of dephosphorylating Thr(P) in p38α/pTpY. These results suggest that ERK2 and activated p38α are capable of binding to MKP3 simultaneously, and MKP3 undergoes catalytic activation toward other protein substrate (phosphorylated p38α) when bound to ERK2. The various phosphorylation states of p38α were also confirmed by Western blot analysis as described above (Fig. 2D).

Finally, we evaluated the effect of the unphosphorylated ERK2 on the MKP3-catalyzed dephosphorylation of ERK2/pTpY because ERK2 is a product of the MKP3 reaction and has high affinity for MKP3. The kinetics of the MKP3-catalyzed dephosphorylation of ERK2/pTpY were examined in the presence of various fixed concentrations of ERK2. The initial rates were measured as a function of ERK2/pTpY concentration keeping the ERK2 concentration constant (0–0.4 μM) (supplemental Fig. S3). Under these conditions, the double reciprocal plots yielded a simple competitive pattern with a Ki value of 0.103 ± 0.018 μM. Thus, ERK2 acts as a competitive inhibitor of the ERK2/pTpY dephosphorylation by MKP3.

Interactions of MKP3 with ERK2 and p38α in Vitro and in Vivo—Given that the dephosphorylation of p38α/pTpY by MKP3 can be regulated by ERK2, we devised an in vitro interaction assay employing size exclusion chromatography to test whether ERK2, MKP3, and p38α/pTpY could form a stable ternary complex in solution. First, we studied the interaction between only two of the three proteins. Due to the transient nature of the enzyme-substrate complex, it is difficult to study the binding interaction between wild-type MKP3 and bisphosphorylated p38α/pTpY (Fig. 3A). Consequently, we employed catalytically inactive MKP3C293S to capture the enzyme-substrate complex, as judged by comparing gel filtration profiles of the individual proteins with their equimolar mixture (Fig. 3B). When an equimolar mixture of p38α/pTpY and ERK2 was sized on gel filtration, only ERK2 showed a slight change in elution volume, indicative of an extremely weak interaction between those two proteins (panel 4). Both ERK2 and p38α/pTpY, but not p38α (data not shown), were able to associate with MKP3 to form a stable heterodimer (panels 5 and 6). To demonstrate the formation of ERK2-MKP3-p38α/pTpY heterotrimer in solution, we next incubated the three proteins in an equimolar ratio and performed gel filtration analysis. Gel filtration profiles showed that purified ERK2, MKP3, and p38α/pTpY co-migrated to earlier fractions than MKP3-p38α/pTpY and MKP3-ERK2 heterodimers (panel 7). The apparent molecular mass of the ERK2-MKP3-p38α/pTpY complex appeared to be ~140 kDa, as assessed from the elution volume of the protein complex peak compared with the protein standards, indicating that these three proteins formed a stable ternary complex in solution. For comparison purposes, we also examined if an ERK2-MKP3-ERK2/pTpY ternary complex could also be formed under the same experimental conditions. In the absence and presence of ERK2, MKP3-ERK2/pTpY binary complex eluted with a similar retention time, and no ERK2-MKP3-ERK2/pTpY ternary complex was observed (supplemental Fig. S4).

In order to study the effects of MKP3 in vivo, 293T cells overexpressing the phosphatases and p38α were stimulated with sorbitol to induce p38 phosphorylation and activation, followed by immunoprecipitation with the anti-FLAG antibody; the phosphorylation state of FLAG-p38α was detected by immunoblot using bisphosphorylated p38 and anti-phosphoTyr antibodies (Fig. 3C, first and second panels). The results showed that like HePTP and MKP5, MKP3 can effectively diminish the level of phosphotyrosine of FLAG-p38α in intact cells. We further examined the association of MKP3 with both p38α and immunoblot using anti-Myc antibody (Fig. 3C, third panel, lane 6 versus lane 7). Consistent with our in vitro data, wild-type MKP3 failed to bind to p38α, but the catalytically inactive MKP3 mutant C293S physically interacted with bisphosphorylated p38α and anti-phosphoTyr antibodies (Fig. 3C, first and second panels). The results showed that like HePTP and MKP5, MKP3 can effectively diminish the level of phosphotyrosine of FLAG-p38α in intact cells. Further, we examined the association of MKP3 with its substrate p38α by immunoblot using anti-Myc antibody (Fig. 3C, third panel, lane 6 versus lane 7). ERK2 can form a tight physical complex with MKP3 via the KIM motif in the N-terminal domain of MKP3 and the docking sites on ERK2. Thus, it would be of interest to know whether p38α binds to MKP3 through the same mechanism. To address this issue, we analyzed the interaction of ERK2 and p38α MAPKs with the N-terminal domain of MKP3 (residues 1–154) using gel filtration assays. It can be seen from Fig. 4, A–C, that in contrast to ERK2, neither the unphosphorylated nor the phosphorylated p38α was able to associate with MKP3(1–154) to form a stable heterodimer. To study the binding of the KIM sequence to ERK2 and p38α further, we synthesized peptide corresponding to the KIM sequences of MKP3 and measured the binding affinities of ERK2 and p38α with the KIM peptide by ITC. The KIM peptide binds to ERK2 with a dissociation
constant ($K_d$) of 65.8 ± 15.4 μM, but no detectable interaction between the KIM peptide and p38α was observed under the same conditions (Fig. 4, D–F). These results support the notion that the KIM sequence in MKP3 is not responsible for p38α/pTyr binding.

Kinetic Characterization of MKP3-catalyzed Reactions—To gain further insight into the mechanism of ERK2-induced MKP3 activation, we conducted a detailed kinetic study of MKP3-catalyzed reaction at pH 7.0 and 25 °C. The dephosphorylation of ERK2 and p38α/pTyr catalyzed by MKP3 obeyed classical Michaelis-Menten kinetics (supplemental Fig. S5A). We were able to determine the kinetic parameters, $k_{cat}$ and $K_m$, for MKP3 with both ERK2 and p38α as a substrate (Table 1). As shown in Table 1, the $k_{cat}/K_m$ value for the MKP3-catalyzed ERK2 dephosphorylation was determined to be $7.3 \times 10^6$ s$^{-1}$ M$^{-1}$, which is similar to those obtained from earlier studies (24). In comparison, the $k_{cat}$ of MKP3-catalyzed p38α/pTyr dephosphorylation is only 3-fold lower than that of ERK2/pTyr, whereas the $K_m$ value for p38α/pTyr substrate is about 8-fold higher than that for ERK2/pTyr. Binding of ERK2 to MKP3 results in a 3-fold increase in $k_{cat}$ for p38α/pTyr substrate with a modest effect on $K_m$. To quantitatively assess the contribution of the N-terminal domain to the MKP3-catalyzed p38α dephosphorylation, we determined the kinetic parameters of MKP3/ΔN151 toward p38α substrate (supplemental Fig. S5B). The $k_{cat}$ value for MKP3/ΔN151 was comparable with that of full-length MKP3. However, its $K_m$ value was more than 20-fold higher than that of full-length MKP3, suggesting that the major function of the MKP3 N-terminal domain is to increase the “effective concentration” of the pTXY motif in the vicinity of the

![FIGURE 3. Direct binding between p38α/pTyr and MKP3.](image-url)
active site of MKP3 for p38α/pTpY dephosphorylation. In contrast to the previous conclusion obtained with small molecule substrates (17, 35), our results suggest that the catalytic domain of MKP3 alone is in a catalytically active conformation, providing the first experimental evidence that substrate specificity of MKP3 can be independent of the capability of substrate-induced MKP3 activation.

Comparison with Other Tyrosine-specific Phosphatases—In addition to MKPs, biochemical and genetic studies indicated that distinct tyrosine-specific phosphatases (PTPases) are involved in MAPK inactivation. The hematopoietic protein-tyrosine phosphatase, HePTP, and its brain-specific homologs, STEP and PTP-SL, can associate with ERK2 and p38/H9251 through the N-terminal domain and inactivate the ERK2 and p38/H9251 MAPK pathways by dephosphorylation of Tyr(P) in the activation loop (36, 37). Fig. 5 shows the time courses of the p38/H9251/pTpY dephosphorylation by these phosphatases. Both HePTP and PTP-SL can effectively dephosphorylate Tyr(P) in p38/H9251/pTpY under the same conditions used for MKP3, whereas MKP3 can be independent of the capability of substrate-induced MKP3 activation.

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similar. The $k_{cat}/K_m$ values for PTP-SL and HePTP are greater than $10^6$ s$^{-1}$ M$^{-1}$ and represent the largest values measured for a protein phosphatase reaction. In contrast, the $k_{cat}$ value for the STEP-catalyzed p38α/pY dephosphorylation was about 20-fold lower than those for the PTP-SL and HePTP-catalyzed reactions. This result is consistent with the previous findings that HePTP and PTP-SL specifically down-regulate the p38α pathway, whereas STEP was unable to inactivate p38α in intact cells (37). With the monotyroline-phosphorylated p38α/pY as a substrate, the kinetic parameters for the p38α/pY dephosphorylation by MKP3 are only slightly different from the corresponding values of the p38α/pTpY dephosphorylation, indicating that dephosphorylation of the Tyr(P) residue by MKP3 does not require the presence of Thr(P) in p38α substrate. Unlike MKP3-catalyzed reactions, however, when the monophosphorylated p38α/pY was used as a substrate for PTP-SL and HePTP, both the $k_{cat}$ and $K_m$ values decreased about 3-fold compared with those of p38α/pTpY, indicating that the presence of Thr(P) had a significant effect on the kinetic properties of the p38α substrate for these two phosphatases. Interestingly, the kinetic parameters for the dephosphorylation of p38α by the MKP3-ERK2 complex are only 2–4-fold lower than those by the most active PTPase, PTP-SL, suggesting that phosphorylated p38α is a physiological substrate for MKP3 in cell signaling.

**Catalytic Activation of MKP3 toward p38α by ERK2**

**TABLE 2**

| Phosphatase | Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-------------|-----------|----------|-------|---------------|
| MKP3       | p38α/pY   | 0.094 ± 0.004 | 0.341 ± 0.033 | 0.27 ± 0.035 |
| MKP3 (+ ERK2) | p38α/pY | 0.084 ± 0.002 | 0.138 ± 0.012 | 0.6 ± 0.071 |
| PTP-SL     | p38α/pY   | 0.28 ± 0.028 | 0.516 ± 0.015 | 0.54 ± 0.07 |
| HePTP      | p38α/pY   | 0.284 ± 0.008 | 0.359 ± 0.037 | 0.79 ± 0.10 |
| STEP       | p38α/pY   | 0.536 ± 0.034 | 0.32 ± 0.03 | 1.67 ± 0.26 |
|            | p38α/pTpY | 1.28 ± 0.03 | 0.98 ± 0.08 | 1.3 ± 0.13 |

STEP was less active toward p38α/pTpY. To investigate whether binding of ERK2 can induce HePTP activation toward p38α/pTpY substrate, we examined the effect of the unphosphorylated ERK2 on the HePTP-catalyzed dephosphorylation of p38α/pTpY. As shown in Fig. 5B, double reciprocal plots displayed the characteristic intersecting line pattern indicative of competitive inhibition. Thus, in contrast to MKP3, ERK2 acts as competitive inhibitor of the p38α/pTpY dephosphorylation by HePTP.

Using the continuous spectrophotometric enzyme-coupled assay, we also quantitatively compared the catalytic efficiency of the MKP3-catalyzed p38α dephosphorylation with HePTP, PTP-SL, and STEP. All kinetic parameters are summarized in Table 2. As shown in this table, the kinetic parameters for the dephosphorylation of p38α by PTP-SL and HePTP were very similar.
the kinase domain exist in an open conformation, and the conformation of the activation loop is incompatible with substrate binding and/or catalysis (Fig. 6C). Phosphorylation of Thr\(^{180}\) and Tyr\(^{182}\) leads to a rearrangement of the lip (Fig. 6D). The phosphate moiety of Thr(P)\(^{180}\) forms 11 hydrogen bonds with four invariant arginines, including Arg\(^{167}\) and Arg\(^{170}\) in helix \(\alpha C\), Arg\(^{149}\) in the catalytic loop, and Arg\(^{173}\) in the activation lip. An additional water molecule-mediated interaction occurs between the phosphate moiety and Lys\(^{66}\) in helix \(\alpha C\). These interactions provide a link between helix \(\alpha C\) and lip promoting the reorientation of catalytic site residues and interlobe closure.

The lip refolding induces a 100° rotation of the side chain of Asp\(^{168}\) in the conserved DFG motif, which adopts a conformation almost identical with that of Asp\(^{184}\) in the active PKA (40). In contrast to Thr(P)\(^{180}\), the phosphate group of Tyr(P)\(^{182}\) makes only two hydrogen bonds to the side chain of His\(^{228}\),...
which is not conserved in ERK2 and JNKs. Upon phosphorylation, Tyr^{182} moves by $\sim$18.1 Å relative to its position in the inactive p38α structure and appears to be positioned to the p + 1 site. The conformational changes of the activation lip disrupt the turn of helix Val^{183}–Arg^{186} near the p + 1 site and relieve steric constraints to substrate binding, thereby helping to form the peptide substrate binding channel (Fig. 6D).

The ATP binding site is formed by the crossover connection, the flexible glycine-rich loop in the N-terminal domain, and the DFG motif in the C-terminal domain. The relative orientation of the two domains of inactive p38α is different from that observed in ERK2 and other kinases. The twisted arrangement of two domains results in misalignment of catalytic residues, such as residues Lys^{33} in the N-terminal domain and Asp^{168} from the C-terminal domain. Residues in the crossover connection (His^{107}–Asp^{112}) are shifted, on average $\sim$2.5 Å relative to ERK2, in toward the ATP binding site. As a consequence, the backbone of Met^{109} blocks the ATP binding site observed in ERK2 (Fig. 6E). In the structure of bisphosphorylated p38α, the crossover connection shifts away from the active site. Met^{109} occupies a new pocket formed in the $\beta_3$-$\beta_4$ reverse turns, and the active site residues are significantly displaced relative to their orientation in the unphosphorylated p38α structure (Fig. 6F). Thus, the crossover connection of p38α is optimally arranged for accommodating ATP during the kinase activation.

In addition, the network of interaction between Thr(P)^{180} and p38α has been two structural reports for the activated form of ERK2 and p38α. In p38α, Thr(P)^{180} and Tyr(P)^{182} in p38α are 2.5 and 3.1 Å away from the location of the corresponding residues in ERK2 and p38γ, respectively, and the X residues (Gly^{181} in p38α, Gly^{184} in p38γ, and Glu^{184} in ERK2) in the pTPγ motif exhibit the largest distance of 6.3 Å. The Gly^{181} in p38α forms hydrogen bonds with Arg^{149} from C-lobe, whereas Glu^{194} in ERK2 and Gly^{184} in p38γ do not hydrogen-bond directly with any other residue. Thus, in contrast to what had been suggested previously, our structure shows that the conformation of the activation lip in the high activity forms of MAPKs is not conserved.

From the biochemical studies given above, it is evident that the phosphotyrosine plays the critical role in p38α recognition by MKP3. To provide a structural explanation of how phosphorylation modulates the interactions between p38α and the MKP3 catalytic domain, first compared the electrostatic potential surfaces of unphosphorylated p38α with that of phosphorylated p38α and estimated the potential surfaces of phosphorylated p38α with which the MKP3 catalytic domain might interact. Surface representations of p38α versus p38α/pTPγ show that the conformational difference in the activation loop significantly alters the hydrophobic and electrostatic surface distribution (Figs. 7, C and D). Protein-protein association results from both hydrophobic and electrostatic/hydrogen binding interactions between interfaces composed of complementary nonpolar and charged/polar residues. A common type of interactive surface contains a hydrophobic patch surrounded by polar groups (42). Inspection of the phosphorylated p38α structure reveals one such potential protein binding site in the C-lobe, a hydrophobic cleft, formed by Thr^{185}, Arg^{186}, His^{228}, and Ile^{229} on one side and Val^{183}, phospho-Tyr^{182}, Met^{194}, and Leu^{195} on the other, that are surrounded by positively charged residues. In unphosphorylated p38α, this site is unformed and further occluded by an acidic patch formed by Asp^{176}, Asp^{177}, and Glu^{178}. Upon phosphorylation, the movement of the activation loop leads to $\sim$23 Å displacement of the acidic patch and exposes the binding site for MKP3 interactions. Additionally, notable shifts and side chain rotations are also observed in residues contributing to the hydrophobic cleft. For example, Tyr(P)^{182} and Val^{183} are shifted by more than 12 Å; Met^{194}, Leu^{195}, and Thr^{185} move by distances between 1.0 and 2.5 Å. This predicts that the activation state of p38α should influence MKP3 binding, by favoring stronger binding interactions; the phosphodonor (phospho-Tyr^{182}) and the hydrophobic cleft of p38α/pTPγ simultaneously contact MKP3.

MKP3 exhibits very high activity toward ERK2/pTPγ and p38α/pTPγ ($k_{cat}/K_m = 7.3 \times 10^6$ M$^{-1}$ s$^{-1}$ and 2.8 $\times 10^5$ M$^{-1}$ s$^{-1}$), as compared with that for the ERK2-derived phosphopeptide harboring the same pTPγ motif ($k_{cat}/K_m = 5$ M$^{-1}$ s$^{-1}$) (43). In contrast, MKP3 is unable to dephosphorylate and bind p38γ/pTPγ (Fig. 1D and supplemental Fig. S6), although the conformation of the phosphorylated activation loop of p38γ is very similar to that of ERK2 (28, 41). This strikingly high su-
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A

B

C

D

E

F

G

H

I

p38α/pTpY

ERK2/pTpY

p38β/pTpY

14 15 16 17 18 19

Elution Volume [mL]

14 15 16 17 18 19

Elution Volume [mL]

14 15 16 17 18 19

Elution Volume [mL]
substrate specificity displayed by MKP3 suggests that there is no structural complementarity between the activation loop of ERK2 and MKP3 and that structural features outside the immediate vicinity of the pTεpY site of ERK2 play an important role for the selective specificity of MKP3 for MAPKs. In addition to the common docking site, phosphorylated ERK2 has a second binding site utilized by transcription factor substrates and phosphatases, the hydrophobic DEF motif binding site formed by Thr<sup>188</sup>, Arg<sup>189</sup>, His<sup>230</sup>, Tyr<sup>231</sup>, and Leu<sup>232</sup> on one side; phospho-Tyr<sup>185</sup>, Val<sup>186</sup>, Met<sup>197</sup>, and Leu<sup>198</sup> on the other; and Asn<sup>260</sup> and Tyr<sup>261</sup> at the base (Fig. 7E) (44–47). Interactions between this site and MKP3 are essential for allosteric activation of MKP3 and formation of a productive complex. Interestingly, the DEF motif binding site in ERK2/pTpY is very similar to the hydrophobic cleft of p38α/pTpY proposed to mediate interactions with MKP3. By comparison, the corresponding region in p38γ/pTpY is occupied by the side chains of Asp<sup>230</sup>, His<sup>231</sup>, Leu<sup>232</sup>, Asp<sup>233</sup>, Asp<sup>256</sup>, Glu<sup>257</sup>, Asn<sup>260</sup>, and Tyr<sup>261</sup> (Fig. 7F).

Thus, the DEF motif interaction pocket in the active p38γ model is unavailable and therefore unceptive for MKP3 binding. In agreement with this notion, both ERK2/pTpY and p38α/pTpY, but not p38γ/pTpY, were able to associate with the catalytic domain of MKP3 (MKP3/ΔN151) to form a stable heterodimer (Fig. 7, G–I). The structural differences in the DEF motif binding sites and the region outside the immediate vicinity of the pTεpY site suggest one possible mechanism for encoding complex target specificity among MAPK isomers.

Further structural analyses show that the phospho-Tyr side chains in both ERK2/pTpY and p38α/pTpY structures are positioned similarly and are easily accessible to the active site of MKP3 (Fig. 7, B, D, and E). In contrast, the phospho-Thr residues of ERK2/pTpY and p38α/pTpY are in different positions and circumstances. Thr<sup>(P)</sup><sup>180</sup> of p38α/pTpY nestles more deeply into the N-lobe and is in the proximity of a negatively charged patch formed by Asp<sup>176</sup>, Asp<sup>177</sup>, and Glu<sup>178</sup>, whereas the corresponding region near Thr<sup>(P)</sup><sup>183</sup> in the ERK2/pTpY is largely hydrophobic (Fig. 7, D and E). Thus, the distinct position and circumstance of Thr<sup>(P)</sup><sup>180</sup> probably explain the lack of MKP3 activity toward the dephosphorylating phosphothreonine residue in p38α.

### DISCUSSION

The most commonly used approaches for studying the substrate specificity of MAPK phosphatases involve overexpression experiments followed by in vitro assay of kinase activity in the immunoprecipitate of cellular extracts. These kinds of experiments are indirect and may not provide definitive information about the identity of protein phosphatases involved in certain cellular functions if the kinase activity does not reliably reflect its phosphorylation state. Based on our previous studies, the monothreonine-phosphorylated p38α/pT is catalytically active, and dephosphorylation of phospho-Tyr<sup>182</sup> in the activation loop of p38α primarily increases its <i>K<sub>m</sub></i> for ATF2 (activating transcription factor 2) substrate, with only a slight decrease in <i>k<sub>cat</sub></i> (25). Consequently, the inhibition extent of p38α activity by tyrosine-specific phosphatase strongly depends on the concentration of ATF2 used in the assay system (supplemental Fig. S7).

Upon increasing the concentration of ATF2, the relative activity gradually increases and reaches a limiting value 0.8 at the high ATF2 concentration. Considering this fact, our result is also consistent with the in vivo experimental observations that MKP3, PTP-SL, and HePTP can only partially inhibit stress-induced p38 activation (10, 13, 36, 37).

MAPK signaling pathways are tightly regulated, and individual MAPKs exhibit exquisite specificity for their cognate regulators and substrates. All MAPK family members share a common phosphorylation site motif, raising questions as to how specific MAPK recognition by its binding partners is achieved. Previous studies (34, 47) suggested that ERK2 is capable of forming a complex with MKP3 through a bipartite mechanism. In this model, one part of MKP3 (e.g. the kinase interaction motif sequence) docks to the D-site located on the back side of the ERK2 active site for high affinity association, whereas the interaction of another structural element with the DEF motif binding site in ERK2 is primarily responsible for ERK2-induced MKP3 activation. In this study, we show that the phosphorylated p38α also contains a MKP3 docking pocket that is located below the active site. This pocket is occluded in unphosphorylated p38α; thus, the MKP3 preferentially binds to the dually phosphorylated, activated isoform of p38α. Based on the crystal structural studies, we propose that the specific interaction between the catalytic domain of MKP3 and the DEF motif docking pocket in a MAPK may ensure the precise orientation and positioning of the catalytic residues in the phosphatase with respect to the TXY motif in the activation lip for efficient dephosphorylation and therefore possibly correlates with MKP3 substrate specificity toward the members of the MAPK.

Using a variety of approaches, including enzyme kinetics, size exclusion chromatography, and cellular transfection studies, we have identified that phosphorylated p38α is an authentic in vitro and in vivo substrate of MKP3. MKP3 catalyzes the specific hydrolysis of phospho-Tyr<sup>182</sup> from activated p38α, resulting in partial inactivation of kinase activity. Together with published structural and biochemical data (35, 48), our experimental observations give rise to a working model for the MKP3-catalyzed p38α dephosphorylation in cells (supplemental Fig. S8). In this model, MKP3 can exist in two distinct conformational states: a low activity ERK2-independent form with a <i>k<sub>cat</sub></i> of 0.09 s<sup>–1</sup> and a high activity ERK2-dependent form with a <i>k<sub>cat</sub></i> of 0.28 s<sup>–1</sup>. Binding of ERK2 to the noncatalytic domain of MKP3 increases its catalytic activity toward the phosphorylated p38α substrate.
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MKP3 results in an optimum arrangement of active site residues with a concomitant 3-fold increase in the $k_{\text{cat}}$ value.

Mounting evidence suggests a cross-talk between the p38 and ERK MAPK pathways in different types of cells. For example, both pathways are simultaneously activated in melanoma, with a positive feedback loop from active ERK to p38 (49). We postulate that, upon activation of both ERK2 and p38 pathways, the active ERK2/pTpY may induce transcriptional activation of the MKP3 gene, and MKP3 in turn dephosphorylates/activates ERK2/pTpY. Unphosphorylated ERK2, the dephosphorylated product of ERK2/pTpY by MKP3, associates with MKP3, and thereby enhances the dephosphorylation of the phospho-Tyr residue in p38α/pTpY to down-regulate the p38α pathway. Therefore, the cross-talk between ERK2 and p38α mediated through MKP3 may play a role in coordinating regulation of these two distinct MAPK pathways, and the ERK2-induced allosteric activation may reflect an additional level of fine tuning of p38α MAPK signaling.

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