Mapping ERK2-MKP3 Binding Interfaces by Hydrogen/Deuterium Exchange Mass Spectrometry*

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ERK2, a prototypic member of the MAPK family, plays a central role in regulating cell growth and differentiation. MKP3, an ERK2-specific phosphatase, terminates ERK2 signaling. To understand the molecular basis of ERK2 recognition by MKP3, we carried out hydrogen/deuterium exchange mass spectrometry experiments to map the interaction surfaces between the two proteins. The results show that the exquisite specificity of MKP3 for ERK2 is governed by two distinctive protein-protein interactions. To increase the “effective concentration” of the interacting molecules, the kinase interaction motif in MKP3 (HRLKGNPVR) and an MKP3-specific segment (NSSDNWE) bind the common docking site in ERK2 defined by residues in L16, L5, β7-β8, and αδ-L8-αε, located opposite the kinase active site. In addition to this “tethering” effect, additional interactions between the 364FTAP367 sequence in MKP3 and the ERK2 substrate-binding site, formed by residues in the activation lip and the P+1 site (β9-αε loop), L13 (αζ-αε loop), and the MAPK insert (L14, α11.4-α21.4), are essential for allosteric activation of MKP3 and formation of a productive complex whereby the MKP3 catalytic site is correctly juxtaposed to carry out the dephosphorylation of phospho-Thr183/phospho-Tyr185 in ERK2. This bipartite protein-protein interaction model may be applicable to the recognition of other MAPKs by their cognate regulators and substrates.

Mitogen-activated protein kinase (MAPK)3 cascades are highly conserved signal transduction modules in eukaryotes that mediate the intracellular transmission and amplification of extracellular stimuli, leading to the induction of appropriate cellular responses to changes in the surrounding environment.

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† Both authors contributed equally to this work.

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§ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK2, extracellular signal-regulated protein kinase 2; MKP3, MAPK phosphatase 3; H/D X-MS, hydrogen/deuterium exchange mass spectrometry; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; MS/MS, tandem mass spectrometry; KIM, kinase interaction motif; SB, substrate-binding; PTP, protein-tyrosine phosphatase; CD, common docking.

(1, 2). The MAPKs are compact enzymes lacking recognizable protein-protein interaction modules normally found in signaling molecules (3, 4), yet they are highly specific in their interactions with substrates, activating kinases, or inactivating phosphatases. Although the importance of MAPKs in cellular signaling is well established, there is limited understanding of the molecular basis for MAPK recognition by its activators, inactivators, and substrates. Such knowledge is essential for comprehension of the ability of MAPKs to integrate diverse biological stimuli and to transmit signals to the nucleus to generate appropriate cellular responses.

Extracellular signal-regulated protein kinase 2 (ERK2), the prototypic member of the MAPK family, has been the subject of intense study. ERK2 is activated by phosphorylation of Thr183 and Tyr185 in the activation loop by the dual specificity MAPK/ERK kinase MEK1. Deactivation of ERK2 activity is carried out by MAPK phosphatase 3 (MKP3) (5, 6). Genetic analyses show that MKP3 plays an important role in modulating a number of ERK-mediated embryonic developmental processes, including oogenesis and wing formation in Drosophila and neural patterning in Xenopus (7, 8). Additional studies have indicated that MKP3 is responsible for the negative feedback regulation of fibroblast growth factor–induced ERK activation in developing limbs, neural plate, and somites in chicken embryos (9–11).

MKP3 forms a physical complex with ERK2 and is highly specific for ERK2 dephosphorylation with a kcat/Km that is 106-fold higher than that for the hydrolysis of p-nitrophenyl phosphate or the bisphosphorylated peptide derived from the ERK2 activation lip (12, 13). Part of the specificity comes from docking interactions between the N-terminal domain of MKP3 and the non-catalytic regions of ERK2 (12, 14, 15). Interestingly, ERK2 can also stimulate the phosphatase activity of MKP3 (16). Biochemical and structural evidence suggests that ERK2 binding elicits allosteric activation of MKP3, resulting in optimum alignment of the general acid and other active-site residues in MKP3 with respect to the substrate for efficient catalysis (12, 17–20). Thus, MKP3 substrate specificity is linked to the ability of the substrate to induce productive orientation in the active site. This provides a powerful mechanism to ensure high fidelity in MKP3-mediated ERK2 inactivation. In this mechanism, MKP3 exists in latent inactive states, and upon association with ERK2, MKP3 is activated, leading to selective inactivation of ERK2.

The structural basis for the exquisite specificity of ERK2 dephosphorylation by MKP3 has not been elucidated. In fact, it has been notoriously difficult to obtain co-crystals of ERK2 in complex with any of its interacting proteins. To better under-
stand the molecular basis of specific ERK2 recognition by MKP3, we have employed hydrogen/deuterium exchange mass spectrometry (H/DX-MS) to map the interaction surfaces between the two proteins concomitant with complex formation. By combining the information gained from the H/DX-MS experiments and direct biochemical analyses, coupled with molecular modeling, we were able to define the structural elements in both MKP3 and ERK2 that are important for specific recognition. The results also yield a structural model for understanding how efficient and precise docking interactions between ERK2 and its cognate substrates and regulators can be achieved.

MATERIALS AND METHODS

Protein Expression and Purification—N-terminally His_6-tagged ERK2 was expressed in Escherichia coli and purified following a published procedure (21). The expression and purification of ERK2/pTpY (ERK2 phosphorylated at both Thr^{183} and Tyr^{185}) were carried out as described (21). Catalytically inactive MKP3 (MKP3/C293S) with a C-terminal His_6 tag and glutathione S-transferase (GST)-MKP3/C293S were expressed in E. coli and purified as described previously (14, 17).

Site-directed Mutagenesis of MKP3 and ERK2—Mutant ERK2 and MKP3 were generated by PCR according to the standard procedure of the QuikChange™ site-directed mutagenesis kit (Stratagene) using pET15b-His_{6}-ERK2 and pET21a-MKP3-His_{6} as templates, respectively. All mutants were verified by DNA sequencing.

GST Pulldown Analysis—The binding affinity of GST-MKP3/C293S for ERK2 or ERK2/pTpY was determined by GST pulldown and Western blot analyses. Five μg of GST-MKP3/C293S in 0.5 ml of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2 mM dithiothreitol, pH 7.3) was immobilized on 20 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) by gentle agitation at 4 °C for 2 h. Different concentrations of ERK2 or ERK2/pTpY in 200 μl of phosphate-buffered saline containing 0.5% Triton X-100 were incubated with 20 μl of GST-MKP3/C293S-bound beads with gentle agitation at 4 °C for 2 h. After washing the beads once with phosphate-buffered saline containing 0.5% Triton X-100 and three more washes with phosphate-buffered saline, 20 μl of 2× SDS sample buffer was added to the beads. GST-MKP3/C293S and bound ERK2 (or ERK2/pTpY) were released by boiling the beads for 5 min at 95 °C. The sample was centrifuged at 10,000 rpm for 2 min. The supernatant (15 μl) was loaded onto 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins on the gel were transferred at 25 mA to nitrocellulose membrane overnight at 4 °C. Rabbit anti-phospho-p44/42 MAPK antibody (catalog no. 9101, Cell Signaling Technology, Inc.) and rabbit anti-p44/42 MAPK antibody (catalog no. 9102, Cell Signaling Technology, Inc.) were used as primary antibodies to detected phospho-ERK2 and ERK2, respectively, on the membrane.

H/DX-MS—Hydrogen/deuterium exchange in MKP3/C293S and ERK2/pTpY, either alone or in complex, was initiated by diluting 1 μl of the stock solution (200 μM) to 19 μl of the D_2O buffer (20 mM Tris and 100 mM NaCl, pH 7.5) on ice. The lower temperature was chosen because exchange was too rapid to be monitored at room temperature by manual quenching. The final protein concentration was 10 μM. The resulting hydrogen/deuterium exchange solutions were maintained at 0 °C by leaving the sample on ice and allowed for exchange for different periods of time. At appropriate intervals, the hydrogen/deuterium exchange reaction was quenched by the addition of an equal volume of cold 0.5 M phosphate buffer, pH 2.5. One μl of the deuterated protein solution was loaded immediately onto a Vydac C_{18} column (1.0 × 50 mm) and analyzed by mass spectrometry for global exchange. Changes in deuterium incorporation within specific regions of proteins can be detected by peptidic digestion of the deuterium-labeled proteins, followed by HPLC separation and mass spectrometric analysis. To analyze hydrogen/deuterium exchange in segments of MKP3/C293S and ERK2/pTpY proteins, the hydrogen/deuterium exchange reaction was quenched by the addition of an equal volume of cold 0.5 M phosphate buffer, pH 2.5, in the presence of 20 μM pepsin. Immediately, 5 μl of the sample was injected into the sample loop, where pepsin digestion proceeded at 0 °C for 4 min. The pepsin digests were subsequently separated on a Vydac C_{18} column (1.0 × 50 mm).

A Shimadzu HPLC system equipped with two LC-10ADVP pumps was used to generate an acetonitrile gradient. Solvent A was 94.9% H_2O containing 5% acetonitrile and 0.1% formic acid, and Solvent B was 95% acetonitrile containing 4.9% H_2O and 0.1% formic acid. A low volume static mixing tee was used to minimize the delay period for HPLC. The solvent pre-cooling, static mixing tee, Rhodyne injector, and column were immersed in an ice bath (0 °C) to minimize back-exchange with HPLC solvents. After being desalted at 5% solvent B for 5 min, the protein eluted with a 2-min 30–50% solvent B gradient, and the peptide peptides eluted with a 0.5–5 min 5–15% solvent B gradient, followed by an 8 min 15–45% solvent B gradient. The column eluent (50 μl/min) was delivered directly to a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer for mass analyses of the deuterated protein and its peptic fragments. MagTran Version 1.0 software (written by Dr. Zhongqi Zhang) was used to determine the centroid value for each peak in the list processed from the mass spectrum. The sequences of the peptide fragments obtained after pepsin digestion were identified by tandem mass spectrometry (MS/MS). The total ion intensity of the peptides was detected in the m/z range of 400–2000. Mass scanning was followed by collision-induced dissociation to acquire a tandem mass spectrum within a peak during the HPLC run. Tandem mass spectra were interpreted by searching peptide data bases using the SEQUEST algorithm.

The extent of hydrogen/deuterium exchange was calculated using Equation 1,

\[ D = \frac{m - m_{100\%}}{m_{100\%} - m_{0\%}} \times H \]  

(Eq. 1)

where \( m \) is the measured mass of the deuterated protein or peptide; \( m_{100\%} \) is the measured mass of a “zero-deuteration” control prepared by adding 1 μl of 200 μM protein solution to a 19:20 (v/v) mixture of deuterated buffer and quenching buffer; \( m_{0\%} \) is the measured mass of a “full-deuteration” control pre-
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RESULTS AND DISCUSSION

H/DX-MS has emerged in recent years as a powerful tool for mapping protein-ligand and protein-protein interfaces as well as identifying conformational and dynamic perturbations in proteins (27). In this study, we sought to define the ERK2/pTpY-MKP3 binding sites by determining the changes in solvent accessibility of the protein backbone amides as a result of complex formation between the full-length proteins in solution. However, given the transient nature of the enzyme-substrate complex, it is difficult to

study the binding interaction between wild-type MKP3 and doubly phosphorylated ERK2/pTpY. Fortunately, catalysis by the protein-tyrosine phosphatase (PTP) superfamily requires an essential nucleophilic Cys (28), and the catalytically inactive Cys-to-Ser mutant (e.g. Cys293 in MKP3) retains the ability to bind substrates, but is unable to carry out substrate dephosphorylation. In fact, the Cys-to-Ser mutant binds substrates/ligands with the same affinity as the wild-type enzyme (29–31). Consequently, we employed catalytically inactive MKP3/C293S to capture the enzyme-substrate complex and to prevent the hydrolysis of ERK2/pTpY during H/DX-MS analysis.

Binding Affinity of MKP3/C293S for ERK2/pTpY—The affinity of MKP3/C293S for ERK2 ($K_d = 0.13 \pm 0.01 \mu M$) is similar to that of wild-type MKP3 ($K_d = 0.17 \pm 0.04 \mu M$) (14). In addition, GST-MKP3 and MKP3 display identical affinity for ERK2 (17). To determine the $K_d$ for MKP3/C293S and ERK2/pTpY, we carried out GST-MKP3/C293S pulldown experiments to measure ERK2/pTpY binding directly. As a control, we also performed GST-MKP3/C293S pulldown of unphosphorylated ERK2. The amount of ERK2 or ERK2/pTpY associated with the protein-tyrosine phosphatase (PTP) superfamily requires an essential nucleophilic Cys (28), and the catalytically inactive Cys-to-Ser mutant (e.g. Cys293 in MKP3) retains the ability to bind substrates, but is unable to carry out substrate dephosphorylation. In fact, the Cys-to-Ser mutant binds substrates/ligands with the same affinity as the wild-type enzyme (29–31). Consequently, we employed catalytically inactive MKP3/C293S to capture the enzyme-substrate complex and to prevent the hydrolysis of ERK2/pTpY during H/DX-MS analysis.

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and ERK2/pTpY should enhance our understanding of ERK2 recognition by MKP3.

Global Hydrogen/Deuterium Exchange in Intact MKP3/C293S and ERK2/pTpY—In a typical H/DX-MS experiment, MKP3/C293S and ERK2/pTpY, either alone or in complex, were incubated in D₂O to allow exchange of protons with solvent deuterium, and mass spectrometry was used to monitor the in-exchange rates. The final concentration for both proteins was 10 μM. Given the Kᵣ (31 nM) for complex formation, 95% of MKP3/C293S and ERK2/pTpY should be in the bound state under our experimental conditions. C-terminally His₆-tagged MKP3/C293S contains 387 amino acids (43,117.5 Da) with 365 exchangeable amide hydrogens, whereas N-terminally His₆-tagged ERK2/pYpT has 364 residues (42,328.0 Da) with 343 exchangeable amide hydrogens. Fig. 2 shows the total deuterium incorporation into MKP3/C293S and ERK2/pTpY in either the free or bound form. Within 40 min, a total of 190 amide hydrogens in MKP3/C293S were replaced with deuterium in the absence of ERK2/pTpY, whereas 136 of the MKP3/C293S amide protons were exchanged with deuterium in the presence of ERK2/pTpY, indicating that 54 amide protons in MKP3/C293S were protected from deuterium exchange upon complex formation. Compared with ERK2/pTpY alone, 30 of the ERK2 amide protons were protected from exchange in the ERK2/pTpY-MKP3/C293S complex. These results indicate that an overall decrease in solvent accessibility occurred in MKP3/C293S and ERK2/pTpY as a result of protein-protein interaction. By determining the changes in solvent accessibility of MKP3/C293S and ERK2/pTpY resulting from complex formation, one can identify the ERK2-MKP3 binding interfaces and the structural changes that accompany the complex formation.

Changes in Solvent Accessibility in Specific Segments of MKP3/C293S and ERK2/pTpY upon Complex Formation—H/DX-MS combined with peptidic mapping permits segment-specific identification of solvent-accessible exchange sites in proteins. The principle for this approach is that ligand binding or protein-protein interaction perturbs protein structure and thus solvent accessibility of the contact regions. The binding interfaces can be localized by comparing the rates of hydrogen/deuterium exchange on proteins in bound and unbound states. To identify the segments of MKP3/C293S and ERK2/pTpY that displayed altered solvent accessibility, deuterium uptake in MKP3/C293S and ERK2/pTpY was locked in place at various times by rapidly lowering the pH and temperature; the proteins were digested with pepsin; and the resulting peptides were separated by HPLC and analyzed by electrospray ionization mass spectrometry. This measurement reports time-dependent changes in weighted average peptide masses, which yields rates of hydrogen/deuterium exchange within different regions of the protein (27). Overall, 49 peptides (including those with overlapping sequences), covering 92% of the MKP3 amino acid sequence, were identified by MS/MS as shown by the coverage map (Fig. 3). Of the 49 peptides, 30 displayed reduced exchange rates, 2 exhibited an increase in exchange rates, and 17 had no change in deuterium incorporation upon binding to ERK2/pTpY. For ERK2/pTpY, 47 peptides were identified, effectively covering 92.5% of the primary structure (Fig. 4). Upon complex formation with MKP3/C293S, there were 22 peptides displaying a decrease in deuterium incorporation, 22 showing no change, and 3 exhibiting an increase in deuterium incorporation.

To directly visualize the hydrogen/deuterium exchange results, we depicted changes in solvent accessibility in MKP3 upon complex formation by color-coding segments of the solution NMR structure of the N-terminal domain (residues 1–154) (20) and the x-ray crystal structure of the C-terminal catalytic domain (residues 204–347) (18) of MKP3 (Fig. 3C). Indicated are peptides observed by H/DX-MS that exhibited a decrease (red), an increase (green), or no change (blue) in hydrogen/deuterium exchange rates upon complex formation. Peptides in black were not identified in the MS/MS experiments. As shown in Fig. 3, ERK2/pTpY-bound MKP3 displayed reduced solvent accessibility within Val¹⁹⁶-Leu²⁰⁶ (N-β₁-α₃), Tyr²¹⁴-Leu¹¹⁵ (N-β₄-α₅), Ser²³⁹-Phe²⁴⁶, Tyr²¹⁵-Phe²⁷⁸ (C-β₂-α₂-β₁-β₅-β₆-3₁₀-α₄), Val²⁹¹-Thr³⁰⁴ (C-β₂-α₅), Met³⁰⁹-Phe³⁴³ (C-α₃-α₆-α₇), and Tyr³⁶¹–Asn³⁶⁹. Interestingly, increased hydrogen/deuterium exchange rates were observed within Thr³⁸⁷–Leu³⁸⁷ (N-α₃-β₂) and Gln³⁴₀–Phe³⁴₃ (N-α₅-β₄).
Changes in solvent accessibility in ERK2/pTpY upon complex formation were depicted by color-coding segments of the x-ray crystal structure of ERK2/pTpY (25). As shown in Fig. 4C, MKP3/C293S-bound ERK2/pTpY exhibited reduced solvent accessibility (red) within Val12–Lys53 (β12-L1-β1-β2-L2-β3), Met106–Tyr126 (α4-L8-α5), Ala156–Leu161 (β7-β8), Phe166–Leu198 (β5-L12), Leu220–Glu248 (α13-L14-α8-L14), Ile263–Leu262 (α21,14), and Leu292–Glu324 (L15-α8-L16) compared with free ERK2/pTpY. Increased solvent accessibility was observed for Asn199–Gly213 (green).

Identification of the ERK2/pTpY-binding Sites in MKP3—The H/DX-MS technique cannot differentiate between changes in solvent accessibility as a result of direct binding and those due to conformational or dynamic perturbations. Thus, peptide segments showing altered hydrogen/deuterium exchange after complex formation could be involved in either ERK2-MKP3 binding or conformational/dynamic changes. Additional biochemical experiments in combination with the H/DX-MS data were required to identify the ERK2-binding sites in MKP3. To this end, we had suggested previously that KIM (RRLQKGNLPVRAL76) in MKP3 plays a major role in high affinity ERK2 binding (14). To provide further evidence that KIM is directly involved in ERK2 binding, we performed GST pull-down assays. Purified His6-tagged MKP3/C293S proteins were incubated with GST-ERK2/pTpY immobilized on glutathione-Sepharose beads. As expected, replacement of Arg65 with Ala (MKP3/R65A) completely disrupted ERK2 binding (Fig. 5). Finally, the crystal structure of ERK2 in complex with the KIM peptide from MKP3 provides a direct visualization of the docking interactions between KIM and ERK2 (22). Together, these findings are fully consistent with the strong solvent protection observed in Val56–Leu76 (N-β3-α3) upon ERK2-MKP3 complex formation (Fig. 3).

Aside from KIM, no other regions in the MKP3 N-terminal domain have been implicated previously in ERK2 binding. The H/DX-MS experiments revealed that, in addition to KIM, the Tyr98–Leu115 (N-β4-α4) segment also displayed a significant decrease in deuterium incorporation, whereas the Thr87–Leu97 (N-α3-β4) and Gln140–Phe143 (N-α5-β5) segments showed increased deuterium uptake in the complex (Fig. 3). To evaluate their potential involvement in ERK2 binding, a number of residues in these regions were mutagenized, and the effect on ERK2 binding was determined by the GST pulldown assay. As shown in Fig. 5, substitution of Leu97, Asn106, and Gln142 with Ala abrogated the
ability of MKP3/C293S to bind GST-ERK2/pTpY, suggesting that these segments in the N-terminal domain of MKP3 may also be important for ERK2 binding.

The existing MKP3 three-dimensional structures do not include residues 155–203 between the N- and C-terminal domains. A previous study suggested that residues 161–177 might contribute to ERK2 binding (14). These residues may also serve as a nuclear export signal for cytoplasmic retention of ERK2 (32). Furthermore, ERK2 can phosphorylate two residues in this region, Ser159 and Ser197, promoting MKP3 degradation by the proteasomal pathway (33). Although no change in solvent accessibility was detected for residues 151–193 upon complex formation, a decrease in deuterium uptake was observed for residues 194–206 (Fig. 3). Surprisingly, replacement of Asp195 or Leu199 with Ala had no significant effect on MKP3/C293S binding to ERK2/pTpY (Fig. 5). Thus, based on the present data, it is not clear whether this region is directly involved in ERK2 binding.

The C-terminal domain of MKP3 is the catalytic domain, which adopts a typical PTP fold (Fig. 3C) (18). The active site is situated within a cleft formed by the PTP loop between C-H295 and C-H295 (291VHCLAGISRS300). In the catalytic mechanism, Cys293 functions as a nucleophile, whereas Arg299 coordinates the substrate phosphoryl group. Substrate turnover is facilitated by a general acid (Asp262 in MKP3) located in a flexible surface loop (the WpD loop, C-H267-310, residues 257–268) in the vicinity of the active site. However, unlike the PTPs, MKP3 exhibits an extremely low basal activity for p-nitrophenyl phosphate (17), and the three-dimensional structures of the catalytic domain of MKP3 show that Cys293 and Arg299 coordinates the substrate phosphoryl group. Substrate turnover is facilitated by a general acid (Asp262 in MKP3) located in a flexible surface loop (the WpD loop, C-β7-310, residues 257–268) in the vicinity of the active site. However, unlike the PTPs, MKP3 exhibits an extremely low basal activity for p-nitrophenyl phosphate (17), and the three-dimensional structures of the catalytic domain of MKP3 show that Cys293 and Arg299 are misaligned and that the WpD loop is in the open conformation such that the general acid Asp262 is positioned away from the active site (18, 20). Interestingly, the MKP3 phosphatase activity can be

FIGURE 4. H/DX-MS analysis of ERK2/pTpY. A, sequence coverage map of ERK2, with secondary structures as described previously (35), showing 47 identified peptides in the MS/MS experiments. Indicated are peptides observed by H/DX-MS that exhibited a decrease (red), an increase (green), or no change (blue) in hydrogen/deuterium exchange rates upon complex formation. Peptides in black were not identified in the MS/MS experiments. B, time courses of deuterium uptake for a number of ERK2/pTpY peptides that underwent significant changes in hydrogen/deuterium exchange rates between the free (C) and MKP3/C293S-bound (F) states. C, structural representation of ERK2/pTpY (25) depicted with the same color scheme described for A.

FIGURE 5. Direct binding between MKP3/C293S and GST-ERK2/pTpY. His6-tagged MKP3/C293 proteins were incubated with GST-ERK2/pTpY bound to glutathione-Sepharose 4B resins. WT, wild-type.
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substantially increased by the presence of ERK2 (16). Kinetic evidence suggests that ERK2 binding elicits MKP3 activation by facilitating the repositioning of active-site residues (Cys253 and Arg299) and WpD loop closure, bringing the carboxyl group of Asp262 close to the leaving group oxygen of a substrate (12, 17, 19).

As shown in Fig. 3, binding of ERK2/pTpY reduces the hydrogen/deuterium exchange rates throughout the MKP3 catalytic domain, including both the PTP and WpD loops. Strong solvent protection in the PTP and WpD loops is consistent with the expectation that the main chain amides and the Arg299 side chain in the PTP loop make numerous hydrogen bonds with the bound phosphoryl oxygens and that the WpD loop should adopt the closed conformation upon substrate binding (28). However, it is unlikely that the decrease in hydrogen/deuterium exchange over the vast majority of the C-terminal domain is due to direct contact with ERK2/pTpY. To determine whether any of the segments displaying reduced deuterium incorporation are important for ERK2 binding, a number of surface-exposed residues in these regions were changed to Ala, and the effect on ERK2 binding was assessed with the GST pulldown assay. Substitution at Asp262 in C-α7, Tyr235 in C-β3, Asp262 and Trp264 in the WpD loop (C-β1–2-3), Glu274 in C-α3, Asn329, Ser331, and Phe334 in the C-α4–α5 loop; and Leu341 in C-α5 had little effect on MKP3/C293S binding to ERK2/pTpY (Fig. 5). The results suggest that these residues are not directly involved in ERK2 binding. On the other hand, elimination of the side chains at Tyr215 in C-β1–2-3, Leu246 in C-β1–2-3, and Tyr255 in C-β2 significantly reduced the binding affinity of MKP3/C293S for ERK2/pTpY (Fig. 5), indicating that these residues may play a role in ERK2 binding. Thus, ERK2/pTpY binding may not only lead to closure of the WpD loop, but also tighten up much of the structures stabilizing the PTP loop, resulting in an overall decrease in protein dynamics and flexibility in the MKP3 catalytic domain. This is consistent with the notion that an ERK2-induced conformational reorganization in the active site is required for MKP3 to achieve full activity (17–19).

No structural information is available for the C-terminal tail of MKP3 (residues 348–381). The only segment in this region that displayed reduced deuterium incorporation upon ERK2 binding encompasses residues 363–378 (Fig. 3B). Interestingly, this segment contains a putative ERK2-docking motif (364FTAP367), which is also found in many ERK2 substrates (34). Similar FXFP sequences can also be found in a number of MKPs that are capable of inactivating ERK2 (14). Although deletion or mutation of 364FTAP367 reduces the affinity of MKP3 for ERK2 by <10-fold, this region is absolutely required for ERK2-induced MKP3 activation (14). Consistent with this finding, we found that conversion of the 364FTAP367 sequence to AAAA (FTAP/A4) completely abolished the association of MKP3/C293S with ERK2/pTpY (Fig. 5). Together, these results provide strong evidence that the FXFP peptide is directly involved in ERK2 binding and possibly in ERK2-induced MKP3 activation.

Identification of the MKP3-binding Sites in ERK2/pTpY—

The ERK2 structure includes an N-terminal lobe (β1–β3 and α3) responsible for ATP binding and a larger C-terminal lobe (α7–α9 and β11–β12) involved in protein substrate recognition (25, 35). The catalytic cleft is situated between the two lobes, which mediates the phosphoryl transfer reaction (Fig. 4C). Phosphorylation of Thr183 and Tyr185 in the activation lip (β11–α9) is important for ERK2 kinase activity and substrate recognition. Phosphorylated ERK2 is a substrate of MKP3, and MKP3 is capable of dephosphorylating both phospho-Thr183 and phospho-Tyr185 (12). A common docking domain in ERK2 (residues 311–324 in L16), decorated with several acidic residues (e.g. Asp316 and Asp319), was proposed to interact with the positively charged residues in KIMs (36).

On the basis of the ability of ERK2 to induce MKP3 activation, we developed a biochemical assay that provides a quantitative assessment of the importance of structural features in ERK2 for MKP3 recognition in terms of both the MKP3 binding affinity and the propensity to activate MKP3 (14). Our previous mutational studies suggested that MKP3 binding and activation likely involve two distinct surface areas in ERK2 (15). One area, termed the common docking (CD) site, is situated opposite the kinase catalytic cleft and includes Glu79, Tyr126, Arg131, Asp160, Tyr314, Asp316, and Asp319. The CD site is important for high affinity MKP3 binding, but is not essential for ERK2-induced MKP3 activation. MKP3 activation appears to require Arg189, Trp190, Glu218, Arg223, Lys229, and His236 in the putative ERK2 substrate binding (SB) site, located distal to the CD site.

The crystal structure of ERK2 bound to the KIM peptide from MKP3 reveals that the KIM peptide binds to a contiguous surface area defined by the CD site, which is nested in the C-terminal domain between L16, L15, α7, β7–β9, α8, and α9 (22). This KIM-docking site consists of a highly acidic patch (Glu79, Tyr126, Asp160, Asp316, and Asp319) in L16, β7, and α8 and a hydrophobic groove (Thr108, Leu110, Leu113, Leu119, Phe127, and Leu155 in α9, β7–β9, and α9), which engage the basic (Arg44 and Arg65) and hydrophobic (Leu71-Pro72-Val73) residues, respectively, in the KIM sequence.

In agreement with the x-ray structure of ERK2-KIM complex and mutational studies, strong protection from hydrogen/deuterium exchange was observed in Met106–Tyr126 (α9–L8–α9), Asn156–Leu161 (β7–β9), and Leu306–Glu324 (α1–L16) upon MKP3/C293S binding (Fig. 4), regions that are known to directly interact with the KIM sequence from MKP3. Decreased deuterium incorporation in these regions was also detected in ERK2/pTpY upon binding the KIM peptide from the transcription factor Elk1 (residues 311–327), a substrate for ERK2 (26). In addition to these peptides, Leu292–Glu315 (L7–L9–α7) also showed a decrease in deuterium uptake upon complex formation. Substitution of Arg299 with Ala reduced the binding affinity of ERK2 for MKP3 by 24-fold (Table 1), suggesting that this region may also be important for MKP3 binding.

Collectively, our biochemical and structural data have defined the CD site for KIM, which is situated on a surface of the ERK2 C-terminal lobe between α9, α9, L16, L16, and the β7–β9 reverse turn. Additional studies indicate that the KIMs from various MAPK substrates and regulators occupy the same docking site in all MAPKs (22, 26, 37–39). This begs the question, how is specificity maintained by individual MAPK-interacting proteins to ensure signaling fidelity? Although differences in the amino acid composition of the CD sites can
partially explain binding selectivity, it is unlikely that the CD site interactions alone can account for the observed pathway specificity in MAPK signaling. The interaction of the ERK2 SB site with other elements in MKP3 may provide additional contacts crucial for MKP3 phosphatase activity and/or specificity (12, 14, 15).

Strong solvent protection was observed in Phe<sup>166</sup>–Leu<sup>198</sup> (β<sub>b</sub>–β<sub>2</sub>–L<sub>12</sub>), Leu<sup>220</sup>–Glu<sup>248</sup> (α<sub>f</sub>–L<sub>13</sub>–α<sub>g</sub>–L<sub>14</sub>–α<sub>11</sub>), and Ile<sup>253</sup>–Leu<sup>262</sup> (α<sub>21,14</sub>) in complex compared with free ERK2/pTpY (Fig. 4). These regions include the phosphorylation sites and the adjacent P+1 site in the activation lip, residues in L<sub>13</sub>–α<sub>g</sub> and the MAPK insert (residues 241–279), all of which have been implicated previously in MAPK substrate binding (15, 25, 35, 40). In addition, interactions between the MKP3 active site and phospho-Thr<sup>183</sup>/phospho-Tyr<sup>185</sup> in ERK2 should limit the solvent accessibility and therefore reduce the rate of hydrogen/deuterium exchange in the ERK2 activation lip. Indeed, within these peptides displaying reduced hydrogen/deuterium exchange, Thr<sup>179</sup>, Arg<sup>189</sup>, Trp<sup>190</sup>, Glu<sup>18</sup>, Arg<sup>23</sup>, Lys<sup>229</sup>, and His<sup>230</sup> have been shown previously to be important for MKP3 binding, ERK2-induced activation, or both (15). To assess the involvement of α<sub>21,14</sub> in the MAPK insert in MKP3 binding, we replaced Lys<sup>257</sup> and Tyr<sup>261</sup> with Ala. No significant effect was observed for ERK2/K257A. In contrast, although ERK2/Y261A displayed the same affinity for MKP3 as wild-type ERK2, it was able to activate MKP3 only to 15% of the maximum activity even at saturating ERK2/Y261A concentrations (Table 1). Thus, the biochemical and hydrogen/deuterium exchange data together suggest that residues in Phe<sup>166</sup>–Leu<sup>198</sup> (β<sub>b</sub>–β<sub>2</sub>–L<sub>12</sub>), Leu<sup>220</sup>–Glu<sup>248</sup> (α<sub>f</sub>–L<sub>13</sub>–α<sub>g</sub>–L<sub>14</sub>–α<sub>11</sub>), and Ile<sup>253</sup>–Leu<sup>262</sup> (α<sub>21,14</sub>) provide additional contacts with MKP3. Interestingly, these peptides (residues 169–213, 220–248, and 257–262) also exhibit decreased hydrogen/deuterium exchange in the presence of an FXF-containing peptide from Elk1 (991 AKLS-FQFPS<sup>209</sup>) (26). This further supports the conclusion that the ERK2 SB site is important for MKP3 recognition.

Val<sup>12</sup>–Lys<sup>53</sup> (spanning β<sub>2</sub>–L<sub>1</sub>–β<sub>1</sub>–L<sub>2</sub>–β<sub>2</sub>–L<sub>3</sub>) compose the third region in ERK2/pTpY in which a decrease in deuterium incorporation upon complex formation with MKP3/C293S was observed (Fig. 4). This region includes the ATP-binding site and is adjacent to the activation lip in the three-dimensional structure. However, substitution of a number of residues in this region, including Val<sup>19</sup>, Thr<sup>25</sup>, Tyr<sup>34</sup>, and Lys<sup>36</sup>, did not significantly affect the ability of ERK2 either to bind MKP3 or to induce MKP3 activation (Table 1). Thus, residues in the N-terminal β-strands may not be in direct contact with MKP3, and the observed protection from hydrogen/deuterium exchange in this region is likely the result of a decrease in backbone dynamics and/or flexibility. The last region of altered hydrogen/deuterium exchange in ERK2/pTpY corresponds to Asn<sup>199</sup>–Gly<sup>213</sup> (L<sub>12</sub>–α<sub>4</sub>), which immediately follows the activation lip (Fig. 4). Because removal of the side chain at either Lys<sup>201</sup> or Lys<sup>205</sup> had little effect on MKP3 recognition (Table 1), the increase in hydrogen/deuterium exchange in Asn<sup>199</sup>–Gly<sup>213</sup> likely reports conformational/dynamic changes within L<sub>12</sub>–α<sub>4</sub> upon MKP3 binding.

**Structural Model for ERK2/pTpY-MKP3 Recognition**—To define the precise interaction surfaces and to identify residues that are directly involved in ERK2/pTpY recognition by MKP3, we constructed a model for the ERK2/pTpY–MKP3 complex (Fig. 6) based on existing three-dimensional structures for ERK2 and MKP3 as well as on the H/DX-MS and mutagenesis data obtained in this study. The ERK2–KIM<sup>MKP3</sup> structure (22) was employed as the starting point for modeling the interaction between the N-terminal domain of MKP3 and ERK2. The KIM peptide from the NMR structure of the N-terminal domain of MKP3 (20) was docked onto the ERK2 structure with molecular interactions analogous to those found in the experimentally determined ERK2–KIM<sup>MKP3</sup> structure (Fig. 6A). Residues 101–107 from the NMR structure were extended and adjusted to optimize contacts with ERK2 in the model using program O (23). The model suggests that MKP3 residues Asn<sup>101</sup>, Ser<sup>102</sup>, and Ser<sup>103</sup> engage in polar interactions with ERK2 residues Asp<sup>122</sup>, His<sup>123</sup>, and Ser<sup>120</sup>. In addition, Asp<sup>106</sup> in MKP3 forms two hydrogen bonds with the side chain of Gln<sup>17</sup> in ERK2, and Trp<sup>105</sup> in MKP3 makes van der Waals contacts with Gln<sup>117</sup> and His<sup>118</sup> in ERK2. Finally, the side chains of Asn<sup>106</sup> and Gln<sup>17</sup> in MKP3 are involved in polar interactions with His<sup>118</sup> and Asn<sup>21</sup> in ERK2, respectively (Fig. 6A). This model is compatible with all available structural, H/DX-MS, and mutational data and defines the interactions between the CD site and the KIM sequence and the N-β<sub>b</sub>–α<sub>4</sub> loop in MKP3.

To model the interaction between ERK2/pTpY and the MKP3 catalytic domain, we utilized the phosphorylated ERK2 structure (25) and the crystal structure of the C-terminal domain of MKP3 (18). The model was created based on the following constraints: phospho-Tyr<sup>185</sup> occupies the MKP3 active site; the WpD loop adopts a closed conformation; there is no contact between MKP3 and ERK2 peptides 54–65 and 330–358 (based on the hydrogen/deuterium exchange results); and the MKP3 peptide 362LYFTAPSN<sup>389</sup> resides in the previously implicated ERK2 SB site (15, 22). As shown in Fig. 6B, the MKP3 peptide 362LYFTAPSN<sup>389</sup> docks into a hydrophobic cleft in ERK2 formed by Thr<sup>188</sup>, Arg<sup>189</sup>, His<sup>230</sup>, Tyr<sup>231</sup>, Leu<sup>232</sup>, and Leu<sup>235</sup> on one side; Ala<sup>187</sup>, Val<sup>186</sup>, phospho-Tyr<sup>185</sup>, Met<sup>197</sup>, and Lys<sup>199</sup> on the other; and Tyr<sup>261</sup> and Asn<sup>260</sup> at the base. In this model, Leu<sup>362</sup> forms weak hydrophobic contacts with Val<sup>186</sup>, Ala<sup>187</sup>, and Thr<sup>188</sup> in ERK2; Tyr<sup>263</sup> stacks with Tyr<sup>231</sup> in ERK2, and its OH group forms hydrogen bonds with the side chains of His<sup>230</sup> and Arg<sup>189</sup> in ERK2; Phe<sup>164</sup> is involved in π–π interactions with phospho-Tyr<sup>185</sup>; Thr<sup>365</sup> and Ala<sup>366</sup> make van der Waals contacts with phospho-Thr<sup>183</sup> and phospho-Tyr<sup>185</sup> in ERK2; and Ser<sup>103</sup> displays a hydrogen bond with Tyr<sup>261</sup> in MKP3, which is compatible with the hydrogen/deuterium exchange data.
FIGURE 6. Structural model for the ERK2/pTpY-MKP3 complex. A and B, interactions between ERK2 and the N- and C-terminal domains of MKP3, respectively. The molecular surface of ERK2 is colored according to electrostatic potential: blue for positive and red for negative. The KIM peptide (residues 64–74), the N–β4–α4 loop (residues 101–107), and the FXFP peptide (residues 362–369) from MKP3 are shown as stick models: oxygen (red); carbon (light cyan); and nitrogen (blue). ERK2 residues are shown in blue (A) or red (B), and MKP3 residues are shown in black. C, interaction of the MKP3 active site with phospho-Tyr185 (pTyr185) in the ERK2 activation lip. A–C were produced using GRASP (41). D, overall model of ERK2/pTpY complexed with MKP3. ERK2/pTpY is shown in gray, the MKP3 N-terminal domain in green, and the MKP3 C-terminal domain in blue. The CD and SB sites are colored yellow and cyan, respectively, and phospho-Tyr185 is colored pink. The KIM peptide, the N–β4–α4 loop, and the FXFP peptide are highlighted in red. The MKP3 active-site residues Asp262, Cys293, and Arg299 are also depicted as stick models in atomic colors. D was created using PyMOL (www.pymol.org) (42). It should be noted that the linker (residues 155–203) between the N- and C-terminal domains of MKP3 is of sufficient length to connect the two domains in the model.
Waals contacts with Tyr<sup>231</sup> and Leu<sup>232</sup> in ERK2; Pro<sup>367</sup> plugs into a hydrophobic pocket surrounded by the side chains of Leu<sup>232</sup>, Tyr<sup>235</sup>, Tyr<sup>261</sup>, Met<sup>197</sup>, and Leu<sup>198</sup>; and finally, Ser<sup>368</sup> and Asn<sup>369</sup> make polar interactions with Tyr<sup>261</sup> and Asn<sup>260</sup> in ERK2 (Fig. 6B). Because the FXFP motif is present in many ERK2 substrates (34), the hydrophobic cleft, delineated by the residues in the ERK2 activation lip and the P↓1 site (β<sub>γ</sub>-α<sub>γ</sub> loop), L<sub>13</sub> (α<sub>γ</sub>-α<sub>δ</sub> loop), and the MAPK insert (L<sub>14</sub>-G<sub>1114</sub>-G<sub>2114</sub>), most likely corresponds to the SB site. Again, the model is in complete agreement with the hydrogen/deuterium exchange and mutagenesis results. Given the close proximity of the SB from the phospho-ERK2 is governed by two distinctive protein-protein interactions (Fig. 6D). The CD site, located opposite the kinase active site, is responsible for high affinity binding to KIM and the MKP3-specific N-β<sub>γ</sub>-α<sub>δ</sub> loop. The role for this docking interaction is to increase the “effective concentration” of the interacting molecules (12). In addition to this “tethering” effect, additional interactions between the SB site and the phospho-Thr<sup>183</sup> sequence are essential for allosteric activation and formation of a productive complex whereby the MKP3 catalytic site is correctly juxtaposed to carry out the dephosphorylation of phospho-Thr<sup>183</sup>/phospho-Tyr<sup>185</sup> in the ERK2 activation lip.

Given the structural similarity among the MAPKs and the fact that the KIM and FXFP sequences are present in many MAPK-interacting proteins (34, 36), we suspect that this bipartite protein-protein interaction model may be applicable to the recognition of other MAPKs by their cognate regulators and substrates. Thus, in addition to the docking interaction between KIM and the CD site, the interaction of the SB site with the FXFP sequence in the substrates may be required to organize the MKP3 active site with respect to the phosphoreceptor in the substrate for efficient phosphoryl transfer. Similarly, specific interaction of the SB site with the FXFP-like peptides in MAPKs or phosphatases may ensure the precise orientation and positioning of the catalytic residues in the MAPK kinases or phosphatases with respect to the TXY motif in the activation lip for efficient phosphorylation or dephosphorylation. It appears that, to ensure pathway specificity, the MAPKs make use of both high affinity docking interactions as well as a binding-induced allosteric mechanism in which specific interactions between the MAPK and its binding proteins enable the attainment of optimum alignment of the catalytic residues with respect to the substrate for efficient catalysis.

**Conclusions and Implications**—Using a combination of H/DX-MS, mutagenesis, and modeling, we have further established the importance of the KIM sequence and the FXFP peptide for high affinity ERK2 binding and ERK2-induced MKP3 activation. This study has also identified a novel ERK2-binding peptide (N-β<sub>γ</sub>-α<sub>δ</sub>) in MKP3 that, together with the KIM sequence, occupies the ERK2 CD site for high affinity binding. Furthermore, this study has uncovered a striking reduction in the conformational/dynamic flexibility of the MKP3 catalytic domain upon association with ERK2, which likely correlates with ERK2-induced MKP3 activation. Finally, in addition to providing solution evidence that the CD site is important for binding the KIM and N-β<sub>γ</sub>-α<sub>δ</sub> peptides in MKP3, this study has also defined the ERK2 SB site, which directly interacts with the phospho-Thr<sup>183</sup> peptide, triggering MKP3 activation. Taken together, our study has elucidated the structural basis for ERK2 recognition by MKP3. The exquisite specificity of MKP3 for phospho-ERK2 is governed by two distinctive protein-protein interactions (Fig. 6D). The CD site, located opposite the kinase active site, is responsible for high affinity binding to KIM and the MKP3-specific N-β<sub>γ</sub>-α<sub>δ</sub> loop. The role for this docking interaction is to increase the “effective concentration” of the interacting molecules (12). In addition to this “tethering” effect, additional interactions between the SB site and the phospho-Thr<sup>183</sup> sequence are essential for allosteric activation and formation of a productive complex whereby the MKP3 catalytic site is correctly juxtaposed to carry out the dephosphorylation of phospho-Thr<sup>183</sup>/phospho-Tyr<sup>185</sup> in the ERK2 activation lip.

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