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

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Running Title: Molecular Basis of ERK2 Recognition by MKP3

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Extracellular signal-regulated protein kinase 2 (ERK2), a prototypic member of the mitogen-activated protein (MAP) kinases family, plays a central role in regulating cell growth and differentiation. MAP kinase phosphatase 3 (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 (64 RRLQKGNLPVR 74) and an MKP3-specific segment (101 NSSDWNE 107) bind the common docking site in ERK2 defined by residues in L16, L5, β7-β8, and α1-L8-αe, located opposite of the kinase active site. In addition to this “tethering” effect, additional interactions between the 364 FTAP 367 sequence in MKP3 and the ERK2 substrate-binding site, formed by residues in the activation lip and the P+1 site (β9-αf loop), L13 (αf-αg loop), and the MAP kinase insert (L14-α1L14-α2L14), 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 pTyr185/pThr183 in ERK2. This bipartite protein-protein interaction model may be applicable to the recognition of other MAP kinases by their cognate regulators and substrates.

Mitogen-activated protein (MAP) kinase cascades are highly conserved signal transduction modules in eukaryotes, which mediate the intracellular transmission and amplification of extracellular stimuli, leading to the induction of appropriate cellular responses to changes in surrounding environment (1, 2). The MAP kinases 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 and inactivating phosphatases. Although the importance of MAP kinases in cellular signaling is well established, there is limited understanding of the molecular basis for MAP kinase recognition by its activators, inactivators, and substrates. Such knowledge is essential for comprehension of the ability of MAP kinases to integrate diverse biological stimuli and to transmit signals to the nucleus, in order to generate appropriate cellular responses.

Extracellular signal-regulated protein kinase 2 (ERK2), the prototypic member of the MAP kinase family, has been the subject of intense study. ERK2 is activated by phosphorylation of Thr183 and Tyr185 in the activation lip by the dual specificity MAP kinase/ERK kinase 1 (MEK1). Deactivation of ERK2 activity is carried out by MAP kinase 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). Further studies indicate that MKP3 is responsible for the negative feedback regulation of FGF-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 $k_{cat}/K_m$ that is $10^6$-fold higher than those for the hydrolysis of p-nitrophenyl phosphate (pNPP) 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 suggest that ERK2 binding elicits allosteric activation of MKP3 resulting in optimal 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 understand 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 are 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-terminal His$_6$-tagged ERK2 was expressed in *E. coli* and purified using a published procedure (21). The expression and purification of ERK2/pT$\gamma$Y (ERK2 phosphorylated on both Thr183 and Tyr185) were carried out as described (21). The catalytic inactive MKP3 (MKP3/C293S) with a C-terminal His$_6$ tag and 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 QuickChange™ 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 Pull-down Analysis.* The binding affinity of GST-MKP3/C293S for ERK2 or ERK2/pT$\gamma$Y was determined by GST pull-down and Western blotting analysis. Five µg of GST-MKP3/C293S in 0.5 ml of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 2 mM dithiothreitol, pH 7.3) was immobilized on 20 µl of glutathione-Sepharose 4B beads (Amersham Bioscience) by gentle agitation at 4 °C for 2 h. Different concentrations of ERK2 or ERK2/pT$\gamma$Y in 200 µl of PBS buffer with 0.5% TX-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 PBS buffer with 0.5% TX-100, and 3 more washes with PBS buffer, 20 µl of 2× SDS sample buffer was added to the beads. GST-MKP3/C293S and the bound ERK2 (or ERK2/pT$\gamma$Y) 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 on 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins on the gel were transferred to nitrocellulose membrane at 25 mA and 4°C overnight. Rabbit anti-phospho-p44/42 MAP kinase antibody (#9101, Cell Signaling...
H-D Exchange Mass Spectrometry. H/D exchange in MKP3/C293S and ERK2/pTpY, either alone or in the complex form, was initiated by diluting 1 µl of the stock solution (200 µM) to 19 µl of the D2O buffer (20 mM Tris, 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 quench. The final protein concentration was 10 µM. The resulting H/D 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, H/D 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 1.0 x 50-mm C4 column and analyzed by mass spectrometry for global exchange. Changes in deuterium incorporation within specific regions of proteins can be detected by peptic digestion of the deuterium labeled proteins, followed by HPLC separation and mass spectrometric analysis. To analyze H/D exchange in segments of MKP3/C293S and ERK2/pTpY, proteins H/D 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 a Vydac 1.0x50 mm C18 column.

A Shimadzu HPLC equipped with two LC-10ADVP pumps was used to generate an acetonitrile gradient. Solvent A was 94.9% H2O containing 5% acetonitrile and 0.1% formic acid, and Solvent B was 95% acetonitrile containing 4.9% H2O and 0.1% formic acid. A low volume static mixing tee was used to minimize the delay period for HPLC. The solvent pre-cooling coil, static mixing tee, Rheodyne injector, and the column were immersed in an ice bath (0°C) to minimize back exchange with HPLC solvents. Desalted at 5% solvent B for 5 min, the protein eluted with a 2-min 30-50% B gradient, and the peptic peptides eluted with a 0.5-min 5-15% B gradient followed by an 8-min 15-45% B gradient. The column effluent (50 µl/min) was delivered directly to a Thermo Finnigan (Riviera Beach, FL) LCQ Deca XP plus ion trap mass spectrometry for mass analyses of deuterated protein and its peptic fragments. MagTran 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 sequence of the peptide fragments obtained after pepsin digestion was identified using 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 MS/MS spectrum within a peak during the HPLC run. MS/MS spectra were interpreted by searching peptide databases using the SEQUEST algorithm.

The extent of H/D exchange was calculated using equation 1:

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

where m is the measured mass of deuterated protein or peptide; \(m_{0\%}\) is the measured mass of a “zero-deuteration” control prepared by adding 1 µl of 200 µM protein solution into a 19:20 (v/v) mixture of deuterated buffer and quenching buffer; \(m_{100\%}\) is the measured mass of a “full-deuteration” control prepared by incubating the protein sample for 16 h at room temperature in 3 M deuterated GnHCl followed by addition of an equal volume of cold quenching buffer; and H is the total exchangeable amide hydrogens.

Molecular Modeling of ERK2/pTpY and MKP3 Interactions. The crystal and NMR structures of ERK2 and MKP3, together with H/DX-MS and mutagenesis data, were used to generate a model for the ERK2-MKP3 complex. The structure of ERK2•KIMKMP3 (22; PDB entry code: 2FYS) was utilized as the starting point for modeling the interaction between the N-terminal domain of MKP3 and ERK2. The KIM peptide (residues 64-74) from the NMR structure (20; PDB entry code: 1HZM) was docked onto the ERK2 structure with contacts analogous to those...
found in the experimentally determined ERK2*KIM\textsuperscript{MKP3} structure (22). Residues 101-107 in the N-terminal domain of MKP3 relative to ERK2 was adjusted based on the H/DX-MS and mutational results. The resulting model was then stereochemically refined by energy minimization using the program CNS (24).

The phosphorylated ERK2 structure (25; PDB entry code: 2ERK) and the crystal structure of the C-terminal domain of MKP3 (18; PDB entry code: 1MKP) were utilized as the starting point for modeling the interaction between ERK2 and the C-terminal domain of MKP3. The ERK2/pTpY structure superimposes with a root mean square deviation of 0.59 Å with ERK2*KIM\textsuperscript{MKP3} and is thus quite similar. The model was created using program O (23) based on the following constraints: pTyr185 of ERK2 binds the MKP3 active site; the WpD loop adopts a closed conformation; no contact between MKP3 and ERK2 peptides 54-65 and 330-358 (based on the H/D exchange results); and the MKP3 peptide \textsuperscript{362}LYFTAPSN\textsuperscript{369} occupies the previously implicated ERK2 substrate-binding site (15, 26). Finally, the model was refined using program CNS (24).

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 MKP3-ERK2/pTpY (ERK2 phosphorylated on both Thr183 and Tyr185) 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 the wild-type MKP3 and the doubly phosphorylated ERK2/pTpY. Fortunately, catalysis by the 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 (29-30). Consequently, we employed the 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 \(K_d = 0.13\pm0.01 \mu\text{M}\) is similar to that of wild-type MKP3 \(K_d = 0.17\pm0.04 \mu\text{M}\) (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 pull-down experiments to measure ERK2/pTpY binding directly. As a control, we also performed GST-MKP3/C293S pull-down of unphosphorylated ERK2. The amount of ERK2 or ERK2/pTpY associated with GST-MKP3/C293S was visualized by anti-ERK2 or anti-phospho-ERK2 antibodies and quantified by densitometry (Figure 1). The dissociation constant \(K_d\) for ERK2 binding to GST-MKP3/C293S is \(0.19\pm0.06 \mu\text{M}\), which is similar to that measured previously by a competition based assay (14). Likewise, the \(K_d\) value determined by the pull-down experiments for GST-MKP3/C293S and ERK2/pTpY is \(31\pm5 \text{nM}\), which is comparable to the \(K_m\) of MKP3 for ERK2/pTpY (22\pm5 N M) (12). Thus, GST-MKP3/C293S binds the double phosphorylated ERK2 6-fold tighter than the unphosphorylated ERK2, consistent with the expectation that the interactions between the MKP3 active site and the phosphoamino acids in ERK2 should further enhance the binding affinity between the two proteins. Obviously, characterization of the interfaces between MKP3/C293S and ERK2/pTpY should enhance our understanding of ERK2 recognition by MKP3.

Global H/D 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 D\(_2\)O 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 \(\mu\text{M}\).
Given the $K_d$ (31 nM) for complex formation, 95% of MKP3/C293S and ERK2/pTpY should be in the bound state under our experimental conditions. The C-terminal His$_6$-tagged MKP3/C293S contains 387 amino acids (MW=43,117.5 Da) with 365 exchangeable amide hydrogens, while the N-terminal His$_6$-tagged ERK2/pYpT has 364 residues (MW= 42,328.0 Da) with 343 exchangeable amide hydrogens. Figure 2 shows the total deuterium incorporation into MKP3/C293S and ERK2/pTpY either in the free or the bound form. Within 40 min, a total of 190 amide hydrogens in MKP3/C293S were replaced with deuterium in the absence of ERK2/pTpY while 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 form deuterium exchange upon complex formation. Compared to ERK2/pTpY alone, 30 of ERK2 amide protons were protected from exchange in the MKP3/C293S•ERK2/pTpY 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 MKP3-ERK2 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 H/D 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 H/D exchange within different regions of the protein (27). Overall, 49 peptides (including those with overlapping sequences), covering 92% of MKP3 amino acid sequence, were identified by tandem mass spectrometry (MS/MS), as shown by the coverage map (Figure 3). Out of the 49 peptides, 30 displayed reduced exchange rates, 2 exhibited an increase in the 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 (Figure 4). Upon complex formation with MKP3/C293S, there were 22 peptides displaying a decrease in deuterium incorporation, 22 showing no change, and 3 peptides exhibiting an increase in deuterium incorporation.

To directly visualize the H/D exchange results, we depict 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) of MKP3 (18) (Figure 3C). Indicated are peptides observed by H/DX-MS that exhibited a decrease (red), an increase (green), or no change (blue) in H/D exchange rates upon complex formation. Peptides in black were not identified in the MS/MS experiments. As shown in Figure 3, the ERK2/pTpY bound MKP3 displayed reduced solvent accessibility in regions Val56-Leu76 (N-$\beta_1$-$\beta_2$-$\alpha_3$), Tyr98-Leu115 (N-$\beta_4$-$\alpha_4$), Ser194-Phe206, Tyr215-Phe278 (C-$\beta_2$-$\alpha_2$-$\beta_3$-$\beta_7$-310-$\alpha_4$), Val291-Thr304 (C-$\beta_8$-$\alpha_5$), Met309-Phe343 (C-$\alpha_5$-$\alpha_6$-$\alpha_7$) and Tyr363-Asn369. Interestingly, increased H/D exchange rates were observed within Thr87-Leu97 (N-$\alpha_3$-$\beta_4$) and Gln140-Phe143 (N-$\alpha_3$-$\beta_6$).

Changes in solvent accessibility in ERK2/pTpY upon complex formation are depicted by color-coding segments of the X-ray crystal structure of ERK2/pTpY (25). As shown in Figure 4C, the MKP3/C293S bound ERK2/pTpY exhibited reduced solvent accessibility in regions
(highlighted in red) from residues Val12-Lys53 \((\beta_{2L2-L12-L17-\beta_{2L2}})\), Met106-Tyr126 \((\alpha_4-L_4)\), Asn156-Leu161 \((\beta_{1-\beta_8})\), Phe166-Leu198 \((\beta_{9}\cdot L_{12})\), Leu220-Glu248 \((\alpha_5-L_{15}\cdot \alpha_7\cdot L_{14})\), Ile253-Leu262 \((\alpha_{2L14})\), and Leu292-Glu324 \((L_{15}\cdot \alpha_4\cdot L_{16})\), as compared to free ERK2/pTpY. Increased solvent accessibility was observed for Asn199-Gly213 \((L_{12}\cdot \alpha_4)\) (highlighted in green).

Identification of the ERK2/pTpY Binding Sites in MKP3. The H/DX-MS technique can not differentiate changes in solvent accessibility as a result of direct binding from those due to conformational or dynamic perturbations. Thus, peptide segments that show altered H/D exchange after complex formation can either be involved in ERK2/MKP3 binding or conformational/dynamic changes. Additional biochemical experiments in combination with the H/DX-MS data are required to identify the ERK2 binding sites in MKP3. To this end, we had previously suggested that the kinase interaction motif (KIM) \(\text{RRLQKGNLPVRAL}^{76}\) in MKP3 plays a major role for high affinity ERK2 binding (14). To provide further evidence that the KIM is directly involved in ERK2 binding, we performed GST pull-down assays. The purified, His\(_6\)-tagged MKP3/C293S proteins were incubated with GST-ERK2/pTpY that was immobilized onto glutathione-Sepharose beads. As expected, replacement of Arg65 by an Ala (MKP3/R65A) completely disrupted ERK2 binding (Figure 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 \((N-\beta_3\cdot \beta_3-\alpha_5)\) upon MKP3-ERK2 complex formation (Figure 3).

Aside from KIM, no other regions in the MKP3 N-terminal domain have previously been implicated for ERK2 binding. The H/DX-MS experiments revealed that in addition to KIM, the Tyr98-Leu115 \((N-\beta_4\cdot \alpha_4)\) segment also displayed a significant decrease in deuterium incorporation, while residues Thr87-Leu97 \((N-\alpha_5\cdot \beta_5)\) and Gln140-Phe143 \((N-\alpha_5\cdot \beta_5)\) showed increased deuterium uptake in the complex (Figure 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 pull-down assay. As shown in Figure 5, substitution of Leu97, Asn106, and Glu142 by an 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 to 203 between the N- and C-terminal domains. Previous studies suggest 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, which promotes MKP3 degradation by the proteasomal pathway (33). While 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 (Figure 3). Surprisingly, replacement of Asp195 or Leu199 with an Ala had no significant effect on MKP3/C293S binding to ERK2/pTpY (Figure 5). Thus, it is not clear based on the current data whether this region is directly involved in ERK2 binding.

The C-terminal domain of MKP3 is the catalytic domain, which adopts a typical protein tyrosine phosphatase (PTP) fold (Figure 3C, 18). The active site is situated within a cleft formed by the PTP loop between C-\(\beta_8\) and C-\(\alpha_5\) \(\text{VHCLAGISRS}^{306}\). In the catalytic mechanism, Cys293 functions as a nucleophile while 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-\(\beta_7\cdot \beta_{10}\)-residues 257-268) in the vicinity of the active site. However, unlike the PTPs, MKP3 exhibits an extremely low basal activity toward \(p\text{NPP}^{(17)}\) and the three dimensional structures of the catalytic domain of MKP3 show that residues Cys293 and Arg299 are misaligned and 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 substantially
increased by the presence of ERK2 (16). Kinetic evidence suggest that ERK2 binding elicits MKP3 activation by facilitating the repositioning of active site residues (Cys293 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 Figure 3, binding of ERK2/pTpY reduces the H/D 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 H 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 H/D exchange over the vast majority of the C-terminal domain is due to direct contact with ERK2/pTpY. To determine if 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 pull-down assay. Substitution at Asp226 in C-α2, Tyr235 in C-β1, Asp262 and Trp264 in the WpD loop (C-β7-310), Glu274 in C-α4, Asn329, Ser331 and Phe334 in the C-α6-α7 loop, and Leu341 in C-α7 had little effect on MKP3/C293S binding to ERK2/pTpY (Figure 5). The results suggest that these residues are not directly involved in ERK2 binding. On the other hand, elimination of the side chains at position Tyr215 in C-β2-α2, Leu246 in C-β3-β3', and Tyr255 in C-β2 significantly reduced the binding affinity of MKP3/C293S for ERK2/pTpY (Figure 5), indicating that these residues may play a role in ERK2 binding. Thus, ERK2/pTpY binding may not only lead to the 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 (Figure 3B). Interestingly, this segment contains a putative ERK2 docking motif 364^FTAP^367, 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 364^FTAP^367 reduces MKP3’s affinity for ERK2 by less than 10-fold, this region is absolutely required for ERK2 induced MKP3 activation (14). Consistent with this finding, we found that conversion of the 364^FTAP^367 sequence to AAAA (MKP3/A4) completely abolished the association of MKP3/C293S with ERK2/pTpY (Figure 5). Together, these results provide strong evidence that the FXFP peptide is directly involved in ERK2 binding and possibly the ERK2-induced MKP3 activation.

Identification of the MKP3 Binding Sites in ERK2/pTpY. The ERK2 structure includes an N-terminal lobe (β1-β5 and α3) responsible for ATP binding and a larger C-terminal lobe (αα'-αα' and βα'-βα') involved in protein substrate recognition (25, 35). The catalytic cleft is situated between the two lobes, which mediates the phosphoryl transfer reaction (Figure 4C). Phosphorylation of Thr183 and Tyr185 in the activation lip (βα-α3) is important for ERK2 kinase activity and substrate recognition. The phosphorylated ERK2 is a substrate of MKP3, and MKP3 is capable of dephosphorylating both pThr183 and pTyr185 (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).

Based on 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, both in terms of the MKP3 binding affinity and the propensity to activate MKP3 (14). Our previous mutational studies suggest that MKP3 binding and activation likely involves two distinct surface areas in ERK2 (15). One area, termed common docking (CD) site, is situated opposite to the kinase catalytic cleft and
includes residues Glu79, Tyr126, Arg133, Asp160, Tyr314, Asp316, and Asp319. The CD site is important for high-affinity MKP3 binding but not essential for ERK2-induced MKP3 activation. MKP3 activation appears to require residues Arg189, Trp190, Glu218, Arg223, Lys229, and His230 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, L5, αe, β7-β8, and αd (22). This KIM docking site consists of a highly acidic patch (residues Glu79, Tyr126, Asp160, Asp316, and Asp319 in L16, L5, β8 and αe) and a hydrophobic groove (residues Thr108, Leu110, Leu113, Leu119, Phe127, and Leu155 in αe, β7-β8, and αd), which engage the basic (Arg64 and Arg65) and hydrophobic (Leu71-Pro72-Val73) residues, respectively, in the KIM sequence.

In agreement with the X-ray structure of ERK2•KIMMKP3 and mutational studies, strong protection from H/D exchange was observed in Met106-Tyr126 (αe-L8-αe), Asn156-Leu161 (β7-β8), and Leu306-Glu324 (αd-L16) upon MKP3/C293S binding (Figure 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, residues Leu292-Gln313 (L15-αl) also showed a decrease in deuterium uptake upon complex formation. Substitution of Arg299 by an 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 ERK2 C-terminal lobe between αd, αe, L16, L5, and the β7 - β8 reverse turn. Additional studies indicate that the KIMs from various MAP kinase substrates and regulators occupy the same docking site in all MAP kinases (22, 26, 37-39). This begs for the question: how is specificity maintained by individual MAP kinase 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 MAP kinase signaling. The interaction of the ERK2 substrate-binding 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 Phe166-Leu198 (β8-β9-L12), Leu220-Glu248 (αr-L13-αg-L14-α114), and Ile253-Leu262 (α214) in the complex as compared to free ERK2/pTpY (Figure 4). These regions include the phosphorylation sites and the adjacent P+1 site in the activation lip, residues in L13-αg and the MAP kinase insert (residues 241-279), all of which have been previously implicated in MAP kinase substrate binding (15, 25, 35, 40). In addition, interactions between the MKP3 active site and pThr183/pTyr185 in ERK2 should limit the solvent accessibility and therefore reduce the rate of H/D exchange in the ERK2 activation lip. Indeed, within these peptides that display reduced H/D exchange, residues Thr179, Arg189, Trp190, Glu218, Arg223, Lys229, and His230 have been previously shown to be important for either MKP3 binding or ERK2-induced activation, or both (15). To assess the involvement of α214 in the MAP kinase insert for MKP3 binding, we replaced Lys257 and Tyr261 with an Ala. No significant effect was observed for K257A. In contrast, although ERK2/Y261A displayed the same affinity for MKP3 as the wild-type ERK2, it was only able to activate MKP3 to 15% of the maximum activity even at saturating ERK2/Y261A concentrations (Table 1). Thus, the biochemical and H/D exchange data together suggest that residues in Phe166-Leu198 (β8-β9-L12), Leu220-Glu248 (αr-L13-αg-L14-α114), and Ile253-Leu262 (α214) provide additional contacts with MKP3. Interestingly, these peptides (residues 169-213, 220-248 and 257-262) also exhibit decreased H/D exchange in the presence of an FXFP-containing peptide from Elk1 (residues 391-399, AKLSFQFPS) (26). This further supports the conclusion that ERK2 substrate-binding site is important for MKP3 recognition.
Residues Val12-Lys53 spanning β12-α, L1, β1, L2, β2, L3, and β3 comprise the third region in ERK2/pTpY in which a decrease in deuterium incorporation upon complex formation with MKP3/C293S was observed (Figure 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 Val19, Thr24, Tyr34, and Lys46, did not significantly affect the ability of ERK2 to either bind MKP3 or 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 H/D exchange in this region is likely the result of a decrease in backbone dynamics and/or flexibility. The last region of altered H/D exchange in ERK2/pTpY corresponds to Asp199-Gly213 (L12-α3), which immediately follows the activation lip (Figure 4). Since removal of the side chain at either Lys201 or Lys205 had little effect on MKP3 recognition (Table 1), the increase in H/D exchange in Asp199-Gly213 likely reports conformational/dynamic changes within L12-α3 upon MKP3 binding.

A Structural Model for MKP3-ERK2/pTpY Recognition. To define the precise interaction surfaces and identify residues that are directly involved in ERK2/pTpY recognition by MKP3, we constructed a model for the MKP3-ERK2/pTpY complex (Figure 6) based on existing three dimensional structures for ERK2 and MKP3, as well as the H/DX-MS and mutagenesis data obtained in this study. The ERK2-KIMMKP3 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 structure (Figure 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 Asn106, Ser102, and Ser103 engage in polar interactions with ERK2 residues Asp122, His123, and Ser120. In addition, Asp104 of MKP3 forms two H-bonds with the side-chain of Gln117 of ERK2; and Thr105 of MKP3 makes van der Waals contacts with Gln117 and His118 in ERK2. Finally, the side-chains of Asn106 and Glu107 of MKP3 are involved in polar interactions with ERK2 residues His118 and Asn121, respectively (Figure 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-β4-α4 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: pTyr185 occupies the MKP3 active site; the WpD loop adopts a closed conformation; no contact between MKP3 and ERK2 peptides 54-65 and 330-358 (based on the H/D exchange results); and the MKP3 peptide LYFTAPSN resides in the previously implicated ERK2 substrate-binding site (15, 22). As shown in Figure 6B, the MKP3 peptide LYFTAPSN docks into a hydrophobic cleft in ERK2 formed by Thr188, Arg189, His230, Tyr231, Leu232, Leu235 on one side, Ala187, Val186, pTyr185, Met197, and Leu198 on the other, and Tyr261 and Asn260 at the base. In this model, Leu362 forms weak hydrophobic contacts with Val188, Ala187, and Thr188 in ERK2; Tyr363 stacks with Tyr231 in ERK2, and its OH group forms H-bonds with the side-chains of His230 and Arg189 in ERK2; Phe364 is involved in π-π interactions with pTyr185; Thr365 and Ala366 makes van der Waals contacts with Tyr231 and Leu232 in ERK2; Pro367 plugs into a hydrophobic pocket surrounded by the side chains of Leu232, Leu235, Tyr261, Met197, and Leu198; and finally, Ser368 and Asn369 makes polar interactions with Tyr261 and Asn260 in ERK2 (Figure 6B). Since 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 (β9-α9 loop), L13 (α9-α9 loop), and the MAP kinase insert (L14-α14-α14), most likely corresponds to the SB site. Again, the model is in complete agreement with the H/D exchange and mutagenesis results. Given the close proximity of the SB site to the MKP3 active site (Figure 6B), the 362FTAP367/SB
site interaction likely triggers dynamic/conformational changes in the catalytic domain of MKP3 responsible for the ERK2-induced allosteric activation of MKP3.

The interaction between ERK2/pTpY and the MKP3 active site in the model is limited to pTyr185 (Figure 6C). The phosphoryl group of pTyr185 makes numerous hydrogen bonds to the backbone amides of the P-loop as well as to the side chain of Arg299. The phenyl ring of pTyr185 is surrounded by the side-chains of Ala295, Pro241, and Phe364 of MKP3, which delineate the boundaries of the MKP3 active site pocket. The WpD loop adopts a closed conformation, placing the general acid Asp262 within hydrogen bonding distance to the phenolic oxygen of pTyr185. The model can also easily accommodate pThr183 in the MKP active site with very minor adjustments in the activation lip. The limited interactions between the MKP3 active site and pTyr185 or pThr183 are expected to increase the overall binding affinity of MKP3 for ERK2. Indeed, MKP3/C293S binds the double phosphorylated ERK2 6-fold tighter than the unphosphorylated ERK2 (Figure 1). However, it is important to point out that the interactions between the MKP3 active site and the phosphoamino acids can not account for the 10^6-fold higher activity of MKP3 for ERK2/pTpY, as compared to that for the ERK2-derived phosphopeptide harboring both pThr183 and pTyr185 (12). Clearly, additional protein-protein interactions between MKP3 and ERK2 are required for the exquisite specificity and efficiency of MKP3 for ERK2/pTpY.

Conclusions and Implications. Using a combination of H/DX-MS, mutagenesis and modeling, the current study further establishes the importance of the KIM sequence and FXFP peptide for high affinity ERK2 binding and the ERK2-induced MKP3 activation. This study also identifies a novel ERK2 binding peptide (N-β_4-α_4) in MKP3 that, together with the KIM sequence, occupies the ERK2 CD site for high affinity binding. Furthermore, the study uncovers a striking reduction in the dynamic/conformational flexibility in the MKP3 catalytic domain upon association with ERK2, which likely associates with the ERK2-induced MKP3 activation. Finally, in addition to providing solution evidence that the CD site is important for binding the KIM and the N-β_4-α_4 peptide in MKP3, the study also defines the ERK2 SB site, which directly interacts with the 364FTAP^367 peptide, triggering MKP3 activation. Taken together, our study elucidates the structural basis for ERK2 recognition by MKP3. The exquisite specificity of MKP3 for phospho-ERK2 is governed by two distinctive protein-protein interactions (Figure 6D). The CD site, located opposite of the kinase active site, is responsible for high affinity binding with KIM and the MKP3-specific N-β_4-α_4 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 364FTAP^367 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 pTyr185/pThr183 in the ERK2 activation lip.

Given the structural similarity among the MAP kinases, and the fact that the KIM and the FXFP sequences are present in many MAP kinase interacting proteins (34, 36), we suspect that this bipartite protein-protein interaction model may be applicable to the recognition of other MAP kinases by their cognate regulators and substrates. Thus, in addition to this docking interaction between the KIM and the CD site, the interaction of the SB site with the FXFP sequence in the substrates may be required to organize the MAP kinase 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 MAP kinase kinases or phosphatases may ensure the precise orientation and positioning of the catalytic residues in the MAP kinase 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 MAP kinases make use of both high-affinity docking interactions as well as an induced allosteric mechanism in which specific interactions between the MAP kinase and its binding proteins enable the attainment of optimal alignment of the catalytic residues with respect to the substrate for efficient catalysis.
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33. Marchetti, S., Gimond, C., Chambard, J. C., Touboul, T., Roux, D., Pouyssegur, J., and Pages, G.


Figure legends.

Figure 1. Binding affinity of GST-MKP3/C293S for ERK2/pTpY and ERK2 determined by GST pull-down and Western blotting. (A) Binding of GST-MKP3/C293S to ERK2/pTpY. (B) Binding of GST-MKP3/C293S to ERK2.

Figure 2. Global H/D exchange for MKP3/C293S (A) and ERK2/pTpY (B) alone (○) or in complex (●).

Figure 3. H/DX-MS analysis of MKP3/C293S. (A). Sequence coverage map of MKP3, with secondary structures as described by (20) for the N-terminal domain (residues 1-154) and by (18) for the C-terminal domain (residues 204-347), showing 49 identified peptides in the MS/MS experiments. Indicated are peptides observed by H/DX-MS that exhibited a decrease (colored in red), an increase (colored in green), or no change (colored in blue) in H/D exchange rates upon complex formation. Peptides in black were not identified in the MS/MS experiments. (B) Time courses of deuterium uptake are shown for a number of peptides in MKP3/C293S that underwent significant changes in H/D exchange rates between the free (○) and ERK2/pTpY bound state (●). (C) Structural representation of the N- and C-terminal domains of MKP3 depicted with the same color scheme in (A).

Figure 4. H/DX-MS analysis of ERK2/pTpY. (A). Sequence coverage map of ERK2, with secondary structure as described by (35), showing 47 identified peptides in the MS/MS experiments. Indicated are peptides observed by H/DX-MS that exhibited a decrease (colored in red), an increase (colored in green), or no change (colored in blue) in H/D exchange rates upon complex formation. Peptides in black were not identified in the MS/MS experiments. (B) Time courses of deuterium uptake are shown for a number of peptides in ERK2/pTpY that underwent significant changes in H/D exchange rates between the free (○) and MKP3/C293S bound state (●). (C) Structural representation of ERK2/pTpY (25) depicted with the same color scheme in (A).

Figure 5. Direct binding between MKP3/C293S and GST-ERK2/pTpY. (His)₆-tagged MKP3/C293 proteins were incubated with GST-ERK2/pTpY bound to glutathione sepharose 4B resins.
**Figure 6.** A Structural Model for the ERK2/pTpY-MKP3 Complex. Interactions between ERK2 and the N- and the C-terminal domain of MKP3 are shown in (A) and (B), respectively. The molecular surface of ERK2 was 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 were shown in stick model: oxygen, red; carbon, light cyan; nitrogen, blue. ERK2 residues were labeled in blue (A) or red (B), and MKP3 residues are shown in black. (C) Interaction of the MKP3 active site with pTyr185 in the ERK2 activation lip. Figures 6A, 6B, and 6C were produced using GRASP (41). (D) The overall model of ERK2/pTpY complexed with MKP3. ERK2/pTpY was shown in gray, MKP3 N-terminal domain in green, and MKP3 C-terminal domain in blue. The CD and SB site were colored yellow and cyan, respectively, and pTry185 was colored pink. The KIM peptide the N-β₄-α₄ loop, and the FXFP peptide were highlighted in red. MKP3 active site residues Asp262, Cys293, and Arg299 were also depicted as stick model in atomic colors. Figure 6D was created using PyMOL (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.

**Abbreviations:**

ERK, extracellular signal-regulated protein kinase; KIM, kinase interaction motif; MAP kinase, mitogen-activated protein kinase; MKP, MAP kinase phosphatase; PTP, protein tyrosine phosphatase; CD, common docking; SB, substrate-binding.
Figure 1
Figure 2
Figure 3

(A) Sequence alignment

(B) Deuterium incorporation

(C) Structural representation

N-terminal domain

C-terminal domain

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Figure 4

(A) Sequence alignment with structural elements.

(B) Deuterium incorporation over time.

(C) Structural model of the protein with labeled regions and activation lip.
<table>
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<tr>
<th>GST</th>
<th>GST-ERK2/pTpY</th>
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<tbody>
<tr>
<td>(His)$_2$-MKP3</td>
<td></td>
</tr>
<tr>
<td>WT</td>
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</table>

**Pull down MKP3**

**Input MKP3**

**Input ERK2**

**Input GST**

**Anti-(His)$_6$**

**Anti-GST**
Figure 6

(A) ERK2 CD Site-MKP3 N-terminal domain
(B) ERK2 SB Site-MKP3 C-terminal FXFP
(C) MKP3 active site-ERK2 pTyr185
(D) ERK2/pTyr

MKP3 N-terminal domain
MKP3 C-terminal domain
KIM
CD site
SB site
N-β4-α4
FXFP peptide
pTyr185
Table 1

Effects of ERK2 mutations on MKP3 binding and activation.

<table>
<thead>
<tr>
<th>ERK2 mutant</th>
<th>$K_d$ (µM)</th>
<th>MKP3 Activation (%)</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>0.18 ± 0.04</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>V19A</td>
<td>0.18 ± 0.06</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>T24A</td>
<td>0.37 ± 0.06</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>Y34A</td>
<td>0.17 ± 0.05</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>K46A</td>
<td>0.19 ± 0.08</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>K201A</td>
<td>0.07 ± 0.04</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>K205A</td>
<td>0.14 ± 0.07</td>
<td>110 ± 15</td>
</tr>
<tr>
<td>N236A</td>
<td>0.18 ± 0.09</td>
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</tr>
<tr>
<td>K257A</td>
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<td>85 ± 5</td>
</tr>
<tr>
<td>Y261A</td>
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<td>15 ± 5</td>
</tr>
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<td>25 ± 5</td>
</tr>
<tr>
<td>L306A</td>
<td>0.28 ± 0.09</td>
<td>70 ± 10</td>
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</tbody>
</table>

The $K_d$ and extent of ERK2-induced MKP3 activation were determined using methods described previously (14).
Mapping ERK2-MKP3 binding interfaces by hydrogen/deuterium exchange mass spectrometry
Bo Zhou, Jialin Zhang, Sijiu Liu, Sharanya Reddy, Fang Wang and Zhong-Yin Zhang

J. Biol. Chem. published online October 17, 2006

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