Substrate Recognition Domains within Extracellular Signal-regulated Kinase Mediated Binding and Catalytic Activation of Mitogen-activated Protein Kinase Phosphatase-3*

Received for publication, February 22, 2000, and in revised form, May 5, 2000
Published, JBC Papers in Press, May 15, 2000, DOI 10.1074/jbc.M001515200

Anthony Nichols, Montserrat Camps, Corine Gillieron, Christian Chabert, Anne Brunet‡, Julie Wilsbacher§, Melanie Cobbs§, Jacques Pouyssegur¶, Jeffrey P. Shaw, and Steve Arkinstall

From the Serono Pharmaceutical Research Institute, Ares-Serono International SA, Plan-les-Ouates 1228, Geneva, Switzerland, the §Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, the ¶Department of Pharmacology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041, and the ‡CNRS-UMR 6543 Centre de Biochimie, Universite de Nice, Parc Valrose, 06108 Nice, France

Mitogen-activated protein (MAP) kinase phosphatase-3 (MKP-3) is a dual specificity phosphatase that inactivates extracellular signal-regulated kinase (ERK) MAP kinases. This reflects tight and specific binding between ERK and the MKP-3 amino terminus with consequent phosphatase activation and dephosphorylation of the bound MAP kinase. We have used a series of p38/ERK chimeric molecules to identify domains within ERK necessary for binding and catalytic activation of MKP-3. These studies demonstrate that ERK kinase subdomains V-XI are necessary and sufficient for binding and catalytic activation of MKP-3. These domains constitute the major COOH-terminal structural lobe of ERK. p38/ERK chimeras possessing these regions display increased sensitivity to inactivation by MKP-3. These data also reveal an overlap between ERK domains interacting with MKP-3 and those known to confer substrat specificity on the ERK MAP kinase. Consistent with this, we show that peptides representing docking sites within the target substrates Elk-1 and p90rsk inhibit ERK-dependent activation of MKP-3. In addition, abolition of ERK-dependent phosphatase activation following mutation of a putative kinase interaction motif (KIM) within the MKP-3 NH2 terminus suggests that key sites of contact for the ERK COOH-terminal structural lobe include residues localized between the Cdc25 homology domains (CH2) found conserved between members of the DSP gene family.

Mitogen-activated protein (MAP)1 kinases represent a subfamily of serine/threonine protein kinases functioning within pathways that become activated following cell exposure to a large number of external signals. In mammalian cells at least four MAP kinase classes have been identified. These are known as the extracellular signal regulated kinase (ERK), the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), the p38/RK/CSBP (p38), and BMK1/ERK5 MAP kinases. In addition to this diversity, multiple genes and splice variants of each MAP kinase class have also been identified (1–3). Crystallization of MAP kinases has revealed two major three-dimensional structural domains (4–7). A smaller NH2-terminal domain comprises kinase subdomains I-IV together with the COOH-terminal tail (L16) and is made up mostly from β-strands. The COOH-terminal domain, by contrast, includes kinase subdomains V-XI and is rich in α-helices. ATP binds deep in the active site cleft formed between the two domains whereas substrate protein is believed to associate with a groove formed on the surface of the COOH-terminal domain. MAP kinases phosphorylate only substrates that contain proline in the P+1 site which binds within a surface pocket formed by residues highly conserved in the MAP kinase family. This P+1 specificity pocket is contiguous with the “activation loop” or “lip” which contains conserved Thr and Tyr residues important for control of MAP kinase activation state (see below).

Different cell stimuli activate preferentially distinct MAP kinases. Hence, while ERKs are highly responsive to growth factors, phorbol esters, and some oncogenes, JNK/SAPK and p38 MAP kinases are activated by inflammatory cytokines and cell stresses (1–3, 8). Recent studies using mutant kinases, chemical inhibitors, or gene deletion in mice indicate a key role for MAP kinases in controlling several diverse cell functions. ERK MAP kinases, for instance, appear important in pathways leading to cellular proliferation, oncogenic transformation, and metastasis, as well as in processes underlying memory and learning. JNK/SAPK and p38 MAP kinases, in contrast, appear to control T cell differentiation, production of inflammatory cytokines and events leading to neuronal apoptotic death (1, 9–18). These observations indicate that mechanisms controlling MAP kinases are likely to be of central importance to several diverse aspects of normal and pathological cell functions.

MAP kinase activation is triggered by phosphorylation on specific Thr and Tyr residues localized within the activation loop TXY motif of kinase domain VIII (where X is Glu, Pro, or Gly in ERK, JNK/SAPK or p38 MAP kinases, respectively). Several upstream kinases are now known to activate different MAP kinases selectively (1–3). Notwithstanding the importance of such stimulatory input, MAP kinase activation is normally reversible in cells indicating that protein phosphatases
also provide an important mechanism for control. Dual specificity phosphatases (DSPs) represent a subclass of the protein-tyrosine phosphatase (PTP) gene superfamily which appear to play an important role in regulating the inactivating MAP kinases. Nine distinct DSPs have now been reported including CI100/MKP-1 (19, 20), PAC1 (21, 22), HVII-2/MKP-2/TPY1 (23–25), hvH3/B23 (26, 27), hvH3/M3–6 (28, 29), MKP-3/PYST1/rvH6 (30–32), B59/PYST2/MKP-X (31–33), MKP-4 (34), and MKP-5 (35). Some DSPs are localized to different subcellular compartments and moreover, several DSP genes undergo powerful induction following exposure to cell stresses and/or growth factors (36). This, together with recent reports of specific MAP kinase inactivation by some DSPs, suggests a sophisticated transcriptional mechanism for inactivation of selected MAP kinase activities.

MKP-3 is a selective DSP that mediates preferential dephosphorylation and inactivation of ERK1 and ERK2 MAP kinases (32, 37). We have reported recently that this reflects tight and specific binding of ERK to non-catalytic regions within the MKP-3 amino terminus and that this triggers a powerful increase in MKP-3 phosphatase activity (38, 39). Other DSPs also display binding and activation by ERK, JNK/SAPK, and p38 suggesting a general mechanism for targeted inactivation of different MAP kinases (39). One important unanswered question is of the molecular domains within MAP kinases important for specific binding and catalytic activation of DSPs. To address this question we have employed a number of p38/ERK chimeric molecules used previously to reveal domains within MAP kinases important for interactions with upstream activating kinases and that target substrate proteins (40, 41). Based on studies with purified MKP-3, it appears that regions localized within the COOH-terminal structural lobe of ERK are essential for binding and catalytic activation of MKP-3. These subdomains include regions believed to be important for substrate binding and consistent with this, peptides based on the ERK substrates Elk-1 and p90(rk) inhibit MKP-3 catalytic activity by this MAP kinase.

MATERIALS AND METHODS

p38/ERK Chimeric Expression in Escherichia coli—Constructs encoding the p38α/ERK1 chimeras (Chim I, III, V, VII, VIII, XI, and p38-ERK-p38) described previously (40) were used as a basis for constructs enabling bacterial expression of these proteins with an N-terminal His$_6$-tag. Subcloning involved inserting a BamHI/XhoI PCR fragment from each p38α/ERK1 chimera containing a 5'-NcoI compatible site and nucleotides encoding a His$_6$-tag into the NcoI/XhoI sites of pET23D. The BamHI/XhoI PCR fragments were obtained by two separate PCR reactions. In the first step the coding region of the different chimeras was amplified using the sense His$_6$-p38α primer 5'-GCTTCAC- CACACACCAACACCAATCATCGAGAAGGCAGCCACG-3' together with the ERK1 antisense primer 5'-ATCTGTCAGATCGAGGGGC- TCTGAGGCCCTCCGCGG-3'. The resulting PCR products were then used as templates in a second amplification using the same antisense primer together with a common BamHI-His$_6$ 5'-sense primer including a NcoI compatible 5'-BamHI followed by nucleotides encoding the His$_6$-tag (5'-TCTATTAAAAAAGATTATCGATCCATCGATCCATCGATCCACA- ACCACACACACAC-3'). The wild type p38α construct was obtained by the same PCR procedure using the primers p38α in combination with an antisense p38α-XhoI primer 5'-ATCTGTCAGATCGAGAC- TCCATTCTTCTTGTCG-3'. A wild type ERK1 bacterial expression construct was obtained directly by one PCR amplification using a 5'-sense primer including a BamHI site followed by nucleotides encoding the His$_6$-tag and p44 ERK1 sequence (5'-TCTATTAAAAAAGATTATCGATCCATCGATCCATCGATCCACA- ACCACACACACAC-3'). The wild type p38α construct was obtained by the same PCR procedure using the primers p38α in combination with an antisense p38α-XhoI primer 5'-ATCTGTCAGATCGAGAC- TCCATTCTTCTTGTCG-3'. The resulting PCR products were then used as templates in a second amplification using the same antisense primer together with a common BamHI-His$_6$ 5'-sense primer including a NcoI compatible 5'-BamHI followed by nucleotides encoding the His$_6$-tag (5'-TCTATTAAAAAAGATTATCGATCCATCGATCCATCGATCCACA- ACCACACACACAC-3'). The wild type p38α construct was obtained directly by one PCR amplification using a 5'-sense primer including a BamHI site followed by nucleotides encoding the His$_6$-tag and p44 ERK1 sequence (5'-TCTATTAAAAAAGATTATCGATCCATCGATCCATCGATCCACA- ACCACACACACAC-3').

For protein purification transformed bacteria were grown overnight to saturation in LB medium containing 100 μg/ml ampicillin, after which growth was resumed by diluting the culture 1:50 and incubating at 37 °C for 1 h. Following transfer to 20 °C for 1 h, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 100 μM and cells were harvested, resuspended in phosphate-buffered saline containing 1% (v/v) Triton X-100, 5 mM dithiotreitol, 2 mM EDTA, 5 mM benzamidine, and 1 mM Pefabloc (Roche Molecular Biochemicals) and broken by passing three times through a French Press at 10,000 psi. The extract was then centrifuged at 10,000 × g for 15 min at 4 °C and purified using Ni$_2$+-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturers instructions. All proteins were >90% pure as assessed by Coomassie Blue staining.

**MKP-3 Interaction Domains on ERK MAP Kinase**

**FIG. 1. Purified p38/ERK chimera binding to MKP-3ΔC.** A series of p38α/ERK1 chimeric molecules (schematically represented in Fig. 6) were expressed in *E. coli* as His$_6$-tagged molecules. A, Coomassie Blue-stained His$_6$-tagged p38α/ERK1 chimeras following their purification by Ni$_2$+-nitrilotriacetic acid-agarose and separation by SDS-polyacrylamide gel electrophoresis using a 12% gel. Chimeras comprise COOH-terminal ERK1 sequence up to and including kinase subdomain I (Chim I), subdomain III (Chim III), subdomain V (Chim V), subdomain VII (Chim VII), subdomain VIII (Chim VIII), and subdomain XI (Chim XI) with p38α residues constituting the remaining NH$_2$ terminus. p38-ERK1 represents an additional chimera constituting p38α sequence except for the activation loop of ERK1. Bovine serum albumin (BSA) was used a standard (10 or 20 μg) for Coomassie staining. B, His$_6$-tagged ERK, p38α, or p38α/ERK1 chimeras (as indicated) were incubated with glutathione-Sepharose beads prebound to GST-MKP-3ΔC. Western analysis of washed beads was performed using anti-His$_6$ monoclonal antibody with goat anti-mouse monoclonal antibody horseradish peroxidase conjugate and chemiluminescence. This binding experiment is representative of three separate experiments.
CGCCCCCGTGCG-3'; and MKP-3 (V73A), 5'-AAGGGCAACCTGCCG-GCGCGCGCTATTCACG-3'. The two PCR products were then mixed and amplified using the sense BamHI primer and the antisense NcoI primer. All pGEX4T3/MKP-3 mutants were verified by sequencing.GST-MKP-3 and the above mutants were expressed and purified from bacteria, assessed for phosphatase activity and binding to MAP kinase chimeras exactly as described previously (38, 39).

MAP Kinase and p38/ERK Chimera Activities—Purified MAP kinases or p38/ERK chimeras (0.5 μg) were activated by incubation for 1 h at 30 °C with 0.1 μg of GST-MEK1 (S218E,S222E) or GST-MKK6 (as indicated) in 60 μl of 50 mM HEPES, pH 7.4, containing 10 mM MgCl₂, 2 mM dithiothreitol, 10 μM [γ-32P]ATP (~10,000 dpm/pmol), and 10 μg of myelin basic protein or GST-ATF-2 (19–96). Inactivation by MKP-3 or MKP-3 mutants was assessed by inclusion of 0.01–10 μg of the GST-MKP-3 proteins as indicated. Reactions were terminated and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described before (38).

RESULTS AND DISCUSSION

MKP-3 Binding to p38α/ERK1 Chimeras—We have reported previously that ERK but not JNK/SAPK or p38 MAP kinases bind tightly to the MKP-3 NH₂ terminus and that this interaction triggers phosphatase activation (38, 39). To investigate which domains within ERK are critical for interacting with MKP-3 we employed a series of p38α/ERK1 chimeras (40) purified following expression in bacteria as His-tagged proteins (see Fig. 6 for schematic representation). Each was estimated as >90% pure (Fig. 1A) and were incubated with the amino-terminal half of MKP-3 (residues 1–221, MKP-3ΔC) (38) expressed as a GST fusion protein and immobilized on beads. Following extensive washing, Western blot analysis was used to indicate binding between different p38α/ERK1 chimeras and the MKP-3 NH₂ terminus. As observed previously, no binding between p38α and MKP-3ΔC was detected (Fig. 1B). Similarly, chimera XI, which contains predominantly the p38α sequence.

**FIG. 2.** MKP-3 phosphatase activation by p38/ERK chimera. Phosphatase activity was assessed by incubating purified full-length GST-MKP-3 (10 μg) together with ERK2, His₆-p38α, or His₆-tagged p38/ERK chimeras (0–20 μg as indicated) in the presence of 20 μM p-nitrophenyl phosphate for 60 min, and measuring absorbance at 405 nm. Chimeras were either p38α/ERK1 as shown in Fig. 1 (A) or chimeras EIIP, EIIPIVE, and PIVECTP of p38α/ERK2 (B) as described (41). Chimera V was retested in B for comparison with this chimera set. A schematic representation of these chimeras is given in Fig. 6. Data points represent the mean of triplicate determinations and are representative of three separate experiments.

**FIG. 3.** Inhibition of MKP-3 activation by the ERK2 CD domain peptide. Purified MKP-3 (5 μg) was incubated alone (control) or with 5 μg of ERK2 and phosphatase activity measured as described in the legend to Fig. 2. Incubation in the presence of 200 μM of a synthetic peptide LEQYYDPSDEPIAE (Erk2 311–324) representing the CD domain of ERK2 (42) inhibits activation of MKP-3 by purified ERK2. Data points are the mean of triplicate determination and representative of two separate experiments.

**FIG. 4.** Inactivation of p38α/ERK1 chimera by MKP-3. Purified p38α/ERK2 chimera (0.5 μg) were activated by 0.1 μg of an appropriate MAP kinase kinase (MEK1 S218E,S222E for Chim I and III and MKK6 for all other chimeras) and incubated with 0.01–3.0 μg of full-length GST-MKP-3. Enzymatic activities of p38α/ERK1 chimera were assessed by phosphorylation in the presence of [γ-32P]ATP of either 10 μg of myelin basic protein (MBP) or 10 μg of GST-ATF-2 (19–96) as indicated. Autoradiogram shows substrate phosphorylation following separation with SDS-polyacrylamide gel electrophoresis using a 12% gel and is representative of three separate experiments.
exception for kinase subdomain XI and the carboxyl terminus of ERK1, was found to bind only very weakly to immobilized GST-MKP-3ΔC (Fig. 1B). Chimeras VIII and VII contain progressively longer carboxyl-terminal sequences from ERK1 including, respectively, either kinase subdomain VIII or subdomain VII together with the activation loop. In contrast to chimera XI, chimeras VIII and VII both bound to immobilized MKP-3ΔC (Fig. 1B). Since chimeras VIII and VII bind MKP-3 similarly, neither the activation loop or kinase domain VII of ERK appear to confer any additional capacity for binding MKP-3. This is consistent with the failure of a p44 ERK1 loop chimera (p38α possessing the activation loop of ERK1) (40) to exhibit any detectable binding to MKP-3ΔC (Fig. 1B). Increasing further the proportion of COOH-terminal ERK1 sequence up to and including kinase subdomains V (chimera V), III (chimera III), or I (chimera I) resulted in a further increase in binding to MKP-3ΔC (Fig. 1B). Similar results were observed when binding was assessed using full-length GST-MKP-3 or when lysates from COS-7 cells transfected with HA-tagged versions of the p38α/ERK1 chimeras were incubated with immobilized GST-MKP-3ΔC (data not shown).

MKP-3 Catalytic Activation by p38/ERK Chimeras—ERK1 or ERK2 binding to the NH₂ terminus of MKP-3 triggers a powerful increase in phosphatase activity. In contrast, neither JNK/SAPK nor p38 MAP kinases bind or activate MKP-3 (38, 39). This suggests a correlation between tight MAP kinase binding and activation of the MKP-3 phosphatase. To test this correlation further, we next assessed the ability of different p38α/ERK1 chimeras to stimulate catalytic activity of purified MKP-3 as assessed by hydrolysis of the artificial substrate p-nitrophenyl phosphate. As anticipated from this relationship, both chimera XI and the p44 ERK1 loop chimera were indistinguishable from p38α and totally ineffective as activators of the MKP-3 phosphatase (Fig. 2A). In contrast, chimeras VIII and VII both elicited weak, but clearly detectable increase in MKP-3 phosphatase activity (Fig. 2A). Chimeras V, III, and I were different again insofar that all three are highly effective stimulators of MKP-3 phosphatase activity and similar to that seen with control purified ERK2 (Fig. 2A). These data indicate

**FIG. 5. Inactivation of p38α/ERK2 chimera by MKP-3.** Purified p38α/ERK1 chimera PIVECTP and EIIPIVE (0.5 μg) were activated by 0.1 μg of MKK6 (ERK2 control was activated by MEK1 S218E,S222E) and incubated with 0.01–10.0 μg of full-length MKP-3. Enzymatic activities of p38α/ERK2 chimera or ERK2 were assessed by phosphorylation in the presence of [γ-32P]ATP of either 10 μg of myelin basic protein (MBP) or 10 μg of GST-ATF-2-(19–96) as indicated. Autoradiogram shows substrate phosphorylation following separation with SDS-polyacrylamide gel electrophoresis using a 12% gel and is representative of two separate experiments.

**FIG. 6. Schematic representation of p38/ERK chimeras.** The chimeras are denoted using the nomenclature employed in the original reports describing these molecules (40, 41). Sequence derived from ERK and p38 are represented by gray and white boxes, respectively. Chimeras Chim I-XI comprise COOH-terminal ERK1 sequence up to and including kinase subdomain I (Chim I), subdomain III (Chim III), subdomain V (Chim V), subdomain VII (Chim VII), subdomain VIII (Chim VIII), and subdomain XI (Chim XI) with p38α residues constituting the remaining NH₂ terminus. Chimera EII contains NH₂-terminal ERK2 residues up to subdomain II and p38α sequence from subdomain III to the COOH terminus. Chimera EIIPIVE comprises ERK2 sequence except for subdomains III and IV that are from p38α. Chimera PIVECTP contains p38α sequence constituting the NH₂ terminus up to subdomain IV as well as the COOH-terminal tail (L16) with the remaining sequence from ERK2. The black line indicates the ATP-binding site whereas TxY represents the phosphorylation site motif localized within the activation loop. Also indicated is a summary of the data shown in Figs. 1–5 on MKP-3 binding, MKP-3 catalytic activation, and the sensitivity of each p38/ERK chimera to inactivation by MKP-3. Values assigned range from not detectable (−) to a value equivalent to that obtained with ERK (+++). ND, not done.
a correlation between binding and catalytic activation of MKP-3 by the p38α/ERK1 chimeras.

To assess further the importance of ERK kinase subdomains in activating MKP-3, we employed some additional chimeras made using sequence from p38α and ERK2 (41). Chimera PIVECTP is similar to chimera V except that it also possesses the COOH-terminal loop L16 of p38α (see Fig. 6 for an illustration of these chimeras). This chimera was designed so that the NH2- and COOH-terminal major structural domains are contributed by p38α or ERK2 sequences, respectively (41). Chimera PIVECTP stimulates powerful activation of MKP-3 and to an extent similar to chimera V (Fig. 2B). Since chimeras V and III stimulate similar activation of MKP-3 (Fig. 2A) the role of ERK kinase subdomains II and III in this activity is unclear. Chimera EIIPIVE is constituted mainly of ERK2 sequence although kinase subdomains III and IV have been substituted with cognate residues from p38α. This chimera stimulates MKP-3 activation in a manner indistinguishable from chimera PIVECTP and chimera V (Fig. 2B). Additional control peptides, QKGMMPMDLPLPSLL (A, open squares), QMGMMPMDLPLPSLL (A, solid squares), MMPRAPAKLSQGPS (B, open squares), and MMPMAPAMLPSQGPS (B, solid squares) in which underlined Met replace charged residues are also ineffective at inhibiting ERK-dependent activation of MKP-3. Data points are the mean of triplicate determination and representative of three separate experiments.

FIG. 7. ERK-stimulated MKP-3 phosphatase inhibition by Elk-1 peptides. Purified MKP-3 (5 μg) was incubated with ERK2 (5 μg) and phosphatase activity measured as described in the legend to Fig. 2. Incubation in the presence of peptide QRKPRDLPLPSLL (Elk-1 amino acids 312–328) (A) or RRPRAPAKLSQGPS (Elk-1 amino acids 387–399) (B) up to 200 μM (open circles) results in an inhibition of ERK-dependent phosphatase activity. Control peptides in which residues (underlined) important for ERK binding have been altered, QKGMMPMDLPLPSLL (A) and RRPRAPAKLSQGPS (B), display reduced inhibition of MKP-3 (solid circles). Additional control peptides, QKGMMPMDLPLPSLL (A, open squares), QMGMMPMDLPLPSLL (A, solid squares), MMPRAPAKLSQGPS (B, open squares), and MMPMAPAMLPSQGPS (B, solid squares) in which underlined Met replace charged residues are also ineffective at inhibiting ERK-dependent activation of MKP-3. Data points are the mean of triplicate determination and representative of three separate experiments.

FIG. 8. ERK-stimulated MKP-3 phosphatase inhibition by the p90<sup>rsk</sup> COOH-terminal peptide 700–724. Purified MKP-3 (5 μg) was incubated with ERK2 (5 μg) and phosphatase activity measured as described in the legend to Fig. 2. Bars show basal (open) and ERK-stimulated (filled) MKP-3 phosphatase activity. Incubation in the presence of a synthetic peptide TPQLKPIESSLLAQRRVRKLPSTTL (p90<sup>rsk</sup> 700–724) up to 200 μM (open circles) inhibits MKP-3 phosphatase activity. A control peptide (filled circles) where residues critical for p90<sup>rsk</sup> binding to ERK (underlined) were exchanged for ASQGA displayed greatly reduced inhibition of the ERK2-activated MKP-3 phosphatase. Points are the mean of triplicate determination and representative of three separate experiments.

MKP-3 Interaction Domains on ERK MAP Kinase

24617
This, the peptide LEQYYDPSDPIAE (Erk2 311–324) representing the CD domain of ERK2 inhibits activation of MKP-3 by purified ERK2 (Fig. 3). This inhibition supports the notion that the CD domain within the COOH-terminal structural lobe of ERK contributes to the binding and catalytic activation of MKP-3. Moreover, chimera XI possessing the COOH-terminal of ERK was observed to bind MKP-3 albeit weakly (Fig. 1B).

Notwithstanding this, our data from p38/ERK chimeras indicate that multiple domains within the COOH-terminal major structural lobe of ERK together provide important surfaces for binding and catalytic activation of the DSP MKP-3.

**MKP-3 Inactivation of p38/ERK Chimeras**—Specific MKP-3 catalytic activation by ERK appears to account for the MAP kinase selectivity of this DSP (38, 39). This model predicts that p38/ERK chimeras should display differential inactivation by MKP-3. To test this, chimeras I-XI were activated by an appropriate upstream MAP kinase kinase (either MEK1 or MKK6) and incubated in the presence of different concentrations of purified GST-MKP-3. Chimeric catalytic activity was measured by 32P-phosphorylation of either myelin basic protein or GST-ATF2 as indicated. While chimeras V, III, and I appear sensitive to inactivation by low concentrations of MKP-3, chimera...
MKP-3 Interaction Domains on ERK MAP Kinase

VIII and particularly chimera XI and p38α are all relatively resistant to this DSP (Fig. 4). As predicted by their ability to stimulate phosphatase activity, chimeras PIVECTP and EIIP-IVE were both highly sensitive to inactivation by low concentrations of MKP-3 (Fig. 5). Chimeras VII and EIIP as well as the p44 loop chimera displayed catalytic activities too low for accurate analysis of MKP-3-dependent inactivation. Together, these results demonstrate a correlation between MAP kinase chimera binding, phosphatase activation, and kinase inhibition by MKP-3 and these observations are summarized schematically in Fig. 6.

**ERK Substrate-binding Domains Mediate MKP-3 Activation**—A previous study using the same p38α/ERK1 chimeras revealed that ERK substrate recognition occurs within regions COOH-terminal to and including kinase subdomain V. Thus, chimera V was shown to display a shift from p38- to ERK-like substrate specificity as indicated by phosphorylation of Myc, binding and activation of p90rsk, and increased transcription by the fos promoter (40). Together with data reported here, this suggests an overlap between ERK regions responsible for substrate recognition and those important for binding and activation of MKP-3. To investigate this further we employed synthetic peptides corresponding to docking sites identified within selected ERK substrates. Elk1 is a member of the ternary complex factor subfamily of ETS-domain transcription factors that binds ERK though two distinct docking sites known as the D-domain and the FXFP motif (also termed DEJL and DEF motifs, respectively). ERK binding to these targeting domains is essential for efficient Elk-1 phosphorylation (43, 44). To test the importance of ERK substrate-binding domains for MKP-3 activation we employed two peptides, QKGRKPRLDEPLPSLL (Elk-1 amino acids 312–328) and RRPRAPKLSFPFS (Elk-1 amino acids 387–399) which encompass, respectively, the D-domain and FXFP motif and which have both been shown to inhibit substrate phosphorylation by ERK (43, 44). Both peptides elicit a dose-dependent inhibition of MKP-3 catalytic activation by ERK2 (Fig. 7, A and B). Control peptides in which critical residues (underlined) have been altered (QKGRKPRDAEAPLPSLL and RRPRAPKLSATAPS) and which are less effective at blocking ERK-dependent substrate phosphorylation (43, 44) also display a similarly reduced inhibition of MKP-3 activity (Fig. 7, A and B).

Positively charged amino acids appear critical for binding to ERK (42). Consistent with this, we find that mutant peptides QKGMMPRDLEPFSLL and QMGMPMDLEPLPSLL (Elk-1 amino acids 312–328; underlined Met replace charged residues) as well as MMPRAPKLSFPFS and MMPMAMPSLFSFPFS (Elk-1 amino acids 312–328; underlined Met replace charged residues) are ineffective at inhibiting ERK-dependent activation of MKP-3 (Fig. 7, A and B). Together these observations indicate that regions within ERK responsible for binding to the Elk-1 D-domain and the FXFP motifs, as well as adjacent charged amino acids within this substrate protein, play an important role in mediating ERK-dependent catalytic activation of MKP-3.

The p90 ribosomal S6 protein kinase-1 (RSK1, p90rsk) is an additional well characterized MAP kinase substrate that is activated following its binding and phosphorylation by ERK. Recently, a docking site within the COOH-terminal 25 amino acids of p90rsk was found to bind ERK and to be essential for p90rsk phosphorylation and activation (45–47). To test whether MKP-3 interacts with ERK at sites overlapping with those responsible for ERK binding to this substrate, we tested a synthetic peptide corresponding to the COOH terminus of p90rsk. Peptide TPQLKPIESSILAQRRVRKLPSTTL (p90rsk 700–724) was found to elicit dose-dependent and complete blockade of ERK-stimulated MKP-3 (Fig. 8). The motif LAXRR (underlined) within this sequence was shown to be critical for ERK binding to p90rsk (45) and consistent with this, a control peptide (where LAQR was changed to ASQGA) failed to elicit significant inhibition of the ERK2-activated MKP-3 phosphatase (Fig. 8). A shorter COOH-terminal p90rsk peptide LPKPIESSILAQRRVRK (p90rsk 703–718), but not its control (underlined residues exchanged for ASQGA), also inhibits activation of MKP-3 by ERK2 with similar potency (not shown).

**Fig. 10. Inhibition of PIVECTP-stimulated MKP-3 by the p90rsk COOH-terminal peptide 703–718**. Purified MKP-3 (5 μg) was incubated with chimera PIVECTP (5 μg) and phosphatase activity measured as described in the legend to Fig. 2. Incubation in the presence of the p90rsk COOH-terminal peptide LPKPIESSILAQRRVRK (p90rsk 703–718) at concentrations up to 200 μM inhibits activation of MKP-3 by PIVECTP (filled circles). Control peptide (open circles) with residues important for p90rsk binding to ERK (underlined) exchanged for ASQGA displayed reduced inhibition of ERK2-activated MKP-3 phosphatase activity. Data points are the mean of triplicate determination and representative of two separate experiments.

**Fig. 11. Mutant MKP-3 R64A,R65A is insensitive to phosphatase activation by ERK2**. Wild type MKP-3 or mutant MKP-3 R64A,R65A (10 μg) were incubated with ERK2 (10 μg) and phosphatase activity measured as described in the legend to Fig. 2. In contrast to wild type MKP-3, MKP-3 R64A,R65A displayed little or no increase in phosphatase activity in the presence of ERK.
The COOH terminus of p90\textsuperscript{rsk} binds ERK but not JNK/SAPK or p38 MAP kinases (46) suggesting that the effects of this peptide sequence should be specific for ERK. To test this we employed MKP-4 (34), an additional DSP gene family member which although closely related to MKP-3 is subject to activation by ERK, JNK/SAPK, and p38 MAP kinases (39). Consistent with an ERK-specific action, the p90\textsuperscript{rsk} peptide 700–724 was found to inhibit MKP-4 activation by ERK2 but not by JNK3 or p38a (Fig. 9). Since our data with chimeric p38/ERK molecules demonstrates an important role for the ERK COOH-terminal structural lobe in activating MKP-3 (see above), we next tested the p90\textsuperscript{rsk} peptides using chimera PIVECTP (Fig. 6). Both peptides inhibit MKP-3 activation by chimera PIVECTP in a manner indistinguishable from their actions on ERK. Fig. 10 illustrates this with an experiment showing does-dependent inhibition of PIVECTP-stimulated MKP-3 by the peptide p90\textsuperscript{rsk} 703–718 and presumably reflects interaction at the COOH-terminal structural lobe of ERK2 that is preserved in this chimeric molecule. Together, these data indicate that COOH-terminal ERK domains important for interaction with docking sites and charged residues within the target substrates Elk-1 and p90\textsuperscript{rsk} are also important for interaction and activation of the DSP MKP-3.

An MKP-3 "Kinase Interaction Motif" Is Responsible for Binding and Activation by ERK—The ERK docking site within the p90\textsuperscript{rsk} COOH-terminal appears to be strictly dependent upon the pentapeptide sequence LAXRR which is also conserved in a number of downstream kinases including Msk1, Msk2, Mk1, and Mnk2. Mutation of these residues abolishes p90\textsuperscript{rsk} binding and phosphorylation by ERK (45). In addition, the PTP family members PTP-SL, STEP, and HepPTP/LC-PTP bind ERK1 and ERK2 through a kinase interaction motif (KIM) which includes within its core the sequence LQERR (48). Mutation of the arginine residues (underlined) within this motif abolishes binding of PTP-SL to ERK MAP kinases (49). Interestingly, examination of the MKP-3 primary amino acid sequence (31) reveals a loosely related pentapeptide sequence IMLRR (amino acids 61–65) localized within the NH\textsubscript{2} terminus. These Arg residues have recently been shown to be important for MKP-3 binding to ERK (42). Consistent with this, the MKP-3 mutant (R65A) and particularly MKP-3 (R64A, R65A) are insensitive to catalytic activation by ERK2 (Fig. 11). This is in contrast to another mutant, MKP-3 (R64A), which undergoes powerful activation by ERK (not shown). As may be anticipated by reciprocal relationship between MKP-3 catalytic activity and MAP kinase inactivation, ERK2 is either resistant (R65A) or totally insensitive (R64A/R65A) to inactivation by these MKP-3 mutants (Fig. 12). Predictably, ERK2 displays similar sensitivity to inactivation by MKP-3 (R64A) as by wild type MKP-3 (Fig. 12). It is of note that wild type and all MKP-3 mutants display similar basal phosphatase activity (not shown) indicating that mutant proteins are all correctly folded and otherwise normal. These observations suggest that as in p90\textsuperscript{rsk}, PTP-SL, STEP, and HepPTP/LC-PTP, a KIM-like domain within the MKP-3 NH\textsubscript{2} terminus also underlies targeted interaction between ERK and MKP-3.

Conclusion—Specific MKP-3 catalytic activation by ERK appears to account for its selectivity between MAP kinase subtypes (39). Consistent with this, experiments presented here demonstrate a correlation between MKP-3 catalytic activation by p38/ERK chimeras and the sensitivity of these MAP kinase variants to inactivation by MKP-3. Together, these studies also indicate that the COOH-terminal major structural lobe of ERK MAP kinases is necessary and sufficient for binding and catalytic activation of the DSP MKP-3. A previous study with these chimeras demonstrated a switch to “ERK-like” substrate specificity when comparing chimera VII with V (40) suggesting an overlap between structural regions important for substrate...
MKP-3 Interaction Domains on ERK MAP Kinase

11. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852

12. Xia, Z., Dickens, M., Raisinga, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331

13. Kirschbaum, J. M., and Greenberg, M. E. (1997) Neuron 18, 839–842

14. Zanke, W. B., Boudreau, K., Rubie, E., Wenning, E., Tibbles, L. A., Zon, L., Kyriakis, J., Liu, F.-F., and Woodgett, J. R. (1996) Curr. Biol. 6, 606–613

15. Ichijo, H., Nishida, E., Irie, K., Dijke, P. T., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyamoto, K., and Gotoh, Y. (1997) Science 275, 93–94

16. Yang, D. K., Duan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) Nature 389, 865–870

17. Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T. D., Davis, R. J., Rincon, M., and Flavell, R. A. (1998) Immunity 9, 575–585

18. Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092–2095

19. Keyse, S. M., and Emalie, E. A. (1992) Nature 353, 644–646

20. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) Cell 75, 487–493

21. Rohan, P. J., Davis, M., Moskaluk, C. A., Kearns, M., Kreutz, H., Siebenlist, U., and Kelly, K. (1993) Science 259, 1763–1766

22. Ward, Y., Gupta, S., Jensen, P., Wartmann, M. D., Davis, R. J., and Kelly, K. (1994) Nature 367, 651–654

23. Guan, K.-L., and Butch, E. (1995) J. Biol. Chem. 270, 7197–7203

24. King, A. G., Ozanne, B. W., Suryan, C., and Ashworth, A. (1996) Oncogene 25, 2553–2563

25. Miura-Pless, A., Rim, C. S., Yao, H., Eberbom, M. S., and Stork, P. J. S. (1995) J. Biol. Chem. 270, 1457–1459

26. Kwak, S. P., and Dixon, J. E. (1995) J. Biol. Chem. 270, 1156–1160

27. Ishibashi, T., Bottaro, D. P., Michieli, P., Kelley, C. A., and Aaronson, S. A. (1994) J. Biol. Chem. 269, 29897–29902

28. Martell, K. J., Seasholtz, A. P., Kwak, S. P., Clemens, K. K., and Dixon, J. E. (1995) J. Neurochem. 65, 1823–1833

29. Theodsiou, A. M., Rodrigues, N. R., Nesbit, M. A., Ambrose, H. J., Paterson, H., McLellan-Arnold, E., Boyd, Y., Leversha, M. A., Owen, N. B., Blake, D. J., Ashworth, A., and Davies, K. E. (1996) Hum. Mol. Genet. 5, 675–684

30. Mourey, R. J., Vega, Q. C., Campbell, J. S., Wenderoth, M. P., Hauschka, S. D., Krebs, E. G., and Dixon, J. E. (1996) J. Biol. Chem. 271, 3795–3802

31. Muda, M., Boschert, U., Dickinson, R., Martinou, J.-C., Martinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996) J. Biol. Chem. 271, 4319–4326

32. Groom, I. A., Sneedon, A. A., Alessi, D. R., Dowd, S., and Keyse, S. M. (1996) EMBO J. 15, 3621–3632

33. Shin, D.-Y., Ishibashi, T., Choi, T. S., Chung, E., Chung, Y. I., Aaronson, S. A., and Bottaro, D. P. (1997) Oncogene 14, 2633–2639

34. Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C., Camps, M., Martinou, I., Ashworth, A., and Arkinstall, S. (1997) J. Biol. Chem. 272, 5141–5151

35. Theodsiou, A., Smith, A., Gillieron, C., Arkinstall, S. and Ashworth, A. (1999) Oncogene 18, 6981–6988

36. Camps, M., Nichols, A., and Arkinstall, S. (2000) FASEB J. 14, 6–16

37. Muda, M., Theodsiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A., and Arkinstall, S. (1996) J. Biol. Chem. 271, 27205–27209

38. Muda, M., Theodsiou, A., Gillieron, C., Smith, A., Chabert, C., Camps, M., Antonsson, B., Muda, M., Chabert, C., and Boschert, U., and Arkinstall, S. (1998) Science 280, 1262–1265

39. Brunet, A., and Pouyssegur, J. (1996) Science 272, 1652–1655

40. Wibraber, J. L., Goldsmith, E. J., and Cobb, M. H. (1999) J. Biol. Chem. 274, 16988–16994

41. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) Nature Cell Biol. 2, 110–116

42. Yang, S.-H., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) EMBO J. 17, 1740–1749

43. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfield, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 163–175

44. Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Kang, B. E., and Karin, M. (1994) Nature Genes 18, 377–378

45. Keyse, S. M., and Ginsburg, M. (1993) Trends Biochem. Sci. 18, 377–378