Docking sites on mitogen-activated protein kinase (MAPK) kinases, MAPK phosphatases and the Elk-1 transcription factor compete for MAPK binding and are crucial for enzymic activity

A. Jane BARDWELL, Mahsa ABDOLLAHI and Lee BARDWELL[1]

Department of Developmental and Cell Biology, 5207 McGaugh Hall, University of California, Irvine, CA 92697-2300, U.S.A.

Mitogen-activated protein kinase (MAPK) cascades control gene expression patterns in response to extracellular stimuli. MAPK/ERK (extracellular-signal-regulated kinase) kinases (MEKs) activate MAPKs by phosphorylating them; activated MAPKs, in turn, phosphorylate target transcription factors, and are deactivated by phosphatases. One mechanism for maintaining signal specificity and efficiency is the interaction of MAPKs with their substrates and regulators through high-affinity docking sites. In the present study, we show that peptides corresponding to the MAPK-docking sites of MEK1, MEK2, Ste7, Elk-1 and MAPK phosphatase (MKP)-2 potently inhibit MEK2 phosphorylation of ERK2, ERK2 phosphorylation of Elk-1, and MKP-1 dephosphorylation of ERK2. Each peptide inhibited multiple reactions; for example, the MEK2 peptide inhibited not only MEK2, but also ERK2 and MKP-1. In addition, these docking-site peptides inhibited MEK2–ERK2 binding. The MAPK-docking site of MEK1 also potently stimulated ERK2-mediated phosphorylation of a target site on the same peptide. Control peptides with mutations of conserved basic and hydrophobic residues of the MAPK-docking site consensus lacked biological activity. We conclude that MEKs, MKPs and the Elk-1 transcription factor compete for binding to the same region of ERK2 via protein–protein interactions that are crucial for kinase/phosphatase activity.

Key words: docking-site peptide, Elk-1, extracellular-signal-related kinase (ERK), MAPK phosphatase (MKP), MAPK/ERK kinase (MEK), mitogen-activated protein kinase (MAPK).

INTRODUCTION

Eukaryotic cells respond to developmental cues, regulatory signals and environmental stimuli with diverse changes in gene expression. Many of these responses are orchestrated by a network of mitogen-activated protein kinase (MAPK) signalling pathways [1,2]. At least four distinct MAPK cascades have been characterized in mammalian cells: the extracellular-signal-regulated kinase (ERK)1/ERK2, ERK5, c-Jun N-terminal kinase (JNK) and p38 pathways. These cascades contain three sequentially acting components: MEKKs [MEK (MAPK/ERK kinase) kinases or MAPK kinase kinases], MEKs [or MAPK kinases (MKKs)] and MAPKs. Upon activation, MEKKs phosphorylate and thereby activate MEKs, which in turn phosphorylate and thereby activate MAPKs. Activated MAPKs phosphorylate protein kinases (MAPK-activated protein kinases or MAPKAPKs), transcription factors and other proteins, leading to changes in gene expression and cell physiology. Activated MAPKs are themselves targets for dephosphorylation by serine/threonine phosphatases, tyrosine phosphatases and dual-specificity MAPK phosphatases [3].

Precise regulation of protein-kinase networks is critical in order for cells to respond appropriately to signals in their environment. Furthermore, signal-transduction-pathway deregulation corresponds to a number of disease states, particularly cancer. Hence elucidating the mechanisms by which signal fidelity and efficiency are maintained may enable the development of novel therapeutics with increased specificity, and consequently, reduced side effects. However, understanding the regulation of MAPK signalling cascades is complicated by the finding that a diverse range of signals and responses utilize the same MAPK pathways, and that there is a high degree of sequence similarity between many of the protein kinases involved.

One mechanism that influences specificity, as well as efficiency, is the interaction of protein kinases with their substrates and regulators through high-affinity protein–protein docking sites [4–7]. These interactions are distinct from the transient interactions that occur between the active site of a protein kinase and the phosphoacceptor site in its substrate. A number of studies have demonstrated a role for regions that contain known or putative MAPK-docking sites in the binding of MAPKs to upstream MEKs [8–15], scaffold proteins [16,17], transcription-factor targets [18–25], protein kinase targets [13,26,27], phosphatases [13,28–37] and other enzymes [38,39].

A MAPK-docking site that is involved in the association of many MAPK-binding proteins to their cognate MAPKs contains the conserved consensus amino acid sequence $(Arg/Lys)_2$-$(Xaa)_{2-6}$-Leu/Ile-Xaa-Leu/Ile [4,6]. In the present study, we have used synthetic peptides corresponding to the MAPK-docking-site regions in upstream MEKs, the target transcription factor Elk-1 and the dual-specificity MAPK phosphatase MKP-2, as competitive inhibitors in order to investigate the role of these docking sites in ERK2 binding. We have also used the same peptides as competitive inhibitors of MEK2 phosphorylation of ERK2, ERK2 phosphorylation of Elk-1 and MKP-1 dephosphorylation of ERK2, in order to examine the importance of the MAPK-docking sites on enzymic activity. Finally, we used a peptide-array-based assay to investigate whether a MAPK-docking site could influence ERK2-mediated phosphorylation of a target site on the same peptide. Using these approaches, we

Abbreviations used: CD, conserved docking; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MKK, MAPK kinase; MKP, MAPK phosphatase.

[1] To whom correspondence should be addressed (e-mail bardwell@uci.edu).

show that upstream MEKs, inactivating phosphatases and downstream phosphorylation substrates can compete with each other for binding to the same protein-binding site on ERK2, and that these protein–protein interactions play a critical role in the catalytic activity of the kinases and phosphatases involved.

EXPERIMENTAL

Kinase assays

Kinase reactions (20 μl) for MEK2 phosphorylation of ERK2 contained kinase-assay buffer [50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol], 1 μM (840 ng) inactive mouse ERK2 ($Lys^{52} \rightarrow Arg$ mutation; New England Biolabs), 0.1 unit (approx. 40 ng) of active, full-length human glutathione S-transferase (GST)–MEK2 (Upstate Biotechnology), 50 μM ATP, 1 μCi of [γ-^{32}P]ATP, and the indicated concentration of peptide. The $Lys^{52} \rightarrow Arg$ mutation in ERK2 lies within the ATP-binding pocket, rendering it catalytically inactive. Thus phosphorylation of ERK2 by MEK2 did not result in activated ERK2 that could autophosphorylate or back-phosphorylate MEK2 in the assays. ERK2 and peptide were pre-incubated in buffer for 10 min at 37 °C, then returned to ice before the addition of ATP and MEK2. Reactions were for 20 min at 30 °C. ERK2 phosphorylation was quantified by SDS/PAGE (12% gels) followed by analysis of relative incorporation using a PhosphorImager™ (Molecular Dynamics, Inc.).

Kinase reactions (20 μl) for ERK2 phosphorylation of Elk-1 contained kinase-assay buffer (see above), 1 μM (820 ng) of GST–Elk-1 (a fusion protein consisting of residues 307–428 of human Elk-1 fused to GST; New England Biolabs), 10 units (approx. 1 ng) of active mouse ERK2 (New England Biolabs), 50 μM ATP, 1 μCi of [γ-^{32}P]ATP, and the indicated concentration of peptide. Reactions were for 20 min at 30 °C. Reactions were analysed and quantified as above, except that 10% gels were used.

Protein-binding assays

The constructs and methods used in the GST co-sedimentation ('pull-down') experiments shown in Figure 4 have been described elsewhere [14]. The indicated concentrations of peptides were added to the GST–glutathione–Sepharose or GST–ERK2–glutathione–Sepharose beads prior to the addition of the radiolabelled protein.

Dephosphorylation assays

Phosphatase reactions (20 μl) for MKP-1 dephosphorylation of ERK2 contained 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 25 nM (approx. 21 ng) active mouse ERK2, 0.5 unit (approx. 7 ng) of active, full-length human GST–MKP-1 (Upstate Biotechnology) and the indicated concentration of peptide. Reactions were for 30 min at 30 °C. Reactions were analysed by SDS/PAGE (12%) followed by Western transfer and immunostaining with anti-(phospho-ERK2) antibody (New England Biolabs).

Peptides and peptide-array assay

Soluble peptides used in this study (see Figure 1) were synthesized by United Biochemical Research, Inc. (Seattle, WA, U.S.A.). Custom synthesis of the peptide arrays used in this study was performed by the ResGen division of Invitrogen Corp. (Carlsbad, CA, U.S.A.). For a recent review of this technology, see [40]. Typically, an approx. 13 cm^2 membrane containing 16 peptide spots was used in an experiment. The membrane was first blocked by incubation with 0.4 ml of kinase-assay buffer (see above) containing 1 mg/ml BSA and 50 μM ATP. The blocking solution was layered directly on to each peptide spot, approx. 20 μl/spot. After 15 min at 30 °C, this solution was removed completely by aspiration and the membrane was then incubated for 30 min at 30 °C with 0.4 ml of a mixture containing 0.5 unit/μl active ERK2 in kinase-assay buffer containing 0.1 mg/ml BSA, 50 μM ATP and 0.05 μCi/μl [γ-^{32}P]ATP. The membrane was then washed four times for 5 min in PBS containing 5 mM EDTA and 0.1% (v/v) Tween 20, allowed to dry, and quantified on a PhosphorImager.

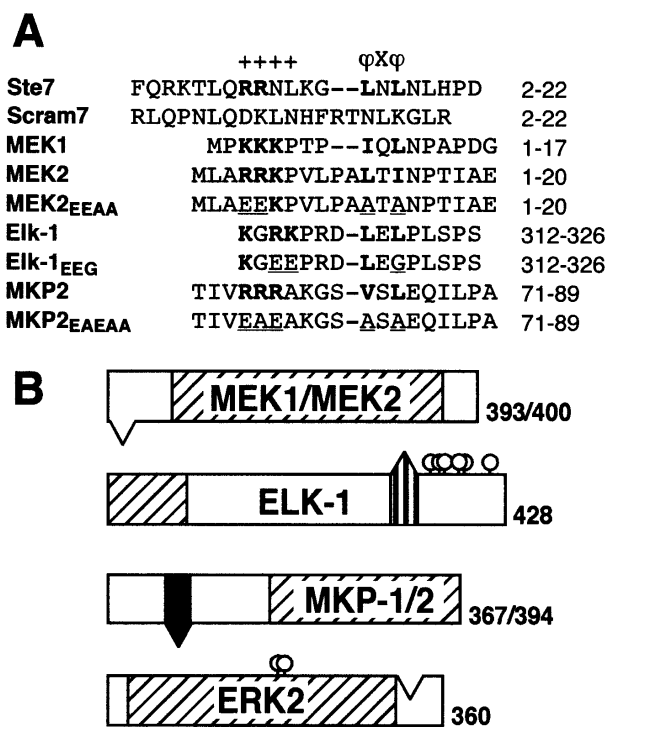

Figure 1 Peptides used in inhibition assays and diagrammatic representation of the full-length proteins from which they are derived

(**A**) Amino acid sequence of all the peptides used. Conserved MAPK-docking-site residues are in bold and marked as either basic (+) or hydrophobic (φ), and mutated residues are underlined. Dashes denote gaps inserted to optimize the alignment. The corresponding residues of the full-length proteins are given on the right. (**B**) Diagrammatic representation of the full-length proteins from which the synthetic peptides are derived. Proteins are shown left to right, N- to C-terminus. Hatched boxes represent the catalytic domains in MEK1/MEK2, MKP-1/MKP-2 and ERK2, and the DNA-binding domain in Elk-1. The phosphoacceptor sites in ERK2 and Elk-1 are shown as circles. The MAPK-docking site is shown as a protruding triangular region on MEK1/MEK2, Elk-1 and MKP-1/MKP-2. The number of amino acid residues in each polypeptide is given on the right.

RESULTS

Peptides containing the MAPK-docking sites of MEK1, MEK2 and Ste7 inhibit MEK2 phosphorylation of ERK2

Several studies have demonstrated the presence of MAPK-docking sites in the N-terminal regions of MEKs that are important for both high-affinity binding to, and phosphorylation of, target MAPKs [8–14]. The core of these docking sites is an amino acid sequence with the consensus $(Arg/Lys)_2$-$(Xaa)_{2-6}$-Leu/Ile-Xaa-Leu/Ile [4–6]; mutation of either the basic or hydrophobic residues in this motif results in impaired MAPK

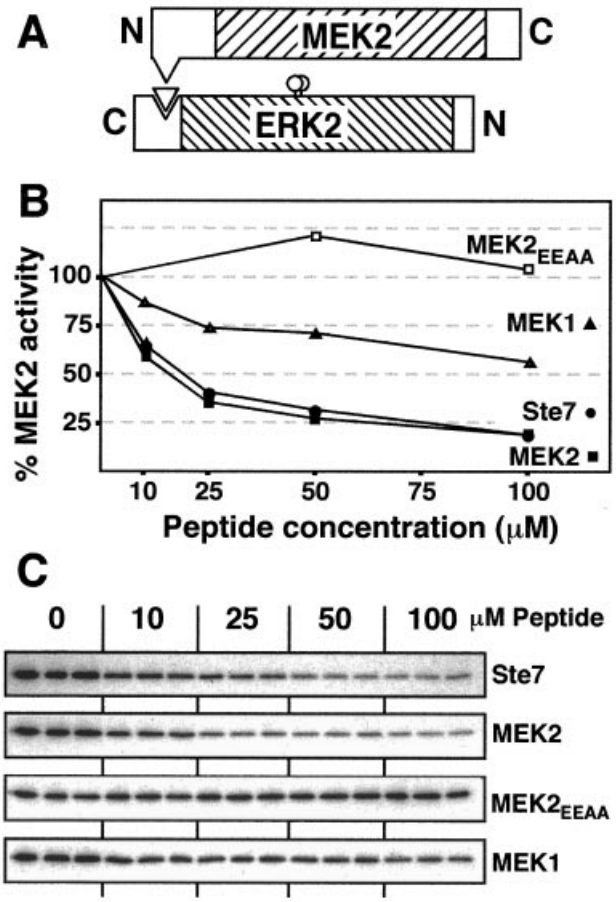

Figure 2 Inhibition of MEK2-dependent phosphorylation of ERK2 by MEK peptides

(**A**) Schematic representation of the experiment: MAPK-docking-site peptides (triangle) were used to inhibit MEK2 phosphorylation of ERK2. The N- and C-terminus of each protein is indicated. (**B**) and (**C**) Purified, catalytically inactive ERK2 (1 μM) was incubated with purified active MEK2 (approx. 30 nM) and [γ-^{32}P]ATP for 20 min, in the absence or presence of the specified concentrations of the indicated peptides. (**B**) Results plotted as percentage phosphorylation relative to that observed in the absence of any added peptide. ERK2 phosphorylation was analysed by SDS/PAGE (12% gels) and quantified on a PhosphorImager. Results are the mean of two to four experiments, with triplicate data points in each experiment. (**C**) Autoradiogram of a representative experiment.

binding and activation [12–15]. Furthermore, synthetic peptides corresponding to this MAPK-docking-site region in MEKs can inhibit MEK1, MKK3 or MKK6 binding to, and activation of, their cognate MAPKs [12–15], presumably because the peptides are able to compete with the MEKs for binding to MAPKs.

Previously, we have shown that the MAPK-docking sites in MEK1 and MEK2 proteins are both necessary and sufficient for the formation of stable protein complexes with ERK1 and ERK2 [14]. Upon activation, MEK1 and MEK2 activate ERK1 and ERK2 by dual phosphorylation of the threonine and tyrosine residues within a Thr-Glu-Tyr motif. In order to examine the importance of the MAPK-docking site in MEK2 for its ability to phosphorylate ERK2, synthetic peptides corresponding to the MAPK-docking sites of three different MEKs were tested for their ability to inhibit MEK2 phosphorylation of ERK2. The peptides used were 17–21 residues in length, and have been previously shown to inhibit MEK1 phosphorylation of ERK2 [14]. The sequences of all the peptides used in this study are shown in Figure 1(A); schematics of the corresponding proteins demonstrating the position of the docking sites are shown in Figure 1(B).

Kinase assays contained active MEK2 and inactive ERK2 as substrate. When peptides corresponding to the MAPK-docking sites of MEK1, MEK2 or Ste7 were titrated into the MEK2 kinase assays, a dose-dependent inhibition of ERK2 phosphorylation was observed (Figure 2). Thus the MAPK-docking site of MEK2 is not only important in binding to ERK2 [14], but also MEK2–ERK2 interaction via this site facilitates ERK2 phosphorylation. In contrast, a MEK2-mutant peptide ($MEK2_{EEAA}$), in which two of the basic residues (Arg^4 and Arg^5) and two of the hydrophobic residues (Leu^{12}, Ile^{14}) were mutated to two glutamic acid and two alanine residues respectively (Figure 1A), did not inhibit the ability of MEK2 to phosphorylate ERK2 (Figure 2). This confirms the requirement for these conserved residues for efficient ERK2 interaction.

MEK1 and MEK2 are closely related proteins, both of which target ERK1 and ERK2 in cells. Intriguingly, the docking sites of MEK1 and MEK2 show less sequence conservation than the rest of these proteins. The IC_{50} of the MEK1 and MEK2 peptides used in these experiments was approx. 100 and 20 μM respectively (Figure 2B). This indicates that the MEK2–ERK docking interaction is stronger than the MEK1–ERK docking interaction, consistent with our previous results [14]. Ste7 is a MEK that phosphorylates the MAPKs Kss1 and Fus3 in the yeast *Saccharomyces cerevisiae*, and was the first MEK found to contain a MAPK-docking site [4,8]. Interestingly, the Ste7 peptide is a very effective inhibitor of ERK2 phosphorylation by MEK2, with an IC_{50} of approx. 20 μM (Figure 2B).

MEK peptides, like the Elk-1 peptide, inhibit ERK2 phosphorylation of Elk-1

Activated MAPKs bind to and phosphorylate target transcription factors, MAPKAPKs, and other proteins. A docking site region (the 'D-domain') that fits the consensus $(Arg/Lys)_2$-$(Xaa)_{2-6}$-Leu/Ile-Xaa-Leu/Ile has been identified in the ERK transcription-factor target Elk-1 [19,20,23]. The finding that MEKs and transcription-factor targets contain MAPK-docking sites with similar sequences (Figure 1) suggests that MEKs and downstream transcription factors may compete for binding to the same protein interaction site on ERKs. To test this possibility, the MEK1, MEK2 and Ste7 peptides, as well as a peptide containing the MAPK-docking site in Elk-1 (Figure 1A), were assessed for their ability to inhibit phosphorylation of GST–Elk-1 by active ERK2 (Figure 3). Elk-1 is phosphorylated by ERK2 on at least six sites in its transcriptional activation domain [41], resulting in a ladder of bands displaying retarded electrophoretic mobility (Figure 3C); peptide-dependent inhibition was revealed both by the progressive collapse of this ladder and by decreased overall phosphate incorporation (Figure 3B).

The Elk-1 peptide inhibited ERK2 phosphorylation of Elk-1 in a dose-dependent manner (IC_{50} of approx. 20 μM), presumably because it was an effective competitor of Elk-1 for binding to ERK2. In contrast, a mutant Elk-1 peptide in which two of the basic residues (Arg^{314}, Lys^{315}) and one of the hydrophobic residues (Leu^{321}) of the MAPK-docking site consensus sequence were replaced by two glutamic acid and one glycine residue respectively, was unable to inhibit ERK2 phosphorylation of Elk-1. Strikingly, the MEK1, MEK2 and Ste7 peptides, with IC_{50} values of < 15, < 10 and < 5 μM respectively, were even more effective inhibitors of Elk-1 phosphorylation by ERK2 than the Elk-1 peptide was itself (Figure 3). The $MEK2_{EEAA}$ control peptide did not inhibit ERK2 phosphorylation of Elk-1,

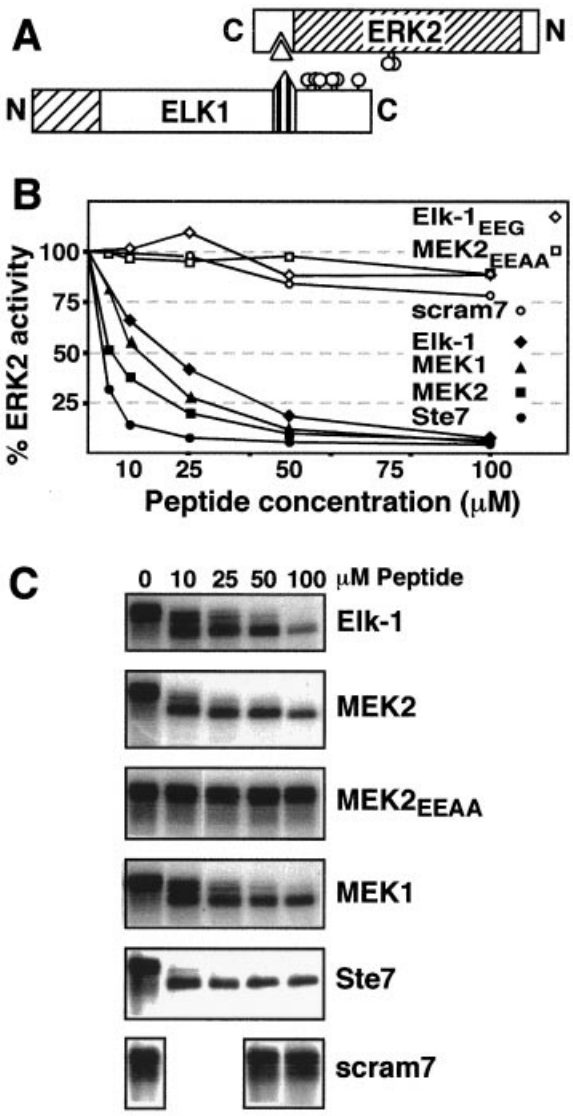

Figure 3 Inhibition of ERK2-dependent phosphorylation of Elk-1 by MEK and Elk-1 peptides

(**A**) Schematic representation of the experiment: MAPK-docking-site peptides (triangle) were used to inhibit ERK2 phosphorylation of Elk-1. The N- and C-terminus of each protein is indicated. (**B**) and (**C**) Purified GST–Elk-1 (1 μM) was incubated with purified, active ERK2 (approx. 1 nM) and [γ-^{32}P]ATP for 20 min, in the absence or presence of the specified concentrations of the indicated peptides. (**B**) Results plotted as percentage phosphorylation relative to that observed in the absence of any added peptide. Elk-1 phosphorylation was analysed by SDS/PAGE (10% gels) and quantified on a PhosphorImager. The fastest migrating band [see (**C**)], which appears unaffected by peptide inhibition, was omitted from the quantification. Results are the mean of three to eleven experiments, with triplicate data points in each experiment. (**C**) Autoradiogram of a representative experiment. Only one of the triplicate points is shown.

just as it was unable to inhibit MEK2 phosphorylation of ERK2. Similarly, a peptide consisting of a scrambled version of the Ste7 sequence (scram7) was not inhibitory (Figure 3). At least one of the bands (the fastest migrating species in Figure 3C) was resistant to peptide inhibition, suggesting that not all target sites were equally sensitive to inhibition of docking. This conclusion is supported by another recent study [23].

The Elk-1 peptide inhibits MEK2 phosphorylation of ERK2

If the MAPK-docking sites on MEK2 and Elk-1 compete for binding to ERK2, then the Elk-1 peptide might also compete with MEK2 for binding to ERK2, and thereby inhibit MEK2 phosphorylation of ERK2. In fact, when the Elk-1 peptide (see Figure 1A) was titrated into a MEK2 kinase assay using inactive ERK2 as substrate, it inhibited MEK2 phosphorylation of ERK2 in a dose-dependent manner (IC_{50} of approx. 40 μM; results not shown). In contrast, the Elk-1_{EEG} mutant peptide showed no inhibitory activity whatsoever, even at a concentration of 100 μM (results not shown).

Docking-site peptides inhibit binding of MEK2 to ERK2

In order to demonstrate directly that the peptides were inhibiting stable protein-complex formation between MEK2 and ERK2, a series of binding assays were performed. The ability of radiolabelled, *in vitro* translated, full-length MEK2 protein to co-sediment with GST–ERK2 bound to glutathione–Sepharose beads was tested in the presence or absence of the various MAPK-docking-site peptides. As we have previously observed [14], MEK2 protein co-sedimented with GST–ERK2 in the absence of peptide inhibitors (Figure 4); this binding was specific because precipitation of MEK2 protein did not occur when GST was used instead of the GST–ERK2 fusion protein, regardless of whether or not peptides were included in the reactions (Figure 4 and results not shown). When the Ste7, MEK2, MEK1 or Elk-1 peptides were added to the reactions, they all inhibited the amount of MEK2 protein that co-sedimented with GST–ERK2 (Figure 4). Thus the MEK1, MEK2, Ste7 and, to a lesser extent, Elk-1 peptides are all able to compete with MEK2 protein for binding to ERK2.

The Ste7 and MEK2 peptides were the most potent inhibitors of MEK2 binding to GST–ERK2 (Figure 4). This correlates well with the finding that these peptides were also the strongest inhibitors of MEK2 phosphorylation of ERK2 (Figure 2). In fact, at 25 μM, the Ste7 peptide inhibited co-sedimentation of MEK2 with ERK2 to a similar degree as did complete removal of the docking site from MEK2 (compare with MEK2Δ4–16 protein, which lacks residues 4–16). In contrast, the scram7, $MEK2_{EEAA}$ or Elk-1_{EEG} control peptides did not inhibit co-sedimentation of MEK2 protein with GST–ERK2. The correlation between the ability of the peptides to block binding and to inhibit enzymic activity strongly suggests that the enzymic inhibition is a consequence of the blocking of docking.

A docking-site peptide from MKP-2 inhibits MEK2 phosphorylation of ERK2 and ERK2 phosphorylation of Elk-1

MKPs, a class of dual-specificity phosphatases that act on MAPKs, remove phosphate groups from both the threonine and tyrosine residues in the Thr-Xaa-Tyr motif [3]. Some MKPs, such as MKP-3, MKP-4, MKP-5 and MKP-7, are located predominantly in the cytoplasm, whereas MKP-1 and MKP-2 are inducible nuclear enzymes [3]. Several of the MKPs have been shown to contain a MEK-like docking site important for MAPK binding [13,29,33–37].

The MAPK-docking site in either MKP-1 or MKP-2 has been shown to be critical for optimal ERK2 binding, and mutation of this site also severely compromises ERK2-dependent activation of the catalytic activity of MKP-1 and MKP-2 [33,35]. Since the MAPK-docking site in these MKPs roughly fits the same consensus as those in the MEKs, it is possible that these ERK-

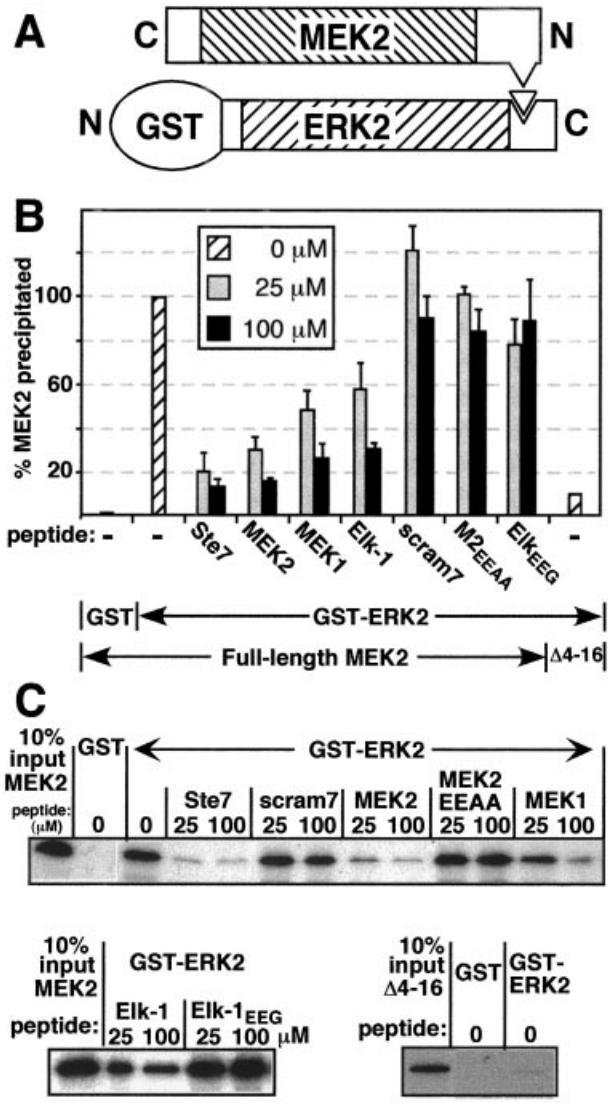

Figure 4 Inhibition of MEK2 binding to GST-ERK2 by MEK and Elk-1 peptides

(**A**) Schematic representation of the experiment: MAPK-docking-site peptides (triangle) were used to inhibit MEK2 binding to GST–ERK2. The N- and C-terminus of each protein is indicated. (**B**) and (**C**) ^{35}S-labelled MEK2 or MEK2$_{\Delta 4-16}$ proteins (approx. 1 pmol) were prepared by *in vitro* translation, partially purified by ammonium sulphate precipitation, and then incubated with 10 μg of purified GST or GST–ERK2 pre-bound to glutathione–Sepharose beads, in the absence or presence of the specified concentrations of the indicated peptides. Bead-bound protein complexes were isolated by sedimentation and resolved by SDS/PAGE (12% gels). Gels were analysed by staining with Coomassie Blue for visualization of the bound GST fusion protein in order to verify equal amounts in each reaction (results not shown) and by autoradiography for visualization of the bound radiolabelled proteins. Relative binding was determined using a PhosphorImager. (**B**) Relative binding of radiolabelled MEK2 or MEK2$_{\Delta 4-16}$ to GST or GST–ERK2 in the absence of peptide (hatched bars), or in the presence of 25 μM (light grey bars) or 100 μM peptide (black bars). Results are the means $\pm$ S.E.M. of three to five experiments. (**C**) Autoradiogram of a representative experiment. A portion of the *in vitro* translated MEK2 or MEK2$_{\Delta 4-16}$ proteins corresponding to 10% of the amount added to the binding reactions is shown in the left-hand lane of each panel.

interacting MKPs utilize this docking site to compete with MEKs for binding to the same site on their MAPK targets (Figure 1). In order to test this hypothesis, a synthetic peptide corresponding to the MAPK-docking site region of MKP-2 (Figure 1A) was tested for its ability to inhibit MEK2 phosphorylation of ERK2. The MKP-2 peptide inhibited MEK2 phosphorylation of ERK2 in a dose-dependent manner (IC_{50} of approx. 50 μM; results not shown), presumably by competing for the same binding site on ERK2. On the other hand, a peptide in which the three conserved arginine residues and the two conserved hydrophobic residues in the MKP-2 MAPK-docking site were mutated to Glu-Ala-Glu and two alanine residues respectively (see Figure 1A) did not inhibit MEK2 phosphorylation of ERK2 ($< 20\%$ inhibition at 100 μM peptide; results not shown).

If the MAPK-docking sites on MKP-2 and Elk-1 compete for binding to ERK2, then the MKP-2 peptide should inhibit ERK2 phosphorylation of Elk-1. Indeed, when the MKP-2 peptide was titrated into an ERK2 kinase assay utilizing GST–Elk-1 as substrate, the MKP-2 peptide inhibited ERK2 phosphorylation of Elk-1 in a dose-dependent manner (IC_{50} of approx. 20 μM; results not shown) whereas the corresponding control peptides (MKP-2$_{\mathrm{EAEAA}}$) had no effect on Elk-1 phosphorylation (results not shown).

Docking-site peptides inhibit MKP-1 dephosphorylation of ERK2

If the MAPK-docking sites on MEKs, MKPs and Elk-1 compete for binding to ERK2, then the MEK and Elk-1 peptides should inhibit MKP-mediated dephosphorylation of ERK2. To explore this possibility, a series of MKP-1 phosphatase assays were performed utilizing activated ERK2 as substrate, in the presence or absence of peptide. MKP-1, like MKP-2, is an inducible, nuclear dual-specificity phosphatase that can utilize ERK2 as substrate [3,35]; MKP-1 and MKP-2 share a high level of sequence similarity, including their MEK-like docking-site regions. Phosphatase assay products were resolved by SDS/PAGE (12% gels), transferred on to membranes, and an antibody specific for phospho-ERK2 was used to detect remaining active ERK2. Under the reaction conditions used, MKP-1 dephosphorylated ERK2 completely when peptide was omitted (Figure 5). In contrast, a dose-dependent inhibition of MKP-1-mediated dephosphorylation of phospho-ERK2 was observed in the presence of the Ste7, MEK2, MEK1, Elk-1 and MKP-2 peptides. The Elk-1$_{\mathrm{EEG,\ scram}}$ and MEK2$_{\mathrm{EEAA}}$ control peptides did not inhibit MKP-1 dephosphorylation of ERK2 (Figure 5).

MAPK-docking sites potentiate ERK2 kinase activity

To complement the experiments above, in which MAPK-docking-site peptides were used to inhibit phosphorylation and dephosphorylation, we wished to determine whether a MAPK-docking site would stimulate or inhibit the phosphorylation of a MAPK-target site on the same peptide. To do so, we used a peptide array phosphorylation assay. A schematic representation of this assay is shown in Figure 6(A). A series of short synthetic peptides were synthesized in place, anchored to a cellulose membrane. Each spot represents nanomolar quantities of a different peptide. Peptides were designed containing the MAPK-docking site of MEK1 immediately adjacent to a preferred target phosphorylation site (Pro-Leu-Ser-Pro) for ERK2 (Figure 6B). The MAPK-docking site of MEK1 was chosen as the best compromise with regard to considerations of length, affinity and specificity. The target phosphorylation site chosen contains optimal residues for ERK-mediated phosphorylation at the $+1$, $+2$, -1, -2 and -3 positions, and also contains a preferred residue at the $+3$ position [42]. The peptides were synthesized in two different configurations with respect to the relative positioning of the phosphoacceptor site and the MAPK-docking site. Control peptides contained either a mutant, unphos-

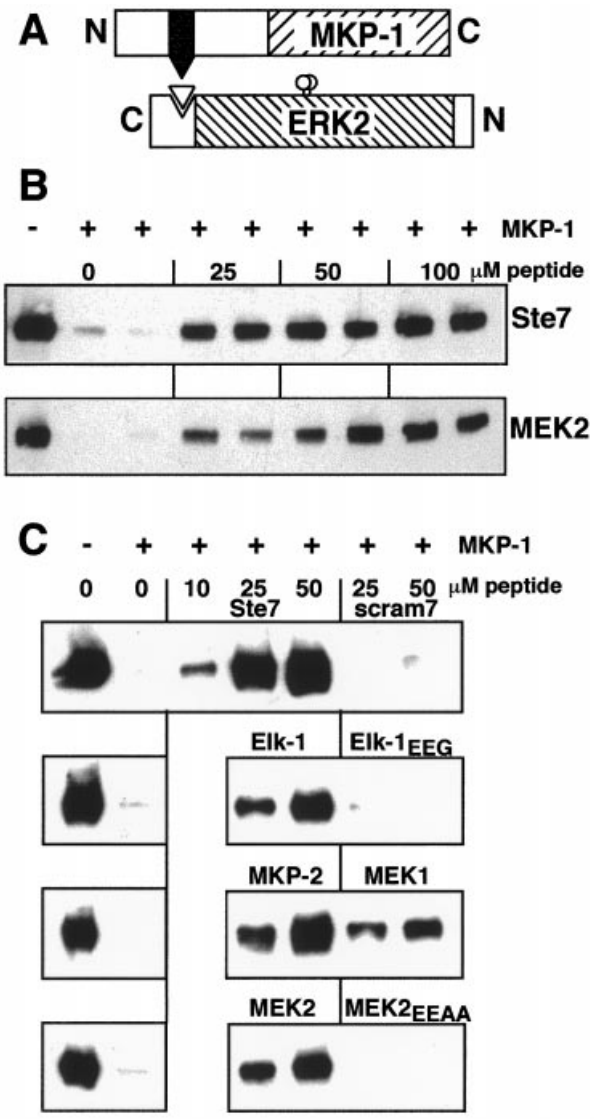

Figure 5 Inhibition of MKP-1-dependent dephosphorylation of ERK2 by MEK, Elk-1 and MKP-2 peptides

(**A**) Schematic representation of the experiment: MAPK-docking site peptides (triangle) were used to inhibit dephosphorylation of ERK2 by MKP-1. The N- and C-terminus of each protein is indicated. (**B**) and (**C**) Purified, dually phosphorylated ERK2 (25 nM) was incubated with purified, active MKP-1 (approx. 5 nM) for 20 min, in the absence or presence of the specified concentrations of the indicated peptides. ERK2 dephosphorylation was quantified by SDS/PAGE (12% gels) followed by Western blotting and immunostaining with anti-(phospho-ERK2). The lanes corresponding to ERK2 with no MKP-1 or no added peptide contain 50% of the relative amounts loaded in all other lanes.

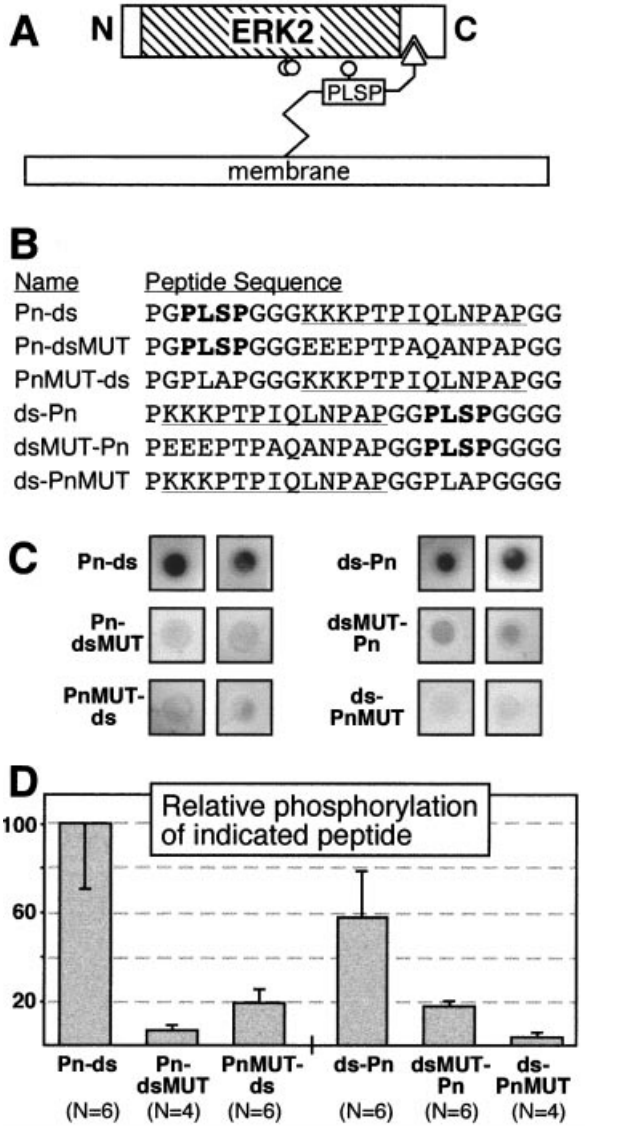

Figure 6 Stimulation of ERK2-dependent phosphorylation of peptides containing a near-optimal target site by an adjacent MAPK-docking site

(**A**) Schematic representation of the experiment: membrane-attached peptides, containing a MAPK-docking site (shown as a triangle) and phosphorylation site (shown as a rectangle containing the sequence Pro-Leu-Ser-Pro), or a mutated version of one or the other, were phosphorylated *in vitro* by ERK2 in the presence of [γ-^{32}P]ATP. (**B**) Sequences of the peptides used. The core of the phosphorylation target site is shown in bold, and the docking site is underlined. The peptides were anchored to the membrane via a chemical linker attached to their C-termini. (**C**) Autoradiogram of representative phosphorylated peptide spots. Of the four or six replicate spots, the ones shown are the second and third most intensely labelled. (**D**) Graphical representation of relative peptide phosphorylation, as quantified on a PhosphorImager. The average phosphorylation of the peptide designated 'Pn-ds' was set to 100, and the other peptides were normalized relative to it. Standard error bars are shown; the number of replicate spots (N) is indicated beneath the histogram.

phorylatable target site (Pro-Leu-Ala-Pro), or a mutant docking site in which the key basic and hydrophobic residues had been mutated to three glutamic acid and two alanine residues respectively (Figure 6B). The filter was incubated with a solution containing active ERK2 and [γ-^{32}P]ATP, and phosphate incorporation into the individual peptide spots was quantified.

A high level of peptide phosphorylation was seen for the peptides that contained the phosphoacceptor site together with the intact MAPK-docking site sequence, regardless of the orientation of the docking site relative to the phosphoacceptor target site (Figures 6C and 6D). Strikingly, considerably less peptide phosphorylation was observed with the peptides containing the mutant MAPK-docking site, despite the fact that these peptides still contained a near-optimal phosphoacceptor site sequence. The peptides containing the mutant target site were also poor substrates, as anticipated. These results indicate that a MAPK-docking site can potently stimulate MAPK-mediated phosphoryl-

ation of a phosphoacceptor-target site on the same peptide, regardless of orientation, and even if that target site is nearly optimal.

DISCUSSION

In the present study, we have used short synthetic peptides corresponding to the MAPK-docking sites of three different MEKs (MEK1, MEK2 and Ste7), the ERK transcription-factor target Elk-1, and the MAPK phosphatase MKP-2, in a series of competition assays. Our major new finding was the extensive cross-inhibition of MAPK-dependent reactions by these MAPK-docking-site peptides. The MEK1, MEK2 and Ste7 peptides, as well the Elk-1 peptide, were effective inhibitors of MEK2 phosphorylation of ERK2 (Figure 2 and results not shown) and of MEK2–ERK2 binding (Figure 4). Conversely, all of these peptides were also effective inhibitors of ERK2 phosphorylation of Elk-1 (Figure 3). These results suggest that upstream MEKs and the downstream transcription-factor target Elk-1 bind to the same site on ERKs. Furthermore, these data demonstrate for the first time that such docking interactions are crucial for optimal MEK2 phosphorylation of ERK2, and support the importance of docking interactions for ERK-mediated phosphorylation of specific target residues in Elk-1 [19,23]. We also found that a peptide corresponding to the MAPK-docking site of the dual-specificity MAPK phosphatase MKP-2 inhibited MEK2 phosphorylation of ERK2, and ERK2 phosphorylation of Elk-1. Furthermore, the MEK, Elk-1 and MKP-2 peptides all inhibited dephosphorylation of ERK2 by MKP-1 (Figure 5). These findings indicate that the MAPK-docking sites in MKP-1 and MKP-2 bind to the same region of ERKs as do the MEKs and Elk-1, and reaffirm the importance of this binding for MKP-1 phosphatase activity [35].

Finally, the requirement of a MAPK-docking site for efficient ERK2-mediated phosphorylation was further demonstrated by the use of a peptide-array phosphorylation assay (Figure 6). Despite the presence of a high-efficiency phosphoacceptor site, only peptides containing an intact MAPK-docking site were phosphorylated effectively by active ERK2. The finding that an adjacent MAPK-docking site could so substantially (3- to 5-fold) stimulate the phosphorylation of a near-optimal target site was unanticipated and suggests that further development of such peptides might lead to a useful set of reagents. For example, efficient and specific MAPK-substrate peptides would be advantageous in a recently developed methodology for monitoring the activity of multiple kinases in single cells [43], with applications to the molecular profiling of diseased tissue.

Potency of inhibition and stimulation

An additional important result of our study is the potency of the enzymic inhibition that we obtained with docking-site peptides. This contrasts, for example, with the rather modest effects on MEK activity that were found to result from mutation of the basic residues of the MAPK-docking site of MEK1, or in the acidic residues of ERK2 thought to interact with these basic residues [13]. The greater inhibition that we observed may be because the MAPK-docking-site peptides were able to simultaneously block the interactions mediated by the basic and hydrophobic submotifs of the MAPK-docking site. Also, a steric blockage of an interaction might be more effective than a simple lack of an interaction due to mutation. Furthermore, *in vivo*, other protein–protein interactions, such as those provided by scaffold proteins, may partially compensate for the loss of direct docking interactions [14]. Regardless of the mechanism, our results support a key role for docking in MAPK-dependent reactions, and suggest that drugs that mimic MAPK-docking-site peptides might be efficacious modulators of MAPK signalling. One trivial explanation for the potent inhibition of enzymic activity by MAPK-docking-site peptides is that these peptides unfold or alter the conformation of ERK2 is such a way as to render it inactive and unrecognizable. This possibility, however, is ruled out by our demonstration that a MAPK-docking site potently stimulates ERK2's ability to phosphorylate a target site on the same peptide (Figure 6).

Implications of competitive docking

The finding that the MAPK-docking sites of MEKs, Elk-1 and MKPs all compete for binding to ERK2 suggests that all three classes of ERK-interacting proteins bind to the same site(s) on ERK2. In agreement with this, a common binding site termed the conserved docking (CD) region has been identified in ERK2 that utilizes acidic residues for binding to several ERK-interacting proteins [13,44]. Also, the hydrophobic residues in the docking sites of both MKK3b and MEF2A have been shown to bind to a hydrophobic docking groove in p38 MAPK [45]. However, other regions of MAPKs have been implicated in binding to the basic residues [46,47], as well as to the hydrophobic residues [15] of the MAPK-docking site, and other regions have been implicated as well [18]. Moreover, the *sevenmaker* mutation in the CD region, which substantially reduces the ERK–MKP interaction, surprisingly has little or no effect on the ERK–MEK and ERK–Elk interactions [48]. Furthermore, multiple regions of MAPKs, in addition to the CD region, have been shown to be important for ERK–MEK binding [49,50]. Finally, both Elk-1 and MKP-3 have been shown to interact with ERK2 by a distinct MAPK-docking site designated Phe-Xaa-Phe-Pro [20,23,36]. Hence our finding of extensive cross-inhibition by MAPK-docking-site peptides, and the competitive and mutually exclusive binding of MAPKs to certain substrates and regulators that this finding implies, were by no means foregone conclusions.

The ability of MEKs, Elk-1 and MKPs to bind to the same site on ERK2 suggests that they may compete with each other for binding to ERK2 *in vivo*. Current understanding of the subcellular localization of these proteins is that MEK1 and MEK2 retain ERK1 and ERK2 in the cytoplasm in the absence of activation [9,51–53]. Upon activation, active ERK translocates to the nucleus, where it phosphorylates Elk-1 and other nuclear targets, and then relocalizes to the cytoplasm. Like Elk-1, MKP-1 and MKP-2 are nuclear proteins. It is therefore possible that MKP-1 and MKP-2 compete with Elk-1 for binding to activated ERKs in the nucleus. If so, perhaps ERK molecules that encounter Elk-1, or other physiological substrates, are temporarily protected from phosphatase action, whereas those molecules that do not find appropriate targets are quickly deactivated. One study [54] has demonstrated that the relocalization of ERK2 back to the cytoplasm is dependent upon its binding to MEK, which shuttles between the nucleus and cytoplasm. Again, ERK molecules that have found an appropriate nuclear target may be protected from this MEK-mediated nuclear export. In this way, the competitive docking of substrates and regulators to MAPKs may play a direct role in enhancing signal fidelity.

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