Mitogen-activated protein (MAP) kinases are activated with great specificity by MAP/ERK kinases (MEKs). The basis for the specific activation is not understood. In this study chimeras composed of two MAP kinases, extracellular signal-regulated protein kinase 2 and p38, were assayed in vitro for phosphorylation and activation by different MEK isoforms to probe the requirements for productive interaction of MAP kinases with MEKs. Experimental results and modeling support the conclusion that the specificity of MEK/MAP kinase phosphorylation results from multiple contacts, including surfaces in both the N- and C-terminal domains.

Mitogen-activated protein (MAP)\(^1\) kinase or extracellular signal-regulated protein kinase (ERK) cascades are present in all eukaryotes and are utilized in almost all signal transduction pathways originating from receptors at the cell surface (1, 2). A plethora of different stimuli, including growth factors, cytokines, heat shock, and ultraviolet light, can initiate signaling through these cascades. Each cascade consists of a three kinase module: a MAP kinase, a MAP kinase/ERK kinase (MEK) that activates the MAP kinase, and a MEK kinase (MEKK) that activates the MEK (3). The MAP kinase in each cascade preferentially phosphorylates substrates with a serine or threonine followed by a proline. There are three mammalian MAP kinase modules that have been extensively studied. These include the ERK1/2 module, the c-Jun N-terminal protein kinase/stress-activated protein kinase module, and the p38 module. ERK3, ERK4, and ERK5 and other p38 isoforms have also been identified, but the cascades leading to activation of these kinases are not well characterized (4–11).

Like other protein kinases, the MAP kinases are folded into two domains (12). The smaller N-terminal domain is composed mostly of \(\beta\) strands, whereas the C-terminal domain is made up of \(\alpha\) helices. ATP binds between the two domains, and protein substrate is believed to bind on the surface of the C-terminal domain. Alignment of the amino acid sequences of many protein kinases reveals a common core catalytic domain of 250–300 residues encoding the two-domain structure (13). Protein kinases possess 12 conserved stretches of amino acids within their catalytic domains known as subdomains (13–15). These conserved elements as well as unique structures contribute to catalysis by and regulation of protein kinases. The functions of several of the conserved and unique structural motifs are known. A glycine-rich loop in the N-terminal domain, termed the phosphate anchor ribbon, has a role in binding ATP. Also in the N-terminal domain, subdomain III encodes the C helix, which contains an invariant Glu involved in binding MgATP (16, 17). This helix is important for maintaining an open domain conformation in unphosphorylated ERK2 (18), aligning catalytic residues in Src (19) and Cdk2 (20), and binding to the cyclin regulatory subunit in Cdk2 (21). The activation loop, known as the phosphorylation lip in MAP kinases, is a poorly conserved element in the C-terminal domain and contains the two MAP kinase phosphorylation site residues within a TXY sequence. Also in the C-terminal domain is the MAP kinase insert. It is composed of 32 residues and is only found in the MAP kinases and the Cdns. The MAP kinase insert and a loop consisting of residues 199–205 of ERK2 interact with the phosphorylation lip in the unphosphorylated form of ERK2. Finally, a loop at the C terminus of the MAP kinases, L16 or the C-terminal tail, wraps around the back of the structure and interacts with the N-terminal domain (18).

The crystal structures of unphosphorylated and phosphorylated ERK2 and unphosphorylated p38 have been solved (18, 22–24). Several important differences exist between the structures of the unphosphorylated forms of ERK2 and p38. There is a wider domain separation in p38 than in ERK2. The phosphorylation lip in p38 is six residues shorter and has a different conformation. In p38 the lip is folded up between the two domains, and the C-terminal portion of the lip forms a turn of helix that blocks the \(P + 1\) specificity pocket. This pocket directs the proline specificity of MAP kinases. The axis of the C helix is rotated, and the N terminus of this helix is shifted by 6 Å relative to the helix in ERK2. The helix at the end of L16 is extended by 7 Å, and there is an increase in the hydrophilicity of residues that form contacts between L16 and the N-terminal domain. This results in a less intimate interaction between L16 and the N-terminal domain in p38 (22, 24).

Within MAP kinase cascades, the MEKs are the most specific enzymes. These dual specificity kinases activate their respective MAP kinase substrates by phosphorylating the threonine and tyrosine of the specific TXY sequence located in the phosphorylation lip. The only known substrates of MEK1 and MEK2 are ERK1 and ERK2 (25). Other MEKs also phosphorylate only a small subset of the MAP kinase family. For example, MEK3 and MEK6 will only phosphorylate p38 isoforms, whereas MEK5 will only phosphorylate ERK5 (26–28). In addition, the MEKs require native MAP kinases as substrates; they will not phosphorylate denatured proteins or pep...
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Experimental Procedures

Generation of Mutants and Purification of Proteins—Restriction sites were inserted at the ends of subdomains II and IV and at residue 326 of ERK2 and residue 322 of p38 with the QuickChange kit (Stratagene, La Jolla, CA). ERK2II—CT contains all the restriction sites as well as sites at the ends of subdomains III and V, and p38III contains the site at subdomain II. The resulting plasmids were used to create ERK2-p38 chimeras. His6-tagged proteins were expressed and purified as described previously (32) or by batch binding to Ni2+-nitrilotriacetic acid-agarose (Qiagen). Protein concentrations were determined with the interface.

Multiple spatially segregated contacts in the MEK/MAP kinase MEK1, GST-MEK2, and His6-MEK3 were purified from a previously described coexpression system (33), and GST-MEK4 and GST-MEK6 were purified and activated by GST-MEKK-C (34) essentially as described (29).

Phosphorylation and Activity Assays—Phosphorylation and activity assays were performed essentially as described (29). Activity of ERK2, p38, or chimeras was determined using myelin basic protein (Sigma) as substrate. One-dimensional phosphoamino acid analysis was performed as described (35).

Time Courses of Phosphorylation and Activity—Assays were performed as described (29), but reactions were stopped at the times indicated in Figs. 5 and 6 for MEK2 and MEK6, respectively. For MEKs 1, 3, and 4, 50-μl aliquots were removed from one large reaction mix and stopped by addition to 15 μl of 5× SDS sample buffer.

Western Blotting—20 ng of ERK2 or each chimera from the time course reactions with MEK2 and MEK6 were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were probed with an antibody specific for phosphorylated ERK2, V803 (Promega), and an antibody that recognizes phosphorylated and unphosphorylated ERK2, Y691.

Phosphatase Treatment—ERK2 or chimeras were incubated with an equimolar amount of PTP1 at 30 °C for 30 min. Sodium orthovanadate was added to one set of reactions at time 0 and to an identical set at 30 min. Kinase assays with MEK2 were then performed as above, but they were stopped after 1 min.

Results

MEKs recognize the native MAP kinase structure with considerable selectivity. The primary sequence surrounding the TXY phosphorylation sites of the ERKs is not sufficient to determine MEK recognition. Neither peptides derived from the double phosphorylation site nor denatured proteins are phosphorylated by MEKs (25). These findings support the conclusion that MEKs require specific conformations or secondary determinants for productive interaction with their MAP kinase substrates. We wished to identify the structural features of MAP kinases that allow MEKs to distinguish among them as substrates. To do this we created chimeras between ERK2 and p38 that contained selected intact structural elements from each enzyme (Fig. 1A). The chimeras were composed of ERK2 and p38 because a related set of chimeras had been tested in transfected cells (31), and the structures of both of these MAP kinases were available for analysis (18, 22, 24). The chimeras were expressed and purified and then tested in vitro for activation by MEK family members, MEKs 1, 2, 3, 4, and 6, which phosphorylate ERK2 or p38 in vitro. Measurements included rate and extent of phosphorylation, phosphoamino acids, recognition by antibodies selective for phosphorylated ERK2, and activated activity relative to the wild type proteins. ERK2 and p38 proteins containing inserted restriction sites were also purified and tested as controls.

The MAP kinase catalytic core contains two domains. The N-terminal domain is composed of N-terminal sequence (blocks in ERK2 and p38. Odd numbers are shown. Subdomains from ERK2 are white, and subdomains from p38 are gray. The chimeras were named according to the subdomains that were exchanged. B, structural representation of MAP kinase domains using ERK2 as the model. The C-terminal domain is shown in blue. The N-terminal domain includes residues in white (subdomains I and II), red (subdomains III and IV, which include the C helix and β strands 4 and 5), and yellow (L16). Chimeras swapped the white, red, and yellow structures as indicated in A and the text.

Fig. 1. Schematic and structural representation of ERK2, p38, and chimeras. A, conserved subdomains I–XI including VIa and VIb are indicated by blocks in ERK2 and p38. Odd numbers are shown. Subdomains from ERK2 are white, and subdomains from p38 are gray. The chimeras were composed of ERK2 and p38 because a related set of chimeras had been tested in transfected cells (31), and the structures of both of these MAP kinases were available for analysis (18, 22, 24). The chimeras were expressed and purified and then tested in vitro for activation by MEK family members, MEKs 1, 2, 3, 4, and 6, which phosphorylate ERK2 or p38 in vitro. Measurements included rate and extent of phosphorylation, phosphoamino acids, recognition by antibodies selective for phosphorylated ERK2, and activated activity relative to the wild type proteins. ERK2 and p38 proteins containing inserted restriction sites were also purified and tested as controls.

The MAP kinase catalytic core contains two domains. The N-terminal domain is composed of N-terminal sequence (white and red in Fig. 1B) and the C-terminal tail, L16 (yellow in Fig. 1B), and is primarily involved in binding ATP. In the MAP kinases L16 lies on the surface of the N-terminal domain. The C-terminal domain (blue in Fig. 1B) binds the protein substrate. To identify important structural features, we first created a chimera, PIVECTP, which contained one of its two major
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Fig. 2. Phosphorylation of chimeras by MEK isoforms. Relative stoichiometry of phosphate incorporation into ERK2, PIVECTP, PIVE, PIIE, EIIPIVE, EIIP, and p38 by the indicated MEKs. Data for each MEK are expressed relative to stoichiometry of phosphorylation of ERK2 by MEK1 and MEK2 and of p38 by MEK3, MEK4, and MEK6. Data are the means ± S.E. of three to five independent experiments.

Fig. 3. Kinase activity of chimeras after phosphorylation by MEK isoforms. Relative myelin basic protein kinase activity of ERK2, PIVECTP, PIVE, PIIE, EIIPIVE, and EIIP after phosphorylation by the indicated MEKs. Data are expressed relative to specific activity of ERK2 after phosphorylation by MEK2, set to 1. Data are the means ± S.E. of three to five independent experiments.

Fig. 4. Phosphoamino acid analysis. Phosphoamino acid analysis of p38 and p38II after incubation with MEK3 and EIIP after incubation with MEK4. The positions of the three phosphoamino acid standards are indicated.

Structural domains from each kinase (Fig. 1). This chimera contained the larger C-terminal structural domain from ERK2 and the smaller N-terminal domain from p38. The p38 elements making up the N-terminal domain are the N terminus and the smaller N-terminal domain from p38. The p38 elements were included in the C-terminal structural domain from ERK2. Maximal activity was about 15% of the activity of wild type ERK2 (Fig. 3). MEK3, MEK4, and MEK6 did not phosphorylate it. Therefore, the C helix, β4, and β5 of MAP kinases were not sufficient for phosphorylation of a MAP kinase by these MEKs. EIIPIVE also was phosphorylated by MEK1 and MEK2 at rates similar to the rates of phosphorylation of ERK2 (see Figs. 5A and 7A). Results from these four chimeras indicated that many p38 residues must be present in the N-terminal domain of each chimera for phosphorylation by the p38-specific MEKs.

We wanted to determine whether any differences existed in the pattern of phosphorylation and activation by MEKs if the other MAP kinase was at the N terminus of the chimera. Thus, we expressed a reciprocal chimera, EIIP. EIIP contained ERK2 residues from the N terminus through subdomain II and ERK2 residues in subdomain III through the C terminus (Fig. 1A). This chimera had activity comparable with that of unphosphorylated ERK2. Once again, only MEK1 and MEK2 phosphorylated and activated this chimera (Figs. 2 and 3). The rates of phosphorylation of PIIE by MEK1 and MEK2 were similar to the rates of phosphorylation of wild type ERK2 by MEK1 and MEK2 (see Fig. 5A and 7A). Results from these four chimeras indicated that many p38 residues must be present in the N-terminal domain of each chimera for phosphorylation by the p38-specific MEKs.

We wanted to determine whether any differences existed in the pattern of phosphorylation and activation by MEKs if the other MAP kinase was at the N terminus of the chimera. Thus, we expressed a reciprocal chimera, EIIP. EIIP contained ERK2 residues from the N terminus through subdomain II (white in Fig. 1B) and p38 residues from subdomain III through the C terminus. This chimera was phosphorylated by MEK4 and MEK6 but not by MEK1, MEK2, or MEK3 (Fig. 2). The time courses of phosphorylation were slightly more rapid (see Fig. 7C). This chimera had very low basal activity, less than 1% of wild type ERK2, suggesting that it was poorly folded. After phosphorylation, this chimera was inactive (Fig. 3). Analysis of its phosphoamino acids after phosphorylation by MEK4 revealed that it was inactive in part because there was little or no threonine phosphorylated (Fig. 4). Domains I and II of ERK2 alter the position of the C helix in the chimera, which may
account for the inability of MEKs to phosphorylate threonine.

The two control proteins were also tested in the in vitro kinase assays to determine whether any residues changed by the insertion of the restriction sites caused the changes in interaction with MEKs. The ERK2 control, ERK2II–CT was phosphorylated and activated only by MEK1 and MEK2 (data not shown). Therefore, residues that were changed by the insertion of restriction sites did not affect the productive interaction of ERK2 chimeras with MEKs. The p38 control, p38II, was phosphorylated by MEK3, MEK4, and MEK6 (data not shown). p38II was activated normally by MEK4/6 but less well by MEK3 (data not shown). The phosphoamino acid analysis (Fig. 4) indicates that p38II is not fully activated by MEK3 because it is poorly phosphorylated on threonine.

The maximum stoichiometries of phosphorylation of the chimeras by MEKs 1–4 and 6 were evaluated (Table I) and were consistent with the extents of activation of the chimeras. With the exception of EIIP, the time courses of phosphorylation of the chimeras by individual MEK family members were similar to their rates with their normal substrates, ERK2 or p38 (Figs. 5–7).

To determine whether phosphorylation occurred on the predicted residues (Thr$^{183}$ and Tyr$^{185}$), chimeras phosphorylated by MEKs for different times were immunoblotted with antibodies that selectively recognize the doubly phosphorylated ERK2 epitope. These antibodies do not detect unphosphorylated or singly phosphorylated ERK2 at equivalent protein concentrations. The immunoblots reveal an excellent parallel between increasing phosphorylation and appearance of the phospho epitope (Figs. 5 and 6). Phosphorylation of the chimeras saturated at the same times as did blotting intensity. This is consistent with the conclusion that phosphorylation occurred largely on Thr$^{183}$ and Tyr$^{185}$ and not on previously unidentified residues.

These immunoblotting findings agree with phosphoamino acid analysis, which showed the majority of phosphate on tyrosine and threonine. However, PIVECTP, for example, apparently contained less phosphotyrosine in these analyses than did wild type ERK2. To determine whether this was due to prior autophosphorylation of the chimera, we compared the stoichiometry of phosphorylation of wild type ERK2 and three of the chimeras before and after dephosphorylation with the tyrosine phosphatase PTP1 (Fig. 8A). The stoichiometry of phosphorylation of ERK2 was unchanged by pretreatment with phosphatase; on the other hand, phosphorylation of two of the chimeras increased by 20–30%. The increase was primarily due to increased tyrosine phosphorylation as was apparent from the increase in phosphotyrosine recovered in the phosphoamino acid analysis (Fig. 8, B and C). This is consistent with the idea that the chimeras have enhanced abilities to autophosphorylate on Tyr$^{185}$ prior to isolation from bacteria. Because this tyrosine is already partly phosphorylated when

![Fig. 5. Time course of phosphorylation and activation of chimeras by MEKs](image-url)

**TABLE I**

Maximum stoichiometry of phosphorylation by MEKs

| MAP Kinase Chimeras | Maximum stoichiometry of phosphorylation by MEKs | ERK2 | p38 | PIIE | PIVE | PIVECTP | EIIP | EIIPVE |
|---------------------|--------------------------------------------------|------|-----|------|------|---------|------|-------|
| Alone               | 0.04                                             | 0.01 | 0.02 | 0.13 | 0.02 | 0.00    | 0.17 |
| MEK1                | 1.35                                             | 0.03 | 0.61 | 2.12 | 0.47 | 0.00    | 0.75 |
| MEK2                | 2.45                                             | 0.06 | 0.89 | 2.69 | 0.91 | 0.02    | 1.16 |
| MEK3                | 0.05                                             | 1.36 | 0.02 | 0.11 | 0.18 | 0.03    | 0.12 |
| MEK4                | 0.05                                             | 2.68 | 0.04 | 0.15 | 0.08 | 1.38    | 0.15 |
| MEK6                | 0.06                                             | 1.45 | 0.07 | 0.88 | 0.28 | 0.46    | 0.08 |
the proteins are purified, less phosphate can be transferred to tyrosine by MEKs.

**DISCUSSION**

MEK isoforms recognize members of the MAP kinase family with selectivity. The residues or structural motifs that are required for this selective recognition have not been defined. ERK1/2 and p38 isoforms share about 40–45% sequence identity (5, 36, 37), and the structures of the unphosphorylated forms of ERK2 and p38 are similar (18, 22). The goal of this project was to use chimeras containing swapped structural elements of ERK2 and p38 to identify regions of the MAP kinases that direct interactions with particular MEKs.

Our findings using ERK2/p38 chimeras indicate that no single MAP kinase structural motif forms a sufficient interaction surface with MEK. The N-terminal structural domain likely forms part of the interface. However, even the entire domain is not sufficient to restrict phosphorylation by MEKs, because the chimera containing the N-terminal domain from p38 was phosphorylated by MEK1 and MEK2 in addition to MEK3/4/6. This demonstrates that there are interaction determinants in the C-terminal domain. In addition, a fairly large surface area within the N-terminal domain must be present for the interaction of the chimeras with MEK3/4/6. Swapping only subdomains I and II or subdomains III and IV was not enough to allow MEK3/4/6 to phosphorylate the chimeras. Based on these data, it appears that MEKs require multiple interacting sites within both domains to specifically phosphorylate different MAP kinases.

Our results with ERK2/p38 chimeras are in part consistent with work from Brunet and Pouységur (31), who expressed p38/ERK1 chimeras in mammalian cells. A p38/ERK1 chimera equivalent to PIVE was activated by both growth factors and cell stress, whereas a chimera equivalent to PIIE was only activated by growth factors. We have shown with further subdivision of the swapped regions that in contrast to the suggestion from the p38/ERK1 studies, the C helix alone is not the key element determining which MEKs will phosphorylate a particular MAP kinase *in vitro*.

Additional findings suggest that the MAP kinase insert is required for phosphorylation by MEKs. To address the function of this insert, it was deleted from wild type ERK2.2 Examination of the crystal structure indicated that deletion of these residues would have a minimal effect on the tertiary structure of ERK2. In the unphosphorylated state, the resulting protein had protein kinase activity equivalent to unphosphorylated wild type ERK2. However, the protein lacking the MAP kinase
BPTP1. Data are expressed as the means ± S.E. for three independent experiments. β: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE without or with pretreatment with PTP1. Data are expressed as the means ± S.E. for three independent experiments. C: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE with PTP1. For B and C, the positions of the three phosphoamino acid standards are indicated.

The insert was no longer phosphorylated by MEK2 in vitro. It is possible that the insert aids in the correct folding of the phosphorylation lip, for binding to MEKs, or that it may be directly involved in interaction with MEKs.

We mapped the currently available data for MEK specificity determinants onto the structure of unphosphorylated ERK2 (Fig. 9). The data reported here indicate that the C helix, β strands 4 and 5, and L16 of MAP kinase are important, because when these motifs are from ERK2, phosphorylation by MEK3/4/6 is lost. The findings noted above suggest a role for the MAP kinase insert. In addition, previous work showed that mutating tyrosine 185 in the phosphorylation lip or deleting six residues from this loop of ERK2 resulted in significantly decreased MEK phosphorylation of PIVE, PIVECTP, and EIIPIVE by MEK2. A relative stoichiometry of phosphate incorporation by MEK2 into ERK2, PIVE, PIVECTP, and EIIPIVE without or with pretreatment with PTP1. Data are expressed as the means ± S.E. for three independent experiments. β: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE not dephosphorylated with PTP1. C: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE first dephosphorylated with PTP1. For B and C, the positions of the three phosphoamino acid standards are indicated.

FIG. 8. Autophosphorylation accounts for decreased tyrosine phosphorylation of PIVE, PIVECTP, and EIIPIVE by MEK2. A relative stoichiometry of phosphate incorporation by MEK2 into ERK2, PIVE, PIVECTP, and EIIPIVE without or with pretreatment with PTP1. Data are expressed as the means ± S.E. for three independent experiments. B: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE not dephosphorylated with PTP1. C: phosphoamino acid analysis of ERK2, PIVE, PIVECTP, and EIIPIVE with PTP1. For B and C, the positions of the three phosphoamino acid standards are indicated.

ERK2 phosphorylation lip was placed on MEK1 in the position of the bound peptide substrate (16). The sites of contact in this hypothetical ERK2/MEK1 model include residues from each of the structural elements implicated experimentally, namely, the tip of the C helix, the loop region of L16, the phosphorylation lip, and the tip of the MAP kinase insert.

The proposed MEK/MAP kinase interface is involved in other protein-protein interactions. Residues in this putative interface overlap with the ERK2 dimerization interface (38). In particular, L16 and the phosphorylation lip are intimately involved in both. Furthermore, it is intriguing that the proposed MEK/MAP kinase interface is similar to the interface between Cdk2 and cyclin A (21). Structural elements of Cdk2 that participate in cyclin A binding include the PSTAIRE helix region, which encompasses the helix equivalent to the C helix of MAP kinases and the β4 and β5 strands. In the C-terminal domain, contacts with cyclin A are made by residues in the region of the T loop, equivalent to the ERK2 phosphorylation lip, and a C-terminal helix, helix 7. The only motif that participates in the proposed MEK/ERK interface but not in the Cdk2/cyclin A interface is the MAP kinase insert.

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