Contributions of the Mitogen-activated Protein (MAP) Kinase Backbone and Phosphorylation Loop to MEK Specificity*

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Intracellular signaling pathways are triggered in response to extracellular stimuli, including growth factors, heat shock, inflammatory cytokines, and ultraviolet light (1, 2). These pathways allow the cell to respond in an appropriate fashion, through proliferation, expression of differentiated properties, or protective responses to environmental insult (1, 2). Mitogen-activated protein (MAP)1 kinase cascades contribute to these responses and are highly conserved in both yeast and mammalian systems (3, 4). MAP kinase cascades consist of three protein kinases acting in series: a MAP kinase, a MAP kinase kinase or MEK, and a MAP/ERK kinase or MAPK. In mammalian cells at least three such cascades have been well described (Fig. 1A): the MEK1/2-ERK1/2 module, the MEK3/6-p38 module, and the MEK4-stress-activated protein kinase/c-Jun-N-terminal protein kinase (SAPK/JNK) module (1, 2). In addition, two less-studied pathways containing ERK3 kinase-ERK3 and MEK5-ERK5 have been identified (5–7).

Within these modules MEKs appear to be singularly specific activators of MAP kinases (8–10). MEKs are a family of dual specificity kinases that phosphorylate MAP kinases on both a threonine and a tyrosine residue (11). MEKs have an unusually restricted substrate specificity in that they have no other known substrates beyond MAP kinases (12). Furthermore, each MEK isoform selectively phosphorylates only a subset of the MAP kinase family members. Finally, MEKs do not recognize denatured protein or peptides derived from the MAP kinases (12); apparently the three-dimensional structure of the MAP kinase is important for MEK recognition.

The sequence identity among ERK1/2, JNKs, and p38 MAP kinases is ~40–45%. These three mammalian MAP kinases have a common regulatory mechanism requiring phosphorylation by MEKs of a threonine and a tyrosine in comparable positions in the loop known as the phosphorylation lip. We examined differences among family members in that loop which might contribute to the specificity of recognition by upstream MEKs. First, the three kinases ERK2, p38, and SAPKβ have a different intervening residue (Glu, Gly, and Pro, respectively) between the two sites of phosphorylation (1). In addition, the yeast MAP kinase SMK1 and other related kinases such as the KKIALRE kinase (1, 13) contain different residues (Asn and Asp, respectively) at this position (Fig. 1B). It has been suggested that this residue determines the ability of a given MEK to phosphorylate a particular MAP kinase (14, 15). Second, the three MAP kinases differ in the length of their phosphorylation lips. ERK2 has the longest lip, at 25 amino acids, following the conserved DFG extending to the conserved APE residues. SAPKβ has a lip length of 21 amino acids, and p38 has the shortest lip length at 19 residues.

To probe features of the MAP kinase phosphorylation lip which are important for MEK recognition and activation, we constructed the following ERK2 mutants: 1) mutations of the sites of MEK phosphorylation; 2) mutations of the residue between the two sites of MEK phosphorylation; and 3) mutations to shorten the length of the phosphorylation lip. We then tested the ability of all six available MEKs as well as constitutively active mutants of MEK1 and MEK2 to phosphorylate and activate ERK2, ERK3, p38, SAPKβ, and ERK2 mutants. Of the MEKs tested, only MEK1 and MEK2 phosphorylated ERK2. None of the lip changes destroyed the recognition of ERK2 by MEK1 and MEK2 or caused ERK2 to be recognized by any other MEK isoforms. Interestingly, however, the mutants...
allowed us to identify specificity differences between MEK1 and MEK2. Thus, although the phosphorylation lip is important for interaction of a MEK isoform with its downstream MAP kinase, other factors must direct the specificity of this interaction.

EXPERIMENTAL PROCEDURES Pro...
MEK Specificity

Fig. 2. Phosphorylation and activity of wild type MAP kinases and mutants after incubation with MEK1. Panel A, stoichiometry of phosphate incorporation into the wild type and mutant kinases by MEK1. Data are an average of 3–15 independent experiments, and standard deviations are shown. Panel B, phosphoamino acid analysis of the same kinases as in panel A after incubation with MEK1. The positions of the three phosphoamino acids, determined from internal phosphoamino acid standards, are indicated. Panel C, MBP kinase activity of the wild type and mutant ERK2s after phosphorylation by MEK1. Results are the average of multiple experiments, and standard deviations are shown.

producing E184D (TDY ERK2), E184G (TGY ERK2), E184N (TNY ERK2), and E184P (TPY ERK2) (Fig. 1B).

Third, to begin to assess the importance of lip length, we removed either four or six nonconserved amino acids from the lip of ERK2 to make it the same length as that of SAPK\(\theta\) or p38, respectively (Fig. 1B). These mutants are designated ERK2\(\Delta 4\) and ERK2\(\Delta 6\). Finally, to produce ERK2 mutants that contained lips that were more like those of p38 and SAPK, we made hybrids of intervening residue point mutants and lip length mutants to create TPY ERK2\(\Delta 4\) and TGY ERK2\(\Delta 6\).

MEK—As reported previously, MEK1 does not phosphorylate the other family members ERK3, p38, or SAPK JNK (Fig. 2A) (7, 24–26). The stoichiometry of phosphorylation of wild type ERK2 by MEK1 is approximately 0.8–1.0 mol/mol, consistent with previous results with in vitro phosphorylation (11, 20). It has been suggested that some percentage of bacterial wild type ERK2 is not correctly folded. Alternatively, MEK1 may require additional factors, such as MEK enhancing factor, to enable MEK to phosphorylate ERK to a stoichiometry of 2 mol/mol in vitro (27).

MEK1 phosphorylates all of the E184 ERK2 mutants nearly equally well as wild type (Fig. 2A). With wild type and the E184D, E184G, and E184N ERK2 mutants, phosphoamino acid analysis indicates that threonine and tyrosine are phosphorylated to approximately equivalent extents (Fig. 2B) with a combined stoichiometry of ~1 mol/mol on both residues. Interestingly, however, MEK1 can only phosphorylate E184P ERK2 on tyrosine (Fig. 2B); thus the stoichiometry on this residue alone must be near 1 mol/mol.

Removing four amino acids from the ERK2 lip has little effect on the ability of MEK1 to phosphorylate ERK2; ERK2\(\Delta 4\) is phosphorylated to a stoichiometry equal to wild type ERK2. The E184P ERK2\(\Delta 4\) protein is phosphorylated significantly less well by MEK1, to about 50% of wild type levels. As with E184P ERK2, only tyrosine is phosphorylated (Fig. 2B). Removal of six amino acids from the lip in ERK2, however, greatly reduces the ability of MEK1 to phosphorylate the mutant.

Changing the phosphorylatable residues of ERK2 has a more profound effect on phosphorylation by MEK1 than the other mutations. T183S/Y185G (SEG) ERK2 is not phosphorylated at all by MEK1 even on serine (Fig. 2, A and B). Serine phosphate seen in SEG ERK2 (Fig. 2B) is due to autophosphorylation. Interestingly, Y185G (TEG) ERK2 is phosphorylated, suggesting that the presence of the extra methyl group in threonine facilitates recognition by MEK1. T183S (SEY) ERK2 is phosphorylated on both serine and tyrosine (Fig. 2B) and retains MBP kinase activity (Fig. 2C). Thus the presence of either the tyrosine or the threonine is essential for phosphorylation by MEK1. Finally, the positioning of the residues is also important to MEK recognition, as the ERK2 mutant T183Y/Y185T (YET), in which the two residues are transposed, is not phosphorylated by MEK1 (data not shown).

MEK2—MEK2 phosphorylates wild type ERK2 to a stoichiometry of ~1.5–2 mol/mol (Fig. 3A). Like MEK1, MEK2 does not phosphorylate p38 or SAPK\(\theta\). Also like MEK1, MEK2 can phosphorylate the Glu\(\theta\) mutants as well as wild type ERK2.

Again, E184P ERK2 is only phosphorylated on tyrosine (data not shown). MEK2 also phosphorylates ERK2\(\Delta 4\) and E184P ERK2\(\Delta 4\) to ~100% and ~50% of wild type, respectively. Furthermore, phosphorylation site residues in the ERK2 lip seem to have an importance similar to that with MEK1 because MEK2 phosphorylates T183S/Y185G (SEG) ERK2, Y185G (TEG) ERK2, and T183S (SEY) ERK2 to the same extent and on the same residues as does MEK1 (Fig. 3A). MEK2 phosphorylates the ERK2\(\Delta 6\) mutants to a higher extent than MEK1, although the differences are small.

An unexpected finding that distinguishes MEK1 (Fig. 2A) from MEK2 (Fig. 3A) is that MEK2 is able to phosphorylate ERK3 and S189T/G191Y (TEY) ERK3. When we mutated the ERK3 phosphorylation region from SEG to TEY, the amount of phosphate incorporated increased to levels nearly equal to wild type ERK2 (Fig. 3A). Incorporation occurred primarily on threonine, as the ERK3 mutant with only a small amount incorporated onto tyrosine (Fig. 3B).

MBP Kinase Activity of ERK2 Mutants—Upon phosphorylation with either MEK1 or MEK2, E184D, E184G, and E184N ERK2 possess about 10–50% of the MBP kinase activity of wild type phosphorylated ERK2 (Figs. 2C and 3C). The E184P-containing mutants do not have MBP kinase activity presumably because they are not phosphorylated on threonine (see Fig. 2B). The lip length mutants have slightly less kinase activity.
than their wild type counterparts. Considering its barely detectable phosphorylation by MEK1, ERK2 Δ6 has sufficient MBP kinase activity to suggest that it might have near wild type activity if it were more highly phosphorylated. Neither T183S/Y185G (SEG) nor Y185G (TEG) ERK2 has protein kinase activity toward MBP determined from internal standards are indicated. The phosphoamino acid analysis of other MAP kinases after MEK2 phosphorylation did not differ significantly from those shown in Fig. 2B. Panel C, MBP kinase activity of the wild type and mutant ERK2s after phosphorylation by MEK2. Results are the average of multiple experiments, and standard deviations are shown.

Constitutively Active MEK1 and MEK2—Previously it has been shown that deletion of a region of the MEK1 N terminus combined with replacement of conserved serines that are sites of activating phosphorylation with glutamic or aspartic acids (MEK1-R4F) greatly increases its basal activity (18). Similar mutations in MEK2 produce a similarly active form of MEK2 (MEK2-KW71) (19).

We compared the activities of these mutant, active MEKs with their phosphorylated, active wild type counterparts. The most noticeable difference between the constitutive MEKs and wild type MEKs was that the constitutively active MEK2-KW71 has now lost the ability to phosphorylate ERK3 and TEY ERK3 (data not shown). Furthermore, in a number of the intervening site mutants and lip deletion mutants, threonine is poorly recognized by both MEK1-R4F and MEK2-KW71 (data not shown). We are currently examining the differences between wild type and constitutively active MEKs in greater detail.

**MEK3**—When tested on our panel of proteins, MEK3 phosphorylated only p38 to high stoichiometry (Fig. 4A). MEK3 did exhibit low but reproducible activity toward SAPKβ, ERK3, and TEY ERK3. Interestingly, MEK3 phosphorylated S189T/G191Y (TEY) ERK3 primarily on tyrosine (Fig. 4B). This is in contrast to MEK2, which phosphorylated TEY ERK3 primarily on threonine (Fig. 3B). MEK3 did not phosphorylate E184G ERK2, ERK2 Δ6, or E184G ERK2 Δ6. This is in spite of the fact that these mutants contain the same residue between the phosphorylation sites and/or a lip of the same length as p38.
MEK Specificity

Fig. 5. Phosphorylation of wild type MAP kinases and ERK2 mutants by MEK4. Panel A, stoichiometry of phosphate incorporation into the kinases. Data are the average of at least three independent experiments, and standard deviations are indicated. Panel B, phosphoamino acid analysis of p38 and SAPKβ. Positions of phosphoamino acid standards are indicated based on internal standards.

ATF2 and c-Jun kinase activity of p38 was similar regardless of whether MEK3, MEK4, or MEK6 was used as the activator (Fig. 4C).

MEK4—MEK4 phosphorylated both p38 and SAPKβ close to or greater than 2 mol of phosphate/mol (Fig. 5A), even though they contain a different intervening residue (TGY versus TPY). This confirms reports in the literature that both MAP kinases may be targets for MEK4 in vivo (28). Like MEK3, MEK4 does not recognize any of the ERK2 mutants, even E184P ERK2, ERK2Δ4, and E184P ERK2Δ4, which are designed to mimic the lip of JNK/SAPK. Phosphoamino acid analysis shows, however, that MEK4 recognizes threonine poorly in the TPY phosphorylation lip of SAPKβ (Fig. 5B). Like p38, SAPKβ phosphorylates both ATF2 and c-Jun with approximately equal specific activities (Fig. 4C).

MEK5 and MEK6—MEK5 was tested with a number of the ERK2 mutants and did not show activity toward any of them. In fact, to date, MEK5 has not been shown to phosphorylate any member of the MAP kinase family, even its putative target MAP kinase ERK5 (5, 6). MEK6 was recently cloned as an activator of p38 (21, 29–31). In our study, p38 was the only MAP kinase phosphorylated by MEK6 (data not shown). This specificity is consistent with a physiological role for MEK6 in phosphorylation of p38.

DISCUSSION

Among the protein kinases, MEKs, the activating enzymes upstream of the MAP kinases, have a remarkable degree of specificity. They are highly selective among MAP kinase family members and recognize only the native conformation of their targets (12). We tested the specificity of the known MEK family members using ERK2, ERK3, and mutants of each as well as SAPK/JNK and p38 to examine the contributions of the phosphorylation sites, intervening residue, lip length, and backbone context of the substrate to the specificity of MEKs.

Different members of the MAP kinase family contain different intervening residues between their threonine and tyrosine phosphorylation sites. Within a MAP kinase subfamily the intervening residue remains conserved. For instance, all the members of the SAPK/JNK subfamily contain proline at this position (1). The multiple p38 kinases and the yeast homolog HOG1 contain glycine at this position (1). Because of the conservation of the intervening residue within a MAP kinase subfamily and the fact that the intervening residue is different among different MAP kinase subfamilies, it has been suggested that the role of the amino acid between the two sites of MEK phosphorylation might be to direct MEK specificity (14, 15). We tested this hypothesis by mutating E184 in ERK2 in the TEY sequence to the corresponding amino acids found in other MAP kinases (see Fig. 1B). We found that both MEK1 and MEK2 phosphorylated the Glu184 mutants as well as wild type ERK2, suggesting that the intervening residue has little or no effect on MEK1 or MEK2 specificity. Further, these changes did not allow ERK2 to be phosphorylated by other MEK family members. Thus, we conclude that the presence of a certain intervening residue will not direct a MEK to phosphorylate a different MAP kinase family member from its natural substrate. It is possible that the residue between the sites of phosphorylation may be important in other ways, such as directing phosphatase specificity, but this remains to be determined.

Members of the MAP kinase family vary in the length of their phosphorylation lip. ERK2 and ERK3 have the longest lip of the MAP kinases we studied, followed by JNK/SAPK and then p38. Structural analysis of the ERK2 phosphorylation lip revealed that it was a highly flexible structure because even the conservative mutation Tyr185 → Phe resulted in a different conformation of this lip (32, 33). Thus, we considered the possibility that the length of the phosphorylation lip might be a significant determinant of lip conformation and thereby MEK specificity. Using lip deletion mutants we found that the lip length does not substantially influence the ability of MEKs to phosphorylate ERK2. One consequence of shortening the lip is that its interactions with underlying structures may be impaired. In the future we hope to probe the importance of backbone versus lip conformation further by stabilizing distinct lip conformations on the same kinase core.

Unanticipated results were obtained when MEK1 and MEK2 were tested for their abilities to phosphorylate the mutants T183S/Y185G ERK2, T183S ERK2, Y185G ERK2, and wild type ERK2. T183S/Y185G ERK2 is not phosphorylated by either MEK1 or MEK2, although Y185G ERK2 is phosphorylated by both MEK1 and MEK2. As threonine contains a β-methyl group not present in serine, perhaps threonine in ERK2 must fit in a well-defined binding pocket in the MEK active site. Phosphorylation of T183S ERK2 occurs both on serine and tyrosine, indicating that the presence of Tyr185 now allows MEK1 and MEK2 to interact with a serine at position 183. T183S/Y185T ERK2 is not phosphorylated detectably by MEK1 or MEK2. These results may reflect structural requirements that are necessary for proper lip conformation (35). Furthermore, SEG ERK2, TEG ERK2, and wild type (SEG) ERK2 have little to no activity toward the substrate MBP. This suggests that defects in MBP kinase activity with the Tyr → Gly mutants could be due to the effect of the mutation itself on MBP kinase activity and that the phosphorylation lip may play a role in substrate specificity. To date, however, we have not found that any of the phosphorylation lip mutants become able to phosphorylate other MAP kinase substrates such as the N terminus of c-Jun or ATF2.

Use of ERK3 allowed us to define a significant difference in specificity between MEK1 and MEK2. Wild type ERK3 contains a SEG sequence in its phosphorylation lip, which is the site of phosphorylation by the ERK3 kinase (7). Although
MEK1 could not phosphorylate ERK3 or TEY ERK3, MEK2 phosphorylated it both on serine and threonine but not tyrosine. It is curious that MEK2 cannot phosphorylate serine in an SEG motif in ERK2 but can phosphorylate serine in that motif in ERK3. The difference between MEK1 and MEK2 in their abilities to phosphorylate ERK3 in vitro is striking given recent data concerning their possible differences in vivo (34).

Our data demonstrate that the MAP kinase phosphorylation lip plays a relatively minor role in directing MEK specificity. In contrast, it appears that the kinase backbone has a major role in MEK interactions, as the backbone was the only major determinant of specificity in our experiments. Consistent with this hypothesis are results from Brunet and Pouyssegur (35), who reported that in vivo pathway specificity determinants may lie in the N-terminal domain of MAP kinases.

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