Hydrophobic as Well as Charged Residues in Both MEK1 and ERK2 Are Important for Their Proper Docking*

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Docking between MEK1 and ERK2 is required for their stable interaction and efficient signal transmission. The MEK1 N terminus contains the ERK docking or D domain that consists of conserved hydrophobic and basic residues. We mutated the hydrophobic and basic residues individually and found that loss of either type reduced MEK1 phosphorylation of ERK2 in vitro and its ability to bind to ERK2 in vivo. Moreover, ERK2 was localized in both the cytoplasm and the nucleus when co-expressed with MEK1 that had mutations in either the hydrophobic or the basic residues. We then identified two conserved hydrophobic residues on ERK2 that play roles in docking with MEK1. Mutating these residues to alanine reduced the interaction of ERK2 with MEK1 in cells. These mutations also reduced the phosphorylation of MEK1 by ERK2 but had little effect on phosphorylation of MBP by ERK2. Finally, we generated docking site mutants in ERK2-MEK1 fusion proteins. Although the mutation of the MEK1 D domain significantly reduced ERK2-MEK1 activity, mutations of the putatively complementary acidic residues and hydrophobic residues on ERK2 did not change its activity. However, both types of mutations decreased the phosphorylation of Elk-1 caused by ERK2-MEK1 fusion proteins. These findings suggest complex interactions of MEK1 D domains with ERK2 that influence its activation and its effects on substrates.

MAP kinase cascades are found in organisms ranging from yeast to human (1–3). A typical MAP kinase pathway consists of at least three kinases: a MEKK that phosphorylates and activates the downstream kinase MEK; a MEK that phosphorylates and activates the MAP kinase; and the MAP kinase that has a variety of targets. The most studied mammalian MAP kinase pathway is the Raf/MEK/ERK pathway. Upon activation of the pathway, Raf-1 phosphorylates and activates the MAP kinase; and the MAP kinase activates the downstream kinase MEK; a MEK that phosphorylates and activates the MAP kinase via its activation loop of MEK1/2, and MEK1/2 in turn will phosphorylate a threonine and a tyrosine in the activation loop of ERK1/2.

MAP kinase docking domains have been identified in ERK1/2 substrates, including transcription factors and protein kinases. The most thoroughly characterized MAP kinase docking domain is the D domain originally identified in the ternary complex factor Elk-1 (4). These docking domains have now been recognized not only in substrates but also in the upstream kinases, MEK1 and MEK2, and in dual specificity and phosphotyrosine phosphatases (5, 6). The typical D domain contains a cluster of basic residues followed by two conserved hydrophobic residues (e.g. KKKPTPIQL in MEK1). The MEK1 D domain is required for efficient phosphorylation and activation of ERK2 and for interaction with ERK2 in cells (7–9). Deletion of the MEK1 D domain decreases not only the feedback phosphorylation of MEK1 by ERK2 but also the phosphorylation of ERK2 by MEK1 in vitro (8). D domains are also necessary for inactivation of MAP kinases by some of the phosphatases. The protein kinase substrates of MAP kinases including RSKs and MNKs contain variants of the D domain motif, having a typical sequence LAQRR (5, 10–12). This domain on RSK interacts with ERK2 directly and is required for intracellular recognition by ERK2. Another ERK docking motif found in transcription factors such as Elk-1, SAP-1, and LIN-1 is the FXFP sequence (13). It can work by itself or together with a D domain to mediate kinase-substrate interactions.

Different experimental approaches have revealed multiple domains on ERKs that are involved in docking interactions with MEKs and substrates. Two groups independently identified a common docking (CD) motif, found in all known MAP kinase family members, that mediates interactions with MEKs, MAP kinase phosphatase 3, and the protein kinase substrate MNK1 (9, 14). The CD domain is located near the C termini of MAP kinases and contains two acidic residues that are thought to interact directly with the basic residues in D domains. Several studies with MAP kinase chimeras suggested that ERK1/2 residues in the N-terminal folding domain of the kinase are also important; these include the C helix and the ERK2 N terminus (8, 15, 16). Weber and co-workers (17) have also recently reported that a short sequence at the ERK2 N terminus is involved in MEK binding.

In this study, we examined the importance of the conserved hydrophobic and basic residues in the MEK1 D domain to its ability to phosphorylate ERK2 in vitro and interact with ERK2 in vivo. In addition, two tyrosine residues near the CD domain on ERK2 were identified as being important for MEK1 interaction. We also tested the effects of the docking site mutations on the activation and activity of ERK2-MEK1 fusion proteins (18).

MATERIALS AND METHODS

DNA, Site-directed Mutagenesis, and Proteins—pCMV5-Myc-MEK1, pCEP-PA-ERK2, pCEP-PA-MEK1, and pCMV5-MEK1-R4F were as described (8, 19). pCMV5-Myc-ERK2-MEK1 and pCMV5-Myc-ERK2-
followed by boiling for 2 min. The reactions were stopped by adding 7.5 M SDS followed by SDS-polyacrylamide gel electrophoresis.

Three times in 1 M NaCl, 20 mM Tris-HCl (pH 7.4), and once in 10 M NaCl, 10 mM NaCl, and 10 mM MgCl2 followed by SDS-polyacrylamide gel electrophoresis.

Immunoprecipitates were then washed with 40% protein A-Sepharose beads. Immunoprecipitated proteins were immunoprecipitated from cell lysates with the anti-Myc antibody and 50 µl of protein A-Sepharose beads. The beads were then washed three times in 100 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 10 mM MgCl2 followed by SDS-polyacrylamide gel electrophoresis.

Immuno-

Fluorescence was performed as described (20, 21). 293 cells were cultured on coverslips coated with either poly-lysin or collagen (Sigma), fixed, and permeabilized. Primary antibodies used were anti-HA and anti-Myc antibody. Goat anti-mouse fluorescein secondary antibody was then used followed by diamidinophenylindole staining to observe the nucleus.

Kinase Assays and Immunoblots—Kinase assays were performed in 30 µl of 1× kinase buffer at 30 °C for the indicated times (8). The reactions were stopped by adding 7.5 µl of 5× SDS sample buffer followed by boiling for 2 min. The reactions (20 µl) were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Immunoblots were developed essentially as described using enhanced chemiluminescence (8).

Cell Culture, Transient Transfections, and Preparation of Cell Lysates—HEK 293 cells were maintained, transfected, and harvested as described (8). Triton X-100 (1%) was present in the lysis buffer except for in MEK1-ERK2 co-immunoprecipitation experiments, in which no detergent was added to the lysis buffer.

Computer Modeling—The unphosphorylated ERK2 structure was created based on the published data using the Insight II (Molecular Biosystems) program.

RESULTS

Both the Hydrophobic and Basic Residues in the MEK1 D Domain Are Required for Its Efficient Phosphorylation of ERK2 in Vitro—The MEK1 N terminus contains an ERK docking or D domain; this domain is also present in phosphatases and substrates of ERK1/2 (5). Within the MEK1 D domain, a cluster of basic residues and two hydrophobic residues are well conserved among MEKs across species (Fig. L4). Previously, we showed that deletion of the MEK1 N terminus, including the D domain, reduced its ability to phosphorylate ERK2 in vitro (8). In the present study, we have examined the individual contributions of these conserved residues in the MEK1 D domain by creating point mutants (Table I).

HEK 293 cells were transfected with Myc-tagged wild type MEK1 or MEK1 mutants. The cells either were left untreated or were treated with 100 ng/ml EGF for 5 min. MEK1 proteins were immunoprecipitated with the anti-Myc antibody and subjected to in vitro kinase assays using GST-ERK2 (K52R) as substrate. An autoradiogram is shown. The lysates were blotted with the anti-Myc antibody to compare protein expression. Bottom panel, plot showing the average incorporation of 32P into ERK2 determined by scintillation counting from three independent experiments.

MEK1 or MEK1 mutants. The cells either were left untreated or were treated with EGF prior to harvest. MEK1 was then immunoprecipitated using the anti-Myc antibody, and its kinase activity was assayed using GST-ERK2 (K52R) as substrate. MEK1 along with the MEK1 mutants toward ERK2 in vitro. However, both the ILAA mutant and the KKMM mutant showed much reduced activity either with or without EGF stimulation, indicating that both the conserved hydrophobic residues and the basic residues in the MEK1 D domain are required for efficient ERK2 phosphorylation. These results are similar to those observed with MEK1 lacking the D domain (8).

A MEK1 N-terminal Peptide Containing the D Domain Residues Can Inhibit MEK1 Phosphorylation of ERK2—We previously demonstrated that a MEK1 N-terminal peptide (P1) that contains the D domain was able to inhibit the feedback phosphorylation of MEK1 by ERK2 (8). Here we examined whether this peptide would inhibit MEK1 phosphorylation of ERK2. His-tagged, constitutively active MEK1 (S218E/S222D) was used to phosphorylate GST-ERK2 (K52R) as the substrate (Fig. 1B). EGF enhanced the activities of wild type MEK1 along with the MEK1 mutants toward ERK2 in vitro.

However, both the ILAA mutant and the KKMM mutant showed much reduced activity either with or without EGF stimulation, indicating that both the conserved hydrophobic residues and the basic residues in the MEK1 D domain are required for efficient ERK2 phosphorylation. These results are similar to those observed with MEK1 lacking the D domain (8).

![Fig. 1](http://www.jbc.org/)

**Table I**

| Protein | Name     | Mutations                        |
|---------|----------|---------------------------------|
| MEK1    | ILAA     | I9A and L11A                    |
|         | KKKM     | K3M and K4M                     |
|         | AAMM     | I9A, L11A, K3M, and K4M         |
| ERK2    | YYAA     | Y314A and Y315A                 |
|         | DDAA     | D316A and D319A                 |
|         | AAA      | D316A, D319A, and E320A         |
|         | 5A       | Y314A, Y315A, D316A, D319A, and E320A |

**TABLE I**

**Mutants used in this study**

| Protein | Name     | Mutations                        |
|---------|----------|---------------------------------|
| MEK1    | ILAA     | I9A and L11A                    |
|         | KKKM     | K3M and K4M                     |
|         | AAMM     | I9A, L11A, K3M, and K4M         |
| ERK2    | YYAA     | Y314A and Y315A                 |
|         | DDAA     | D316A and D319A                 |
|         | AAA      | D316A, D319A, and E320A         |
|         | 5A       | Y314A, Y315A, D316A, D319A, and E320A |

**Table I**

| Protein | Name     | Mutations                        |
|---------|----------|---------------------------------|
| MEK1    | ILAA     | I9A and L11A                    |
|         | KKKM     | K3M and K4M                     |
|         | AAMM     | I9A, L11A, K3M, and K4M         |
| ERK2    | YYAA     | Y314A and Y315A                 |
|         | DDAA     | D316A and D319A                 |
|         | AAA      | D316A, D319A, and E320A         |
|         | 5A       | Y314A, Y315A, D316A, D319A, and E320A |

**Table II**

| Protein | Name     | Mutations                        |
|---------|----------|---------------------------------|
| MEK1    | ILAA     | I9A and L11A                    |
|         | KKKM     | K3M and K4M                     |
|         | AAMM     | I9A, L11A, K3M, and K4M         |
| ERK2    | YYAA     | Y314A and Y315A                 |
|         | DDAA     | D316A and D319A                 |
|         | AAA      | D316A, D319A, and E320A         |
|         | 5A       | Y314A, Y315A, D316A, D319A, and E320A |
the P1 peptide at 20 μM. At 200 μM P1, nearly complete inhibition was achieved, similar to what was seen with the feedback phosphorylation (8). On the other hand, the control P2 peptide had little effect on MEK1 phosphorylation of ERK2 even at the highest concentration (200 μM). In contrast, neither P1 nor P2 had any effect on the ability of MEK1-DN1, which does not contain the D domain, to phosphorylate GST-ERK2 (K52R) in vitro (data not shown). These results indicated that a functional D domain peptide could efficiently inhibit MEK1 phosphorylation of ERK2.

Mutating Either the Hydrophobic or Basic Residues in the MEK1 D Domain Reduces Its Interaction with ERK2 in Vivo—The N-terminal D domain of MEK1 is apparently both required and sufficient for binding to ERK2 (7, 9). We previously showed that a MEK1 N-terminal deletion mutant (MEK1-DN1) failed to co-immunoprecipitate with ERK2 (8). Here we tested the ability of the MEK1 ILAA and KKMM mutants to co-immunoprecipitate with ERK2. 293 cells were co-transfected with HA-ERK2 and pCMV5-Myc vector control, wild type MEK1, or the MEK1 mutants. Myc-tagged MEK1 proteins were immunoprecipitated (IP) with the anti-Myc antibody, and the precipitates were blotted with the anti-HA antibody. The same blot was then stripped and re-probed with the anti-Myc antibody. The cell lysates were also blotted with the anti-HA antibody. B, 293 cells were co-transfected with pCEP4-HA-ERK2 and various Myc-MEK1 constructs. The cells were fixed and subjected to immunofluorescence. The localization of HA-ERK2 was visualized by staining the cells with the anti-HA antibody, and the nucleus was detected by diamidino-phenylindole (DAPI) staining. One of five similar experiments is shown.

FIG. 3. The conserved residues of MEK1 D domain are required for MEK1-ERK2 interaction in vivo. A, 293 cells were co-transfected with pCEP4-HA-ERK2 and pCMV5-Myc vector control or various MEK1 constructs. Myc-MEK1 proteins were immunoprecipitated (IP) with the anti-Myc antibody, and the precipitates were blotted with the anti-HA antibody. The cell lysates were also blotted with the anti-HA antibody. B, 293 cells were co-transfected with pCEP4-HA-ERK2 and various Myc-MEK1 constructs. The cells were fixed and subjected to immunofluorescence. The localization of HA-ERK2 was visualized by staining the cells with the anti-HA antibody, and the nucleus was detected by diamidino-phenylindole (DAPI) staining. One of five similar experiments is shown.

Two Conserved Hydrophobic Residues near the CD Domain on ERK2 Play Roles in Docking with MEK1—Several regions on ERK2 have been identified as being important for MEK1 interaction (8, 9, 14–17). Among these, the so-called CD domain near the C terminus of ERK2 that contains two acidic residues in the MEK1 D domain are required for its association with ERK2.

The MEK1 ILAA and KKMM Mutants Are Defective in Sequestering Overexpressed ERK2 in the Cytoplasm in Quiescent Cells—Overexpressed ERK2 accumulates in the nucleus (7, 14, 17). MEK1 can restrict ERK2 to a cytoplasmic localization in quiescent cells. Because the MEK1 D domain mutants were defective in interacting with ERK2, we tested whether they would still be able to cause ERK2 to be in the cytoplasm. 293 cells were co-transfected with HA-ERK2 and either the vector control or the Myc-MEK1 (wild type and mutants). The subcellular localization of HA-ERK2 was visualized by immunofluorescence using the anti-HA antibody (Fig. 3B). In the absence of MEK1, overexpressed ERK2 was present in both cytoplasm and nucleus as previously reported. In the presence of wild type MEK1, ERK2 was predominantly in the cytoplasm, consistent with the ability of MEK1 to remove ERK2 from the nucleus. However, in the presence of either the ILAA MEK1 mutant or the KKMM MEK1 mutant, ERK2 was distributed throughout the cell, with a substantial amount in the nucleus. The localization is similar to that in cells in which MEK1 was not overexpressed. These data indicate that both the hydrophobic and basic residues are necessary for MEK1 to exclude overexpressed ERK2 from the nucleus in unstimulated cells.
residues (Asp\textsuperscript{316} and Asp\textsuperscript{319}) has been proposed to interact directly with the basic residues in the MEK1 D domain (9, 14). Because the two hydrophobic residues in the MEK1 D domain are important for MEK1-ERK2 interaction, we speculated that there would be hydrophobic residues near the CD domain on ERK2 that could play roles in docking with MEK1. By examining the primary sequence of ERK2 and other MAP kinases, we noticed that Tyr\textsuperscript{314} and Tyr\textsuperscript{315} in ERK2 are residues with large side chains (Tyr, His, or Trp) in all MAP kinase family members (Fig. 4A). The crystal structure of ERK2 shows that the side chains of both Tyr\textsuperscript{314} and Tyr\textsuperscript{315} are exposed on the surface (22), not buried inside the molecule, suggesting that they may be involved in interacting with other proteins (Fig. 4B).

Tyr\textsuperscript{314} and Tyr\textsuperscript{315} were mutated to alanine to generate the YYAA ERK2 mutant. The CD domain residues Asp\textsuperscript{316} and Asp\textsuperscript{319} along with Glu\textsuperscript{320} were also mutated to alanine to generate the AAA ERK2 mutant. Asp\textsuperscript{316} and Asp\textsuperscript{319} alone were mutated to alanine to generate the DDAA ERK2 mutant. Finally, all five residues were mutated to alanine to generate the 5A ERK2 mutant (Table I). Vector control, Myc-tagged wild

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**Fig. 4. Identification of two conserved hydrophobic residues near the CD domain in ERK2.** A, sequence alignment of the CD domain sequence among several MAP kinases ranging from budding yeast to human. The numbers mark the residues in the ERK2 sequence. The two conserved acidic residues (Asp\textsuperscript{316} and Asp\textsuperscript{319} in ERK2) are in bold type, and the two conserved hydrophobic residues (Tyr\textsuperscript{314} and Tyr\textsuperscript{315} in ERK2) are underlined. B, ERK2 structure with Asp\textsuperscript{316} and Asp\textsuperscript{319} shown in red and Tyr\textsuperscript{314} and Tyr\textsuperscript{315} shown in yellow. The figure was generated with the Insight II program.

**Fig. 5. Tyr\textsuperscript{314} and Tyr\textsuperscript{315} in ERK2 are important for MEK1-ERK2 docking.** A, 293 cells were co-transfected with pCEP4-HA-MEK1 and pCMV5-Myc vector control or various ERK2 constructs. Myc-tagged ERK2 proteins were immunoprecipitated (IP) with the anti-Myc antibody, and the immunoprecipitates were blotted with the anti-HA antibody. The blot was stripped and reprobed with the anti-Myc antibody to compare the amounts of ERK2 proteins present in the precipitates. The lysates were also blotted with the anti-HA antibody to compare the expression of HA-MEK1. B, left panel, 293 cells were co-transfected with a constitutively active form of MEK1 and pCMV5-Myc-ERK2 constructs. The wild type (WT) and mutant ERK2 proteins were immunoprecipitated with the anti-Myc antibody and subjected to kinase assays using either MBP or His-tagged, kinase dead MEK1 as substrates. Cells lysates were blotted with the anti-Myc antibody to compare the expression of ERK2. Right panel, plot of \textsuperscript{32}P incorporation from three experiments is shown.
type ERK2, or ERK2 mutants were co-transfected into 293 cells with HA-MEK1. ERK2 proteins were immunoprecipitated with the anti-Myc antibody, and the presence of HA-MEK1 in the ERK2 immunoprecipitates was examined by blotting with the anti-HA antibody. As expected, wild type ERK2 precipitated a significant amount of MEK1 protein. In contrast, all three ERK2 mutants tested in the assay extracted less MEK1 from the lysates (Fig. 5A). These findings suggest that both Tyr314/Tyr315 and Asp316/Asp319/Glu320 are important for interacting with MEK1 in cells.

We next examined the effects of these mutations on the kinase activity of ERK2. Myc-tagged ERK2, wild type and mutants, were co-transfected into 293 cells with a constitutively active form of MEK1. ERK2 proteins were then immunoprecipitated with the anti-Myc antibody and assayed for their kinase activity using either MBP or His-MEK1 (K97M) as substrates (Fig. 5B). Wild type ERK2 and the ERK2 mutants showed comparable activity toward MBP, indicating that all of the ERK2 mutants were phosphorylated and activated in cells by overexpressed, active MEK1. When His-MEK1 was used as the substrate, the YYAA mutant showed a modest but reproducibly decreased kinase activity toward it compared with that catalyzed by wild type ERK2. Although the AAA mutant showed a clearly decreased phosphorylation, the 5A mutant had even less activity toward MEK1. Similar results were achieved when lysates from EGF-treated or Ras-V12 co-transfected cells were used instead (data not shown). These results indicate that not only the acidic residues of the CD domain but also the tyrosine residues near the CD domain are important for the interaction of ERK2 with MEK1.

Mutations of the Docking Residues in ERK2-MEK1 Fusion Proteins Reduce Their Activity—To examine the MEK1-ERK2 docking interaction under circumstances in which the proteins are covalently bound, we introduced specific mutations into ERK2-MEK1 fusion proteins. A constitutively active form of ERK2 was created by covalently linking ERK2 to MEK1 (18). A mutant form of the fusion protein ERK2-MEK1 (LA), which replaces four leucine residues in the MEK1 nuclear export sequence with alanine, has higher activity than the wild type fusion protein. To test whether docking domain mutations would affect the ERK2-MEK1 fusion proteins, we made fusion protein mutants in both the MEK1 D domain and the ERK2 CD domain.

Myc-tagged ERK2-MEK1 fusions (wild type and mutants) were immunoprecipitated from transfected 293 cell lysates and assayed for kinase activity using MBP as the substrate, so that effects of ERK2 activation could be assessed using a substrate that does not contain a D domain (Fig. 6A). Surprisingly, MEK1 ILAA mutations greatly reduced ERK2-MEK1 (LA) activity, whereas the KKMM mutations reduced its activity modestly. These results indicated that the MEK1-ERK2 docking interaction is required for ERK2 activation even when ERK2 and MEK1 are covalently linked to each other. Interestingly, neither the ERK2 YYAA nor the DDAA mutations had much effect on ERK2 activity in the ERK2-MEK1 (LA) fusion. To confirm results from these activity assays, we also performed anti-phospho-ERK1/2 blots on the ERK2-MEK1 fusion proteins with the same set of protein lysates. Overall, results were comparable with those from the kinase assays with MBP as substrate. Similar results were obtained with both ERK2-MEK1 and ERK2-MEK1 (LA) fusion proteins and mutants.

To examine whether these docking site mutations in ERK2-MEK1 fusion proteins affect their ability to phosphorylate and activate the ternary complex transcription factor Elk-1, wild type and mutant ERK2-MEK1 fusion constructs were transfected into 293 cells along with pCMV5-His-Elk-1. An antibody that specifically recognizes Elk-1 phosphorylated on Ser383 was used to detect the phosphorylated, active forms of Elk-1 (Fig. 6B). As shown before, ERK2-MEK1 (LA) caused significant activation of Elk-1, whereas the ERK2-MEK1 fusion, which is excluded from the nucleus, had no effect on Elk-1 (18). In contrast, both the MEK1 D domain mutants (ILAA and KKMM) and the ERK2 mutants (YYAA and AAA) decreased Elk-1 activation caused by ERK2-MEK1 (LA). Because the fusion proteins with D domain mutations contain ERK2 that is less active than the wild type ERK2-MEK1 (LA), the reduced phosphorylation of Elk-1 may simply be a consequence of reduced ERK2 activity in these mutant fusion proteins. However, the activities of the fusion proteins containing the ERK2 mutations YYAA and AAA are the same as that of wild type ERK2-MEK1 (LA). Thus, their reduced activities toward Elk-1 suggest that these mutations impair the interaction of ERK2 with Elk-1.

**DISCUSSION**

Recently, docking between MAP kinases and their substrates has emerged as a recurring theme to ensure efficient enzymatic reactions and signal transmission (5, 6). The selective recognition of the binding motifs on substrates by MAP kinases also increases the specificity of each pathway (11). It
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appears that multiple regions on MAP kinases are involved in substrate recognition and interactions (8, 9, 14–17, 23).

Here we present evidence that both the hydrophobic and basic residues conserved in the MEK1 D domain are required for its efficient phosphorylation of ERK2 in vitro. A MEK1 N-terminal peptide containing the D domain motif not only inhibits the feedback phosphorylation of MEK1 by ERK2; it also inhibits MEK1 phosphorylation of ERK2 in vitro. Mutants that lack either the hydrophobic or the basic residues are defective in binding to ERK2 in vivo, as shown by weaker co-immunoprecipitation compared with wild type MEK1. Although wild type MEK1 is able to retain overexpressed ERK2 in the cytoplasm of quiescent cells, these MEK1 mutants cannot.

We also identified two conserved hydrophobic residues near the CD domain of ERK2 that contribute to the interaction of ERK2 with MEK1. First, mutation of the previously identified acidic residues or these hydrophobic residues weakens binding to MEK1 compared with that displayed by wild type ERK2. Second, both types of mutants phosphorylate MEK1 less well in vitro. The results suggest that both hydrophobic and electrostatic interactions are involved in MEK1-ERK2 docking.

The hydrophobic residues we identified in ERK2, tyrosine 314 and 315, are in close proximity to the CD domain aspartic acids, residues 316 and 319, in the three-dimensional structure, and their side chains are exposed. Interestingly, Rubinfeld et al. (14) identified the CD domain on ERK2 as a cytoplasmic retention sequence. They observed a stronger defect with the mutant that has alanine substituted for residues 312–319 than the mutant that has alanine substituted only for the three acidic residues, Asp316, Asp319, and Glu320, suggesting that there are additional residues between 312 and 318 involved in MEK1 interaction. Our findings here are consistent with their results. Tyr314 and Tyr315 may interact directly with the hydrophobic residues in the MEK1 D domain. Alternatively, they could affect MEK1-ERK2 docking indirectly, either by interaction with other residues on MEK1 or by altering the interface on ERK2 that comprises the docking site.

ERK2 also docks with its substrates and phosphatases that inactivate it, presumably via the same sites that recognize MEK1. Our results showing reduced phosphorylation of Elk-1 by the ERK2-MEK1 fusion protein with these tyrosine residues were mutated suggest that these hydrophobic residues are also important for substrate interactions. It will be interesting to determine the extent to which Tyr314 and Tyr315 interact with D domain-like docking motifs on other proteins. For instance, RSK contains a different docking domain from MEK1 or Elk-1, characterized as the LQARR motif, and Elk-1 also contains the distinct FXFP docking motif (5).

By examining the primary sequences of MAP kinases ranging from yeast to human, we found that residues corresponding to Tyr314 and Tyr315 in ERK2 are conserved in that all have large side chains at these positions. It is tempting to speculate that the corresponding residues in other MAP kinases may also be involved in docking with their MEKs. The crystal structure of p38 shows that Tyr311 and His312 are also near the two aspartic acids in the CD domain, and their side chains are exposed (24), suggesting they might have similar functions to Tyr314 and Tyr315 on ERK2.

An interesting but somewhat surprising result came from our studies using ERK2-MEK1 fusion proteins. Mutations in the MEK1 D domain resulted in a significant decrease of ERK2-MEK1 activity, whereas mutations in the ERK2 CD domain had little effect. With the fusion proteins, ERK2 and MEK1 are covalently bound, so their affinity is not an issue. The fact that MEK1 D domain mutations decreased the fusion protein activity suggests that the orientation of MEK1-ERK2 docking is critical for the efficient phosphorylation and activation of ERK2. On the other hand, neither mutation of Asp316 and Asp319 nor mutation of Tyr314 and Tyr315 of ERK2 in the fusion proteins affected their activity. These results suggest that unlike the D domain, which is the major docking site on MEK1, the CD domain and its vicinity on ERK2 are apparently not significant for this orientation effect, perhaps because of the presence of multiple docking domains on ERK2. Consistent with this idea, our data and results from other groups showed that the ERK2 CD domain mutants are still phosphorylated and activated in cells properly, despite defects they display in various other assays shown here and elsewhere (9, 14). Work from Resing and Ahn (25) using deuterium exchange mass spectrometry shows that the MEK1 N terminus has increased flexibility upon activation. Perhaps the MEK1 D domain helps to orient the otherwise floating MEK1 N terminus onto ERK2, so that disruption of the D domain would have a dramatic effect on MEK1-ERK2 docking. In contrast, the ERK2 structure is relatively rigid so that disruption of the CD domain may not affect MEK1-ERK2 docking in the fusion protein context.

An open and evolving question is whether there are additional domains or residues on ERK2 necessary for docking with MEK1. Nishida and co-workers (23) recently reported the identification of the ED domain on p38 and ERK2 near the substrate binding pocket that mediates docking interactions with substrates along with the CD domain. However, they did not examine the interaction with MEKs. Eben et al. (17) recently showed that residues 19–25 at the ERK2 N terminus are involved in docking with MEKs. Studies of ERK-p38 chimera also suggested motifs in the N-terminal domain of MAP kinases that are necessary for specific interactions with MEKs (8, 15, 16). A model of the interaction of ERK2 with MEK1 suggests that essential points of contact include regions from both of the folded domains of the kinase core as well as the MAP kinase insert in the C-terminal domain (16). Because the properly folded structure of ERK2 is essential for its recognition by MEK1 (26), mutations that impact the global structure of ERK2 may impair MEK recognition in a nonspecific manner. In any case, determination of the crystal structure of the docked proteins should provide substantial information still lacking from mutagenesis studies.

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