A Docking Site in MKK4 Mediates High Affinity Binding to JNK MAPKs and Competes with Similar Docking Sites in JNK Substrates*

David T. Ho, A. Jane Bardwell, Mahsa Abdollahi, and Lee Bardwell‡

From the Department of Developmental and Cell Biology, University of California, Irvine, California 92697

Received for publication, April 22, 2003, and in revised form, June 2, 2003
Published, JBC Papers in Press, June 3, 2003, DOI 10.1074/jbc.M304229200

Mitogen-activated protein kinases (MAPKs)† are activated by a wide variety of stimuli and contribute to the regulation of diverse responses, often in a cell context-dependent manner. Their ubiquity and versatility raise the issue of how they achieve specific coupling of signal to cellular response (1).

Central to these cascades is a MAPK kinase (MKK) that phosphorylates and thereby activates the MAPK. Activated MAPKs phosphorylate multiple targets, including transcription factors, other kinases, and other enzymes. In mammals, there are three major MAPK pathways (the extracellular signal-regulated kinase (ERK)-1/2, c-Jun N-terminal kinase (JNK), and p38 modules) that each contain two to four different MAPKs regulated by two different MKKs (2). (Some MAPKs are also called ERKs, and some MKKs are also called MAPK/ERK kinases (MEKs)). In general, the MKKs in one pathway do not phosphorylate MAPKs in other pathways. How this selectivity is achieved is incompletely understood.

The JNK pathway is activated primarily in response to cytokines and environmental stress and plays an important role in the regulation of stress-induced apoptosis (reviewed in Refs. 3–5). Key targets phosphorylated by JNK include components of the AP-1 transcription factor such as c-Jun and ATF2. Current thinking suggests that the JNK cascade and AP-1 can cooperate in the pathogenesis of many human diseases, including cancer (8), obesity and insulin resistance (9), muscular dystrophy (10), arthritis (11), aspects of heart disease (12), Parkinson’s disease (13), and other neurological disorders characterized by abnormal cell death (14).

There are three different JNK proteins (JNK1, JNK2, and JNK3) encoded by separate genes. Two protein kinases that activate JNK have been identified: MKK4 (also known as JNKK1 and SEK1) and MKK7/JNKK2. Although both MKK4 and MKK7 activate JNK, they are functionally distinct in several ways (4). First, although both kinases activate JNK by dual phosphorylation at Thr and Tyr, MKK4 prefers Tyr, and MKK7 prefers Thr (15). Second, MKK4 is activated primarily by environmental stress, and MKK7 by cytokines. Third, MKK4 (but not MKK7) can also activate the p38 MAPK. Finally, MKK4 (but not yet MKK7) is a candidate tumor/metastasis suppressor, as inactivating mutations in the MKK4 gene (MAP2K4) have been found in ~5% of a wide variety of tumor types (16–18).

As in other MAPK pathways, the formation of stable complexes between MKKs and MAPKs is thought to contribute to signal transmission and specificity in the JNK pathway (19, 20). Full-length MKK4 has been shown to form complexes with JNK1/SAPKα (20), JNK2/SAPKα (21), and JNK3/SAPKβ (22, 23). Like other MKKs, MKK4 consists of a highly conserved catalytic domain and an N-terminal extension that exhibits substantially less conservation (see Fig. 1A). The N-terminal extension has been found to mediate MAPK binding by many MKKs, including yeast Ste7 (24) as well as mammalian MKE1 (25), MEK2 (26), MKK3/MKK6 (27), and MKK7 (28). In keeping with this pattern, the N-terminal extension of MKK4 has been shown to be both necessary and sufficient for JNK binding (20, 23).

* This work was supported by National Institutes of Health Training Grant LM07443 (to D. T. H.) and by seed funds from the University of California, Irvine, Institute for Genomics and Bioinformatics, by a Burroughs Wellcome Foundation New Investigator Award, by a Beckman Foundation Young Investigator Award, and by National Institutes of Health Research Grant GM60366 (all to L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Developmental and Cell Biology, 5205 Maughall Hall, University of California, Irvine, CA 92697-2300. Tel.: 949-824-6902; Fax: 949-824-4709; E-mail: bardwell@uci.edu.

The abbreviations used are: MAPKs, mitogen-activated protein kinases; MKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; JNKK, JNK kinase; SEK, SAPK/ERK kinase; SAPK, stress-activated protein kinase; GST, glutathione S-transferase; SH3, Src homology-3; MKP, MAPK phosphatase.
The N-termini of many MKKs contain a conserved MAPK-docking site (consensus sequence (KR)_{2,3}X_{1,6}-L(X)L-X(LL)), herein referred to as the “D-site” that was first recognized based on sequence similarity to yeast Ste7 (26, 29). Indeed, the D-site in Ste7 (24, 26) and those in mammalian MEK1 (26, 30), MEK2 (26), and MKK3 and MKK6 (27) have been shown to mediate high affinity binding to their cognate MAPRs. Similar and related D-sites were also found in the transcription factors c-Jun (31) and Elk-1 (32) as well as in other transcription factors, phosphatases, scaffolds, other kinases, and other proteins (see Fig. 1B) (reviewed in Refs. 33 and 34). D-sites appear to be portable modular motifs that mediate the interaction of MAPKs with multiple binding partners, contributing to both signal transmission and specificity. Yet, the fact that distinct docking sites share a core consensus sequence suggests that there might be limits to the specificity intrinsic to docking interactions.

The D-sites in MEK1, MEK2, MKK3, and MKK6 have been shown to be substrates for cleavage by the lethal factor protease of Bacillus anthracis (anthrax) (35); furthermore, lethal factor cleavage has been shown to compromise the ability of some of these enzymes to activate their cognate MAPKs (36, 37). MKK4 is also cleaved by lethal factor at two sites in its N terminus (see Fig. 1A) (35), but whether one or both of these cleavage sites are also MAPK-docking sites has not heretofore been examined. A putative D-site that fits the consensus sequence (KR)_{2,3}X_{1,6}-L(X)L-X(LL) has been identified in MKK4 (26), but has not previously been shown to be functional; this D-site corresponds to the first of the two lethal factor cleavage sites. Here, we show that this D-site is indeed functional, necessary, and sufficient for JNK binding; quantify its selectivity for JNK versus ERK2; and provide evidence that it competes with D-site-containing substrates for binding to JNK.

EXPERIMENTAL PROCEDURES

Genes—The following human genes were used in this study: MEK1 (MAP2K1; GenBankTM/EBI accession number NM_002755), MEK2 (MAP2K2; accession number NM_030662), MKK4 (MAP2K4; accession number NM_003010), JNK3a1 (MAPK10; accession number NM_002753), p38α (MAPK14; L35253), and p38δ (MAPK11; accession number NM_002751).

Proteolysis, lysis, and lysis—Several purified or partially purified proteins used in this study were purchased from Cell Signaling Technology, Upstate Biotechnology, Inc., Calbiochem, New England Biolabs Inc., and Santa Cruz Biotechnology. These included fused of glutathione S-transferase (GST) to human c-Jun(1–89), ATF2(19–96), and Elk-1(307–428) all from Cell Signaling Technology; the GenBankTM/EBI accession numbers of the corresponding full-length proteins are NM_002298, NM_001850, and NM_002299, respectively; inactive (K52R mutation) mouse ERK2 (Cell Signaling Technology; accession number BAA01733); unactivated rat ERK2 (Calbiochem; accession number M64300); unactivated human JNK1a1 (Upstate Biotechnology, Inc. or Santa Cruz Biotechnology; accession number L26318), JNK1p1 (Calbiochem; accession number U35904), and JNK2a2 (Upstate Biotechnology, Inc.; accession number L31951) and mouse ERK2 (New England Biolabs Inc.; accession number BAA01733) and activated human JNK2a2 (Upstate Biotechnology, Inc.; accession number L31951) and mouse ERK2 (New England Biolabs Inc.; accession number BAA01733); and activated human MEK2 (Upstate Biotechnology, Inc.; accession number A46723) and mouse MKK4 (35–397) (Upstate Biotechnology, Inc.; accession number U18310). Anti-ERK2 and anti-JNK antisera was from Santa Cruz Biotechnology. These included fusions of glutathione-

Plasmids for the Production of GST Fusion Proteins—The vector used for generating the GST fusion proteins was pGEXLB (26), a derivative of pGEX-4T-1 (Amersham Biosciences). In pGEXLB, an encoded Pro residue is replaced with a Gly-Gly-Gly-Gly-Ser-Gly sequence to promote the independent functioning of the GST and fusion moieties.

To construct pGEXLB-JNK3 (a gift from Douglas M. Molina), the JNK3 coding region was excised from pGEM4Z-JNK3 by restriction digest with BamHI and SalI and inserted into the corresponding sites of pGEXLB. The MEK1 (1–86) coding sequence was amplified by PCR with primers SP6PL (26) and JB804 (see Table 1) using pGEM-MEK1 (Upstate Biotechnology, Inc.; accession number L31951) as the template; digested with BamHI and SalI with EcoRI and SalI and inserted into the corresponding sites of pGEXLB, yielding pGEXLB-MEK1 (1–69). Plasmid pGEXLB-MEK2 (1–69), containing MEK2 residues 1–69, was generated by a similar procedure using primers SP6PL and JB805, pGEM-MEK2 (26) as the template, and cutting with EcoRI and SalI.

Plasmid pGEXLB-MKK4-(1–94) was generated by PCR amplification using primers DH709 and DH701 and the template pSRE–JNK1k1 (a gift from Michael Karin, University of California at San Diego). The PCR product was subsequently digested with BamHI and SalI and inserted into the corresponding sites of pGEXLB. The pGEXLB-MKK4-(37–94) wild-type and mutant constructs were generated using the pGEM-MKK4-(1–94) construct as the template. The wild-type construct was obtained using the forward primer MKK4F and the reverse primer MKK4RevNew (see Table I). The mutant construct was generated using the forward primer MKK4F(37–94)Fmut and the reverse primer MKK4RevNew (see Table I). The other mutants (see Fig. 5A) before being inserted into the corresponding sites of pGEM4Z.

To generate the MKK4 D-site mutant constructs, an adaptor oligonucleotide approach was utilized. Site-directed mutagenesis using oligonucleotide pGEXBSIIIIdestroyFwd and its complement was carried out to destroy a BsiIII site at bases 4102–4107 within the β-lactamase gene of pGEXLB–MKK4-(1–94). A unique BsiIII site was then introduced by silent substitution at codons 57 and 58 of the MKK4(1–94) open reading frame; this was accomplished by site-directed mutagenesis using oligonucleotide pGEXBSIIIFormingFwd and its complement. This new plasmid, pGEX–2SDM–MKK4(1–94), was then digested with BamHI and BsiIII to excise a 168-bp fragment encoding residues 1–58. The excised fragment was replaced with annealed oligonucleotide pairs encoding residues 37–58 and containing BamHI and BsiIII sticky ends. These oligonucleotide pairs contained the particular docking site mutations. For example, the K40A mutant of the pGEXLB–MKK4(37–94)Fmut (see Table I). The other mutants (see Fig. 5A) were constructed by the same procedure, except that oligonucleotides encoding the indicated substitutions were employed.

Translation and Translation in Vitro—Proteins labeled with [35S]methionine were produced coupled transcription and translation reactions (SPT3, Novagen). Translation products were partially purified by ammonium sulfate precipitation and quantified as described previously (24, 26).

Binding Assays—GST fusion proteins were expressed in bacteria, purified by affinity chromatography using glutathione-Sepharose (Amersham Biosciences), and quantified as described previously (24, 26). Specific binding measurements were performed and analyzed as described previously (24, 26). Binding constants were calculated as described previously (24, 26) based on the known input concentrations and a determination of the amount of complex formed, assuming a simple bimolecular binding reaction. An additional assumption was that 100% of the input molecules were complexed. The data were fitted to the following model: B + AB (where B is the known input concentration and AB is the complex capable of binding). For the reaction A + B ⇔ AB (where A is the “S-labeled protein, and B is the GST fusion protein), K_B = ([A]_0 × [B]_0)/[AB]_eq, where [A]_0 = [A]_i – [AB]_i, [B]_0 = [B]_i – [AB]_i, and [AB]_i are the input concentrations of A and B, respectively. [AB]_eq is the percentage of A that co-sediments with B multiplied by...
A Conserved Motif Near the N Terminus of MKK4—Based on sequence similarity to known MAPK-docking sites in other MKKs, we (26) and others (40, 41) identified a putative JNK-docking site near the N terminus of MKK4 (Fig. 1A). The characteristic feature of MAPK-docking sites found in MKKs, including the putative JNK-docking site in MKK4, is a basic submotif, which is separated by a short spacer from a hydrophobic submotif. Also, there is usually a basic/aromatic residue at the beginning of the basic tripeptide or more complex sequences.

### RESULTS

**A Conserved Motif Near the N Terminus of MKK4**—Based on sequence similarity to known MAPK-docking sites in other MKKs, we (26) and others (40, 41) identified a putative JNK-docking site near the N terminus of MKK4 (Fig. 1A).
analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2, the MKK4 D-site is required for JNK binding. A, 35S-labeled MKK4 derivatives were tested for binding to GST-JNK3. B, 35S-radiolabeled full-length MKK4 protein and N-terminal truncations thereof were prepared by in vitro translation and partially purified by ammonium sulfate precipitation, and portions (10% of the amount added in the binding reactions) were resolved on a 10% SDS-polyacrylamide gel (lane 1). Samples (~1 pmol) of the same proteins were incubated with 40 μg of GST (lanes 2) or with 10 or 40 μg of GST-JNK3 (lanes 3 and 4, respectively) bound to glutathione-Sepharose beads, and the resulting bead-bound protein complexes were isolated by sedimentation and resolved by 10% SDS-PAGE on the same gel. The gel was analyzed by staining with Coomassie Blue for visualization of the bound radiola­beled protein (upper three panels).

has detectable similarity to known JNK-docking sites in c-Jun (the δ-domain) (31), ATF2 (34), Elk-1 (the D-domain) (32), and the JIP-1 scaffold protein (42) (Fig. 1B). The term D-site, an amalgam of δ-domain, D-domain, and docking site, will be frequently used herein to refer to this class of related JNK-docking sites as well as to similar docking sites for other MAPKs.

The D-site of MKK4 Is Necessary for JNK Binding—As an initial test of the hypothesis that this region of MKK4 is a bona fide JNK-docking site, the ability of MKK4 to bind to JNK3 was assessed in an in vitro assay. Human JNK3 was fused at its N terminus to Schistosoma japonicum GST, and the resulting fusion protein was expressed in bacteria and purified by adsorption to glutathione-Sepharose beads. GST-JNK3 (or GST alone as a control) was then incubated with either full-length MKK4 or N-terminal truncation mutants of MKK4 that had been produced in radiolabeled form by in vitro translation. Bead-bound complexes were collected by sedimentation and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2, full-length MKK4 bound efficiently to JNK3 (Kd ~ 35 μM) (Table II); this binding was specific because precipitation of MKK4 protein did not occur when GST was used instead of the GST-JNK3 fusion protein. MKK4(37–399), which lacks the first 36 residues of full-length MKK4, also bound to GST-JNK3. In contrast, MKK4(47–399), which lacks the first 46 residues, did not bind to JNK3. Hence, residues 37–46, which contain the putative JNK-docking site and the first anchor lethal factor cleavage site, are necessary for high affinity MKK4/JNK binding. Furthermore, the second anchor lethal factor cleavage site (approximately residues 54–59) is not sufficient for JNK3 binding.

Localization of the MKK4 D-site—To confirm a previous report that the N-terminal region of MKK4 is sufficient for binding to JNK (20), a fusion of GST to the first 94 residues of MKK4 was constructed (designated GST-MKK4(1–94)). Residues 1–94 comprise the entire portion of MKK4 that is N-terminal to the kinase domain. To explore the specificity of binding, similar fusions of GST to the N-terminal domains of MEK1 (residues 1–60) and MEK2 (residues 1–64) were also constructed. There are three JNK genes (JNK1, JNK2, and JNK3) that are alternately spliced into at least 13 isoforms (43). The α- and β-isoforms differ in their amino acid sequence between kinase subdomains IX and X; the 2 isoforms have a mass of 54 kDa and differ from the 46-kDa ‘‘1’’ isoforms in their C-terminal extension. We assessed the binding of the JNK1a1, JNK1a1, JNK2a2, and JNK3a1 isoforms to the N-terminal domains of MEK1, MEK2, and MKK4 (Fig. 3A). The binding of ERK2 to all three MKKs was also examined. As shown in Fig. 3B, all JNK isoforms tested bound efficiently to GST-MKK4(1–94), but did not bind to GST-MEK1(1–60) or GST-MEK2(1–64). Conversely, ERK2 showed a preference for binding to the N terminus of its cognate M KK MEK2. Hence, the M KK N-terminal domains are selective for binding to their cognate MAPKs: the MKK4 N-terminal domain prefers JNK, and the MEK2 N-terminal domain prefers ERK2. However, this specificity is not absolute, as detectable binding of ERK2 to GST-MKK4(1–94) was observed; indeed, MKK4(1–94) bound to ERK2 just as well as, if not better, than MEK1(1–60) did. This incomplete specificity was also seen in experiments measuring the binding of full-length MEK1, MEK2, and MKK4 to GST-ERK1, GST-ERK2, and GST-JNK3 (data not shown).

The binding of in vitro translated, 35S-labeled JNK3 to GST-MKK4(1–94) was quantified (Kd ~ 25 μM) (Table II). This Kd agrees rather well with the value (Kd ~ 35 μM) obtained above for the binding of 35S-labeled full-length MKK4 to GST-JNK3.

In vitro translated JNK3 migrated on SDS-polyacrylamide gels as a distinct doublet (Fig. 3B); the upper band presumably corresponded to full-length JNK3, whereas the lower band, which was ~4 kDa smaller, presumably represented the product of an internal translation initiation or a premature termini.

Table II

| Experiment | 35S-protein (5 nm) | GST fusion | μg | μM | Bindinga | Kd (nM) |
|------------|-------------------|------------|----|----|----------|---------|
| A-1 MKK4   | GST-JNK3         | 40         | 4.0| 5.4| 70.0     |         |
| A-2 MKK4   | GST-JNK3         | 40         | 8.6| 42.6|          |         |
| A-3 MKK4   | GST-JNK3         | 40         | 16.9| 19.7|          |         |
| A-4 MKK4   | GST-JNK3         | 40         | 12.1| 29.1|          |         |
| A-5 MKK4   | GST-JNK3         | 40         | 9.7| 37.1|          |         |
| A-6 MKK4   | GST-JNK3         | 10         | 3.6| 27.0|          |         |
| A-7 MKK4   | GST-JNK3         | 10         | 5.0| 19.0|          |         |
| B-1 JNK3   | GST-MKK4(1–94)   | 50         | 6.9| 12.6| 48.3     |         |
| B-2 JNK3   | GST-MKK4(1–94)   | 50         | 12.1| 10.8|          |         |
| B-3 JNK3   | GST-MKK4(1–94)   | 50         | 15.2| 38.9|          |         |
| B-4 JNK3   | GST-MKK4(1–94)   | 40         | 12.3| 39.4|          |         |
| B-5 JNK3   | GST-MKK4(1–94)   | 10         | 7.3| 17.6|          |         |
| B-6 JNK3   | GST-MKK4(1–94)   | 10         | 10.5| 11.8|          |         |
| B-7 JNK3   | GST-MKK4(1–94)   | 10         | 8.6| 14.8|          |         |
| B-8 JNK3   | GST-MKK4(1–94)   | 10         | 6.7| 19.2|          |         |

a Percent of the input 35S-labeled protein that bound to the GST fusion protein.

b Calculated based on the known input concentrations and percent binding (see “Experimental Procedures” for details).

Fig. 2 The MKK4 D-site is required for JNK binding. A, 35S-labeled MKK4 derivatives were tested for binding to GST-JNK3. B, 35S-radiolabeled full-length MKK4 protein and N-terminal truncations thereof were prepared by in vitro translation and partially purified by ammonium sulfate precipitation, and portions (10% of the amount added in the binding reactions) were resolved on a 10% SDS-polyacrylamide gel (lane 1). Samples (~1 pmol) of the same proteins were incubated with ~40 μg of GST (lane 2) or with 10 or 40 μg of GST-JNK3 (lanes 3 and 4, respectively) bound to glutathione-Sepharose beads, and the resulting bead-bound protein complexes were isolated by sedimentation and resolved by 10% SDS-PAGE on the same gel. The gel was analyzed by staining with Coomassie Blue for visualization of the bound radiolabeled protein (upper three panels).
To delineate the contribution to the MKK4/MAPK interaction of the residues comprising the basic submotif of the putative D-site in MKK4, Lys\(^{40}\) and Lys\(^{42}\) were both changed to glutamate; Arg\(^{41}\) was changed to alanine; and this mutant, designated EAE, was fused to GST, yielding MKK4-(37–94)(EAE). As shown in Fig. 3C, this mutation abolished binding to all three JNK proteins and also to p\(^{38}\) and even to ERK2. Several conclusions can be drawn from these data. First, the basic submotif of the putative D-site in MKK4 is necessary for binding to its cognate MAPKs JNK and p\(^{38}\). Second, the weak non-cognate interaction of MKK4 with ERK2 is not a completely nonspecific interaction mediated by some “nonspecifically sticky” portions of each polypeptide, but is in fact dependent upon the same structural feature as the cognate interactions. In other words, ERK2 apparently binds weakly to the MKK4 D-site, indicating that this D-site has only limited ability to discriminate cognate MAPKs from non-cognate MAPKs (or that ERK2 has only a limited ability to discriminate cognate from non-cognate D-sites, or both). Finally, as the EAE mutation did not affect the integrity of the second lethal factor cleavage site (residues 54–59), yet completely abolished binding of JNK, ERK, and p\(^{38}\), it can be concluded that the second lethal factor cleavage site is not a high affinity MAPK-docking site. This is the first example of a lethal factor cleavage site that is not also a MAPK-docking site.

**The D-site of MKK4 Is Sufficient for JNK Binding**—To determine whether the D-site of MKK4 is sufficient for JNK binding, a peptide comprising residues 37–51 was synthesized and anchored to a cellulose membrane (44). A control peptide containing mutations of all three of the conserved basic residues, as well as of the second of the two conserved hydrophobic residues, was also synthesized and attached to the same membrane. These peptide spots were then incubated with radiolabeled JNK3 protein, and binding was quantified. As shown in Fig. 4, JNK3 bound very strongly to the wild-type MKK4 D-site, but exhibited no detectable binding to the mutant peptide. Hence, the D-site of MKK4 is sufficient for JNK binding.

To investigate the N- and C-terminal borders of the MKK4 D-site, a series of shortened MKK4 peptides were assayed using the same peptide array method (data not shown). Removal of Met\(^{37}\) did not affect JNK3 binding; however, removal of Gln\(^{38}\) did somewhat diminish JNK3 binding. Similarly, removal of Pro\(^{31}\), Pro\(^{31}\) plus Asn\(^{36}\), or Ala\(^{39}\)–Pro\(^{31}\) did not affect JNK3 binding; but removal of the next residue, Phe\(^{48}\), reduced JNK3 binding very strongly. The Lys-Arg-Lys basic submotif of MKK4 had only moderate effects on JNK binding, reducing it to 60 and 90% of the wild-type level, respectively (Fig. 5B). In contrast, mutation of the Arg residue to alanine reduced JNK3 binding more substantially (37% of the wild-type level). Interestingly, double mutation of the Arg residue and either Lys residue almost completely abolished JNK3 binding, yet mutation of both lysines had much less of an effect (40% of the wild-type level).

**Analysis of Conserved Residues Required for Docking**—To delineate the sequence features in the JNK-docking site of MKK4 that contribute to its stable and selective binding to JNK isoforms, amino acid substitutions were introduced into D-site consensus residues in this region (Fig. 5A). The D-site is a composite motif, composed of basic and hydrophobic submotifs (Fig. 1B). Single alanine substitution of either Lys residue in the Lys-Arg-Lys basic submotif of MKK4 had only moderate effects on JNK binding, reducing it to 60 and 90% of the wild-type level, respectively (Fig. 5B). In contrast, mutation of the Arg residue to alanine reduced JNK3 binding more substantially (37% of the wild-type level). Interestingly, double mutation of the Arg residue and either Lys residue almost completely abolished JNK3 binding, yet mutation of both lysines had much less of an effect (40% of the wild-type level).
Substitution of all three basic residues with alanine eliminated JNK3 binding. These data suggest, first, that the basic submotif of the MKK4 D-site is critical for JNK binding and, second, that the three residues of the basic submotif bind in a partially cooperative manner to JNK3. Arg^41 apparently participates in the most critical contact, but Lys^40 and Lys^42 are able to somewhat compensate for its loss.

Substitution of Leu^44 or Leu^46 of the conserved hydrophobic-X-hydrophobic submotif with alanine either alone or in combination also substantially reduced MKK4-JNK3 complex formation (Fig. 5, B and C). Hence, the hydrophobic submotif of the MKK4 D-site is also critical for JNK binding. These results collectively establish that residues 38–48 of MKK4 constitute a bona fide D-site.

D-site Peptides Inhibit MKK4 Binding to and Phosphorylation of JNK—To explore the role that MKK4/JNK docking plays in signal transmission from MKK4 to JNK, we tested whether synthetic peptides corresponding to D-sites are able to act as competitors of MKK4-mediated phosphorylation of JNK1 in vitro (Fig. 6A). Five different peptides were tested (Fig. 6B). The MKK4 D-site peptide corresponds to residues 37–52 of wild-type MKK4, and the MKK4(EAG) control peptide carries substitution mutations of three residues (K40E, R41A, and L46G) that are critical for JNK binding (Fig. 5). The JIP-1 peptide corresponds to residues 156–167 of the human JIP-1 scaffold protein (which is identical to residues 152–163 of mouse JIP-1); this region has been shown to bind to JNK (42). The MEK1 and MEK2 D-site peptides correspond to the MAPK-docking sites of the respective MKKs. Since the cognate MAPKs of MEK1 and MEK2 are ERK1 and ERK2, the MEK1 and MEK2 D-sites would not be expected to bind to JNK1. Indeed, this expected lack of interaction has been confirmed in GST co-sedimentation assays (Fig. 2B) (26), although it has not heretofore been examined in kinase inhibition assays, which can be more sensitive. Phosphorylation of unactivated JNK1 by limiting amounts of MKK4 was measured in the absence and presence of 200 μM peptide. As shown in Fig. 6C, the MKK4 D-site peptide was able to partially inhibit JNK1 phosphorylation, presumably because it was able to compete with full-length MKK4 for binding to JNK1 and thereby block MKK4/JNK docking. In contrast, the MKK4(EAG) mutant peptide had little or no inhibitory effect. In addition, the JIP-1 D-site peptide was also able to inhibit JNK1 phosphorylation, suggesting that full-length MKK4 and JIP-1 may compete for JNK binding. Notably, the MEK1 and MEK2 D-site peptides did not inhibit MKK4-mediated phosphorylation of JNK1. We have previously demonstrated that these two peptides can effectively inhibit the ability of both MEK1 (26) and MEK2 (39) (also see below) to phosphorylate ERK2. Hence, these two
peptides exhibit good specificity for ERK2 versus JNK1.

To further examine the specificity of the D-site peptides, we performed the reciprocal experiment and examined their ability to inhibit MEK2-mediated phosphorylation of ERK2 (Fig. 6D). As we have previously shown (39), the MEK2 D-site peptide was a potent inhibitor of this reaction (IC50 = 1.5 μM), whereas the MEK1 D-site peptide was less effective (IC50 = 100 μM). In contrast, a mutant version of the MEK2 peptide, MEK2(EAA) (Fig. 6B), had no inhibitory effect. Somewhat surprisingly, the MKK4 and JIP-1 D-site peptides were almost as effective inhibitors of MKK2 phosphorylation of ERK2 as the MEK1 D-site peptide was. Hence, these JNK-docking sites are not completely specific for their cognate MAPKs JNK1, JNK2, and JNK3 (p88); they are also able to bind with reasonable affinity to ERK2, a non-cognate MAPK.

It is notable that the inhibition of MEK2 phosphorylation of ERK2 by D-site peptides was considerably more extensive than the inhibition of MKK4 phosphorylation of JNK1 (Fig. 6, compare C and D). Indeed, dose-response profiles (data not shown) showed that D-site-mediated inhibition of MKK4 leveled out at ~50% maximal inhibition. In contrast, D-site-mediated inhibition of MEK1 and MEK2 levels out at ~75–80% inhibition (26, 39). One possible explanation for this difference is that relative to MEK2, MKK4 has a reduced dependence on docking interactions with its cognate MAPKs. Another, more trivial possibility is that a significant fraction of the particular preparation of active MKK4 used in Fig. 6 was in a lower activity, uninhibitable state.

To demonstrate directly that the MKK4 and JIP-1 D-site peptides inhibit stable protein complex formation between MKK4 and JNK, a series of binding assays were performed. The ability of in vitro translated, radio-labeled full-length MKK4 protein to co-sediment with GST-JNK3 bound to glutathione-Sepharose beads was tested in the presence or absence of various D-site peptides. When the MKK4 and JIP-1 D-site peptides were added to the reactions, they inhibited the amount of MKK4 protein that co-sedimented with GST-JNK3 (Fig. 7). In contrast, the MKK4 mutant peptide did not appreciably inhibit MKK4 binding. Thus, the MKK4 and JIP-1 D-site peptides are both able to compete with MKK4 protein for binding to JNK3. In addition, the MEK2 D-site peptide did not appreciably inhibit MKK4 binding to JNK3 (Fig. 7), although it does substantially inhibit MEK2 binding to ERK2 (39), again...
Fig. 8. Inhibition of MAPK-dependent phosphorylation of transcription factors by D-site peptides. A, D-site peptides (triangle) were used to inhibit JNK2 phosphorylation of c-Jun or ATF2 or ERK2 phosphorylation of Elk-1. B and C, purified GST-c-Jun (1 μM) was incubated with purified active JNK2 (~5 nM) and [γ-32P]ATP for 20 min in the absence or presence of the specified concentrations of the indicated peptides (see Fig. 6B). B, results are plotted as percent phosphorylation relative to that observed in the absence of any added peptide. c-Jun phosphorylation was analyzed by SDS-PAGE and quantified on a PhosphorImager. Data are the average of two to four experiments, with duplicate or triplicate data points in each experiment. C, shown is an autoradiogram of a representative experiment. D and E, purified GST-ATF2 (1 μM) was incubated with purified active JNK2 (~5 nM); other details are as described for B and C. F and G, shown is the inhibition of ERK2-dependent phosphorylation of Elk-1 by D-site peptides. Purified GST-Elk-1 (1 μM) was incubated with purified active ERK2 (~1 nM) and [γ-32P]ATP for 20 min in the absence or presence of the specified concentrations of the indicated peptides. F, results are plotted as percent phosphorylation relative to that observed in the absence of any added peptide. Elk-1 phosphorylation was analyzed by SDS-PAGE and quantified on a PhosphorImager. The fastest migrating band (see G), which appeared to be unaffected by peptide inhibition, was omitted from the quantification. Data are the average of two to four experiments, with duplicate or triplicate data points in each experiment. G, shown is an autoradiogram of a representative experiment.

Demonstrating that these ERK-docking sites are specific for ERK versus JNK.

D-site Peptides Inhibit JNK2 Phosphorylation of c-Jun and ATF2—We have shown previously that peptides based on the D-sites of MEK1 and MEK2 are able to inhibit not only MEK phosphorylation of ERK, but also other ERK-dependent reactions as well, such as ERK2-mediated phosphorylation of the Elk-1 transcription factor (39). This cross-inhibition strongly suggests that the D-sites in MEK1, MEK2, and Elk-1 all bind to the same region of ERKs. Likewise, the JNK-docking site of MKK4 characterized in this study displays noticeable sequence similarity to known JNK-docking sites in transcription factors (Fig. 1B), suggesting that MKK4 and downstream transcription factors may compete for binding to the same protein interaction site on JNKs. To test this possibility, the MKK D-site peptides were assessed for their ability to inhibit phosphorylation of GST-c-Jun by active JNK2 (Fig. 8, B and C). JNK phosphorylates c-Jun at Ser63 and Ser73 (31). The MKK4 D-site potently inhibited JNK2 phosphorylation of c-Jun in a dose-dependent manner (IC50 ~ 7.5 μM), whereas the MKK4(EAG) control peptide showed no inhibitory activity whatsoever, indicating that the MKK4 D-site and c-Jun compete for binding to JNK2. The JIP-1 D-site peptide was also quite an effective inhibitor of JNK phosphorylation of c-Jun, as has been previously reported (45). In contrast, the MEK1 D-site peptide did not inhibit JNK2 (~20% inhibition at 100 μM), and the MEK2 D-site peptide displayed a weak inhibition (IC50 ~ 105 μM).

Like c-Jun, the ATF2 transcription factor is also a target of JNK (46) and also contains a JNK-docking site that displays some similarity to the MKK4 D-site characterized in this study (Fig. 1B). JNK phosphorylates ATF2 at Thr69 and Thr71 (46). When the MKK4 D-site peptide was titrated into a JNK2 kinase assay using GST-ATF2 as a substrate, it inhibited JNK2 phosphorylation of ATF2 in a dose-dependent manner (IC50 ~ 15 μM) (Fig. 8, D and E). Hence, the MKK4 D-site and ATF2 compete for binding to JNK2. The JIP-1 D-site peptide was also a potent inhibitor of ATF2 phosphorylation. In contrast, neither the MKK4(EAG) mutant peptide nor the MEK1 or MEK2 D-site peptide was able to inhibit ATF2 phosphorylation (~75% inhibition at 100 μM).

The Elk-1 transcription factor contains a docking site for the ERK2 MAPK (32), and this D-site also exhibits similarity to the D-sites in MKks (Fig. 1B). To gain further insight into the specificity of the MKK4 D-site for JNK versus ERK, the panel of D-site peptides were also tested for their ability to inhibit ERK2 phosphorylation of Elk-1. Elk-1 is phosphorylated by ERK2 on at least six sites in its transcriptional activation domain (47), resulting in a ladder of bands displaying retarded electrophoretic mobility (Fig. 8G); peptide-dependent inhibition was revealed both by the progressive collapsing of this
were also reasonably effective inhibitors (IC$_{50}$ and $F_D$-site/cognate D-site IC$_{50}$ ratio (Table III, part A). For exam-
ple, the selectivity of the MKK4 D-site to inhibit ERK2 required conserved residues of the D-site consensus sequence, as
the MKK4(EA) mutant peptide was not inhibitory. These data indicate that the MKK4 D-site does bind to ERK2, con-
sistent with other results shown above (Figs. 3 and 6).

**Selectivity of the MEK1, MEK2, and MKK4 D-sites**—The above results suggest that the specificity of MKK/MAPK dock-
ing is somewhat limited, i.e. the specificity of binding between MAPKs and D-sites in their cognate MKKs is quantitative, but
not typically qualitative. Typically, quantitative estimates of relative specificity are expressed as selectivity ratios. Hence, to see
what insights could be gained from this approach, the selectivity of the D-sites in MEK1, MEK2, and MKK4 for their appro-
priate (cognate) versus inappropriate (non-cognate) MAPKs ERK2 versus JNK2 was estimated in two ways.

First, the selectivity of ERK and JNK for their cognate D-
sites in a given reaction was expressed as the non-cognate D-site/cognate D-site IC$_{50}$ ratio (Table III, part A). For example, the selectivity of the MKK4 D-site versus the MEK2 D-site for JNK2 was $>10$, estimated from the MEK2/MKK4 IC$_{50}$ ratio in the c-Jun phosphorylation reaction. Indeed, selectivities of at least 10 were obtained comparing the MKK4/JNK2 interaction with the MEK1/JNK2 and MEK2/JNK2 interactions. These selectivities appear to be driven by the relatively strong bind-
ing of the MKK4 D-site to JNK2 and the minimal binding of the MEK1/JNK2 D-sites to JNK2. In contrast, lower selectivities (be-
tween 3 and 6) were obtained comparing the MEK2/MKK4 IC$_{50}$ ratio in the c-Jun phosphorylation reaction. Furthermore, selectivities of at least 10 were obtained comparing the MKK4/JNK2 interaction with the MEK1/JNK2 and MEK2/JNK2 interactions. These lower selectivities are attributable to the moderate affinity binding of the MKK4 D-site to ERK2. Finally, the MEK1/ERK2 interaction displayed little or no selectivity compared with the MKK4/ERK2 interaction because the MEK1 D-site does not appear to bind to ERK2 that much better than the MKK4 D-site does.

As a second means to estimate specificity, the selectivity of a given D-site for binding to its cognate MAPK, instead of to a non-cognate MAPK, was expressed as the non-cognate MAPK/ cognate MAPK IC$_{50}$ ratio. For example, the selectivity of the MKK4 D-site for JNK2 versus ERK2 was estimated by dividing the IC$_{50}$ for its inhibition of ERK2 phosphorylation of Elk-1 (50 $\mu$m) by the IC$_{50}$ for its inhibition of JNK2 phosphorylation of c-Jun (7.5 $\mu$m) or ATF2 (15 $\mu$m). The results of these calcula-
tions revealed that the MKK4 D-site had a selectivity of between 3 and 6 for JNK2 versus ERK2, whereas the MEK1 and MEK2 D-sites had a selectivity of at least 16 for ERK2 versus JNK2 (Table III, part B). Again, these numbers highlight the nontrivial propensity of the MKK4 D-site to bind to ERK2.

**DISCUSSION**

Docking sites on MAPK regulators and substrates are key components of a web of links in a conserved signaling network.
In this study, we have investigated stable, high affinity complex formation between human MKK4 and its target kinases JNK1, JNK2, and JNK3. A short N-terminal region (the JNK- docking site) in MKK4, corresponding to residues 38–48, was shown to be both necessary and sufficient for the formation of stable MKK4-JNK complexes. This JNK-docking site conforms to the consensus sequence found in MAPK-docking sites (or D-sites) in other MKKs and is related to docking sites found in certain substrates and regulators of JNK. Mutation of conserved residues in the D-site of MKK4 substantially reduced complex formation between MKK4 and JNK. In addition, a peptide corresponding to the D-site of MKK4 (but not a mutant version thereof) inhibited both MKK4/JNK3 binding and MKK4-mediated phosphorylation of JNK1. Furthermore, this peptide also inhibited JNK2-mediated phosphorylation of the c-Jun and ATF2 transcription factors, suggesting that MKK4 competes with these JNK targets for binding to JNK. We con-
clude that MKK4 contains a conserved docking site that contributes to signal transmission and specificity in the JNK cascade.

**Ubiquity of MAPK-docking Sites in MKKs**—Docking sites for MAPKs have now been identified in MEK1 (26, 30), MEK2 (26), MKK3 and MKK6 (27), and MKK4 (this work). Hence, of the seven MKKs in the human genome (48), MAPK-docking sites have so far been found in all but MEK5 and MKK7. The large N-terminal domain of MEK5 has been shown to bind to its cognate MAPK ERK5; however, this region does not contain a clear match to the D-site consensus sequence. The N-terminal

---

**Table III**

| Reaction Peptide | Selectivity | IC$_{50}$ |
|------------------|-------------|-----------|
| **MKK4 phosphorylation of JNK1** | | |
| MEK2 | >1000 | >3 |
| MEK1 | >1000 | >3 |
| JIP-1 | 225 | 0.6 |
| MKK4 | 350 | 1.0 |
| **MEK2 phosphorylation of ERK2** | | |
| MEK2 | 15 | 1.0 |
| MEK1 | 100 | 6.7 |
| JIP-1 | 100 | 6.7 |
| MKK4 | 150 | 10.0 |
| **JNK2 phosphorylation of c-Jun** | | |
| MEK2 | 105 | 14.0 |
| MEK1 | >300 | >30 |
| JIP-1 | 5 | 0.7 |
| MKK4 | 7.5 | 1.0 |
| **JNK2 phosphorylation of ATF2** | | |
| MEK2 | >150 | >10 |
| MEK1 | >300 | >20 |
| JIP-1 | 5 | 0.3 |
| MKK4 | 15 | 1.0 |
| **ERK2 phosphorylation of Elk-1** | | |
| MEK2 | 6.5 | 1.0 |
| MEK1 | 18.5 | 2.8 |
| JIP-1 | 65 | 10.0 |
| MKK4 | 50 | 7.7 |

**B. Selectivity ratios of D-sites for MAPKs**

| D-site | MAPKs | Reaction | Selectivity ratio |
|--------|-------|----------|------------------|
| MKK4 | JNK2 vs ERK2 | ATF2 | 3.3 |
| MKK4 | JNK2 vs ERK2 | c-Jun | 6.7 |
| MEK1 | ERK2 vs JNK2 | ATF2 | >16 |
| MEK1 | ERK2 vs JNK2 | c-Jun | >16 |
| MEK2 | ERK2 vs JNK2 | ATF2 | >20 |
| MEK2 | ERK2 vs JNK2 | c-Jun | >20 |

---

D. T. Ho and L. Bardwell, unpublished data.
MEK1-ERK1, MEK1-ERK2, MEK2-ERK1, and MEK2-ERK2 such competition presumably occurs and the cytoplasm of stimulated and unstimulated cells (28), JNK. Since MKK4 (like MKK7) is found in both the nucleus and the cytoplasm of stimulated and unstimulated cells (28), MKK4 and c-Jun/ATF2 do indeed compete for docking to transcription factor substrates. Hence, it appears that the D-sites binding to JNK2 and blocking it from docking with its tran-

Almost certainly, this peptide inhibited these reactions by inhibiting JNK2-mediated phosphorylation of c-Jun and ATF2. Nevertheless, we found that an MKK4 D-site peptide could inhibit JNK2-mediated phosphorylation of c-Jun. In contrast, the MKK4 and JIP-1 D-sites did not bind to JNK and did not appreciably inhibit JNK-dependent reactions, although the MKK2 peptide did show some ability to inhibit JNK2 phosphorylation of c-Jun. In contrast, the MKK4 and JIP-1 D-sites were relatively good inhibitors of ERK2-dependent reactions, and the MKK4 N terminus displayed significant, D-site-dependent binding to ERK2. Hence, there appears to be appreciable, undiscriminating binding of the MKK4 D-site to a non-cognate MAPK, ERK2. When the selectivity of D-site/MKK interactions was quantified, it ranged from ~20-fold or more at best (MEK2 D-site for ERK2 versus JNK2) to ~3-fold or less at worst (MKK4 D-site for JNK2 versus ERK2). Ladbury and Arol (49) have argued that selectivities of this magnitude cannot, by themselves, account for the requisite signaling specificity.

The limited specificity of D-site-mediated interactions is perhaps not surprising given the sequence similarity among D-sites. Indeed, the D-site of Elk-1 has apparently evolved to bind to the same protein domain of MKK7 has also been shown to bind to JNK (28), and a putative D-site in this domain has been predicted based on similarity to the consensus sequence (26, 40, 41). However, this region is not sufficient for JNK binding; as such, it does not appear to be a bona fide JNK-docking site.

The characterized MAPK-docking sites in mammalian MKKs, including the JNK-docking site in MKK4 characterized here, have the following properties in common. 1) They mediate the formation of a relatively stable MKK-MAPK complex; 2) they are necessary and sufficient for the formation of this complex; and 3) they share the core consensus sequence (K/R)(L/I)-(L/I)-(L/I). We have estimated the affinities of the MEK1-ERK1, MEK1-ERK2, MEK2-ERK1, and MEK2-ERK2 complexes (Kd ~ 30, 15, 20, and 10 μM, respectively) (26) and of the MKK4-JNK3 complex (Kd ~ 30 μM) (this work). These estimates, obtained from binding assays, are consistent with IC50 values obtained in peptide inhibition assays (e.g. Table III). Thus, human MKK-MAPK complexes appear to have affinities with Kd values in the low micromolar range, consistent with the affinities of protein/protein interactions mediated by certain other modular domains or motifs involved in signaling, e.g. those mediated by SH3 domains (49).

Relationship of MKK D-sites to Anthrax Lethal Factor Cleavage Sites—The MAPK-docking sites in MEK1, MEK2, MKK3, and MKK6 have been shown to be substrates for cleavage by the anthrax lethal factor protease (35). MKK4 is also cleaved by lethal factor at two sites in its N terminus (35); the first of these corresponds to the JNK-docking site identified herein. Thus, this study extends the correlation that all proven MAPK-docking sites found in the N termini of mammalian MKKs are also cleavage sites for anthrax lethal factor. However, we also demonstrated, by counterexample, that not all lethal factor cleavage sites are high affinity MAPK-docking sites. For instance, MKK4 (37–94) (EAE), which contains the second lethal factor cleavage site intact, did not bind with high affinity to the ERK, JNK, and p38 MAPKs.

Competition for JNK Docking—The D-sites in MKKs are related to D-sites found in other MAPK-interacting proteins, including certain transcription factors, MAPK phosphatases, and scaffolds (34). This broader class of D-sites fits the rough consensus sequence (K/R)(L/I)-(L/I)-(L/I)-(L/I), where φ represents a hydrophobic residue, typically L or I, often V (particularly in phosphatases), occasionally M. The similar primary structure of D-sites suggests that they may bind to the same protein interaction sites (s) on their cognate MAPKs. Indeed, the MEK1 and MEK2 D-site peptides inhibit MEK phosphorylation of ERK2, ERK2 phosphorylation of Elk-1, and dephosphorylation of ERK2 by the MKP-1 phosphatase (39); this extensive cross-inhibition indicates that the D-sites in MEK1, MEK2, Elk-1, and MKPα compete for binding to ERK. It was not clear, however, if the same situation would apply to the JNK group of MAPKs because JNK2 mutations that reduce its interaction with MKK4 lie on the opposite face of its tertiary structure from mutations that reduce its interaction with c-Jun (40, 50). Nevertheless, we found that an MKK4 D-site peptide could inhibit JNK2-mediated phosphorylation of c-Jun and ATF2. Almost certainly, this peptide inhibited these reactions by binding to JNK2 and blocking it from docking with its transcription factor substrates. Hence, it appears that the D-sites in MKK4 and c-Jun/ATF2 do indeed compete for docking to JNK. Since MKK4 (like MKK7) is found in both the nucleus and the cytoplasm of stimulated and unstimulated cells (28), such competition presumably occurs in vivo, at least to some extent. We have suggested previously that the competitive docking of substrates and regulators to MAPKs may play a role in enhancing signaling fidelity (39). For example, ERK2 molecules that are docked to an appropriate physiological substrate, such as Elk-1, may be protected from dephosphorylation by MAPK phosphatases as well as from MEK1-mediated nuclear export. Similar mechanisms may also be relevant to JNK signaling.

We also found that the D-site from the cytoplasmic protein JIP-1 could inhibit both MKK4/JNK binding and MKK4-mediated phosphorylation of JNK. These results suggest that, in the cell cytoplasm, JIP-1 competes with MKK4 for docking to JNK. JIP-1 is a putative scaffold protein that binds to JNK, MKK7, and certain upstream kinases (51). The mutually exclusive docking of JIP-1 and MKK4 to JNK may prevent the recruitment of MKK4 to the JIP-JNK complex and may also prevent MKK4-mediated activation of JIP-bound JNK. More generally, the competitive docking of scaffolds and MKKs to MAPKs would seem inconsistent with the proposed role of scaffold proteins as potentiators of MAPK activation (52). We speculate that under some circumstances, scaffold proteins may in fact function to inhibit MAPK activation. For example, JIP proteins and their cargo are transported by microtubule-based motors, and it may be useful to inhibit JNK activity while this is happening (53). We further speculate that some signal-regulated event, such as release from motor proteins or oligomerization, may switch the activity of scaffolds from inhibitors to activators of MAPK cascade signaling.

Specificity of D-site-mediated Interactions—To explore the specificity of MKK D-sites for their cognate MAPKs, we tested the ability of the MEK1 and MEK2 D-sites to inhibit JNK-dependent reactions and of the MKK4 and JIP-1 D-sites to inhibit ERK-dependent reactions. By and large, we found that the MEK1 and MEK2 D-sites did not bind to JNK and did not appreciably inhibit JNK-dependent reactions, although the MEK2 peptide did show some ability to inhibit JNK2 phosphorylation of c-Jun. In contrast, the MKK4 and JIP-1 D-sites were relatively good inhibitors of ERK2-dependent reactions, and the MKK4 N terminus displayed significant, D-site-dependent binding to ERK2. Hence, there appears to be appreciable, undiscriminating binding of the MKK4 D-site to a non-cognate MAPK, ERK2. When the selectivity of D-site/MKK interactions was quantified, it ranged from ~20-fold or more at best (MEK2 D-site for ERK2 versus JNK2) to ~3-fold or less at worst (MKK4 D-site for JNK2 versus ERK2). Ladbury and Arol (49) have argued that selectivities of this magnitude cannot, by themselves, account for the requisite signaling specificity.

The limited specificity of D-site-mediated interactions is perhaps not surprising given the sequence similarity among D-sites. Indeed, the D-site of Elk-1 has apparently evolved to bind to both ERK and JNK so that Elk-1 can be regulated by both certain D-site-mediated interactions.

What are the physiological implications of the relative lack of specificity in D-site-mediated interactions? These interactions are best viewed as one component of a “double selection” for fidelity in MAPK-dependent transactions (24, 26). For example, effective selectivity is presumably increased by the combined effects of multiple separate interactions between MKKs.

3 A. J. Bardwell and L. Bardwell, unpublished data.
MKK4 JNK-Docking Site

and their cognate MAPKs (56). Consequently, even though MKK4 docks to ERK2, it does not phosphorylate ERK2. Another mechanism that may enhance the specificity of relatively nonspecific pairwise interactions is the assembly of multiprotein complexes (54, 57). The binding of both MKK and MAPK to a scaffold protein might function in such a manner, for instance (52). It is not clear, however, if this conception is entirely consistent with the observed competitive docking of MKKs and scaffolds to MAPKs. Such considerations highlight the theme that multiple, incompletely understood mechanisms contribute to specificity in cell signaling.

Acknowledgments—We thank Roger Davis, Kun-Liang Guan, and Michael Karin for generous gifts of reagents.

REFERENCES

1. Chang, L., and Karin, M. (2001) Nature 410, 37–40
2. Garrington, T., and Johnson, G. (1999) Curr. Opin. Cell Biol. 11, 211–218
3. Kyrakis, J. M., and Avruch, J. (2001) Physiol. Rev. 81, 807–869
4. Davis, R. J. (2000) Cell 103, 239–252
5. Weston, C. R., and Davis, R. J. (2002) Curr. Opin. Genet. Dev. 12, 14–21
6. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136
7. Lin, A. (2002) Bioessays 25, 17–24
8. Kennedy, N. J., Sluss, H. K., Jones, S. N., Bar-Sagi, D., Flavell, R. A., and Davis, R. J. (2003) Genes Dev. 17, 629–637
9. Hriseuski, J., Tuneman, G., Chang, L., Gergen, C. Z., Uysal, K. T., Maeda, K., Karin, M., and Hotamisligil, G. S. (2002) Nature 420, 331–336
10. Kolodziejczyk, S. M., Walsh, G. S., Balazs, K., Seale, P., Sandoz, J., Hierlihy, A. M., Rudnicki, M. A., Chamberlain, J. S., Miller, F. D., and Megeney, L. A. (2001)Curr. Biol. 11, 1278–1282
11. Han, Z., Boyle, D. L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A. M., and Firestein, G. S. (2003) J. Clin. Invest. 108, 78–81
12. Bishoprie, N. H., Andraka, P., Slepak, T., and Webster, K. A. (2001) Curr. Opin. Pharmacol. 1, 141–150
13. Xia, Y., Hardigg, T., Weller, M., Bieneman, A., Uney, J. B., and Schulz, J. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10435–10438
14. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) Nature 386, 865–870
15. Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998) Curr. Biol. 8, 1387–1390
16. Yoshida, B. A., Dubauskas, Z., Chekmareva, M. A., Christiano, T. R., Stadler, W. M., and Rinkers-Schaefler, C. W. (1999) Cancer Res. 59, 5483–5487
17. Teng, D. H., Perry, W. L., III, Hogan, J. K., Baumgard, M., Bell, R., Berry, S., Davis, T., Frank, D., Frye, C., Hattier, T., Hu, R., Jammula, B., Janecki, T., Leavitt, A., Mitchell, J. T., Pero, R., Sexton, D., Schroeder, M., Su, P. H., Swedlund, B., Kyrakis, J. M., Avruch, J., Bartel, P., Wong, A. K., and Tavtigan, S. V. (1997) Cancer Res. 57, 4177–4182
18. Su, G. H., Hilgers, W., Shekher, M. C., Tang, D. J., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1998) Cancer Res. 58, 2339–2342
19. Cheng, J., Yang, J., Xia, Y., Karin, M., and Su, B. (2000) Mol. Cell. Biol. 20, 2334–2342
20. Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998) Genes Dev. 12, 3369–3381
21. Zanke, B. W., Rubic, E. A., Winnett, E., Chan, J., Randall, S., Parsons, M., Boudreau, K., McInnis, M., Yan, M., Templeton, D. J., and Woodgett, J. R. (1996) J. Biol. Chem. 271, 28767–28774
22. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyrakis, J. M., and Zen, L. I. (1994) Nature 372, 794–798
23. Kieran, M. W., Katz, S., Vail, B., Zon, L. I., and Mayer, B. J. (1999) Oncogene 18, 6647–6657
24. Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R., and Thorner, J. (1996) Mol. Cell. Biol. 16, 3637–3650
25. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) EMBO J. 16, 1901–1908
26. Bardwell, A. J., Flatauer, L. J., Matsukuma, K., Thorner, J., and Bardwell, L. (2001) J. Biol. Chem. 276, 10574–10586
27. Ensen, H., Branco, D. M., and Davis, R. J. (2000)EMBO J. 19, 1301–1311
28. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1999) Mol. Cell. Biol. 19, 1569–1581
29. Bardwell, L., and Thorner, J. (1996) Trends Biochem. Sci. 21, 373–374
30. Xu, B., Wilsbacher, J. L., Collisson, T., and Cobb, M. H. (1999) J. Biol. Chem. 274, 34029–34035
31. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) Cell 87, 929–939
32. Yang, S. H., Whitmarsh, A. J., Davis, R. J., and Sharrack, A. D. (1998)EMBO J. 17, 1740–1749
33. Ensen, H., and Davis, R. J. (2001) Biol. Cell 93, 5–14
34. Sharrack, A. D., Yang, S. H., and Galanis, A. (2000) Trends Biochem. Sci. 25, 448–453
35. Vitale, G., Bernardi, L., Napolitani, G., Mock, M., and Montecucco, C. (2000) Biochem. J. 352, 739–745
36. Duesbery, N. S., Webb, C. P., Leppila, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. D., Aha, N. G., Oskarson, M. K., Fukasawa, K., Pashal, K. D., and Vande Woude, G. F. (1998)Science 280, 734–737
37. Park, J. M., Greten, F. R., Li, Z. W., and Karin, M. (2002)Science 297, 2048–2051
38. Lennon, G., Auffray, C., Polymenopoulos, M., and Soares, M. B. (1996)Genomics 33, 151–152
39. Bardwell, A. J., Abdollahi, M., and Bardwell, L. (2003)Biochem. J. 370, 1077–1085
40. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000)Nat. Cell Biol. 2, 110–116
41. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999)Genes Dev. 13, 163–175
42. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halperton, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997)Science 277, 693–696
43. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. L., Derijard, B., and Davis, R. J. (1996)EMBO J. 15, 2766–2770
44. Beinke, M., Volcani-Kengrav, R., and Schneider-Mergener, J. (2001) Curr. Opin. Biotechnol. 12, 59–64
45. Barr, R. K., Kendrick, T. S., and Bogoyevitch, M. A. (2002)J. Biol. Chem. 277, 10987–10997
46. Livingstone, C., Patel, G., and Jones, N. (1995)Proc. Natl. Acad. Sci. U. S. A. 92, 10435–10438
47. Lim, W. A. (2000)Acc. Chem. Res. 33, 1278–1282