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Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions*

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ABSTRACT

Mitogen-activated protein (MAP) kinases comprise a family of ubiquitous proline-directed, protein-serine/threonine kinases, which participate in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms. MAP kinases lie in protein kinase cascades. This review discusses the regulation and functions of mammalian MAP kinases. Nonenzymatic mechanisms that impact MAP kinase functions and findings from gene disruption studies are highlighted. Particular emphasis is on ERK1/2. (*Endocrine Reviews* **22:** 153–183, 2001)

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I. Introduction

PROTEIN kinases and other messenger systems form highly interactive networks to achieve the integrated function of cells in an organism. To understand the signaling mechanism for any agent, its repertoire of signal transducers and their interactions within this network must be defined within the cellular context. This includes the production of second messengers, activation of protein kinases, and the subcellular distribution of these transducers to bring them into contact with appropriate targets. Within the repertoire of signaling molecules in the network is a family of protein kinase cascades known as mitogen-activated protein (MAP) kinase modules. These cascades contain at least three protein kinases in series that culminate in the activation of a multifunctional MAP kinase (1–3). MAP kinases are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation, and cell death. This review contains a historical overview of the mammalian MAP kinases that have been studied to date, their regulatory cascades, and some of their functions. Current research on these pathways is described in detail, and emphasis is on nonenzymatic mechanisms and findings from gene disruption studies. Much of the review highlights work on extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). Some mechanisms in yeast MAP kinase cascades that might offer insight into the mammalian pathways are also included.

II. Overview of Regulation and Properties of MAP Kinases

Between 1989 and 1991 the sequences of the first MAP kinases, Kss1p and Fus3p in the pheromone response pathway of the budding yeast and the mammalian MAP kinases ERK1, ERK2 and ERK3, became available, revealing that these enzymes were members of a newly identified protein kinase family (4–8). The activities of ERK1 and ERK2 had been routinely measured with two substrates, myelin basic protein (MBP) and microtubule-associated protein-2 (MAP2); as a result, they had been called MBP and MAP2

kinases (9, 10). The MAP acronym was retained, but with a different meaning: the name mitogen-activated protein kinase was assigned to these enzymes to acknowledge the fact that they had first been detected as mitogen-stimulated tyrosine phosphoproteins in the early 1980s, during an intense search for tyrosine kinase substrates (11).

The concept that there were multiple MAP kinases with distinct regulation and functions arose from the description of additional pathways found initially in yeast, the high osmolarity glycerol (HOG) pathway containing the MAP kinase HOG1 and the cell wall pathway containing the kinase MPK1, and then in metazoans with the discovery of c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), p38 enzymes, and others discussed below (12–18). Extensive analyses of sequence relationships among these kinases have been published recently (19–21).

MAP kinases have some features in common with the cyclin-dependent kinases (cdks). These include an insert of unknown function between subdomains X and XI of the catalytic core and a preference for serine or threonine residues followed by proline in their substrates. Among the distinguishing features of the MAP kinases are activation directly by phosphorylation in the absence of a regulatory subunit, and usually two activating phosphorylation sites in the kinase activation loop, one a tyrosine and one a threonine, separated by a single, variable residue (Fig. 1 and Table 1). Kinases such as KKIALRE, for which cDNAs were first cloned as homologs of the cdk cdc2, KKIAMRE, and the nemo-like kinase NLK, identified by its similarity to *Drosophila* nemo, appear intermediate between the MAP kinase and cdk families and may function in a manner distinct from the majority of MAP kinases discussed in this review (22–25). Analysis of the sequence of the *Caenorhabditis elegans* genome reveals 15 MAP kinase family members (26). Nearly 20 MAP kinases are now known in mammals and more are anticipated (Table 1).

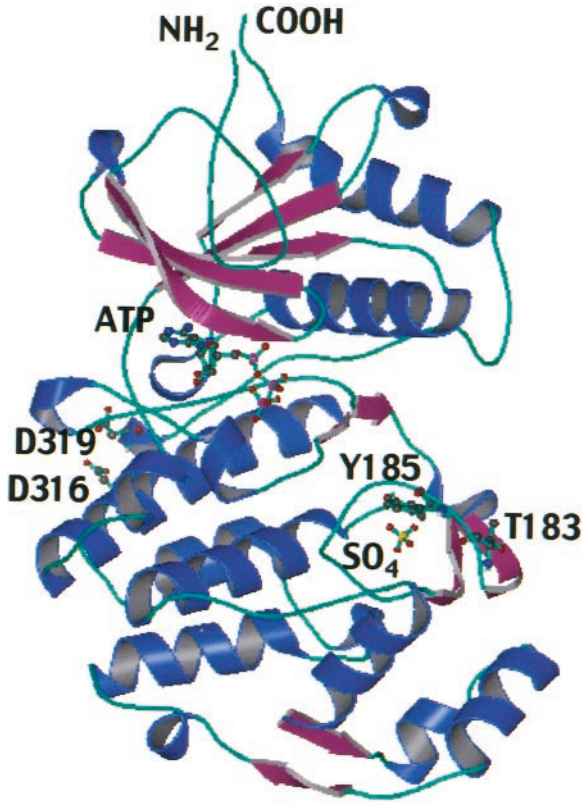

FIG. 1. Unphosphorylated structure of ERK2. ATP binds in the interior of the active site at the domain interface and protein substrates are bound on the surface. MAP kinase activity is controlled by phosphorylation of two residues, a tyrosine (185) and a threonine (183), that are in a surface loop known as the activation loop or phosphorylation lip. Phosphorylation of ERK2 or other MAP kinases on a single residue does not cause a substantial increase in activity, nor does replacement of the phosphorylation sites with acidic amino acids (77;445). This is probably because of the nature of the conformational changes that must occur upon phosphorylation. The sulfate ion that lies in the position occupied by phosphotyrosine in the active structure is shown. The aspartic acid residues (D316 and D319) in the proposed binding site for D domains are also indicated.

III. MAP Kinases Are Activated by Phosphorylation Cascades

MAP kinases are regulated by phosphorylation cascades. Two upstream protein kinases activated in series lead to activation of a MAP kinase, and additional kinases may also be required upstream of this three-kinase module (Fig. 2). In all currently known MAP kinase cascades, the kinase immediately upstream of the MAP kinase is a member of the MAP/ERK kinase (MEK or MKK) family. These are dual specificity enzymes that can phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues in their MAP kinase substrates (27–31). In spite of their ability to phosphorylate proteins on both aliphatic and aromatic side chains in the appropriate context, the substrate specificity of the known MEKs is very narrow: each MEK phosphorylates only one or a few of the MAP kinases.

There are several characteristics of MAP kinases that result from their activation by kinase cascades. Important among these is that the intermediates provide distinct mechanisms for detecting inputs from other signaling pathways to enhance or suppress the signal to the MAP kinase (32–34). Another is signal amplification. Amplification can occur if each successive protein in the cascade is more abundant than its regulator. This may be true at one or both steps within MAP kinase modules. Studies combining overexpression and immunoblotting might be interpreted to indicate that each step in the MAP kinase module of the pheromone response pathway in yeast is represented by a successively more abundant protein (2, 35), so that the signal may be amplified at both steps within the module. In the case of the ERK1/2 pathway, amplification occurs at the Raf-MEK step, because MEK1 is much more abundant (perhaps as high as 1 μM) than Raf, but is not the major function of the MEK-ERK step because the relevant MEKs (MEK1/2) and ERK1/2 are present at approximately the same concentrations (36, 37).

Another feature of MAP kinase cascades derives in part from the dual phosphorylation of the MAP kinase by the MEK. In the case of ERK1/2, the kinases are phosphorylated on tyrosine before threonine is phosphorylated both *in vitro* and in cells (38, 39). The result of this nonprocessive phosphorylation is the establishment of a threshold (40, 41). The

TABLE 1. Mammalian MAP Kinases

MAP Kinase	Other names	Comments	P Site motif	References
ERK1	p44 MAPK	>80% identical to ERK2; abundant and ubiquitous	TEY	(5)
ERK2	p42 MAPK	Abundant and ubiquitous	TEY	(7)
ERK3α	p63, rat ERK3	Immunoblotting detects 63K and full-length 95–100K species; α is present in many species including human	SEG	(7)
ERK3β	Human ERK3	~75% identical to ERK3α	SEG	(59)
ERK1b	(ERK4)	46K splice form of ERK1; comigrates with band originally named ERK4	TEY	(60)
JNK1	SAPKγ	Multiple spliced forms	TPY	(14, 15, 121)
JNK2	SAPKα	Multiple spliced forms	TPY	(14, 15, 121)
JNK3	SAPKβ	Multiple spliced forms	TPY	(14, 15, 121)
p38α	p38, CSBP, SAPK2	Sensitive to SB203580	TGY	(16–18)
p38β	p38-2	Partially sensitive to SB203580	TGY	(138, 137)
p38β2		Sensitive to SB203580; lacks the 8-amino acid insertion unique to p38β	TGY	(142)
p38γ	ERK6, SAPK3	Insensitive to SB203580	TGY	(140, 143)
p38δ	SAPK4	Insensitive to SB203580	TGY	(139, 141, 142)
Mxi		p38α splice form lacking 80 C-t residues and containing 17 novel ones	TGY	(146)
ERK5		Involved in proliferation	TEY	(160, 161)
ERK7		May have a role in cell proliferation	TEY	(175)
NLK	Nemo-like kinase	Regulation of Wnt pathway; ortholog of *C. elegans* LIT-1; relative of *Drosophila* nemo	TQE[a]	(24)
MAK	Male germ cell associated kinase	Expressed in cells undergoing meiosis in the testis but not ovary	TDY	(179)
MRK	MAK-related kinase	Expressed in embryonic myocardium; ubiquitous in adult tissues	TDY	(180)
MOK		Phorbol ester sensitive	TEY	(178)
KKIALRE		Cdc2-related kinase	TDY	(22)
KKIAMRE		T, Y mutants still activated in cells	TDY	(23)

[a] The CDK phosphorylation site motif is THE and the sequence of the nematode homolog of NLK is THE.

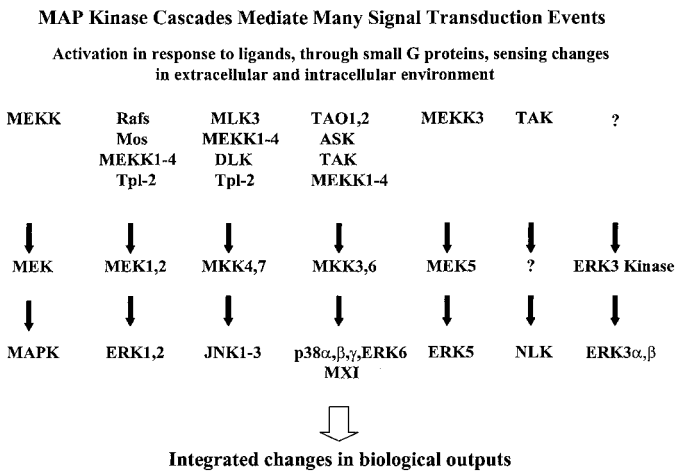

FIG. 2. MAP kinase cascades. Enzyme cascades shown are described in the text.

tyrosine-phosphorylated proteins are not active but must accumulate before phosphorylation of threonine. Once this accumulation threshold has been reached, the kinases are rapidly converted to the active state, as threonine is phosphorylated. It may be generally true that the MEK-MAP kinase step exists to enhance the cooperativity of activation of the MAP kinase and to allow modulation by other signaling events, in addition to or rather than amplifying the MEK signal.

MEKs are also activated by phosphorylation of two residues, either serine or threonine, in their activation loops (42, 43). At least in the case of MEK1, either phosphorylation alone significantly increases activity, in contrast to the effects of the phosphorylations on the MAP kinase. Nevertheless, activation of MEK also displays cooperativity at least in the *Xenopus* oocyte system as elucidated in detail by Ferrell and Machleder (44).

The MEK kinases (MEKKs) that activate MEKs are many and diverse. Enzymes with MEKK activity in metazoans include several relatives of the yeast MEKK Ste11p; several distant relatives of another yeast kinase Ste20p, which lies upstream of Ste11p; and Raf isoforms and Mos, which have no homologs in yeast (45–47). Few generalizations can yet be made about regulation of these MEKKs themselves, except that they may be subject to multiple regulatory inputs. Most, if not all, of these MEKKs are not abundant, suggesting that the MEKK-MEK step amplifies the signal emanating from a given MEKK.

IV. Signal Integration and Specificity

Interactions among the cascades occur in numerous ways to integrate responses and moderate outputs. Abundant evidence demonstrates that MAP kinases have overlapping substrate specificities (1, 48, 49). The resulting activities of the substrates reflect the cumulative extent of phosphorylation on all regulatory sites, which may be shared among multiple protein kinases. MAP kinase cascades form complexes that facilitate their activation and impact their localization, specificity, and targets (50–52). Potential scaffold proteins and adaptor or linker molecules have been found for some of the pathways. Regulation of complex formation provides yet another site for cross-talk between signaling pathways. Sev-

eral MEK family members contain sites that are phosphorylated by kinases in other pathways; these events may influence the ability of MEKs to interact in complexes, for instance (32, 53, 54). Integration may also occur early in the signaling pathway and at the top of the kinase module. Some MEKKs may regulate more than one MAP kinase cascade, and some cascades may be controlled by several, unrelated MEKKs.

V. Mammalian MAP Kinase Cascades

A. The ERK1 and ERK2 cascades

ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates (5, 7). The two phosphoacceptor sites, tyrosine and threonine, which are phosphorylated to activate the kinases, are separated by a glutamate residue in both ERK1 and ERK2 to give the motif TEY in the activation loop (55). Both are ubiquitously expressed, although their relative abundance in tissues is variable. For example, in many immune cells ERK2 is the predominant species, while in several cells of neuroendocrine origin they may be equally expressed. They are stimulated to some extent by a vast number of ligands and cellular perturbations, with some cell type specificity (1). In fibroblasts (the cell type in which the generalizations about their behavior and functions have been developed) they are activated by serum, growth factors, cytokines, certain stresses, ligands for G protein-coupled receptors (GPCRs), and transforming agents, to name a few. They are highly expressed in postmitotic neurons and other highly differentiated cells (7). In these cells they are often involved in adaptive responses such as long-term potentiation (56–58).

Recently an ERK1 splice variant, ERK1b, was found as an immunoreactive band that migrates more slowly than the ubiquitously expressed form of ERK1 (60). It is possible that ERK1b corresponds to the protein species originally named ERK4 (62). An alternatively spliced form of ERK2, lacking some residues from the N terminus, has also been reported; overexpression suggested that it was selectively membrane localized (59, 61). The three-dimensional structures of ERK2 in its unphosphorylated and phosphorylated states have been determined and reviewed elsewhere (63–67).

1. MEK1 and 2. ERK1 and ERK2 are activated by a pair of closely related MEKs, MEK1 and MEK2 (28–30, 68–71). Both of these MEKs have been shown to fully activate ERK1/2 *in vitro* (72, 73). Upon dual phosphorylation, ERK1/2 activities increase by well over 1,000-fold to specific activities of 1–2 μmol/min/mg protein. The largest effect appears to be due to an increase in V_{max}; changes in K_m for substrates are small (74, 75). The stoichiometry of phosphorylation of ERK1/2 by MEK2 more readily approaches 2 mol phosphate/mol ERK than does phosphorylation by MEK1. Haystead and coworkers (76) purified a factor that enhances phosphorylation of ERKs by MEK1. The biological importance of this molecule remains uncertain. Replacement of the two ERK2 phosphorylation sites with acidic residues does not elevate the activity of the protein (77).

Phosphorylation of MEK1 on both sites has been reported to stimulate its activity by more than 7,000-fold; as noted above, phosphorylation of either site alone produces a significant increase in activity (42, 78). Both V_{max} and K_m values change; K_m decreases by nearly 100-fold. Substitution of the two sites of phosphorylation with acidic residues increases their activity; deletions in the N terminus increase activity even more. The combination of these two changes yields constitutive MEK1/2 mutants nearly as active as phosphorylated wild-type proteins (78). These MEK mutants, most often MEK1R4F, have been used in many systems to infer events associated exclusively with the ERK cascade (79, 80). It has been assumed, from lack of evidence to the contrary, that MEK1/2 have no other substrates. Although this may not be the case, at this time no other MEK1/2 substrates have been identified.

2. Raf isoforms. Of all the known MEKKs, Raf isoforms and Mos are perhaps the only ones that phosphorylate MEKs in a single cascade. These proteins appear to phosphorylate only two MEK family members, MEK1 and MEK2, placing these MEKKs exclusively in the ERK1/2 MAP kinase cascade (81–83).

The Raf family of protein kinases is composed of A-Raf, B-Raf, and Raf-1 (or c-Raf) (84, 85). Each isoform contains three conserved regions, termed CR1, CR2, and CR3. The first two conserved regions are located in the amino terminus and have been implicated in regulating the Raf catalytic domain, because their deletion creates a mutant of Raf-1 that either has constitutively high activity or can be activated in a Ras-independent manner (see below) (86, 87). The kinase domain is located in CR3. Raf-1 is ubiquitous; highest expression of B-Raf occurs in neuronal tissue and testis; and A-Raf appears to function primarily in urogenital tissue.

Most studies have focused on Raf-1. Raf-1 regulation is complex, involving protein-protein interactions, phosphorylation of tyrosine, threonine, and serine residues, and cellular localization (84). These multiple modes of regulation allow Raf-1 to fluctuate through a number of graded activity states. Raf exists as part of a multiprotein complex composed of Raf-1 or B-Raf, heat shock protein 90 (hsp90), p50, and an indeterminate number of 14–3-3 proteins (88–95). 14–3-3 Appears to stabilize Raf-1 in both low and high activity conformations depending upon Raf phosphorylation state and interaction with other regulatory proteins such as GTP-liganded Ras. 14–3-3 May also serve to regulate Raf-1 signaling specificity by recruiting Raf-1 to higher order protein complexes. Disruption of hsp90-p50 binding to Raf, through the use of pharmacological agents such as geldanamycin and dexamethasone and mutants of p50 that are deficient for hsp90 binding, disrupt Raf-dependent signaling to downstream effectors (92, 93, 96). Multiple lines of evidence indicate that geldanamycin's effects are due to a depletion of Raf in the cell. Geldanamycin does not affect the ability of Raf to form complexes with an upstream activator Ras or reduce its specific activity upon epidermal growth factor (EGF) stimulation. Coexpression of p50 with Raf in Sf9 cells increases Raf activity and potentiates v-*src* activation of Raf (92). It is uncertain as yet whether p50 is an active regulator

or whether it works passively in concert with hsp90 to stabilize Raf.

There are significant differences in regulation of Raf isoforms. One notable difference between Raf-1 and B-Raf is their differential regulation by the small G proteins Ras and Rap1a (97–101). Raf-1 is activated by H-, K-, and N-Ras. It has been suggested that proliferation in nontransformed cells may be controlled primarily by N-Ras, but most studies have employed H- or K-Ras (102). Although Raf-1 also interacts with Rap1a, the function of this interaction is uncertain, because no increase in activity is seen. On the other hand, B-Raf is activated by both Ras and Rap. In neuronal model systems such as PC12 cells, activation of B-Raf by Rap1 may be the dominant mechanism (Refs. 97 and 100; G. Landreth, personal communication). This functional difference has been attributed to the cysteine-rich domains (CRDs) of these proteins. Swapping the Raf-1 and B-Raf CRDs allows for activation of Raf-1 by Rap1 and eliminates Rap activation of B-Raf (103).

The phosphorylation state of Raf-1 is influenced by multiple protein kinases, including Src, protein kinase C (PKC) family members, the p21 (Rac/Cdc42)-activated protein kinase PAK, and Akt (also called protein kinase B). The PAK and Src phosphorylation sites are located N-terminal to the catalytic domain at serine 338 and tyrosines 340 and 341, respectively (104–106). These sites have each been found to increase activity when phosphorylated and may do so in an interactive manner, depending on the signal context (105, 107, 108). The activation loop residues, serine 497 and 499, were the originally reported PKC phosphorylation sites (109); however, mutation of these sites has no discernible impact on Raf stimulation by serum (83). Wolfman and colleagues have recently found that PKCε forms a stable complex with Raf-1 and phosphorylates serine 338 (Hamilton, M., M. K. Cathcart, and A. Wolfman, submitted), the same site as PAK (105, 110). Other serine 338 kinases have been proposed. Down-regulation of PKCε blocks the phorbol ester activation of Raf-1 but has no effect on activation by EGF, one of many lines of evidence indicating multiple, independent mechanisms for activation of Raf-1.

Serine 259 is part of a putative 14–3-3 binding site (111–113). Phosphorylation of this serine may stimulate binding of 14–3-3 which, when bound to this region of Raf, has an inhibitory effect on Raf-1 activity. Mutation of this *in vivo* phosphorylation site to alanine creates an active mutant of Raf-1 (104, 114). Akt has been shown to phosphorylate serine 259 in MCF-7 breast cancer cells (115). Forced down-regulation of ERK1/2 in C2C12 cells cultured in serum can stimulate early stages of myotube differentiation (116). Akt may reduce Raf-1 activity in a number of contexts such as during C2C12 myoblast differentiation (117). The site of Raf-1 phosphorylation by Akt in C2C12 cells was not directly mapped. Instead, the authors show that in insulin-like growth factor I (IGF-I)-treated, postdifferentiated myotubes, there is reduced phosphorylation of serine 338 when a kinase active mutant of Akt is expressed. These methods of regulation are not mutually exclusive; however, Akt's ability to inhibit Raf-1 activity may vary depending on cell type. It is also interesting to note that in C2C12 cells an Akt-Raf-1 association only occurs during differentiation and is dependent on Akt kinase activity whereas in MCF-7 breast cancer cells the association of the two proteins appears to be constitutive. Further study is required to reconcile these differences and determine the generality of Akt-mediated down-regulation of Raf-1 during physiological processes.

TC21, a Ras family member, was previously thought to use a Raf-1-independent mechanism to activate ERKs 1 and 2; however, both B-Raf and Raf-1 displayed increased kinase activity in TC21-transformed NIH 3T3 cells (118). Also, overexpressed TC21 coimmunoprecipitated with overexpressed Raf-1 or B-Raf; it interacts with the two isoforms in a directed two-hybrid assay; and disruption of the TC21-Raf-1 interaction abolished the ability of TC21 to transform cells.

The three-kinase cascade, so well defined for the ERK1/2 module, is more difficult to identify as a discrete unit for other MAP kinase cascades at the present time. This is in part due to the capacity of many MEKKs to phosphorylate many MEKs *in vitro* and to activate many MAP kinases when overexpressed. Thus, the other MEKKs that are currently known will be discussed as a group after the description of the MAP kinases and their probable MEKs. The MAP kinases and related enzymes are listed in Table 1. Those not mentioned below appear in the overview section.

B. c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK)

A form of JNK/SAPK was first purified as a 54-kDa MBP kinase from livers of cycloheximide-treated rats (119). Shortly thereafter, JNK/SAPKs of 46 and 54 kDa were purified by affinity adsorption to a c-Jun fusion protein (120). Isolation of cDNAs encoding these enzymes and subsequent analysis of their expression revealed three genes encoding proteins with 10 or more alternatively spliced forms (14, 15, 121). Within the core catalytic domains, JNK1/SAPKγ, JNK2/SAPKα, and JNK3/SAPKβ are more than 85% identical. Based on mutagenesis studies, JNK/SAPKs are activated upon phosphorylation of two sites, a tyrosine and threonine, like other MAP kinases (15). In all JNK/SAPKs these residues are separated by a proline residue to give the motif TPY in the activation loop. They are activated by cytokines, certain ligands for GPCRs, agents that interfere with DNA and protein synthesis, many other stresses, and to some extent by serum, growth factors, and transforming agents. The alternatively spliced forms and their properties have been reviewed in detail elsewhere (121a).

1. MKK4 and MKK7. Two MEK family members, MKK4 (SEK1, MEK4, JNKK1, SKK1) and MKK7 (MEK7, JNKK2, SKK4), have been implicated in JNK/SAPK pathways. Both were identified initially by cDNA cloning strategies rather than by purification (122–132). One approach identified MKK7 as a two-hybrid binding partner of MEK1, although the significance of their association is not known. Unlike MKK4, MKK7 will rescue a lethal mutation in a *Drosophila* MEK (*hemipterous*) that is required for dorsal closure (126). Both MKK4 and MKK7 have the ability to phosphorylate p38 family members *in vitro* and when overexpressed, although JNK/SAPKs are the preferred substrates (133). JNK/SAPK

activation is impaired in cells from animals in which the MKK4 gene was disrupted, but changes in p38 activation have been found that are dependent on cell type (Ref. 134; see below). The prevailing view that MKK4 acts exclusively in JNK/SAPK cascades remains an open question. JNK/SAPKs are still activated by certain stimuli in MKK4 −/− cells, consistent with the conclusion that MKK7 is also linked to JNK/SAPK cascades. *In vitro* MKK4 preferentially phosphorylates the tyrosine residue in the TPY activation loop motif of JNK/SAPKs, and MKK7 preferentially phosphorylates the threonine residue. Based on these specificity differences, it has been suggested that these kinases cooperate to activate JNK/SAPKs, perhaps allowing for signal integration (135, 136). Results also indicate that phosphorylation of threonine may be most important for activity changes of JNK3 (136).

C. p38 Pathways

p38α was discovered independently in three contexts. It was found as a tyrosine phosphoprotein present in extracts of cells treated with inflammatory cytokines (17); as the target of a pyridinyl imidazole drug that blocked production of tumor necrosis factor-α (TNFα) and as such was called cytokine-suppressive antiinflammatory drug-binding protein or CSBP (16); and as a reactivating kinase for MAP kinase-activated protein (MAPKAP) kinase-2 (18). Cloning strategies rather than biological approaches were used to identify the other three genes that encode members of the p38 subfamily: p38β (or p38–2), p38γ (ERK6 or SAPK3), and p38δ (SAPK4) (137–143). All of these kinases contain the sequence TGY in their activation loops. A splice variant of p38β lacks the eight-amino acid insertion unique to β. p38α And β-isoforms are sensitive to pyridinyl imidazole inhibitors, but γ- and δ-isoforms are resistant to these drugs (141, 142). A variety of agents including cytokines, hormones, GPCRs, osmotic and heat shock, and other stresses activate p38 family members. In some contexts p38 family members have apparently opposite actions (144, 145).

A sixth protein, Mxi, is a splice variant of p38α in which the last 80 residues have been replaced by a novel 17-residue C terminus (146). Mxi was isolated from a two-hybrid screen with the c-Myc binding partner Max. Both Mxi and p38α bind Myc. The change in the C-terminal residues confers unique properties on Mxi. Unlike p38α, Mxi is activated not only by stresses but also by growth factors (146a). In contrast to p38α, Mxi is relatively insensitive to pyridinyl imidazole compounds; Mxi also displays a reduced affinity for p38α substrates. Crespo and colleagues showed that deletion of the 80 C-terminal residues from p38α yielded a mutant with properties similar to Mxi. An explanation may be proposed for these findings from the crystal structures of MAP kinases (64, 147–149). In these enzymes the C-terminal residues, deleted in Mxi, make intimate contacts with the N-terminal domain of the kinase catalytic core. These contacts undoubtedly influence the interaction with ATP and other compounds that bind in the ATP pocket, such as pyridinyl imidazoles.

Two MEK family members, MEK3 and MEK6, have high activity toward p38 MAP kinases (123, 150–152). MEK3 appears to favor phosphorylation of p38α and p38β isoforms, while MEK6 phosphorylates all p38 family members well (150). Both will also phosphorylate JNK/SAPK isoforms. MEK6 phosphorylates p38/ERK2 chimeras, and NLK (see below) *in vitro*, suggesting that it has a broader specificity than other MEKs (153, 154). The physiological implications of this broader specificity are not clear at this time.

D. Other MAP kinases

1. ERK3 isoforms. cDNAs encoding rat ERK3 were isolated from a library using a probe derived from ERK1 (7). A human cDNA predicted a second ERK3-like kinase, also 63 kDa, about 75% identical to ERK3 (59). These kinases are nearly 50% identical to ERK1 and ERK2 in the core catalytic domain, and both contain C-terminal extensions of approximately 200 residues. For the purposes of discussing them here, the first of these will be designated as ERK3α and the second as ERK3β. Subsequently, Flier and colleagues isolated a human cDNA that predicted a 97-kDa protein 100% identical to ERK3α over their shared lengths but lacking a stop codon and longer by nearly 300 residues (155). Immunoblotting with antibodies specific for ERK3α revealed proteins of 63, 95, and 160 kDa in multiple rat tissues and several cell lines, consistent with multiple species predicted by the cDNAs (156). A clone encoding a 100-kDa form of ERK3α was recently isolated by Meloche and colleagues (157) from mouse and a single genomic locus was mapped. Database analysis indicates that there may be several loci encoding ERK3-like molecules. Genes encoding ERK3 homologs have not been found in the genomes of yeast or nematodes, suggesting that ERK3α and β may have arisen from a relatively late gene duplication (26, 158).

Despite the similarity to ERK1/2, ERK3α and -β have some features that are different from other family members. The phosphorylation site motif in the activation loop of ERK3 isoforms has a single phosphoacceptor site, serine189 in ERK3α in the sequence SEG. Glycine replaces the usual tyrosine phosphorylation site found in most other MAP kinases. ERK3α autophosphorylates, but data for other ERK3 substrates are weak (156). Several MAP kinases are largely cytoplasmic in unstimulated cells and translocate to the nucleus when cells are stimulated. In contrast, ERK3α is highly concentrated in the nucleus under all conditions examined (156) but the mechanism is unknown; ERK3 lacks a consensus nuclear localization sequence. A kinase that binds to and phosphorylates ERK3α on serine189 has been described but its molecular identity is unknown (159). This activity phosphorylates ERK3 but not other MAP kinases.

2. ERK5. ERK5 was identified independently by two groups. One used a two-hybrid screen with an upstream activator MEK5 as the bait; the other used a degenerate PCR strategy to clone novel MAP kinases (160, 161). Thus, the putative upstream activator MEK5 was found ahead of this MAP kinase. Among the most intriguing features of ERK5 is its size, 816 amino acids, due to a stretch of approximately 400 amino acids C-terminal to the kinase domain. When comparing the primary sequence of the catalytic domain of ERK5 to other mammalian MAP kinases, it appears to be most like

ERK2. The 400-residue C terminus, however, neither displays sequence similarity to any known proteins nor has a known function, although it contains 10 consensus sites for MAP kinase phosphorylation. These phosphorylation sites may be autophosphorylated, consistent with the dramatic increase in autophosphorylation ERK5 displays when it is in a high activity state (162). Whether autophosphorylation plays an integral role in ERK5 function within the cell remains to be seen. The C terminus also contains a potential cytoskeletal targeting motif; however, there is no evidence supporting this putative function (160).

In mammals, ERK5 is ubiquitously expressed. Like the other MAP kinases, ERK5 activity is regulated by a wide variety of proliferative and cell-stressing agents. The proliferative stimuli include serum, EGF, nerve growth factor (NGF), lysophosphatidic acid (LPA), and phorbol ester (163–165). The ability of these agonists to activate ERK5 is Ras-dependent in some cell types; EGF activation of ERK5 requires MEKK3 activity in HeLa cells (Refs. 163, 164, 166; see below). The stress stimuli include sorbitol, H_2O_2, UV irradiation, vascular shear stress, and ischemia (164, 165, 167–169). These stimuli may sometimes exert their activity through Src (170). Cellular requirements for ERK5 activity have been better defined in proliferation models. Dominant negative forms of ERK5 can inhibit EGF-stimulated proliferation and RafBXB-stimulated focus formation in 3T3 cells (163, 171).

English and colleagues (162) examined the regulation of the catalytic domain through truncation of its C terminus. The ERK5 catalytic domain is activated by V^{12}Ras and an active mutant of MEK5, MEK5DD (the two sites of activating phosphorylation are replaced with acidic residues), as determined by an increase in activity toward substrates. *In vitro*, the ERK5 catalytic domain expressed in bacteria is phosphorylated by immunoprecipitated MEK5DD on its TEY motif and displays an increased activity toward substrate, consistent with the behavior of the majority of MAP kinase family members, which are only slightly larger than a core catalytic domain (Pearson, G., and M. H. Cobb, unpublished). Coexpression of ERK5 with MEK5DD in cells increases ERK5 activity. The kinase domain displays the expected specificity of activation in that other MEK family members such as MEK1 fail to phosphorylate it *in vitro* or increase its activity when coexpressed in 293 cells.

ERK5 can affect cellular activity through phosphorylation of the MADS box transcription factors, myocyte enhancer factor 2A and C (MEF2A and C), and the ETS-like transcription factor SAP1a (164, 165, 172). The ability of ERK5 to activate MEF2 isoforms appears to allow it to positively regulate intracellular concentrations of c-Jun (172). Additional downstream effectors are likely to exist.

MEK5 is upstream of ERK5. MEK5 was identified by two groups using cDNA cloning strategies (160, 173). There are multiple splice variants including 50-kDa α- and 40-kDa β-isoforms. MEK5 α is particulate and primarily expressed in liver and brain; the ubiquitously expressed β-isoform is cytosolic. The only known substrate of MEK5 is ERK5; thus, effects of MEK5 have been attributed to its ability to activate ERK5.

According to primary sequence alignment, MEK5 is most closely related to MEKs 1 and 2. Perhaps as a consequence of this relationship, it is also inhibited by PD98059 and U0126, two compounds that have been considered highly selective inhibitors of MEK1 and MEK2 (Ref. 164; see below). At low concentrations, the effects of these inhibitors may be primarily on MEK1/2, since the K_i for MEK5 is significantly higher.

In spite of the similarity to MEK1/2, MEK5 is not phosphorylated or activated by Raf-1 (162). Although Raf-1 is unable to increase MEK5 activity, MEK5 is intimately involved in Raf-1 signaling. Kinase-defective MEK5, MEK5KM, can inhibit RafBXB-stimulated focus formation in 3T3 cells, whereas a constitutively active form of MEK5, MEK5DD, can synergize with RafBXB to form foci (171). MEK5DD cannot stimulate focus formation when expressed alone. MEK5KM can also inhibit focus formation induced by the Cot protooncogene product, also known as Tpl-2, and coexpression of Tpl-2 with MEK5 increases the phosphoserine content of MEK5 (174). Direct effects of Tpl-2 on MEK5 activity have not been demonstrated. The only MEK5 kinase identified thus far is MEKK3 (166).

3. ERK7. A cDNA encoding ERK7 was isolated by Rosner and colleagues (175). ERK7 is a 61-kDa MAP kinase with a TEY motif in the activation loop, like ERK1, ERK2, and ERK5. ERK7 is not activated by stimuli that activate ERK2 or the stress-responsive kinases, but appears to be constitutively activity in serum-starved cells. A role in growth inhibition has been proposed for ERK7. Its long C terminus has been suggested to be required for the localization and high basal activity of this protein. A cDNA encoding the protein CLIC3 was isolated using the tail of ERK7 as bait in a yeast two-hybrid screen (176). CLIC3 is related to human intracellular chloride channel proteins.

4. NLK. NLK was identified by Erikson's group (24) as a mammalian relative of *Drosophila* nemo. This kinase has properties that place it between the MAP kinases and the cdks. Although it is nearly 45% identical to ERK2, the dual phosphorylation motif TXY in the activation loop is absent, and instead a single phosphorylation site in the sequence TQE, most similar to the cdks, is present. Nevertheless, NLK appears to lie in a MAP kinase cascade that negatively regulates Wnt signaling (154, 177). Studies in *C. elegans* have demonstrated that an NLK homolog lit-1 is activated by the MEKK Mom-4. Mom-4 is a homolog of TAK1, described below as an MEKK for the p38 MAP kinase module. In transfected cells TAK1 can enhance the activity of cotransfected NLK. Although a MEK specific for NLK has not been reported, NLK is activated *in vitro* by MKK6, a TAK substrate. Thus, it is possible that TAK1 and MKK6 may be normal cellular regulators of both p38 and NLK.

5. MOK. MOK has approximately 30% identity to members of the MAP kinase family and equivalent identity to the cdk family (178). Strikingly, however, MOK contains the TEY motif in its activation loop that is typical of MAP kinases. It has been shown to be activated by okadaic acid and phorbol ester, suggesting that it may be controlled by a kinase cascade. Its relatives include male germ cell-associated kinase (MAK) and the MAK-related kinase, MRK (25, 179, 180).

E. MEKKs, the first tier in the kinase cascade

A specific MEKK enzyme may regulate either a single or multiple MEKs depending upon the enzymatic specificity of the MEKK, the cellular and subcellular distribution of the signaling components, the formation of protein complexes, and the activating stimuli. Consequently, significant differences in both the magnitude and kinetics of MAP kinase activation may occur in response to a given agent under different circumstances. Many kinases acting at the MEKK level have been identified, adding to the complexity of unraveling signaling mechanisms. There is no apparent similarity among these proteins outside of their kinase catalytic domains. The relative contribution of each MEKK to the activation of individual MAP kinases, with the possible exception of Raf in the ERK1/2 module, is unclear.

Aside from Raf isoforms, the first of these to be isolated was the 195-kDa protein MEKK1. It is one of a family of molecules most closely related to the yeast kinase Ste11p, all of which contain C-terminal kinase domains and N-terminal regions of variable length (45). In their catalytic domains, MEKK2 and MEKK3, each approximately 70 kDa, and MEKK4, about 150 kDa, are nearly 50% identical to MEKK1 (181–184).

The other enzymes with MEKK activity mentioned next are less similar with identities to MEKK1 generally in the 30–40% range. The following MEKK level kinases activate JNK/SAPKs when overexpressed or by *in vitro* reconstitution with MEKs: MEKKs(1–4) (181–184), MAP three kinase (MTK1) (181, 183–186), Tpl-2/Cot (187), dual leucine zipper kinase (DLK) (188), mixed lineage kinase MLK2/MST (189), MLK3/PTK-1/SPRK (190, 191), transforming growth factor-β (TGFβ)-activated kinase (TAK1) (192), apoptosis signal-regulating kinases (ASK1)/MAPKKK5 (193, 194) and ASK2/MAPKKK6 (195), and thousand and one amino acid kinases 1,2 (TAOs1, 2) (196, 197). Of these, MEKKs(1–3) and Tpl-2 can also activate the ERK1/2 pathway (187); MEKK3 and Tpl-2 also activate the ERK5 pathway (172, 174); and TAK1, ASK1, TAOs1/2, and MTK1 also activate the p38 pathway (194, 196, 197).

Unraveling the relationships of these MEKKs to the MAP kinases they activate has been a daunting task. Identification of the intrinsic enzymatic specificities, the distribution, and the phenotypes of animals and cells with these MEKK genes disrupted should begin to help decipher their cascade specificity and their functions. The function of MEKK1, the first of these enzymes isolated, is still in question. It has been implicated in activation of JNK/SAPK, ERK, and p38 MAP kinase pathways, as noted above, and in the activation of nuclear factor-κB (NF-κB) (198, 199). *In vitro* MEKK1 phosphorylates MEKs 1, 2, 3, 4, 6, and 7 (73, 200–203). However, despite the fact that the recombinant protein phosphorylates MEKs 1 and 2 on the same sites as Raf-1, it does so poorly relative to the phosphorylation of MEK4 in the JNK/SAPK pathway, consistent with the finding that signaling to JNK/SAPKs is most affected in cells lacking MEKK1 (Refs. 204–206; see below).

Although the classical MAPK module is a three-tiered kinase cascade, a fourth kinase may act directly upstream as an activator of the MEKKs. This was discussed earlier for Raf. Kinases implicated in JNK/SAPK activation at the MEKK kinase level include PAKs 1–4 (207–209), germinal center kinase (GCK) (210, 211), GCK-related kinase (KHS/GCKR) (212), GCK-like kinase (213, 214), hematopoietic progenitor kinase 1 (HPK1) (215), and Nck-interacting kinase (NIK) (216).

Both small G proteins and heterotrimeric G proteins can activate MAP kinase cascades as discussed in more detail for ERK1/2 below (217). Activation of JNK/SAPKs and p38 in response to interleukin (IL)-1β, muscarine, bradykinin, and heterotrimeric G protein $\beta\gamma$ subunit complexes may be mediated by Rho family members Rac and Cdc42 (209, 218–220).

VI. Activation of ERK1/2 and Other MAP Kinases from the Cell Surface

Perhaps the most well defined signaling pathway from the cell membrane to ERK1 and ERK2 is that used by receptor tyrosine kinases (reviewed in Refs. 221 and 222). Stimulation of these receptors by the appropriate ligand results in an increase in receptor catalytic activity and subsequent autophosphorylation on tyrosine residues. Phosphorylation of these receptors results in the formation of multiprotein complexes whose organization dictates further downstream signaling events. Quite often one of these functions is the activation of the monomeric G protein Ras. This is achieved by the recruitment of adaptor proteins, such as Shc and Grb2, to the receptor through interactions between their SH2 domains and phosphotyrosine residues. The guanine nucleotide exchange factor (GEF) Son of Sevenless (Sos) then becomes engaged with the complex and induces Ras to exchange GDP for GTP. GTP-liganded Ras is capable of directly interacting with a number of effectors, including Raf isoforms, of which the best characterized is Raf-1. As discussed before, Ras binding to Raf may result in conformational changes in Raf that increase its kinase activity or simply provide the proper environment for Raf-1 signaling (223–228). Localization of Raf to the plasma membrane may also allow protein kinases such as Src, PKC, and PAK to further modify Raf to increase its activity (105, 106, 109, 110, 228). The increase in Raf activity is subsequently transduced through the MEK-ERK module.

Signaling to ERKs by GPCRs also involves modulation of Raf activity; however, the mechanisms employed by these receptors are widely varied. The existence of multiple classes of G proteins, the ability of some receptors to activate more than one class of G protein, and cell type-specific mechanisms contribute to the diversity. For clarity, only the general trends observed for a few specific classes of G proteins will be discussed. There are several more detailed recent reviews (1, 67).

Signals transmitted from receptors through Gαs are particularly diverse, consistent with the variety of effects on ERK activity evoked by elevation of cAMP concentration. cAMP-dependent protein kinase (PKA) has been reported to reduce Raf-1 activity through direct phosphorylation of serine 43 and serine 621 in some situations (229–232). On the other hand, PKA can also phosphorylate Rap1a, which may positively influence ERK through activation of B-Raf in cells of neuronal origin (97, 233). Activation of Rap1a by a cAMP-

binding Rap1 GEF, or by some other means, has been suggested to inhibit ERK activity through Rap1a-dependent sequestration of Raf-1 (99, 234). The particular effect of Rap1a activation on ERK may be determined by the expression level of B-Raf. Lefkowitz and colleagues (235) have reported that isoproterenol treatment of 293 cells overexpressing β2-adrenergic receptors stimulates a PKA-dependent switch of receptor coupling form Gαs to Gαi, and that ERK activation is through the Gi pathway.

In Gαi-dependent ERK activation, free $\beta\gamma$-subunits may be the active signal transducers, reminiscent of their role in the yeast mating response. This is evidenced by overexpression studies showing $\beta\gamma$-subunits are sufficient to activate ERKs and a $\beta\gamma$ sequestering peptide reduces ERK stimulation by Gαi-coupled receptors (236, 237). In one proposed model, $\beta\gamma$ stimulates a Src family kinase activity in a PI-3 kinase γ-dependent manner (238). The Src family kinase may then phosphorylate a tyrosine kinase receptor, PYK2, or focal adhesion kinase (FAK), to create SH2 domain binding motifs (239–241). Then, analogous to the signaling mechanism used by receptor tyrosine kinases described above, a Shc-, Grb2-, and Sos-containing complex is formed at the membrane to activate Ras and, in turn, Raf-1. ERK activation in cell types where PI-3 kinase expression is low may be dependent on alternative means to activate Src or PYK2 (239, 242).

Gαq activation of ERK2 is often a PKC-dependent process, which may be Ras-dependent or independent (240, 243–247). The Gαq effector is PLCβ, which generates inositol triphosphate (IP_3) and diacylglycerol (DAG) through the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2). Some isoforms of PKC are activated by DAG and the intracellular Ca released as a result of IP_3 production. PKC may then regulate Raf through direct phosphorylation, although this mechanism has not been fully characterized (109). As noted above, Wolfman and colleagues have found that PKCϵ phosphorylates Raf-1 and increases its activity (Hamilton, M., M. K. Cathcart, and A. Wolfman, submitted). In PC12 cells, stimulation of Gαq by receptors results in PYK2 and Shc phosphorylation. In Rat-1 cells phosphorylation of the EGF receptor, Neu, and Shc increases after treatment with endothelin. Thus, G proteins appear able to access multiple tyrosine kinases to activate the ERK pathway.

Overexpression of Gα12 activates Ras in 293 cells, although a strong link to the ERK pathway has not been made (245). GTPase-deficient mutants of Gα12 and Gα13 can stimulate focus formation in 3T3 cells, perhaps suggesting that some subset of the MAPK family is involved. Slight increases in JNK and p38α and -β activity are seen when coexpressed with Gα13. JNK activity is also increased by Gα12 (248). Based on blocking experiments, Rac may be an intermediate (220).

VII. Scaffolding and Its Role in Organization, Localization, and Specificity in MAP Kinase Cascades

A. Complexes predicted from studies in yeast

1. The scaffold Ste5p. The first scaffolding protein identified that binds the kinase components of a MAP kinase pathway was the *S. cerevisiae* protein Ste5p. Mutants lacking Ste5p, as suggested by its name, are sterile. They fail to progress through the pheromone-induced mating pathway.

Two-hybrid studies from several laboratories revealed that Ste5p interacts with the three protein kinases of the MAP kinase module, either Fus3p or the similar MAP kinase, Kss1p, the MEK, Ste7p, and the MEKK, Ste11p (50, 51, 249). These results supported earlier biochemical studies with overexpressed protein, which also showed that Ste5p is a Fus3p substrate (250). Deletion analysis indicated that the binding sites for these kinases on Ste5p are distinct, suggesting that a multiprotein complex can form (50, 51, 249). Phosphorylation and activity states affect association of the kinases with Ste5p (251).

Epistasis analysis is consistent with the idea that Ste5p has an important function at more than one step of the cascade (252). Four properties of Ste5p have been discovered that are likely to be keys to its function. As discussed above, the first is its capacity to bind the components of a MAP kinase module. Second is its ability to interact with the upstream signal transducers. These signaling intermediates include the heterotrimeric G protein that is activated by pheromone binding to its receptor. In this pathway the $\beta\gamma$-subunits (Ste4p and Ste18p) transduce the signal and do so in a manner that requires Ste5p (253); this is consistent with the finding that, when overexpressed, the α-subunit inhibits the pheromone signal (254, 255). The interaction of Ste5p with the Gβ-subunit is essential for activation of the MEKK Ste11p (255, 256). A close parallel exists in mammalian MAP kinase modules, which can be regulated by the Gi family through β-subunit interactions. Third, Ste5p forms oligomers; these may promote complex activation (256, 257). Finally, Ste5p may also be an essential feature of the mechanism of localization of the kinases in the complex, because it must localize to the plasma membrane for cascade activation, yet its entry and exit from the nucleus are also required for pheromone-induced signaling (258).

2. Pbs2p. The formation of protein complexes may determine the regulation and functions of the associated MAP kinases. This idea was strongly suggested by findings in a second yeast MAP kinase module, which is part of a homeostatic response to osmotic shock (12, 259, 260). The HOG pathway contains the MAP kinase Hog1p, a relative of mammalian p38 (12). The MEK upstream in the pathway is Pbs2p (12, 260). Two osmosensors can activate the pathway through one of three different MEKKs—Ste11p, Ssk2p, or Ssk22p (261). A transmembrane osmosensor, Sho1p, activates Ste11p, the same MEKK that works in the pheromone response pathway (260). Thus, the mating and osmotic stress pathways share a common MEKK. When the osmotic pathway is activated, Ste11p binds to Pbs2p, which apparently scaffolds the MAP kinase module of the HOG pathway. Pbs2p binds Sho1p, Ste11p, and Hog1p (260). Ste5p is either absent or present in very low concentrations in diploid cells. Its presence may be required for recognition of Ste7p by Ste11p. Its absence may be an important factor in the specificity of Ste11p for Pbs2p, both the MEK and the scaffold, rather than Ste7p. Thus, the binding partners of Ste11p seem to determine the signals it transmits. This sort of mechanism may well hold in mammalian MAP kinase cascades.

B. Protein associations in mammalian MAP kinase pathways

Although the roles of Ste5p may not yet have been fully elucidated, the fact that Ste5p is required for the function of the MAP kinase module of the pheromone response pathway focused attention on the importance of assembly of cascade complexes. Furthermore, the control of specificity of Ste11p that appears to be exerted by its binding to either Ste5p or Pbs2p indicates that signal reception and transmission can be channeled by the formation of protein complexes. Another apparently essential function of Ste5p is its ability to move and become appropriately localized within cells.

Extrapolating from these findings in yeast, we expect that scaffold proteins have one or more key functions: 1) they may organize MAP kinase cascades for the efficient serial activation of the components; 2) they may restrict signal reception by recognizing signals from only a subset of possible receptor systems; 3) they may restrict the specificity of signal transmission by interacting with a limited repertoire of potential components of MAP kinase cascades; and 4) they may determine the output signal not only as a consequence of selectivity among MAP kinases, but also by localizing the cascade to selected sites of action, *e.g.*, the transcription machinery, the microtubule cytoskeleton, etc.

While the inherent enzymatic specificity of Raf isoforms and MEK1/2 may be sufficient to account for their selectivity for ERK1/2 in cells, some of the mammalian MEKKs and MEKs implicated in the stress pathways appear less specific *in vitro* and when overexpressed in cells. For example, overexpression of Tpl-2 has been linked to the activation of at least five MAP kinase pathways, and MKK6 phosphorylates at least seven different MAP kinases *in vitro*. This apparent lack of enzymatic selectivity suggests that the assembly of these enzymes in complexes may restrict their actions to the MAP kinase or kinases in the complex and thereby determine their output signal. As a result of these considerations, the search for scaffolds for MAP kinase cascades has been intense.

1. Protein-protein interactions in the ERK1/2 cascade. Evidence from binding studies, cloning, and the behavior of mutant MEKs suggests that several protein-protein interactions are required for intracellular signal transmission through the ERK1/2 pathway. These interactions have proposed functions that lead to the localization of the kinases for signal reception, movement of the kinases to sites of action, substrate specificity and recognition, and temporal control of kinase activation. Several of these are described next.

2. Raf-1 forms complexes with Ras and MEK1. Wolfman and colleagues (223) showed that pull-down assays could be used to isolate Ras-Raf-1 complexes. MEK1 was also present in these complexes by virtue of a tight interaction with Raf-1, which can be demonstrated by coimmunoprecipitation. Raf-1 has been the subject of most studies in part because it is ubiquitous. Other Raf isoforms may display distinct properties. Less is known about binding interactions of Raf isoforms with MEK2.

3. Binding domains on MEK1/MEK2. MEK1 and MEK2 display one unique feature and one feature conserved in other MEK family members: both are required for efficient activation of their downstream MAP kinases in cells. The conserved feature is a stable binding site for MAP kinases, specifically ERK1/2, which is located at the N terminus of MEK1 and 2 in a short basic region. This sequence has all the hallmarks of a MAP kinase substrate-docking domain known as the D domain. An extensive list and examination of the presence of this domain in many proteins were presented by Nishida and colleagues (262) and others (263, 264) (see below). This docking site on MEK1 is not only required for ERK2 activation *in vitro* but is also necessary for its activation of ERK2 in cells. Several types of experiments support this conclusion. A MEK1 deletion mutant lacking N-terminal sequence including the docking domain interferes with activation of ERK2 by EGF (263). When introduced into cells, an N-terminal peptide derived from MEK1, which contains the docking site, inhibits progress through the cell cycle (265). Anthrax lethal factor cleaves the D domain from MEK and inhibits ERK activation (266). Using mutagenesis and deletion analysis, a binding site on ERK1/2 for this D domain has been localized to a pair of aspartate residues in the C-terminus of ERK2, just outside the catalytic core (Refs. 262 and 267; Fig. 1 and Table 2). Additional sites of interaction on ERK2 have also been proposed (153, 263, 268).

The unique feature is a proline-rich region inserted between protein kinase subdomains IX and X of MEK1 and MEK2 (269, 270). This proline-rich insert is present in the MEKs in the ERK pathway, but not in any other MEK family members identified thus far. Deletion studies indicate that this domain is also required for stimulation of ERK1/2 intracellularly, although its absence has no effect on the enzymatic activities of these kinases *in vitro* (269, 270). Expression of a peptide that encompasses the insert inhibits activation of ERK2 by EGF, suggesting that binding of the insert to other proteins is important for signaling through the cascade. This insert region contains multiple potential bind-

TABLE 2. Substrate and kinase interaction domains found on multiple proteins

Domain	Sequence motif	Proteins containing motif	Proteins binding to motif
On substrates	(R/K)X (R/K)X_{2-4}(L/I)X(L/I) (D domain)	Elk-1, c-Jun, MEF2, MEK1, many others	ERK1/2, JNK/SAPKs, p38 (binds to CD site)
	LAQRR	Rsks, Mnk2	ERK1/2
	LA(K/R)RR	Mnk1, MSK1	ERK1/2, p38
	LX(K/R)(R/K)R/K	PRAK, MAPKAP kinases 2 and 3, MSK2	p38
	FXFP	LIN-1, SAP-1, Elk-1, Ksr-1, A-Raf, MKP-1, DUS4, others	ERK1/2 (unknown binding site)
On MAP kinases	DXXD (CD site)	Most MAPKs	Proteins containing D domains

ing sites for SH3 domains and is phosphorylated by several protein kinases (32, 53, 200, 271); this may be a mechanism for feedback control and for modulation by other signaling pathways.

4. MP-1. MP-1, a protein of approximately 13 kDa, was identified by Weber and colleagues (272) in a two-hybrid screen with MEK1. Deletion of the MEK1 proline-rich insert eliminates binding, suggesting that the insert is the primary site of interaction between MEK1 and MP-1. It has been suggested that MP-1 is a scaffold that enhances formation of protein complexes, because it also binds to ERK1. Interestingly, it binds much less well to ERK2, indicating an unexpected selectivity between these two very similar MAP kinases. Cellular studies demonstrated that MP-1 increases the activation of ERK1, consistent with the interpretation that it binds both MEK1 and ERK1 (272). Because of its small size, MP-1 is unlikely to be a functional equivalent of Ste5p. However, MP-1 may be one unit of a modular scaffolding system that may facilitate the formation of a smorgasbord of complexes with minor differences in protein composition.

5. Grb10. Nantel and co-workers (273) have shown that the proline-rich insert of MEK1 binds to Grb10. Grb10 is usually viewed as an adapter molecule. It was originally isolated in a screen for proteins that bound to the tyrosine-phosphorylated, C-terminal domain of the EGF receptor. Grb10-MEK1 complexes have been identified in association with mitochondria and may be involved in cell survival signals that can be generated by this pathway (274). MEK1 also binds to Grb2 (A. Dang and M. H. Cobb, unpublished data), a common adapter that links receptors to the Ras GEF Sos. The significance of these associations is unknown.

6. Kinase suppressor of Ras (KSR). Eye development in *Drosophila* and vulval induction in the nematode *C. elegans* have proven to be valuable systems in which to discover Ras signaling mechanisms and components of the ERK1/2 MAP kinase signaling cascade using genetics. In each system the MAP kinase cascade is regulated by a receptor tyrosine kinase—Sevenless in flies and the EGF receptor in worms. Each works through Ras to control cell fate. To identify molecules that were required for the function of Ras, mutants with impaired Ras signaling without effect on Raf or downstream molecules were sought using these two systems (275–277). Kinase suppressor of Ras or KSR resulted from these screens and was found to act in numerous tyrosine kinase pathways. KSR, like Raf, has an N-terminal cysteine-rich region and a C-terminal kinase domain. Also in common with Raf, KSR homologs have been found in numerous animal species but not in yeast (158).

Substantial evidence indicates that KSR acts as a scaffold to bind the kinases of the ERK1/2 MAP kinase module (278–282). On the other hand, there is little evidence to indicate that it is a protein kinase. It has strong primary sequence similarity to the protein kinase family, but has arginine in place of the lysine in kinase subdomain II that is required for catalysis (283). There is also little to suggest that its functions depend on protein kinase activity, although its kinase domain is required for binding to both Raf-1 and MEK1, and mutation of the above mentioned arginine impairs its function (278, 280–282). The autophosphorylating activity of KSR, the only reported evidence of its catalytic function, is most likely due to the association with MEK and ERK (D. K. Morrison, personal communication), raising further questions about its protein kinase activity.

The ability of the catalytic domain to bind to MEK1 is essential for the function of KSR (282). The CRD of KSR binds to ERK2 (278–280). One function of KSR, like Ste5p, may be to localize the MAP kinase module at the membrane to be activated by transmembrane cues. Also similar to Ste5p, KSR binds to γ-subunits of heterotrimeric G proteins, suggesting that KSR may have roles in signaling by G protein-coupled as well as tyrosine kinase receptors (284).

7. Raf kinase inhibitor protein (RKIP). A Raf-1-interacting protein, named RKIP, was isolated from a two-hybrid screen using Raf-1 as bait (285). As suggested by its name, RKIP inhibits the phosphorylation and activation of MEK by Raf-1. RKIP appears to disrupt the formation of Raf-MEK complexes. RKIP binds directly to Raf-1, MEK, and ERK as assessed by *in vitro* binding and coimmunoprecipitation from cell lysates, apparently preventing their productive interactions. Based on overexpression studies and the use of antisense RNA and inhibitory antibodies, it was concluded that RKIP functions physiologically to shut off the activation of the ERK1/2 module. It is possible that RKIP may have other functions, *e.g.*, as a scaffold that promotes activation of the cascade under a select group of circumstances or to localize the cascade to a specialized organelle. Although there are no data supporting this idea currently, both JNK inhibitory proteins (JIPs, discussed below) and the inhibitor protein for PKA (PKI) were originally identified as inhibitors and are now believed to have additional functions. JIP is apparently a Ste5p-like scaffold and PKI terminates the nuclear activity of PKA by forming a complex that promotes the export of the catalytic subunit of PKA from the nucleus (286).

8. YopJ. Orth *et al.* (287) have identified a virulence factor from the bacterial pathogen *Yersinia pestis* that binds to multiple MEKs so that host signaling responses can be usurped or interrupted. YopJ blocks phosphorylation and activation of MEKs and thereby inhibits ERKs, JNK/SAPKs, p38 MAP kinases, and other signaling pathways. Among the consequences are prevention of cytokine biosynthesis and promotion of apoptosis. YopJ-related proteins exist in some other bacterial pathogens, but mammalian homologs of YopJ have not been reported.

9. STYX. Dixon's group (288, 289) also identified a tyrosine phosphatase-related molecule, STYX, which lacks the cysteine required for phosphatase catalytic activity. When cysteine was introduced into the appropriate position in the molecule, it displayed phosphatase activity toward ERK1/2. STYX bound tightly to ERK1/2, suggesting that it may act as an inhibitor either of ERK1/2 activity or their dephosphorylation.

10. Sur-8. Sur-8 was identified as a loss-of-function mutation that can suppress the multivulval phenotype in *C. elegans* in the presence of an activated let-60 (ras) gene (290, 291). Loss of Sur-8 function in a wild-type genetic background pro-

duced no observable phenotype. When worms with mutated Sur-8 were crossed with worms deficient in either mpk-1 (ERK1/2 ortholog) or ksr-1, vulval induction was severely compromised. Ectopic expression of wild-type Sur-8 enhanced the multivulval phenotype caused by an activated let-60 mutation and also increased Raf-1 activity. Epistatic analysis in worms placed Sur-8 at the same level of the pathway or downstream of Ras. This was consistent with two-hybrid experiments that showed that Sur-8 interacts with Ras mutants. Overexpressed Sur-8 coimmunoprecipitated with complexes of Ras and Raf. Point mutations (cysteine 260 tyrosine, glutamate 457 lysine) in Sur-8 reduced its association with Ras and Raf-1 and its ability to enhance Raf kinase activity.

11. Connector enhancer of KSR (CNK). To identify molecules that modified the function of KSR in the Sevenless/photoreceptor system, Rubin and colleagues (292, 293) created a line of flies expressing only the putative catalytic domain of KSR, which they named KDN. Expression of KDN produced a mild rough-eye phenotype. These flies were subsequently mutagenized to identify enhancers or suppressors of the rough-eye phenotype. CNK was identified as a gene that enhanced the KDN phenotype and suppressed phenotypes caused by expression of activated alleles of sevenless or ras. *Drosophila* CNK is a protein of 1,557 residues, containing individual sterile α motif (SAM), CRIC, PDZ, and PH domains. There are also two potential SH3 binding sites. This domain structure and the ability of *Drosophila* CNK to interact with Raf-1 indicate that it may be an adaptor protein. As is the case with KSR, overexpression of CNK can inhibit Raf function; however, this may be due to an unproductive interaction of the two proteins in the absence of an activating signal. Further domain analysis has shown that the C-terminal domain of CNK interacts with the kinase domain of Raf. Consistent with this finding, a candidate human homolog of CNK is much shorter, lacks the extended C terminus, and is unable to interact with Raf. This suggests distinctions in function of CNK in flies and its putative homolog in mammals. In flies, the SAM and CRIC domains in the CNK N terminus are sufficient to cooperate with $V^{12}G^{37}$ Ras signaling during *Drosophila* eye development. These domains are conserved in the putative human homolog; thus, it is possible that human protein may regulate Ras function independently of the MAPK cascade.

12. The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) module and the JIP family of scaffolding proteins. No Ste5p orthologs have thus far been identified in mammalian DNA sequences. However, JIP1, a protein found in a two-hybrid screen and originally identified as an inhibitor of the cellular effects of JNK/SAPKs, binds kinases at each level of this mammalian MAP kinase pathway (294, 295). When expressed in cells JIP1 immunoprecipitates complexes that contain JNK1/2, the MEK, MKK7, and MEKK level enzymes of the mixed-lineage kinase subgroup, MLK3 and DLK. In contrast, JIP1 does not bind MKK4 or MEKK1, MEKK3, or MEKK4. JIP1 also associates with the Ste20p-related kinase, hematopoietic progenitor kinase-1 (HPK1), which is believed to be an upstream regulator of MEKKs. A D domain-like sequence within an N-terminal collagen homology domain of JIP1 appears to constitute the JNK-binding domain. MKK7 and MLK3 bind directly to JIP1, in a region C-terminal to the JNK binding site. In addition to binding sites for the protein kinases, JIP1 also contains an SH3 domain.

Additional proteins with related functions have been found (52, 296–298). A JIP1 relative, JIP2, has properties similar to JIP1. JIP2 oligomerizes with JIP1 to form higher order complexes, and JIP2 complexes appear to be concentrated in the cytoplasm, perhaps near the cell surface. JIP3 is expressed most highly in brain. Like JIP1 and JIP2, JIP3 binds components of the JNK/SAPK module and is cytoplasmic. JIP3 was found to accumulate in growth cones within neurites. Another protein, termed JNK/SAPK-associated protein 1 (JSAP1) was also identified by a yeast two-hybrid screen, using JNK3 as bait (297). In transfected cells JSAP1 preferentially associated with the JNK3 compared with JNK1 and JNK2. In contrast to JIPs, JSAP1 interacted with MKK4/SEK1 and MEKK1. Although JNK and MEKK1 binding was direct, only active MKK4/SEK1 bound to JSAP1 in cells. Interestingly, JSAP1 also coprecipitated with MEK1 and Raf-1, but not MKK6 or MKK7. Overexpression of JSAP1 enhanced activation of JNK3 and, to a lesser extent, JNK1 and JNK2. The significance of binding to MEK1 and Raf-1 is unknown.

13. MEKK1. MEKK1 is a large protein that has binding sites for multiple components of MAP kinase modules. JNK/SAPK binds to the N-terminal 220 amino acids of MEKK1 through a D domain-like sequence, and MKK4 associates with its catalytic domain (205, 299). Both ERK2 and MEK1 bind to its N-terminal noncatalytic domain (M. Karandikar, S. Xu, and M. H. Cobb, submitted). MEKK1, like Raf-1, is less abundant than the downstream kinases (Ref. 44; and M. Karandikar and M. H. Cobb, submitted), indicating that only a small fraction of these enzymes will be associated with MEKK1 at any given time. MEKK1 does not bind to p38, ERK3, or TAO, suggesting that its associations with JNK and ERK modules are specific. A role of MEKK1 in regulating these two pathways has been supported by gene disruption studies (204).

Endogenous MEKK1 colocalizes with α-actinin in stress fibers, focal adhesions, with the focal adhesion protein paxillin, and on microtubules (300). Thus, the cytoskeleton may send or receive signals from MEKK1. MEKK1 also binds to the Nck-interacting kinase NIK, which may be a MEKK kinase. Skolnik and co-workers (216) cloned NIK, through a two-hybrid screen with the adapter molecule Nck. Nck binds to tyrosine phosphoproteins, the Ras GEF Sos, and a number of other proteins such as the Rac/Cdc42-activated kinase PAK, indicating that it may transmit signals from tyrosine kinase and other small G proteins to the Ras pathway (301). Overexpression of NIK in fibroblasts increased JNK/SAPK activity. NIK is distantly related to GCK, which has also been shown to bind to MEKK1 and to activate JNK/SAPKs (211). Interactions with these kinases may offer additional mechanisms of regulating MEKK1 by linking it to membrane receptors.

14. TAOs. TAO1 and 2 were isolated as mammalian relatives of the yeast kinase Ste20p (196, 197). TAO2 was also iden-

tified as prostate-derived Ste20-like kinase or PSK, a human kinase expressed in prostate carcinoma (302). TAO1 and 2 have MEKK activity and can activate MEKs 3, 4, and 6 *in vitro*. TAO1/2 bind to MEK3 and MEK6, but not to MEK4, in spite of the fact that MEK4 is an *in vitro* substrate. The N terminus of the MEK is required for this binding, while the C terminus is dispensable. The stable association of MEK3 or MEK6 with TAO proteins may link their physiological functions to p38 but not JNK/SAPK pathways by restricting their intracellular targets. TAOs may be regulated by the Gi family of heterotrimeric G proteins (Chen, L., Z. Chen, M. H. Cobb, and A. G. Gilman, submitted). A third TAO-like kinase, JNK inhibitory kinase or JIK, is nearly 90% identical to TAO1 in its catalytic domain; effects of JIK on the p38 pathway have not been reported (303).

15. Generality of stable association of MEKs with their MAP kinase targets. The stable association of a MEK with its MAP kinase may be a general property of MAP kinase modules and has been found in yeast and mammalian MAP kinase pathways (150, 262, 263, 304–307). In addition to the interaction of MEK1 and ERK2, MEKs in stress cascades also associate with their downstream MAP kinases. Mayer has shown a stable association between JNK/SAPK and one of its MEKs, SEK1/MKK4 (307). The function of this association is unclear; however, the data suggest that an inhibitory complex forms that must dissociate before JNK/SAPK activation. p38 Family members interact differentially with MEK3 and MEK6 (150). Nishida's group has found D domains in several MEK family members that may mediate the interaction with their MAP kinase targets, suggesting that this is a general feature of these cascades (262).

VIII. Regulation of MAP Kinase Localization

The spatial organization of kinases and substrates determines what signals may be transmitted and received at various possible sites of action. The complement of cellular signaling proteins and cell state together determine the distribution of MAP kinases and other signaling molecules in a manner that can be regulated acutely and long term by extracellular signals. Functionally distinct populations of a MAP kinase may restrict potential targets. This may occur through highly specialized complexes, concentration of a MAP kinase at a site of action, such as the membrane, or compartmentalization of a population of a MAP kinase to a diffusion-limited site such as a membrane-bound vesicle or the nucleus. For example, distinct populations of ERKs have been shown to associate with the microtubule cytoskeleton (308, 309), membrane specializations (310–311), and the nucleus (312–314). Active JNK has been localized to punctate structures along microtubules and to nuclear speckle populations (315).

Much effort has been focused on understanding the consequences of localization of ERK1 and 2 to the nucleus. Stimulus-dependent nuclear localization appears to be essential for morphological transformation of fibroblasts and differentiation of PC12 cells (316, 317), for instance. Although all phosphorylation-dependent transcriptional regulation might be expected to require nuclear localization of the relevant kinases, in a number of cases, key transcription factors are cytoplasmic until activated, and, as a result, may be covalently modified while in the cytoplasm (318).

Nuclear translocation may occur by distinct mechanisms depending on the MAP kinase and the setting. The process has been most extensively studied for ERK1 and ERK2. Several events may cooperatively determine the amount of ERK1 and 2 in the nucleus. Some evidence has been reported for each of the following: 1) cytoplasmic anchoring; 2) nuclear entry by diffusion; 3) phosphorylation and subsequent dimerization; 4) active transport of protein monomers, dimers, or complexes across the nuclear membrane; 5) nuclear export of ERK1/2 alone or in complexes; and 6) binding to retention sites in the nucleus (306, 313, 314, 319, 320). A diverse group of experimental approaches suggest that interactions with MEK1/2, in particular, play a prominent role in the stimulus-dependent nuclear accumulation of ERK1/2 and their redistribution after stimulus termination (267, 306, 317, 320, 321).

The significance of ERK-MEK binding on ERK localization has been addressed using MEK1 mutations, MEK1-derived peptides, and ERK2-MEK1 fusion proteins. Localization studies of MEK1 have demonstrated that it is largely a cytoplasmic protein (322–324). MEK1 contains a nuclear export sequence (NES), which is functional based on the finding that its deletion results in the dramatic accumulation of MEK1 in the nucleus. Thus, MEK1 enters the nucleus but is exported due to its NES (325). A MEK1 peptide that contains both the D domain that mediates MEK-ERK docking and the NES causes retention of ERK2 in the cytoplasm (306). Furthermore, an ERK2-MEK1 fusion protein is excluded from the nucleus, if the MEK1 NES is intact, but accumulates in the nucleus, if the leucine residues within the NES are mutated to alanine (317). ERK2 also binds stably to a number of other proteins that may restrict its access to the nucleus. One of these is the phosphatase PTP-SL (326). Activation-induced release of the ERK2-PTP-SL interaction has also been proposed to enhance the nuclear accumulation of ERK2.

MAP kinases can also regulate subcellular distribution of downstream effectors, thereby affecting the signaling properties of these proteins. The calcineurin-activated transcription factor, NFAT4, is phosphorylated by JNK and thus retained in the cytoplasm (327). Dephosphorylation of NFAT4 is required for its nuclear translocation and hence its activity.

IX. Inactivation of MAP Kinases

The duration and amplitude of MAP kinase activation represents the balance between the activating signal and inactivation mechanisms. Both are influenced by negative feedback triggered by the activating signal upstream of the MAP kinase. As has been discussed, ERK activity is tightly regulated through phosphorylation of tyrosine and threonine residues on the activation lip. The removal of one or both of these phosphates by tyrosine, serine/threonine, or dual-specificity phosphatases dramatically decreases MAP kinase activity. The specificity of phosphatases is strongly dictated by intracellular localization. Thus, clear-cut evidence implicating them in MAP kinase regulation has been

difficult to obtain. Nevertheless, analysis of activities in fractionated cell lysates with specificity toward MAP kinases, genetic analysis, and substrate trapping mutants, in which the catalytic cysteine is mutated to serine, have implicated phosphoprotein phosphatases from each of the three major phosphatase categories in inactivating MAP kinases (328–331).

A substantial number of dual-specificity phosphatases known as MAP kinase phosphatases (MKPs) are largely dedicated to the inactivation of MAP kinases at the appropriate times and locations (70, 332–338). For extensive reviews see Refs. 339 and 340. The MKPs fall largely into two groups: they are either encoded by growth factor or stress-inducible genes and are located primarily in the nucleus, or they are not acutely regulated by transcription and are located in the cytosol. Both classes have a similar domain organization, an N-terminal regulatory domain and C-terminal catalytic domain that displays sequence homology to the dual-specificity phosphatase VH1 (341). The differences in localization and induction imply differences in the temporal and spatial inactivation that they may produce. Specificity of different MKPs for different MAP kinase family members has been suggested by *in vitro* and cellular studies that sometimes have different conclusions (336, 342, 343).

Consistent with the concept of MKPs being involved in negative feedback, two distinct mechanisms for positive regulation of MKP activity by ERK1/2 have been identified. MKP1 is phosphorylated by ERK1/2, which may protect it from proteosomal degradation (344). The activity of MKP3 is increased as a consequence of the binding of ERK2 to its regulatory domain (345). There is also evidence that MKPs may regulate localization of MAPKs, which is noted above.

X. Substrate Recognition and Stable Binding of Substrates to MAP Kinases

MAP kinases have overlapping specificities for substrates. Some proteins are substrates for two or more MAP kinase family members *in vitro* and *in vivo*, indicating that signal integration takes place at the substrate level, as well as in upstream signaling pathways. MAP kinases phosphorylate serine and threonine residues followed by proline residues. Proline at the P + 1 position is the most reliable primary sequence determinant that can be used to identify MAP kinase substrates. This requirement arises from the nature of the binding site for the P + 1 residue. Many protein kinases contain a pocket for a large hydrophobic residue immediately after the serine/threonine residue to be phosphorylated (346). The three-dimensional structure of phosphorylated ERK2 reveals a surface depression, not a large pocket, because the ERK2 phosphotyrosine occupies this pocket (64). Proline is preferred because its favored backbone conformations place the side chain away from the kinase surface. In some substrates, the acceptor site is followed by glycine, not proline, at the P+1 residue (1). Because glycine lacks a side chain, it could be accommodated on the P + 1 binding surface.

MAP kinases distinguish among sequences with proline at the P + 1 site; only a few are substrates, suggesting that there are extended regions of interaction that enhance substrate recognition. In the case of ERK1/2 in particular, substrates often also contain proline at the P-2 position, giving the motif PX(T/S)P. Further specificity parameters have not been delineated for JNK/SAPKs and p38 MAP kinases. Peptide library screening does not reveal any strong ERK1 preferences for residues at the P − 5, 4, or 3 positions or at the P + 2, 3, or 4 positions (347). However, conserved motifs have been identified that mediate interactions of some MAP kinases with their substrates. It is becoming increasingly clear that these motifs have a major impact on signaling through MAP kinases.

The first docking motif that was identified is the δ domain, residues 30–79, of the transcription factor c-Jun (348, 349). JNK/SAPKs bind tightly to certain Jun family members, but not to the oncogenic form v-Jun. v-Jun lacks the docking site which is present in many Jun family members. This sequence was found to confer tight binding of certain Jun species to JNK/SAPKs.

The best characterized docking motif is a short sequence related to the δ domain called the docking or D domain (350). The D domain may appear at some distance from and in apparently any orientation with respect to the phosphoacceptor site in the substrate. The typical D domain sequence is a cluster of basic residues, usually two or more, followed within a few residues by (L/I)X(L/I) (Table 2). D domains interact with ERK1/2, JNK/SAPK, p38 family members, and perhaps other MAP kinases; modest sequence differences may result in recognition by only one or two of these types of MAP kinases (262, 264, 350, 351). D domains are present in numerous substrates including the transcription factors Elk-1, c-Jun and the MEF2 family, upstream activators such as MEK1/2, and phosphotyrosine phosphatases. Removing or mutating these domains within substrates markedly reduces their phosphorylation by MAP kinases. As noted above, the D domain on MEK1 is required for efficient signal transmission.

Another targeting motif that may be related to the D domain is typified by the sequence LAQRR and its variants, and is present in several protein kinases that are MAP kinase substrates. LAQRR is thought to be recognized specifically by ERK1/2 and is found in Rsk isoforms and Mnk2 (352, 353). This domain on Rsk binds to ERK1/2 directly and is required for Rsk activation by ERK. The related sequence LA(K/R)RR has been suggested to bind to both ERK1/2 and p38 and is found in the protein kinases MSK1 and Mnk1. A variation, LX(K/R)(R/K)RK is targeted by p38 and is found in several downstream protein kinases including MSK2, PRAK, MAPKAP kinase-2, and MAPKAP kinase-3.

A distinct motif is the FXFP sequence that is thought to interact only with ERK1/2 (264, 350, 354). This motif is present in transcription factors such as LIN-1, SAP-1 and Elk-1, protein kinases such as KSR and A-Raf, and dual-specificity protein phosphatases such as MKP-1 and DUS4. This motif has been shown to work independently or in combination with the D domain to mediate kinase-substrate binding. Its importance has been tested by mutation in *C. elegans* proteins as well as in mammalian systems.

Complementary domains on MAP kinases that may bind the substrate targeting domains have been proposed. The

first of these to be identified was mapped by Kallunki and associates (348) in a splice form of the JNK/SAPKs. One splice variant in these enzymes introduces an insert between conserved kinase subdomains IX and X. This insert lies very near the protein substrate binding region on the surface of the C-terminal half of the kinase catalytic core and increases the affinity for Jun.

Recently two laboratories identified a sequence, DXXD-E, in ERKs that is important for ERK binding and activation by MEK (262, 267). The name common docking (CD) domain has been suggested for this sequence. The CD domain lies just C-terminal to the protein kinase catalytic core within a C-terminal extension shared by the MAP kinase family. This sequence is believed to be a putative docking site to allow D domains not only of MEK but also of other MAP kinase substrates to associate with MAP kinases (262). The sequence is conserved in the MAP kinase family. The acidic residues in the CD motif are thought to interact with the basic cluster in the D domain. It seems highly unlikely that the interaction between MAP kinases and substrates can be limited to electrostatic interactions provided by two acidic residues alone. In this regard, the L/I × L/I sequence in the D domain is critical for efficient MAP kinase-substrate binding. This suggests that other regions on MAP kinases must also participate in the association with D domains. The N-terminus of ERK2 has also been implicated in MEK binding by two groups, although the mechanism is unknown (Ref. 263 and M. J. Weber, personal communication).

XI. Substrates of MAP Kinases

Our understanding of the functions of MAP kinases is still expanding as more cellular substrates are identified. ERK1/2 as well as other MAP kinases target membrane proteins, such as phospholipase A2, cytoplasmic proteins, such as downstream kinases and cytoskeletal proteins, and nuclear proteins, such as transcription factors. A brief overview of MAP kinase substrates follows.

1. Protein kinases. Protein kinases form a substantial subset of ERK1/2 targets. These include Rsk1, Rsk2, Rsk3, MAPKAP kinase-2, MAP kinase-interacting kinase (Mnk) 1 and Mnk2 (48, 49, 353, 355–358). Rsk1 and Rsk2 were initially isolated from *Xenopus* and identified as pp90 ribosomal S6 kinases [also known as MAPKAP kinase 1α and β, respectively (355)]. ERK2 phosphorylates the Rsk proteins on serine 363 in the linker between two distinct yet highly conserved catalytic domains and on threonine 573 in the activation loop of the C-terminal kinase domain (359, 360). Other events may also be involved in Rsk activation. Once activated, Rsk1, 2, and 3 phosphorylate downstream targets involved in transcriptional activation, such as the cAMP-response element binding protein (CREB) (361–363), the coactivator CBP (364), c-Fos (365, 366), the serum response factor (363, 366, 367), and the estrogen receptor (368). In a comparative study, ERK2 coimmunoprecipitated with Rsk2 and Rsk3 isozymes, but not with Rsk1 (357). The carboxyl-terminal residues of Rsks proved to be critical for the binding interaction and contain the D domain-like motif described above (357).

Many ERK substrates have been identified by testing logical candidates. More recently, novel ERK substrates have been discovered from two-hybrid and protein phosphorylation screens using rat ERK2 as bait or enzyme (48, 49). Among several substrates identified in this fashion were Mnk1 and Mnk2. Mnk1 and 2 are serine/threonine kinases that have putative MAP kinase phosphorylation sites within the activation loops of their catalytic domains and conserved C-terminal ERK-interacting domains. As is the case for Rsk, ERK2 did not phosphorylate and activate a C-terminal truncation (residues 334–424) mutant of Mnk2 due to the lack of the interaction domain (48).

Mnk1 and Mnk2 serve as common substrates for growth factor-stimulated ERK2 and stress-activated p38 (48) and may thereby integrate signals from multiple cellular stimuli as alluded to earlier. *In vitro* findings suggest that there may be some selectivity in activation of the enzymes; Mnk2 is a good substrate for both kinases *in vitro*, whereas Mnk1 is a better substrate for p38 (48). JNK/SAPK does not interact with either Mnk1 or Mnk2. Once activated, Mnk1 and Mnk2 phosphorylate the eukaryotic initiation factor 4E (eIF-4E) on serine 209 *in vitro* (48). As a result, protein-synthesizing ribosomes and additional protein synthesis initiation factors are recruited to mRNA. Mnk1 is activated by both mitogenic (phorbol esters) and stress factors (NaCl and anisomycin), and these effects can be blocked by inhibitors of MEK1 (PD98059) and p38 (SB203580) (48), supporting the idea that Mnk1 integrates signals from these two kinase pathways.

Both p38 and ERK share other common kinase substrates, including MAPKAP kinase-2 and -3 (356). MAPKAP kinases are serine/threonine kinases with proline-rich N termini, highly conserved catalytic domains, and C-terminal autoinhibitory regions (356, 369). MAPKAP kinase-2 is phosphorylated on residues within the catalytic domain and the C-terminal autoinhibitory domain (370). Stress signals mediated via p38 have the greatest effect on MAPKAP kinase-2 phosphorylation (18, 369). In turn, MAPKAP kinase-2 phosphorylates heat shock protein 27 (hsp27) (18, 371, 372). In human neutrophils, MAPKAP kinase-2 also phosphorylates lymphocyte-specific protein-1 (LSP1) (373). Both hsp27 and LSP1 are F-actin binding proteins involved in cytoskeletal structure (373). NGF and fibroblast growth factor stimulate p38 and ERK2 phosphorylation of MAPKAP kinase-2, which induces phosphorylation of both CREB and ATF-1 in cells (361, 374). Msk is another protein kinase target of both ERK1/2 and p38 MAP kinases (375). Inhibitor and gene disruption studies suggest that Msk may also be a significant CREB kinase.

2. Membrane and cytoplasmic substrates. In addition, other physiological substrates of ERK1/2 have been identified. Cytosolic phospholipase A_2 is phosphorylated on serine 505 *in vivo* in response to EGF stimulation of ERK activity (376). As with Mnks, PLA_2 is also subject to regulation by p38 (377). In the protein phosphorylation screen, several potential clones activated by ERK2 were identified as heat shock factor transcription factor 1 (hsp1), topoisomerase II-b (378), Ral-GDS, and ZNF7, a zinc finger protein (49).

3. Nuclear substrates. MAP kinases are capable of modulating gene expression by phosphorylating transcription factors di-

rectly and by activating other protein kinases (Rsks, Mnks), which then phosphorylate proteins involved in gene expression. For instance, Rsk2 can phosphorylate histone H3, a protein involved in regulating the structure of chromatin (379). Msk1, another ERK2 kinase substrate, has also been shown to phosphorylate histone H3 and high mobility group protein, HMG-14 (380). Since these proteins are involved in packaging DNA into chromatin, activation of histone and HMG via the ERK pathway could result in increased transcription factor accessibility to DNA binding sites (381).

Recently, ERK2 has been shown to phosphorylate steroid receptor coactivator-1 (SRC-1), which possesses an intrinsic histone acetyltransferase activity and is a coactivator that enhances the activation of steroid nuclear receptors (382). SRC-1 also interacts with CREB to enhance estrogen and progesterone receptor-mediated gene activation (383, 384) and another HAT protein p300/CBP-associated factor (385). Cumulatively, these downstream effects of ERK2 could influence chromatin remodeling and activation of gene expression.

Not only are MAP kinases capable of affecting gene expression via intermediary kinases and by phosphorylating proteins in the cytoplasm, but MAP kinases translocate to the nucleus where they are able to phosphorylate transcription factors to regulate their activities (see above). ERK1/2 phosphorylates and alters the properties of several subclasses of transcription factors. The AP-1 family (activating protein-1) of transcription factors that are phosphorylated by ERK1/2 include c-Jun (15, 120), c-Fos (365, 366), and ATF-2 (activating transcription factor), although the *in vivo* relevance of phosphorylation by ERK1/2 is not certain (386). These proteins are leucine zipper proteins that form homodimers and heterodimers when activated to bind to DNA (387). c-Jun is phosphorylated on serine 63 and 73 by JNK/SAPKs and on C-terminal inhibitory sites by ERK1/2 (15, 120). Phosphorylation at the N-terminal sites results in increased stability of c-Jun and an increase in its transactivation potential and DNA binding affinity (15, 120); phosphorylation of the C-terminal sites inhibits DNA binding (388, 389).

Another class of transcription factors, the ternary complex factors (TCFs), are MAP kinase substrates. These Ets-domain proteins, such as Elk-1, mediate transcription from serum response elements (SREs) contained in the promoters of genes for c-Fos and other serum-induced genes. Elk-1 appears to be phosphorylated *in vivo* by ERK1/2, JNK/SAPK, and p38, although results with p38 are contradictory (390, 391). Elk-1 is phosphorylated on several residues within the C-terminal transactivating domain, notably serine 389. Phosphorylation of these sites results in increased formation of ternary complexes with serum response factor and DNA (391). Two other TCF family members, SAP-1 and SAP-2, are also activated by MAP kinases. SAP-1a and SAP-2 are each phosphorylated by both ERK and p38, but not JNK/SAPK (392–395). In addition, Spi-B and Spi-1/PU.1 transcription factors are phosphorylated by JNK1 *in vitro*, but ERK phosphorylates only Spi-B (396). The physiological relevance of these phosphorylations has not yet been established.

Many other transcription factors have also been identified as MAP kinase substrates. For example, multiple MAP kinase pathways are used to phosphorylate STAT3 (signal transducers and activators of transcription) on serine 727 (397). This is an example of the cooperation of MAP kinase pathways with cytokine signaling systems to bring about the activation of an important transcriptional regulator. ERK1/2 also phosphorylate Beta2/NeuroD1 and enhance glucose-dependent insulin gene transcription in islets (S. Khoo, S. C. Griffin, M. S. German, and M. H. Cobb, submitted).

XII. Biology of MAP Kinase Pathways

A. Development of inhibitors

The functions of MAP kinase pathways have most often been probed by correlating their activities with particular biochemical and cellular responses. Inhibitors have been used where available. Many such studies have been reviewed recently (1). Nonfunctional mutants of components in signal transduction pathways, which often act as dominant inhibitors, have been used extensively to probe the relationships among components and the functions of signal transduction pathways. Early among these were dominant negative mutants of Ras. Some processes blocked by these mutants have been directly linked to ERKs, initially including proliferation of fibroblasts and more recently long-term potentiation in neurons (56, 57). Mutants of protein kinases that have defective catalytic activity have been widely used to infer roles of kinase cascades. Residues required for phosphoryl transfer, including the lysine in protein kinase subdomain II and the aspartic acids that bind magnesium or serve as the catalytic base in protein kinase subdomains VI and VII, are commonly mutated to create kinase-defective mutants. All MAP kinase pathways have been probed using these sorts of mutants.

Pharmacological inhibitors are invaluable to the study of signal transduction pathways. Most known protein kinase inhibitors bind in the ATP site. Of course, in addition to protein kinases, many other enzymes use ATP as a substrate. One difference between the small-molecule kinases, such as hexokinase, and the protein kinases is the orientation of substrate binding. The small-molecule kinases bind the substrate other than ATP in the active site interior, while protein kinases bind ATP in the interior of the active site. Nevertheless, protein kinase inhibitors that bind in the ATP site are likely to have unrecognized actions on other enzymes, including those that control metabolism, transport, etc.

The determination of the crystal structures of several protein kinases has revealed structural differences in protein substrate and nucleotide binding pockets that are consistent with the previously unanticipated selectivity of some of these inhibitory agents (64, 65, 147–149, 398–406). The most selective inhibitors for components of MAP kinase pathways target either MEK1/2 or p38 α and β isoforms. Inhibitors of each type have been used extensively to implicate the ERK or p38 pathways in a wide array of biological events.

Two companies developed inhibitors of MEK1/2 that are commercially available. One class typified by PD98059 was found using the ERK pathway as an *in vitro* target (407, 408). A second type, of which U0126 is the prototype, was identified in a cell-based screen seeking inhibitors of phorbol ester-stimulated AP-1 transactivation (409); MEK1/2 were

later identified as the targets of this drug. These drugs have been used to demonstrate the requirement for ERK1/2 in numerous processes ranging from neurite extension in PC12 cells and proliferation in fibroblasts and some cancer cells to cell motility, and circadian rhythm (1, 410–412). Although these compounds have little inhibitory activity toward many other protein kinases, including several other MEK family members, they have significant effects on MEK5 (164), as discussed above. These inhibitors are not competitive with respect to ATP, suggesting that they do not bind in the ATP site (407, 409). The elucidation of the basis for the interaction of these inhibitors with MEK1/2 has been retarded by the lack of a crystal structure of any MEK family member. A second generation MEK inhibitor (PD184352) has been synthesized with enhanced bioavailability (413).

As noted above, p38 was identified as the target of compound discovered in a cell-based screen for inhibitors of lipopolysaccharide (LPS)-induced TNFα and IL-1β production in monocytes (16). SB203580 is representative of these pyridinyl imidazole compounds and inhibits the α- and β-isoforms of p38 with selectivity. Cells treated with SB203580 had reduced intracellular amounts of both cytokines, due primarily to blockade of their translation, with no appreciable effect on total DNA, RNA, or protein synthesis. This and related drugs have been used to show that p38 is necessary for numerous translational and transcriptional responses (414).

The three-dimensional structure of p38 has aided in the design of better pyridinyl imidazole inhibitors (147, 148, 401). Inhibitors with subnanomolar K_i values have been synthesized (415). Threonine 106, located in the ATP binding site, is a major determinant of inhibitor sensitivity, as has been confirmed by mutagenesis studies on p38 and other kinases (402–404, 416). Larger side chains interfere with drug binding. A few other protein kinases, notably Raf-1, also contain threonine or residues with smaller side chains at the equivalent position (417). Raf-1 may be inhibited or paradoxically activated by these compounds (417). SB203580 blocks a number of processes in cells without inhibiting the activity of ERK1/2, suggesting that the effects of this inhibitor are largely attributable to inhibition of p38, in spite of its ability to interact with Raf.

Binding studies using tritiated pyridinyl imidazoles showed that the inhibitor bound unphosphorylated p38 as well as it bound phosphorylated p38 (418). The unphosphorylated kinase bound ATP poorly, because it competed poorly with drug binding to inactive enzyme. Thus, the inhibitor will bind unphosphorylated p38, even in the presence of a huge excess of ATP. These observations suggest a rationale for the activity of this inhibitor in cellular assays even though the intracellular concentration of ATP is in the millimolar range.

XIII. Gene Disruption Experiments

In the last 4 yr, the functions of MAP kinase pathways have also been studied in mice by gene knock-out. In the final section of this review these studies will be discussed. As with many gene knock-out studies, it is most difficult to deduce the ultimate functions of genes that are also required for development, and animals lacking genes whose products may have largely redundant functions often display little or no phenotypic change. When specific phenotypes are observed, there is sometimes insufficient information to connect the observed phenotype to specific biochemical and molecular events. In the future, tissue-specific and/or inducible gene knock-outs may allow investigators to overcome embryonic lethality and assess the physiological roles of specific MAP kinase pathway components in specific tissues. One caveat in interpreting knock-out studies is that the gene targeting strategy may result in the production of some protein fragment, whose function may itself produce a phenotype distinct from that which would be caused by total loss of the gene product. Finally, some of the studies on MAP kinase pathways that are summarized below must be viewed as controversial or preliminary because conclusions from different laboratories are, in several cases, contradictory.

A. The ERK1/2 pathway

The genes encoding all three Raf proteins have been disrupted in mice. Although subject to reevaluation, all three genes are required for survival. Unique and redundant functions of Raf isoforms are suggested. Knock-outs of genes encoding MEK1 and ERK1 but not MEK2 or ERK2 have been reported. Disruption of the MEK1 gene caused embryonic lethality, while disruption of the ERK1 gene was associated with minimal phenotypic manifestations. In several of the ERK pathway knock-outs, although not in the ERK1 knock-out animals, significant changes were observed in angiogenesis and the development of the placenta.

1. Raf-1. The gene encoding Raf-1 (c-Raf) was disrupted by replacing the first coding exon (exon 2) with a neomycin-resistance gene (419). While this strategy eliminated the full-length (74 kDa) Raf-1 protein, alternative splicing resulted in the production of a transcript in which the mutated exon 2 had been removed. Upon translation, this transcript yielded a 62-kDa Raf-1 protein with the N terminus truncated. This mutant Raf-1 protein was produced at low levels (~15% of full-length Raf-1), and retained about 10% of wild-type kinase activity after serum or phorbol ester treatment of cells (419). Raf-1 mut/mut mice showed retarded growth beginning at embryonic day (E) 10.5 and died between E 10.5–E 12.5. Histological examination of E 10.5 Raf-1 mut/mut mice revealed placental defects characterized by a reduction in the size of both the spongiotrophoblast and labyrinthine layers. The labyrinthine layer contained fewer than normal blood vessels and large numbers of undifferentiated mesenchymal cells. The authors concluded that the midgestational lethality observed in Raf-1 mut/mut mice likely resulted from compromised placental function. The severity of the Raf-1 mutant phenotype was dramatically influenced by the genetic background on which the mutation was carried. On a 129/C57B6 background, nearly all Raf-1 mut/mut embryos died between E 10.5 and E 12.5; on the outbred CD1 background, about two-thirds of Raf-1 mut/mut mice survived to term, dying just after birth. Raf-1 mut/mut mice surviving beyond midgestation displayed specific developmental defects. While most organs appeared normal, the eyelids failed to

fuse properly, and the dermis and epidermis were abnormally thin and poorly differentiated. Defects in lung maturation were also observed; lungs were smaller and failed to inflate at birth. This was believed to be the cause of neonatal death. Fibroblasts isolated from Raf-1 mut/mut embryos displayed reduced proliferation in response to serum.

2. B-Raf. The B-Raf gene was disrupted using a gene targeting strategy that replaced exon 3, which codes for the N-terminal portion of the Ras-binding domain. This strategy eliminated the B-Raf mRNA (420). B-Raf −/− mice died at E 10.5–12.5 from vascular defects. At the time of death, B-Raf −/− embryos displayed vascular abnormalities characterized by enlarged vessels, an overabundance of endothelial cells in vessels, and incomplete lining of vessels with endothelial cells. This structurally compromised endothelial cell layer resulted in vessel ruptures that allowed blood to spill into surrounding tissues. Altered patterns of apoptosis were observed throughout B-Raf-deficient embryos, though net increases in the number of apoptotic cells were only observed in the vascular endothelium. The authors concluded that B-Raf plays a critical role in the development of the vascular system.

3. Raf-1 and B-Raf. The individual Raf-1 and B-Raf knock-outs suggested that these genes have essential, nonoverlapping developmental functions beginning at midgestation (419, 420). Animals heterozygous for the disruptions described above were crossed to determine the impact of disrupting both genes (421). The resulting homozygous embryos failed to develop properly beyond the blastocyst stage, supporting the authors' conclusion that Raf-1 and B-Raf have an essential, redundant function in very early embryonic development. Additionally, the developmental defects observed in either Raf-1 mut/mut or B-Raf −/− mice were each exacerbated by the loss of one copy of the other Raf isoform (*i.e.*, Raf-1 mut/mut, B-Raf +/− embryos were more severely affected than Raf-1 mut/mut, B-Raf +/+ embryos).

4. A-Raf. The gene targeting strategy eliminated A-Raf protein expression as deduced by Western blot (422). The survival of A-Raf −/− mice varied according to mouse strain. On a C57B6 background, A-Raf −/− animals were of normal size at birth, but displayed retarded growth by 2–3 days and died 1–3 weeks after birth. Neurological deficiencies were manifested as abnormal movement and proprioception, although no neuronal defects were apparent histologically. These mice displayed megacolon, although enteric innervation appeared normal. When the A-Raf mutation was maintained on a largely 129/OLA genetic background, about 50% of A-Raf-deficient animals survived to adulthood. These mice, although runted, were fertile and showed no intestinal abnormalities and few of the neurological defects displayed by the more severely affected young mice.

5. MEK1. The mouse MEK1 gene was disrupted by insertional mutagenesis. MEK1 − / − mice died at about E 10.5 (423). At the time of death, these mice were undersized, had distended blood vessels lacking erythrocytes in the yolk sac, and showed signs of necrosis in some tissues. Placental defects were identified, including a poorly defined spongiotrophoblast layer, and a vessel-poor, compacted labyrinthine layer, consistent with failed angiogenesis into the labyrinthine layer. MEK1-deficient fibroblasts migrated more slowly than wild-type cells on a fibronectin substrate, but behaved normally on a collagen substrate. These findings are consistent with a requirement for the ERK pathway for stimulation of migration and angiogenesis by extracellular matrix. Transient transfection of MEK1 into MEK1-deficient cells rescued the migration defect. Surprisingly, ERK2 activation in response to plating on fibronectin appeared normal in cells from −/− animals.

6. ERK1. The mouse ERK1 gene was disrupted via deletion of the sequences coding for protein kinase subdomains V and VI; Western blotting showed that ERK1 protein was not detectable (424). Consistent with this finding, total serum-stimulated ERK activity in lysates from −/− embryonic fibroblasts was 50% of that from wild-type cells, also suggesting that ERK2 protein does not increase to compensate. A similar result was observed by Landreth and co-workers (424a). ERK2 activation in ERK1 −/− cells was more sustained in response to serum than in cells from normal animals. The knock-out mice were normal by numerous measures and were fertile. Fibroblasts from these animals proliferated normally in response to serum, α-thrombin, or PDGF-B. Isolated thymocytes from ERK1 −/− mice showed reduced proliferation in response to ligation of the T cell receptor. Thymocytes from ERK1 −/− mice showed decreased rates of maturation into singlepositive (CD8+ or CD4+) thymocytes. Expression of α- and β-chains of T cell receptors was reduced by about 50% in thymocytes from knock-out mice. Activation-induced apoptosis in thymocytes from ERK1 −/− mice was unaffected, suggesting that the defect in thymocyte maturation occurred at the level of positive thymic selection.

B. The JNK/SAPK pathways

The three genes encoding JNK/SAPK isoforms, one of the two MEKs, and several MEKKs have been disrupted. Apoptosis is commonly affected, although both positive and negative effects are observed in the knock-outs. Distinct immunological deficiencies are found to arise from the deletion of specific JNK/SAPK isoforms and MKK4/SEK1.

1. MEKK1. Two groups have disrupted the gene encoding MEKK1. Although the wild-type protein was not detected in either study, MEKK1 fragments, which have a variety of activities on their own, may have been produced. The targeting strategy used by Johnson and co-workers (204) eliminated sequence encoding residues 1–132; Western analysis using antibodies to multiple MEKK1 epitopes did not detect any common bands, suggesting that smaller MEKK1 proteins were not synthesized. This gene disruption led to embryonic lethality. MEKK1-deficient cells underwent apoptosis more readily than control cells in response to 0.2 M sorbitol or 25–50 ng/ml nocodazole, consistent with the interpretation that MEKK1 transduces an antiapoptotic signal. This is opposite to what was concluded from the behavior of overexpressed MEKK1, which has been reported to be cleaved by caspases and to induce apoptosis (425, 426).

MEKK1 −/− embryonic stem (ES) cells displayed normal activation of JNK/SAPKs in response to UV, heat shock, and anisomycin, but dramatic decreases in JNK activity in response to nocodazole, serum, cold stress, and LPA, and a slight defect in JNK activity in response to 0.2 M sorbitol. Interestingly, these MEKK1 −/− ES cells also showed modest decreases in activation of ERK in response to 0.2 M sorbitol, serum, and LPA, but did not differ from control cells in the activation of ERK by phorbol ester. Serum-starved MEKK1 −/− ES cells possessed slightly lower basal ERK activity than control cells. Activation of p38 was unaffected by deletion of MEKK1. These results suggest a connection of MEKK1 to both the JNK/SAPK and ERK pathways.

In contrast, Karin and colleagues, who used a targeting strategy that disrupted the catalytic domain at the C terminus of MEKK1, found that MEKK1 was not necessary for activation of ERKs by growth factors or serum, but was necessary for maximal JNK/SAPK activation by growth factors, TNFα, IL-1, double-stranded RNA, and LPS (206). The reason for the apparent discrepancy with respect to the role of MEKK1 in ERK activation is not clear at this time. Additionally, these investigators found that MEKK1 was essential for induction of ES cell migration by serum factors.

2. MKK4/SEK1. The gene encoding MKK4/SEK1 has been disrupted by three groups of investigators (134, 427, 428). In each case, the resulting mice were not viable past E 12.5. Two groups reported that MKK4/SEK1 −/− embryos were anemic (134, 427) and that hematopoiesis in the yolk sac and vascular development appeared normal. Developing livers of MKK4/SEK1-deficient embryos contained fewer hepatocytes than those of the controls. Livers and isolated hepatocytes from knock-out animals displayed enhanced apoptosis relative to wild-type controls. The authors concluded that MKK4/SEK1 likely transduces signals critical for hepatocyte proliferation and/or survival.

ES cells and MEF cells from MKK4/SEK1 −/− animals were used to study the importance of this kinase to JNK/SAPK and p38 activation (134). In ES cells, loss of MKK4/SEK1 eliminated JNK/SAPK phosphorylation and activity in response to anisomycin and heat shock and dramatically reduced its phosphorylation in response to UV radiation. No changes were observed in the activation of p38 in response to these same stimuli. In contrast, in MEF cells, the loss of MKK4/SEK1 dramatically reduced activation of both JNK/SAPK and p38 in response to TNFα or IL-1 treatment (134). Both p38 and JNK/SAPK phosphorylation were eliminated in response to anisomycin treatment of MKK4/SEK1 −/− MEF cells. JNK/SAPK activation was lost in MEF cells treated with sorbitol, but p38 activation was unaffected.

A third group also examined defects in JNK/SAPK signaling in ES cells lacking MKK4/SEK1 (428). Activation of JNK/SAPK by MEKK1, anisomycin, and heat shock were completely blocked; and activation by osmotic shock and UV-C radiation were partially blocked in MKK4/SEK1 −/− ES cells. Consistent with these results, activation of an AP-1 transcriptional reporter was also blocked but could be restored by transfection of a cDNA encoding MKK4/SEK1. p38 Activation was not examined in this study.

Taken together, these studies strongly link MKK4/SEK1 to the regulation of JNK/SAPK in response to some but not all activators of this pathway. The results are consistent with the likelihood that another MEK family member, probably MKK7, based on biochemical and genetic studies in flies, regulates JNK/SAPK under certain circumstances; the possibility that MKK4/SEK1 and MKK7 cooperate to regulate JNK/SAPKs, as suggested by biochemical data, is not tested by these studies. The results of these experiments also suggest a link between MKK4/SEK1 and p38, at least in MEF cells. The findings challenge the exclusive connection, suggested by kinetic data, between MKK4/SEK1 and JNK/SAPKs. This is discussed further below.

Two groups have pursued a functional analysis of MKK4/SEK1 in B and T lymphocytes by generating MKK4/SEK1 −/−, Rag2 −/− somatic chimera mice (429–432). These studies have examined the role of MKK4/SEK1 in B and T cell development, activation, and proliferation and in T cell apoptosis after activation. Some conflicting conclusions have been drawn from these studies and a thorough discussion of this work will not be provided here.

3. JNK1. The gene for JNK1 was disrupted by deleting four exons that encode the C-terminal half of the catalytic core of the protein (433). The JNK1 −/− mice developed normally and were fertile. JNK1 −/− mice exhibited alterations in CD4+ T cell activation, affecting differentiation to the T_H1 or T_H2 subset of effector cells. Specifically, antigen-stimulated CD4+ T cells from JNK1-deficient mice preferentially differentiated into the T_H2, not the T_H1, subset of effector cells; T_H2 cells mediate humoral immune responses. The authors conclude that T cell receptor-initiated signaling inhibits CD4+ T cell differentiation to T_H2 cells by a mechanism that involves JNK1.

4. JNK2. Two groups have generated mice lacking an intact JNK2 gene (434, 435). Both found JNK2 −/− mice to be viable and fertile. Both groups also concluded that B and T cell development was normal in JNK2 −/− mice. However, other findings were not in agreement or have been interpreted differently by the authors. Yang *et al.* (434) reported a specific deficiency in the ability of mature (peripheral) CD4+ T cells from JNK2 −/− mice to differentiate into the T_H1 class of effector cells after antigen stimulation. These cells exhibited impaired secretion of the T_H1-specific cytokine interferon (IFN)γ in response to treatment with the T_H1-polarizing cytokine IL-12. Sabapathy *et al.* (435) found that JNK2 −/− mice had a more general defect in T cell activation, as well as altered apoptotic responses to antigen stimulation. In this study, peripheral T cells from JNK2 −/− mice secreted drastically less IL-2, IL-4 (T_H2-specific), and IFNγ (T_H1-specific) in response to *in vitro* stimulation with anti-CD3 antibodies. Sabapathy *et al.* also performed an *in vivo* experiment in which anti-CD3 antibody was injected into wild-type and JNK2 −/− mice. They found that immature CD4+, CD8+ thymocytes were resistant to apoptosis triggered by anti-CD3 antibody. Thymocytes isolated from JNK2 −/− mice were resistant to apoptosis stimulated by anti-CD3 but not to cell death resulting from treatment with UV radiation, dexamethasone, or anti-Fas antibody. Mature, peripheral T cells from JNK2 −/− mice were not resistant to

apoptosis stimulated by anti-CD3 antibodies. Peripheral T cells from JNK2 −/− mice proliferated more slowly in response to stimulation with anti-CD3 antibodies; this effect was overcome by addition of exogenous IL-2.

5. JNK3. The JNK3 gene was disrupted by eliminating the sequences coding for amino acids 211–267 of JNK3, which includes the core catalytic residues and TPY phosphorylation motif (436). Phenotypic characterization of JNK3 −/− mice focused on the brain, as this JNK isoform is selectively expressed in the nervous system. JNK3-specific kinase activity was absent from the hippocampus in JNK3 −/− mice, as deduced by an activity assay. JNK3 −/− mice were of normal size and fertile, and all tissues, including brain, were histologically normal.

The investigators examined the role of JNK3 in excitotoxicity-induced hippocampal apoptosis. Kainate-induced seizures cause excitotoxic neuronal damage; the hippocampus is particularly sensitive to this type of damage. Kainic acid stimulates JNK activity in cultured neurons. This and other evidence has suggested that JNK, Jun proteins, and AP-1 may be involved in kainate-induced nerve damage. Thus, the loss of JNK3 was anticipated to have an impact on the kainate response. JNK3 −/− mice displayed less severe seizures after systemic injection of moderate doses of kainate (30 mg/kg). At higher doses (45 mg/kg), JNK3 −/− and wild-type mice had seizures of similar severity, but JNK3 −/− mice had a higher survival rate after seizures than wild -type mice. JNK3 −/− mice showed dramatically reduced c-Jun phosphorylation and AP-1 transcriptional activity in the hippocampus after kainate injection and, in contrast to wild-type mice, little or no kainate-stimulated apoptosis in the hippocampus. This study provides strong evidence that JNK3 transduces a proapoptotic signal in certain neurons in response to excitotoxic stress.

6. JNK double knock-outs. As described above, mice in which genes encoding JNK1, JNK2, or JNK3 were disrupted developed and survived normally. Two groups have examined the impact of disrupting combinations of these genes (437, 438). JNK1/JNK3 and JNK2/JNK3 double mutant mice developed normally (437). However, JNK1/JNK2 double mutants had severe defects in the regulation of apoptosis during brain development and died at E 10.5–11.5. Neural tube closure was defective leading to hindbrain exencephaly in both studies. The apparent cause of this defect proposed in one study of JNK1/JNK2-deficient embryos was a reduction in apoptosis required for development within the hindbrain, leading to the conclusion that either JNK1 or JNK2 provided a critical proapoptotic signal in the hindbrain (437). The other study reported augmented apoptosis in the hindbrains of JNK1/JNK2-deficient mice at E 10.5 (438). The explanation for this apparent discrepancy is unknown. JNK1/JNK2-deficient mice also showed excessive apoptosis in the forebrain at E 10.5 to 11.5, indicating that JNK1/2 can also provide an antiapoptotic signal (433, 438).

C. The p38 pathways

1. MKK3. Mice lacking the gene encoding MKK3 were viable and fertile (439). Macrophages and dendritic cells from MKK3 −/− mice displayed reduced (50%) p38 activation compared with cells from wild-type animals in response to LPS. The ability of sorbitol to activate p38 in macrophages was not significantly affected by the loss of MKK3, nor was JNK activation by LPS. Both accumulation of IL-12 mRNA (enhanced transcription and perhaps stabilization) and secretion of IL-12 in response to LPS were inhibited in macrophages from MKK3 −/− mice. Dendritic cells were also defective in IL-12 production in response to CD40 stimulation. Secretion of TNFα and IL-6 after LPS treatment was similar in MKK3−/− and wild-type macrophages, whereas secretion of IL-1α and IL-1β was decreased. In cultured T_H1 type CD4+ T cells mixed with antigen-presenting cells, the loss of MKK3 resulted in defective IFNγ production.

In a second study, p38 and JNK were found to be activated normally in response to UV, sorbitol, and IL-1 in MEF cells from MKK3 −/− mice (440). The activation of p38 but not JNK by TNFα was reduced in MKK3 −/− MEFs, and the secretion of both IL-1 and IL-6 in response to TNFα was severely impaired. Accumulation of the mRNAs encoding IL-1, IL-6, and TNFα was lost in MKK3 −/− MEF cells. Both reports link MKK3 and p38 in proinflammatory cytokine production, consistent with other studies of p38 function (439, 440).

2. p38α. The p38α gene-targeting strategy replaced an 8- to 10-kb piece of DNA including the exon encoding the TGY motif required for p38 activation (441). Western blots showed no p38α protein in the −/− ES cells. p38α-Deficient mice died during embryogenesis; further details were not reported. Signaling was examined in p38α −/− ES cells. Sodium arsenite was no longer able to stimulate activation of MAPKAP kinase 2 (95% reduction in activity). This is consistent with considerable earlier data demonstrating that p38 is an upstream activator of MAPKAP kinase 2 (18). The residual MAPKAP kinase 2 activating activity in p38α-deficient ES cells was insensitive to the p38 inhibitor SB203580. Wild-type and p38α −/− ES cells were differentiated into embryoid bodies and then cultured for 12 days in the presence of IL-3, IL-12, and granulocyte macrophage-colony stimulating factor (GM-CSF). Cells were sorted by fluorescence-activated cell sorting (FACS) to isolate those expressing the IL-1 receptor. Loss of p38 had no effect on this process. Both types of cells expressing the IL-1 receptor secreted IL-6 when challenged with IL-1, but the response in the p38α −/− cells was greatly reduced. IL-1-stimulated secretion of IL-6 was sensitive to SB203580 in wild-type cells, but not in p38α −/− cells. Consistent with earlier findings, these studies suggest that p38α mediates the major pathway leading to production of IL-6 (442, 443).

D. Other components of MAP kinase pathways

1. MEKK3. The gene targeting strategy eliminated a portion of the MEKK3 catalytic domain. MEKK3 −/− mice died around day 11 of gestation, and showed defects in early, developmentally essential angiogenesis (444). Most smaller vessels were affected, particularly in the labyrinthine layer of the placenta, where fetal blood vessels failed to intermingle with maternal. The authors concluded that there was an

intrinsic defect in endothelial cells from MEKK3-deficient animals that impaired angiogenesis in response to certain signals. mRNAs for major angiogenic markers were unaffected, suggesting that MEKK3 may function either further downstream or on as yet undefined events.

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