Mammalian MAP kinase signalling cascades

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Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes, unique to eukaryotes, that are involved in many facets of cellular regulation. Initial research concentrated on defining the components and organization of MAPK signalling cascades, but recent studies have begun to shed light on the physiological functions of these cascades in the control of gene expression, cell proliferation and programmed cell death.

MAPKs are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. MAPKs also respond to chemical and physical stresses, thereby controlling cell survival and adaptation. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK)[1]. These modules may be activated by STE20 kinases or small GTP-binding proteins[2]. Many MAPKs activate specific effector kinases—MAPK-activated protein kinases (MAPKAPKs)—and are inactivated by MAPK phosphatases.

As the regulation of MAPK signalling has been discussed elsewhere[1,2], we focus on recent progress in understanding MAPK function in mammals. Because of the ease of genetic analysis, the functions and regulation of MAPK cascades are best understood in budding yeast[3], but the analysis of targeted mutations in mice and development of specific inhibitors have begun to shed light on their functions in mammals. It is becoming clear that MAPKs regulate almost all cellular processes, from gene expression to cell death.

Mammalian MAPKs

Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ) and ERK5, that are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling. Presumably each MAPKKK confers responsiveness to distinct stimuli. For example, activation of ERK1/2 by growth factors depends on the MAPKKK c-Raf, but other MAPKKKs may activate ERK1/2 in response to pro-inflammatory stimuli.

The situation is quite complex with the JNK and p38 cascades, which respond to many stimuli and can be activated on overexpression of at least a dozen MAPKKKs, whose physiological functions and specificities remain elusive. Dominant-negative catalytically inactive MAPKKKs can be used to address these issues, but the results of such experiments can be confusing. For example, at least four MAPKKKs were implicated in JNK activation by tumour necrosis factor (TNF) and interleukin (IL)-1, including ASK1 (MEKK5), TAK1, MLK and MEKK1 (reviewed in ref. 4). These difficulties are probably generated by loss of specificity caused by overexpression of dominant-negative mutants.

Systematic genetic ablation of MAPKKKs may provide definitive answers to many of these questions. For instance, it has been shown that MEKK1 is required for JNK activation in embryonic stem cells or fibroblasts by microtubule-disrupting agents, lysophosphatidic acid, viral infection, double-stranded RNA and serum[4,5]. MEKK1 also is required for activation of JNK by TNF and IL-1 in embryonic stem cells[4], but not in fibroblasts[5]. These experiments revealed that MEKK1 is dispensable for activation of JNK by ultraviolet radiation and protein-synthesis inhibitors and is not essential for ERK1/2 or p38 activation. Such results suggest considerable specificity in MAPK activation even at the MAPKKK level.

Specificity in MAPK activation and function

The control of diverse cellular processes in response to a plethora of extracellular stimuli by a few MAPKs implies that considerable specificity is built into MAPK activation and function. Several distinct but not mutually exclusive mechanisms secure specificity in MAPK activation. In yeast, the STE5 protein functions as a scaffold that organizes the three components of a pheromone-responsive MAPK cascade and its upstream activators into a specific module[3]. A search for analogous mammalian scaffolds led to JIP1, which organizes JNK1/2, MKK7 and the MAPKKK MLK1 into a specific signalling cassette (Fig. 1a)[6]. MP1, another mammalian scaffold protein not related to JIP-1, interacts with ERK1 and MEK1, thereby potentiating ERK1 activation[7].

A second mechanism ensuring specific MAPK activation depends on sequential physical interactions between members of a given cascade[8]. For instance, JNK1/2 is bound by the N-terminal extension of MKK4 (JNKK1), which also interacts with the catalytic domain of MEKK1. Each interaction is disrupted on activation of the downstream kinase (Fig. 1b). Whereas scaffolds increase specificity at the expense of signal amplification, sequential interactions provide amplification without sacrificing specificity[8]. Both mechanisms may operate in parallel, leading to differential activation of the same MAPK in response to distinct stimuli.

A third and more complex mechanism for signal amplification is based on the ability of MAPKs to regulate indirectly the expression of both ligands and inhibitors for cell-surface receptors that feed into MAPK cascades[9]. Such positive and negative autocrine loops can generate specific spatial patterns of MAPK activation (Fig. 1c).

Once activated, MAPKs need to find their targets. Although it is necessary to limit the phosphorylation of irrelevant substrates, it is also important that each MAPK recognizes a number of substrates to allow the regulation of many processes. This problem seems to have been solved through a bipartite enzyme–substrate interaction. All MAPKs recognize similar phosphoacceptor sites composed of serine or theonine followed by a proline, and the amino acids that surround these sites further increase the specificity of recognition by the catalytic pocket of the enzyme. Full specificity is ensured through a docking interaction mediated by another site on the kinase that recognizes a distinct site on the substrate (Fig. 1d). As first described for c-Jun, a short sequence that precedes the two principal JNK phosphoacceptor sites, Ser 63 and Ser 73, is recognized by an interaction surface on JNK, outside its catalytic pocket[10]. This interaction determines the efficiency of c-Jun phosphorylation and the choice of phosphoacceptor sites (Fig. 1d). Similar interactions involving distinct phosphoacceptor and docking sites determine substrate recognition by other MAPKs[11].

Spatial localization of signalling molecules further augments specificity in signal transduction. For instance, MEKK1 colocalizes with elements of the cytoskeleton[1], and cytoskeletal rearrangements

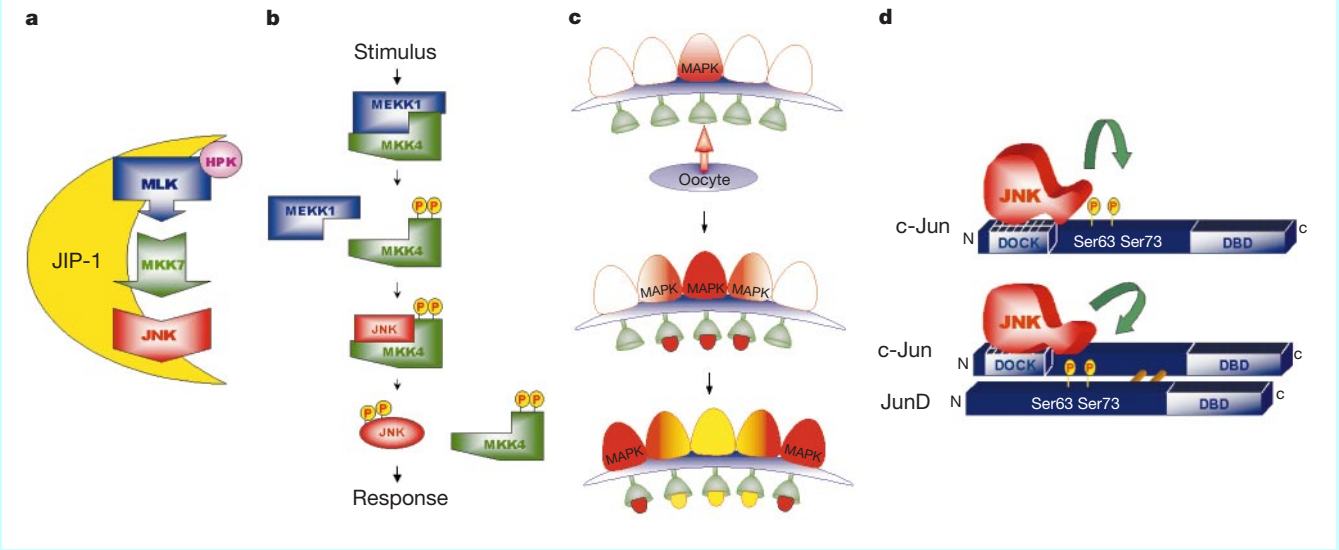

Figure 1 Mechanisms for generation of specificity in MAPK activation and function. **a**, JIP-1 acts as a scaffold that ties together the upstream kinase HPK-1, the MAPKKK MLK1, the MAPKK MKK7 and the MAPK JNK into a specific signalling module. **b**, Sequential and specific interaction between members of a MAPK cascade. MEKK1 interacts with inactive MKK4 to form a MEKK1–MKK4 complex. MEKK1 phosphorylates and activates MKK4, resulting in dissociation of the complex. The free and active MKK4 then specifically interacts with JNK. Once JNK is activated, the MKK4–JNK complex is disrupted and the active JNK is now freed to phosphorylate downstream effectors. **c**, Specific spatial patterns of MAPK activation by positive and negative autocrine loops in the *Drosophila* oocyte. A ligand called Gurken (red arrow) that is localized in the underlying oocyte activates a MAPK cascade (red gradient) in follicle cells, through an EGF receptor (green). This results in upregulation of another EGF receptor agonist, 'Spitz' (red), that activates MAPK in neighbouring cells. This is followed by expression of the inhibitory ligand 'Argos' (yellow) in midline cells with higher levels of MAPK activity, which eventually inhibits of MAPK activity in this region. **d**, The JNK docking region (DOCK) of c-Jun determines its specific phosphorylaton at Ser 63 and Ser 73 by JNK. A substrate lacking a functional docking site, such as JunD, can be phosphorylated by JNK that is recruited through the DNA-binding domain (DBD) of c-Jun.

may stimulate MEKK1 activity[4,5]. Such localized JNK activation may allow MEKK1 to modulate cell motility[4,5].

General functions of MAPK cascades

One of the most explored functions of MAPK signalling modules is regulation of gene expression in response to extracellular stimuli[12]. JNKs phosphorylate Jun proteins and thereby enhance their ability to activate transcription without affecting DNA binding[10]. Most MAPKs phosphorylate Ets transcription factors that are involved in induction of *fos* genes, whose products heterodimerize with Jun proteins to form activation protein 1 (AP-1) complexes[12]. The p38 proteins phosphorylate and enhance the activity of MEF2C and related family members[13]. In all of these cases, the MAPKs function inside the nucleus and target transcription factors that are pre-bound to DNA. Most transcription factors that are regulated by MAPKs are dimers, and structural analysis indicates that some MAPKs dimerize on activation[1]; however, MAPK dimerization may facilitate phosphorylation of dimeric transcription factors.

Although transcription factors are important MAPK targets, only part of the active MAPK pool translocates to the nucleus and much remains in the cytoplasm and other subcellular compartments. Correspondingly, MAPKs also regulate gene expression through post-transcriptional mechanisms involving cytoplasmic targets. JNK, for instance, is involved in IL-2 messenger RNA stabilization in activated T cells[14]. The target for JNK action appears to be a cytoplasmic protein that is recruited to IL-2 mRNA through two other proteins that bind to the 5′ untranslated region[14]. Other mRNA species are stabilized in response to p38 activation[15,16]. MAPKs are also involved in translational control, but in this case (as well as in some cases of mRNA stabilization[15]) their action is mediated by the MAPKAPK-2 and MNK1 effector kinases[17,18]. MNK1 phosphorylates translation initiation factor 4e (eIF-4e), augmenting the interaction of the cap-binding complex with capped mRNAs, thereby enhancing polysome assembly.

MAPKs regulate cell proliferation. ERK1/2 stimulates DNA synthesis through phosphorylation of carbamoyl phosphate synthetase II, a rate-limiting enzyme in pyrimidine nucleotide biosynthesis[19]. In addition, the ERKs, probably through MAPKAPKs such as RSK, promote cell-cycle progression by inactivating MYT1, a cell-cycle inhibitory kinase[20], but arrest meiotic cells at metaphase II by activating a cytostatic factor[21,22]. The ERKs can also stimulate cell proliferation indirectly by enhancing AP-1 activity, resulting in cyclin D1 induction[23]. That pathway alone is insufficient for initiation of DNA synthesis and needs assistance from phosphatidylinositol-3-OH kinase, which is indirectly activated by autocrine growth factors whose expression is ERK responsive[23]. Autocrine factors are important in cellular responses to MAPKs[9] and can provide a means for one MAPK cascade to activate other signalling pathways[24]. In T cells, the JNK pathway may stimulate proliferation by inducing IL-2 expression[25].

MAPKs also control cell survival. By and large, activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are linked to induction of apoptosis[26]. This dichotomy, however, is an oversimplification, and the actual roles of each MAPK cascade are highly cell type and context dependent. A pro-apoptotic function has been established for prolonged JNK activation in certain neuronal cells, especially in the CA1 layer of the hippocampus after intense activation of glutamate receptors[26–28]. This process does not occur in *Jnk3*$^{-/-}$ mice[28] or in mice expressing a mutant form of c-Jun (c-Jun$^{A63/73}$) lacking the JNK phosphoacceptor sites[29]; however, transient and more modest JNK activation in response to physiological stimulation does not result in neuronal apoptosis[30]. JNK-induced neuronal apoptosis partially depends on induction of death cytokines, such as Fas ligand, whose promoter contains an AP-1 site, explaining the dependence on c-Jun phosphorylation[27,29]. JNK1 and JNK2 are required for apoptosis of ultraviolet-irradiated fibroblasts, in which they have been suggested to activate directly the apoptotic machinery[31]. However, the combined JNK1/2 deficiency only prevents ultraviolet-induced apoptosis and does not hinder other pro-apoptotic stimuli[31]. c-Jun-deficient fibroblasts also exhibit specific resistance to ultraviolet-induced apoptosis[32]. Given that JNKs are vigorously activated after ultraviolet exposure and needed

for c-Jun induction[31], it is likely that the pro-apoptotic function of JNK1/2 in ultraviolet-irradiated fibroblasts is mediated through c-Jun[32]. Nevertheless, in cells exposed to physiologically relevant doses of ultraviolet radiation, JNK-induced c-Jun stimulates cell-cycle re-entry rather than inducing apoptosis[32].

The mechanism by which ERK protects cells from apoptosis is complex and Ras, a potent ERK activator, may also promote apoptosis[33]. In cerebellar granular cells, ERK activation by survival factors prevents apoptosis through RSK, which inactivates the pro-apoptotic protein BAD[34]. ERK may also induce growth factors that promote cell survival.

In vivo analysis of MAPK and MAPKK function

Although much can be learned about MAPK functions from *ex vivo* studies, it is important to investigate both their normal and their pathophysiological functions in the whole animal. Such analysis, accomplished through generation of mutant mouse strains by gene targeting, is also useful for determining the organization and regulation of MAPK signalling cascades. As the different MAPKKs exhibit high levels of specificity, it should also be possible to learn about MAPK function by targeting relevant MAPKKs. Some of the findings and the conclusions derived from analyses of MAPK- and MAPKK-deficient mice are summarized in Table 1.

ERK1/2. Only the knockout of ERK1 has been described[35]. *Erk1*$^{-/-}$ mice are viable and appear normal and with a modest defect in T-cell development (positive selection)[35]. It is likely that most ERK1 functions are equally served by ERK2. A similar and more marked defect is present in transgenic mice expressing dominant-negative MEK1 in thymocytes[36]; and *Mek1*$^{-/-}$ mice die *in utero*, exhibiting defective placental vascularization[37].

Despite impairment of positive selection and loss of ERK activity, thymocytes expressing dominant-negative MEK1 exhibit normal IL-2 induction and a mitogenic response[36]. Thus, other MAPKs are probably involved in IL-2 induction. As suggested by the *Mek1*$^{-/-}$ phenotype, MEK1 is required for migration of vascular endothelial cells as well as fibroblasts plated on fibronectin[37]. However, fibronectin-induced ERK activity remains normal[37]. An ERK1/2–MEK1 module may target specific substrates involved in cell migration while making only a small contribution to total ERK1/2 activity.

JNKs. All three *Jnk* loci have been knocked out. On mixed genetic backgrounds, none of the mutations results in lethality or obvious defects. However, *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ double mutants die at mid-gestation, exhibiting defective neural-tube closure[38,39]. Thus, JNK functions needed for development and viability are not isoform specific. Unexpectedly, deletion of MKK4 results in a more severe phenotype than the combined loss of JNK1/JNK2: mid-gestational lethality caused by abnormal liver development[40]. The same phenotype is caused by complete loss of c-Jun[42].

Although JNK1 and JNK2 are widely expressed, much attention has been paid to their functions in T cells. Unfortunately, owing to different experimental protocols and genetic backgrounds, the results of these MKK4 knockout experiments are confusing. In T thymocytes, JNK activity is synergistically stimulated by occupancy of the T-cell receptor and the CD28 auxiliary receptor and this activation parallels IL-2 induction[42]. Induction of anergy, which prevents T-cell activation, interferes with JNK activation[43]. Congruently, two groups found defective IL-2 production and proliferation after co-stimulation in JNK1/2- or MKK4-deficient T cells[44,45].

This defect was also observed in *Jnk1*$^{+/-}$*Jnk2*$^{+/-}$ double heterozygote T cells[44], suggesting that partially reduced JNK1/2 activity—which is expected to occur in *Mkk4*$^{-/-}$ cells—is sufficient to decrease T-cell activation. At any rate, this defect is partial and bypassed by strong stimulation or antigen-presenting cells[44]; therefore, it is possible that neither JNK1 nor JNK2 has a major role in controlling T-cell proliferation *in vivo*. Unexpectedly, JNK1/2-deficient T cells exhibit normal AP-1 activity, but display a partial defect in activation of NF-AT, another transcription factor required for IL-2 induction[45].

Table 1 Phenotypes of MAPKK and MAPK knockout mice

MAPKK/MAPK	Phenotypes	Similar to
MEK1	Defective placental vascularization[37]	ERK2?
MKK4	Defective liver development[40]	c-Jun knockout[41]
MKK7	Embryonic lethality of unknown cause[48]	
MKK3	Defective IL-12 production[52]	
ERK1	Defective T-cell development (positive selection)[35]	MEK1 dn negative transgenics
JNK1	Defective T-cell differentiation to Th2 cells[46]	
JNK2	Defective T-cell differentiation to Th1 cells[47]	
JNK1 or JNK2	Defective T-cell proliferation and IL-2 production[25]	JNK1 dn negative transgenics MKK4 knockouts
JNK1 or JNK2	Defective activation induced death of thymocytes[25]	JNK1 dn negative transgenics
JNK1 & JNK2	IL-2 overproduction[48]	MKK7 knockout
JNK1 & JNK2	Neural tube disclosure[38,39]	
JNK3	Resistance to excitotoxic neuronal cell death[28]	c-Jun$^{A63/73}$ knockin[29]
p38α	Placental defect[51] (trophoblast cells)	
p38α	Insufficient production of erythropoietein[50]	

dn, dominant-negative

Another group, however, found that *Jnk1*$^{-/-}$ or *Jnk2*$^{-/-}$ T cells produce IL-2 normally but undergo aberrant differentiation to effector cells[46,47]. Furthermore, an experiment in which *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ double knockout ES cells were used to reconstitute the lymphoid lineage of immunodeficient mice suggests that JNK1/2 negatively regulated IL-2 production[48].

These results are supported by analysis of *Mkk7*$^{-/-}$ T cells, which exhibit defective JNK activation[48], but they contradict experiments in which inhibition of JNK activity correlates with decreased IL-2 expression[42,43]. Although this discrepancy is not fully explainable, each experiment relied on a different approach and *Mkk7*$^{-/-}$ T cells also exhibit much higher p38 activity than wild-type cells[48]. It is therefore worthwhile to evaluate whether the overproduction of IL-2 is sensitive to p38 inhibitors.

Nevertheless, instead of T-cell activation JNK1 and JNK2 may be more intimately involved in formation of effector T cells[46,47]. As T cells lacking both JNK1 and JNK2 differentiate along the T-helper cell type-2 (Th2) path[48], the most important function of JNK1/2 in T cells could be to direct non-differentiated cells to the Th1 fate. JNK1/2 may also be involved in negative selection of T cells[25,44]. JNK-deficient cells, however, remain sensitive to other inducers of apoptosis, providing another example of the cell type- and stimulus-specific involvement of JNK in apoptosis. Surprisingly, no B-cell defects were detected in any of the JNK-deficient mice[25,44,46–48], but a partial proliferation defect was found in *Mkk4*$^{-/-}$ B cells[49].

In contrast to JNK1/2, the JNK3 isoform is expressed predominantly in the nervous system. *Jnk3*$^{-/-}$ mice are resistant to exitotoxic killing of neurons in the CA1 layer of the hippocampus[28]. The same phenotype is exhibited by c-Jun$^{A63/73}$ mice[29]. Thus, in this apoptotic pathway as in ultraviolet-irradiated fibroblasts[32], the function of JNK is exerted through its effects on gene expression.

p38. The only p38 isozyme whose *in vivo* function has been examined genetically is p38α. Unlike deletions of individual *Jnk* loci or *Erk1*, inactivation of *p38*α results in embryonic lethality[50,51]. It is not clear whether the lack of compensation by other isoforms is indicative of distinct biochemical functions or a marked difference in expression patterns. The severity of the phenotype and the cause of death vary with the genetic background in which the *p38*α deficiency is examined. In an inbred C57Bl6/J background, p38α deficiency results in lethality before day 11 of gestation, owing to defective placental development[51]. However, in a mixed background, some *p38*α$^{-/-}$ mice survive longer[50].

Analysis of these mice suggests that after passing the earlier hurdle imposed by development of placental trophoblasts, p38α-deficient

mice face a second hurdle owing to insufficient production of erythropoietin (Epo). Experiments with human hepatoma cells—a surrogate for fetal liver, the main site of fetal Epo production—indicate that p38α is activated in response to hypoxia and that its activation is required for hypoxia-induced stabilization of *Epo* mRNA[50]. Regardless of the molecular processes in which p38α participates, these experiments show that a MAPK previously thought to be mostly involved in inflammation and stress does have critical developmental functions.

Consistent with the presence of two MAPKKs that activate p38 MAPKs, *Mkk3*$^{-/-}$ mice are viable without obvious abnormalities[52]. Although *Mkk3*$^{-/-}$macrophages exhibit reduced endotoxin-induced p38 activity, most cytokines in these cells are normally induced following endotoxin exposure with the exception of IL-12 p35 and p40 subunits[52]. Although this defect occurs at the transcriptional level, the p38 responsive transcription factor is unknown.

Conclusions and prospects

In mammals, MAPK signalling cascades regulate important cellular processes including gene expression, cell proliferation, cell survival and death, and cell motility. With a few exceptions (mostly transcription factors), the MAPK substrates that regulate these processes are not known and their identification is a major challenge. The definition of physiologically relevant MAPK substrates should result in a thorough understanding of MAPK action. Currently, the only process in which a target (c-Jun) for a MAPK (JNK3) has been genetically defined is excitotoxic neuronal apoptosis[28,29]. As revealed by the involvement of c-Jun, this response is mediated through an effect on gene expression.

Similar analysis can be used to determine how many other biological responses to MAPK activation are mediated through changes in gene expression. As different cells express distinct sets of transcription factors, transcriptional control can easily account for much of the cell-type specificity in MAPK action. Techniques such as DNA microarray analysis should enable the definition of genes that are regulated in response to individual MAPKs, whereas proteomic analyses can be applied to the identification of substrates. Finally, specific inhibitors of MAPK signalling are becoming available. In addition to their utility in identifying MAPK functions, it is likely that they will prove useful in treating conditions, such as inflammation or cancer, caused by deregulation of MAPK cascades. □

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