



# Signaling by dual specificity kinases

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**Dual specificity kinases that phosphorylate the Thr- and Tyr-residues within the TXY motif of MAP-kinases of play a central role in the regulation of various processes of cell growth. These dual specificity kinases also known as MAP kinase kinases are constituents of the sequential kinase signaling modules. Seven distinct mammalian MAP kinases kinases have been identified. Some of the unique signaling properties of these kinases are discussed here.**

**Keywords:** oncogene; apoptosis; cell transformation; MAP kinase; MEKK; MEK; MKK; ERK; JNK; signal transduction

## Introduction

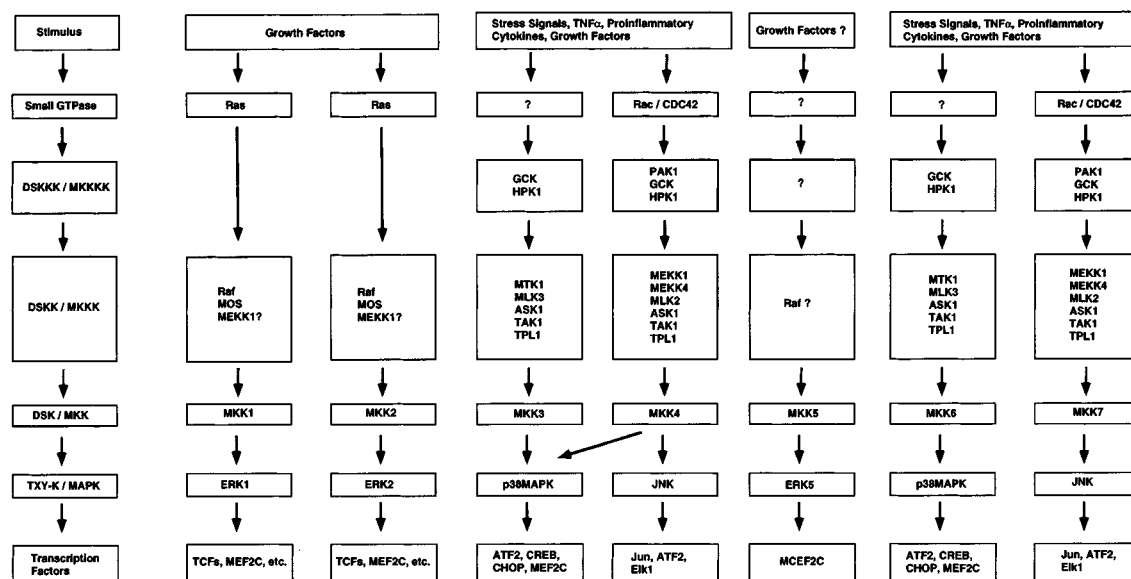
Regulation of cell growth is mediated by a complex array of signaling pathways precisely coordinated by different families of cell surface receptors. These signaling pathways regulate all the critical phases of cell growth including cell proliferation, differentiation, and apoptosis. In many instances, the signal coupling between the receptors to the nucleus is mediated by a series of sequentially activating protein kinases. Protein kinases add a phosphate group to specific amino acid residues. The alcoholic group of serine and threonine as well as the phenolic group of tyrosine provide the major phosphorylation-sites for many of these kinases. Depending on the specificity of the substrate amino acids, the protein kinases are either known as serine/threonine (Ser/Thr) kinases or tyrosine kinases (Tyr). However, a third group of protein kinases that can phosphorylate both Ser/Thr- as well as Tyr-residues has been identified and the kinases that belong to this group are known as dual specificity kinases. Such dual-specificity kinases play a central role in the 'kinase cascade' that regulate cell proliferation, differentiation, and apoptosis. The focus of the review is to summarize the present state of knowledge of the signaling modules regulated by these dual specific kinases.

### *Superfamily of dual-specificity kinases*

The dual specificity kinases (DSK) are unique in that they share the consensus kinases motifs of both Ser/Thr and Tyr-kinases (Lindberg *et al.*, 1992). Their ability to autophosphorylate Ser/Thr- as well as Tyr-

residues in their amino acid sequences rather than those of the exogenous substrates initially identified most of the dual specificity kinases. An earlier classification of these kinases was based on their ability to dual-phosphorylate the exogenous substrates (Lindberg *et al.*, 1992) and it divided these kinases into three groups: (1) kinases that show 'true' dual specificity by phosphorylating Tyr- and Thr-residues of exogenous substrates; (2) kinases that exhibit dual specificity only through autophosphorylation; and (3) kinases that possess the structural motif characteristic of dual specificity kinases. However, it has been observed that as the kinases belonging to group two and three get fully characterized, they turn out to be the 'true dual specificity kinases' of group one. Of the different dual specificity kinases that have been identified to date, the kinases that phosphorylate the family of proline directed protein kinases are of great interest. In a prototypical kinase-signaling module, the kinase-core consists of a minimum of three kinases, an upstream Ser/Thr kinase, middle dual specificity kinases and a downstream Ser/Thr kinase. An activated receptor stimulates a Ser/Thr kinase *via* Ras or Rho family of GTPases. The upstream kinases, in turn stimulate the dual specificity kinase through phosphorylation. Consequently, the phosphorylated DSK activates the downstream Ser/Thr kinase, which contain the TXY motif by dual phosphorylation at the Thr- and Tyr-residues (Figure 1). Such *dual specificity kinase kinase-dual specificity kinase-TXY* kinase (DSKK-DSK-TXY) signaling module appears to be well-conserved in eukaryotes ranging from yeast to mammalian cells (Davis, 1994; Johnson and Vaillancourt, 1994; Cano and Mahadevan, 1995; Fanger *et al.*, 1997). To date, seven such signaling modules have been identified in mammalian cells (Figure 1). Based on the number of newly discovered kinases that can 'communicate' to these kinase-modules, it is likely that many more such 'modules' are remaining to be identified in mammalian cells.

Since a considerable degree of confusion persists regarding the nomenclature of these dual specificity kinases, a clarification of the nomenclature is in order. The dual specificity kinase that phosphorylates ERK is known as either MEK (*Mitogen/Extracellular-signal regulated kinase kinase*) or simply *MAP kinase kinase* (MKK). Thus MEK1/MKK1 and MEK2/MKK2 denotes the dual specificity kinase that phosphorylate ERK1 and ERK2 respectively (Ahn *et al.*, 1991; Crews *et al.*, 1992; Zheng and Guan, 1993). In some instances, as in the case of a *Xenopus* homolog, MAP kinase kinase (MAPKK) also refers to the kinase that activates ERKs (Matsuda *et al.*, 1992). MKK3 has been used to denote the dual specificity kinase that phosphorylate and activate p38MAPK (Derijard *et al.*,



**Figure 1** The mammalian kinase signaling modules. The seven known mammalian signaling modules involving the dual specificity kinases are compared. The question marks denote the signaling components that remain to be established

1995). MKK4 refers to the dual specificity kinase that phosphorylates JNK (Derijard *et al.*, 1995), and it is also known as JNK kinase I or abbreviated as JNKK1 (Lin *et al.*, 1995). Since JNK is also known as stress activated protein kinase (SAPK), a mouse homolog of MKK4/JNKK is known as SAPK/ERK kinase-1 or SEK1 (Sanchez *et al.*, 1994). The dual specificity kinase that phosphorylate ERK5 is known as MEK5 or MKK5 (Zhou *et al.*, 1995; Tournier *et al.*, 1997). The recently identified dual specificity kinase that specifically involved in the regulation of p38MAPK is known as MKK6 (Raugeaud *et al.*, 1996; Moriguchi *et al.*, 1996; Han *et al.*, 1996; Stein *et al.*, 1996). Likewise, newly characterized dual specificity kinase that specifically activates JNK through phosphorylation is named MKK7 (Moriguchi *et al.*, 1997; Yao *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997). It has been also referred as JNKK2 (Wu *et al.*, 1997). For the sake of clarity and conformity, only the MKK-nomenclature is being used here.

It has been identified that both the Thr- as well as Tyr- phosphorylating activities of the dual specificity kinases are required for the signaling of the respective signaling modules. The structure and function relationship of these kinases has been widely studied using MKK1 and MKK2 as prototypic kinases (Wu *et al.*, 1993; Brott *et al.*, 1993; Pages *et al.*, 1994). Selective mutations inhibiting either of the kinase activities have been shown to disrupt the signaling function of the respective signaling module (Brott *et al.*, 1993; Pages *et al.*, 1994; Huang *et al.*, 1995). Of the known kinase signaling modules, only those involved in the activation of the extracellular-signal regulated kinases 1 and 2 (ERK1 and ERK2) are more thoroughly characterized. However, the kinase modules leading to the activation of c-Jun N-terminal kinase (JNK) and p38-mitogen activated protein kinase (p38MAPK) are beginning to be understood.

#### *MAP kinase kinase-1 and MAP kinase kinase-2*

In a typical signaling pathway involving tyrosine kinase receptors, the ligand binding to the receptor leads to receptor-dimerization and subsequent autophosphorylation. The phosphorylated tyrosine residues of the receptor act as a docking bay for various signaling proteins that get activated by phosphorylation. One such effector molecule that link the activated EGFR to Ras-signaling pathway is Shc. Phosphorylation of Shc leads to its interaction with GRB2 through the SH2 domains. The EGFR-Shc-GRB2 complex recruits a guanine nucleotide exchange factor SOS which catalyzes the exchange of guanine nucleotides in Ras. The GTP-bound Ras facilitates the translocation of the Ser/Thr kinase Raf-1 to the plasma membrane for activation through phosphorylation. The activated Raf-1 stimulates the dual specificity kinase MKK1 and MKK2 through the phosphorylation of Ser-217/218 and Ser-221. MKK1 and MKK2 in turn activate their respective ERKs through phosphorylation of specific Thr- and Tyr-residues within the characteristic TPY motif. The phosphorylated ERKs activate other signaling proteins such as cPLA<sub>2</sub> (Nemenoff *et al.*, 1993; Lin *et al.*, 1993) as well as other kinases (Boulton *et al.*, 1991; Sturgill *et al.*, 1988; Stokoe *et al.*, 1992). In addition, the phosphorylated ERKs translocate to nucleus (Chen *et al.*, 1992; Seth *et al.*, 1992; Sanghera *et al.*, 1992; Lenormand *et al.*, 1993) where they activate transcription factors such as TCFs through phosphorylation (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991; Baker *et al.*, 1992; Gille *et al.*, 1992) which leads to the activation of specific genes involved in cell proliferation (Davis, 1992; Johnson and Vaillancourt, 1994).

MKK1 and MKK2 are of 43–46 kDa proteins encoded by two distinct genes located at different chromosomes (Brott *et al.*, 1993). An analysis of the primary sequence of MKK indicate that the N-terminus (first 30 amino acids) show a low sequence

homology in comparison with other DSKs and this region has been speculated to be involved in the differential interaction of specific MKKs with its substrates or activators. One interesting feature of MKK1 is the proline clusters found in the N- and C-terminal regions. Although the functional significance of this proline-rich region is not known, it has been speculated that these domains may direct these kinases to the Ser/Thr residues of the downstream kinases. It is also likely that these proline-rich domains may act as the recognition-motifs for the upstream kinases that activate MKKs through phosphorylation. The most significant feature that distinguishes MKK1/2 from other dual specificity kinases is the high level of stringency exhibited by MKKs in phosphorylating their substrates. MKK1 and MKK2 phosphorylate Thr-183 and Tyr-185 of ERK1 and ERK2 respectively. Phosphorylation of both of these residues is essential for the activation of ERK1 and ERK2. The activated ERKs initiate the nuclear events that regulate cell proliferation. Consistent with this view, it has been observed that the sustained activation of MKK1-ERK1 was essential for the PDGF-stimulated G1 to S phase transition and cell proliferation in various cell types (Cowley *et al.*, 1994, Weber *et al.*, 1997). In these cells expression of dominant negative MKK1 (Cowley *et al.*, 1994) or the treatment of cells with a specific MKK1/2-inhibitor PD98059 (Weber *et al.*, 1997), have been shown to inhibit ERK activity as well as cell growth.

Ras plays a major role in integrating the signaling between the receptors and the kinase modules since the expression of a dominant negative, competitively inhibitory mutant of Ras, (N17-Ras) inhibits Raf-MKK-ERK signaling in diverse cell types (de Vries-Smits *et al.*, 1992; Robbins *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). Raf-1 is the most predominant upstream kinase that is involved in the activation of MKKs (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). However, it is worth noting that MKK1/2 can also be activated through a Ras-Raf independent pathway. MKK1/2 can be phosphorylated by other dual specificity kinase kinase such as cMOS (Nebreda *et al.*, 1993; Shibuya and Rudersman, 1993; Posada *et al.*, 1993) or MEKK1 (Lange-Carter *et al.*, 1993). Phosphorylation of MKK1 by cMOS has been observed only in *Xenopus* oocytes suggesting cell-type specificity of MOS-MKK1 interaction. (Posada *et al.*, 1993., Matsuda *et al.*, 1992; 1993; 1994). However, MOS can phosphorylate and activate MKK1/2 from other tissue *in vitro* (Posada *et al.*, 1993). Although MEKK1 can phosphorylate MKK2 at Ser-217 and Ser-221, the phosphorylation does not lead to the activation of MKK2 as indicated by its inability to phosphorylate ERKs (Cobb and Goldsmith, 1995). This study underscores an important point, which proposes that additional tissue-specific factors may be required for the efficient activation of MKK2. This has led to the speculation that there may be a scaffolding protein or a nucleation factor that physically integrates distinct DSKs to specific DSKs. It is significant to note here that there is precedence for the presence of such scaffolding protein in budding yeast. In *S. cerevisiae*, the protein encoded by STE5 appears to act as a scaffolding protein for the kinase module consisting of STE20, STE11, and STE7 kinases. In

addition, the findings that MAPKK and MAPK exist as a high molecular weight complex in *Xenopus* oocyte (Matsuda *et al.*, 1993) lend credence to the view that a scaffolding protein may be present to physically integrate the MKK1/2-ERK1/2 modules of the higher eukaryotes. Interestingly, 14-3-3 family of proteins that interact with Rafs and MEKKs appear to be a prospective candidate for this role in mammalian cells (Fanger *et al.*, 1998). However, this does not rule out the presence of other proteins that may be involved in this process. Using a yeast two-hybrid screen, it has been observed that the carboxyl terminus of KSR (Kinase Suppressor of Ras) interacts with MKK1 whereas the amino terminus interacts with ERK1/2. Based on these physical interactions of KSR with both MKK1 and ERK1, it has been proposed that KSR may act as a scaffolding protein that links MKK1 to its substrate (Yu *et al.*, 1998).

MKK1 and MKK2 are ubiquitously expressed. They are critically involved in the regulation of signaling pathways involved in cell proliferation and differentiation. Expression of the constitutively active MEK has been shown to stimulate neuronal differentiation of PC-12 even in the absence of NGF in the growth medium (Cowley *et al.*, 1994). Similar expression in NIH3T3 cells results in the activation of cell proliferation and subsequent cell transformation (Cowley *et al.*, 1994). The expression of the inhibitory mutants of MKK1 inhibited serum-induced DNA synthesis in NIH3T3 cells. Interestingly, Src- as well as Ras-mediated transformations were also blocked by the dominant negative mutants MKK1 identifying the principal role of MKK1 in these pathways (Cowley *et al.*, 1994). Such critical role for MKK1 has been also demonstrated in the meiotic maturation of oocytes (Posada *et al.*, 1993; Matsuda *et al.*, 1994). Microinjection of an antibody raised against MKK1 completely inhibited oocyte maturation (Matsuda *et al.*, 1994). Furthermore, the microinjection of purified constitutively active MEK1 induced meiotic maturation as indicated by the germinal vesicle breakdown (GVBD). It has been observed that the activated MKK1 stimulates GVBD as well as the activity of MPF presumably through MKK1. It is worth noting that in these oocytes only a homolog of MKK1 is present and MOS is upstream of the MKK1 (Posada *et al.*, 1993; Matsuda *et al.*, 1992, 1993, 1994). In addition, MKK1 of the *Xenopus* oocytes has been shown to be phosphorylated by ERK1 *in vitro* (Matsuda *et al.*, 1993). MKK1 contain the ERK-phosphorylation motif PX(S/T)P and ERKs phosphorylate MKK1/2 at Thr-388 within this motif. It has been speculated that ERK-phosphorylation of this consensus Thr-residue may exert a negative feedback regulation on the activity of MKK1 (Matsuda *et al.*, 1993, 1994). In the budding yeast, it appears that such phosphorylation of STE7 (MKK-homolog) by FUS3 (ERK homolog) attenuate the activity of STE7 (Errede *et al.*, 1993). However, such negative regulation of mammalian MKK1/2 by ERK1/2 is yet to be established.

Although MKK1 and MKK2 appear to be activated by same or similar upstream kinases, they appear to be differentially regulated. A significant difference between MKK1 and MKK2 can be observed in their kinetics of activation by serum growth factors. While MKK1 shows a sustained activation upon serum stimulation of

fibroblasts such as Rat1 cells, MKK2 shows a transient activation. Although these differences can be traced to an unique phosphorylation site present in MKK1, the physiological significance of the closely related but differentially regulated MKK1 and MKK2 pathways in cell proliferation and differentiation is largely unknown at present (Catling *et al.*, 1995).

#### MAP kinase Kinase-3

MAP kinase Kinase-3 (MKK3) has been identified as the dual specificity kinase that is involved in the phosphorylation of p38MAPK (Derijard *et al.*, 1995). The p38 MAPK was initially identified as a kinase that was rapidly phosphorylated upon exposure of cells of monocytic lineage to the endotoxin, lipopolysaccharides (Han *et al.*, 1993). Subsequent characterization of this 38 kDa kinase revealed that is closely related to the osmotic stress activated-HOG 1 kinase of yeast (Han *et al.*, 1994; Rouse *et al.*, 1994). In yeast HOG-1 was phosphorylated by an upstream kinases PBS2. A search for the mammalian homologue of PBS2 yielded MKK3 that can activate p38MAPK and MKK4 that can activate both p38MAPK and JNK (Derijard *et al.*, 1995). MKK3 activates p38MAPK through the phosphorylation of Thr-180 and Tyr-residue-182 in the T-X-Y sequence motif (TGY). The ability of pyridinyl imidazole derivatives to inhibit p38MAPK has been widely used to identify the physiological role of this kinase (Lee *et al.*, 1994). Diverse stimuli including inflammatory cytokines, osmotic stress, UV-irradiation and heat-shock have been shown to activate MKK3 (Derijard *et al.*, 1995). Activation of MEKK3-p38MAPK signaling pathway leads to the activation of different kinases such as MAPKAP-2 (Stokoe *et al.*, 1992; Rouse *et al.*, 1994; Freshney *et al.*, 1994), MAPKAP-3 (Ludwig *et al.*, 1996), and MNK1/2 kinases (Waskiewicz *et al.*, 1997; Fukunaga and Hunter, 1997). Similar to ERKs the phosphorylated p38MAPK translocates to the nucleus and phosphorylates the transcription factors such as ATF2 (Raingeaud *et al.*, 1995), CREB (Tan *et al.*, 1996), CHOP (Wang and Ron, 1996), and MEF2C (Han *et al.*, 1997a). Unlike the signaling pathways regulated by MKK1/2-ERK1/2, the pathways regulated by MKK3-p38MAPK appear to be involved in the regulation and/or progression of apoptosis. It has been shown that the apoptosis induced by serum or growth factor deprivation principally involves the activation of p38MAPK rather than JNK (Kummer *et al.*, 1997). In some instances, MKK3-p38MAPK signaling pathways actively oppose ERK-signaling pathway and induce apoptosis (Xia *et al.*, 1995). One of the mechanisms through which MKK3/p38MAPK can arrest cell growth and induce apoptosis is through its effect on cyclin D1 expression during cell cycle. It has been demonstrated that the coexpression of MKK3 along with p38MAPK inhibits mitogen-induced cyclin D1 expression (Lavoie *et al.*, 1996).

MKK3 is activated through phosphorylation on Ser-189 and Thr-193 residues by a MKKK. MEKK1 does not activate MKK3 (Han *et al.*, 1997). Similarly, The small GTPases such as Rac and CDC 42 fail to activate MKK3 (Han *et al.*, 1996). However, the recently identified MKKKs such as apoptosis signal regulating kinase or ASK1 (Ichijo *et al.*, 1997),

MEKK4/MTK1 (Takekawa *et al.*, 1997), TGF $\beta$ -activated kinase-1 or TAK1 (Shirakabe *et al.*, 1997) and mixed lineage kinase-3 or MLK3 (Rana *et al.*, 1996; Tibbles *et al.*, 1996) appear to activate MKK3. The identity of the upstream small GTPases and kinase(s) involved in the activation of MKK3 is yet to be defined. The observation that the expression of the constitutively activated Rac or CDC42 fails to activate MKK3, these findings suggest that Rac and CDC42 are not involved in the activation of MKK3 (Han *et al.*, 1996). To date, four distinct forms of p38MAPK-p38MAPK $\alpha$ , p38MAPK $\beta$ , p38MAPK $\gamma$  and p38MAPK $\delta$ -have been cloned. Of these isoforms, MKK3 appears to be activate p38MAPK $\alpha$ , p38MAPK $\gamma$  and p38MAPK $\delta$  (Enslin *et al.*, 1998; Wang *et al.*, 1997). Two alternatively spliced forms of MKK3 known as MKK3 and MKK3b have been identified (Han *et al.*, 1997). Although the MKK3b isoform is shown to be more potent in activating p38MAPK, the physiological significance of this finding remains to be defined.

#### MAP kinase kinase-4

Initial search for the kinase that phosphorylates the N-terminal Ser-63 and Ser-73 residues of c-Jun identified Jun N-terminal kinase-1 or JNK1 (Derijard *et al.*, 1994). Subsequent characterization of this kinase indicated that the activation of this kinase required a dual phosphorylation at Thr-183 and Tyr-185 of the TPY motif (Derijard *et al.*, 1995) by a dual specificity kinase. Later the DSK involved in the activation of JNK1 has been identified to be MKK4 (Derijard *et al.*, 1995; Lin *et al.*, 1995). Similar to other proline directed protein kinases, upon phosphorylation by MKK4, the activated JNK translocates to the nucleus to phosphorylate c-Jun and other transcription factors such as ATF2 (Gupta *et al.*, 1995) and Elk1 (Whitmarsh *et al.*, 1995). To date, ten different alternatively spliced isoforms of JNK derived from three distinct genes, JNK1, JNK2, and JNK3, have been identified (Gupta *et al.*, 1996). Since all of them are activated by the same or similar stimuli, it is likely that MKK4 can phosphorylate all the different isoforms of JNK. In addition, MKK4 can also phosphorylate and activate p38MAPK (Derijard *et al.*, 1995; Lin *et al.*, 1995). Perhaps this is more significant in the physiological contexts in which the coordinated and synergistic activation of JNK and p38MAP are required. Diverse growth factors, environmental stimuli, and cytotoxic as well as genotoxic stress signals activate JNK signaling pathways. Signal coupling between these stimuli and the MKK4-JNK signaling module is provided by an upstream small molecular weight GTPase(s) and Ser/Thr kinases (Minden *et al.*, 1994). Rac and CDC42, two members of the Rho family GTPases, couple different receptors and diverse stimuli to MKK4-JNK signaling module (Coso *et al.*, 1995; Minden *et al.*, 1995). Rac and CDC42 have been known to regulate the organization of actin cytoskeleton (Hall, 1994). While Rac regulates lamellipodia and membrane ruffling through actin fibers CDC42 regulates the formation of filopodia in cells. However, the activation of MKK3-p38MAPK by Rac/CDC42 is independent of their effect on cytoskeleton. The initial event leading to the activation of MKK4-JNK involves a

guanine nucleotide exchange in Rac or CDC42. The GTP-bound Rac or CDC42 bind to and stimulate the Ser/Thr kinase of 65–68 kDa. These kinases known as p21-activated kinases (PAKs), a mammalian homologue of yeast STE20 kinase involved in the mating pheromone response pathway, presumably stimulate the downstream MEKK. The phosphorylated and stimulated MEKK activate the dual specificity kinase MKK4 through Ser/Thr phosphorylation (Figure 1). Since MEKK1 can activate both MKK3 and MKK4, it has been observed that interfering the signaling pathway upstream of MEKK1 or MEKK1 itself inhibits the activation of both MKK3-p38MAPK and MKK4-JNK pathways (Yan *et al.*, 1994; Lin *et al.*, 1995; Derijard *et al.*, 1995; Zhang *et al.*, 1995).

Although it has been observed that functional Rac or CDC42 is required for the activation of JNKK-JNK signaling module, it has been observed that Ras is also involved in the activation JNK signaling pathway (Smeal, 1991, Minden *et al.*, 1994; Kyriakis *et al.*, 1994). Recent studies indicate that in the specific instances in which Ras is involved in the activation of JNKK/JNK module, it appears that Rac or CDC42 is downstream of Ras mediating the 'true' coupling (Coso *et al.*, 1995; Minden *et al.*, 1995). In addition to Ras, Rac, and CDC 42 there has been reports that Rho also mediates the activation of JNK through a PAK-independent pathway (Teramoto *et al.*, 1996; Atfi *et al.*, 1997). Furthermore, a role for ceramide in mediating the apoptotic signals from TNF $\alpha$  to JNK signaling pathway *via* MKK4 has been shown (Verheij *et al.*, 1996). A specific PAK involved in the activation of MEKK1 and subsequently MKK4 and JNK remains to be identified. Considering the general similarity between MKK3-p38MAPK and MKK4-JNK modules, PAK1 itself or a closely related dual specificity kinase kinase kinase is involved in the activation of MEKK1 and subsequently MKK3 as well as MKK4. Additional MKKKs that can activate MKK4 by phosphorylations have been identified. They include MEKK4, ASK1, tumor progression locus-1 (Tp11), MLK2, and TGF $\beta$ -activated kinase-1 (Fanger *et al.*, 1997). The observation that the dominant negative MEKK4 inhibits Rac/CDC42-stimulated JNK suggests that MEKK4 is likely to be involved in the activation of MKK4 (Gerwins *et al.*, 1997). Similarly, the recently cloned MEKK5, which is abundantly expressed in human heart and pancreas, also activates MKK4. (Wang *et al.*, 1996). MKK4 can also be activated by other MKKKs such as MLK2 and TGF $\beta$ -activated kinase-1 (TAK1). MLK2 has been shown to preferentially activate MKK4 (Hirai *et al.*, 1997). TAK1 activates MKK4 independent of MEKK1 or MLK3 suggesting that TAK-1 is an upstream kinase that can activate MKK4 (Wang *et al.*, 1997). It is interesting to note that similar to MEKK1, TAK1 activates both MKK4 as well as MKK3. It is equally interesting to note that TAK1 has been observed to mediate ceramide-activated MKK4-JNK activity (Shirakabe *et al.*, 1997). The observation that Rho mediates TGF $\beta$ -stimulated activation of JNK is of significance here (Afti *et al.*, 1997). Since TGF $\beta$  activation preferentially involves TAK1, it will be interesting to test whether Rho-activation of MKK4/JNK is specifically through TAK1.

While the role of MEK-ERK pathways in cell proliferation and differentiation pathways have been well characterized, the role of MKK4-JNK in different physiological contexts are largely unknown. It is clearly established that both JNK- and p38MAPK-modules are activated in response to cellular stress. Several studies suggest that JNKK-JNK pathway may be involved in the regulation of cell volume. Interestingly it has been observed that either JNK1 or p38MAPK can rescue the HOG-1 mutant of yeast which is deficient in regulating the cell volume in response to osmotic shock. Several other stimuli including mitogenic ones also activate JNKK/JNK as well as MKK3/p38MAPK pathway. Initial observations indicated that JNK activation is essential for Ras-transformation of REF3T3 cells, thus suggesting a mitogenic role for JNK. However, the recent observation that the expression of MEKK1, the upstream kinase that activates MKK4 strongly inhibits cell growth in NIH3T3 cells contradicts this view. Based on these findings, it has been proposed that the activation of MKK4/JNK module may lead to the inhibition of cell growth and possibly apoptosis. However, it has been observed that the activation of JNK enhances the transforming ability of Ras (Smeal *et al.*, 1991). Moreover, v-Src transformed and the heterotrimeric G-protein G $\alpha$  12/13 subunit-transformed NIH3T3 cells show an increased JNK (Vara Prasad *et al.*, 1995). Taken together, these findings suggest that the ability of MKK4-JNK module to inhibit cell growth depends upon the cell-type and/or other signaling inputs present. Several lines of evidence support this view. The observation that MKK4 (-/-) homozygous animals die before embryonic day-14 suggest the critical role played by MKK4 in embryonic survival (Yang *et al.*, 1997). Similarly, MKK4 has been shown to protect thymocytes from Fas and CD3-mediated apoptosis (Nishima *et al.*, 1997). These apparently contradictory effects of MKK4/JNK suggest that the final commitment of the cells to divide, differentiate, or die may depend upon the other parallel signaling inputs.

#### MAP kinase Kinase-5

A dual specificity kinase kinase closely related to the other MKKs has been recently cloned from human fetal brain library and it is known as MKK5 (Zhou *et al.*, 1995). Using the sequence of MKK5 in a yeast two-hybrid system identified a novel downstream kinase, which is termed as ERK-5 or big MAP kinase (BMK). Although the upstream and downstream signaling proteins that interact with these kinases or the stimulus that activate these kinases have not been identified, the physical interaction between MKK5-ERK5 suggest that they are part of a distinct MKK5-ERKs signaling module. Analysis of the primary structure of MKK5 indicates its unique structural features possibly having a critical role in the regulation of the cytoskeletal organization of the cell. MKK5 contain all of the 11 kinase domains, a hallmark of DSKs. Similar to MKK1 (S218XXXS222), MKK5 contain a Raf-1 phosphorylation motif (S311XXXT315), suggesting that the upstream kinase in this pathway may be Raf. However, in contrast to MKK1 and MKK2 the proline rich region between

domains IX and X is absent. In this regard, MKK5 resembles more like MKK3 and MEK4. Despite the overall structural similarity with MKK1, MKK2, MKK3, and MKK4, it distinctly differs from them in the unique domain present in its N-terminal 150 amino acid residues. Sequence analysis of the N-terminal sequence of MKK5 with other known proteins identified a close similarity between this domain with yeast cell division protein 24 (CDC24) of *S. cerevisiae* and its *S. pombe* homolog (SCD1). Previous studies with CDC24 and SCD1 proteins have indicated that these proteins act as guanine nucleotide exchange factor for CDC42, a Rho related small molecular weight GTPase that is involved in the cytoskeletal organization of the cell. Sequence alignment of N-terminus of MKK5 with CDC24 and SCD1 indicate that the MKK5-N-terminus shows significant identity to C-terminal, putative CDC42-interacting region of CDC24 and SCD1. On the basis of this observation it has been speculated that MKK5 may be interacting with mammalian CDC42/Rac and this N-terminal domain may provide the physical basis for the coupling. It is noteworthy that Rac and CDC42 have been shown to activate MKK3- as well as MKK4-modules through PAK1 and MEKK. Therefore it will be of interest to know whether MKK5 bypasses analogous DSKK and DSKK in interacting with Rac/CDC42, thus providing a novel pathway involved in cytoskeletal organization regulated by CDC42/Rac.

Two distinct alternatively spliced isoforms of MKK5 have been identified (English *et al.*, 1995). The larger 50-kDa isoform is known as MKK5 $\alpha$  whereas the 40-kDa shorter isoform is known as MKK5 $\beta$ . The MKK5 $\beta$ -isoforms is ubiquitously expressed and cytosolic. MKK5 $\alpha$  shows more restricted expression. It is interesting to note that the 23-amino acids encoded by 5' exon in the larger  $\alpha$ -isoform is similar to the sequence found in proteins which associate with the actin cytoskeleton. This may be indicative of the isoform-specific subcellular localization of MKK5 (English *et al.*, 1995). Interestingly, ERK5 also shows a distinct structural motif consisting of Pro-Ala repeats. The (PA) $n$  repeats have been identified to be involved in directing proteins to cytoskeletal actin filaments. Based on this observation it has been proposed that the (PA) $n$  repeats may be targeting ERK5 to cytoskeleton where MKK5 $\alpha$  is localized. On the basis of these observations, it is likely that MKK5-ERK5 signaling module is closely associated with the regulation of cytoskeletal organization and cell morphology.

It has been recently observed that the osmotic- and oxidative-stress can activate ERK5 (Abe *et al.*, 1996). Analysis of the signaling mechanism indicates that MKK5-ERK5 activation results in the phosphorylation of MEF2C, the transcription factors belonging to the myocyte enhancer factor-2 (MEF2)-family (Kato *et al.*, 1997). Further studies have indicated that MEF2C is a protein substrate for ERK5 and MEF2c activation is involved in the expression of several immediate early genes including c-Jun (Kato *et al.*, 1997). Although, the functional significance of this pathway in relation to other MKK-signaling pathway remains unclear, it is significant to note here that p38MAPK and ERK2 can also phosphorylate and activate MEF2C. In this context, it is interesting to note that MKK5 and

ERK5 are highly expressed in cardiac skeletal muscle (Zhou *et al.*, 1995; English *et al.*, 1995) while MEF2 family of transcription factors are mainly expressed in skeletal muscle and brain tissue (Martinet *et al.*, 1994; McDermott *et al.*, 1994). Taken together, these observation may be indicative of the critical role played by MKK5-ERK5 signaling pathway in cardiac physiology (Zhou *et al.*, 1995).

#### MAP kinase Kinase-6

MKK6 is a dual specificity kinases showing close similarity to MKK3. The 334 amino acid protein is found to be 80% identical to MKK3 and strongly activated by UV, anisomycin, osmotic shock (Stein *et al.*, 1996; Moriguchi *et al.*, 1996). Unlike MKK3, MKK6 is a common activator of all the different isoforms of p38MAPK that include p38MAPK $\alpha$ , p38MAPK $\beta$ , p38MAPK $\gamma$ , and p39MAPK $\delta$  (Enslin *et al.*, 1998; Wang *et al.*, 1997). Expression of MKK6 has been shown to activate p38MAPK (Raingeaud *et al.*, 1996). Similar to MKK3, Rac and CDC42 fail to activate MKK6. Likewise, the identity of the PAK-like kinases that activate MKK3 and MKK6 is not known. However, it has been observed that germinal center kinase (GCK; Pombo *et al.*, 1995) and hematopoietic protein kinase-1 (HPK1; Keifer *et al.*, 1996) have been shown to regulate the activity of the MLK3, a MKKK that can activate MKK3/4/6/7 kinases. Therefore it is likely that an upstream kinases hitherto uncharacterized is involved in the activation of the MKKK upstream of MKK3/6 (Tibbles *et al.*, 1996). The signaling pathways regulated by MKK3 and MKK6 often overlap with each other. This is not surprising in the light of the observation that the similar upstream signaling molecules activate them and both of them in turn, activate p38MAPKs. Accordingly, it has been observed that MLK3, and upstream DSKK, activates p38MAPK through phosphorylation *in vitro* (Figure 1). Furthermore, it has been seen that the immunoprecipitation of MLK3 results in the coimmunoprecipitation of both MKK3 and MKK6. This finding suggests that MLK3 activates both MKK3 as well as MKK6. Similarly, expression of either MKK3 or MKK6 stimulates p38 MAPK activity and the resultant increase in the reporter gene expression mediated by ATF2 and Elk1 (Raingeaud *et al.*, 1996).

MKK6 exists in a variety of alternatively spliced isoforms with tissue-specific patterns of expression (Han *et al.*, 1996). At least in some cell types, a specific isoform of MKK6, MKK6b has been shown to be involved in transmitting apoptotic signal. It has been known that MKK6-p38MAPK signaling is involved in Fas-mediated apoptosis (Toyoshima *et al.*, 1997). In T-cells, Fas-activation of apoptosis involves the stimulation of a distinct isoform of MKK6b. More interestingly, the activation of MKK6b in these cells leads to little or no activation of p38 MAPK (Huang *et al.*, 1997). It has been speculated that there may be a divergence of apoptotic signaling at the level of MKK6b independent of p38MAPK. In addition, MKK6 seems to play a critical role in cardiac physiology. Cardiac hypertrophic growth can be characterized by three distinct features such as (1) increases in cell size; (2) sarcomeric organization; and (3) induction of cardiac-specific genes (for natriuretic

peptides A and B; alpha-skeletal actin). Coexpression of activated MKK6 along with p38MAPK in cultured myocardial cells resulted in inducing all the three characteristic features of cardiac hypertrophic growth suggesting a central role for MKK6-p38MAPK in myocardial cell hypertrophy (Zechner *et al.*, 1997).

#### MAP kinase Kinase-7

MKK7 is identified as the mammalian homolog of the Hep gene product of *Drosophila* (Moriguchi *et al.*, 1997; Yao *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997). In *Drosophila*, Rac/CDC42 regulates the activity of Hep and subsequently the *Drosophila* JNK. Rac/CDC42-Hep-JNK pathways regulate the dorsal closure of *Drosophila* embryo (Holland *et al.*, 1997). Interestingly, MKK7 can functionally rescue *hep* mutant flies (Holland *et al.*, 1997). Analyses of mammalian MKK7 has indicated that its highly specific for JNK and does not phosphorylate p38MAPK (Wu *et al.*, 1997). MKK7 is expressed in all adult and embryonic tissue. Increased expression has been seen in epithelial tissues at later stages of development (Yao *et al.*, 1997, the significance of which is not known at present. It will be interesting to see whether MKK7 is a true mammalian homolog of *hep* in mediating embryonic developmental program.

MKK7 is activated by proinflammatory cytokines and osmotic stress (Finch *et al.*, 1997). In addition, it has been shown that Fas-induced apoptosis in T-cells specifically involve the activation of MKK7-JNK and MKK6-p38MAPK (Toyoshima *et al.*, 1997). A dominant negative mutant of MKK7 inhibits the MEKK-stimulated JNK activity suggesting that MKK7 is downstream of MEKK1. Since Rac/CDC42 and PAK mediate the signal coupling between various stimuli and MEKK1, it is likely that Rac/CDC42-PAK1-MEKK signaling similar to that of MKK4 regulates MKK7. The analogous *hep*-pathway in *Drosophila* also support a role for Rac/CDC in the activation of MKK7 (Holland *et al.*, 1997). Since the dominant negative MEKK4 inhibits Rac/CDC42-stimulated JNK (Gerwins *et al.*, 1997), it is likely that MEKK4 is also involved in the activation of MKK7. It is possible that many of the upstream kinase that activate MKK4 may also activate MKK7. However, their affinity to MKK7 versus MKK4 and physiological contexts in which they activate either of them may vary.

Although both MKK4 and MKK7 activate JNK, they appear to be differentially regulated. MKK7 is preferentially activated by TNF $\alpha$  whereas MKK4 is not. MKK7 is highly specific to JNK whereas MKK4 can activate both JNK and p38MAPK (Moriguchi *et al.*, 1997; Yao *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997). Similarly, proinflammatory cytokines specifically activate MKK7 whereas other diverse stress stimuli activate both MKK7 and MKK4 (Lawler *et al.*, 1997). These findings suggest an interesting possibility that different stimuli recruit MKK4 and MKK7 based on their need to co-stimulate p38MAPK or not.

#### Conclusion

Cell growth is regulated by the integration of different signaling pathways resulting in the generation of a net

signaling input. Thus, the observed cross talk, permissiveness, and synergism seen among these pathways at the molecular level may be of greater physiological significance at the cellular level. The recent past has shown the role of dual specificity kinases and their signaling modules in diverse signaling pathways. An emerging view is that these kinases regulate diverse responses by integrating the signals from each other. Evidence for such signal integration is best exemplified in the case of MKK4 through which the coordinated activation of p38MAPK and JNK can be mediated during apoptotic and proinflammatory responses. The co-operation between MKK6/p38MAPK and MKK7/JNK in Fas-induced apoptosis may be a representative example of such a global signaling network. There are other instances in which the cells prefer to differentially regulate specific signaling pathways as in the case p38MAPK-specific MKK3/6 and JNK-specific MKK7. It is likely that the scaffolding proteins such as 14-3-3 or the yet to be identified mammalian counterpart of STE5 may insulate the respective signaling pathways from the 'cross-talk' of the adjacent signaling pathways. It is equally important that various critical signaling pathways are coordinated so as to generate a signaling network. Especially, the critical signaling programs involved cell proliferation, differentiation, and apoptosis require such global networking between different signaling pathways. In fact, the generation of such multiple signal-inputs through an 'intentional' cross-talk between different signaling modules, may be a preferred mechanism by which cells insure that the required complement of signals is present prior to committing themselves to critical responses.

The discussions presented here have mainly focussed on the known mammalian kinase signaling modules. The cloning of different MEKKs, MLKs, MTKs, and TAKs suggest that there may be other signaling modules and constituent dual specificity kinases remaining to be defined. Even among the known signaling modules some of the constituent kinases are yet to be identified. In addition, the dual specificity phosphatases are bound to have a major role in regulating these pathways. The identification and characterization of these kinases and their unique substrates should further resolve the signaling network mediated by these kinases.

#### Abbreviations

ASK1, apoptosis signal regulating kinase; DSK, dual specificity kinase; DSKK, dual specificity kinase kinase; ERK, Extracellular-signal regulated kinase; GCK, germinal center kinase; HPK1, hematopoietic protein kinase-1; IL, interleukin; JNK, Jun N-terminal kinase; KSR, kinase suppressor of Ras; MAP kinase, mitogen activated protein kinase; MAPK, mitogen activated protein kinase; MEKK, MAPK/ERK kinase kinase; MTK1, MAP three kinase; MKK, MAP kinase kinase; MKKK, MAP kinase kinase kinase; MLK, mixed lineage kinase; PAK, p21-activated kinase; TAK 1, TGF $\beta$ -activated kinase 1; TGF, tumor growth factor; TNF, tumor necrosis factor; TPL-1, tumor progression locus-1, TXY-K, TXY-motif kinase.

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