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20

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Stress-Activated Protein Kinases

With 41 Figures, 15 in Color; and 12 Tables

Springer

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The cover illustration depicts pseudohyphal filaments of the ascomycete *Saccharomyces cerevisiae* that enable this organism to forage for nutrients. Pseudohyphal filaments were induced here in a wild-type haploid MATa Σ1278b strain by an unknown readily diffusible factor provided by growth in confrontation with an isogenic petite yeast strain in a sealed petri dish for two weeks and photographed at 100X magnification (provided by Xuewen Pan and Joseph Heitman).

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MAPK kinase kinase regulation of SAPK/JNK pathways

Lisa Stalheim and Gary L. Johnson

Abstract

SAPK/JNK members of the MAPK family are regulated by at least fourteen known MAPK kinase kinases (MKKKs). In addition to the kinase domain, each MKKK encodes different protein interaction domains and motifs to control their interaction with upstream GTPases such as Rho, Rac and Cdc42, downstream MAPK kinases, and scaffold proteins that assemble the MKKKs into signaling complexes for the control of physiological responses to a plethora of different stimuli. Several MKKKs coordinately regulate the SAPK/JNK pathway with other MAPKs including p38, ERK1/2 and ERK5. It is the diversity of MKKKs within the MAPK signaling network that provides the signaling specificity for activation of MAPKs including SAPK/JNKs and the integration with other signaling pathways within cells.

1 Introduction

SAPKs are MAPKs shown to be activated by many different stress stimuli, hence their name stress-activated protein kinases (SAPKs) (Kyriakis et al. 1994). The same kinases were shown to phosphorylate c-Jun at Ser 64 and 73 (Pulverer et al. 1991; Smeal et al. 1992; Derijard et al. 1994), hence the name Jun N-terminal kinases (JNKs). There are three SAPK/JNK genes (JNK1, JNK2, JNK3). Herein, for simplicity they are referred to as JNKs. JNK1 and JNK2 are expressed ubiquitously while JNK3 has a more limited expression primarily in brain, heart, and testis (Pulverer et al. 1991; Derijard et al. 1994; Kyriakis et al. 1994; Yang et al. 1997). Including c-Jun, several members of the AP-1 transcription factors are substrates for JNKs including JunD, ATF2, and ATF3 (Behrens et al. 1999; Shaulian and Karin 2001). Phosphorylation of AP-1 members by JNKs enhances AP-1 transcriptional control of specific gene expression. The importance of AP-1 in the transcriptional control of many different genes involved in homeostasis and the role of JNKs in regulating AP-1 activity led to an intense study of JNK regulation and it is now clear that JNKs have many substrates in addition to AP-1. The targeted gene disruption of JNK1, JNK2, and JNK3 has defined tissue-specific functions for each isoform including the control of metabolism, apoptosis, motility, proliferation, DNA repair, and the regulation of genes involved in homeostasis

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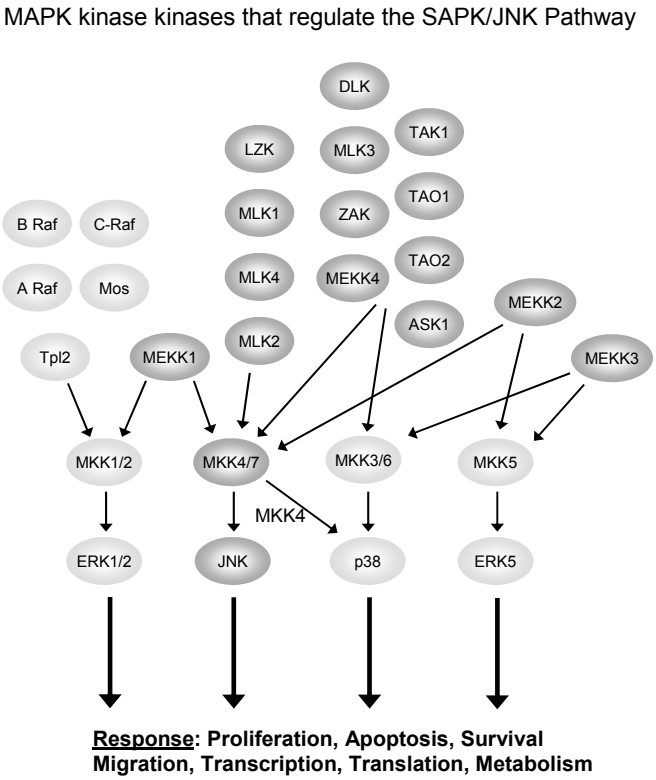

Fig. 1. MKKKs that control the MAPK pathways. There are twenty defined MKKKs known to regulate MAPK pathways. MKKKs phosphorylate and activate specific MKKs. Activated MKKs phosphorylate and activate specific MAPKs. The MKKKs and MKKs that regulate JNKs are highlighted in dark grey.

such as proteases and cytokines (Yang et al. 1997; Kuan et al. 1999; Sabapathy et al. 1999; Chang et al. 2003).

JNKs, like all MAPKs, are part of a three kinase signaling module (Fig. 1). JNKs are phosphorylated and activated by the MAPK kinases, MKK4 and MKK7. MKK4 and MKK7 are phosphorylated and activated by MAPK kinase kinases (MKKKs). Interestingly, whereas there are eleven MAPKs (JNK1/2/3, ERK1/2, p38, α/β/γ/δ, ERK5 and ERK7), there are only seven MKKs and at least twenty MKKKs. It is noteworthy that fourteen of the twenty defined MKKKs activate the MKK4/7→JNK1/2/3 pathway, demonstrating the importance of the JNK signaling pathway in the cellular response to stimuli that frequently involve potentially harmful or lethal consequences for the cell. Such stress stimuli include irradiation, toxins, drugs, osmolarity, temperature, changes in cell shape, adherence, cytoskeletal dynamics, and responses to antigens, growth factors and cytokines. Table 1 shows a partial list of substrates for JNKs that control adaptive responses of the cell to these different stimuli.

Table 1. Phosphorylation substrates for JNKs

Category	Substrate
Transcription factors	c-Jun
	JunD
	ATF2
	ATF3
	Elk-1
	Elk-3
	P53
	NFAT4
	HSF-1
	c-Myc
	Androgen receptor
	RXRα
	RARα
Signaling proteins	IRS-1
	Paxillin
	14-3-3
Microtubule-associated proteins	MAP1
	MAP2A
	Tau
	Doublecortin (DCX)
	Amyloid β precursor protein
Bcl family proteins	Bcl-2
	Bcl-xl
	Mcl-1
	Smac
	Bim
	Bmf
Nuclear core complex	Nup214

2 Organization of the MKKK-MKK4/7-JNK1/2/3 signaling module

Specificity in the organization of JNK signaling modules is controlled in part by recognition motifs for MKKK-MKK4/7 and MKK4/7-JNK1/2/3 interactions. A docking site referred to as DVD (domain for versatile docking) encoded near the C-terminus of MKK4 and 7 interacts with the N-lobe of the kinase domain of the specific MKKK (Takekawa et al. 2005), providing a docking mechanism for selective interaction of MKKKs and MKKs. Docking sites between the JNKs and MKK4 and 7 provide specificity in the interaction of the MKK and MAPK (Jacobs et al. 1999; Sharrocks et al. 2000; Fantz et al. 2001; Ho et al. 2003; Mooney and Whitmarsh 2004; Ho et al. 2006). Similar recognition motifs are present in JNK substrates such as c-Jun and ATF-2 (Sharrocks et al. 2000).

There are also several scaffolding proteins that organize specific JNK signaling modules. These scaffold proteins generally have no catalytic function, but rather

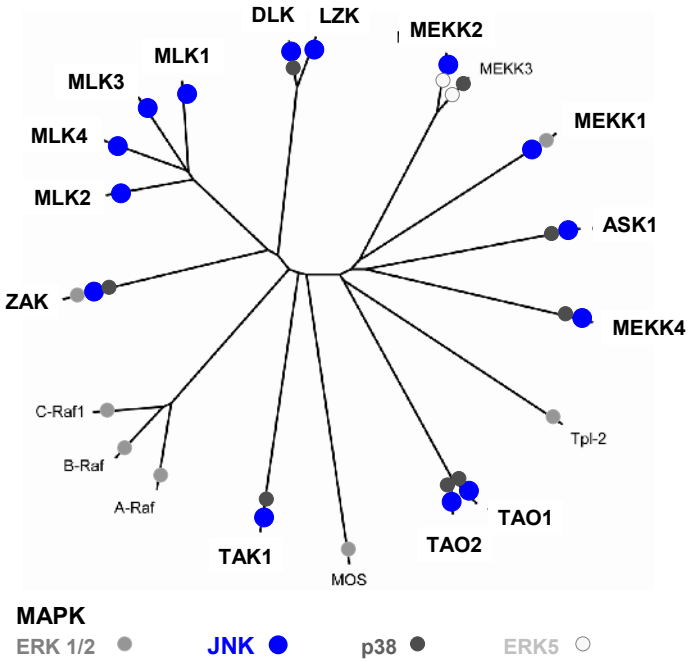

Fig. 2. Dendrogram showing the twenty MKKKs based on homology of their kinase domain primary amino acid sequences. The MAPKs activated by each MKKK are shown in the highlighted circles on the dendrogram.

have docking sites for binding specific MKKKs, MKKs, and MAPKs. Scaffold proteins that regulate the JNK signaling module include the JIP (JNK Interaction Proteins) 1-4 proteins, POSH (Plenty of SH3s), JKAP1 (SKRP), filamin, CrkII and IKAP (Morrison and Davis 2003). Scaffold proteins play an important regulatory role in controlling JNK signaling because they frequently bind specific MKKKs and localize the signaling module within the cell. Thus, scaffold proteins can regulate the spatio-temporal dynamics of JNK signaling.

3 MKKKs as signaling hubs controlling JNK activation

Figure 1 shows the MKKKs that have been defined to regulate MKK-MAPK modules. Of the twenty MKKKs, fourteen have been shown to regulate JNK activity. Six MKKKs regulate the ERK1/2 pathway while only two MKKKs are defined to regulate the ERK5 pathway. Nine MKKKs are known to regulate the p38 pathway. The restricted number of MKKKs regulating the ERK1/2 and ERK5 pathways implies a more restricted response and function for these MAPKs. For example, ERK1/2 is important in regulating cell proliferation in response to tyrosine kinases. The fact that ERK5 has a single MKK and only two defined MKKKs

shown to physiologically regulate ERK5 activity suggests a rather restricted function for this MAPK. Physiologically, ERK5 appears important in regulating vascular development and maintenance of the vasculature in adults. In contrast, the large number of MKKKs that regulate JNK and p38 indicates a role for these MAPKs in the response to diverse stress stimuli.

Figure 2 shows a dendrogram for the relationship of the different MKKKs based on sequence homology of their kinase domains. Based on the kinase homologies, the MKKKs can be divided into six groups: MEKK, MLK, Raf, Tao/Tpl2, Mos, and TAK1. Among these twenty known MKKKs, members of the MEKK, MLK, TAO, and TAK1 groups regulate JNK activation. The properties of each group of MKKKs controlling JNK activation is discussed below.

3.1 MLKs (mixed lineage kinases)

The MLK group has seven members that can be further divided into the MLKs (MLK1, 2, 3, 4), DLKs (DLK, LZK), and ZAK (Gallo and Johnson 2002). The members of the MLK subgroup each have an N-terminal Src-homology-3 (SH3) domain, kinase domain, leucine zipper region and a Cdc42/Rac interactive binding (CRIB) domain. DLKs and ZAK have kinase domains and leucine zipper regions but lack the CRIB and SH3 domains. ZAK is structurally similar to the DLKs but also encodes a sterile-alpha motif that mediates homo- or hetero-dimerization.

3.2 MEKKs (MAPK-ERK kinase kinases)

MEKK1, MEKK2, and MEKK4 have each been shown to regulate the JNK pathway in response to different stimuli. MEKK1 is a large 196 kDa protein with complex regulation. MEKK1 appears to be regulated by both Rac/Cdc42 and RhoA GTPases (Fanger et al. 1997; Gallagher et al. 2004). Furthermore, MEKK1 is the only member of the MKKK-MKK-MAPK signaling network that has a caspase 3 cleavage site. MEKK1 is also the only member of the MAPK signaling network to encode a RING domain containing E3 ubiquitin ligase function. The MEKK1 RING domain has been shown to regulate auto-ubiquitination of MEKK1 that inhibits its kinase activity as well as ubiquitinate and stimulate the degradation of ERK1 and c-Jun (Lu et al. 2002; Witowsky and Johnson 2003; Xia et al. 2007). MEKK1 is also one of only a few proteins in defined proteomes to encode a SWIM domain whose function in MEKK1 remains undefined (Makarova et al. 2002).

Whereas MEKK1 regulates both the ERK1/2 and JNK pathways, MEKK2 regulates the ERK5 and JNK pathways. MEKK2 is only one of two MKKKs, the other being MEKK3, which regulate the MEK5-ERK5 pathway (Nakamura and Johnson 2003; Uhlik et al. 2004; Nakamura et al. 2006). MEKK2 and MEKK3 encode PB1 (Phox-Bem1p) domains that selectively heterodimerize with the MEK5 PB1 domain to form a functional MEKK2 (or MEKK3)–MEK5-ERK5 ternary complex (Nakamura and Johnson 2003; Nakamura et al. 2006). The C-

terminal moiety of the MEKK2 domain is also capable of binding MKK7 for JNK activation. In contrast, the MEKK3 PB1 domain does not bind MKK7. Thus, MEKK2, but not MEKK3, regulates JNK activation.

MEKK4 selectively phosphorylates MKK3, MKK4, and MKK6 leading to the activation of both JNK and p38. MEKK4 binds Cdc42 and Rac via a CRIB domain and kinase-inactive MEKK4 inhibits Cdc42 and Rac activation of JNK (Fanger et al. 1997; Gerwins et al. 1997). MEKK4 also binds GADD45 α, β and γ proteins (Mita et al. 2002; Chi et al. 2004; Miyake et al. 2007), resulting in activation of MEKK4 kinase activity. MEKK4 and MEKK1 also bind the scaffold protein Axin, leading to JNK activation (Zhang et al. 1999; Zhang et al. 2001; Luo et al. 2003; Wong et al. 2004), suggesting MEKK4 and MEKK1 control JNK activation in the non-canonical Wnt signaling pathway.

3.3 ASK1 (apoptosis signal-regulating kinase 1)

ASK1 binds thioredoxin near its N-terminus and is activated in response to reactive oxygen species that cause the release of thioredoxin (Hayakawa et al. 2006). In addition, ASK1 binds JIP scaffold proteins and phosphorylates MKK4, MKK7, and MKK3, thereby activating both JNK and p38. ASK1 has been found to be activated by LPS and various pro-inflammatory cytokines.

3.4 TAK1 (TGFβ-activated kinase 1)

TAK1 is activated in response to IL-1, TNFα, and LPS stimulation of cells. In response to pro-inflammatory stimuli, TAK1 forms a complex with TAB1, which forms a complex with TAB2, TAB3, and the E3 ubiquitin ligase TRAF6 (Wang et al. 2001; Kanayama et al. 2004). This activated TAK1 complex coordinates the activation of MKK4, MKK6, and IKK leading to the activation of JNK, p38, and NF-κB.

3.5 TAO1 (thousand and one-amino acid kinase 1)

TAO1 and the related kinase TAO2 were cloned using degenerate oligonucleotide-based PCR cloning strategies for kinases related to the yeast Ste20 kinase (Chen et al. 1999; Zhou et al. 2004). Other than overexpression studies showing TAO kinases activate JNKs and p38, little is known about their function.

The different MKKKs described above that regulate the JNK pathway should be thought of in the greater context of the MAPK signaling network. The differing regulatory domains and motifs encoded in each MKKK selectively control their activation, inactivation, and association with regulatory proteins and scaffold proteins to mediate localization within the cell. As we see with most MKKKs that regulate the JNK pathway, MKKKs are often able to phosphorylate more than one MKK and thus regulate more than one MAPK pathway (see Fig. 1). Many

MKKKs that regulate JNK also regulate p38 consistent with a coordinated activation of JNK and p38 in response to stress stimuli. In contrast, MEKK1 activates JNK and ERK1/2, and MEKK2 activates JNK and ERK5. It is the differential control of MKKK activation and the organization of MKKKs with other proteins in signaling complexes that provide an amazing combinatorial diversity for the integration of MAPK networks for control of cellular responses to many different stimuli.

4 Insight into the function of MKKKs regulating the JNK pathway from targeted gene knockouts

The function of MKKKs controlling the JNK pathways have been defined using biochemical and cell biological approaches, but elucidation of physiological functions has mostly come from targeted gene knockouts. Of the MKKKs that regulate the JNK pathway the phenotypes of MEKK1, MEKK2, MEKK4, ASK1, TAK1, and MLK3 knockouts in mice have been reported. A brief description of the phenotypes of these knockout mice demonstrates their selective function in cellular and animal physiology (see Table 2 for summaries of phenotypes).

4.1 MEKK1

Mice having the targeted deletion of MEKK1 generally appear normal at birth and are fertile. In a SVEV129 mouse genetic background, MEKK1-null neonates have open eyes at birth, indicative of an epithelial morphogenesis defect (Yujiri et al. 1998, 2000; Zhang et al. 2003; Xia and Kao 2004; Xia and Karin 2004). In addition, MEKK1 deficient mice have wound healing and homeostasis defects associated with defective tissue remodeling. MEKK1-deficient cells have defective migration due to a loss of calpain activation required for release of focal adhesions at the rear of migrating cells (Cuevas et al. 2003). MEKK1 has also been shown to be required for activin-dependent epithelial cell migration (Zhang et al. 2005). In human breast cancer cell lines and mouse fibroblasts, MEKK1 has also been shown to be a primary regulator of urokinase-type plasminogen activator (uPA), which is required for cell invasion of the extracellular matrix (Witowsky et al. 2003). MEKK1 has been shown to regulate c-Jun, JunB, and Fra-2 expression and degradation and hence is a key regulator of AP-1 function (Cuevas et al. 2005). The role MEKK1 plays in the control of AP-1 function, protease expression, cell migration, and invasion is consistent with its involvement in wound healing, tissue remodeling, and tumor metastasis. In a transgenic model of metastatic breast cancer, MEKK1 deficiency markedly delayed tumor metastasis to the lungs. This was found to be due to a delay in dissemination of tumor cells because of an inability of the tumor to breakdown the basement membrane surrounding the tumor cell-filled ducts of the mammary gland (Cuevas et al. 2006). Recently, MEKK1 deficiency in a C57/Bl6 background has also been shown to be involved in fetal liver

Table 2. Phenotypes of MKKKs Regulating JNK

MKK	Described knockout phenotypes
MEKK1	Epithelial morphogenesis defects (open eyes at birth, defective tissue remodeling)
	Defective cell migration, loss of calpain activation
	Inhibition of urokinase-type plasminogen activator expression
	Suppressed cell invasion and dissemination of tumor cells
	Defective fetal liver hematopoiesis
	CD40 control of germinal center formation and B cell antibody production
	TH2 T cell tolerance
MEKK2	Mice normal and fertile at birth
	Suppressed TNFα, IL1α & β and IL-6 production in response to FGF-2
	Defective IgE-Fc□R1 signaling in mast cells
	Role in osteoclast function
MEKK4	Neural tube closure defects (exencephaly with enhanced apoptosis)
	Neuronal migration defects
	Skeletal defects
ASK1	Viable and fertile
	Fibroblasts have altered response to reactive oxygen species
	Decreased TNFα production in response to LPS
	Resistant to LPS-induced toxic shock
TAK1	Embryonic lethal due to vascularization defects
	Critical for IL-1 and TNFα signaling
MLK3	Viable and fertile
	Mild defect in epidermal tissue of the dorsal midline

hematopoiesis (Bonnesen et al. 2005), CD40 regulation of germinal center formation and B cell antibody production (Gallagher et al. 2007), and a JNK-Itch E3 ubiquitin ligase-mediated TH2 process in TH2 tolerance and lung inflammation (Venuprasad et al. 2006).

4.2 MEKK2

Mice having the targeted deletion of MEKK2 appear normal at birth and are fertile (Kesavan et al. 2004). MEKK2 deficiency has been shown to inhibit activation of ERK5 in response to growth factors such as FGF-2 in fibroblasts and IgE stimulation of the FcεR1 in mast cells (Garrington et al. 2000; Kesavan et al. 2004). Like MEKK1, MEKK2 has been shown to regulate the repertoire of proteins in the AP-1 complex. MEKK2-/- fibroblasts are inhibited in the induction of c-Jun, Fra-1, and Fra-2 mRNA in response to FGF-2 (Kesavan et al. 2004). FGF-2-induced expression of TNFα, IL1α and β, and IL-6 was inhibited in MEKK2-/- fibroblasts as is the expression of specific mast cell cytokines in response to IgE (Garrington et al. 2000). Interestingly, the knockout of MEKK1 and MEKK2 both alter the control of AP-1 components but do so differently, rendering distinct phenotypes. It was also shown that in osteoblasts, TGFβ or bone morphogenesis protein (BMP)

activates MEKK2 and JNK. Smurf-1, a HECT domain ubiquitin ligase, binds MEKK2 to promote its degradation and negatively regulate osteoclast function to suppress osteogenic activity (Yamashita et al. 2005).

4.3 MEKK4

The MEKK4 protein is highly expressed in the developing central nervous system. MEKK4 knockout mice have been generated by homologous recombination and MEKK4 knockin mice have been generated by genetic mutation to generate a kinase-inactive MEKK4 (Abell et al. 2005; Chi et al. 2005). The phenotypes of MEKK4 knockout and knockin mice are overlapping but not identical. Prenatal lethality is often seen due to severe defects in both the neural tube and skeleton. Both the knockout and kinase-inactive MEKK4 have exencepahly associated with enhanced apoptosis in the neural tube and loss of both JNK and p38 activity. The phenotype of the MEKK4 knockout and kinase-inactive knockin are extremely similar to the knockout phenotype for the adaptor protein TRAF4 (Abell et al. 2005; Abell 2005). Abell et al. (Abell and Johnson 2005) showed that MEKK4 binds Traf4, promoting MEKK4 oligomerization and activation. This indicates that MEKK4 and TRAF4 are in a common biochemical and genetic pathway. Recently, MEKK4 was shown to be involved in regulating filamin-A expression and controlling neuronal migration in developing forebrain (Sarkisian et al. 2006). Filamin-A is an actin-binding protein essential for cytoskeletal rearrangement and cell locomotion. Loss of MEKK4 expression disrupts filamin-A expression and phosphorylation. The loss of MEKK4 and the resulting phenotype seen in the developing forebrain is similar to that seen with filamin-A mutations that contribute to periventricular heterotopia (PVH), a congenital malformation of the human cerebral cortex.

4.4 ASK1

Mice deficient in ASK1 are viable and fertile. ASK1-/- mouse embryonic fibroblasts respond to reactive oxygen species (H_2O_2) with a normal transient activation of JNK and p38. However, the prolonged activation of these two MAPKs is lost, demonstrating that ASK1 is required for the prolonged phase of JNK and p38 activity in response to H_2O_2 (Tobiume et al. 2001). A similar loss of prolonged JNK and p38 activation is seen in ASK1-/- fibroblasts in response to TNFα. ASK1-/- mice also have diminished TNFα production in response to LPS and are resistant to LPS-induced toxic shock (Matsuzawa et al. 2005). The regulation of ASK1 in response to LPS is through the MyD88/TRAF6 signaling pathway and plays an important role in the control of cytokines for the innate immune response.

4.5 TAK1

The TAK1 knockout mouse is embryonic lethal (Shim et al. 2005; Omori et al. 2006). There is defective vascularization of TAK1-/- embryos and yolk sacs, indicating that TAK1 plays an essential role in vascular development (Jadrich et al. 2006). The role of TAK1 in IL-1 and TNFα signaling suggests it plays an important role in the inflammatory response (Sato et al. 2005, 2006). TAK1-/- fibroblasts show decreased IL-1β-induced IL-6 production (Sato et al. 2005) and TAK1-/- keratinocytes have a reduced survival when challenged with TNFα (Omori et al. 2006). Conditional keratinocyte deletion of TAK1 results in a severe post-natal inflammation with elevated levels of inflammatory cytokines and keratinocyte apoptosis (Omori et al. 2006).

4.6 MLK3

MLK3-/- mice have no obvious phenotype except for a mild defect in the epidermal tissue of the dorsal midline (Brancho et al. 2005). MLK3-/- mice are viable and fertile. TNFα -stimulated JNK activation was partially inhibited in MLK3-/- fibroblasts, but there was no measurable inhibition of ERK1/2 or p38 signaling. The response to UV irradiation, sorbitol, anisomycin, and ceramide was normal in regards to JNK and p38 activation.

Therapeutically, MLKs have been intensely studied. Inhibition of MLK kinase activity by small molecules such as CEP-1347, an indolocarbazole that is a derivative of K252a, protects neurons from apoptosis. The compound was well-tolerated in humans but performed poorly in trials for neurodegenerative diseases including Parkinson's and Alzheimer's disease (Wang et al. 2004).

5 Conclusions

MKKKs are upstream regulators of the MAPKs that integrate MAPK signaling networks with the complex cellular response to many different stimuli. MKKKs that regulate the JNK pathway are clearly involved in neural and vascular development, immune response, and inflammation. MKKKs as therapeutic drug targets are now being explored for small molecule inhibition. Inhibition of a specific MKKK has the potential to selectively inhibit stimulus-specific activation of MAPKs (Johnson et al. 2005). The continued characterization of MKKKs and their function in cells and animal physiology is needed to define their utility as therapeutic targets in human disease.

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