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DYNAMIC REGULATION OF MIXED LINEAGE KINASE 3 BY c-JUN N-TERMINAL KINASE-MEDIATED PHOSPHORYLATION

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KAREN ALEXANDRA SCHACHTER

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Kathleen A. Gallo

Major Professor's Signature

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DYNAMIC REGULATION OF MIXED-LINEAGE KINASE 3 BY c-JUN N-TERMINAL KINASE-MEDIATED PHOSPHORYLATION

By

Karen Alexandra Schachter

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ABSTRACT

DYNAMIC REGULATION OF MIXED-LINEAGE KINASE 3 BY c-JUN N-TERMINAL KINASE-MEDIATED PHOSPHORYLATION

By

Karen Alexandra Schachter

Mixed-Lineage Kinase 3 (MLK3) is a widely expressed mammalian serine/threonine protein kinase. MLK3 functions as a Mitogen Activated Protein Kinase (MAPK) Kinase Kinase (MAPKKK) to activate the c-Jun N-terminal kinase (JNK) pathway. In addition to its kinase domain, MLK3 contains a Src-homology 3 (SH3) domain, a leucine zipper domain, a Cdc42/Rac interactive binding (CRIB) motif, and a C-terminal region which is rich in proline, serine and threonine residues.

The small GTPase Cdc42 in its activated state binds to MLK3 through the CRIB motif. As a result of this interaction, the catalytic activity of MLK3 increases. Several lines of evidence suggest that Cdc42 induces dimerization of MLK3 and trans-autophosphorylation of MLK3 in its activation loop sites, and targets MLK3 to the plasma membrane. Additionally, Cdc42 alters the *in vivo* phosphorylation pattern of MLK3. Mass spectrometry coupled with phosphopeptide mapping was used to identify 11 phosphorylation sites on MLK3. Six of the identified sites correspond to phosphorylated serines followed by proline residues, thus conforming to the consensus for phosphorylation by proline-directed kinases. The work described in this dissertation examines the role of proline-directed phosphorylation of MLK3.

The group of proline-directed kinases comprises the MAPKs, the cyclin-dependent kinases (Cdks) and Glycogen Synthase Kinase 3 (GSK-3). Since MLK3 is an

upstream activator of the MAPKs, it is possible that MAPKs feedback-phosphorylate MLK3 on the proline-directed sites. Pharmacological inhibition of JNK, but not other MAPK pathways, decreases the *in vivo* phosphorylation of MLK3 on proline-directed sites, suggesting that JNK phosphorylates MLK3. In fact, an *in vitro* phosphorylation assay using recombinant, active JNK demonstrates that MLK3 is a *bona fide* substrate of JNK.

Interestingly, the degree of proline-directed phosphorylation of MLK3 correlates with its levels in cellular lysates. Inactivation of JNK in cells, by using dominant negative versions of MKK4 and MKK7, the upstream activators of JNK, results in a reduction on the levels of MLK3 in cellular lysates. On the other hand, overexpression of a constitutively active form of JNK in cells results in an increase on the levels of MLK3 in cellular lysates. These results were corroborated using a pharmacological inhibitor of JNK, and extended to demonstrate that inhibition of JNK allows (a) phosphatase(s) to dephosphorylate MLK3; the dephosphorylated MLK3 is inactive, and redistributes to a Triton-X100 insoluble fraction. Since long term JNK inhibition does not result in degradation of MLK3 in this experimental system, and given that this redistribution process is reversible, a possible mechanism to modulate MLK3 signaling in cells could rely on sequestering MLK3 in a "stand-by" mode in a dephosphorylated, Triton-X100 resistant compartment.

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"Looking back, I realize that I have been favored extraordinarily by external circumstances, the proper place at the proper time in terms of my PhD thesis, my first employment in the USA (...) and in particular having had incredibly brilliant coworkers."

Richard Ernst

Working towards a PhD has been more than just running experiments at the bench on my own; I have had the input and help from various people and have interacted with many more, and they all deserve a big Thank You.

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KEY TO ABBREVIATIONS

AGC	containing Protein Kinase A, Protein Kinase G, Protein Kinase C families
AP-1	Activating Protein 1
ASK-1	Apoptosis Signal-regulating Kinase 1
ATF-2	Activating Transcription Factor 2
Bsk	Basket
CAMK	Calcium/calmodulin protein Kinase
CDK	Cyclin Dependent protein Kinase
CMBG	containing CDK, MAPK, GSK3, CLK families
CNK 1/2	Connector Enhancer of KSR 1/2
CREB	c-AMP-Response Element Binding protein
CRIB	Cdc42/Rac interactive binding
CK1	Casein Kinase 1
COX4	Cytochrome Oxidase 4
DLK	Dual leucine zipper protein kinase
DMEM	Dulbecco's Modified Eagle's Medium
DRMs	Detergent Resistant Membranes
Dpp	Decapentaplegic
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
ERK	Extracellular signal-Regulated Kinase
GEF	Guanine nucleotide Exchange Factor

GFP	Green Fluorescent Protein
GSK	Glycogen Synthase Kinase
GST	Glutathion S Transferase
HA	Hemagglutinin
HEK	Human Embryionic Kidney
Hep	Hemipterous
HPK1	Hematopoietic Progenitor Kinase 1
Hsp	Heat shock protein
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IMP	Impedes Mitogenic signal Progression
JIP	JNK interacting protein
JNK	c-Jun N-terminal Kinase
JNKK	JNK Kinase
Kay	Kayak
KIF3	Kinesin superfamily motor protein 3
KSR	Kinase Suppresor of Ras
Lamp1	Lysosomal associated membrane protein 1
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LPS	Lipopolysaccharide
LZK	Leucine Zipper-bearing Kinase
MALDI-MS	Matrix-Assisted Laser Desorption/Ionization- Mass Spectrometry

MAPK	Mitogen Activated Protein Kinase
MAPKAPK2	MAPK activated Protein Kinase 2
MAPKKK	MAPK kinase kinase
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen-activated protein/Erk Kinase
MEKK	MEK Kinase
MKK	MAPK Kinase
MLK	Mixed Lineage Kinase
Mnk	MAPK-interacting kinase
MP1	MEK Partner 1
Msn	Misshapen
NGF	Nerve Growth Factor
NIMA	Never In Mitosis A
PAGE	Polyacrylamide Gel Electrophoresis
PAK	p21 Activated protein Kinase
PDGF	Platelet-derived Growth Factor
PI3K	Phosphoinositide-3 Kinase
PIP_3	Phosphatidylinositol 3,4,5 triphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol Myristoyl Acetate
PMSF	phenylmethylsulfonylfluoride
POSH	Plenty of SH3s

PP2A	Protein Phosphatase 2A
PSD-95	Post-Synaptic Density protein 95
SCG	Superior Cervical Ganglion
RGC	Receptor Guanylate Cyclase
RNAi	RNA intereference
Rsk	Ribosomal S6-Kinase
SAM	Sterile Alpha Motif
SH2	Src-Homology 2
SH3	Src-Homology 3
STE	homologs of yeast Sterile 7, 11, 20 kinases
TAK1	TGF-β Activated protein Kinase 1
TGF	Transforming Growth Factor
TK	Tyrosine Kinase
TKL	Tyrosine Kinase Like
TLC	Thin Layer Chromatography
TLE	Thin Layer Electrophoresis
TNF	Tumor Necrosis Factor
Tpl2	Tumor progression locus 2
ZAK	leucine-Zipper and sterile-Alpha motif Kinase

I. Literature Review

1. Overview of protein kinase classification, structure and function

Protein kinases are enzymes that catalyze the transfer of the γ-phosphate of ATP to a substrate. A comprehensive analysis of human protein kinase genes reveals that 518 protein kinases are encoded in the human genome (Manning et al., 2002). The human kinome is divided in nine groups: AGC (PKA, PKG and PKC families), CAMK (calcium/calmodulin dependent protein kinases), CMGC (CDK, MAPK, GSK3 and CLK families), TK (tyrosine kinases), STE (homologs of yeast Sterile 7, 11, 20 kinases), CK1 (casein kinase 1), TKL (tyrosine kinase-like), RGC (receptor guanylate cyclase), and atypical kinases (Manning et al., 2002). Mutations and deregulation of protein kinases play underlying roles in human diseases. The availability of the complete list of human protein kinases will help in the identification of genes involved in human diseases and in the development of therapies to treat them.

Phosphorylation is an important posttranslational modification that regulates most of the signal transduction in cells. Modification of substrate activity by phosphorylation allows the modulation of cellular processes such as transcription, metabolism, cell cycle progression, cytoskeleton rearrangements, differentiation, and apoptosis. Protein phosphorylation also regulates key aspects of development, and the general physiological homeostasis.

Protein kinases phosphorylate their substrates in a sequence-specific manner. In this "consensus sequence" model, the kinase recognizes residues directly flanking the phosphoacceptor site (Kemp & Pearson, 1990; Pearson & Kemp, 1991). This has

allowed *in vitro* studies using synthetic peptides containing the recognition motif for a protein kinase. However, it seems unlikely that substrate specificity inside the cells would rely exclusively on recognition motifs; several kinases share a similar consensus sequence for phosphorylation, and *in vitro* studies using synthetic peptides do not always reflect the phosphorylation kinetics observed for the full length protein substrate *in vivo*. In fact, docking interactions between the kinase and the substrate are usually required for efficient substrate phosphorylation (reviewed in (Biondi & Nebreda, 2003)).

Most mammalian protein kinases phosphorylate hydroxyl amino acids, and are therefore classified as either Ser/Thr kinases or Tyr kinases. A small group of kinases can phosphorylate Ser, Thr and Tyr residues, and are hence called dual-specificity kinases (Dhanasekaran & Premkumar Reddy, 1998; Lindberg et al., 1992). Based on sequence similarity and structural information, Ser/Thr and Tyr kinases form a superfamily that is distinct from other eukaryotic kinases, like inositol and inositol phosphate kinases. Crystal structures of the kinase domains of several protein kinases have been solved, and the structure is well conserved within the superfamily. Figure 1 shows the structure of the kinase domain of B-Raf in complex with a small molecule inhibitor, BAY 43-9006 (Wan et al., 2004). Like other protein kinases, the Raf kinase fold consists of a NH_2-terminal small lobe and a COOH-terminal large lobe (Zoller et al., 1991). In the interface between the two lobes Mg^{2+}, ATP and the protein substrate bind. BAY 43-9006 is an ATP analogue, and therefore is located where ATP would normally bind.

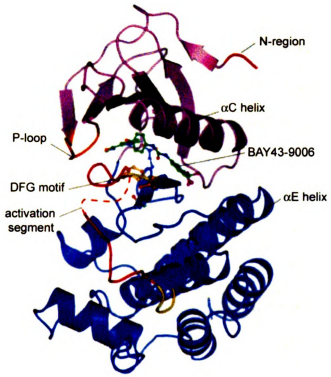


Figure 1. **Crystal structure of the kinase domain of B-Raf in complex with a small molecule inhibitor, BAY 43-9006.** The NH_2-terminal lobe is in magenta, and the COOH-terminal lobe is in marine. The P-loop is in orange. The DFG and APE motifs are in yellow, the rest of the activation segment and the N-terminus are in red. Dashed lines indicate a disordered region in the activation loop (Reprinted from Cell (116) 855-67, Wan P.T.C. *et al. Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF*, Copyright 2004 with permission from Elsevier). Images in this dissertation are presented in color.

The catalytic mechanism of phosphorylation involves direct transfer of the γ-phosphoryl group from ATP to the hydroxyl group in the protein substrate (reviewed in (Kaiser, 1987)). A number of conserved residues or motifs are found in all protein kinases and they are required for proper substrate orientation and for the stabilization of ATP (Figure 1). The glycine loop or P-loop, whose consensus sequence is G*X*G*XX*G, is found at the top of the cleft between lobes. The Gly residues of this loop coordinate the phosphates of ATP via backbone interactions. The P-loop is very flexible in the absence of ATP, and this allows small molecule inhibitors to bind. An invariant Lys also participates in the orientation of the phosphate groups of ATP for catalysis, and a conserved Glu stabilizes this Lys by ionic interactions. Mutation of this conserved Lys renders protein kinases inactive. At the bottom of the cleft is located the catalytic loop. A conserved Asp residue interacts with the nucleophilic hydroxyl group on the substrate, and an Asn residue orients this Asp. Another Asp residue, located in the conserved motif DFG, makes crucial contacts with the Mg^{2+} ions, which are coordinated with the phosphate groups of ATP (Hanks et al., 1988).

Within physiological systems, most protein kinases are normally maintained in an inactive state. One of the mechanisms by which protein kinases are "turned on" is through phosphorylation of the activation loop. The activation loop or activation segment, provides a stage for peptide binding (Huse & Kuriyan, 2002; Morgan & De Bondt, 1994). The activation loop is flanked by the motifs DFG and APE, present in nearly all protein kinases. Upon phosphorylation, the activation loop is stabilized in an open and extended conformation that allows the binding of the substrate (Huse & Kuriyan, 2002).

Most protein kinases contain non-catalytic domains that assist in the regulation of the protein function, by binding activators or inhibitors, by controlling subcellular localization, by mediating the interaction with other proteins, or by influencing protein stability. These regulatory mechanisms are required to provide specificity in response to different extracellular signals. In addition, protein kinases participate in signaling networks, in which, as a result of the stimulation of a particular signaling pathway, feedback mechanisms are initiated to regulate the activity of protein kinases in the same or in different pathways.

2. Mitogen Activated Protein Kinase pathways

The Mitogen Activated Protein Kinases (MAPKs) are a family of protein kinases that respond to extracellular stimuli, such as growth factors, cytokines, irradiation, high osmolarity, etc. (reviewed in (Johnson & Lapadat, 2002; Raman & Cobb, 2003)), to coordinate a variety of cellular processes. MAPKs are conserved from yeast to humans. The MAPK signaling pathways operate by sequential phosphorylation and activation: an upstream MAPK kinase kinase (MAPKKK) phosphorylates the activation loop of the MAPK kinase (MAPKK). MAPKKs are dual-specificity kinases; upon activation, they phosphorylate the MAPKs on both the Thr and Tyr residues of a conserved T*X*Y motif in the MAPK activation loop. Although activated MAPKs have mainly been associated with the phosphorylation and activation of transcription factors in the cell nucleus, MAPKs also have cytosolic substrates. MAPKs are proline-directed kinases; they phosphorylate Ser or Thr residues that are followed by a Pro residue in their substrates (Aitken, 1999).

In multicellular organisms, there are three major MAPK cascades, each of which includes multiple isoforms (Figure 2): the extracellular-signal regulated kinase (ERK), ERK1 and ERK2; four p38 kinases, p38α, p38β, p38γ and p38δ; and c-Jun N-terminal Kinase (JNK), JNK1, JNK2 and JNK3. A few other MAPKs have been discovered, namely ERK3 and ERK5.

2.1 The MAPK pathways

2.1.1 The ERK pathway

The ERK pathway is described as the canonical signaling cascade that is activated by growth factors. At the top of the pathway, the Raf family of MAPKKKs is activated by their association with the small GTPase Ras (reviewed in (Pearson et al., 2001)). Activated Raf phosphorylates the dual specificity kinases MEK1 and MEK2 in their activation loops. MEK1 and MEK2 in turn, phosphorylate ERK1 and ERK2 in the Thr and Tyr residues of the TEY motif in their activation loop (Zheng & Guan, 1993). Additional MAPKKKs that activate the ERK pathways have been described, such as MAPK/extracellular-signal regulated kinase kinase kinase 1 (MEKK1) (Xu et al., 1996), MEKK2 and MEKK3 (Blank et al., 1996), tumor progression locus-2 (Tpl2) (Ceci et al., 1997) and Mos (Nebreda & Hunt, 1993).

The ERK pathway is generally associated with proliferation, and deregulation of the components of the pathway often results in cellular transformation. ERK is hyperactivated in 30% of cancers (Allen et al., 2003). B-Raf is also mutated in human cancers; the most common mutation is a substitution of Val 599 by Glu in the activation loop of B-Raf, which generates a gain-of-function, activated kinase (Davies et al., 2002).

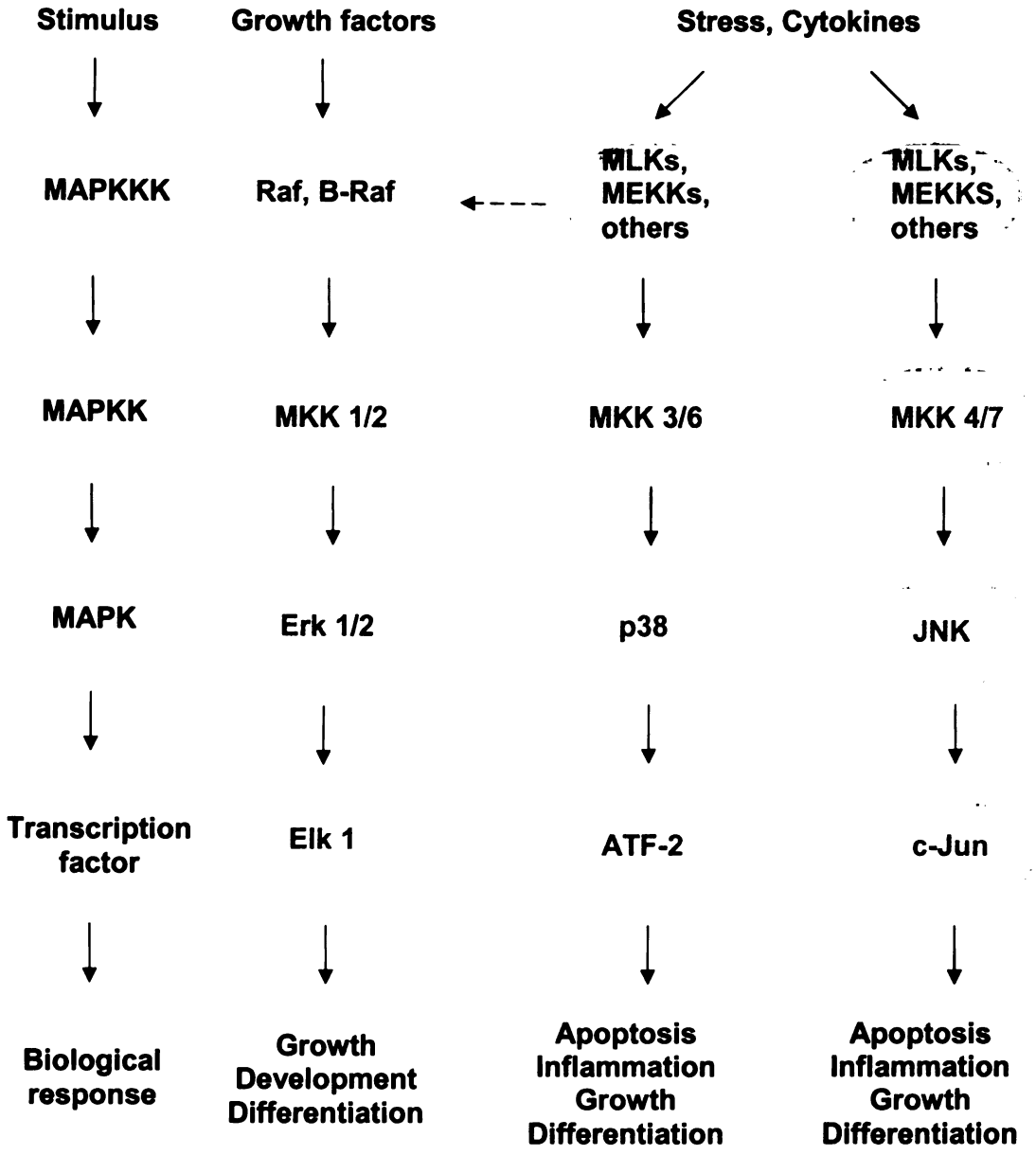


Figure 2. **Mammalian MAPK signaling cascades.** The three best-defined mammalian MAPK pathways are the ERK, p38 and JNK pathways. The core module of the MAPK pathway is composed of three kinases that are sequentially activated by phosphorylation.

Consequently, inhibitors of the ERK pathway are been tested in clinical trials as potential anti-cancer agents.

The regulation of gene expression by ERK is accomplished at two levels. ERK phosphorylates a number of protein kinases, including Ribosomal S6-kinase 1-3 (Rsk1-3) and MAPK-interacting kinase 1 (Mnk1) and Mnk2 (reviewed in (Pearson et al., 2001)). Rsk proteins activate gene expression through phosphorylation of several transcriptional regulators, such as c-AMP-response element binding protein (CREB), serum response factor, and the coactivator CBP (Pearson et al., 2001). In addition, ERK proteins directly phosphorylate transcription factors, such as the activating protein 1(AP-1) family members c-Jun, c-Fos and activating transcription factor 2 (ATF-2), and members of the ternary complex factors (TCFs) like Elk-1 (reviewed in (Chen et al., 2001)).

Among the numerous cytosolic substrates for ERK are other signaling molecules, like phosphodiesterase 4D3 (Hoffmann et al., 1999) and cytoskeletal elements such as neurofilament subunits (Veeranna et al., 1998).

2.1.2 The p38 pathway

The human p38α isoform was identified as a target for drugs that block tumor necrosis factor alpha (TNF-α) and interleukin1 (IL-1) release from monocytes stimulated with lipopolysaccharide (LPS) (Lee et al., 1994). Subsequentially, other isoforms were cloned by homology. Both p38α and p38β have splicing variants, making a total of six different p38 MAPK isoforms. The p38 MAPKs are activated by phosphorylation on their activation loops by MKK3 and MKK6 (Enslen et al., 1998). MAPKKKs that activate the p38 pathways include transforming growth factor beta (TGF-β)-activated

kinase 1 (TAK1) (Moriguchi et al., 1996), apoptosis signal-regulating kinase 1 (ASK1) (Ichijo et al., 1997), and some Mixed Lineage Kinases (MLKs) (Gallo & Johnson, 2002), among others.

Isoforms of p38 differ in their expression patterns, extracellular activators and cellular substrates; for instance p38α, but not p38β, is activated by LPS (Nick et al., 1999). The p38 MAPKs play a major role in immunity, regulating the expression of several cytokines and activating the immune response (Crawley et al., 1997). Death receptors, such as Fas and TNF-α receptors, trigger the activation of the p38 pathways, implicating p38 in apoptosis (Beyaert et al., 1996; Juo et al., 1997).

p38 phosphorylates a variety of different cellular substrates that include phospholipase A2 (Kramer et al., 1996) and the microtubule associated protein Tau (Reynolds et al., 1997). Analogous to ERK, p38 regulates gene expression directly, by phosphorylating transcription factors like ATF-1 and -2, Ets-1, Myocyte Enhancer Factor 2A (MEF2A) and p53 (reviewed in (Kyriakis & Avruch, 2001)), and by phosphorylating other protein kinases involved in gene regulation like Msk1-2 and MAPK activated protein kinase 2 (MAPKAPK 2) (reviewed in (Roux & Blenis, 2004)).

2.1.3 The JNK pathway

JNKs, or stress-activated protein kinases (SAPKs), as their name imply, are activated by environmental stress, such as heat shock, UV and osmotic stress (reviewed in (Ip & Davis, 1998; Weston & Davis, 2002)). Ten JNK isoforms, generated by alternative splicing from the *Jnk1*, *Jnk2* and *Jnk3* genes, exist. JNK1 and JNK2 are

ubiquitously expressed, while JNK3 is restricted to brain, heart and testis (Gupta et al., 1996).

Regulation of the JNK pathway is very complex, and many MAPKKK have been implicated in its activation, including MEKK1-4 (Blank et al., 1996; Gerwins et al., 1997; Xu et al., 1996), TAK1 (Yamaguchi et al., 1995), ASK1 (Ichijo et al., 1997), and the MLKs (reviewed in (Gallo & Johnson, 2002)). Dual phosphorylation of the activation loop of JNK is accomplished by the MAPKKs MKK4 and MKK7 (Lin et al., 1995; Tournier et al., 1997; Tournier et al., 1999).

The best characterized substrate of JNK is the transcription factor c-Jun. Activated JNK phosphorylates c-Jun on serine 63 and serine 73 of its transactivation domain, increasing the half life of c-Jun by inhibiting its ubiquitination and degradation through the 26S proteasome pathway (Fuchs et al., 1996). c-Jun is a component of the AP-1 transcription complex, an important regulator of gene expression. Cellular responses to environmental stresses, which activate JNK, are controlled through AP-1-mediated transcriptional regulation.

JNK has been implicated in promoting apoptosis. Mouse embryonic fibroblasts derived from *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ knockout mice are resistant to UV-induced apoptosis (Tournier et al., 2000). Apoptosis induced by nerve growth factor (NGF) withdrawal in rat PC-12 cells is suppressed when the JNK pathway is inhibited (Le-Niculescu et al., 1999). However, in pro-B lymphocytes, JNK promotes cell survival. In response to IL3, JNK phosphorylates the pro-apoptotic Bcl-2 family protein BAD, which is then sequestered in the cytosol by 14-3-3, inhibiting apoptosis (Yu et al., 2004). Thus, it

seems likely that JNK activation modulates apoptotic responses in a cell type and stimulus dependent manner.

2.1.4 The ERK3 and ERK5 pathways

ERK3 displays high similarity in its kinase domain to ERK1/2; however, it has a unique COOH-terminal extension and instead of the TEY motif in its activation loop, ERK3 possesses a SEG motif, where the Ser residue is constitutively phosphorylated (Cheng et al., 1996). ERK3 mRNA is widely expressed, and its expression is upregulated during development. ERK3 is a highly unstable protein with a fast turnover rate regulated through ubiquitin-mediated degradation (Coulombe et al., 2003). Interestingly, ERK3 is stabilized and accumulates during the differentiation of PC12 cells into neuronal cells, and of C2C12 cells into muscle cells (Coulombe et al., 2003), suggesting that protein abundance, rather than activation loop phosphorylation, might regulate ERK3 activities. There is not much information on physiological substrates of ERK3; it does not phosphorylate ERK1/2 substrates *in vitro* or *in vivo*. Recently, MAPKAPK5 has been identified in a yeast-two-hybrid screen as an ERK3 interacting partner that can also be phosphorylated by ERK3 (Seternes et al., 2004). The interaction between these proteins seems to stabilize ERK3 and results in nuclear exclusion and accumulation in the cytoplasm of cells. The biological function of this interaction remains to be elucidated.

ERK5, also called Big MAPK 1 (BMK1), is two times the size of ERK1/2, and like ERK3, it has a unique COOH-terminal extension. ERK5 follows the classical MAPK activation cascade. Its activation loop segment contains the TEY motif that is phosphorylated by an upstream MAPKK, MEK5 (Kato et al., 1997; Zhou et al., 1995).

At the MAPKKK level, MEKK2 and MEKK3 have been shown to activate ERK5 (Chao et al., 1999; Sun et al., 2001). The repertoire of ERK5 substrates includes transcription factors from the MEF2 family, serum- and glucocorticoid-inducible kinase (SGK), connexin 43 and the pro-apoptotic protein Bad (reviewed in (Hayashi & Lee, 2004)). Like ERK1/2, ERK5 is activated by growth factors; the ERK5 knockout is lethal, and the embryos die of cardiovascular defects. Studies in ERK5 conditional knockout mice indicate that ERK5 is required for survival of endothelial cells and the maintenance of vascular integrity (Hayashi et al., 2004).

2.2 Specificity on the MAPK pathways

MAPK cascades respond to extracellular stimuli in an organized manner; yet the great variety of MAPKKKs and MAPKKs that can be utilized in response to a signal raises the question of how specificity is achieved. Part of the answer comes from the identification of scaffold proteins for each MAPK family. Scaffold proteins interact through different domains with specific subsets of kinases, as well as with other molecules (reviewed in (Dard & Peter, 2006; Morrison & Davis, 2003; Nicolas Dard, 2006; Raman & Cobb, 2003)). In addition to promoting kinase-substrate interactions, scaffolds can spatially and temporally regulate the activation of MAPKs and can modulate the kinetics of activation by recruiting phosphatases (reviewed in (Dard & Peter, 2006)). For example, kinase suppressor of Ras (KSR) is a scaffold protein for the ERK pathway (Figure 3) (Roy et al., 2002). KSR is constitutively associated with MEK and 14-3-3 through phosphorylated Ser residues. In addition, KSR is associated with

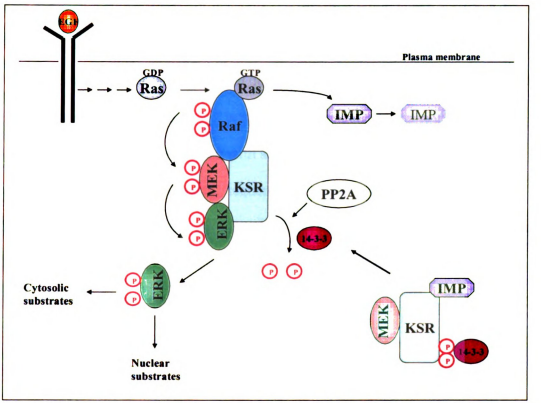


Figure 3. **Regulation of ERK activation by the scaffold KSR**. Cytosolic KSR is kept inactive through its interaction with 14-3-3 proteins and Impedes Mitogenic signal Propagation (IMP). Upon binding of EGF to its receptor, Ras is activated (Ras-GTP). Ras-GTP induces dephosphorylation of KSR by PP2A, releasing 14-3-3 and translocating KSR to the membrane; in addition, Ras-GTP also induces ubiquitination and degradation of IMP. At the membrane, KSR facilitates the phosphorylation of MEK by Raf, and the phosphorylation of ERK by MEK. Activated ERK translocates to the nucleus to activate transcription factors, or phosphorylates cytosolic substrates (adapted from (Kolch, 2005)).

Impedes Mitogenic signal Propagation (IMP) and this interaction keeps KSR in a Triton-insoluble compartment (Matheny et al., 2004). In response to cell stimulation, IMP is degraded through ubiquitination-mediated degradation. KSR is dephosphorylated by the protein phosphatase PP2A, and 14-3-3 is released. KSR translocates to the plasma membrane, and brings MEK in contact with activated Raf. In addition, KSR provides a docking stage for ERK, facilitating the sequential phosphorylation events required for ERK activation (Ory & Morrison, 2004). Additional scaffold proteins that regulate the ERK pathway include MEK partner 1 (MP1), β-arrestins, and connector enhancer of KSR 1/2 (CNK1/2) (reviewed in (Kolch, 2005; Morrison & Davis, 2003)). Scaffold proteins for the JNK and p38 pathways have also been described, and will be discussed in detail later in the chapter.

In summary, the MAPKs are a complex family of protein kinases with many different subcellular functions. Various upstream activators impinge in the different pathways to regulate a different subset of biological responses. For the rest of the chapter, detailed consideration will be given to one of the families of MAPKKKs, the MLKs; their functions in the MAPK pathways; the regulation of their activities.

3. Mixed Lineage Kinases

Mixed Lineage Kinases are a family of serine/threonine kinases that constitutes one group of MAPKKKs. The MLKs, which share sequence similarity with both Ser/Thr kinases and Tyr kinases in their catalytic domains, are found in the TKL branch of the human kinome. To date Tyr phosphorylation activity has not been demonstrated for the

MLKs. The best described function of the MLKs is as upstream activators of the JNK and p38 pathways. MLKs have been implicated in neuronal apoptosis, and a pan MLK inhibitor, CEP-1347 (Maroney et al., 1998), was in clinical trials for Parkinson's disease (Parkinson Study, 2004). A derivative of CEP-1347, CEP-11004 (Murakata et al., 2002), is currently available for research studies.

3.1 The MLK family members

The MLK family can be divided into 3 subgroups (Figure 4): MLK1-4; the Dual Leucine zipper bearing Kinases (DLKs); and Zipper sterile-α motif Kinase (ZAK) (reviewed in (Gallo & Johnson, 2002)).

3.1.1 MLK1-4

The MLK subfamily includes MLK1 (Dorow et al., 1993), MLK2 (also called MST, (Dorow et al., 1995; Katoh et al., 1995)), MLK3 (also known as SPRK/PTK1, (Ezoe et al., 1994; Gallo et al., 1994; Ing et al., 1994)) and the splice isoforms MLK4α and MLK4β (Kvasha et al, from Genebank). MLK1-4 contain a NH_2-terminal SH3 domain followed by the catalytic domain, a centrally located leucine zipper region, and a Cdc42/Rac interactive binding (CRIB) motif. MLK1-4 share over 75% sequence identity in their kinase domain, and 65% sequence identity from the SH3 through their CRIB motif. The COOH-terminal regions of the MLKs diverge, but they are all proline-rich.

In spite of their similarities, MLK1-4 have different cellular expression patterns. MLK1 mRNA has been detected in epithelial cell lines of breast, colonic and esophageal origin (Dorow et al., 1993). MLK1 protein is detected in an immature β-cell line, RIN-

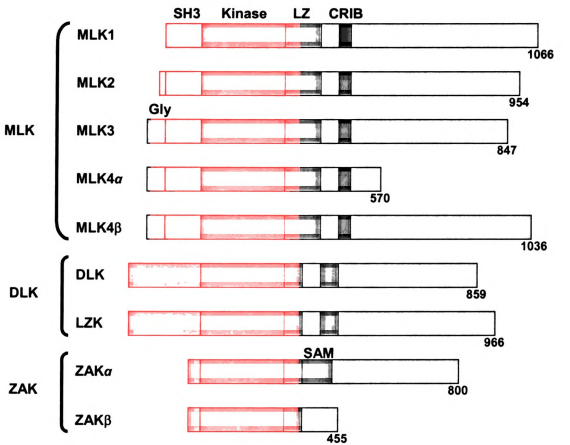


Figure 4. **Block diagram structure of human Mixed Lineage Kinases**. The relative position of the SH3 domain, leucine zipper (LZ), CRIB motif, and sterile α motif (SAM) is indicated (Adapted from (Gallo & Johnson, 2002))

5AH cells, but is absent in a more mature β-cell line, PIN-A12 cells, suggesting that MLK1 might be regulated during β-cell development (Dorow et al., 1993). MLK2 mRNA is predominantly expressed in brain, testis and skeletal muscle, and at lower levels in pancreas (Dorow et al., 1995; Katoh et al., 1995). MLK3 mRNA is widely expressed in human tissues, with lower expression in brain and heart (Ezoe et al., 1994; Gallo et al., 1994; Ing et al., 1994). There is currently no published information on the expression pattern of MLK4.

3.1.2 DLK and LZK

The DLK subfamily includes DLK (also called ZPK/MUK, (Holzman et al., 1994)) and Leucine-Zipper Kinase (LZK) (Sakuma et al., 1997). While DLKs lack an SH3 domain and a CRIB motif, they do have two small leucine zipper motifs separated by 31 amino acids, which follow the catalytic domain. The catalytic domains of DLK and LZK share 87% sequence identity with each other, but only 35% sequence identity with MLK1-4. DLK mRNA is highly expressed in the adult mouse brain, primarily in the hippocampus, cerebral cortex and the Purkinje cells of the cerebellum (Blouin et al., 1996; Holzman et al., 1994). LZK mRNA is widely expressed, with the highest expression in the pancreas (Sakuma et al., 1997).

3.1.3 ZAK

The only member of the third MLK subfamily is ZAK (Gotoh et al., 2001; Gross et al., 2002; Liu et al., 2000a). Two splicing forms of ZAK have been described, ZAKα (also known as MRK) and ZAKβ (also called MLTK/MLK7/ZAK). In addition to

the catalytic domain, ZAKα contains a leucine zipper region and a sterile-α motif (SAM). Due to the differential splicing, ZAKβ lacks the SAM. ZAK mRNA is widely expressed in human tissues, with highest expression in heart and skeletal muscle (Gotoh et al., 2001; Gross et al., 2002).

3.1.4 MLK orthologs

MLKs are absent from yeast, but MLK orthologs are found in *Drosophila*, *C. elegans* and *Xenopus*. The *Drosophila* ortholog of the MLK subfamily is called Slipper (Slpr), and is involved in regulating the process of dorsal closure in the fly embryo (Stronach & Perrimon, 2002). The expression of the *Xenopus* MLK ortholog, which, among the human MLKs, is most similar to MLK2, varies during differentiation (Poitras et al., 2003), and antisense inactivation of *Xenopus* MLK2 impairs normal tubule development of the cement gland (Poitras et al., 2003). By analysis of the sequences from the catalytic domain and zipper regions of DLK against the National Center for Biotechnology Information (NCBI) protein database, both *Drosophila* and *C.elegans* have a DLK ortholog (Gallo & Johnson, 2002). *C. elegans* also has an ortholog of ZAK, and another distantly related SH3-containing kinase whose catalytic domain is similar to the MLKs.

3.2 MLKs signaling activities

3.2.1 Activation of the JNK pathway

Of the seven mammalian MLKs, all except MLK1 and MLK4 have been reported to activate the JNK pathway when overexpressed in cells (Cuenda & Dorow,

1998; Fan et al., 1996; Gotoh et al., 2001; Liu et al., 2000a; Rana et al., 1996; Sakuma et al., 1997). MLK2 and MLK3 phosphorylate the MAPKKs MKK4 and MKK7 in *in vitro* kinase assays (Cuenda & Dorow, 1998; Rana et al., 1996), while DLK only phosphorylates MKK7 *in vitro* (Merritt et al., 1999), suggesting that DLK may only utilize MKK7 to transduce signals to JNK *in vivo*. Overexpression of ZAK enhances the catalytic activity of MKK7, and to a lesser degree, that of MKK4 (Gotoh et al., 2001). In addition, a catalytically inactive form of MKK7, but not a catalytically inactive MKK4, can block ZAK-mediated JNK activation, (Yang, 2002), suggesting that the relevant substrate for ZAK *in vivo* may be MKK7.

3.2.2 Activation of the p38 pathway

Concomitant to the activation of JNK, a modest activation of the p38 pathway, through phosphorylation of MKK3 and MKK6, was initially observed for overexpressed MLK3 (Tibbles et al., 1996), and later for DLK (Fan et al., 1996), MLK2 (Cuenda & Dorow, 1998; Hirai et al., 1997), and ZAKα (Gotoh et al., 2001; Gross et al., 2002). The scaffold protein JIP2 has been shown to mediate the MLK-induced p38 activation (Buchsbaum et al., 2002; Schoorlemmer & Goldfarb, 2001). Endogenous MLK3 can activate the p38 pathway in hepatoma cell lines upon TGF-β treatment (Kim et al., 2004), although the slow kinetics of activation suggest that p38 activation by MLK3 might occur indirectly, perhaps as a result of activating some other signaling pathway. Suppression of MLK3 expression by RNAi reduces TNF-α-induced activation of p38 on CCD-18Co human colon fibroblasts and Jurkat T lymphocytes (Chadee & Kyriakis, 2004).

3.2.3 Activation of the ERK pathway

ERK activation can be detected in cells overexpressing MLK2 (Hirai et al., 1997; Nagata et al., 1998) and ZAK (Gotoh et al., 2001). The data regarding the activation of the ERK pathway by ectopically expressed MLK3 are conflicting. Overexpression of MLK3 can result in ERK activation (Gallo KA, unpublished observations, and (Kim et al., 2004)). In contrast to these results, some data indicate that overexpression of MLK3 blocks ERK activation in response to Phorbol Myristoyl Acetate (PMA) and Platelet-Derived Growth Factor (PDGF) in mesangial cells (Parameswaran et al., 2002a; Parameswaran et al., 2002b). Although MLK3 can phosphorylate and activate MEK1 *in vitro* and *in vivo*, a report indicates that overexpression of MLK3, which results in sustained JNK activation, blocks mitogenic activation of ERK (Shen et al., 2003). The proposed mechanism suggests that negative cross-talk between JNK and ERK exists, in which c-Jun mediated gene transcription is required to somehow uncouple the MLK3-mediated MEK1 activation from ERK activation.

Studies using RNA interference (RNAi) to silence MLK3 expression in human and murine cell lines had reveal a novel role of MLK3 in the activation of the ERK pathway. The results indicate that MLK3 is required for the activation of ERK upon various extracellular stimuli, and for the proliferation of human tumor cell lines (Chadee & Kyriakis, 2004). MLK3 appears to play a scaffolding role, rather than a catalytic role in mediating ERK activation. Specifically, MLK3 is proposed to form a complex with B-Raf and Raf-1 that is required for efficient signaling to ERK.

3.2.4 Activation of NF-κB

NF-κB is a transcription factor involved in immune, inflammatory and anti-apoptotic responses. NF-κB is normally sequestered in the cytosol through the interaction with IκB. Phosphorylation of IκB by the Ser/Thr kinases IκB kinases (IKKs), IKKα and IKKβ, results in its proteasomal-mediated degradation, allowing NF-κB to translocate to the nucleus and activate transcription (reviewed in (Israel, 2000)). In Jurkat cells, overexpression of MLK3 induces phosphorylation and activation of IKKα and IKKβ (Hehner et al., 2000). Catalytically inactive MLK3 blocks NF-κB dependent transcription in response to CD3/CD28 T-cell costimulation. Other MLKs that have been reported to activate NF-κB are ZAK (Liu et al., 2000a) and LZK (Masaki et al., 2003).

3.2.5 Other MLK substrates

Most MLK substrates are protein kinases whose activation loops are phosphorylated by the MLKs. Recently, it has been shown that MLK3 can phosphorylate golgin-160 *in vitro* (Cha et al., 2004). Golgin-160 is a peripheral membrane protein localized to the cytoplasmic face of the Golgi complex (Hicks & Machamer, 2005), which upon genotoxic stress, is cleaved by caspases, resulting in the disassembly of the Golgi complex during programmed cell death (Mancini et al., 2000). Coexpression of MLK3 and golgin-160 in CHO cells results in a decrease in the mobility of golgin-160 on SDS-PAGE, suggesting that MLK3 can promote phosphorylation of golgin-160 *in vivo* (Cha et al., 2004). The function of this phosphorylation is not clear; overexpression of MLK3 on HeLa cells that stably overexpress golgin-160 results in the caspase-induced

cleavage of a very small fraction of golgin-160, but its effect on the induction of apoptosis was not tested.

Using a yeast-two-hybrid system, the nuclear receptor corepressor Alien was identified as an interacting partner of MLK2 (Eckey et al., 2003). In the absence of thyroid hormone, Alien is recruited by thyroid hormone receptor through its silencing domain located in its COOH-terminus. Thyroid hormone receptor-corepressor complexes repress target gene expression partly through association with histone deacetylases (Eckey et al., 2003). MLK2 can phosphorylate Alien *in vitro*, and reporter-gene assays indicated that coexpression of MLK2 with Alien results in increased transcriptional repression when compared to Alien expressed alone (Eckey et al., 2003). The relevance of the MLK2-Alien interaction as well as the effect of the phosphorylation of Alien by MLK2 needs further investigation.

Another MLK2-interactive partner discovered in a yeast-two-hybrid screen is NeuroD. NeuroD is a member of the basic helix-loop-helix family of transcription factors, and it plays a key role in neuronal development and survival in vertebrates (Lee et al., 1995). NeuroD and MLK2 can be coprecipitated from transfected mouse neuroblastoma N2A cells, and coexpression of MLK2 with NeuroD induces a retardation in the mobility of NeuroD in SDS-PAGE (Marcora et al., 2003). Furthermore, purified recombinant MLK2 phosphorylates NeuroD isolated from cells in an *in vitro* assay. However, purified recombinant NeruroD is a poor substrate for MLK2 *in vitro*, suggesting that additional cofactors or previous modification of NeuroD in cells is required for the phosphorylation by MLK2. Microinjection of the NeuroD mRNA into *Xenopus* embryos results in ectopic neurogenesis, which is used as an indicator of

NeuroD activity. Coinjection of NeuroD and MLK2 into *Xenopus* embyos further potentiates the ability of NeuroD to ectopically induce neurogenesis (Marcora et al., 2003).

3.3 Regulation of MLKs activities

A considerable amount of work has focused on deciphering the biochemical mechanisms that regulate MLKs signaling, by analyzing the roles that the different domains of the MLKs play on MLKs activities. Recently, the efforts have been concentrated in understanding the biological contexts in which MLKs are activated. The following sections summarize the current knowledge on how MLKs are regulated.

3.3.1 Regulation of MLKs by extracellular signals

Upon overexpression, the MLKs display relatively high catalytic activity judged by their ability to autophosphorylate in *in vitro* kinase assays. However, the extracellular signals that activate and regulate endogenous MLKs have remained largely unknown. Most of the work has focused on MLK3, for which there are commercially available antibodies. Figure 5 summarizes some of the data.

Several studies have been undertaken to look at the effects of extracellular signals on MLK3, using as a readout changes in electrophoretic mobility on SDS-PAGE gels, as an indication of phosphorylation. For instance, in Jurkat T lymphocytes, PMA treatment and co-stimulation with CD3/CD28 antibodies induce retardation in the electrophoretic mobility of MLK3, implying that activation of lymphocytes results in MLK3 phosphorylation (Hehner et al., 2000). Incubation of PMA-treated Jurkat T

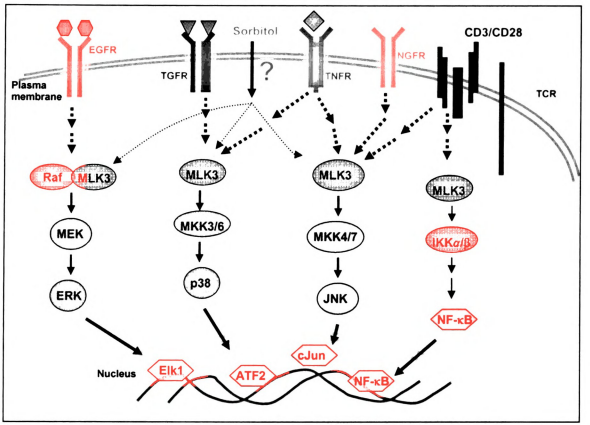


Figure 5. **Regulation of MLK3 by extracellular signals**. Several extracellular stimuli have been shown to activate MLK3. Cross-linking of the T-cell receptor (TCR) with anti CD3/CD28 antibodies results in MLK3 activation and signaling through the JNK and/or NF-κB pathways. NGF withdrawal results in MLK3 activation and JNK-mediated apoptosis. Activation of the TNF-α receptor (TNFR) also initiates MLK3-mediated signaling to the JNK and p38 pathways that results in program cell death. TGF-β binding to its receptor (TGFR) triggers MLK3-mediated activation of the p38 pathway. Growth factors like EGF, upon binding to their receptors, initiate a signaling cascade that requires the interaction of Raf and MLK3 for proper activation of the ERK pathway. Stressers like sorbitol can also activate MLK3 signaling. The precise mechanisms that connect MLK3 to the activation of the different receptors are not clear. MLK3-mediated activation of the MAPK pathways initiates a wave of transcriptionally-regulated gene expression that elicits a variety of different biological responses.

lymphocytes with inorganic phosphate results in an increase in the incorporation of radioactive phosphate into endogenous MLK3, confirming that MLK3 is phosphorylated *in vivo* upon PMA treatment (W. Chen, unpublished results). It is unclear whether the phosphorylation changes detected *in vivo* correlate with changes in the catalytic activity of MLK3.

Incubation of Jurkat T lymphocytes with sorbitol, which results in hyperosmotic stress and activation of all the MAPK pathways, dramatically retards the mobility of MLK3 in SDS-PAGE (unpublished results). Recently, a phospho-specific antibody directed against the activation loop of MLK3 has become commercially available. Using this antibody, it has been possible to detect activation of endogenous MLK3 in Jurkat T lymphocytes treated with sorbitol (unpublished results), as well as in A431 human epidermoid carcinoma cells (Xu et al., 2005). When subjected to hyperosmotic conditions, cells turn on the expression of several proteins that regulate the intracellular levels of osmolytes, like inositol transporters, as well as the expression of Hsp70 to protect proteins from unfolding and aggregating (reviewed in (Sheikh-Hamad & Gustin, 2004)). Although the mechanism by which cells sense hyperosmolarity is not clear, it has been established that hyperosmotic stress activates several signaling pathways, including the p38, JNK, PKA and the tyrosine kinase Fyn, resulting in the transcription of the osmolyte regulators (Sheikh-Hamad & Gustin, 2004). It is possible that MLK3 plays a role in the transduction of the signal from the osmotic sensor to the MAPK pathways. In addition, other stress conditions, like DNA damage induced with camptothecin, a topoisomerase inhibitor, results in activation of endogenous MLK3 in HeLa cells and in PC12 cells as judged by reactivity with the phospho-specific MLK3 antibody (Xu et al.,

2005). Overall, the data indicate that MLK3 might be playing a role in cellular responses against stress insults.

Also in Jurkat T lymphocytes, TNF-α stimulation results in MLK3 activation, as judged by a two fold increase in the phosphorylation of recombinant GST-MKK4 by MLK3 *in vitro* (Sathyanarayana et al., 2002). Similar results have been obtained in MCF-7 cells (Zhang et al., 2004). Interestingly, no changes in the electrophoretic mobility of MLK3 can be detected upon stimulation of cells with TNF-α. In addition, treatment of Jurkat T cells with the lipid C6-ceramide increases endogenous MLK3 catalytic activity as judged by *in vitro* kinase assay (Sathyanarayana et al., 2002). Ceramide is generated in cells challenged with TNF-α through the activation of sphingomyelinases that degrade sphingomyelin into ceramide and phosphocholine. Ceramide promotes apoptosis by inducing permeabilization of the mitochondria membranes (reviewed in (Ding & Yin, 2004)). The MLK inhibitor CEP-11004 attenuates both TNF-α-induced and ceramide-induced JNK activation (Sathyanarayana et al., 2002). These data suggest that MLK3 participates in the activation of apoptotic pathways in response to TNF-α.

It has been reported that TGF-β treatment of FaO rat hepatoma cells and Hep3B human hepatoma cell lines induces activation of MLK3 as judged by reactivity with the phospho-specific antibody (Kim et al., 2004), although the slow kinetics of MLK3 activation suggest that MLK3 does not lie directly under the TGF-β receptor signaling cascade, but is rather indirectly activated. As with TNF-α, no change in the electrophoretic mobility of total MLK3 is observed upon TGF-β treatment. Overexpression of kinase defective MLK1, MLK2 or MLK3 in these hepatoma cell lines

diminishes TGF-β induced apoptosis (Kim et al., 2004), suggesting that activation of the MLKs is a required step in the process of TGF-β-induced cell death.

Recent work has looked at the effect of growth factors on the activation of MLK3. Incubation of normal human colon cells CCD-18Co with epidermal growth factor (EGF) activates endogenous MLK3, as judged by *in vitro* kinase assay of immunopurified MLK3 using recombinant GST-MEK1 as a substrate (Chadee & Kyriakis, 2004). However, MLK3 has been reported to be in a complex with B-Raf and Raf-1(Chadee & Kyriakis, 2004; Chadee et al., 2006), which are upstream activators of MEK1. Therefore, it is possible that the ascribed ability of MLK3 to phosphorylate MEK1 *in vitro* might be due to the presence of B-Raf and Raf-1 in complex with the immunoprecipitated MLK3. Nonetheless, RNAi against MLK3 abolishes the EGF-induced activation of ERK, JNK and p38, indicating that MLK3 is required to transduce signals from the EGF receptor (Chadee & Kyriakis, 2004).

Treatment of neuronal-glial cultures from rat embryonic telencephalon with okadaic acid, a serine/threonine phosphatase inhibitor, results in a retarded electrophoretic mobility of DLK (Mata et al., 1996). In addition, JNK is activated, presumably by inhibition of phosphatases that negatively regulate DLK and/or JNK.

The mechanisms by which extracellular ligands regulate the activation of different MLKs are still unsolved. Specifically, how the signals are transmitted from the plasma membrane to the MLKs, and what determines which MLK is activated, are puzzling questions still unresolved. It is likely that the expression levels of the different MLKs as well as cell type specific interacting partners are modulating the specificity of the activation of the MLKs.

3.3.2 Regulation of MLKs by autoinhibition

MLK1-MLK4 have an NH_2-terminal SH3 domain. SH3 domains are independently folded modules of about 60 amino acids. SH3 domains bind proline-rich sequences, the consensus for binding being P*XX*P, preceded or followed by basic amino acids (Yu et al., 1994). One prediction is that the SH3 domain of MLK1-MLK4 would mediate the interaction with other proteins and/or the localization to different cellular compartments, to regulate signaling events. The SH3 domain of MLK3 has been shown to interact with a proline-rich region in hematopoietic progenitor protein kinase 1 (HPK1), and HPK1 can phosphorylate kinase inactive MLK3 *in vitro* (Kiefer et al., 1996). However, no effect of the HPK1 interaction with MLK3, as well as of the HPK1-mediated phosphorylation of MLK3, on MLK3 activity has been reported in the literature or has been observed in our laboratory.

The SH3 domain of the protein kinase Src is involved in an autoinhibitory, intramolecular interaction (Hubbard et al., 1998). Similarly, the SH3 domain of MLK3 has been shown to negatively regulate its kinase activity, by means of an interaction with a region of MLK3 located between the zipper and the CRIB motif (Zhang & Gallo, 2001). Disruption of the SH3 domain of MLK3 by mutation of a conserved Tyr to Ala results in increased MLK3 catalytic activity. Interestingly, the SH3 domain binding region contains only one Pro residue. Mutation of that Pro to Ala also results in increased MLK3 kinase activity. The critical Pro in the SH3-binding region is conserved in MLK1, MLK2, MLK4, Slpr and the frog ortholog of MLK2, suggesting that all the MLK subfamily members may use a common mechanism of SH3-mediated autoinhibition.

3.3.3 Regulation of MLKs by dimerization

All the MLK family members have a leucine zipper. Leucine zippers mediate protein oligomerization by forming coiled coil structures. These structures are stabilized mainly by leucine residues spaced seven residues apart, which interact at the interface of opposing helices (Hu et al., 1990; O'Shea et al., 1991). The data available indicate that the leucine zipper region of MLK3 mediates homodimerization (Leung & Lassam, 1998). Although MLK3 and DLK can be coimmunoprecipitated from cells (Tanaka & Hanafusa, 1998), the DLK leucine zipper does not form efficient heterodimers with other MLKs (Nihalani et al., 2000), suggesting that heterodimerization of MLKs is unlikely to occur through this region.

The leucine zipper region of MLK3 is necessary for MLK3 oligomerization (Bock et al., 2000), and deletion of the entire zipper region generates a MLK3 variant that is catalytically inactive *in vitro* and that fails to activate the JNK pathway (Leung & Lassam, 1998). Destabilization of the leucine zipper region by mutating one of the Leu residues to Pro, L410P, disrupts dimerization and MLK3-mediated JNK activation (Vacratsis & Gallo, 2000). Strikingly, although the leucine zipper mutant displays nearly null *in vitro* autophosphorylation activity, coexpression of the small GTPase Cdc42 greatly stimulates this activity (Vacratsis & Gallo, 2000). Similarly, the leucine zipper mutant does not display reactivity with the phospho-MLK3 antibody, while coexpression with Cdc42 potently induces activation loop phosphorylation (Yan Du, unpublished observations). A possible explanation for this observation is that Cdc42 itself can dimerize (Zhang & Zheng, 1998), and dimerized Cdc42 can force two molecules of MLK3 into close proximity, so that transphosphorylation and activation can occur.

However, even in the presence of activated Cdc42, the leucine zipper mutant fails to phosphorylate Thr 258 in the activation loop of MKK4 when used as an *in vitro* substrate. These results indicate that MLK3 dimerization is required for proper substrate phosphorylation and activation of the downstream pathway.

Leucine zipper-mediated DLK dimerization is necessary for DLK activation and stimulation of the JNK pathway (Nihalani et al., 2000). Induced dimerization of DLK promotes autophosphorylation and activation of JNK (Nihalani et al., 2001). Consistent with a model in which dimerization results in transphosphorylation and activation, overexpression of the DLK leucine zipper inhibits dimerization and activation of full length DLK, by forming inactive complexes (Nihalani et al., 2001). LZK also forms oligomers, and deletion of the LZK leucine zipper region prevents LZK-induced JNK activation (Ikeda et al., 2001).

In summary, leucine zipper-mediated dimerization is a common regulatory mechanism among the members of the MLK family. Dimerization precedes autophosphorylation, and is required for subsequent JNK activation.

3.3.4 Regulation of MLKs by small GTPases

Small GTPases are molecular switches that cycle between an active, GTP-bound state, and an inactive, GDP-bound state. The Rho family of small GTPases includes RhoA-E and RhoG; Rac1 and Rac2; Cdc42; and TC10 (Mackay & Hall, 1998). They regulate many cellular processes, including cell growth, cellular transport, cell motility and cytoskeleton rearrangements (Bar-Sagi & Hall, 2000). Constitutively active

forms of Rac and Cdc42 have been shown to activate the JNK and p38 pathways (Coso et al., 1995; Minden et al., 1995).

MLK1-4 contain a CRIB motif. CRIB motifs are composed of 14-16 amino acids, eight of which are conserved and mediate binding with activated forms of Rac and Cdc42 (Burbelo et al., 1995). MLK3, which has six of the eight conserved residues in its CRIB motif, binds to activated Cdc42 and Rac (Bock et al., 2000; Burbelo et al., 1995; Teramoto et al., 1996). Ablation of MLK3 expression by RNAi blocks ectopically expressed Cdc42-induced JNK activation (Du et al., 2005), reaffirming the notion that Cdc42 is an upstream activator of MLK3, and that MLK3 is a relevant interactor of Cdc42 in cells. Coexpression of MLK3 with activated Cdc42 promotes MLK3 oligomerization (Leung & Lassam, 1998; Nihalani et al., 2001), increases MLK3 catalytic activity, potentiates MLK3-induced JNK activation (Bock et al., 2000), and increases the *in vivo* phosphorylation of MLK3, which is accompanied by a retarded mobility of MLK3 in SDS-PAGE (Bock et al., 2000). Coexpression of MLK3 with Cdc42 also results in the phosphorylation of the activation loop of MLK3, translocation of MLK3 to membranes, and further phosphorylation of MLK3 (Du et al., 2005). Maximal JNK activation by MLK3 is dependent on its localization to membranes, suggesting that full activation of MLK3 is achieved once targeted by Cdc42 to membranes (Du et al., 2005).

The binding region for Cdc42 lies in close proximity to the autoinhibitory binding site. Although the mechanism by which Cdc42 activates MLK3 is not completely understood, the following model has been proposed: binding of Cdc42 to the CRIB motif on MLK3 disrupts the SH3-mediated intramolecular inhibition, resulting in induced

dimerization, transphosphorylation on the activation loop, and relocalization to membranes. Here, MLK3 undergoes full activation and utmost JNK activation is obtained.

3.3.5 Regulation of MLKs by scaffold proteins

JNK interacting proteins 1-4 (JIPs) are a group of scaffold proteins encoded by four genes. These proteins can be divided into two sub-groups based on sequence similarities, but all of the JIPs share common biochemical properties, including potential functions as cargo molecules for kinesin motor proteins and as scaffold proteins for JNK and p38 (Morrison & Davis, 2003).

JIP1 and JIP2 are structurally similar. JIP1 is a scaffold protein that binds some members of the JNK signaling cascade. In addition to JNKs and MKK7, several MLKs, including MLK2, MLK3, DLK and LZK, have been shown to interact with JIP1 (Whitmarsh et al., 1998; Yasuda et al., 1999). Furthermore, guanine nucleotide exchange factors (GEFs) like Tiam, kinases like AKT, and phosphatases like MAPK phosphatase 7 (MKP7) are also found in association with the JIP scaffolds (reviewed in (Morrison & Davis, 2003)). Coexpression of JIP1 with MLK3 potentiates JNK activation, suggesting that the role of scaffolds is to facilitate signaling by organizing the cascade module. However, the proposed mechanism for JIP-mediated DLK activation of JNK is more complex than just providing docking regions for the different components of the signaling pathway. Under basal conditions, DLK is bound to JIP in a monomeric, inactive form. Upon stimulation, JNK is recruited to JIP1 and phosphorylates it. DLK now dissociates from JIP1, dimerizes and becomes catalytically competent. Activated

DLK phosphorylates JIP1-bound MKK7, which in turn phosphorylates JIP1-bound JNK and activates it (Nihalani et al., 2001; Nihalani et al., 2003).

Although initial studies suggested that JIP1 and JIP2 bound the same repertoire of proteins of the JNK pathway, later experiments indicated that the binding of JIP2 to JNK is weaker than that of JIP1, and that JIP2 instead prefers p38α and p38δ as binding partners (Buchsbaum et al., 2002; Schoorlemmer & Goldfarb, 2001). JIP2 associates with MLK3, MKK3 and p38 isoforms. In addition, JIP2 associates with Tiam1 and Ras-GRF, which are GEFs for the small GTPase Rac. Expression of these GEFs enhances the association between JIP2 and MLK3 (Buchsbaum et al., 2002), presumably by facilitating the activation of MLK3 through Rac and the formation of an activated complex between MLK3 and JIP2.

JIP3 and JIP4 are similar to each other. JIP3 (also known as JNK/stress-activated protein kinase-associated protein 1 (JSAP1)) has been shown to interact with JNK, MKK7 and members of the MLKs (Kelkar et al., 2000). However, JIP3 can also associate with the MAPKKK MEK kinase 1 (MEKK1) and MKK4 (Ito et al., 1999), and with ASK1 (Matsuura et al., 2002). Similarly, JIP4 interacts with MEKK3 and MKK4 (Lee et al., 2002).

Because all of the JIP proteins associate with kinesin motor complexes, it is possible that the JIP proteins play a role in kinesin-mediated transport and could have roles in regulating the subcellular localization of the JNK signalosome.

In addition to JIPs, Plenty of SH3s (POSH) is another scaffold protein that associates with the small GTPase Rac, and in overexpression studies it promotes apoptosis by activating the JNK pathway (Tapon et al., 1998). POSH interacts directly

with activated Rac1, MLKs (MLK1-3 and DLK), and indirectly with MKK4/7 and JNKs (Xu et al., 2003). POSH-induced apoptosis can be blocked by treatment with the MLK inhibitor CEP-1347 (Xu et al., 2003). Akt2 has also been reported to bind to the POSH-MLK-MKK-JNK complex, and the interaction of Akt2 with POSH seems to disassemble the JNK signalosome and to downregulate JNK signaling (Figueroa et al., 2003). Recently, it has been shown that POSH and JIPs directly bind to each other to form a multiprotein complex that includes all of the known kinase components of the pathway (Kukekov et al., 2006).

Post-synaptic density protein 95 (PSD-95) is a neuronal protein composed of three PDZ domains, an SH3 domain, and a catalytically inactive guanylate kinase domain (Garcia et al., 1998). PSD-95 interacts with glutamate receptor 6 (GluR6) through one of the PDZ domains. The SH3 domain of PSD-95 interacts with MLK2 and MLK3. Coexpression of kinase defective MLK2 or MLK3 significantly diminishes JNK activation and neuronal toxicity mediated by GluR6. These data suggest that PSD-95 acts as a scaffold protein to mediate signaling from ion channels to JNK by anchoring MLK2 or MLK3 to the GluR6 receptor complex (Savinainen et al., 2001).

Connector enhancer of KSR (CNK) was identified in a genetic screen for genes that could enhance the phenotype observed in the fly when the kinase domain of KSR is overexpressed (reviewed in (Kolch, 2005)). There are three mammalian homologues of CNK, CNK1-3. The widely expressed CNK1 interacts with Raf-1 and c-Src (Ziogas et al., 2005) to enhance Raf-1 activity. However, another report indicates that CNK1 can also bind members of the JNK pathway, namely MLK2 and MLK3, and MKK7, to induce c-Jun phosphorylation downstream of the small GTPase Rho (Jaffe et al., 2005).

At present, it is not known whether these activities are performed by different pools of CNK molecules, or whether overlapping binding partners link the different regulatory functions of CNK1.

3.3.6 Regulation of MLKs by chaperone proteins

Recently, an affinity purification procedure was used to isolate MLK3 protein complexes from cells inducibly expressing MLK3. Electrospray/Mass spectrometry analysis of the mixture identified Hsp90 and Cdc37 as binding partners of MLK3 (Zhang et al., 2004). Hsp90 is one of the most abundant proteins in eukaryotic cells. It functions as a molecular chaperone to prevent aggregation of its client proteins. In mammalian cells, there are two genes encoding cytosolic Hsp90 homologs, and there are also distinct isoforms localized in the endoplasmic reticulum (Grp94) and in mitochondria (TRAP-1). Hsp90 consists of three major domains: a highly conserved NH_2-terminal ATPase domain, a middle domain, and a COOH-terminal dimerization domain. The clients of Hsp90 include steroid hormone receptors and protein kinases, such as Src, Raf, and Cdk4 among others. The co-chaperone Cdc37 is required for the interaction of Hsp90 with its client protein kinases, which occurs through the kinase domain (reviewed in (Pearl, 2005; Wegele et al., 2004)).

MLK3 also associates with Hsp90/Cdc37 through the kinase domain, and like other protein kinases, the interaction is not dependent on the activation state of MLK3. Inhibition of the ATPase activity of Hsp90 with geldanamycin decreases the protein levels of endogenous MLK3, which suggests that Hsp90 helps MLK3 to fold properly, and that in the absence of chaperone activity MLK3 is unstable (Zhang et al., 2004).

3.3.7 Regulation of MLKs by phosphorylation

Phosphorylation is a versatile mechanism to regulate protein kinase activities. Activation loop phosphorylation is the predominant event involved in the activation of most protein kinases. As mentioned before, the activation loop is flanked by two motifs very conserved among protein kinases, DFG and APE. Analysis of the sequence of the activation loop of MLK3 indicates that there are four potential phosphorylation sites within the loop: Thr 277, Thr 278, Ser 281 and Thr 285. Replacement of Thr 277 or Ser 281, but not Thr 278, with Ala generates a form of MLK3 with low kinase activity, whereas replacement of the Thr 277 and Ser 281 with Asp residues results in a kinase as active as wild type MLK3, suggesting that Thr 277 and Ser 281 could be activation loop sites (Leung & Lassam, 2001). A commercially available phospho-specific antibody has been generated against a peptide containing the activation loop sequence of MLK3 phosphorylated at Thr 277 and Ser 281. This antibody recognizes MLK3 when it has been coexpressed in cells with activated Cdc42 (Du et al., 2005). Under the same experimental conditions, a kinase defective form of MLK3 does not react with the phospho-specific antibody, consistent with the idea that MLK3 autophosphorylates its activation loop sites. The reactivity with the phospho-specific antibody correlates with increased catalytic activity of MLK3, supporting the idea that Thr 277 and Ser 281 are the *bona fide* activation loop sites of MLK3.

An alignment of the sequences of the activation loop of the MLK subfamily is shown in Figure 6. The sequences of the activation loop of MLK1 and of the activation loop of MLK3 differ in only 3 out of 26 residues, with the MLK3 activation loop sites conserved in MLK1. Surprisingly, mutagenesis studies indicate that the activation of

Figure 6. **Alignment of the sequences of the activation loop of the MLK subfamily members**. The activation loop sequences from the human MLKs, as well as mouse MLK3 (mMLK3) and the *Drosophila* (Slpr) and *Xenopus* (xMLK) orthologs of the MLKs are displayed. The phosphorylation sites identified in human MLK3 are highlighted in *sky blue* box. The phosphorylation site identified in human MLK1 is highlighted in *yellow* box.

MLK1 requires phosphorylation of Thr 312, which is equivalent to Thr 285 in MLK3 (Durkin et al., 2004). The availability of the crystal structures of the kinase domains of the MLKs might enlighten the reasons for this difference.

In HepG2 liver cancer cells, insulin treatment results in the activation of phosphatidylinositol 3-kinase (PI3K), which generates phosphoinositol 3 phosphate (PIP_3) at the cell membrane. The protein kinase Akt is activated by its association with PIP_3, as well as by phosphorylation by other protein kinases at the membrane. Akt is suggested to have a positive impact on cell survival, blocking the processes that lead to apoptosis. It has been shown that upon insulin treatment, Akt is activated; under those conditions, MLK3, MKK7 and JNK catalytic activities are attenuated. The data suggest that Akt phosphorylates Ser 674 on MLK3, negatively affecting MLK3 activity and MLK3-mediated JNK activation and apoptosis (Barthwal et al., 2003).

Mass spectrometry coupled with phosphopeptide mapping analysis was used to identify *in vivo* phosphorylation sites on MLK3 (Vacratsis et al., 2002). One of the sites is located between amino acids 11-37 of MLK3, but the exact residue could not be assigned. Eleven serines were positively identified as phosphorylation sites; all of them are located beyond the CRIB motif on the COOH-terminal region of MLK3. From these eleven sites, seven correspond to Ser residues immediately followed by a Pro residue, which suggests that MLK3 might be a substrate for proline-directed kinases. This idea will be discussed in detail in Chapter II.

3.3.8 Regulation of MLKs by subcellular localization

One way to regulate the activity of protein kinases is by modulating their accessibility to their substrates. Changes in subcellular localization of protein kinases might bring them in close proximity to a particular target, thus regulating signal specificity.

Initial immunohistochemical studies in NIH 3T3 cells indicate that endogenous DLK localizes to a perinuclear region. Treatment of cells with Brefeldin A, a fungal toxin that dismembers the stacks of the Golgi apparatus, results in a dispersed cytosolic localization of DLK, strongly suggesting that DLK is localized to the Golgi (Douziech et al., 1999). DLK present in a crude membrane fraction preparation could only be solubilized in the presence of Triton-X114, indicating that DLK is a peripheral protein associated with the cytosolic face of the Golgi membranes (Douziech et al., 1999). The activation status of the Golgi-associated DLK has not been examined; therefore, it is not currently known how changes in DLK activity correlate with its localization to the Golgi apparatus. Similarly, ectopically expressed MLK3 shows some localization to a perinuclear region in HeLa cells (Du et al., 2005). Since both the wild type form and a kinase defective mutant form of MLK3 have similar subcellular distributions, at least in biochemical fractionations (Du et al., 2005), the presence of MLK3 in the Golgi/perinuclear region is unlikely to be regulated through activation of MLK3. However, as mentioned in the previous section, maximal activation of MLK3 upon coexpression with Cdc42 requires translocation to the plasma membrane.

MLK2 microinjected into the nuclei of Swiss 3T3 cells localizes along microtubules (Nagata et al., 1998). The staining pattern is punctate, and radial.

Interestingly, a truncated form of MLK2 which lacks the SH3 domain and the kinase domain retains the same punctuate cellular distribution. However, only when wild type MLK2, but not the truncated form of MLK2, is microinjected into cells, does activated JNK colocalize to microtubules, supporting the idea that they form a functional signaling complex which might play a role in cellular trafficking. Yeast-two-hybrid studies using MLK2 as a bait identified members of the kinesin superfamily proteins (KIFs) as interacting partners of MLK2 (Nagata et al., 1998). KIFs are molecular motor proteins that transport organelles, mRNAs and protein complexes in vesicles along microtubules to different locations within the cell, in an ATP-dependent manner (Miki et al., 2001). MLK2 and MLK3 were shown to interact with the kinesin motor protein KIF3, and with the putative cargo recognition molecule KAP3. These data suggest that MLK2 and MLK3 could be involved in regulating vesicular trafficking.

3.4 The physiological roles of the MLKs

Studies regarding the function of the MLKs have been related to their role as upstream activators of the JNK pathway and their effect in apoptosis, specifically in neuronal cells. Some of the studies are outlined below.

3.4.1. The *Drosophila* MLK, Slpr

In *Drosophila,* the process of dorsal closure is regulated by the JNK pathway. In this process, the dorsal ectoderm moves from a lateral position to the dorsal midline, to cover the embryo in a continuous protective epidermis (Noselli, 1998). The components of the signaling pathway required for dorsal closure are the GTPase dRac1, a

MAPKKKK called Misshapen (Msn), Slpr, the MKK7 ortholog Hemipterous (Hep), the JNK ortholog Basket (Bsk), and the AP-1 transcription factors dJun and dFos, which are encoded by *Jun-related antigen* (*Jra*) and *kayak* (*kay*), respectively (reviewed in (Stronach, 2005; Xia & Karin, 2004)).

JNK has two functions during epithelial cell-sheet morphogenesis. One of them is the expression of the cytokine Decapentaplegic (Dpp), which is orthologous to bone morphogenic protein 4, at the leading edge of the epithelial sheet. AP-1 activation is required for the expression of Dpp. *Slpr* mutants fail to express *dpp*, consistent with the lack of activation of Bsk (Stronach & Perrimon, 2002). The other function of JNK is the control of stretched epithelial cell morphology. Mutants in the *slpr* gene cannot maintain the stretched morphology and the cells round up, indicating that this MLK is involved in the regulation of cell shape during dorsal closure. Interestingly, whole-mount *in situ* hybridization of *Xenopus laevis* embryos shows early expression of the *Xenopus* ortholog of the MLKs (xMLK2) in the cement gland, a mucus-secreting epithelium that forms from the extreme anterior ectoderm (Poitras et al., 2003). In later stages, xMLK2 is found in the brain and pronephros. In the case of the cement gland and pronephros, the expression of xMLK2 correlates with the formation of epithelia (Poitras et al., 2003). Thus, it seems that the MLKs might be playing a general role during differentiation of epithelial layers.

3.4.2 Mammalian MLKs

Using RNAi, it has been shown that MLK3 is required for the proliferation of tumor cell lines (Chadee & Kyriakis, 2004). The data indicate that MLK3 and B-Raf

exist in a complex in cells, and that MLK3 is required for the activation of B-Raf, which in turn results in the activation of ERK and in the induction of cell proliferation. Recently, the generation of the *mlk3* knockout mice has been reported (Brancho et al., 2005). Mice derived from targeted gene disruption of the *mlk3* gene are viable and show no obvious defects. In primary mouse embryonic fibroblasts (MEFs) derived from *mlk3* knockout mice, there is a 50% reduction in TNF-α mediated activation of JNK, suggesting that MLK3 is necessary for full activation of the JNK pathway upon TNF-α stimulation, but that other MAPKKKs may also partially contribute or at least compensate for the lack of MLK3 (Brancho et al., 2005). MEFs from *mlk3*$^{-/-}$ mice do not display deficient activation of the MAPKs in response to various growth factors (Brancho et al., 2005), whereas the RNAi studies indicate that the activation by cytokines and mitogens of JNK, ERK and p38 is dependent on MLK3 (Chadee & Kyriakis, 2004). One possible explanation for this discrepancy is that in the knockout mouse, compensatory mechanisms are prone to occur during development. If other MLKs can substitute for MLK3 functions, then only non-redundant roles for MLK3 will emerge. However during transient knock down of MLK3 expression by RNAi, the cellular phenotypes observed would correspond to more immediate effects of MLK3 ablation.

A cumulative body of work implicates the MLKs in JNK-mediated apoptosis in neuronal cells. NGF deprivation induces apoptosis, and this event is dependent on the activation of the JNK pathway (Mielke & Herdegen, 2000). Overexpression of MLK2, MLK3 or DLK induces apoptosis in neuronal-like PC12 cells (Xu et al., 2001). Overexpression of MLK3 in superior cervical ganglion (SCG) sympathetic neurons activates JNK and induces apoptosis, while expression of a dominant negative MLK3

blocks apoptosis induced by NGF deprivation (Mota et al., 2001). Likewise, the MLK inhibitor CEP-1347 protects primary neurons in culture from NGF-deprivation-induced apoptosis (Maroney et al., 1998). In addition, animal models of chemical-induced neurotoxicity show that CEP-1347 effectively protects *in vivo* (Saporito et al., 1999). Altogether the data suggest that the MLKs are activated by NGF withdrawal and this turns on the JNK pathway and promotes apoptosis.

Huntington's disease is a neurodegenerative disorder produced by mutations in the gene of the Huntingtin protein. Polyglutamine stretches are added at the NH_2-terminus of the protein causing it to aggregate inside neuronal bodies (Morfini et al., 2005). The SH3 domain of MLK2 has been shown to interact with normal Huntingtin, but not with the polyglutamine expanded mutant protein. Likewise, MLK3 also interacts with normal Huntingtin in GST-pulldown assays using the SH3 domain of MLK3 (Geou-Yiarh Liou, Gallo lab). Expression of catalytically inactive MLK2 can diminish neuronal apoptosis induced by mutant Huntingtin (Liu et al., 2000b). The proposed model suggests that normal Huntingtin and MLK2 interact in neuronal cells, and that this association keeps MLK2 inactive. In Huntington's disease, polyglutamine expanded Huntingtin can no longer associate with MLK2. This free MLK2 can now be activated and can promote apoptosis. However, the inability of MLK2 to interact with polyglutamine expanded Huntingtin may just be due to the aggregation of the mutated Huntingtin. Further experiments are needed to clarify the role of the MLKs in Huntington's disease.

Finally, the MLKs have also been involved in the regulation of the cell cycle. Cell cycle studies in the filamentous fungus *Aspergillus nidulans* have demonstrated that

proper control of the G2/M transition requires the activity of a serine/threonine protein kinase called never in mitosis A (NIMA). Loss of NIMA function causes cell cycle arrest in G2 phase (Osmani et al., 1991). In addition to its kinase catalytic domain, NIMA has a long COOH-terminal region that is necessary and sufficient to induce G2/M arrest when overexpressed in human cells (Lu & Hunter, 1995). The COOH-terminal region of NIMA, and the region between amino acids 365-847 of MLK3 were found to share 16% identity and 22% similarity (Swenson et al., 2003). In essence, the sequence similarities between NIMA and MLK3 are due to the presence of a kinase domain, central domains predicted to be coiled-coils, and a COOH-terminal region rich in Ser/Thr-Pro motifs. The catalytic activity of NIMA is enhanced during G2/M; during early mitosis, the electrophoretic mobility of MLK3 is retarded due to phosphorylation, which correlates with increased catalytic activity of MLK3. However, this MLK3 activation does not translate into JNK activation, suggesting a JNK-independent role for MLK3 during the cell cycle. Analysis of the subcellular distribution of ectopically expressed NIMA or MLK3 revealed that they both localize to the centrosome, as judged by costaining with γ-tubulin. Interestingly, the subcellular distribution of endogenous MLK3 varies in the different stages of the cell cycle; during interphase and prophase, MLK3 appears as a very bright spot located near the nucleus that corresponds to the centrosomes. During metaphase, MLK3 staining becomes totally cytosolic, and it only relocates to the centrosome after cytokinesis is complete (Swenson et al., 2003). Other reports indicate that MLK3 localizes to a Golgi/perinuclear region (Cha et al., 2004; Du et al., 2005), but the bright spot of centrosomal localization has not been reproduced in our hands. Loss of NIMA leads to cell cycle arrest; however, ablation of MLK3 expression by RNAi does

not result in a cell cycle arrest in SAOS human osteosarcoma cells (Swenson et al., 2003). In contrast, the MLK inhibitor CEP-11004 delays mitotic progression and promotes the formation of aberrant mitotic spindles in HeLa cells (Cha et al., 2006). The results obtained using CEP-11004 should be carefully considered, since its ability to inhibit other protein kinases has not been well characterized and therefore the effects seen may very well correspond to off target events. Interestingly enough, the levels of the MLK ZAK increase during G2/M in MDCK cells stably expressing ZAK (Gross et al., 2002; Liu et al., 2000a). Catalytically inactive ZAK attenuates γ-irradiation-induced cell cycle arrest. At present, the exact role of the MLKs in the regulation of cell cycle progression is not clear.

4. Objective of the Thesis

Protein kinases are key regulators of a variety of cellular processes. Regulation of their activities is at the center of cellular homeostasis. My thesis work has been directed towards understanding the molecular mechanisms that regulate the various activities of MLK3.

As described in the previous sections, MLK3 is a widely expressed serine/threonine kinase whose major function has been ascribed as an upstream activator of the JNK pathway. MLK3 contains several domains that regulate its activity, including an SH3 domain, a leucine zipper region and a CRIB motif. Our current working model for MLK3 activation proposes that disruption of the SH3-mediated autoinhibitory intramolecular interaction allows MLK3 to dimerize and transphosphorylate in its activation loop. Activated MLK3 is competent to modulate JNK activation through

phosphorylation of MKK4 and MKK7. The interaction of MLK3 with the small GTPase Cdc42 might trigger the activation process, and also results in relocalization of MLK3 to the plasma membrane.

MLK3 is a multiphosphorylated protein. Activation of MLK3 relies on phosphorylation of its activation loop. In addition, several other phosphorylation sites have been identified in MLK3, most of which correspond to phosphorylated Ser residues followed by Pro residues (SP sites) that could potentially be phosphorylated by proline-directed kinases. The research work presented in this dissertation investigates whether MLK3 is regulated by proline-directed kinases.

Chapter II investigates which proline-directed kinase phosphorylates MLK3 on SP sites. This study also evaluates the conditions in which phosphorylation at the SP sites occurs and the dynamics of the process. Chapter III examines the changes in the subcellular distribution of MLK3 as a result of phosphorylation by proline-directed kinases. Chapter IV summarizes the results presented in this work and presents the most current working model for the regulation of MLK3, as well as final remarks and future directions. Finally, an Appendix compiles the information obtained from site-directed mutagenesis studies of some of the phosphorylation sites on MLK3 and their effect on MLK3 catalytic activity.

II. Phosphorylation of Mixed Lineage Kinase 3 by Proline-directed Kinases

1. Abstract

Mixed-lineage kinase 3 (MLK3) is a widely expressed, mammalian serine/threonine protein kinase that activates multiple MAPK pathways. MLK3 induces JNK activation through phosphorylation of MKK4 and MKK7. MLK3, and other MLKs, have been implicated in JNK-mediated neuronal cell death in response to trophic factor withdrawal. However, MLK3 also appears to play key roles in cellular proliferation, especially in the context of cancer cells. Previously our lab used *in vivo* labeling/mass spectrometry to identify phosphorylation sites of activated MLK3. Seven of eleven identified sites correspond to the consensus motif for phosphorylation by proline-directed kinases. Based on these results, I hypothesized that JNK, or another proline-directed kinase, phosphorylates MLK3 as part of a feedback loop. Using a combination of pharmacological inhibitors and modulators of JNK activity, I show that MLK3 can be phosphorylated by JNK *in vitro* and *in vivo*. Blockade of JNK results in dephosphorylation of MLK3. My data indicate that COOH-terminal JNK phosphorylation is critical for the regulation of MLK3 levels in cellular lysates. This work describes a novel mode of regulation of MLK3 and its signaling to JNK. My findings have important implications for processes involving sustained JNK activation, including apoptosis.

2. Introduction

Protein kinases and their signaling cascades are dynamically regulated through protein-protein interactions, subcellular targeting, and phosphorylation. By differential integration of these regulatory mechanisms, a protein kinase and its signaling pathway can lead to distinct cellular responses. In addition, many protein kinases are subjected to feedback regulation, and biological signaling pathways are modulated by positive and negative regulatory feedback loops.

Our lab has focused on understanding the mechanisms that regulate MLK3 activity and signaling. In addition to its catalytic domain, MLK3 contains several other regions important for its regulation, including an SH3 domain and a centrally located zipper followed by a Cdc42/Rac Interactive Binding (CRIB) motif. Notably, the COOH-terminal region of MLK3 is rich in Ser, Thr, and Pro residues, with the final 220 amino acids of MLK3 being comprised of 12%, 13%, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, retard MLK3 electrophoretic mobility, increase MLK3 catalytic activity and potentiate signaling to JNK (Bock et al., 2000; Burbelo et al., 1995; Teramoto et al., 1996; Tibbles et al., 1996). Our recent work demonstrates that binding of activated Cdc42 promotes phosphorylation of Thr 277 and Ser 281 within the activation segment of MLK3 leading to increased MLK3 activity (Du et al., 2005). Additionally Cdc42, in a prenylation-dependent fashion, induces translocation of MLK3 to cellular membranes (Du et al., 2005).

MLK3 is multiphosphorylated *in vivo*. In addition, data from our lab indicate that coexpression of activated Cdc42 changes the *in vivo* phosphorylation pattern of MLK3

(Bock et al., 2000). Several *in vivo* phosphorylation sites of MLK3 have been identified using metabolic labeling coupled with mass spectrometry (Figure 1) (Vacratsis et al., 2002). Strikingly, seven of the identified sites, serines 524, 705, 727, 740, 758, 770 and 793, are followed immediately by a proline, thus conforming to the consensus for phosphorylation by proline-directed kinases. There are three well-characterized groups of proline directed kinases: the MAPKs, the Cyclin-dependent kinases (Cdks), and Glycogen Synthase Kinase 3 (GSK3). GSK3 is special in that it phosphorylates Ser or Thr residues in a "Ser-Pro" motif in proteins that have been already primed, by phosphorylation of another Ser or Thr (reviewed in (Frame & Cohen, 2001)). For the MAPKs and Cdks, some effort has been directed towards identifying the precise sequence requirements for substrate phosphorylation, but it seems that in most cases the exact sequence surrounding the phosphorylation site is not as relevant as the presence of a Ser/Thr-Pro motif.

The identification of sites of proline-directed phosphorylation of MLK3, together with the ability of MLK3 to activate multiple MAPK pathways, led me to hypothesize that MLK3 might be regulated through feedback phosphorylation by MAPKs. In this chapter, data is presented supporting JNK-mediated phosphorylation of MLK3 in cells. JNK also phosphorylates MLK3 *in vitro*. Manipulation of JNK activity in cells dramatically impacts net phosphorylation and the levels of MLK3 in cellular lysates. Taken altogether the data provides evidence for a novel positive feedback loop in which the MAPKKK, MLK3, is phosphorylated by its downstream MAPK, JNK, leading to stabilization of MLK3 and enhanced signaling through JNK.

3. Materials and Methods

3.1 Reagents and Antibodies

The following reagents were purchased from Calbiochem: SP600125, SB203580, U0126, Roscovitine, and Kenpaullone. Calyculin A was obtained from BioMol. The MLK inhibitor CEP-11004 was generously provided by Cephalon, Inc. Mouse monoclonal antibodies against phospho-c-Jun (KM-1) and GFP, were purchased from Santa Cruz Biotechnology, Inc. The phospho-MLK3 antibody and phospho-JNK antibody were from Cell Signaling Technology. Other antibodies used were the MLK3 rabbit polyclonal antibody (Bock et al., 2000), actin and Flag mouse monoclonal antibody (Sigma), hemagglutinin (HA) mouse monoclonal antibody (BAbCO), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

3.2 Construction of mammalian expression vectors and site-directed mutagenesis

Construction of the expression vectors pRK5-MLK3, pRK5-MLK3 K144R, pRK5-Flag-MLK3, pRK5-Flag-MLK3 1-598, pCGN-HA-MLK3 and pRK5 N-Flag $Cdc42^{V12}$ has been described elsewhere (Bock et al., 2000; Zhang & Gallo, 2001). MLK3 K144M was generated by the Quick Change Site-directed Mutagenesis method (Stratagene) using the following oligonucleotide: 5'CTGGTGGCTGTGATGGCAGCTCGCC 3', and its reverse complement. The MLK3 variant containing six point mutations was constructed by introducing successive point mutations into pCGN-HA-MLK3. The primers used for mutagenesis were: 5'-GACCTCGGCCCGCGCCCCTTCGCAG-3' (S770A);

5'-ACCCCACGTGCACCACCCCTG-3' (S758A);

5'-GGTCAGCCAAGGCCCCCCGACGTGA-3' (S727A);

5'-TCTCCCCTGCCAGCACCACAGCCTGCACCC-3' (S793A);

5'-CTCAAGACGCCCGCAGCCCCGCCCAC-3' (S705A) and

5'-GGAGGCACTGTCGCACCCCCACCGG-3' (S740A), and their reverse complements.

Flag-MKK7 K165A and GST-MKK4 K129R were generated using pCDNA3-Flag-MKK7 and pEBG-GST-MKK4 as templates, and the following oligonucleotides and their reverse complements: 5'- CATTGCTGTTGCGCAAATGCG-3' for MKK7, and 5'- CAGATAATGGCAGTTAGAATTCGGTCAACT-3' for MKK4. Other mammalian expression vectors include pSRα3HA-JNKK2-JNK1 (kindly provided by Dr. Anning Lin, U. of Chicago), pCDNA3-Flag-ubiquitin (kindly provided by Dr. Kun-Liang Guan, U. of Michigan) and pCMS-EGFP (kindly provided by Dr. Lloyd Greene, Columbia University).

3.3 Cell culture and transfections

Human embryonic kidney (HEK) 293 cells were maintained in Ham's F12:low glucose Dulbecco's modified Eagle's media (DMEM) (1:1) supplemented with 8% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. Transfections were performed using the calcium phosphate technique or using LipofectAMINE 2000 (Invitrogen), following manufacturer's instructions. MCF-7/iFLAG-MLK3 cells inducibly expressing Flag-MLK3 have been

described elsewhere (Zhang et al., 2004), and were maintained in DMEM supplemented with 5% fetal bovine serum, 40 μg/mL neomycin and 10 μg/mL hygromycin. MCF7 cells inducibly expressing Flag-Estrogen Receptor were kindly provided by Dr. Susan Conrad (Michigan State University). Flag-MLK3 expression as well as Flag-Estrogen Receptor expression was induced by the addition of 50 nM AP21967 for 20 h.

3.4 Cell lysis and immunoprecipitation

Cells were washed with ice cold PBS and lysed by addition of lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na_4PP_i, 100 μM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM PMSF) complemented with a cocktail of protease inhibitors (Sigma). Lysates were clarified by centrifugation for 15 min at 14,000 rpm at 4°C. For immunoprecipitations, antibodies against the proteins of interest were prebound to Protein A-agarose beads at room temperature for 30 min and incubated with clarified cell lysates for 90 min at 4°C. The immunoprecipitates were washed with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol).

3.5 Gel electrophoresis and western blot analysis

Cellular lysates and immunoprecipitates were separated by SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and immunoblotted using appropriate antibodies. Western blots were developed by the chemiluminescence method as described previously (Bock et al., 2000).

3.6 Pro-Q Diamond and Silver Staining

HEK 293 cells were transfected with vectors encoding MLK3 or MLK3 K144M as described. MLK3 was immunoprecipitated as described above and resolved by SDS-PAGE. The gel was stained with Pro-Q Diamond stain (Invitrogen) following the manufacturer's instructions. The image was acquired using a Molecular Imager FX Pro Plus scanner and PDQuest v7.4 (Bio-Rad). The gel was then silver stained using SilverQuest (Invitrogen) following manufacturer's instructions. Quantitations were done by densitometry (ImageJ, http://rsb.info.nih.gov/ij/).

3.7 *In vivo* labeling and phosphopeptide mapping

Twelve hours after transfection, HEK 293 cells were washed twice with phosphate-free medium (Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum (Summit Biotechnology)), and incubated at 37 °C for 1 h. The cells were then pre-treated with the different MAPK pathway inhibitors (40 μM SP600125; 6 μM SB203580; 10 μM U0126) or vehicle DMSO for 1 h before addition of 1 mCi/ml [^{32}P]-orthophosphate (PerkinElmer Life Sciences, Inc), and further incubated for 4 h at 37°C. Cells were washed twice with ice-cold PBS and then lysed as described above. MLK3 was immunoprecipitated as described above and resolved by SDS-PAGE. Incorporation of radiolabel into MLK3 was quantified by phosphorimaging, and normalized to the MLK3 expression levels, determined by densitometry.

Radiolabeled bands were excised from the gel, transferred to siliconized eppendorf tubes and rehydrated with 4 μl of water. The gel pieces were washed twice with 500 μl of 0.1 M NH_4HCO_3, 50% acetonitrile at 30°C for 50 min. The gel pieces

were completely dried using a SpeedVac, partially rehydrated with 4 µl of 0.1 M NH_4HCO_3, 0.02% Tween 20, and incubated with 40 µl of 50 mM NH_4HCO_3 containing 2 µg of trypsin (Roche) overnight at 30°C. The supernatant was then collected, and the gel pieces were sonicated in 100 ul of a 60% acetonitrile, 0.1% trifluoroacetic acid solution, for 30 min. The supernatant was collected and the sonication repeated in 50 µl of the same solution. The supernatants were combined and concentrated using a speedvac. The peptides were separated on cellulose thin layer chromatography (TLC) plates by thin layer electrophoresis (TLE) in the first dimension in a pH 1.9 buffer (formic acid/glacial acetic acid/water, 25:78:897, v/v/v) at 0°C and 1000 V for 30 min, and separated by TLC in *n*-butanol/pyridine/glacial acetic acid/water, 15:10:3:12, v/v/v/v. The radiolabeled phosphopeptides were visualized and quantitated using a PhosphorImager.

3.8 *In vitro* phosphorylation of MLK3

HEK 293 cells were transfected with a vector encoding MLK3 K144M. Twenty hours after transfection the cells were washed and lysed in Lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). MLK3 K144M was immunopurified from clarified cellular lysates. Kinase reactions were carried out in 50 µl Kinase buffer (50 mM Tric-Cl pH 7.5, 0.1 mM EGTA, 10 mM $MgCl_2$, 0.1% β-mercaptoethanol) containing 10 µM ATP, 10 µCi [γ-^{32}P]-ATP (3000 Ci/mmol, PerkinElmer Life Sciences, Inc) and 0.5 µg (57 mU) of recombinant JNK1α1 (Upstate), at 37°C for 30 min. Proteins were fractionated by SDS-PAGE, and the incorporation of radioactivity into MLK3 was measured by phosphorimaging.

3.9 Northern blot analysis

Flag-MLK3 expression was induced by incubation of MCF-7/iFLAG-MLK3 cells with 50 nM AP21967 for 20 h. Cells were then incubated with 15 μM SP600125 for different periods of time. Total RNA was extracted using TRIzol (Invitrogen) following manufacturer's instructions. Forty micrograms of RNA were fractionated on a 1% formaldehyde/agarose gel and transferred to a nitrocellulose membrane. pRK-MLK3 was digested with BstEII to generate a probe encompassing nucleotides 198-1836 of the MLK3 gene. The random primer DNA labeling system (Invitrogen) was used together with 50 μCi of [α-^{32}P]dCTP to radiolabel the probe, following manufacturer's instructions. The membrane was pre-hybridized in 15 mL of pre-hybridization solution (50% formamide, 5X SSPE (20X SSPE: 3M NaCl, 0.23M NaH_2PO_4, 0.02M EDTA, pH 7.7), 2X Denhardt's, 0.1% SDS, 100 μg/mL salmon sperm DNA) 1 h at 42°C. The radiolabeled probe was then added to the solution and the membrane was incubated overnight at 42°C. The membrane was then washed once at room temperature in 1X SSC (20X SSC: 3M NaCl, 0.3M sodium citrate, pH 7.0)/0.1 % SDS, and twice at 42°C in 0.2X SSC/0.1% SDS. The radiolabeled Flag-MLK3 mRNA was visualized by phosphorimaging.

4. Results

4.1 JNK Is Required for *in vivo* Phosphorylation of MLK3

Using mass spectrometry coupled with comparative phosphotryptic peptide mapping, we previously identified eleven *in vivo* phosphorylation sites on MLK3 (Figure 1) (Vacratsis et al., 2002). In our strategy, HEK 293 cells expressing MLK3 with its activator $Cdc42^{V12}$ for 16 h were subsequently labeled with [^{32}P]-orthophosphate for an additional 4 h. After affinity purification and trypsin digestion of MLK3, the resultant radiolabeled phosphotryptic peptides were resolved by chromatography and their sequences were determined using a combination of MALDI-MS and LC-MS/MS. Thus only those sites which are relatively labile (undergo dephosphorylation) and incorporate substantial radiolabel (undergo rephosphorylation) during the 4 h labeling period could potentially be identified.

MLK3 contains 27 potential proline-directed kinase sites, most of which are found in the COOH-terminal region, which is rich in proline, serine, and threonine (Figure 1) (Gallo et al., 1994). Examination of the identified phosphorylation sites revealed that seven of the eleven sites conform to the consensus sequence for phosphorylation by proline-directed kinases; and six of the seven sites reside in the COOH-terminal region. This suggests that MLK3 is phosphorylated by proline-directed kinases. Additionally we have found that, whereas wild type MLK3 incorporates substantial radiolabel under our experimental conditions, a kinase-defective MLK3 variant incorporates very little radiolabel (P.O. Vacratsis and K.A. Gallo, unpublished data).

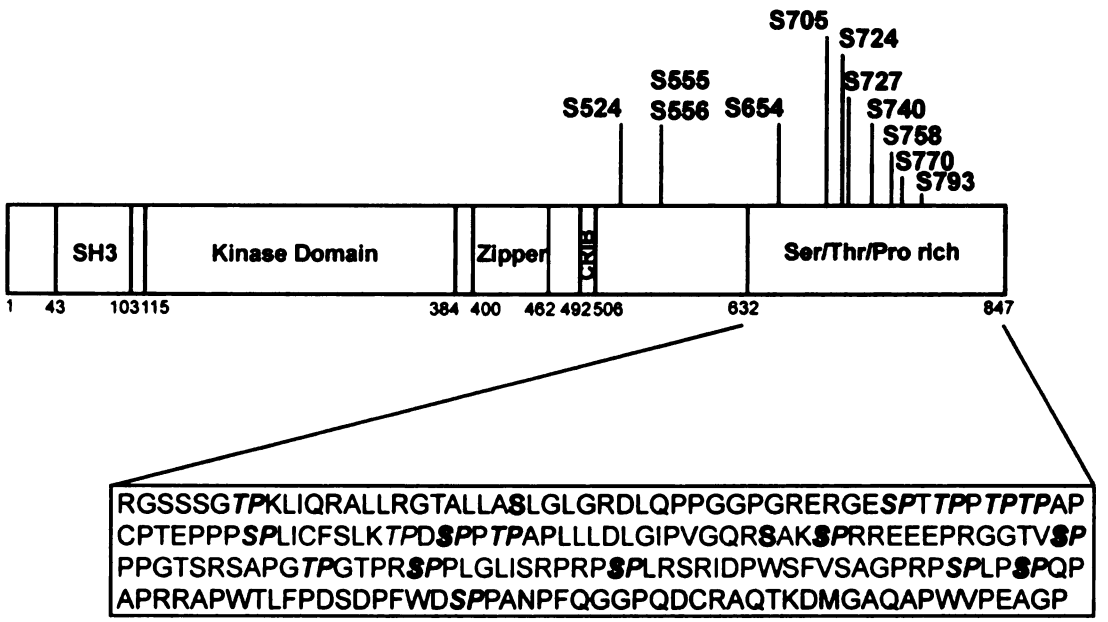


Figure 1. **Schematic representation of MLK3**. The Ser residues of the identified phosphorylation sites on MLK3 are indicated above. Numbers below the diagram correspond to amino acid numbers. The amino acid sequence of the proline/serine/threonine-rich region is shown below the diagram. TP and SP motifs are highlighted in *bold italic*. Identified phosphorylation sites are highlighted in *red*.

Total phosphate incorporation into MLK3 was determined using the Pro-Q diamond dye. This fluorescent stain detects phosphate groups attached to serine, threonine or tyrosine residues in a protein (Schulenberg et al., 2004; Steinberg et al., 2003). MLK3 or a kinase inactive version MLK3 K144M was expressed in HEK 293 cells. Immunopurified MLK3 variants were resolved by SDS-PAGE. After staining the gel with Pro-Q Diamond, densitometry was used to quantitate the relative incorporation of Pro-Q Diamond stain into the MLK3 variants (Figure 2). After correcting for expression levels, the extent of Pro-Q diamond staining was determined to be 40% less for kinase-defective MLK3 than for wild type MLK3. The increased phosphorylation of wild type MLK3 over that of inactive MLK3 is consistent with MLK3 autophosphorylation and/or feedback phosphorylation emanating from active MLK3. Given that MLK3 is implicated in the activation of multiple MAPK pathways, it is reasonable to hypothesize that, upon MLK3-induced activation, MAPKs might phosphorylate MLK3.

To decipher which MAPK(s) might contribute to proline-directed phosphorylation of MLK3, the impact of MAPK pathway inhibitors on the incorporation of radiolabeled phosphate into MLK3 was determined using the same conditions that were used in the original mass spectrometry studies. Concentrations of SP600125, U0126, and SB203580 required for inhibition of JNK, ERK (through inhibition of its upstream activator MEK), and p38, respectively, were experimentally determined in HEK 293 cells coexpressing MLK3 and activated Cdc42. Cells were pretreated with MAPK pathway inhibitors prior to and during *in vivo* labeling and, after affinity purification and SDS-PAGE, the incorporation of radiolabel into MLK3 was quantitated by phosphorimaging. Based on

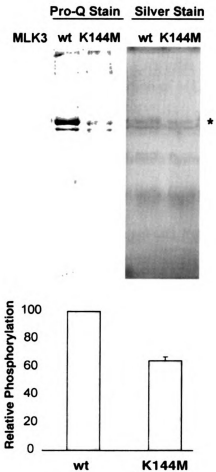


Figure 2. **Phosphorylation levels of MLK3**. Ectopically expressed MLK3 and MLK3 K144M were immunopurified from HEK 293 cells and resolved by SDS-PAGE. Pro-Q Diamond staining was used to detect total phosphorylation levels (*top panel, left*). Total amounts of proteins were detected by silver staining of the same gel (*top panel, right*). Asterisk denotes the position of the MLK3 band. Data from three independent experiments were normalized to total protein levels and quantitated as described under "Materials and Methods" (*bottom panel*).

three independent experiments, incorporation of radiolabel into MLK3 was reduced by 70% in the presence of SP600125 (Figure 3). In contrast, inhibition of the p38 and ERK pathways does not reduce *in vivo* phosphorylation of MLK3 (Figure 3). In fact, treatment with SB203580 enhances the phosphorylation of MLK3 by about 2-fold. This may reflect the established antagonism between p38 and JNK signaling (Hall & Davis, 2002). Phosphotryptic peptide maps of MLK3 isolated from cells treated with each of the MAPK pathway inhibitors separately, as well as all three together, were generated. The radiolabeled MLK3 was excised from the gel and trypsin-digested, and identical cellular equivalents of the phosphotryptic peptides were resolved by TLE/TLC, and radioactive phosphopeptides were detected by phosphorimaging. Phosphotryptic peptide maps from the different samples were processed and imaged in parallel so that the intensities of spots between maps could be directly compared. Treatment of cells with SP600125, but not with other MAPK pathway inhibitors, yielded phosphopeptide maps that showed a decrease in intensity of essentially all of the spots corresponding to identified phosphorylation sites (Figure 4). These findings are not altogether unexpected; phosphopeptide mapping analysis of a mutant version of MLK3, in which six of the serines phosphorylated on Ser-Pro motifs previously identified by mass spectrometry were mutated to alanine (MLK3 6A), indicated that proline-directed phosphorylation accounts for most of the phosphorylation detected on MLK3 when coexpressed with activated Cdc42 (Figure 5). Thus, the activity of JNK, but not of other MAPKs, is apparently responsible for the dynamic COOH terminal proline-directed phosphorylation of MLK3.

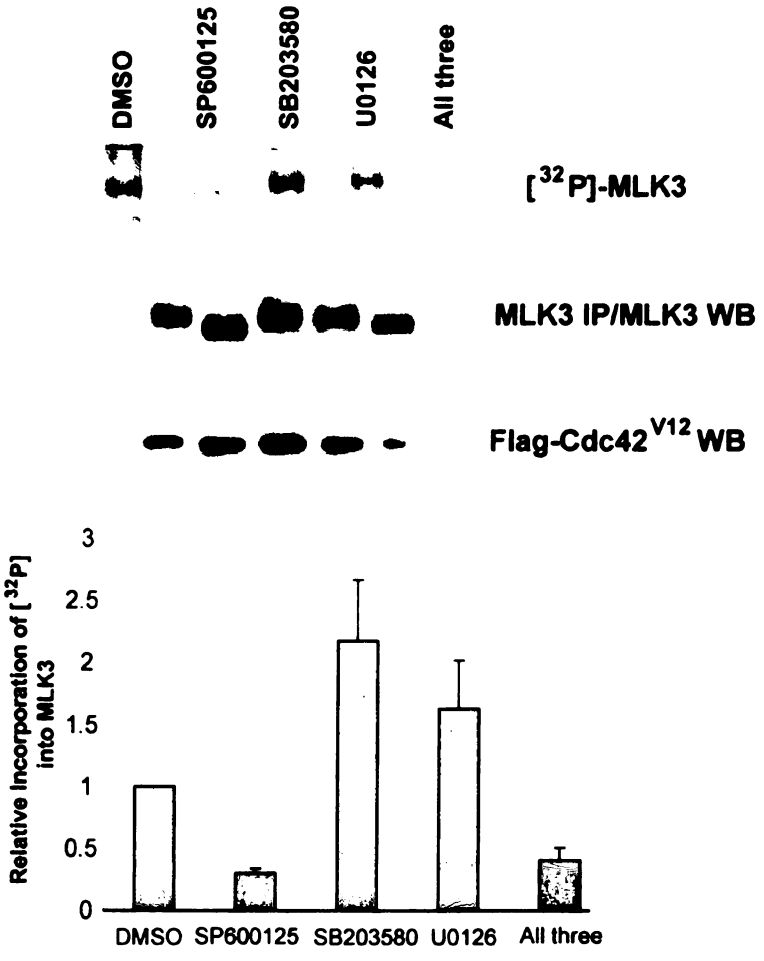
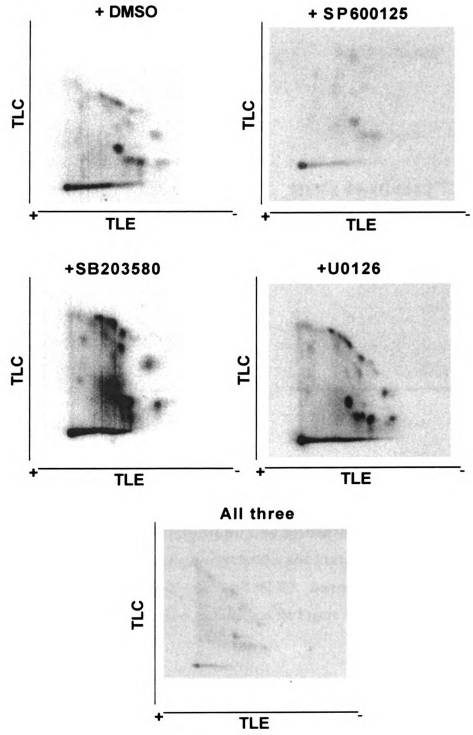


Figure 3. **Effect of MAPK pathway inhibitors on *in vivo* phosphorylation of MLK3.** HEK 293 cells transiently coexpressing MLK3 and Flag-Cdc42^{V12} were pre-incubated in phosphate-free medium containing either SP600125, SB203580, U0126, the combination of all three, or carrier (DMSO) as described in "Materials and Methods". After *in vivo* labeling with [^{32}P]-inorganic phosphate for 4 h in the presence of the MAPK pathway inhibitors, MLK3 was immunoprecipitated from cleared lysates and resolved by SDS-PAGE. *Top panel*, autoradiogram of immunoprecipitated MLK3. *Middle panel*, western blot of a fraction of the immunoprecipitated MLK3 using an MLK3 antibody. *Bottom panel*, western blot of Flag-Cdc42^{V12} in cellular lysates using a Flag antibody. Incorporation of radioactivity into MLK3 was quantified by phosphorimaging and normalized to MLK3 expression levels as described in "Materials and Methods". The means ± S.E. of three independent experiments are shown.

Figure 4. **Phosphotryptic peptide maps of MLK3 from cells metabolically labeled in the presence of MAPK inhibitors.** Radiolabeled MLK3 from HEK 293 cells incubated with the different MAPK inhibitors was fractionated by SDS-PAGE and the bands corresponding to MLK3 were excised from the gel and subjected to trypsin digestion. Portions of the recovered peptides corresponding to equal cellular equivalents were analyzed by thin-layer electrophoresis (TLE) in the first dimension and thin-layer chromatography (TLC) in the second dimension. Identical exposure times of the phosphopetide maps were analyzed by phosphorimaging. + and – indicate positive and negative poles, respectively.


+ DMSO
TLC
+
TLE
-
+ SP600125
TLC
+
TLE
-
+SB203580
TLC
+
TLE
-
+U0126
TLC
+
TLE
-
All three
TLC
+
TLE
-

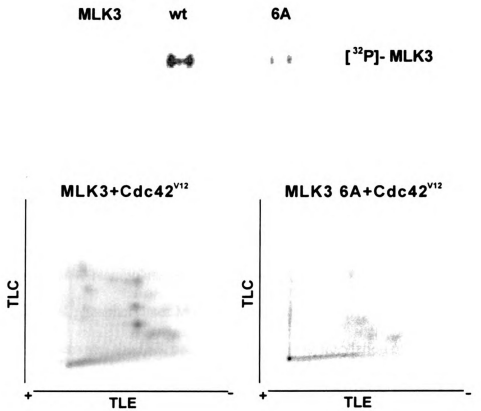


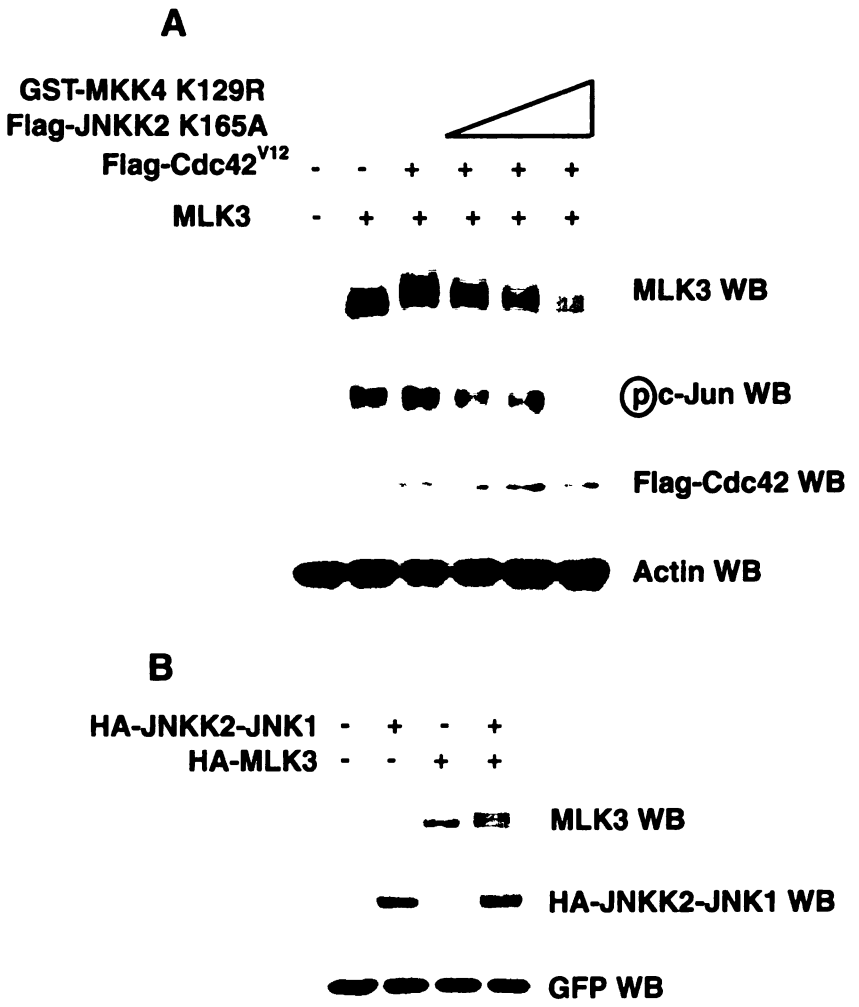
Figure 5. **Phosphopeptide mapping of MLK3 6A**. HEK 293 cells were transfected with expression vectors for MLK3 or MLK3 6A, together with activated Cdc42. Cells were *in vivo* labeled as described for Figure 3, except that the inhibitors were omitted. MLK3 was immunopurified from clarified lysates and fractionated by SDS-PAGE. *Top panel*, autoradiogram of immunoprecipitated MLK3. *Bottom panels*, phosphopeptide maps were developed and analyzed as described for Figure 4.

4.2 Manipulation of JNK Activity Alters the Phosphorylation of MLK3

Coexpression with activated Cdc42 retards the electrophoretic mobility of MLK3 due to phosphorylation at sites other than in the activation segment (Du et al., 2005). The reduced *in vivo* phosphorylation of Cdc42-activated MLK3 observed upon treatment of cells coexpressing activated Cdc42 and MLK3 with SP600125 is correlated with a more rapidly migrating, hypophosphorylated form of MLK3 (Figure 3). Because SP600125 may target other protein kinases in addition to JNK (Bain et al., 2003), I sought alternative means to manipulate cellular JNK activity so as to provide further support for the idea that JNK phosphorylates MLK3 *in vivo*.

MLK3 activates the JNK pathway through phosphorylation of MKK4 (Rana et al., 1996) and MKK7/JNKK2 (Tournier et al., 1997). Catalytically inactive versions of MKK4 (MKK4 K129R) and MKK7/JNKK2 (MKK7 K165A) can function as dominant-negative proteins to block JNK activity in cells. The blockade of JNK activation is most efficient when the inactive versions of MKK4 and MKK7 are coexpressed (Liu et al., 2000a). Therefore increasing amounts of expression vectors for MKK4 K129R and MKK7 K165A were simultaneously transfected into HEK 293 cells, together with vectors encoding MLK3 and activated Cdc42. Upon introduction of increasing amounts of dominant negative MKK4 and MKK7, efficient inhibition of the Cdc42-MLK3-induced JNK activity in cells was achieved, as judged by the decrease in phospho-c-Jun levels (Figure 6A, second panel). Under these conditions of efficient JNK inhibition, the electrophoretic mobility of MLK3 was restored to that observed for MLK3 in the absence of Cdc42 (Figure 6A, first panel). These results are similar to the effects of SP600125 on MLK3, suggesting that the effects of SP600125 are, indeed, mediated through inhibition

Figure 6. **JNK phosphorylation of MLK3**. *A*, HEK 293 cells were cotransfected with expression vectors for MLK3 (0.5 μg), Flag-Cdc42^{V12} (1 μg), and increasing amounts of Flag-MKK7/JNKK2 K165A and GST-MKK4 K129R (lanes 4, 5 and 6; 10,15 and 20 μg each, respectively). Cellular lysates were analyzed by western blotting using the indicated antibodies. *B*, HeLa cells were cotransfected with expression vectors for HA-MLK3, HA-JNKK2-JNK1 and GFP. Twenty four hours post-transfection the cells were lysed, and clarified lysates were analyzed by western blotting using the indicated antibodies. *C*, *in vitro* JNK phosphorylation of MLK3. Immunopurified MLK3 K144M was incubated with [^{32}P]-ATP in the presence (lane 1) or absence (lane 2) of recombinant, activated JNK1α1. Data shown is representative of three independent experiments.


A
GST-MKK4 K129R
Flag-JNKK2 K165A
Flag-Cdc42V12 - - + + + +
MLK3 - + + + + +
MLK3 WB
pc-Jun WB
Flag-Cdc42 WB
Actin WB
B
HA-JNKK2-JNK1 - + - +
HA-MLK3 - - + +
MLK3 WB
HA-JNKK2-JNK1 WB
GFP WB
C
MLK3 K144M
JNK1α1 - +
[32P]-MLK3
MLK3 IP/
MLK3 WB

of JNK, and that the retarded mobility of MLK3 observed upon coexpression with Cdc42 is mediated, at least in part, through JNK phosphorylation.

In a complementary approach, I sought to determine whether selective activation of the JNK pathway would promote phosphorylation of MLK3. However, the numerous extracellular stimuli that activate the JNK pathway invariably activate other MAPK pathways as well. The only MKK that has been shown to activate exclusively the JNK pathway is MKK7/JNKK2 (Tournier et al., 1999). Mutant forms of the appropriate upstream MKKs (MEK1, and MKK3 and MKK6), in which the activation loop sites have been replaced with acidic residues to mimic phosphorylation, can selectively activate ERK and p38, respectively (Cowley et al., 1994; Huang et al., 1997) whereas the corresponding mutant of MKK7/JNKK2 is not highly active (Zheng et al., 1999). However fusion of MKK7/JNKK2 to JNK1 results in a constitutively active JNK when expressed in cells (Zheng et al., 1999). To determine whether selective activation of JNK promotes MLK3 phosphorylation, HeLa cells were transfected with expression vectors for MLK3 and the fusion protein JNKK2-JNK1. As shown in Figure 6B, the introduction of activated JNK in cells results in the appearance of slower migrating forms of MLK3, indicative of phosphorylation of MLK3.

Since the majority of the identified phosphorylation sites of MLK3 conform to the consensus for proline-directed kinases, the simplest explanation for my data is that JNK directly phosphorylates MLK3 in cells. To determine whether recombinant, activated JNK can directly phosphorylate MLK3 *in vitro*, it was necessary to eliminate the background of MLK3 autophosphorylation, and therefore a kinase-defective version of MLK3 (K144M) was expressed, immunopurified from HEK 293 cells, and used as a

substrate for recombinant, active JNK in an *in vitro* kinase assay. As shown in Figure 6C, JNK phosphorylates MLK3 K144M *in vitro*, and this phosphorylation results in a decreased electrophoretic mobility of MLK3 (Figure 6C, bottom panel). These data support the hypothesis that MLK3 is a direct substrate of JNK.

4.3 Feedback Phosphorylation Regulates MLK3 Protein Levels in Cellular Lysates

One intriguing observation in the experiments described above was an apparent correlation between the cellular levels of activated JNK and the levels of MLK3 protein present in cellular lysates. As shown in Figure 6A, when JNK activity is diminished, less MLK3 is detected. This effect is specific to MLK3 since the levels of Flag-Cdc42 in cellular lysates, which is expressed using an identical vector, are unchanged in response to JNK inhibition. Likewise the amount of MLK3 in cellular lysates (Figure 6B) increases when the corresponding cells express the constitutively active JNK fusion protein, whereas the levels of GFP used as a transfection control remain constant. These observations suggest the existence of a positive feedback loop involving MLK3-mediated JNK activation and JNK-mediated phosphorylation of MLK3, leading to increased hyperphosphorylated, active MLK3 in cellular lysates and sustained activation of the JNK pathway.

The JNK-phosphorylated MLK3 is activated, as judged by activation loop phosphorylation. Activation loop phosphorylation due at least in part to autophosphorylation (Du et al., 2005), COOH-terminal phosphorylation by JNK, or both, may be required for the increased levels of MLK3 in cellular lysates. As shown previously (Du et al., 2005), Cdc42 promotes activation loop phosphorylation of wild

type MLK3, increases MLK3 protein levels in cellular lysates, and potentiates JNK activation (Figure 7A). However, catalytically inactive MLK3 K144R is refractory to regulation by Cdc42 (Figure 7A), indicating that the enhanced levels of MLK3 in cellular lysates caused by coexpression with the activated GTPase require activation loop phosphorylation and/or JNK activation. To dissociate activation loop phosphorylation, which requires MLK3 kinase activity, from JNK-mediated phosphorylation of MLK3, the constitutively active JNKK2-JNK1 fusion protein was employed. As shown in Figure 7B, expression of the constitutively active JNKK2-JNK1 fusion protein increases the protein levels in cellular lysates and retards the mobility, of both wild type and kinase-defective MLK3 indicating that feedback phosphorylation by JNK, rather than activation segment phosphorylation *per se*, is the critical determinant in dictating JNK-mediated enhanced levels of MLK3 in cellular lysates.

4.4 Inhibition of JNK Reduces the Levels of Activated MLK3 in Cellular Lysates

To study the regulation of MLK3 by JNK phosphorylation in a more reproducible, homogeneous system, a stable clone of a human breast cancer cell line, termed MCF7/iFlag-MLK3, was used (Zhang et al., 2004). In this cell line, transcription of Flag-MLK3 is controlled by the small molecule dimerizer AP21967. The induced Flag-MLK3 potently activates the JNK pathway (Zhang et al., 2004). Consistent with the high activity of MLK3 in this system, the electrophoretic mobility of MLK3 is retarded to a degree comparable to that observed when activated Cdc42 is transiently coexpressed with MLK3 (Figure 8A).

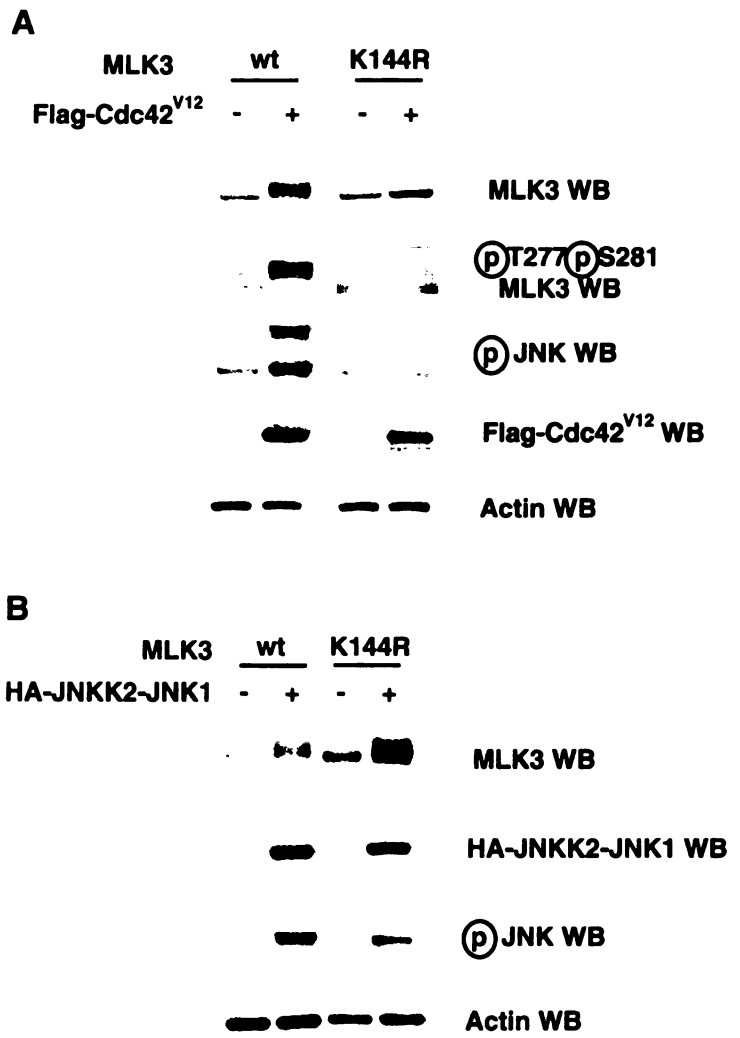
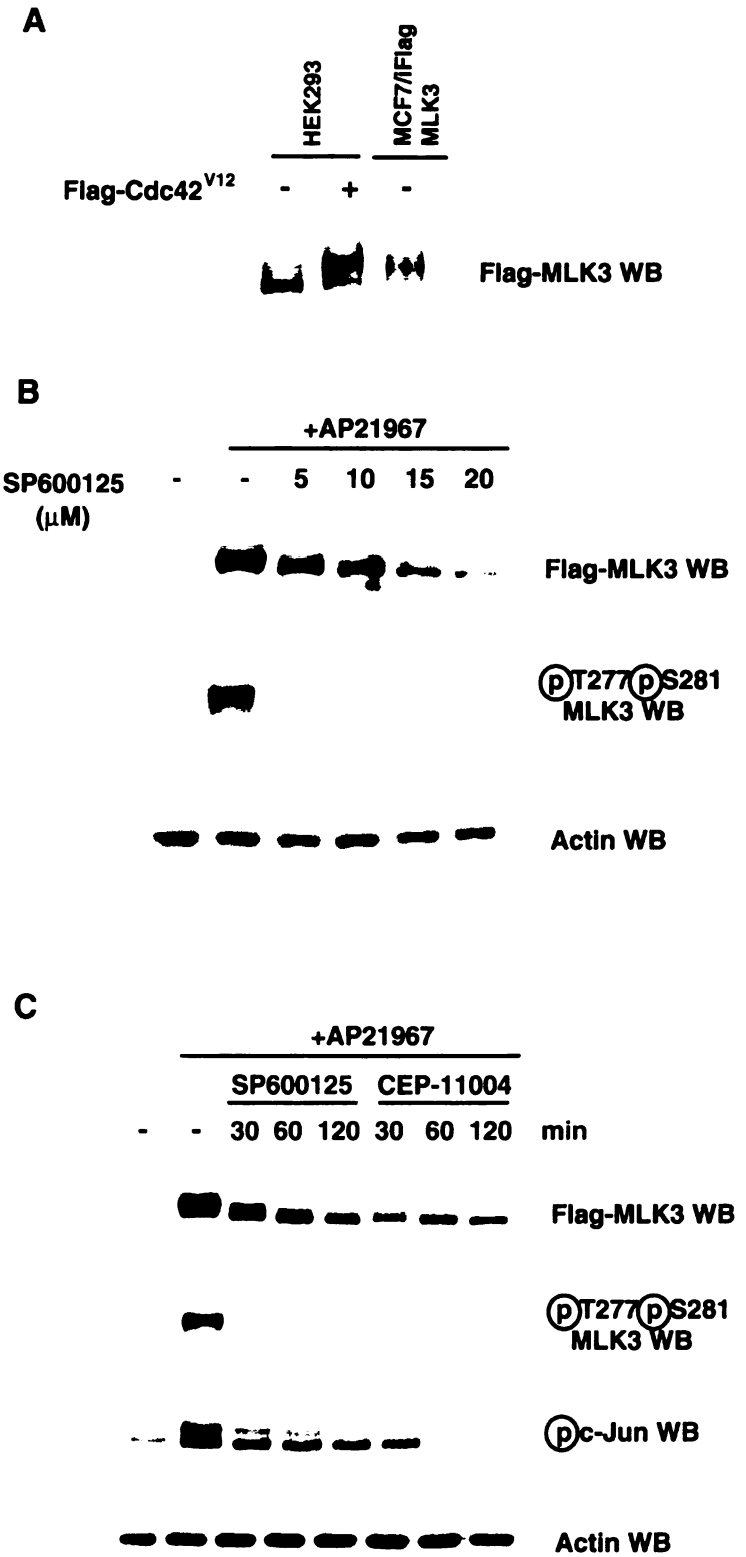


Figure 7. **Regulation of the levels of MLK3 variants in cellular lysates in response to modulation of JNK signaling**. HeLa cells transiently coexpressing the indicated MLK3 variants and JNK signaling modulators were harvested and cellular lysates were prepared. The status of MLK3 activation loop phosphorylation and of JNK activation was assessed by western blotting of cellular lysates using appropriate antibodies. *A*, HeLa cells were transfected with vectors for MLK3 or MLK3 K144R with or without Flag-Cdc42^{V12}. *B*, HeLa cells were cotransfected with expression vectors for MLK3 or MLK3 K144R with or without HA-JNKK2-JNK1. Levels of expressed proteins, and of endogenous actin as a loading control, were detected by western blotting using the indicated antibodies. Panel *A* is provided by Yan Du, Gallo lab.

Figure 8. **Electrophoretic mobility of activated MLK3, and impact of pharmacological inhibition of JNK on MLK3 levels in cellular lysates.** *A*, Electrophoretic mobility of Flag-MLK3. The electrophoretic mobility of Flag-MLK3 expressed alone or with Flag-Cdc42^{V12} in HEK 293 cells and Flag-MLK3 inducibly expressed in engineered MCF-7 cells (MCF7/iFlag-MLK3) was assessed by western blotting of cellular lysates after SDS-PAGE. *B*, Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20h. Cells were then treated with various concentrations of SP600125 for 2h. Clarified lysates were subjected to western blotting analysis using the indicated antibodies. *C*, MCF7/iFlag-MLK3 cells inducibly expressing Flag-MLK3 as described in *B* were treated with 15 μM SP600125 or 400 nM CEP-11004 for the indicated times. Clarified lysates were subjected to western blotting analysis using the indicated antibodies.


A
HEK293
MCF7/IFlag MLK3
Flag-Cdc42V12 - + -
Flag-MLK3 WB
B
+AP21967
SP600125 (μM) - - 5 10 15 20
Flag-MLK3 WB
pT277 pS281 MLK3 WB
Actin WB
C
+AP21967
SP600125 CEP-11004
- - 30 60 120 30 60 120 min
Flag-MLK3 WB
pT277 pS281 MLK3 WB
pc-Jun WB
Actin WB

To test the effects of JNK inhibition on MLK3, MCF7/iFlag-MLK3 cells were induced for 20 h with AP21967, washed to remove the inducer, and then treated with different concentrations of SP600125 for 2 h. Figure 8B shows that inducibly expressed Flag-MLK3 is active as judged by western blotting with an antibody specific for activation loop phosphorylation of MLK3. The levels of Flag-MLK3 in cellular lysates are dramatically decreased upon treatment with the JNK inhibitor, in a concentration dependent manner. Interestingly, the activation loop phosphorylated form seems to disappear more quickly than the net Flag-MLK3, which may indicate that the active form of MLK3 is most sensitive to regulation by JNK.

Recently a pharmacological inhibitor of the MLK family, CEP-11004, has been shown in animal models to attenuate JNK-induced apoptotic death of dopaminergic neurons of the substantia nigra (Ganguly et al., 2004). Therefore, CEP-11004 was used in our inducible expression system to corroborate the effects on MLK3 protein levels observed using the JNK inhibitor SP600125. MCF-7 cells inducibly expressing Flag-MLK3 were incubated with 400 nM CEP-11004 and, as shown in Figure 8C, the levels of phosphorylated c-Jun are decreased dramatically, indicating that JNK activity is potently inhibited. In addition, CEP-11004 reduces the levels of activated and total MLK3 protein in cellular lysates, in a similar manner to that of the JNK inhibitor. The effect of SP600125 appears to be specific for MLK3, since it had no impact on the levels of a Flag-tagged estrogen receptor inducibly expressed in MCF-7 cells using the identical expression system and vectors (Figure 9A). Northern blots using mRNA derived from the MCF-7 cells inducibly expressing Flag-MLK3 showed that SP600125 did not alter

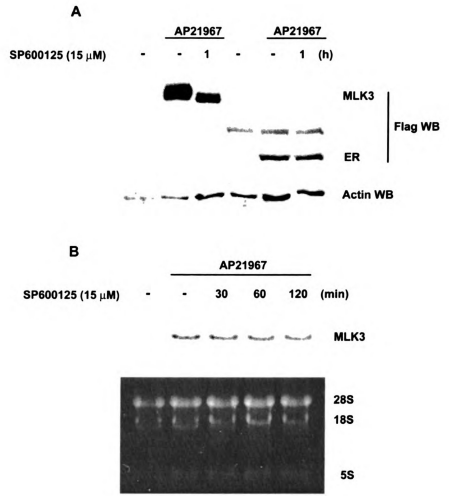


Figure 9. **Effect of SP600125 on inducibly expressed Flag-ER and on Flag-MLK3 mRNA levels.** *A*, Flag-MLK3 expression and Flag-estrogen receptor expression were induced using AP21967 for 20 h. Cells were then treated with 15 μM SP600125 for 1 h. Clarified lysates were subjected to western blotting analysis using the indicated antibodies. *B*, Northern blot analysis of Flag-MLK3 in MCF7/iFlag-MLK3. Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 for the indicated times. Total RNA was then extracted and analyzed by northern blotting. The ethidium bromide-stained gel, displaying the 28S, 18S and 5S ribosomal RNA bands, is shown as a loading control.

the mRNA level of Flag-MLK3 (Figure 9B). These data argue for posttranslational modulation of activated MLK3 by JNK.

4.5 Inhibition of Other Proline-Directed Kinase Pathways Has No Impact on MLK3 Levels

In addition to the MAPKs, Cdks and GSK-3 are proline-directed kinases. To test whether other MAPKs might directly or indirectly regulate protein levels of MLK3, the p38 and ERK pathways were blocked using pharmacological inhibitors. As shown in Figure 10A, neither the p38 inhibitor, SB203580, nor the MEK inhibitor, U0126, altered the levels of Flag-MLK3 protein, in agreement with the results from the *in vivo* labeling experiment. In addition, reactivity with the MLK3 activation loop phosphorylation-specific antibody was maintained, indicating that active MLK3 was unaffected. Likewise, inhibition of the Cdks with roscovitine (Meijer et al., 1997) or of GSK-3 with kenpaullone (Leclerc et al., 2001) failed to impact levels of total MLK3 or active MLK3 found in cellular lysates (Figure 10B). Together these data support the idea that inhibition of JNK, but not of other proline-directed kinases, reduces the level of active MLK3.

4.6 Dephosphorylation of MLK3 Reduces Its Levels in Cellular Lysates

My data provide evidence for positive feedback phosphorylation of MLK3 by JNK. I surmise that the JNK phosphorylation sites are relatively labile, given that they incorporate substantial radioactivity during a 4 h labeling period. This raises the idea that

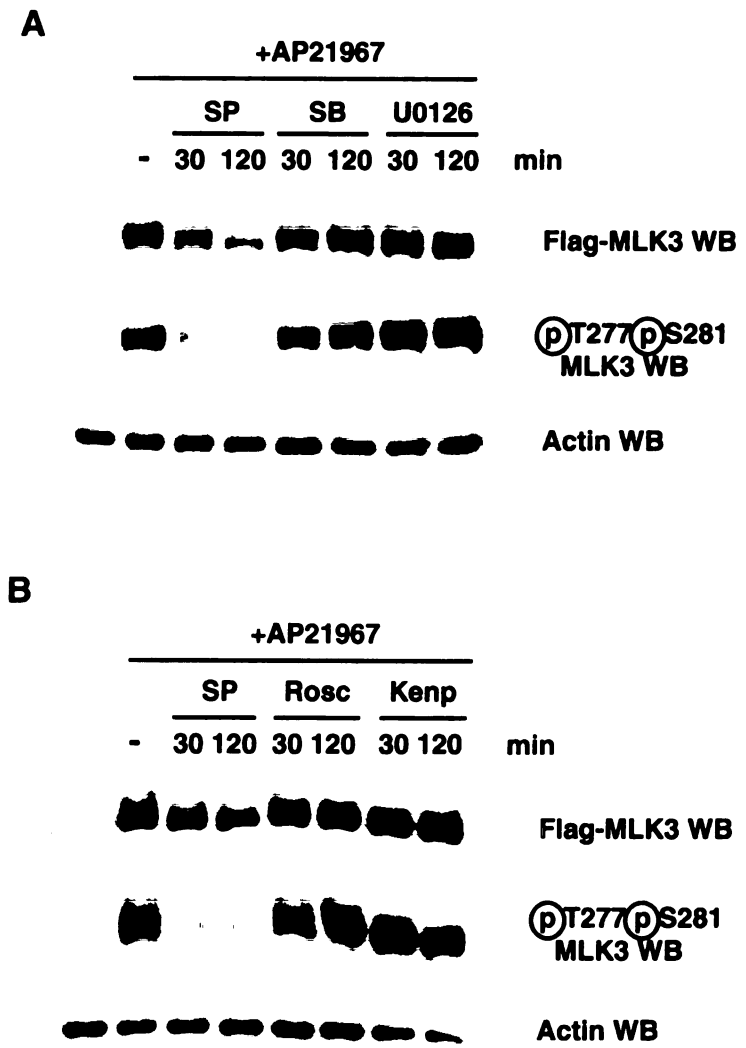


Figure 10. **Effect of proline-directed kinase inhibitors on MLK3 levels in cellular lysates and activation loop phosphorylation.** *A*, Flag-MLK3 expression was induced in MCF7/iFlag-MLK3 cells using AP21967 for 20h. Cells were then treated with 15 μM SP600125 (SP), 6 μM SB203580 (SB) or 10 μM U0126 for the indicated times. Clarified lysates were analyzed by western blotting using appropriate antibodies. *B*, Same as in *A*, but cells were treated with 15 μM SP, 10 μM Roscovitine (Rosc) or 10 μM Kenpaullone (Kenp), and analyzed as described above.

JNK and an opposing phosphatase target the same COOH-terminal phosphorylation sites and that MLK3 levels are regulated by the balance between the two. JNK inhibition would be expected to shift the balance towards dephosphorylation and reduction of MLK3 in cellular lysates. If COOH-terminal dephosphorylation signals to downregulate active MLK3 levels, inhibition of the responsible phosphatase should protect MLK3 levels from JNK inhibition. To test this hypothesis, the Flag-MLK3-expressing cells were treated with calyculin A, a broad-spectrum Ser/Thr phosphatase inhibitor (Dawson & Holmes, 1999; Millward et al., 1999), together with the JNK inhibitor. As predicted, calyculin A blocked the effect of SP600125 on MLK3 protein levels and MLK3 activity (Figure 11), consistent with the model that dephosphorylation of JNK phosphorylation sites somehow downregulates the levels of MLK3 found in cellular lysates.

4.7 Dephosphorylation of MLK3 upon JNK inhibition does not result in its degradation

Transcription factors, such as c-Jun and ATF2, have been reported to be stabilized by JNK phosphorylation (Fuchs et al., 1998; Fuchs et al., 1997; Musti et al., 1997). Recent evidence indicates that proteasome-mediated degradation of c-Jun by specific E3 ligases is dependent on JNK-mediated phosphorylation (Laine & Ronai, 2005). For instance, in PC12 cells, the E3 ligase SCF^{Fbw7} interacts with c-Jun once c-Jun has been phosphorylated by JNK, promoting its degradation (Nateri et al., 2004). This mechanism is thought to allow the cells to tolerate the neurotoxic effects of activated JNK. Another E3 ligase that promotes c-Jun degradation is Itch (Gao et al., 2004). In this case, activation of JNK in T cells results in phosphorylation of Itch and degradation of c-Jun

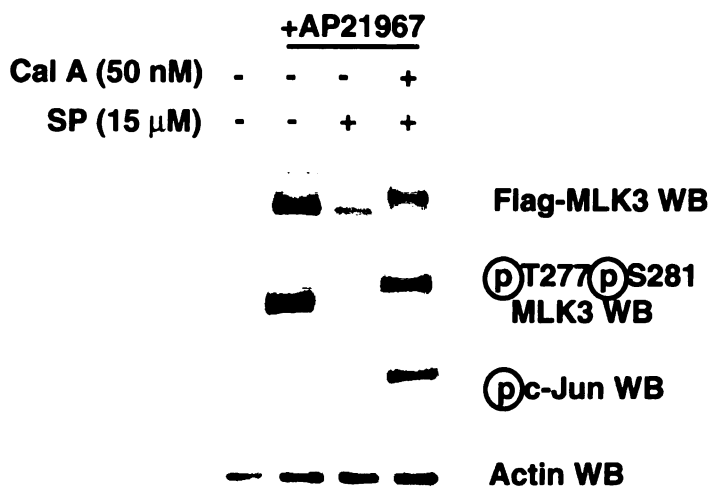


Figure 11. **Impact of phosphatase inhibition on MLK3 levels on cellular lysates.** Flag-MLK3 expression was induced in MCF7/iFlag-MLK3 cells using AP21967 for 20 h. Cells were then treated with 15 μM SP600125 for 1 h, in the presence or absence of 50 nM calyculin A. MLK3 protein levels and electrophoretic mobility, as well as activation loop phosphorylation, were assessed by western blotting using the indicated antibodies. JNK activation was assessed by western blotting for phospho-c-Jun levels.

and JunB, although it is not clear whether Itch recognizes phosphorylated c-Jun or not. It is conceivable that the changes in MLK3 levels in cellular lysates observed upon JNK inhibition are due to increased degradation of the dephosphorylated form of MLK3.

Ubiquitin-mediated degradation is commonly used to regulate protein turnover. Therefore, I set out to investigate whether JNK inhibition results in ubiquitin-mediated degradation of MLK3. Flag-MLK3-expressing cells were incubated with MG132, a proteasome inhibitor, prior to treatment with SP600125. The prediction is that ubiquitination of MLK3 induced upon JNK inhibition would result in the accumulation of MLK3 in the cells; conversely, phosphatase inhibition, which preserves the phosphorylated form of MLK3 even when JNK has been inhibited, would override the effect of MG132. As shown in Figure 12, I did not observe a substantial increase in the amount of MLK3 upon MG132 treatment. To assess whether MLK3 can be regulated through ubiquitination, MLK3 and Flag-ubiquitin were ectopically expressed in HEK 293 cells. To (potentially) increase the accumulation of ubiquitinated MLK3, JNK was pharmacologically inhibited with SP600125, either in the presence or absence of lactacystin, another type of proteasome inhibitor. The ubiquitinated proteins were immunoprecipitated from clarified lysates with a Flag antibody, and the presence of ubiquitin-modified MLK3 was determined by western blot. In accordance with previous results, SP600125 dramatically reduces the levels of MLK3 in cellular lysates, and proteasomal inhibition does not protect MLK3 (Figure 13). Interestingly, a single band corresponding to MLK3 was detected in the Flag pull down, suggesting that MLK3 is monoubiquitinated. Recently, it has been recognized that while polyubiquitination usually targets modified substrates for proteasome mediated degradation,

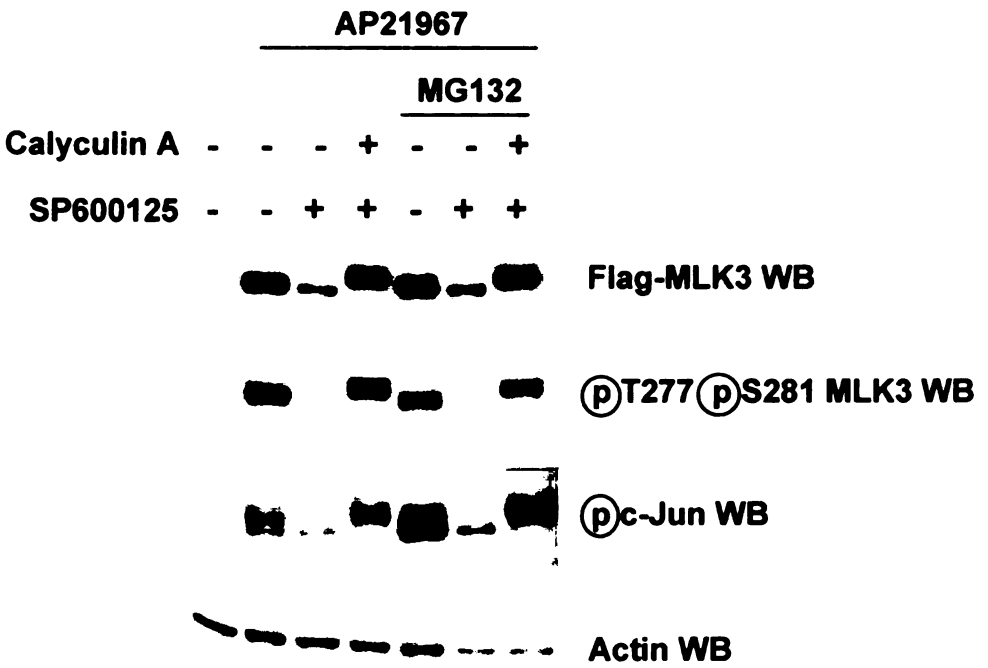
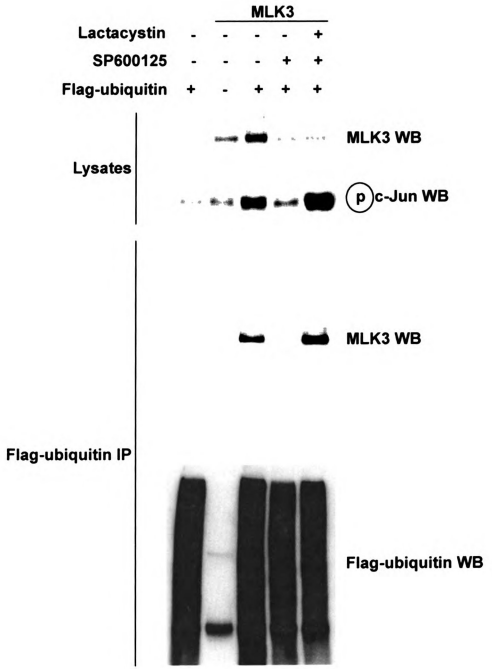


Figure 12. **Effect of MG132 on the protein levels of MLK3 on cellular lysates.** Flag-MLK3 expression was induced in MCF7/iFlag-MLK3 cells using AP21967 for 20h. Cells were incubated with 25 μM MG132 for 2h, and then treated with 15 μM SP600125 for 1h, in the presence or absence of 50 nM calyculin A. MLK3 protein levels and electrophoretic mobility, as well as activation loop phosphorylation, were assessed by western blotting using the indicated antibodies. JNK activation was assessed by western blotting for phospho-c-Jun levels.

Figure 13. **Ubiquitination of MLK3 in HEK 293 cells**. MLK3 was transiently expressed with or without Flag-ubiquitin in HEK 293 cells. After 24 h, cells were harvested and ubiquitin-modified proteins immunoprecipitated from cellular lysates were subjected to western blotting with MLK3 and Flag antibodies to detect ubiquitinated MLK3 and total ubiquitination. An aliquot from the cellular lysates was analyzed for the expression levels of MLK3, and the activation of the JNK pathway was indirectly measured by western blotting of phospho-c-Jun levels.


MLK3
Lactacystin - - - - +
SP600125 - - - + +
Flag-ubiquitin + - + + +
Lysates
MLK3 WB
p c-Jun WB
MLK3 WB
Flag-ubiquitin IP
Flag-ubiquitin WB

monoubiquitination regulates location and activity of modified proteins, the best example being the downregulation and internalization of monoubiquitinated plasma membrane receptors into endocytic vesicles (Haglund et al., 2003; Hicke, 2001; Hicke & Dunn, 2003). These data altogether suggest that the reduction in MLK3 levels observed upon JNK inhibition is not due to degradation through the 26S proteasome pathway.

5. Discussion

In this work I provide evidence that MLK3 is regulated by JNK-mediated COOH-terminal phosphorylation. These studies were prompted by earlier identification of multiple, proline-directed *in vivo* phosphorylation sites on Cdc42-activated MLK3 (Vacratsis et al., 2002). Since MLK3 acts as a MAPKKK to induce multiple MAPK pathways, the possibility of feedback phosphorylation was addressed. Inhibition of JNK, but not other MAPKs, dramatically reduced the dynamic, *in vivo* phosphorylation of MLK3. Activated JNK promotes MLK3 phosphorylation *in vivo* and phosphorylates MLK3 *in vitro*. A kinase defective version of MLK3 is less phosphorylated in cells than wild type MLK3; furthermore, JNK-mediated phosphorylation of MLK3 requires MLK3 activity, consistent with the existence of a feedback loop.

Feedback phosphorylation of MAPKKKs by their downstream MAPKs is not without precedence. For instance, in yeast, pheromone induces the three component MAPK pathway of Ste11 (MAPKKK)-Ste7(MAPKK)-Fus3/Kss1(MAPK). Fus3/Kss1-mediated feedback phosphorylation of active Ste11 targets this MAPKKK for proteasome-mediated degradation (Esch & Errede, 2002). In the Raf-MEK-ERK pathway, both B-Raf and Raf-1 are negatively regulated by ERK phosphorylation (Brummer et al., 2003; Wartmann & Davis, 1994; Wartmann et al., 1997). Recently, ERK-mediated feedback phosphorylation was shown to inactivate and desensitize Raf-1, rather than target Raf-1 for degradation. Hyperphosphorylated Raf is recycled through dephosphorylation by PP2A after Pin1-dependent isomerization (Dougherty et al., 2005).

In contrast to these examples of negative feedback phosphorylation of MAPKKKs by their downstream MAPKs, in the MLK3-MKK4/7-JNK pathway, our evidence

indicates that JNK-mediated phosphorylation does not downregulate or drive degradation of active MLK3, but rather enhances its levels in cellular lysates. In Xenopus oocyte maturation, progesterone treatment leads to the accumulation of the MAPKKK Mos (Gotoh et al., 1995; Matten et al., 1996; Roy et al., 1996). This positive feedback of the Mos-MEK-ERK pathway is manifested primarily through stabilization of *mos* mRNA and accompanying increased translation of Mos. JNK has been reported to phosphorylate other MAPKKKs. For instance, JNK binds and phosphorylates the MAPKKK, MEKK1, in the MEKK1-MKK4-JNK (Xu & Cobb, 1997) pathway, but this apparently does not regulate MEKK1 stability. Interestingly, MEKK1 functions not only as a MAPKKK and scaffold, but also as an E3 ubiquitin ligase and undergoes ubiquitination. However, ubiquitinated MEKK1 is not rapidly degraded, but instead is rendered catalytically inactive (Witowsky & Johnson, 2003). MLK2 can also be phosphorylated by JNK, but precise phosphorylation sites and whether they impact MLK2 stability was not reported (Phelan et al., 2001).

The incorporation of substantial radiolabel into multiple JNK phosphorylation sites of Cdc42-activated MLK3 indicates that these sites are relatively labile. Inhibition of phosphatase activity with calyculin A resulted in a marked reduction in the electrophoretic mobility of MLK3, indicating that in fact, MLK3 is subjected to dynamic cycles of phosphorylation-dephosphorylation. Interestingly, the activation loop phosphorylation sites of MLK3 did not incorporate significant radiolabel under our experimental conditions, as judged by phosphotryptic peptide mapping (Vacratsis et al., 2002). This unexpected finding may indicate that the activation loop phosphorylation sites are quite stable, and do not undergo rapid phosphatase-mediated exchange resulting

in meager incorporation of radiolabel. In response to JNK inhibition, the disappearance of activation loop phosphorylated MLK3 precedes the loss of net MLK3. This is consistent with the notion that activation loop phosphorylated MLK3 is inherently unstable in the absence of additional COOH-terminal phosphorylation by JNK. I cannot, however, rule out the possibility that an activation loop phosphatase for MLK3 exists, as it has been described for ASK1 (Morita et al., 2001), but I would hypothesize that it would be inhibited by JNK.

A MLK3 variant in which the phosphorylated serines from the proline-directed kinase sites located in the COOH-terminal region of MLK3 were substituted with alanine residues has an incomplete phenotype: it not longer incorporates radioactivity during an *in vivo* labeling experiment, but yet can still be phosphorylated *in vitro* by JNK, although to a much lesser degree than wild type MLK3 (data not shown). Likewise, it can be phosphorylated *in vivo* by JNK when using the fusion protein between MKK7 and JNK1, but again, to a lesser degree than wild type MLK3 (data not shown). In addition, activated Cdc42 can still promote a slight decrease in its electrophoretic mobility (data not shown). All these data suggest that there might be other feedback phosphorylation sites on MLK3 that have not been identified.

My finding that JNK phosphorylation regulates the cellular levels of a MAPKKK is novel. Interestingly, it had been previously reported that NGF-withdrawal or UV treatment of neuronally differentiated PC12 cells results in an increase in the protein levels of DLK, and in the appearance of slowing migrating forms that correspond to phosphorylated DLK (Xu et al., 2001). Those studies have been extended, and it has been recently shown that cellular stresses like camptothecin, a topoisomerase inhibitor,

and sorbitol, an osmotic stresser, can induce activation and stabilization of endogenous MLK3. Ablation of JNK expression by siRNA or with dominant negative JNK1 blocked camptothecin-induced increase in MLK3 protein levels (Xu et al., 2005). Changes in the cellular levels of other MLK family members, as well as changes in the levels of the scaffold proteins JIP1 and POSH were also observed upon modulation of JNK activation status. Although changes in protein stability were not directly measured, the authors observed changes in the levels of exogenous MLK3 driven by a constitutive promoter, but not for a kinase defective form of MLK3 expressed under the same conditions, concluding that the regulation of MLK3 levels is transcription-independent. In my experimental system, inhibition of JNK reduces the levels of MLK3 in cellular lysates, and northern blot analysis indicates that this is not regulated at the mRNA level. To be in consonance with a model for the regulation of MLK3 stability through JNK-mediated phosphorylation, blockade of JNK would be expected to increase MLK3 degradation. This could be accomplished through ubiquitination and proteasome-mediated degradation of dephosphorylated MLK3. Although MLK3 can be ubiquitinated when ectopically expressed with ubiquitin in HEK 293 cells, I have not detected increased ubiquitination of MLK3 upon JNK inhibition, even in the presence of proteasome inhibitors. These observations indicate that instead of being degraded, MLK3 might have become "unavailable", perhaps sequestered in a different subcellular compartment that is resistant to the cell lysis conditions.

In the next Chapter, I will address this question by looking at the subcellular distribution of the dephosphorylated MLK3.

III. Feedback Phosphorylation of MLK3 by JNK Regulates its Distribution to Triton-insoluble Fractions

1. Abstract

Mixed-lineage kinase 3 (MLK3) is a widely expressed, mammalian serine/threonine protein kinase that activates multiple MAPK pathways. Previously our lab used *in vivo* labeling/mass spectrometry to identify phosphorylation sites on activated MLK3. Seven of eleven identified sites correspond to the consensus motif for phosphorylation by proline-directed kinases. JNK phosphorylates MLK3 *in vitro*, and it phosphorylates MLK3 on the COOH-terminal sites *in vivo*. When JNK is inhibited, MLK3 is rapidly dephosphorylated, consistent with the idea that phosphorylation of the COOH-terminal sites occurs through a dynamic, JNK-mediated, feedback mechanism. Herein I present evidence that the hypophosphorylated form of MLK3 is inactive, and redistributes to a Triton-insoluble fraction. Recovery from JNK inhibition restores MLK3 solubility and activity, indicating that the redistribution process is reversible. Sucrose density gradient experiments demonstrate that the Triton-insoluble MLK3 is not largely associated with lipid rafts. Immunofluoresence microscopy experiments indicate that the dephosphorylated MLK3 is present in a vesicle-like pattern throughout the cells, and does not colocalize with major organelle markers. My work describes a novel mode of regulation of MLK3, by which JNK-mediated feedback phosphorylation of MLK3 regulates its activation and deactivation states by cycling between Triton-soluble and Triton-insoluble forms.

2. Introduction

Our interest in the role of phosphorylation in regulating MLK3 activities led us to undertake a mass spectrometry/phosphopeptide mapping approach to identify *in vivo* phosphorylation sites on MLK3. Cells expressing MLK3 and Cdc42 were radiolabeled with inorganic phosphate, and the residues that incorporated radioactivity were identified by mass spectrometry. Eleven phosphorylation sites were found, most of them clustered at the COOH-terminal region of MLK3. Seven of these sites correspond to serine residues that are followed by proline residues, a consensus sequence that is recognized by the MAPKs. The work described in Chapter II shows that JNK phosphorylates MLK3 *in vivo* and *in vitro*, and that inhibition of JNK-mediated phosphorylation of MLK3 reduces MLK3 protein levels in cellular lysates. I hypothesized that the decrease in MLK3 levels could result from ubiquitination and proteasome-mediated degradation of the hypophosphorylated MLK3. The data presented in Chapter II indicate that even in the presence of a proteasome inhibitor, the levels of MLK3 in cellular lysates decrease when JNK is blocked in my experimental setting. This raises the possibility that MLK3 had redistributed to the Triton-insoluble fraction, which had not been previously analyzed for the presence of MLK3.

Intracellular translocation of signaling proteins is one way to regulate their actions and fate. Redistribution to different subcellular compartments results in their exposure to different subsets of molecules, and thus adds specificity to cellular responses. For instance, upon ligand stimulation of a plasma membrane receptor, signaling molecules like Src family tyrosine kinases, scaffolds and adaptor molecules like Grb2 and SOS are recruited to the cytoplasmic leaflet of the plasma membrane (reviewed in (Hunter,

2000)). The ability of the recruited proteins to generate productive signaling complexes by increasing the local concentration of proteins at the membrane has been termed induced protein proximity. The recruitment of selective signaling molecules to the plasma membrane generates new micro-environments, which are also characterized by the presence of specific lipids (Simons & Toomre, 2000). These specialized regions of the plasma membrane are referred to as ordered lipid microdomains, and include rafts and caveolae. Due to their lipid content, rafts and caveolae are insoluble in non-ionic detergents, and are therefore also called detergent-resistant membranes (DRMs) (Morris et al., 2004).

In this chapter, the finding that dephosphorylated MLK3 redistributes to a Triton X100-insoluble fraction and that it displays a punctate localization pattern in cells is described. The nature of the Triton X100-insoluble fractions is analyzed and the importance of this localization for MLK3 regulation is discussed.

3. Materials and Methods

3.1 Reagents and Antibodies

SP600125 was purchased from Calbiochem. Calyculin A was obtained from BioMol. AP21967 was generously provided by Ariad Pharmaceuticals. Mouse monoclonal antibodies against phospho-c-Jun (KM-1), as well as antibodies against Hsp90 and Cdc37, were purchased from Santa Cruz Biotechnology, Inc. The phospho-MLK3 antibody and the phospho-JNK antibody were from Cell Signaling Technology. The monoclonal antibody against cytochrome oxidase subunit IV (COX IV) was from Invitrogen. The antibody against ERp72 was purchased from Stressgen. The antibody against flotillin-1 was from BD-Transduction Labs. Antibodies against EEA1 were obtained from Affinity BioReagents (for immunoblotting) and from BD-Transduction Labs (for immunofluorescence). Antibodies against the Golgi marker GM130, Integrin α2 and Nucleoporin p62 were from BD-transduction Labs. The antibody against clathrin was from Affinity BioReagents. Antibodies against β-tubulin, γ-tubulin and vinculin were from Sigma. The antibody against Histone 3 was from Abcam. The antibody against p27 is from Santa Cruz Biotechnology. Other antibodies used were the MLK3 rabbit polyclonal antibody (Bock et al., 2000), actin and Flag mouse monoclonal antibody (Sigma), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Alexa-488 and Alexa-543 and Alexa-555 conjugated secondary antibodies, TOPRO iodide and MitoTracker Red were from Molecular Probes.

3.2 Cell culture

MCF-7/iFLAG-MLK3 cells inducibly expressing Flag-MLK3 have been described in Chapter II (Zhang et al., 2004), and were maintained in DMEM supplemented with 5% fetal bovine serum, 40 μg/mL neomycin and 10 μg/mL hygromycin. Flag-MLK3 expression was induced by the addition of 50 nM AP21967 for 20 h.

3.3 Cell lysis and western blotting

Cells were lysed in 1% Triton lysis buffer, containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na_4PP_i, 100 μM β-glycerophosphate, 1 mM Na_3VO_4 and 2 mM phenylmethylsulfonylfluoride (PMSF), and complemented with a cocktail of protease inhibitors (Sigma). Following sonication for 40 s in a sonication bath to enhance disruption of organelles, lysates were clarified by centrifugation at 14,000 rpm at 4°C for 15 min. For the Triton X-100 biochemical fractionation, the cells were lysed as described above, and the Triton-insoluble pellets were washed in lysis buffer and then resuspended in 2X SDS loading buffer and boiled prior to SDS-PAGE.

3.4 Preparation of detergent-resistant membrane fractions by sucrose-density gradient

Flag-MLK3 expression was induced in MCF7/iFlag-MLK3 for 20 h. Cells were then treated with carrier or 15 μM SP600125 for 1 h, washed twice in ice-cold PBS, and lysed in cold TNE buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA) containing

1% Triton X-100. Cells were incubated on ice for 20 min and then homogenized using 12 strokes in a Dounce homogenizer. Homogenates were mixed with an equal volume of an 80% sucrose solution prepared in TNE, to yield a final concentration of 40% sucrose, and placed at the bottom of an ultracentrifuge tube. A 5%-30% discontinuous sucrose gradient was layered on top of the homogenate by adding 2 volumes of a 30% sucrose solution prepared in TNE, and 1 volume of a 5% sucrose solution prepared in TNE. Samples were spun at 200,000 x *g* (45,000 rpm in a Beckman TH660 rotor) for 18h at 4°C. Ten fractions (0.4 mL) were collected from the top of the gradient. The insoluble material was resuspended in 400 μL of 2X SDS loading buffer. Equal volumes from each fraction were separated by SDS-PAGE and subjected to western blotting analysis.

3.5 Immunofluorescence Confocal Microscopy

MCF-7/iFlag-MLK3 cells were plated into 6-well plates containing cover slips coated with poly-D-lysine (Sigma). Flag-MLK3 expression was induced for 20 h, and the cells were treated with 15 μM SP600125 or carrier for 1 h. When costaining for mitochondria, MitoTracker was added to the cells 30 min prior to fixation at a final concentration of 50 nM. The cells were rinsed twice with PBS, fixed in 2% formaldehyde (Polyscience, Inc.) for 30 min at room temperature, and washed three times with PBS. The cells were permeabilized with PBS containing 0.2% Triton X-100 or 1% Triton X-100 for 10 min. Where indicated, the cells were first washed in PBS containing 1% Triton X-100 for 5 min and then fixed in 2% formaldehyde for 30 min. Blocking was performed in PBS containing 10% fetal calf serum, 2% bovine serum albumin, and RNase A (100 μg/ml) for 1 h at room temperature. Primary antibodies were prepared in

PBS containing 5% bovine serum albumin (MLK3 rabbit polyclonal antibody 1:1500; Flag M2 monoclonal antibody 1:1500; β-tubulin mouse monoclonal 1:200; vinculin mouse monoclonal 1:400; GM130 mouse monoclonal 1:100; EEA1 mouse monoclonal antibody 1:250; clathrin X22 mouse monoclonal antibody 1:1000) and were incubated for 1 h at room temperature. The cells were then washed three times with PBS containing 2% fetal calf serum and incubated for 1 h with the appropriate Alexa Fluor 488-, Alexa Fluor 546-, Alexa Fluor 555-conjugated secondary antibody. After five washes with PBS containing 2% fetal calf serum, the nuclei were stained for 30 min with TOPRO-3 iodide (2 μM in PBS) and mounted onto glass slides. The fluorescently labeled cells were examined with a Zeiss LSM Pascal confocal laser scanning microscope. Three separate tracks were used for capturing fluorescent images; excitation was done using 488-, 543-, and 633-nm laser lines with BP 500-530 nm, BP 560-615 nm and LP 650 nm, respectively, as emission filters.

4. Results

4.1 JNK inhibition redistributes MLK3 to a Triton-insoluble fraction

The data presented in Chapter II demonstrate that JNK inhibition results in a decrease in the levels of Flag-MLK3 in cellular lysates. Although MLK3 can be ubiquitinated when coexpressed in cells with ubiquitin, increased ubiquitination of MLK3 in the presence of the JNK inhibitor was not observed. This suggests that ubiquitin-mediated degradation of Flag-MLK3 could not account for the large reduction in Flag-MLK3 protein levels observed upon JNK inhibition. To explain the reduction in Flag-MLK3 protein levels in cellular lysates, I speculated that JNK-mediated phosphorylation of MLK3 might impact the distribution, rather than the protein stability, of MLK3.

To test this idea, the Triton X-100-insoluble pellets obtained after cellular lysis were examined for both the presence as well as the activation state of MLK3. Briefly, cells inducibly expressing Flag-MLK3 were incubated with SP600125 alone or with SP600125 in the presence of calyculin A. Cells were disrupted in the standard 1% Triton X-100-containing buffer, the cellular lysates were removed after centrifugation and, after rinsing in lysis buffer, the Triton-insoluble pellets were solubilized in SDS-containing buffer. Equal cellular equivalents from the Triton X-100-soluble fractions and the SDS-resolubilized Triton X-100 pellets were examined by western blotting using identical exposure times and conditions. As shown in Figure 1, JNK inhibition results in a dramatic shift of Flag-MLK3 from a Triton-soluble to a Triton-insoluble fraction. This redistribution is prevented by the addition of the phosphatase inhibitor calyculin A.

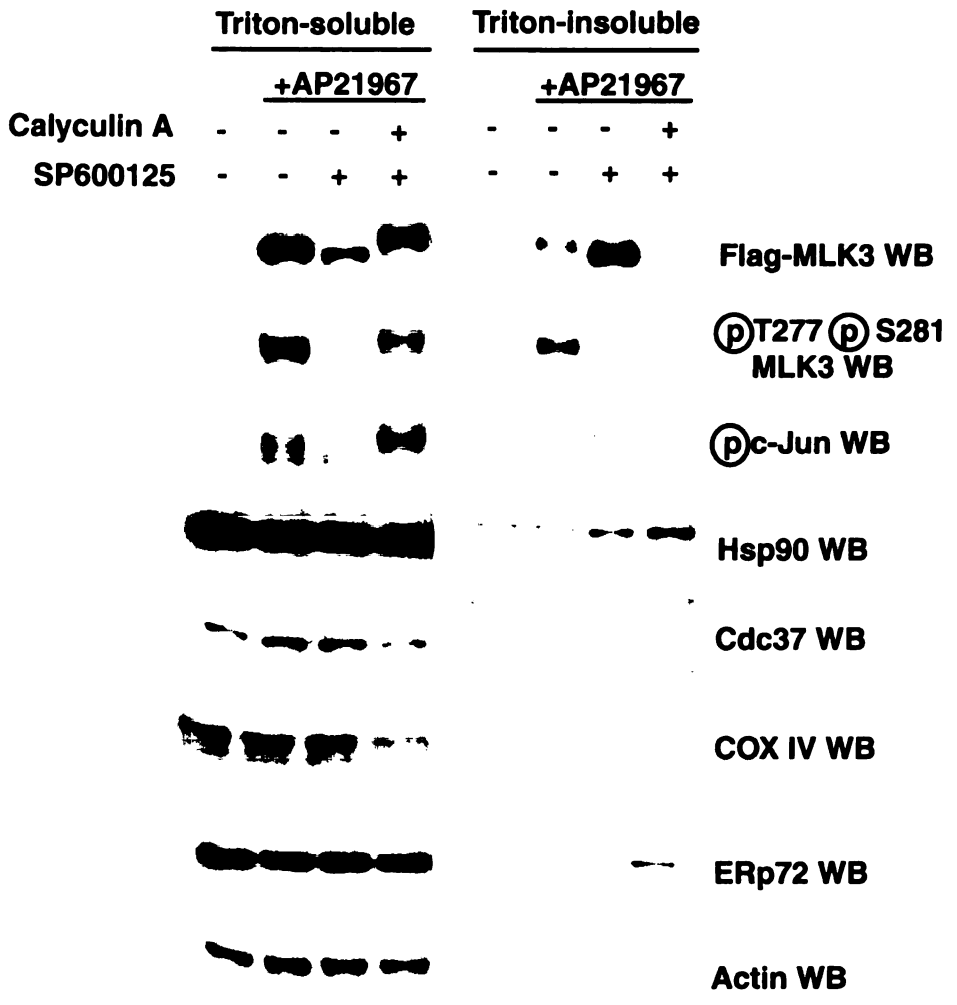


Figure 1. **Redistribution of MLK3 to detergent-insoluble fractions.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 for 1 h, in the presence or absence of 50 nM calyculin A. The cells were lysed as described in "Materials and Methods" and identical cellular equivalents from the Triton-soluble and Triton-insoluble fractions were analyzed by western blotting, using appropriate antibodies and identical exposure times.

Thus, while the total cellular amount of Flag-MLK3 is largely unaffected by the manipulation of the phosphorylation state of Flag-MLK3, the distribution of Flag-MLK3 is profoundly impacted by its phosphorylation status. The Flag-MLK3 that redistributes to the Triton-insoluble fraction upon JNK inhibition is largely inactive, as judged by its trace reactivity with the phospho-MLK3 antibody. This is consistent with a JNK-mediated positive feedback loop.

To analyze the solubility in Triton X-100 of different organelles under the cellular lysis procedure, the distribution of various subcellular markers in the crude fractionation was analyzed by western blotting. The Triton-soluble fractions contain phospho-c-Jun, cytochrome oxidase IV (COX IV), and ERp72, but these proteins are largely absent from the Triton-insoluble fractions, indicating that the nucleus, mitochondria, and endoplasmic reticulum membranes are efficiently solubilized.

In a recent study from our lab, Hsp90 and its co-chaperone Cdc37 were identified as cochaperones of MLK3 (Zhang et al., 2004). As shown in Figure 1, the Triton-insoluble fractions are largely devoid of Hsp90 and Cdc37, which may suggest that the dephosphorylation-dependent redistribution of Flag-MLK3 to detergent-insoluble domains requires disruption of the MLK3-Hsp90/Cdc37 complex.

4.2 Distribution of MLK3 between Triton-soluble and Triton-insoluble fractions is reversible

Treatment of cells with geldanamycin, an Hsp90 inhibitor, reduces the protein levels of MLK3, suggesting that the interaction of Hsp90/Cdc37 with MLK3 stabilizes MLK3 (Zhang et al., 2004). My findings that the Triton-insoluble domains contain

inactive Flag-MLK3 and largely lack the MLK3 chaperones Hsp90/Cdc37 might indicate that the redistributed, insoluble, hypophosphorylated MLK3 is irreversibly aggregated or inactivated. It is possible that aggregated MLK3 could be destroyed after a long term inhibition of JNK. However, as shown in Figure 2, hypophosphorylated, inactive Flag-MLK3 is still present in a Triton-insoluble compartment, with no evidence of enhanced degradation even after 12 h of continuous treatment with 15 μM SP600125. This raises the possibility that the sequestration of Flag-MLK3 is reversible. To test whether hypophosphorylated, inactive Flag-MLK3 could be reactivated by altering its phosphorylation status, Flag-MLK3 expression was induced and the cells were treated with SP600125 for 1 h. The JNK inhibitor was removed by placing the cells in fresh media either with or without calyculin A for different periods of time. The cells were then lysed and the distribution of Flag-MLK3 was analyzed by western blotting. As shown in Figure 3, upon removal of the JNK inhibitor a significant portion of Flag-MLK3 redistributed to the soluble fraction and regained its activation status, as judged by activation loop phosphorylation. The portion of Flag-MLK3 that remained insoluble also recovered its activation status. Removal of the JNK inhibitor, followed by phosphatase inhibition with calyculin A resulted in quantitative reactivation of Flag-MLK3 and redistribution to the soluble fraction. These data indicate that the phosphorylation/dephosphorylation cycle of Flag-MLK3 modulates the distribution of Flag-MLK3 in a reversible manner.

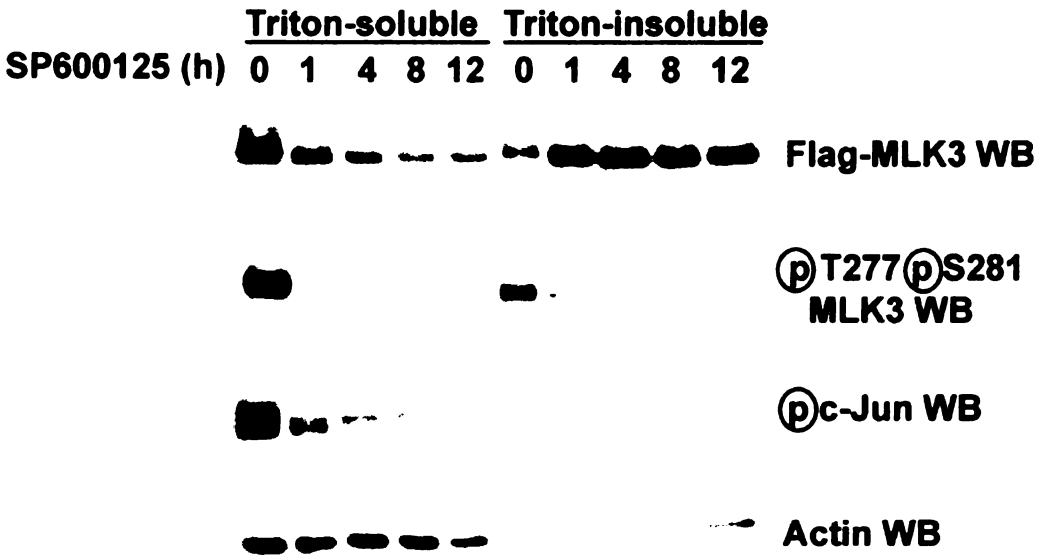


Figure 2. **Effect of long-term JNK inhibition on the distribution of MLK3 in Triton X-100.** Flag-MLK3 expression was induced using 50 nM AP21967 in MCF7/iFlag-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 for different periods of time. The cells were lysed as described in "Materials and Methods" and identical cellular equivalents from the Triton-soluble and Triton-insoluble fractions were analyzed by western blotting, using appropriate antibodies.

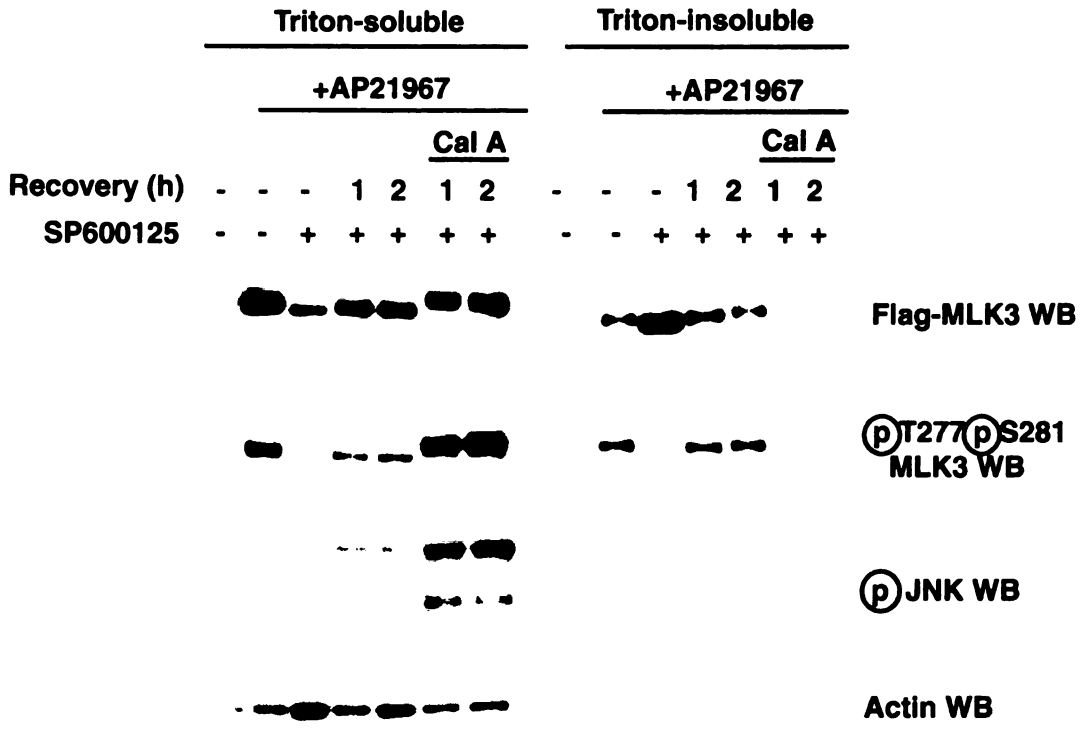


Figure 3. **Effect of SP600125 removal on MLK3 distribution.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 for 1 h, washed and incubated in fresh media with or without 50 nM calyculin A for the indicated times. Triton-soluble and Triton-insoluble fractions were prepared and analyzed as described for Figure 1.

4.3 Triton-insoluble MLK3 is not associated with lipid rafts

It is possible that the Triton-insoluble, Flag-MLK3-containing fraction represents either cytoskeletal elements or lipid rafts. MLK3 has been shown in cotransfection/coimmunoprecipitation experiments to interact with the actin cytoskeleton linker protein, Merlin, which is the product of the *neurofibromatosis-2* gene (Chadee et al., 2006). In addition to its association with cytoskeleton (Bretscher et al., 2002), Merlin has been recently reported to associate with lipid rafts (Stickney et al., 2004). Detergent-resistant membranes have been described as microdomains within membranes, highly enriched in cholesterol and with a distinct protein composition. In particular, lipid rafts are thought to transiently organize a signaling microdomain, clustering receptor tyrosine kinases, adaptor molecules and endocytic proteins, among others. Because of their high cholesterol levels, lipid rafts can be easily isolated by density gradient centrifugation.

To determine whether the hypophosphorylated, inactive form of MLK3 generated upon JNK inhibition localizes to lipid rafts, a sucrose-density centrifugation assay was implemented. Flag-MLK3 expression was induced for 20 h, and the cells were then treated with or without 15 μM SP600125 for 1 h. Homogenates were fractionated by sucrose-density centrifugation; fractions were collected from top (lighter) to bottom (heavier) and analyzed by western blotting. Flotillin 1, a component of lipid rafts, was used as a marker. As shown in Figure 4, Flotillin 1, which is present along the entire gradient, is enriched in the lighter fractions (fractions 3 and 4). Since equal volumes, and not equal amounts of proteins, were resolved in the gel, the presence of Flotillin 1 in the heavier fractions is probably due to the proportionally higher protein content of the fractions at the bottom of the gradient. Overall, the data indicate that the gradient was

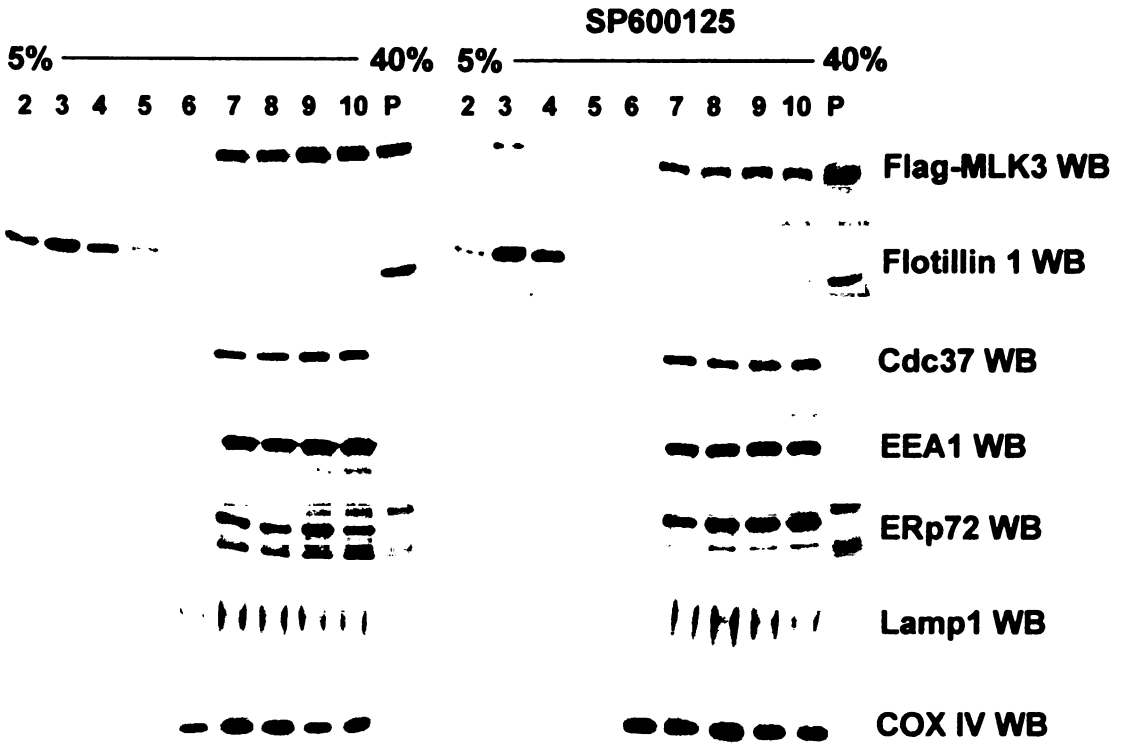


Figure 4. **Distribution of MLK3 in a sucrose-density gradient.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 for 1 h and homogenates were loaded onto a 5%-40% sucrose gradient. After centrifugation at 200,000 x *g* for 18 h at 4°C, fractions of equal volume were collected from top to bottom of the gradient and analyzed by western blotting using the indicated antibodies. *Numbers* on top indicate fraction number from top to bottom of the gradient. *P*, pellet.

successfully generated. Flag-MLK3 was found predominantly at the bottom of the gradient (fractions 7-10), where it cofractionated with disrupted endocytic vesicles, endoplasmic reticulum, lysosomes and mitochondria, as determined by the presence of early endosome antigen 1 (EEA1), ERp72, Lysosomal-associated membrane protein 1 (Lamp1) and COX IV, respectively. However, only a very small portion of Flag-MLK3 (approximately 6% of the total Flag-MLK3 as determined by densitometry) was redistributed to a lighter fraction (fraction 3) upon JNK inhibition, while a substantial amount of MLK3 (approximately 37% of the total Flag-MLK3) remained in the insoluble pellet. Therefore, relocalization of Flag-MLK3 to lipid rafts cannot explain the dramatic change in solubility of Flag-MLK3 in Triton X-100 upon inhibition of JNK.

4.4 Inhibition of JNK results in the localization of MLK3 to punctate, vesicle-like structures

To gain further insight into the redistribution of the hypophosphorylated MLK3 in cells, immunofluorescence microscopy was used. MCF-7 cells inducibly expressing Flag-MLK3 were incubated either in the presence or absence of 15 μM SP600125 for 1 h. Cells were then fixed, permeabilized with 1% Triton X-100 and stained for MLK3. Inducibly expressed Flag-MLK3 is cytosolic, and evenly distributed in the cells (Figure 5). No nuclear localization of Flag-MLK3 was observed. Upon JNK inhibition, Flag-MLK3 displays a more punctate pattern, and seems to be absent from the plasma membrane and cellular projections like filopodia. In a parallel experiment, cells were first incubated in 1% Triton X-100 prior to fixation and staining. Under those conditions, all the cellular compartments that are solubilized in Triton X-100 are washed away before

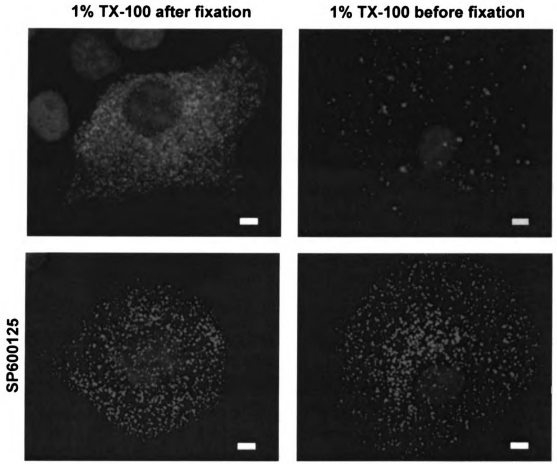


Figure 5. **Subcellular localization of MLK3 in MCF-7/iFlag-MLK3 cells**. Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h. The cells were incubated in 1% Triton X-100 before or after fixation, and MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 543. The nuclei were stained with TOPRO-3 iodide. Representative images are shown with MLK3 and the nuclei represented in *red* and *blue*, respectively. *Bar*, 5 μm.

fixation, and the MLK3 staining is practically lost. However, in the presence of the JNK inhibitor, the MLK3 staining is preserved, and is undistinguishable from the pattern observed when the cells are permeabilized after fixation, suggesting that the punctate, dot-like structures correspond to the MLK3 present in the Triton X-100 insoluble fraction from the cellular lysates.

The colocalization of MLK3 with other subcellular markers was analyzed. Like MLK3, cytoskeletal elements are, under certain conditions, Triton X-100 insoluble. To determine whether Flag-MLK3 could localize along cytoskeletal elements, a β-tubulin staining was performed in cells inducibly expressing Flag-MLK3. As shown in Figure 6, no obvious colocalization of Flag-MLK3 and β-tubulin was observed. In the presence of the JNK inhibitor, the MLK3 pattern changed to the punctate structures seen before; however, these structures did not colocalize with β-tubulin either.

I also analyzed the possibility that MLK3 could colocalize with focal adhesions, Golgi vesicles and mitochondria. Vinculin is a cytoskeletal protein associated with the cytoplasmic face of cell junctions, where it anchors the actin lattice to the plasma membrane (reviewed in (Bailly, 2003). As shown in Figure 7, no colocalization of MLK3 and vinculin was observed, either in the presence or absence of SP600125.

Golgi Matrix protein 130 (GM130) is peripherally associated with the cis-compartment of the Golgi and was initially isolated as a Triton X-100 insoluble component of the Golgi matrix (Slusarewicz et al., 1994). When the localization of GM130 was analyzed in the MCF-7/iFlag-MLK3 cells, it was found that MLK3, even after JNK inhibition, does not colocalize with GM130 (Figure 8). Finally, I used

Figure 6. **Costaining of MLK3 and β-tubulin in MCF-7/iFlag-MLK3 cells.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and fixed. MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 488. β-tubulin was stained with a mouse β-tubulin antibody and a secondary antibody conjugated with Alexa Fluor 555. The nuclei were stained with TOPRO-3 iodide. Representative images are shown with MLK3, β-tubulin and nuclei represented in *green, red* and *blue*, respectively. *Bar*, 5 μm.

MLK3
β-tubulin
TOPRO
Merge
SP600125

Figure 7. **Costaining of MLK3 and vinculin in MCF-7/iFlag-MLK3 cells.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and fixed. MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 488. Vinculin was stained with a mouse vinculin antibody and a secondary antibody conjugated with Alexa Fluor 555. Representative images are shown with MLK3 and vinculin represented in *green* and *red*, respectively. *Bar*, 5 μm.

MLK3
Vinculin
Merge
SP600125

Figure 8. **Costaining of MLK3 and GM130 in MCF-7/iFlag-MLK3 cells.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and fixed. MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 488. GM130 was stained with a mouse GM130 antibody and a secondary antibody conjugated with Alexa Fluor 555. Representative images are shown with MLK3 and GM130 represented in *green* and *red*, respectively. *Bar*, 5 μm.

MLK3
GM130
Merge
SP600125

Mitotracker Red, a dye that metabolically labels mitochondria, to analyze their distribution in the inducible cell line. No colocalization of Flag-MLK3 and mitotracker Red was observed, irrespective of JNK inhibition, even when different focal planes were analyzed (Figure 9).

Because of the distinctly punctate cytoplasmic distribution of MLK3, I turned my attention to endocytic vesicles. Endosomes are cellular compartments that internalize extracellular molecules into the cytosol. Early endosomes receive the endocytosed material, and sort it to late endosomes and lysosomes, or send it for recycling to the plasma membrane. EEA1 is an evolutionarly conserved protein associated with the membrane of the early endocytic vesicles (Mu et al., 1995). To determine whether the punctate pattern corresponds to endosomes, cells were costained for MLK3 and EEA1. As shown in Figure 10, there is a small amount of colocalization of signaling-competent MLK3 with EEA1. Upon JNK inhibition, the subcellular distribution of the hypophosphorylated MLK3 looks, at first glance, very similar to the distribution of EEA1. Some structures seem to harbor both MLK3 and EEA1 staining. However, the majority of the vesicles are either exclusively MLK3-positive or EEA1-positive. Triton-soluble and Triton-insoluble fractions from cellular lysates prepared from MCF7/iFlag-MLK3 cells were analyzed for the presence of early endosomes; EEA1 was found exclusively in the Triton-soluble fractions (see Table 1). These data indicate that JNK inhibition does not result in quantitative targeting of the dephosphorylated, inactive MLK3 to endosomes.

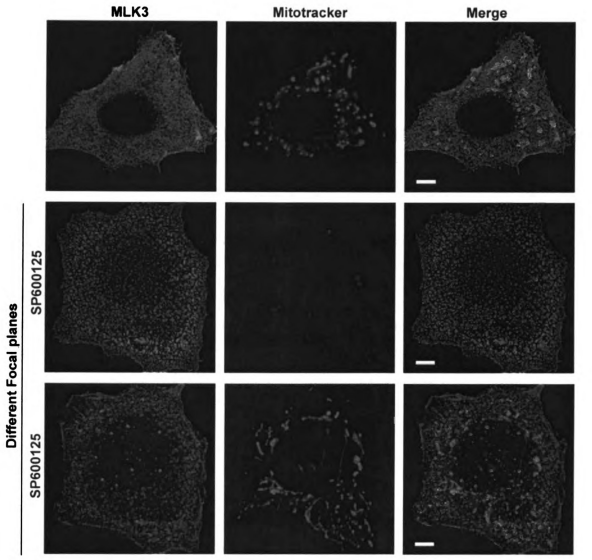
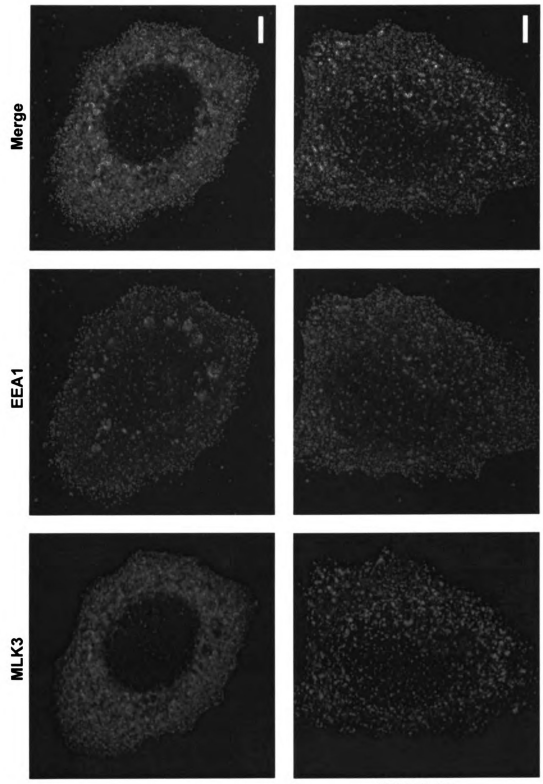


Figure 9. **Costaining of MLK3 and mitochondria in MCF-7/iFlag-MLK3 cells**. Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and incubated with Mitotracker Red 30 min before fixation. MLK3 was stained using a Flag antibody and a secondary antibody conjugated with Alexa Fluor 488. Representative images are shown with Flag-MLK3 and mitochondria represented in *green* and *red*, respectively. Two different focal planes from the same cell treated with SP600125 are shown. *Bar*, 5 μm.

Figure 10. **Costaining of MLK3 and endocytic vesicles in MCF-7/iFlag-MLK3 cells.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and fixed. MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 546. Endocytic vesicles were stained with a mouse EEA1 antibody and a secondary antibody conjgated with Alexa Fluor 488. Representative images are shown with MLK3 and EEA1 represented in *red* and *green*, respectively. *Bar*, 5 μm.


Merge
EEA1
MLK3
SP600125

I then investigated whether MLK3 could localize to other type of vesicles, such as clathrin-coated vesicles. Figure 11 shows cells inducibly expressing MLK3, which display a substantial amount of colocalization between MLK3 and clathrin, except at the cell periphery, where only MLK3 could be detected. When the cells are incubated with SP600125, a dramatic change in the subcellular distribution of MLK3 is observed, with loss of staining at the cell edge, as described before. However, there is no substantial change in the amount of colocalization between MLK3 and clathrin under conditions of JNK inhibition, when compared with untreated cells. These data indicate that MLK3 and clathrin can colocalize in cells, but that JNK does not cause a dramatic redistribution of MLK3 to clathrin-coated vesicles.

Table 1 summarizes the distribution of different subcellular markers in the crude Triron X-100 fractionation. Most of the markers analyzed are soluble in Triton X-100. The data presented in this Chapter indicate that upon JNK inhibition, MLK3 becomes hypophosphorylted and redistributes to a Triton X-100 insoluble fraction that appears as small dot-like vesicles throughout the cell. The nature of those vesicles could not be precisely determined, but it does not seem to correspond to any major organelle.

Figure 11. **Costaining of MLK3 and clathrin in MCF-7/iFlag-MLK3 cells.** Flag-MLK3 expression was induced using AP21967 in MCF7/iFlag-MLK3 cells for 20 h. The cells were then treated with or without 15 μM SP600125 for 1 h and fixed. MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 546. Clathrin was stained with a mouse clathrin heavy chain antibody and a secondary antibody conjugated with Alexa Fluor 488. Representative images are shown with MLK3 and clathrin represented in *red* and *green*, respectively. *Bar*, 5 μm.

Merge
Clathrin
MLK3
SP600125

Table 1. **Distribution of organelle markers in a crude Triton X-100 fractionation.**

Abbreviations: COX IV, cytochrome oxidase IV; ERp72, endoplasmic reticulum protein 72; Lamp1, lysosome associated membrane protein 1; EEA1, early endosome antigen 1.

Organelle	**Marker**	**TX-100 soluble**	**TX-100 insoluble**
Mitochondria	COX IV	++	--
Endoplasmic Reticulum	ERp72	++	--
Golgi	GM130	++	--
Lysosome	Lamp1	++	--
Plasma membrane	Integrin α2	++	--
Endocytic vesicles	EEA1	++	--
Lipid Rafts	Flotillin 1	++	+/-
Cytoskeleton	γ-tubulin	++	--
	Actin	++	+/-
	Vinculin	++	--
Nucleus	phospho-c-Jun	++	--
	Histone 3	+/-	++
	p27	++	--
	Nucleoporin p62	++	++
Cytosolic	Hsp90	++	+/-
	Cdc37	++	--

5. Discussion

Many signal transduction pathways are modulated by feedback loops. In the case of MLK3, activation of the JNK pathway results in positive feedback phosphorylation of MLK3 by JNK. In this chapter I show that when JNK is inhibited, MLK3 is rapidly inactivated and is redistributed to a Triton-insoluble fraction. This JNK phosphorylation-dependent, reversible redistribution of MLK3 represents a novel mode by which MLK3 is regulated.

In my studies I made use of MCF-7 cells engineered to inducibly express wild type Flag-MLK3 which is highly active, and phosphorylated both in its activation loop (most likely through autophosphorylation) and in its COOH-terminal region by JNK. This hyperphosphorylated Flag-MLK3 is present in Triton-soluble fractions and in turn potently activates JNK. Inhibition of JNK results in a rapid redistribution of the majority of Flag-MLK3 to a Triton-insoluble fraction. Under conditions of JNK blockade, all of the Flag-MLK3 in the Triton-insoluble fraction, as well as the small portion of Flag-MLK3 that remains in the Triton-soluble fraction, is inactive, as judged by activation loop phosphorylation status. The complete loss of reactivity with the activation loop specific phospho-MLK3 antibody upon JNK inhibition may suggest the existence of an activation loop phosphatase that is either repressed by JNK or restricted to the cellular compartment corresponding to the MLK3-containing Triton-insoluble fraction. Although, activation loop phosphatases that target MAPKKKs do not seem to be commonplace, an activation loop phosphatase for ASK1 has been described (Morita et al., 2001).

MLK3 distribution depends upon its phosphorylation status. The serine/threonine phosphatase inhibitor, calyculin A, renders Flag-MLK3 highly phosphorylated and completely soluble. JNK inhibition results in inactivation and redistribution of Flag-MLK3 to a Triton-insoluble fraction. Removal of the JNK inhibitor results in active, solubilized Flag-MLK3, providing evidence for reversibility of this process, at least in short term treatments.

The finding that JNK phosphorylation regulates the solubility of a MAPKKK is novel. It has been reported that cellular stressers like camptothecin and sorbitol can induce MLK3 activation and stabilization. Ablation of JNK expression by siRNA or with dominant negative JNK1 blocked camptothecin-induced increase in MLK3 protein levels, supporting a model for a positive feedback regulation of MLK3 stability by JNK (Xu et al., 2005). However, the subcellular distribution of the stabilized MLK3 was not analyzed. In my experimental setting, JNK inhibition does not reduce the total amount of MLK3, but rather changes its subcellular distribution. It is unclear whether the camptothecin-dependent increase in soluble MLK3 is a result of increased protein stability or increased protein solubility.

Our lab recently published that Hsp90/Cdc37 act as cochaperones for MLK3 (Zhang et al., 2004). The Triton-insoluble fraction containing inactive Flag-MLK3 is largely devoid of Hsp90 and lacks detectable Cdc37, suggesting that Hsp90/Cdc37 dissociation from Flag-MLK3 might be involved in its redistribution to Triton-insoluble fractions. Dissociation of Hsp90 from the IKK complex was recently shown to result in transient, reversible Triton-insolubility of the IKKs (Pittet et al., 2005), whereas long term stress signals result in degradation of IKK. A role for IKK phosphorylation in this

process has not been established. In my case, long term (12 h) inhibition of JNK did not result in increased degradation of Flag-MLK3. Furthermore, the fact that removal of the JNK inhibitor results in MLK3 reactivation and solubilization indicates that the MLK3 present in the Triton-insoluble fraction is not irreversibly aggregated, raising the possibility that there may be other chaperones interacting with Flag-MLK3 that keep it folded when present in Triton-insoluble compartments. For instance, small Heat shock proteins (sHsps) help in resolubilizing proteins that become unfolded upon stress, protecting them from irreversible aggregation (reviewed in (Sun & MacRae, 2005)). In yeast, upon heat stress sHsps like Hsp26 or Hsp42 become insoluble and bind to unfolded proteins, keeping the whole complex in a state that allows the Hsp104/Hsp40/Hsp70 machinery to reactivate the proteins (Cashikar et al., 2005). Additionally, translation of new MLK3 molecules must have occurred during the extended treatment with the JNK inhibitor; these newly synthesized MLK3 molecules were also targeted to the Triton-insoluble fraction, suggesting that under non-inhibiting conditions JNK phosphorylation occurs rapidly, and reinforcing the idea that JNK phosphorylation of MLK3 is required to keep MLK3 soluble.

Since MLK3 is not degraded, to where has it been redistributed? I analyzed the Triton-soluble and Triton-insoluble fractions for the presence of markers of different organelles. The data, summarized in Table 1, indicate that only flotillin-1, which is present in cholesterol-enriched membranes like lipid rafts and nuclear components fractionate with MLK3. However, density gradient centrifugation experiments indicate that MLK3 does not partition to lipid rafts upon JNK inhibition. Confocal microscopy studies demonstrate that treatment with the pharmacological inhibitor of JNK changes the

MLK3 localization pattern from diffuse cytosolic to vesicular. MLK3 does not localize to the nucleus, and incubation with the JNK inhibitor does not result in the total translocation of MLK3 into the nucleus, although some MLK3-containing structures seem to be perinuclear.

Inducibly expressed MLK3 colocalizes with vesicle markers to some degree. Yet I did not detect increased colocalization to endocytic vesicles or clathrin-coated vesicles upon JNK inhibition. In addition, under my cellular lysis conditions, the vesicular markers fractionate to the Triton-soluble supernatant. The ERK pathway scaffold protein KSR has been found to fractionate to Triton-insoluble cellular compartments. c-Tak-1-mediated phosphorylation of KSR, and/or its interaction with the Ras effector protein IMP, results in hyperphosphorylation and inactivation of KSR, and redistribution of KSR to a Triton-insoluble fraction (Matheny et al., 2004; Stewart et al., 1999). Interestingly, coexpression of KSR with IMP results in colocalization of both proteins to Triton-resistant punctate structures, similar to the ones observed for hyposphopshorylated MLK3 (Matheny et al., 2004). The authors speculate that KSR could be sequestered into microdomains where it becomes inaccessible to activators. Like in the case of MLK3, this distribution is reversible and can be overcome by expression of oncogenic Ras. However, the nature of the punctate structures was not analyzed.

In summary, this work describes a novel way of regulating MLK3, by targeting its inactive, dephosphorylated form to a Triton-resistant fraction. The determination of the exact nature of the Triton-insoluble MLK3, as well as the presence of interactive partners of MLK3 in the Triton-insoluble fractions requires further investigation.

IV. Concluding Remarks

The purpose of the work described in this dissertation was to gain additional insight into the mechanism by which phosphorylation regulates MLK3 activities. Of particular interest were the roles of a subset of phosphorylation sites identified on MLK3, which correspond to proline-directed kinase sites.

The work presented here is the natural continuation of previous studies done in the laboratory. The identification of *in vivo* phosphorylation sites on MLK3 was thought as a first step towards understanding how phosphorylation regulates MLK3. The experiment was designed as follows: MLK3 and its upstream activator Cdc42 were transfected into cells. Sixteen hours after transfection, the cells were labeled with inorganic phosphate for four hours. Subsequently, the phosphorylation sites on MLK3 that incorporated radioactivity were determined by mass spectrometry. As a result, eleven sites were identified. Seven of them contained a proline residue immediately following the phosphorylated serine residue, suggesting that MLK3 is regulated by proline-directed kinases.

The work in Chapter II reveals that JNK is responsible for phosphorylating those sites on MLK3. Since phosphorylation of the proline-directed sites requires activation of the JNK pathway by MLK3, the data is consistent with positive feedback phosphorylation of MLK3 as the regulatory mechanism. The rapid exchange of phosphate into those sites is also indicative of dynamic phosphorylation. Feedback phosphorylation of MLK3 generates a slower migrating form of MLK3 in SDS-PAGE, which is also abolished when JNK is inhibited.

In retrospect, the experimental design that led to the discovery of phosphorylation sites resulted in the identification of sites that occurred as a consequence of MLK3 activation, rather than phosphorylation events that caused MLK3 activation. This helps explain, for instance, why the activation loop phosphorylation sites were not detected even though, by means of a phospho-specific antibody, they are real phosphorylation sites on MLK3: phosphorylation of the activation segment is thought to be one of the first required steps in the activation of a protein kinase. Therefore, it would have occurred long before the radiolabeled orthophosphate was added to the cells. This hypothesis implicitly states that the activation loop sites are quite stable, with a very slow turnover, resulting in very little incorporation of radioactivity during the labeling period. Likewise, I believe that the decreased electrophoretic mobility of MLK3 observed upon coexpression with activated Cdc42 correspond to feedback phosphorylation by JNK, rather than being a direct effect of Cdc42 on MLK3.

The work in Chapters II and III demonstrate that feedback phosphorylation of MLK3 is a dynamic process. Inhibition of JNK activity not only impaired feedback phosphorylation of MLK3, but it also dramatically reduces the levels of MLK3 in cellular lysates. This is not likely due to degradation of MLK3. As shown in Chapter III, the dephosphorylated MLK3 was rendered Triton-insoluble. However, if the inhibition of JNK is released, MLK3 moves back to a Triton-soluble fraction, indicating that the process is reversible and dependent on the phosphorylation state of MLK3. By confocal microscopy it was shown that the Triton-insoluble MLK3 localizes to dot-type structures in the cytosol of the cells, although the exact nature of those punctate structures remains to be determined.

An important question that remains to be answered is whether the regulation of MLK3 by phosphorylation observed in the inducible expression system can be applied to endogenous MLK3. Under basal conditions, endogenous MLK3 is phosphorylated, based on phosphatase treatment of cellular lysates, which increases the electrophoretic mobility of MLK3 (K. Viswanathan). However, activation loop phosphorylation of MLK3 has not been detected in resting cells. TNF-α treatment, which transiently activates JNK, does not result in altered mobility of MLK3, suggesting that feedback phosphorylation has not occurred. However, under conditions of sustained JNK activation, like a 4 h treatment with camptothecin, feedback phosphorylation of MLK3 has been proposed to occur. In this regard, phospho-specific antibodies generated against the proline-directed kinase phosphorylation sites would be predicted to recognize MLK3 phosphorylated under sustained, but not transient, JNK activation. Development of such antibodies becomes necessary to fully clarify this aspect of the molecular regulation of MLK3. However, for practical reason, it might be necessary to make only one of the phospho-specific antibody. In that case, selecting a peptide containing phosphorylated Ser 740 may be the best option; the phosphopeptide maps indicate that Ser 740 is highly phosphorylated when *in vivo* labeled, which would likely make it easier to detect changes in the phosphorylation status of the endogenous protein. Whether MLK3 is phosphorylated on other yet unidentified sites during transient activation also need to be determined. Mass spectrometry could be applied on immunopurified MLK3 from cells that have been treated with TNF-α, which has been documented to activate endogenous MLK3, to identify phosphorylation sites that regulate transient MLK3 activation.

The redistribution of inactive, hypophosphorylated MLK3 to a Triton-insoluble fraction was somewhat surprising. Even more, the fact that MLK3 is kept insoluble, rather than degraded, is unexpected. It is conceivable that MLK3 is associated with other molecule(s) that keep MLK3 insoluble, but stable. In fact, during a long term (12 h) treatment with the JNK inhibitor, the steady state level of MLK3 does not change and MLK3 does not accumulate in the insoluble fraction, suggesting that MLK3 can be recognized and turned over normally. One possibility is that the redistribution of MLK3 to Triton-insoluble vesicles is a normal process used by the cell to inactivate MLK3. It would be inportant to analyze the distribution of endogenous MLK3 in Triton X-100 upon JNK activation and after JNK activation has ceased.

Appendix A: Determination of the *in vitro* kinase activity of various MLK3 mutants.

This appendix describes the generation of phosphorylation site mutants of MLK3 and the analysis of their impact on the kinase catalytic activity of MLK3.

Two regions of MLK3 were analyzed: the glycine-rich region, where serines 11, 15 and 35 were independently mutated to either alanine or the phosphomimetic glutamic acid, and the Cdc42-inducible sites, where serines 555 and 556 were both mutated to either alanine or glutamic acid.

Generation of phosphorylation site mutants.

Site directed mutagenesis was performed by polymerase chain reaction (PCR) using the Quick Change method (Stratagene). The oligonucleotides used for PCR, as well as the DNA template are described in Table 1. The double mutants of the Cdc42 inducible sites were constructed as followed: oligonucleotides containing the S555A or the S555E point mutation were used in a PCR reaction using pRK5-MLK3 as a template. The presence of the mutation was confirmed by DNA sequencing (MSU DNA Sequencing Facility). A second PCR reaction over this mutated DNA was done using oligonucleotides containing the S555A,S556A or the S555E,S556E mutations and the presence of the mutations was confirmed by DNA sequencing.

Table 1. **Oligonucleotides used for Site-directed mutagenesis.**

Mutant	Oligonucleotide	Template
S11A	5'-AGCCTCTTCCTCAAGGCACCTCTAGGGTCATGG-3'	pRK5-MLK3
S11E	5'-AGCCTCTTCCTCAAGGAGCCTCTAGGGTCATGG-3'	pRK5-MLK3
S15A	5'-AAGAGCCCTCTAGGGGCATGGAATGGCAGTGGC-3'	pRK5-MLK3
S15E	5'-CCTCTAGGGGAATGGAATGGC-3'	pRK5-MLK3
S35A	5'-CCTGAGGGGGCTCCAAAGGCA-3'	pRK5-MLK3
S35E	5'-CGGCCTGGAGGGGGAACCAAAGGCAGCG-3'	pRK5-MLK3
S555A	5'-CTGGAGGACGCAAGCAATGGAGAG-3'	pRK5-MLK3
S555A,S556A	5'-CTGGAGGACGCAGCCAATGGAGAG-3'	pRK5-MLK3 S555A
S555E	5'-CTGGAGGACGAAAGCAATGGAGAG-3'	pRK5-MLK3
S555E,S556E	5'-CTGGAGGACGAAGAAAATGGAGAG-3'	pRK5-MLK3 S555E

Effect of mutagenesis on MLK3 *in vitro* kinase activity

1. Mutagenesis of the glycine-rich region

Using mass spectrometry coupled with phosphopeptide mapping analysis, *in vivo* phosphorylation sites of MLK3 were previously identified, under conditions of ectopic expression of MLK3 with its upstream activator, Cdc42. Eleven sites were identified; all are serine residues located towards the COOH-terminal region of MLK3. An additional phosphorylation site was found in a peptide encompassing Ser 11-Lys 37 of MLK3, whose sequence is ^{11}SPLGSWNGSGSGGGGGGGGGRPEGSPK^{37} (Vacratsis et al., 2002). Five serines are present in this peptide, and although the mass spectrometry data indicates that only one phosphate group is present, it was not possible to specifically assign the phosphorylation site, presumably due to the poor fragmentation of the peptide on the ion source of the MALDI mass spectrometer (Vacratsis et al., 2002).

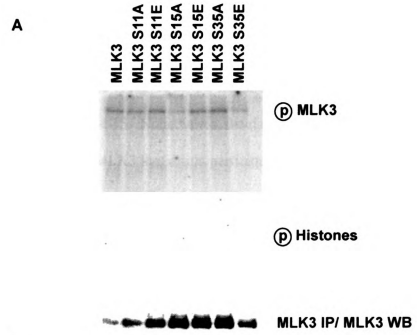
In an attempt to implicate one of the Ser residues in that peptide as a phosphorylation site, I individually mutated Ser 11, Ser 15 and Ser 35 to Ala or to Glu residues. Ala mutants are used to mimic non-phosphorylated form because the side chain of alanine cannot be modified with a phosphate moiety. To mimic the effect of having a serine residue constitutively phosphorylated, Glu or Asp are used to substitute the phosphorylation site, the idea being that the carboxylate can mimic both the structure and the charge of a phosphate group. However, substitution of a carboxylate for a phosphate group is not always successful in reproducing the phenotype of the phosphorylated state of a protein.

To determine if phosphorylation on any of those serines on the glycine-rich region could affect MLK3 kinase activity, an *in vitro* kinase assay was performed. HEK 293

cells were transfected using the calcium phosphate technique with expression vectors for the different MLK3 variants. Twenty hours post-transfection the cells were lysed, and MLK3 was immunoprecipitated from clarified lysates with a MLK3 antibody. The immunoprecipitates were washed three times with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) containing 1 M LiCl, three times with HNTG buffer, and twice with kinase assay buffer (50 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.1 mM Na_3VO_4). MLK3 kinase assays were performed in 20 μl kinase assay buffer using 50 μM ATP and 5 μCi [γ-^{32}P] ATP (3000 Ci/mmol) (NEN Life Science Products). The immunoprecipitates were incubated with 10 μg of mixed histones (Roche Molecular Biochemicals) for 15 min at room temperature. The kinase reaction was terminated by adding 40 μl of 1.5x SDS loading buffer (100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue, 100 mM dithiothreitol, and 1% β-mercaptothanol, 50 mM EDTA (pH 8.0)). The proteins were resolved by SDS-PAGE. The gels were rinsed in PBS, dried, and the incorporation of radioactivity into kinase or substrates was determined using PhosphorImaging (Molecular Dynamics). The data was normalized to the levels of MLK3 in the immunoprecipitates.

The results shown in Figure 1 indicate that substitution of serine 11 or serine 35 of MLK3 with alanine or glutamic acid residues did not affect MLK3 catalytic activities *in vitro*. On the other hand, the S15A substitution on MLK3 reduced its ability to autophosphorylate as well as its ability to phosphorylate an exogenous substrate by 40%. However, the correspondent glutamic acid mutation did not increase MLK3 catalytic

Figure 1. ***In vitro* kinase assay of MLK3 mutants in the glycine-rich region.** HEK 293 cells were transfected with expression vectors for different variants of MLK3 as indicated. MLK3 was immunoprecipitated from clarified lysates and subjected to *in vitro* kinase assay using [γ-^{32}P] ATP and histones as substrates. (*A*) MLK3 and histones were fractionated by SDS-PAGE. *Top panel*, autoradiogram showing MLK3 autophosphorylation and histone phosphorylation. *Bottom panel*, a fraction of the immunoprecipitated MLK3 was subjected to western blotting using a MLK3 antibody. (*B*) The incorporation of radioactivity into MLK3 and into histones was quantified by phosphorimaging, and normalized to the levels of immunoprecipitated MLK3. Data from two or three independent experiments are shown as mean ± S.E.


A
MLK3
MLK3 S11A
MLK3 S11E
MLK3 S15A
MLK3 S15E
MLK3 S35A
MLK3 S35E
(p) MLK3
(p) Histones
MLK3 IP/ MLK3 WB
B
Fold increase in autophosphorylation
3.5
3
2.5
2
1.5
1
0.5
0
wt
S11A
S11E
S15A
S15E
S35A
S35E
Fold increase in histone phosphorylation
2.5
2
1.5
1
0.5
0
wt
S11A
S11E
S15A
S15E
S35A
S35E

activities, the autophosphorylation activity being identical to that of the wild type kinase, and the substrate phosphorylation activity being even lower than wild type MLK3. It is possible that the change to glutamic acid is not adequately mimicking the effect of a phosphate group on serine 15. Nonetheless, the data suggest that Ser 15 is a potential phosphorylation site on MLK3 that might participate in the regulation of MLK3 catalytic activity.

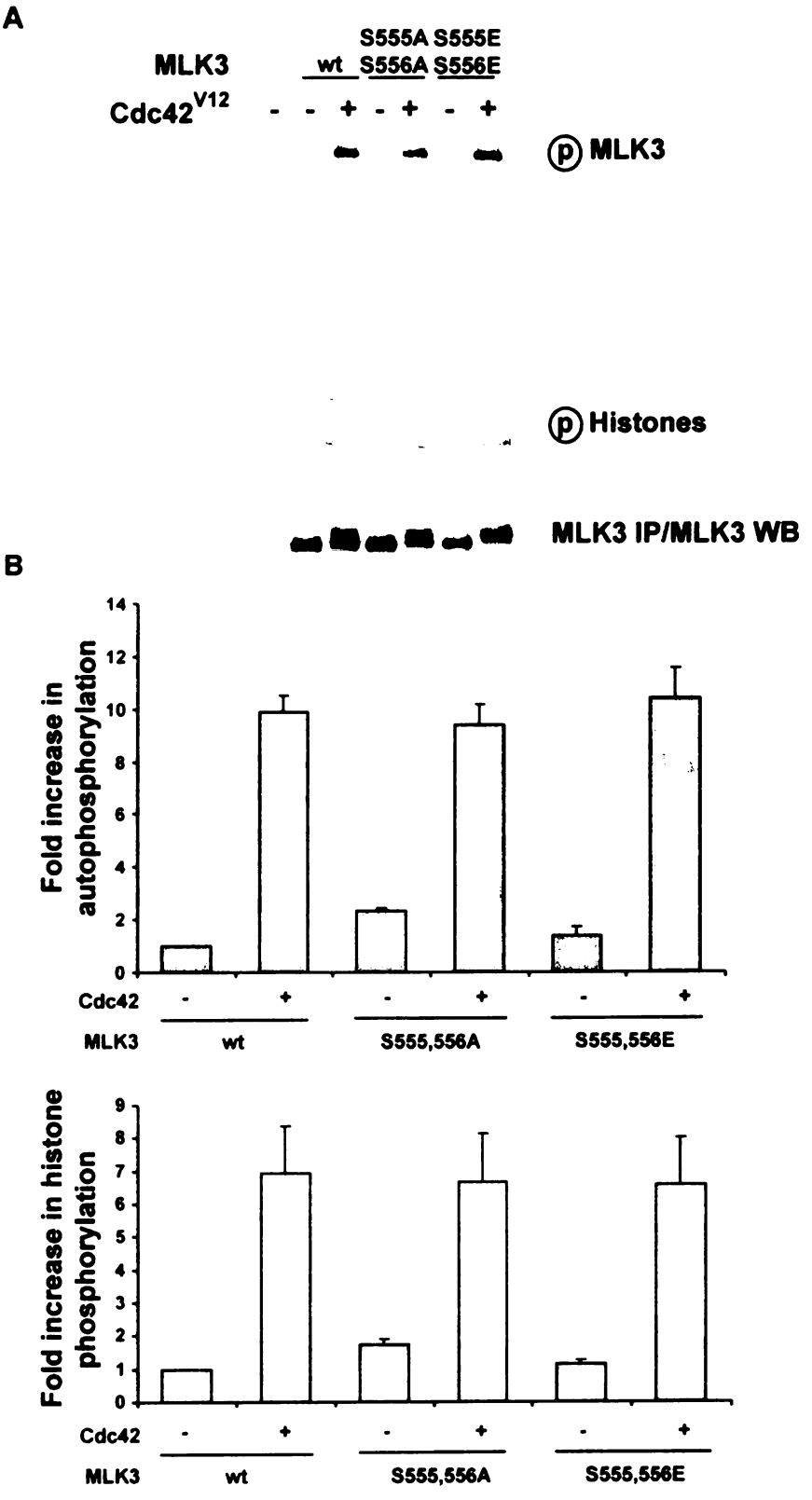
2. Mutagenesis of the Cdc42-inducible sites

Cdc42 is a small GTPase that binds, in its activated state, to the CRIB motif of MLK3. Coexpression of a constitutively active mutant form of Cdc42, $Cdc42^{V12}$, with MLK3, increases the *in vitro* catalytic activity of MLK3 as judged by autophosphorylation and histones phosphorylation (Bock et al., 2000). In addition, activated Cdc42 changes the *in vivo* phosphorylation pattern of MLK3. Two of the identified phosphorylation sites of MLK3, serine 555 and serine 556, reside in phosphopeptides that appear at high levels in phosphopeptides maps of MLK3 when MLK3 is co-expressed with $Cdc42^{V12}$, and for that reason they were called "Cdc42 inducible sites".

To test whether the Cdc42-inducible phosphorylation sites are responsible for the potentiation of MLK3 catalytic activity by the small GTPase, those sites were mutated to alanine and to glutamic acid using site-directed mutagenesis. Vectors encoding MLK3, MLK3 S555A,S556A (double mutation to alanine) and MLK3 S555E,S556E (double mutation to glutamic acid) were transfected into HEK 293 cells and the different variants of MLK3 were immunoprecipitated from cellular lysates. An *in vitro* kinase assay using

histones as a substrate was used to test the activity of the variants. If phosphorylation at serine 555 and serine 556 were responsible for the increase in MLK3 catalytic activity, then the double alanine mutant should fail to potentiate MLK3 activity when co-expressed with $Cdc42^{V12}$. Similarly, the double glutamic acid mutant would be expected to exhibit higher catalytic activity than wild type MLK3 when expressed alone. Furthermore, the catalytic activity of the double glutamate mutant should not be further increased by coexpression with activated Cdc42. The result shown in Figure 2 did not match any of these expectations. MLK3 S555A,S556A had a basal autophosphorylation level higher than with wild type MLK3, and its catalytic activity was potentiated upon co-expression with $Cdc42^{V12}$ at a similar level as wild type MLK3 (Figure 2A, compare lanes 2 and 4, 3 and 5). Similarly, MLK3 S555E,S556E showed increased basal autophosphorylation activity compared to wild type MLK3, and could still be potentiated by coexpression with activated Cdc42 (Figure 5A, compare lanes 2 and 6, 3 and 7). These results suggest that the phosphorylation of the Cdc42 inducible sites is not required for the increase in MLK3 catalytic activity observed upon coexpression with activated Cdc42. It is possible that phosphorylation of Ser^{555} and Ser^{556} regulates some other function of MLK3 such as protein-protein interaction or subcellular localization. Alternatively the phosphorylation of Ser^{555} and Ser^{556} may result from, rather than cause, the increased catalytic activity of MLK3 with Cdc42. Finally, there may be other sites of Cdc42-induced phosphorylation, as yet unindentified, that are responsible for the Cdc42-induced effect in MLK3 catalytic activity.

Figure 2. ***In vitro* kinase activity of the Cdc42-inducible sites mutants of MLK3.** HEK 293 cells were transfected with expression vectors for different variants of MLK3, with or without activated Cdc42 as indicated. MLK3 was immunoprecipitated from clarified lysates and subjected to *in vitro* kinase assay using [γ-^{32}P] ATP and histones as substrates. (*A*) MLK3 and histones were fractionated by SDS-PAGE. *Top panel*, autoradiogram showing MLK3 autophosphorylation and histone phosphorylation. *Bottom panel*, a fraction of the immunoprecipitated MLK3 was subjected to western blotting using a MLK3 antibody. (*B*) The incorporation of radioactivity into MLK3 and into histones was quantified by phosphorimaging, and normalized to the levels of immunoprecipitated MLK3. Data from three independent experiments are shown as mean ± S.E.


A
S555A S555E
MLK3
wt S556A S556E
Cdc42V12
- - + - + - +
ⓟ MLK3
ⓟ Histones
MLK3 IP/MLK3 WB
B
Fold increase in autophosphorylation
14
12
10
8
6
4
2
0
Cdc42 - + - + - +
MLK3 wt S555,556A S555,556E
Fold increase in histone phosphorylation
9
8
7
6
5
4
3
2
1
0
Cdc42 - + - + - +
MLK3 wt S555,556A S555,556E

VI. References

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