This is to certify that the
dissertation entitled

REGULATION OF MIXED LINEAGE KINASE 3 BY AUTOINHIBITION, PROTEOLYSIS AND BINDING PARTNERS

presented by

Hua Zhang

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Cell and Molecular Biology Program

Kathleen A. Gallo
Major Professor's Signature

August 21, 2003
Date

REGULATION OF MIXED LINEAGE KINASE 3 BY AUTOINHIBITION, PROTEOLYSIS AND BINDING PARTNERS

By

Hua Zhang

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology Program

2003

ABSTRACT

REGULATION OF MIXED LINEAGE KINASE 3 BY AUTOINHIBITION, PROTEOLYSIS AND BINDING PARTNERS

By

Hua Zhang

Mixed lineage kinase 3 (MLK3) is a serine/threonine protein kinase widely expressed in human tissues. MLK3 acts as a mitogen activated protein kinase (MAPK) kinase kinase (MAPKKK) to phosphorylate and activate the dual specific kinases, MAPK kinase 4 (MKK4) and MKK7, which, in turn, can phosphorylate and activate the c-Jun NH_2-terminal kinase (JNK) pathway. Overexpressed MLK3 also modestly activates the p38 MAPK pathway.

In addition to its catalytic domain, MLK3 contains several domains, including an amino-terminal Src Homology 3 (SH3) domain, a centrally located zipper region, a Cdc42/Rac interactive binding (CRIB) motif and a Pro/Thr/Ser rich carboxyl terminus, which may mediate protein-protein interactions to regulate its activity and/or signaling. The work presented herein demonstrated that MLK3 is autoinhibited through an interaction between its amino terminal SH3 domain and a sequence located between the zipper and CRIB motifs of MLK3. A single proline residue in the SH3-binding site is required for this intramolecular autoinhibition. Mutation of either the SH3 domain or the proline residue in the SH3–binding site increases MLK3 catalytic activity. The SH3 domain and the critical proline residue in the binding site are conserved in the closely

related family members, MLK1, MLK2, MLK4 and the *Drosophila* homologue, Slipper, suggesting a common autoinhibitory mechanism among these kinases.

MLK3 undergoes proteolysis to generate a stable carboxyl terminal fragment (CTF) *in vivo* in an activity dependent fashion in HEK 293 cells and in a PMA –induced fashion in Jurkat T Ag cells. The amino terminus of the CTF is Pro 252, suggesting that the "MLK3 protease" cleaves between Gln 251 and Pro 252 within the kinase domain. Site directed mutagenesis revealed that Leu 250 and Gln 251, but not Pro 252, are required for this proteolytic event. Interestingly, the coronavirus main protease shares a strikingly similar substrate specificity, where it cleaves immediately after Leu-Gln sequences in its viral polyprotein substrates. Chapter III of this thesis presents data that indicate mammalian cells contain a coronavirus protease-like activity. In addition, the protease inhibitor, MG 132, blocks the PMA-induced proteolysis of MLK3.

Affinity purification coupled with mass spectrometry was employed to isolate and identify MLK3 binding partners. In-gel trypsin digestion together with in-solution trypsin digestion have revealed a total of 23 potential interacting proteins of MLK3. These findings certainly will lead to new directions in the study of the regulations and biological functions of MLK3.

In addition, the functional significance of the interaction between the molecular chaperones Hsp90/Cdc37 and MLK3 was studied. Hsp90/Cdc37 was shown to bind to the catalytic domain of MLK3 in a MLK3 activity-independent manner. Disruption of Hsp90 function with geldanamycin decreases the protein level of MLK3 in MCF-7 cells. Furthermore, Hsp90/Cdc37 regulate TNFα-induced JNK activation at the MAPKKK (MLK3) level, but not at MKK4/MKK7 or JNK level.

To my family

ACKNOWLEDGMENTS

I would like to thank my committee members Dr. Kathy Gallo, Dr. Susan Conrad, Dr. John Wang, Dr. David Dewitt and Dr. Robert Hausinger for their guidance and support. Especially I deeply appreciate my mentor, Dr. Gallo's guidance, support and encouragement throughout my graduate career. I would like to thank Wei Wu and Dr. Watson for their collaboration on the mass spectrometry project. And I thank Weiqin Chen and Dr. Walter Esselman for their collaboration on the MLK3 proteolysis project.

I am very thankful for the friendships with the members in Dr. Gallo's lab during my graduate training, especially Dr. Otis Vacratsis, Mary Chao, Karen Schatcher, Yan Du, Geouyarh (Stancy) Liou, Ritesh Agrawal, and Dr.Barbara Bock. I thank them for their valuable discussion and co-operation.

TABLE OF CONTENTS

List of Tables

List of Figures

Chapter V

Key to Abbreviations

$3C^{pro}$	3C-like proteinase
AGC	containing Protein Kinase A, Protein Kinase G, Protein Kinase C families
ANT-2	adenine nucleotide translocator-2
AP50	clathrin assembly protein complex 2 medium chain
ASK1	apoptosis signal- regulating kinase 1
CaM	Calcium /Calmodulin
CAMK	calcium/calmodulin dependent protein kinases
CBP	calmodulin-binding peptide
CDK	cyclin dependent protein kinases
CHC	clathrin heavy chain
CK1	casein kinase 1
CKI	CDK inhibitors
CMGC	containing CDK, MAPK, GSK3, CLK families
CRIB	Cdc42/Rac interactive binding motif
DeltaCn	1.0-normalized correlation score
DLK	dual leucine zipper protein kinase
DMEM	Dulbecco's Modified Eagle's Medium
ERK	extracellular signal-regulated kinase
ES	nanoelectrospray
ESI	electrospray ionization
FKBP	FK binding proteins
FnIII	fibronectin type III
FRET	fluorescence resonance energy transfer
GA	geldanamycin
GluR6	Kainate receptor glutamate receptor
GST	glutathione S-transferase
HA	hemagglutinin
HPK1	hematopoietic progenitor protein kinase-1

Hsc70	heat shock cognate 71 kD protein
HSP	heat shock protein
IκBα	inhibitor of NF-κB alpha
IB	islet brain
Ig	immunoglobulin
IKK	IκB kinase
JIP	JNK interacting protein
JNK	c-jun N-terminal kinase
KIF3	kinesin superfamily motor KIF3
LC	liquid chromatography
LZK	leucine zipper-bearing kinase
MALDI	matrix assisted laser desorption /ionization
MAPK	mitogen activated protein kinase
MAPKKK	MAPK kinase kinase
MEK	mitogen-activated protein/ERK kinase
MEKK	MEK kinase
MKK	MAPK kinase
MLK	mixed lineage kinase
MLTK	MLK-like mitogen-activated protein triple kinase
MP1	MEK partner 1
M^{pro}	main proteinase
MRK	MLK-related kinase
MS	mass spectrometry
NFAT4	nuclear factor of activated T cells 4
NGF	nerve growth factor
NIMA	never in mitosis A
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated protein kinase
PDGF	platelet-derived growth factor

Pin1	peptidyl-prolyl isomerase Pin1
PKA	protein kinase A
PKC	protein kinase C
POSH	plenty of SH3s
PSD-95	post-synaptic density protein 95
PTPC	permeability transition pore
RGC	receptor guanylate cyclase
RNAi	RNA interference
RSp	rank/preliminary score
SAM	sterile alpha motif
SAPK	stress-activated protein kinases
SARS	severe acute respiratory syndrome
SH2	src-homology 2
SH3	src-homology 3
SPRK	SH3 domain containing proline rich protein kinase
STE	homologs of yeast Sterile 7, 11, 20 kinases
Syd	Sunday driver
TAK1	TGFβ activated protein kinase 1
TAP	tandem affinity purification
TD	terminal domain
TEV	tobacco etch virus
TGF	transforming growth factor
TK	tyrosine kinases
TKL	tyrosine kinase-like
TNF	tumor necrosis factor
TOF	time of Flight
Xcorr	cross-correlation score
ZAK	leucine-zipper and sterile-alpha motif kinase

I. Literature Review

1. Mammalian protein kinases

Mammalian protein kinases catalyze the transfer of the γ-phosphate of ATP to the amino acid residues of protein substrates. Protein phosphorylation can be reversed through the action of protein phosphatases. Mammalian protein kinases can be divided mainly into three groups based on amino acid specificity. The tyrosine kinases phosphorylate only Tyr residues within proteins, whereas the serine/threonine protein kinases are specific for phosphorylation of Ser or Thr residues. The dual specificity kinases are a small group of kinases capable of phosphorylating Tyr as well as Ser and Thr in their protein substrates [1] [2]. In addition, histidine kinases are newly discovered protein kinases in mammalian systems, which phosphorylate the 1 and/or 3 nitrogen atoms in the histidine residues by forming a phosphoramidate bond. Histidine kinases are better defined in bacteria and plants and the function of histidine phosphorylation in mammals has remained obscure [3].

All eukaryotic protein kinases share a highly conserved catalytic domain, often referred as the kinase domain. This kinase domain, of approximately 250-300 amino acids, can be divided into 12 subdomains, which share conserved sequences among protein kinases. The kinase domain folds into a two lobed structure. The smaller N-terminal lobe consists of subdomains I-IV of the kinase. Its secondary structure is mainly composed of antiparallel beta sheets with one conserved helix called the C-helix. The primary function of the N-terminal lobe is to orient and bind to Mg^{2+} and ATP. The C-terminal lobe consists of subdomains VIA-XI. Its secondary structure is mainly alpha

helical. The C-terminal lobe functions mainly in protein substrate binding and in initiating the phospho-transfer from ATP to the substrate. Subdomain V connects the two lobes and plays an important role in recognition of the peptide substrate and in the anchoring of the Mg^{2+}ATP (reviewed in [4, 5]). Within the kinase domain, there is a so-called "activation loop" which is located between two highly conserved sequence motifs Asp-Phe-Gly of subdomain VII and Ala-Pro-Glu of subdomain VIII [6]. This activation loop often contains site(s) which, upon phosphorylation, undergo significant conformational changes to increase the catalytic activity of the protein kinases.

In addition to their catalytic domains, most kinases contain non-catalytic regions or domains, which may control the subcellular location of protein kinases, mediate their interaction with other proteins or molecules, affect their enzymatic activity, and influence their stability for degradation by proteases. For example, Src homology 3 (SH3) domains normally bind to proline-rich sequences to mediate protein-protein interactions. In addition, a few protein kinases contain leucine zipper motifs. Leucine zipper motifs mediate protein oligomerization by forming coiled coil structures, in which the leucine residues spaced seven residues apart interact at the interface of opposing helices [7]. Several protein kinases contain a Cdc42-Rac interactive binding (CRIB) motif to which small GTPase Cdc42 or Rac can bind [8].

The human genome encodes 518 protein kinases, making the protein kinases one of the largest families of proteins. Based on sequence homology within the catalytic domains, and assisted by information of domain arrangement outside of the catalytic domain, mammalian protein kinases are classified into nine groups: AGC (containing PKA, PKG, PKC families), CAMK (calcium/calmodulin dependent protein kinases),

CMGC (containing CDK, MAPK, GSK3, 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 [9].

Protein kinases control many important physiological processes, such as energy metabolism, cell proliferation, cell differentiation, and apoptosis. In addition, protein kinases play critical roles in the operation of the nervous and immune systems and during the development in cell-cell communication.

2. Regulation of protein kinases

Since virtually all physiological processes are regulated by reversible protein phosphorylation, protein kinases should be precisely controlled. Most protein kinases are normally kept in an inactive form, and in response to extracellular stimuli, such as growth factors, hormones, or osmotic stress, certain protein kinases are activated and serve to transduce signals from the outside to within the cell. A variety of mechanisms regulate protein kinases, including phosphorylation and other post-translational modifications, binding of activators or inhibitors, and changes in subcellular localization. Herein, two well studied protein kinases, the serine/threonine protein kinase PKC (protein kinase C) and the tyrosine protein kinase Src will be used as examples to discuss regulatory mechanisms that are often utilized by protein kinases.

The PKCs are a family of protein kinases involved in modulating diverse cellular responses, including immune responses, cell proliferation, and learning [10]. The mammalian PKC family contains ten isozymes that cluster into three groups based on their domain arrangements: conventional (α, γ and the alternatively spliced βI and βII), novel (δ, ε, η/L, θ), and atypical (ζ, ι/λ) PKC isozymes (Fig.1). In addition to the kinase domain in the carboxyl terminus, PKC contains domains, which regulate its subcellular localization as well as catalytic activity. Conventional and novel PKC isoforms contain a typical Cysteine-rich C1 domain, which binds to diacylglycerol/ phorbol esters. The C2 domain in conventional PKC, upon binding to Ca^{2+}, facilitates PKC translocation to the plasma membrane [11].

The non-receptor tyrosine kinase c-Src, encoded by the *c-src* gene is involved in many cellular processes, such as cell cycle, cell proliferation and migration [12]. Src, and

its family members, Hck and Fyn, share a similar domain arrangement. The amino terminal SH4 domain of Src is critical for Src association with the plasma membrane. Src also contains an SH3 domain and an SH2 domain, which are characterized as binding region for proline-rich sequences or sequences containing phosphorylated tyrosine residues respectively. The kinase domain of Src is located at its carboxyl terminus [13] (Fig. 2).

2.1 Regulation of protein kinases by autoinhibition

Many protein kinases are kept in an inactive state through autoinhibitory intramolecular interactions. Some protein kinases contain pseudosubstrate domains that bind the active site of the catalytic domain, blocking substrate access [14]. For example, a pseudosubstrate autoinhibitory domain that precedes the C1 region of PKC, binds to the kinase domain of PKC, inhibiting substrate binding and keeping PKC in an inactive state [10].

Protein kinases can also be autoinhibited through intramolecular interactions without the participation of the catalytic domain. One interesting example is provided by the non-receptor tyrosine kinases of the Src family (Fig. 2), in which an autoinhibitory intramolecular association involves both the SH2 domain and the SH3 domain of Src. In the autoinhibited state of Src, the carboxyl terminal phosphorylated Tyr 527 interacts with the SH2 domain while the SH3 domain of Src binds to a polyproline type II helix sequence that is located between the SH2 domain and the kinase domain. These two intramolecular interactions constrain the motion of the kinase domain and block the binding of ATP and substrate, thus keeping Src in an inactive form [15, 16].

2.2. Regulation of protein kinases by intermolecular interactions

In the process of signal transduction, intermolecular interactions between small molecules and protein play a critical role. For example, binding of diacylglycerol or phorbol esters to the C1 domain of PKC disrupts the autoinhibitory interaction, and leads to its recruitment to the plasma membrane and its activation [11].

Autoinhibition within some protein kinases can be released through binding to another protein. For example, a proline-rich sequence in the Nef protein of the human immunodeficiency virus-1 (HIV-1) can bind to the SH3 domain of Hck, a Src family member, thus releasing Hck autoinhibition, and increasing Hck activity dramatically [17].

The activity of cyclin dependent protein kinases (CDKs) is regulated by binding to either activating or inhibitory proteins. CDKs are nuclear serine-threonine kinases that control the cell cycle in eukaryotes. CDKs are catalytically active only when bound to cyclins. CDKs are negatively regulated through binding to CDK inhibitors (CKI) such as $p27^{Kip1}$ or p16 [18, 19].

2.3. Regulation of protein kinases by proteolysis

Ubiquitin-mediated degradation is a highly conserved mechanism for selective protein degradation in eukaryotes. Ubiquitin is a 76 amino acid protein of 8.6 kDa that is covalently attached to proteins targeted for degradation. Three major enzymes are required in ubiquitination. Ubiquitin is activated in an ATP-dependent step by a specific activating enzyme (E1). The activated ubiquitin molecule is then transferred to a ubiquitin-conjugating enzyme (E2). Then the ubiquitin protein ligase (E3) links the

ubiquitin through its carboxyl terminus to the ε-amino group of a Lys residue in the substrate protein. The 26S proteasome can recognize and degrade the ubiquitinated proteins (reviewed in [20]). Many proteins involved in signal transduction and cell cycle progression, including protein kinases, are subject to ubiquitin-mediated degradation. For example, upon treatment of the renal epithelial cells with a diacylglycerol analogue, PKCα undergoes ubiquitin-mediated degradation [21]. The non-receptor tyrosine kinases Src and Blk, have also been reported to undergo ubiquitin mediated degradation [22] [23]. Furthermore, this ubiquitination is activity dependent, as activated forms of Src are less stable than wild type or kinase-inactive Src. The steady state level of Src can be up-regulated by the proteasome inhibitors [22]. Interestingly, the activities of CDKs are indirectly regulated by ubiquitin-mediated degradation pathway. The protein levels of cyclins and CKIs are controlled by the proteasome during the cell cycle, thus influencing the activity of CDKs along cell cycle [18, 19].

Many protein kinases are substrates for caspases, and several protein kinases become activated after proteolytic cleavage by the caspases. Caspases are cysteine proteases, which are activated during apoptosis and cleave before an aspartic acid within the substrate [24-26]. For example, PKCε[27] and PKCδ [28] are activated by caspases-mediated cleavage. In contrast, other protein kinases that control anti-apoptotic pathways, are inactivated upon caspases cleavage [29].

Calpains are responsible for the degradation of some proteins in signal transduction. Calpains are Ca^{2+}-dependent intracellular cysteine proteases, widely expressed in various mammalian tissues. There are two major forms of calpains: m-calpain and μ-calpain. Calpains show no strict sequence preferences. It has been

suggested that calpain-mediated cleavage may be dependent on protein structure [30]. The substrates of calpain include PKC isoforms [31]. Recent studies show that calpain can be activated through ERK pathway [32, 33] and the precise mechanism of how this activation occurs remains to be discovered.

2.4. Regulation of protein kinases by heat shock proteins

Heat shock proteins are molecular chaperones that assist in the folding of newly synthesized proteins and maintain protein homeostasis in cells. Heat shock proteins are constitutively expressed under normal growth conditions. Upon exposure to stress, such as heat shock, the expression of some heat shock proteins is greatly enhanced [34]. Heat shock proteins, Hsp90 and Hsp70, are two of the most highly expressed molecular chaperones. Hsp70 interacts with unfolded substrates and assists them in folding to intermediate states, thus preventing irreversible aggregation. In contrast, Hsp90 can prevent the aggregation of intermediately folded proteins, but lacks the ability to refold denatured proteins [34].

Most of the Hsp90 client proteins are involved in signal transduction. Studies indicate that some protein kinases interact with Hsp90, and the Hsp90 co-chaperone Cdc37 is required for this interaction. Association with Hsp90/Cdc37 stabilizes the client protein kinase and prevents its degradation by the proteasome. The ansamycin antibiotics, such as geldanamycin (GA), occupy the ATP/ADP pocket of Hsp90, thus inhibiting Hsp90 chaperone activity. Geldanamycin treatment destabilizes several protein kinases, such as Src, Raf-1 and Akt [35] [36, 37], because geldanamycin treatment dissociates dysfunctional Hsp90 from protein kinases and protein kinases undergo ubiquitin-

mediated degradation. However, some of the protein kinases, such as IκB kinase (IKK), although they have been reported to associate with Hsp90/Cdc37, are not destabilized by geldanamycin [38].

Relatively less is known about how Hsp70 may regulate protein kinases. Recent studies show that Hsp70 interacts with PKC. Hsp70 prolongs the lifetime of mature PKC by stabilizing the protein and allowing re-phosphorylation of the kinase. Disruption of Hsp70-PKC interaction prevents re-phosphorylation and targets PKC for down-regulation [39].

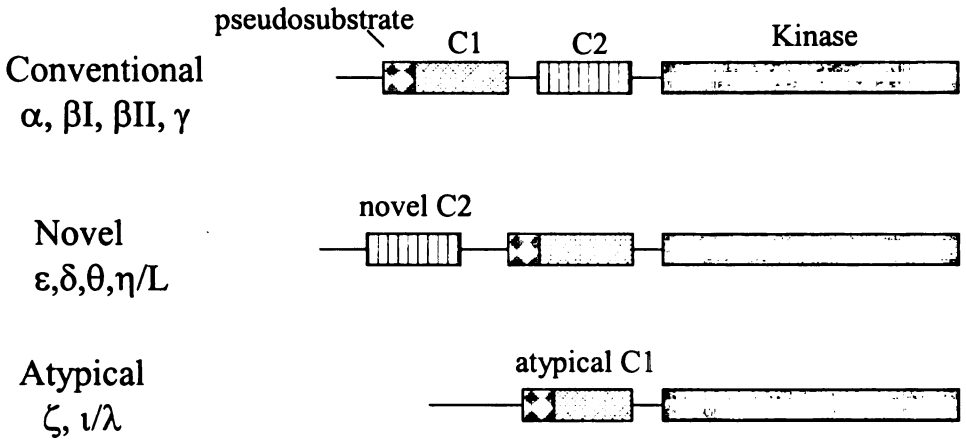


Fig. 1. **Domain structures of protein kinase C family members.** The C-terminal kinase domains are shown in *solid bar*. The regulatory region or domains are shown as following: the pseudosubstrate ( ); the C1 domain ( ); the C2 domain ( ). (Adapted from [11])

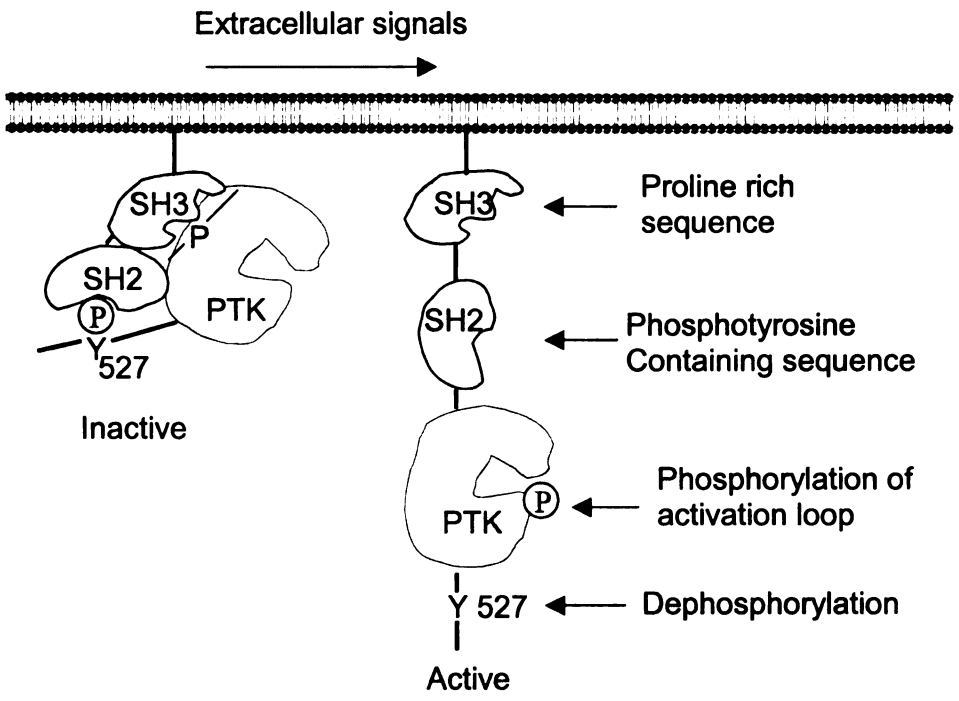


Fig. 2. **Autoinhibition and activation of tyrosine kinase Src**. Src is autoinhibited by two intermolecular interactions. In response to extracellular signals, the activity of Src increases by either releasing the autoinhibition through high affinity SH2 or SH3 ligands, or by dephosphorylation of Tyr 527. *PTK* stands for protein tyrosine kinase. (Adapted from [40])

3. Mitogen activated protein kinase pathways

Mitogen activated protein kinase (MAPK) pathways are composed of three sequentially activated protein kinases, and the basic model is conserved from yeast to humans (reviewed in [41-43]). MAPK pathways are activated in response to extraordinarily diverse stimuli such as growth factors, cytokines, irradiation, and high osmolarity (Fig. 3). The first kinase of the MAPK module is referred as a MAPK kinase kinase (MAPKKK), which may be activated through phosphorylation by a MAPK kinase kinase kinase (MAPKKKK) or through interaction with and, sometimes translocation by an activated small GTPase such as Ras, Rac or Cdc42. An activated MAPKKK can then phosphorylate and activate a dual specific MAPK kinase (MKK), which, in turn, phosphorylates a specific Tyr and a Thr residue in the activation loop of the kinase domain of a terminal MAPK. Phosphorylated activated MAPK have cytosolic substrates but can also translocate to the nucleus, where it phosphorylates nuclear substrates including transcription factors. There are three well-defined major mammalian MAPKs: the extracellular signal-regulated kinase (ERK), ERK1 and ERK2; the c-jun N-terminal kinase (JNK), JNK1, JNK2, and JNK3; and the p38 isoforms, p38α, p38β, p38γ, and p38δ (Fig. 4). In addition, a fourth MAPK, ERK5 has been recently described [44]. All MAPKs are proline-directed kinases, phosphorylating their substrates on Ser or Thr residues immediately precede a Pro residue [45].

3.1. The ERK pathway

In 1990, the first MAPK, *erk1* cDNA was isolated by Cobb's lab [46]. ERK1 and ERK2 (reviewed in [47]) are activated by a variety of extracellular signals, such as growth factors, cytokines, virus infections, and lipopolysaccharides (LPS). MAPKKKs that activate the ERK pathway include Raf-1 [48], A-Raf [49] and B-Raf [50]. The activity of Raf can be elevated by the activated small GTPase Ras [51]. Additionally, the MAPKKKs, MEKK1[52], MEKK2, MEKK3 [53], and Tpl2[54] have been reported to activate ERK. In contrast to the diversity of MAPKKKs, there are only two MKKs that activate ERK: mitogen-activated protein/ERK kinase 1(MEK1) and MEK2. Activated MEK1/2 phosphorylates a Thr and Tyr in the activation loop of the catalytic domain of ERK1/2 [55].

The ERK pathway is generally associated with proliferation and deregulation of the components in the ERK pathway often leads to cellular transformation. Systematic genome-wide screen from 15 cancer cell lines revealed Ras is mutated to an oncogenic form in about 15% of human cancer. It was recently reported that 66% of malignant melanomas contain mutations in the *B-raf* gene, which results in amino acid substitutions in the kinase domain. A single substitution (V599E) in the activation loop accounts for 80% of the mutations. Some of the mutated B-Raf proteins have enhanced *in vitro* kinase activity and can transform NIH3T3 cells [56]. Activated MEK1/2 transforms fibroblasts and produces tumors in nude mice [57], and activation of ERK2 transforms immortalized cells [58]. Therefore, the ERK pathway has been targeted for anti-cancer therapies.

ERK1 knockout animals are viable with a modest defect in T-cell development [59], suggesting that there might be some functional compensation by ERK2.

3.2. The JNK pathway

JNKs, also known as stress-activated protein kinases (SAPK), are a group of MAPKs that are activated by cytokines and environmental stresses, such as heat shock, ultraviolet (UV), osmotic stress (reviewed in [60, 61]). Ten JNK isoforms, which correspond to alternatively spliced isoforms derived from the *Jnk1, Jnk2* and *Jnk3* genes, exist. JNK1 and JNK2 are ubiquitously expressed, while JNK3 is selectively expressed in the brain, heart, and testis [62]. Regulation of JNK at the MAPKKK level is very complex and multiple MAPKKKs, at least upon ectopic expression, activate the JNK pathway, including MEKK1-4 (reviewed in [63]), transforming growth factor (TGF) β activated protein kinase 1 (TAK1) [64], apoptosis signal- regulating kinase 1 (ASK1) [65], the seven mixed lineage kinases (MLKs) (reviewed in [66]). JNK activation is directly mediated by dual phosphorylation on Thr and Tyr residues by either MKK4 or MKK7 [67]. In contrast to ERK, which has substrates both in the cytosol and nucleus, the substrates of JNK are mainly transcription factors in the nucleus. JNK phosphorylates the transcriptional activation domains of several transcription factors including c-Jun, JunD, Elk1 and ATF2. For instance, c-Jun is a transcription factor with relatively short half-life and is rapidly degraded by the ubiquitin-mediated degradation pathway. Activated JNK phosphorylates c-Jun on Ser 63 and Ser 73 and these phosphorylation events stabilize c-Jun and inhibit ubiquitination and degradation by the proteasome [68]. In contrast, phosphorylation of nuclear factor of activated T cells 4 (NFAT4) by JNK causes nuclear exclusion of NFAT4, thus inhibiting NFAT transcriptional activity [69].

JNK is involved in immune responses, development and apoptosis. Apoptosis is a form of cell suicide that is triggered by a variety of stimuli and involves the activation of

members of the caspase family of cysteine proteases. During the process of apoptosis, cytochrome *c* is released from mitochondria into the cytosol to activate caspase-9 which subsequently activates the downstream caspases [70]. JNK is required for UV-induced apoptosis in primary murine embryonic fibroblasts. *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ mouse fibroblasts have a defect in UV-induced cytochrome *c* release and subsequent mitochondrial death signaling, thus protecting cells against UV-stimulated apoptosis [71]. The targeted knockout of three JNK genes (*Jnk1, Jnk2, Jnk3*) individually results in viable mice that have defects in immune responses and apoptosis [61, 72], suggesting that different JNK isoforms may have overlapping functions. Indeed, Jnk1$^{-/-}$/Jnk2$^{-/-}$ double knockout mutants die at mid-gestation, and have defects in neural-tube closure [73]. The *Jnk3* knockout mice shows a reduction in seizure activity and decreased susceptibility to hippocampal neuron apoptosis in response to excitotoxicity [74].

3.3. The p38 MAPK pathway

The p38 MAPKs were first identified as targets for drugs that inhibit TNFα-mediated inflammatory responses [75]. Four p38 isoforms were identified: p38α, p38β, p38γ, and p38δ. The p38 MAPKs can be activated by cytokines, hormones, stresses such as heat shock and osmotic shock [29]. Similar to ERK and JNK, p38 is activated by dual phosphorylation of Thr and Tyr in the activation loop of the kinase domain by MKK3 and MKK6. MAPKKKs that can activate the p38 pathway includes TAK1 [76], ASK1 [65], and some MLKs [66].

The p38 pathway is involved in the regulation of cytokine production and in cytokine-stimulated cell proliferation [77]. Inhibition of p38 by a specific inhibitor, SB

203580, blocks interleukin (IL)-2 and IL-7 induced T cell proliferation. In addition, p38 is also involved in apoptosis. Death receptors, such as Fas and TNF receptors, trigger the activation of the p38 pathway [78]. Several studies have shown that different p38 isoforms have overlapping, but also distinct, physiological roles. In the human cervical carcinoma cell line, HeLa, p38α induces apoptosis, whereas p38β promotes cell survival [79]. Among the four p38 isoforms, p38α is the best studied and most widely expressed. Disrupting the p38α gene in mice causes embryonic lethality [80, 81]. It is quite interesting that the other p38 isoforms are unable to compensate the deletion of the *p38α* gene, reiterating that the different p38 isoforms likely perform distinct biological functions.

3.4. Scaffold proteins and specificity of MAPK pathways

MAPK signaling is complex at many levels. First, diverse extracellular signals activate MAPK pathways. In addition, multiple protein kinases, particularly at the MAPKKK level, have been implicated in the activation of a single MAPK pathway. Finally, a single MAPKKK can often activates multiple MAPKs. All of these issues raise the question as to how signaling specificity is achieved. This question can be answered, at least partially, by considering protein expression patterns, the differential activation mechanisms and selective interactions with MAPK scaffold proteins.

Although the catalytic domains of all these MAPKKKs are fairly similar, the divergent sequences in the non-catalytic regions of these kinases may allow for their differential activation in response to different upstream signals. Signal connectivity from extracellular stimuli to MAPKKKs has been addressed by using RNA interference

(RNAi) in *Drosophila* Schneider S2 cells in Cobb's lab [82]. In contrast to more than a dozen MAPKKKs activating JNK in mammalian cells, four such MAPKKKs are expressed in *Drosophila* S2 cells. RNAi experiments revealed that maximal activation of JNK by lipopolysaccharide requires TAK1. However, all four MAPKKKs (TAK1, MLK2, ASK1, and MEKK1) are required to cause maximal JNK activation by sorbitol. One could imagine that the selectivity of signaling is more complex in mammalian MAPK pathways due to the involvement of multiple MAPKKKs. These data indicate that specific stimuli utilize different mechanisms to recruit different MAPKKKs to regulate the JNK pathway [82]. The molecular details by which different upstream signals regulate distinct MAPKKKs are still largely undefined.

Several scaffold proteins that co-ordinate MAPK signaling have been identified. Scaffold proteins selectively bind to multiple components of the MAPK pathways and, in some cases, may increase the local concentration of the signaling components, thus enhancing the speed of signal transduction. For example, MEK partner 1(MP1) is a scaffold protein involved in the activation of ERK pathway. MP1 binds MEK1 and ERK1, but not ERK2. Upon overexpression, MP1 enhances ERK1 activation and reporter gene expression driven by the transcription factor Elk-1 [83]. JNK interacting proteins (JIP) are another group of scaffold proteins involved in the JNK and p38 pathway (This content will be discussed in detail in the next section).

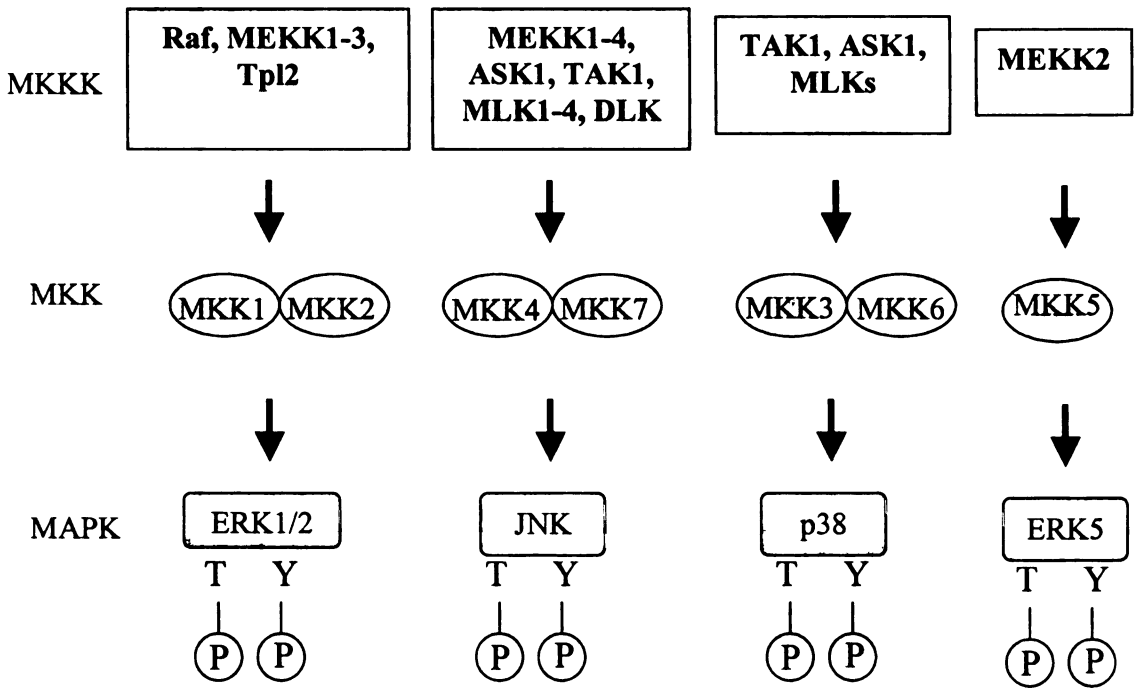


Fig. 3. **The mammalian MAPK pathways**. In mammalian cells, there are four major MAPK pathways: ERK, JNK, p38 and ERK5 pathways. MAPKs are activated by dual phosphorylation of Thr and Tyr in the activation loop.

MAPK	Isoforms	Molecular weight	Primary accession number
ERK	ERK1	43 kDa	P27361
	ERK2	41 kDa	P28482
JNK	JNK1α1	46 kDa	AAA36131
	JNK1α2	55 kDa	AAC50607
	JNK1β1	46 kDa	AAC50610
	JNK1β2	55 kDa	AAC50611
	JNK2α1	46 kDa	AAC50606
	JNK2α2	55 kDa	AAA56831
	JNK2β1	46 kDa	HSU35002
	JNK2β2	55 kDa	HSU35003
	JNK3α1	46 kDa	AAC50605
	JNK3α2	55 kDa	HSU34819
P38	P38 α	41 kDa	Q16539
	P38 β	42 kDa	Q15759
	P38 γ	42 kDa	P53778
	P38 δ	42 kDa	O15264

Fig. 4. **Human MAPKs**. The molecular weight and primary accession number of different MAPK isoforms are shown.

4. Mixed lineage kinases

Mixed lineage kinases (MLKs) are a family of serine/threonine protein kinases (Fig. 5) that were first described in mammals. MLKs belong to the tyrosine kinase like (TKL) group of protein kinases [9]. Their catalytic domains share sequence similarities to both tyrosine and serine/threonine protein kinases, giving rise to the name mixed lineage kinase. However, to date, only serine/threonine phosphorylation activity has been documented in MLKs. Biochemical and genetic studies have revealed that MLKs function as MAPKKKs to activate the JNK and p38 pathways. MLKs have been implicated in neuronal apoptosis and are therapeutic targets for many neurodegenerative diseases such as Parkinson's disease.

4.1. Family members of mixed lineage kinases

Over the past several years, seven human MLK genes have been identified. Based on the domain arrangement and sequence homology within the catalytic domains, MLKs cluster into 3 subfamilies: the MLKs; the dual leucine zipper bearing kinases (DLKs); and zipper sterile-α-motif kinase (ZAK).

The MLK subfamily includes MLK1, MLK2, MLK3, and MLK4. MLK4 has two splicing forms, designated MLK4α and MLK4β. MLKs all contain an amino terminal SH3 domain followed by a catalytic domain, a centrally located zipper region and a CRIB motif. The kinase domains of MLKs share more than 75% sequence identity. The carboxyl terminal regions of MLKs are quite divergent except that they are all proline rich. This diversity indicates that different MLKs might be regulated by distinct upstream signals through this region. MLK3 mRNA is widely expressed in adult and fetal human

tissues [84] and protein is detected by Western blotting in a wide variety of hematopoietic and epithelial cell lines (Gallo lab, unpublished data). MLK1 mRNA is detected in epithelial tumor cell lines of breast, colonic and esophageal origin [85]. Both the RNA and protein levels of MLK1 are detected in an immature β-cell line, RIN-5AH cells, but are absent in a more mature RIN-A12 cells, suggesting that MLK1 is developmentally regulated in β-cell development [86]. The mRNA for MLK2 is present in brain, testis, and skeletal muscle and is expressed at a lower level in pancreas [87, 88]. There is currently no published information on the MLK4 mRNA distribution. Of the four MLKs, only MLK2 and MLK3 have been biochemically characterized.

The DLKs include DLK (also called zipper protein kinase (ZPK) [89]) and leucine-zipper kinase (LZK). DLKs lack an SH3 domain and a CRIB motif, but do have two small leucine-zipper motifs that are separated by a 31 amino acid spacer that follow the catalytic domain. Analogous to the MLKs, DLKs have a proline-rich carboxyl terminal region, whose function has not been well defined. The catalytic domains of DLK and LZK are 87% identical. DLK mRNA is highly expressed in adult mouse brain [90], and is detected primarily in the hippocampus, the cerebral cortex, and the Purkinje cell layer of the cerebellum by *in situ* hybridization analysis of mouse brain sections [89]. LZK mRNA is widely expressed, with the highest expression in the pancreas [91]. There is relatively little information on the protein level of DLK and LZK, presumably because the endogenous protein level may be too low to be detected by Western blotting.

The sole member of the third subfamily is ZAK [92], which has two different splicing forms, ZAKα and ZAKβ. Other names for ZAK includes MLK-like mitogen-activated protein triple kinase (MLTK) [93], and MLK-related kinase (MRK) [94]. In

addition to the catalytic domain, ZAKα contains leucine zipper region and a sterile-α motif (SAM). Due to different mRNA splicing, ZAKβ lacks the SAM motif. ZAK mRNA is expressed widely in human tissues, with highest expression in heart and skeletal muscle [93, 94].

MLKs are absent from yeast, but MLK homologues are found in *Drosophila* and *C. elegans* [66]. Specifically, *Drosophila* have a DLK and MLK homologue and *C. elegans* have a DLK and ZAK homologue. In addition, *C. elegans* also contains a distantly related SH3 containing MLK, lacking the zipper region and the CRIB motif.

4.2. Pathways regulated by MLKs.

4.2.1. The JNK pathway.

Upon overexpression in mammalian cells, all MLKs tested, including MLK2 [95], MLK3 [96], DLK [97], LZK [91], and ZAK [92] [93], activate the JNK pathway. MLK2 and MLK3 are capable of phosphorylating both MKK4 and MKK7 in an *in vitro* kinase assay [96] [95]. However, DLK phosphorylates and activates recombinant MKK7, but not MKK4, *in vitro* [97], suggesting that DLK specifically utilizes MKK7 to transduce the signals and activate the JNK pathway *in vivo*. Overexpression of ZAK enhances both the *in vitro* catalytic activity of MKK4 and MKK7 [93]. However, another study indicated that co-expression of dominant negative MKK7, but not MKK4, with ZAK, blocks ZAK mediated JNK activation [98]. Further studies are necessary to clarify this issue.

4.2.2. The p38 pathway.

MLK2 and MLK3, when overexpressed in COS cells, modestly activate the p38 kinase. [99] [96]. Immunoprecipitated p38 from cells co-transfected with DLK and p38 shows increased activity in an *in vitro* kinase assay [100]. In addition, ZAKβ modestly activates p38 upon overexpression [94].

In general, upon overexpression of MLKs, the fold activation of JNK is much higher than the fold activation of p38, suggesting that MLKs may serve as a potent activator of JNK pathway, but it only partially activates the p38 pathway.

4.2.3. The ERK pathway.

When cotransfected with ERK2 in COS cells, MLK2 enhances ERK *in vitro* kinase activity by 11 fold [99]. Our lab also observed that overexpression of MLK3 in HEK (human embryonic kidney epithelial) 293 cells activates the ERK pathway (unpublished data). However, another report shows that MLK3 fails to enhance ERK1 activity when both MLK3 and ERK are overexpressed in COS cells [96]. Overexpressed ZAK activates the ERK pathway in COS cells [93, 94]. However, overexpression of DLK in COS 7 cells and in NIH 3T3 cells fails to increase the *in vitro* kinase activity of ERK2 [100]. It is unclear whether the activation of ERK pathway by some of the MLKs is cell type specific. The contradictory results from different labs using the same cell line make this question more complex. Further investigations are necessary to clarify these issues.

4.2.4. The NF-κB pathway.

NF-κB is a transcription factor involved in immune, inflammatory, and anti-apoptotic responses. NF-κB is normally sequestered in the cytosol by binding to the inhibitor of NF-κB alpha (IκBα). Phosphorylation of IκBα induces its degradation, allowing NF-κB to translocate to the nucleus, bind to its target promoters, and activate transcription. Phosphorylation of IκBα is mediated by the serine/threonine protein kinases IκB kinase alpha (IKKα) and IKKβ (reviewed in [101]). In Jurkat cells, overexpressed MLK3 induces phosphorylation and activation of IKKα and IKKβ [102]. A kinase-defective mutant of MLK3 strongly blocks the NF-κB dependent transcription in response to T cell activation by CD3/CD28 antibodies.

LZK induces NF-κB activation weakly (1.4 fold) as judged by a reporter gene assay. Cotransfection of LZK with IKKβ increases IKKβ *in vitro* catalytic activity. Furthermore, LZK associates with the IKK complex through the kinase domain as shown by a coimmunoprecipitation experiment [103]. In addition, ZAK was also reported to activate the NF-κB pathway as judged by transcription reporter assay [92].

In summary, almost all the studies regarding the activation of MAPK pathways and NF-κB pathway by MLKs were carried out using overexpressed systems in mammalian cells. There are technical difficulties to obtain purified, fully active MLKs to study their enzymology to determine the k_{cat} and K_m towards their putative substrates *in vitro*. Many issue further complicate the *in vivo* situation, including phosphorylation and activation states of MLKs, the subcellular localization of the MLK and its substrate, and the possible signaling specificity derived from scaffold proteins.

4.3 The regulation of MLKs

4.3.1. Regulation of MLKs by extracellular signals

The extracellular signals that activate endogenous MLKs are not well defined. Serum, platelet-derived growth factor (PDGF), anisomycin, PMA, CD3/CD28 antibodies, but not TNFα induce endogenous MLK3 mobility changes, suggesting of phosphorylation of MLK3. In addition, the phosphopeptide map of *in vivo* [^{32}P] labeled endogenous MLK3 from PMA-treated Jurkat cells is virtually identical to that obtained from MLK3 overexpressed with activated Cdc42 in HEK 293 cells, indicating that PMA induces the same phosphorylation events on MLK3 (Gallo lab and Esselman lab, unpublished data). However, it is unclear whether these changes correspond to changes in MLK3 activity.

Recently, TNFα has been shown to increase the activity of endogenous MLK3 about two fold, as judged by the ability of MLK3 to phosphorylate recombinant GST-MKK4 *in vitro* [104]. In addition, the lipid C6-ceramide treatment in Jurkat T cells is reported to increase endogenous MLK3 catalytic activity to about the same extent. The ability of the MLKs inhibitor, CEP-11004 to attenuate ceramide-induced JNK activation, suggests that activation of JNK by ceramide is accomplished at least partially through MLKs. The activity of MLK3 is also reported to increase in the presence of ceramide in an *in vitro* kinase assay system, suggesting that MLK3 is a direct target of ceramide [104]. However, it will be important for other researchers to confirm these reports.

Treatment of mammalian cell lines with the Ser/Thr phosphatase inhibitor, okadaic acid, results in retarded mobility of DLK [105, 106] and induces JNK activation, presumably through inhibition of phosphatases that negatively regulate DLK and /or JNK

pathway [106, 107]. However, it is unclear whether okadaic acid may affect the activity of DLK.

After transient expression in COS-7 cells, ZAK has relatively high basal activity. Treatment with osmotic shock, such as sorbitol, KCl or NaCl, increases the activity of ZAK. However, the activity of ZAK remains unchanged upon treatment with fetal calf serum, TGF-β, anisomycin, or UV, which are also upstream signals that activate JNK pathway [93].

Taken together, it seems that the activation of different subfamilies of MLKs requires distinct upstream stimuli. This specificity may be mainly due to the different domain arrangement and sequence diversity in the carboxyl terminal regions among the subfamily members.

4.3.2. Regulation of MLKs by phosphorylation

Phosphorylation of protein kinases may rapidly and reversibly change the activities of kinases, regulate its association with other molecules, and affect its subcellular localization. Activation loop phosphorylation is a prominent regulatory mechanism of protein kinases. Mutation of Thr 277 and Ser 281 to Glu (to mimic the phosphorylated Ser and Thr) in the activation loop region in MLK3 results in fully functional kinase, whereas the MLK3 T277A or S281A mutant has low activity, suggesting that these two residues may serve as the phosphorylation sites to regulate MLK3 activity [108]. Since the Thr and Ser residues in the activation loop are highly conserved, phosphorylation in the activation loop is very likely to be a regulatory mechanisms for all the MLKs.

Using mass spectrometry and phosphopeptide mapping techniques, our lab identified eleven *in vivo* phosphorylation sites of MLK3 overexpressed with activated Cdc42 in HEK 293 cells [109]. Seven of the identified phosphorylation sites are followed immediately by a proline residue, suggesting that MLK3 is a target for proline-directed kinases. Since MLK3 activates JNK, ERK and p38 to some extent, one interesting idea is that MLK3 may be phosphorylated by MAPKs in a feedback mechanism. The precise mechanism is under investigation in our lab. In addition, It has been shown that overexpressed MLK2 can be phosphorylated in its carboxyl terminal region by JNK *in vitro* [110]. However, how phosphorylation by JNK or other MAPKs regulates MLK2 or MLK3 is still unclear. It is necessary to generate antibodies directly against specific phosphorylation sites to more carefully probe their function.

4.3.3. Regulation of MLKs by oligomerization

All seven MLK family members contain some type of leucine zipper. MLK3 is reported to form disulfide-linked dimers upon overexpression in HEK 293 cells. The leucine zipper region in MLK3 is necessary and sufficient for MLK3 oligomerization [111]. Using site directed mutagenesis to deliberately introduce a helix disrupting proline mutation in the zipper region, work in our lab has shown that the zipper mediated oligomerization of MLK3 is required for proper phosphorylation of its substrate MKK4 and subsequent activation of JNK pathway [112].

Leucine zipper mediated DLK dimerization is necessary for DLK activity and subsequent JNK activation [113]. Using chimeric DLK–FK-binding protein (FKBP) constructs, artificially induced dimerization of DLK monomers is sufficient to induce

DLK phosphorylation and JNK activation[106]. LZK forms dimers/oligomers and deletion of a zipper motif of LZK prevents activation on JNK [114]. The regulation of ZAK by its zipper region has not been determined.

Zipper-mediated oligomerization is very likely to be a common regulatory mechanism to modulate the activity of all MLKs. Oligomerization may trigger the trans-autophosphorylation of MLKs, leading to MLK activation and subsequent JNK activation.

4.3.4. Regulation of MLKs by Rho GTPase

The mammalian Rho GTPase family contains seven distinct proteins: Rho (A, B, and C isoforms), Rac (1 and 2 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), RhoD, RhoG, RhoE, and TC10 [115]. Rho GTPases cycle between active, GTP-bound states and inactive, GDP-bound states, acting as molecular switches to control many cellular processes, such as cytoskeletal organization, gene transcription, and proliferation [116]. Constitutively active forms of Rac and Cdc42 have been shown to activate the JNK and p38 pathways [117]. Only MLK subfamily members (MLK1-4) contain a CRIB motif. The CRIB motif is a 14-16 amino acid sequence with eight consensus residues that mediates binding with activated form of Cdc42/Rac [8]. The CRIB motif is necessary but not sufficient for MLK3's interaction with Cdc42 [118]. Coexpression of MLK3 with activated Cdc42 increases MLK3 catalytic activity and potentiates MLK3-mediated JNK activation, and this is accompanied by a change in the *in vivo* phosphorylation pattern of MLK3 [118, 119]. Attempts to activate MLK3 by Cdc42 in an *in vitro* system using purified, recombinant proteins have so far failed, suggesting that an additional component

or the cellular environment may be required for MLK3's activation [118]. Recent work in our lab has shown that prenylated activated Cdc42 induces translocation of MLK3 to a plasma-membrane enriched fraction (Gallo lab, manuscript in preparation).

Activated Cdc42 seems to promote the oligomerization of MLK3, suggesting that the activation mechanism by Cdc42 involves oligomerization of MLK3 [111] [106]. Although most studies on the regulation of MLKs by small GTPases have been focused on Cdc42, activated Rac also binds to and potentiates MLK3 activity [119].

4.3.5. Regulation of MLKs by scaffold proteins

JIPs are scaffold proteins that function in the JNK and p38 pathways. JIP1 was first identified in a two-hybrid screen for JNK-interacting proteins [120]. Subsequently it was shown that JIP1 binds MLKs, but not other MAPKKKs; MKK7, but not MKK4; and multiple JNK isoforms [121]. To date, three JIPs have been identified. While JIP1 is ubiquitously expressed in human tissues, JIP2 is predominantly expressed in brain [122]. JIP3 is structurally distinct from JIP1/2 and is highly expressed in brain, heart and lung [123].

Several MLKs, including MLK2, MLK3, DLK and LZK have been shown to interact with JIP1 [121, 122] (Fig. 6). It has been suggested that JIPs facilitate the JNK signaling by assembling the kinase components in the signaling pathway. This idea is supported by the finding that coexpression of JIPs with MLK3 potentiates JNK activation [121-123]. Indeed, recent work with yeast MAPK scaffolds supports the idea that scaffolds may simply function to increase the local concentration of signaling components [124]. However, later studies indicate that the regulation of the JNK

pathway by JIPs may be more complex. These data suggest that JIP1 bound DLK is monomeric, unphosphorylated and catalytically inactive. JNK promotes the dimerization, phosphorylation and activation of JIP-associated DLK [106].

Compared with JIP1, JIP2 binds JNK more weakly [122], suggesting that JIP2 may have other binding partners in cells. Indeed, JIP2 has been shown to act as a scaffold protein in the p38 pathway. JIP2 associates with MLK3, MKK3 and p38. In addition, JIP2 associates with Tiam1 and Ras-GRF, guanine nucleotide exchange factors for Rac. Expression of JIP2 potentiates Tiam1 or Ras-GRF1 induced p38 activation. Expression of Tiam1 or Ras-GRF1 also enhances association of JIP2 with MLK3, MKK3 and p38 [125].

Although the JNK binding region is conserved, JIP3 is structurally distinct from JIP1/2. The only MAPKKK reported to interact with JIP3 is MLK3. JIP3 interacts with MKK7 and multiple JNK isoforms to facilitate JNK signaling [123].

Genetic studies revealed that the Drosophila homologue of JIP3, Sunday driver (Syd), is involved in kinesin-dependent axonal transport [126]. In addition, JIPs have been shown to bind several other proteins that have diverse biological functions, including the kinesin light chain. JIPs may associate with kinesin motor proteins and serve as the loading dock for JNK signaling components moving along microtubules [127].

In addition to the JIPs, a protein named POSH (plenty of SH3s) also acts as a scaffold for the JNK pathway to trigger neuronal apoptosis. POSH interacts directly with activated Rac1, MLKs (MLK1-3 and DLK), and indirectly with MKK4/7 and JNKs, presumably through another protein. POSH overexpression induces neuronal apoptosis

and this can be suppressed by dominant negative forms of MLKs, MKK4/7 and c-Jun, and by the MLK inhibitor, CEP-1347 [128].

Post-synaptic density protein 95 (PSD-95) is a scaffold protein that contains three PDZ domains, a SH3 domain, and an enzymatically inactive guanylate kinase domain [129]. The PDZ domain of PSD-95 binds to and clusters ion channels, including the kainate receptor glutamate receptor 6 (GluR6), in the postsynaptic density membrane of neurons [130]. Expression of GluR6 activates the JNK pathway and induces neuronal apoptosis. The SH3 domain of PSD-95 binds to MLK2 and MLK3. Co-expression of kinase defective MLK2 or MLK3 significantly inhibits GluR6-mediated JNK activation and neuronal toxicity [131], suggesting that the PSD-95 acts as a scaffold protein to mediate the signaling from ion channels to MLKs induced JNK activation and neuronal apoptosis.

4.4. The biological functions of MLKs in mammalian cells.

4.4.1. MLKs in neuronal apoptosis.

Nerve growth factor (NGF) deprivation-induced neuronal apoptosis requires both the activity of the small GTPase Cdc42 and the activation of JNK pathway [132]. Recently, several studies in neuronal systems indicate that MLKs are involved in neuronal apoptosis induced by NGF deprivation. Overexpression of MLK family members, including MLK1-3 and DLK, effectively induces apoptosis in neuronal-like PC12 cells and sympathetic neurons. NGF deprivation-induced apoptosis can be blocked by expression of catalytically inactive forms of MLKs [133, 134].

The presence of polyglutamine-expanded huntingtin is a hallmark in the neuronal cells of Huntington's disease patients. The SH3 domain of MLK2 has been shown to interact with normal huntingtin protein, but not polyglutamine-expanded huntingtin in transfection experiments. Expression of catalytically inactive MLK2 attenuates neuronal apoptosis induced by the polyglutamine-expanded huntingtin, suggesting that MLK2 may be involved in polyglutamine-expanded huntingtin-mediated neuronal apoptosis [135].

The JNK pathway has become a target for drugs against neurodegenerative diseases. Cephalon has developed inhibitors that block JNK activation and neuronal apoptosis in cells and animal models. *In vitro* and *in vivo* studies indicate that the direct targets of these drugs are not JNK, but MLKs. K252a [136] and its derivative, CEP-1347 [137], act as competitors of ATP to inhibit the kinase activity of recombinant MLKs *in vitro*. CEP-1347 blocks JNK1 activation induced by MLKs (MLK1-3, DLK, LZK), but not MEKK1. In addition, K252a and CEP-1347 also activate Akt and ERK in a MLK-independent manner, suggesting that the neuroprotective and neurotrophic effects of K252a and CEP1347 may involve modulation of several neurotrophic signaling pathways [136].

Overexpression of gp120, the major coat protein of the HIV-1, induces apoptosis in primary hippocampal neurons, which is blocked by CEP-1347. In addition, overexpression of MLK3 in hippocampal pyramidal neurons potentiates gp120IIIB-induced neuronal apoptosis, whereas expression of a catalytically inactive MLK3 blocks the apoptosis induced by gp120IIIB. These results indicate that MLKs may mediate gp120IIIB-induced neuronal death, suggesting potential clinical utility of CEP-1347 in inhibiting AIDS-related dementia [138].

4.4.2. MLKs in cellular transformation, cell morphology changes and cancer.

Overexpression of wild-type MLK3 leads to morphological transformation of NIH 3T3 fibroblasts, whereas overexpression of catalytically inactive MLK3 fails to do so. The MEK inhibitor PD 098059 partially reverts the MLK3-transformed phenotype [139]. However, another recent report showed that MLK3 inhibits Rac-mediated cellular transformation [140]. Recently, comprehensive screening of 35 colorectal cancer cell lines for mutations of genes encoding all the TK and TKL protein kinases in the human genome was performed. Mutations in MLK4, but not other MLKs, were detected in several cancer cell lines [141]. The identified mutations in the kinase domain of MLK4 occur in highly conserved residues (H261, G291, A293, and W296), suggesting that these mutations may render MLK4 catalytically inactive. Therefore, it is conceivable that MLK4 act as a tumor suppressor gene.

Overexpression of ZAKα, but not ZAKβ, in Swiss 3T3 (mouse embryonic fibroblast) cells and 10T1/2 (mouse embryonic fibroblast) cells, causes disruption of actin stress fibers and cell shrinkage. The kinase defective mutant of ZAKα does not cause this phenomenon [93] [98]. This suggests that ZAK may play a role in the regulation of actin organization.

4.4.3. MLKs in cell cycle.

Using a bioinformatics approach, MLK3 was identified as a NIMA (never in mitosis A)-related protein kinase. NIMA is a Ser/Thr kinase involved in cell cycle regulation. Loss of NIMA function causes cell arrest in G2 phase [142]. During the G2/M

phase of synchronized HeLa cells, MLK3 is localized to the centrosome and there is a retarded mobility of endogenous MLK3 due to hyperphosphorylation, which correlates with an increase of MLK3 catalytic activity. However, JNK activity remains unchanged during the cell cycle, suggesting that MLK3 may function in a JNK independent pathway during cell cycle progression. In addition, overexpresed MLK3 causes profound disruption of cytoplasmic microtubules and a nuclear distortion, suggesting that endogenous MLK3 may perform a similar function during G2/M [143].

Interestingly, expression of ZAK increases the G2/M cell population [94, 98], suggesting that ZAK may be involved in the regulation of the G2 checkpoint control. Furthermore, catalytically inactive ZAK attenuates the γ-radiation induced cell cycle arrest [94]. Taken together, these data suggest a role for MLKs in cell cycle regulation.

4.5. The biological functions of MLKs in *Drosophila*.

The *Drosophila melanogaster* homologue of MLK1-4, Slipper (Slpr), is involved in JNK mediated dorsal closure during embryogenesis. Dorsal closure is a process of cell sheet movement, in which the dorsal ectoderm moves from a lateral position to the dorsal midline, thus enclosing the embryo in a continuous protective epidermis [144]. Dorsal closure involves a pathway that requires small GTPase dRac1, the MKK7 homologue Hep, the JNK homologue Bsk and the transcription factor dJun and dFos [145]. Genetic studies revealed that the *slpr* mutant embryos have a dorsal open cuticle phenotype and fail to maintain the dramatic cell shape changes within the dorsal epithelium. Genetic epistasis tests indicate that Slpr functions at the downstream of GTPase dRac and upstream of Hep and Bsk to regulate dorsal closure [146].

4.6. MLK3

MLK3, also called SH3 domain-containing proline-rich protein kinase (SPRK) or protein tyrosine kinase 1 (PTK-1) is a serine/threonine kinase with a predicted molecular weight of 93 kDa [84, 147, 148]. MLK3 contains a unique NH_2-terminal glycine-rich region followed by an SH3 domain and a kinase catalytic domain. Following the kinase domain, there are two closely spaced leucine/isoleucine zipper motifs followed by a CRIB motif and a stretch of basic amino acids. The final 220 amino acids of MLK3 are rich in proline (24%), serine (12%) and threonine (13%) [84].

Our lab has been investigating the molecular mechanisms that regulate MLKs activities using MLK3 as a paradigm. While several laboratories have focused on the downstream effects of MLK3 expression in different cell types, less is known about the molecular mechanisms regulating its activity and its intracellular signaling pathways. Many aspects of MLK3 regulation are likely to hold for other family members that share homologous domains. Understanding the molecular mechanisms that regulate MLK3 activity should provide insight into the regulation of protein kinases and aid in the design of isoform-specific MLK inhibitors that may be useful therapeutics in the future.

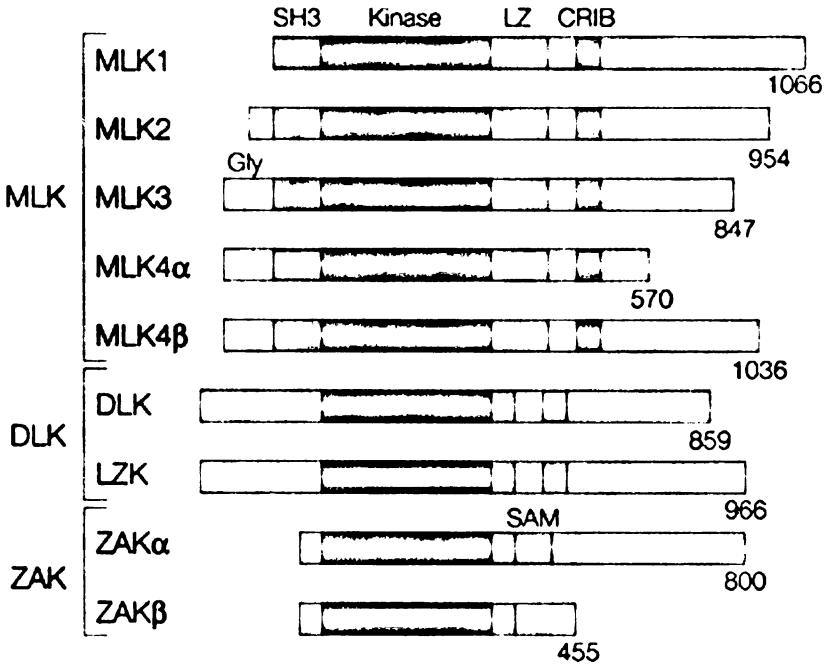


Fig. 5. **Composite structure of human mixed lineage kinases**. The relative position of SH3 domain, leucine zipper (LZ), CRIB motif, and sterile α motif (SAM) was shown. (Adapted from [66])

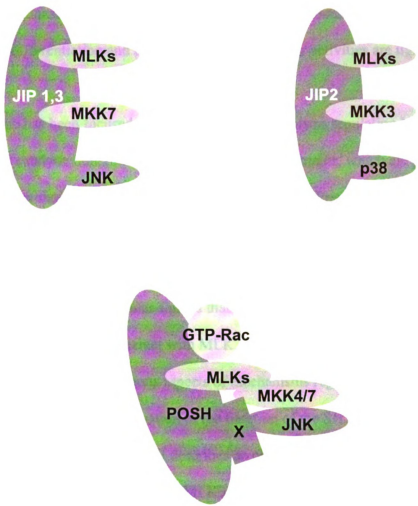


Fig. 6. **Scaffold proteins that interact with MLKs**. JIP1/3 specifically bind to MLKs, MKK7, and JNK to coordinate JNK pathway. JIP2 binds to MLKs, MKK3 and p38. POSH interacts with activated Rac and MLKs directly. Interaction of MKK4/7 and JNKs with POSH may be mediated by an additional protein, designated here as 'X'.

5. Objective of Thesis

Protein kinases are involved in many physiological processes, such as proliferation, differentiation and apoptosis. Therefore, their activities are tightly regulated.

Our lab has been focused on the molecular mechanisms regulating MLK3. In addition to its catalytic domain, MLK3 contains several domains, which are predicted to be critical for regulating the activity and signaling of MLK3. Previous work in the lab has explored the importance of the zipper region and CRIB motif. However, relatively little is known about the function of the SH3 domain and the carboxyl terminal proline rich region. In addition, the extracellular signals that affect MLK3 activity have not been well defined, and the proteins that interact with MLK3 are largely unknown. This thesis describes the examination of various aspects of mechanisms regulating MLK3.

Chapter II presents investigation of the function of the SH3 domain of MLK3 in regulating MLK3 activity. Chapter III describes an observed proteolytic event of MLK3. The study examines the cleavage site of MLK3, the specificity of the proteolysis, as well as the protease that is involved in the proteolysis. Chapter IV details the identification of MLK3 binding partners by affinity purification and mass spectrometry. Chapter V describes the investigation of the regulation of MLK3 by heat shock protein Hsp90/Cdc37. The study examines whether Hsp90/Cdc37 affect MLK3 protein level, as well as MLK3 mediated JNK signaling.

6. References:

1. Lindberg, R.A., A.M. Quinn, and T. Hunter, *Dual-specificity protein kinases: will any hydroxyl do?* Trends Biochem Sci, 1992. **17**(3): p. 114-9.

2. Dhanasekaran, N. and E. Premkumar Reddy, *Signaling by dual specificity kinases.* Oncogene, 1998. **17**(11 Reviews): p. 1447-55.

3. Steeg, P.S., et al., *Histidine kinases and histidine phosphorylated proteins in mammalian cell biology, signal transduction and cancer.* Cancer Lett, 2003. **190**(1): p. 1-12.

4. Hanks, S.K. and T. Hunter, *Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification.* Faseb J, 1995. **9**(8): p. 576-96.

5. Johnson, L.N., et al., *The Eleventh Datta Lecture. The structural basis for substrate recognition and control by protein kinases.* FEBS Lett, 1998. **430**(1-2): p. 1-11.

6. Bossemeyer, D., *Protein kinases--structure and function.* FEBS Lett, 1995. **369**(1): p. 57-61.

7. O'Shea, E.K., et al., *X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil.* Science, 1991. **254**(5031): p. 539-44.

8. Burbelo, P.D., D. Drechsel, and A. Hall, *A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases.* J Biol Chem, 1995. **270**(49): p. 29071-4.

9. Manning, G., et al., *The protein kinase complement of the human genome.* Science, 2002. **298**(5600): p. 1912-34.

10. Newton, A.C., *Protein kinase C: structure, function, and regulation.* J Biol Chem, 1995. **270**(48): p. 28495-8.

11. Newton, A.C., *Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions.* Chem Rev, 2001. **101**(8): p. 2353-64.

12. Tatosyan, A.G. and O.A. Mizenina, *Kinases of the Src family: structure and functions.* Biochemistry (Mosc), 2000. **65**(1): p. 49-58.

13. Xu, W., S.C. Harrison, and M.J. Eck, *Three-dimensional structure of the tyrosine kinase c-Src.* Nature, 1997. **385**(6617): p. 595-602.

14. Soderling, T.R., *Protein kinases. Regulation by autoinhibitory domains.* J Biol Chem, 1990. **265**(4): p. 1823-6.

15. Hubbard, S.R., *Src autoinhibition: let us count the ways.* Nat Struct Biol, 1999. **6**(8): p. 711-4.

16. Williams, J.C., R.K. Wierenga, and M. Saraste, *Insights into Src kinase functions: structural comparisons.* Trends Biochem Sci, 1998. **23**(5): p. 179-84.

17. Moarefi, I., et al., *Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement.* Nature, 1997. **385**(6617): p. 650-3.

18. Olashaw, N. and W.J. Pledger, *Paradigms of growth control: relation to Cdk activation.* Sci STKE, 2002. **2002**(134): p. RE7.

19. Steinman, R.A., *Cell cycle regulators and hematopoiesis.* Oncogene, 2002. **21**(21): p. 3403-13.

20. Hershko, A. and A. Ciechanover, *The ubiquitin system.* 1998.

21. Lee, H.W., et al., *Ubiquitination of protein kinase C-alpha and degradation by the proteasome.* J Biol Chem, 1996. **271**(35): p. 20973-6.

22. Harris, K.F., et al., *Ubiquitin-mediated degradation of active Src tyrosine kinase.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13738-43.

23. Oda, H., S. Kumar, and P.M. Howley, *Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9557-62.

24. Earnshaw, W.C., L.M. Martins, and S.H. Kaufmann, *Mammalian caspases: structure, activation, substrates, and functions during apoptosis.* Annu Rev Biochem, 1999. **68**: p. 383-424.

25. Wolf, B.B. and D.R. Green, *Suicidal tendencies: apoptotic cell death by caspase family proteinases.* J Biol Chem, 1999. **274**(29): p. 20049-52.

26. Shi, Y., *Mechanisms of caspase activation and inhibition during apoptosis.* Mol Cell, 2002. **9**(3): p. 459-70.

27. Basu, A., et al., *Proteolytic activation of protein kinase C-epsilon by caspase-mediated processing and transduction of antiapoptotic signals.* J Biol Chem, 2002. **277**(44): p. 41850-6.

28. Emoto, Y., et al., *Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells.* Embo J, 1995. **14**(24): p. 6148-56.

29. Widmann, C., S. Gibson, and G.L. Johnson, *Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals.* J Biol Chem, 1998. **273**(12): p. 7141-7.

30. Sorimachi, H., S. Ishiura, and K. Suzuki, *Structure and physiological function of calpains.* Biochem J, 1997. **328**(Pt 3): p. 721-32.

31. Wang, K.K., *Calpain and caspase: can you tell the difference?, by kevin K.W. WangVol. 23, pp. 20-26.* Trends Neurosci, 2000. **23**(2): p. 59.

32. Glading, A., et al., *Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway.* J Biol Chem, 2000. **275**(4): p. 2390-8.

33. Glading, A., et al., *Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling.* J Biol Chem, 2001. **276**(26): p. 23341-8.

34. Nollen, E.A. and R.I. Morimoto, *Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins.* J Cell Sci, 2002. **115**(Pt 14): p. 2809-16.

35. An, W.G., T.W. Schulte, and L.M. Neckers, *The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome.* Cell Growth Differ, 2000. **11**(7): p. 355-60.

36. Schulte, T.W., et al., *Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association.* J Biol Chem, 1995. **270**(41): p. 24585-8.

37. Basso, A.D., et al., *Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function.* J Biol Chem, 2002. **277**(42): p. 39858-66.

38. Chen, G., P. Cao, and D.V. Goeddel, *TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90.* Mol Cell, 2002. **9**(2): p. 401-10.

39. Gao, T. and A.C. Newton, *The turn motif is a phosphorylation switch that regulates the binding of Hsp70 to protein kinase C.* J Biol Chem, 2002. **277**(35): p. 31585-92.

40. Hubbard, S.R., M. Mohammadi, and J. Schlessinger, *Autoregulatory mechanisms in protein-tyrosine kinases.* J Biol Chem, 1998. **273**(20): p. 11987-90.

41. Widmann, C., et al., *Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human.* Physiol Rev, 1999. **79**(1): p. 143-80.

42. Johnson, G.L. and R. Lapadat, *Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.* Science, 2002. **298**(5600): p. 1911-2.

43. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**(6824): p. 37-40.

44. Zhou, G., Z.Q. Bao, and J.E. Dixon, *Components of a new human protein kinase signal transduction pathway.* J Biol Chem, 1995. **270**(21): p. 12665-9.

45. Su, B. and M. Karin, *Mitogen-activated protein kinase cascades and regulation of gene expression.* Curr Opin Immunol, 1996. **8**(3): p. 402-11.

46. Boulton, T.G., et al., *An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control.* Science, 1990. **249**(4964): p. 64-7.

47. Cobb, M.H., *MAP kinase pathways.* Prog Biophys Mol Biol, 1999. **71**(3-4): p. 479-500.

48. Kyriakis, J.M., et al., *Raf-1 activates MAP kinase-kinase.* Nature, 1992. **358**(6385): p. 417-21.

49. Wu, X., et al., *Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells.* J Biol Chem, 1996. **271**(6): p. 3265-71.

50. Vaillancourt, R.R., A.M. Gardner, and G.L. Johnson, *B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP.* Mol Cell Biol, 1994. **14**(10): p. 6522-30.

51. Vojtek, A.B., S.M. Hollenberg, and J.A. Cooper, *Mammalian Ras interacts directly with the serine/threonine kinase Raf.* Cell, 1993. **74**(1): p. 205-14.

52. Lange-Carter, C.A., et al., *A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf.* Science, 1993. **260**(5106): p. 315-9.

53. Blank, J.L., et al., *Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase.* J Biol Chem, 1996. **271**(10): p. 5361-8.

54. Ceci, J.D., et al., *Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation.* Genes Dev, 1997. **11**(6): p. 688-700.

55. Zheng, C.F. and K.L. Guan, *Cloning and characterization of two distinct human extracellular signal- regulated kinase activator kinases, MEK1 and MEK2.* J Biol Chem, 1993. **268**(15): p. 11435-9.

56. Davies, H., et al., *Mutations of the BRAF gene in human cancer.* Nature, 2002. **417**(6892): p. 949-54.

57. Mansour, S.J., et al., *Transformation of mammalian cells by constitutively active MAP kinase kinase.* Science, 1994. **265**(5174): p. 966-70.

58. Robinson, M.J., et al., *A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation.* Curr Biol, 1998. **8**(21): p. 1141-50.

59. Pages, G., et al., *Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice.* Science, 1999. **286**(5443): p. 1374-7.

60. Ip, Y.T. and R.J. Davis, *Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development.* Curr Opin Cell Biol, 1998. **10**(2): p. 205-19.

61. Weston, C.R. and R.J. Davis, *The JNK signal transduction pathway.* Curr Opin Genet Dev, 2002. **12**(1): p. 14-21.

62. Gupta, S., et al., *Selective interaction of JNK protein kinase isoforms with transcription factors.* Embo J, 1996. **15**(11): p. 2760-70.

63. Schlesinger, T.K., et al., *The TAO of MEKK.* Front Biosci, 1998. **3**: p. D1181-6.

64. Yamaguchi, K., et al., *Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction.* Science, 1995. **270**(5244): p. 2008-11.

65. Ichijo, H., et al., *Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways.* Science, 1997. **275**(5296): p. 90-4.

66. Gallo, K.A. and G.L. Johnson, *Mixed-lineage kinase control of JNK and p38 MAPK pathways.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 663-72.

67. Whitmarsh, A.J. and R.J. Davis, *Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways.* J Mol Med, 1996. **74**(10): p. 589-607.

68. Fuchs, S.Y., et al., *Phosphorylation-dependent targeting of c-Jun ubiquitination by Jun N- kinase.* Oncogene, 1996. **13**(7): p. 1531-5.

69. Chow, C.W., et al., *Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway.* Science, 1997. **278**(5343): p. 1638-41.

70. Martinou, J.C., S. Desagher, and B. Antonsson, *Cytochrome c release from mitochondria: all or nothing.* Nat Cell Biol, 2000. **2**(3): p. E41-3.

71. Tournier, C., et al., *Requirement of JNK for stress-induced activation of the cytochrome c- mediated death pathway.* Science, 2000. **288**(5467): p. 870-4.

72. Davis, R.J., *Signal transduction by the JNK group of MAP kinases.* Cell, 2000. **103**(2): p. 239-52.

73. Sabapathy, K., et al., *Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2.* Mech Dev, 1999. **89**(1-2): p. 115-24.

74. Yang, D.D., et al., *Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene.* Nature, 1997. **389**(6653): p. 865-70.

75. Lee, J.C., et al., *A protein kinase involved in the regulation of inflammatory cytokine biosynthesis.* Nature, 1994. **372**(6508): p. 739-46.

76. Moriguchi, T., et al., *A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3.* J Biol Chem, 1996. **271**(23): p. 13675-9.

77. Crawley, J.B., et al., *T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation.* J Biol Chem, 1997. **272**(23): p. 15023-7.

78. Brenner, B., et al., *Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153.* J Biol Chem, 1997. **272**(35): p. 22173-81.

79. Nemoto, S., et al., *Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase.* J Biol Chem, 1998. **273**(26): p. 16415-20.

80. Tamura, K., et al., *Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis.* Cell, 2000. **102**(2): p. 221-31.

81. Adams, R.H., et al., *Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development.* Mol Cell, 2000. **6**(1): p. 109-16.

82. Chen, W., M.A. White, and M.H. Cobb, *Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway.* J Biol Chem, 2002. **277**(51): p. 49105-10.

83. Schaeffer, H.J., et al., *MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade.* Science, 1998. **281**(5383): p. 1668-71.

84. Gallo, K.A., et al., *Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity.* J. Biol. Chem., 1994. **269**(21): p. 15092-100.

85. Dorow, D.S., et al., *Identification of a new family of human epithelial protein kinases containing two leucine/isoleucine-zipper domains.* Eur J Biochem, 1993. **213**(2): p. 701-10.

86. DeAizpurua, H.J., et al., *Expression of mixed lineage kinase-1 in pancreatic beta-cell lines at different stages of maturation and during embryonic pancreas development.* J Biol Chem, 1997. **272**(26): p. 16364-73.

87. Dorow, D.S., et al., *Complete nucleotide sequence, expression, and chromosomal localisation of human mixed-lineage kinase 2.* Eur J Biochem, 1995. **234**(2): p. 492-500.

88. Katoh, M., et al., *Cloning and characterization of MST, a novel (putative) serine/threonine kinase with SH3 domain.* Oncogene, 1995. **10**(7): p. 1447-51.

89. Blouin, R., et al., *Cell-specific expression of the ZPK gene in adult mouse tissues.* DNA Cell Biol, 1996. **15**(8): p. 631-42.

90. Holzman, L.B., S.E. Merritt, and G. Fan, *Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein kinase that defines a second subfamily of mixed lineage kinases.* J Biol Chem, 1994. **269**(49): p. 30808-17.

91. Sakuma, H., et al., *Molecular cloning and functional expression of a cDNA encoding a new member of mixed lineage protein kinase from human brain.* J Biol Chem, 1997. **272**(45): p. 28622-9.

92. Liu, T.C., et al., *Cloning and expression of ZAK, a mixed lineage kinase-like protein containing a leucine-zipper and a sterile-alpha motif.* Biochem Biophys Res Commun, 2000. **274**(3): p. 811-6.

93. Gotoh, I., M. Adachi, and E. Nishida, *Identification and characterization of a novel MAP kinase kinase kinase, MLTK.* J Biol Chem, 2001. **276**(6): p. 4276-86.

94. Gross, E.A., et al., *MRK, a mixed lineage kinase-related molecule that plays a role in gamma- radiation-induced cell cycle arrest.* J Biol Chem, 2002. **277**(16): p. 13873-82.

95. Cuenda, A. and D.S. Dorow, *Differential activation of stress-activated protein kinase kinases SKK4/MKK7 and SKK1/MKK4 by the mixed-lineage kinase-2 and mitogen- activated protein kinase kinase (MKK) kinase-1.* Biochem J, 1998. **333**(Pt 1): p. 11-5.

96. Rana, A., et al., *The mixed lineage kinase SPRK phosphorylates and activates the stress- activated protein kinase activator, SEK-1.* J Biol Chem, 1996. **271**(32): p. 19025-8.

97. Merritt, S.E., et al., *The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate.* J Biol Chem, 1999. **274**(15): p. 10195-202.

98. Yang, J.J., *Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c- Jun N-terminal kinase and playing a role in the cell arrest.* Biochem Biophys Res Commun, 2002. **297**(1): p. 105-10.

99. Nagata, K., et al., *The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3.* Embo J, 1998. **17**(1): p. 149-58.

100. Fan, G., et al., *Dual leucine zipper-bearing kinase (DLK) activates p46SAPK and p38mapk but not ERK2.* J Biol Chem, 1996. **271**(40): p. 24788-93.

101. Israel, A., *The IKK complex: an integrator of all signals that activate NF-kappaB?* Trends Cell Biol, 2000. **10**(4): p. 129-33.

102. Hehner, S.P., et al., *Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex.* Mol Cell Biol, 2000. **20**(7): p. 2556-68.

103. Masaki, M., et al., *Mixed lineage kinase LZK and antioxidant protein-1 activate NF-kappaB synergistically.* Eur J Biochem, 2003. **270**(1): p. 76-83.

104. Sathyanarayana, P., et al., *Activation of the Drosophila MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3.* Mol Cell, 2002. **10**(6): p. 1527-33.

105. Mata, M., et al., *Characterization of dual leucine zipper-bearing kinase, a mixed lineage kinase present in synaptic terminals whose phosphorylation state is regulated by membrane depolarization via calcineurin.* J Biol Chem, 1996. **271**(28): p. 16888-96.

106. Nihalani, D., et al., *Mixed lineage kinase-dependent JNK activation is governed by interactions of scaffold protein JIP with MAPK module components.* Embo J, 2001. **20**(13): p. 3447-58.

107. Barancik, M., P. Htun, and W. Schaper, *Okadaic acid and anisomycin are protective and stimulate the SAPK/JNK pathway.* J Cardiovasc Pharmacol, 1999. **34**(2): p. 182-90.

108. Leung, I.W. and N. Lassam, *The kinase activation loop is the key to mixed lineage kinase-3 activation via both autophosphorylation and hematopoietic progenitor kinase 1 phosphorylation.* J Biol Chem, 2001. **276**(3): p. 1961-7.

109. Vacratsis, P.O., et al., *Identification of in vivo phosphorylation sites of MLK3 by mass spectrometry and phosphopeptide mapping.* Biochemistry, 2002. **41**(17): p. 5613-24.

110. Phelan, D.R., et al., *Activated JNK phosphorylates the c-terminal domain of MLK2 that is required for MLK2-induced apoptosis.* J Biol Chem, 2001. **276**(14): p. 10801-10.

111. Leung, I.W. and N. Lassam, *Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3.* J Biol Chem, 1998. **273**(49): p. 32408-15.

112. Vacratsis, P.O. and K.A. Gallo, *Zipper-mediated oligomerization of the mixed lineage kinase SPRK/MLK-3 is not required for its activation by the GTPase cdc 42 but Is necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not catalyze the activating phosphorylation of Thr258 of murine MITOGEN-ACTIVATED protein kinase kinase 4.* J Biol Chem, 2000. **275**(36): p. 27893-900.

113. Nihalani, D., S. Merritt, and L.B. Holzman, *Identification of structural and functional domains in mixed lineage kinase dual leucine zipper-bearing kinase required for complex formation and stress-activated protein kinase activation.* J Biol Chem, 2000. **275**(10): p. 7273-9.

114. Ikeda, A., et al., *Identification and characterization of functional domains in a mixed lineage kinase LZK.* FEBS Lett, 2001. **488**(3): p. 190-5.

115. Mackay, D.J. and A. Hall, *Rho GTPases.* J Biol Chem, 1998. **273**(33): p. 20685-8.

116. Bar-Sagi, D. and A. Hall, *Ras and Rho GTPases: a family reunion.* Cell, 2000. **103**(2): p. 227-38.

117. Coso, O.A., et al., *The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway.* Cell, 1995. **81**(7): p. 1137-46.

118. Bock, B.C., et al., *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation.* J Biol Chem, 2000. **275**(19): p. 14231-41.

119. Teramoto, H., et al., *Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c- Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family.* J Biol Chem, 1996. **271**(44): p. 27225-8.

120. Dickens, M., et al., *A cytoplasmic inhibitor of the JNK signal transduction pathway.* Science, 1997. **277**(5326): p. 693-6.

121. Whitmarsh, A.J., et al., *A mammalian scaffold complex that selectively mediates MAP kinase activation.* Science, 1998. **281**(5383): p. 1671-4.

122. Yasuda, J., et al., *The JIP group of mitogen-activated protein kinase scaffold proteins.* Mol Cell Biol, 1999. **19**(10): p. 7245-54.

123. Kelkar, N., et al., *Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3.* Mol Cell Biol, 2000. **20**(3): p. 1030-43.

124. Park, S.H., A. Zarrinpar, and W.A. Lim, *Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms.* Science, 2003. **299**(5609): p. 1061-4.

125. Buchsbaum, R.J., B.A. Connolly, and L.A. Feig, *Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade.* Mol Cell Biol, 2002. **22**(12): p. 4073-85.

126. Bowman, A.B., et al., *Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein.* Cell, 2000. **103**(4): p. 583-94.

127. Verhey, K.J., et al., *Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules.* J Cell Biol, 2001. **152**(5): p. 959-70.

128. Xu, Z., N.V. Kukekov, and L.A. Greene, *POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis.* Embo J, 2003. **22**(2): p. 252-61.

129. Kim, E., et al., *Heteromultimerization and NMDA receptor-clustering activity of Chapsyn- 110, a member of the PSD-95 family of proteins.* Neuron, 1996. **17**(1): p. 103-13.

130. Garcia, E.P., et al., *SAP90 binds and clusters kainate receptors causing incomplete desensitization.* Neuron, 1998. **21**(4): p. 727-39.

131. Savinainen, A., et al., *Kainate receptor activation induces mixed lineage kinase-mediated cellular signaling cascades via post-synaptic density protein 95.* J Biol Chem, 2001. **276**(14): p. 11382-6.

132. Mielke, K. and T. Herdegen, *JNK and p38 stresskinases--degenerative effectors of signal- transduction-cascades in the nervous system.* Prog Neurobiol, 2000. **61**(1): p. 45-60.

133. Mota, M., et al., *Evidence for a role of mixed lineage kinases in neuronal apoptosis.* J Neurosci, 2001. **21**(14): p. 4949-57.

134. Xu, Z., et al., *The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis.* Mol Cell Biol, 2001. **21**(14): p. 4713-24.

135. Liu, Y.F., D. Dorow, and J. Marshall, *Activation of MLK2-mediated signaling cascades by polyglutamine- expanded huntingtin.* J Biol Chem, 2000. **275**(25): p. 19035-40.

136. Roux, P.P., et al., *K252a and CEP1347 are neuroprotective compounds that inhibit mixed-lineage kinase-3 and induce activation of Akt and ERK.* J Biol Chem, 2002. **277**(51): p. 49473-80.

137. Maroney, A.C., et al., *Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family.* J Biol Chem, 2001. **276**(27): p. 25302-8.

138. Bodner, A., et al., *Mixed lineage kinase 3 mediates gp120IIIB-induced neurotoxicity.* J Neurochem, 2002. **82**(6): p. 1424-34.

139. Hartkamp, J., J. Troppmair, and U.R. Rapp, *The JNK/SAPK activator mixed lineage kinase 3 (MLK3) transforms NIH 3T3 cells in a MEK-dependent fashion.* Cancer Res, 1999. **59**(9): p. 2195-202.

140. Lambert, J.M., et al., *Role of MLK3-mediated activation of p70 S6 kinase in Rac1 transformation.* J Biol Chem, 2002. **277**(7): p. 4770-7.

141. Bardelli, A., et al., *Mutational analysis of the tyrosine kinome in colorectal cancers.* Science, 2003. **300**(5621): p. 949.

142. Osmani, A.H., S.L. McGuire, and S.A. Osmani, *Parallel activation of the NIMA and p34cdc2 cell cycle-regulated protein kinases is required to initiate mitosis in A. nidulans.* Cell, 1991. **67**(2): p. 283-91.

143. Swenson, K.I., K.E. Winkler, and A.R. Means, *A New Identity for MLK3 as an NIMA-related, Cell Cycle-regulated Kinase That Is Localized near Centrosomes and Influences Microtubule Organization.* Mol Biol Cell, 2003. **14**(1): p. 156-72.

144. Knust, E., *Drosophila morphogenesis: movements behind the edge.* Curr Biol, 1997. **7**(9): p. R558-61.

145. Noselli, S., *JNK signaling and morphogenesis in Drosophila.* Trends Genet, 1998. **14**(1): p. 33-8.

146. Stronach, B. and N. Perrimon, *Activation of the JNK pathway during dorsal closure in Drosophila requires the mixed lineage kinase, slipper.* Genes Dev, 2002. **16**(3): p. 377-87.

147. Ing, Y.L., et al., *MLK-3: identification of a widely-expressed protein kinase bearing an SH3 domain and a leucine zipper-basic region domain.* Oncogene, 1994. **9**(6): p. 1745-50.

148. Ezoe, K., et al., *PTK1, a novel protein kinase required for proliferation of human melanocytes.* Oncogene, 1994. **9**(3): p. 935-8.

II. Autoinhibition of Mixed Lineage Kinase 3 Through Its SH3 Domain

1. Abstract

Mixed lineage kinase 3 (MLK3) is a serine/threonine protein kinase that functions as a mitogen-activated protein kinase kinase kinase to activate the c-Jun NH_2-terminal kinase pathway. MLK3 has also been implicated as an IκB kinase kinase in the activation of NF-κB. Amino-terminal to its catalytic domain, MLK3 contains a *src*-homology 3 (SH3) domain. SH3 domains harbor three highly conserved aromatic amino acids that are important for ligand binding. In this study, we mutated one of these corresponding residues within MLK3 to deliberately disrupt the function of its SH3 domain. This SH3-defective mutant of MLK3 exhibited increased catalytic activity compared to wildtype MLK3 suggesting that the SH3 domain negatively regulates MLK3 activity. We report herein that the SH3 domain of MLK3 interacts with full length MLK3, and we have mapped the site of interaction to a region between the zipper and the Cdc42/Rac Interactive Binding (CRIB) motif. Interestingly, the SH3 binding region contains not a proline-rich sequence but, rather, a single proline residue. Mutation of this sole proline abrogates SH3 binding and increases MLK3 catalytic activity. Taken together, these data demonstrate that MLK3 is autoinhibited through its SH3 domain. The critical proline residue in the SH3 binding site of MLK3 is conserved in the closely related family members, MLK1 and MLK2, suggesting a common autoinhibitory mechanism among these kinases. Our study has revealed the first example of SH3 domain-mediated autoinhibition of a serine/threonine kinase and provides insight into the regulation of the mixed lineage family of protein kinases.

2. Introduction

Virtually all physiological processes are regulated by reversible protein phosphorylation. Therefore protein kinases and phosphatases should be highly regulated enzymes. Physical interactions with other signaling molecules can modulate protein kinase activity by changing subcellular location, autophosphorylation state, or conformation of a protein kinase. In the absence of intermolecular associations, many kinases are kept in an inhibited state that is maintained by intramolecular interactions.

Mixed lineage kinase 3 (MLK3)[1], also called *src* homology 3 (SH3) domain-containing proline-rich kinase (SPRK)[1], is a serine/threonine kinase that functions as a mitogen-activated protein kinase (MAPK) kinase kinase (MKKK) to phosphorylate and activate the dual specific kinases, MAPK kinase 4 (MKK4) [2]and MKK7 [3] which, in turn, can phosphorylate and activate c-Jun NH_2-terminal kinase (JNK). Recent evidence suggests that IκB kinase α (IKKα) and IKKβ are also substrates of MLK3, and that MLK3 acts as an IKK kinase to activate the NF-κB pathway in response to T cell receptor costimulation [4].

MLK3 contains several domains that are predicted to mediate protein-protein interactions including a *src*-homology 3 (SH3) domain, a leucine zipper, and a Cdc42/Rac Interactive Binding (CRIB) motif (Fig. 1). The small GTPase Cdc42, in its activated state, binds to MLK3 through the centrally located CRIB motif, and increases MLK3 catalytic activity [5-7]. This activation is accompanied by a change in the phosphopeptide map pattern of *in vivo* labeled MLK3, suggesting that Cdc42 binding induces activating phosphorylation event(s) on MLK3 [7]. A leucine zipper that resides NH_2-terminal to the CRIB motif is necessary for MLK3 homo-oligomerization [8].

Work in our lab has shown that zipper-mediated oligomerization is not required for MLK3 activation by Cdc42, but instead is critical for proper interaction and phosphorylation of a downstream target, MKK4, and subsequent JNK activation [9].

The NH_2-terminal portion of MLK3 contains an SH3 domain. SH3 domains are independently folding modules of about 60 amino acids. Although different SH3 domains have distinct ligand preferences [10], the consensus binding site is composed of a short proline-rich sequence, often Pro-Xxx-Xxx-Pro preceded or followed by basic amino acids [11-14]. The SH3 domain of MLK3 has been shown to interact with a proline-rich region in hematopoietic progenitor protein kinase-1 (HPK1)[15], but an effect on MLK3 activity has not been demonstrated.

Since MLK3 contains a large proline-rich COOH-terminal region (Fig. 1), we were interested in the possibility that the SH3 domain of MLK3 might bind to this region in an autoregulatory fashion. In the work presented here, we show that the SH3 domain of MLK3 does indeed play a critical role in autoregulation. However, unexpectedly, the SH3 domain binds to a region between the zipper and CRIB motifs that lacks a classical SH3 binding site. Point mutations, either in the SH3 domain or in the newly identified SH3 binding site of MLK3, which disrupt this interaction, result in increased MLK3 activity. The data presented here indicate that the catalytic activity of MLK3 is negatively regulated by its SH3 domain. This is the first demonstration of SH3-mediated autoinhibition of a serine/threonine kinase.

3. Materials and Methods

3.1 DNA Constructs and Mutagenesis

Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wildtype MLK3 (pRK5-NFlag.*mlk3*), MLK3 L410P, and MLK3 Δ430-486 has been described elsewhere [7, 9]. A series of truncation variants of MLK3 were generated by 15 cycles of amplification of pRK5-NFlag.*mlk3* using the polymerase chain reaction (PCR) with *Pfx* polymerase (Invitrogen). The same 5' oligonucleotide was used for the construction of a series of variants with successive COOH-terminal deletions: 5'-GCATTAGCTAGCACCATGGACTACAAG-3'.

The following oligonucleotides were used as 3' primers:

pRK5-NFlag.*mlk3* 1-635: 5'-CGTTAAGGATCCTCAAGAGCTGCTACCGCG-3';

pRK5-NFlag.*mlk3* 1-598: 5'-CGTTAAGGATCCTCAGGATGAGTCATCTGA-3';

pRK5-NFlag.*mlk3* 1-529: 5'-CGTTAAGGATCCTCACCGGGGAAAGGTGGG-3';

pRK5-NFlag.*mlk3* 1-485: 5'-CGTTAAGGATCCTCACGCCCGGAGCTTGCT-3';

pRK5-NFlag.*mlk3* 1-386: 5'-CGTTAAGGATCCTCATTCCCGTAGGACCTG-3'.

The amplified *mlk3* fragments were subcloned in-frame with the *Flag* coding sequence into pRK5-NFlag.*mlk3* using *Nhe*I and *Bam*HI.

For the construction of the hemagglutinin (HA)- tagged MLK3 variants, the following oligonucleotides were used in the PCR reactions with pRK5-NFlag.*mlk3* as the template:

pCGN-HA.*mlk3* 1-114: 5'-CGTTAGTCTAGAATGGAGCCCTTGAAGAG-3' and 5'-GCATTAGGATCCTCAGAAGCTGGCCACCTCGC-3'

pCGN-HA.*mlk3* 115-399: 5'-CGTTAGTCTAGACAGGAGCTGCGGCTGG-3' and 5'-GCATTAGGATCCTCACCAGCCTTCCTGCATGG-3'

pCGN-HA.*mlk3* 400-591: 5'-CGTTAGTCTAGAAAGCGCGAGATCCAGGG-3' and 5'-GCATTAGGATCCTCAGTACCATGTGGCTTCG-3'

pCGN-HA.*mlk3* 592-847: 5'-CGAATGTCTAGACTGGATTCAGATGACTC-3' and 5'-GCATTAGGATCCTCAAGGCCCCGCTTCCGGC-3'

The amplified *mlk3* fragments were subcloned in-frame with the HA coding sequence into the pCGN mammalian expression vector [16] using *Xba*I and *Bam*HI .

Variants of MLK3 containing point mutations were constructed using the Quick Change site-directed mutagenesis method (Stratagene) with pRK5-NFlag.*mlk3* as the template DNA using *Pfx* polymerase (Invitrogen) and 15 cycles of amplification. The following oligonucleotides, and their reverse complements, were used as mutagenesis primers:

pRK5-NFlag.*mlk3* Y52A: 5'-GGACAGCCCTGTTCGACGCCGAGCCCAGTGG-3'

pRK5-NFlag.*mlk3* P469A: 5'-GTGGACCGCGAGCGAGCGCACGTGC-3'

The presence of the appropriate mutations was confirmed by DNA sequencing (MSU DNA Sequencing Facility).

A Glutathione S-transferase (GST) expression vector (pGEM-2T-*SH3*) carrying the cDNA encoding the SH3 domain of MLK3 (amino acids 43-104) was constructed by PCR-mediated amplification of the corresponding coding sequence from pRK5-*mlk3* followed by subcloning into the pGEM-2T vector. The Y52A mutation was introduced by site-directed mutagenesis of pGEM-2T-*SH3* using the same oligonucleotides that were used to construct pRK5-NFlag.*mlk3* Y52A.

3.2 Expression and Purification of GST Fusion Proteins

GST and GST fusion proteins (GST-SH3 and GST-SH3 Y52A) were expressed in *E. coli* and purified using glutathione Sepharose 4B, according to the manufacturer's protocol (Amersham Phamacia Biotech). Eluted fractions containing the GST fusion protein, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining, were pooled and concentrated to about 1 mg/ml using a Centriprep concentrator (Amicon).

3.3 Cell Culture, Transfections, and Lysis

Human fetal kidney 293 cells were cultured on 100-mm dishes and transfected using the calcium phosphate method as previously described [7]. Cells were harvested 16 hours after transfection, and lysed by the addition of 1 ml 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 sodium fluoride, 1 mM Na_4PP_i, 100 μM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, and 0.15 units/ml aprotinin) as described previously [7].

3.4 Immunoprecipitations and GST Pulldown Assays

Antibodies against the proteins of interest were prebound to protein A-agarose beads for 30 min at room temperature: MLK3 antiserum (0.25 μg/μl slurry), M2 monoclonal antibody (Sigma) directed against the Flag epitope (0.45 μg/μl slurry). For the GST-pulldown experiment, the GST fusion proteins were preincubated with glutathione Sepharose 4B resin for 30 min. Clarified lysate (400 μl) was incubated with

20 μl of antibody-bound Protein A-agarose or with 20 μl of glutathione Sepharose 4B resin prebound with 5 μg GST fusion protein for 90 min at 4 °C. Immunoprecipitates and GST-pulldowns were washed with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton-X-100, and 10% glycerol). Immunoprecipitates used for kinase assays were washed three times with HNTG buffer containing 1 M LiCl, three times with HNTG buffer, and twice with kinase assay buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4).

3.5 Gel Electrophoresis and Western Blot Analysis

Proteins from lysates and immunoprecipitates were resolved by SDS-PAGE according to Laemmli [17] and transferred to nitrocellulose membranes. The membranes were immunoblotted using MLK3 antiserum (1 μg/ml), M2 Flag monoclonal antibody (9 μg/ml) or HA antibody (BAbCO) (5μg/ml), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Invitrogen). Western blots were developed by the chemiluminescence method. Multiple exposures of the Western blots were developed, and densitometry (NIH Image) of unsaturated films was used to determine relative expression levels.

3.6 *In vitro* Kinase Assays

Kinase assays were performed in 20 μl of kinase assay buffer containing 50 μM ATP and 5 μCi [γ-^{32}P]-ATP (3000 Ci/mmol) (NEN Life Science Products), 10 μg of mixed histones (Roche), and the reactions were carried out for either 15 min or 30 min at room temperature as previously described [7]. Following the kinase assay, proteins were

separated by SDS-PAGE. Gels were rinsed in phosphate-buffered saline, dried, and the incorporation of radioactivity into the kinase or substrates was determined by PhosphorImaging (Molecular Dynamics).

4. Results

4.1 Point Mutation in the SH3 Domain Increases the *In Vitro* Kinase Activity of MLK3

Sequence and structural analyses of SH3 domains reveal three highly conserved aromatic amino acids that participate in ligand binding [18, 19]. Replacement of any one of these conserved residues with alanine in the SH3 domain of the adaptor protein Sem-5/Grb2 abolishes binding to its proline-rich targets [18]. The corresponding conserved residues in the SH3 domain of MLK3 are Tyr 52, Trp 83, and Tyr 99. To deliberately disrupt the function of the SH3 domain of MLK3, site-directed mutagenesis was used to substitute the tyrosine residue at position 52 with an alanine residue. The catalytic activity of this SH3 mutant, MLK3 Y52A, was compared with that of wildtype MLK3.

Cells transiently expressing MLK3 or MLK3 Y52A were lysed, the MLK3 variants were immunoprecipitated, and *in vitro* kinase assays were performed using histones as an exogenous substrate. Data from a representative experiment are shown in Fig. 2. Based on three independent experiments, MLK3 Y52A exhibits a two-fold increase in autophosphorylation activity and a 2.5-fold increase in histone phosphorylation activity when compared with wildtype MLK3. These data suggest that the catalytic activity of MLK3 is negatively regulated by its functional SH3 domain.

4.2 The SH3 Domain of MLK3 Interacts with MLK3

The finding that a disruptive mutation in its SH3 domain increases MLK3 activity, coupled with the fact that MLK3 contains nine potential SH3 binding sequences (Pro-Xxx-Xxx-Pro) in its COOH terminal 220 amino acids, led us to investigate whether

the SH3 domain may bind to MLK3 itself. The SH3 domain of MLK3 (amino acids 43-104) was expressed as a fusion protein with GST in *E. coli* and purified. The ability of MLK3 to interact with its SH3 domain in an intermolecular fashion was assessed in GST pulldown experiments. As shown in Fig. 3, both wildtype MLK3 and MLK3 Y52A associate with GST-SH3 but not with GST. The observation that wildtype MLK3 associates with GST-SH3 to a lesser extent than does MLK3 Y52A suggests that an SH3-mediated intramolecular interaction within wildtype MLK3 competes with the intermolecular binding of GST-SH3.

4.3 Identification of the SH3 Binding Region Within MLK3

Variants with progressive deletions from the COOH-terminus of MLK3 were constructed with an NH_2-terminal Flag tag, expressed, and tested for their ability to associate with GST-SH3. MLK3 1-635 and MLK3 1-598 lack the Pro/Ser/Thr-rich region; MLK3 1-529 ends 23 amino acids after the CRIB motif; MLK3 1-485 terminates just after the zipper motif; and MLK3 1-386 contains the NH_2-terminus through the kinase domain. All variants expressed at similar levels in 293 cells (Fig. 4*A, bottom panel).* In order to create a more appropriate negative control for the GST pulldown experiment, the Tyr residue in GST-SH3 that corresponds to Tyr 52 in MLK3 was replaced with Ala, thus giving rise to GST-SH3 Y52A. Both fusion proteins were expressed in *E. coli*, purified, and used in GST pulldown assays to test their binding to the Flag-tagged MLK3 variants. Surprisingly, as shown in Fig. 4*A*, deletion of the Pro/Ser/Thr-rich COOH-terminal region does not prevent association with GST-SH3. In fact, of all the truncation variants tested, only MLK3 1-386 fails to detectably bind to

GST-SH3. This suggests that the SH3 binding site is within amino acids 387-485 of MLK3. However, it is also conceivable that the SH3 binding site may be present but masked in MLK3 1-386. In any case, one can conservatively conclude that a binding site for MLK3's SH3 domain is present in amino acids 1-485 of MLK3. All MLK3 variants fail to associate with GST-SH3 Y52A, indicating that introduction of this point mutation successfully blocks the binding ability of the SH3 domain.

To further define the SH3 binding site within MLK3, individual or tandem domains of MLK3 were constructed as NH_2-terminal HA-tagged variants, expressed, and analyzed for their capacity to interact with the SH3 domain of MLK3. MLK3 1-114 contains the NH_2-terminal glycine-rich region and the SH3 domain; MLK3 115-399 contains the kinase domain; MLK3 400-591 includes the zipper region, the CRIB motif and an adjacent stretch of basic amino acids; and MLK3 592-847 contains the COOH-terminal Pro/Ser/Thr-rich region. All variants expressed at similar levels although MLK3 592-847 apparently undergoes some proteolytic degradation (Fig. 4*B*, *bottom panel*). Data from GST pulldown assays show that, of these variants, only MLK3 400-591 retains the ability to interact with GST-SH3 (Fig. 4*B*). As expected, none of the variants associates with GST-SH3 Y52A. Taking together all of the results from the GST pulldown experiments, we conclude that the binding site for MLK3's SH3 domain is within amino acids 400-485 of MLK3.

To confirm that the SH3 binding region lies within MLK3 400-485, we took advantage of a previously constructed deletion mutant, MLK3 Δ430-486, which lacks the COOH-terminal half of the zipper and the flanking stretch of basic amino acids [7]. As shown in Fig. 5, MLK3 Δ430-486 fails to associate with GST-SH3. These data suggest

that the critical SH3 binding determinants lie within amino acids 430-485 of MLK3. However, an alternative explanation is that disruption of zipper-mediated oligomerization prevents access to the SH3 binding site. We recently reported that a leucine zipper point mutant, MLK3 L410P, behaves as a monomer [9]. Our results show that GST-SH3 associates with both MLK3 L410P and wildtype MLK3 to approximately the same extent (Fig. 5).

4.4 Point Mutation in the SH3 Binding Site Abolishes Binding to the SH3 Domain and Increases MLK3 Activity

An SH3 binding region has been mapped to amino acids 400-485 of MLK3. The predicted leucine zipper which comprises amino acids 400-463 of MLK3 is devoid of proline residues, and GST-SH3 binding does not require zipper-mediated homo-oligomerization of MLK3 (Fig. 5), suggesting that the zipper domain should not contain the SH3 binding region. Amino acids 463-485 of MLK3 are therefore predicted to be crucial for the binding of the SH3 domain. As shown in Fig. 6A, no typical SH3 binding motif of Pro-Xxx-Xxx-Pro is found in this region of MLK3, although a single proline residue is found at position 469 and four contiguous arginine residues (472-475) are present. Substitution of all four arginine residues with neutral glutamine residues reduces, but does not abolish, binding of MLK3 to GST-SH3 (data not shown). The sole proline residue in this region was substituted with an alanine residue by site-directed mutagenesis and the ability of this mutant, MLK3 P469A, to associate with MLK3's SH3 domain was tested in a GST-pulldown assay. As shown in Fig. 6*B*, MLK3 P469A does

not detectably associate with GST-SH3, indicating that Pro 469 is critical for SH3 binding.

A point mutation in the SH3 domain increases MLK3 activity (Fig. 2), presumably because the mutation disrupts an autoinhibitory interaction. It has now been determined that Pro 469 is critical for binding to MLK3's SH3 domain. A reasonable prediction, therefore, is that mutation of Pro 469 should likewise disrupt an autoinhibitory interaction and increase MLK3 activity. Indeed, as measured in an *in vitro* kinase assay (Fig. 7), MLK3 P469A has about four-fold higher autophosphorylation and histone phosphorylation activity than does wildtype MLK3. These data argue that MLK3 is autoinhibited by a physical interaction between its SH3 domain and a SH3 binding site that is located between its zipper and CRIB motif.

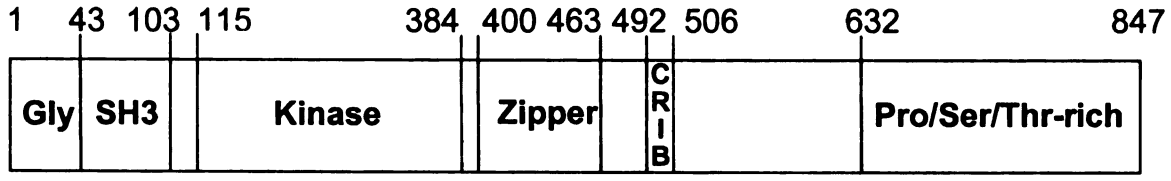


Fig. 1. **Schematic of MLK3**. The *numbers* in the diagram represent amino acid number. The glycine-rich region (amino acid 1-42) is denoted by *Gly*. *CRIB* stands for "Cdc42/Rac interactive binding" motif.

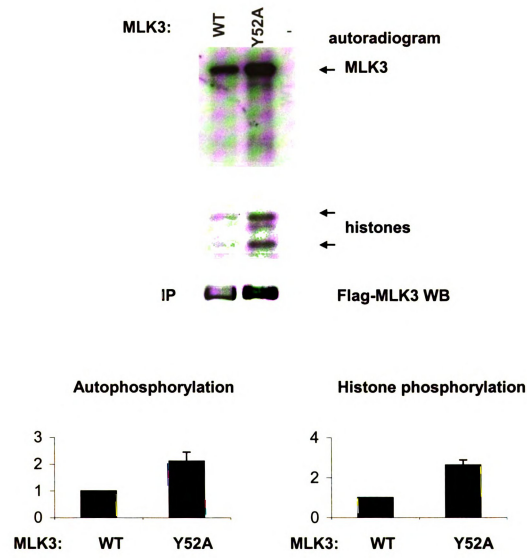


Fig. 2. **Effect of a point mutation in the SH3 domain of MLK3 on catalytic activity**. Cells were transfected with expression vectors containing the cDNAs for Flag-tagged wildtype (*WT*) MLK3 or MLK3 Y52A. A *minus sign* indicates that a control empty vector was transfected. The MLK3 variants were isolated from cellular lysates by immunoprecipitation (*IP*) using the Flag antibody, and kinase activity was assessed *in vitro* as described under "Experimental Procedures". *A, in vitro* kinase assay of MLK3 and MLK3 Y52A using histones as a substrate. The *top panel* shows an autoradiogram with bands corresponding to MLK3 autophosphorylation and histone phosphorylation indicated by *arrows*. A Western blot (*WB*) of the immunoprecipitated MLK3 variants using the Flag antibody is shown in the *bottom panel*. *B* and *C*, the mean $\pm$ standard errror for fold increase in MLK3 autophosphorylation and histone phosphorylation from three independent experiments is shown. Data were quantitated by phosphorimaging and normalized to MLK3 expression levels as described under "Experimental Procedures".

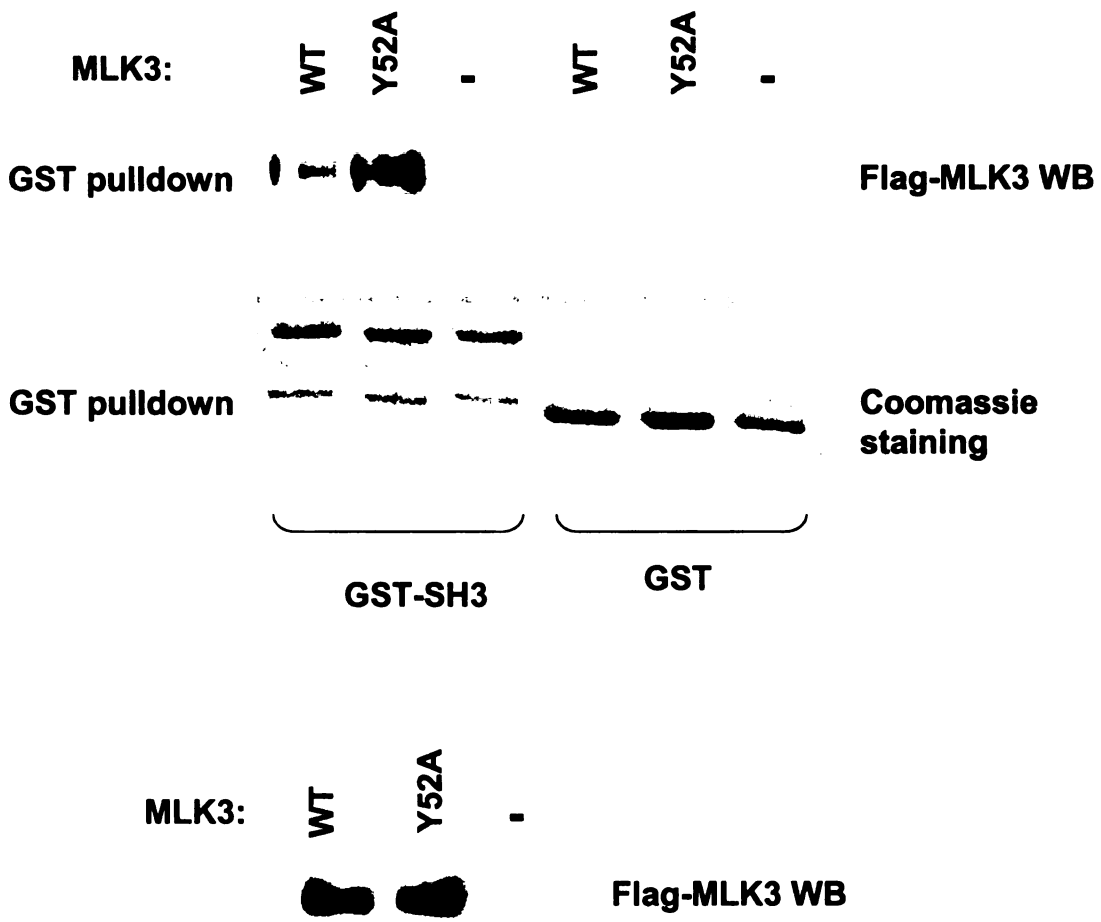
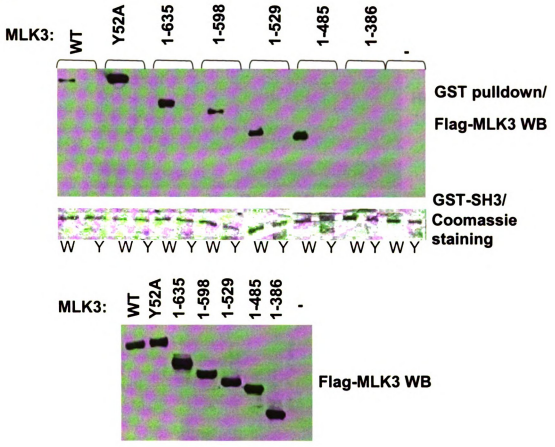


Fig. 3. **Assay for association of the SH3 domain of MLK3 with full length MLK3.** Cells were transfected with expression vectors containing the cDNAs for MLK3 variants. Cellular lysates expressing the indicated MLK3 variants were incubated with glutathione Sepharose 4B resin to which purified GST-SH3 or GST had been prebound. *Top panel*, the presence or absence of bound MLK3 variants was assessed by Western blotting with the Flag antibody. *Middle panel*, equal loading of GST-SH3 or GST on the glutathione Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with the Flag antibody. The data shown is representative of four independent experiments.

A.


MLK3:
WT
Y52A
1-635
1-598
1-529
1-485
1-386
-
GST pulldown/
Flag-MLK3 WB
GST-SH3/
Coomassie
staining
W Y W Y W Y W Y W Y W Y W Y W Y
MLK3:
WT
Y52A
1-635
1-598
1-529
1-485
1-386
-
Flag-MLK3 WB

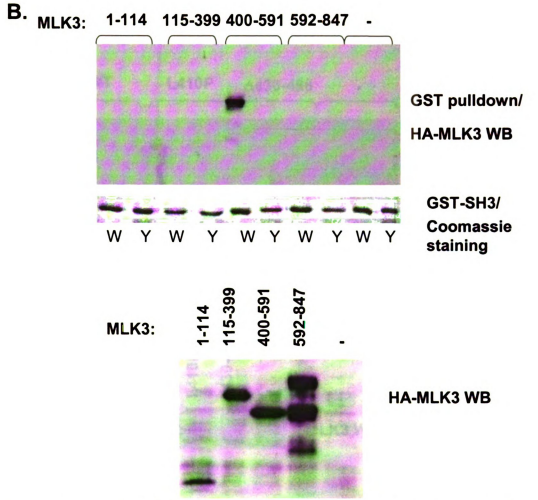


Fig. 4. **Mapping of the SH3 binding region within MLK3.** Cells were transfected with expression vectors containing the cDNAs for MLK3 truncation variants. The *numbers* in the Figure represent amino acid number in MLK3. *A, B*, cellular lysates expressing the indicated MLK3 truncation variants were incubated with glutathione Sepharose 4B resin to which purified GST-SH3 (*W*) or GST-SH3 Y52A (*Y*) had been prebound. *Top panels*, the presence or absence of bound MLK3 variants was assessed by Western blotting with the Flag antibody (*A*) or the HA antibody (*B*). *Middle panels*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panels*, the expression of MLK3 truncation variants was assessed by Western blotting of cellular lysates with the Flag antibody (*A*) or the HA antibody (*B*). The data shown is representative of at least four independent experiments.

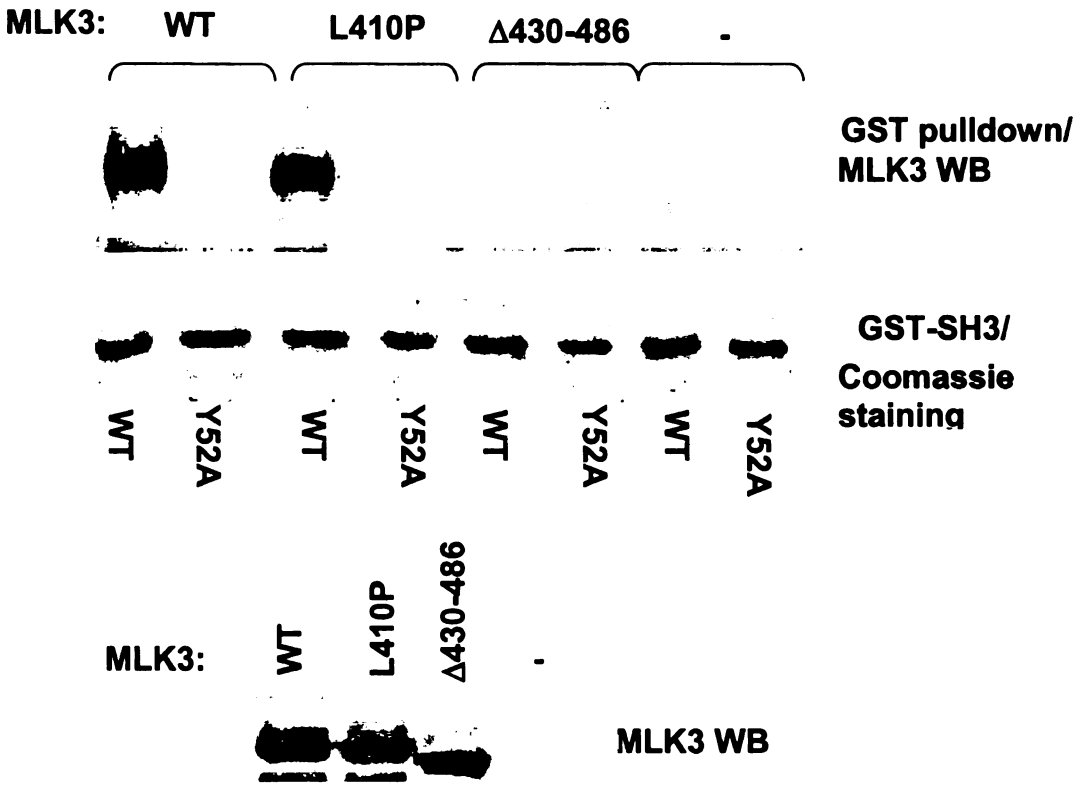


Fig. 5. **Effect of zipper mutations on the SH3 binding.** Cells were transfected with expression vectors containing the cDNAs for MLK3, MLK3 L410P, and MLK3 Δ430-486. Cellular lysates expressing the indicated MLK3 variants were incubated with glutathione Sepharose 4B resin to which purified GST-SH3 or GST-SH3 Y52A had been prebound. *Top panel*, the presence or absence of bound MLK3 variants was assessed by Western blotting with the MLK3 antibody. *Middle panel*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with MLK3 antibody. The data shown is representative of three independent experiments.

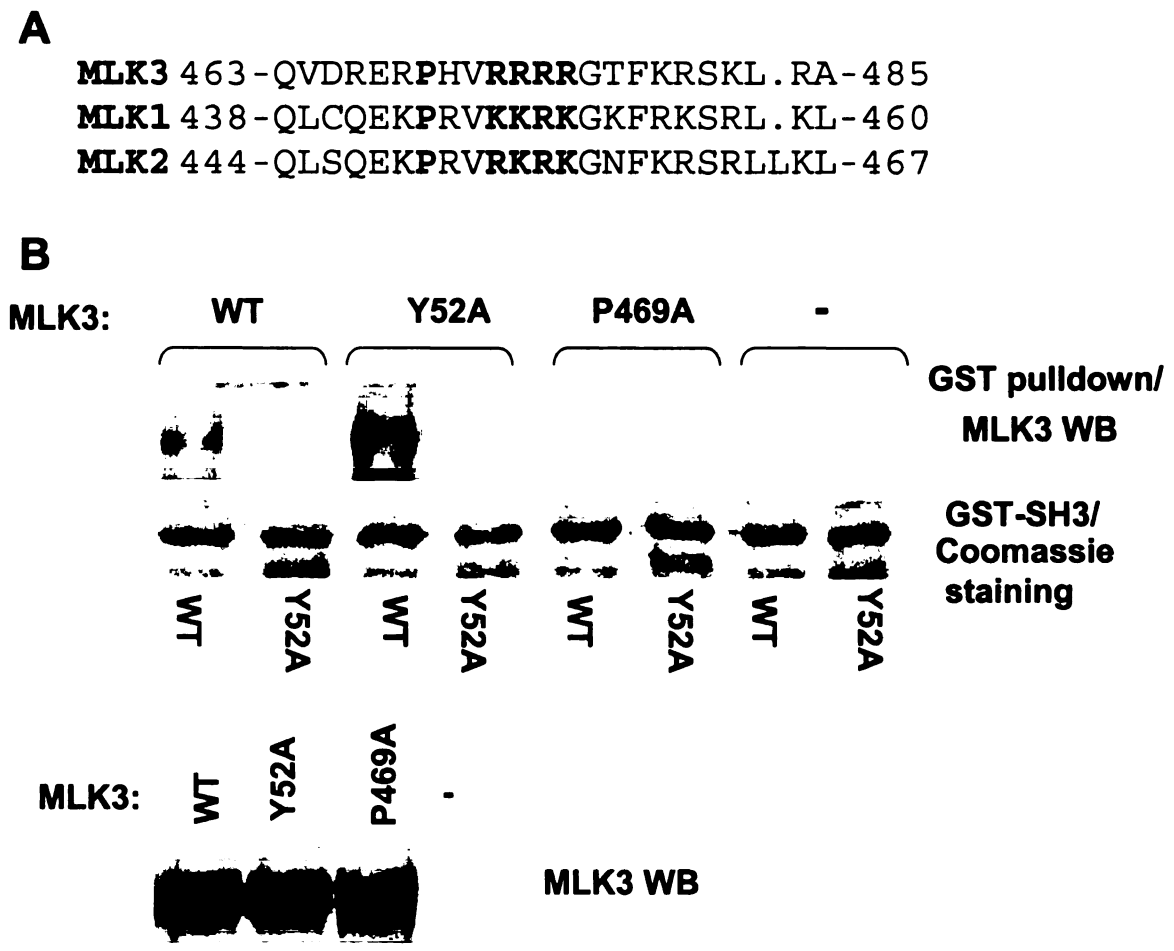


Fig. 6. **Alignment of the SH3 binding region of MLK3 with MLK1 and MLK2 and effect of MLK3 P469A on binding of SH3 domain.** *A*, alignment of the SH3 binding region of MLK3 with MLK family members, MLK1 and MLK2. Amino acid numbers are indicated to the *left* and *right* of each sequence. The sole proline residue and the four contiguous basic residues are shown in *bold*. *B*, cells were transfected with expression vectors containing the cDNAs for the indicated MLK3 variants. Cellular lysates expressing the MLK3 variants were incubated with glutathione Sepharose 4B resin to which purified GST-SH3 or GST-SH3 Y52A had been prebound. *Top panel*, the presence or absence of bound MLK3 variants was assessed by Western blotting with MLK3 antibody. *Middle panel*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with MLK3 antibody. The data shown is representative of four independent experiments.

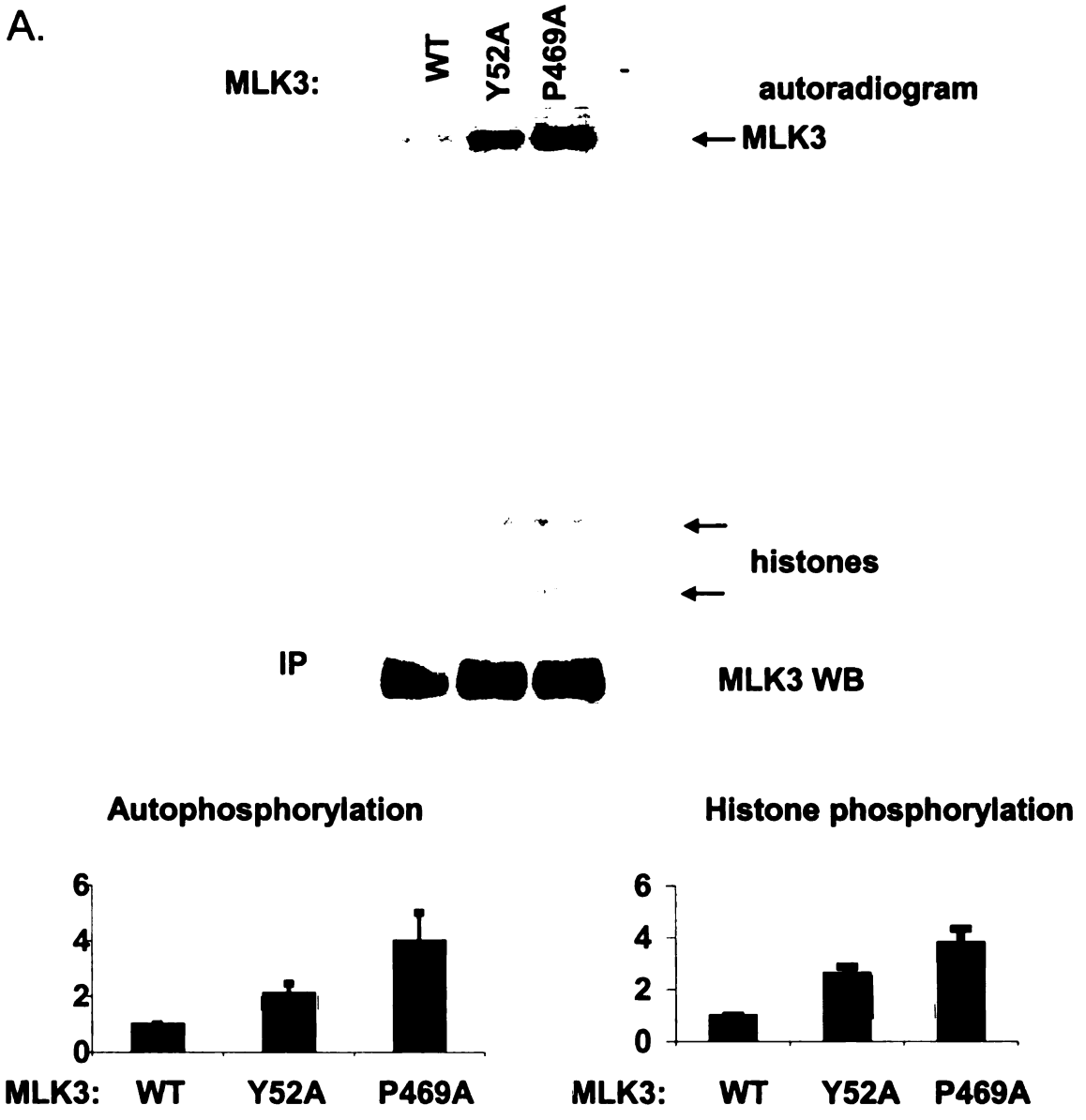


Fig. 7. **Catalytic activity of MLK3 P469A.** *A, in vitro* kinase assay of MLK3 variants using histone as a substrate. The *top panel* shows an autoradiogram with bands corresponding to MLK3 autophosphorylation and histone phosphorylation indicated by *arrows*. A Western blot of the immunoprecipitated samples using a Flag antibody is shown in the *bottom panel*. *B* and *C*, the mean ± standard error for fold increase in MLK3 autophosphorylation and histone phosphorylation from three independent experiments is shown. Data was quantitated by phosphorimaging and normalized to MLK3 expression levels as described under "Experimental Procedures".

5. Discussion

Protein phosphorylation is a ubiquitous regulatory event in biology. Many pathological processes are associated with dysregulation of protein kinase activity. It is not surprising, therefore, that nature has evolved and coordinated multiple mechanisms to exquisitely control the activity of protein kinases. Many protein kinases are maintained in low activity or inactive states through intermolecular or intramolecular association with inhibitory molecules or domains. These inhibitory interactions may be disrupted in response to appropriate stimuli, allowing the protein kinase to adopt an active conformation.

SH3 domains were first identified as targeting domains that through binding to proline-rich sequences mediate intermolecular associations among signaling molecules [13, 14]. Extensive structural information has revealed three aromatic residues conserved in SH3 domains that participate in binding to proline-rich ligands which usually adopt left-handed polyproline type II helices [18, 19]. MLK3 contains an NH_2-terminal SH3 domain. Notably, proline residues comprise 24% of the COOH-terminal 220 amino acids. Our finding that the mutation of one of the conserved aromatic residues in MLK3, Tyr 52, to Ala renders MLK3 more active (Fig. 2), suggested to us that MLK3's SH3 domain might bind to MLK3 itself to negatively regulate its activity. Pulldown experiments showed that full-length MLK3 associates with the SH3 domain of MLK3 fused to GST (Fig. 3). However, the extent of association of MLK3 Y52A with the GST-SH3 fusion protein is much greater than that of wildtype MLK3 (Fig. 3), suggesting that the functional SH3 domain of wildtype MLK3 competes with exogenous GST-SH3. These results argue that an intramolecular association involving the SH3 domain of wildtype MLK3 negatively regulates MLK3 activity.

Despite the fact that the COOH-terminal Pro/Ser/Thr-rich region of MLK3 contains nine Pro-Xxx-Xxx-Pro sequences, deletion of this region has no effect on association of MLK3 with GST-SH3 (Fig. 4*A*). Instead, a series of mapping experiments has identified a sequence located between the zipper and CRIB motifs that is important for the interaction of MLK3 with its SH3 domain (Fig. 4*B*). Unexpectedly, this sequence is not proline-rich but, rather, contains a single proline residue at position 469. Mutation of this sole proline residue to alanine in MLK3 abolishes binding to GST-SH3. In accord with the supposition that MLK3 is autoinhibited by an SH3-mediated intramolecular interaction, replacement of Pro 469 with Ala increases the catalytic activity of MLK3.

While MLK3 represents the first demonstrated example of SH3-mediated autoinhibition of a serine/threonine kinase, the well-studied tyrosine kinases of the Src family offer some interesting parallels. Crystal structure and biochemical studies have revealed an autoinhibitory intramolecular association that involves both the SH2 domain and the SH3 domain of Src [20-22]. A COOH-terminal phosphorylated Tyr provides a ligand for the SH2 domain, and the SH3 domain of Src binds to its so-called "SH2-kinase linker" region, a sequence located between the SH2 and kinase domains. These intramolecular interactions constrain the movement of the two lobes of the kinase domain and therefore, block the binding of ATP, keeping Src in an inactive form (reviewed in [23-25]). Interestingly, the SH3 binding sequence in Src, and also in its relative Fyn, harbors only a single proline residue, analogous to what we have discovered in MLK3, even though the preferred ligand of Src's SH3 domain was found by phage display methods to be the proline-rich sequence Leu-Xxx-Xxx-Arg-Pro-Leu-Pro-Xxx-Pro [10].

One might imagine that the SH3 binding sequence in the "zipper-CRIB linker" region of MLK3 would provide a suboptimal ligand for MLK3's SH3 domain. The high effective concentration afforded in intramolecular associations in all likelihood allows this otherwise weak interaction to occur. Indeed, evolutionary selection may have given rise to a relatively low affinity intramolecular ligand for MLK3's SH3 domain so that it might be outcompeted by the presentation, in response to an appropriate physiological signal, of a high affinity proline-rich ligand on another signaling molecule. For instance, binding of a proline rich sequence in the Nef protein of human immunodeficiency virus-1 to the SH3 domain of Hck, a Src family member expressed primarily in myeloid cells, overcomes autoinhibition, dramatically increasing the activity of Hck [26, 27].

Signaling molecules that overcome SH3-mediated autoinhibition of MLK3 have yet to be identified. One potential candidate is the MKKK kinase HPK1 that contains proline-rich sequences through which it interacts with the SH3 domain of MLK3 [15] and that, like MLK3, can activate both the JNK pathway and the NF-κB pathway [28, 29]. Furthermore, HPK1 phosphorylates kinase-inactive MLK3 in an *in vitro* kinase assay [15]. However, no effect of HPK1 on MLK3 activity has been observed in our hands or reported in the literature.

The JNK pathway scaffold proteins, JNK interacting protein 1 (JIP1), JIP2 and JIP3, have been shown to bind to mixed lineage kinases including MLK3 [30-32]. Cotransfection of JIP1, JIP2 or JIP3 with MLK3 in COS-7 cells enhances the activation of JNK by MLK3 [31, 32]. This may suggest that binding of JIP increases MLK3 activity. It is conceivable that JIP binding might relieve SH3-mediated autoinhibition of MLK3. However, the mixed lineage kinase family member, dual leucine zipper kinase

(DLK), which lacks an SH3 domain, also binds to JIP1 and JIP2 [33], and recent data from Holzman's lab suggests that JIP-associated DLK is catalytically inactive [34]. Further studies are necessary to clarify these issues.

We previously reported that activated Cdc42 binds to MLK3 in a CRIB-dependent manner and results in a change in the *in vivo* phosphorylation pattern of MLK3 as judged by phosphopeptide mapping [7]. The close proximity of the newly identified SH3 binding sequence of MLK3 to its CRIB motif may suggest interplay between Cdc42-mediated activation and SH3-mediated autoinhibition of MLK3. The precise mechanistic relationship is likely to be complex and is currently under investigation in our laboratory.

The mixed lineage family of serine/threonine kinases are so-named for the sequence similarity in their catalytic domains to both tyrosine and serine/threonine kinases. The major function ascribed to this group of protein kinases is activation of the JNK pathway, specifically by acting as MKKKs to phosphorylate and activate MKK4 or MKK7. MLK1 [35], MLK2 [36, 37], and MLK3 share the same general domain arrangement, which includes an NH_2-terminal SH3 domain and centrally located zipper and CRIB motifs. However, the sequences in their COOH-termini diverge. The more distantly related mixed lineage kinases include leucine zipper-bearing kinase (LZK) [38], DLK/ZPK/MUK [39-41], leucine-zipper and sterile-alpha motif kinase (ZAK) [42], and MLK-like mitogen-activated protein triple kinase (MLTK) [43]. These kinases lack both SH3 domains and CRIB motifs. In this report we have identified a single proline residue in MLK3 that is required for binding and autoinhibition through MLK3's SH3 domain. Interestingly, this proline residue is conserved in the closely related family members

MLK1 and MLK2 (Fig. 6*A*), but not in the more distantly related mixed lineage kinases. Thus this newly discovered mechanism of SH3-mediated autoinhibition of MLK3 is predicted to apply to the regulation of MLK1 and MLK2 as well.

Footnotes

Acknowledgements- We are grateful to Dr. Ronald William Henry (Michigan State University) for providing the pCGN vector.

6. References

1. Gallo, K.A., *et al.*, *Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity.* J. Biol. Chem., 1994. **269**(21): p. 15092-100.

2. Rana, A., *et al.*, *The mixed lineage kinase SPRK phosphorylates and activates the stress- activated protein kinase activator, SEK-1.* J Biol Chem, 1996. **271**(32): p. 19025-8.

3. Merritt, S.E., *et al.*, *The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate.* J Biol Chem, 1999. **274**(15): p. 10195-202.

4. Hehner, S.P., *et al.*, *Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex.* Mol Cell Biol, 2000. **20**(7): p. 2556-68.

5. Burbelo, P.D., D. Drechsel, and A. Hall, *A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases.* J Biol Chem, 1995. **270**(49): p. 29071-4.

6. Teramoto, H., *et al.*, *Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c- Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family.* J Biol Chem, 1996. **271**(44): p. 27225-8.

7. Bock, B.C., *et al.*, *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation.* J Biol Chem, 2000. **275**(19): p. 14231-41.

8. Leung, I.W. and N. Lassam, *Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3.* J Biol Chem, 1998. **273**(49): p. 32408-15.

9. Vacratsis, P.O. and K.A. Gallo, *Zipper-mediated oligomerization of the mixed lineage kinase SPRK/MLK-3 is not required for its activation by the GTPase cdc 42 but Is necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not catalyze the activating phosphorylation of Thr258 of murine MITOGEN-ACTIVATED protein kinase kinase 4.* J Biol Chem, 2000. **275**(36): p. 27893-900.

10. Sparks, A.B., *et al.*, *Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCgamma, Crk, and Grb2.* Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1540-4.

11. Ren, R., *et al.*, *Identification of a ten-amino acid proline-rich SH3 binding site.* Science, 1993. **259**(5098): p. 1157-61.

12. Yu, H., *et al.*, *Structural basis for the binding of proline-rich peptides to SH3 domains.* Cell, 1994. **76**(5): p. 933-45.

13. Feller, S.M., *et al.*, *SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl.* Trends Biochem Sci, 1994. **19**(11): p. 453-8.

14. Mayer, B.J. and M.J. Eck, *SH3 domains. Minding your p's and q's.* Curr Biol, 1995. **5**(4): p. 364-7.

15. Kiefer, F., *et al.*, *HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway.* Embo J, 1996. **15**(24): p. 7013-25.

16. Tanaka, M. and W. Herr, *Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation.* Cell, 1990. **60**(3): p. 375-86.

17. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(259): p. 680-5.

18. Lim, W.A. and F.M. Richards, *Critical residues in an SH3 domain from Sem-5 suggest a mechanism for proline-rich peptide recognition.* Nat Struct Biol, 1994. **1**(4): p. 221-5.

19. Weng, Z., *et al.*, *Structure-function analysis of SH3 domains: SH3 binding specificity altered by single amino acid substitutions.* Mol Cell Biol, 1995. **15**(10): p. 5627-34.

20. Williams, J.C., *et al.*, *The 2.35 A crystal structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions.* J Mol Biol, 1997. **274**(5): p. 757-75.

21. Xu, W., S.C. Harrison, and M.J. Eck, *Three-dimensional structure of the tyrosine kinase c-Src.* Nature, 1997. **385**(6617): p. 595-602.

22. Sicheri, F., I. Moarefi, and J. Kuriyan, *Crystal structure of the Src family tyrosine kinase Hck.* Nature, 1997. **385**(6617): p. 602-9.

23. Hubbard, S.R., M. Mohammadi, and J. Schlessinger, *Autoregulatory mechanisms in protein-tyrosine kinases.* J Biol Chem, 1998. **273**(20): p. 11987-90.

24. Williams, J.C., R.K. Wierenga, and M. Saraste, *Insights into Src kinase functions: structural comparisons.* Trends Biochem Sci, 1998. **23**(5): p. 179-84.

25. Hubbard, S.R., *Src autoinhibition: let us count the ways.* Nat Struct Biol, 1999. **6**(8): p. 711-4.

26. Moarefi, I., *et al.*, *Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement.* Nature, 1997. **385**(6617): p. 650-3.

27. Briggs, S.D., *et al.*, *SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1.* J Biol Chem, 1997. **272**(29): p. 17899-902.

28. Hu, M.C., *et al.*, *Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade.* Genes Dev, 1996. **10**(18): p. 2251-64.

29. Hu, M.C., *et al.*, *Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates IkappaB kinases (IKK-alpha/beta) and IKK-beta is a developmentally regulated protein kinase.* Oncogene, 1999. **18**(40): p. 5514-24.

30. Whitmarsh, A.J., *et al.*, *A mammalian scaffold complex that selectively mediates MAP kinase activation.* Science, 1998. **281**(5383): p. 1671-4.

31. Yasuda, J., *et al.*, *The JIP group of mitogen-activated protein kinase scaffold proteins.* Mol Cell Biol, 1999. **19**(10): p. 7245-54.

32. Kelkar, N., *et al.*, *Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3.* Mol Cell Biol, 2000. **20**(3): p. 1030-43.

33. Nihalani, D., S. Merritt, and L.B. Holzman, *Identification of structural and functional domains in mixed lineage kinase dual leucine zipper-bearing kinase required for complex formation and stress-activated protein kinase activation.* J Biol Chem, 2000. **275**(10): p. 7273-9.

34. Nihalani, D., *et al.*, *Mixed lineage kinase-dependent JNK activation is governed by interactions of scaffold protein JIP with MAPK module components.* Embo J, 2001. **20**(13): p. 3447-58.

35. Dorow, D.S., *et al.*, *Identification of a new family of human epithelial protein kinases containing two leucine/isoleucine-zipper domains.* Eur J Biochem, 1993. **213**(2): p. 701-10.

36. Dorow, D.S., *et al.*, *Complete nucleotide sequence, expression, and chromosomal localisation of human mixed-lineage kinase 2.* Eur J Biochem, 1995. **234**(2): p. 492-500.

37. Katoh, M., *et al.*, *Cloning and characterization of MST, a novel (putative) serine/threonine kinase with SH3 domain.* Oncogene, 1995. **10**(7): p. 1447-51.

38. Sakuma, H., *et al.*, *Molecular cloning and functional expression of a cDNA encoding a new member of mixed lineage protein kinase from human brain.* J Biol Chem, 1997. **272**(45): p. 28622-9.

39. Hirai, S., *et al.*, *Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK.* Oncogene, 1996. **12**(3): p. 641-50.

40. Holzman, L.B., S.E. Merritt, and G. Fan, *Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein kinase that defines a second subfamily of mixed lineage kinases.* J Biol Chem, 1994. **269**(49): p. 30808-17.

41. Reddy, U.R. and D. Pleasure, *Cloning of a novel putative protein kinase having a leucine zipper domain from human brain.* Biochem Biophys Res Commun, 1994. **202**(1): p. 613-20.

42. Liu, T.C., *et al.*, *Cloning and expression of ZAK, a mixed lineage kinase-like protein containing a leucine-zipper and a sterile-alpha motif.* Biochem Biophys Res Commun, 2000. **274**(3): p. 811-6.

43. Gotoh, I.I., M. Adachi, and E. Nishida, *Identification and characterization of a novel MAP kinase kinase kinase, MLTK.* J Biol Chem, 2000. **19**: p. 19.

III. Characterization of a Proteolytic Event of Mixed Lineage Kinase 3

1. Abstract

Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates MAPK pathways, including the JNK and p38 pathways. When MLK3 is transiently expressed in HEK 293 cells, MLK3 undergoes proteolysis to generate a stable carboxyl terminal fragment (CTF). The site of proteolysis has been deduced to be Gln 251-Pro 252, which is located within the kinase domain of MLK3. Based on the predicted structure of the kinase domain of MLK3 from homology modeling, the cleavage site is in a solvent accessible region. Site-directed mutagenesis studies revealed that Gln 251 and Leu 250 are required for this proteolytic event of MLK3. Interestingly, the substrates of coronavirus main protease share the same sequence determinants, with a required sequence Leu-Gln just amino terminus to the cleavage site. Our data suggest that a protease with substrate specificity similar to that of the coronavirus protease exists in mammalian cells. It is also conceivable that MLK3 may be a cellular target of the coronavirus protease during viral infection.

Overexpressed MLK3 undergoes phorbol 12-myristate 13-acetate (PMA) (or TNFα, or anti-CD3 antibody) induced proteolysis in Jurkat T antigen cells. The ability of various protease inhibitors to block PMA-induced proteolysis of MLK3 was examined. Among the many commercially available protease inhibitors tested, only MG 132 blocks the generation of CTF, presumably through competitive inhibition by mimicking the sequence at the cleavage site and occupying the catalytic core of the protease.

In addition, pulse chase experiments suggest that MLK3 undergoes proteolysis shortly after synthesis and the protein synthesis inhibitor cycloheximide efficiently blocks PMA-induced proteolysis of MLK3 in Jurkat T Ag cells.

MLK3 undergoes proteolysis in an activity dependent manner in HEK 293 cells, whereas the catalytic activity of MLK3 is not required for PMA induced generation of the CTF in Jurkat T Ag cells. We hypothesized that activation of a particular pathway or several pathways by MLK3 (293 cells) or by PMA (Jurkat T Ag cells) is required for MLK3 proteolysis. Using specific inhibitors that target the ERK, p38 or JNK pathways respectively, our data suggest that ERK activation may be required for the generation of the CTF.

2. Introduction

Protein kinases and other molecules involved in signaling transduction are often regulated by proteolysis. According to enzyme nomenclature, proteases are divided into five subclasses based on their catalytic mechanisms, including serine proteases, cysteine proteases, aspartic proteases, metalloproteases, and a subclass of proteases for which the catalytic mechanism is yet unknown [1]. The amino acid residues at the cleavage sites of the substrates are designated as ···P3, P2, P1-P1', P2', P3'···, where the cleavage site is denoted as P_1- P_1' [2]. Several proteases, which have been implicated in regulating signaling pathways, are described in details in the following.

Ubiquitin-mediated degradation is a highly conserved mechanism for selective protein degradation in eukaryotes. Ubiquitin is an 8.6 kDa protein that is covalently attached to proteins targeted for degradation. There are three major enzymes that are involved in ubiquitination. Ubiquitin is activated in an ATP-dependent step by a specific activating enzyme (E1). The activated ubiquitin molecule is then transferred to a ubiquitin-conjugating enzyme (E2). Then the ubiquitin protein ligase (E3) links the ubiquitin through its carboxyl terminus to an ε-amino group of a Lys residue in the substrate protein. The 26S proteasome then recognizes and degrades the ubiquitinated proteins. The 26S proteasome is a large multisubunit complex, consisting of a central catalytic 20S proteasome and a 19S regulatory complex. The tertiary structure of the 20S proteasome is highly conserved from yeast to mammals and consists of a stack of four rings, each containing seven subunits, $\alpha_7\beta_7\beta_7\alpha_7$ (reviewed in [3-5]). The 20S proteasome is a novel "threonine" protease, in which the Thr residue in the β subunit acts as nucleophile to attack the carbonyl carbon of the substrate [6]. Many proteins involved in

signal transduction and cell cycle progression, such as protein kinase Cα, p53, cyclins, CDK inhibitors and transcription factors, are degraded through the ubiquitin-mediated degradation pathway. Lactacystin, a natural metabolite of Streptomyces, upon entering the cells, undergoes a spontaneous conversion (lactonization) to the clasto-lactacystin β-lactone, which specifically inhibits the activity of proteasome by covalently modifying the active site Thr residue [7].

Caspases are cysteine proteases involved in apoptosis. Caspases are synthesized as inactive zymogens and become activated through proteolytic processing and self-association of two procaspases in the apoptotic cascade. The P1 position in the substrate of the caspases is restricted to Asp. Caspase substrates include cytoskeletal proteins, nuclear proteins, cell cycle regulators, and protein kinases. In contrast to the ubiquitin-mediated degradation pathway, which generally degrades its substrates to inactive, small fragments, caspases cleavage can either render a protein kinase inactive, or constitutively active by removal of autoinhibitory domains. Interestingly, upon cleavage by caspases, some protein kinases, such as protein kinase Cδ, further contribute to the apoptotic response (reviewed in [8]). Thus caspase-mediated proteolysis can set in motion a positive feedback loop.

The calpains constitute another family of proteases that regulate signaling pathways. Calpains are Ca^{2+}-dependent intracellular cysteine proteases, widely expressed in various mammalian tissues. There are two major forms of calpains: m-calpain and μ-calpain. Calpains show no strict sequence preferences. Instead, it has been suggested that calpain may recognize and cleave specific tertiary conformations of proteins.

Calpain substrates include cytoskeletal proteins such as actin, protein kinases such as protein kinase C, nuclear transcription factors, and caspases (reviewed in [9]).

The secretases are a family of aspartyl proteases that exist as complexes in the transmembrane space. γ-secretase is an intramembrane proteolytic complex that is involved in the proteolysis of amyloid precursor protein. The proteolytic product, amyloid β-protein, is a major constituent of the abundant neuritic plaques which are the hallmark of Alzheimer's disease [10]. The catalytic core of γ-secretase is presenilin. In addition to amyloid precursor protein, many substrates of presenilin, including the cell surface receptor Notch [11] and the receptor tyrosine kinase ErbB4 [12], localize predominantly at the plasma membrane [13]. Presenilin inhibitors are being developed by many pharmaceutical companies as possible therapeutics for Alzheimer's disease. A commercially available hydroxyethylene dipeptide isostere, L-685, 458, functions as a transition state analogue and potently inhibit the activity of presenilin [14].

Herein, we report that MLK3 undergoes proteolysis to generate a stable carboxyl terminal fragment *in vivo* and describe the characterization of this proteolytic event.

3. Materials and Methods

3.1. Reagents

Lactacystin, MG132, MDL 28170, leupeptin, L-685, 458, ZVAD-FMK, E64d, U 0126, SP 600125, and SB 203580 were purchased from Calbiochem. Cycloheximide, pepstatin A, chloroquine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma.

3.2. Construction of Mammalian Expression Vectors and Site-Directed Mutagenesis

Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wild type MLK3 (pRK-*mlk3*) and for the kinase defective mutant of MLK3 (pRK- *mlk3* K144A) and monomeric form of MLK3 (pRK *mlk3* L410P) has been described elsewhere [15, 16]. The expression plasmid encoding amino-terminal Flag epitope-tagged constitutively active variant (pRK5-NFlag.*Cdc42* $^{Val\ 12}$) of Cdc42 was kindly provided by Avi Ashkenazi (Genentech, Inc.). The wildtype MLK3 expression vector was used as a template to create mutants (*mlk3* L250A, L250V, Q251A, Q251E, Q251L, Q251N, P252A, P252L, and P252D) using the Quick Change Site-directed Mutagenesis method (Stratagene). The presence of the desired mutation was confirmed by automated DNA sequencing in the Genomics Technology Support Facility at Michigan State University.

3.3. Cell Culture, Transfections, and Lysis

Human embryonic kidney (HEK) 293 cells were cultured in Ham's F12:low glucose Dulbecco's modified Eagle's media (1:1) (Invitrogen) supplemented with 8%

fetal bovine serum (Invitrogen), 2 mM L-glutamine, and penicillin/streptomycin (Invitrogen). Jurkat T antigen cells were cultured in RPMI 1640 (Invitrogen) supplemented with 8% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and penicillin/streptomycin. HEK 293 cells were transfected with plasmid DNA using the calcium phosphate method as previously described . Jurkat T antigen cells were transfected by electroporation (250 V, 950 μF) using a Bio-Rad Gene Pulser electroporator. Cells were harvested, washed with ice cold PBS and lysed for 5 min on ice by the addition of 1 ml of lysis buffer (50 mM HEPES (pH7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM sodium fluoride, 1 mM Na_4PP_i, 100 μM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, and 0.15 units/ml aprotinin). To disrupt cells under denaturing conditions, 0.2% SDS and/or 8 M urea were added to the lysis buffer.

3.4. Immunoprecipitations

MLK3 antibody (0.4 μg/μl slurry) was prebound to protein A-agarose beads for 0.5 h at room temperature. Clarified lysate (300 μl) was incubated with 20 μl of antibody bound Protein A-agarose for 90 min at 4 °C. Immunoprecipitates were washed with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol).

3.5. SDS-PAGE and Western Blot Analysis

Lysates and immunoprecipitates of proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted using MLK3

antibody (7.7ug/ml), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-rad). Western blots were developed by chemiluminescence.

3.6. Pulse Chase Analysis

16 h post transfection, HEK 293 cells were washed twice with PBS, and the culture medium was replaced with methionine/cysteine free high glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 10% dialysed fetal bovine serum (Invitrogen) and penicillin /streptomycin. After 1 h incubation, cells were pulse-labeled by the addition of 150 μCi/ml [^{35}S] methionine /cysteine to the culture medium for an additional 1 h. Cells were washed twice with PBS and then either lysed immediately or incubated in complete medium containing 100 fold excess of methionine (3 mg/ml) for the indicated times prior to cell lysis. MLK3 was immunoprecipitated from the cleared lysates with anti-MLK3 antibody as described above and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and the relative amounts of radiolabel in full length and CTF of MLK3 were measured by phosphorimaging.

3.7. Amino Terminal Sequencing

HEK 293 cells (in 16 X 150 mm tissue culture plates) transiently expressing MLK3 were disrupted in lysis buffer and immunoprecipitated with 77 μg of MLK3 antibody (0.4 μg/μl slurry) for 1.5 h as described above. Immunoprecipitates were washed six times with HNTG buffer and resolved by SDS/PAGE. Proteins were transferred to a nitrocellulose membrane and stained with Coomassie Blue. The membrane slice corresponding to the MLK3 CTF was excised from the membrane and

washed several times with water. The amino terminus of the fragment was sequenced by Edman degradation in the Genomics Technology Support Facility at the Michigan State University.

3.8. MLK3 Kinase Domain Homology Modeling

A three dimensional model of MLK3's catalytic domain was constructed with the program *Homology* based onthe kinase domain of Src. This work was done by Ritesh Agrawal with the assistance from Dr. Leslie Kuhn at Michigan State University.

4. Results

4.1. MLK3 undergoes activity dependent proteolysis in HEK 293 cells.

After transient expression of MLK3 in HEK 293 cells followed by Western blot analysis, a band corresponding to full length MLK3 as well as an immuno-reactive band of about 66 kDa were observed (Fig. 1A). Since the MLK3 antibody was raised against the carboxyl terminal eight amino acids of MLK3, this suggests that MLK3 undergoes proteolysis to generate a stable carboxyl terminal fragment (CTF). We refer to generation of the CTF as the "proteolytic event". When we used an antibody against the amino terminal SH3 domain of MLK3, we were unable to detect a corresponding 27 kDa amino terminal fragment of MLK3 (data not shown). It is possible that the amino terminal fragment is rapidly degraded after it is produced.

The proteolytic event of MLK3 may occur *in vivo* or may occur after cell lysis. However, when urea and SDS, in addition to several protease inhibitors, were included in the cell lysis buffer, the CTF was still observed (Fig. 1A). This is consistent with the idea that the proteolytic event occurs *in vivo.*

In our lab, several variants of MLK3 have been constructed to study its function. Interestingly, when MLK3 mutants with diminished catalytic activity were expressed in HEK 293 cells, little or no CTF was observed (Fig. 1B). For instance, expression of the kinase-defective variant MLK3 K144A fails to generate the CTF. Perhaps, the most interesting mutant is MLK3 L410P, which has a helix disrupting point mutation in its zipper domain and fails to oligomerize [16]. When transfected alone in HEK 293 cells (lane 5), its catalytic activity is low and no CTF is observed. When cotransfected with constitutively active Cdc42, the activity of MLK3 L410P increases as judged by

autophosphorylation and histone phosphorylation in an *in vitro* kinase assay [16], and the CTF is observed (lane 6). This is consistent with the idea that MLK3 activity is required for proteolysis in HEK 293 cells.

4.2. Proteolysis occurs between amino acids 251-252 of MLK3

In order to determine the cleavage site within MLK3, the CTF was isolated and subjected to amino terminal sequencing by Edman degradation. No amino acid was detected in the first cycle, but the following cycles revealed the correct sequence of MLK3 from amino acids 253-273 (Fig. 2A). This is consistent with the idea that the proteolytic event occurs between Gln 251 and Pro 252 of MLK3. It is unclear why the proline was not detected in the first cycle. Perhaps this proline is modified either *in vivo* or *in vitro* under the conditions of isolation. The CTF contains an incomplete kinase domain and thus, this proteolytic event should render MLK3 inactive.

Among the solved structures of protein kinase domains, the kinase domain of Src shares the closest similarity with that of MLK3 with 28% identity . A three dimensional model of the kinase domain of MLK3 was generated based on the crystal structure of Src using Homology modeling [18]. As indicated by the arrow in Fig 2B, Gln 251 and Pro 252 are located in the loop region between the amino terminal lobe and carboxyl terminal lobe of the kinase domain, thus this bond is predicted to be a solvent accessible region. This model suggests that the site of proteolysis in MLK3 should be available to an "MLK3 protease".

4.3. Gln 251 and Leu 250 are critical for MLK3 proteolysis.

Since MLK3 is cleaved between Gln 251-Pro 252, one might hypothesize that Gln 251 and Pro 252 are important for the recognition by the "MLK3 protease". Therefore, a series of MLK3 variants containing amino acid substitutions at Gln 251 or Pro 252, were constructed and transiently expressed in HEK 293 cells. As shown in Fig. 3A, upon substitution of Gln 251 with Ala, Glu, Leu or Asn of MLK3, no CTF was generated. In contrast, substitution of Pro 252 of MLK3 with Ala, Leu, or Asp resulted in the generation of the CTF. These data suggest that the "MLK3 protease" may specifically recognize Gln 251 at the P1 cleavage site. However, Pro 252 at P1' is not essential for recognition. In addition, the Leu 250 was mutated to Ala and Val respectively and the effect on MLK3 proteolysis was tested. As shown in Fig. 3B, L250A and L250V do not generate CTF, suggesting that the Leu 250 at the P2 position is also critical for recognition by the MLK3 protease.

4.4. Inducible proteolysis of MLK3 by extracellular stimuli in Jurkat T antigen (Ag) cells.

The CTF of MLK3 is not present when MLK3 is transiently expressed in Jurkat T Ag cells. However, Weiqin Chen (Esselman lab) observed the CTF of MLK3 upon treatment of cells with PMA. We have extended this initial observation to demonstrate the appearance of the CTF as soon as 1 h after PMA treatment, and the accumulation of the CTF in a time dependent manner. In addition, Stancy Liou (Gallo lab) has observed that anti-CD3 antibody, which clusters and activates CD3 receptors, as well as TNFα treatment results in the generation of the CTF (Fig 4).

4.5. Inducible proteolysis of MLK3 is independent of MLK3 activity in Jurkat T Ag cells

Expression of the kinase defective variant MLK3 K144A or the monomeric variant MLK3 L410P in Jurkat T Ag cells results in the generation of the CTF in response to PMA treatment (Fig. 5). This suggests that generation of the CTF does not require an active conformation of MLK3, but rather requires signaling events that can emanate from PMA, CD3 antibody, or TNFα. MLK3 Q251A, L250A and L250V do not produce the CTF upon PMA treatment, consistent with the idea that Gln 251 and Leu 250 are retained as the sequence determinants for the MLK3 protease.

4.6. Protease inhibitor MG 132 blocks the generation of CTF of MLK3 in Jurkat T Ag cells.

The inducible proteolytic event of MLK3 in Jurkat T Ag cells provides a model system to investigate the attributes/identity of the MLK3 protease. The ability of several protease inhibitors to block PMA-induced generation of the CTF was tested in Jurkat T Ag cells (Table 1). Treatment of MLK3-transfected Jurkat T Ag cells with a general aspartyl protease inhibitor (pepstatin A), general cysteine protease inhibitors leupeptin and E64d, a caspases inhibitor (ZVAD-FMK), a calpain inhibitor (MDL 28170), and a lysosomal protease inhibitor (chloroquine) all failed to block the PMA-induced generation of the CTF. Surprisingly, only MG132, a peptide aldehyde that is marketed as a proteasome inhibitor [19], is able to block the PMA-induced MLK3 proteolytic event. To determine whether generation of the CTF reflects a proteasome-mediated event, the more specific inhibitor Lactacystin was used, but failed to block this proteolytic event. In

addition, MG 132 has been reported to inhibit secretases [20, 21]. However, a specific inhibitor of γ-secretase, L685, 458, failed to prevent PMA-induced generation of the CTF (Fig. 6). These data suggest that MG 132 is targeting a protease other than the proteasome or γ-secretase to inhibit MLK3 proteolysis.

4.7. The CTF is generated from newly synthesized MLK3

To demonstrate a precursor: product relationship between full length MLK3 and the CTF, pulse chase experiments in HEK 293 cells were performed. After transiently expressing wildtype MLK3 and the cleavage site mutant MLK3 Q251A, HEK 293 cells were pulse-labeled with [^{35}S]-Met/Cys for 1 h and chased for up to 6 h. Fig. 8 shows representative results from four independent experiments. Surprisingly, the CTF is apparent at the end of the 1 h pulse period, yet during the chase period, the labeled CTF did not accumulate. This suggests that the MLK3 proteolysis occurs rapidly after MLK3 synthesis, and the proteolysis does not occur or occurs very slowly afterwards.

Furthermore, when Jurkat T Ag cells transiently expressing MLK3 were treated with the protein synthesis inhibitor cycloheximide along with PMA for 3 h, the CTF was not detected (Fig. 9). Taken together, these two sets of experiments are consistent with the idea that only newly synthesized MLK3 is a substrate for the "MLK3 protease".

4.8. ERK activation is likely required for MLK3 proteolysis

MLK3 undergoes proteolysis to generate the CTF in an activity dependent manner in HEK 293 cells. Only active MLK3 variants produce the CTF, whereas catalytically inactive variants do not. The occurrence of MLK3 proteolytic event may

depend on the conformation of MLK3 and the MLK3 protease might specifically recognize the active conformation of MLK3. Another possibility is that proteolysis of MLK3 may occur through a signaling pathway that is activated by MLK3 or other factors. The data obtained from the Jurkat T Ag cells support the second hypothesis. When MLK3 is overexpressed in Jurkat T Ag cells, its catalytic activity is low compared with that of MLK3 overexpressed in HEK 293 cells. PMA induces MLK3 proteolysis, but does not change MLK3 catalytic activity (data not shown, from Weiqin Chen). Furthermore, PMA also induces the proteolysis of catalytically inactive mutants of MLK3. It is reasonable to hypothesize that proteolysis of MLK3 may require a pathway that can be activated by overexpressed MLK3 in HEK 293 cells or induced by PMA in Jurkat T Ag cells.

If this hypothesis is true, a reasonable prediction, therefore, is that PMA should trigger proteolysis of the kinase defective mutant MLK3 K144A in HEK 293 cells. Indeed, we observed the corresponding CTF of MLK3 K144A after PMA treatment (data not shown).

It has been reported that PMA activates the ERK, the JNK and the p38 pathways [22]. MLK3 has been reported to activate both the JNK and p38 pathways [23]. Unpublished data from our lab has shown that upon overexpression, MLK3 activates the ERK pathway in HEK 293 cells (data not shown). It is possible that activation of one or more of the MAPK pathways is required for the proteolytic event of MLK3. To test this hypothesis, the ability of specific inhibitors of ERK, JNK or p38 pathways to block PMA-induced MLK3 proteolysis was assessed in Jurkat T Ag cells. Jurkat T Ag cells transiently expressing MLK3 were pretreated with the MEK inhibitor U0126 [24], the

JNK inhibitor SP 60012 [25], the p38 inhibitor SB 203580 [26] (Table 2), or a combination of them for 0.5 h prior to treatment with PMA for 3 h. As shown in Fig. 10, the MEK inhibitor U0126 alone partially blocks the generation of the CTF. Neither the JNK inhibitor nor the p38 inhibitor alone was effective in blocking the MLK3 proteolysis. Generation of the CTF is potently inhibited by using all three inhibitors. These results suggest that ERK activation is likely required for MLK3 proteolytic event. The p38 and/or JNK activation may partially contribute to the proteolytic event in MLK3. Finally, we cannot rule out the possibility that another MAPK independent pathway, activated by MLK3 in HEK 293 cells, or by PMA in Jurkat T Ag cells, might be critical for generation of the CTF.

Table 1. Effect of protease inhibitors on PMA-induced generation of MLK3 CTF.

Inhibitor	Targeted protease	Concentration used	CTF detected
Pepstatin A	aspartyl protease	20 μM	Yes
Leupeptin	cysteine protease	100 μM	Yes
E64d	cysteine protease	20 μM	Yes
ZVAD-FMK	caspases	200 μM	Yes
MDL 28170 [27]	calpain	5 μM	Yes
Chloroquine [28]	lysosomal protease	100 μM	Yes
Lactacystin [7]	proteasome	5 μM	Yes
L-685, 458 [14]	γ-secretase	100 nM	Yes
MG132 [19-21]	proteasome γ-secretase	10 μM	No

Table 2. MAPK inhibitors.

Inhibitor	Targeted kinase	Targeted pathway	Concentration
U0126	MEK1,2	ERK	10 μM
SP600125	JNK1,2,3	JNK	20 μM
SB203580	p38 MAPK	p38	6 μM

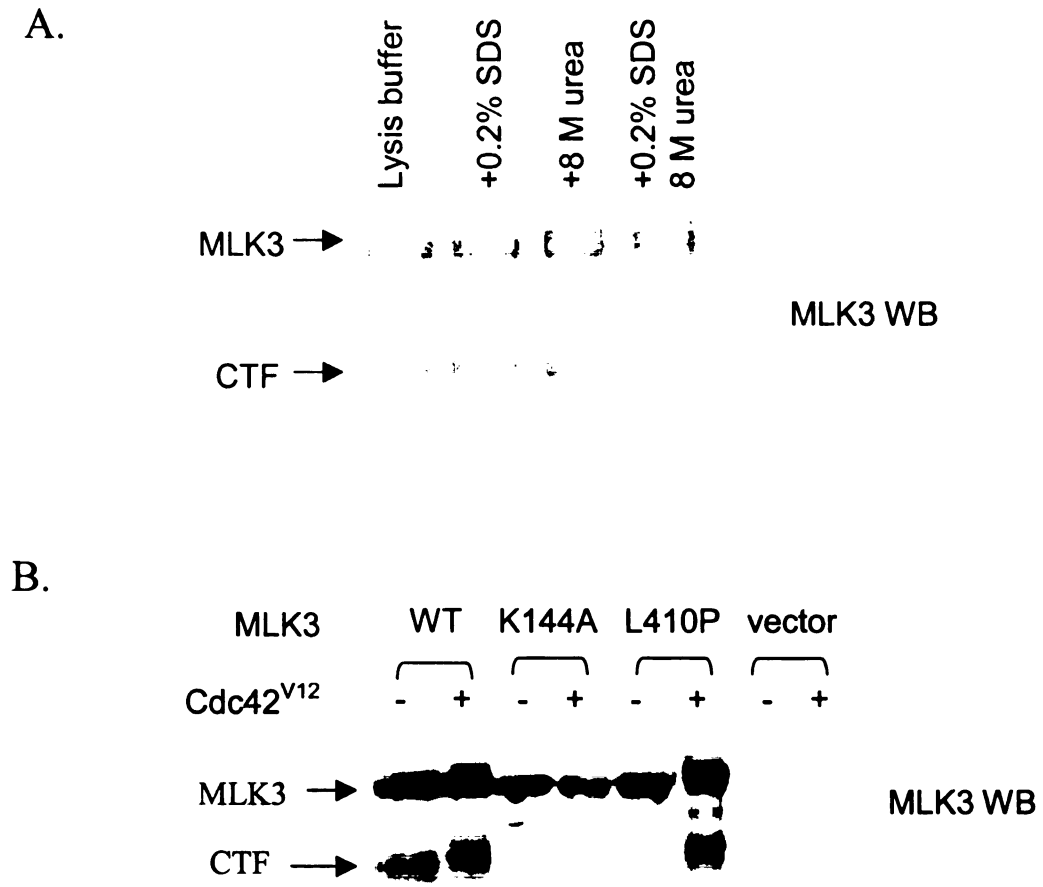
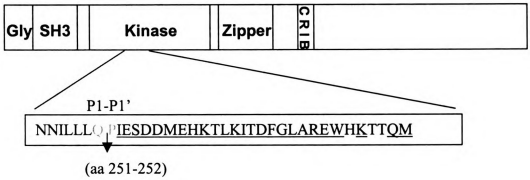


Fig. 1. ***In vivo* proteolysis of MLK3 in HEK 293 cells**. HEK 293 cells were transfected with expression vectors containing the cDNA for MLK3 in the presence or absence of cDNA for activated Cdc42. Cells were lysed using lysis buffer containing the protease inhibitors aprotinin and PMSF along with EGTA. Strong denaturants were added to the lysis buffer as indicated. A Western blot (WB) is shown with full length MLK3 and the CTF of MLK3 indicated by arrows. *A*. MLK3 proteolysis pattern in different lysis conditions. *B*. Generation of CTF of MLK3 variants.

A.



B.

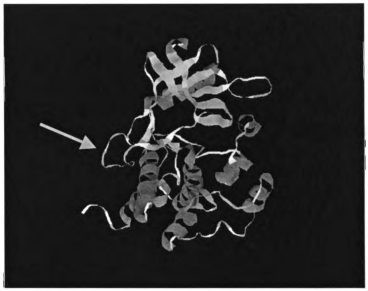


Fig. 2. **MLK3 is cleaved within the kinase domain**. *A*. MLK3 is cleaved between Gln 251 and Pro 252. The amino acids determined by Edman degradation are *underlined*. *B*. MLK3 kinase domain model based on Homology modeling. The *arrow* indicates the cleavage site Gln 251-Pro 252.

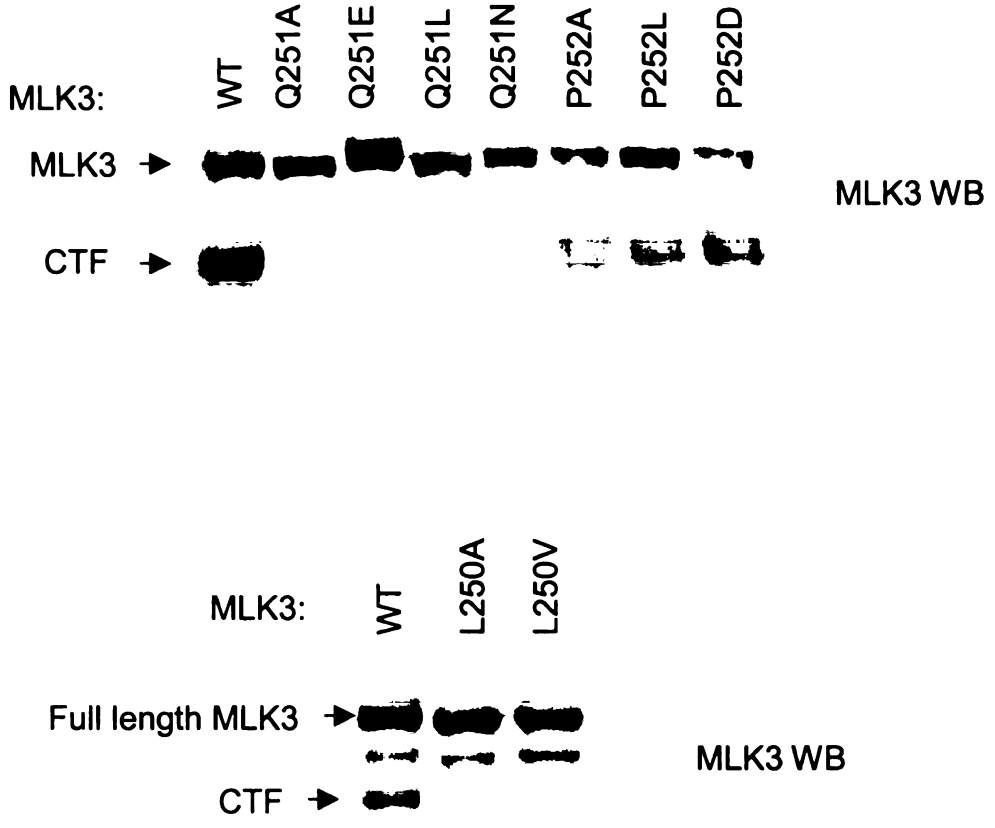


Fig. 3. **The proteolytic pattern of MLK3 variants.** HEK 293 cells were transfected with expression vectors containing the cDNAs as indicated above each lane. After 20 h, cells were lysed and Western blotting using the MLK3 antibody was performed. Bands corresponding to full length MLK3 and the CTF are indicated.

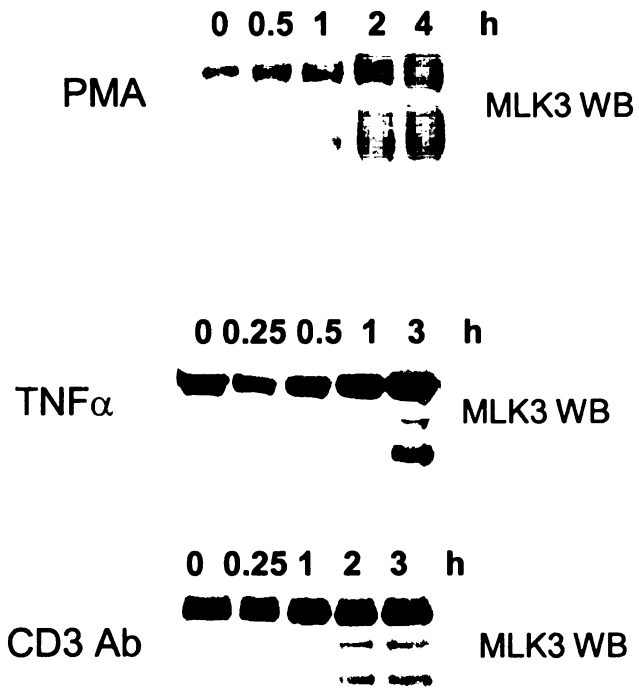


Fig. 4. **Inducible proteolysis of MLK3 in Jurkat T Ag cells.** Jurkat T Ag cells were transfected with expression vectors containing the cDNA Forty hour post-transfection, cells were treated with 50 ng/ml PMA, 10 nM of TNFα, or 0.5 μg/ml CD3 antibody for the indicated times and cells were lysed. MLK3 and CTF were detected by Western blotting using the MLK3 antibody. (TNFα and CD3 antibody data from Stancy Liou, Gallo lab)

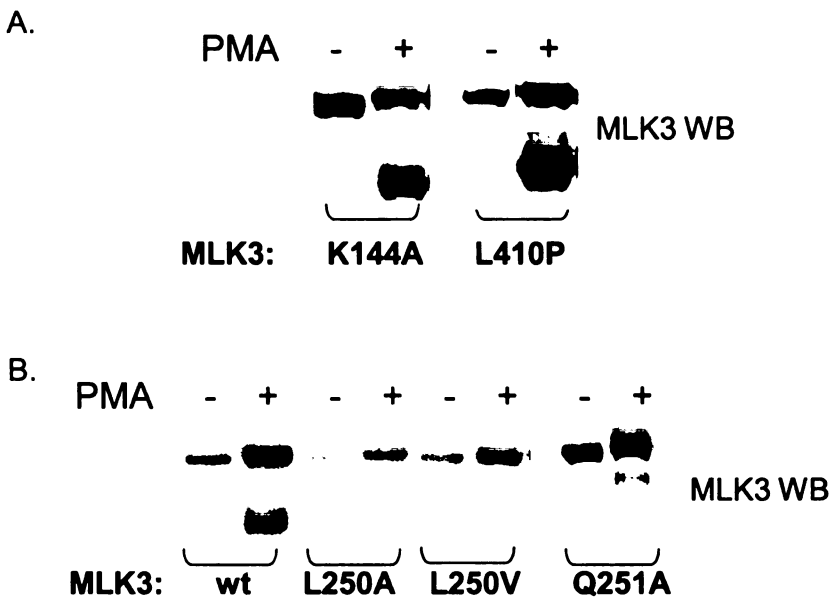


Fig. 5. **The proteolytic pattern of transfected MLK3 variants in PMA-treated Jurkat T Ag cells.** Jurkat T Ag cells were transfected by electroporation with expression vectors containing the cDNAs for the indicated MLK3 variants. After 40 h, the tranfected Jurkat T Ag cells were treated with solvent control (DMSO) or 50 ng/ml PMA for 2 h and cells were lysed. The presence or the absence of the CTF was detected by Western blotting using the MLK3 antibody. *A.* proteolytic pattern of catalytically inactive MLK3 and monomeric MLK3. *B.* proteolytic pattern of cleavage site MLK3 variants.

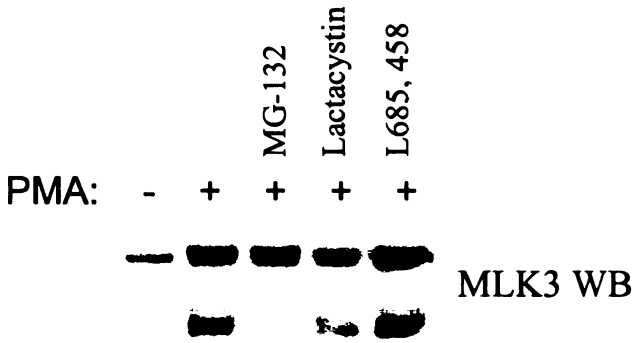
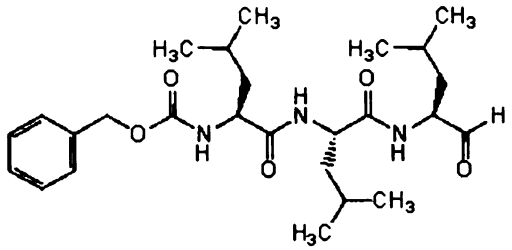


Fig. 6. **Effect of protease inhibitors on PMA-induced generation of the CTF of MLK3 in Jurkat T Ag cells.** Jurkat T Ag cells were transfected with expression vectors containing the cDNAs for MLK3. After 40 h, cells were pretreated with vehicle control (DMSO), MG132 (10 μM), lactacystin (5μM), or L685, 458 (100 nM) for 0.5 h, then treated with 50 ng/ml PMA for an additional 2 h. Cells were lysed and Western blotting was performed using the MLK3 antibody.

A.

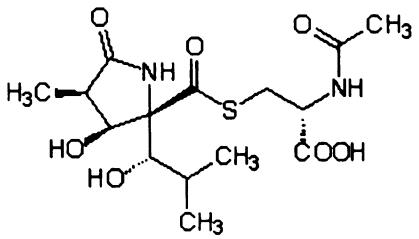


MG-132

Carbobenzoxy-Leu-Leu-Leu-CHO

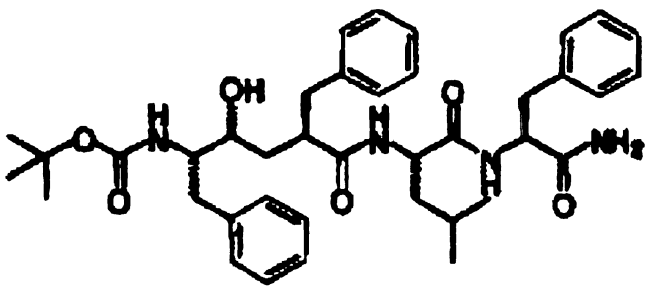
Cleavage site in MLK3: Leu-Leu-Leu-Gln-Pro

B.



Lactacystin

C.



L-685, 458

Fig. 7. **Chemical structure of selected protease inhitors and comparison with the sequence before the cleavage site in MLK3.** *A*, the chemical structure of MG-132 in comparison with the cleavage site within MLK3. The *arrow* indicates the cleavage site. *B*, the chemical structure of lactacystin. *C*, the chemical structure of L-685, 458.

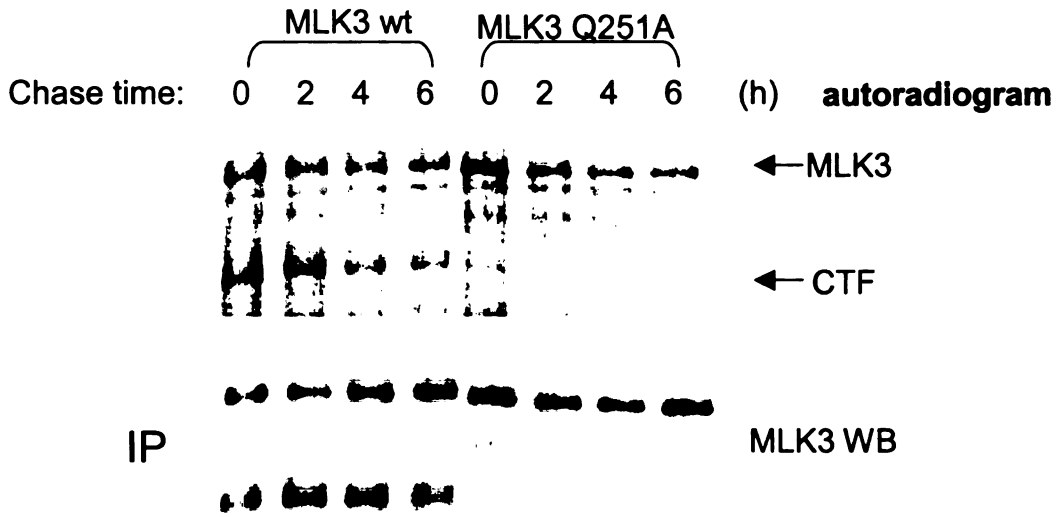


Fig. 8. **Pulse chase experiments in HEK 293 cells**. Cells were transfected with MLK3 wildtype and MLK3 Q251A cDNA. After 16 h post-transfection, cells were incubated with methionine-cysteine-free media for 1 hour, then supplemented with 150 μCi of [^{35}S] Met/Cys label mix in the same media. After 1 hour, cells were placed in fresh media containing 300-fold excess of unlabeled methionine. Cells were harvested and lysed at various time points. Cell lysates were immunoprecipitated with the MLK3 antibody and immunoblotted MLK3 was resolved by SDS-PAGE and transferred to a PVDF membrane. The radiolabeled protein was detected by phosphorimaging and the total immunoprecipitated MLK3 was assessed by Western blot. The *top panel* shows the radiolabeled MLK3, and the *bottom panel* shows levels of immunoprecipitated MLK3 variants.

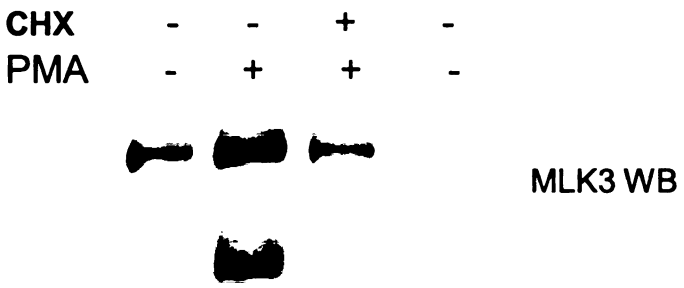


Fig. 9. **Cycloheximide blocks PMA-induced generation of the CTF of MLK3 in Jurkat T Ag cells.** Jurkat T Ag cells were transfected with expression vectors containing the cDNAs for MLK3. After 40 h, cells were treated with 10 ng/ml PMA in the presence or absence of 10 μg/ml cycloheximide (CHX) for 3 h. Cells were lysed and Western blotting using the MLK3 antibody was performed.

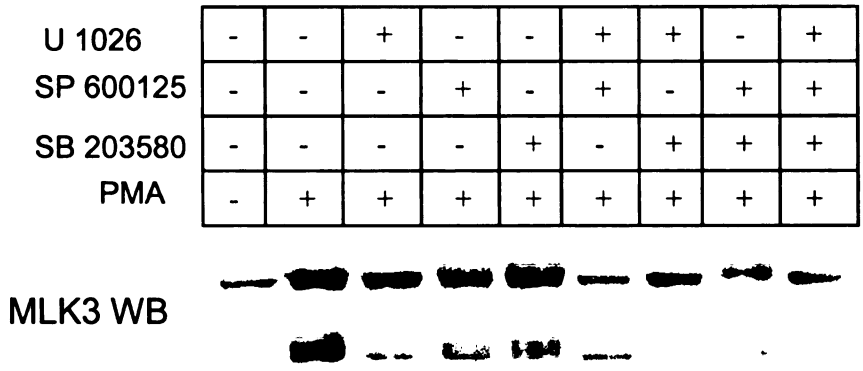


Fig. 10. **Effect of MAPK pathway inhibitors on the PMA-induced generation of the CTF of MLK3 in Jurkat T Ag cells.** Jurkat T Ag cells were transfected with expression vectors containing the cDNAs for MLK3. After 40 h, cells were pretreated with vehicle control (DMSO), 10 μM U0126 (MEK inhibitor), 20 μM SP 600125 (JNK inhibitor), and/or 6 μM SB 203580 (p38 inhibitor) as indicated for 0.5 h prior to treatment with 50 ng/ml PMA for 2 h. Cells were lysed and Western blotting using the MLK3 antibody was performed.

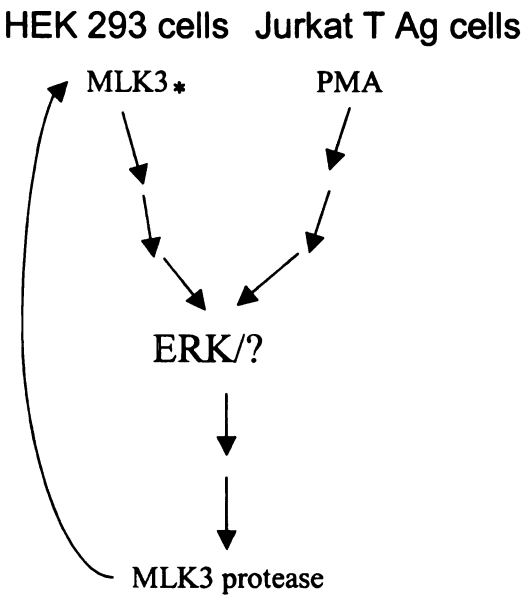


Fig. 11. **Model for generation of CTF of MLK3.** The CTF of MLK3 may be generated through activation of one or more pathways by MLK3 (in HEK 293 cells) or PMA, TNFα and anti-CD3 antibody (in Jurkat T Ag cells). ERK pathway is likely required for the generation of the CTF.

5. Discussion

We have characterized a proteolytic event of MLK3. MLK3 undergoes proteolysis to generate a stable CTF of 66 kDa. Based on our observations in both HEK 293 cells and Jurkat T Ag cells, we hypothesize that proteolysis of MLK3 may occur by a feedback mechanism. Therefore, specific inhibitors targeting the ERK, JNK and p38 pathways were used separately or in combination to test whether they may block proteolysis. Inhibitor studies suggest that ERK activation may be required for this proteolytic event as an ERK inhibitor diminished MLK3 proteolysis. However, p38 activation and JNK activation may contribute to the generation of the CTF of MLK3 as well. Since PMA is a potent activator of all three MAPK pathways, we cannot rule out the possibility of cross talk among the different pathways. It might be useful to test these MAPK inhibitors in TNFα-triggered proteolysis to find out whether JNK or p38 pathways actually play a role in this MLK3 proteolytic event.

Data presented thus far is derived from transiently overexpressed MLK3. We have not reproducibly observed the production of CTF from endogenous MLK3. It could be due to the fact that endogenous MLK3 is present at relatively low levels, making it technically difficult to detect the CTF using the immunoblotting method. It is also conceivable that we have not identified an appropriate signal to trigger endogenous MLK3 proteolysis. The strict sequence specificity of the MLK3 proteolytic event largely rules out the possibility that the proteolysis is just an artifact of MLK3 overexpression. It is conceivable that, under physiological condition, ERK activation promotes cell proliferation, and may trigger the activation or expression of the MLK3 protease, which cleaves and inactivates MLK3, thus blocking MLK3-mediated apoptosis.

Pulse chase experiments in HEK 293 cells support the idea that proteolysis occurs in newly synthesized MLK3. Cycloheximide, a protein synthesis inhibitor, blocks PMA induced proteolysis, further strengthening this idea. We are unaware of reports of the proteases that act on newly synthesized proteins, except that the 26S proteasome located in the cytosol degrades misfolded or unassembled newly synthesized proteins [29]. It is unclear why the MLK3 protease selectively chooses newly synthesized MLK3. Interestingly, we observe that the 5' untranslated region (UTR) in *mlk3* gene is required for this proteolytic event, since an MLK3 construct lacking the 5' UTR does not generate the CTF (data not shown). It is conceivable that the MLK3 protease may physically interact with the translational machinery near the 5' UTR.

Western blotting using an antibody raised against the amino terminal SH3 domain of MLK3 fails to detect the predicted 27 kDa amino terminal fragment of MLK3, suggesting that the amino terminal fragment may be unstable. The existence of the stable CTF suggests that CTF may have some biological function. Since the CTF does not contain a complete kinase domain, it should render MLK3 catalytically inactive. However, co-immunoprecipitation experiments using the CTF and full length MLK3 reveals that the CTF can associate with MLK3, presumably through its zipper region (data not shown). It has been shown that zipper-mediated homo-oligomerization is required for MLK3 activation and JNK signaling [16, 30]. This leads to the possibility that the CTF may modulate MLK3 catalytic activity through heterodimerization. In addition, CTF contains the CRIB motif responsible for association with small GTPase Rac and Cdc42. Thus, although speculative, it may act as dominant negative effector to regulate GTPase signaling.

Site-directed mutagenesis studies indicate that the Leu 250 and Gln 251 at the P2 and P1 sites are critical for generation of the CTF. Among the four MLK family members MLK1-4, the Leu and Gln are only conserved in MLK1 and MLK3, suggesting that it may be a unique regulatory mechanism for these two kinases. Extensive searches of the literature have failed to reveal a mammalian protease that cleaves after a Gln residue. However, a group of related coronavirus proteases does display a requirement for Gln at the P1 site. Coronaviruses are positive-strand RNA viruses with exceptionally large genomes (27 to 31 kb) and one strain of human coronavirus, 229E, causes upper respiratory tract illness in humans, in particular, the common cold [31]. The coronavirus main proteinase (M^{pro}), also called 3C-like proteinase ($3C^{pro}$), is a cysteine protease [32] that cleaves protein substrate at Leu-Gln ↓(Ser, Ala, Gly) sequences (the cleavage site is denoted by ↓). M^{pro} functions in proteolysis and maturation of the coronavirus replicase polyproteins. Cellular substrates for M^{pro} have not been identified [33]. The newly identified severe acute respiratory syndrome (SARS) M^{pro} also requires a Gln residue at the P1 site, with some flexibility at the P2 site [34]. Our data indicate that a coronavirus protease-like activity exist in mammalian cells. There are a couple of interesting thoughts regarding this finding. First, several labs have developed inhibitors against the M^{pro} of SARS for therapeutical application. It would be intriguing to test whether those inhibitors actually block the MLK3 proteolytic event. It is also conceivable that MLK3 may be a cellular target for M^{pro} upon viral infection. Based on the literature [35] and the simple BLAST search performed by us, there are no mammalian homologues of M^{pro}. The MLK3 protease may share little primary sequence homology with M^{pro}, but shares sufficient structural similarity to retain the same recognition site specificity.

The ability of several commercially available protease inhibitors to block the PMA-induced proteolytic event of MLK3 was tested. General cysteine or aspartyl protease inhibitors failed to block the generation of the CTF. However, one protease inhibitor, MG-132 is able to block the PMA-induced proteolysis. In the literature, MG-132 has been reported to inhibit the activity of proteasome and secretases. However, a specific inhibitor of proteasome, lactacystin, and a specific inhibitor of γ-secretase, L-685, 458 are unable to block the proteolysis. These findings suggest that MG-132 blocks the proteolytic event of MLK3, by inhibiting a protease other than the proteasome or the γ-secretase. The structure of MG-132 is carbobenzoxy-Leu-Leu-Leu-CHO. The sequence before the cleavage site of MLK3 is Leu-Leu-Leu-Gln (Fig. 7). It is very likely that MG-132 acts as a competitive inhibitor that binds to the catalytic core of the MLK3 protease, thus preventing MLK3 binding and subsequent cleavage by the protease. To gain further support for this idea, the peptide Ac-Ile-Leu-Leu-Leu-Gln-CO-NH_2 is being synthesized and its ability to block this proteolytic event of MLK3 will be tested. If this proves successful, it may be possible to use the peptide inhibitor or another such compound as an affinity tag to isolate the MLK3 protease from cellular lysates so that the identity of the protease can be determined by mass spectrometry.

Footnotes

Acknowledgements:

1. The kinase domain model of MLK3 was done by Ritesh Agrawal.
2. The PMA induced MLK3 proteolysis was first observed by Weiqin Chen in Dr. Esselman's lab.
3. TNFα and CD3 antibody induced MLK3 proteolysis was observed by Geou-yarh (Stancy) Liou.

6. Reference:

1. Keil, B., *Specificity of proteolysis.* 1992: Berlin ; New York : Springer-Verlag.

2. Schechter, I. and A. Berger, *On the size of the active site in proteases. I. Papain.* Biochem Biophys Res Commun, 1967. **27**(2): p. 157-62.

3. Seemuller, E., et al., *Proteasome from Thermoplasma acidophilum: a threonine protease.* Science, 1995. **268**(5210): p. 579-82.

4. Groll, M., et al., *Structure of 20S proteasome from yeast at 2.4 A resolution.* Nature, 1997. **386**(6624): p. 463-71.

5. Unno, M., et al., *The structure of the mammalian 20S proteasome at 2.75 A resolution.* Structure (Camb), 2002. **10**(5): p. 609-18.

6. Hershko, A. and A. Ciechanover, *The ubiquitin system.* 1998.

7. Dick, L.R., et al., *Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells.* J Biol Chem, 1997. **272**(1): p. 182-8.

8. Earnshaw, W.C., L.M. Martins, and S.H. Kaufmann, *Mammalian caspases: structure, activation, substrates, and functions during apoptosis.* Annu Rev Biochem, 1999. **68**: p. 383-424.

9. Sorimachi, H., S. Ishiura, and K. Suzuki, *Structure and physiological function of calpains.* Biochem J, 1997. **328**(Pt 3): p. 721-32.

10. Marks, N. and M.J. Berg, *APP processing enzymes (secretases) as therapeutic targets: insights from the use of transgenics (Tgs) and transfected cells.* Neurochem Res, 2003. **28**(7): p. 1049-62.

11. Fortini, M.E., *Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 673-84.

12. Lee, H.J., et al., *Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4.* J Biol Chem, 2002. **277**(8): p. 6318-23.

13. Ray, W.J., et al., *Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch.* J Biol Chem, 1999. **274**(51): p. 36801-7.

14. Shearman, M.S., et al., *L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity.* Biochemistry, 2000. **39**(30): p. 8698-704.

15. Bock, B.C., et al., *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation.* J Biol Chem, 2000. **275**(19): p. 14231-41.

16. Vacratsis, P.O. and K.A. Gallo, *Zipper-mediated oligomerization of the mixed lineage kinase SPRK/MLK-3 is not required for its activation by the GTPase cdc 42 but Is necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not catalyze the activating phosphorylation of Thr258 of murine MITOGEN-ACTIVATED protein kinase kinase 4.* J Biol Chem, 2000. **275**(36): p. 27893-900.

17. Zhang, H. and K.A. Gallo, *Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain.* J Biol Chem, 2001. **276**(49): p. 45598-603.

18. Xu, W., S.C. Harrison, and M.J. Eck, *Three-dimensional structure of the tyrosine kinase c-Src.* Nature, 1997. **385**(6617): p. 595-602.

19. Rock, K.L., et al., *Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules.* Cell, 1994. **78**(5): p. 761-71.

20. Klafki, H., et al., *The carboxyl termini of beta-amyloid peptides 1-40 and 1-42 are generated by distinct gamma-secretase activities.* J Biol Chem, 1996. **271**(45): p. 28655-9.

21. Steinhilb, M.L., R.S. Turner, and J.R. Gaut, *The protease inhibitor, MG132, blocks maturation of the amyloid precursor protein Swedish mutant preventing cleavage by beta-Secretase.* J Biol Chem, 2001. **276**(6): p. 4476-84.

22. Widmann, C., et al., *Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human.* Physiol Rev, 1999. **79**(1): p. 143-80.

23. Gallo, K.A. and G.L. Johnson, *Mixed-lineage kinase control of JNK and p38 MAPK pathways.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 663-72.

24. DeSilva, D.R., et al., *Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy.* J Immunol, 1998. **160**(9): p. 4175-81.

25. Bennett, B.L., et al., *SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13681-6.

26. Davies, S.P., et al., *Specificity and mechanism of action of some commonly used protein kinase inhibitors.* Biochem J, 2000. **351**(Pt 1): p. 95-105.

27. Rami, A., D. Ferger, and J. Krieglstein, *Blockade of calpain proteolytic activity rescues neurons from glutamate excitotoxicity.* Neurosci Res, 1997. **27**(1): p. 93-7.

28. Ohkuma, S. and B. Poole, *Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents.* Proc Natl Acad Sci U S A, 1978. **75**(7): p. 3327-31.

29. Wickner, S., M.R. Maurizi, and S. Gottesman, *Posttranslational quality control: folding, refolding, and degrading proteins.* Science, 1999. **286**(5446): p. 1888-93.

30. Leung, I.W. and N. Lassam, *Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3.* J Biol Chem, 1998. **273**(49): p. 32408-15.

31. Myint, S., *The coronavirdae.* 1995: Plenum, New York.

32. Ziebuhr, J., G. Heusipp, and S.G. Siddell, *Biosynthesis, purification, and characterization of the human coronavirus 229E 3C-like proteinase.* J Virol, 1997. **71**(5): p. 3992-7.

33. Hegyi, A. and J. Ziebuhr, *Conservation of substrate specificities among coronavirus main proteases.* J Gen Virol, 2002. **83**(Pt 3): p. 595-9.

34. Anand, K., et al., *Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs.* Science, 2003. **300**(5626): p. 1763-7.

35. Anand, K., et al., *Structure of coronavirus main proteinase reveals combination of a chymotrypsin fold with an extra alpha-helical domain.* Embo J, 2002. **21**(13): p. 3213-24.

IV. Identification of Mixed Lineage Kinase 3 Binding Partners by Affinity Purification and Mass Spectrometry

1.Abstract

Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates MAPK pathways, including the JNK and p38 pathways. Western blotting of cellular lysates from human cell lines revealed that MLK3 is present at high levels in breast cancer cell lines. Thus the human breast cancer cell line, MCF-7, was used as a source for the identification of proteins that interact with MLK3. MCF-7 cells were engineered to inducibly express Flag-tagged MLK3. Flag-MLK3 protein complexes were affinity purified from MCF-7 cellular lysates using anti-Flag M2 affinity resin. Flag-MLK3 and associated proteins were resolved on SDS-polyacrylamide gels and stained with Coomassie Blue. Bands representing potential MLK3 binding partners were identified by trypsin digestion followed by liquid chromatography/ nanoelectrospray tandem mass spectrometry (LC/MS/MS). Ten proteins were identified using this strategy, including heat shock proteins Hsp90 α,β, Hsc70, Hsp70, and Hsp90 co-chaperone Cdc37; clathrin heavy chain, clathrin assembly protein complex 2 medium chain; tubulin, adenine nucleotide translocator-2, and ribosomal protein S3. The strategy described in this chapter should be applicable to the identification of binding partners of other proteins expressed in mammalian cells.

As a second approach, in-solution digestion coupled with LC/MS/MS was also performed to identify MLK3 binding partners. This method not only confirmed the identity of most binding partners discovered by in-gel digestion, but also identified

thirteen additional potential MLK3 binding partners. These findings provide new avenues to explore that may help us further understand the regulation and biological function of MLK3.

2. Introduction

Cells respond to a variety of extracellular stimuli such as growth factors, osmotic stress, hormones, and nutritional deprivation. Many of these responses are mediated by cell surface receptors, and activation of these receptors triggers cellular responses, such as proliferation, differentiation, or apoptosis. In these signaling cascades, physical protein-protein interactions are often critical in transducing the signal. The activity of a protein kinase can be modulated by physical interactions with other signaling molecules. For instance, binding of an activator or inhibitor may directly impact the catalytic activity of protein kinases. Phosphorylation by other kinase may alter the properties of a protein kinase. Conversely, protein kinases can interact with and phosphorylate downstream substrate proteins. In addition, protein interactions may dictate the subcellular location of a particular protein kinase. Finally, chaperone proteins can influence the stability of protein kinases.

MLK3 is a serine/threonine kinase that functions as a mitogen activated protein kinase (MAPK) kinase kinase (MAPKKK) to activate the JNK and p38 pathways [1-4]. Though more controversial, it has also been published that MLK3 is capable of phosphorylating IKK to positively regulate the NFκB pathway in response to T cell costimulation [5]. In addition to its catalytic domain, MLK3 contains an SH3 domain, a leucine zipper, a CRIB motif and a carboxyl terminal Pro/Ser/Thr-rich region. These regions may mediate interactions with other proteins, which either regulate MLK3 activity, or impact MLK3 signaling.

MLK3 belongs to the mixed lineage kinase subfamily of protein kinases. The four MLK members, MLK1-4, all contain an SH3 domain, a kinase domain, a zipper region

and a CRIB motif. Of the four MLK subfamily members, only MLK3 and MLK2 have been biochemically characterized. Using a variety of approaches, several proteins have been shown to interact with MLK2 and/or MLK3 as summarized in Table 1. However, the mechanisms regulating MLK3 activity are still not well understood, and the biological functions of MLK3 are not well defined. The identification of additional, physiologically relevant MLK3 binding should shed light on the biological roles of MLK3.

Affinity purification coupled with mass spectrometry has emerged as a popular tool for isolating protein complexes and identifying their components. Two groups have successfully applied a single step purification using the anti-Flag M2 affinity resin to fish out protein complexes [6, 7].

Mass spectrometry (MS) has become a critical component in the study of biological systems. It can be applied to many aspects of biological research, such as the study of protein conformational changes by hydrogen/deuterium exchange, the identification of posttranslational modifications, and the identification of unknown proteins. In addition, methods are being developed to use mass spectrometry to determine relative levels of specific cellular proteins in different biological/pathological states.

All mass spectrometers are composed of three basic components: an ionization source wherein molecules are ionized and vaporized, a mass analyzer where the ions are separated based on the mass to charge ratios, and a detector. Matrix assisted laser desorption /ionization (MALDI) and electrospray ionization (ESI) are the two most commonly used ionization methods applied to proteins and peptides. MALDI uses a laser pulse to induce the formation of singly charged peptide ions [8], and is often coupled to a

Time of Flight (TOF) mass analyzer for peptide mass mapping or analysis of posttranslational modification, including phosphorylation.

In ESI, molecules form a beam of ions in the presence of a strong electrical field by first forming a spray of droplets, which quickly form smaller droplets upon solvent evaporation. This process continues until individual charged peptide ions are formed [9].

ESI is often coupled with a tandem MS (MS/MS) instrument to derive sequence information of ionized peptides. In an MS/MS experiment, a precursor ion is mass-selected by the first mass analyzer and focused into a collision region in which the precursor ion is induced to fragment thus yielding product ions. Product ions are mass analyzed by a second mass analyzer. There are two commonly used tandem MS instruments. The triple quadruple mass analyzer is arranged in series in space, whereas the ion trap mass analyzer is arranged in series in time [9].

Several programs exist to analyze the raw MS/MS data of peptides, including *PROFOUND*, *MASCOT*, and *SEQUEST*. Using the *SEQUEST* algorithm, acquired fragmentation spectra of peptides are correlated with the predicted amino acid sequences in protein databases. The proteins can be identified by the resulting list of peptide sequences [10]. Several parameters are used in *SEQUEST* to indicate the accuracy of the prediction. The cross-correlation score (XCorr) measures the similarity between the mass-to-charge ratios for the fragment ions predicted from amino acid sequences obtained from the database and the fragment ions observed in the tandem mass spectrum. A peptide with an XCorr above 2.5 is generally considered reliable. The DeltaCn (1.0-normalized correlation score) indicates the normalized numerical difference between the top and second highest XCorr in the database. RSp(Rank/preliminary score) indicates a

preliminary ranking based on the number of matched ion peaks. A subjective combination of these scores, as well as other factors such as the charge of the precursor ion, and the number of peptides that map to a given protein, is typically used to evaluate the accuracy of each prediction [11]. Finally, further biochemical analysis must be performed to validate these findings.

Using affinity purification coupled with electrospray/MS/MS, more than twenty potential MLK3 binding partners were identified, suggesting MLK3 forms complexes with other proteins *in vivo*.

3. Materials and Methods

3.1. Plasmid Constructs and Mutagenesis

Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wild type MLK3 (pRK5-NFlag.*mlk3*), and MLK3 truncation variants (pCGN-HA *mlk3* 1-114, 115-399, 400-591, 592-847) have been described elsewhere [12]. The expression vector encoding pGEX-2T GST-clathrin 1-579 was kindly provided by Dr. James H. Keen at Thomas Jefferson University (Philadelphia, PA). The expression vector encoding wildtype MLK3 was used as a template to create *mlk3* K144R using the Quick Change Site-directed Mutagenesis method (Stratagene). The presence of the desired mutation was confirmed by automated DNA sequencing in the Genomics Technology Support Facility at Michigan State University.

3.2. Construction of MCF-7 Inducible Cell Lines

MCF-7 stable cell line expressing the transcription factor vector pL_2N_2-R_HS3H/ZF3 (ARIAD) was kindly provided by Dr. Susan Conrad. The target gene vector pLH-Z_{12}I-PL *mlk3* was constructed by polymerase chain reaction-mediated amplification of the corresponding coding sequence from pRK5-NFlag.*mlk3* followed by subcloning into the target gene vector at the *Hind* III and *Cla* I sites. The pLH-Z_{12}I-PL *mlk3* plasmid was transfected into the stable MCF-7 cell line using the calcium phosphate method as described elsewhere [13]. Stable clones were selected under neomycin (40 μg/ml) (Invitrogen) and hygromycin (50 μg/ml) (Invitrogen) containing Dulbecco's Modified Eagle's Medium (DMEM) (5% fetal bovine serum) (Invitrogen) for about 2 weeks. Individual colonies that formed after about 4 weeks were picked and cultured separately.

AP21967 was added to induce the expression of MLK3 and the expression level was confirmed by resolving cellular lysates on SDS-PAGE followed by immunoblotting using Flag antibody. Individual clones inducibly expressing MLK3 were maintained under neomycin (40 μg/ml) and hygromycin (10 μg/ml) containing DMEM (5% fetal bovine serum).

3.3. Purification of Flag-MLK3 Complexes

MCF-7/ iFlag-MLK3 (inducibly expressing Flag-MLK3) cells (1X 10^8) in eight 15 cm dishes were cultured in the presence of 100 nM AP21967 for 20 h to induce Flag-MLK3 expression. Equal amounts of cells were cultured in the absence of the AP21967 as a negative control. The cells were washed once with PBS and lysed in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) containing a cocktail of protease inhibitors (Sigma). The lysates were pooled, centrifuged at 14,000 rpm for 15 min, and the supernatants passed through a 0.22 μM filter to remove any remaining cells and particulates. Cleared cellular lysates were then incubated with 400 μl of anti-Flag M2 agarose gel (Sigma) (previously equilibrated in TBS (50mM Tris HCl, pH 7.4, 150 mM NaCl) buffer) for 90 min with gentle mixing at 4°C. The resin was collected by centrifugation at 1000x *g* for 5 min and was washed with TBS buffer three times to remove non-specific binding proteins. Flag-MLK3 complexes were eluted from the resin by incubation for 60 min at 4°C with 400 μl TBS containing 300 ng/μl 3X Flag peptide (Sigma). The proteins collected in the supernatant were then precipitated by ice-cold acetone (3:1 acetone: supernatant).

3.4. Nupage Gel Electrophoresis and Coomassie Staining

After acetone precipitation, the Flag-MLK3 complexes were dissolved in 30 μl of water and Nupage Lithium Dodecyl Sulfate (LDS) sample buffer (4X) (Invitrogen) and 50 mM DTT was added. Protein mixtures were denatured at 70°C for 10 min and then separated on Nupage 4-12 % Bis-Tris Gels (Invitrogen). Gels were stained with SimplyBlue SafeStain (Coomassie G-250) (Invitrogen).

3.5. In-gel Trypsin Digestion

The protein bands were excised from the Coomassie stained gel and chopped into 1 mm^3 pieces. Gel pieces were transferred to siliconized 1.5 ml Eppendorf tubes (Dot Scientific), and proteins were reduced with 10 mM dithiotheritol (DTT) by incubating at 56°C for 30 min, and then alkylated with 55 mM iodoacetamide in the dark at room temperature for 20 min. The gel pieces were dehydrated with 100% acetonitrile and dried using a Speed-Vac. Gel pieces were covered with sequencing grade trypsin (13 ng/μl) (Promega) and incubated at 37°C overnight. Tryptic peptides were extracted with 60% acetonitrile/1% trifluoroacetic acid and were completely dried using a Speed-Vac. The dried sample was reconstituted by adding 8 μl of 1% formic acid.

3.6. In-solution Trypsin Digestion

After acetone precipitation, proteins were dissolved in 15 μl buffer containing 6M urea, 50 mM Tris pH 8, 2 mM DTT and incubated at 60°C for 45 min to reduce and denature the proteins. Sequencing grade trypsin (100 ng in 85 μl of 50 mM ammonium

bicarbonate) was added to digest the protein mixture at 37°C overnight. Tryptic peptides were concentrated to approximately 20 μl by Speed-Vac evaporation.

3.7. LC/MS/MS

A portion of the tryptic peptides (typically 6 μl out of 20 μl sample) was loaded onto a peptide capillary trap (Michrom 004/25108/32) through the auto sampler of the CapLC system (Waters Co., Milford, MA). An auxiliary pump was used to wash the trap with 1% aqueous formic acid at a flow rate of 20 μl/min for 5 min. At the end of the first 5 min wash, an automated valve on the CapLC system was switched allowing the gradient mixture of solvent A and B (Solvent A: 1% formic acid in water; Solvent B: 1% formic acid in acetonitrile) to flow through the trap and onto the nano-spray column (PicoFrit column from NewObjective, PFC7515-AQ--5, 15 μm tip id, 75 μm column id, 5 cm of C18 packing resin before the frit at the tip). The chromatography was run isocratically for 5 min at 5% B/95%A and then was increased to 60% B in a linear gradient over 25 minutes. For in solution digests, the chromatography was run isocratically for 10 min at 5% B and then increased in a linear gradient to 60% B over 140 minutes. The flow rate from the PicoFrit column tip was 0.1 μl/min. The flow from the pump was 9 μl/min, allowing the gradient to be ramped up or down smoothly. A micro T-splitter (Upchurch Scientific, Oak Harbor, WA) between the CapLC pumps for solvent A/B mixture and the capillary trap interfaced the two flow rates. The flow from the PicoFrit column tip was directly sprayed into a Finnigan LCQ-Deca mass spectrometer (Thermo Finnigan, San Jose, CA). The source voltage was set at 2.7 kV and the heated capillary temperature was set at 200°C. The quadruple ion trap mass spectrometer scanned from m/z 200 to m/z 2000 at a rate of 1.5 sec/scan. Data-dependent

scan methods were used. The dynamic exclusion scan was set so that MS/MS would be performed on the three highest peaks over the set threshold. The same m/z detected over 5 min was scanned only three times. Zoom scan was not applied in order to perform more MS/MS scans. The calibration was accomplished by infusion of 1 pmol/μl of angiotensin peptide at the same flow rate (0.1 μl/min). The *SEQUEST* software (version 2, TurboSequest) (Thermo Finnigan, San Jose, CA) was used to extract the MS/MS files from the raw data file, and to match the peptides in the human Fasta protein database. The precursor ion mass tolerance was set at 2.5 Da. A smaller deviation between the experimental mass and the calculated mass results in a higher final score. Scoring of all the candidate peptides was based on the matching of the observed *b, y* ions in an MS/MS spectrum with the calculated counterparts from the candidate sequences.

3.8. Transfection and Lysis of HEK 293 cells

Human embryonic kidney (HEK) 293 cells were cultured on 100-mm dishes and transfected using the calcium phosphate method as previously described [13]. Cells were harvested 16 h after transfection, and lysed as described previously[12].

3.9. Purification of GST Fusion Proteins and GST Pulldown Assays

GST and GST-clathrin heavy chain (1-579) were expressed in DE3 *E. coli* and purified on glutathione-Sepharose 4B, according to the manufacturer's protocol (Amersham Phamacia Biotech). Clarified lysate (400 μl) was incubated with 20 μl of glutathione-Sepharose 4B resin prebound with 5 μg of GST fusion protein for 90 min at

4 °C. GST pull-downs were washed twice with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol).

3.10. SDS-PAGE and Western Blot Analysis

Lysates and GST pulldowns were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted using the Flag antibody (4 μg/ml), actin mouse monoclonal antibody (Sigma), or phospho-c-Jun (KM-1) mouse monoclonal antibody (2 μg/ml) (Santa Cruz), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Western blots were developed by chemiluminescence.

4. Results

4.1. Survey of endogenous levels of MLK3.

Total cellular lysates from a variety of human cell lines, HEK 293 cells (human embryonic kidney epithelial cells), Jurkat T lymphocyte cells, MCF-7 cells (human breast carcinoma), A549 (human lung carcinoma), and HeLa (human cervix adenocarcinoma) were surveyed for their levels of endogenous MLK3. As shown in Fig. 1, of the cell lines tested, MLK3 is present at highest levels in MCF-7 cells, suggesting that MCF-7 cells may be a good source of physiologically relevant binding partners of MLK3.

4.2. Inducible expression of MLK3 in MCF-7 cells

To establish a reliable system for affinity purification of MLK3 complexes, MCF-7 cells were engineered to inducibly express Flag-tagged MLK3 using the Ariad system (Fig. 2). As shown in Fig. 3, Flag-MLK3 was inducibly expressed upon the addition of the dimerizer AP21967. Since MLK3 activates the JNK pathway, the phosphorylation of the transcription factor c-Jun was used as readout of MLK3 activity *in vivo*. The phosphorylation of c-Jun dramatically increased upon induced expression of Flag-MLK3, suggesting that inducibly expressed Flag-MLK3 is catalytically active.

4.3. Purification of Flag-MLK3 complexes from MCF-7 cells

Inducibly expressed Flag-MLK3 in MCF-7/iFlag-MLK3 cells was immunoprecipitated with anti-Flag M2 affinity gel as described in "Materials and Methods". Flag-MLK3 complexes were eluted by addition of 3X Flag peptide and were analyzed by SDS-PAGE and Coomassie staining. Uninduced MCF-7/iFlag-MLK3 cells

were used as a negative control. As shown in Fig. 4, in addition to the band representing Flag-MLK3, there are multiple bands representing proteins that co-purified with Flag-MLK3, but were absent in the fraction purified from control cells. Protein bands were assigned numbers as indicated in Fig. 4.

4.4. Identification of putative MLK3 binding partners by in-gel trypsin digestion and MS/MS

The bands of interest were excised from the gel and trypsin digestion was performed as described in "Materials and Methods". After digestion, tryptic peptides were analyzed directly by LC/MS/MS to obtain sequence information. Analysis of band 1 is shown as an example in Fig. 5. Tryptic peptides were separated based on hydrophobicity by reverse phase HPLC, which greatly reduces the complexity of the tryptic peptide mixture, and eluting peptides were ionized by electrospray under a high voltage field. Ionized peptides enter the ion trap sequentially to generate mass spectra. The mass spectrum taken at 25.36 min is shown as an example in Fig. 5B. In this spectrum, there are three peaks with high intensity. The three corresponding peptides all undergo collision-induced dissociation and produce MS/MS. The MS/MS of peptide peak m/z=617.21 and peak m/z=668.13 are shown in Fig. 5C and 5D. The *SEQUEST* algorithm was used to analyze the raw MS/MS data and to match the peptide sequences to proteins in human Fasta protein database. Based on the sequence information obtained by MS/MS, these two peptides with a m/z =617.21 or 668.13, respectively, match clathrin heavy chain in the protein database. For protein band 1, fourteen peptide

sequences determined by MS/MS match the clathrin heavy chain in the human protein database. Therefore, protein band 1 represents clathrin heavy chain.

Using in-gel digestion followed by LC/MS/MS, ten proteins were identified as potential MLK3 binding partners (Table 2). Three of the bands (band 2, 3 and 6) excised from the gel contained heat shock proteins. These proteins are heat shock protein 90 (Hsp90) α, β and its co-chaperone Cdc37, heat shock 70 kD protein 1 (Hsp70) and heat shock cognate 71 kD protein (Hsc70). Two bands (band 1 and 4) excised from the gel represent clathrin heavy chain (CHC) and clathrin assembly protein complex 2 medium chain (AP2-μ2). In addition, α-tubulin, adenine nucleotide translocator-2 (ANT2), and ribosomal protein S3 were identified in Flag-MLK3 complexes.

4.5. In-solution digestion and MS/MS

In-gel digestion requires separation of protein samples in advance and the catalytic activity of trypsin is reduced in gel. Yates *et al.* have successfully performed in-solution trypsin digestion coupled with multidimensional liquid chromatography and MS/MS to identify large numbers of proteins in complex mixtures [14] [15]. Since MLK3 complexes are relatively simple after affinity purification, we utilized an in-solution digestion coupled with one-dimensional HPLC to separate tryptic peptides derived from Flag-MLK3 complexes. Three independent experiments were performed. As shown in Table 3, except for AP2-μ2, all other proteins that were identified by in-gel digestion were detected in these experiments. Although α-tubulin was not identified, β-tubulin was detected. This suggests that MLK3 may associate with microtubules which are composed of α/β tubulin heterodimers [16]. Thirteen additional potential MLK3

binding partners were also identified. However, since each of these proteins was identified in only one experiment, they might represent false positives rather than bona fide binding partners. Further biochemical analysis is required to analyze and confirm their interactions with MLK3.

4.6. Association of MLK3 with clathrin heavy chain

Band 1 was identified as clathrin heavy chain (CHC). Clathrin is a main component of the clathrin-coated vesicles involved in endocytosis. Clathrin is a complex of trimeric heavy chains, each of which is associated with a regulatory light chain. The amino terminus of CHC consists a globular terminal domain (TD), which has been shown to interact with a variety of proteins that function in the clathrin mediated endocytosis [17] [18]. We were interested to test whether MLK3 interacts with CHC through its TD. The TD of CHC (amino acids 1-579) was expressed as a fusion protein with GST in *E. coli* and purified with glutathione Sepharose 4B beads. The ability of MLK3 to interact with GST-CHC TD was assessed in GST pulldown experiments. As shown in Fig. 6, MLK3 associates with GST-CHC TD but not with GST. A kinase inactive form of MLK3, K144R, retains the ability to bind to GST-CHC TD, suggesting the kinase activity is not required for this interaction.

A series of previously constructed amino terminal HA-tagged MLK3 variants, which contains individual or tandem domains of MLK3, were used to define the clathrin binding region within MLK3. These variants were expressed and analyzed for their capacity to interact with the TD domain of CHC. Data from GST pull down assays show that, of these variants, only MLK3 592-847 retains the ability to interact with GST-CHC TD (Fig. 7). Thus we conclude that the binding site for clathrin TD is within amino acids

592-847 of MLK3. Interestingly, MLK3 592-847 apparently undergoes some proteolytic degradation and the amino terminal proteolytic fragment associates with GST-CHC TD, suggesting that clathrin binding region is not within the extreme carboxyl terminus of MLK3.

Some proteins that function in clathrin-mediated endocytosis, such as the adaptor proteins (AP), arrestin and epsin1, interact with the terminal domains of CHC through a "clathrin box" motif with a consensus sequence of "LLpL-" (where p and – denote a polar and a negatively charged residue respectively). This clathrin box motif is critical for binding to the TD domain of CHC [18]. In the carboxyl terminus of MLK3, there is a "clathrin box" like sequence "LLDVG" (aa713-717). However, mutation of one of the five amino acid residue, Asp 715 to Ala in MLK3 did not abolish the binding ability of MLK3 with clathrin heavy chain as judged by the GST pulldown assay (data not shown). The nature and the consequences of this interaction are under investigation.

TABLE 1. **Previously identified binding partners of MLK1, MLK2, and MLK 3**

Binding partners	Reports on	Binding region in MLK1,2,3	Effect of interaction
14-3-3 ε [3]	MLK2		No detectable effect on MLK2 induced activation of JNK
Akt [19]	MLK3	511-847	Akt phosphorylates MLK3 S674 to negatively regulate MLK3 activity and prevent MLK3 induced cell death
Clathrin heavy chain [20]	MLK2	MLK2 931-935 Clathrin box motif	Overexpression of MLK2 may negatively influence clathrin mediated endocytosis
Dynamin [21]	MLK2	SH3 domain	MLK2 SH3 domain synergistically stimulates dynamin activity with PtdSer
Hippocalcin [3]	MLK2		No detectable effect on MLK2 induced activation of JNK
HPK1 [22]	MLK3	SH3 domain	HPK1 phosphorylates kinase inactive MLK3 and but no report showing it could alter MLK3 kinase activity
Huntingtin [23]	MLK2	SH3 domain	MLK2 associates with normal huntingtin, but not polyglutamine-expanded Huntington. Normal huntington may negatively regulate MLK2 activity.
JIP1 [24, 25]	MLK2, 3		JIP1 enhances MLK3-mediated JNK activation
JIP2/IB2 [4, 25];	MLK2, 3	MLK3 1-204	JIP2 enhances MLK3-mediated JNK activation Tiam1 enhances association of JIP2 with MLK3 and JIP2 enhances Tiam1 activation of p38.
JIP3 [26]	MLK3		JIP3 enhances MLK3 mediated JNK activation (JIP3 is reported to not associate with MLK2)
KIF 3 [3]	MLK2, 3	MLK2 497-953 MLK3 348-847	No detectable effect of MLK2 induced activation of JNK. MLK2 co-localize with JNK1/2 to punctate structures along microtubules.
NeuroD [27]	MLK2		MLK2 phosphorylates NeuroD and stimulate its transcription activity.
Pin1 [28]	MLK3		Pin1 may preferentially bind to hyperphosphorylated MLK3
POSH [29]	MLK1,2,3		POSH act as a scaffold protein for Rac1 and MLKs to facilitate JNK mediated apoptosis.
PSD-95 [30]	MLK2,3		The SH3 domain of PSD-95 associates with MLK2, 3 during GluR6 mediated JNK activation.
Rac/Cdc42 [3, 31]	MLK2, 3	CRIB	Enhances MLK3 autophosphorylation and substrate phosphorylation

TABLE 2. **Proteins identified by in-gel trypsin digestion and LC/MS/MS**

The table lists the proteins, their abbreviations, GI (Gene Identifier) numbers in NCBI database and molecular weight. Identified peptides and sequence coverage from one out of three sets of experiments are indicated.

Protein band	Protein name	Abbreviat-ions	GI	M.W. (kDa)	Identified peptides	Sequence Coverage
1	Clathrin heavy chain	CHC	4758012	191.6	14	10.8%
2	Heat shock protein 90 α	Hsp90α	123678	84.7	12	15.4%
	Heat shock protein 90 β	Hsp90β	6680307	83.3	9	12%
3	Heat shock 70 kD protein 1	Hsp70	123648	70.0	10	18.9%
	Heat shock cognate 71 kDa protein	Hsc70	462325	70.9	2	2.9%
4	Clathrin assembly protein complex 2 medium chain	AP2-μ2 /AP50	113332	49.7	3	6.7%
5	α-tubulin		20455316	50.0	2	7.3%
6	P50 ^{Cdc37}	Cdc37	21542000	44.5	1	4.5%
7	Adenine nucleotide translocator-2	ANT2	4502099	32.9	7	23.5%
8	Ribosomal protein S3	S3	417794	26.7	9	39.9%

TABLE 3. **Proteins identified by in-solution trypsin digestion and LC/MS/MS in three independent experiments.**

Identified proteins	Gene Identifier	M.W. (kDa)	Identified Peptides	Seq cov.	Detected in Exp.
MLK3	GI:4505195	92.6	28	48.3%	1,2,3
Hsp90α*	GI:123678	84.6	10	17.9%	1,2,3
Hsp90β*	GI:11277141	84.8	7	14.0%	1,2
Hsc70*	GI:5729877	70.9	9	34.4%	1,2
Hsp70*	GI:2119712	69.9	5	14.1%	1,2
CDC37*	GI:5901922	44.4	5	19.0%	3
Ribosomal protein S3*	GI:13640373	26.7	3	18.5%	3
ANT2*	GI:4502099	32.9	4	18.5%	1,3
Clathrin heavy chain*	GI:4758012	191.6	2	3.0%	1
β-tubulin*	GI:135471	49.7	2	9.2%	1,3
Clathrin assembly protein complex 2 α-A large chain	GI:20178274	107.6	2	6.3%	1
Ribosomal protein S5	GI:4506729	22.8	1	13.0%	1
Sorting nexin 12	GI:24418866	18.9	1	12.8%	2
Voltage-dependent N-type calcium channel α-1B subunit	GI:1705854	262.5	1	0.9%	2
Methylosome subunit t pICln	GI:1708393	26.2	1	10.4%	3
Fibronectin receptor beta subunit	GI:124963	88.5	1	2.4%	3
Calcineurin-binding protein Cabin 1	GI:6685261	246.4	1	1.8%	3
Transcription factor SL1	GI:2136304	95.3	1	3.9%	3
Glycoprotein gC1qBP	GI:730772	31.4	1	9.1%	3
Son of sevenless protein homolog 2 (SOS-2)	GI:6175038	153.0	1	1.9%	3
SRPK1a protein kinase (SRPK1)	GI:14252988	92.4	1	4.8%	3
Interferon-induced 35 kDa protein (IFP 35)	GI:19861692	31.5	1	13.3%	3
Death receptor 5, TRAIL receptor-2	GI:17380321	47.9	1	8.3%	3

Table 3 (cont'd)

Also detected in negative control:					
Titin	GI:2136280	299.2	30	3.0%	1,2,3
Fission yeast Skb1 protein homolog	GI:5174683	72.7	6	21.7%	2,3
Eukaryotic translation initiation factor 4B	GI:4503533	69.2	3	9.0%	1,2,3
Cystathionine beta-synthase	GI:543959	60.5	1	5.6%	3

Notes:

1. The *asterisk* indicates proteins that were also identified by in-gel digestion and LC/MS/MS.
2. This table includes the identified potential MLK3 binding partners that contain at least one peptide with a "XCorr" (cross-correlation score) above 2.5.
3. For the proteins that were identified in more than one experiment, the sequence coverage from one set of experiments was listed.

Fig. 1. **Expression of MLK3 in several human cell lines.** Cellular lysates containing equal amounts of total protein (20 μg) from the indicated cell lines were resolved by SDS-PAGE and immunoblotted for MLK3.

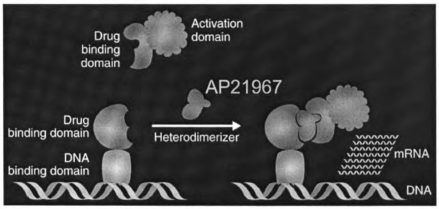


Fig. 2. **Construction of inducible cell lines.** The cDNA encoding Flag-tagged *mlk3* was inserted into pLH-Z12I-PL vector and transfected into an MCF-7 cell line that stably expresses the transcription factor vector pL2N2-RHS3H/ZF3. Stable clones were selected using neomycin (40 μg/ml) and hygromycin (50 μg/ml) containing media. Clones were isolated and tested for AP21967-induced expression of Flag-MLK3. (Figure adapted from ARIAD)

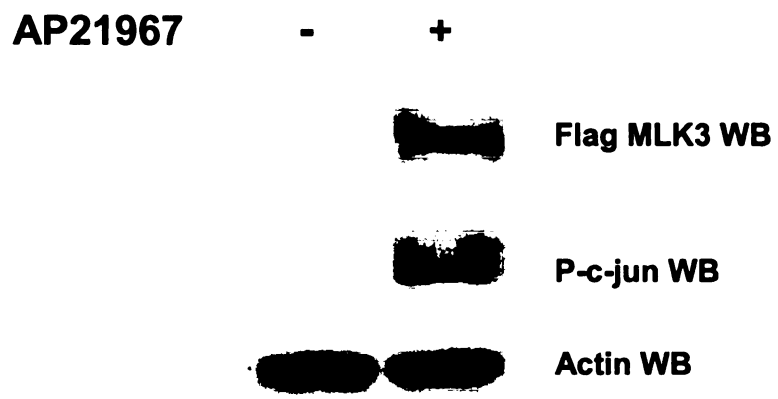


Fig. 3. **Inducible expression of Flag tagged MLK3.** A selected clone from the MCF-7 cells which inducibly expresses Flag-MLK3 was cultured either in the presence or in the absence of AP21967 (100 nM) for 20 h. Cells were lysed and cellular lysates were subjected to Western blot analysis. *Top panel*, the presence of Flag-MLK3 in the total cellular lysates was assessed by immunoblotting using the Flag antibody. *Middle panel*, the level of phosphorylated c-Jun in the total cellular lysates was assessed by immunoblotting using an antibody directed against phospho-c-Jun. *Bottom panel*, equal loading of the cellular lysates was confirmed by immunoblotting using an antibody directed against actin.

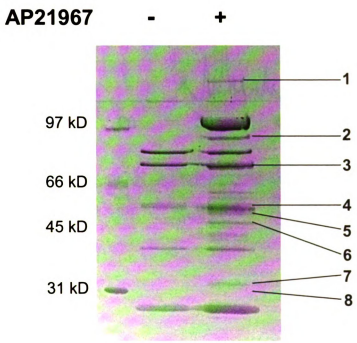
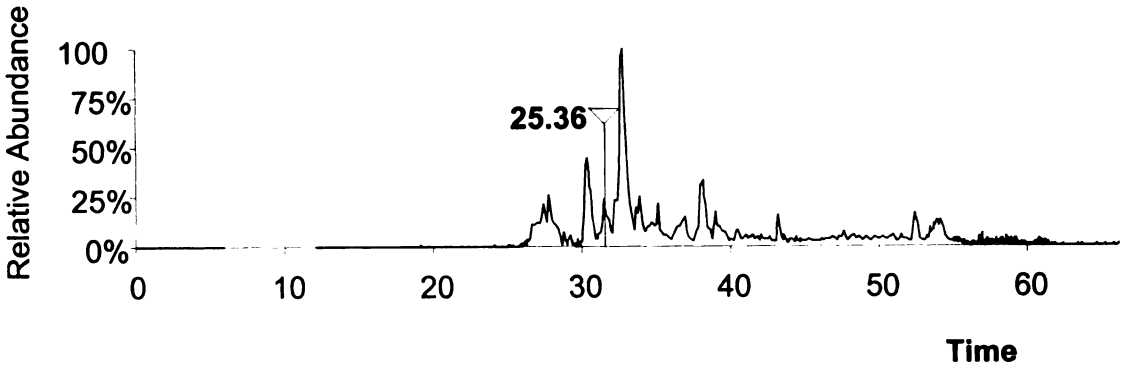
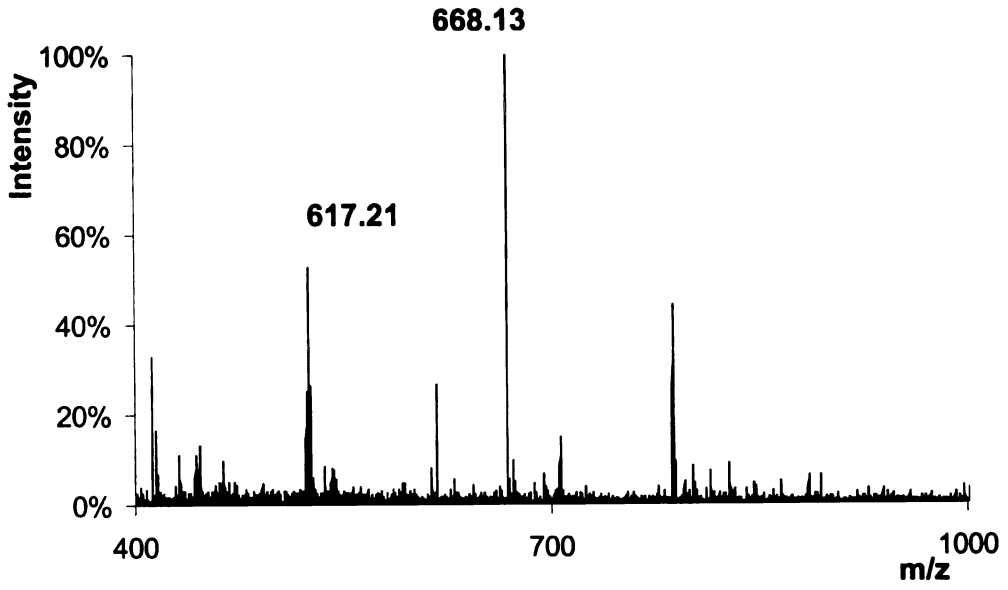


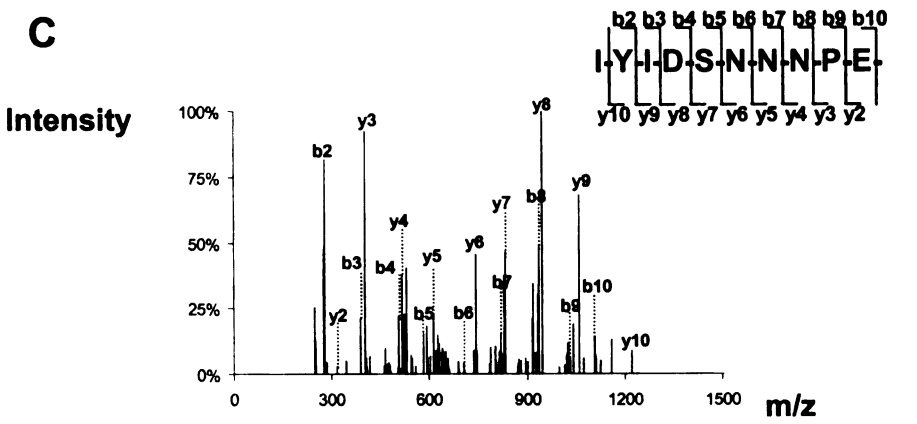
Fig. 4. **Affinity purification of Flag MLK3 complexes**. MCF-7 cells inducibly expressing Flag-MLK3 were cultured either in the presence or in the absence of AP21967 for 20 h. Cleared cellular lysates were immunoprecipitated with anti-Flag M2 agarose. Immunoprecipitates were washed and Flag-MLK3 complexes were eluted with 3X Flag peptide. After acetone precipitation, Flag-MLK3 and associated proteins were resolved by SDS-PAGE on a 4-12% acrylamide gradient gel and stained with Coomassie Blue. Molecular mass markers are indicated on the *left* in kilodaltons. The lines with *band numbers* indicate the positions of the proteins that were excised, digested with trypsin and analyzed by mass spectrometry.

A


Relative Abundance
100
75%
50%
25%
0%
25.36
0
10
20
30
40
50
60
Time

B


668.13
Intensity
100%
80%
617.21
60%
40%
20%
0%
400
700
1000
m/z


C
Intensity
b2 b3 b4 b5 b6 b7 b8 b9 b10
I-Y-I-D-S-N-N-N-P-E
y10 y9 y8 y7 y6 y5 y4 y3 y2
100%
75%
50%
25%
0%
0
300
600
900
1200
1500
m/z
b2
y3
y8
y9
y7
b8
y4
y6
b3
b4
y5
b7
b10
y2
b5
b6
b9
y10

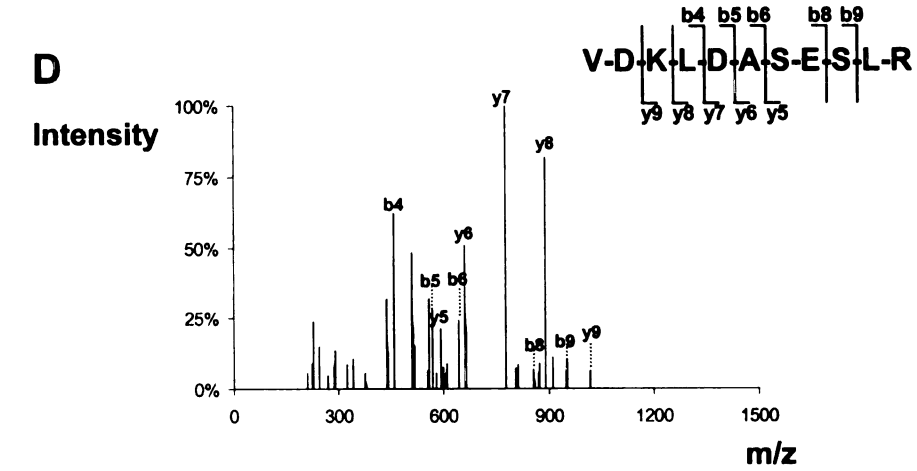

D
Intensity
b4 b5 b6 b8 b9
V-D-K-L-D-A-S-E-S-L-R
y9 y8 y7 y6 y5
100%
75%
50%
25%
0%
0
300
600
900
1200
1500
m/z
y7
y8
b4
y6
b5
b6
y5
b8
b9
y9

Fig. 5. **Identification of band 1 as clathrin heavy chain by LC/MS/MS.** *A,* Reconstructed chromatogram of an LC/MS/MS run of a tryptic digest from an excised gel band. *x* axis represents time; *y* axis represents the intensity of the base peak in the mass spectrum acquired at that time. *B*, mass spectrum taken at 25.36 min (scan number: 810). There are at least three peaks representing three different peptides in this spectrum. MS/MS was performed on each of them. The two MS/MS spectra that show adequate fragmentation are plotted in *C* and *D*. *C*, MS/MS spectrum of the doubly charged tryptic peptide IYIDSNNNPER (m/z 668.13). A nearly complete series of b ions and a nearly complete series of y ions can be identified. *D,* MS/MS spectrum of another doubly charged peptide VDKLDASESLR (m/z 617.21). The observed y and b fragment ions corresponding to the indicated peptide sequence are shown.

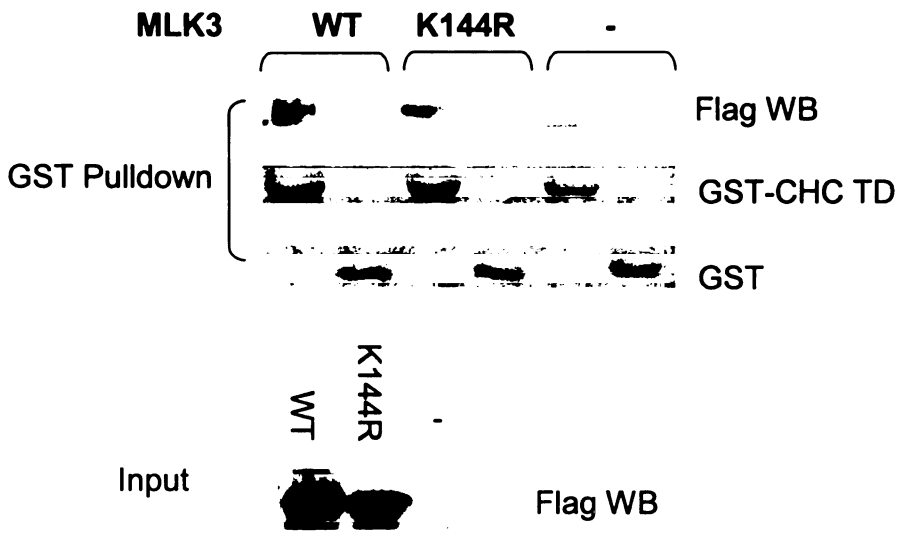


Fig. 6. **Association of MLK3 with clathrin heavy chain terminal domain**. HEK 293 Cells were transfected with expression vectors containing the cDNAs for MLK3 and MLK3 K144R. Cellular lysates expressing the indicated MLK3 variants were incubated with glutathione-Sepharose 4B resin to which purified GST-CHC TD or GST had been bound. *Top panel,* the presence or absence of bound MLK3 variants was assessed by Western blotting using the Flag antibody. *Middle two panels,* equal loading of GST-Clathrin TD on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates using the Flag antibody. The data shown are representative of three independent experiments.

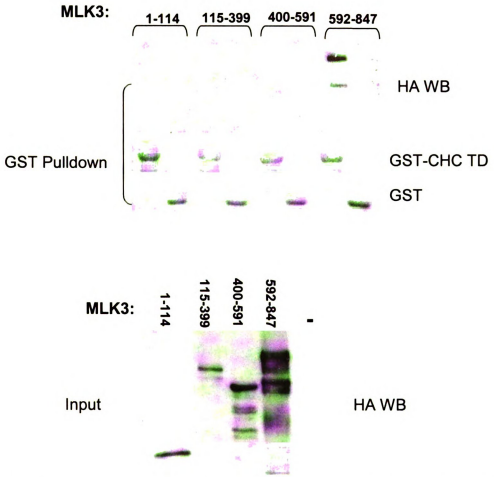


Fig. 7. **Interactions of MLK3 variants with clathrin heavy chain**. After transfection, GST pulldown assays were performed as described in the "Material and Methods". *Top panel,* the presence or absence of bound MLK3 variants was assessed by Western blotting using the HA antibody. *Middle two panels,* equal loading of GST-CHC TD on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates using the HA antibody. The data shown are representative of three independent experiments.

5. Discussion

In the signal transduction cascades, many signals are transduced through protein-protein interactions. The data presented here document how the affinity purification coupled with mass spectrometry can be used to isolate interacting proteins. Based on its generic nature, this method can be widely applicable to the identification and characterization of interacting proteins in mammalian cells.

The anti-Flag M2 antibody covalently linked to the agarose was used in the isolation of Flag-MLK3 complexes. Addition of the 3X Flag peptide to the affinity agarose results in quantitative elution of MLK3 (data not shown), which not only reduces contamination with light and heavy chains of the antibody, but also allows the eluted proteins to be precipitated with acetone. Acetone precipitation significantly reduces the volume of the sample, making it feasible to load large amounts of total proteins onto the polyacrylamide gels.

In this study, tandem affinity purification (TAP) was not applied. The TAP methodology is a protein tag-based affinity purification technique, which was originally developed in yeast [32, 33]. One TAP tag consists of two immunoglobulin-binding domains of protein A, followed by a cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin-binding peptide (CBP). Bait protein and its interacting proteins in the cellular extract are first recovered by affinity selection on an IgG agarose. After washing, the TEV protease is added to release the bound complexes. The eluate is incubated with calmodulin-coated beads in the presence of calcium. After washing, the bound complexes are released with EGTA. This two-step purification greatly reduces

non-specific binding, but also requires high-affinity binding among the complex proteins and is most suitable for stable protein complexes, such as translational machinery or transcription complexes. However, MLK3 is a protein kinase involved in signal transduction and its interactions with other proteins may be weak or transient. The affinity purification and elution procedure applied in this study are relatively gentle, which enhances the capture of MLK3 complexes.

Electrospray coupled with ion trap mass spectrometer was used in the identification of potential MLK3 binding partners. In contrast to MALDI, electrospray ionizes peptide samples directly from solution; thus peptides mixture can be resolved by capillary HPLC, which greatly reduces the complexity of the peptide mixtures. Ion trap mass spectrometry is capable of sequencing the peptides ions by collision-induced dissociation and this significantly facilitates the identification of unknown proteins. For example, the peptide sequence with the highest XCorr score obtained from in-gel digestion of band 6 matches a peptide sequence in Cdc37 in the human Fasta protein database. Therefore, Cdc37 was assigned as an MLK3 binding partner.

We noticed that the identified MLK3 binding partners are relatively abundant proteins in cell using the in-gel digestion approach. This could be due to the fact that only Coomassie stainable bands were analyzed. Certainly, ES/MS/MS is capable of identifying less abundant proteins. In-solution digestion coupled with one-dimensional reverse phase C18 HPLC was attempted three times to identify additional MLK3 binding partners. In these experiments, thirteen proteins were identified in the MLK3 complexes (Table 3), including some signaling proteins, such as the guanine nucleotide exchange factor SOS2, SRPK1a protein kinase, interferon-induced 35 kDa protein, and death

receptor 5. This suggests that MLK3 may form complexes with those signaling proteins to mediate signal transduction. However, the in-solution digestion experiment is unable to provide the molecular weight information of the unknown proteins. In addition, most proteins, including some proteins already identified by in-gel digestion, were not detected consistently in the three experiments. Immunoblotting of gels of resolved MLK3 complexes with specific antibodies against each potential candidate is necessary to examine their presence in the MLK3 complexes. Due to the uncertainty of their interaction with MLK3, the various proteins that have been identified using the in-solution digestion will not be discussed in details.

Several additional approaches to increase the sensitivity of the identification of MLK3 binding partners can be applied in future studies. For example, a cross-linking reagent can be used to capture the transient interacting proteins of MLK3. Cellular fractionation may reduce the complexity of MLK3 complexes. Additionally, in the in-solution digestion followed by LC/MS/MS experiment, the peptides mixture may overwhelm the resolution capacity of the single-dimensional chromatography system. Orthogonal two-dimensional separation methods (cation exchange chromatography followed by reverse-phase chromatography) have been reported to dramatically improve the resolution of complex mixtures compared with any one-dimensional separation [14]. In the future, in-solution digestion coupled with two-dimensional chromatography may be a practical way to identify low abundance MLK3 binding partners.

Clathrin heavy chain was identified as a potential MLK3 binding partner by mass spectrometry and its interaction with MLK3 was confirmed by GST pulldown assay using a fusion protein of clathrin heavy chain terminal domain with GST. In addition, the

carboxyl terminus of MLK3 (amino acids 592-847), which contains one potential clathrin box motif, is capable of interacting with CHC. However, mutation of one of the five conserved amino acids in this clathrin box motif, Asp 715 to Ala, does not abolish the binding to GST-CHC TD, suggesting that the aspartic acid residue is not critical for the interaction between MLK3 and CHC. The clathrin binding region within MLK3 needs to be further defined. Previous studies showed that MLK2 interacts with clathrin heavy chain through the extreme carboxyl terminal clathrin box motif within MLK2 [20]. However, interestingly, sequence alignment of MLK1, 2, 3 and 4 indicates that the clathrin box motif identified in MLK2 is conserved in the extreme carboxyl terminus of MLK1 and MLK4, but not in MLK3. This suggests that MLK3 has a distinct clathrin binding motif.

In addition to clathrin, there are large numbers of other proteins that participate in the process of endocytosis. In this study, three other proteins that are involved in the clathrin mediated endocytosis were identified as MLK3 binding partners. AP2-μ2 and Hsc70 were identified in the in-gel digestion. AP2-α, together with clathrin heavy chain was discovered in the first set of in-solution digestion experiments. AP2 are heterotetrameric protein complexes of subunits α (100-110 kDa), β2 (105 kDa), μ2 (50 kDa) and σ2 (20 kDa). AP2 is the second most abundant protein in coated vesicles after clathrin, which links the clathrin shell to the membrane through interaction of its μ2 and α subunits with membrane proteins and lipids [34]. *In vivo* studies have shown that the μ2 subunit is phosphorylated and this phosphorylation is essential for clathrin-mediated endocytosis [35]. Adaptor-associated kinase 1 (AAK1) has been reported to phosphorylate μ2 *in vitro* [36]. However, the identity of the "μ2 kinase" *in vivo* has not

been defined. It is conceivable that MLK3 may phosphorylate AP2-μ2. Hsc70 is involved in the disassembly of clathrin lattices and also contributes to adaptor uncoating [37]. The uncoating of clathrin coated vesicles is inhibited *in vivo* by overexpressing a dominant negative mutant of Hsc70 [38]. However, since Hsc70 is a constitutive member of the heat shock protein family, we cannot rule out the possibility that the sole role of Hsc70 is to fold and maintain the conformation of MLK3. Further studies are necessary to elucidate the potential biological function of MLK3 on clathrin mediated endocytosis.

In addition to Hsc70, Hsp70 and Hsp90 and its cochaperone Cdc37, were identified as MLK3 binding partners. Heat shock proteins play critical roles in protein folding and maturation. They also control protein homeostasis by regulating protein stability [39]. Hsp70 has been reported to associate with the unphosphorylated variant of PKC βII, thus prolonging the lifetime of mature PKC [40]. Whether phosphorylation of MLK3 regulates its association with Hsp70 has not yet been determined. The interaction of Hsp90/Cdc37 with MLK3 will be discussed extensively in the next chapter.

ANT2 was identified as a potential MLK3 binding partner. There are three ANT isoforms, ANT1, ANT2 and ANT3. ANT1 is expressed mainly in heart and skeletal muscle and ANT3 is ubiquitously expressed in all differentiated tissue [41]. Interestingly, ANT2 has relatively low expression level in most mature tissues [41, 42], with the highest expression levels detected in myoblasts and tumor cells. ANT is synthesized in the cytosol before export to the inner membrane of mitochondria where it catalyzes the ADP/ATP transport. In addition, mounting evidence suggests that ANT is involved in apoptosis, by controlling the opening of the permeability transition pore (PTPC) (reviewed in [43]). It has been reported that some protein kinases, such as PKCδ [44],

and Raf-1 [45] can translocate to mitochondria to modulate the mitochondrial mediated apoptosis. Further experiments are needed to understand the association of ANT2 with MLK3 and the potential role of this interaction in regulating apoptosis through the mitochondrial membrane permeabilization.

In summary, affinity purification coupled with mass spectrometry has been used to identify potential MLK3 binding partners. Confirmation of these interactions and further analysis of the consequences of these interactions will be the subject of future studies.

Footnotes

Acknowledgements:

1. The LC/MS/MS experiments were performed in collaboration with Wei Wu in Dr. Jack Watson's lab.
2. Endogenous MLK3 expression levels (Fig.1) were screened by Yan Du.
3. The MCF-7 cell line inducibly expressing MLK3 was constructed by Sarah Santos (Dr. Conrad Lab) and Yan Du.
4. The GST-CHC (1-579) construct (pGEX-2T GST-clathrin 1-579) was kindly provided by Dr. James H. Keen in Thomas Jefferson University.

6. References

1. Rana, A., et al., *The mixed lineage kinase SPRK phosphorylates and activates the stress- activated protein kinase activator, SEK-1.* J Biol Chem, 1996. **271**(32): p. 19025-8.

2. Merritt, S.E., et al., *The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate.* J Biol Chem, 1999. **274**(15): p. 10195-202.

3. Nagata, K., et al., *The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3.* Embo J, 1998. **17**(1): p. 149-58.

4. Buchsbaum, R.J., B.A. Connolly, and L.A. Feig, *Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade.* Mol Cell Biol, 2002. **22**(12): p. 4073-85.

5. Hehner, S.P., et al., *Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex.* Mol Cell Biol, 2000. **20**(7): p. 2556-68.

6. Westermarck, J., et al., *The DEXD/H-box RNA helicase RHII/Gu is a co-factor for c-Jun-activated transcription.* Embo J, 2002. **21**(3): p. 451-60.

7. Boudeau, J., et al., *Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability.* Biochem J, 2003. **370**(Pt 3): p. 849-57.

8. Hillenkamp, F., et al., *Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers.* Anal Chem, 1991. **63**(24): p. 1193A-1203A.

9. Griffiths, W.J., et al., *Electrospray and tandem mass spectrometry in biochemistry.* Biochem J, 2001. **355**(Pt 3): p. 545-61.

10. Eng JK, M.A., Yates, JR III, *An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database.* Journal of the American Society for Mass Spectrometry, 1994. **5**(11): p. 976-989.

11. Jimmy Eng, J.Y., *SEQUEST web homepage.* http://fields.scripps.edu/sequest/index.html.

12. Zhang, H. and K.A. Gallo, *Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain.* J Biol Chem, 2001. **276**(49): p. 45598-603.

13. Bock, B.C., et al., *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation.* J Biol Chem, 2000. **275**(19): p. 14231-41.

14. Link, A.J., et al., *Direct analysis of protein complexes using mass spectrometry.* Nat Biotechnol, 1999. **17**(7): p. 676-82.

15. Washburn, M.P., D. Wolters, and J.R. Yates, 3rd, *Large-scale analysis of the yeast proteome by multidimensional protein identification technology.* Nat Biotechnol, 2001. **19**(3): p. 242-7.

16. Nogales, E., *Structural insight into microtubule function.* Annu Rev Biophys Biomol Struct, 2001. **30**: p. 397-420.

17. Dell'Angelica, E.C., et al., *Association of the AP-3 adaptor complex with clathrin.* Science, 1998. **280**(5362): p. 431-4.

18. Kirchhausen, T., *Clathrin.* Annu Rev Biochem, 2000. **69**: p. 699-727.

19. Barthwal, M.K., et al., *Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival.* J Biol Chem, 2003. **278**(6): p. 3897-902.

20. Akbarzadeh, S., et al., *Mixed lineage kinase 2 interacts with clathrin and influences clathrin- coated vesicle trafficking.* J Biol Chem, 2002. **277**(39): p. 36280-7.

21. Rasmussen, R.K., et al., *Mixed-lineage kinase 2-SH3 domain binds dynamin and greatly enhances activation of GTPase by phospholipid.* Biochem J, 1998. **335**(Pt 1): p. 119-24.

22. Kiefer, F., et al., *HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway.* Embo J, 1996. **15**(24): p. 7013-25.

23. Liu, Y.F., D. Dorow, and J. Marshall, *Activation of MLK2-mediated signaling cascades by polyglutamine- expanded huntingtin.* J Biol Chem, 2000. **275**(25): p. 19035-40.

24. Whitmarsh, A.J., et al., *A mammalian scaffold complex that selectively mediates MAP kinase activation.* Science, 1998. **281**(5383): p. 1671-4.

25. Yasuda, J., et al., *The JIP group of mitogen-activated protein kinase scaffold proteins.* Mol Cell Biol, 1999. **19**(10): p. 7245-54.

26. Kelkar, N., et al., *Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3.* Mol Cell Biol, 2000. **20**(3): p. 1030-43.

27. Marcora, E., K. Gowan, and J.E. Lee, *Stimulation of NeuroD activity by huntingtin and huntingtin-associated proteins HAP1 and MLK2.* Proc Natl Acad Sci U S A, 2003.

28. Swenson, K.I., K.E. Winkler, and A.R. Means, *A New Identity for MLK3 as an NIMA-related, Cell Cycle-regulated Kinase That Is Localized near Centrosomes and Influences Microtubule Organization.* Mol Biol Cell, 2003. **14**(1): p. 156-72.

29. Xu, Z., N.V. Kukekov, and L.A. Greene, *POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis.* Embo J, 2003. **22**(2): p. 252-61.

30. Savinainen, A., et al., *Kainate receptor activation induces mixed lineage kinase-mediated cellular signaling cascades via post-synaptic density protein 95.* J Biol Chem, 2001. **276**(14): p. 11382-6.

31. Teramoto, H., et al., *Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c- Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family.* J Biol Chem, 1996. **271**(44): p. 27225-8.

32. Rigaut, G., et al., *A generic protein purification method for protein complex characterization and proteome exploration.* Nat Biotechnol, 1999. **17**(10): p. 1030-2.

33. Puig, O., et al., *The tandem affinity purification (TAP) method: a general procedure of protein complex purification.* Methods, 2001. **24**(3): p. 218-29.

34. Takei, K. and V. Haucke, *Clathrin-mediated endocytosis: membrane factors pull the trigger.* Trends Cell Biol, 2001. **11**(9): p. 385-91.

35. Olusanya, O., et al., *Phosphorylation of threonine 156 of the mu2 subunit of the AP2 complex is essential for endocytosis in vitro and in vivo.* Curr Biol, 2001. **11**(11): p. 896-900.

36. Conner, S.D. and S.L. Schmid, *Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis.* J Cell Biol, 2002. **156**(5): p. 921-9.

37. Wakeham, D.E., et al., *Molecular structures of proteins involved in vesicle coat formation.* Traffic, 2000. **1**(5): p. 393-8.

38. Newmyer, S.L. and S.L. Schmid, *Dominant-interfering Hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo.* J Cell Biol, 2001. **152**(3): p. 607-20.

39. Nollen, E.A. and R.I. Morimoto, *Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins.* J Cell Sci, 2002. **115**(Pt 14): p. 2809-16.

40. Gao, T. and A.C. Newton, *The turn motif is a phosphorylation switch that regulates the binding of Hsp70 to protein kinase C.* J Biol Chem, 2002. **277**(35): p. 31585-92.

41. Stepien, G., et al., *Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation.* J Biol Chem, 1992. **267**(21): p. 14592-7.

42. Lunardi, J., et al., *The multiple ADP/ATP translocase genes are differentially expressed during human muscle development.* J Biol Chem, 1992. **267**(22): p. 15267-70.

43. Belzacq, A.S., et al., *The adenine nucleotide translocator in apoptosis.* Biochimie, 2002. **84**(2-3): p. 167-76.

44. Majumder, P.K., et al., *Mitochondrial translocation of protein kinase C delta in phorbol ester- induced cytochrome c release and apoptosis.* J Biol Chem, 2000. **275**(29): p. 21793-6.

45. Wang, H.G., U.R. Rapp, and J.C. Reed, *Bcl-2 targets the protein kinase Raf-1 to mitochondria.* Cell, 1996. **87**(4): p. 629-38.

V. Regulation of Mixed Lineage Kinase 3 by Hsp90 and Cdc37

1. Abstract

Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) that activates MAPK pathways, including the JNK and p38 pathways. In Chapter IV, affinity purification coupled with LC/MS/MS revealed that components of Flag-MLK3 complexes include heat shock protein 90 α, β (Hsp90), and its co-chaperone Cdc37. The association of endogenous MLK3 with Hsp90/Cdc37 was confirmed by co-immunoprecipitation and immunoblotting. Domain mapping experiments demonstrated that Hsp90/Cdc37 binds to the catalytic domain of MLK3. The specific inhibitor for Hsp90, geldanamycin, has been shown to modulate the stability of steroid hormone receptors and certain protein kinases including Raf and Src. Upon treatment of MCF-7 cells with geldanamycin, MLK3 levels decrease dramatically. TNFα treatment increases the catalytic activity of endogenous MLK3 and JNK. Pretreatment of MCF-7 cells with geldanamycin abolishes TNFα-induced activation of MLK3 and JNK. Taken together, the data presented in this chapter demonstrate a role for molecular chaperones Hsp90/Cdc37 in MLK3 signaling.

2. Introduction

MLK3 is a serine/threonine protein kinase that acts as a MAPKKK to phosphorylate and activate the dual specific kinases, MKK4 [1] and MKK7 [2] which, in turn, can phosphorylate and activate JNK pathway. Overexpression of MLK3 also modestly activates the p38 MAPK pathway [3, 4].

JNKs and p38 MAPKs are activated by diverse extracellular signals, including cytokines, osmotic stress, UV and heat shock [5]. However, there are more than a dozen MAPKKKs demonstrated to activate the JNK and p38 pathways. The stimuli that can specifically activate endogenous MLK3 are not well characterized. Recently, it has been shown that treatment of Jurkat T lymphocytes with tumor necrosis factor-α (TNFα) activates endogenous MLK3 as judged by the ability of MLK3 to phosphorylate recombinant MKK4 *in vitro* [6].

In addition to its catalytic domain, MLK3 contains several domains, which may mediate protein-protein interactions to regulate its activity and/or signaling. The small GTPases, Cdc42 and Rac, in their activated states, bind to MLK3 in a Cdc42/Rac interactive binding (CRIB) motif-dependent manner and increase MLK3 catalytic activity [7]. Work described in Chapter II shows that MLK3 is autoinhibited through an interaction between its amino terminal SH3 domain and a sequence located between the zipper and CRIB motifs of MLK3 [8].

Hsp90 is a heat shock protein highly conserved in all eukaryotic cells. Hsp90 functions as a molecular chaperone to prevent the aggregation and to maintain the homeostasis of client proteins. The client proteins of Hsp90 include steroid hormone receptors and protein kinases (reviewed in [9, 10]). The co-chaperone Cdc37 is required

for the interaction between Hsp90 and it client protein kinases. The identified clients of Hsp90/Cdc37 include several protein kinases involved in proliferation, cell cycle control, and tumorigenesis, including Src [11, 12], Raf [13, 14], and CDK4 [15].

As detailed in Chapter IV, using affinity purification coupled with mass spectrometry, heat shock protein Hsp90 α,β and its co-chaperone Cdc37 were identified as binding partners of MLK3 in breast cancer cell line MCF-7 cells. In this chapter, the functional importance of the association of molecular chaperones Hsp90/Cdc37 on the stability and signaling of MLK3 is presented and discussed.

3. Materials and Methods

3.1. Plasmid Constructs and Site Directed Mutagenesis

Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wild type (pRK5-NFlag.*mlk3*) and inactive (pRK5-NFlag.*mlk3* K144A) has been described elsewhere [7]. The mammalian expression vector pGST-Cdc37 was kindly provided by Dr. Nicholas Grammatikakis in Tufts University School of Medicine.

Construction of pCGN-HA.*mlk3* 115-399 was described in Chapter II. For the construction of the additional hemagglutinin (HA)-tagged MLK3 variants, the following oligonucleotides were used in the polymerase chain reactions with pRK5-NFlag.*mlk3* as the template;

pCGN-HA.*mlk3* 1-399, 5'-CGTTAGTCTAGAATGGAGCCCTTGAAGAG-3' and 5'- GCATTAGGATCCTCACCAGCCTTCCTGCATGG-3';

pCGN-HA.*mlk3* 400-847, 5'-CGTTAGTCTAGAAAGCGCGAGATCCAGGG-3' and 5'-GCATTAGGATCCTCAAGGCCCCGCTTCCGGC-3'.

The amplified *mlk3* fragments were subcloned in-frame with the HA coding sequence into the pCGN mammalian expression vector using *Xba*I and *Bam*HI.

3.2. Cell lines and Transfections

MCF-7 cells were maintained in high glucose Dulbecco's modified Eagle's media (1:1) (Invitrogen) supplemented with 8% fetal bovine serum (Invitrogen), 2 mM glutamine, and penicillin/streptomycin (Invitrogen). MCF-7 cells were plated at a density of 1 X 10^6 cells/ ml and after one day, were pretreated for 24 h with 5 μM of geldanamycin or with DMSO. Cells were then treated with TNFα for the indicated

periods of time, harvested, and lysed as described previously[8]. A portion of the pelleted cells was boiled with SDS-loading buffer and used for immunoblotting of the phospho-c Jun level.

Human fetal kidney 293 cells were maintained in Ham's F-12/low glucose Dulbecco's modified Eagle's media (1:1) (Invitrogen) supplemented with 8% fetal bovine serum (Invitrogen), 2 mM glutamine, and penicillin/streptomycin (Invitrogen). HEK 293 cells were transfected using the calcium phosphate method as previously described [7]. Cells were harvested 16 h after transfection, and lysed as described previously[8].

3.3. GST Pulldown Assays

Mammalian constructs (pGST or pGST-*cdc37*) encoding GST or GST-Cdc37 respectively were cotransfected with expression vectors encoding MLK3 truncation variants in HEK 293 cells. Cells were lysed 16 h after transfection and the clarified lysate (400 μl) was incubated with 20 μl of glutathione-Sepharose 4B resin (Amersham Phamacia Biotech) for 90 min at 4 °C. The pelleted resin was washed twice with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), and bound proteins were resolved by SDS-PAGE.

3.4. Immunoprecipitations

Antibodies against the proteins of interest were prebound to protein A-agarose beads for 30 min at room temperature: MLK3 antibody (0.25 μg/μl slurry), GST antibody (0.25 μg/μl slurry). Clarified lysates (400 μl- 600 μl) were incubated with 20 μl of antibody-bound Protein A-agarose for 90 min at 4 °C. For detection of interactions between endogenous proteins, 2 ml of clarified lysates from MCF-7 cells were incubated

with 50 μl of antibody-bound Protein A-agarose. Immunoprecipitates were washed with HNTG buffer. Immunoprecipitates used for kinase assays and endogenous associations were washed twice with lysis buffer, and twice with kinase assay buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4).

3.5. *In vitro* Kinase Assays

Immunoprecipitated MLK3 was incubated in 20 μl of kinase assay buffer containing 10 μM ATP and 5 μCi (γ-^{32}P)-ATP (3000 Ci/mmol) (NEN Life Science Products), 6 μg of recombinant GST-MKK7 K165A for 30 min at room temperature. Following the kinase assay, proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane, and the incorporation of radioactivity into the GST-MKK7 was determined by PhosphorImaging (Molecular Dynamics). The membrane was Western blotted with MLK3 antibody and GST antibody sequentially to detect the MLK3 and GST-MKK7 level.

3.6. SDS-PAGE and Western Blot Analysis

Lysates and GST-pulldowns of proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted using anti-MLK3 rabbit polyclonal antibody (4μg/ml) [7], anti-actin mouse monoclonal antibody (Sigma), HA mouse monoclonal antibody (5 μg/ml)(BAbCO), phospho-c-Jun (KM-1) mouse monoclonal antibody (0.2 μg/ml), MKK4 rabbit polyclonal antibody (0.2 μg/ml), MKK7 goat polyclonal antibody (0.4 μg/ml), JNK rabbit polyclonal antibody (0.2 μg/ml), Hsp90 rabbit polyclonal antibody (0.4 μg/ml), Cdc37 mouse monoclonal antibody (0.8 μg/ml) GST mouse monoclonal antibody (0.2 μg/ml) (all of the above antibodies were purchased

from Santa Cruz, unless indicated), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-rad). Western blots were developed by chemiluminescence.

4. Results

4.1. Demonstration of the interaction between endogenous Hsp90/Cdc37 and Flag-MLK3.

In Chapter IV of this thesis, affinity purification of Flag-MLK3 complexes from MCF-7 cells coupled with mass spectrometry identified Hsp90/Cdc37 as potential MLK3 binding partners. To verify the interactions between Hsp90/Cdc37 and MLK3, Flag-MLK3 complexes were isolated from MCF-7 cells inducibly expressing Flag-MLK3 and subjected to Western blot analysis using antibodies directly against Hsp90 and Cdc37. As shown in Fig. 1, Hsp90 and Cdc37 were coimmunoprecipitated when MLK3 expression was induced. Hsp90 and Cdc37 could not be detected in the un-induced control, confirming that the interaction is specific for Flag-MLK3.

4.2. Endogenous MLK3 is associated with Hsp90/Cdc37.

To exclude the possibility that the association of MLK3 with Hsp90/Cdc37 is due to an artifact of Flag-MLK3 overexpression in these cells, endogenous MLK3 was immunoprecipitated from MCF-7 cell lysates. As shown in Fig. 2, endogenous Hsp90/Cdc37 coimmunoprecipitated with endogenous MLK3 as judged by immunoblotting. We also observed that only a very small proportion of the total cellular pool of Hsp90/Cdc37 is associated with MLK3 (data not shown). This observation is not surprising as the Hsp90 and Cdc37 are relatively abundant proteins that associate with many other cellular protein kinases.

4.3. Hsp90/Cdc37 associates with the kinase domain of MLK3.

Previous studies have revealed that Hsp90/Cdc37 binds to client protein kinases IκB kinase (IKK)[16] and Raf [17] through their catalytic domains. To map the region in MLK3 that interacts with Hsp90/Cdc37, two NH_2-terminal HA-tagged MLK3 truncation variants were constructed. MLK3 1-399 contains the glycine rich region, SH3 domain and the kinase domain, whereas MLK3 400-847 includes the zipper region, CRIB motif and the proline rich carboxyl terminus. These two variants, together with a previously constructed variant MLK3 115-399 (which contains the kinase domain only) were used to map the region of MLK3 that interacts with Hsp90/Cdc37. MLK3 variants were either expressed together with GST-tagged Cdc37 (GST-Cdc37) or GST, and GST pulldown experiments were performed to pull down GST-Cdc37, endogenous Hsp90 and associated MLK3 variants. All variants expressed at similar levels as shown in Fig. 3B. Data from GST pulldown assays show that, of these variants, MLK3 1-399 and MLK3 115-399 are able to interact with GST-Cdc37 and endogenous Hsp90 (Fig. 3A). As expected, none of the variants associates with GST. Based on these experiments, MLK3 115-399 is sufficient to interact with GST-Cdc37. Therefore, we conclude that MLK3 interacts with Hsp90/Cdc37 through its kinase domain.

4.4. MLK3 is associated with Hsp90/Cdc37 in an MLK3 activity independent manner.

We were interested to test whether the activation state of MLK3 affects its interaction with Hsp90/Cdc37. We selected three forms of MLK3 that reflect different activation states. MLK3 K144A is catalytically inactive. Previous work from our lab has shown that, when wildtype MLK3 is overexpressed alone, it has basal catalytic activity

[18]; when it is coexpressed with activated Cdc42, the activity of MLK3 increases about 4 folds [7]. After transient expression of MLK3 variants with or without activated Cdc42, MLK3 variants were immunoprecipitated and the presence of endogenous Hsp90/Cdc37 was assessed by Western blotting using appropriate antibodies. As shown in Fig. 4, equal amount of Hsp90/Cdc37 immunoprecipitates with the MLK3 variants under these conditions. These data are consistent with the idea that MLK3 activity does not influence its association with Hsp90/Cdc37.

4.5. Hsp90/Cdc37 regulates the protein level of MLK3 in MCF-7 cells.

The ansamycin antibiotics, such as geldanamycin (GA), inhibit Hsp90 chaperone activity by occupying its ATP/ADP pocket [19, 20]. Upon GA treatment, the protein level of several protein kinases, such as Raf [14] and Akt [21], decreases dramatically. However, GA does not affect the protein level of IKK although IKK proteins have been shown to interact with Hsp90/Cdc37 [16]. We tested whether GA alters the expression level of endogenous MLK3. MCF-7 cells were treated with increasing concentration of GA for 24 h, and levels of endogenous MLK3 were assessed by Western blotting. We observed a loss in the cellular levels of MLK3. GA (1 μM) is sufficient to trigger the loss of MLK3 in MCF-7 cells (Fig. 5A). In addition, GA treatment causes a decline in the level of MLK3 in a time dependent manner (Fig. 5B). On average, levels of MLK3 were reduced by more than 80% at 24 h. These results indicate that Hsp90/Cdc37 regulate the protein levels of MLK3 in MCF-7 cells.

4.6. Hsp90/Cdc37 regulate TNFα-induced activation and signaling of MLK3 in MCF-7 cells.

TNFα treatment has been shown to increase MLK3 catalytic activity in Jurkat T cells [6]. We examined whether TNFα increases MLK3 activity in MCF-7 cells. After 30 min treatment with TNFα, endogenous MLK3 was immunoprecipitated and its activity was assessed by an *in vitro* kinase assay using recombinant GST-MKK7 as a substrate. The basal activity of endogenous MLK3 is low. Upon TNFα treatment, the catalytic activity of MLK3 increases about 2.5 fold (Fig. 6A). Consistent with the activation of endogenous MLK3, JNK activity in cells, as measured by the phosphorylation of transcription factor c-Jun in the cell lysates, was greatly enhanced with parallel kinetics (Fig. 6C). This suggests that TNFα-induced JNK activation is through the activation of MLK3. We then examined the effect of GA on TNFα signaling to MLK3 and JNK. MCF-7 cells were pretreated with or without GA for 24 h, and cells were treated with TNFα for 30 min. As shown in Fig. 6, GA pretreatment completely abolished the TNFα induced activation of MLK3 although there is about 20% of residual MLK3 protein in the cellular lysates. In addition, pretreatment with GA not only lowered the basal level of c-Jun phosphorylation in MCF-7 cells, but also abolished the TNFα-induced JNK activation. Since the TNFα induced JNK activation requires the three modules of the MAPK cascades, we also examined the protein level of MKK4, MKK7, and JNK upon GA treatment. The levels of MKK4, MKK7 and JNK were not affected by GA. Taken together, these results support the idea that Hsp90/Cdc37 regulate TNFα induced JNK activation through controlling the protein level of MAPKKKs, including MLK3.

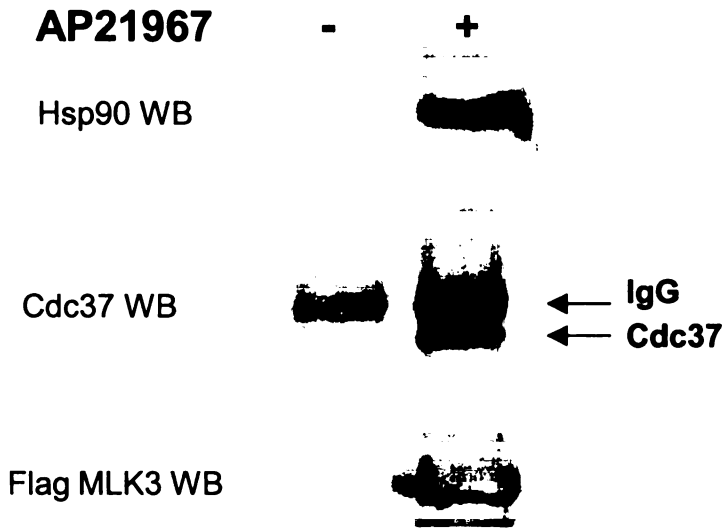


Fig.1. **Association of Flag-MLK3 with Hsp90/Cdc37**. Affinity purified Flag-MLK3 complexes from MCF7 cells inducibly expressing Flag-MLK3 were separated by SDS-PAGE. The presence of endogenous Hsp90, Cdc37 and Flag-MLK3 was assessed by immunoblotting with Hsp90, Cdc37, and Flag antibody respectively. In addition to Cdc37, the presence of the anti-Flag M2 antibody (IgG) heavy chain is indicated by *arrow* in the *middle panel.*

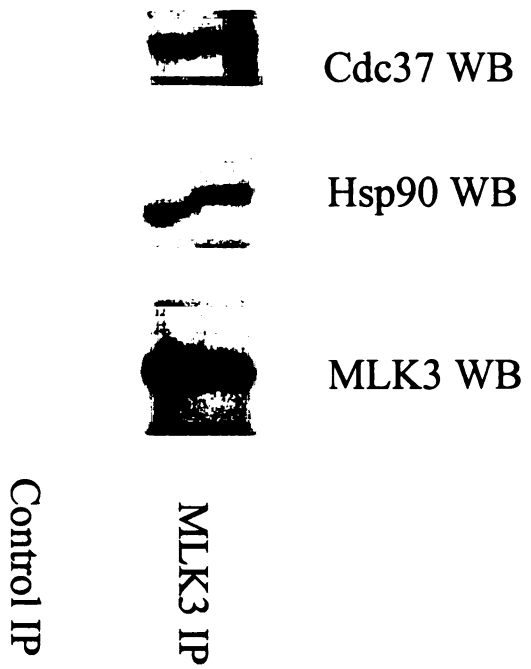


Fig. 2. **Endogenous MLK3 is associated with Hsp90 and Cdc37**. MLK3 was immunoprecipitated (IP) from MCF-7 cell lysates using an MLK3 antibody prebound to protein A agarose beads. A control immunoprecipitation was performed in parallel using a GST antibody. The presence of Cdc37, Hsp90, and MLK3 was assessed by Western blotting using the indicated antibodies.

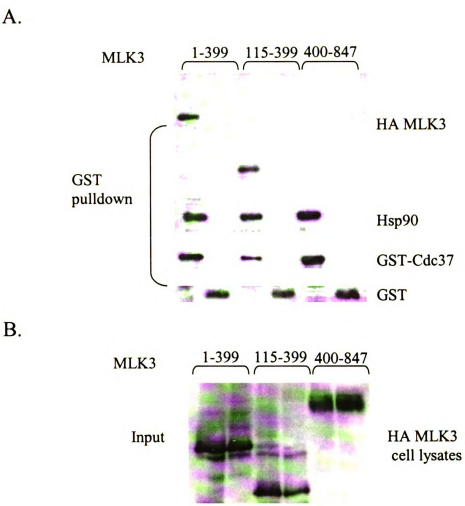


Fig. 3. **Hsp90 and Cdc37 associate with the catalytic domain of MLK3**. HEK 293 cells were transfected with expression vectors containing the cDNA for MLK3 truncation variants and GST-Cdc37 (or GST). The *numbers* in the figure represent amino acid numbers in MLK3. Cellular lysates expressing the indicated MLK3 truncation variants and GST-Cdc37 (or GST) were incubated with glutathione-Sepharose 4B resin. *A, top two panels,* the presence or absence of bound MLK3 variants and Hsp90 was assessed by Western blotting with the HA antibody and Hsp90 antibody respectively. *Bottom two panels,* equal binding of GST-Cdc37 or GST on the glutathione-Sepharose 4B resin was confirmed by Western blotting with GST antibody. *B,* the expression of MLK3 variants in cellular lysates probed with the HA antibody. The data shown are representative of three independent experiments.

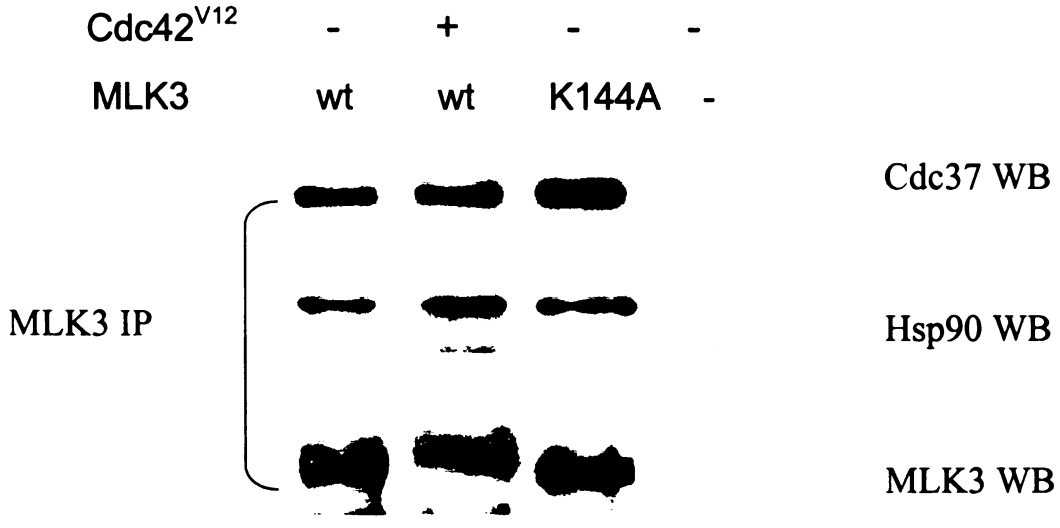
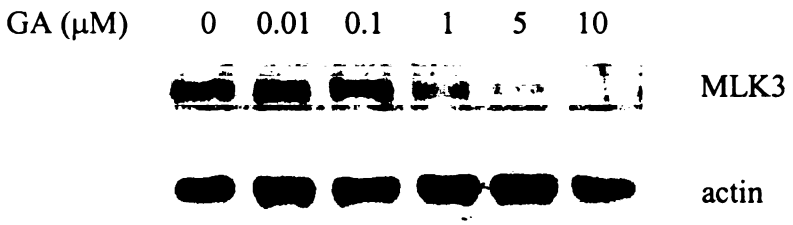


Fig. 4. **Association of MLK3 variants with Hsp90/Cdc37**. HEK 293 cells were transfected with expression vectors containing the cDNA for MLK3 wildtype (wt), K144A in the presence or absence of $Cdc42^{V12}$. MLK3 was immunoprecipitated from cellular lysates using MLK3 antibody preincubated with protein A agarose beads. The presence or absence of Cdc37, Hsp90 and MLK3 was assessed by Western blotting using the indicated antibodies.

A.



B.

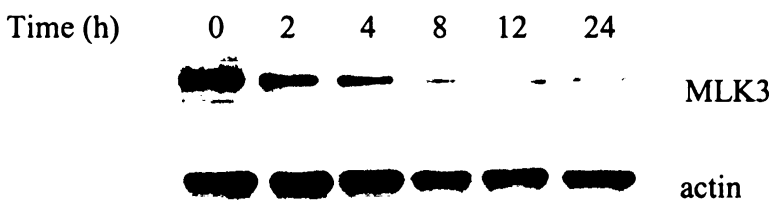
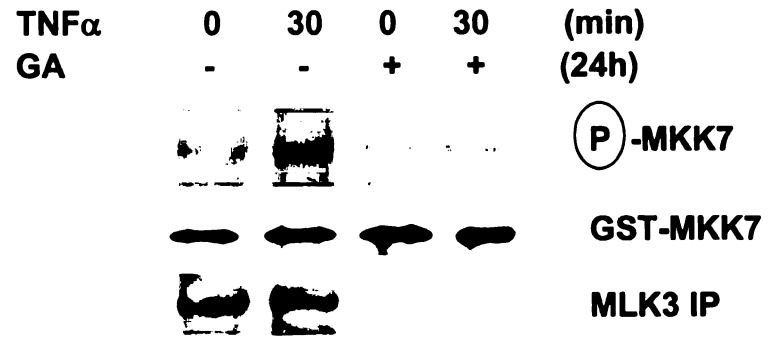
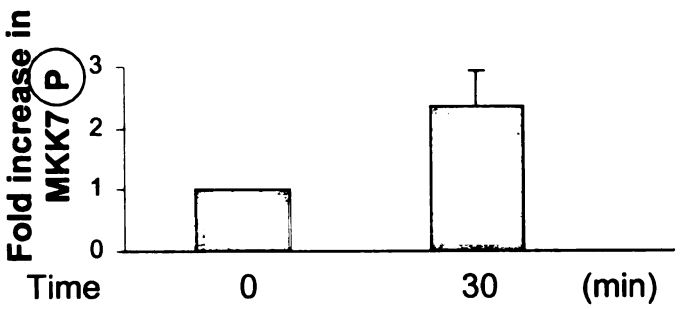


Fig. 5. **Geldanamycin (GA) decreases the endogenous level of MLK3**. MCF-7 cells were treated with various concentrations of GA for 24 h *(A)* or with 5 μM of GA for the indicated times (*B*). The endogenous level of MLK3 was assessed by Western blotting of cellular lysates using the MLK3 antibody. Western blotting for endogenous actin was used to confirm that equal cell equivalents were loaded.

A


TNFα 0 30 0 30 (min)
GA - - + + (24h)
P-MKK7
GST-MKK7
MLK3 IP

B


Fold increase in P-MKK7
3
2
1
0
Time
0
30
(min)

C

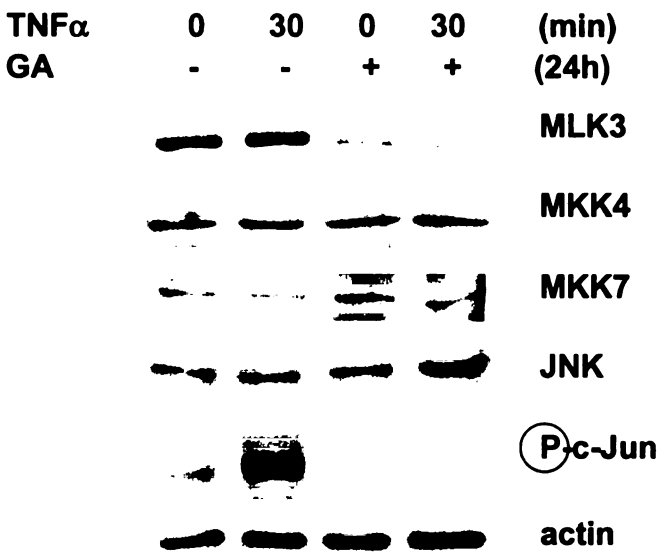


Fig. 6. **GA abolishes TNFα-mediated activation of MLK3 and JNK.** MCF-7 cells were pretreated with GA or the vehicle DMSO for 24 h, then treated with TNFα for the indicated time, and lysed. *A*, *in vitro* kinase assay of MLK3 using GST-MKK7 K165A as a substrate. MLK3 was isolated from cellular lysates by immunoprecipitation using an MLK3 antibody, and kinase activity was assessed *in vitro* using recombinant GST-MKK7 K165A as a substrate. *Top panel* shows a phosphorimage with bands corresponding to MKK7 phosphorylation. A Western blot of the immunoprecipitated MLK3 is shown in the *bottom panel*. The equal loading of GST-MKK7 substrate in the kinase assay was confirmed by immunoblotting using an antibody against GST as shown in the *middle panel*. *B*, the mean $\pm$ S.E. for fold increase in GST-MKK7 K165A phosphorylation from three independent experiments is shown. *C*, equal amounts of protein from cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies.

5. Discussion

Using affinity purification coupled with mass spectrometry, we detected MLK3 in association with Hsp90/Cdc37 and as well other proteins as described in Chapter IV. Herein, we confirm that endogenous MLK3 is associated with Hsp90/Cdc37 in MCF-7 cells. Our finding that Hsp90/Cdc37 interact with MLK3 suggests that Hsp90/Cdc37 play an important role for the physiological function of MLK3.

Several protein kinases have been reported to associate with Hsp90 and Cdc37. In the few cases where domain interactions have been mapped, Hsp90/Cdc37 associates with the catalytic domains of its client protein kinases. In accord with these studies, we also find that the catalytic domain of MLK3 is sufficient for association with recombinant Cdc37. In addition to MLK3, other MLKs exist [22]. Given their highly homologous catalytic domains, it seems likely that Hsp90/Cdc37 may associate with and regulate the other MLKs as well. The lack of commercially available antibodies that recognize the other MLKs makes it difficult to test this prediction.

Despite the consistent findings that the catalytic domain of protein kinases mediates binding to Hsp90/Cdc37, whether Hsp90 or Cdc37, or both, directly interact with client protein kinases is less clear. Coomassie stained gels of Flag-MLK3 complexes suggest that Hsp90 and Cdc37 associate with MLK3 at equal molar ratios. However, our experiments were not able to assess whether both Hsp90 and Cdc37 directly associate with MLK3. According to Grammatikakis et al., both Cdc37 and Hsp90 interact directly with recombinant protein kinase Raf-1 *in vitro* and the association between Hsp90 and Raf-1 was enhanced in the presence of Cdc37 [23]. Similar results were obtained from Goeddel's lab when studying Hsp90/Cdc37 association with IKK. Recombinant Cdc37 binds to IKK weakly and Hsp90 itself binds poorly to IKK. However, Cdc37 interacts

more strongly with IKK in the presence of recombinant Hsp90 [16]. It is likely that Hsp9/Cdc37 bind in a cooperative fashion to MLK3, but this issue deserves further study.

Our data suggest that MLK3 associates with Hsp90/Cdc37 in an activity independent manner, as both a kinase defective variant of MLK3, and a Cdc42 activated form of MLK3, associate with Hsp90/Cdc37 equally well. The MAPKKK Raf-1 constitutively associates with Hsp90/Cdc37 as well. The Raf-Hsp90-Cdc37 complex was not only observed in serum deprived cells, but also upon serum or phorbol ester stimulation conditions, whereas the small GTPase Ras recruits the Raf-Hsp90-Cdc37 complex to the plasma membrane [24]. These findings suggest that Hsp90/Cdc37 interaction with Raf is also independent of kinase activity. In contrast, Hsp90/Cdc37 only associates with tyrosine kinase v-Src during its biosynthesis. Once v-Src is inserted into the plasma membrane by virtue of myristoylation, it no longer associated with Hsp90 [11].

An important role of Hsp90 is to stabilize its client proteins and maintain their correctly folded states, thus preventing their degradation through the proteasome. The stability of many, but not all, client protein kinases, such as Src and Raf-1, are regulated by Hsp90. Treatment of cells with geldanamycin, a specific inhibitor of Hsp90, decreases the protein level of MLK3. In the previous chapter, we have shown that MLK3 is present at high levels in MCF-7 cells. Interestingly, many cancer cell lines, including MCF-7, have high levels of Hsp90 [9, 25]. Conceivably, it is these high levels of Hsp90 that are responsible for the high levels of MLK3 in breast cancer cells. Disruption of the function of Hsp90 does not affect the level of many protein kinases, such as MKK4, MKK7 and JNK studied here. It is likely that Hsp90 does not associate with these kinases. However,

IKK has been detected to form a complex with Hsp90/Cdc37, yet geldanamycin does not affect the protein level of IKK [16].

The extracellular stimuli that activate MLK3 have not been well defined. Recently, it has been reported that TNFα activates endogenous MLK3, leading to JNK activation in Jurkat T cells. In the study presented here, we have shown that TNFα activates endogenous MLK3 in MCF-7 cells. In addition, the phosphorylation of c-Jun is greatly enhanced in response to TNFα with similar kinetics. Pretreatment of the MCF-7 cells with GA abolishes TNFα-induced MLK3 activation, in parallel with the loss of MLK3 in cells. This data indicates that geldanamycin inhibits MLK3 activity primarily by decreasing MLK3 stability. In addition to MLK3, another MAPKKK, apoptosis signal-regulating kinase 1 (ASK1), has been shown to mediate TNFα induced JNK activation [26]. Therefore, we could not rule out the possibility that GA may inhibit the protein level of ASK1, and therefore indirectly its activity as well. Our data indicate that Hsp90/Cdc37 regulates TNFα-induced JNK signaling at the MAPKKK level, but not at the MAPKK (MKK4/MKK7) or MAPK (JNK) level. Our findings share some interesting parallels with the Hsp90/Cdc37 regulation of the ERK pathway. Hsp90/Cdc37 controls the stability of Raf-1, but not MEK1, MEK2 or ERK1/2. Destabilization of Raf-1 by geldanamycin abolishes PMA induced ERK activation [14]. Taken together, it is consistent with the idea that Hsp90/Cdc37 regulates MAPK pathways at the MAPKKK level.

Most of the previously identified Hsp90/Cdc37 clients are involved in cell proliferation, cell cycle control and tumorigenesis. Therefore, Hsp90 has been targeted for cancer therapy. The geldanamycin analogue, 17-AAG is currently in phase I clinical

trials for many types of cancer. Although the biological function of MLK3 in MCF-7 cells is not defined so far, MLK3 has been implicated in neuronal apoptosis and activates JNK pathway, which mediates apoptosis in many cell types. Our findings indicate that the Hsp90/Cdc37 not only regulates the protein kinases that promote proliferation and/or tumorigenesis, but it appears that anti-proliferative /proapoptotic protein kinases are also clients for Hsp90/Cdc37. This is supported by the recent report that LKB1, a serine/threonine protein kinase that act as tumor suppressor, associates with Hsp90/Cdc37 [27]. This finding, taken together with the data presented in this chapter, indicate that the use of Hsp90 inhibitor in cancer therapy should be approached with caution.

Footnotes

Acknowledgements:

1. We are grateful to Dr. Nicholas Grammatikakis (Tufts University School of Medicine) for providing the mammalian GST-Cdc37 and GST vectors
2. The bacteria expression vector of GST-MKK7 was kindly provided by Dr. Roger J. Davis at University of Massachusetts Medical School. Geou-yarh (Stancy) Liou engineered the GST-MKK7 K165A mutant and purified the corresponding recombinant protein.

6. References:

1. Rana, A., et al., *The mixed lineage kinase SPRK phosphorylates and activates the stress- activated protein kinase activator, SEK-1.* J Biol Chem, 1996. **271**(32): p. 19025-8.

2. Merritt, S.E., et al., *The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate.* J Biol Chem, 1999. **274**(15): p. 10195-202.

3. Teramoto, H., et al., *Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c- Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family.* J Biol Chem, 1996. **271**(44): p. 27225-8.

4. Buchsbaum, R.J., B.A. Connolly, and L.A. Feig, *Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade.* Mol Cell Biol, 2002. **22**(12): p. 4073-85.

5. Johnson, G.L. and R. Lapadat, *Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.* Science, 2002. **298**(5600): p. 1911-2.

6. Sathyanarayana, P., et al., *Activation of the Drosophila MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3.* Mol Cell, 2002. **10**(6): p. 1527-33.

7. Bock, B.C., et al., *Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation.* J Biol Chem, 2000. **275**(19): p. 14231-41.

8. Zhang, H. and K.A. Gallo, *Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain.* J Biol Chem, 2001. **276**(49): p. 45598-603.

9. Nollen, E.A. and R.I. Morimoto, *Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins.* J Cell Sci, 2002. **115**(Pt 14): p. 2809-16.

10. Jolly, C. and R.I. Morimoto, *Role of the heat shock response and molecular chaperones in oncogenesis and cell death.* J Natl Cancer Inst, 2000. **92**(19): p. 1564-72.

11. Xu, Y. and S. Lindquist, *Heat-shock protein hsp90 governs the activity of pp60v-src kinase.* Proc Natl Acad Sci U S A, 1993. **90**(15): p. 7074-8.

12. Whitesell, L., et al., *Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for*

stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A, 1994. **91**(18): p. 8324-8.

13. Stancato, L.F., et al., *Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system.* J Biol Chem, 1993. **268**(29): p. 21711-6.

14. Schulte, T.W., et al., *Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association.* J Biol Chem, 1995. **270**(41): p. 24585-8.

15. Dai, K., R. Kobayashi, and D. Beach, *Physical interaction of mammalian CDC37 with CDK4.* J Biol Chem, 1996. **271**(36): p. 22030-4.

16. Chen, G., P. Cao, and D.V. Goeddel, *TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90.* Mol Cell, 2002. **9**(2): p. 401-10.

17. Silverstein, A.M., et al., *p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site.* J Biol Chem, 1998. **273**(32): p. 20090-5.

18. Gallo, K.A., et al., *Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity.* J. Biol. Chem., 1994. **269**(21): p. 15092-100.

19. Stebbins, C.E., et al., *Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent.* Cell, 1997. **89**(2): p. 239-50.

20. Roe, S.M., et al., *Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin.* J Med Chem, 1999. **42**(2): p. 260-6.

21. Basso, A.D., et al., *Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function.* J Biol Chem, 2002. **277**(42): p. 39858-66.

22. Gallo, K.A. and G.L. Johnson, *Mixed-lineage kinase control of JNK and p38 MAPK pathways.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 663-72.

23. Grammatikakis, N., et al., *p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function.* Mol Cell Biol, 1999. **19**(3): p. 1661-72.

24. Wartmann, M. and R.J. Davis, *The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex.* J Biol Chem, 1994. **269**(9): p. 6695-701.

25. Vercoutter-Edouart, A.S., et al., *Proteomic detection of changes in protein synthesis induced by fibroblast growth factor-2 in MCF-7 human breast cancer cells.* Exp Cell Res, 2001. **262**(1): p. 59-68.

26. Ichijo, H., et al., *Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways.* Science, 1997. **275**(5296): p. 90-4.

27. Boudeau, J., et al., *Heat-shock protein 90 and Cdc37 interact with LKB1 and regulate its stability.* Biochem J, 2003. **370**(Pt 3): p. 849-57.

VI. Concluding Remarks

This thesis describes work aimed at the elucidation of the molecular mechanisms that regulate MLK3. In particular, the regulatory role of SH3 domain of MLK3 was examined, and a proteolytic event of MLK3 was characterized. In addition, twenty-three potential binding partners of MLK3 were identified by affinity purification coupled with mass spectrometry.

MLK3 contains an amino terminal SH3 domain and a large proline-rich carboxyl-terminal region. The finding that mutation of the SH3 domain increases MLK3 activity led to the original hypothesis that the SH3 domain of MLK3 might bind to this proline rich region in an autoregulatory fashion. However, unexpectedly, the SH3 domain does not bind to the extreme carboxyl terminal region, but rather binds to a region between the zipper and CRIB motifs that lacks a classical SH3 binding site. Point mutations, either in the SH3 domain or in the newly identified SH3 binding site of MLK3, which disrupt this interaction, result in increased MLK3 activity. There are some important implications regarding this work. This is the first finding of a SH3 domain mediated autoinhibition in a serine/threonine kinase. Second, the proline residue in the SH3 binding site is conserved in other MLK family members including MLK1,2,4 and the *Drosophila* homolog Slipper. Thus this mechanism of SH3 mediated autoinhibition of MLK3 is predicted to apply to the regulation of other MLKs as well. Third, there is no classical proline rich sequence in the binding region. One might imagine that this sequence would provide a suboptimal ligand for MLK3's SH3 domain so this autoinhibition could be released by the presentation of a high affinity ligand in response to an upstream signal.

This discovery also refined our working hypothesis of how small GTPases Cdc42/Rac regulate MLK3 activity. The close proximity of the SH3 binding site and the CRIB motif imply that the Cdc42 mediated activation of MLK3 should disrupt the autoinhibition. Together with the published data, it seems that Cdc42/Rac activation of MLK3 is a multi-step process which includes release of the SH3 mediated autoinhibition, promotion of zipper-mediated oligomerization, induction of hyperphosphorylation on MLK3 and presumably translocation of MLK3 to plasma membrane. The precise mechanism is under investigation by Karen Schatcher in our lab using fluorescence resonance energy transfer (FRET) techniques.

Chapter III described the characterization of a proteolytic event of MLK3. This proteolytic event holds several unique features. First, MLK3 undergoes proteolysis to generate a stable carboxyl terminal fragment (CTF) and the cleavage site has been mapped to Gln 251-Pro 252 within the kinase domain. Thus, this proteolysis would render MLK3 inactive. Second, evidence is presented that suggests the proteolysis occurs shortly after MLK3 is synthesized. Third, MLK3 proteolysis appears to occur by a feedback mechanism that requires the activation of ERK pathway. The CTF seems relatively stable, suggesting that it might have some as yet unclarified biological function. The protease responsible for generation of the CTF of MLK3 has not yet been identified. MG 132 inhibition of the proteolysis has prompted us to consider fishing out the protease by affinity purification. Stancy Liou in our lab is currently working on the project to identify the MLK3 protease. We were unable to observe the generation of the CTF consistently and reliable in endogenous MLK3, which may be due to the low level of endogenous MLK3. It is also possible that we have not been able to find the appropriate

upstream signal to trigger endogenous MLK3 proteolysis. In order to show the physiological significance of the MLK3 proteolysis, it will be important to establish conditions under which the endogenous event occurs.

Using affinity purification coupled with mass spectrometry, more than twenty potential MLK3 interacting proteins were identified. This certainly provides new directions for the study of the regulation as well as the biological functions of MLK3. The identification of clathrin and associated adaptor proteins in MLK3 complexes suggests that MLK3 may be involved in clathrin-mediated endocytosis. This is an area that requires further study. In addition, it seems that the stability and activity of MLK3 may be regulated by multiple molecular chaperones including Hsp70, Hsc 70 and Hsp90/Cdc37. The function of Hsp90/Cdc37 on MLK3 signaling has been examined in detail. It may be necessary to characterize the role of Hsp70 and Hsc70 on MLK3 signaling in the future. Finally, many of MLK3 binding partners have not been reproducibly identified. Verification of these interactions by coimmunoprecipitation and immunoblotting using specific antibodies is required.

In conclusion, the research findings presented in this thesis constitute important contributions to the understanding of the molecular mechanisms regulating MLK3 and of the biological function of MLK3. In addition, this work aids in the overall understanding of the regulation of the protein kinases.