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REGULATION OF MIXED LINEAGE KINASE 3 BY SMALL GTPASE, GUANINE NUCLEOTIDE EXCHANGE FACTOR AND PROTEIN-PROTEIN INTERACTIONS

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Yan Du

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Kathleen A. Gallo
Major Professor's Signature

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REGULATION OF MIXED LINEAGE KINASE 3 BY SMALL GTPASE, GUANINE NUCLEOTIDE EXCHANGE FACTOR AND PROTEIN-PROTEIN INTERACTIONS

By

Yan Du

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ABSTRACT

REGULATION OF MIXED LINEAGE KINASE 3 BY SMALL GTPASE, GUANINE NUCLEOTIDE EXCHANGE FACTOR AND PROTEIN-PROTEIN INTERACTIONS

By

Yan Du

Mixed-lineage kinase 3 (MLK3) is a widely expressed, mammalian serine/threonine protein kinase that functions as a mitogen-activated protein kinase kinase kinase (MAPKKK) and activates multiple MAPK pathways. MLK3 is not only implicated in JNK-mediated neuronal apoptosis in response to trophic factor withdrawal, but also is critical for epidermal growth factor (EGF) induced B-Raf activation and cell proliferation. MLK3 contains multiple protein interaction domains or motifs that may be involved in inter- or intra-molecular interactions, including an N-terminal Src Homology 3 (SH3) domain, a centrally located zipper region, a Cdc42/Rac interactive binding (CRIB) motif and a Pro/Thr/Ser rich COOH-terminus.

The data presented in Chapter 2 demonstrate that Cdc42 is a physiological activator of MLK3. The binding of activated Cdc42 induces the activation loop autophosphorylation of MLK3. Prenylation-competent, activated Cdc42 targets MLK3 to cellular membranes, leading to the full activation of MLK3 and signaling to JNK. These data from our lab and other labs help to establish the current working model for

Cdc42-induced MLK3 activation. Binding of the activated form of Cdc42 is proposed to disrupt the autoinhibitory conformation of MLK3, promoting the homo-dimerization of MLK3 which leads to the trans-autophosphorylation of MLK3 on its activation loop. The membrane targeting of MLK3 induced by Cdc42 further promotes its full activation as well as the downstream signaling to JNK.

Since Rac/Cdc42 promote MLK3 activation, the GEFs specific for these GTPases may regulate MLK3 as well. Vav, a GEF for Rho-GTPases, is demonstrated to increase MLK3 activity as judged by MLK3 autophosphorylation and substrate phosphorylation. Surprisingly, Vav also interacts with MLK3 through at least two distinct binding sites within MLK3, indicating that it regulates MLK3 activity not only through its GEF activity, but also by forming a Vav/GTPase/MLK3 signaling complex. Furthermore, the MLK inhibitor, CEP-11004, dramatically blocks JNK activation induced by ectopically expressed Vav, suggesting that MLK family kinases are involved in Vav-induced JNK activation. However, the specific disruption of MLK3 expression by siRNA does not impact JNK activation induced by ectopic expression of Vav in HeLa cells.

Finally, the Appendix describes the generation of a cell line to inducibly express MLK3 in the human breast cancer cell line, MCF-7. Flag-MLK3 signaling complexes were isolated from this cell line by affinity purification, and were subjected to liquid chromatography/nanoelectrospray tandem mass spectrometry (LC/MS/MS) analysis to identify proteins in Flag-MLK3 signaling complexes.

To my family

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Key to Abbreviations

AGC	containing Protein Kinase A/G/C families
ANT-2	adenine nucleotide translocator-2
AP50	clathrin assembly protein complex 2 medium chain
ASK1	apoptosis signal- regulating kinase 1
BSA	bovine serum albumin;
CAAX	Cys-aliphatic-aliphatic-any amino acid;
CAMK	calcium/calmodulin dependent protein kinases
CDK	cyclin dependent protein kinases
CK1	casein kinase 1
CMGC	containing CDK, MAPK, GSK3, CLK families
CRIB	Cdc42/Rac interactive binding motif
DLK	dual leucine zipper protein kinase
DMEM	Dulbecco's Modified Eagle's Medium
ERK	extracellular signal-regulated kinase
GluR6	Kainate receptor glutamate receptor
GST	glutathione S-transferase
HA	hemagglutinin
HEK	human embryonic kidney;
HPK1	hematopoietic progenitor protein kinase-1
Hsc70	heat shock cognate 71 kD protein
HSP	heat shock protein
IB	islet brain
Ig	immunoglobulin

JIP	JNK interacting protein
JNK	c-jun N-terminal kinase
LC	liquid chromatography
LZK	leucine zipper-bearing kinase
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
MS	mass spectrometry
NGF	nerve growth factor
NIMA	never in mitosis A
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated protein kinase
PBD	p21-binding domain;
PBS	phosphate-buffered saline;
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
RGC	receptor guanylate cyclase
RNAi	RNA interference

SAM	sterile alpha motif
SAPK	stress-activated protein kinases
SDS-PAGE	SDS-polyacrylamide gel electrophoresis;
SH2	src-homology 2
SH3	src-homology 3
siRNA	small interfering RNA;
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
TGF	transforming growth factor
TK	tyrosine kinases
TKL	tyrosine kinase-like
TNF	tumor necrosis factor
ZAK	leucine-zipper and sterile-alpha motif kinase

Chapter 1. Literature review

1. Protein kinases

Protein phosphorylation is an essential mechanism of cellular signal transduction and protein function regulation. Protein phosphorylation is catalyzed by protein kinases, a family of evolutionarily conserved enzymes that transfer the γ-phosphate of ATP to certain Ser, Thr or Tyr residues of their protein substrates. In physiological settings, protein phosphorylation is reversed through the action of protein phosphatases.

Protein kinases make up one of the largest protein superfamilies in eukaryotes. The human kinome consists of 518 protein kinase genes, which represent about 1.7% of all human genes[1]. Protein kinases control a variety of physiological processes, such as metabolism, cell cycle regulation, proliferation, differentiation, gene transcription, cell morphology and motility, and apoptosis.

Based on substrate specificity, mammalian protein kinases are mainly divided into three major groups: the tyrosine kinases, which phosphorylate only Tyr residues within their protein substrates; the serine/threonine protein kinases, which phosphorylate Ser or Thr residues; and the dual specificity kinases, which are capable of phosphorylating Tyr, as well as Ser and Thr, residues [2, 3].

The catalytic domains of eukaryotic protein kinases are highly conserved both in their primary amino acid sequence and in the tertiary structure. They consist of approximately 250-300 amino acids, which fold into a two-lobed structure. The N-terminal lobe is smaller and is formed by several antiparallel beta sheets and one

conserved helix. In the catalyzed reaction, the binding of Mg^{2+} and ATP occurs in the N-terminal lobe. The C-terminal lobe structure, which is mainly composed of alpha helices, primarily functions in protein substrate recognition and binding and also initiates the phosphate transfer from ATP to the substrate [4, 5]. The sequences involved in active site stability and ATP binding are usually highly conserved among protein kinases, whereas the substrate recognition and binding residues diverge, suggesting that protein kinases share a similar catalytic mechanism while having specific substrate specificity [6].

Within the catalytic domains of each protein kinase resides the so-called activation loop, which is located between two highly conserved sequence motifs Asp-Phe-Gly and Ala-Pro-Glu [6]. The phosphorylation of certain sites within this activation loop often alters its conformation rendering the kinase catalytically active [7-10]. The regulatory mechanism by which protein kinases are activated through activation-loop phosphorylation is still not completely understood [10-12]. By comparing the structures of the active state to the inactive state of protein kinases, several models have been proposed including formation of a substrate binding site [12-14], regulation of the phosphoryl transfer step [11] and control of the active conformation [10].

Based on sequence homology of the catalytic domains, aided by sequence similarity and domain arrangement of the full proteins, and known biological functions, mammalian protein kinases are classified into nine groups: AGC (containing PKA, PKG, PKC families), CAMK (calcium/calmodulin dependent protein kinases), CK1 (casein kinase 1), CMGC (containing CDK, MAPK, GSK3, CLK families), STE (homologs of yeast Sterile 7, 11, 20 kinases), TK (tyrosine kinases), TKL (tyrosine kinase-like), RGC (receptor guanylate cyclase), and atypical kinases [15, 16].

2. The mitogen-activated protein kinase pathways

The mitogen-activated protein kinase (MAPK) pathways play important roles in signal transduction networks in eukaryotic cells. MAPK pathways are involved in transducing a large variety of external signals into cells leading to corresponding cellular responses, such as gene expression, metabolism, cell division, cell morphology, inflammation, cell proliferation, differentiation, and apoptosis. MAPK substrates include transcription factors, other kinases, enzymes, and cytoskeletal proteins. MAPK pathways are organized in three-kinase modules consisting of a terminal MAP kinase (MAPK), which is activated through the phosphorylation of a specific Thr and a Tyr residue within the activation loop of the kinase by a MAPK activator, the dual specific MAPK kinase (MKK). In a similar fashion, a MKK kinase (MAPKKK) phosphorylates and activates the MKK (Fig. 1). These three-tiered cascade modules are conserved from yeast to humans [17-19]. In some cases, a MAPKKK kinase (MKKKK) exists and activates the MAPKKK. Upon extracellular stimulation, the MAPKKKK or MAPKKK can often be recruited to the plasma membrane. Frequently, this is accomplished through the association with a membrane anchored small GTPase.

MAPKs are activated through the dual phosphorylation of conserved Thr and Tyr residues within the activation loop. Once MAPKs are activated, they can either phosphorylate substrates within the cytosol or translocate into the nucleus to phosphorylate nuclear substrates including transcription factors, ultimately leading to appropriate cellular responses. MAPKs are proline-directed kinases whose consensus sequence for substrate phosphorylation is PXT/SP motif, although the first Pro residue is not strictly required [20].

MKKs (MAPKKs) have very high specificity towards their substrate MAPKs. In contrast, there are many different mammalian MAPKKKs, including Raf isoforms, MEKK1-MEKK4, and mixed lineage kinases (MLK1–MLK4, DLK, LZK and ZAK). The MAPKKKs are usually large proteins with many domains or motifs important for inter- or intra-molecular protein interactions that regulate the activity and signaling of the kinase.

In mammals, there are five MAPK pathways: the extracellular signal-regulated kinases, ERK1/2 (also known as MAPKs), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), the p38 kinases (p38α, p38β, p38γ, and p38δ), ERK3/ERK4, and ERK5 [18, 19, 21, 22]. The first three have been well studied.

2.1. The ERK pathway

The Raf/MEK/ERK signaling pathway was the first MAP kinase cascade to be characterized. It is activated in response to mitogenic stimuli such as growth factors and cytokines, and is involved in regulation of many fundamental cellular processes, such as proliferation, differentiation and cell cycle progression. Depending on the strength and duration of the signal, activation of the ERK1/ERK2 can lead to either proliferation or differentiation[23]. Targeted disruption of the *ERK1* gene results in viable mice with modest defects in T-cell development, indicating ERK1 is important for T cell responses [24].

ERK1/2 have a large variety of substrates that include the transcription factors such as Elk and NF-κB, kinases such as Rsk, and other structural or signaling proteins such as cell survival regulator, Bcl-2, and the cytoskeletal scaffold, paxillin.

The activation of ERK is directly mediated by two MKKs, mitogen-activated protein/ERK kinase 1/2 (MEK1 and MEK2). Activated MEK1/2 activates ERK1/2 through the phosphorylation of Thr and Tyr within the activation loop of ERK1/2 [25].

The upstream activation of the ERK pathway at the MAPKKK level and above is much more complicated. For instance, growth factors or cytokines bind to cell surface receptor tyrosine kinases (RTKs), G-protein-coupled receptors or cytokine receptors [26]. This leads to activation of Ras. Activated Ras then recruits Raf isoforms to the plasma membrane and induces their activation [27], followed by phosphorylation and activation of MEK1/MEK2. MAPKKKs that activate the ERK pathway include Raf-1 [28], B-Raf [29] and A-Raf [30]. Additionally, the MAPKKKs, MLK3, MEKK1 [31], MEKK2, MEKK3 [32], Tpl2 [33] have been reported to activate ERK 1/2. Interestingly, it has been reported recently that gene silencing of mixed-lineage kinase 3 (MLK3) blocks EGF-induced B-Raf activation and cell proliferation[34, 35]. It has been proposed that MLK3 is required for maintenance of a MLK3/B-Raf/Raf-1 complex[36].

Ras is mutated to an oncogenic form in about 15% of human cancers and mutations in the *B-raf* gene have been found in more than 60% of malignant melanomas [37]. In addition, activated MEK1/2 transforms fibroblasts and in xenograft studies, MEK-transformed fibroblasts produce tumors in nude mice [38]. Furthermore, activation of ERK2 is sufficient to transform mammalian cells [39]. All of these indicate the critical relationship between ERK pathway and cancer.

2.2. The JNK pathway

The JNK family of MAPKs regulates responses to various stresses, and plays crucial roles in inflammation, proliferation, development, apoptosis and metabolism. JNKs are activated by cytokines and environmental stresses, such as heat shock, ultraviolet (UV), radiation, osmotic stress, as well as by growth factors[40, 41].

JNK isoforms are encoded by three genes (*Jnk1*, *Jnk2*, and *Jnk3*). Ten JNK isoforms are derived from alternative splicing of the three genes. JNK1 and JNK2 are ubiquitously expressed in many tissues, while JNK3 is specifically expressed in the brain [42]. Mice with targeted disruption of only one of the three JNK genes are viable, suggesting that the different JNK isoforms may have overlapping functions, but they do show defects in apoptosis and immune responses regardless of which is knocked out [21, 41]. $Jnk1^{-/-}$ and $Jnk2^{-/-}$ double knockout mice die in early embryogenesis with an open neural tube, which is associated with decreased apoptosis in the hindbrain, and increased apoptosis in the forebrain [43]. However, $Jnk1^{-/-}$ $Jnk2^{-/-}$ fibroblasts were isolated from these mice, suggesting that JNK is not required for inherent cell viability. The $Jnk1^{-/-}$ $Jnk2^{-/-}$ fibroblasts display defects in AP-1 transcriptional activation, proliferation, and fail to undergo apoptosis in response to certain stresses such as UV light [44]. The *Jnk3* -/- mice show reduced seizure activity and decreased susceptibility to hippocampal neuron apoptosis in response to excitotoxicity [45].

Nuclear substrates of JNK include c-Jun, JunD, Elk1 and ATF2. Activated JNK phosphorylates c-Jun on two sites (Ser63 and Ser73). JNK also phosphorylates anti-apoptotic members of the Bcl family, including Bcl-2, Bcl-XL and Bim.

Two direct activators of JNK have been identified: MKK4 (also call SEK1) and MKK7 [46, 47]. There is some evidence that MKK4 and MKK7 act synergistically to phosphorylate and activate the JNKs. MKK4 preferentially phosphorylates Tyr185 within the JNK1 activation loop, whereas MKK7 preferentially phosphorylates Thr183 [47]. Because dual phosphorylation of JNK on the Tyr and Thr is required for its full activation, both MKK4 and MKK7 are required to achieve the full activation of JNK. Indeed, single knockout of either *Mkk4* or *Mkk7* results in partial defects in stress-stimulated JNK activation, whereas disruption of both genes eliminates JNK activation [48]. In addition, it has been suggested that these two kinases mediate JNK activation in response to different stimuli; MKK7 is activated primarily by cytokines (such as tumor necrosis factor, TNF, and interleukin-1, IL-1), whereas MKK4 is primarily activated by environmental stresses [21]. When one of these two kinases is preferentially activated by certain stimuli, the basal activity of the other kinase seems to be required for full activation of JNK. For example, targeted disruption studies demonstrate that MKK7 is essential for TNF-stimulated JNK activation whereas MKK4 knockout only results in a partial decrease in JNK activation[48], indicating that TNF-induced JNK activation is through MKK7, but the basal activity of MKK4 is required for full activation of JNK in response to TNF.

The regulation of the JNK pathway at the MAPKKK level is much more complex due to the existence of at least 13 MAPKKKs, including MEKK1-MEKK4 [49], apoptosis signal- regulating kinase 1 (ASK1) [50], transforming growth factor (TGF) β activated protein kinase 1 (TAK1) [51], and the MLKs [52]).

2.3. The p38 pathway

The p38 MAPKs are activated by numerous physical and chemical stresses, including the cytokines interleukin-1 and tumor necrosis factor, hormones, UV irradiation, ischemia, osmotic shock and heat shock [53]. The p38 MAPK pathway appears to play an important role in apoptosis, cytokine production, transcriptional regulation, and cytoskeletal reorganization. The availability of specific p38 inhibitor, SB 203580, helps to clarify the role that p38 MAPK plays in these processes. Inhibition of p38 by SB 203580, blocks interleukin-2 and -7 (IL-2 and IL-7) induced T cell proliferation.

Four p38 isoforms exist: p38α, p38β, p38γ, and p38δ. Most studies have been performed on p38α. Disruption of the p38α gene in mice causes embryonic lethality between embryonic days 11.5 and 12.5. Those mice which develop past this stage have normal morphology but are anemic due to failure in erythropoiesis [54, 55]. These findings suggest that different p38 isoforms have nonredundant biological functions.

There are only two MAPKKs, MKK3 and MKK6, that can activate p38 MAPKs. In contrast, numerous MAPKKKs have been demonstrated to participate in p38 pathway regulation, including TAK1 [56], ASK1 [50], MEKK 4, MLK2, MLK3, and DLK [52].

3. The mixed lineage kinase family

Mixed lineage kinases (MLKs) are a family of widely expressed, serine/threonine protein kinases that act as MAPKKKs. The name "Mixed Lineage Kinase" derives from the sequence similarity of its catalytic domain to both tyrosine and serine/threonine

protein kinases [57]. Experimentally, however, only serine/threonine kinase activity has been demonstrated [58]. MLKs have mainly been implicated in the activation of JNK pathway by phosphorylation and activation of the dual specific kinases MKK4/7 [41, 59-61], and of the p38 pathway through the phosphorylation and activation of MKK3/6[62, 63]. Interestingly, in addition to its function as a MAPKKK in the activation of JNK and p38 pathways, a recent report suggests that MLK3 is involved in mitogen-stimulated B-Raf activation and cell proliferation in cancer cell lines, by forming and maintaining MLK3/B-Raf/Raf-1 complex. In mammalian cells, MLKs have been implicated in regulating many physiological processes, including apoptosis, proliferation and differentiation. Studies using small molecule MLK inhibitors implicated MLKs in neuronal apoptosis. The MLKs are therefore potential therapeutic targets for many neurodegenerative diseases such as Parkinson's disease [52].

3.1. The Members of Mixed lineage kinase family.

According to their domain organization and sequence similarity within the catalytic domains, MLKs can be clustered into three subfamilies: the MLKs; the dual leucine zipper bearing kinases (DLKs); and zipper sterile-α-motif kinase (ZAK) (Fig.2).

The MLK subfamily consists of MLK1, MLK2, MLK3, and MLK4. In addition to the catalytic domain, MLKs contain several conserved domains that are important for inter- or intra- molecular interactions, including an amino terminal Src homology 3 (SH3) domain, a centrally located leucine zipper region and a Cdc42/Rac-interactive binding (CRIB) motif. MLK1-4 share more than 75% sequence identity within their catalytic domains. However, the COOH-terminal regions of MLKs, which are rich in proline,

serine and threonine, are highly divergent, indicating that this region might be involved in regulating isoform specific functions of MLK. MLK1 mRNA and/or protein expression have been detected in epithelial tumor cell lines of colonic, breast and esophageal origin [57], and in an immature β-cell line (RIN-5AH), but not in the more mature RIN-A12 cells, suggesting that MLK1 may be involved in the differentiation of β-cells [64]. MLK2 mRNA is present in brain, skeletal muscle and testis [65, 66], whereas MLK3 transcripts are detected in wide variety of adult and fetal human tissues [58].

The DLK subfamily contains DLK (also called zipper protein kinase (ZPK) [67]) and leucine-zipper kinase (LZK). DLKs lack an SH3 domain and CRIB motif. Follow the catalytic domain of the DLKs are two consecutive leucine-zipper motifs separated by a 31 amino acid spacer. Like MLK1-4, the COOH-terminal regions of DLK and LZK are proline rich, but lack significant sequence similarity. DLK mRNA is present at high levels in the brain of adult mice and also in the epithelial layers of various organ systems, including stomach, small intestine, liver, pancreas, and the seminiferous tubules of mature testes [67, 68]. Based on northern blot analysis, LZK is widely expressed, with the highest expression level in the pancreas [69].

The third subfamily, ZAK, contains a leucine zipper and a sterile-α motif (SAM) [70-72]. There are two alternatively spliced isoforms of ZAK (ZAKα and β) [71, 72].

MLKs are absent from yeast, but MLK homologues are found in *Drosophila* and *C. elegans* [52]. The *Drosophila* MLK Slipper (Slpr) contains a similar domain arrangement as MLK1-4 including a SH3 domain, a kinase domain, a leucine zipper and a CRIB motif, and shows significant sequence similarity. *Slipper* is required for

basket/JNK activation during dorsal closure. *C. elegans* has DLK and ZAK homologues [52].

3.2. Pathways regulated by MLKs.

3.2.1. The JNK pathway.

When ectopically expressed in mammalian cell lines, MLK2 [61], MLK3 [59], DLK [73], LZK [69], and ZAK [70, 71] have all been shown to activate JNK. The data presented in Chapter 2 demonstrates that knockdown of the MLK3 expression by siRNA blocks Cdc42-induced JNK activation, suggesting that Cdc42 regulates endogenous JNK through endogenous MLK3. A number of *in vitro* experiments have been performed to assess the ability of different MLKs to phosphorylate and activate MKK4/7. MLK2 and MLK3 phosphorylate both MKK4 and MKK7[59, 61]. In contrast, DLK phosphorylates and activates MKK7, but not MKK4[73], suggesting that DLK regulates JNK activation solely through MKK7. Co-expression of dominant-negative MKK7 but not MKK4 dramatically blocks ZAK induced JNK activation, indicating that ZAK activates JNK pathway primarily through MKK7[74].

3.2.2. The p38 pathway.

The importance of MLKs in the activation of p38 pathway is not defined. Upon overexpression, MLK2, MLK3, DLK and ZAKβ have been shown to modestly activate the p38 pathway[59, 72, 75, 76]. Unpublished data from our lab show that ectopically expressed MLK3 dramatically activates both JNK and p38 in human embryonic kidney (HEK) 293 cells, but induces the activation of JNK, but not p38, in HeLa cells. MLK3's

role in p38 activation might be cell-type dependent. Indeed, JIP2 (JNK Interacting Protein 2), which is specifically expressed in brain, has been shown to associate with MLK3, MKK3 and p38 to facilitate MLK-mediated activation of p38 [77, 78]. It should be note that these data also relied on ectopic expression.

3.2.3. The ERK pathway.

The function of MLKs in ERK activation is controversial. In certain cell lines, overexpression of MLK2 and ZAK, but not DLK or MLK3, has been shown to activate ERK [59, 71, 72, 75, 76]. However, MLK3 has also been shown to contribute to ERK pathway activation as well [79]; and recent reports have demonstrated that gene silencing of MLK3 using siRNA blocks B-Raf-mediated ERK activation and proliferation [34, 35]. Interestingly, the activity of MLK3 is not required for B-Raf activation, but rather instead of functioning as a MAPKKK, MLK3 is involved in mitogen stimulated B-Raf activation and cell proliferation, by forming and maintaining a MLK3/B-Raf/Raf-1 complex [36].

3.3 The regulation of MLKs

3.3.1. Regulation of MLKs by extracellular signals

The extracellular stimuli that activate endogenous MLKs in mammalian cells are not completely known. Tumor necrosis factor α (TNF-α) activates endogenous MLK3 in Jurkat T lymphocytes [80] and in the human breast cancer cell line (MCF-7) [81], as judged by *in vitro* kinase assays using recombinant GST-MKK4/7 as substrates. Consistent with these finding, MLK3 -/- murine fibroblasts display decreased TNF-

induced JNK activation. Ceramide is also reported to increase endogenous MLK3 activity *in vivo* and *in vitro* [80]. Finally, transforming growth factor beta (TGF-β), as well as camptothecin, has shown to induce the phosphorylation of MLK3 in hepatoma cell lines and HeLa cells, respectively [82, 83].

3.3.2. Regulation of MLKs by phosphorylation

The majority of protein kinases are regulated by phosphorylation. Within the catalytic domain of protein kinases resides an activation loop, which often contains highly conserved regulatory phosphorylation sites. Phosphorylation within the activation loop alters its conformation rendering the kinase catalytically active [7-10]. Indeed, site-directed mutagenesis studies suggest that phosphorylation of Thr277 and Ser281 within the activation loop of MLK3 is critical for its activity [84]. Because the residues analogous to Thr277 and Ser281 are conserved in the activation loops of all mammalian MLKs, the other MLKs may be regulated by activation loop phosphorylation as well. A phosphospecific antibody directed against the phosphorylated activation loop-phosphorylation sites of MLK3 (pThr277/pSer281) serves as a valuable tool to assess MLK3 activation status.

In vivo labeling coupled with mass spectrometry has revealed eleven phosphorylation sites within activated MLK3, most of which are clustered at the COOH-terminus [85]. Seven of these eleven identified sites are serines followed immediately by a proline, and thus correspond to the consensus motif for phosphorylation by proline-directed kinases. Indeed, recent data from our lab has demonstrated that activated JNK phosphorylates MLK3 in a positive feedback-loop to regulate MLK3's distribution to

Triton X-100 soluble fractions of cells [86]. JNK can phosphorylate overexpressed MLK2 at its COOH terminal region *in vitro* as well [87]. Finally, MLK3 is reported to be negatively regulated by Akt phosphorylation [88].

3.3.3. Regulation of MLKs by oligomerization

Protein oligomerization is often involved in protein kinase regulation. For instance, receptor tyrosine kinases are activated through growth factor induced dimeriztion and transautophophorylation [89]. All MLKs contain some type of leucine zipper domain, suggesting that leucine zipper-mediated oligomerization might be important in regulating MLKs. No evidence for heterodimerizations of MLKs currently exists [52]. Indeed, formation of disulfide-linked MLK3 dimers requires its leucine zipper region and deletion of the leucine zipper domain abolishes its ability to autophosphorylate and to activate the JNK pathway [90]. Further data suggests that zipper-mediated homo-oligomerization is required for full activity of MLK3, proper dual phosphorylation of the downstream MKK, and subsequent activation of the JNK pathway [91].

The leucine zipper of DLK mediates its homodimerization, which is important for its phosphorylation, activation and stimulation of the JNK pathway [92]. Enforced dimerization of DLK using an engineered dimerization strategy is sufficient to induce DLK autophosphorylation and JNK activation [93]. Consistent with the model that homodimerization of DLK promotes its transphosphorylation and activation, overexpression of the DLK leucine zipper effectively inhibits the dimerization and activation of full-length DLK, presumably by forming inactive leucine zipper-DLK

hetero-complexes [93]. LZK has been reported to form dimers as well, and deletion of a zipper from LZK prevents the induction of JNK activity [94], suggesting its leucine-zipper domain might have the same function as that in other MLKs. In summary, the model, in which leucine zipper-mediated dimerization/oligomerization leads to trans-autophosphorylation and subsequent JNK activation, most likely applies to all MLKs.

3.3.4. Regulation of MLKs by autoinhibition

Most protein kinases, including the MLKs, contain multiple regulatory domains and motifs, which not only are critical in interacting with other signaling proteins, but also may be involved in intramolecular interactions to regulate catalytic activity. For example, PKC (protein kinase C) contains a pseudosubstrate autoinhibitory domain, which binds the active site of PKC kinase domain, therefore blocks substrates access [95, 96]. Another well known example of intramolecular autoinhibition is tyrosine kinases of the Src family. The SH2 domain of Src associates with a COOH-terminal phosphorylated Tyr, and the SH3 domain of Src binds to a sequence located between the SH2 and kinase domains. Src is kept in an autoinhibitory inactive state by these two intramolecular interactions, because these interactions restrict the movement of the kinase domain of Src, thus block the binding of ATP and substrates [97, 98].

MLK3 is autoinhibited through an interaction between its SH3 domain and a short proline-containing sequence located between its leucine zipper and the CRIB motif (Fig. 3) [99] . Mutation of either the conserved Tyr52 in the SH3 domain or the single proline residue that involved in SH3 binding disrupts the autoinhibition leads to increased catalytic activity of MLK3. The sole proline residue in the SH3-binding region of MLK3

is conserved in MLK1, MLK2, MLK4 and the *Drosophila* MLK, *Slpr*, suggesting that SH3-mediated autoinhibition is a common regulatory mechanism among these kinases [52, 99].

3.3.5. Regulation of MLKs by Rho GTPases

Rho family GTPases regulate MAPK pathways. Like Ras, Rho GTPases act as binary switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) conformation [100, 101]. Only in the active GTP-bound state can these GTPases associate with their downstream effector proteins. The Rho family of small GTPases, which includes Rho and Rac isoforms, Cdc42, and TC10 [102], are involved in regulating many eukaryotic processes, such as proliferation, gene transcription, cellular transport, cell motility and cytoskeletal remodeling [103]. Constitutively active forms of Rac and Cdc42 have been shown to activate the JNK and p38 pathways [104].

MLK1-4 contain a so called Cdc42/Rac interactive binding (CRIB) motif. The CRIB motif is composed of 14-16 amino acids, eight of which are consensus residues that mediate the binding of GTP bound-Cdc42/Rac [105]. The CRIB motif of MLK3 contains six of the eight conserved residues, and the activated forms of Cdc42 and Rac have been shown to interact with MLK3 in a CRIB motif-dependent manner to increase MLK3's autophosphorylation and substrate phosphorylation activity [105-107], and to potentiate MLK3-induced activation of JNK [63, 106, 107]. In addition, it has been suggested that activated Cdc42 promotes dimerization of MLK3 [93, 108]. The detailed mechanism through which MLK3 is activated by GTPases will be addressed in Chapter 2.

3.3.6. Regulation of MLKs by scaffold proteins

By assembling signaling complexes, scaffold proteins often facilitate and provide specificity for the signal transduction of MAPK pathways. Scaffolds may not only target the MAPK modules to different subcellular loci, but increasing evidence supports the idea that scaffolds contribute to kinase activation and/or substrate selection. For example: the scaffold protein KSR (Kinase Suppressor of Ras) links the three core kinases of Raf, MEK, and ERK to Ras, thereby facilitating ERK activation [109]. Other scaffold proteins involved in the activation of the ERK pathway include ß-arrestin1/2 and MP-1 [110].

JIPs (JNK Interacting Protein) are a group of scaffold proteins that can interact with components of the JNK and/or p38 signaling pathway to activate these pathways. In mammalian cells, four JIP proteins have been identified: JIP1, JIP2, JIP3, and JIP4 (fig. 4). JIP1 is ubiquitously expressed, while JIP2 is specifically expressed in brain [78]. JIP3 and the newly identified JIP4 are most closely related based on their primary sequence and are structurally distinct from JIP1/2. JIP3 is selectively expressed at high levels in brain and at low levels in heart and testis [111]. In contrast, JIP4 is widely expressed in many tissues including brain, kidney, liver, heart, testis and other tissues[112].

JIP1 has been demonstrated to bind several MLKs, including MLK2, MLK3, DLK and LZK [78, 113]. MKK7, but not MKK4, has been shown to interact with JIP1 [113], indicating that JIP1 may regulate JNK activation specifically through MKK7. Another report suggests that JIP1 regulates DLK activation by preventing its oligomerization, because JIP1 bound DLK is monomeric, unphosphorylated and inactive,

and the recruitment of JNK to JIP1 promotes the dimerization, phosphorylation and activation of JIP-associated DLK [93].

Originally JIP2 was reported to bind JNK1 but its affinity is relatively weak[78]. Subsequently it was shown that JIP2 interacts with p38 and apparently functions as a scaffold protein in MLK-mediated activation of p38 pathway. JIP2 has been shown to associate with MLK3, MKK3 and p38 [77, 78]. JIP2 has also been shown to associate with the upstream activators of the p38 pathway, including Tiam1 and Ras-GRF1, which are Rac-specific guanine nucleotide exchange factors [77]. Expression of JIP2 potentiates Tiam1- or Ras-GRF1- induced p38 activation [77].

JIP3 and JIP4 are structurally distinct from JIP1 and JIP2. Still, both of them contain a JNK binding domain [112]. JIP3 interacts with MLK3, MKK7 and multiple JNK isoforms to facilitate JNK signaling [111]. However, even though JIP4 associates with JNK, it does not bind MKK7 or MLK3 and does not facilitate the activation JNK pathway. In contrast, JIP4 interacts with p38 MAP kinase to potentiate the activation of the p38 pathway [112]. Interestingly, none of the MAPKKs that activate JNK or p38 kinase (MKK4/7 and MKK3/6) has been shown to interact with JIP4. However, MKK3/6 are required for the regulation of p38 by JIP4, because JIP4 fails to activate p38 in Mkk3–/– Mkk6–/– compound mutant fibroblasts [112].

All JIPs interact with kinesin light chain, a component of the microtubule motor protein kinesin-1[78, 112], indicating that JIPs might serve as the loading dock for JNK signaling components moving along microtubules and cargoes for kinesin-mediated

transport [114]. Indeed, genetic studies showed that the *Drosophila* homologue of JIP3, named as Sunday driver (Syd), is important for kinesin-dependent axonal transport [115].

POSH (Plenty of SH3s) is another scaffold protein involved in the regulation of the JNK pathway (fig. 4). Coimmunoprecipitation and pulldown assays demonstrated that POSH interacts directly with activated Rac1, MLK1-3 and DLK, and indirectly with MKK4/7 and JNK, suggesting that POSH may act as a scaffold for Rac-mediated JNK activation. Furthermore, POSH-induced apoptosis in PC-12 cells can be blocked by dominant negative forms of MLKs, MKK4/7 or c-Jun, and by the MLK inhibitor, CEP-1347 [116]. Finally, Akt2 has been reported to associate with POSH and negatively regulate assembly of the POSH/MLK/MKK/JNK signaling complex[117].

3.3.7. Regulation of MLKs by heat shock proteins

Heat shock proteins (HSPs) are ubiquitously expressed highly conserved molecular chaperones that are involved in many cellular functions such as protein folding, assembly, secretion, trafficking and maintaining protein homeostasis. Many heat shock proteins interact with multiple key components of signal transduction pathways [118].

Hsp90 and its kinase-specific co-chaperone $p50^{cdc37}$ have been identified as MLK3 associating proteins by affinity purification coupled with mass spectrometry [81]. Further experiments demonstrate that endogenous MLK3 complexes with Hsp90 and $p50^{cdc37}$ and the Hsps interact through the catalytic domain of MLK3 independent of MLK3 activity. Treatment of MCF-7 cells with the Hsp90 inhibitor, geldanamycin,

dramatically decreases MLK3 protein levels in cell lines, suggesting that MLK3 is stabilized through association with Hsp90.

3.4. The biological functions of MLKs in mammalian cells.

3.4.1. MLKs in neuronal apoptosis.

Nerve growth factor (NGF) withdrawal-induced apoptosis of superior cervical ganglion (SCG) sympathetic neurons requires both the activity of the small GTPase Cdc42 and the activation of JNK pathway [119], indicating that CRIB motif containing MLKs might be involved in NGF deprivation-induced apoptosis. Indeed, ectopic expression of MLK family members (MLK1-3 or DLK) induces apoptosis in neuronal-like PC12 cells and sympathetic neurons. Furthermore, NGF deprivation-induced apoptosis can be blocked by expression of dominant negative forms of MLKs [120, 121] or by the MLK inhibitor, CEP-1347 [116].

Another study demonstrated the post-synaptic density protein (PSD-95) associates with MLK2 and MLK3 in HN33 cells and rat brain preparations. This association, which requires the SH3 domain of PSD-95, recruits MLK2/3 to kainate receptor glutamate receptor 6 (GluR6) complex [122]. Co-expression of catalytically inactive forms of MLK2 or MLK3 significantly blocks JNK activation and neuronal apoptosis mediated by GluR6/PSD-95. Co-expression of a mutant form of PSD-95, in which its SH3 domain has been deleted, also inhibits GluR6-induced JNK activation and neuronal toxicity, suggesting that MLK2/3 are involved in GluR6-mediated JNK activation and neuronal apoptosis.

Finally, apoptosis of hippocampal neurons induced by gp120, the major coat protein of the HIV-1 virus, can be inhibited by CEP-1347 [123] . Overexpression of MLK3 in hippocampal pyramidal neurons enhances gp120IIIB-induced neuronal apoptosis, whereas overexpression of kinase dead form of MLK3 blocks gp120IIIB-induced toxic effects. These data indicate that MLKs may be involved in gp120IIIB-induced neuronal apoptosis and are potential targets for inhibiting AIDS-related dementia [123].

3.4.2. MLKs in cell proliferation

MLKs have been largely implicated in JNK-mediated apoptosis of neuronal cells. However, recent reports reveal that MLK3 might also contribute to ERK pathway activation and proliferation [36, 79]. Knockdown of MLK3 using siRNA blocks B-Raf-mediated ERK activation and proliferation [34, 35]. Further experiments demonstrated that catalytic activity of MLK3 is not required for its ability to regulate B-Raf activation. Instead, it is proposed that MLK3 regulates B-Raf and ERK activation is through forming a MLK3/B-Raf/Raf-1 signaling complex. Interestingly MLK3, B-Raf1, and Raf1 are all chaperoned by Hsp90/Ccd37, suggesting that these MAPKKKs may form multiprotein signaling complexes and that removal of a component may result in disassembly of the entire complex. These new findings also suggest that there might be some cross talk between ERK and JNK pathway to regulate the balance between survival and apoptosis.

3.4.3. MLKs in cell cycle.

Based on homology of the noncatalytic region, MLK3 was identified as a NIMA (never in mitosis A) like protein kinase [124]. NIMA is a fungal Ser/Thr kinase that is

involved in regulating G2/M transition during cell cycle regulation [124, 125]. During the G2/M phase, MLK3 is localized to the centrosome and has increased phosphorylation and activity. However, JNK activity remains low during the cell cycle, suggesting that MLK3 may function in a JNK-independent manner to regulate cell cycle progression. In addition, when overexpressed, MLK3 localizes to the centrosomal region, and induces profound disruption of cytoplasmic microtubules and a nuclear distortion phenotype [124]. It is also reported that cells treated with the MLK inhibitor CEP-11004 are defective in exiting mitosis, due to disruption of mitotic spindle formation[126]. The overexpression of MLK3 can partially rescue the CEP-11004-induced accumulation of cells in G2/M.

Expression of ZAK has been reported to increase the population of cells in G2/M phase [72, 74], whereas overexpression of catalytically inactive ZAK attenuates γ-radiation-induced G2 arrest [72], suggesting that ZAK, like MLK3 is involved in cell cycle regulation.

3.5. The biological functions of MLKs in *Drosophila* development.

The *Drosophila* MLK, called Slipper (*Slpr*), is critical for the JNK-dependent process of dorsal closure during fly embryogenesis [127]. Dorsal closure is the process in which the dorsal ectoderm moves from a lateral position to the dorsal midline and ultimately fuses at the dorsal midline to enclose the embryo in a continuous protective epidermis [128]. Genetic studies of the dorsal closure process in flies led to identification of a complete MAPK pathway composed of the small GTPase *dRac1*, an MKKKK called Misshapen (*Msn*), the MKK7 homologue Hemipterous (*Hep*), the JNK homologue

Basket (*Bsk*) and the transcription factors *dJun* and *dFos* [129]. *Slpr* functions at the downstream of *dRac1* and *Msn*, and upstream of *hep* and *bsk* in the *Drosophila* JNK pathway [127]. The *slpr* mutant embryos fail to maintain the proper cell shape within the dorsal epithelium resulting in a dorsal open cuticle phenotype. These data suggest a role for MLKs in controlling cell morphogenesis and development.

3.6. MLK3

MLK3, formerly called SH3 domain-containing proline-rich protein kinase (SPRK) and protein tyrosine kinase 1 (PTK-1), is a serine/threonine kinase consisting of 847 amino acids, with a predicted molecular weight of 93 kDa [58, 130, 131]. MLK3 mRNA is widely expressed in various tissues [58]. MLK3 contains multiple domains and motifs that may be playing regulatory roles, including a unique NH_2-terminal glycine-rich region, followed by an SH3 domain and the catalytic domain, a centrally located leucine zipper followed by a CRIB motif and a stretch of basic amino acids. The COOH-terminal 220 amino acids of MLK3 are rich in proline (24%), serine (12%) and threonine (13%) [58].

In addition to activating JNK, ectopically expressed MLK3 in certain experimental settings has also been shown to activate ERK, p38 and NF-κB pathways [59, 63, 73, 132, 133]. MLKs have been implicated in promoting apoptosis and the MLK inhibitor, CEP-1347, partially rescues cells from neuronal apoptosis induced by nerve growth factor withdrawal [52, 121]. Recent evidence suggests that MLK3 may play critical roles in B-Raf-induced ERK activation and, therefore, cell proliferation, by

serving as a scaffold protein to hold together a MLK/B-Raf/Raf-1 complex that is required for Raf transphosphorylation and activation [34-36].

Our lab has focused on understanding the mechanisms that regulate MLK3 activity and signaling. Zipper-mediated homo-oligomerization is required for full activation and proper substrate phosphorylation of MLK3 [91, 108]. Work from our lab indicates that MLK3 is autoinhibited through an intramolecular interaction between its SH3 domain and a short proline-containing sequence located between its zipper and CRIB motif (fig. 3) [99]. In accord with this model, mutation of a conserved tyrosine residue within the SH3 domain or mutation of the proline residue involved in the interaction disrupts the autoinhibition and results in increased MLK3 activity.

Phosphorylation is also important in the regulation of MLK3. Site-directed mutagenesis indicates that phosphorylation of Thr277 and Ser281 within the activation loop is critical for MLK3 activity [84]. In addition, MLK3 is reported to be negatively regulated by Akt phosphorylation [88]. Finally, MLK3 has a large COOH-terminus that is rich in proline, serine and threonine. Previous data from our lab has revealed multiple phosphorylation sites of MLK3, most of which are clustered at the COOH-terminal region [85]. Recent work from our lab suggests JNK can phosphorylate MLK3 *in vitro* and *in vivo*. Inhibition of JNK results in a hypophosphorylated form of MLK3, which is inactive, and redistributes to a Triton X-100-insoluble fraction. Recovery of JNK activity leads to phosphorylation of MLK3, which restores its solubility and activity [86].

The upstream activators of MLK3 are less defined. Activated forms of the small GTPases Cdc42 and Rac interact with MLK3 through the CRIB motif, increase

autophosphorylation and substrate phosphorylation activity of MLK3 [105-107] and potentiate MLK3-induced activation of JNK [63, 106, 107]. However, the detailed mechanism of how GTPases activate MLK3 is still incomplete.

4. Rho family small GTPases

Rho (Ras-homologous) family GTPases are key regulators of numerous cellular functions, including morphogenesis, polarity, motility, vesicular transport, endocytosis, and cell cycle progression [134-138]. They belong to a subfamily of the superfamily of Ras-related small GTPases and are found in all eukaryotic cells. In mammalian cells, 22 Rho GTPases have been identified, including Rho isoforms A, B, and C; Rac isoforms 1, 2, and 3; Cdc42, RhoD, Rnd1, Rnd2, RhoE/Rnd3, RhoG, TC10, and TCL; RhoH/TTF; Chp and Wrch-1; Rif, RhoBTB1, and 2; and Miro-1 and 2 [139, 140].

Like other GTPases, Rho GTPases act as binary switches cycling between an inactive GDP-bound state and an active GTP-bound state (Fig. 5) [100, 101, 140]. Only in the GTP-bound active state, do these GTPases adapt a conformation that allows them to bind downstream effectors to transmit signals. To date, over 50 effectors have been identified for Rho, Rac, and Cdc42, including kinases, lipases, oxidases, and scaffold proteins [140]. Structural analysis suggests that the binding of the small GTPase usually causes conformational changes in the effector proteins to release the effector from a usually closed, inactive conformation [141]. However, it is possible that GTPases might also regulate the subcellular localization of effectors by targeting them to specific locations. The GTPase switch is regulated by three types of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide

dissociation inhibitors (GDIs). GEFs catalyze the exchange of GDP for GTP to generate the activated form of the GTPase. GAPs stimulate the intrinsic GTPase activity of Rho family members to inactivate the switch. Finally, GDIs interact with the prenylated, GDP-bound form of GTPase to block spontaneous activation of the GTPases and to regulate GTPases cycling between membrane and cytosol [142].

Since Ras is the most extensively characterized GTPase, the knowledge from Ras has been applied to study other GTPases. Ras is an oncogene which is mutated in about 30% of human tumors [6]. Oncogenic forms of Ras are created by point mutations that results in amino acid substitutions at position 12, 13 or 61, which render Ras insensitive to GAPs and thus unable to hydrolyze GTP, resulting in a constitutively activated GTPase [143, 144]. Ras G12V is the most common one among these mutations. A so-called "dominant negative" form of Ras, generated by substitution of Ser17 with Asn, is inactive due to its preferential binding of GDP. Based on sequence and structural homology, the analogous amino acid substitutions have been made for many other small GTPases, including Rac and Cdc42, in order to generate constitutively active or inactive forms of GTPases; and these GTPases variants have become indispensable tools in studying the signaling of these GTPases.

Like all members of the Ras superfamily of GTPases, Rho family GTPases are able to associate with cellular membranes through the posttranslational prenylation of the Cys residues of their COOH-terminal CAAX (Cys-aliphatic-aliphatic-any amino acid) motifs [145, 146]. Protein prenylation is a type of lipid modification in which either a 15-carbon farnesyl or 20-carbon geranylgeranyl lipid is attached to the Cys of the CAAX via a thioester bond. This process is catalyzed by one of the two prenyltransferases

(farnesyltransferase or geranylgeranyltransferase). The residue in the X position of the CAAX motif determines whether a farnesyl or a geranylgeranyl lipid group will be attached[146]. If the X is a leucine or a methionine residue, a geranylgeranyl group is added. After the prenylation, the AAX residues are cleaved by a specific protease. The prenylcysteine at the new COOH terminus is then recognized by a prenylcysteine carboxyl methyltransferase (pcCMT) that methylesterifies the α carboxyl group [146].

A second signal for membrane localization, found in the so-called hypervariable region immediately upstream of the CAAX motif, is often present as well. It typically contains either palmitoylation sites [147] or a series of basic residues [148]. For example, in N-Ras or H-Ras, the hypervariable region immediately upstream of the CAAX motif contains two cysteine residues, which can be reversibly modified by palmitic acid via a labile thioester linkage to generate a hydrophobic COOH terminus [145, 147]. In contrast, the hypervariable region in K-Ras does not have the palmitoylation sites; instead it contains a polybasic sequence, serving as the second membrane targeting signal [148]. Similar to K-Ras, Cdc42 contains four basic residues in its COOH-terminal hypervariable region, and Rac has six basic residues. Prenylation within the CAAX motif coupled with the secondary palmitoylation sites or polybasic sequences in the hypervariable region is sufficient for membrane targeting of the GTPase[145].

5. Objective of thesis

MLK3 is an activator of multiple MAPK pathways. MLKs have been implicated in JNK-mediated apoptosis in some cells including neuronal cells. However, recent evidence suggests MLK3 is also critical for ERK activation and cell proliferation. Indeed, ample levels of MLK3 are present in human cancer cell lines, including breast cancer cell lines. Therefore, understanding how MLK3 and its signaling pathways are regulated may open up new therapeutic avenues for treating not only neurodegenerative diseases, such as Parkinson's disease (PD), but also cancers.

Activated forms of the small GTPases Cdc42 and Rac interact with MLK3 in a CRIB motif-dependent manner to increase MLK3 activity [105-107] and to potentiate MLK3-induced activation of JNK [63, 106, 107]. However, little is known about the detailed mechanism by which small GTPases activate MLK3. In Chapter 2, the molecular mechanisms through which Cdc42 regulates MLK3, is investigated.

Upstream stimuli/activator of MLK3 are less defined. Since GEFs are the direct activators of the small GTPases, Cdc42 and Rac, the regulation of MLK3 by one of the GEFs, Vav, is examined in Chapter 3.

Finally, proteomic/mass spectrometry methods have been used to identify the components of MLK3 signaling complexes isolated from breast cancer cells. These data are presented in the Appendix.

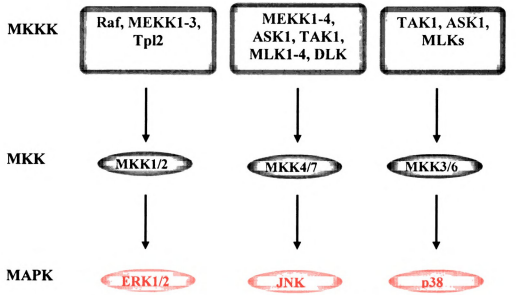

Fig. 1. **The mammalian MAPK pathways**. In mammalian cells, the three well defined MAPK pathways include ERK, JNK and p38 pathways. (Adapted from [52]) See text for details.

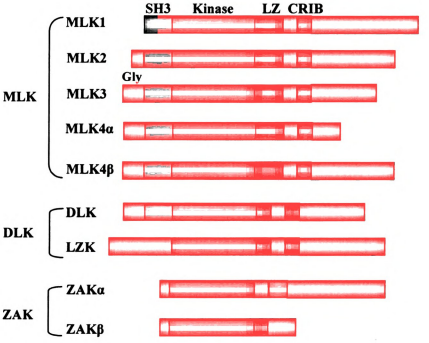

Fig. 2. **Members of the human Mixed-Lineage Kinases (MLK) family**. This diagram demonstrates the relative positions of the Src-homology-3 (SH3) domain(light blue), kinase domain (red), leucine-zipper (LZ) (green) and Cdc42/Rac-interactive binding (CRIB) motif (dark blue), and the sterile-α motif (SAM) (pink). (Adapted from [52])

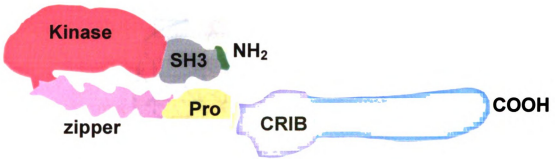

Fig. 3. **Model of autoinhibition of MLK3.** MLK3 is autoinhibited through the association between its SH3 domain and a proline-containing sequence located between the leucine zipper and the CRIB motif [99]. See text for details.

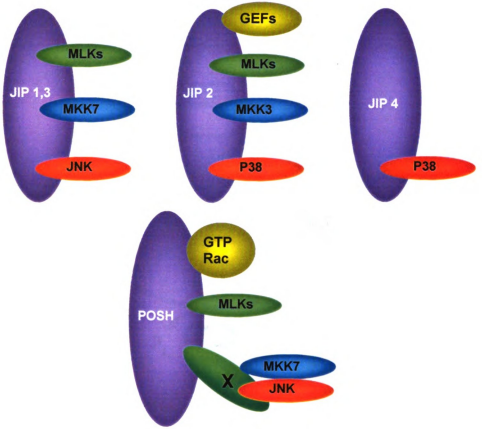

Fig. 4. **Scaffold proteins that interact with MLKs.** JIP1 specifically binds several MLKs, MKK7, but not MKK4 and JNK isoforms to facilitate JNK activation. JIP2 associates with MLK3, MKK3, p38 and the upstream activators of p38 pathways, including Tiam1 and Ras-GRF1. JIP2 binds to MLKs, MKK3 and p38. POSH interacts with activated Rac and MLKs directly. JIP4 interacts with p38 MAP kinase and potentiate the activation of p38 pathway. None of the MAP2Ks that activates JNK or p38 kinase (MKK4/7 and MKK3/6) interact with JIP4. POSH interacts directly with activated Rac1, MLK1-3 and DLK, and indirectly with MKK4/7 and JNK.

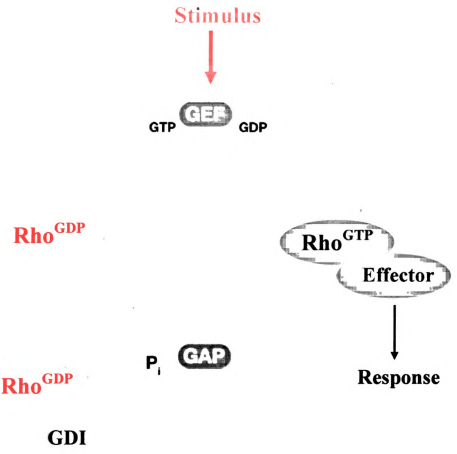

Fig. 5. **The regulation of GTPase.** Rho family small GTPases act as biological switches by cycling between an inactive GDP-bound state and an active GTP-bound state. Only the active GTPases can interact with effector proteins to mediate cellular responses. The activity of GTPases is regulated by GEFs, GAPs, and GDIs. (Adapted from [140]). See text for details.

Chapter 2. Cdc42 induces activation loop phosphorylation and membrane targeting of MLK3

1. Abstract

Mixed-lineage kinase 3 (MLK3) functions as a MAPKKK to activate multiple MAPK pathways. Our current studies demonstrate that lack of MLK3 blocks signaling of activated Cdc42 to JNK, giving strong support for the idea that Cdc42 is a physiological activator of MLK3. We show herein that Cdc42, in a prenylation-dependent manner, targets MLK3 from a perinuclear region to membranes, including the plasma membrane. Cdc42-induced membrane targeting of MLK3 is independent of MLK3 catalytic activity, but depends upon an intact Cdc42/Rac Interactive Binding (CRIB) motif, consistent with MLK3 membrane translocation being mediated through direct binding of Cdc42. Phosphorylation of MLK3's activation loop requires MLK3 catalytic activity and is induced by Cdc42 in a prenylation-independent manner, arguing that Cdc42 binding is sufficient for activation loop autophosphorylation of MLK3. However, membrane targeting is necessary for full activation of MLK3 and maximal signaling to JNK. We previously reported that MLK3 is autoinhibited through an interaction between its N-terminal SH3 domain and a proline-containing sequence found between the leucine zipper and the CRIB motif of MLK3. Thus we propose a model in which GTP-bound Cdc42/Rac binds MLK3, disrupts SH3-mediated autoinhibition

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leading to dimerization and activation loop autophosphorylation. Targeting of this partially active MLK3 to membranes likely results in additional phosphorylation events that fully activate MLK3 and its ability to maximally signal through the JNK pathway.

2. Introduction

The activation of protein kinases and the specification of their signaling pathways is a highly orchestrated process that is accomplished through dynamic and reversible events including phosphorylation, molecular interactions with proteins or other effector molecules, and subcellular targeting. Altered subcellular localization may impact protein kinase activation, signaling, or both. For instance, whether a protein kinase encounters an activating protein may depend upon its subcellular localization. Alternatively, many protein kinases have multiple *in vivo* substrates and signaling pathways, and spatiotemporal localization of protein kinases provides a mechanism by which substrate and signaling specificity can be achieved.

Mixed-lineage kinase 3 (MLK3) was first characterized as a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates the c-Jun N-terminal kinase (JNK) pathway through the dual phosphorylation of mitogen-activated protein kinase kinases 4/7 (MKK4/7) [59, 62]. The *Drosophila* MLK, called Slipper, is critical for the JNK-dependent process of dorsal closure in the fly embryo [127]. MLK3-induced JNK activation is implicated in apoptosis of neuronal cells in response to trophic factor withdrawal [120, 149-151]. MLK3 also activates JNK in Jurkat T lymphocytes [80] and MCF-7 breast cancer cells [81] in response to tumor necrosis factor alpha (TNF-α) treatment. MLK3 activates the p38 pathway through phosphorylation of MKK3/6 [63], although this activity may be dependent upon the scaffold JIP2 [77, 152]. Transforming growth factor beta (TGF-β)-induced apoptosis of hepatocytes is reportedly dependent on MLK3-induced activation of p38 [83]. MLK3 also contributes to extracellular signal-

regulated kinase (ERK) activation [79]; and gene silencing of MLK3 using siRNA blocks B-Raf-mediated ERK activation and proliferation [34, 35].

Deciphering how MLK3 is regulated is critical to our ultimate understanding of how MLK3 integrates different MAPK signaling pathways. Autoregulatory interactions are key to controlling MLK3 activity and signaling. MLK3's catalytic domain is flanked by an N-terminal SH3 domain and a centrally located zipper and Cdc42/Rac interactive binding (CRIB) motif. Zipper-mediated homo-oligomerization is required for full activity of MLK3, proper substrate phosphorylation, and activation of the JNK pathway [91, 108]. Work from our lab indicates that MLK3 is autoinhibited through an interaction between its SH3 domain and a proline-containing sequence within MLK3 [99].

Phosphorylation also contributes to the regulation of MLK3. Site-directed mutagenesis data indicate that activation loop phosphorylation of Thr277 and Ser281 is critical for MLK3 activity [84]. In addition MLK3 is reported to be negatively regulated by Akt phosphorylation [88]. Finally *in vivo* labeling coupled with mass spectrometry has revealed multiple sites of phosphorylation of MLK3, most of which are clustered at the COOH-terminus [85], whose functions are currently under study.

Cdc42 and Rac are Rho family GTPases that regulate diverse cellular processes including actin cytoskeleton remodeling, vesicular transport, endocytosis, cell cycle progression, cellular transformation, motility, and cell polarity [134-138]. Like all members of the Ras superfamily of GTPases, Cdc42 and Rac are able to associate with cellular membranes by virtue of posttranslational prenylation of the Cys of the COOH-terminal CAAX (Cys-aliphatic-aliphatic-any amino acid) motif [145, 146]. A second

signal for membrane localization, found in the so-called hypervariable region immediately upstream of the CAAX motif, typically contains either palmitoylation sites [147] or a series of basic residues [148] (Fig. 2).

Activated forms of the small GTPases Cdc42 and Rac interact with MLK3 in a CRIB motif-dependent manner to increase MLK3's autophosphorylation and substrate phosphorylation activity [105-107] and to potentiate MLK3-induced activation of JNK [63, 106, 107]. However little is known about how GTPases activate MLK3.

Herein we provide evidence that Cdc42 induces JNK signaling through endogenous MLK3. Furthermore we show that activated Cdc42 translocates MLK3 to membranes and induces activation loop phosphorylation of MLK3. The data presented support a mechanism whereby Cdc42 binding is sufficient for activation loop autophosphorylation of MLK3, but that prenylation-dependent, Cdc42-induced membrane targeting of MLK3, which does not, in itself, require MLK3 activity, is required for full activation of MLK3 and signaling to JNK. This work provides important insight into the molecular mechanism by which Cdc42 activates MLK3 and its signaling pathways.

3. Materials and Methods

3.1. Antibodies

The phospho-MLK3 (Thr277/Ser281), phospho-SEK1/MKK4 (Thr261), phospho-MKK7 (Ser271/Thr275), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and phospho-ERK (Thr202/Tyr204) monoclonal antibodies were purchased from Cell Signaling Technology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. The Flag M2 monoclonal antibody and actin mouse monoclonal antibody were purchased from Sigma-Aldrich. Other antibodies used were the MLK3 rabbit polyclonal antibody [107], and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

3.2. siRNA

The human *mlk3* siRNA sequence is derived from the sequence 5'-GGGCAGTGACGTCTGGAGTTT-3' as described previously [35]. The negative control siRNA sequence is derived from the sequence 5'-GGGCAGCGACGTGTCGAGCTT-3'. HeLa cells were plated in 6-well plates and transfected with 250 nmol of siRNA oligonucleotide per well using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 20 h, transfections using Flag-Cdc42^{V12} expression vector were performed; and after another 20 h, cells were lysed.

3.3. Expression Vectors and Site-Directed Mutagenesis

The construction of the cytomegalovirus-based expression vectors containing the cDNA for the wildtype MLK3 (pRK5-*mlk3*) has been described elsewhere [58]. The

expression plasmid construct encoding the N-terminal Flag epitope-tagged constitutively active Cdc42 (pRK5-N-Flag.*cdc42*^{V12}) was kindly provided by Avi Ashkenazi (Genentech, Inc.).

Variants of Cdc42^{V12} containing point mutations were constructed using the Quick Change site-directed mutagenesis method (Stratagene) using *Pfx* polymerase (Invitrogen) and 15 cycles of amplification. To generate pRK5-NFlag.*cdc42*^{V12}C188S, 5'-GATGTTCATAGCAGCACAGATCTGCGGCTCTTCTTCG-3' and its reverse complement were used as primers and pRK5-NFlag.*cdc42*^{V12} was used as the template. To substitute Lys183, Lys184, Arg186, Arg187 of Cdc42 with neutral Gln residues, pRK5-NFlag.*cdc42*^{V12} C188S, was used as a template in two successive rounds of mutagenesis using the following primers and their reverse complements: 5'-CCAGAACCGAAGAAGAGCCAGAGGTCTGTGCTGCTATGAAC-3' for the first round; and 5'-GCCTCCAGAACCGCAGCAGCAGAGCCAGCAGTCTGTGCTATGAACGCT-3' for the second round. The presence of the desired mutations was confirmed by DNA sequencing (MSU Genomics Technology Support Facility).

3.4. Cell Lines and Transfections

Human embryonic kidney (HEK) 293 cells were cultured and transfected using the calcium phosphate method as previously described [107]. HeLa cells were cultured in high glucose Dulbecco's modified Eagle's media containing 8% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen) and were transfected using Lipofectamine 2000 (Invitrogen).

3.5. Subcellular Fractionation

Plasma membrane-enriched fractions were generated according to Stokoe *et al.* with minor modifications [153]. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and disrupted in hypotonic buffer (10 mM Tris (pH 7.5) containing 35 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_4PP_i, 1 mM Na_3VO_4, 100 μM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, and 0.15 U/ml aprotinin). The cells were homogenized with 60 strokes in a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 500 x *g* for 5 min at 4°C. Centrifugation of the supernatant at 4°C at 16,900 x *g* yielded a plasma membrane-enriched pellet (P16.9) fraction and a soluble (S16.9) fraction. The pellet fractions were resuspended in lysis buffer (50 mM HEPES (pH 7.5) containing 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na_4PP_i, 10 mM NaF, 100 μM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, and 0.15 U/ml aprotinin). Protein concentrations in each fraction were determined using Bradford assays according to the manufacturer's instructions (Bio-Rad). Either equal amounts of total protein or equal cellular equivalents from the S16.9 and P16.9 fractions were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as indicated in figure legends, and Western blotting for MLK3 and Flag-Cdc42 was performed as described below.

3.6. Cell Lysis and Immunoprecipitations

Cells were harvested and lysed as described previously [91] . Clarified lysates were incubated with antibody-bound protein A-agarose for 90 min at 4 °C.

Immunoprecipitates were washed three times with lysis buffer and analyzed by Western blotting.

3.7. SDS-PAGE and Western Blot Analysis

Lysates and immunoprecipitates of proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and Western blotted using appropriate antibodies. Western blots were developed by the chemiluminescence method. Multiple exposures of the Western blots were developed, and densitometry (ImageJ, http://rsb.info.nih.gov/ij/) of unsaturated films was used to determine relative expression levels/phosphorylation. Statistics were calculated using an unpaired Student's *t* test. A *p* value less than 0.05 was considered statistically significant.

3.8. Immunofluorescence and Confocal Microscopy

HeLa cells were plated onto 6-well plates containing cover slips (1×10^5 cells per well) and transiently transfected using Lipofectamine 2000 (Invitrogen). After transfection for 16-18 h, the cells were rinsed twice with PBS, fixed in 2% formaldehyde (Polyscience, Inc.) for 30 min at room temperature and washed three times with PBS. The cells were permeabilized with PBS containing 0.2% Triton X-100 for 10 min at 37°C and blocked in PBS containing 10% fetal calf serum (FCS), 2% bovine serum albumin (BSA) and RNAse A (100 μg/ml) for 60 min at room temperature. After incubation with the primary antibody (5 μg/ml of the MLK3 rabbit polyclonal antibody or 3.5 μg/ml Flag M2 monoclonal antibody, in PBS containing 5% BSA) for 1 h at room temperature, cells were washed three times with PBS containing 2% FCS and incubated for 1 h with the appropriate Alexa Fluor 488 or Alexa Fluor 546-conjugated secondary antibody

(Molecular Probes). After extensive washing with PBS containing 2% FCS, the nuclei were stained with TO-PRO-3 iodide (2 μM, Molecular Probes, Inc.) and mounted onto glass slides. The fluorescently labeled cells were examined with a Zeiss LSM Pascal confocal laser scanning microscope. Three separate tracks were used for capturing fluorescent images; excitation was done using 488 nm, 543 nm and 633 nm lasers with BP 500-530 nm (Flag-Cdc42, represented in Green), BP 560-615 nm (MLK3, represented in Red) and LP 650 nm (TO-PRO-3 iodide, represented in Blue) respectively as emission filters.

3.9. Immune Complex Kinase Assays

For measurement of MLK3 activity in the plasma-enriched fractions, an immune complex kinase assay of the solubilized P16.9 fractions was performed. The relative amounts of MLK3 in the P16.9 fractions from cells expressing MLK3 alone or MLK3 plus $Cdc42^{V12}$ were determined by densitometry (NIH image) and adjusted to ensure equal amounts of immunoprecipitated MLK3 from the P16.9 fractions. Solubilized portions of the P16.9 fractions were incubated with 20 μl of Protein A-agarose beads pre-bound with MLK3 antibody for 90 min at 4°C. The MLK3 kinase assay was carried out as described previously [91], except that 10 μg of purified GST-MKK4 was used as substrate. The extent of GST-MKK4 phosphorylation was determined by Western blotting with an anti-phospho-MKK4 antibody.

For measurement of MLK3 activity in total cellular lysates, the MLK3 precipitates were washed twice with lysis buffer and twice with kinase buffer. 8 μg of recombinant, catalytically inactive GST-MKK7 K165A [81] was used as substrate, and

the reaction was carried out for 20 min at room temperature. The extent of GST-MKK7 phosphorylation was determined by Western blotting with a phospho-MKK7 antibody.

4. Results

4.1. Cdc42-induced JNK Activation Requires MLK3

Previous studies have demonstrated that activated Cdc42 binds MLK3 and increases its catalytic activity [106, 107]. However, these studies have relied upon ectopically expressed MLK3. To determine whether Cdc42 regulates JNK signaling through endogenous MLK3, Cdc42-induced JNK activation was measured in HeLa cells in which MLK3 expression was abolished using RNA interference[35]. As shown in Fig. 1, Cdc42-induced JNK activation is reduced at least 5-fold upon transfection with human-specific, but not the control siRNA, indicating that MLK3 is a major physiological target of Cdc42 in signaling to JNK.

4.2. Membrane Targeting-defective Variants of Activated Cdc42 Are Able to Interact with MLK3

The COOH-terminal CAAX motif of Cdc42 serves as a target for geranylgeranylation, thus endowing Cdc42 with the ability to associate with membranes. Therefore it is tempting to speculate that prenylated, activated Cdc42 localizes MLK3 to cellular membranes. To study the potential of Cdc42-induced membrane targeting of MLK3, variants of Cdc42 that fail to undergo prenylation and/or lack the basic secondary membrane targeting motifs were constructed as diagrammed in Fig. 2A. Specifically, the prenylation site Cys188 in the CAAX motif of $Cdc42^{V12}$ was mutated to Ser (Fig.2A). In yeast, this analogous change of a sulfhydryl to a hydroxyl group prevents prenylation and rescues the lethal phenotype induced by constitutively active $Cdc42^{V12}$ [154].

In initial fractionation experiments, the prenylation-defective mutant of $Cdc42^{V12}$ C188S was found primarily in a soluble fraction, but a small amount was still retained in a membrane-enriched fraction (data not shown). This observation suggests that, even in the absence of prenylation, the hypervariable region of Cdc42 mediates residual membrane binding, as has been shown for K-Ras [148]. Consequently the four basic residues in the hypervariable region of Cdc42, Lys183, Lys184, Arg186 and Arg187 were mutated to neutral glutamine residues to generate $Cdc42^{V12}$C188S, K/R4Q (Fig. 2A). Two of these residues, Lys183 and Lys184, are critical for endomembrane binding of Cdc42 through γ-COP [155]. This variant, $Cdc42^{V12}$C188S, K/R4Q, was present only in the soluble fraction (Fig. 3A). As has been observed for Ras [147, 148, 156], the posttranslationally modified Cdc42 migrates more rapidly than the unprenylated mutant form in SDS-PAGE.

To rule out the possibility that the introduced mutations interfere with the ability of $Cdc42^{V12}$ to interact with MLK3, coimmunoprecipitation experiments were performed. As shown in Fig. 2B, both prenylation-defective variants of $Cdc42^{V12}$ associate with MLK3 to the same extent, indicating that the geranylgeranyl group and the COOH-terminal basic residues of Cdc42 are not required for association with MLK3.

4.3. Activated Cdc42 Targets MLK3 to a Plasma Membrane-enriched Fraction

To determine whether Cdc42 impacts the subcellular distribution of MLK3, biochemical fractionation experiments were performed using HEK293 cells transiently expressing MLK3 and variants of constitutively active $Cdc42^{V12}$. The distribution of MLK3 between a soluble (S16.9) and a plasma membrane-enriched pellet (P16.9)

fraction was assessed by Western blotting. Data from a representative experiment are shown in Fig. 3A. Based on five independent experiments, MLK3 is distributed approximately equally between the S16.9 and P16.9 fractions when expressed alone. In contrast, upon co-expression with activated $Cdc42^{V12}$, MLK3 is found predominantly in the P16.9 fraction. However, activated $Cdc42^{V12}$ is present in both fractions, as has been reported for overexpressed Ras^{V12} in COS cells [153]. Coexpression of MLK3 with the membrane targeting-defective, activated GTPase, $Cdc42^{V12}$C188S, K/R4Q, yielded a fractionation pattern identical to that of MLK3 alone (Fig. 3A), indicating that activated $Cdc42^{V12}$ alters the subcellular localization of MLK3 in a prenylation-dependent manner.

To confirm that MLK3 is only activated at the plasma membrane upon targeting by Cdc42, the catalytic activity of MLK3 in the P16.9 fractions was measured in *in vitro* kinase assays. The P16.9 fractions were solublized in Triton X-100-containing buffer and equal amounts of immunoprecipitated MLK3 were subjected to *in vitro* kinase assays using unlabeled ATP and recombinant GST-MKK4 as substrates. Phosphorylation of the activation segment of MKK4 was monitored using a phospho-specific antibody against MKK4. Data from three independent experiments demonstrate that MLK3 activity is present in the plasma membrane-enriched fractions only in the presence of active, prenylated Cdc42 (Fig. 3B). When MLK3 is expressed alone or with the prenylation-defective $Cdc42^{V12}$C188S, K/R4Q, negligible MLK3 activity resides in the P16.9 fraction. Taken together, these data support the hypothesis that functional prenylation is required for Cdc42-mediated targeting and activation of MLK3 at the plasma membrane.

4.4. Activated Cdc42 and MLK3 Colocalize at the Plasma Membrane

To examine the subcellular localization of $Cdc42^{V12}$ and MLK3 in cells, confocal microscopy experiments were performed using HeLa cells transfected with vectors encoding MLK3 and $Cdc42^{V12}$ variants. MLK3 alone displays prominant perinuclear staining along with punctate, vesicular patterns and limited diffuse cytosolic staining (Fig. 4, *top* panel). These data are in general agreement with reports that MLK3 localizes to the Golgi [157] and centrosome regions [124]. $Cdc42^{V12}$ has been previously shown to localize to the plasma membrane [158]. When MLK3 is coexpressed with $Cdc42^{V12}$, they clearly colocalize at the plasma membrane (Fig. 4, *center* panel). In contrast the prenylation-defective, activated $Cdc42^{V12}$ consistently displays cytosolic staining. In the presence of prenylation-defective $Cdc42^{V12}$, MLK3 fails to localize to the plasma membrane and largely maintains perinuclear staining (Fig. 4, *bottom* panel). Taken altogether, these data further support the idea that activated $Cdc42^{V12}$ targets MLK3 to the plasma membrane.

4.5. Requirements of MLK3 Membrane Targeting by Activated Cdc42

To determine the requirements for Cdc42-induced membrane targeting of MLK3, variants of MLK3 were expressed alone and with activated Cdc42 in HEK293 cells and the subcellular distribution of MLK3 was examined by Western blotting of equal cellular equivalents after biochemical fractionation. As shown in Fig. 5A, both wild type MLK3 and the catalytically inactive variant, MLK3 K144R, are efficiently targeted to the plasma membrane-enriched fraction when expressed with $Cdc42^{V12}$, indicating that MLK3 activity is not required for Cdc42-induced targeting.

MLK3 translocation may depend on direct physical association with Cdc42 or, alternatively, may be an indirect effect of activated Cdc42 signaling. To discern between these possibilities, we made use of the variant MLK3 I492A,S493A in which two of the conserved residues in the CRIB motif have been changed to Ala residues. This variant, which has been shown to be defective in $Cdc42^{V12}$ binding [107], fails to undergo Cdc42-mediated translocation as shown in Fig. 5A. These data strongly support a mechanism whereby direct interaction with activated Cdc42 is responsible for membrane targeting of MLK3.

4.6. Cdc42 Induces Activation Loop (Auto)-Phosphorylation of MLK3

Within the catalytic domain of protein kinases resides the so-called activation loop, whose phosphorylation often alters its conformation rendering the kinase (usually) catalytically active [7-10]. Site-directed mutagenesis data support phosphorylation of the activation loop within the kinase domain of MLK3 is critical for MLK3 activity [84]. Activation loop phosphorylation of MLK3 was assessed by Western blotting of cellular lysates with a phosphospecific antibody directed against activation loop-phosphorylated MLK3 (pThr277/pSer281). As shown in Fig. 5B, wildtype MLK3 lacks activation loop phosphorylation, which is markedly enhanced upon coexpression with activated Cdc42. The kinase-defective variant of MLK3 is effectively targeted by Cdc42 to the membrane fraction (Fig. 5A) but fails to undergo activation loop phosphorylation (Fig. 5B), indicating that activation loop phosphorylation is due, at least in part, to autophosphorylation.

If direct association with Cdc42 is required for MLK3 translocation, as our data above indicate, and if translocation is needed for MLK3 activation, it is reasonable to predict that the CRIB mutant of MLK3 would not be activated by Cdc42. In accord with this hypothesis, MLK3 I492A,S493A exhibits very minimal activation loop phosphorylation whether expressed with or without activated Cdc42 (Fig. 5B). As shown in Fig. 5B, activation loop phosphorylation, to a first approximation, mirrors MLK3-induced JNK activation, as judged by Western blotting of phospho-JNK in cellular lysates.

4.7. Cdc42 Induces Activation Loop Phosphorylation Independent of Membrane Targeting

Catalytic activity and GTPase binding are required for Cdc42-induced activation loop phosphorylation of MLK3. As expected, the activation loop phosphorylated MLK3 resides in the membrane fraction (Fig. 6A). One reasonable hypothesis is that Cdc42-induced membrane targeting is critical for MLK3 activation loop phosphorylation. However we find that both the prenylation-defective and -competent versions of Cdc42 induce a comparable increase in activation loop phosphorylation of MLK3, indicating that the Cdc42-mediated increase in activation loop phosphorylation of MLK3 does not depend upon membrane targeting (Fig. 6B). Taken altogether, these data imply that Cdc42 activates MLK3, at least in part, by inducing activation loop (auto)phosphorylation. Furthermore, this function of Cdc42 is independent of its ability to associate with cellular membranes and likely ensues from the physical association of the kinase with the small GTPase.

4.8. Membrane Targeting Contributes to Full Activation of MLK3

Both prenylation-competent and prenylation-defective variants of $Cdc42^{V12}$ promote activation loop phosphorylation of MLK3 to the same extent. However, activation loop phosphorylation may not be sufficient for full activation of MLK3. To determine whether membrane targeting contributes to the activation of MLK3 by Cdc42, *in vitro* MLK3 immune complex catalytic activity assays were performed from HeLa cells expressing MLK3 and $Cdc42^{V12}$ variants. Recombinant, catalytically inactive GST-MKK7 was used as a substrate; and the extent of phosphorylation of the activation segment of MKK7 was measured using a phospho-specific antibody directed against MKK7. Control experiments were performed to confirm that a linear relationship exists between the amount of phospho-GST-MKK7 loaded and the resulting signal quantitated using ImageJ software (data not shown).

As demonstrated previously, coexpression of MLK3 with activated $Cdc42^{V12}$ enhances the *in vitro* catalytic activity of MLK3 (Fig. 7). We have consistently observed that coexpression with Cdc42 increases the protein levels of MLK3 and thus it is necessary to correct the MLK3 activity for the amount of protein present. Based on three independent experiments using HeLa cells and normalizing for the amount of immunoprecipitated MLK3, MLK3 catalytic activity is increased 5.5-fold upon coexpression with prenylation-competent $Cdc42^{V12}$ over that of MLK3 alone, whereas the prenylation-defective $Cdc42^{V12}$C188S, K/R4Q induces only a 2.5-fold activation of MLK3. These data indicate membrane targeting is required for the full activation of MLK3 induced by Cdc42.

Prior work from our lab identified Ser555 and Ser556 of MLK3 as sites that incorporate radiolabeled phosphate *in vivo* upon coexpression of MLK3 with activated Cdc42 [85], leading us to hypothesize that these might be membrane targeting-dependent, activating phosphorylation sites. However, an MLK3 variant in which these phosphorylation sites have been substituted with alanine was fully activated by Cdc42-induced membrane targeting (Y. Du and K. Schachter, data not shown), suggesting that other phosphorylation events/posttranslational modifications are responsible for the enhanced activation of MLK3 in response to membrane targeting.

4.9. Enhanced MLK3 Signaling to JNK Requires Cdc42-induced Membrane Targeting

Since full activation of MLK3 requires Cdc42-induced membrane targeting one might expect membrane targeting to affect MLK3 signaling. One of the best-described functions of MLK3 is as a MAPKKK that activates the JNK pathway [62]. In addition, activated Cdc42 potentiates MLK3-induced JNK activation [107]. To determine the impact of membrane targeting of MLK3 by Cdc42 on JNK activation, MLK3-induced JNK activation in cells was assessed using either the phospho-specific antibodies directed against activated JNK (pThr183/pTyr185) and/or c-Jun (pSer63). MLK3 alone induces basal phosphorylation JNK and c-Jun that is markedly enhanced upon coexpression with prenylation-competent $Cdc42^{V12}$ in HeLa cells (Fig. 8A). In contrast, despite its full ability to induce activation loop phosphorylation of MLK3, prenylation-defective $Cdc42^{V12}$C188S, K/R4Q only weakly potentiated MLK3 activation of JNK and c-Jun in cells.

Additional experiments show that prenylated Cdc42 alone, or MLK3 alone, only weakly activate JNK, whereas expression of the two together results in synergistic JNK activation (Fig. 8B, *fourth* panel), as judged by phospho-JNK Western blotting. Furthermore, only when MLK3 has been activated by prenylated Cdc42, are there significant levels of activated MKK7 in cells, as measured by immunoblotting of cellular lysates with a phospho-MKK7 antibody (Fig. 8B, *third* panel). From these data we conclude that functional targeting to the plasma membrane contributes to MLK3-induced activation of JNK by Cdc42, most likely reflecting the increased catalytic activity of membrane-targeted MLK3.

5. Discussion

In this study we investigated the mechanism by which the small GTPase Cdc42 activates MLK3. Depletion of cellular MLK3 using RNA interference prevents Cdc42-induced JNK activation implicating MLK3 as a physiological target of Cdc42. Previous work from our lab showed that GTP-bound Cdc42 associates with and activates MLK3 in a CRIB motif-dependent manner [107]. In order to assess the contribution of Cdc42-induced membrane targeting to MLK3 activation and signaling, a membrane-targeting defective variant of $Cdc42^{V12}$ was engineered. This membrane targeting-defective counterpart has no detectable membrane association yet retains the ability to associate with MLK3 in coimmunoprecipitation assays. In these studies, biochemical fractionation experiments, along with confocal microscopy, indicate that prenylation-competent, but not prenylation-defective, activated Cdc42 targets MLK3 from a perinuclear region to heavy membranes, including the plasma membrane. These results are consistent with the idea that Cdc42 and MLK3 signal at the plasma membrane.

Recently a biosensor approach revealed that active Cdc42 is present not only at the cell periphery, but also within the cell body near the cell periphery and at the trans-Golgi apparatus [159]. Given recent data regarding Ras signaling on endosomes as well as on the plasma membrane [160-163] and considering the ability of both Cdc42 [159, 164] and MLK3 to localize to endomembrane structures [157], it would not at all be surprising if, under certain conditions, Cdc42 may activate MLK3 on these cellular membranes.

Phosphorylation within the so-called "activation loop" promotes the active conformation of many protein kinases. Substitution of phosphomimetic Asp residues for Thr277 and Ser281 within the activation loop results in active MLK3 [84]. Interestingly, using a phospho-specific antibody, we found that GTP-bound Cdc42 potently induces activation loop phosphorylation of wildtype MLK3. However, Cdc42-induced membrane targeting is not required for activation loop phosphorylation since both prenylation-competent and prenylation-defective variants of $Cdc42^{V12}$ promote activation loop phosphorylation of MLK3 to the same extent. Furthermore, we show that Cdc42-induced activation loop phosphorylation requires MLK3 activity. These data imply that physical association with activated Cdc42 causes activation loop (auto)phosphorylation of MLK3.

It is possible that Cdc42 activates MLK3 indirectly through one of the MAPKKK kinases, such as the p21-activated kinase (PAK), which is known to be activated by Cdc42 and Rac [165-168]. However our finding that a GTPase binding-defective version of MLK3 cannot be activated by Cdc42 strongly argues for Cdc42 as a direct effector of MLK3.

The N-terminal region of PAK harbors a CRIB motif-containing p21-binding domain (PBD) which overlaps with an autoinhibitory domain [165-167]. The binding of the active, small GTPase disrupts this autoinhibitory conformation resulting in autophosphorylation of the activation loop and activation of PAK [165, 166]. Our lab has previously reported that MLK3 is autoinhibited through association of its N-terminal SH3 domain with a sequence containing Pro469 which, based at least on primary sequence, is situated in close proximity between the zipper domain (amino acids 400-462) and CRIB

motif (amino acids 498-514) [99]. Thus, although they utilize different structural domains/elements, PAK and MLK3 appear to share a common theme of GTPase-mediated disruption of autoinhibition. In the case of PAK, crystal structures reveal a *trans* autoinhibited dimeric form [168]; and experimental evidence indicates that activated Cdc42 dissociates the autoinhibited PAK dimer leading to *cis* autophosphorylation. In contrast, zipper-mediated dimerization/oligomerization is critical for MLK3 substrate phosphorylation and signaling [91, 108]. While the precise oligomerization state of neither the autoinhibited nor the activated form of MLK3 has been experimentally determined, it has been suggested, based upon co-immunoprecipitation of differentially tagged versions of MLK3, that Cdc42 induces dimerization/oligomerization of MLK3 [93, 108].

From the results discussed above, it might be concluded that physical association with Cdc42 is sufficient for MLK3 activation. However, *in vitro* kinase assays for MLK3 activity revealed that maximal activation of MLK3 requires Cdc42-induced membrane targeting. The inference is that activation loop (auto)phosphorylation is required, but insufficient, for full activation of MLK3, and is consistent with the idea that membrane-targeting dependent phosphorylation events, are necessary for the full activation of MLK3 and its signaling to JNK. It is also conceivable that enhanced accessibility of cellular substrates to MLK3 might contribute to its signaling to JNK.

The process of Ras-induced Raf activation shares some similar features with Cdc42-induced MLK3 activation. GTP-bound Ras binds and translocates c-Raf to the plasma membrane [27, 153, 169-171] and endomembranes [160-163]. Full activation of c-Raf requires activation loop phosphorylation [172] as well as membrane-dependent

phosphorylation, reportedly by PAK [173, 174] and Src [175-177]. However, while prenylation-defective, activated Cdc42 induces activation loop phosphorylation and partial activation of MLK3, prenylation-defective variants of Ras fail to activate c-Raf [156, 176]. A prenylation-defective variant of Ras has been shown to bind Raf [156], but binding-induced activation loop phosphorylation has not been examined.

The protein kinase(s) responsible for the membrane targeting-dependent phosphorylation of MLK3 are as yet unidentified, but two MAPKKKKs, germinal center kinase (GCK) [178] and hematopoietic progenitor protein kinase 1 (HPK1) [179], which have been implicated in MLK3 activation, emerge as candidate MLK3 kinases. While HPK1 is restricted to cells of hematopoietic lineage, both MLK3 and GCK are widely expressed in cell and tissue types [58, 180]. Interestingly it has been shown that HPK1 can be recruited to lipid rafts [181] and GCK is found at Golgi and plasma membranes [182].

One of the major functions ascribed to MLK3 is as a MAPKKK for activation of the JNK pathway. Our finding that the prenylation-defective, activated Cdc42 variant fails to fully potentiate MLK3-induced JNK activation supports the idea that plasma membrane localization of MLK3 is an important facet of the mechanism by which Cdc42 activates MLK3-induced JNK signaling. We propose that activated Cdc42 associates with autoinhibited MLK3 through its CRIB motif, disrupting SH3-mediated autoinhibition, inducing zipper-mediated dimerization of MLK3 and subsequent (auto)phosphorylation within the activation loop of MLK3. This localization-independent, allosteric activation and activation loop phosphorylation accounts for about half of the full activation of MLK3. As a consequence of Cdc42-induced membrane

targeting, MLK3 undergoes additional phosphorylation events that are required for its full activation.

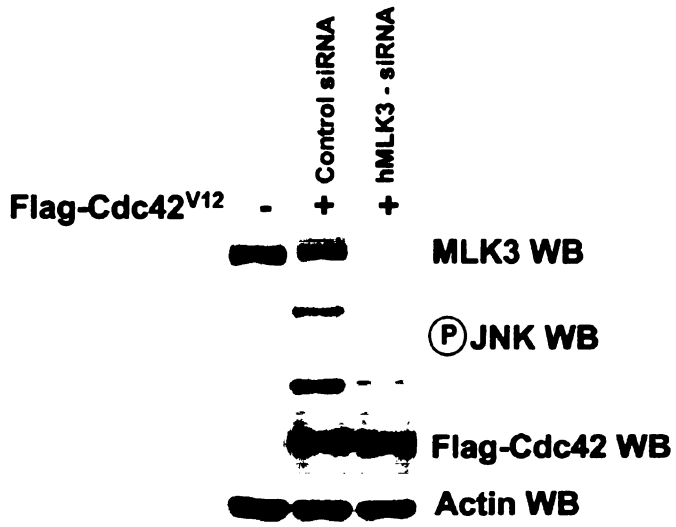


Fig. 1. **Cdc42-induced JNK activation requires MLK3.** HeLa cells were transfected with human *MLK3* siRNA or control siRNA followed by Flag-Cdc42^{V12} transfections, 20 h later. Cell were lysed and cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies. The data shown are representative of three independent experiments.

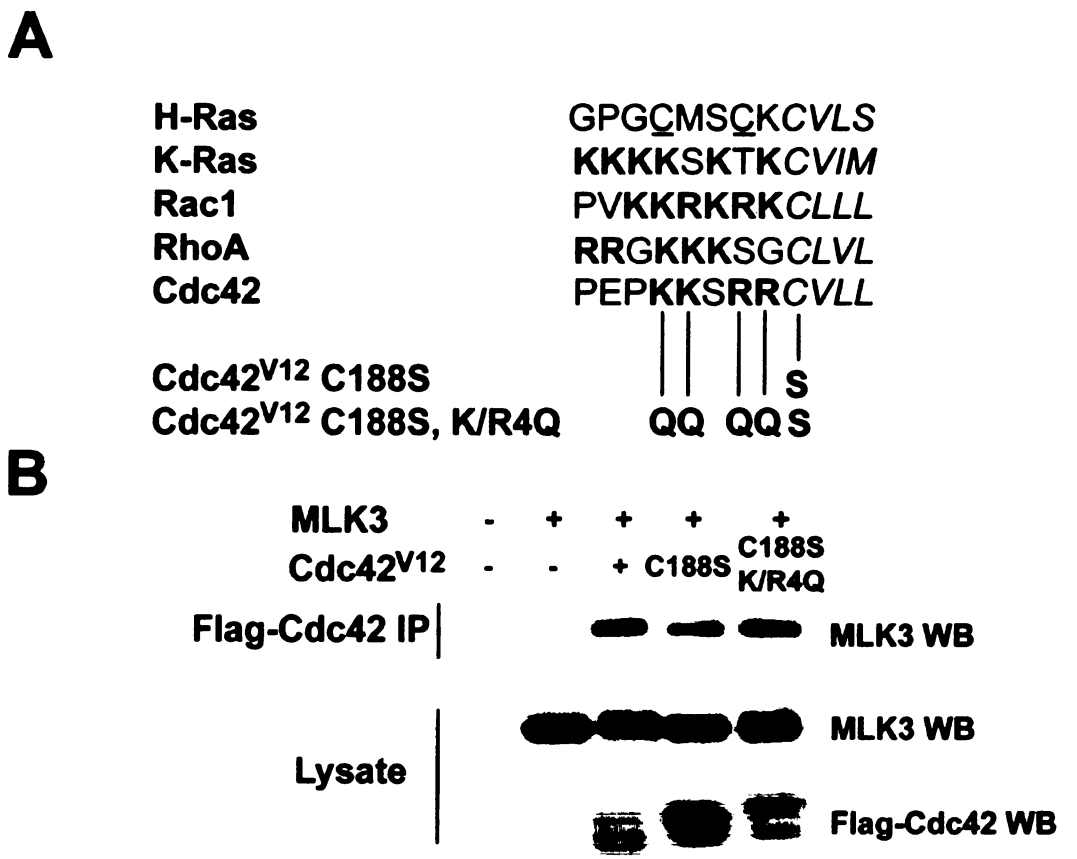


Fig. 2. **Construction of prenylation-defective variants of Cdc42^{V12} and the ability of Cdc42^{V12} variants to associate with MLK3.** A. Alignment of the COOH-termini of small GTPases and Cdc42^{V12} mutants. The twelve COOH-terminal amino acids of H-Ras, K-Ras, Rac1, RhoA and Cdc42 are aligned. The CAAX motif is *italicized.* Basic residues are shown in *bold* and palmitoylation sites are *underlined.* The mutations in the engineered Cdc42^{V12} variants are shown with the Lys/Arg to Gln and/or Cys to Ser changes indicated by *bold* letters. *B*, Association of Cdc42^{V12} variants with MLK3. Flag-Cdc42^{V12} variants were immunoprecipitated from total cellular lysates using the Flag antibody and associated MLK3 was detected by immunoblotting with an MLK3 antibody. (Barbara C. Böck, 2000)

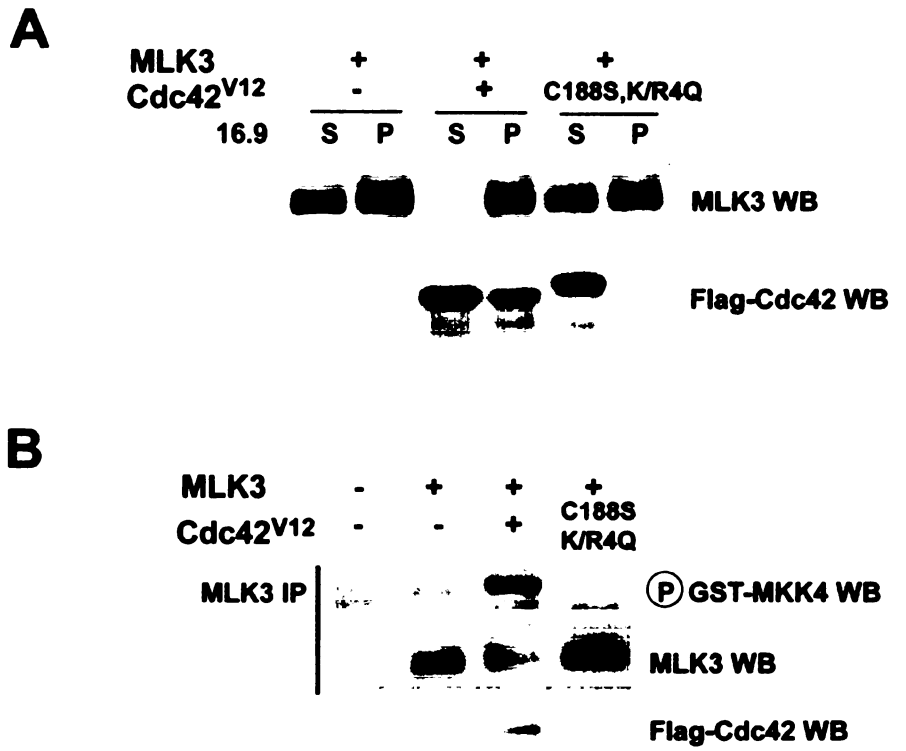


Fig. 3. **Effect of Cdc42^{V12} variants on the subcellular distribution of MLK3 and MLK3 activity in plasma membrane-enriched fractions.** HEK293 cells were transiently transfected with expression vectors containing cDNAs indicated above each figure. *A*, Subcellular distribution of MLK3 upon coexpression with Cdc42^{V12} variants. Cells expressing MLK3 in the presence and absence of Cdc42^{V12} variants were disrupted by Dounce homogenization in hypotonic buffer. The postnuclear fractions were centrifuged at 16,900 x *g* to yield soluble (S16.9) fractions and pellet (P16.9) fractions. Equal amounts of total protein were loaded in each lane. Western blots used to determine the distribution of MLK3 and Flag-Cdc42, using the MLK3 or Flag antibody, are shown in the *top* and *lower* panels, respectively. The data shown is representative of five independent experiments. *B.* Catalytic activity of MLK3 in P16.9 fractions. An *in vitro*

assay of MLK3 activity using equal amounts of MLK3 immunoprecipitated from solubilized P16.9 fractions was performed using GST-MKK4 as a substrate. Phosphorylation of GST-MKK4 was assessed using an antibody that recognizes phosphorylated Thr261 of MKK4. (Barbara C. Böck, 2000)

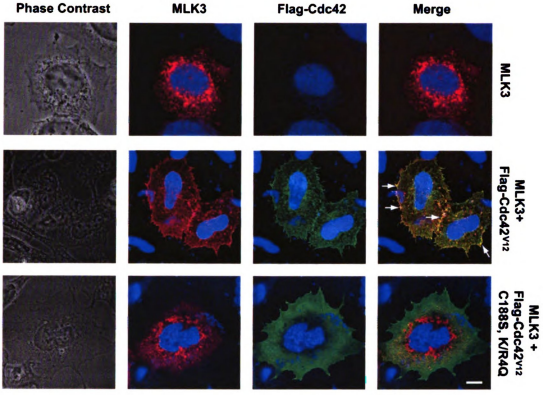


Fig. 4. **Subcellular localization of MLK3.** HeLa cells, transiently expressing MLK3 with or without Flag-tagged Cdc42^{V12} variants, were fixed and MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 546. Flag-tagged Cdc42^{V12} variants were detected by mouse monoclonal Flag antibody and a secondary antibody conjugated with Alexa Fluor 488. The nuclei were stained with TO-PRO-3 iodide. Using confocal laser scanning microscopy, more than 50 cells were examined for each transfection. Representative images are shown with Flag-Cdc42, MLK3, and nuclei represented in *green, red* and *blue,* respectively. Bar = 10 μm.

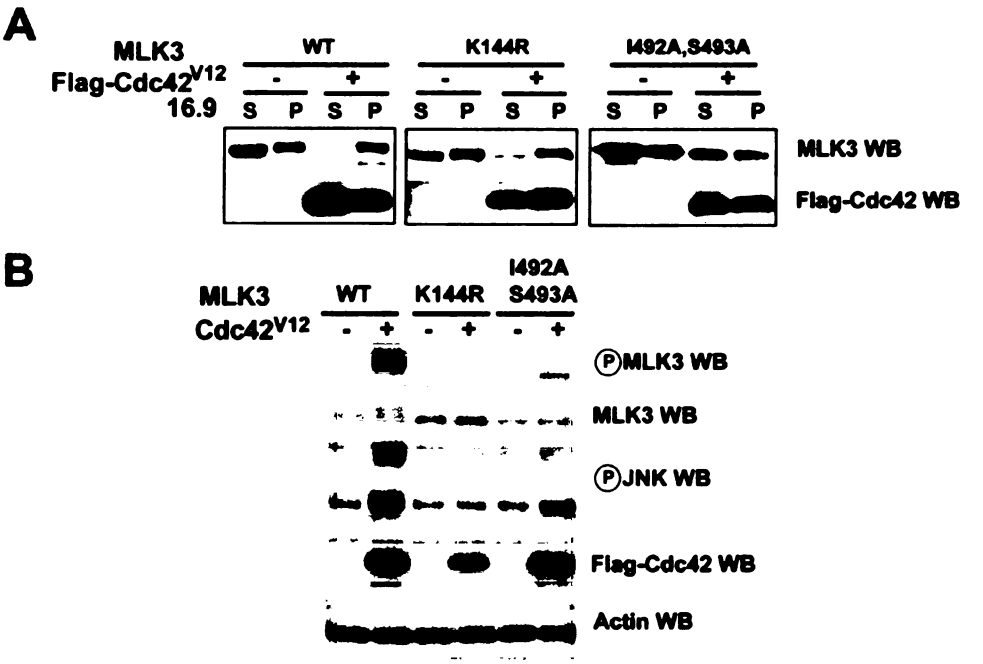


Fig. 5. **Requirements of MLK3 membrane targeting by activated Cdc42 and activation loop phosphorylation of MLK3.** *A*, Requirements of MLK3 membrane targeting by activated Cdc42. HEK 293 cells were transiently transfected with expression vectors containing cDNAs as indicated. Subcellular fractionation experiments were performed as described in Fig. 3. The samples for all the fractions were loaded as equal cellular equivalents. The distribution of MLK3 and Flag-Cdc42 were shown by Western blotting using the MLK3 or Flag antibody respectively. *B*. Cdc42-induces activation loop (auto)phosphorylation of MLK3. HeLa cells were transiently transfected with expression vectors containing cDNAs for MLK3 variants with or without Flag-$Cdc42^{V12}$, as indicated. Equal amounts of total protein from cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies. The data shown are representative of three independent experiments. (The experiments in panel A were performed in collaboration with Karen Schachter.)

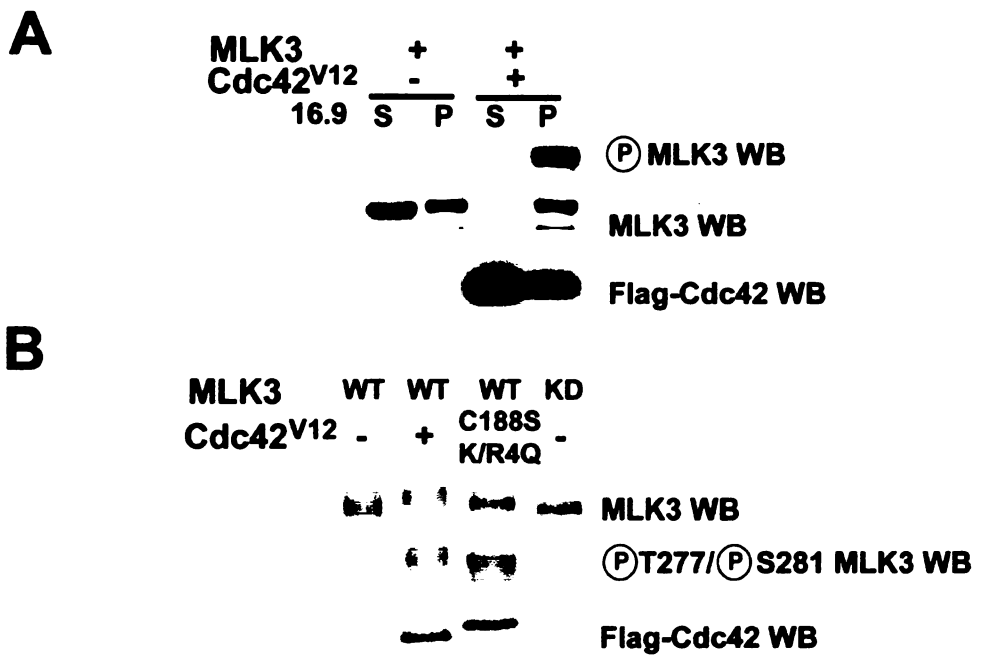


Fig. 6. **Cdc42 induces activation loop phosphorylation of MLK3 independent of membrane targeting.** *A.* Distribution of the activation loop phosphorylated MLK3. HEK 293 cells were transiently transfected with expression vectors for MLK3, with or without Flag-Cdc42^{V12}. Biochemical fractionation experiments were performed as described in Fig. 3. The distribution of MLK3, Flag-Cdc42 were examined by Western blotting. The activation loop phosphorylation of MLK3 in these different fractions was determined by Western blotting using a specific phospho-MLK3 antibody. *B.* The effect of Cdc42^{V12} variants on activation loop phosphorylation of MLK3. HEK293 cells transiently expressing wildtype (WT) MLK3 and Cdc42^{V12} variants, or MLK3 K144A (KD), were lysed and equal amounts of total protein were resolved by SDS-PAGE. Western blots of cellular lysates using MLK3 and phospho-MLK3 antibodies are shown in the *top* and *middle* panels, respectively. The data shown are representative of three independent experiments.

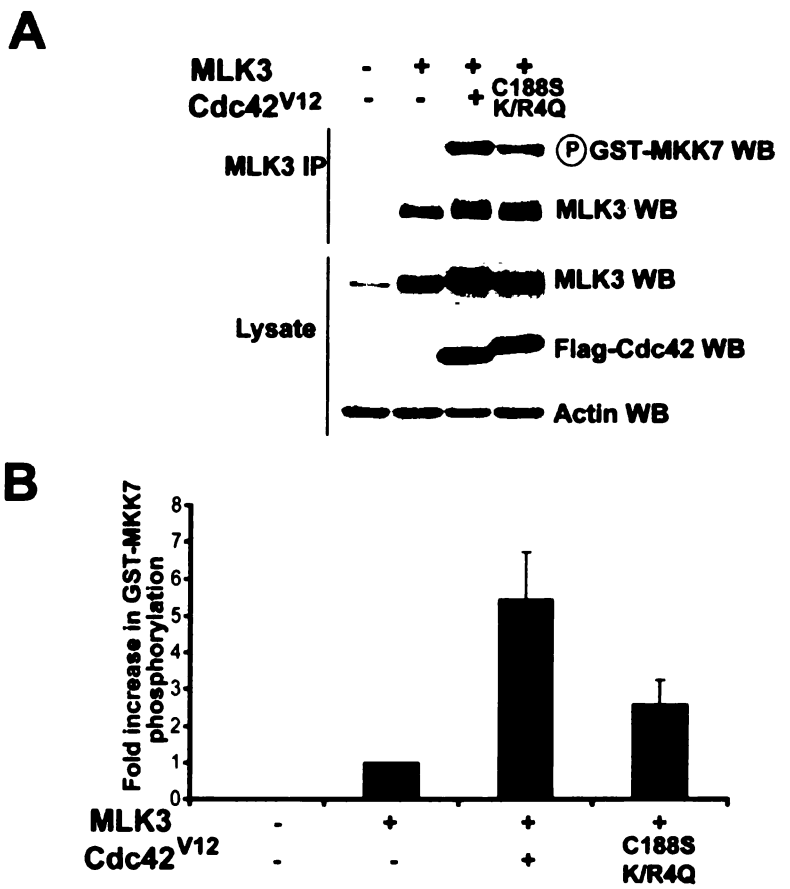


Fig. 7. **MLK3 activity induced by Cdc42^{V12} variants.** HeLa cells were transfected with or without Cdc42^{V12} variants as indicated. *A, in vitro* immune complex kinase assay of MLK3 using recombinant GST-MKK7 K165A as a substrate. The *top* panel shows phosphorylation of GST-MKK7 K165A as determined by Western blotting using a phospho-specific antibody against MKK7; the *second* panel shows a Western blot of the immunoprecipitated MLK3 from the *in vitro* kinase assays; the *third, fourth* and *fifth* panels are Western blots indicating the levels in cellular lysates of MLK3, Flag-Cdc42^{V12} variants and, as a loading control, actin. *B*, Quantitation of MLK3 activity assay. Phosphorylation of GST-MKK7 was quantitated by densitometry and

normalized to levels of immunoprecipitated MLK3 as described under "Experimental Procedures". The means ± S.E. for fold-increase in phosphorylation of GST-MKK7 K165A from three independent experiments are shown.

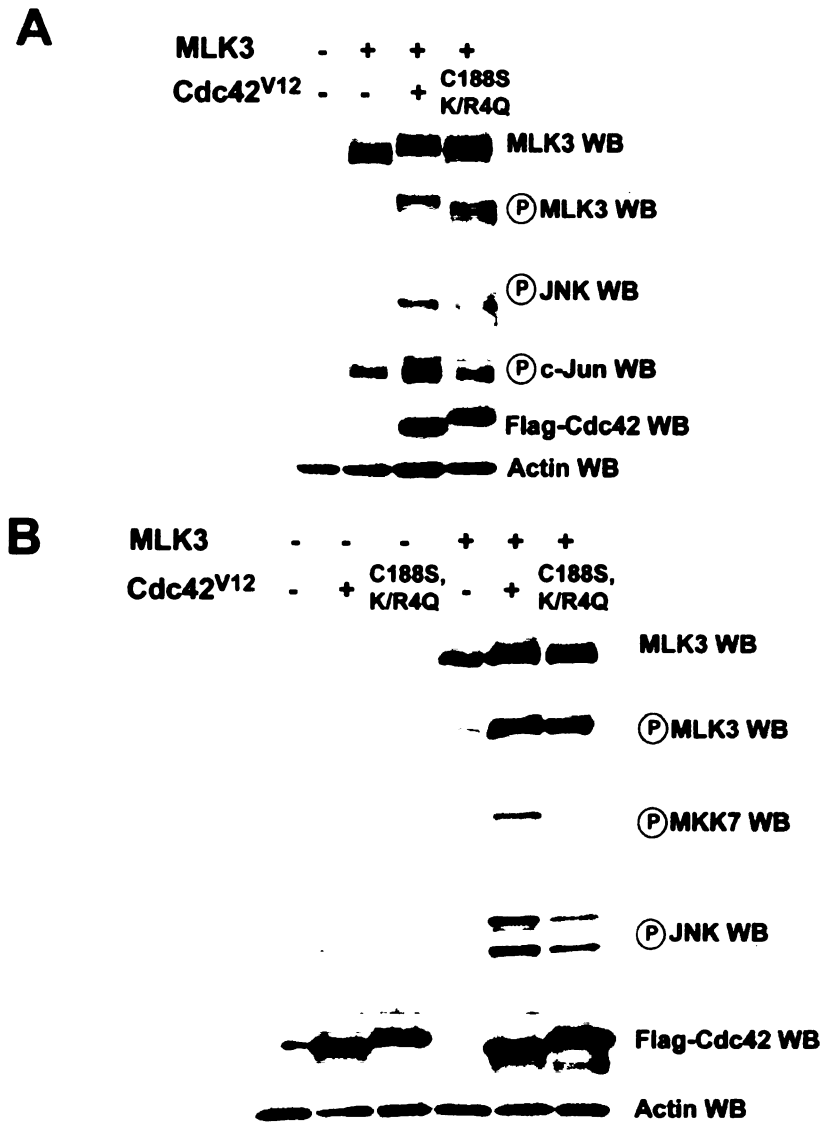


Fig. 8. **Effect of Cdc42^{V12} variants on MLK3-induced JNK signaling.** HeLa cells were transfected with expression vectors as indicated. Equal amounts of protein from cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies. The data shown are representative of three independent experiments.

Chapter 3. Regulation of mixed-lineage kinase 3 by guanine nucleotide exchange factor, Vav

1. Abstract

Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates multiple MAPK pathways. Whereas the downstream signaling components that are regulated by MLK3 have been largely defined, less is known about the upstream regulators of MLK3. Data presented in Chapter 2 demonstrated that the small GTPase Cdc42 is a physiological regulator of MLK3. In this study the role of Vav, one of the guanine nucleotide exchange factors that activates Cdc42, in the regulation of MLK3 was examined. Herein, we report that the expression of Vav increases MLK3 activity as judged by MLK3 autophosphorylation and substrate phosphorylation. Furthermore, Vav associates with MLK3 via at least two distinct sites within MLK3. The MLK family inhibitor, CEP-11004 dramatically inhibits Vav-induced JNK activation. However, silencing of the MLK3 gene by siRNA has no effect on activation of JNK-induced by expressed Vav. These data suggest Vav may signal through multiple MLKs to activate JNK.

2. Introduction

The immediate downstream signaling components of MLK3 have been largely defined, but the identification of the upstream activators of MLK3 is incomplete. Multiple lines of evidence point to a role for both Rac and Cdc42 as activators of MLK3. For instance *Slpr*, the *Drosophila* MLK homolog, is critical for Rac signaling to JNK during dorsal closure in fly embryogenesis [127]. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3 through the CRIB motif and increase MLK3 catalytic activity [63, 105, 107, 183]. The data in Chapter 2 demonstrates that binding of activated Cdc42 promotes activation loop phosphorylation of MLK3 and partial activation of MLK3. Furthermore, activated Cdc42, in a prenylation-dependent fashion, targets MLK3 to the plasma membrane; and this prenylation-dependent membrane targeting is required for full activation of MLK3 and its signaling to JNK [184].

Guanine nucleotide exchange factors (GEFs) activate GTPases by stimulating the exchange of the bound GDP for GTP [100, 101]. Only in their active, GTP-bound state, are GTPases capable of interacting with downstream effector proteins. Since MLK3 is activated by Cdc42 and Rac, the GEFs that activate Cdc42 and Rac might also promote MLK3 signaling. There are multiple GEFs that can activate Cdc42 and/or Rac. Vav was determined to be one of the most potent activators of MLK3 in an MLK3 activity assay, where several different GEFs were coexpressed with MLK3 (M. Chao 2002, unpublished data).

The Vav proteins are GEFs for Rho-family GTPases. In mammals, three isoforms of Vav are Vav1, Vav2 and Vav3. Whereas Vav1 is predominantly expressed in the

haemopoietic system, Vav2 and Vav3 are more ubiquitously expressed [185-188]. The spectrum of GTPase targets for different Vav proteins is diverse. It has been reported that Vav1 is primarily a GEF for Rac1, Rac2, RhoA and RhoG; that Vav2 acts on RhoA, RhoB and RhoG; and that Vav3 activates RhoA, RhoG and, modestly, Rac1 [186-191]. However, others have reported that Vav1 may also activate Cdc42 and that Vav2 may serve as a GEF for Rac1 and Cdc42[188, 190, 192-194]. In addition, activation of Rac1 is absent in *Vav1*$^{-/-}$ thymocytes and in *Vav3*$^{-/-}$ DT40 B cells [195, 196]. Activation of Cdc42 also appears to be defective in *Vav1*$^{-/-}$ T cells [197]. However, structural analysis revealed that the features that are important for the interaction with Vav, and proper Vav-induced GDP/GTP exchange are present in Rac1 and RhoA, but not in Cdc42 [198].

The classical Rho family GEFs, including Vav proteins, contain a pleckstrin homology (PH) domain immediately followed by a Dbl homology (DH) domain. The DH domain is essential for GEF activity, whereas the PH domain has been suggested to directly affect the catalytic activity of the DH domain and to target the GEF to its appropriate intracellular locations [191, 198, 199]. Besides the tandem DH and PH domains, all Vavs also contain a number of other domains and motifs, which can contribute to protein-protein interactions [191, 198, 199] (Fig. 1). The NH_2-terminus of Vav proteins contain a calponin homology (CH) domain which inhibits GEF activity, perhaps through an interaction with the cysteine-rich C1 domain[199]. The acidic region (Ac) contains at least three tyrosine phosphorylation sites [200]. Among them, Tyr174 has been shown to bind to the DH domain, thus inhibiting its GEF activity by keeping Vav1 in an autoinhibited conformation [201]. Phosphorylation of Tyr174 results in the disruption of its intramolecular interaction with the DH domain, promoting the active

conformation of Vav [201]. The autoinhibition of Vav may be regulated by Tyr174-DH interaction in collaboration with CH-C1 interaction. The COOH-terminus of Vav has two SH3 domains flanking a single SH2 domain. Because of these multiple protein interaction domains, Vavs might also serve as an adaptor protein to facilitate the assembly of signaling complexes and to regulate signaling cascades in a GEF activity independent manner [188, 198].

Results from mice with targeted disruption of single or multiple Vav isoforms have suggested that they play important roles in T-cell development and T-cell antigen receptor (TCR) signaling[188, 202]. Whereas disruption of Vav1 results in decreased numbers of thymocytes and peripheral T cells in mice, mice deficient in either Vav2 or Vav3 alone appear to have normal T-cell development[202]. However, there is a further significant decrease in the numbers of thymocytes and peripheral T cells in Vav1/2/3 triple knockout mice, suggesting the different Vav isoforms have redundant functions. Further analysis reveals that Vav1-null cells have decreased TCR signal-induced proliferation and cytokine secretion[188, 202].

Vav proteins plays important roles in antigen receptor-induced cytoskeletal reorganization, and in activation of stress-activated protein kinases and several important transcription factors, such as the nuclear factor of activated T cells (NF-AT) and NF-κB [191, 203-205].

T cell activation *in vivo* is achieved through the interaction of specific antigens with the TCR along with co-stimulatory signals provided by antigen-presenting cells (APC). Cultured T cells can be activated by stimulation with a combination of a

monoclonal antibody specific for CD3, which mimics antigen-mediated TCR/CD3 complex signaling [206], and an anti-CD28 monoclonal antibody which provides the required co-stimulatory signals [207, 208]. Upon TCR stimulation in Jurkat T lymphocytes, Vav1 is rapidly recruited to the activated receptors via its SH2 domains and is transiently phosphorylated by members of the Src and Syk tyrosine kinase families, resulting in the stimulation of its GEF activity[191, 209, 210]. Vav2 and Vav3 have also been shown to undergo TCR-induced phosphorylation[202, 203, 211, 212]. In addition to their involvement in TCR signaling, it has also been reported that Vav proteins undergo phosphorylation and activation in response to the stimulation of a variety of receptors, including the B-cell antigen receptor, cytokine receptors, chemokine receptors, integrin, and growth factor receptors [188, 213]

It is well-established that TCR co-stimulation results in JNK activation in T cells [204, 214-217]. Expression of dominant negative versions of Vav1 blocks CD3/CD28-induced activation of JNK [216]. Furthermore, TCR-induced activation of JNK is reduced in Vav1-deficient Jurkat T lymphocytes, and expression of a GEF-defective version of Vav1 is unable to rescue TCR-induced activation of JNK [218], suggesting that the GEF activity of Vav1 is critical for TCR-induced JNK activation. MKK4 (SEK1)-deficient T cells have reduced proliferation and IL-2 production in response to TCR costimulation [219]. Finally, in Jurkat T cells TCR engagement by anti-CD3/CD28 stimulation retards the electrophoretic mobility of MLK3 consistent with phosphorylation and a kinase inactive form of MLK3 blocks TCR-induced activation of the NF-κB pathway [133]. Recently, silencing of the *mlk3* gene has been shown to abolish CD3/CD28 co-stimulation induced activation of JNK in Jurkat T lymphocytes [35].

Data presented in this Chapter shows that Vav can increase MLK3 catalytic activity, leading to enhanced JNK activity. Furthermore, work presented in this thesis demonstrates that Vav can associate with MLK3 through two distinct binding sites within MLK3. An MLK inhibitor, but not silencing of the *mlk3* gene blocks JNK activation induced by ectopically expressed Vav, suggesting that MLKs may play redundant roles in mediating signals from Vav to JNK.

3. Materials and Methods

3.1. Plasmids

Expression vectors carrying the cDNAs for wild type MLK3 including pRK5-*mlk3* and pRK5-NFlag.*mlk3*, a series of NH_2-terminal Flag-tagged MLK3 variants with COOH-terminal truncations including pRK5-NFlag.*mlk3* 1-635, pRK5-NFlag.*mlk3* 1-598, pRK5-NFlag.*mlk3* 1-529, pRK5-NFlag.*mlk3* 1-485, pRK5-NFlag.*mlk3* 1-386, and a series of the hemagglutinin (HA)-tagged MLK3 variants, including pCGN-HA.*mlk3* 1-114, pCGN-HA.*mlk3* 115-399, pCGN-HA.*mlk3* 400-591, pCGN-HA.*mlk3* 592-847, were constructed previously in the lab [99, 107]. The bacterial expression vector of the kinase defective mutant GST-MKK7 K165A was constructed by YG. Liou in the lab. The mammalian expression vectors including pCF1-HA-Vav1, pCF1-HA-Vav2 and pCF1-HA-Vav3 were kindly provided by Dr. Joan Brugge (Department of Cell Biology, Harvard Medical School, Boston, Massachusetts). pCF1-Flag-Vav1 was constructed by replacing the HA-tag with a linker coding the 8 amino acid Flag epitope at the *Spe I* and *BamH*I sites.

3.2. Reagents and Antibodies

MLK3 rabbit polyclonal antibody was raised against the COOH terminal of MLK3 [107]. Other antibodies used were anti-Flag M2 monoclonal antibody (Sigma), anti-HA monoclonal antibody (BAbCO), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Anti-Flag M2 affinity gel was purchased from Sigma. The MLK inhibitor CEP-11004 was generously provided by Cephalon, Inc.

3.3. Cell Culture, Transfections, and Lysis

HEK 293 cells were maintained in Ham's F-12/low glucose DMEM (1:1) (Invitrogen) supplemented with 8% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. HEK 293 cells were transfected using the calcium phosphate method. Cells were harvested 16-18 h 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). HeLa cells were maintained in high glucose DMEM supplemented with 8% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (Invitrogen).

3.4. Immunoprecipitations

Antibodies against the proteins of interest and protein A-agarose beads were added to clarified lysate (450 μl) and incubated for 2 h at 4°C. Immunoprecipitates were then washed with lysis buffer three times. Immunoprecipitates used for kinase assays 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). In the co-immunoprecipitation experiments where Flag-MLK3 variants were immunoprecipitated, the Flag-MLK3/Vav complexes were eluted from the beads by incubation for 30 min at 4°C with 300 ng/μl 3X Flag peptide (Sigma) in TBS

3.5. Gel Electrophoresis and Western Blot Analysis

Proteins from lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted using primary antibodies against the proteins of interest, followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Bio Rad). Western blots were developed by the chemiluminescence method. The concentrations of primary antibodies used were 0.8μg/ml MLK3 polyclonal antibody, 1μg/ml anti-Flag M2 monoclonal antibody, and 1μg/ml anti-HA antibody.

3.6. *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 catalytic inactive MKK7 (GST-MKK7 K165A) for 20 min at room temperature. Following the kinase assay, proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and the incorporation of radioactivity into the GST-MKK7 was quantitated by PhosphorImaging (Molecular Dynamics). The membrane was probed with MLK3 antibody to confirm equal amounts of immunoprecipitated MLK3 in each lane.

3.7. *siRNA*

The human *mlk3* siRNA sequence is derived from the sequence 5'-GGGCAGTGACGTCTGGAGTTT-3' as described previously [35]. The negative control siRNA sequence is derived from the sequence 5'-GGGCAGCGACGTGTCGAGCTT-3'. HeLa cells were plated in 6-well plates and transfected with 250 nmol of siRNA oligonucleotide per well using 5 μl Lipofectamine 2000 (Invitrogen) according to the

manufacturer's instructions. After 20 h, transfections using Flag-Cdc42^{V12} expression vector were performed, and after another 20 h, cells were lysed and processed for analysis.

4. Results

4.1. Vav increases the catalytic activity of MLK3.

To determine whether Vav can regulate MLK3 activity, transient co-expression experiments in conjunction with *in vitro* kinase assays were performed. Expression vectors for MLK3 with or without expression vectors for hemagglutinin (HA) tagged versions of each of the 3 different isoforms of Vav were transiently transfected into HEK293 cells. MLK3 was immunoprecipitated from the cellular lysates and its activity was assessed by an *in vitro* kinase assay using purified, recombinant, catalytically inactive GST-MKK7 as a substrate. As shown in Fig. 2A, equal amounts of MLK3 are present in the immunoprecipitated samples. Upon co-expression with each of the Vav isoforms, the catalytic activity of MLK3 increases about 2- to 4-fold as judged by both MLK3 autophosphorylation and substrate MKK7 phosphorylation (Fig. 2). These data indicate that Vav proteins can increase MLK3 catalytic activity, at least when overexpressed (K. Schachter, 2003, unpublished data).

4.2. Vavs associate with MLK3.

In addition to their exchange factor activity, Vavs also interact with many signaling molecules. To determine whether Vavs can associate with MLK3, coimmunoprecipitation experiments were performed. HA-tagged versions of Vavs were transiently co-expressed with MLK3 in HEK293 cells. MLK3 was immunoprecipitated using MLK3 antibody. The presence of associated Vav was detected using an antibody directed against the HA-tag. As shown in Fig. 3, all three isoforms of Vav complex with MLK3 under these conditions. These experiments indicate that Vavs are able to associate

with MLK3, but they do not address whether this association is direct or whether it might be mediated through the GTPases, Cdc42 or Rac, or some other proteins.

4.3. Two distinct regions of MLK3 mediate binding to Vav

To better understand how Vav binding might increase MLK3 activity, the regions of MLK3 that interact with Vav were determined using variants of MLK3. Flag-tagged MLK3 variants with COOH-terminal truncations [99] were transiently co-expressed with HA-tagged Vav1 into HEK293 cells and tested for their ability to associate with Vav in co-immunoprecipitation experiments. The molecular weight of some of these MLK3 variants is similar to the IgG heavy chain or light chain. Therefore, in order to avoid the interference of IgG heavy chain or light chain in Western blotting, MLK3-Vav complexes were eluted by the addition of excess amount of triple Flag peptide, a peptide containing 3 tandem repeats of the Flag epitope. The Flag-MLK3 variants are depicted in Fig 4A. All MLK3 variants express in HEK293 cells (Fig. 4B). Deletion of the extreme COOH-terminal Pro/Ser/Thr-rich region (Flag-MLK3-(1-635) has no impact on the ability of Flag-MLK3 to associate with HA-Vav, whereas further deletions (Flag-MLK3-(1-598), Flag-MLK3-(1-529) and Flag-MLK3-(1-485)) have dramatically reduced binding to HA-Vav. Interestingly, the binding activity is largely recovered in a variant Flag-MLK3-(1-386) containing the NH_2-terminus through the kinase domain. Taken together these data indicate that MLK3 may contain two independent Vav-binding sites, one requiring amino acids in the region of 598-635 of MLK3 and a second binding site in the NH_2-terminal region of MLK3. One way to reconcile these findings is that the NH_2-terminal binding site within MLK3 must be somehow obscured in the MLK3 variants,

(Flag-MLK3-(1-598), Flag-MLK3-(1-529), and Flag-MLK3-(1-485)), which fail to bind Vav.

To further define the Vav-binding site(s) within MLK3, individual domains or regions of MLK3 tagged at their N-termini to HA epitopes were used (Fig 4A) [99]. Each of these variants was transiently co-transfected with Flag-Vav1 into HEK 293 cells, and their capacities to interact with Vav1 were examined by immunoprecipitation of Flag-Vav1 and western blot for associated HA tagged MLK3 fragments. All variants expressed at similar levels except that MLK3-(592-847) apparently undergoes some proteolytic degradation (Fig. 4C). The co- immunoprecipitation experiments demonstrated that HA-MLK3-(115-399), the kinase domain, and the COOH-terminal Pro/Ser/Thr-rich region, HA-MLK3-(592-847), interact with Vav1, whereas HA-MLK3-(1-114), containing the NH_2-terminal glycine-rich region and the SH3 domain, and HA-MLK3-(400-591), containing the zipper region, the CRIB motif, and an adjacent stretch of basic amino acids, failed to do so (Fig. 4C). These data suggest that the kinase domain (MLK3 155-399) and the COOH-terminal Pro/Ser/Thr-rich region (MLK3 592-847) of MLK3 are constitute independent binding sites Vav1.

Results from these two sets of experiments, suggest that MLK3 associates with Vav through two distinct interaction sites – one within the kinase domain and one that includes amino acid 598-635 of MLK3. Interestingly the association of full length Vav with full length MLK3 appears to be of high affinity since it is resistant to multiple washes with 300 mM NaCl containing 1% Triton X-100. Perhaps, it is these multiple interactions that result in the salt resistant, high affinity interaction between full length

MLK3 and full length Vav. The Vav binding regions within MLK3 do not include the CRIB motif, indicating that it is unlikely that Rac or Cdc42 mediate the binding of Vav to MLK3. However, it is still possible that GTPase binding may contribute to the association of MLK3 with Vav.

4.4. Vav-induced JNK activation is largely blocked by the MLK inhibitor, CEP-11004

All the studies described above have relied upon ectopically expressed MLK3. To determine whether Vav activates JNK through endogenous MLKs, we made use of a pharmacological inhibitor of the MLK family, CEP-11004. HA-Δvav (a constitutively active form of Vav) or wild type HA-Vav was expressed in HeLa cells in which the activation of MLKs was inhibited by CEP-11004. The downstream JNK activation was examined by Western blotting using a specific antibody directed against activation loop-phosphorylated JNK. As judged by the decreased levels of phospho-JNK, CEP-11004 treatment reduces JNK activation induced by expression of Vav1 or Δvav to approximately 25% of that of untreated cells expressing Vav1 or ΔVav (Fig.5). These data argue that the JNK activation induced by ectopically expressed Vav1 is mediated largely through MLK family members.

4.5. MLK3 is not required for Vav-induced JNK activation

To specifically test whether ectopically expressed Vav regulates JNK signaling through endogenous MLK3, Vav-induced JNK activation was measured in HeLa cells in which endogenous MLK3 expression was abolished using MLK3 siRNA [35]. The knockdown of MLK3 expression did not impact JNK activation induced by expressed

Vav (Fig 6), indicating that MLK3 is not essential for Vav-mediated JNK signaling, at least in this experimental context.

5. Discussion

Detailed studies performed in our lab and by others have lead to a model for MLK3 regulation in which MLK3 is captured in an inactive comfirmation through its SH3-mediated autoinhibition. The binding of the activated GTPase is proposed to induce a conformational change which leads to release of the autoinhibition, induced dimerization and trans-autophosphorylation of the activation loop of MLK3. Membrane targeting of MLK3 by Cdc42 promotes MLK3 full activation and is accompanied by additional phosphorylation events [63, 105, 107, 183, 184].

Since MLK3 is activated by the small GTPases, Cdc42 and Rac, a specific GEFs that regulates MLK3 should exist. Vav emerged as a potential MLK3 regulator from a survey in which the effect of activated forms of several different GEFs on MLK3 activity was assessed. Expression of each of the three isoforms of Vav promotes MLK3 activity to about 2-4 fold (Fig 2). In Jurkat T lymphocytes, the GEF activity of Vav1 has been shown to be critical for TCR-induced JNK activation [218]. The simplest explanation for these findings is that Vav promotes MLK3 activation through its GEF activity. However, experiments presented in this Chapter demonstrate that MLK3 is also able to associate with Vav. Thus it is possible that Vav may modulate MLK3 activity not only through its GEF activity, but may also facilitate GTPase-induced MLK3 activation by assembling a signaling competent MLK3/GTPase/Vav complex.

My data argues for two independent Vav binding sites within MLK3. MLK3-(1-485), which lacks the COOH-terminal Vav-binding site, fails to interact with Vav, yet the isolated kinase domain of MLK3 binds Vav. One possible way to explain these findings

is that the kinase binding site of Vav may be masked in the SH3-mediated autoinhibited conformation of MLK3[99]. When the SH3-binding region of MLK3 has been removed in Flag-MLK3-(1-386), the NH_2-terminal binding site may be revealed and become accessible for Vav binding. If this is true, the disruption of the autoinhibition should rescue the binding of the kinase domain within MLK3-(1-485) to Vav. Taken together, it is possible that the Vav-MLK3 association via MLK3-(598-635) region may facilitate the binding of activated GTPase to MLK3, by binding to both MLK3 and the GTPase. After the kinase domain binding site is exposed due to the disruption of the SH3-mediated autoinhibition by binding of the GTPase, Vav may further associate with MLK3 to stabilize the MLK3-GTPase binding. Further experimentation will be required to fully understand the detailed mechanism by which Vav association may regulate MLK activation and signling.

CEP-11004 is a small-molecule derived from the natural product K252a. It inhibits MLK family members by competing for the binding of ATP to the kinases, with an IC_{50} values of 45, 31, and 89 nM for MLK1, MLK2, and MLK3 respectively[220, 221]. Ihe related MLK inhibitor, CEP-1347, which has been in clinical trials for Parkinson's disease, blocks activation of the JNK pathway and protects neurons exposed to various stresses in a number of *in vitro* and *in vivo* models[222-225]. In this work, I have found that CEP-11004 blocks ectopically expressed Vav-induced JNK activation. These data argue for a role for the MLK family of kinases in Vav-induced JNK activation in HeLa cells.

Interestingly, while gene silencing of MLK3 in Jurkat T cells is reported to block anti-CD3/anti-CD28-induced JNK activation [34, 35], which is thought to be mediated

through Vav, knockdown of MLK3 has no impact on JNK activation in HeLa cells ectopically expressing Vav. The most reasonable explanation for these disparate findings may be that in Jurkat T cells MLK3 is the predominant mediator of Vav signaling to JNK, whereas in HeLa cells multiple MLKs are functionally redundant in signaling from Vav to JNK. It will be important to perform studies in Jurkat T cells to clarify the role of MLK3 in Vav-induced JNK signaling in response to TCR activation.

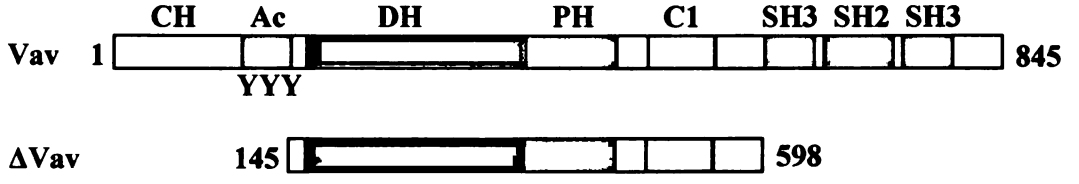


Fig. 1. **Domain structure of Vav proteins and ΔVav.** The N-terminal calponin homology (CH) domain inhibits GEF activity, through an interaction with the cysteine-rich C1 domain. The acidic (Ac) domain contains three tyrosine residues, which are involved in the autoinhibition of Vav by binding the Dbl homology (DH) domain. DH domain carries GEF catalytic activity towards Rho GTPases. The pleckstrin homology (PH) domain may regulate its GEF activity by binding of the phospholipids PIP_2 and PIP_3. The C1 (zinc finger) domain may associate with the GTPases. The COOH-terminal of Vav has a SH2 domain flanked by two SH3 domains (Adapted from [188, 191]). Deletion of the inhibitory CH domain and the Ac motif result in constitutively active form of Vav. A truncated, activated form of Vav, known as ΔVav contains the DH, PH and C1 regions.

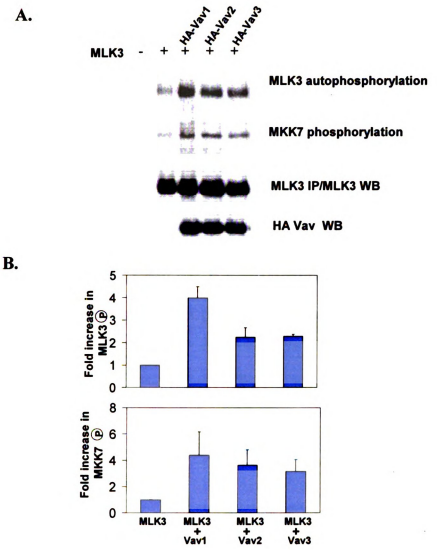


Fig. 2. **Ectopically expressed Vav isoforms potentiate MLK3 catalytic activity in HEK293 cells.**

Expression vectors for either MLK3 with or without expression vectors for HA-tagged versions of each of the 3 different isoforms of Vav were transiently transfected into HEK293 cells. A minus sign indicates that a control empty vector was transfected, while a plus sign indicates that an expression vector for MLK3 was transfected. MLK3 was

immunoprecipitated (*IP*) from the cellular lysates and its activity was assessed by an *in vitro* kinase assay using the purified, recombinant catalytically inactive GST-MKK7 as a substrate. *A, in vitro* kinase assay of MLK3 using GST-MKK7 K165A as a substrate. The *top panel* shows an autoradiogram with bands corresponding to MLK3 autophosphorylation and GST-MKK7 phosphorylation as indicated. A Western blot (*WB*) of the immunoprecipitated MLK3 using the MLK3 antibody is shown in the *middle panel. Bottom panel* shows the expression level of HA-Vav isoforms. *B,* the mean ± S.E. for fold increase in MLK3 autophosphorylation and GST-MKK7 phosphorylation from three independent experiments is shown. Data was quantitated by phosphorimaging and normalized to MLK3 expression levels. (K. Schachter, unpublished data)

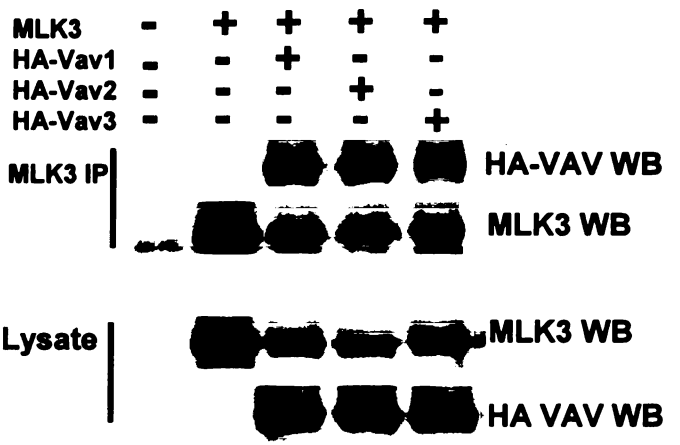


Fig. 3. **Full length Vav isoforms associate with MLK3 in HEK293 cells.** Expression vectors for HA-tagged Vav isoforms were transiently co-transfected with expression vector for MLK3 into HEK293 cells. A minus sign indicates that a control empty vector was transfected, while a plus sign indicates an expression vector for that listed at left side was transfected. MLK3 was immunoprecipitated from cellular lysates using MLK3 antibody. *Top panel,* approximately equal amounts of MLK3 were immunoprecipitated and the presence of associated HA-Vavs was detected using an antibody directed against the HA-tag. *Bottom panel,* expression levels of MLK3 and various HA-Vav were assessed by western blotting using the antibodies as indicated.

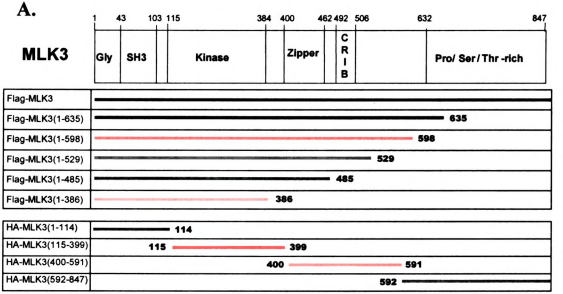


Fig. 4. **Interaction of MLK3 with Vav.**

A, schematic diagrams show MLK3 variants used in co-immunoprecipitation experiments to map Vav binding sites within MLK3. They are NH_2-terminal Flag-tagged MLK3 variants with COOH-terminal truncations and NH_2-terminal HA-tagged individual domains or regions of MLK3. The *numbers* in the figure represent amino acid numbers in MLK3. *Top panel,* schematic diagrams *of* MLK3. *Middle panel,* Flag-MLK3 variants. *Bottom panel,* HA-MLK3 variants. *B,* Expression vectors containing the sequence of Flag-tagged MLK3 variants with COOH-terminal truncations were transiently co-transfected with an expression vector for HA-tagged Vav1 into HEK293 cells. Flag-tagged MLK3 variants were immunoprecipitated from cellular lysates using anti-Flag M2 affinity gel and were eluted by an addition of excess amount of triple Flag peptide. *Top panels,* the presence or absence of associated HA-Vav1 in the immunoprecipitates was assessed by Western blotting with the HA antibody. Immunoprecipitations of Flag-tagged MLK3 variants were detecteded by western blotting using the the Flag antibody.

Fig. 4. continued

Bottom panels, expression levels of Flag-MLK3 variants and HA-Vav1 were assessed by western blotting of cellular lysates with the antibodies as indicated. *C,* Expression vectors containing the sequence of NH2-terminal HA-tagged version of MLK3 variants were transiently co-transfected with an expression vector for Flag-tagged Vav1 into HEK293 cells. Flag-tagged Vav1 was immunoprecipitated from cellular lysates using anti-Flag M2 affinity gel and was eluted by an addition of excess amount of triple Flag peptide. *Top panels,* the presence or absence of associated HA-tagged MLK3 variants was assessed by Western blotting with the HA antibody. The levels of immunoprecipitated Flag-tagged Vav1 were determined by western blotting using the Flag antibody. *Bottom panels,* expression levels of HA-MLK3 variants and Flag-Vav1 were assessed by western blotting of cellular lysates with the indicated antibodies.

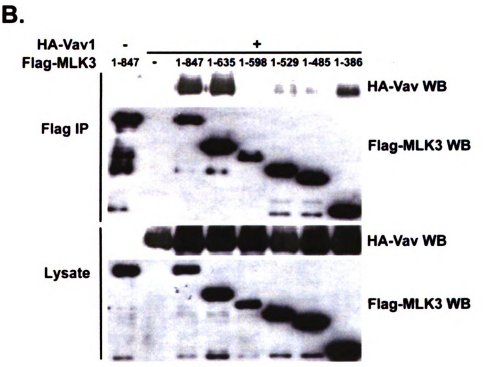
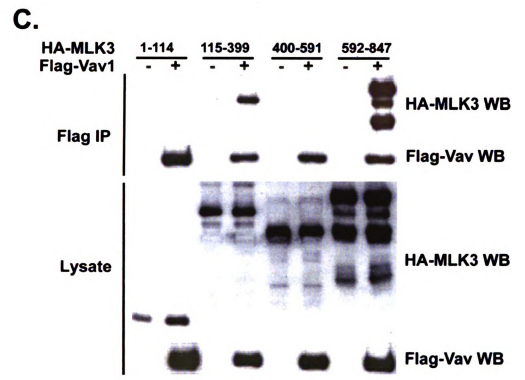


Fig. 4. continued



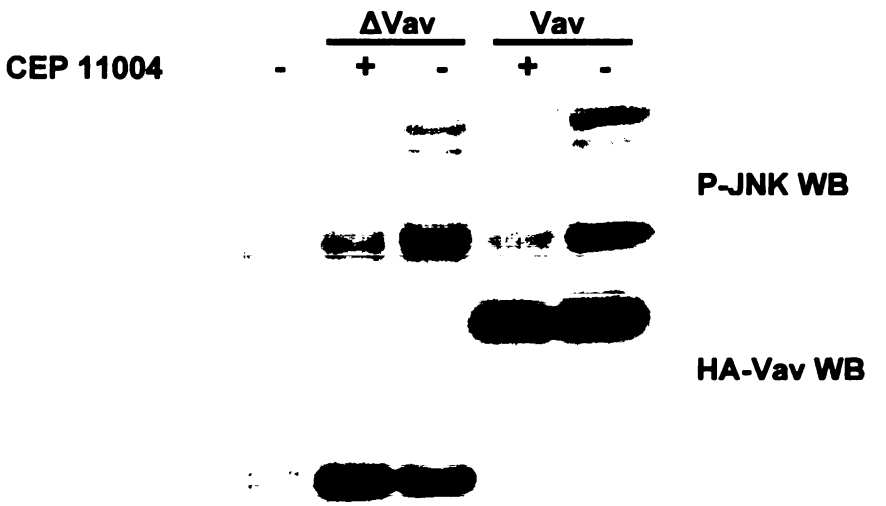


Fig. 5. **CEP-11004 largely blocks Vav-induced JNK activation**

HeLa cells were transfected for 4 h with expression vectors for either the constitutively active Vav, HA-Δvav, or wild type HA-Vav1, and incubated with fresh media in the presence or absence of 400 nM CEP-11004 for another 20 h. Cells were lysed and clarified lysates were analyzed by Western blotting using the antibodies as indicated.

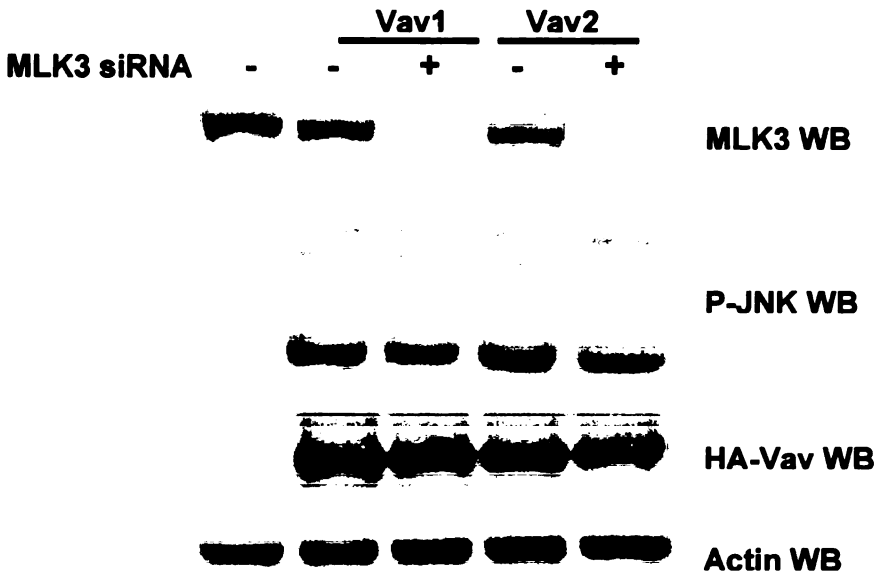


Fig. 6. **Effect of *mlk3* gene silencing on Vav-induced JNK activation**

HeLa cells were transfected with human *MLK3* siRNA (+) or control siRNA (-) followed by HA-Vav1 or HA-Vav2 transfections, 20 h later. Cells were lysed and cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies.

Chapter 4. Concluding remarks

The aim of this work is to elucidate the molecular mechanisms that regulate MLK3 signaling. The data presented in this dissertation describes the molecular mechanism by which Cdc42 activates MLK3 and its signaling pathways. The regulation of MLK3 by Vav, a GEF for Rho GTPases, is also examined. In addition, affinity purification coupled with mass spectrometry has been utilized in an effort to identify the protein components of MLK3 signaling complexes.

Evidence presented in Chapter 2 demonstrates that Cdc42 is a physiological activator of MLK3. The binding of activated Cdc42 is sufficient to promote the activation loop autophosphorylation of MLK3, but full activation of MLK3 and signaling to JNK requires membrane targeting by prenylation-competent, activated Cdc42. Taken together with the previous data from the lab, our current model for MLK3 activation is that the binding of the activated form of the small GTPases disrupts the autoinhibitory conformation of MLK3, promotes the homo-dimerization of MLK3 and leads to the trans-autophosphorylation of MLK3 on its activation loop, which renders MLK3 partially active. Membrane targeting induced by Cdc42 promotes full activation of MLK3 as well the downstream signaling to JNK.

The reason that the membrane targeting results in full activity of MLK3, might be due to the existence of a membrane localized MAPKKKK, which phosphorylates MLK3 at other regulatory sites that in collaboration with the activation loop

phosphorylation induced by Cdc42, fully activates MLK3. It will be interesting to identify the upstream MAPKKKK(s) that may regulate MLK3.

Since the activation of Cdc42 is promoted by GEFs and only the GTP-bound active form of Cdc42 is able to interact with MLK3, certain GEFs might be critical in the regulation of MLK3. Therefore, the regulation of MLK3 by Vav, one of the GEFs that can activate Cdc42/Rac, is investigated in Chapter 3. The work suggests that the expression of Vav indeed increases MLK3 activity as judged by MLK3 autophosphorylation and substrate phosphorylation. Surprisingly, Vav also interacts with MLK3 via at least two distinct sites within MLK3. However, Vav appears to regulate JNK activation through multiple MLKs in our experimental settings in which Vav proteins are ectopically expressed in HeLa cells.

In Vav1-/- T lymphocytes, TCR signaling to JNK is defective. Furthermore dominant negative Vav1 blocks TCR-induced JNK activation in Jurkat T cells. Recently, it has been reported that silencing of MLK3 expression by siRNA blocks the TCR-induced JNK activation in Jurkat T lymphocytes, suggesting that MLK3 is required for Vav-induced JNK activation. Further experiments need to be performed in the physiological setting of Jurkat cells will help to reveal the role of MLK3 in Vav mediated JNK activation.

The identification of the protein components of MLK3 signaling will certainly aid in understanding the regulation and signaling of MLK3. Recently, affinity purification, coupled with tandem mass spectrometry has emerged as an important technique for the identification of individual components of multiprotein complexes. Previously in the lab,

Hsp90, p50cdc37, ANT2 and clathrin heavy chain were identified as MLK3 associating proteins by using an affinity purification followed by identification of proteins corresponding to bands that were visible on Coomassie Blue staining of SDS-PAGE. Thus these identified proteins are fairly abundant in cells. To maximize the possibility of identifying low abundance protein present in MLK3 complexes, the entire lane of an SDS-PAGE for each sample was divided into 22 slices and analyzed. Indeed, many more proteins were identified from our samples. The newly identified proteins have a large variety of fuctions. They include some signaling proteins, protein phosphatases, proteins involved in cellular trafficking and protein degradation, co-chaperons of Heat shock proteins, many mitochondrial proteins and nuclear proteins. The interactions of MLK3 with some of these proteins are being confirmed in the lab and their role in MLK3 signaling is under investigation as well. These data provide new directions for studying the signaling of MLK3.

In conclusion, this work presented in this thesis provides important insight into the molecular mechanisms by which MLK3 is regulated by small GTPases and GEFs. The identification of the components of MLK3 signaling complexes accelerates the study of MLK3 signaling and leads to the understanding of the biological functions of MLK3.

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