This is to certify that the
thesis entitled

THE REGULATION OF MIXED LINEAGE KINASE 3 BY SUBCELLULAR LOCALIZATION

presented by

MARY CHAO

has been accepted towards fulfillment
of the requirements for the

MASTER OF SCIENCE degree in BIOCHEMISTRY AND MOLECULAR BIOLOGY

Kathleen A Gallo
Major Professor's Signature

11/24/03
Date

MSU is an Affirmative Action/Equal Opportunity Institution

THE REGULATION OF THE MIXED LINEAGE KINASE 3 BY SUBCELLULAR LOCALIZATION

By

Mary Chao

A THESIS

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

MASTER OF SCIENCE

Department of Biochemistry and Molecular Biology

2003

ABSTRACT

THE REGULATION OF THE MIXED LINEAGE KINASE 3 BY SUBCELLULAR LOCALIZATION

By

Mary Chao

Mixed lineage kinase 3 (MLK3) is a widely expressed mammalian serine/threonine kinase that functions as a mitogen activated protein kinase (MAPK) kinase kinase (MAPKKK) to activate the c-Jun NH_2-terminal kinase (JNK) signaling pathway. In addition, MLK3 contains various domains, including an amino terminal *Src* -homology 3 (SH3) domain, a leucine zipper, a Cdc42/Rac interactive binding (CRIB) motif and a carboxyl terminal region rich in proline, serine, and threonine.

Previous studies have demonstrated that MLK3 can associate with an activated form of the small GTPase, Cdc42, and this association requires a functional CRIB motif. Coexpression of constitutively active Cdc42 with MLK3 potentiates JNK activation and alters the *in vivo* phosphorylation pattern of MLK3. Interestingly, the activation of MLK3 by Cdc42 cannot be reproduced in an *in vitro* system using purified recombinant proteins, suggesting that a cellular environment is required.

The work presented herein demonstrates that MLK3 localizes to the perinuclear/Golgi region. Upon coexpression with prenylated, activated Cdc42, MLK3 is targeted to the plasma membrane. Activated Cdc42 that cannot be prenylated fails to target MLK3 to the plasma membrane and fails to potentiate JNK activation. This report offers important information regarding MLK3's subcellular location and provides an initial understanding on how subcellular distribution regulates MLK3 function.

To my family

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Veronica Maher, Dr. Justin McCormick, Dr. John Wang, Dr. William Kopachik, and Dr. Kathy Gallo for their valuable discussions and encouragement. I am very thankful to work in a friendly and professional environment at the Gallo lab. I am grateful for the support and the valuable discussion provided by the members of the lab, including Dr. Barbara Bock, Dr. Panayiotis Vacratsis, Dr. Hua Zhang, Karen Schachter, Yan Du, and Stancy Liou.

I am especially thankful for the endless support and guidance of Dr. Veronica Maher and my mentor Dr. Kathleen Gallo.

TABLE OF CONTENTS

List of Figures..... vii

Key to Abbreviations..... viii

I. Literature Review..... 1

1. Mammalian Protein Kinases..... 1
2. Mechanism Regulating Protein Kinases..... 3
    2.1. The Binding of Activating and Inhibiting Molecules..... 3
    2.2. Subcellular Localization..... 4
3. The c-Jun NH_2-terminal Kinase Pathway..... 6
    3.1. Mammalian MAPK Pathways..... 6
    3.2. The JNK Pathway..... 6
4. The Mixed Lineage Kinase Family..... 11
    4.1. MLK Family Members..... 11
    4.2. The Biological Functions of MLKs in Mammalian Cells..... 12
        4.2.1. MLKs in Vesicular Transport..... 12
        4.2.2. MLKs in Neuronal Apoptosis..... 13
        4.2.3. MLKs in Cell Cycle..... 14
    4.3. MLK in *Drasophila* Development..... 14
    4.4. Mixed-Lineage Kinase 3..... 14
5. Rho Family GTPases..... 18
    5.1. Functions of Rho GTPases..... 20
    5.2. Regulators of Rho GTPases..... 20
        5.2.1. Guanine Nucleotide Exchange Factors..... 21
        5.2.2. GTPase Activating Protein..... 22
        5.2.3. Guanine Nucleotide Dissociation Inhibitor..... 23
6. Objective of Thesis..... 25
7. References..... 26

II. Prenylated, GTP-bound Cdc42 Targets Mixed Lineage Kinase 3 to the Plasma Membrane to Potentiate Jnk Signaling..... 33

1. Abstract..... 33
2. Introduction..... 34
3. Experimental Procedures..... 37
    3.1. Expression Vectors, Site-Directed Mutgenensis, and Antibodies..... 37

3.2. Cell Lines and Transfections..... 38
3.3 Subcellular Fractionation..... 38
3.4. Antibodies, Immunoprecipitations, Western Blotting..... 39
3.5. Immune Complex Kinase Assay.....40
3.6. Immunofluorescence and Confocal Microsocpy..... 41
4. Results..... 42
4.1. Activated Cdc42 Targets MLK3 to a Plasma Membrane-enriched Fraction..... 42
4.2. Activated Cdc42 Targets MLK3 from an Endomembrane-enriched Fraction to a Plasma Membraen-enriched Fraction..... 43
4.3. Prenylated Cdc42V12 Directs Active MLK3 to a Plasma Membrane-enriched Fraction..... 45
4.4. Colocalization of MLK3 and Markers for the Golgi Apparatus..... 46
4.5. Prenylated, Activated Cdc42 Targets MLK3 to the Plasma Membrane.....46
4.6. Functional Targeting to the Plasma Membrane is Required for Potentiation of MLK3-induced Activation of JNK by Ccd42..... 47
5. Discussion.....57
6. References.....61

III. Concluding Remarks.....66

LIST OF FIGURES

Figure Page

1. The mammalian MAPK pathways.....9

2. Mammalian JNK scaffold complexes.....10

3. *Schematic diagram of the mixed-lineage kinase (MLK) family.....17

4. *Regulation of small GTPases.....24

5. Alignment of the COOH-termini of small GTPases and Cdc42^{Val12}.....49

6. *Effect of Cdc42 expression on the subcellular distribution and electrophoretic mobility of MLK3.....50

7. *Subcellular distribution and catalytic activity of MLK3 upon coexpression with Cdc42^{Val12} variants.....51

8. Ability of Cdc42 variants to associate with MLK3.....53

9. Effect of Cdc42^{Val12} on subcellular localization of MLK3.....54

10. Effect of prenylation competence of activated Cdc42 on subcellular localization of MLK3.....55

11. Effect of Cdc42^{Val12} variants on MLK3-induced JNK activation.....56

* Images are presented in color

KEYS TO ABBREVIATIONS

ACK	Cdc42HS-associated kinase
AKAP	A-kinase anchoring protein
ASK1	Apoptosis signal regulating kinase
ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3'-5' cyclic monophosphate
CDK	Cyclin dependent protein kinase
CH	Calponin homology
COP	Coatamer complex protein
CRIB	Cdc42/Rac interactive binding
Dbl	Diffuse B-cell lymphoma
DLK	Dual-leucine zipper bearing kinase
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine5'-diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine 5'-triphosphate
IKK	IκB-kinase
JIP	JNK-interacting protein
JNK	c-Jun NH_2-terminal kinase
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MLK	Mixed lineage kinase
NGF	Neuronal growth factor
PAK	p21-activated kinase
PH	Pleckstrin homology
PKA	Protein kinase A
POSH	Plenty of SH3
SAPK	Stress-activated protein kinase
SH3	Src-homology 3
TAK1	Transforming growth factor activating kinase
WASP	Wiskott Aldrich syndrome protein
ZAK	Zipper sterile-α-motif kinase

I. Literature Review

1. Mammalian Protein Kinases

Cellular signal transduction refers to the network of events that allows cells to respond coordinately to multiple, divergent extracellular stimuli. A relatively simple signal transmission event is exemplified by ion channels, which upon ligand binding, allow the movement of ions into a cell or a cellular organelle that, in turn, changes the electrical potential of the cell. The coupling of ligand-receptor interactions to many intracellular signaling newtworks, including protein kinase cascades, is much more complex.

Protein kinases represent one of the largest protein superfamilies. Based on the current draft of the human genome, it is estimated that the human genome encodes 518 kinases [1]. Protein kinases catalyze the transfer of the γ-phosphate of ATP to amino acid residues in protein substrates. In physiological systems, protein phosphorylation is rendered reversible through the action of protein phosphatases. Most mammalian protein kinases phosphorylate hydroxy amino acids of their protein substrates. The serine/threonine protein kinases catalyze the phosphorylation of serine/threonine residues in protein substrates, whereas tyrosine protein kinases are specific for phosphorylating only tyrosine residues in their substrates. A small group of protein kinases, capable of targeting both serine/threonine residues as well as tyrosine residues, are known as the dual specific kinases [2, 3]. Finally, there is some evidence, though still controversial, for the existence of mammalian histidine kinases, which phosphorylate the nitrogen atoms at position 1 and/or 3 in the imidazole ring of the histidine residue, forming a

phosphoramidate bond. While histidine kinases have been definitively identified in prokaryotes, fungi and plants, the function and existence of histidine kinases in mammals is still under debate [4, 5].

The catalytic domains of eukaryotic tyrosine and serine/threonine kinases are highly conserved both in primary sequence and in tertiary structure. Based on crystal structures of the catalytic domains of several protein kinases, the kinase domain, of approximately 250 amino acids forms a two-lobed structure with conserved critical catalytic residues. The amino-terminal lobe contains mainly β strands with one conserved helix and includes the critical residues for interaction with ATP. The carboxyl terminal lobe contains mainly α helices and functions in substrate recognition and binding. Residues associated with substrate binding diverge among different protein kinases, while the sequences involved in the active site stability and MgATP-binding are highly conserved, supporting the idea that protein kinases are evolutionarily conserved and share a similar catalytic mechanism (reviewed in [6]).

2. Mechanisms Regulating Protein Kinases

Protein kinases regulate virtually all cellular processes, including transcription, cell cycle progression, metabolism, cell motility, apoptosis, and cytoskeletal organization. Since protein kinases direct such critical cellular functions, it is not surprising that protein kinases themselves are tightly regulated. Protein kinases are commonly maintained in an inactive state in cells until activated in response to an extracellular signal or environmental stimulus. The activities of protein kinases are regulated through several different mechanisms including the binding of activators or inhibitors, post-translational modifications such as phosphorylation, and changes in subcellular localization.

2.1. The Binding of Activating and Inhibiting Molecules

The allosteric binding of activating and inhibiting molecules is a well-characterized mechanism regulating the activities of protein kinases. For example, cyclin dependent protein kinases (CDKs) are nuclear serine/threonine kinases that control cell cycle progression in eukaryotic cells. CDK is active when bound to cyclins whereas binding to CDK inhibitors (CDKI), such as p27 or p16, inhibits CDK activity [7, 8].

In addition, certain protein kinases can be activated by binding to small molecules, such as cyclic nucleotides, cAMP and cGMP. Protein kinase A (PKA) is a tetrameric holoenzyme consist of two regulatory and two catalytic subunits [9]. PKA is maintained in an inactive state by binding of its regulatory subunit to the substrate binding site in the absence of cAMP. Binding of cAMP to the regulatory subunit allows the dissociation of the catalytic subunit from the regulatory subunit and the catalytic subunit is thus rendered active [9].

2.2. Subcellular Localization

Compartmentalization may restrict the access of a protein kinase to its substrate to achieve specificity of a ligand-mediated response. Distinct protein modules have been demonstrated to coordinate the subcellular localization of protein kinases. Herein, two well-studied protein kinases, protein kinase A (PKA) and the serine/threonine protein kinase, Raf, will be discussed as examples of protein kinases that are regulated by subcellular localization.

The serine/threonine kinase, Raf, participates in a growth promoting signal transduction pathway. For instance, activation by epidermal growth factor (EGF) results in EGF receptor dimerization and trans-autophosphorylation of the tyrosine residues in the cytoplasmic tail of the receptor. The activated receptor recruits the adaptor-guanine nucleotide exchanger factor complex, Grb2-Sos, which activates Ras at the plasma membrane. The activated Ras, in turn, recruits Raf to the plasma membrane (reviewed in [10, 11]). The membrane targeting of Raf is required for its full activation. Appending a membrane-targeting motif to Raf (Raf-*CAAX*) keeps it constitutively active. In addition, Raf-*CAAX* is insensitive to both dominant negative Ras and activated Ras [12, 13], suggesting that membrane-targeting is a key mechanism regulating Raf activation.

PKA, on the other hands, utilizes the A-kinase anchoring proteins (AKAPs) to ensure proper association with subcellular components. AKAPs are a family of signaling molecules that contain an anchoring motif that interacts with the regulatory subunit of PKA, and a targeting domain that associates with various subcellular structures. AKAPs vary in size and affinity for various subcellular components [14, 15]. The specific

subcellular targeting of a kinase enables one enzyme to regulate a variety of physiological processes in a coordinated manner.

3. The c-Jun NH_2-terminal Kinase Pathway

3.1. Mammalian MAPK Pathways

Mitogen-activated protein kinase (MAPK) signal transduction pathways are evolutionarily conserved in eukaryotic cells. They are important mediators of cell signaling in response to changes in the extracellular environments. The MAPK pathways regulate a variety of cellular processes including proliferation, differentiation, and gene expression (reviewed in [16-18]). The best-characterized MAPK pathways in mammalian cells are the extracellular signal regulated kinase (ERK), p38, and the c-Jun NH_2-terminal kinase (JNK) pathways (Fig.1). MAPK cascades involve the sequential activation of a MAPK kinase kinase (MAPKKK), a dual specific MAPKK (MKK), and a MAPK. Extracellular stimulation leads to MAPKKK phosphorylation and activation. This activation event usually is mediated through a small GTPase. The activated MAPKKK phosphorylates a serine and a threonine residue in the activation segment of a dual specific MAP kinase kinase (MAPKK) leading to its activation, which, in turn, phosphorylates a threonine and a tyrosine residue within the activation segment of the MAPK, leading to its activation.

3.2. The JNK pathway

JNK, also known as stress-activated protein kinase (SAPK), was initially characterized as being activated in the liver upon treatment with a protein synthesis inhibitor, cycloheximide. Subsequent studies illustrate that JNK is activated in response to various forms of environmental stress, such as heat shock, exposure to inflammatory cytokines, hyperosmolarity, and ultraviolet irradiation (reviewed in [19-22]. Ten

isoforms of JNK resulting from alternative splicing of three mammalian genes, *Jnk1*, *Jnk2*, and *Jnk3*, exist. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is predominantly expressed in the brain [23]. Only two MKKs, MKK4 and MKK7, have been shown to phosphorylate and activate JNK. However, MKK4/7 have been shown to be phosphorylated by several MAPKKKs, including MLK family members [24], transforming growth factor activating kinase (TAK1) [25], apoptosis signal-regulating kinase (ASK1) [26], and mitogen-activated protein/ERK kinase kinase (family members MEKK1-4) [27], demonstrating the complexity of JNK signaling. The substrates for JNK are predominantly transcription factors that reside in the nucleus. JNK phosphorylates the activation domains of several transcription factors including, c-Jun, Elk1, and ATF2 (reviewed in [28]).

Genetic studies of *Drosophila* have demonstrated that JNK is required for early embryonic development [29]. In mammals, JNK has been implicated in oncogenic transformation [30], apoptosis [31], and immune responses [31]. JNK knockout mice show various defects in immune responses and apoptosis. However, none of the targeted knockouts of individual JNK genes (*Jnk1, Jnk2, Jnk3*) results in lethality, suggesting that different JNK isoforms may have overlapping functions [21].

As an emerging feature of signal transduction pathways in general, that applies to the JNK pathway as well, is organization through scaffold proteins. Although scaffold proteins lack enzymatic activity, they often function to promote efficient activation of their specific components. JNK-interacting protein (JIP) was first identified as a binding partner for JNK1 in a yeast two-hybrid analysis [32]. It contains an NH_2-terminal JNK binding domain and a potential SH3 domain at the COOH-terminus. Whitmarsh *et al.*

demonstrated that JIP-1 facilitates JNK signal transmission through specific binding to three components of the pathway including MLKs, MKK7 but not MKK4, and JNK [33] (Fig. 2).

Currently, three JIPs have been identified. JIP1 is ubiquitously expressed in human tissues, while JIP2 is predominantly expressed in the brain [34]. JIP3 is expressed in the brain, lung and heart [35]. JIP1 and JIP2 are structurally related, whereas JIP3 is structurally distinct. It has been suggested that JIPs potentiate the activation of JNK by facilitating the assembling of signaling pathway components [34, 35]. Other studies suggest that JIP1-associated dual leucine zipper-bearing kinase (DLK) is monomeric and catalytically inactive. JNK facilitates the dimerization and activation of JIP-bound DLK. Regulation of JNK signaling through JIPs is not completely understood and is likely to be complex [36].

In addition to JIPs, Plenty of SH3s (POSH) has been identified as a scaffold protein linking active, GTP-bound Rac to JNK signal transduction pathways triggering neuronal apoptosis [37]. POSH complexes directly with activated Rac1, MLKs, and indirectly with MKK4/7 and JNKs. The overexpression of POSH promotes apoptotic neuronal death which can be suppressed by dominant negative forms of MLKs, MKK4/7, and c-Jun [37].

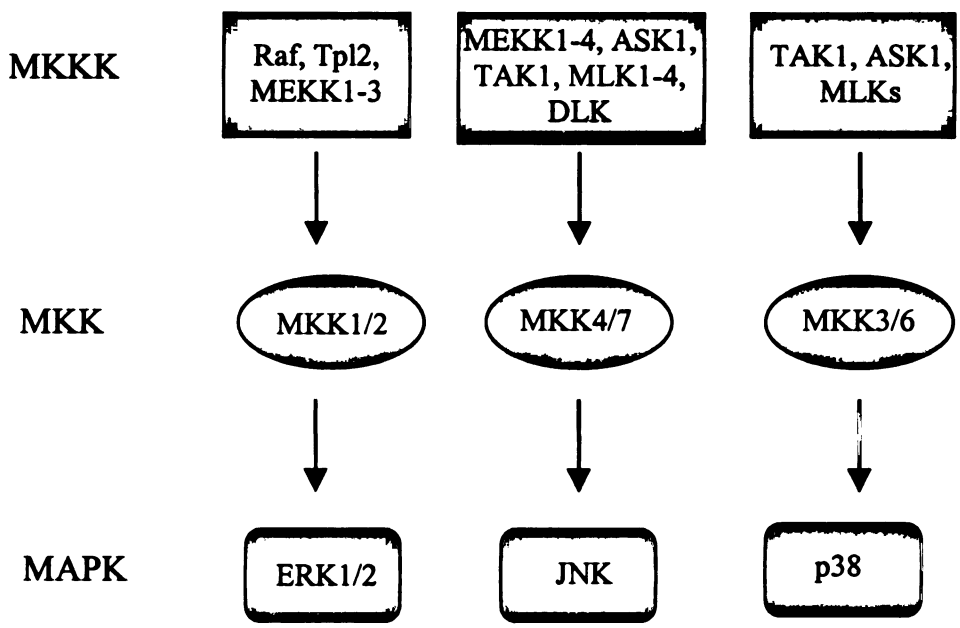


Fig. 1. **The Mammalian MAPK Pathways.** In mammalian cells, there are three well-defined MAPK pathways: ERK, JNK, and p38. The MAPK pathways are composed of three sequentially activated protein kinases: MKKK, MKK, and MAPK.

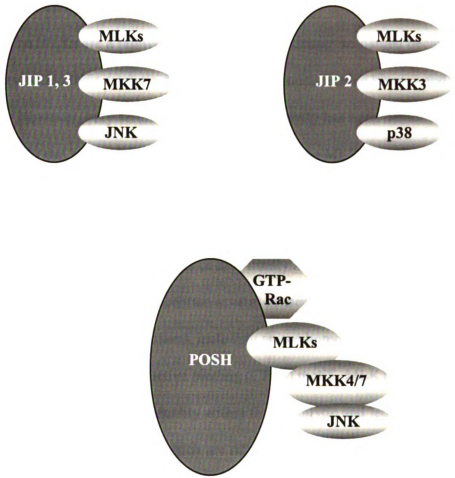


Fig. 2. **Mammalian JNK Scaffold Complexes**. The scaffold proteins JIP1/3 specifically bind to MLKs, MKK7, and JNK to coordinate the JNK signaling cascade. JIP2 binds to MLKs, MKK3, and p38. POSH interacts with activated GTP-bound Rac and MLKs directly and MKK4/7 and JNK indirectly.

4. The Mixed Lineage Kinase Family

4.1. MLK Family Members

The MLKs are a family of serine/threonine kinases so-named because of sequence similarity within their catalytic domain to both serine/threonine kinases and tyrosine kinases. However, only serine/threonine kinase activity has been demonstrated. The MLK family can be clustered into three subfamilies: the MLKs; the dual-leucine-zipper-bearing kinases (DLKs); and zipper sterile-α-motif kinase (ZAK) (reviewed in [24]) (Fig. 3).

The MLK subfamily consists of four family members, MLK1-4. In addition to a kinase catalytic domain, MLKs contain several conserved structural domains that may mediate protein-protein interactions, including an *src*-homology (SH3) domain, a leucine zipper, and a Cdc42/Rac interactive binding (CRIB) motif. MLK1-MLK4 share approximately 75% sequence identity within their catalytic and leucine zipper domains. However, their COOH termini, which are rich in serine, threonine, and proline, are highly divergent, indicating a possible role in regulatory functions.

The MLK family members function as MAPKKKs to activate the JNK pathway via phosphorylation and activation of an MKK, such as MKK4 and MKK7 [38-40]. Of the MLK subfamily, only MLK2 and MLK3 have been studied in any detail. MLK1 mRNA has been detected in epithelial cell lines of breast, colonic and esophageal origin [41] and shows differential expression of mRNA in pancreatic β-cells as a function of development. MLK2 mRNA has been detected at high levels in human brain, skeletal muscle, and testis [41]. Northern blot analysis indicated that the MLK3 mRNA is widely expressed in various tissues, with lower expression in the heart and brain [55].

4.2. The Biological Functions of MLKs in Mammalian Cells

4.2.1. MLKs in Vesicular Transport

Immunostaining of MLK2 expressed in Swiss 3T3 cells demonstrated that MLK2 colocalizes with active, dually phosphorylated JNK along microtubules [42]. Yeast two-hybrid analysis identified KIF3A, B, and X and KAP3A as potential MLK2 interacting proteins. KIF3s are members of the kinesin superfamily of motor proteins that transport vesicles along microtubules toward the plus end, whereas KAP3A recruits cargo to the motor protein complex [43]. It appears that MLK2 can associate with both the motor molecule itself and the putative cargo recognition component of the KIF motor complex. Furthermore, MLK2, together with MKK7 and JNK, binds to the JNK scaffolding protein, JIP, which can associate with kinesin light chain, as well as with the transmembrane receptor molecule ApoER2 [44, 45]. These results suggest that the JIP scaffolding complex may be transported along microtubules to potential site of activity and thus regulating the subcellular distribution.

MLK2 can bind to dynamin [46], a GTPase which is involved in vesicle formation during endocytosis, synaptic transmission, and secretion [47]. The function of this interaction has not been well described. Finally, mass spectrometric analysis has identified clathrin as a MLK2 binding protein in cultured cells and mammalian brain [48]. MLK2 binds to clathrin heavy chain and is enriched in clathrin-coated vesicles, suggesting a role for MLK2 in vesiclar transport processes.

4.2.2. MLKs in Neuronal Apoptosis

MLK3, along with MLK2 and DLK, have been implicated in neuronal apoptosis involving the JNK pathway. Ectopic expression of MLK2, MLK3 or DLK induces apoptosis in neuronal-like PC12 cells, whereas a catalytically inactive MLK3 effectively blocked neuronal death caused by neuronal growth factor (NGF) deprivation [49]. In addition, CEP-1347, an inhibitor of the MLK family, protected neuronal PC12 cells from MLK-mediated apoptosis induced by overexpression of MLK family members. Overexpression of MLK3 in superior cervical ganglion (SCG) sympathetic neurons activated JNK and induced apoptosis, whereas catalytically inactive MLK3 blocked SCG neuron apoptosis in response to NGF withdrawal [50].

Another study has indicated that MLK2/3 can associate with the SH3 domain of the postsynaptic density protein (PSD-95), which is involved in kainate receptor glutamate receptor 6 (GluR6)-mediated JNK activation and apoptosis [51]. Coexpression of catalytically inactive MLK2/3 significantly attenuated GluR6/PSD-95-mediated neuronal apoptosis. PSD-95 with a deficient SH3 domain inhibited GluR-induced JNK activation and neuronal apoptosis.

Finally, a yeast two-hybrid study has identified plenty of SH3s (POSH) as a scaffold protein that binds activated Rac1 and MLKs to potentiate JNK signaling [52]. Overexpression of POSH induced JNK activation and promoted neuronal apoptosis in PC12 cells and in sympathetic neurons. Catalytically inactive MLKs and CEP-1347 suppressed neuronal apoptosis induced by POSH overexpression [37].

4.2.3. MLKs in Cell Cycle

Distinct from its function in the JNK pathway, MLK3 may be involved in cell cycle regulation, inducing microtubule instability at the G_2/M transition [53]. Using a bioinformatics approach, MLK3 was identified as homologous to the non-catalytic region of NIMA, a fungal serine/threonine kinase that is essential for regulating G_2/M transition in *A. nidulans*. The study showed that phosphorylation and activity of MLK3 are enhanced at G_2/M, while JNK remains inactive. MLK3 is localized at the centrosome during interphase of mitosis and its overexpression disrupts microtubules.

4.3. MLKs in *Drosophila* Development

Recent genetic analysis of *slpr* indicated that it encodes a *Drosophila* MLK homologous to the mammalian MLKs. Using mutants in the *slpr* gene, Stronach *et al.* demonstrated that Slpr is required for JNK activation during dorsal closure in *Drosophila* embryonic development [54], suggesting a role for MLK in controlling epithelial cell morphogenesis and development..

4.4. Mixed-Lineage Kinase 3

MLK3, formerly called SH3-domain-containing proline-rich kinase (SPRK) and protein tyrosine kinase 1 (PTK1), consists of 847 amino acid residues and has a predicted molecular weight of 93 kDa [55-57]. Northern blot analysis indicated that the MLK3 mRNA is widely expressed in various tissues, with lower expression in the heart and brain [55]. The overexpression of MLK3 in mammalian cells can activate JNK via MKK4 and/or MKK7. In addition, it binds to JNK scaffold proteins, JNK interacting protein 1 (JIP), JIP2, and JIP3. JIP proteins organize JNK1/2, MLKs, and MKK7 into a

specific signaling module and thereby potentiate JNK activation. MLK3 has also been shown to activate the MAPK p38 via MKK3/6 [58] as well as ERK [59]. Ceramide and TNF-α have been recently reported as agonists of mammalian MLK3, resulting in increased MLK3 kinase activity and JNK activation which was blocked by a pan-MLK inhibitor, CEP-1347 [60]. In some cell types, MLK3 has been shown to function as an IκB kinase kinase(IKKK) to positively regulate the NF-κB pathway in response to Jurkat T cell receptor costimulation [61].

MLK3 contains several domains that can potentially mediate protein-protein interactions including an NH_2-terminal SH3 domain, a leucine zipper, a CRIB motif, and COOH-terminal proline, serine, and threonine-rich region. The activities of MLK3 are regulated, at least in part, by protein-protein interactions. MLK3 is autoinhibited by an interaction between its SH3 domain and a short proline-containing sequence that is located between its zipper and CRIB motif [62]. Mutation of the conserved tyrosine residue 52 to an alanine in the SH3 domain, disrupts SH3 ligand binding and increases the catalytic activity of MLK3. Mutation of the single proline residue in the SH3 binding region prevents SH3 binding and increases the catalytic activity of MLK3. SH3-mediated autoinhibition is likely a characteristic of MLK1-4 since the proline residue critical for this interaction is conserved in all of these MLKs.

Zipper-mediated oligomerization is critical for MLK3 activation of JNK [63, 64]. The autophosphorylation and histone phosphorylation activity of a monomeric MLK3 containing a mutated leucine zipper can be induced when coexpressed with activated Cdc42. However, this monomeric, activated MLK3 fails to induce JNK signaling due to its inability to properly phosphorylate a downstream substrate, MKK4.

Coexpression with activated variants of the small Rho family GTPases, Cdc42 and Rac, potentiates MLK3 activity [65, 66]. The precise mechanism by which small GTPases activate MLK3, and presumably other CRIB-containing MLKs, is yet to be elucidated. It would be interesting to study the prospect that an SH3-mediated intramolecular interaction could inhibit MLK3 dimerization, while relief of autoinhibition, possibly by activated Cdc42, could induce dimerization.

A role for phosphorylation is suggested by the finding that the *in vivo* phosphorylation of MLK3 is altered by expression with activated Cdc42 [65, 67]. MLK3's CRIB motif is required for the Cdc42-mediated potentiation of MLK3 activity [65] and MLK3 and activated forms of Cdc42 associate in coimmunoprecipitation assays [68] suggesting that the GTPase directly associates with MLK3, at least transiently, to impact MLK3 activity.

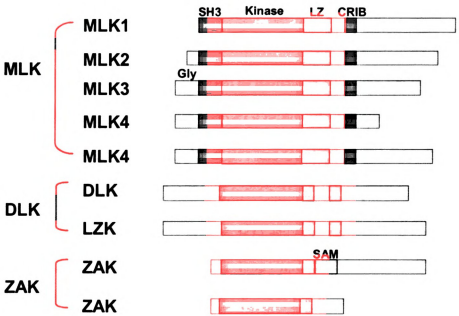


Fig. 3. **Schematic diagram of the Mixed-Lineage Kinase (MLK) family**. *Gly* denotes glycine-rich region. *SH3* represents *src*-homology 3 domain. *L* denotes leucine zipper. *CRIB* stands for Cdc42/Rac Interactive Binding. *SAM* represents sterile alpha motif.

5. Rho Family GTPases

The Rho GTPases are a subfamily of the Ras superfamily of small GTP-binding proteins that have been implicated in a wide variety of cellular functions. These proteins with molecular masses of 20-40 kDa are found in all eukaryotes from yeast to man (reviewed in [69-72]). The Rho GTPases function as molecular switches, cycling between an active GTP-bound form and an inactive GDP-bound form (Fig. 4). Among the 16 members of the Rho GTPase family, Rho, Rac and Cdc42, are best characterized.

All small GTP-binding proteins have consensus amino acid sequences responsible for specific interaction with GDP and GTP and for GTPase activity, which hydrolyzes bound GTP to GDP and P_i. The switch I, II, and p-loop regions constitute the GTP-binding core of the GTPases. Mutations in regions involved in the GTP binding and hydrolysis such as Cdc42G12V and Cdc42Q61L decrease the intrinsic GTPase activity rendering the small GTPases constitutively active [69].

The effector domain of small GTPases is required for downstream signaling. For example, a predominant binding partner for the effector domain of Cdc42 and Rac is the Cdc42/Rac interacting binding (CRIB) motif present in downstream effectors, such as the p21-activated kinase (PAK) family of protein kinases, Wiskott Aldrich Syndrome protein (WASP), activated Cdc42HS-assoicated kinase (ACK) and MLKs [68]. However, Cdc42 can associate with other signaling proteins through regions other than CRIB motifs. In addition, Rho GTPases contain an unique Rho insert domain that can interact with downstream effectors as well as with its guanine nucleotide dissociation inhibitors (GDIs) [72].

Small GTPases contain *CAAX* (*C* is the cysteine residue, *A* represents aliphatic residues, and *X* can be any amino acid residue) motifs at their extreme COOH terminus. Prenylation is a posttranslational lipidation process whereby a farnesyl or geranylgeranyl isoprenoid is covalently attached to the COOH-terminal cysteine residues in the *CAAX* motif of a target protein through the action of a prenyl transferase [73]. When X of the *CAAX* motif is a leucine or a methionine residue, a geranylgeranyl moiety will be added to the small GTPase. Prenylation allows the association of the small GTPase with cellular membranes. After prenylation, small GTPases are proteolytically processed by a carboxypeptidase that removes the –AAX residues. Finally the terminal carboxylate is methylated by a carboxymethyl transferase [76]. The prenylation step occurs in the cytosol [76], whereas the proteolysis and carboxymethylation steps occur at the surface of the endoplasmic reticulum [77].

However, prenylation does not stably anchor a protein into the lipid biolayer. This requires a second membrane-targeting signal in the so-called hypervariable region immediately upstream of the *CAAX* motif [78]. The second component of membrane localization signals typically contains either a site for reversible palmitoylation, as found in H-Ras and N-Ras [79] or a series of basic residues, as found in K-Ras [78]. In the case of the well-studied Ras family, the *CAAX* modification may provide initial weak association of the Ras protein with the plasma membrane and allowing H-Ras and N-Ras access to the palmitoylation machinery, whereas the positively charged polybasic sequences in K-Ras can interact electrostatically with the head groups of anionic phospholipids in the plasma membrane. The combination of the *CAAX* motif and a

palmitoylation site or polybasic sequences in the hypervariable region is sufficient for plasma membrane targeting [80].

Interestingly, a Cdc42 variant, in which the cysteine in the *CAAX* motif is mutated to serine, can rescue the lethal phenotype induced by constitutively active Cdc42 in yeast [74]. The disruption of prenylation of the small GTPase Ras prevents its membrane targeting, keeping it inactive and cytosolic.

5.1. Functions of Rho GTPases

Cellular functions of various Rho GTPases are multiple and complex. Constitutively activated mutants of Rho were found to induce the assembly of contractile actin and myosin filaments (stress fibers) when introduced into fibroblasts. Activated Rac was shown to promote the formation of lamellipodia, while Cdc42 induces the formation of finger-like membrane filopodia [69, 70]. Rac is required for the correct assembly of extracellular laminin and therefore influences the orientation of the apical-basal axis. Cdc42 and Rac positively regulate neurite outgrowth by promoting membrane protrusion whereas Rho inhibits neurite extension by promoting membrane retraction. In addition to their effects on cellular morphology, Rho family members also have been implicated in apoptosis, cell motility, cell cycle progression, and phagocytosis [81].

5.2. Regulators of Rho GTPases

Three classes of regulatory proteins control the active versus the inactive states of Rho GTPases: guanine nucleotide exchange factors (GEFs) which act as a positive regulator of Rho GTPase function by promoting the active GTP-bound form of

RhoGTPase; GTPase-activating proteins (GAPs) which enhance the intrinsic GTP hydrolysis and lead to the inactive GDP-bound Rho GTPase; guanine nucleotide dissociation inhibitors (GDIs) which sequester the inactive GDP-bound Rho GTPase and regulate intracellular distribution.

5.2.1. *Guanine Nucleotide Exchange Factors*

Since small GTPases control many aspects of cellular processes, it is not surprising that they are themselves highly regulated. GEFs facilitate the exchange of GDP for GTP to promote the activated form of small GTPases. The first mammalian GEF, Dbl, was isolated as an oncogene in an NIH 3T3 focus formation assay [82]. Dbl, which contains a conserved DH (Dbl homology) domain, can catalyze nucleotide exchange on human Cdc42 [83]. The DH domain was subsequently shown to be necessary for GEF activity [84]. Two conserved helices of the DH domains participate in the formation of the GTPase interaction pocket where the GDP-bound form of small GTPase binds to the GEF. The association with the GEF destabilizes the GDP-GTPase complex while stabilizing a nucleotide-free reaction intermediate. The ten-fold higher intracellular concentration of GTP versus that of GDP, therefore, facilitates the GTP-bound, active form of the GTPase.

Many GEFs are autoinhibited through intramolecular interactions. It is assumed that activation of full length GEFs is achieved through the relief of autoinhibition by phosphorylation or by binding to other proteins. One of the best-understood examples is Vav. Vav possesses a calponin homology (CH) domain and an acidic region, followed by the DH-PH module, a zinc finger domain, a proline-rich region and an SH2 domain flanked by two SH3 domains. Truncation of the first 66 amino acid of Vav renders it

constitutively active. The three conserved tyrosine residues, Tyr142, Tyr160, and Tyr174, in the acidic region are critical for phosphorylation-dependent activation of Vav. Structural analysis reveals that the N-terminal region of Vav interacts directly with the GTPase-interaction pocket of the DH domain and block substrate access. Phosphorylation of Tyr174 relieves the inhibition and allowing access to small GTPases (Reviewed in [85]).

Almost all GEFs also possess a pleckstrin homology (PH) domain that is located immediately downstream of the DH domain. The PH domains may target GEFs to their appropriate subcellular locations through phospholipids binding that may spatially control GEF activity [86].

Aside from the DH-PH modules, the distinct domains including SH2 and SH3 domains that are present in each Dbl family of GEFs suggest that they participate in the complicated signaling networks via interaction with other proteins.

5.2.2. *GTPase Activating Protein*

One common feature of the small GTPases is that they have low intrinsic GTPase activity. However, GAPs can stimulate GTP hydrolysis on Rho GTPases to down regulate the activities of the small GTPases. RhoGAPs contain a conserved RhoGAP domain that is distinct from the GAP domains of other GAPs, such as Ras GAP. The RhoGAP domain is sufficient for binding to the GTP-bound form of Rho GTPase to augment GTP hydrolysis and, hence, inactivate the Rho GTPases.

GAPs directly participate in the hydrolysis of GTP by inserting a highly conserved arginine residue to the active site of Rho GTPases, which comprises the switch I, II, and p-loop regions (reviewed in [87, 88]). The residue Gln 61 of the Rho GTPase

positions the water molecule for catalysis. The interaction between arginine residue of GAPs and Gln 61 of the GTPase reduces the energy barrier and accelerates GTP hydrolysis. Interestingly, the switch II region is in close proximity with the *CAAX* motif, suggesting that the nucleotide status of the GTPase may also influence its membrane association.

5.2.3. *Guanine Nucleotide Dissociation Inhibitors*

A number of studies have indicated that Rho GTPase activation is accompanied by its intracellular translocation. One means to regulate a Rho GTPase may be to control its association with the membrane compartments. GDIs have been demonstrated to sequester the posttranslationally modified GDP-bound Rho GTPase via binding to the prenylated COOH-terminus preventing the interaction with membranes of the Rho GTPase [89]. Therefore, the Rho GTPase-GDI complex constitutes a cytosolic population of inactive GDP-bound Rho GTPase. The membrane association and activation of Rho GTPases require dissociation from their corresponding GDIs (reviewed in [90]).

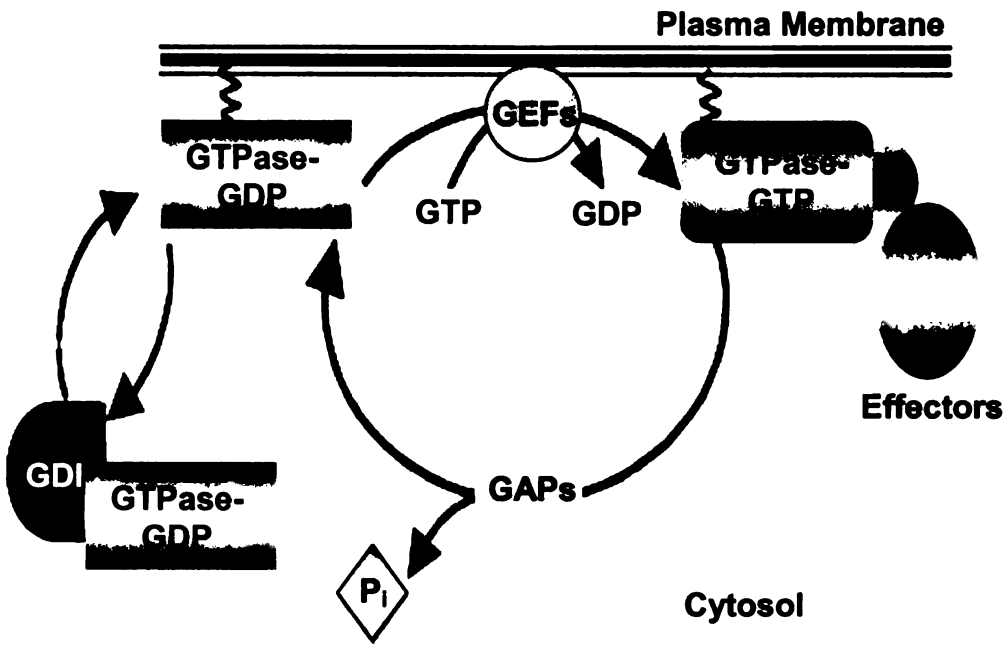


Fig. 4. **Regulation of Small GTPases.** The Rho GTPases function as molecular switches, cycling between active GTP-bound forms and inactive GDP-bound forms. GEFs facilitate the exchange of GDP to GTP to promote the activated form of small GTPases. GAPs stimulate GTP hydrolysis of Rho GTPases. GDIs sequester the GDP-bound Rho GTPases in the cytosol.

6. Objective of Thesis

Protein kinases regulate a variety of cellular processes, ranging from cellular motility to gene expression. Thus, the mechanisms that control the activities of protein kinases are critical to the function and survival of cells.

The serine/threonine kinase MLK3, in addition to its catalytic domain, contains several domains that have the potential for regulating the activities and signaling of MLK3. Previous work has explored the functions of the SH3 domain, zipper region, and the CRIB motif. However, relatively little is know about the subcellular localization of MLK3. This thesis describes the work that explores the regulation of MLK3 by subcellular targeting.

Chapter I briefly describes our understanding of protein kinase regulation. A brief synopsis of MAPK pathways focuses on the JNK signaling pathway and its regulation by scaffold proteins. The current state of knowledge regarding the various MLK members and their biological functions is described. The final portion of the literature review summarizes the functions of the Rho family of small GTPases and the mechanisms that regulate their activities.

Chapter II presents the investigation of the effects of Cdc42 on MLK3's subcellular localization and addresses the role of Cdc42-mediated plasma membrane targeting of MLK3 in the activation of MLK3 and its downstream activation of JNK.

7. Refe

1. M
S

2. L
a

3. D
k

4. C
M

5. S
n
1

6. B
3

7. C
a

8. S
2

9. T
fr
5

10. S
g

11. H
M

12. L
a
3

13. St
m

14. Fr
A

7. References

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

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

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

4. Grebe, T.W. and J.B. Stock, *The histidine protein kinase superfamily.* Adv Microb Physiol, 1999. **41**: p. 139-227.

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

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

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

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

9. Taylor, S.S., J.A. Buechler, and W. Yonemoto, *cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes.* Annu Rev Biochem, 1990. **59**: p. 971-1005.

10. Schrader, J.W., et al., *Activation of small GTPases of the Ras and Rho family by growth factors active on mast cells.* Mol Immunol, 2002. **38**(16-18): p. 1181-6.

11. Hancock, J.F., *Ras proteins: different signals from different locations.* Nat Rev Mol Cell Biol, 2003. **4**(5): p. 373-84.

12. Leevers, S.J., H.F. Paterson, and C.J. Marshall, *Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane.* Nature, 1994. **369**(6479): p. 411-4.

13. Stokoe, D., et al., *Activation of Raf as a result of recruitment to the plasma membrane.* Science, 1994. **264**(5164): p. 1463-7.

14. Fraser, I.D. and J.D. Scott, *Modulation of ion channels: a "current" view of AKAPs.* Neuron, 1999. **23**(3): p. 423-6.

15.

16.

17.

18.

19.

20.

21.

22.

23.

24.

25.

26.

27.

28.

29.

15. Colledge, M. and J.D. Scott, *AKAPs: from structure to function.* Trends Cell Biol, 1999. **9**(6): p. 216-21.

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

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

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

19. Barr, R.K. and M.A. Bogoyevitch, *The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs).* Int J Biochem Cell Biol, 2001. **33**(11): p. 1047-63.

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

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

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

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

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

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

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

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

28. Davis, R.J., *Transcriptional regulation by MAP kinases.* Mol Reprod Dev, 1995. **42**(4): p. 459-67.

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

30.

31.

32.

33.

34.

35.

36.

37.

38.

39.

40

4

30. Rennefahrt, U.E., et al., *Constitutive JNK activation in NIH 3T3 fibroblasts induces a partially transformed phenotype.* J Biol Chem, 2002. **277**(33): p. 29510-8.

31. Lin, A. and B. Dibling, *The true face of JNK activation in apoptosis.* Aging Cell, 2002. **1**(2): p. 112-6.

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

33. Whitmarsh, A.J., et al., *Requirement of the JIP1 scaffold protein for stress-induced JNK activation.* Genes Dev, 2001. **15**(18): p. 2421-32.

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

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

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

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

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

39. Hirai, S., et al., *MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase.* J Biol Chem, 1997. **272**(24): p. 15167-73.

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

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

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

43. Y
st
9

44. S
P

45. V

46.

47.

48.

49.

50.

51.

52.

53.

54.

55

43. Yamazaki, H., et al., *Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B.* Proc Natl Acad Sci U S A, 1996. **93**(16): p. 8443-8.

44. Stockinger, W., et al., *The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2.* J Biol Chem, 2000. **275**(33): p. 25625-32.

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

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

47. Urrutia, R., et al., *The dynamins: redundant or distinct functions for an expanding family of related GTPases?* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 377-84.

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

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

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

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

52. Tapon, N., et al., *A new rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-kappaB signalling pathways.* Embo J, 1998. **17**(5): p. 1395-404.

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

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

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

56. I
b
1

57. E
n

58. T
S

59. H
l
C

60. S
R
2

61. H
i

62. Z
S

63. L
f
E

64. V
l
4
L
M
2

65. B
v
p

66. T
C
r
m

67. V
m
Sc

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

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

58. Tibbles, L.A., et al., *MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6.* Embo J, 1996. **15**(24): p. 7026-35.

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

60. Sathyanarayana, P., et al., *Activation of the Drosophila MLK by Ceramide Reveals TNF-alpha and Ceramide as Agonists of Mammalian MLK3.* Mol Cell, 2002. **10**(6): p. 1527-33.

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

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

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

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

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

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

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

68.

69.

70.

71.

72.

73.

74.

75.

76.

77.

78.

79.

80.

81.

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

69. Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. **420**(6916): p. 629-35.

70. Hall, A. and C.D. Nobes, *Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton.* Philos Trans R Soc Lond B Biol Sci, 2000. **355**(1399): p. 965-70.

71. Van Aelst, L. and C. D'Souza-Schorey, *Rho GTPases and signaling networks.* Genes Dev, 1997. **11**(18): p. 2295-322.

72. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.

73. Hancock, J.F., et al., *A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins.* Embo J, 1991. **10**(13): p. 4033-9.

74. Ziman, M., et al., *Mutational analysis of CDC42Sc, a Saccharomyces cerevisiae gene that encodes a putative GTP-binding protein involved in the control of cell polarity.* Mol Cell Biol, 1991. **11**(7): p. 3537-44.

75. Seabra, M.C., *Membrane association and targeting of prenylated Ras-like GTPases.* Cell Signal, 1998. **10**(3): p. 167-72.

76. Casey, P.J. and M.C. Seabra, *Protein prenyltransferases.* J Biol Chem, 1996. **271**(10): p. 5289-92.

77. Pillinger, M.H., et al., *Characterization of a plasma membrane-associated prenylcysteine- directed alpha carboxyl methyltransferase in human neutrophils.* J Biol Chem, 1994. **269**(2): p. 1486-92.

78. Hancock, J.F., H. Paterson, and C.J. Marshall, *A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane.* Cell, 1990. **63**(1): p. 133-9.

79. Kloc, M., X.X. Li, and L.D. Etkin, *Two upstream cysteines and the CAAX motif but not the polybasic domain are required for membrane association of Xlcaax in Xenopus oocytes.* Biochemistry, 1993. **32**(32): p. 8207-12.

80. Choy, E., et al., *Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi.* Cell, 1999. **98**(1): p. 69-80.

81. Qualmann, B. and H. Mellor, *Regulation of endocytic traffic by Rho GTPases.* Biochem J, 2003. **4**.

82. Eva, A., et al., *Independently activated dbl oncogenes exhibit similar yet distinct structural alterations.* Oncogene, 1987. **1**(4): p. 355-60.

83. Hart, M.J., et al., *Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product.* Nature, 1991. **354**(6351): p. 311-4.

84. Hart, M.J., et al., *Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product.* J Biol Chem, 1994. **269**(1): p. 62-5.

85. Bustelo, X.R., *Regulation of Vav proteins by intramolecular events.* Front Biosci, 2002. **7**: p. d24-30.

86. Hoffman, G.R. and R.A. Cerione, *Signaling to the Rho GTPases: networking with the DH domain.* FEBS Lett, 2002. **513**(1): p. 85-91.

87. Donovan, S., K.M. Shannon, and G. Bollag, *GTPase activating proteins: critical regulators of intracellular signaling.* Biochim Biophys Acta, 2002. **1602**(1): p. 23-45.

88. Moon, S.Y. and Y. Zheng, *Rho GTPase-activating proteins in cell regulation.* Trends Cell Biol, 2003. **13**(1): p. 13-22.

89. Geyer, M. and A. Wittinghofer, *GEFs, GAPs, GDIs and effectors: taking a closer (3D) look at the regulation of Ras-related GTP-binding proteins.* Curr Opin Struct Biol, 1997. **7**(6): p. 786-92.

90. Olofsson, B., *Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling.* Cell Signal, 1999. **11**(8): p. 545-54.

II. Prenylated, GTP-bound Cdc42 Targets Mixed Lineage Kinase 3 to the Plasma Membrane to Potentiate JNK Signaling

1. Abstract

MLK3 functions as a MAPKKK to activate the JNK pathway. In addition to an SH3 domain and zipper region, MLK3 contains a centrally located Cdc42/Rac interactive binding (CRIB) motif through which activated Cdc42 can bind. Coexpression of constitutively active Cdc42 with MLK3 potentiates JNK activation and alters the *in vivo* phosphorylation pattern of MLK3. However, GTP-bound Cdc42 did not activate MLK3 *in vitro* using purified recombinant proteins, suggesting a role for the cellular environment. In this study, using biochemical fractionation experiments, coupled with confocal microscopy, we demonstrated that MLK3 alone localizes to the perinuclear/Golgi region. Upon coexpression with prenylated active Cdc42, MLK3 is localized to the plasma membrane. A prenylation-defective mutant of activated Cdc42, $Cdc42^{Val12}C188SK/R4Q$, fails to target MLK3 to the plasma membrane and fails to potentiate JNK activation. These studies suggest that prenylation-dependent Cdc42-induced translocation of MLK3 to the plasma membrane is required for full activation of MLK3 and for its downstream activation of JNK.

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. Many protein kinases have multiple *in vivo* substrates and signaling pathways. Spatiotemporal localization of protein kinases provides a mechanism by which substrate and signaling specificity can be achieved.

The mixed lineage kinases are a family of serine/threonine protein kinases that activate mitogen-activated protein kinase (MAPK) pathways [1]. The mammalian mixed lineage kinase 3 (MLK3) functions as a mitogen-activated protein kinase (MAPK) kinase kinase (MKKK) to activate the c-Jun N-terminal kinase (JNK) pathway through phosphorylation of MKK4/7 [2, 3]. MLK3 and family members are implicated in JNK activation leading to neuronal apoptosis in response to trophic factor deprivation [4-6]. TNF-α and ceramide have also been identified as agonists of MLK3 and MLK3-induced JNK signaling leading to apoptosis [7] in Jurkat T lymphocytes. In addition to their roles in apoptosis, MLKs impact development. For instance, the *Drosophila* MLK, called Slipper, is critical for the JNK-dependent process of dorsal closure in the fly embryo [8]. Furthermore, MLK3 reportedly activates the p38 pathway through phosphorylation of MKK3/6 [3]; this activity may be dependent upon the scaffold JIP2 [9, 10]. In addition to MKKs, IKKα and IKKβ have been reported as substrates of MLK3 in the activation of the NF-κB pathway in response to T cell receptor costimulation [11].

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 (reviewed by [12-21]). Rho family GTPases can modulate the catalytic activities and subcellular localization of protein kinases. For example, the CRIB-containing serine/threonine kinase PAK can be targeted to the plasma membrane by prenylated activated Rac [22-24].

Cdc42 and Rac are able to associate with cellular membranes by virtue of posttranslational processing. A soluble prenyltransferase [25] adds a geranylgeranyl group to the Cys of the COOH-terminal *CAAX* motif of the Rho family GTPase. Based on studies of the posttranslational processing of Ras [26], the prenylated GTPase is then targeted to cytoplasmic face of endomembranes where, as a substrate for a prenyl-*CAAX* protease, the *AAX* tripeptide is released and the GTPase with a free COOH-terminal prenylcysteine is generated. Finally the new COOH-terminus is methylated by an endomembrane-associated prenylcysteine carboxyl methyltransferase. A second signal for membrane localization, found in the so-called hypervariable region immediately upstream of the *CAAX* motif, typically contains either palmitoylation sites [27] or a series of basic residues [28] (Fig. 5).

The activities of MLK3 are regulated, at least in part, by protein-protein interaction domains. We recently reported that MLK3 is autoinhibited by an interaction between its SH3 domain and a short sequence that is located between its zipper and CRIB motif [29]. Zipper-mediated oligomerization is critical for MLK3 activation of JNK [30, 31] and, in particular, for proper phosphorylation of a downstream substrate [31].

MLK3 harbors a centrally located Cdc42/Rac Interactive Binding (CRIB) motif. We [32] and others [33] have demonstrated that coexpression with activated variants of the small Rho family GTPases Cdc42 and Rac potentiates MLK3 activity. The precise

mechanism by which small GTPases activate MLK3, and presumably other CRIB-containing MLKs, is yet to be elucidated. A role for phosphorylation is suggested by the finding that the *in vivo* phosphorylation of MLK3 is altered by expression with activated Cdc42 [32] [34]. In addition, coexpression with activated Cdc42 promotes MLK3 oligomerization [30, 35]. MLK3 and activated forms of Cdc42 and Rac associate in coimmunoprecipitation [32, 33] and yeast two hybrid [36] assays suggesting that the GTPase directly associates with MLK3 to impact MLK3 activity. However, recapitulation of the Cdc42-induced activation of MLK3 *in vitro* using recombinant proteins has not been successful [32]. This indicates that GTPase regulation of MLK3 might require a specific cellular context.

Since posttranslational prenylation allows Cdc42 and other small GTPases to associate with cellular membranes, we wondered whether Cdc42 might affect compartmentalization of MLK3 to impact MLK3 activity and its downstream signaling pathways. Herein we report that activated Cdc42 targets MLK3 to the plasma membrane in a prenylation-dependent manner, as supported by both biochemical fractionation and confocal microscopy. Furthermore, plasma membrane-enriched fractions are devoid of MLK3 activity except when activated, prenylation-competent Cdc42 is coexpressed. Prenylation-defective mutants of activated Cdc42 do not colocalize with MLK3 and fail to potentiate MLK3-induced JNK activation. This work supports a role for Cdc42-mediated plasma membrane targeting of MLK3 in the activation of MLK3 and its signaling to JNK.

3. Experimental Procedures

3.1. Expression Vectors, Site-Directed Mutagenesis, and Antibodies

The construction of the cytomegalovirus-based expression vectors containing the cDNA for the wild type MLK3 (pRK5-*mlk3*) has been described elsewhere [37]. The expression plasmid construct encoding the NH_2-terminal Flag epitope-tagged constitutively active Cdc42 (pRK5-N-Flag.$cdc42^{Val12}$) was kindly provided by Avi Ashkenazi (Genentech, Inc.). The plasmids encoding the enhanced yellow fluorescent protein (EYFP) fused to subcellular targeting sequences for the Golgi apparatus (pEYFP-*golgi*) and for the plasma membrane, pEYFP-Mem, were from Clontech. A plasmid encoding green fluorescent protein (GFP) fused to a targeting sequence for the endoplasmic reticulum (ER), pEF-*myc*/ER/GFP was from Invitrogen.

Variants of $Cdc42^{Val12}$ 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.$cdc42^{Val12}$ C188S, 5'-GATGTTCATAGCAGCACAGATCTGCGGCTCTTCTTCG-3' and its reverse complement were used as primers and pRK5-NFlag.$cdc42^{Val12}$ was used as the template. To substitute Lys 183, Lys 184, Arg 186, Arg 187 of Cdc42 with neutral Gln residues, pRK5-NFlag.$cdc42^{Val12}$ 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 DNA Sequencing Facility).

3.2. Cell Lines and Transfections

Human embryonic kidney (HEK) 293 cells were cultured and transfected using the calcium phosphate method as previously described [32]. HeLa cells were cultured in low glucose Dulbecco's modified Eagle's media containing 8% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen). For microscopy experiments, HeLa cells were cultured on glass cover slips in 6-well plates and transfected with 2 μg of plasmid using the calcium phosphate method as previously described [32]. Transiently transfected HEK 293 cells were lysed 18 h posttransfection and immunoprecipitation and/or Western blot analysis of MLK3, Flag-tagged Cdc42, or JNK was carried out as previously described [32].

3.3. Subcellular Fractionation

Crude plasma membrane-enriched fractions of transiently transfected HEK 293 cells were generated according to Stokoe *et al.* with minor modifications [38]. 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 sodium orthovanadate, 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 P16.9 fraction was 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 sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.15 U/ml aprotinin). The differences in the contents of the hypotonic buffer and lysis buffer were corrected to ensure identical buffer components in all fractions. Protein concentrations in each fraction were determined using Bradford assays according to the manufacturer's instructions (Biorad). Equal amounts of total protein from the S16.9 and the solubilized P16.9 fraction were resolved by SDS- polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting for MLK3 and Flag-Cdc42 was performed as described previously [32].

The fractionation procedure described above was further refined by subjecting the S16.9 fraction to centrifugation at 210,000 x *g* for 1.5 h at 4°C to generate an endomembrane-enriched pellet (P210) fraction and a soluble (S210) fraction. Pellet fractions were resuspended in lysis buffer and the buffer contents of all subcellular fractions were corrected as above. Equal amounts of total protein from the S210, P210, and P16.9 fractions were separated by SDS-PAGE and MLK3 and Flag-tagged Cdc42 were detected by Western blotting as previously described [32].

3.4. Antibodies, Immunoprecipitations, Western Blotting

Transiently transfected HEK 293 cells were lysed 18 h posttransfection and immunoprecipitation and/or Western blot analysis of MLK3, Flag-tagged Cdc42, or JNK

was carried out as previously described [32]. Rabbit polyclonal antiserum against lactate dehydrogenase (LDH) from pig muscle was a gift from Dr. John Wilson (Michigan State University); the mouse monoclonal antibody M3A5 against the Golgi coatamer protein β-COP was from Sigma; a rabbit polyclonal antibody directed against the protein ERp72 was from StressGen Biotechnologies Corp.; and rabbit polyclonal antibody against the transmembrane Na^+/K^+ ATPase was a gift from Dr. Julia Busik (Michigan State University).

3.5. Immune Complex Kinase Assay

A radioactive immune complex assay for JNK activity was performed and analyzed as previously described [32]. Reactions were carried out for 15 min at room temperature using 8 μg of GST-c-Jun as the substrate in a total reaction volume of 20 μl.

The activity of biochemically fractionated MLK3 was determined by an immune complex kinase assay of the solubilized P16.9 fractions. The relative amounts of MLK3 in the P16.9 fractions from cells expressing MLK3 alone or MLK3 and $Cdc42^{Val12}$ were determined by densitometry (NIH image) and adjusted to ensure equal amounts of immunoprecipated 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 [31] using 10 μg of purified GST-MKK4 as substrate in a total reaction volume of 20 μl containing 50 μM ATP. The extent of GST-MKK4 phosphorylation was determined by Western blotting with an anti-phospho-MKK4 antibody (New England Biolabs).

3.6. Immunofluorescence and Confocal Microscopy

HeLa cells for immunofluorescence microscopy were plated onto 6-well plates containing cover slips (1 x 10^5 cells per well) and transiently transfected by the calcium phosphate method as previously described [32]. Eighteen hours posttransfection, the cells were rinsed twice in PBS, fixed in 2% formaldehyde (Polyscience, Inc.) for 30 min at room temperature, followed by washing three times with 10 mM glycine in PBS and three times with PBS. The cells were permeablized with PBS containing 0.1% Triton X-100 for 10 min at 37°C and blocked in PBS containing 5% Bovine Serum Albumin (BSA) for 10 min at 37°C. After incubation with the primary antibody (15 ng/ml of the MLK3 rabbit polyclonal antibody or 8 ng/ml M2 Flag monoclonal antibody, in PBS containing 5% BSA) for 1h at 37°C, cells were washed three times with PBS and incubated for 1 h at 37 °C with the appropriate FluoroLink™Cy3 ™-labeled secondary antibody (Amersham Pharmarcia) or Alexa 488-conjugated secondary antibody (Molecular Probes). Finally, the cells were washed four times with PBS prior to mounting onto glass slides. The fluorescently labeled cells were examined with a Noran Odyssey confocal laser scanning microscope. For analysis of the FluoroLink Cy3-labeled cells, an argon laser with an excitation at 514 nm was used. Cells labeled with Alexa 488, EYFP, and EGFP were examined at 488 nm. For dual color labeling, the emission signals from each fluorescent probes were collected separately and merged. Control experiments using only one of the two fluorescent probes were performed. The antibody concentrations and laser intensity were adjusted to ensure that there was no significant signal bleedthrough from either of the two fluorescent probes.

4. Results

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

Previous studies have demonstrated that activated Cdc42 potentiates MLK3 activity. To assess whether Cdc42 impacts the subcellular distribution of MLK3, biochemical fractionation experiments were performed using HEK 293 cells that were transiently transfected with expression vectors for MLK3 and constitutively active variants of the GTPase $Cdc42^{Val12}$. The relative distribution of MLK3 between a soluble (S16.9) and a plasma membrane-enriched (P16.9) fraction was assessed by Western blotting. Data from a representative experiment are shown in Fig. 6A. Based on five independent experiments, MLK3 when expressed alone is distributed approximately equally between the S16.9 and P16.9 fractions, but when coexpressed with activated Cdc42, MLK3 is found almost exclusively in the P16.9 fraction. To determine whether the Cdc42-induced transfer of MLK3 to the P16.9 fraction depends upon prenylation of the small GTPase, the site of prenylation, Cys^{188} in the CAAX motif, of $Cdc42^{Val12}$ was mutated to Ser (Fig.5). The analogous mutation in yeast prevents prenylation and rescues the lethal phenotype induced by constitutively active $Cdc42^{Val12}$ [39]. Coexpression of MLK3 with the prenylation-defective activated GTPase, $Cdc42^{Val12}$C188S, yielded a fractionation pattern identical to that of MLK3 alone (Fig. 2A). The activated Cdc42 is found in both fractions, analogous to what has been reported by Stokoe *et al.* [38] for the fractionation of overexpressed Ras^{Val12} in COS cells. The prenylation-defective mutant of $Cdc42^{Val12}$ is found primarily in the S16.9 fraction, but a small amount is present in the P16.9 fraction. It is likely that the polybasic sequence in the hypervariable region (Fig. 5) affords some residual membrane binding in the absence of prenylation.

Coexpression with $Cdc42^{Val12}$ has been demonstrated to alter the *in vivo* phosphorylation pattern of MLK3 [32, 34]. In these experiments, when MLK3 has been coexpressed with activated Cdc42 the electrophoretic mobility of MLK3 is retarded, consistent with Cdc42-induced phosphorylation of MLK3. However, after coexpression with a prenylation-defective mutant of $Cdc42^{Val12}$ the electrophoretic mobility of MLK3 is not altered (Fig. 6B). Taken altogether, these data suggest that activated Cdc42 alters MLK3 subcellular localization and phosphorylation in a prenylation-dependent fashion.

4.2. Activated Cdc42 Targets MLK3 from an Endomembrane-enriched Fraction to a Plasma Membrane-enriched Fraction

To more precisely define the subcellular distribution of MLK3, the biochemical fractionation method was expanded to include an additional centrifugation of the S16.9 fractions at 210,000 x *g* to yield soluble (S210) fractions and pellet (P210) fractions. The efficacy of the fractionation procedure was verified by Western blotting analysis of fractions using antibodies directed against proteins that localize to specific subcellular organelles. A representative Western blot is shown in Fig. 7A. The cytosolic marker protein, LDH, is detected only in the S210 fraction. The transmembrane Na^+/K^+ ATPase is found exclusively in the P16.9 fraction. A Golgi vesicle-associated coatamer protein, β-COP, was detected only in the P210 fraction, whereas the ER marker, ERp72 was detected in both the P210 and P16.9 fractions. Thus plasma membrane-associated proteins are present in the P16.9 fractions; Golgi-associated proteins are in the P210 fractions; and cytosolic proteins are in the S210 fractions. However, ER-associated proteins are found in both the P16.9 and S16.9 fractions. The fractionation pattern of the

marker proteins was not influenced by transfection of MLK3 or Cdc42 variants (data not shown).

This improved biochemical fractionation procedure was utilized to examine the effect of Cdc42 variants on MLK3 localization. Since a small portion of the prenylation-defective variant $Cdc42^{Val12}$ C188S was present in the plasma membrane-enriched fraction (Fig. 6A), the four basic residues in the hypervariable region that have been implicated in contributing to membrane binding [28] were mutated to neutral Gln residues to generate $Cdc42^{Val12}$C188S K/R4Q. Representative data from seven independent experiments are shown in Fig. 7B. Consistent with our earlier experiments, $Cdc42^{Val12}$ is found in the P16.9 and the S210 fractions; however $Cdc42^{Val12}$C188S, K/R4Q is found only in the S210 fraction. These data indicate that, as intended, $Cdc42^{Val12}$C188S, K/R4Q is unable to target to the plasma membrane.

When expressed alone, MLK3 is distributed approximately equally between the P210 and P16.9 fractions. However, when coexpressed with $Cdc42^{Val12}$, very little MLK3 partitions with the P210 fraction; instead, essentially all of the MLK3 is found in the plasma membrane-enriched P16.9 fraction. Coexpression of MLK3 with the $Cdc42^{Val12}$C188S, K/R4Q yielded a fractionation pattern similar to that of MLK3 expressed alone. These data confirm that prenylation of activated Cdc42 is required for the observed changes in MLK3's subcellular distribution.

Under all transfection conditions, MLK3 appears to be absent from the cytosolic (S210) fractions. However, since equal amounts of proteins were loaded in each lane, the percentage of total cytosolic (S210) protein loaded is about four times less than the percentage of total endomembrane (P210) and of total plasma membrane (P16.9)

proteins. Based on analysis of Western blots by densitometry, coupled with determination of protein concentrations, we estimate that less than 15% of MLK3 in the postnuclear fraction resides in the cytosolic fraction, regardless of whether the small GTPase has been expressed.

4.3. Prenylated Cdc42^{Val12} Directs Active MLK3 to a Plasma Membrane-enriched Fraction

Activated, prenylated Cdc42^{Val12} alters MLK3's subcellular distribution in HEK 293 cells. To assess whether Cdc42 targeting affects the catalytic activity of plasma membrane-associated MLK3, *in vitro* kinase assays of MLK3 present in the P16.9 fractions were performed. The P16.9 fractions were solublized in Triton X-100-containing buffer and equal amounts of immunoprecipitated MLK3 were subjected to an *in vitro* kinase assay using unlabeled ATP and recombinant GST-MKK4 as substrates. Phosphorylation at Thr^{258} of the activation segment of MKK4 was monitored using a phospho-specific antibody against MKK4. Data from three independent experiments show that MLK3 activity is present in the plasma membrane-enriched fractions only when active, prenylated Cdc42^{Val12} had been coexpressed (Fig. 7C). When MLK3 is expressed alone or with the prenylation-defective Cdc42^{Val12}C188SK/R4Q, there is negligible MLK3 activity in the P16.9 fraction.

It is plausible that the mutations intended to eliminate the plasma membrane targeting of Cdc42 might also have somehow disrupted the inherent ability of the Cdc42 variants to complex with, and thus activate, MLK3. To rule out this possibility, cotransfected HEK 293 cells were lysed in Triton X-100-containing buffer in order to

disrupt subcellular organelles, and coimmunoprecipitation experiments were performed. The prenylation-defective $Cdc42^{Val12}$ variants retain the ability to associate with MLK3 (Fig. 8), indicating that the prenyl group and the polybasic residues of $Cdc42^{Val12}$ are not required for binding of the activated GTPase by MLK3. Taken together, these data support the hypothesis that functional prenylation is required for Cdc42-mediated targeting of activated MLK3 to the plasma membrane.

4.4. Colocalization of MLK3 and Markers for the Golgi Apparatus

To confirm the data obtained by biochemical fractionation, confocal microscopy experiments were performed using enhanced yellow fluorescent protein (EYFP) tagged with targeting sequences to specific subcellular organelles. HeLa cells were cotransfected with plasmids encoding MLK3 and an EYFP-tagged Golgi marker that carries the Golgi targeting sequence of the integral Golgi protein, β-galactosyltransferase. A perinuclear staining pattern characteristic of the Golgi apparatus was observed using an MLK3 antibody. Fluorescence from the EYFP-tagged Golgi marker overlays with the MLK3 staining, although the MLK3 staining extends beyond that of the Golgi marker in vesicular-like structures that stain with the Golgi-selective lipid, BODIPY-ceramide (data not shown) (Fig. 9A).

4.5. Prenylated, Activated Cdc42 Targets MLK3 to the Plasma Membrane

To determine whether $Cdc42^{Val12}$ can target MLK3 to the plasma membrane, HeLa cells were triply transfected with vectors encoding MLK3, $Cdc42^{Val12}$ and an EYFP-tagged plasma membrane marker. The $Cdc42^{Val12}$ vector was provided in excess

to ensure that all cells expressing MLK3 and the EYFP marker also expressed $Cdc42^{Val12}$. When expressed alone, MLK3 displays a perinuclear/Golgi localization. However, upon coexpression with $Cdc42^{Val12}$, the localization of MLK3 overlaps with that of the EYFP-plasma membrane marker (Fig. 9B). These data suggest that activated Cdc42 and MLK3 colocalize at the plasma membrane.

To evaluate the extent of colocalization of MLK3 and Cdc42 variants, HeLa cells were cotransfected with an MLK3 expression vector and an excess of the Cdc42 variant expression vectors. Indeed the images of cells stained for prenylation-competent activated $Cdc42^{Val12}$ and MLK3 yield overlapping fluorescence at the cell periphery consistent with plasma membrane localization (Fig. 10A). The staining pattern of MLK3 and the membrane targeting-defective variant of Cdc42 do not coincide (Fig. 10B). Instead, MLK3 retains its perinuclear staining and the $Cdc42^{Val12}$C188SK/R4Q variant shows diffuse cytosolic staining. These data indicate that expression of prenylated, activated Cdc42 targets MLK3 to the plasma membrane.

4.6. Functional Targeting to the Plasma Membrane is Required for Potentiation of MLK3-induced Activation of JNK by Cdc42

Subcellular localization may be one means of controlling either substrate selection or access to effector proteins. One of the best-described functions of MLK3 is as a MAPKKK that activates the JNK pathway. We [31, 32] and others [33] have previously shown that activated Cdc42 potentiates MLK3-induced JNK activation. The activity of endogenous JNK from total cellular lysates of HEK 293 cells transiently expressing MLK3 alone or MLK3 and Cdc42 variants was determined. JNK was

immunoprecipitated, and *in vitro* kinase assays were performed using GST-c-Jun as an exogenous substrate. Based on three independent experiments, JNK activity is increased approximately 3-fold in cells that coexpress MLK3 with prenylation-competent $Cdc42^{Val12}$ compared with those that express MLK3 alone (Fig. 11). However, the prenylation-defective $Cdc42^{Val12}$C188S K/R4Q has no effect on MLK3-induced JNK activation. These data indicate that membrane targeting of MLK3 is critical for the Cdc42-dependent increase in JNK activation.

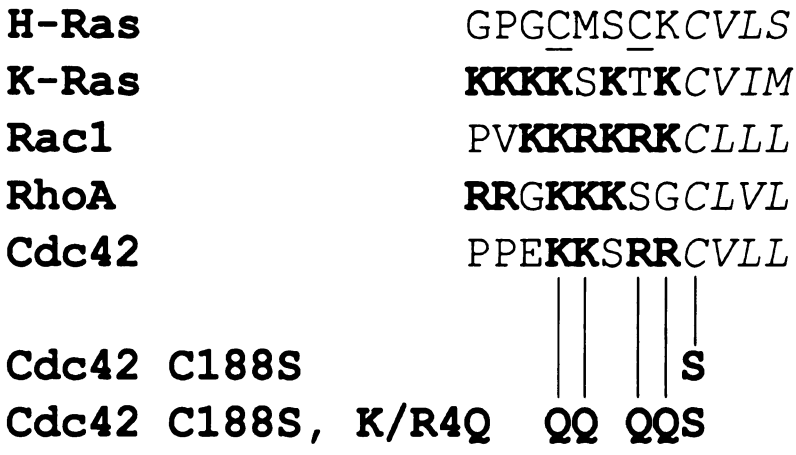


Fig. 5. **Alignment of the COOH-termini of small GTPases and Cdc42^{Val12} mutants**. The twelve COOH-terminal amino acids of H-Ras, K-Ras, Rac1, RhoA and Cdc42 are aligned. The CAAX (cysteine-aliphatic-aliphatic-any amino acid) motif is *italicized*. Basic residues are shown in *bold* and the palmitoylation site is underlined. The mutations in the engineered Cdc42^{Val12} variants are shown with the lysine to glutamine and/or cysteine to serine changes indicated by *bold* letters.

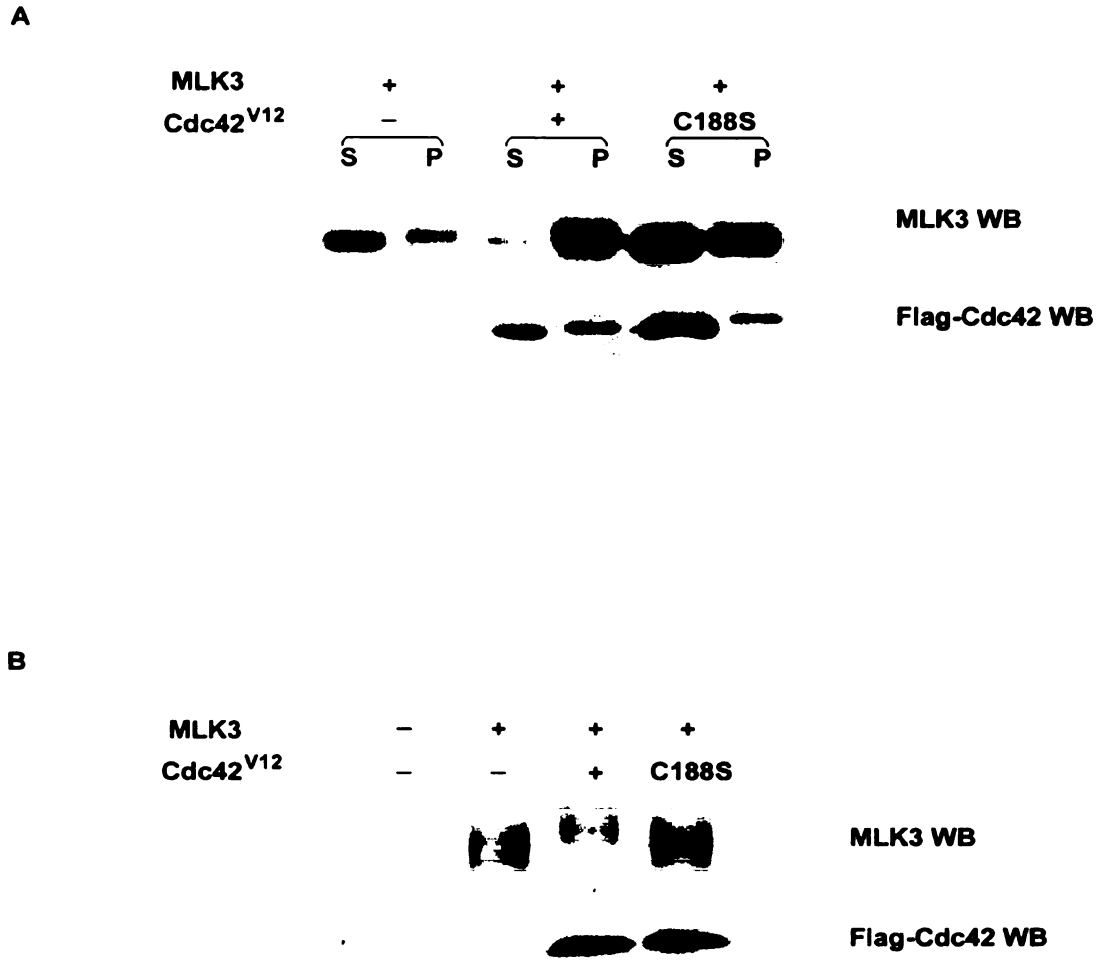
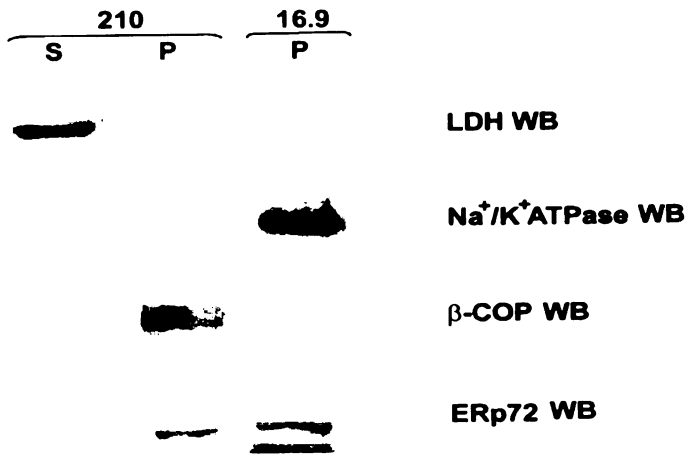
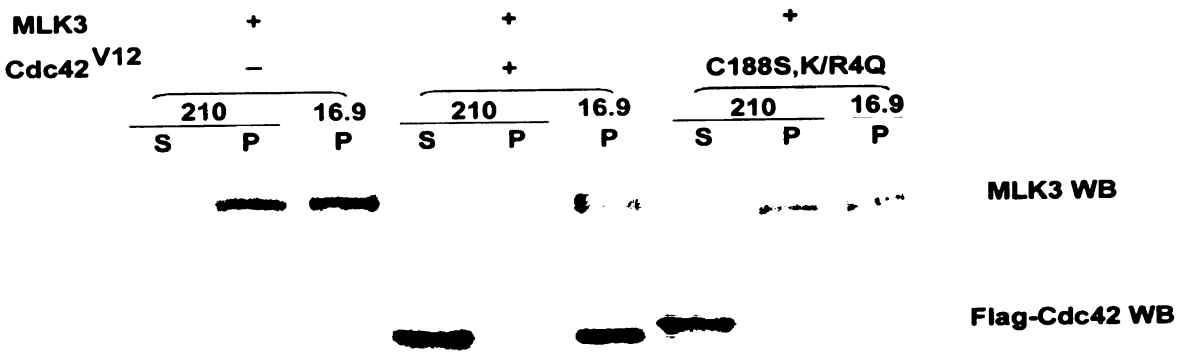


Fig. 6. **Effect of Cdc42 expression on the subcellular distribution and electrophoretic mobility of MLK3.** HEK 293 cells were transiently transfected with expression vectors containing cDNAs indicated above each figure. A minus sign indicates that a control empty vector was transfected. *A*, biochemical fractionation of MLK3. Cells expressing MLK3 in the presence and absence of $Cdc42^{Val12}$ 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 using used to determine the distributions 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 three independent experiments. *B*, electrophoretic mobility of MLK3. Western blots of MLK3 and Flag-Cdc42 from cellular lysates are shown in the *top* and *bottom panels*, respectively. These experiments were performed by Dr. Barbara Bock.

A


210
16.9
S
P
P
LDH WB
Na+/K+ATPase WB
β-COP WB
ERp72 WB

B


MLK3
Cdc42V12
+
–
+
+
+
C188S,K/R4Q
210
16.9
210
16.9
210
16.9
S
P
P
S
P
P
S
P
P
MLK3 WB
Flag-Cdc42 WB

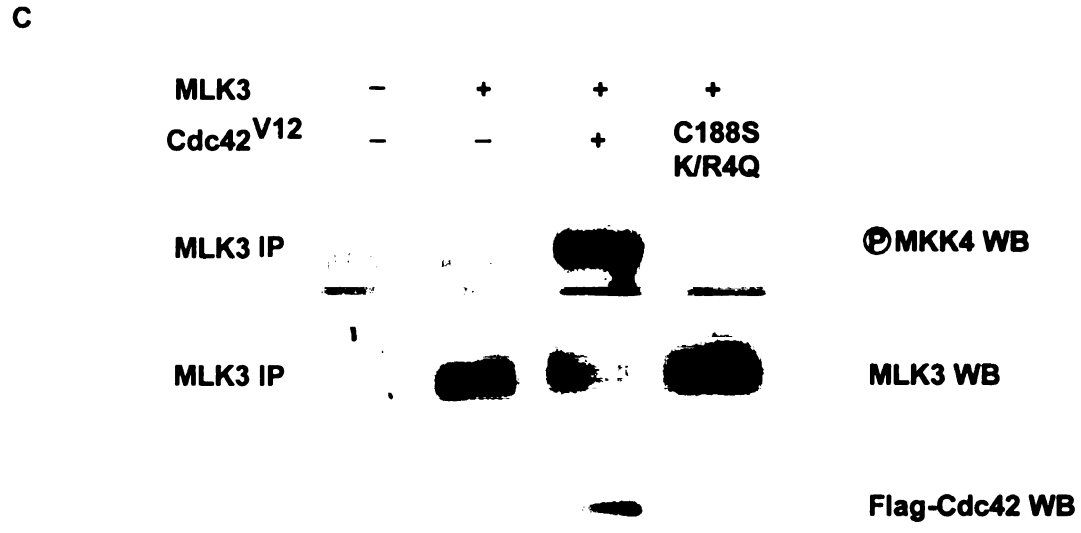


Fig. 7. **Subcellular distribution and catalytic activity of MLK3 upon coexpression with Cdc42^{Val12} variants.** *A*, presence of subcellular marker proteins in biochemical fractions. The biochemical fractionations were performed as described previously except that an additional centrifugation of the S16.9 fraction at 210,000 x *g* was performed to yield soluble (S210) fractions and pellet (P210) fractions. Western blots were performed using antibodies directed against marker proteins for specific subcellular compartments. LDH denotes lactate dehydrogenase. ER stands for endoplasmic reticulum. β-COP denotes coatmer protein complex. *B*, biochemical fractionation of MLK3. HEK 293 cells were transfected with the indicated expression vectors. The distributions of MLK3 and Cdc42 were assessed by Western blotting with an MLK3 antibody and Flag antibody, respectively, as described previously. *C*, *in vitro* kinase assay of MLK3 immunoprecipitated from the P16.9 fractions using GST-MKK4 as a substrate. MKK4 phosphorylation was assessed using an antibody that recognizes phosphorylated Thr^{258} of MKK4. The data shown is representative of three independent experiments. The Cdc42 ^{Val12} variants were constructed by Dr. Barbara Bock. The experiment in Fig. 3C was performed by Dr. Barbara Bock.

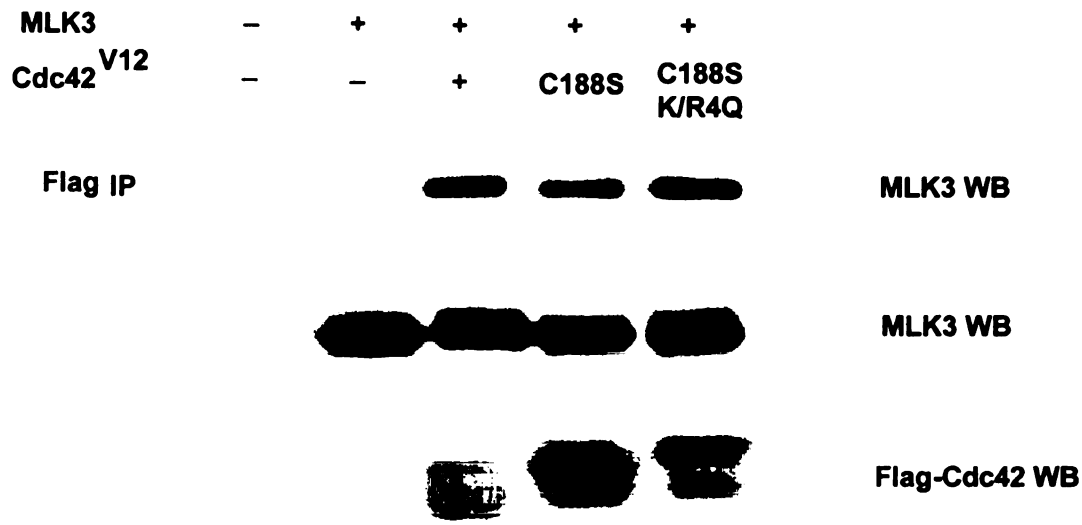


Fig. 8 **Ability of Cdc42 variants to associate with MLK3.** Flag-$Cdc42^{Val12}$ variants were immunoprecipitated from total cellular lysates using the Flag antibody and associated MLK3 was detected by immunoblotting with an MLK3 antibody. This experiment was performed by Dr. Barbara Bock.

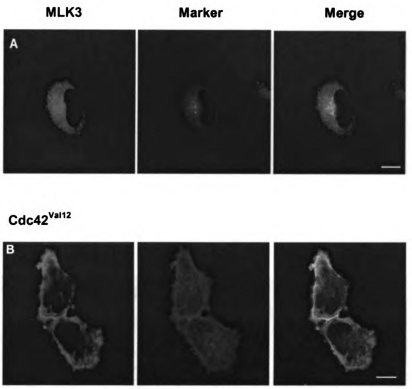


Fig. 9. **Effect of Cdc42 ^{Val12} on subcellular localization of MLK3.** Transfected HeLa cells were analyzed by confocal microscopy. Cells expressing MLK3 and the fluorescence-tagged subcellular marker were stained for MLK3 and images were merged. Scale bar represents 10 μm. *A*, MLK3 and Golgi marker localization. HeLa cells were transfected with expression vectors encoding MLK3 and an enhanced yellow fluorescence protein (EYFP)-tagged Golgi marker and stained for MLK3 using an MLK3 antibody and a secondary antibody conjugated with FluoroLink Cy3. *B*, MLK3 and plasma membrane marker localization. HeLa cells were triply transfected with vectors expressing MLK3, Cdc42 ^{Val12} and EYFP-tagged plasma membrane marker. Excess Cdc42 vector was used.

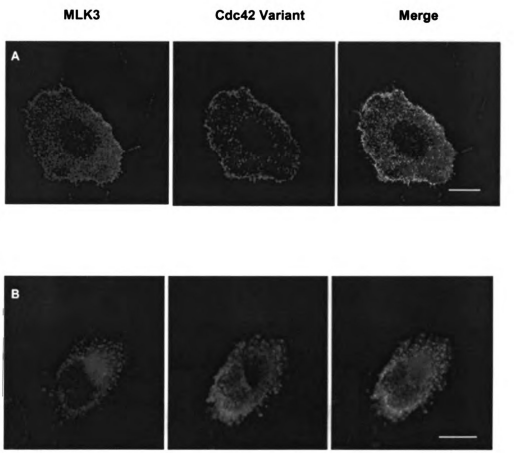


Fig. 10. **Effect of prenylation competence of activated Cdc42 on subcellular localization of MLK3.** HeLa cells were cotransfected with vectors expressing MLK3, and Flag-Cdc42 ^{Val12} or Flag-Cdc42 ^{Val12}C188S, K/R4Q. MLK3 was detected using an MLK3 antibody followed by Alexa 488-conjugated secondary antibody. Flag-Cdc42 was detected using Flag antibody followed by a FluoroLink Cy3-conjugated secondary antibody. Scale bar represents 10 μm. *A*, localization of MLK3 and Cdc42^{Val12}. *B*, localization of MLK3 upon coexpression with the prenylation-defective variant, Cdc42 ^{Val12}C188S, K/R4Q.

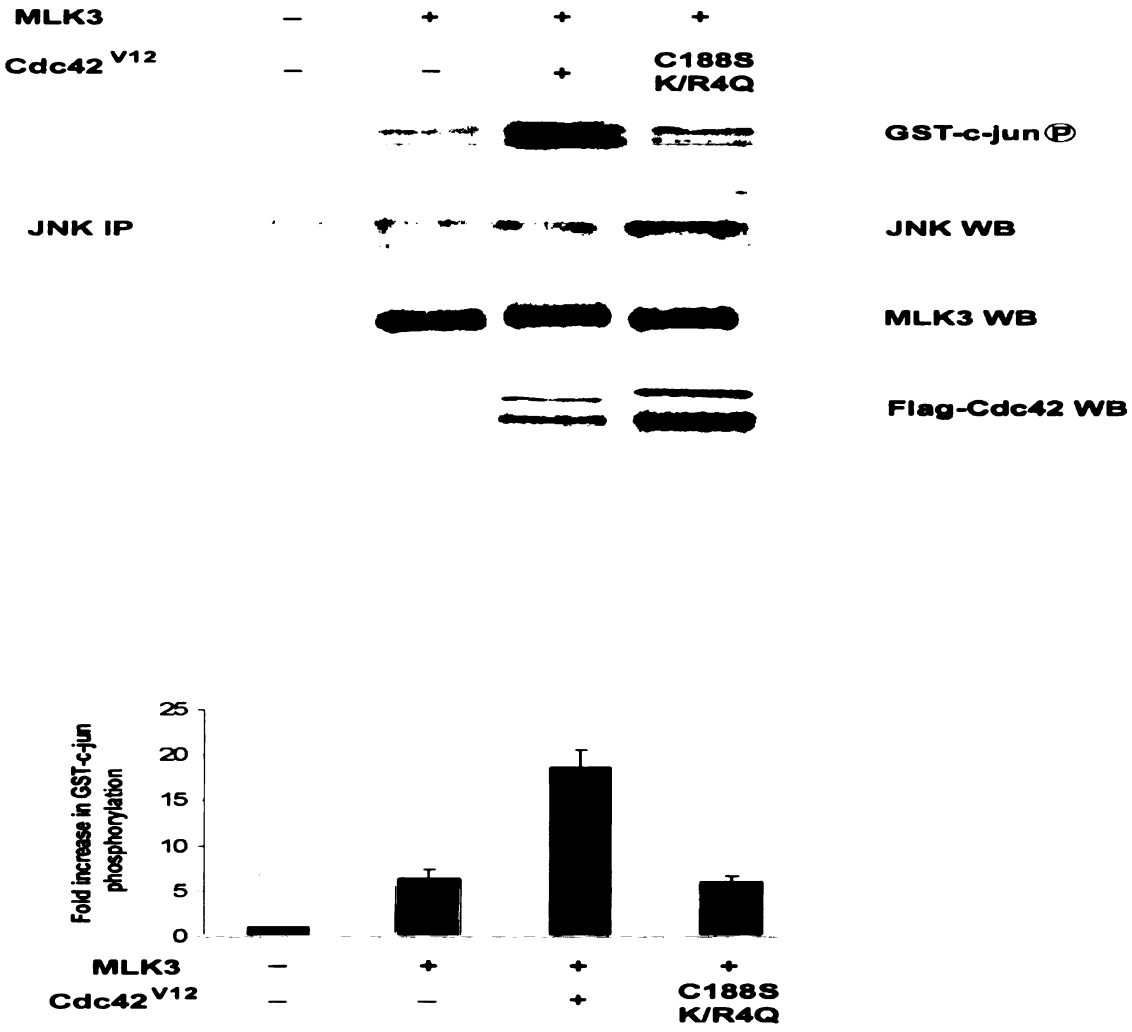


Fig. 11. **Effect of Cdc42^{Val12} variants on MLK3-induced JNK activation.** HEK 293 cells were cotransfected with expression vectors for MLK3 and for Cdc42 ^{Val12} or Cdc42 ^{Val12}C188S, K/R4Q. Endogenous JNK was immunoprecipitated from total cellular lysates expressing MLK3 alone or with the indicated Cdc42 ^{Val12} variant and subjected to an in vitro kinase assay using GST-c-Jun as a substrate. *A*, an autoradiogram showing GST-c-Jun phosphorylation. An immunoblot for JNK from the same immunoprecipitated samples, and immunoblots for MLK3 and Flag-Cdc42 are shown below the autoradiogram. *B*, The means ± S.E. for fold increase in GST-c-Jun phosphorylation of three independent experiments are shown. Only experiments with equal expression levels of MLK3 were included. GST-c-Jun phosphorylation was measured by phosphorimaging.

5. Discussion

The detailed mechanism by which small Rho family GTPases, like Cdc42, can activate MLK3 is not understood. The research described in this Chapter was designed to investigate whether Cdc42 might modulate MLK3's subcellular localization and signaling pathways.

MLK3 expressed alone is largely absent from the cytosol, as judged by both biochemical fractionation experiments and by confocal microscopy. Instead MLK3 staining is perinuclear and overlays partially with a GFP-tagged Golgi integral membrane protein β-galactosyl transferase. Electron microscopy studies have demonstrated that the MLK family member, dual leucine zipper kinase (DLK), localizes to the cytoplasmic face of the trans Golgi apparatus of NIH3T3 cells [40]. Indeed, MLK3 lacks an internal Golgi targeting sequence, suggesting that MLK3 may associate with the cytoplasmic face of the Golgi apparatus.

It has recently been reported that, during the G2/M phase of the cell cycle, endogenous MLK3 localizes to centrosomes and is active, but fails to induce JNK activation [41]. As reviewed by Rios and Bornens [42], the Golgi apparatus surrounds the centrosome of non-polarized mammalian cells where it maintains an intimate relationship with microtubules. Distinct Golgi and centrosomal targeting motifs have been identified on the protein kinase A-anchoring protein 350 (AKAP 350) [43], providing precedence for the idea that MLK3 may be able to associate, depending on the cellular context, with these distinct, yet interrelated, perinuclear structures.

Wildtype Cdc42 is found at the Golgi [44], whereas the constitutively active Cdc42 mutant, $Cdc42^{Val12}$, localizes to the plasma membrane [45]. To generate a

cytosolic form of $Cdc42^{Val12}$, Cys 188 of the *CAAX* motif (Fig. 5) was mutated to a Ser. Biochemical fractionation experiments revealed a small portion of the unprenylated $Cdc42^{Val12}$ C188S in the plasma membrane-enriched fraction (Fig. 6A), which may be attributed to its polybasic region upstream of the CAAX motif. Substitution of the basic residues near the CAAX motif results in decreased membrane association of yeast $Cdc42^{Val12}$ [46]. Therefore, to completely abolish membrane association of $Cdc42^{Val12}$, in addition to the Cys to Ser mutation, the four basic residues near the CRIB motif were substituted with neutral glutamine residues. Two of these four basic residues have been shown to be critical for the binding of prenylated Cdc42 to γ-COP, a Golgi coatamer complex protein [47]. Indeed the $Cdc42^{Val12}$ mutant lacking the prenylation site and basic residues is restricted to the cytosol, as demonstrated by both confocal microscopy (Fig. 10B) and biochemical fractionation (Fig. 7B). The finding that MLK3 retains perinuclear/Golgi-like staining in the presence of the cytosol-restricted, activated Cdc42 variant suggests that targeting of MLK3 to the Golgi/perinuclear region is independent of Cdc42 and that there should exist a distinct targeting sequence within MLK3 that directs it to the Golgi/perinuclear region.

When coexpressed with prenylation-competent, activated Cdc42, MLK3 is targeted to the plasma membrane, as judged by biochemical fractionation experiments (Fig. 6A and Fig. 7B) and by confocal microscopy (Fig. 9B). The mobility of MLK3 on SDS-PAGE gels is retarded only when prenylation-competent, activated Cdc42 is coexpressed (Fig. 6B), suggest the Cdc42-induced *in vivo* phosphorylation of MLK3 depends upon plasma membrane targeting. Furthermore, resolubilization of the plasma membrane-enriched fractions followed by an *in vitro* immune complex assay reveals that

MLK3 in those fractions is active only when activated, prenylation-competent Cdc42 has been coexpressed (Fig.7C).

The data presented in this Chapter indicate that activated Cdc42 targets MLK3 to the plasma membrane in a prenylation-dependent manner thus localizing active MLK3. 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 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 induces JNK pathway activation.

The process by which prenylated, activated Cdc42 directs MLK3 to the plasma membrane is unknown. However several lines of evidence implicate a microtubule-associated transport process. MLK2, which is closely related to MLK3, has been reported to colocalize with microtubules [48]. A yeast two hybrid screen revealed that MLK2 and MLK3 can associate with KIF3A, KIF3B, KIF3X, and kinesin-associated protein 3 (KAP3) [48]. MLK2 interactions with KIF3A and KAP3 were confirmed by coimmunoprecipitation in transfected cells. KIF3 family members function as plus-end directed microtubule associated motors for transport of membrane-bound organelles (reviewed in [49]). Association of KAP3 with KIF3 heterodimers is believed to regulate cargo binding. Thus, it is conceivable that MLK3 might associate directly with kinesins and travel along microtubules, either in a protein complex or in association with vesicles, to the plasma membrane.

JIPs are MAPK scaffolds that bind MLKs as well as their downstream MKKs and MAPKs [9, 10, 35, 50]. Recent work has shown that JIPs interact directly with kinesin

light chain (KLC) [51, 52] and act as cargoes for kinesin-mediated transport to neurite tips [52, 53]. Thus kinesins may function in the transport of the MLK/JNK signaling complex to the plasma membrane. Additional evidence suggests that JIPs might act as adaptors between kinesins and selected cargoes [54]. MLK3 may be sequestered at the perinuclear/Golgi region and activated Cdc42 may induce its relocalization to the plasma membrane in a kinesin-dependent fashion. It is also conceivable that MLK3 may regulate kinesin-mediated transport processes.

Activation of Cdc42 is required for its interaction with and activation of MLK3. Thus, the spatial and temporal control of Cdc42 activation by guanine nucleotide exchange factors (GEFS) is likely to be key in the activation and localization of MLK3. For instance, faciogenital dysplasia-1 (Fgd1) is a Cdc42-specific GEF that localizes to Golgi membranes [55]. An activated form of the GEF, diffuse B-cell lymphoma oncogene (Dbl), activates and translocates wildtype Cdc42 to the plasma membrane [45]. Furthermore, a yeast two hybrid screen for Dbl-interacting proteins being carried out by the Alliance for Cell Signaling has identified the Golgi-associated protein, Golgin, (Signaling Gateway) as a binding partner of Dbl. It is tempting to speculate that Dbl or another Golgi-associated GEF might induce MLK3 activation and localization to the plasma membrane.

6. References

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

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

3. Tibbles, L.A., et al., *MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6.* Embo J, 1996. **15**(24): p. 7026-35.

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

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

6. Harris, C.A., et al., *Inhibition of the c-Jun N-terminal kinase signaling pathway by the mixed lineage kinase inhibitor CEP-1347 (KT7515) preserves metabolism and growth of trophic factor-deprived neurons.* J Neurosci, 2002. **22**(1): p. 103-13.

7. Sathyanarayana, P., et al., *Activation of the Drosophila MLK by Ceramide Reveals TNF-alpha and Ceramide as Agonists of Mammalian MLK3.* Mol Cell, 2002. **10**(6): p. 1527-33.

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

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

10. Schoorlemmer, J. and M. Goldfarb, *Fibroblast growth factor homologous factors and the islet brain-2 scaffold protein regulate activation of a stress-activated protein kinase.* J Biol Chem, 2002. **277**(51): p. 49111-9.

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

12. Ridley, A.J., *Rho family proteins: coordinating cell responses.* Trends Cell Biol, 2001. **11**(12): p. 471-7.

13. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.

14. Van Aelst, L. and C. D'Souza-Schorey, *Rho GTPases and signaling networks.* Genes Dev, 1997. **11**(18): p. 2295-322.

15. Qualmann, B. and H. Mellor, *Regulation of endocytic traffic by Rho GTPases.* Biochem J, 2003. **4**.

16. Manser, E., *Small GTPases take the stage.* Dev Cell, 2002. **3**(3): p. 323-8.

17. Kaibuchi, K., S. Kuroda, and M. Amano, *Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells.* Annu Rev Biochem, 1999. **68**: p. 459-86.

18. Johnson, D.I., *Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity.* Microbiol Mol Biol Rev, 1999. **63**(1): p. 54-105.

19. Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. **420**(6916): p. 629-35.

20. Erickson, J.W. and R.A. Cerione, *Multiple roles for Cdc42 in cell regulation.* Curr Opin Cell Biol, 2001. **13**(2): p. 153-7.

21. Wittmann, T. and C.M. Waterman-Storer, *Cell motility: can Rho GTPases and microtubules point the way?* J Cell Sci, 2001. **114**(Pt 21): p. 3795-803.

22. Schwartz, M.A., J.E. Meredith, and W.B. Kiosses, *An activated Rac mutant functions as a dominant negative for membrane ruffling.* Oncogene, 1998. **17**(5): p. 625-9.

23. Bokoch, G.M., *Regulation of cell function by Rho family GTPases.* Immunol Res, 2000. **21**(2-3): p. 139-48.

24. Lu, W. and B.J. Mayer, *Mechanism of activation of Pak1 kinase by membrane localization.* Oncogene, 1999. **18**(3): p. 797-806.

25. Casey, P.J. and M.C. Seabra, *Protein prenyltransferases.* J Biol Chem, 1996. **271**(10): p. 5289-92.

26. Choy, E., et al., *Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi.* Cell, 1999. **98**(1): p. 69-80.

27. Hancock, J.F., et al., *All ras proteins are polyisoprenylated but only some are palmitoylated.* Cell, 1989. **57**(7): p. 1167-77.

28. Hancock, J.F., H. Paterson, and C.J. Marshall, *A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane.* Cell, 1990. **63**(1): p. 133-9.

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

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

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

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

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

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

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

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

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

38. Stokoe, D., et al., *Activation of Raf as a result of recruitment to the plasma membrane.* Science, 1994. **264**(5164): p. 1463-7.

39. Ziman, M., et al., *Mutational analysis of CDC42Sc, a Saccharomyces cerevisiae gene that encodes a putative GTP-binding protein involved in the control of cell polarity.* Mol Cell Biol, 1991. **11**(7): p. 3537-44.

40. Douziech, M., et al., *Localization of the mixed-lineage kinase DLK/MUK/ZPK to the Golgi apparatus in NIH 3T3 cells.* J Histochem Cytochem, 1999. **47**(10): p. 1287-96.

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

42. Rios, R.M. and M. Bornens, *The Golgi apparatus at the cell centre.* Curr Opin Cell Biol, 2003. **15**(1): p. 60-6.

43. Shanks, R.A., et al., *AKAP350 at the Golgi apparatus. I. Identification of a distinct Golgi apparatus targeting motif in AKAP350.* J Biol Chem, 2002. **277**(43): p. 40967-72.

44. Erickson, J.W., et al., *Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus.* J Biol Chem, 1996. **271**(43): p. 26850-4.

45. Michaelson, D., et al., *Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding.* J Cell Biol, 2001. **152**(1): p. 111-26.

46. Davis, C.R., et al., *Analysis of the mechanisms of action of the Saccharomyces cerevisiae dominant lethal cdc42G12V and dominant negative cdc42D118A mutations.* J Biol Chem, 1998. **273**(2): p. 849-58.

47. Wu, W.J., et al., *The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation.* Nature, 2000. **405**(6788): p. 800-4.

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

49. Hirokawa, N., *Stirring up development with the heterotrimeric kinesin KIF3.* Traffic, 2000. **1**(1): p. 29-34.

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

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

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

53. Meyer, D., A. Liu, and B. Margolis, *Interaction of c-Jun amino-terminal kinase interacting protein-1 with p190 rhoGEF and its localization in differentiated neurons.* J Biol Chem, 1999. **274**(49): p. 35113-8.

54. Verhey, K.J. and T.A. Rapoport, *Kinesin carries the signal.* Trends Biochem Sci, 2001. **26**(9): p. 545-50.

55. Estrada, L., E. Caron, and J.L. Gorski, *Fgd1, the Cdc42 guanine nucleotide exchange factor responsible for faciogenital dysplasia, is localized to the subcortical actin cytoskeleton and Golgi membrane.* Hum Mol Genet, 2001. **10**(5): p. 485-95.

III. Concluding Remarks

The work described in this thesis examined the role of Cdc42 in subcellular distribution of MLK3. The hypothesis was formulated based on previous observations that the comparative two-dimensional *in vivo* phosphorylation map demonstrates that Cdc42 alters the *in vivo* phosphorylation pattern of MLK3, which is correlated with an increase in kinase activity. However, the Cdc42-induced increase in MLK3's kinase activity was not reproduced in an *in vitro* kinase assay using purified recombinant proteins, suggesting that Cdc42-induced activation of MLK3 requires a cellular context.

The study presented in Chapter II suggests that GTP-bound Cdc42 targets MLK3 to the plasma membrane in a prenylation-dependent manner, as supported by both biochemical fractionation and confocal microscopy. Furthermore, MLK3 can phosphorylate its downstream substrate MKK4, only when it is targeted to the plasma membrane and when coexpressed with prenylation-competent Cdc42. While the prenylated activated Cdc42 potentiates MLK3-induced JNK activation, the prenylation-defective mutant of activated Cdc42 fails to do so, supporting the idea that Cdc42 targets MLK3 to the plasma membrane and mediates its activation and downstream signaling to JNK.

In the absence of activated Cdc42MLK3 resides in the perinuclear region indicating that targeting of MLK3 to the perinuclear region is independent of Cdc42 and that MLK3 may contain a distinct sequence that directs it to the perinuclear region. The process by which the prenylated, activated Cdc42 directs MLK3 to the plasma membrane is unknown, although several evidence point to a microtubule-associated transport

process. It would be interesting to explore the Cdc42-directed subcellular localization of MLK3 upon treatement with microtubule-disrupting and/or -stabilizing agents.

Only the GTP-bound form Cdc42 is able to interact with and associate with MLK3. Thus, the spatiotemporal control of activated Cdc42 by guanine nucleotide exchange factors (GEFs) may be critical in the activation and localization of MLK3. It would be interesting to determine the effects of GEFs on the activation and localization of MLK3.

In conclusion, accumulating evidence indicates that the regulation of MLK3 is a complex and multi-step process. The work presented in this thesis contributes to the understanding of the molecular mechanisms regulating MLK3 and its biological functions. Understanding the mechanism by which MLK3 is regulated may provide clues for useful therapeutic intervention in pathological processes, like neurodegenerative diseases.