November 2000

The Role of MKK3 in Mediating Signals to the p38 MAP Kinase Pathway: A Dissertation

Mark Allen Wysk
University of Massachusetts Medical School

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THE ROLE OF MKK3 IN MEDIATING SIGNALING TO THE p38 MAP KINASE PATHWAY

A DISSERTATION PRESENTED

BY

MARK ALLEN WYSK

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirement for the degree of:

DOCTOR OF PHILOSOPHY

November 8, 2000

Department of Biochemistry and Molecular Biology
THE ROLE OF MKK3 IN MEDIATING SIGNALING TO THE p38 MAP KINASE PATHWAY

A DISSERTATION PRESENTED

BY

MARK ALLEN WYSK

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of Biomedical Sciences

Department of Biochemistry
and Molecular Biology
November, 8 2000
DEDICATION

This dissertation is dedicated to the loving memory of my mother, Beatrice (Stearns) Wysk without whom this dissertation would not have been possible.

I also wish to acknowledge other members of my family here especially, my brother (and best friend) Steve his wife Laurie and my nieces and nephews: Scott, Eric Krysta and Loran. My sister Judy her husband Carlo and their family (including six nieces, one nephew 13 grand-nieces and grand-nephews, one great-grand-niece and one great-grand-nephew) and of course Aunt Mary. Your loving support kept me going all these years.
ACKNOWLEDGMENTS

During my time in graduate school I have encountered many people and made many friends, too many in fact to fully mention here. Nonetheless, there are a few people I do wish to acknowledge.

First and foremost I gratefully acknowledge my mentor, Roger Davis, for his encouragement and support during the course of my thesis research.

I would like to thank all members of Roger Davis' lab, past and present with special thanks to the following: Drs. Zoya Galcheva-Gargova and Laxman Gangwani for your friendship, patience and understanding. I wish to thank Dr. Perry Hall for scientific correspondence and patiently listening to me complain about grad school. To my former roommate and bench-mate Jeff Rogers for friendship, sushi, trips to O'Connors and for keeping me sane. Thanks to Victor Lazaron, Benoit Derijard and Martin Dickens, for just being friends. Thanks to all past and present members of the Peterson, Doxsey, Stephenson, Czech, Corvera, Ip, Theurkauf, and Lambright labs (did I forget anybody?) for many scientific conversations and great TGIF's. Finally, I wish to thank all the graduate students for the many good times we have shared (you know who you are).
ACKNOWLEDGMENT

The data presented in this Dissertation was generated in collaboration with Hong-Tao Lu, Ph. D., Howard Hughes Medical Institute and Section of Immunobiology, Yale University School of Medicine. I gratefully acknowledge Hong-Tao's contributions to the material presented in chapters three and four of this thesis.
Portions of this dissertation appear in the following publications:


*Equally contributed
The work presented in this Dissertation also contributed to the following publications:


*Equally contributed
ABSTRACT

p38 mitogen-activated protein (MAP) kinases represent a subgroup of MAP kinases that respond to environmental stress and inflammatory cytokines. p38 MAPK is activated by two upstream kinases, MKK3 and MKK6, by dual phosphorylation on threonine and tyrosine in conserved kinase subdomain VII. Until recently the relative roles of MKK3 and MKK6 have remained unclear. I have undertaken two strategies in an effort to understand the importance of MKK3 as a p38 MAPK activator. First, I cloned and characterized the murine mkk3 gene and determined the structure of the 5'-terminus. Comparison of the murine and human mkk3 genes revealed that the mouse gene encodes a single MKK3 isoform, MKK3b, and the human gene encodes two isoforms, MKK3a and MKK3b. Comparison of the mouse and human mkk3 genes suggests that expression of MKK3a and MKK3b is regulated from different promoters. Analysis of the mkk3 promoter demonstrates that muscle specific expression of murine MKK3b is controlled, in part, by the transcription factors MEF2 and MyoD. Second, I have utilized a gene targeting strategy to disrupt the murine mkk3 gene and to examine the effect on p38 MAPK signaling. I found that there is a p38-specific signaling defect in MKK3 deficient primary mouse embryo fibroblasts (MEF) which correlates with deficits in interleukin (IL)-1 and IL-6 production in
response to tumor necrosis factor-α (TNFα) stimulation. In addition there is a
defect in TNFα mediated expression of TNFα and macrophage inflammatory
proteins (MIP) 1α, MIP1β and MIP2. p38 MAPK-specific signaling defects were
also observed in lipopolysaccharide (LPS) stimulated mkk3 (-/-) macrophages.
Additionally, mkk3 (-/-) macrophages exhibit defects in LPS and CD40-ligand
(CD40L) stimulated IL-12 biosynthesis. Similar data were obtained from CD40L-
stimulated mkk3 (-/-) dendritic cells. I also observe that interferon (Ifn)-γ
production is diminished during T-helper-1 (T_H1) differentiation of CD4^+ T-cells
derived from mkk3 (-/-) mice. Taken together these data demonstrate a crucial
role for p38 MAPK activation by MKK3 in response to the inflammatory cytokine,
TNFα and during a T_H1 inflammatory response.
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<td>EGF</td>
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<td>eIF-4E</td>
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<td>ELISA</td>
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<td>Elk</td>
<td>Ets like extracellular signal regulated kinase</td>
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<tr>
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<td>MAPK</td>
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<td>NH₂</td>
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<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<td>PRAK</td>
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<tr>
<td>RANTES</td>
<td>regulated on activation of normal T-cells expressed and secreted</td>
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<tr>
<td>TRADD</td>
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SPECIFIC AIMS

1). To understand how the *mkk3* gene encodes two isoforms, MKK3a and MKK3b. In addition, to understand how expression of the murine MKK3b is regulated.

2). To understand the contribution of MKK3 in regulating signaling to the p38 mitogen-activated protein (MAP) kinase pathway in response to stress and the inflammatory cytokines tumor necrosis factor (TNF)-α interleukin (IL)-1.

3). To understand the contribution of the MKK3/p38 MAP kinase pathway during the development of CD4⁺ T-cell-dependent inflammatory response.
CHAPTER I

Introduction

Cells in multicellular organism must communicate with each other in order to regulate growth, development, and to coordinate their functions. This is accomplished by means of signaling molecules that interact with receptors located on the surface of cells. A receptor is a protein or complex of proteins, which recognizes a molecule in the cell’s immediate environment. It must be capable of transmitting information about the molecule to the interior of the cell—is the molecule harmful, helpful or unimportant? In turn the cell must interpret this information in such a way that it can mount an appropriate response. Cells also respond to a wide variety of other chemical and physical stimuli in their environment including UV irradiation, osmotic shock, heat shock and DNA damage.

Most cells in a complex organism are specialized to perform a small set of primary functions. A specific signaling molecule (or stimulus) may have different effects on different target cells. This is due in part to different receptors for the same molecule and in part to differences in internal signaling machinery between
different cell types. The mechanism by which a cell converts a stimulus in its environment into a biological response is known as signal transduction. Signal transduction is a universal process by which cells respond to stimuli in their environment and underlies the growth and development of all organisms. Understanding how signal transduction pathways program cells to perform specific functions is crucial to our understanding of how cells communicate with each other in the context of a tissue, organ or an entire organism. Additionally, dissecting specific pathways is essential for understanding and treating human diseases including cancer, autoimmune and many other diseases that result from perturbation of signal transduction pathways.

**Identification of p38 MAP kinases.** p38 MAP kinase, also known as CSBP and reactivating kinase (RK), was first identified by several independent groups using different strategies: it was shown to be a major tyrosine-phosphorylated 38 kD protein induced by lipopolysaccharide (LPS) in murine macrophage cell lines (59); to be the target for a group of anti-inflammatory drugs which inhibit IL-1 and TNFα synthesis in human monocytes (CSBP, (100)) and an IL-1-induced protein kinase that activates the protein kinase MAPKAPK kinase 2 (RK, (44, 170)). The p38 MAP kinase is similar to the yeast HOG1 MAP kinase which is involved in osmolarity regulation (Fig1.1a) (67). Two p38 MAP kinase isoforms were
identified in *Drosophila* that appear to regulate immunity gene expression (63).

There are four mammalian isoforms of p38 MAP kinase: p38α, p38β, p38γ, and p38δ (Fig 1.1b) (27, 38, 44, 53, 59, 81, 82, 100, 103, 122, 170, 187, 214). The biochemical mechanisms that account for the effects of the p38 MAP kinase signaling pathway remain unclear. However, recent studies have led to the identification of substrates for the p38 MAP kinase that may be physiologically relevant. These include the transcription factors ATF2 (161, 162), CHOP (215), ELK-1 (158, 228), MEF2C (58), and SAP-1 (158, 228) which are phosphorylated and activated by p38 MAP kinase. In addition, the p38 MAP kinase also has been reported to regulate NF-kB transcriptional activity by a mechanism that has not yet been defined (212, 253). The p38 MAP kinase has also been reported to phosphorylate and activate other protein kinases, including MNK1, MNK2, MAPKAPK2, MAPKAPK3, MSK1, and PRAK (44, 45, 109, 118, 144, 170, 218). It is likely that these protein kinases may function as effectors for some actions of the p38 MAP kinase pathway. For example, MAPKAPK2 and MSK1 may mediate the effects of p38 MAP kinase signaling on the phosphorylation and activation of the transcription factors ATF1 and CREB (78, 144, 201); MAPKAPK2 may mediate the effects of p38 MAP kinase signaling on the phosphorylation of tyrosine hydroxylase and the small heat shock protein hsp27 (44, 170, 205), and the MNK1 and MNK2 protein kinases may contribute to the effects of the p38
signaling pathway on translation through phosphorylation of eIF-4E (218). There are many MAPKKK's that can activate MKK3 and MKK6 \textit{in vitro}, but the physiologically relevant activators that mediate the effects of specific stimuli remain to be determined (40).

The specific upstream MAPK kinases for p38 MAP kinase are MKK3 and MKK6 (25, 31, 60, 61, 133, 162, 186), although MKK4, an upstream kinase for JNKs, has also been implicated in the activation of the p38 MAP kinase pathway (Fig 1.1a) (31, 46, 104). Both MKK3 and MKK6 are activated upon phosphorylation on Ser and Thr residues within subdomain VIII by upstream MAPKK kinases (MAPKKKs). Recent investigations suggest that MKK3 and MKK6 may differentially regulate p38 MAP kinase isoforms (38). However there is an isoform of MKK3 (MKK3b) that exhibits similar specificity towards p38 MAP kinase isoforms as MKK6 (37, 61, 133). The primary aim of this Dissertation is to examine the role of MKK3 in mediating signaling to the p38 MAP kinase pathway.
Signaling from Growth Factor and Cytokine Receptors

**Signaling from growth factor receptors.** Binding of ligand to some receptors activates intrinsic protein kinase activity. These receptors are grouped into two categories— receptor-tyrosine and receptor-serine/threonine kinases. The remainder of this discussion will focus on receptor tyrosine kinases (RTK's). Several growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), activate intracellular signaling pathways by binding to and activating RTK's. Generally, ligand binding triggers structural changes of RTK's including dimerization and stimulation of kinase activity (175). Activated RTK dimers then auto-phosphorylate (and likely trans-phosphorylate) residues in their cytoplasmic domains (80, 102, 174). Tyrosine auto-phosphorylation creates docking sites for cellular proteins including adaptor proteins and other enzymes. These docking sites are crucial for the recruitment and activation of a variety of signaling molecules (173). Other docking sites are required for initiating the formation of signaling complexes that communicate with MAP kinase modules through cascades of protein phosphorylation.

One class of signaling molecules recruited to activated RTK's are the Src homology-2 (SH2) and SH3 domain containing proteins. These include a large
number of proteins with different enzymatic activities. Some of these proteins are responsible for generating lipid second messengers including PLC-γ. Another class of signaling molecules that are recruited to RTK’s is the adaptor proteins (Grb2, Shc, Crk etc.) that contain only SH2 and/or SH3 domains (reviewed in (173)). These proteins link receptor activation to downstream signaling events. For example, Grb2 recruits the Ras guanine releasing factor son-of-sevenless (SOS) to Ras stimulating GTP for GDP exchange and thus Ras activation (154, 176). Once activated, Ras interacts with effector proteins including PI-3 kinase and the MAP kinase kinase kinase Raf (124, 173, 217).

**Signaling from Cytokine Receptors.** Tumor necrosis factor-α (TNFα), interleukin-1 (IL-1) and lipopolysaccharide (LPS) have a broad range of physiological effects. These include lymphocyte and leukocyte activation and trafficking, fever, acute-phase response and cartilage remodeling (7). TNF and IL-1 trigger the activation of transcription factors NF-κB and AP-1 (among others) which regulate expression of genes involved in the inflammatory response (179). Unlike growth factors, receptors for the inflammatory cytokines IL-1 and TNFα lack intrinsic enzymatic activity. Ligand binding triggers oligomerization resulting in the recruitment of signaling molecules largely through homotypic interactions.
**TNFα signaling.** The type 1 TNF receptor (TNFR1) represents a superfamily of cell surface receptors including FAS, CD27, CD30, CD40 and many others (73, 74). This class of receptors mediates cellular responses through the recruitment of adaptor proteins (Fig 1.2). The TNFR1 contains a structure known as a death domain in the cytoplasmic tail (203). As it's name suggests, the TNFR1 death domain mediates the death (apoptosis) signal in response to TNF-α (203). The death domain of TNFR1 interacts directly with TNFR1-associated death domain protein (TRADD) (73, 74). TRADD functions as a molecular scaffold recruiting additional proteins including Fas-associated death domain protein (FADD), TNFR1-associated factor-2 (TRAF2), and receptor interacting protein kinase (RIP) (72, 88, 250). FADD communicates a signal from TRADD to the caspase pathway stimulating apoptosis (19, 247). Another bifurcation of the TNFR1 signaling pathway just downstream of TRAF2 results in activation of NFκB and AP1 dependant gene transcription (17). TRAF2 is likely to be responsible for recruiting RIP and NFκB-inducing kinase (NIK) to the TNFR1 signaling complex (197). Both RIP and NIK are implicated in initiating kinase cascades leading to nuclear translocation of NFκB and activating the SAPK p38 and JNK. However, RIP deficient cells are selectively defective in mediating TNF induced NFκB activation (88). In addition, dominant negative NIK selectively blocks activation of NFκB by TNF without affecting p38 or JNK activity (185). However, nik (-/-) cells
signal normally to NFκB in response to TNF-α. These observations are consistent with a report that suggests that NIK may be redundant with MEKK1 in the NFκB pathway (7). In contrast, TRAF2 appears to play a minor role in cytokine induced NFκB activation (145, 248). The primary role for TRAF2 may be to activate JNK and p38 signaling modules in the TNFR1 signaling pathway.

**IL-1 and LPS signaling.** Binding of IL-1 to its receptor initiates the formation of a signaling complex that is substantially different from that formed by TNF. IL-1 binding induces IL-1R1 heterodimerization with IL-1R1 accessory protein (IL-1R1AcP). This heterodimer binds the adaptor protein Myd88 mediating the association of the complex to IL-1 receptor-associated kinase (IRAK) and TRAF6. TRAF6 then mediates many of the downstream events including NFκB, JNK and p38 MAP kinase activation. Many of the same proteins implicated in IL-1 signaling are involved in LPS signaling and are discussed below.

LPS signaling through the LPS receptor CD14 utilizes the human toll-like receptors, TLR2 and TLR4 (Fig 1.2). Expression of human TLR2 renders cells responsive to LPS (89). Recent reports suggest that TLR4 may be LPS-specific whereas TLR2 may be the receptor for gram-positive bacterial cell wall components (198). Binding of LPS to CD14 triggers TLR oligomerization and
activates NFκB together with the JNK and p38 MAP kinase signaling pathways (141, 198). Oligomerized TLR recruits adaptor proteins by homotypic interactions utilizing the Toll/IL-R1 (TIR) homology domain (Fig 1.2) (120). TLR utilize the TIR protein Myd88 to recruit the pelle-related protein kinase IRAK to the receptor (142, 224). The function of Myd88 is illustrated by the observation that mice with a targeted disruption of the myd88 gene are unresponsive to IL-1 and LPS (2, 86). Finally, TRAF6 is recruited, likely through a direct interaction with IRAK (16).

Many of the same proteins involved in IL-1R signaling are recruited to activated CD14/ hTLR upon LPS binding suggesting that downstream signaling events are likely to be similar between the two pathways (Fig 1.2). In addition, IL-1 and TLR2 signaling share common features with TNF receptor signaling although there are some fundamental differences.

One primary difference between the TNF and IL-1/TLR pathways is the molecular ordering at the level of TRAF2 and TRAF6 respectively. In the IL-1/TLR pathway, TRAF6 appears to be primarily responsible for activating the transcription factor, NFκB. The signal may be relayed from TRAF6 to NFκB by a succession of protein kinases including TGFβ-associated kinase-1 (TAK1), NIK and IκB-kinase (IKK) (105, 148). IKK phosphorylates IκB targeting it to the proteasome for degradation (121, 192, 252). This frees NFκB for nuclear
translocation and transcriptional activation. IRAK is also responsible for transmitting the IL-1 signal to the p38 and JNK cascades and appears to involve TAK1(148). Thus, like the TNF pathway, signaling from the IL-1 receptor and TLR's bifurcates at the level of a TRAF protein. However, the functions of IRAK and TRAF6 in transmitting the signal from the receptor to JNK and p38 MAP kinase modules are different from those of RIP and TRAF2. Events downstream of IRAK in the IL-1/TLR pathways leading to JNK and p38 MAP kinase activation remain unclear. For example, dominant negative TRAF6 fails to block hToll-induced activation of stress-activated protein kinase/c-Jun NH2-terminal kinases (141). In addition, traf6 (-/-) cells fail to activate JNK or NFκB in response to LPS (107). Also, TRAF6 may directly target IκB for proteolysis by a MAP kinase kinase kinase-independent IKK/ubiquitin dependent mechanism (30). Finally, a recent report identified ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) as a novel component of the IL-1/Toll signaling pathway that links TRAF6 to MEKK1 and activation of the JNK and NFκB pathways (91). TRAF2 is required for activation of the JNK signaling pathway in response to TNF-α (248). TRAF2 was reported to interact with the MAPKK MEKK1 and ASK1, two MAPKKK that activate the JNK signaling pathway (7, 150, 237). The mechanism by which TRAF2 interactions with MEKK1 and ASK1 activate the JNK signaling pathway is unclear. Similarly, it is not known if there are functions for other
MAPKKK in this signaling pathway (28). One possible mechanism linking cytokine induced GCK and MAPKKK activation to JNK and p38 MAP kinase signaling is through the formation of specific signaling complexes.

Signal Transduction by MAP kinase pathways

Mitogen-activated protein kinase signal transduction pathways have been implicated in multiple physiological and pathophysiological processes, including growth, differentiation, cell survival and cell death (79, 123, 227). In the yeast Saccharomyces cerevisiae, MAP kinase signaling pathways are required for mating of haploid cells. Binding of mating pheromone to a G protein-coupled receptor stimulates a MAP kinase module comprised of Ste11, Ste7 and Fus3 (Fig. 1.1) (reviewed in (229)). Fus3 activates transcription of mating genes promoting differentiation of haploid S. cerevisiae into mating-competent cells. In mammals, three groups of MAP kinases have been identified: the extracellular signal-regulated protein kinases (ERK), the c-Jun NH₂-terminal kinases (JNK), and the p38 MAP kinases. These MAP kinases are activated by conserved protein kinase signaling modules which include a MAP kinase kinase kinase (MKKK), and a dual-specificity MAP kinase kinase (MKK). The MKKK
phosphorylates and activates the MKK, which in turn, activates the MAP kinase by dual phosphorylation on threonine and tyrosine residues within a Thr-Xaa-Tyr motif located in protein kinase subdomain VIII (Fig 1.1b) (123, 227). Separate protein kinase signaling modules are employed to activate different groups of MAP kinases (Fig. 1.1a) (31). These signaling modules are coupled to different environmental stimuli. For example, the ERK MAP kinases are activated by a Ras-dependant pathway in response to many growth factors and hormones (227). In contrast, the JNK and p38 MAP kinases are activated by environmental stresses, such as UV radiation, osmotic shock, heat shock, and chemical factors including protein synthesis inhibitors and lipopolysaccharide (79, 123). The JNK and p38 MAP kinases also are activated by treatment of cells with proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor (TNF) (79, 123).

The JNK and p38 MAP kinase pathways are referred to as stress-activated MAP kinase (SAPK) pathways since they are both activated by environmental perturbation (e.g. osmotic changes, heat shock) and by inflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). The physiological functions of the JNK and p38 MAP kinase pathways might therefore be overlapping (79). Biochemical and genetic studies have
revealed the roles of the JNK pathway as regulators of apoptosis (32, 239, 243), development (182, 241), cell transformation, T-cell activation and differentiation (34, 188, 242) and cytokine production (194). Similarly, the p38 MAP kinase pathway has been proposed to function in the regulation of cytokine production (9, 100, 167), B cell and T cell proliferation and differentiation (23, 24, 167, 189), the innate immune response (63, 79), cell cycle control (130, 196), and apoptosis (75, 83, 216, 239).

The immediate upstream activators of the JNK group of MAP kinases are MKK4 and MKK7. Recent evidence suggests that signaling from cytokine receptors to JNK may selectively utilize MKK7 (41, 134). In addition MKK7 is activated by the GTPase Rac (69). MKK7 but not MKK4 selectively interacts with the JIP group of molecular scaffold proteins (87, 226, 246). JIP also directly interacts with JNK, MLK3 and the Ste20 related protein kinase, HPK1 (87, 226, 246). These observations suggest that the formation of specific complexes may define selectivity of JNK activation by MKK4 and MKK7 in response to various stimuli. Similar selectivity in the p38 MAP kinase signaling pathway regarding activation by MKK3 versus MKK6 has not been identified. However, two recent reports demonstrate that MKK6 and MKK3b (but not MKK3a) contain an amino-
terminal domain which may mediate selectivity in the p38 MAP kinase signaling pathway (37, 38).

**Signaling in the immune system.** The adaptive branch of the immune system is an important area to study signaling by the stress-activated protein kinases. Both B and T-cells undergo complicated patterns of development from naive precursor cells to highly specialized effector cells. Upon antigen stimulation, CD4+ T-cells differentiate into two distinct subsets (137, 138). These two subsets, TH1 and TH2 cells, are responsible for cell-mediated inflammatory and humoral immune responses, respectively. (136, 138, 151, 153). The signature cytokines of TH1 cells are IFN-γ, IL-2 and TNF-β which are involved in cell mediated inflammatory reactions (153). TH2 cells secrete mainly IL-4, IL-5, IL-6, IL-10 and IL-13 mediating B-cell activation and antibody production (153). The selective differentiation of naive T-cells into TH1 or TH2 cells depends largely on antigen dose, the source of co-stimulation and the cytokine environment (22). The most potent polarizing factor is the cytokine environment during stimulation. IL-4 drives the differentiation of TH2 cells whereas IL-12 promotes TH1 development (98, 193). Recent reports have demonstrated that the JNK and p38 MAP kinase pathways are involved in the developmental decision between a TH1 and a TH2 response (167, 188, 242). Targeted disruption of the jnk1 or the jnk2...
genes resulted in attenuated T\textsubscript{H}1 responses (34, 242). This was a result of the inability to down-regulate production of T\textsubscript{H}2 cytokines (\textit{jnk1} (-/-)) or decreased IL-12-stimulated IFN-\gamma production (\textit{jnk2} (-/-)) (34, 242). The p38 MAP kinase signaling pathway has also been implicated in the T\textsubscript{H}1 response. Transgenic mice expressing a dominant negative p38 MAP kinase exhibit a selective defect in the T\textsubscript{H}1 response (167). Pyridinyl imidazoles (inhibitors of p38 MAP kinase) block IFN-\gamma production by T\textsubscript{H}1 cells without affecting IL-4 production by T\textsubscript{H}2 cells (167). In contrast, transgenic mice expressing constitutively active MKK6 exhibited increased production of IFN-\gamma during differentiation and activation of T\textsubscript{H}1 cells (167). Thus the immune system provides useful models for examining the contribution of different MAP kinase signaling pathways to the execution of an inflammatory response.
Figure 1.1 MAP kinase modules: The Erk, JNK and p38 pathways.

Five MAP kinase signaling pathways have been identified in mammals, three of which have been extensively characterized: the Erk, JNK and p38 MAP kinase pathways. (A) The three-tiered MAP kinase module is conserved from the yeast *Saccharomyces cerevisiae* to humans. Illustrated are the three kinase modules comprising the MAP kinase signaling pathways. (B) The p38 group of MAP kinases is comprised of four members: p38α; p38β; p38γ; and p38δ. Illustrated is the conservation between p38 MAP kinase isoforms in the activation domain.
A

Yeast

MAPK Pathways

MAPKK

STE11

RAF

?

MEKK1

MLK

MAPKK

STE7

MEK1/2

MKK3/6

MKK4

MKK7

MAPK

FUS3

ERK1/2

p38

JNK

Proliferation, Differentiation Development

Inflammation, Apoptosis Development

B

Human p38α

EMTGYVATRWYRAPE

Human p38β

EMTGYVATRWYRAPE

Murine p38β

EMTGYVATRWYRAPE

Human p38δ

EMTGYVVTTRWYRAPE

Murine p38δ

EMTGYVVTTRWYRAPE

Human p38γ

EMTGYVVTTRWYRAPE

conseusus

EMTGYV - TRWYRAPE
Figure 1.2 Cytokine signaling pathways.

Inflammatory cytokines and LPS activate the JNK and p38 MAP kinase signaling pathways. Illustrated is a diagram of signaling events occurring upstream of the conserved JNK and p38 MAP kinase modules in response to TNF-α and LPS (adapted from Tony Pawson and Piers Nash, Genes and Dev. 2000 14: 1027-1047)
TNFα

TNFR1

TRADD

FADD

pro-caspase 8

active caspase 8

apoptosis

TRADD

DD

DD

DD

DD

RIP

NF-kB pathway

TRAF2

JNK and p38 pathways

TRAF6

ECSIT

MEKK1

NF-kB pathway

JNK and p38 pathways

LPS

CD14

Toll2/4

MYD88

IRAK

Adapted from Tony Pawson and Piers Nash

Genes and Dev. 2000 14: 1027-1047
CHAPTER II

STRUCTURE AND REGULATION OF THE MURINE Mkk3 GENE

Abstract

... Molecular cloning studies identified two human isoforms of MKK3, (MKK3a and MKK3b) which exhibited different substrate specificity (31, 61, 133). Sequence comparison of the MKK3 cDNA identified a 29 amino acid NH₂-terminal extension in MKK3b relative to MKK3a (Fig 2.1a) (37, 61, 133). Cloning and characterization of the 23.6-kilobase (kb) murine mkk3 gene illustrated that it consists of 12 exons interrupted by 11 introns. Comparison of the human and murine mkk3 genes demonstrated that they diverge in the 5'-region. The murine mkk3 gene encodes a single MKK3 isoform, MKK3b, whereas the human gene encodes both MKK3a and MKK3b. The DNA sequence upstream of the translation initiation codon in the murine mkk3 gene encodes multiple cis-acting elements predicted to interact with many transcription factors. These include potential binding sites for the transcription factors MyoD, MEF2, AP1/PEA1, AP2, NF-κB, and PEA3/Ets. The structure and sequence of the human and murine...
mkk3 genes suggest mechanisms for generation of alternate isoforms of MKK3 in humans and for maintenance of robust MKK3b expression in skeletal muscle.
The p38 MAP kinase kinases MKK3 and MKK6 selectively activate the p38 group of MAP kinases. p38α is also activated by MKK4 in vitro but the significance of this observation is unclear (31). MKK6 is a common activator of p38α, p38β2; p38γ and p38δ MAP kinase, while MKK3a activated only p38α, p38γ and p38δ MAP kinase (27, 37, 38, 53, 81, 82, 214). However, recent reports identified cDNA encoding two isoforms of MKK3 (MKK3a, and MKK3b) that have different substrate specificities (61, 133). MKK3b, unlike MKK3a, activates p38β2 together with p38α, p38γ and p38δ MAP kinase (37, 38). The amino acid sequences of MKK3a and MKK3b are identical with one exception. MKK3b has a 29 amino acid NH₂-terminal extension relative to MKK3a (Fig. 2.1a) (37, 61, 133). The NH₂-terminal extension of MKK3b encodes a docking domain, conserved in MKK6 but absent in MKK3a, that mediates MKK interactions with p38α and p38β2 MAP kinase (37). These observations demonstrate that MKK3 isoforms differentially activate the p38 MAP kinase signaling pathway. Expression of two MKK3 isoforms with different substrate specificity suggests that they may differ in their physiological function. These MKK may be coupled to different upstream signaling pathways. Alternatively, these MKK3 isoforms may differ in their tissue distribution. Northern blot analysis has demonstrated that MKK3 mRNA is
Figure 2.1. Comparison of MKK3a and MKK3b.

(A) Amino Acid sequence of human and murine were compared and aligned using the GCG (University of Wisconsin Genetics Computer Group) programs Pileup and Pretty. The initiating methionine in human MKK3a is indicated by a star. Amino acids comprising the p38 MAP kinase-docking site are indicated by the symbol delta. (B) Nucleotide sequence of human mkk3 cDNA and murine mkk3 gene (exon 2) were compared and aligned as described above. The initiation codon (ATG) in human MKK3a is indicated by a star.
A

mmk3b  MESPASSQPASMPQSKGSKRKKDLRISCVSKPPVSNN
hmkk3b  ..........A.P.L.T..........M......AP...
hmkk3a  ..........M......AP...
Consensus  GKSRRKKDLRISC-SKPP--NPT

B

mmk3a  AGGAAAATCCAAGAGGAAGGATCTACGGATATCCTGCATGTC
hmkk3b  ..........A..........CT..........G...
Exon 2  AGGAAAATCCAA-AGGAAGAGA--TACGGATATCCTGC-TGTC
Consensus  AGGAAAATCCAA-AGGAAGAGA--TACGGATATCCTGC-TGTC

CAAGCCACCCGCCCAACCACCACC
..........T.TGT...........
..........G...........
CAAGCCACC-G---CCACCACC
ubiquitously expressed and is enriched in skeletal muscle (31). Thus, MKK3 expression may be positively regulated in skeletal muscle although the significance of this observation remains to be tested.

MyoD is a member of the bHLH group transcription factors that includes myogenin, Myf5 (15) and Myf6 (14, 125, 165). These transcription factors are also known as muscle regulatory factors (MRFs). MRFs promote skeletal muscle differentiation by binding to a consensus sequence termed an E-box, present in the promoters of many muscle specific genes (6, 8, 114, 128, 180, 225, 251). Induction of MRF activities may be the result of extracellular signals such as insulin or insulin like growth factors known to promote terminal differentiation of myoblasts by activating multiple MAP kinase pathways including the p38 pathway (131). The p38 MAP kinase enhances the ability of MyoD to convert fibroblasts to muscle (235, 254). Treatment with the p38 inhibitor, SB203580, blocks the myogenic conversion of L8 and C2C12 cells to multinucleated myotubes and also inhibits MyoD activity in transfected fibroblasts (26, 99, 235, 254).

Several lines of evidence suggest that MRF require a co-factor for muscle specific gene expression. One class of candidates for MRF co-regulator activities is the myocyte enhancer factor-2 (MEF2) family of transcription factors.
MEF2 proteins are encoded by four genes in mice and humans and are referred to as *mef2A*, *mef2B*, *mef2C* and *mef2D* (36, 62, 77, 116, 135, 191). MEF2 proteins alone cannot induce myogenesis in transfected fibroblasts. However when co-expressed with MyoD or myogenin they enhance the extent of myogenic conversion above that seen with either MRF alone (127, 129, 143).

MEF2A and MEF2C are both phosphorylated by p38 MAP kinase within the MEF2 transactivation domain. These phosphorylations regulate MEF2 transcriptional activity and thus play a positive role in regulating MEF2-dependent gene expression. The p38 MAP kinase may enhance MyoD induced myogenic conversion by activation of MEF2A or MEF2C (245, 256). MyoD may also be regulated directly by the p38 MAP kinase signaling pathway (160). The mechanism by which p38 MAP kinase activity is modulated during muscle differentiation remains unclear. One possibility is that regulation of p38 MAP kinase activity occurs at the level of expression of its upstream activators. For example, the JNK MAP kinase activators MKK4 and MKK7 are differentially expressed during T-cell development (223).

The purpose of this study was to examine the structure and regulation of the murine *mkk3* gene. These data show that two factors contribute to the regulation of *mkk3* expression: (i) differential promoter utilization resulting in the
expression of the MKK3a and MKK3b isoforms in humans; and (ii) sequences in
mkk3 5'-flanking region that support a mechanism for maintaining robust
eexpression of murine MKK3 in skeletal muscle. Together these data provide
insight into the mechanisms regulating mkk3 expression.
Materials and Methods

Materials. 10T1/2 fibroblasts were from American Type Culture Collection (ATCC). \([\alpha^{-32}\text{P}]\text{ATP}, [\alpha^{-32}\text{P}]\text{UTP}\) and \([\alpha^{35}\text{S}]\text{ATP}\) were obtained from DuPont-NEN. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Klenow enzyme were obtained from Boehringer-Mannheim. The expression vector pCDNA3-MKK6(glu) has been described elsewhere (162). The expression vectors pCS2, pCSA-E12, pCSA-MyoD, and pCDNA3-MEF2C (117) were provided by A. Lassar. The expression vectors pCDNA1Amp-MEF2A, pCDNA1Amp-MEF2B, pCEFL-MEF2C, and pCEFL-MEF2D (256) were provided by E. Olsen. The expression vectors pCDNA3-MEK5(glu) and pCDNA-ERK5 (115, 240, 258) were provided A. Sharrocks.

Cloning and Characterization of the murine \(mkk3\) gene. Genomic clones corresponding to the \(mkk3\) locus were isolated by screening a \(\lambda\)Fix II murine genomic library (Stratagene) using a human MKK3a cDNA as a probe. Three overlapping clones encompassing the sequence of the entire murine MKK3b cDNA were characterized by a combination of restriction digestion, Southern blotting and DNA sequencing analysis using an Applied Biosystems model 373A instrument.
Characterization of the human mkk3 gene. The human mkk3 gene sequence was assembled from overlapping contigs deposited into Genbank from the human genome sequencing project (Eric Lander, MIT).

FISH (fluorescence in situ hybridization). Lymphocytes isolated from male mouse spleen were used for chromosomal FISH analysis (65, 66). Metaphase chromosomes spread on a glass slide were air dried, baked at 55°C (1h), and digested with RNase A. The DNA was denatured for 3 min at 70°C in 70% formamide in 2X SSC (1X SSC is 0.15 M sodium citrate) and dehydrated with ethanol. DNA prepared from mouse mkk3 genomic clones was biotinylated and used as a hybridization probe (65). The FISH and 4',6-diamidino-2-phenylindole (DAPI) staining were recorded on film. The assignment of the FISH mapping data with chromosomal bands was achieved by superimposing the FISH signals with images of the DAPI-banded chromosomes.

Plasmid construction. The murine mkk3 promoter-luciferase fusion vector pGLUC-MKK3.5 was made by ligating a 3,404 bp Xba I to Rsr II restriction fragment of the 5'-flanking region into pGL3-Basic (Promega) digested with Xba I and Nco I. The mkk3 promoter deletion series pGLUC-MKK3.6, pGLUC-MKK3.7, pGLUC-MKK3.8, pGLUC-MKK3.9, pGLUC-MKK3.10 and pGLUC-
MKK3.11 were constructed by removing progressively larger fragments of the mkk3 5' region from the reporter plasmid pGLUC-MKK3.5 using standard techniques.

**Cell culture.** 10T1/2 fibroblasts were cultured in minimal Eagle's medium (MEM) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO₂. Transient transfections were performed with the LipofectAMINE reagent (Life Technologies) according to the manufacturer's recommendations.

**Mkk3 reporter gene expression.** Plasmids encoding a luciferase gene driven by the murine mkk3 promoter were co-transfected into 10T1/2 fibroblasts together with expression constructs encoding MyoD, E12, MEF2A, MEF2B, MEF2C, MEF2D without or with constitutively active forms of MKK5, MKK6 (MKK5(glu), and MKK6(glu)) or ERK5. Cells were grown in 6-well multi-well plates (Costar) and transiently transfected with 2 μg of total plasmid DNA per well. A β-galactosidase expression plasmid (pCH110, Pharmacia LKB) was used to control for transfection efficiency. The total amount of DNA was kept constant by using the empty vector pCS2 (A. Lassar). After 36 hours the medium was changed to differentiation medium (MEM/ 2% heat inactivated horse serum/ 2
mM glutamine/ 100 U penicillin per ml/100 units streptomycin per ml/ 10 μg/ml insulin/ 5 μg/ml transferrin/ 10 nMol sodium selenate). 60 hours after transfection cell extracts were prepared and the activities of β-galactosidase and luciferase were measured as described below.

**Luciferase and β-glactosidase assays.** Cells were scraped from each well of a six-well tissue culture plate in 300 μl of 0.1M potassium phosphate (pH 7.4)/ 1 mM PMSF. Cells were lysed by four freeze thaw cycles (freeze on crushed dry ice, thaw at 37°C). Cell debris was pelleted by centrifugation at 14,000 rpm in a microcentrifuge at 4°C for 15 minutes. Luciferase reactions were performed by adding 10 μl clarified cell lysate to 350 μl of assay buffer (25 mM glycylglycine pH 7.8, 15 mM KPO₄ pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 2 mM ATP, 1 mM DTT) and 100 ul D-luciferin (1 mM D-luciferin, 25 mM mM glycylglycine pH 7.8, 1 mM DTT). Luciferase activity was measured using the Monolight 2010 luminometer (Pharmingen). β-galactosidase activity was measured on a SpectraMAX 250 plate reader (Molecular Devices).

**RNA isolation and primer extension analysis.** Total RNA was purified from 0.5 g (wet weight) of mouse liver using RNA-Stat reagent (TelTest) according to the manufacturer’s recommendations. The 18 residue oligonucleotide MKK3.91
(5'-TCATGGCTGCTGGTGTCT-3') complementary to nucleotides +259 / +242 in the murine mkk3 5' flanking region was end-labeled using T4 polynucleotide kinase (Promega) and [α-32P]ATP. The labeled primer was annealed to yeast tRNA (20 μg) (control) or adult mouse liver total RNA (20 μg) and extended with AMV reverse transcriptase (Promega) according to manufacturer's recommendations. Chain termination sequencing reactions were performed with the above primer and a segment of the mouse genomic DNA containing the mkk3 5' flanking sequences using a T7 Sequenase Quick-Denature Plasmid Sequencing kit (Amersham Corp.) and [α-35S]ATP. Reverse transcripts were analyzed on a 6% acrylamide, 8M urea gel in parallel with chain termination sequencing reactions.
Results

Structure of the murine mkk3 gene. MKK3 has been identified as a specific activator of the p38 group of MAP kinases. Several overlapping genomic clones encoding MKK3 were isolated. Sequencing of the murine mkk3 gene (23.6 kb) revealed the presence of 12 exons interrupted by 11 introns (Fig. 2.2a, 2.3a). Initiation codons in-frame with upstream termination codons are located in exon one of the murine mkk3 gene. Exon one also encodes the first 16 residues of the amino terminal extension found in MKK3b together with the 5'-untranslated region (UTR) and includes potential transcriptional regulatory elements (see below). Exon two of the murine mkk3 gene encodes the amino terminus of MKK3a but does not encode the initiation codon (ATG) found in human MKK3a. Instead the murine mkk3 gene contains a valine codon (GTG) at this position. Sequences upstream of the valine codon in exon 2 share significant homology with the 5'-sequences of human MKK3 cDNAs and encode 13 amino acids of the NH2 terminus found in murine MKK3b.

Murine genomic clones encoding MKK3 and a MKK3 pseudo-gene were isolated. FISH analysis led to mapping of mkk3 to mouse chromosome 11 region B1.3 whereas the mkk3 pseudogene maps to chromosome 4 region C6 (Fig.
2.2b). Data from the Genome Project maps human mkk3 to chromosome 17q11.2. The human mkk3 gene consists of 15 exons interrupted by 14 introns (Fig 2.2a). Comparison of the human and murine mkk3 genes reveals differences in the structure of the 5'-terminus. Like the murine gene, exon one of the human mkk3 gene encodes the first 16 residues of the amino terminal extension found in MKK3b. However, the human mkk3 gene contains three additional exons encoding the 5'-UTR of MKK3a (Fig 2.3a). Exon 5 of the human gene shares homology with exon 2 of the mouse gene and encodes the translation initiation codon found in the human MKK3a cDNA. These observations suggest that the human MKK3a and MKK3b messages are transcribed from different promoters.

**Determination of transcription start sites for murine mkk3.** Exon 1 of the murine mkk3 gene encodes the translation initiation codon and the first sixteen amino acids of MKK3b. Identity between the cDNA and genomic sequences extends 153 nucleotides upstream of the initiation codon (ATG). To determine the 5'-terminal region of MKK3b mRNA primer extension analysis was performed on yeast tRNA (control) or mouse liver total RNA using anti-sense oligonucleotides complementary to the 5' UTR of MKK3b. Primer extension with the oligonucleotide MKK3.91 (5'-TCATGGCTGCTGGTCT-3') resulted in one
Figure 2.2. Identification and chromosomal localization of the murine mkk3 gene

(A) The murine mkk3 gene was characterized by a combination of Southern blot analysis and DNA sequencing using an Applied Biosystems model 373A instrument. There are 12 exons interrupted by 11 introns. The human mkk3 gene was compiled from sequences deposited in Genbank (Eric Lander, MIT). The human mkk3 gene has 15 exons interrupted by 14 introns. Exons are represented by black boxes and designated with numbers. Introns are represented by a line between exons are designated in letter code. Grey boxes adjacent to exons 1 and 12 (mouse) and 15 (human) represent 5' UTR and 3' UTR respectively. Hatched boxes indicate exons removed by splicing of mkk3 mRNA. (B) Fish analysis of murine mkk3 was performed on murine lymphocyte metaphase chromosomes using DNA prepared from mkk3 and mkk3-pseudogene genomic clones as probes. White numbers indicate the chromosomal localization.
mkk3

FISH

DAPI
major and three minor reverse transcripts (Fig. 2.4). The major transcript started 324 nucleotides upstream of the translation initiation codon. The nucleotide corresponding to the 5’ terminus of the major transcript was designated as +1 and will be used as a reference to describe the relative positions of promoter elements and restriction enzyme sites used to make various reporter constructs.

**Regulatory elements in the 5’-flanking sequence of the murine mkk3 gene.**

To identify cis-acting elements that may regulate murine MKK3b transcription 3,404 bp of of the 5’ flanking sequence was determined (Fig. 2.5). The 300 bp of sequence surrounding the transcription start sites has high GC-content (68%). There was no canonical TATA or CCAAT box near the start site. However there were six Sp1 core binding sites distributed around the transcription start sites. One Sp1 site is located upstream of the major start site (at −18 nucleotides) with the five other Sp1 sites located downstream (at +62, +77, +93, +113 and +169 respectively).

A computer-assisted search of the mkk3 5’-flanking region utilizing the University of Wisconsin Genetics Computer Group (GCG) program FindPatterns together with TFSITE database revealed the presence of multiple cis-acting elements with homology to known binding sequences for a variety of transcription
Figure 2.3. Structure of the murine mkk3 gene.

(A) The murine mkk3 gene was characterized as described above. Illustrated are 23.6 kb of genomic sequence. Introns are represented by a line between exons and are designated in letter code. Grey boxes adjacent to exons 1 and 12 represent 5' UTR and 3' UTR respectively. Individual genomic clones are illustrated by lines below the genomic structure. (B) Represented are the predicted mRNA splice junctions in the murine mkk3 gene. Also illustrated is the relationship between the intron-exon and domain structures of the mkk3 gene and MKK3 protein respectively.
**A**

![Diagram of gene structure](image)

- **MKK3 3D**
- **MKK3 2G**
- **MKK3 3E**

**B**

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Figure 2.4. Determination of transcription start sites for murine MKK3b.

Primer extension analysis was performed on yeast tRNA (20 μg) (control) or adult mouse liver total RNA (20 μg) as described in Materials and Methods. (A) Reverse transcripts were resolved on a 6% denaturing polyacrylamide gel together with chain termination sequencing reactions generated by the same oligonucleotide used for the primer extension reactions. Extension products are indicated by arrows. The single major transcript is indicated by a heavy arrow. (B) An aliquot of the samples described above were electrophoresed for an extended time to better resolve the sequencing ladder in the area of the major reverse transcript. The major reverse transcript is indicated with an arrow. The sequence surrounding the start site is shown with the initiating nucleotide indicated with a star.
factors (Fig. 2.5). These include six MyoD/E-box sites, three MEF2 sites, two AP1/PEA1 sites, one AP2 site, one NF-κB site, and four PEA3/Ets sites.

**Promoter activity of the 5’ flanking region of the murine mkk3 gene.** MKK3 mRNA (together with MKK6) is enriched in skeletal muscle (31, 61, 133). Furthermore the MyoD and MEF2 families of muscle specific transcription factors have been implicated as regulators of skeletal muscle development (10, 36, 70, 143, 152, 202, 219-222, 232, 233). These observations, together with the presence of MyoD and MEF2 binding sites in the 5’ flanking region of the mkk3 gene, suggested that MKK3b expression may be regulated during skeletal muscle development or regeneration. Weintraub and others have described an *in vitro* differentiation protocol during which forced expression of MyoD and E12 in 10T1/2 fibroblasts activated a developmental program reminiscent of skeletal muscle development (15, 29). A similar approach was utilized to examine regulation of murine mkk3 reporter gene expression. A reporter plasmid containing the -3086 to +317 region of the murine mkk3 gene driving a firefly luciferase gene was transfected into 10T1/2 fibroblasts and luciferase activity was examined during *in vitro* differentiation. Luciferase activity was strongly induced by co-transfection with MyoD and E12 (Fig. 2.6a). In contrast, expression of the different MEF2 proteins alone had little or no effect on mkk3
promoter activity (Fig. 2.6b). These data demonstrate that mkk3 expression can be regulated by MyoD and E12. The absence of MEF2 stimulation of mkk3 promoter activity may be due to the presence of endogenous MEF2 proteins in 10T1/2 fibroblasts.

Members of the MEF2 family of transcription factors induce myogenesis in transfected fibroblasts only when co-expressed with myogenic bHLH proteins MyoD or myogenin. Furthermore, MEF2A and MEF2C are phosphorylated and activated by p38 MAP kinase (58, 245, 256). Therefore the ability of MKK6(glu) and different MEF2 proteins to activate expression of an MKK3b-luciferase reporter was tested. A recent report also indicates that MyoD is activated by the p38 MAP kinase signal transduction pathway (160). MyoD transactivation of the MKK3b promoter was enhanced by co-expression with MKK6(glu) (Fig. 2.7a). In addition, transactivation of the MKK3b promoter was greatly diminished by treatment of cells with the p38 MAP kinase inhibitor SB203580 (Fig. 2.7a). In contrast, co-expression of MEK5(glu) and Erk5 had no effect on MyoD and MEF2 transactivation of the MKK3b promoter (Fig. 2.7b). Co-expression of MEF2 isoforms with MyoD/E12 and MKK6(glu) had little additional effect on MKK3b promoter activity. Thus the p38 MAP kinase signaling pathway may enhance
E-box

PEA3

PEA3
Figure 2.5. \textit{mkk3} gene 5'-flanking region: Potential transcriptional regulatory elements.

3404 bp of 5' flanking sequence of the \textit{mkk3} gene was searched for cis-acting transcriptional elements with the program FindPatterns (GCG) using the TFSITE database as the pattern.dat file. Illustrated are 1611 bp of sequence indicated to be important for regulation of MKK3 expression (see Fig. 2.8). Potential transcription factor binding sites are indicated by black boxes with white text just below the sequence. Transcription start sites are indicated with arrows. The major transcription start site is indicated with a heavy arrow and in designated +1. The translation initiation codon is illustrated in bold text.
Figure 2.6. Regulation of MKK3 expression by MyoD in 10T1/2 fibroblasts.

10T1/2 fibroblasts were cultured and subjected to *in vitro* differentiation as described in Materials and Methods. Luciferase assays were performed on cells transfected with (A) MyoD and E12 or with (B) MEF2 expression plasmids. A β-galactosidase expression plasmid (pCH110, Pharmacia LKB) was used to control for transfection efficiency.
MyoD-stimulated expression of mkk3. This may occur by activation of endogenous MEF2 proteins or by direct activation of MyoD by the p38 MAP kinase signaling pathway.

Next, the minimal region of the MKK3b promoter required for activation by MyoD, MEF2C and MKK6(glu) was determined. Deletions of the 3.4 kb murine mkk3 5'-flanking region were made by deleting successive E-box motifs (Fig. 2.8). The 10T1/2 fibroblasts were transfected with each reporter plasmid without or with expression plasmids for MyoD, E12, MKK6(glu), and MEF2C. Luciferase activity was measured as previously described. Each successive 5' deletion resulted in a loss of basal and stimulated promoter activity. A deletion to -950 bp of the transcription start site resulted in 22% of basal promoter activity (pGLUC-MKK3.8) (Fig. 2.8). However, MyoD can still stimulate luciferase expression from this promoter fragment. In addition, MyoD stimulated expression from this promoter fragment was still enhanced by co-expression with MKK6(glu) (Fig 2.8). Deleting an additional 160 nucleotides of promoter sequences from pGLUC-MKK3.8 (pGLUC-MKK3.8b) corresponding to the removal of an extended MEF2 binding site and a consensus E-box had little effect on basal or MyoD, MEF2 or MKK6(glu) stimulated promoter activity (Fig. 2.8). A deletion to -444 bp of the transcription start site (pGLUC-MKK3.9) reduced the basal promoter activity to
7% and MyoD stimulated activity was also greatly diminished (Fig. 2.8). These data define a region required for MyoD/E12 regulation of murine MKK3b expression. These observations suggest that MEF2 binding sites may not be required for MyoD stimulation of mkk3 expression and define region from -950 to -444 of the murine mkk3 5'-flanking region required for response to MyoD/E12 and the p38 MAP kinase signaling pathway.
Figure 2.7. The p38 MAP kinase signaling pathway stimulates MyoD induced MKK3 expression in 10T1/2 fibroblasts.

10T1/2 fibroblasts were cultured and subjected to in vitro differentiation as described in Materials and Methods. Cells were transfected with MyoD and E12 (A) without or with MKK6(glu), treated with DMSO (control) or SB203580 immediately prior to differentiation or (B) without or with MKK5(glu) and Erk5 and assayed for luciferase and β-galactosidase activity.
Figure 2.8. Deletion analysis of the murine mkk3 promoter.

The structure of the mkk3-reporter plasmid pGLUC-MKK3.5 is indicated. In the top illustration MyoD binding sites / E-boxes are indicated by vertical lines and are labeled. MEF2 sites are indicated by lines above and below the top illustration and are also labeled. Additional transcription factor binding sites are indicated in the bottom illustration. 10T1/2 fibroblasts were cultured and subjected to in vitro differentiation as described in Materials and Methods. Cells were transfected with MyoD and E12 and mkk3-reporter plasmids without or with MKK6 (glu) and MEF2C expression plasmids. Cell extracts were assayed for luciferase and β-galactosidase activity as previously described in Materials and Methods.
Promoter Constructs

MKK3.5

MKK3.6

MKK3.7

MKK3.8

MKK3.8b

MKK3.9

Luciferase Activity
(arbitrary units)

0 20 40 60 80 100

Control
MyoD/E12
MyoD/E12/MKK6(E)
MyoD/E12/MKK6(E) MEF2C
Discussion

**Chromosomal localization of the mkk3 gene.** Sequencing of the human and mouse genomes identified the *mkk3, mkk4, mkk6* and *mkk7* genes in both species. FISH analysis has mapped the murine *mkk* genes to the following chromosomal loci: *mkk3*, chromosome 11 region B1.3 (this study); *mkk4*, chromosome 11 region B1.3 (Mouse Genome Database, Jackson Labs); *mkk6*, chromosome 11 region E1 (Tanaka et al., unpublished observation); and *mkk7*, chromosome 11 region A2 (207). These cytogenetic loci on mouse chromosome 11 are syntenic to human chromosome 17. In fact, human data map the *mkk3, mkk4* and *mkk6* genes to the following cytogenetic loci: *mkk3*, 17q11.2 (163); *mkk4*, 17p11.2 (Mouse Genome Database, Jackson Labs); and *mkk6*, 17q23.1-q24.2 (163). These data show that the *mkk3, mkk4, mkk6* and *mkk7* are linked on mouse chromosome 11 and that this genomic organization is conserved in humans. It is interesting that *mkk3* and *mkk4* located less than 1 centimorgan (cM) apart on mouse chromosome 11. The significance of this observation remains to be tested.
**Genomic organization and expression of the mkk3 gene.** The p38 MAP kinase group consists of four isoforms: p38α; p38β; p38γ; and p38δ (27, 38, 44, 53, 59, 81, 82, 100, 103, 122, 170, 187, 214). p38 MAP kinases are activated by dual phosphorylation on threonine and tyrosine by MKK3, MKK4, and MKK6 (25, 27, 31, 53, 60, 132, 133, 162, 186). While MKK4 also activates the JNK group of MAP kinases, MKK3 and MKK6 are specific for p38 MAP kinases (31). MKK3 and MKK6 selectively activate the various p38 MAP kinase isoforms. MKK3a activates p38α, p38γ and p38δ whereas MKK6 activates all four p38 MAP kinase isoforms (38). MKK3b contains an additional 29 amino acids in the NH₂-terminal region compared to MKK3a and also activates all four p38 MAP kinase isoforms (37, 61, 133). Comparison of human MKK3a, human MKK3b and murine MKK3b cDNA revealed that the 5'-UTR of MKK3a is identical to part of the coding sequence of MKK3b (Fig. 2.1a). One exception is that the translation initiation codon (ATG) is not conserved but rather is a valine (GTG) codon in murine MKK3 (Fig. 2.1b). Sequence identity between MKK3a and MKK3b extends for 39 bp upstream of the putative translation initiation codon and encodes 13 amino acids of the MKK3b NH₂-terminal region (Fig. 2.1b). Exon 1 of the murine mkk3 gene encodes the extreme 5' terminus of MKK3b and these sequences are conserved in the human mkk3 gene. These observations strongly suggest that
MKK3b is the primary isoform of MKK3 in the mouse and that human MKK3a and b are expressed from different promoters.

One potential means of regulation for the p38 MAP kinase pathway is at the level of transcription. It has been shown previously that MKK3 is expressed in many tissues but is enriched in skeletal muscle (31, 61). The work presented here demonstrates that the promoter governing MKK3b expression contains elements implicated in muscle specific expression. These cis-acting sequence elements confer muscle specific regulation of a luciferase reporter in an *in vitro* muscle differentiation system. These results explain, at least partially, the mechanism for robust MKK3b expression in skeletal muscle. The observation that *mkk3*(-/-) mice develop normally and have no obvious developmental defects indicates that the product of the *mkk3* gene was not required for muscle development. The presence of sequence elements not necessary for muscle specific expression raises the possibility that MKK3 expression may be regulated in other cellular contexts. This in turn may provide a mechanism for the regulation of p38 activation through the tissue specific regulation of MKK3 and MKK6 expression. Indeed such a case has been reported for the JNK MAP kinase activators MKK4 and MKK7 (223).
CHAPTER III

REQUIREMENT OF MITOGEN ACTIVATED PROTEIN KINASE KINASE 3 (MKK3) FOR TNF-\(\alpha\)-INDUCED CYTOKINE EXPRESSION

Abstract

The p38 MAP kinase is activated by treatment of cells with cytokines and exposure to environmental stress. The effects of these stimuli on p38 MAP kinase are mediated by the MAP kinase kinases (MKK) MKK3, MKK4 and MKK6. The function of the p38 MAP kinase signaling pathway has been examined by investigating the effect of targeted disruption of the mkk3 gene. These data demonstrate that mkk3 gene disruption caused a selective defect in the response of fibroblasts to the proinflammatory cytokine TNF-\(\alpha\), including reduced p38 MAP kinase activation and cytokine and chemokine expression. These data demonstrate that the MKK3 protein kinase is a critical component of a TNF\(\alpha\)-stimulated signaling pathway that causes increased expression of inflammatory cytokines and \(\beta\)-chemokines.
Introduction

Functional analysis of the role of the p38 MAP kinase signaling pathway has been facilitated by the identification of pyridinyl imidazole derivatives that inhibit p38 MAP kinase (100, 101). This inhibition is mediated by specific interactions between the drug molecule and the ATP-binding site on the p38 MAP kinase (206, 230, 249). Interestingly, these drugs exert anti-inflammatory effects because they inhibit the expression of cytokines, including IL-1, IL-6 and TNF (100, 101). Based on this observation it has been proposed that the p38 MAP signaling pathway is a physiologically important mediator of increased cytokine biosynthesis in response to the exposure of cells to stress (100, 101). The p38 MAP kinase therefore represents a possible target for the design of novel anti-inflammatory drugs.

Many studies of the p38 MAP kinase signaling pathway have relied extensively on the use of pyridinyl imidazole drugs to inhibit the function of p38 MAP kinase in vivo. An alternative approach to studying the p38 MAP kinase signaling pathway would be desirable because the drugs employed would inhibit other protein kinases under some experimental conditions (20, 38, 39, 81, 228). The need for an alternative approach to complement pharmacological studies was highlighted by the recent finding that pyridinyl imidazole drugs, including
SB203580 directly inhibit thromboxane synthase and cyclooxygenase-1 and cyclooxygenase-2 (13). This observation indicates that some of the actions of the p38 MAP kinase inhibitors may be secondary to changes in prostaglandin metabolism that occur independently of p38 MAP kinase inhibition.

The genetically tractable model organism Drosophila is likely to be useful for studies of the functional role of the p38 MAP kinase signaling pathway (63). Similar genetic analysis in mammals is difficult. However, targeted gene disruption strategies can be employed to study the function of signaling pathways in mice. Because there are four genes that encode p38 MAP kinases in mammals (27, 38, 53, 81, 82, 103, 187, 214), which may have partially redundant functions, we have investigated the effect of targeted disruption of the genes that encode MKK that activate the p38 MAP kinase. Three genes have been identified: Mkk3 (31), mkk4 (31, 104), and mkk6 (60, 132, 162, 186). The MKK3 and MKK6 protein kinases are specific for p38 MAP kinase whereas the MKK4 protein kinase can activate both p38 MAP kinase and JNK. Disruption of the mkk4 gene causes marked defects in JNK activation and early embryonic death (46, 149, 241). Mkk4 (-/-) cells therefore do not provide a good model for studies of p38 MAP kinase signaling. However, it was likely that the disruption of a gene that encodes a specific activator of p38 MAP kinase might cause a selective
defect in p38 MAP kinase signaling. The studies presented here examine the effect of targeted disruption of the mkk3 gene on the p38 MAP kinase signaling pathway in murine embryo fibroblasts.
Experimental Procedures

Targeted Disruption of the mkk3 gene. DNA clones corresponding to the mkk3 locus were cloned from a λFix II phage library prepared from genomic DNA isolated from mouse strain 129/sv (Stratagene). Positive clones were characterized by Southern blot analysis and DNA sequencing (Applied Biosystems). The PGK-neo cassette and herpes simplex (HSV)-thymidine kinase gene vector pBSNTK2 was constructed as previously described (241). A 4.4-kb Bgl II-Bgl II fragment from the 5’ end of the mkk3 genomic clone was excised with Not I and Spe I, and ligated into the Not I and Xba I sites of pBSNTK2. Finally, a 0.92-kb Eco RV-Hinc II fragment derived from the 3’ end of the mkk3 gene was inserted into the vector at the Xho I site using Xho I linkers. The resulting targeting vector was linearized with Not I and electroporated into W9.5 embryonic stem cells. Genomic DNA from transfectants resistant to G418 (0.2 mg/ml) (Life Technologies, Gaithersburg, MD) and gancyclovir (2 μM) (Syntex, Palo Alto, CA) was isolated and screened by Southern blot analysis. The probe used was a 240-bp fragment of the mkk3 genomic sequence external to the targeting construct that hybridizes to a 20-kb Eco RI fragment of the endogenous mkk3 gene and to a 7-kb fragment of the targeted allele. Heterozygous mkk3 (-/+ ) cells were injected into C57BL/6 blastocysts to create chimeric mice, which
were crossed to obtain germ-line transmission of the disrupted mkk3 allele. Heterozygous mkk3 (-/+ ) mice were intercrossed to obtain wild type mkk3 (+/+ ) and mutant mkk3 (-/-) mice.

**Characterization of mkk3 (-/-) mice.** Genomic DNA was isolated from mouse tails, digested with Eco RI, and subjected to Southern Blot analysis as described above. Northern blot analysis was carried out by isolating total RNA from kidney and liver using the TRIZol reagent and hybridized to a RT-PCR fragment corresponding to nucleotides 777-1231 of the MKK3 cDNA.

**Preparation of murine embryo fibroblasts.** Embryonic day 14 mkk3 (-/-) and wild type embryos were employed to prepare murine embryo fibroblasts (MEF). The cells were grown in DMEM supplemented with 10% heat-inactivated FBS/2 mM glutamine/100 units/ml penicillin/100 μg/ml of streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO₂. Control experiments demonstrated that the wild type (+/+ ) and mkk3 (-/-) MEF proliferated at similar rates. The cells were treated with 10 μM SB203580 (Calbiochem), 10 ng/ml TNF-α (Genzyme), 10 ng/ml IL-1β (Genzyme), 300 mM sorbitol, or 40 J/m² UV radiation (UV-C).
Immuno precipitation. Cells were solubilized with lysis buffer (20 mM Tris, pH 7.4/ 1% Triton X-100/ 10% glycerol/ 137 mM NaCl/ 2 mM EDTA/ 25 mM β-glycerophosphate/ 1 mM Na orthovanadate/ 2 mM pyrophosphate/ 1 mM phenylmethylsulfonylfluoride/ 10 μg/ml leupeptin) and centrifuged at 14000-x g for 15 minutes at 4°C. Protein kinases were immunoprecipitated by incubation with polyclonal antibodies to JNK or p38 MAP kinases bound to protein-A Sepharose (Pharmacia-LKB).

Protein kinase assays. Protein kinase assays were performed in kinase buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4/ 25 mM β-glycerophosphate/ 25 mM MgCl₂/ 2 mM dithiothreitol/ 0.1 mM orthovanadate]. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer. The assays were initiated by the addition of 0.5 μg substrate proteins and 50 μM [γ-32P]ATP (10 Ci/ mMol; 1Ci = 37 GBq) in a final volume of 25 μl. The reactions were terminated after 30 minutes at 30°C by the addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after SDS-PAGE by autoradiography and Phosphorimager analysis.

Cytokine ELISA. IL-1β and IL-6 were measured with a kit, using procedures recommended by the manufacturer. Cells were treated with TNF-α for 24 hours
in vitro and culture medium harvested for IL-6 ELISA. IL-1β ELISA was performed on cells solubilized in lysis buffer.

**RNase protection assay (RPA).** MEF were treated with TNF-α in vitro for six hours and total RNA was isolated using RNA-Stat reagent (TelTest). RPA was performed using 10 μg total RNA under conditions suggested by the manufacturer. The RiboQuant RPA kit was purchased from Pharmingen (San Diego, CA). Probe sets mCK-2b, mCK-3b, mCR-4 and mCK-5 were used to detect mRNA expression. Protected RNA fragments were fractionated by denaturing PAGE (6% polyacrylamide 8M urea) and analyzed by autoradiography and Phosphorimager analysis.

**Immunoblot analysis.** Cell lysates (30 μg) were fractionated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore Inc.) and probed with a polyclonal antibody to p38 MAP kinase (Santa Cruz Biotechnology), a mAb to JNK (PharMingen), and rabbit polyclonal antibodies to MKK3, MKK4, and MKK6. The MKK antibodies were prepared by immunizing rabbits with purified, recombinant human protein kinases expressed in bacteria. Immunocomplexes were detected by enhanced chemiluminescence (Amersham).
Results

Generation of mkk3 (-/-) mice. The MKK3 protein kinase was identified previously as a specific activator of the p38 MAP kinase (31). The mkk3 gene was isolated by screening a mouse genomic library with the human MKK3 cDNA. Disruption of the murine mkk3 gene in W9.5 embryonic stem (ES) cells was accomplished by replacement of mkk3 sequences with a NeoR cassette by homologous recombination (Fig. 3.1a) utilizing a strategy of positive and negative selection. A targeting vector constructed by standard techniques is shown in Fig.3.1a. Homologous recombination with the endogenous mkk3 gene will replace an internal 1.5-kb Bgl II-EcoR V genomic fragment with a neo gene cassette. The deleted region includes exons 8 and 9, which encode amino acids 217-221 of the murine MKK3 protein. This region includes the sequence Ser-Val-Ala-Lys-Thr containing the dual phosphorylation sites (Ser and Thr) that are required for MKK3 activation (31). The deleted region also encompasses sequences that are highly conserved among all protein kinases (64). These observations led us to anticipate that the predicted targeted disruption of the mkk3 gene would result in a null allele.

The linearized targeting construct was transfected into W9.5 ES cells. Disruption of one mkk3 allele was confirmed by Southern blot analysis of
genomic DNA. Analysis of 159 independent G418 and gancyclovir-resistant clones identified 14 positive clones. The frequency of homologous recombinants among the G418 and gancyclovir-resistant clones was 9%. Chimeric mice were created by injecting six independent clones of heterozygous mkk3 (+/+) stem cells into C57BL/6 blastocysts. Two clones resulted in germ line transmission of the disrupted mkk3 allele. Heterozygotes were intercrossed to obtain homozygous mkk3 (+/-), which were identified by Southern blot analysis of genomic DNA. Crosses of the mkk3 (+/-) mice resulted in progeny with the expected Mendelian frequencies. Northern blot analysis of kidney and liver RNA confirmed that the homozygous mkk3 (+/-) mice did not express detectable levels of mkk3 mRNA (Fig. 3.1c). The mkk3 (+/-) mice were viable and fertile with no observed developmental defects. No gross histological abnormalities of the lymphoid organs were apparent in young mice. Gross histological analyses of hematoxylin- and eosin-stained sections of other non-lymphoid organs, including liver and lung, also revealed no obvious abnormalities. The absence of a profound defect in the mkk3 (+/-) animals may reflect a redundant function of the MKK3 protein kinase in the presence of the p38 MAP kinase activators MKK4 (31) and MKK6 (122, 187, 188, 214).
Figure 3.1. Disruption of the mkk3 gene by homologous recombination.

(A) Structure of targeted vector, mkk3 gene and the mutated mkk3 gene following homologous recombination. Relevant restriction enzyme sites are indicated. (N: NotI; RV:EcoRV; Sm:Smal; B:BamHI; R:EcoRI; Bg:BglII; Hc:HincII). Exons 7, 8 and 9 are depicted as closed boxes. SVAKT is the protein sequence (single letter code) encoding the dual phosphorylation sites required for MKK3 activation. The diagnostic probe used for Southern analysis is illustrated.

(B). Southern blot analysis. Genomic DNA from mouse tails was digested with EcoRI, and blots were hybridized with the probe shown in (A). The wild-type allele corresponds to a 20-kb fragment and the mutated allele is a 7-kb fragment.

(C). Northern blot analysis. Total RNA isolated from kidneys and livers of wild-type and homozygous mice was hybridized with a MKK3 cDNA fragment. The blots were also probed for β-actin mRNA which was used as an internal control.

(D). Western blot analysis. Protein lysates from primary mouse embryo fibroblasts (MEF) of wild-type and homozygous mice were used to examine the expression of MKK3, MKK4, MKK6, JNK and p38 MAP kinase.
Targeting vector MKK3 locus (MKK3+)

Mutated MKK3 locus (MKK3-)

Homologous Recombination

EcoRI digestion

Kidney Liver

β-actin

28S

18S
Biochemical analysis of the effect of mkk3 gene disruption was facilitated by the preparation of primary MEF. Protein immunoblot analysis of extracts prepared from wild type (+/+) and mkk3 (-/-) MEF demonstrated that, in contrast to wild type cells, the mkk3 (-/-) cells do not express the MKK3 protein kinase (Fig. 3.1d). However, both wild type (+/+) and mkk3 (-/-) cells express similar levels of the stress-activated MKK MKK4 and MKK6 (Fig. 3.1d). These data demonstrate that the mkk3 gene disruption caused a selective defect in the expression of the protein kinase MKK3 in the absence of changes in the expression of the other stress-activated MKK. In addition, the mkk3 gene disruption caused no change in the expression of the stress-activated MAP kinases p38 and JNK (Fig. 3.1d).

The loss of MKK3 was predicted to cause defects in the p38 MAP kinase signaling pathway. Studies were therefore performed to examine the regulation of p38 MAP kinase in wild-type (+/+) and mkk3 (-/-) MEF. The basal activity of p38 MAP kinase was reduced slightly (approximately 40% lower) in mkk3 (-/-) MEF compared with wild-type (+/+) MEF. Exposure to environmental stress (UV radiation and osmotic shock) and the inflammatory cytokine IL-1 caused activation of p38 MAP kinase in both wild-type (+/+) and mkk3 (-/-) MEF (Fig. 3.2a). These stimuli also caused similar activation of the stress-activated JNK
MAP kinase in wild-type (+/+ and mkk3 (-/-) MEF (Fig. 3.2a). In contrast, studies of the effect of TNF-α demonstrated that the mkk3 (-/-) were selectively defective in the activation of p38 MAP kinase (Fig. 3.2b).

The defect in TNF-α-stimulated p38 MAP kinase activity observed in mkk3 (-/-) MEF might reflect a general deficiency of these cells in their response to TNF-α signaling. However, ribonuclease protection assays demonstrated that the expression of the p55 and p75 TNF receptors was equal in wild-type (+/+ and mkk3 (-/-) MEF. This observation suggested that the mkk3 (-/-) MEF may have a selective defect in their response to TNF-α. To test this hypothesis the effect of TNF-α on the stress-activated MAP kinase JNK was examined. These data demonstrated that TNF-α caused similar JNK activation in wild-type (+/+ and mkk3 (-/-) MEF (Fig. 3.2b). These observations demonstrated that the defect in p38 MAP kinase activation in mkk3 (-/-) MEF represents a selective impairment of TNF-α signaling to the p38 MAP kinase pathway. The conclusion from these studies is that the MKK3 protein kinase is required for efficient TNF-α-stimulated p38 MAP kinase activation in MEF.

The stress-activated p38 MAP kinase signaling pathway has been implicated as a regulatory mechanism that controls the production of cytokines
Figure 3.2. Effect of \textit{mkk3} gene disruption on stress-activated MAP kinase activity in MEF.

\textit{(A)} Wild-type (+/+) and MKK3 (-/-) MEF were untreated, or treated with UV-C (80 J/m\textsuperscript{2}), osmotic shock (300 mM sorbitol, 30 min.), or IL-1 (10 ng/ml, 15 min). \textit{(B)} Wild-type (+/+) and MKK3 (-/-) MEF were untreated or treated with TNF-\alpha (10 ng/ml). The cells were harvested at the indicated times. The p38 MAP kinase and JNK activity were measured using an immunecomplex kinase assay with the substrates ATF2 and c-Jun, respectively (161). Radioactivity incorporated into each substrate was quantitated after SDS-PAGE by Phosphorimager analysis. The data are presented as the relative protein kinase activity.
by cells exposed to stress. Previous studies have established that p38 MAP kinase may mediate the effect of TNF-α to induce the expression of IL-1 (239) and IL-6 (228). I therefore examined cytokine expression by wild-type (+/+) and mkk3 (-/-) MEF treated with TNF-α. Treatment of wild-type (+/+) MEF with TNF-α increased the production of IL-1 and IL-6 (Fig 3.3). The p38 MAP kinase inhibitor SB203580 (158) partially inhibited TNF-α stimulated IL-1 and IL-6 synthesis (Fig. 3.3a-b). In contrast, disruption of the mkk3 gene markedly decreased the TNF-α stimulated production of IL-1 and IL-6 by MEF (Fig. 3.3a-b). These data demonstrate that mkk3 gene disruption caused a defect in TNF-α-stimulated IL-1 and IL-6 expression, indicating that the MKK3 protein kinase is an essential component of the TNF signaling pathway that regulates cytokine expression.

The induction of cytokine expression caused by TNF-α may be mediated through the regulation of several steps in the cytokine biosynthetic pathway, including increased mRNA expression and increased mRNA translation (32, 228, 239). The defect in cytokine expression by mkk3 (-/-) MEF therefore may result from alterations in either of these processes. To examine the mechanism that accounted for the defective production of cytokines by mkk3 (-/-) MEF the ability of TNF-α to induce cytokine mRNA expression was measured by ribonuclease
Figure 3.3. Effect of mkk3 gene disruption on IL-1 and IL-6 production in MEF.

Wild-type (+/+) and MKK3 (-/-) MEF were untreated, or treated with SB203850 (10 μM) for 2 hours and subsequently treated with TNF-α (10 ng/ml). A) Cell lysates were made at the indicated times and IL-1 was measured by ELISA. (B). Culture medium was collected at the indicated times and IL-6 was measured by ELISA.
Chemokines are a class of soluble inflammatory mediators that control leukocyte function and trafficking (169). C-C chemokines (or β-chemokines) are a subclass of chemokines whose expression was induced by LPS and TNF-α stimulation (169). Examination of chemokine expression by ribonuclease protection assay reveals that TNF-α treatment results in increased accumulation of MIP1α, 1β, 2 and RANTES (regulated on activation of normal T-cells expressed
Figure 3.4. Effect of mkk3 gene disruption on cytokine mRNA expression.

Wild-type (+/+) and MKK3 (-/-) MEF were untreated or treated with TNF-α (10 ng/ml) for 24 hours and cytokine mRNA was measured by RPA. Radioactivity incorporated into each protected RNA was quantitated after denaturing polyacrylamide gel electrophoresis by Phosphorimager analysis. Control studies demonstrated equal levels of GAPDH mRNA.
E

**IL-1RA**

![IL-1RA Bar Graph](image)

- Control
- TNF

F

**GAPDH**

![GAPDH Bar Graph](image)

- Control
- TNF
and secreted) β-chemokine mRNA in wild type (+/+) MEF (Fig. 3.5). In contrast, disruption of the mkk3 gene resulted in decreased TNF-α-stimulated accumulation of MIP1α, 1β and 2 mRNA by MEF (Fig. 3.5). Surprisingly, mkk3 (-/-) MEF exhibited markedly increased accumulation of RANTES mRNA in response to TNF-α treatment (Fig. 3.5). TNF-α-stimulated accumulation of TCA3 β-chemokine mRNA was similar between wild type (+/+) and mkk3 (-/-) MEF (see above). These data demonstrate that the mkk3 (-/-) MEF were selectively defective in TNF-α induced expression of MIP1α, 1β and 2 mRNA.

No defects in the expression of cytokine mRNA were detected in control cultures of mkk3 (-/-) MEF treated without TNF-α (Fig. 3.4). However, increased expression of the IL-1 receptor antagonist (IL-1RA) was observed in mkk3 (-/-) MEF compared to wild-type cells (Fig. 3.4e). This observation suggests that MKK3 functions genetically to down-regulate IL-1RA expression. The biochemical basis for this effect is unclear, but may be related to the altered basal p38 MAP kinase activity in mkk3 (-/-) MEF. In addition, mkk3 (-/-) MEF over-accumulate RANTES mRNA in response to TNF-α (Fig. 3.5a). Thus, MKK3 may also function to down-regulate TNF-α-stimulated RANTES expression. Further studies are required to test these hypotheses.
Together these data indicate that the defective cytokine expression by mkk3 (-/-) MEF was accounted for, at least in part, by the failure of TNF-α to induce the expression of cytokine mRNA (Fig. 3.4).
Figure 3.5. Effect of mkk3 gene disruption on chemokine mRNA expression.

Wild-type (+/+) and MKK3 (-/-) MEF were untreated or treated with TNF-α (10 ng/ml) for 24 hours and chemokine mRNA was measured by RPA. Radioactivity incorporated into each protected RNA was quantitated after denaturing polyacrylamide gel electrophoresis by Phosphorimager analysis. Control studies demonstrated equal levels of GAPDH mRNA.
Discussion

It has been proposed that the p38 MAP kinase signaling pathway is an important mediator of inflammatory processes. Several lines of experimental evidence support this contention. First, endotoxic lipopolysaccharide and proinflammatory cytokines cause marked activation of p38 MAP kinase (161). Second, specific inhibitors of p38 MAP kinase (pyridinyl imidazole drugs) inhibit the secretion of inflammatory cytokines (100). Third, transgenic animal models demonstrate that the p38 MAP kinase regulates transcription of the interferon-γ gene by T helper 1 cells (167). The results of the present study provide further support to the hypothesis the p38 MAP kinase signaling pathway contributes to inflammatory processes. Disruption of the mkk3 gene, which encodes an activator of p38 MAP kinase, caused a selective defect in TNF-α stimulated p38 MAP kinase activation (Fig. 3.2) and inflammatory cytokine expression (Figs. 3.3 and 3.4). Together, these observations strongly implicate the p38 MAP kinase pathway as a mediator of inflammatory signaling.

The conclusion that p38 MAP kinase is a mediator of inflammation in mammals is in contrast with observations that have been reported concerning the role of p38 MAP kinase in the innate immune response of Drosophila. Two
genes that encode p38 MAP kinase and one gene that encodes a p38 MAP kinase activator have been identified in *Drosophila* by molecular cloning (63). Inhibition of the *Drosophila* p38 MAP kinase signaling pathway caused increased expression of antibacterial and antifungal genes. This observation suggested that the p38 MAP kinase, which is activated during the innate immune response in the fly, normally functions to down-regulate this response (63). Further studies are required to define whether this apparent difference between mammals and insects reflects an evolutionary change in the function of the p38 MAP kinase or whether the different observations indicate that the role of p38 MAP kinase can be altered in highly differentiated tissues.

Another possibility is that regulation of inflammatory cytokine expression in mammals does not accurately reflect the innate immune response in *Drosophila*. Chemokine expression is known to be modulated by the transcription factor, NFκB. RelB (−/−) fibroblasts exhibit a persistent induction of a number of chemokines including RANTES, MIP1α, β, and 2 in response to LPS (238). This observation suggests that RelB functions to dampen the inflammatory response in fibroblasts. The observation that *mkk3* (−/−) MEF exhibit a dramatic overexpression of RANTES mRNA in response to TNF-α (Fig. 3.5) suggests that, under some circumstances, the p38 MAP kinase pathway may also function to
dampen the inflammatory response by modulating chemokine expression. This was consistent with the observation that \textit{mkk3 (-/-)} MEF also over-accumulate IL-1RA mRNA.

\textbf{Mkk3 gene disruption causes a selective defect in p38 MAP kinase signaling.} The targeted disruption of the \textit{mkk3} gene caused only a selective defect in the p38 MAP kinase signal transduction pathway (Fig. 3.2). It was likely that a more profound defect in p38 MAP kinase signaling was not observed because the function of the MKK3 protein kinase is partially redundant to the MKK4 and MKK6 protein kinases. Indeed, many studies have documented that MKK6 is a strong activator of p38 MAP kinase (60, 162, 186). For example, MKK6 is the major activator of p38 MAP kinase in cells exposed to osmotic stress (133). Interestingly, \textit{mkk3 (-/-)} MEF were selectively defective in their response to TNF-\textalpha whereas JNK activation was comparable to that observed in wild-type MEF (Fig. 3.2). Thus, the MKK3 protein kinase was required for full activation of p38 MAP kinase in response to the treatment of MEF with TNF-\textalpha. The observation that TNF-\textalpha does not activate MKK4 (79) can account for the lack of complementation of the MKK3 deficiency by MKK4. In contrast, MKK6 can be activated by TNF-\textalpha (133). The low level of p38 MAP kinase activation observed in TNF-\textalpha treated \textit{mkk3 (-/-)} MEF therefore may be mediated by MKK6. Further
studies of other cell types derived from the mkk3 (-/-) mice are warranted to document more fully this partial complementation by MKK6.

The MKK3 protein kinase is required for TNF-α regulated cytokine and chemokine expression. Studies using pyridinyl imidazole drugs, which inhibit p38 MAP kinase, indicate that these compounds may regulate the translation of cytokine mRNA's (94, 100, 101, 159). The mechanism by which the p38 MAP kinase pathway regulates cytokine mRNA translation requires further study, but recent progress has been achieved through the identification of cytokine-responsive translational regulatory elements (94) and the identification of a group of eIF-4E protein kinases that are activated by p38 MAP kinase (45, 218). Although the effect of mkk3 gene disruption to block TNF-α stimulated expression of IL-1, IL-6, TNF-α and MIP1α, 1β, and 2 mRNA indicates that the regulation of cytokine and chemokine mRNA expression is the primary target of the MKK3 protein kinase signaling pathway in MEF (Fig. 3.4, Fig 3.5), these data do not exclude the possibility that this signaling pathway may also regulate later steps in the cytokine and chemokine biosynthetic pathways, including translation. It is also possible that the relative importance of regulated mRNA expression and translational regulation for TNF-α-stimulated cytokine and chemokine expression may differ between cell types.
Previous studies have established that the p38 MAP kinase signaling pathway mediates activation of several transcription factors, including ATF1 (78, 201), ATF2 (161, 162), CHOP (215), CREB (78, 201), ELK1 (158, 228), MEF2C (58), NF-κB (212, 253) and SAP-1 (158, 228). Defects in the activation of one or more of these transcription factors may contribute to the reduced TNF-α-stimulated expression of cytokine mRNA in these cells. Indeed, treatment with pyridinyl imidazole drugs inhibits the induction of IL-6 mRNA in TNF-α stimulated fibroblasts (9). The biochemical mechanism that accounts for the p38 MAP kinase-regulated expression of IL-6 mRNA may be caused by increased stability of the IL-6 mRNA (126) or by increased transcription of the IL-6 gene mediated, in part, by NF-κB (212). Further studies are required to identify the specific defects that are present in mkk3 (-/-) MEF.
CHAPTER IV

DEFECTIVE IL-12 PRODUCTION IN mkk3 (-/-) MICE

Abstract

The p38 mitogen-activated protein kinase (MAPK) pathway, like the c-Jun N-terminal kinase (JNK) MAPK pathway, is activated in response to cellular stress and inflammation and is involved in many fundamental biological processes. To study the role of the p38 MAP kinase pathway in vivo homologous recombination was used to inactivate the mkk3 gene, one of the two specific MAPK kinases (MKK) that activate p38 MAP kinase. Targeted disruption of the mkk3 gene resulted in mice that were viable and fertile. However, mkk3 (-/-) mice exhibited defects in LPS-stimulated interleukin-12 (IL-12) production and CD40-ligand (CD40L) stimulated IL-12 production by macrophages and dendritic cells respectively. The effect of the p38 MAP kinase pathway on IL-12 expression was in part transcriptional since inhibition of this pathway blocks IL-12 p40 promoter activity in macrophage cell lines and IL-12 p40 mRNA was reduced in mkk3 (-/-) mice. Interferon (IFN)-γ production following immunization with protein antigens and in vitro differentiation of naive T cells are greatly reduced in mkk3 (-/-) mice.
/-) mice, suggesting an impaired type-1 cytokine immune response. The conclusion drawn from these studies is that the p38 MAP kinase, activated through MKK3, is required for the production of inflammatory cytokines by both antigen-presenting cells and CD4⁺ T cells.
Introduction

LPS is a cell wall component of Gram-positive bacteria that induces pathologic responses including inflammation and sepsis (213). Induction of the inflammatory response is due in part to the activation of the transcription factors NFκB and AP-1 that regulate expression of pro-inflammatory cytokines (21, 33, 164, 178, 210). Recent reports have identified AP-1 and NFκB as targets of the p38 MAP kinase signaling pathway in response to LPS (reviewed in chapter 1). Activation of the p38 MAP kinase signaling pathway by LPS stimulates expression of the inflammatory cytokines IL-1 and TNF-α by monocytes and macrophages (25, 27, 146). In contrast, treatment of cells with the p38 inhibitor SB203580 blocks LPS-stimulated cytokine expression (100, 101, 159). These observations implicate the p38 MAP kinase signaling pathway as an important mediator of the inflammatory response.

Accumulating evidence also implicates the p38 MAP kinase pathway in mediating cellular responses to the TNF family member CD40L (101,(54, 190). Exposure of antigen presenting cells (APC) to CD40L activates pathways culminating in the expression of inflammatory cytokines, most notably IL-12 (18, 55, 85, 90). CD40L-stimulated APC, especially dendrite cells (DC) are an
important source of IL-12 which is essential for mediating differentiation of naive CD4+ T-cells into Th1 cells and stimulates production of IFN-γ (1, 48, 49, 71, 151, 168, 231). Genetic studies identify the p38 MAP kinase pathway as an important mediator of the Th1 specific cytotoxic immune response (167). Transgenic mice expressing dominant negative p38 MAP kinase exhibit specific impairments in the production of IFN-γ by Th1 cells (29) In contrast, activation of the p38 MAP kinase pathway by expression of constitutively active MKK6 resulted in exacerbated Th1 responses (167).

The specific upstream MAPK kinases for p38 MAPK are MKK3 and MKK6 (25, 31, 58, 60, 132, 133, 162, 186), although MKK4, an upstream kinase for JNK, has also been implicated in the activation of the p38 MAPK pathway (31, 46, 104). The studies presented here examine the effect of targeted disruption of the mkk3 gene on the p38 MAP kinase signaling pathway during inflammatory and type-1 cytokine responses. MKK3-deficient mice are viable and fertile. Nevertheless, these mice have defective p38 MAPK activation and defects in the production of IL-12 and IFN-γ, resulting in impaired Th1 CD4+ immune response. These results suggest that MKK3 activates the p38 MAPK signaling pathway in response to LPS and during a Th1 immune response.
Experimental Procedures

Reagents. LPS (E. coli strain 0127:B8), Concanavalin A (Con A), G418 and Keyhole limpet hemocyanin (KLH) were purchased from Sigma Chemical Co. (St. Louis, MO). Complete Freund's adjuvant (CFA) was purchased from Life Technologies, Inc. (Gaithersburg, MD). Gancyclovir was purchased from Syntex (Palo Alto, CA). Sorbitol was purchased from Sigma (St. Louis, MO). The p38 inhibitors SB 203580 and SB 202190 and the control chemical SB 202474 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Brewer thioglycollate was purchased from Difco (Detroit, MI). The GM-CSF-producing hybridoma was kindly provided by Dr. David Gray (Royal Postgraduate Hospital, London). The membrane-bound CD40L was kindly provided by Dr. Marilyn Kehry (Boehringer-Ingelheim, Danbury, CT). Human recombinant IL-2 was kindly provided by Biogen Inc.. Murine IL-12 was kindly provided by Genetics Institute. CD44-biotin, CD45RB-FITC antibodies and Streptavidin-PE were purchased from Pharmingen (San Diego, CA).

Plasmids. The IL-12 p40 promoter reporter plasmid, kindly provided by Dr. Steve Smale (UCLA), contains the -350 to +50 region of the p40 promoter-driving a firefly luciferase gene (156). The PRL-SV40 Renilla luciferase plasmid was
purchased from Promega (Madison, WI). MKK3(ala) and p38(afg), dominant negative constructs for MKK3 and p38 respectively, MKK3(glu) and MKK6(glu), constitutive active constructs of MKK3 and MKK6 respectively, and p38α, p38β, p38γ and p38δ expression constructs were previously described (38, 162). All plasmid DNA was isolated by using Endotoxin-free Maxi-prep kit (Qiagen Inc., Valencia, CA).

**Isolation and culture of peritoneal macrophages.** 5-6 weeks old mice were injected with 3 ml of 3% Brewer thioglycollate i.p. and 72 hours later, peritoneal exudate cells (PECs) were isolated from the peritoneum. The thioglycollate-elicited PECs were cultured at 37°C and 5% CO₂ in 48-well plates at 5 x 10⁵ cells/well in 1 ml/well of high-glucose DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin. Two hours later, non-adherent cells were removed and the adherent macrophages were subjected to various treatments. Peritoneal macrophages were treated with LPS (100 ng/ml) for different times, the supernatants were collected and immediately analyzed for cytokine production by ELISA. For analysis of IL-1α and β production, the macrophages were treated with LPS for 4 hr, ATP (5 mM) was then added into the culture for an additional
30 min to release IL-1 into the media (68). The supernatants were collected for ELISA.

**Measurement of MKK3 and p38 MAP kinase activity.** p38 MAP kinase activity in cell lysates was measured using immunecomplex kinase assays (161). The activity of MKK3 was measured in transfected cultured macrophages. RAW264.7 cells were grown in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 5% FBS (HyClone Inc., Logan, UT), 2 mM L-glutamine, and antibiotics (penicillin/streptomycin). Flag-tagged MKK3 was expressed by transfection of 2 x 10^6 cells using the Superfect reagent (Qiagen Inc., Valencia, CA). After transfection (3 hrs), the cells were divided into two groups and incubated for 24 hrs. The cells were treated (30 min.) without or with LPS (1 µg/ml). Cell lysates were prepared and MKK3 was isolated by immunoprecipitation using the M2 monoclonal antibody to the Flag epitope (Sigma). MKK3 protein kinase activity was measured in the immunoprecipitates in a coupled kinase assay (30 min., 30°C) using 0.5 µg GST-p38γ, 0.5 µg GST-ATF2, and 50 µM [γ-32P]ATP. The phosphorylation of ATF2 was quantitated following SDS-PAGE by Phosphorimager analysis (Molecular Dynamics Inc.).

**RNase protection assay (RPA).** Peritoneal macrophages were treated with
LPS *in vitro* for various times and total RNA was isolated using TRIzol reagent (Gibco BRL, Gaithersburg, MD). RPA was performed using 5 μg total RNA under conditions suggested by the manufacturer. The RiboQuant RPA kit was purchased from Pharmingen (San Diego, CA). Probe sets mCK-2b and mCK-3 were used to detect cytokine expression. Normalization was calculated by determination of the ratio of individual cytokine mRNA against GAPDH mRNA on a phosphorimager screen. The percent inhibition was determined by the following formula:

\[
\text{percent inhibition (\%)} = 100 \times \left(\frac{(+/- \text{ cytokine:GAPDH}) - (-/- \text{ cytokine:GAPDH})}{(+/- \text{ cytokine:GAPDH})}\right)
\]

**Isolation and culture of bone marrow-derived dendritic cells.** Bone marrow cells were isolated from the femur and tibia of male mice of 6-8 weeks of age as described (155). The cells were treated with a combination of depleting antibodies (anti-B220, anti-MHC class II, anti-CD4 and anti-CD8) and complement for 1 hr at 37°C (257). The remaining live cells were cultured at 1 X 10^6 cells/ml in 2 ml/well RPMI supplemented with 5%FCS and GM-CSF (666 U/ml) in a 24-well plate. The culture medium was changed every two days to remove non-adherent granulocytes. On day 8, cells in suspension were collected replated and subjected to various treatments. Bone marrow-derived dendritic
cells were treated with membrane-bound CD40L for 48 hr, the supernatants were collected and subjected to ELISA analysis for cytokine production.

**Enzyme-linked immunosorbent assay (ELISA).** The ELISA was performed as previously described (167). Mouse ELISA paired antibodies for TNF-α, IL-6, IFN-γ and IL-4 were purchased from Pharamingen (San Diego, CA). A mouse IL-12 ELISA kit was purchased from Biosource International (Camarillo, CA), which detects the mature IL-12 p70 heterodimer. Mouse IL-1α ELISA kit was purchased from Endogen (Cambridge, MA), The mouse IL-1β DuosetTM paired antibodies and mouse IL-1β standard were purchased from Genzyme (Cambridge, MA). The concentration of the coating antibody for TNF-α and IL-6 was 4 μg/ml and the secondary biotinylated antibody was 2 μg/ml. The concentration of the coating antibody used for IFN-γ and IL-4 was 0.625 μg/ml and 1 μg/ml respectively and the secondary biotinylated antibody was 2 μg/ml and 1 μg/ml respectively. The ELISA for IL-12, IL-1α and IL-1β was performed according to the manufacturer's instructions. Mouse IL-6 and TNF-α (R&D Systems, Minneapolis, MN), IFN-γ (Gibco BRL, Gaithersburg, MD) and IL-4 (DNAX, Los Angeles, CA) were used as standards. Horseradish peroxidase-conjugated avidin D was purchased from Vector Laboratories (Burlingame, CA). The TMB microwell
peroxidase substrate and stop solution were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

**Measurement of IL-12 p40 promoter-driven luciferase activity.** The RAW264.7 murine macrophage line was grown in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 5% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin and streptomycin. 2X10^6 cells/well in 6-well plate was transiently transfected by Superfect (Qiagen Inc., Valencia, CA) according to manufacturer’s protocol with 4 μg of IL-12 p40 promoter-driven firefly luciferase reporter plasmid and 0.4 μg of PRL-SV40 Renilla luciferase expression vector which was co-transfected to normalize the transfection efficiency. Some cells were also co-transfected with 4 μg of either MKK3(ala), p38(agf), MKK3(glu) or MKK6(glu) or empty expression vector. Three hours after transfection, the cells were harvested and split evenly into two wells. 24 hours later, one group of cells was left untreated and the other group was incubated with LPS (1 μg/ml). In some experiments, the transfected cells were pretreated with SB202190 (2 μM) for one hour before LPS treatment. 24 hours after LPS treatment, cell extracts were prepared and luciferase activity was measured by using the Dual-Luciferase system (Promega Corp., Madison, WI).
In vitro T cell differentiation. CD4⁺ T cells were isolated from spleen and lymph nodes of six-week old mice by negative selection as described (167, 257). Naive CD4⁺ T cells were then isolated by sorting for CD44low CD45RBhigh cells. APCs were obtained by gamma-irradiation and negative selection (257).

5x10⁵ naive CD4⁺ T cells were incubated with an equal number of APCs in Bruff medium with 5% FCS (Life Technologies, Inc. Gaithersburg, MD), 2 mM L-glutamine, and penicillin/streptomycin in the presence of Con A (2.5 μg/ml), IL-2 (20 U/ml) and anti-IL-4 (Clone 11B11) with or without IL-12 (3.5 ng/ml) for 4 days. The cells were exhaustively washed, counted and restimulated at 5x10⁵ cells/ml with Con A (2.5 μg/ml) for 20 h. The supernatants were collected and IFN-γ production was measured by ELISA.

Measurement and induction of the KLH recall response. Mice were immunized with 50 μg/ml of KLH in CFA in each of the hind footpads. Draining lymph nodes were isolated 9 days later. The lymphocytes were cultured in 96-well plates (5X10⁵ cells/well) in the presence of KLH of different concentrations in vitro. One group was incubated with 1 μCi/well ³H-thymidine at day 3. At day 4, proliferation was assayed by determining the incorporation of ³H-thymidine. The
supernatants of other groups were assayed at day 5 by ELISA for IFN-γ production.
Results

**Normal development of mkk3 (-/-) lymphocytes.** mkk3 (-/-) mice have normal numbers of thymocytes and splenocytes. Major cell surface markers of T and B lymphocytes were examined by flow cytometric analysis of cells derived from freshly isolated thymus, spleen and lymph nodes. The wild-type and knockout mice have similar expression of CD3, CD4, CD8, CD25, CD44, CD69, TCR α/β and γδ B220, F4/80 and major histocompatibility complex class II antigen I-Ab. The number of bone marrow-derived dendritic cells (DCs), as well as CD11c, I-Ab and B7-2 surface markers also did not differ between DCs from wild-type (+/+) and mkk3 (-/-). No obvious defects in lymphocyte development were therefore evident in mkk3 (-/-) mice.

**Defective p38 MAP kinase activity in mkk3 (-/-) macrophages.** By the use of the chemical inhibitors, the p38 kinase pathway has been implicated in the regulation of the expression of TNF-α, IL-1, IL-6, and GM-CSF (9, 100). Since many of the inflammatory cytokines are produced by macrophages upon activation by LPS, a potent activator of the p38 MAP kinase pathway, LPS-activated p38 MAP kinase activity in mkk3 (-/-) macrophages was studied. There was reduced p38 MAP kinase activation in mkk3 (-/-) macrophages in
comparison to wild-type macrophages (Fig. 4.1a); interestingly however, the activation of p38 MAP kinase in response to sorbitol was similar in the wild-type and knockout macrophages (Fig. 4.1b), indicating that LPS-induced p38 MAP kinase activation was selectively defective in the M KK3-deficient macrophages. On the other hand, JNK activity induced by LPS in mkk3 (-/-) macrophages was not reduced (Fig 4.1a).

In order for M KK3 deficiency to account for the defect in p38 activation in mkk3 (-/-) macrophages, it is necessary that LPS activates M KK3. To test this directly, RAW264.7 macrophages were transfected with an M KK3 expression vector and activated with LPS. M KK3 was then immunoprecipitated and p38 activity was measured by a coupled kinase assay (Fig. 4.1c). M KK3-directed p38 activation was stimulated more than 4 fold by LPS, showing that LPS indeed activates M KK3.

Defective IL-12 production in mkk3 (-/-) antigen-presenting cells (APCs). To test whether the expression of inflammatory cytokines was affected in mkk3 (-/-) mice, LPS-stimulated macrophage RNA was isolated and subjected to RNase protection assay (RPA) by using a panel of inflammatory cytokine probe sets. IL-6, TNF-α, IL-1α and IL-1β mRNAs accumulated upon LPS stimulation in the wild-
Figure 4.1  p38 MAPK activity in mkk3 (-/-) and wild-type primary macrophages and the macrophage cell line RAW264.7.

Peritoneal macrophages from wild-type (+/+) and mkk3 (-/-) mice were left untreated (control) or treated with (A) LPS (100 ng/ml) or (B) sorbitol (300 mM) for 5, 15 and 30 min. The p38 MAPK activity was measured using an immunocomplex kinase assay with the substrate GST-ATF2. (C) RAW264.7 cells were transiently transfected with an MKK3 expression vector. 24 hours later, the cells were left untreated or treated with LPS for 30 min. MKK3 was immunoprecipitated from the cell lysates. Kinase assays were performed using the immunoprecipitates, GST-p38γ, GST-ATF2 and γ^32P-ATP. The radioactivity incorporated into GST-ATF2 was quantitated after SDS/PAGE by Phosphorlmager analysis and presented as relative p38 MAPK activity. The results shown were obtained in a single experiment and are representative of two separate experiments with similar results.
A

![Graph of relative kinase activity over time for p38 and JNK with LPS treatment.]

B

![Graph of relative kinase activity over time for p38 and JNK with sorbitol treatment.]

- Control
- LPS (min)
- Sorbitol (min)
type and the knockout macrophages to a similar degree (Fig. 4.2), indicating that MKK3 deficiency did not affect the expression of these four cytokine mRNAs. Both IL-12 p40 and p35 mRNAs were up regulated in wild-type macrophages, but unexpectedly, the level of p40 mRNA was barely detectable and p35 mRNA expression was also greatly reduced in the mkk3 (-/-) macrophages (Figs. 4.2a and c). There was also a small decrease of TGFβ2 mRNA in the mkk3 (-/-) (20% reduction) (Fig. 4.2b and c). Relative inhibition of cytokine expression in knockout versus wild-type mice is shown in Fig. 4.2c.

IL-12 is secreted by antigen-presenting cells (APCs), including macrophages and dendritic cells, when microbial pathogens are encountered. To examine the status of IL-12 protein production by APCs in mkk3 (-/-) mice, peritoneal macrophages from both wild-type and knockout mice were activated with LPS and IL-12 production was measured by ELISA after stimulation. There was a marked reduction in IL-12 expressed by the knockout macrophages when compared to the wild-type macrophages (Fig. 4.3a) consistent with the RPA data. This LPS-induced production of IL-12 was also inhibited by SB 203580 and SB 202190, two inhibitors of p38 MAP kinase, but not by SB 202474, a chemical with similar structure that was used as a negative control (Fig. 4.3a). Together, these
Figure 4.2 LPS induced inflammatory cytokine mRNA expression is reduced in mkk (-/-) macrophages

Peritoneal macrophages from wild-type (+/+) and mkk3 (-/-) mice were left untreated (control) or treated with LPS for 6 hours. Total RNA was extracted and subjected to RNAase protection assay (Pharmingen) utilizing the template sets (A) mCK-2b or (B) mCK-3 (C) Protected bands were quantitated by Phosphorimager analysis using the housekeeping GAPDH as an internal control. Percent inhibition was calculated by the following formula:

\[
\text{percent inhibition} \, (\%) = 100 \times \left( \frac{\text{+/+ cytokine:GAPDH} - \text{+- cytokine:GAPDH}}{\text{+/+ cytokine:GAPDH}} \right)
\]
data indicate that MKK3-directed p38 activation is required for LPS-induced IL-12 production in macrophages.

Dendritic cells, another major APC type, are a key source of IL-12 in response to stimulation with CD40L in the presence of low amounts of LPS (18, 55, 85, 90, 184). Therefore IL-12 production by DCs was also measured. IL-12 production was highly induced by CD40L in wild type DCs in a dose-dependent manner; however, the induction of IL-12 production by CD40-CD40L engagement was markedly reduced in the mkk3 (-/-) DCs (Fig. 4.3b). These results demonstrate that there was a general impairment of IL-12 production by mkk3 (-/-) APC's.

The amount of protein secretion of TNF-α and IL-6 in mkk3 (-/-) macrophages was comparable to that of the wild-type (+/+) macrophages upon LPS stimulation (Figs. 4.4a and b). Together with the RPA data which showed little difference in the amounts of mRNA of these two cytokines between the wild-type (+/+) and the mkk3 (-/-) macrophages, these results suggested that MKK3 was not required for the production of IL-6 and TNF-α mRNA in LPS-stimulated macrophages. In contrast, the secretion of both IL-1α and IL-1β protein was reduced in mkk3 (-/-) macrophages (Fig. 4.4 c and d). This contrasts however
Figure 4.3 Inflammatory cytokine production induced by LPS is reduced in mkk3 (-/-) mice.

(A). IL-12 production in wild type (+/+ and mkk3 (-/-) macrophages. Peritoneal macrophages were left untreated, treated with LPS (100 ng/ml) for 20 hr, or pretreated with SB 203580 (10 μM), SB 202190 (10 μM) or SB 202474 (10μM) for 2 hr before LPS (100 ng/ml) was added for an additional 20 hr. Supernatants were collected and IL-12 production was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of six separate experiments with similar results.

(B). IL-12 production by CD40-CD40L engagement in bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells were left untreated or treated with different concentrations of membrane-bound CD40L (1:100 and 1:500) for 48 hr. Supernatants were collected and IL-12 production was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of two separate experiments with similar results.
A. Macrophages

- Control
- LPS
- LPS + SB203580
- LPS + SB202190
- LPS + SB202474

B. Dendritic Cells

- Control
- CD40L 1:100
- CD40L 1:500
Figure 4.4  Reduction in LPS induced IL-1 production in mkk3 -/- macrophages

(A) IL-6 and TNF-α production wild type (+/+) and mkk3 (-/-) macrophages. Peritoneal macrophages were left untreated or treated with LPS (100 ng/ml) for 20 hr or pretreated with SB 202190 (10 μM) for 2 hr before LPS (100 ng/ml) was added for an additional 20 hr. Supernatants were collected and IL-6 and TNF-α production were measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of three separate experiments with similar results.

(B) IL-1α and IL-1β production in wild type (+/+) and mkk3 (-/-) macrophages. Peritoneal macrophages were left untreated or treated with LPS (100 ng/ml) for 4 hr or pretreated with SB 202190 (10 μM) for 2 hr before LPS (100 ng/ml) was added for an additional 4 hr. ATP (5 mM) was added to the culture for 30 min to induce the release of IL-1 into the supernatant. The supernatants were collected and IL-1 (α and β) production was measured by ELISA. The mean and standard error are shown. The results shown were obtained in a single experiment and are representative of three separate experiments with similar results.
with the RPA analysis for IL-1α and IL-1β mRNA (Fig. 4.2a and c), suggesting that MKK3-directed p38 MAP kinase might be involved in IL-1 translational or post-translational regulation.

**Regulation of the IL-12 p40 promoter by the p38 MAP kinase pathway.** To further study the molecular mechanisms underlying the regulation of IL-12 by the MKK3-directed p38 MAP kinase pathway, IL-12 p40 reporter gene expression was examined in macrophages. Since it is very difficult to transfect primary macrophages, the murine macrophage cell line RAW264.7 was used for these co-transfection experiments. An IL-12 p40 reporter construct, containing the −350 to +55 region of the IL-12 p40 promoter driving a firefly luciferase gene (156), was transiently transfected into RAW264.7 cells. Luciferase activity was strongly induced by LPS and was dependent on the p38 MAP kinase pathway since this induction was blocked by SB202190 (Fig. 4.5a), but not by the control drug SB202474. This LPS-induced IL-12 p40 promoter-driven luciferase expression was also suppressed when a dominant negative expression construct of MKK3 or p38 MAP kinase was co-transfected (Fig. 4.5a); thus the p38 MAP kinase pathway is required for LPS-induced IL-12 p40 reporter expression, acting at least in part at the transcriptional level. Since the p40 promoter used for these experiments contains the 5'-UTR, these observations do not exclude the
possibility that p38 MAP kinase may also regulate IL-12 p40 expression post-transcriptionally by acting through the 5' UTR.

To examine which isoform of p38 contributes to the regulation of IL-12 expression, activated MKK3 and MKK6, MKK3 (glu) and MKK6 (glu) respectively, were co-transfected with different p38 isoforms and their ability to activate the p40 promoter was studied. It appears that MKK3(glu) activates the IL-12 p40 promoter through p38α but less well through p38β, p38γ and p38δ; while MKK6(glu) primarily acted through p38α and p38β (Fig. 4.5b). These data indicated that p38α was directly involved in the regulation of IL-12 p40 expression.

**Impaired type 1 cytokine immune response in mkk3 (-/-) mice.** The p38 MAP kinase pathway is activated upon TCR ligation and T cell activation (167, 171, 177). Recently it has been demonstrated that p38 MAP kinase mediates interferon-γ (IFN-γ) expression in T helper 1 (TH1) effector cells (167). IL-12 is an inflammatory cytokine linking the innate and adaptive immune responses. It induces the production of IFN-γ and is therefore a critical mediator of the proinflammatory antigen-specific TH1, cytotoxic T lymphocyte (CTL) and natural killer (NK) cell cytotoxic response (1, 49, 71, 151, 168, 231). To determine if IFN-γ
Figure 4.5  IL-12 p40 promoter activity is regulated by the p38 MAPK signaling pathway in RAW264.7 macrophages.

RAW264.7 macrophages were transiently transfected with the IL-12 p40-Luciferase reporter (A) with or without MKK3(ala) or p38(AGF) or (B) together with MKK3(glu) or MKK6(glu) with or without p38α, p38β, p38γ, or p38δ respectively. Transfected cells were split into two wells and left untreated or were treated with LPS for 24 hours in the presence or absence of SB202190. The luciferase activity was measured by standard techniques.
production was impaired in mkk3 (-/-) mice, naive CD4⁺T-cells were differentiated into T_H⁺ cells in vitro using standard techniques (42, 167, 168, 257). Sorted CD44low CD45RBhigh naive CD4⁺ T cells were cultured with APCs in the presence of Concanavalin-A (Con-A), interleukin-2 (IL-2), and anti-IL-4 antibody for 4 days. After extensive washing, the cells were replated and cultured for another day in the presence of Con A. IFN-γ production was then measured. Wild-type T cells incubated with wild-type APC produce large amounts of IFN-γ, whereas knockout T cells incubated with knockout APC produced little IFN-γ (Fig. 4.6), indicating that IFN-γ production was impaired in mkk3 (-/-) mice. To test whether this defect resulted from an intrinsic deficiency in the property of T_H⁺ CD4⁺ T cells with reduced p38 activity that was described previously (167), or also from an APC deficiency, as described here resulting from impaired IL-12 production, wild-type naive CD4⁺ T cells were incubated with knockout APCs and similarly, knockout naive CD4⁺ cells were incubated with wild-type APCs under the same conditions. In both cases, IFN-γ production was greatly reduced compared to that of the wild-type T cells and APC, but was still substantially higher than that of knockout T cells plus knockout APC (Fig. 4.6). This result suggested that both knockout T cells and knockout APCs were intrinsically defective, and that both contribute to the diminished production of IFN-γ in the knockout mice.
Figure 4.6 IFN-γ production after in vitro differentiation of naive CD4+ T cells.

Sorted CD44^{low}CD45RB^{high} naive CD4+ cells from wild-type (T+) and MKK3-deficient mice (T-) were cultured with either the wild-type APCs (APC+) or knockout APCs (APC-) in the presence of Con A, IL-2 and anti-IL-4 antibody for 4 days. The cells were then washed extensively and re-stimulated with Con A for another day. IFN-γ production in the supernatants was measured by ELISA. The mean and standard error are shown. The results shown in this experiment were representative of three separate experiments with similar outcomes.
To further examine whether T\textsubscript{H}1 CD\textsuperscript{4}+ T cell responses were defective in the mkk3 (-/-) mice \textit{in vivo}, the KLH recall response was examined by measuring IFN-\gamma secretion from lymphocytes after secondary antigenic challenge \textit{ex vivo}. Wild type (+/+ ) mice exhibited dose-dependent increase in IFN-\gamma production in response to KLH. In contrast mkk3 (-/-) littermates produced little IFN-\gamma during re-stimulation with KLH (Fig. 4.7a). To rule out the possibility that this was due to unresponsiveness of mkk3 (-/-) lymphocytes to KLH, T cell proliferation was examined by \textsuperscript{3}H-thymidine incorporation assays. Wild type (+/+ ) and mkk3 (-/-) T-cells incorporated similar amounts of \textsuperscript{3}H-thymidine in response to KLH restimulation. (Fig. 4.7b). Thus the ability to secrete IFN-\gamma in response to antigens was therefore greatly impaired, but not completely absent, in mkk3 (-/-) mice.
Figure 4.7 KLH-induced IFN-γ production is reduced in mkk3 (-/-) mice.

(A) IFN-γ production induced by KLH. Mice were immunized with Keyhole limpet hemocyanin (KLH) in complete Freund’s adjuvant (CFA) in the footpads. Nine days later, lymphocytes from the draining lymph nodes in the treated mice were isolated and incubated in vitro with different concentrations of KLH for 4 days. The supernatants were collected and ELISA was performed to examine the induction of IFN-γ in the supernatants. The mean and standard error are shown. The results obtained in this experiment were representative of two separate experiments with similar results.

b. Proliferation of lymphocytes in response to KLH in vitro. Lymphocytes isolated from draining lymph nodes after nine days of initial challenge with KLH-CFA were incubated in vitro with different concentrations of KLH. At day 3, [3H]thymidine was added to the culture media. The proliferation response was measured at day 4 by examining the incorporation of [3H]thymidine. The mean and standard error are shown. The results shown in this experiment were representative of two separate experiments with similar results.
Discussion

Mice with a targeted disruption of the mkk3 gene were generated and shown to have fundamental defects in the inflammatory response and the T\textsubscript{H}1 CD4\textsuperscript{+} T cell response. Three upstream kinases that activate p38 MAP kinase have been reported: MKK3, MKK4 and MKK6 (227). MKK4 activates JNK and p38 MAP kinase in vitro (31, 104), however, there was no defect in p38 MAP kinase activation in MKK4-deficient embryonic stem (ES) cells although p38 MAP kinase activation in Mkk4 (-/-) MEF was reduced (46, 149, 241). At present, MKK3 and MKK6 are the only known specific p38 MAP kinase activators. By using transient transfection of genes encoding MKK3 or MKK6 and other biochemical characterization in vitro, many reports suggested that MKK6 is the most potent activator of p38 MAP kinase (25, 133, 162). The relative contribution of MKK3 versus MKK6 in vivo is, however, undefined; the generation of mkk3 (-/-) mice therefore provides an opportunity to determine the role of MKK3 versus MKK6 in vivo. LPS-activated p38 MAP kinase activity was reduced, but not absent, in mkk3 (-/-) macrophages (Fig. 4.1a). On the other hand, p38 MAP kinase activity induced by sorbitol in MKK3-deficient macrophages was intact suggesting that MKK3 was required for full activation of p38 MAP kinase by LPS (Fig. 4.1b). The biological significance of this LPS-induced p38 MAP kinase defect in mkk3 (-/-)
mice was illustrated by the demonstration that LPS-induced IL-12 production and IL-12 p40 mRNA expression by macrophages was almost completely blocked in mkk3 (-/-) mice (Figs. 4.2 and 4.3).

Bacterial LPS is one of the most potent activators of cells of the monocyte lineage. LPS forms a complex with the serum protein LPS-binding protein (LBP), the LPS-LBP complex then binds to CD14 on the cell surface to induce a signal within the cell, probably through a Toll-like receptor (89, 157, 211, 244). LPS has been shown to induce activation of all three MAPK pathways including ERK, JNK and p38, in addition to PKC, ceramide and PKA; however, the significance of each pathway in connecting LPS to intracellular gene activation is unknown (195). It has been shown that the p38 MAP kinase pathway is involved in the LPS-induced biosynthesis of TNF-α, IL-1, IL-6 and GM-CSF (9, 100) and that the JNK and ERK pathways are also involved in TNF-α production induced by LPS (194, 255). mkk3 (-/-) mice have enabled us to study the contribution of the MKK3-directed p38 MAP kinase pathway to the regulation of LPS-induced cytokine production in macrophages. Secretion of IL-6 and TNF-α by peritoneal macrophages in response to LPS stimulation in vitro revealed little defect, if any, in the mkk3 (-/-) mice, suggesting that MKK3 is not required for these processes. The biosynthesis of IL-6 and TNF-α was partially inhibited by the p38 MAP
kinase inhibitor SB 202190 (Figs. 4.4a and b). Thus, there is an SB 202190-sensitive pathway that contributes to IL-6 and TNF-α production that is not M KK3-dependent; presumably M KK6 may compensate for the M KK3 defect, or be wholly responsible for the induction of these cytokines. On the other hand, IL-1α and IL-1β production (but not mRNA expression) was reduced in mkk3 (-/-) (Figs. 4.4c and d), indicative of a role of the M KK3-directed p38 MAP kinase pathway in the production of IL-1α and IL-1β that acts at the translational or post-translational level, as suggested previously (100). The most dramatic effect observed in mkk3 (-/-) mice, however, was the almost complete absence of IL-12 production caused by LPS in macrophages and by CD40-CD40L interactions in dendritic cells (Fig. 4.3 a-b). This was surprising, because IL-12 biosynthesis had not been previously known to be regulated by the p38 MAP kinase pathway.

Interleukin-12 is a heterodimeric cytokine that consists of p35 and p40 subunits. It plays a central role in driving naive CD4 T cells into differentiated T H 1 cells by inducing the production of IFN-γ, an important effector in both adaptive cellular immunity and innate immunity (48, 208). Among its many immunomodulatory effects in both innate and adaptive immunity, IFN-γ up-regulates major histocompatibility complex (MHC) class I and II antigen expression, stimulates specific CD8 T cell-mediated cytotoxic immunity through
recognition of specific MHC class I and antigen complexes, and enhances innate immunity by activation of macrophages and NK cells (11, 209). IL-12 therefore serves as a bridge connecting innate immunity to adaptive immunity (209). IL-12 is secreted mainly by APCs upon innate immune recognition of pathogen-associated molecular patterns including LPS (119). However, the signaling pathway leading to IL-12 production was hitherto undefined (151). The p38 MAP kinase pathway was induced by LPS and peptidoglycan, the major molecular recognition pattern among Gram-negative and Gram-positive bacteria, respectively (35, 59). This suggested that the p38 MAP kinase pathway is activated during the innate immune recognition process. It is particularly satisfying therefore that one of the downstream targets of the p38 MAP kinase pathway is IL-12, which, upon induction by microbial products via the p38 MAP kinase pathway, would initiate antigen-specific adaptive immunity. The type I immune response to the protein antigen KLH (in CFA), an adjuvant which favors TH1 responses through the production of IL-12 (43), was compromised in mkk3 (-/-) mice (Fig. 4.7a). IFN-γ production by differentiated CD4+ T cells was also greatly reduced in mkk3 (-/-) mice. Intrinsic defects in both mkk3 (-/-) T-cells and APC contributed to this impairment (Fig. 4.6). The APC defect was likely caused by the impairment of IL-12 production since mice deficient for IL-12-p40, IL-12 receptor beta1 and Stat 4, a signaling molecule that is required for IL-12
responsiveness, also exhibit defective type I cytokine immune responses (84, 112, 204, 234). Taken together, these results suggest that MKK3 plays a determining role in driving downstream p38 MAP kinase activation to regulate IL-12 production in APC.

Analysis of mRNA demonstrated that IL-12 p40 gene expression was almost absent in the mkk3 (-/-) mice (Fig. 4.2a and c); IL-12 p35 mRNA level was also reduced (Fig. 4.2a and 4.2c). Likewise, p40 promoter-driven reporter expression was blocked by SB202190 and dominant negative expression constructs for MKK3 and p38 MAP kinase (Fig. 4.5a). These results indicated that p38 MAP kinase regulates IL-12 p40 transcriptionally, at least in part. In comparison to the IL-12 p35 promoter, the IL-12 p40 promoter and the transcription factors that bind to it are better characterized. C/EBP and NF-kB family members together with an ets-2-related factor have been shown to bind to their corresponding sites in the p40 promoter and regulate the expression of this gene (56, 110, 111, 140, 156). By the use of gene disruptions in mice, IRF-1 and ICSBP, another member of the IRF-1 family, have been shown to be required for IL-12 p40 expression (51, 106, 172, 199). Since many of the identified substrates for p38 MAP kinase are transcription factors, and furthermore, since the p38 MAP kinase pathway can contribute to NF-kB-mediated transactivation (9, 212), it
is tempting to speculate that the p38 MAP kinase pathway may regulate IL-12 p40 transcription by regulating the activity of either IRF-1 family members, C/EBP, NF-κB or the ets-2-related factor, directly or indirectly. Further work will be required to test this hypothesis. It was also possible that the inhibition of IL-12 production in \textit{mkk3} (-/-) mice was mediated by IL-10, IL-4, or TGF-β, cytokines that suppress the production of IL-12 (181). This was considered unlikely since a reduction rather than an increase of TGFβ2 production in \textit{mkk3} (-/-) macrophages was demonstrated using a sensitive RNase protection assay (Fig. 4.2b and c), and because the production of IL-4 and IL-10 mRNA response to antigen was similar in wild-type (+/+ ) and \textit{mkk3} (-/-) T cells. It was also observed that LPS-induced IL-12 production was inhibited by SB 203580 and SB 202190 (Fig. 4a). Since p38α and p38β are inhibited by these drugs, the LPS-induced, MKK3-directed IL-12 production was likely mediated, in part, by the activation of p38α. This conclusion was supported by the observation that p38α potentiates the transactivation of an IL-12 p40 luciferase reporter mediated by constitutive active MKK3a in RAW264.7 macrophages (Fig. 4.5b).

An interesting parallel can be drawn between the role of the p38 MAP kinase pathway in inflammatory APC, such as macrophages and DC, and its role in T cells. The production of IL-12 in APC and the production of IFN-γ in T cells
all require the p38 MAP kinase pathway, shown by us here and elsewhere (167). The ancient p38 MAP kinase pathway appears to have been used repeatedly during evolution for a variety of stress responses, ranging from osmotic stress in single-cell organisms like yeast (67), regulation of antimicrobial peptide expression in insects(63), to the production of inflammatory cytokines by fibroblasts (236) macrophages and dendritic cells in the innate immune response and finally in the proinflammatory T_{H}1 adaptive immune response (167)(this study) developed in higher vertebrates. The conservation of function was unlikely to be fortuitous and may be repeated for other signaling pathways.
CHAPTER V.

CONCLUSIONS

Structure of the \textit{mkk3} gene. Molecular cloning studies have led to the identification of two isoforms of MKK3 in humans (MKK3a and b) that differ by 29 amino acids in the NH$_2$-terminal region (31, 133). However, analysis of mRNA and protein expression in mouse tissues identified only the MKK3b isoform (31, 61, 133) (Fig 3.1d). Analysis of the 5'-terminal region of the human \textit{mkk3} gene revealed that it encodes both MKK3a and MKK3b (Fig. 2.1a). In contrast, the murine \textit{mkk3} gene encodes a single MKK3 isoform, MKK3b (Fig. 2.1). The 5'-UTR of human MKK3a is encoded by three exons unique to MKK3a and one exon that is shared by MKK3a and MKK3b. Alignment of human MKK3a and MKK3b cDNA sequences demonstrates that the 5'-UTR's are distinct. These observations suggest that MKK3a and MKK3b are expressed from different promoters in humans and that MKK3b may be the sole murine isoform.

Regulation of MKK3 expression. MKK3 mRNA is ubiquitously expressed and is enriched in skeletal muscle (31, 61). Characterization of the murine \textit{mkk3} gene reveals a potential mechanism for maintaining robust expression of MKK3b
mRNA in skeletal muscle. The murine *mkk3* 5'-flanking region contains multiple cis-acting sequence elements encoding binding sites for the transcription factors MyoD and MEF2. Transient transfection studies demonstrated that murine *mkk3* expression was stimulated by MyoD and E12 during *in vitro* myogenic conversion of 10T1/2 fibroblasts (Fig. 2.5a). MyoD stimulated MKK3b expression was enhanced by activation of the p38 MAP kinase signaling pathway (Fig. 2.6a). In contrast, activation of the Erk5 signaling pathway had no effect on *mkk3* expression (Fig 2.6b). This effect could be mediated by the transcription factors MEF2A or MEF2C, both of which are targets of the p38 MAP kinase signaling pathway and cooperate with MyoD to mediate muscle specific gene expression (245, 254, 256). However, MEF2C does not activate expression of an *mkk3* reporter plasmid (Fig. 2.6, Fig 2.8).

Expression of MKK6 (glu) activates MyoD-dependent *mkk3*-reporter expression independent of MEF2C coexpression in 10T1/2 fibroblasts (Fig. 2.8). Thus, p38 MAP kinase signaling pathway may activate MyoD-dependent gene expression in the absence of MEF2 (160, 235). It is also possible that MEF2 proteins are important in other cellular contexts. For example, MEF2C has been shown to regulate gene expression in macrophages, T-cells and primary cortical neurons (58, 93). MEF2C is also activated by LPS in the macrophage cell line
RAW 264.7 resulting in increased transcription of c-Jun (58). Thus MEF2C may regulate expression of mkk3 in a similar manner. Further studies are required to test the hypothesis that MKK3 expression is regulated my MEF2 in macrophages.

**Fibroblasts.** Targeted disruption of the mkk3 gene was predicted to cause defects in the p38 MAP kinase signaling pathway. The mkk3 (-/-) mice were viable and fertile with no observed developmental defects. Primary embryo fibroblasts (MEF) derived from wild type and mkk3 (-/-) embryos were therefore used to examine regulation of the p38 MAP kinase signaling pathway I found a specific defect in the response of mkk3 (-/-) MEF to the inflammatory cytokine TNF-α. This results in diminished expression and production of IL-6 and IL-1 by TNF-α treated MEF. In addition, there are defects in TNF-α-induced accumulation of MIP1α, Iβ and 2 mRNA in mkk3 (-/-) MEF (Fig 3.5). In contrast, accumulation of IL-1RA (Fig 3.4b) and RANTES mRNA (Fig. 3.5) are increased by mkk3 (-/-) MEF. As its name suggests IL-1RA dampens the inflammatory response by acting an antagonist for the type 1 IL-1 receptor. These data demonstrate that MKK3 is a critical mediator of signaling from cytokine receptors to the p38 MAP kinase pathway in fibroblasts. They also suggest that the p38
MAP kinase signaling modulates the inflammatory response by suppressing IL-1RA and RANTES expression.

Chemokines are soluble inflammatory mediators with molecular weights in the 8- to 20 kd range (with few exceptions) (169). There are two major chemokine sub-groups called CXC and CC. The β-chemokines belong in the CC sub-group because of the arrangement of two conserved cysteines in the NH₂-terminal region (169). There are seven chemokine receptors that selectively interact with β-chemokines: RANTES interacts with the CC chemokine receptors (CCR) CCR1, CCR3, CCR4 and CCR5; MIP1α interacts with CCR1, CCR4 and CCR5; and MIP1β interacts with CCR5 (reviewed in (169)). Targeted disruption studies demonstrated that CCR1, CCR2, and CCR5 modulate T H 1 and T H 2 responses (4, 12, 47, 50, 96). Examination of ccr1 (-/-) mice revealed a diminished T H 2 response, decreased IL-4 production and increased IFN-γ production (47). In contrast, ccr2 (-/-) and ccr5 (-/-) mice exhibit diminished IL-12 and IFN-γ production consistent with a T H 1 defect (4, 12, 96). CCR5 positively regulates IL-12 production by CD8α⁺ dendritic cells in response to microbial pathogens and RANTES, MIP1α, and 1β (4). The observations that mkk3 (-/-) MEF exhibit reductions in MIP1α, 1β and 2 expression and increased RANTES expression
suggest that the p38 signaling pathway may modulate the immune response by regulating fibroblast expression of β-chemokines.

Until recently, fibroblasts have been considered structural cells that provide scaffolding on which other cells function and migrate (183). It has become apparent that fibroblasts are important mediators of the immune response through the regulated biosynthesis of cytokines and chemokines. Assuming that fibroblasts contribute to the T\textsubscript{H}1 versus T\textsubscript{H}2 decision and that MEF are representative of fibroblasts in general then there is evidence linking cytokine secretion by fibroblasts to the T\textsubscript{H}1 inflammatory response. The inability of \textit{mkk3} (-/-) fibroblasts to express MIP1\textalpha,1β and 2 could help explain the diminished T\textsubscript{H}1 response of \textit{mkk3} (-/-) mice (see below).

**Macrophages.** Targeted disruption of the \textit{mkk3} gene was also predicted to cause defects in the p38 MAP kinase pathway in macrophages. LPS activation of p38 MAP kinase was greatly diminished in \textit{mkk3} (-/-) macrophages (Fig. 4.1). LPS-stimulated IL-1\textalpha and β production by \textit{mkk3} (-/-) macrophages was also diminished (Fig. 4.4c-d). The studies presented here also demonstrated that LPS induced TNF-α and IL-6 production was comparable between \textit{mkk3} (-/-) and wild-type (+/+) macrophages (Fig. 4.4a). In addition, LPS stimulated TNF-α
accumulation was modestly reduced by SB203580 (Fig 4.4a). These observations suggest that TNF-α expression is regulated by an MKK3-independent signaling pathway in macrophages. Alternatively, the low level of p38α activity observed in mkk3 (-/-) macrophages may be sufficient to support TNF-α expression.

The most profound phenotype observed in the mkk3 (-/-) mice was the almost complete lack of LPS-stimulated IL-12 production by macrophages. (Fig. 4.3a). Pretreatment of macrophages with SB203680 also dramatically diminished LPS-stimulated IL-12 production (Fig 4.3a). These observations suggest that LPS induced IL-12 biosynthesis is regulated by a p38α dependent pathway, although contributions by p38β cannot be eliminated. This is in agreement with transient transfection studies in the macrophage cell line RAW 264.7 in which MKK3 and MKK6 both stimulate expression of an IL-12 p40 promoter luciferase reporter construct, primarily through p38α (Fig. 4.5b). LPS also activates ectopically expressed MKK3 and MKK6 in RAW 264.7 cells (Fig. 4.1b). Thus, MKK3 and MKK6 are likely equally required for p38 activation by LPS in macrophages.
**TH1 inflammatory response.** The decision to mount a TH1 or TH2 response is made largely as a result of the local cytokine environment at the site of insult or injury. This is partly due to cytokines secreted by APC, particularly macrophages and dendritic cells. Previous observations have strongly implicated the inflammatory cytokine, IL-12, as an important mediator of the TH1 response (48, 208, 209). The p38 MAP kinase pathway has also been implicated in the developmental switch to a TH1 immune response, primarily through the regulation of IFN-γ expression (167). The observation that MKK3-deficient macrophages and dendritic cells exhibit defects in IL-12 biosynthesis predicts that mkk3 (-/-) mice would have a defect in the TH1-specific immune response (Fig. 4.3a-b). This in fact what was observed during *in vivo* and *in vitro* development of mkk3 (-/-) TH1 cells (Figs. 4.6, 4.7). These data describe a direct link between the activation of the p38 MAP kinase pathway and stimulation of IL-12 production resulting in development of a TH1 response and IFN-γ production. Thus, the p38 MAP kinase pathway is critical for the proper execution of a TH1 response including regulating TH1-specific cytokine production (108, 167).

Another cytokine implicated in mediating T-cell responses, IL-6, is secreted by antigen presenting cells and polarizes naive CD4+ T-cells into TH2 cells through stimulation of IL-4 expression (166). There was little difference in
LPS stimulated IL-6 production between mkk3 (-/-) and wild type (+/+)
macrophages (Fig 4.4) In addition IL-6 biosynthesis was not significantly
affected by pre-treatment with SB203580 suggesting that IL-6 production is regulated by a
mechanism distinct from IL-12. During development of a T cell response, p38
MAP kinase is selectively activated in TH1 cells with little activation seen in TH2
cells (167). Mkk3 (-/-) mice exhibited diminished IFN-γ secretion by APC and T-
cells consistent with a TH1 specific defect (Figs. 4.6, 4.7).

Although these studies create a sound basis for understanding the relative
contribution of MKK3 to p38 MAP kinase signaling, some fundamental questions
remain to be addressed. It is still unclear how specificity is maintained in the p38
MAP kinase pathway. There are recent reports of stimuli that selectively activate
a subset of p38 MAP kinases. This selectivity could be due to differential
expression of MAP kinases and MAP kinase kinases in the tissues tested. In
cardiomyocytes, for instance, expression of various combinations of MKK3 and
MKK6 with p38α and p38β have different effects on the expression of
hypertrophic genes versus apoptotic genes (216). There are also examples of
selective activation of p38 isoforms, MKK3 and MKK6 in natural killer (NK) cells
and neutrophils. For example, α2/β1 integrin ligation on human peripheral blood
natural killer (NK) cells activated a Rac/MKK3/p38 MAP kinase pathway resulting
in stimulation of IL-8 production (113). Ligation of α2/β1-integrin induces tyrosine phosphorylation of the guanine nucleotide exchange factor (GEF) p95-VAV by an unidentified tyrosine kinase (113). VAV in turn activates the small GTP binding protein, Rac, by catalyzing GTP for GDP exchange (113). Activated Rac stimulates the kinase activity of the Ste20-related protein kinase, PAK, which then activates the dual specificity MAP kinase kinase, MKK3 through an unknown MAPKKK (113). MKK3 activates a SB203580 sensitive p38 MAP kinase isoform (either p38α or p38β) stimulating IL-8 production (113). In these same cells, α2β1-integrin ligation activates the FAK-related non-receptor tyrosine kinase, Pyk2 resulting in paxillin phosphorylation, elevation of intracellular calcium and activation of NK cytotoxic functions (52). Human neutrophils selectively express p38α and p38δ without expressing detectable levels of p38β or p38γ (57). LPS selectively activates p38α and this activation was attenuated by expression of a dominant negative mutant of MKK3 (146, 147). Expression of dominant negative MKK6 however has no effect on LPS activation of p38α in human neutrophils (146, 147). These data suggest that MKK3 and MKK6 are not functionally redundant in their activation of the p38 MAP kinase pathway. Indeed, the phenotypes of the mkk3 (-/-) mouse are consistent with this conclusion. Similar experiments performed with p38 (-/-), mkk3 (-/-), mkk6 (-/-) and mkk3/6 (-/-) may be useful to examine MKK3 function in the immune system.
Concluding remarks

The p38 MAP kinase pathway is regulated by multiple inputs and is specifically activated by two MAP kinase kinases, MKK3 and MKK6 (31, 60, 133, 162, 186). The p38 MAP kinase signaling pathway is also activated by MKK4 in vitro but the significance of this observation remains unclear (31, 38). Focusing on a single p38 MAP kinase activator allows biochemical studies in particular model systems to be applied to understanding the role of MKK3 in activating the p38 MAP kinase signaling pathway. Examination of mkk3 (-/-) mice demonstrated the importance of MKK3 for activating p38 MAP kinase pathway in response to TNF, LPS and during a T_H1 immune response. The challenge now is to integrate these findings with observations from mice with targeted disruptions in other genes involved in the p38 MAP kinase signaling pathway including: mkk6; p38α; p38β; p38γ; and p38δ; as well as downstream targets of p38 MAP kinase such as: MAPKAPK-2; PRAK; and others.

There are several protein kinases that are phosphorylated and activated by p38 MAP kinase including MNK1, MNK2, MAPKAPK2, MAPKAPK3, MSK1, and PRAK (44, 45, 109, 118, 144, 170, 218). Activation of one of these, MAPKAPKK2 (MK2), is inhibited by SB203580 indicating that it lies downstream
of p38α or p38β (97, 201). Mk2 (-/-) mice, much like mkk3 (-/-) mice, are phenotypically normal by several criteria (92). However, mk2 (-/-) mice have an inflammatory defect similar to that observed in mkk3 (-/-) mice. There are also defects in TNF-α and IFN-γ biosynthesis from spleen cells of mk2 (-/-) mice injected with LPS (92). These assays, although different from those preformed in the mkk3 (-/-) mice, point to a Th1 defect in the mk2 (-/-) mice. More modest defects are observed in biosynthesis of IL-6 and IL-1β by cultured mk2 (-/-) spleen cells (92). It is an interesting hypothesis that the p38 signaling pathway may modulate inflammatory cytokine expression by activating MAPKAPK-2 in vivo.

The p38 MAP kinase pathway is also important for normal embryonic development. A targeted disruption of the p38α gene results in early embryonic lethality in mice. (3, 5, 139, 200). However, the exact contribution of p38α to mouse development is controversial (3, 5, 76, 139, 200). Embryonic lethality has been attributed to a placental defect in p38α (-/-) mice (3, 139). In addition, p38α (-/-) embryos had significant heart wall defects associated with a reduction in the myocardial cell population (3). The heart wall abnormality may be secondary to the placental defect because no significant differences were observed between wild type and p38α (-/-) cardiomyocytes (3). Embryonic lethality was rescued by
tetraploid fusion of a wild type embryo to a \( p38\alpha \ (-/-) \) embryo resulting in viable and outwardly normal \( p38\alpha \ (-/-) \) embryos that survive to term (3). These studies suggest that the p38 MAP kinase signaling pathway is essential for normal placenta morphogenesis but may be dispensable for the development of other organ systems. Studies of mice with targeted disruption of other p38 MAP kinase isoforms are necessary to test this hypothesis.

Mice with a targeted disruption of the \( mkk6 \) gene in a wild type and \( mkk3 \ (-/-) \) background will be useful to better elaborate the relative roles of MKK3 and MKK6. \( Mkk6 \ (-/-) \) mice may have a phenotype similar to the \( mkk3 \ (-/-) \) mouse. This result would argue that MKK3 and MKK6 are equally required for activation of the p38 MAP kinase. Thus it would be necessary to examine the effect of \( mkk3/6 \) double knockout on activation of p38 MAP kinase pathway. A prediction can be made that the \( mkk3/6 \) double knockout mouse will be early embryonic lethal. This is based on the observation that the \( p38\alpha \) knockout mice become non-viable \textit{in utero} after approximately day 12 \textit{post-coitum} (3, 5, 139, 200). However, \( p38\alpha \) was also activated by MKK4 \textit{in vitro} (31). Targeted disruption of the \( mkk4 \) gene is also early embryonic lethal (before gestation day 14 \textit{post-coitum}) and was associated with abnormal hepatogenesis and hemorrhage (46, 241). In contrast, the \( jnk1/jnk2 \) embryos die between gestation days 11 and 12.
from a hindbrain neural tube defect with no observed abnormalities in cranial mesenchyme, yolk sac, embryonic body wall, developing fetal heart or liver (95). It is possible that early embryonic lethality of the mkk4 (-/-) mouse may be due, in part, to reduced p38α activity during development.

It is apparent that MAP kinase signaling pathways are interconnected with other signaling pathways and are subject to multiple positive and negative feedback mechanisms. Targeted disruption of the mkk3, jnk1 and jnk2 genes demonstrate cooperativity between the JNK and p38 MAP kinase pathways. Mice with null alleles for these three genes have defective T_H1 immune responses (34, 108, 242). The JNK signaling pathway regulates the developmental switch between a T_H1 and T_H2 response. In the absence of JNK1 there was a defect in the down-regulation of T_H2 cytokine expression in T_H1 cells. This was due, in part, to the inability of jnk1 (-/-) cells to inhibit the nuclear accumulation of the transcription factor NFATc resulting in diminished IL-4 expression (34). In contrast, JNK2-deficiency results in a diminished T_H1 response due primarily to the inability of IL-12 to upregulate IFN-γ expression.

The conclusion from these observations is that the JNK and p38 stress-activated protein kinase pathways cooperate in the execution of a T_H1 inflammatory response. This occurs, in part, by inhibition of the T_H2-specific
transcription factor, NFATc, by increasing the expression of $T_H1$ specific cytokines and cytokine receptors (34, 242).

Despite considerable progress there is still uncertainty regarding the biological function of the p38 MAP kinase pathway \textit{in vivo}. The studies presented here demonstrated that MKK3 has a critical, nonredundant role for mediating activation of the p38 MAP kinase signaling pathway in response to inflammatory cytokines, LPS and during a $T_H1$ immune response. MKK3 specific signaling may be controlled, in part, by regulated expression or by differential interaction of MKK3 isoforms with upstream signaling pathways. Further studies are needed to test these hypotheses.


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