REGULATION OF THE STRESS-ACTIVATED PROTEIN KINASE PATHWAYS IN HEMATOPOIETIC CELLS

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B.Sc., The University of Guelph, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE STUDIES Experimental Medicine Program

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March 1999
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Date **April 19, 1999**
ABSTRACT

The regulation of the p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways in hematopoietic cells is largely uncharacterized. We demonstrated the tyrosine phosphorylation and activation of p38 MAPK by interleukin (IL) -3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and Steel locus factor (SLF). We also showed the activation of p38 MAPK is required for the activation of MAPKAP kinase-2 by these cytokines. The activation of JNK often parallels the activation of p38 MAPK. We found that these cytokines also activated the 46 and 55 kDa splice variants of JNK1 and JNK2, and induced threonine phosphorylation of MAPK kinase 4 (MKK4), an upstream activator of JNK. Together, these results demonstrated that p38 MAPK and JNK are activated not only by environmental stresses, but also by hematopoietic growth factors. In contrast, a related cytokine IL-4 failed to activate p38 MAPK, MAPKAP kinase-2, JNK1, JNK2 or MKK4. Therefore, IL-4 was unique in failing to activate any MAPK pathways in hematopoietic cells.

Both SLF and GM-CSF activated JNK comparably with similar kinetics, however SLF induced greater phosphorylation of MKK4 than GM-CSF, suggesting the existence of other JNK kinases. A cDNA with homology to MKK4 was identified in the Expressed Sequence Tag database, and the full-length cDNA was cloned using Rapid-Amplification of Cohesive Ends (RACE) PCR. This cDNA encoded a 419 amino acid protein, hereafter termed MKK7, that contained a putative kinase domain. We identified several splice variants of MKK7, and transcripts encoding MKK7 were expressed ubiquitously. Co-expression of MKK7 with either JNK1 or p38α2 MAPK led to the activation of JNK1, but not p38α2 MAPK. Epitope-tagged MKK7 was activated by IL-3, TNFα, UV light, hyperosmolarity, heat shock and anisomycin, but not by IL-4 or EGF. We also showed that endogenous MKK4 and MKK7 were activated by IL-3, TNFα or the Fc receptor for IgG. MKK7 was also activated by constitutively-active Rac and Cdc42 when expressed in HeLa or Ba/F3 cells. In contrast, constitutively-active Ras activated MKK7 in HeLa cells, but not in Ba/F3 cells, demonstrating that signalling from Ras to MKK7
varies between cell types. In conclusion, MKK7 is regulated by diverse stresses and physiological stimuli in hematopoietic cells.
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PREFACE

My thesis is divided into six chapters and organized into the following format. Chapter one is a general overview of protein kinases, the mitogen-activated protein kinase cascades and a current review of the stress-activated protein kinase pathways. Chapter two is a summary of the techniques used during my thesis. Chapters three to five describe my findings in the field of the stress-activated protein kinase pathways. Due to the competitive nature of the field, I have included a brief introduction for these chapters describing our knowledge at the time the work was being conducted. Similarly, the conclusions of these chapters represent those drawn when the work was done. Chapter six gives a summary of the data and their implications for the field in general.

It is important to state that I have used the term "stress-activated protein kinase" to refer to both JNK and p38 MAPK. I have also used the human nomenclature for JNK and MKK4 (instead of SAPK and SEK1) throughout my thesis. These changes were made simply to reduce confusion for those who are reading the thesis and not intimately versed in the field.

Publications Arising From The Work Of This Thesis


<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ATF-2</td>
<td>Activating Transcription Factor 2</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCR</td>
<td>B Cell Antigen Receptor</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CSF-1</td>
<td>Colony Stimulating Factor - 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FcR</td>
<td>Receptor for the Fc Fragment of Immunoglobulin G</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/Macrophage Colony Stimulating Factor</td>
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<tr>
<td>GTPase</td>
<td>Guanosine Triphosphatase</td>
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<td>HEPES</td>
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<td>MBP</td>
<td>Myelin Basic Protein</td>
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<td>MMLV</td>
<td>Murine Maloney Leukemia Virus</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PBS</td>
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<td>SLF</td>
<td><em>Steel</em> locus factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor alpha</td>
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ACKNOWLEDGMENTS

I am indebted to several people for their support and guidance during my time at the Biomedical Research Centre. I would like to thank my supervisor John Schrader for his enthusiastic "kid in a candy store" approach to science, and for giving me the resources and the freedom to pursue my own research interests. I would also like to extend my appreciation and thanks to the entire staff of the BRC, who have not only shared their scientific expertise, but also made coming to the BRC a pleasure. In particular, I have to thank James Wieler for our enthusiastic discussions of science and teaching me the importance of rounds down range, Melanie Welham for teaching me the art of protein biochemistry, Sam Abraham for our lengthy coffee breaks, Kevin Leslie for making every Christmas a Doozie, and Robert Gerl for his assistance on the MKK7 project. I am also thankful to several other members of the JWS lab including Megan Levings, Frances Lee, Goetz Ehrhardt, John Babcook, Joshua Schafer, Ruth Salmon and Michael Luckach for their invaluable assistance, their patience and their friendship.

I have also been fortunate to have the support of many people outside of the BRC. My family has provided a great deal of support and love during my studies at both the University of Guelph and the University of British Columbia. I have to especially thank my girlfriend Lynda Duncan for her understanding, her overwhelming support and her endless patience during my five minute hours. I would also like to thank Mike Scheid for teaching me how to lose gracefully at pool, and for our long scientific discussions over a pint. Several collaborators have also provided invaluable reagents to me, and without their assistance most of this work would not have been possible. In that light, I would like to thank Drs. Peter Young and John Lee at Smith-Kline Beecham, Dr. Leonard Zon at Harvard University Medical School, Dr. Richard Cerione at Cornell University, Dr. Frank McCormick at Onyx Pharmaceuticals, and Dr. Rob Kay at the Terry Fox Research Laboratories.
CHAPTER 1

1.1 General Overview

Life as we know it likely began as a single-cellular organism similar to the bacteria of today. This organism had to acquire nutrients for growth and survival, remove dangerous metabolic byproducts, and replicate its genetic material for future generations. Besides these problems, this early life form had to respond to and overcome harsh environmental conditions such as osmolarity of its environment, temperature changes and ultraviolet irradiation. As life evolved from an anaerobic to an aerobic state, these organisms had to overcome the problems inherent to aerobic life such as oxygen free-radicals. These early organisms needed to interpret and respond to their environment to overcome all these problems. The ability to sense and respond to the external environment was in essence the evolution of signal transduction.

With the evolution of multicellular organisms, the requirement for cells to respond to their environment was as important as for the earliest forms of life. However, these organisms had many new problems to overcome. They needed to devise a means to provide nutrients and oxygen to all their cells, to remove waste from these cells, to communicate between cells, and to keep their cells associated. The cells became less individualized and more specialized during this process, and evolved to form specific organs and organ systems to overcome these problems. Vasculature systems were developed to supply nutrients and remove waste from cells in the organism when simple diffusion was no longer adequate to meet the organisms needs. Cell to cell communication is observed in single-cellular organisms such as yeast and multicellular organisms exploited similar systems to co-ordinate many aspects of life. Chemokines evolved to direct cells throughout the body, and growth factors, transforming growth factors, and cytokines evolved to control cellular growth, proliferation and differentiation. Multicellular organisms also evolved an extracellular matrix not only act to keep cells together, but also to prevent their death.
Besides these problems, the requirement to control total cell number was possibly the biggest hurdle facing multicellular life. This dynamic process involves the controlled production and the elimination of cells. A failure in this process leading to an over-production of cells is known as cancer, and involves a dysregulation of the same genes, known as proto-oncogenes, normally involved in cellular growth, proliferation and survival. The deregulated expression or mutation of proto-oncogenes, forming oncogenes, has been implicated in most forms of cancers. For example, the p21 Ras family of small GTPases are constitutively active in as many as 90% of human pancreatic cancers. Other genes encoding tumor suppressor proteins such as p53 that function to inhibit cellular proliferation, and are often deleted or non-functional in human cancers. Besides proliferation, total cell number is regulated by the elimination of cells, known as apoptosis.

My thesis focuses on two families of stress-activated protein kinases that are activated by pro-inflammatory cytokines, hyperosmolarity, oxygen free-radicals, ultraviolet irradiation, or basically any cellular insult imaginable. When I began working in this field with the exception of the proinflammatory cytokines, no physiological stimuli were known to activate these kinases. I examined the regulation of the stress-activated protein kinases in hematopoietic cells by growth factors that regulate their growth, proliferation and survival. The fact that hematopoietic cells living inside a multicellular organism sense and respond to environmental stresses that they would never encounter was strange. I reasoned this phenomenon might reflect a remnant of an ancient signalling cascade leftover from single-cellular life as these enzymes do exist in yeast. As new pathways were evolved to co-ordinate the diverse functions of multicellular life, it seems logical for a cell to harness the pre-existing protein machinery instead of re-inventing the wheel. We hypothesized that the stress-activated protein kinases would play many physiological roles in cells of multicellular organisms. Consistent with this notion, the stress-activated protein kinase pathways are now known to be activated by an incredibly diverse range of stimuli. Clearly, these kinases are not only important as effectors of ancient signals regulating single cellular life, but also
have been adapted to regulate many aspects of multicellular life including apoptosis, proliferation, tumorigenesis, chemotaxis, organogenesis, embryogenesis, and the immune system.

1.2 Signal Transduction by Cytokines and Growth Factors

Hematopoietic growth factors including Steel locus factor (SLF), interleukin (IL) -3, IL-4, and granulocyte/macrophage colony-stimulating factor (GM-CSF), are members of a large structural family of polypeptide growth factors or cytokines that regulate the growth, survival, and differentiation of cells in many tissues, including the immune system. SLF acts on pluripotent hematopoietic stem cells, the progenitors of many hematopoietic lineages, and mature mast cells, and also has an important developmental role on cells of non-hematopoietic origin, including germ cells and cells derived from the neural crest (Fleischman, 1993). SLF is produced constitutively by stromal cells, and is essential for the development of the hematopoietic system.

IL-3, GM-CSF and IL-4 are released by activated cells of the immune system and link the immune system to the regulation of the growth and function of cells of hematopoietic origin. IL-3 stimulates the growth, survival and differentiation of pluripotent hematopoietic stem cells, progenitors of all erythroid and myeloid cells, mature macrophages, eosinophils, megakaryocytes, mast cells, or basophils (Miyajima et al., 1993). GM-CSF is more restricted in its activity, and targets the progenitors and mature cells of the macrophage, neutrophil, and eosinophil lineages (Miyajima et al., 1993). Mice lacking GM-CSF have normal hematopoiesis, but surprisingly exhibit altered lung homeostasis and chronic pulmonary infections (Dranoff and Mulligan, 1994; Stanley et al., 1994). IL-4 is active on cells of both hematopoietic and non-hematopoietic origin, and is involved in the differentiation of specific classes of B- and T-lymphocytes and the downregulation of macrophage activity (Taniguchi, 1995). Mice lacking IL-4 exhibit reduced serum levels of IgG1 and IgE due to a failure of immunoglobulin class switching in B cells, fail to differentiate CD4+ T cells to the Th2 subclass, and fail to produce germinal centers in Peyer's patches (Kopf et al., 1993; Vajdy et al., 1995; von der Weid et al., 1994).
The receptor for SLF, c-Kit, is a homodimeric receptor tyrosine kinase, resembling the platelet-derived growth factor receptor (Fantl et al., 1993). The cytoplasmic tail of c-Kit contains an insert within its kinase domain, and the extracellular domain contains five immunoglobulin-like domains (Fantl et al., 1993). In contrast the receptors for IL-3, GM-CSF or IL-4 are distinctive heterodimers, the subunits of which lack enzymatic activity and belong to the hematopoietin receptor superfamily. The extracellular domains of these receptors contain four conserved cysteine residues, and the characteristic WSXWS motif that may be involved in protein folding or interaction with ligand (Bazan, 1990; Hilton et al., 1995; Yoshimura et al., 1992). IL-3, IL-5 and GM-CSF share a common β-chain of their receptor complex known as KH97 (now referred to as β-common) in humans or AIC2B in mice. Each cytokine also binds to a different low-affinity α-chain, and the resulting complex interacts with the common β-chain to form the high-affinity receptor. The α-chains have very short cytoplasmic tails and require the β-common chain to transduce biological signals. In mice, IL-3 also signals through AIC2A, another β-chain arising from gene duplication of AIC2B. However mice lacking AIC2A are phenotypically normal (Nishinakamura et al., 1995). Mice lacking AIC2B show the same lung pathology as mice lacking GM-CSF, and have reduced numbers of eosinophils as seen in mice lacking IL-5 (Nishinakamura et al., 1995). Similarly, mice lacking both AIC2B and IL-3 are phenotypically identical to mice lacking AIC2B, and respond normally to immunological challenges such as infection with Listeria monocytogenes, raising the question of the physiological role of AIC2A (Nishinakamura et al., 1996).

The IL-4 receptor consists of two distinct receptor complexes that use the IL-4 receptor α-chain (IL4Rα) as their low-affinity receptor. One high affinity receptor for IL-4 consists of the IL4Rα chain and the γ-common chain of the IL-2R. The γ-common chain is widely expressed and transduces IL-4 signals in most cells. The other high-affinity receptor for IL-4 consists of the IL4Rα chain and the α-chain of the IL-13 receptor (IL-13Rα). In fact, expression of the IL-13Rα lacking the cytoplasmic domain inhibits IL-4 signalling through the γ-common chain in a dominant
negative fashion (Orchansky et al., 1997). IL-4 and IL-13 share many biological functions, most likely due to shared receptor subunits.

Cytokine signal transduction is initiated by ligand-induced dimerization of their receptors and the subsequent activation of associated tyrosine kinases, including members of the JAK and src protein kinase families (Miyajima et al., 1993; Taniguchi, 1995). The JAK tyrosine kinases are constitutively associated with conserved domains in the cytoplasmic tails of these receptors known as box1 and box2. JAK activity is required for the tyrosine phosphorylation of specific residues in the cytoplasmic domains of these receptors that act as interaction sites for SH2 domains, a protein-protein interaction domain found in proteins such as the signal transducers and activators of transcription (STAT). IL-3 or GM-CSF induced signalling activates JAK2, a tyrosine kinase required for the biological activity of these receptors (Parganas et al., 1998), and subsequently activates STAT5 (Fig. 1.1). IL-4 signalling through the IL4Rα chain and γ-common activates JAK1 and JAK3, whereas signalling through IL4Rα and IL13Rα activates JAK1 and Tyk2. Thymocytes derived from mice lacking JAK1 are unresponsive to IL-4, demonstrating the importance of this tyrosine kinase for IL-4 signalling (Rodig et al., 1998). JAK3 is constitutively associated with γ-common, and loss of either of these proteins results in X-linked severe-combined immunodeficiency (SCID) syndrome (Leonard et al., 1995; Macchi et al., 1995; Nosaka et al., 1995; Russell et al., 1995b). IL-4 induced signalling activates STAT6, a protein that mediates many of the biological activities of IL-4 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). SLF induced signalling activates JAK2, and subsequently induces tyrosine phosphorylation of STAT1 (Deberry et al., 1997).

Besides the JAK/STAT pathways, IL-3, GM-CSF, IL-4 or SLF activate PI-3 kinase through the relocalization of PI-3 kinase to its substrate at the plasma membrane (Gold et al., 1994). PI-3 kinase phosphorylates phosphatidylinositol (PI), PI 4-phosphate, or PI 4,5-bisphosphate on the 3'-position to generate PI 3-phosphate, PI 3,4-biphosphate or PI 3,4,5-triphosphate (PIP3) respectively. PIP3 provides membrane docking sites for pleckstrin homology (PH) domains in proteins such as SOS, Akt, Pdk1 and Pdk2. The recruitment of Pdk1 and Pdk2
Figure 1.1 - Comparison of Signalling Pathways Initiated by Stimulation of Cells with IL-3, GM-CSF, SLF or IL-4.
to the plasma membrane by PIP3 activates p70S6K and Akt (Burgering and Coffer, 1995; Scheid et al., 1996). The activation of p70S6K is required for efficient protein translation through phosphorylation of the S6 peptide. Akt is the mammalian homolog of v-Akt, a transforming oncogene that acts by preventing apoptosis (Coffer and Woodgett, 1991; Staal, 1987). IL-3, SLF or IL-4 activates Akt (del Peso et al., 1997; Scheid and Duronio, 1998; Songyang et al., 1997), and over-expression of catalytically inactive Akt induces apoptosis even in the presence of IL-3 (Songyang et al., 1997). Furthermore, the inhibition of PI-3 kinase prevents the activation of Akt and induces apoptosis of hematopoietic cells, suggesting that Akt may mediate the anti-apoptotic effects of hematopoietic growth factors (Scheid and Duronio, 1998).

Cellular growth and proliferation are normally associated with the activation of the GTPase Ras and the ERK MAPK pathway. Accordingly, IL-3, GM-CSF or SLF activates both Ras and ERK MAPK (Duronio et al., 1992; Welham et al., 1992). Tyrosine kinase activity is required for the activation of Ras by cytokines (Duronio et al., 1992), possibly to provide docking sites for the SH2-containing proteins Shc or Grb2. SOS, an exchange factor for Ras and Rac/Cdc42, interacts with Grb2 through its SH3 domains, a protein-protein interaction domain that recognizes polyproline rich peptides (Chardin et al., 1993; Egan et al., 1993; Li et al., 1993). Grb2 recruits SOS by either directly binding the receptor, or indirectly binding receptor-associated Shc. SOS requires its PH domain to activate Rac or Cdc42 (Nimmual et al., 1998; Zheng et al., 1997), indicating that PI-3 kinase activity may regulate the activation of Rac or Cdc42 by IL-3, GM-CSF or SLF. These cytokines not only activate Ras and PI3K, but also lead to the activation of JNK and p38 MAPK (Foltz et al., 1997; Foltz and Schrader, 1997), two kinases proposed to be downstream of the Rac or Cdc42 (Bagrodia et al., 1995; Coso et al., 1995; Zhang et al., 1995). The activation of JNK by IL-3 requires activation of Ras (Rausch and Marshall, 1997; Terada et al., 1997), and may be prevented by inhibitors of PI-3 kinase (Ishizuka et al., 1997; Kawakami et al., 1998; Logan et al., 1997). While SOS is not necessarily upstream of JNK, these findings suggest that Ras and PI-3 kinase synergize to activate JNK by IL-3. IL-4 and IL-13 are unique among cytokines for their failure to activate Ras and the MAPK pathway (Duronio et al., 1992; Welham et al., 1992; Welham
et al., 1994). IL-4 also fails to activate JNK or p38 MAPK (Foltz et al., 1997; Foltz and Schrader, 1997; Salmon et al., 1997), suggesting that PI-3 kinase activity is not sufficient to activate Rac or Cdc42. Consistent with this hypothesis, a mutant form of SOS containing only the PH domain and the Dbl domain, which activates Rac, requires the expression of activated Ras to activate Rac or Cdc42 (Nimnual et al., 1998). Therefore, both PI-3 kinase and Ras activity appear to be required to activate Rac or Cdc42.

SLF differs from IL-3, GM-CSF or IL-4 in its ability to regulate intracellular calcium channels by activating phospholipase (PL) C (Hallek et al., 1992). PLC cleaves PI 4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Bennett et al., 1988; Exton, 1994). IP3 releases intracellular calcium stores that subsequently allows the entry of extracellular calcium into the cell, effectively increasing the cellular concentration of free calcium. DAG and intracellular calcium activate several enzymes including conventional and novel PKC isoforms, calcium/calmodulin dependent protein kinases, the small GTPase Rap1, and the calcium sensitive phosphatase calcineurin (Crabtree and Clipstone, 1994; Huang and Huang, 1993; M'Rabet et al., 1998; Schonwasser et al., 1998). In summary, these highly related cytokines provide distinct biological functions that reflects the integrated signal provided through these receptors.

1.3 Phosphorylation in Signal Transduction

1.3.1. Protein Kinases - Kinases are enzymes that catalyze the transfer of the γ-phosphate of adenosine triphosphate (ATP) to a hydroxyl group on either its protein or lipid substrate. A single phosphate group adds a large negative charge and drastically changes the appearance of the substrate. The first oncogene identified was the tyrosine kinase v-Src, the transforming agent of the Rous sarcoma virus. v-Src was later discovered to be a homolog of the mammalian protein c-Src. The Src family of proteins are membrane-associated tyrosine kinases, meaning these enzymes specifically phosphorylate proteins on the hydroxyl group of tyrosine residues. Several other
cytosolic tyrosine kinases have been identified including the Janus kinases (JAK), the Tec kinase family, and c-Abl. The other class of tyrosine kinases are the receptor tyrosine kinases that include the receptors for EGF, PDGF, FGF and SLF. These receptors are activated by ligand-induced dimerization or oligomerization, and become trans or autophosphorylated. This phosphorylation creates binding sites for SH2 domains of adapter proteins or other kinases. Tyrosine kinases act, for the most part, as membrane proximal signal transducers and represent less than 1% of total cellular phosphorylation.

Most phosphorylation occurs on serine, and to a lesser extent on threonine residues, although phosphorylation may occur on histidine, aspartate, and lysine residues. As with tyrosine kinases, these enzymes are classified as cytosolic and receptor serine/threonine kinases. The receptors for transforming growth factors are the only known receptor serine/threonine kinases, and as such the majority of serine/threonine kinases are cytosolic proteins. The mammalian homologs of Ste11 and Ste20 including Raf, MEKK1 and PAK, as well as the broad-specificity ERK, JNK and p38 mitogen-activated protein kinases (MAPK) are all serine/threonine specific kinases. Besides the MAPK cascades, the protein kinase A, B and C families are specific for serine and threonine residues. Typically these enzymes are activated downstream of tyrosine kinases and provide a link from the plasma membrane of a cell to the nucleus.

Most kinases are defined based on their substrate specificity, however a few dual-specificity kinases have been identified. The best characterized dual-specificity kinases are the mammalian homologs of Ste7. These enzymes phosphorylate MAPK proteins on tyrosine and threonine residues within their activation loops. Another family of nuclear dual-specificity kinases known as STY, or Clk have also been identified, and appear to regulate mRNA splicing by phosphorylating serine/arginine-rich proteins (Duncan et al., 1997; Lee et al., 1996). In contrast to many kinases, the dual-specificity kinases are very selective enzymes, and have a small number of highly-related physiological substrates.
1.3.2. Mechanism of Phosphotransfer by Kinase Domains - The catalytic domain of protein kinases has been highly conserved through evolution, and forms a highly globular structure consisting of 250 to 300 amino acids. The first crystal structure of a kinase domain was determined for cAMP-dependent protein kinase in 1991 (Figure 1.2), and has provided a structural model for other kinases (Knighton et al., 1991a,b). The catalytic domain consists of a β-stranded N-terminal lobe and an α-helical C-terminal lobe. The active site lies at the interface of the two lobes with the ATP-binding site being formed by residues on both lobes. The adenine moiety binds to a hydrophobic pocket, and the ribose group is stabilized by hydrogen bonds. A kinase catalyzes the transfer of the γ-phosphate of ATP onto a hydroxyl group of its respective substrate. This reaction requires a magnesium ion as a co-factor to co-ordinate the β- and γ-phosphates of ATP.

Alignment of the primary structure of the catalytic domain of protein kinases reveals eleven conserved sub-domains (Hanks et al., 1988). These sub-domains are important for catalytic function and are involved either directly in the active site or indirectly in a structural role. The alignment of catalytic domains has also identified several invariant or nearly invariant amino acids that are critical for phosphotransferase activity. The alignment of the primary structures of catalytic domains has also defined peptides that predict the kinase specificity from their primary sequence. For example, serine/threonine kinases have (T/S)xx(Y/F)xAPE in sub-domain VIII, and DLKP(E/S)N in sub-domain VI, whereas tyrosine kinases have P(I/V)(K/R)W(T/M)APE in sub-domain VIII, and DLRA(A/R)N in sub-domain VI (Hanks et al., 1988).

Sub-domain I contains a GxGxxG motif, and the first two glycines of this motif are nearly invariant residues (Hanks et al., 1988; Johnson et al., 1996a). This motif is present in many nucleotide binding proteins including GTPases, and is involved in the proper co-ordination of ATP. The first glycine contacts the ribose group and the second glycine lies near the α-phosphate of ATP. Sub-domain I also contains a nearly invariant valine residue two amino acids C-terminal to the GxGxxG motif that is believed to properly position the glycines within the kinase domain.
Figure 1.2 - Crystal structure of cAMP dependent protein kinase. The structure of cAMP-dependent protein kinase (cAPK) in complex with an inhibitor peptide and ATP is shown. The N-terminal lobe of cAPK is predominantly β-stranded, whereas the C-terminal lobe is primarily α-helical in nature. The ATP molecule lies between the N- and C-terminal lobes. The serine residue that is the target of phosphorylation is depicted as PO. The activation segment is noted on the right hand side of the diagram. The catalytic base of cAPK is D166, and D184 interacts with the β- and γ-phosphates of ATP. The critical lysine (K72) is positioned above the ATP molecule. This figure was adapted from Johnson et al., 1996.
Sub-domain II contains an invariant lysine residue that is directly involved in the coordination of the α- and β-phosphates of ATP (Johnson et al., 1996a). This lysine (K72, Fig. 1.3) is located within the active site and hydrogen bonds with an invariant glutamate residue (E91, Fig. 1.3) in sub-domain III. The importance of this residue in catalysis was initially discovered with the ATP analog, p-fluoro sulfonyl 5'-benzoyl adenosine, that reacts with the lysine residue and inhibits kinase activity. Site-directed mutagenesis supports a critical role for this lysine in catalysis as its mutation to any amino acid ablates kinase activity (Hanks et al., 1988).

The central core of the catalytic domain lies in the C-terminal lobe, and is highly conserved between kinases. Sub-domain VI contains a highly conserved aspartate group found in an RD motif that is indicative of kinases regulated by phosphorylation (RD kinases), and site-directed mutagenesis of this aspartate group ablates phosphotransferase activity. (Johnson et al., 1996a). An oxygen group on the aspartate side chain (D166, Fig. 1.3) undergoes a base-catalyzed attack on the proton of the hydroxyl group on the substrate. This hydroxyl-group is highly reactive when deprotonated and attacks the γ-phosphate of ATP mediating phosphotransfer. The arginine group (R165, Fig. 1.3) immediately N-terminal to the catalytic aspartate forms a salt bridge with the catalytic aspartate residue and keeps the kinase inactivated.

Sub-domain VII is defined by the presence of the DFG motif that marks the N-terminus of the activation loop (Hanks et al., 1988; Johnson et al., 1996a). The aspartate (D184, Fig. 1.3) of the DFG motif is an invariant amino acid and is critical for kinase activity. The aspartyl side chain interacts with the magnesium ion that is complexed with ATP, and properly orients the β- and γ-phosphates of ATP for catalysis. Phosphotransferase activity is removed by mutation of this aspartate to any amino acid.

Sub-domain VIII contains the APE motif that defines the end of the activation loop (Hanks et al., 1988; Johnson et al., 1996a). The glutamate residue in this motif is invariant, and co-ordinates the invariant arginine in sub-domain XI (Johnson et al., 1996a). The activation loop undergoes dramatic structural changes upon kinase activation, and contains residues that require phosphorylation to activate RD kinases. The phosphorylation dramatically alters the charge of the...
Figure 1.3 - Model of the active site of cAMP dependent protein kinase.
residue and creates new interactions with other positively charged residues. A hydrogen bond forms between the phosphorylated residue (T197, Fig. 1.3) and the arginine residue N-terminal of the catalytic aspartate residue removing its inhibition on catalysis, and allowing the aspartate group to attack the proton of the hydroxyl-group of its substrate. This phosphorylation may change the overall conformation of the kinase to correctly orient the catalytic base, may determine the conformation of residues in the activation loop involved in substrate recognition, and may convert the enzyme from an open to a closed conformation. An open conformation allows ATP into the catalytic site, and allows the release of ADP after catalysis. A closed conformation brings residues into the correct conformation to promote catalysis (Johnson et al., 1996a).

1.3.3. Phosphatases - The advantages of phosphorylation as a post-translational modification lie not only in the addition of a small highly negatively charge, but also in its reversibility. The removal of phosphate groups is catalyzed by a group of enzymes known as phosphatases. As with kinases, phosphatases can be specific for phosphotyrosine, phosphoserine and phosphothreonine or both.

Protein tyrosine phosphatases exist as both transmembrane receptors and cytosolic proteins. Receptor tyrosine phosphatases are fairly well characterized and include CD45 and PTPα. These phosphatases generally contain two cytoplasmic phosphatase domains of which the N-terminal domain is more active. In contrast to the receptor tyrosine kinases, the receptor tyrosine phosphatases are inhibited by dimerization, and are active as monomers. These enzymes are very divergent in their extracellular domains, suggesting the possibility of many distinct ligands, however as yet no ligands that would dimerize and inhibit PTPase activity are known.

The cytosolic protein tyrosine phosphatases and serine/threonine specific phosphatases generally contain a single phosphatase domain. The protein tyrosine phosphatases include the proteins SHP1, SHP2 and PTP1B, while the serine/threonine phosphatases include calcineurin and protein phosphatase 2A. These enzymes often contain domains involved in intracellular localization such as SH2 domains in the case of SHP1 and SHP2, or endoplasmic reticulum
retention sequences in the case of PTP1B. SHP1 and SHP2 are recruited to receptors after signalling and likely act to either attenuate or initiate signalling through dephosphorylation.

The dual specificity phosphatases are characterized as either VH1-like or Cdc25-like phosphatases. The Cdc25-like phosphatases regulate the activity of cyclin-dependent protein kinases by removing inhibitory phosphate groups. The VH1-like phosphatases including MKP1, PAC1 and Pyst1, are classified for their homology to the VH1 phosphatase from vaccinia virus, and function to dephosphorylate and inactivate MAP kinases. The expression of these enzymes is generally regulated at the transcriptional level and these PTPases differentially inactivate the ERK, JNK and p38 MAPK families of protein kinases.

1.4 The Mitogen-Activated Protein Kinase Cascades

1.4.1. The MAPK Cascades - Yeast exist as haploid organisms that, in the presence of peptide pheromones, fuse to become diploid organisms through a process known as the mating response. Mutations either preventing the mating response or inducing the mating response in the absence of pheromones led to the identification of protein kinases in the MAPK cascades. The ability of these different mutant proteins to enforce or prevent the mating response allowed the sequential determination of kinases in the pathway. In summary, this work defined Sterile (Ste) 20 gene as the kinase responsible for activating Ste11. In turn, Ste11 acts upstream of Ste7, a kinase that phosphorylates the mitogen-activated protein kinase Fus3 (Fig. 1.4). This signalling cassette is held together by a scaffolding protein known as Ste5. Proteins in the mammalian MAPK cascades are placed into linear cascades based on their homology to these kinases.

The canonical MAPK cascade in mammalian cells is the ERK MAPK pathway. I will describe this pathway in some detail as much that has been discovered has proven relevant for other MAPK cascades. Growth factors such as EGF or IL-3 activate this pathway downstream of Ras or PKC. The Ras superfamily consists of 5 genes that encode N-Ras, H-Ras, 2 splice variants of Ki-Ras, TC21 and M-Ras. Ras proteins are molecular switches that are inactive when
Figure 1.4 - The MAPK Cascade. Summary of the signal transduction molecules presently identified as being involved or belonging to a MAPK cascade.
they bind GDP, and are active when they bind GTP. These highly related molecules contain two
domains, switch 1 and switch 2, that undergo conformational changes upon binding GTP and are
involved in binding to effectors. Functional differences between the Ras proteins are poorly
characterized, however exchange factors such as Ras-GRF can selectively activate specific Ras
proteins indicating that these proteins may be differentially regulated in vivo (Jones and Jackson,
1998). The greatest differences in the primary structure of the Ras proteins lies in their C-terminal
tails. All Ras proteins are anchored to the membrane by prenylation within a CAAX-motif at their
C-terminus. The C-termini of Ras proteins are also either modified by palmitoylation, or contain a
poly-basic region likely involved in either membrane association or perhaps in recruiting GDP
dissociation factors (Mizuno et al., 1991).

The best described effectors of GTP-bound Ras are the serine/threonine protein kinases
Raf-1, A-Raf and B-Raf. Raf proteins contain a Ras-interaction domain in their N-termini (Vojtek
et al., 1993), and have approximately 1000-fold higher affinity for GTP-bound Ras than GDP-
bound Ras. Ras binding to Raf recruits Raf to the plasma membrane, however localization to the
plasma membrane by Ras is only sufficient to activate B-Raf (Stanton et al., 1989; Yamamori et
al., 1995). Experiments localizing Raf-1 to the plasma membrane suggest that Ras functions to
recruit Raf to the plasma membrane (Stokoe et al., 1994), however these results have been
contentious (Mineo et al., 1997; Tamada et al., 1997). Besides membrane recruitment, Ras may
function to aggregate Raf. This hypothesis is supported by elegant experiments using drugs that
artificially dimerize and activate Raf-1 (Farrar et al., 1996; Luo et al., 1996). Raf also requires
phosphorylation for activation (Morrison et al., 1993), that may occur as a result of
autophosphorylation, or phosphorylation by conventional and novel PKC isoforms (Schonwasser
et al., 1998). Raf is phosphorylated within a highly conserved RSXSXP motif that provides an
interaction domain for 14-3-3β and is required for activity in vitro and in vivo (Thorson et al.,
1998). The best characterized substrates for Raf are the dual-specificity kinases MEK1 and
MEK2. Raf1 and B-Raf are capable of activating both MEK1 and MEK2, while A-Raf appears to
only activate MEK1 (Wu et al., 1996). The Raf gene products have non-redundant functions in
vivo as mice lacking A-Raf display strain specific intestinal defects and neurological abnormalities (Pritchard et al., 1996). Mice lacking B-Raf die in utero at mid-gestation due to a premature apoptotic death of differentiated endothelial cells (Wojnowski et al., 1997). Furthermore, conditionally active mutants of A-Raf and B-Raf differ in their ability to promote the entry of cells into the cell cycle (Pritchard et al., 1995).

MEK1 and MEK2 are dual-specificity kinases that are activated by phosphorylation on two serine residues within their activation loop by Raf. The recognition of MEK1 or MEK2 by Raf requires a large proline-rich insert in their C-terminus (Catling et al., 1995). Both MEK1 and MEK2 activate ERK1 or ERK2 by phosphorylating both threonine and tyrosine residues of the TEY motif in sub-domain VIII (Brott et al., 1993; Crews et al., 1992; Zheng and Guan, 1993). ERK is initially phosphorylated on tyrosine, and then on threonine (Burack and Sturgill, 1997; Ferrell and Bhatt, 1997). The phosphorylation of both residues is required for activation of ERK (Canagarajah et al., 1997). The activation of ERK by MEK is enhanced by a protein characterized as MEF, or MEK-enhancing factor (Scott et al., 1995), and cloned as MP-1 (Schaeffer et al., 1998). Interestingly, MEK2, but not MEK1, also activates ERK3, but the relevance of this observation in vivo is unclear (Robinson et al., 1996). An activator of ERK3 has been characterized biochemically, but remains to be identified (Cheng et al., 1996b).

The ERK MAP kinases are proline-directed serine/threonine kinases (Boulton et al., 1991; Charest et al., 1993; Clark-Lewis et al., 1991) and their activity may to regulate mitogenesis, differentiation and apoptosis. The ERK MAPK pathway activates a broad spectrum of downstream kinases including p90rsk (Blenis, 1993), Mnk1 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), Mnk2 (Waskiewicz et al., 1997), Rsk2 (Xing et al., 1996) and MAPKAP kinase 5 (Ni et al., 1998). The functions of these kinases are largely unknown however Mnk1 phosphorylates eIF-4e possibly linking ERK activity with the regulation of translation (Waskiewicz et al., 1997). ERK MAPK also regulates the transcriptional activity of Elk1, Sap1 and c-Myc, and in turn regulates the expression of many genes critical to mitogenesis such as cyclin D1 (Greulich and Erikson, 1998; Lavoie et al., 1996). The ERK MAPK pathway is also
activated by non-mitogenic stimuli including chemokines and pro-inflammatory cytokines indicating that ERK activity is not sufficient for mitosis. Mice lacking MEK1 die in utero due to a failure in vascular endothelial cell migration within the placenta (Jean Charron, unpublished data). This finding suggests either non-redundant functions or insufficient gene dosages of MEK1 and MEK2 as vascular endothelial cells normally express both enzymes. Upon activation, ERK1 and ERK2 translocate from the cytoplasm to the nucleus in an event that requires neither kinase activity nor phosphorylation on the activation loop (Chen et al., 1992; Lenormand et al., 1993), but may require homodimerization of ERK (Cobb, M., unpublished data). Unlike ERK1 and ERK2, ERK3 is constitutively localized to the nucleus (Cheng et al., 1996a).

Besides the Raf enzymes, MEK1 and MEK2 are activated by the serine/threonine kinases c-Mos, Tpl-2 and MEKK1. c-Mos is expressed predominantly in germline cells and is required for the activation of M-phase promoting factor (MPF) (Haccard et al., 1993; Yew et al., 1992). Genetic disruption of c-Mos supports its role in early meiosis for the activation of MPF and MAPK (Araki et al., 1996). Tpl-2, or tumor progression locus-2, was discovered as a protooncogene in MMLV-derived T-lymphomas in rats, and shares 90% identity with the human gene Cot (Patriotis et al., 1994). Tpl-2 directly phosphorylates and activates MEK1 and MKK4 in vitro (Salmeron et al., 1996). Supporting these observations, the over-expression of Tpl-2 activates both the ERK and JNK pathways, with little effect on p38 MAPK activity. The upstream regulators of c-Mos and Tpl-2 are poorly understood.

MEKK1 was identified based on its homology with the Ste11 and Byr2 kinases from yeast (Lange-Carter et al., 1993; Xu et al., 1996a). Since then five related MEKK enzymes have been identified (Blank et al., 1996; Deacon and Blank, 1997; Gerwins et al., 1997; Ichijo et al., 1997; Wang et al., 1996). MEKK1 was initially characterized by an activator of MEK1 (Lange-Carter et al., 1993), however a role for MEKK1 upstream of MEK1 and MEK2 in vivo is contentious (Gardner et al., 1994; Minden et al., 1994a; Xu et al., 1995). Like Raf, MEKK1 is an effector for GTP-bound Ras (Russell et al., 1995a), and Ras function is required for MEKK1 activation by EGF (Lange-Carter and Johnson, 1994). MEKK1 and MEKK4 bind and may be regulated by
Racl or Cdc42 (Fanger et al., 1997b; Gerwins et al., 1997). However, the regulation of MEKK2, MEKK3 or ASK1/MEKK5 by small GTPases of the Ras or Rho family has not been described. Over-expression of MEKK1 potently activates the c-Jun N-terminal kinases (JNK) (Minden et al., 1994a; Yan et al., 1994) through the activation of MKK4 (Lin et al., 1995; Yang et al., 1997b) and MKK7 (Lu et al., 1997; Xu and Cobb, 1997; Yao et al., 1997). Furthermore, mice lacking MEKK1 fail to activate JNK and also ERK MAPK downstream of specific stimuli indicating that MEKK1 functions to activate JNK \textit{in vivo} (Yujiri et al., 1998). Enforced expression of MEKK2, MEKK3, or MEKK4 activates the JNK pathway (Blank et al., 1996; Deacon and Blank, 1997; Gerwins et al., 1997; Wang et al., 1996). MEKK5/ASK1 activates both JNK and p38 MAPK, but not ERK MAPK, by directly phosphorylating MKK3, MKK4 and MKK6 (Ichijo et al., 1997). Overexpression of MEKK2 or MEKK3 also activates the ERK pathway (Blank et al., 1996) and fails to activate p38 MAPK. Interestingly, MEKK3 activates MKK3, but not p38a MAPK, possibly demonstrating \textit{in vivo} substrate specificity of MKK3 for p38 isoforms (Deacon and Blank, 1997).

The JNK family of MAPK are proline-directed serine-threonine kinases that phosphorylate the N-terminus of c-Jun (Derijard et al., 1994; Kyriakis et al., 1995). These enzymes are activated by dual phosphorylation on threonine and tyrosine residues in a TPY motif (Derijard et al., 1994) by the dual specificity kinases MKK4 and MKK7 (Derijard et al., 1995; Foltz et al., 1998; Holland et al., 1997; Lawler et al., 1997; Lu et al., 1997; Moriguchi et al., 1997; Sanchez et al., 1994; Tournier et al., 1997; Wu et al., 1997; Yang et al., 1998b; Yao et al., 1997). Mice lacking MKK4 die \textit{in utero} suggesting differential functions for MKK4 and MKK7. In support of this notion, GCK and MEKK5 preferentially activate MKK7 in over-expression studies, however MEKK1 and MEKK2 activate both enzymes similarly (Wu et al., 1997). MLK2 also preferentially activates MKK7 over MKK4 \textit{in vitro} under conditions where MEKK1 activated both enzymes comparably (Hirai et al., 1998). However, MEKK1 preferentially activates MKK4 in KB cells (Cuenda and Dorow, 1998), suggesting the activation of MKK4 and MKK7 is modulated by other molecules such as scaffolding proteins (Fanger et al., 1998).
Like JNK and ERK MAPK, the p38 MAPK family are proline-directed serine/threonine kinases (Goedert et al., 1997; Han et al., 1994; Han et al., 1995; Jiang et al., 1996; Jiang et al., 1997; Kumar et al., 1997; Lechner et al., 1996; Lee et al., 1994; Li et al., 1996; Mertens et al., 1996; Rouse et al., 1994; Stein et al., 1997; Wang et al., 1997b). These enzymes are activated by dual-phosphorylation on tyrosine and threonine residues within a TGY motif on their activation loops (Doza et al., 1995; Raingeaud et al., 1995). Proteins of the p38 MAPK family are activated by two dual specificity kinases, MKK3 and MKK6 (Cuenda et al., 1996; Derijard et al., 1995; Han et al., 1996; Han et al., 1997; Lin et al., 1995; Moriguchi et al., 1996; Raingeaud et al., 1996). MKK4 also activates p38 MAPK in vitro, however the relevance of this activation has yet to be determined (Derijard et al., 1995; Lin et al., 1995).

Several homologs of Ste20 and Ste11 activate the JNK and p38 MAPK pathways. The mixed lineage kinases MLK1, MLK2/MST, MLK3/SPRK/PTK1 and DLK/MUK are serine/threonine kinases that are characterized by catalytic domains with similarity to both tyrosine and serine/threonine kinases (Creasy and Chernoff, 1995; Dorow et al., 1993; Ezoe et al., 1994; Gallo et al., 1994; Hirai et al., 1996; Holzman et al., 1994; Ing et al., 1994; Katoh et al., 1995). The C-termini of these enzymes have leucine zippers that are likely involved in protein-protein interactions (Fanger et al., 1997b). Both MLK3 and DLK contain Cdc42/Rac interaction domains (CRIB), however MLK3 interacts weakly and DLK fails to interact with Rac or Cdc42 (Lamarche et al., 1996; Nagata et al., 1998; Teramoto et al., 1996). MLK2, MLK3 and DLK activate MKK4 directly, and over-expression of these enzymes increase JNK activity (Cuenda and Dorow, 1998; Hirai et al., 1996; Hirai et al., 1997; Hirai et al., 1998; Nagata et al., 1998; Tibbles et al., 1996). MLK2 co-localizes with activated JNK1 and JNK2 on microtubules, implying some biologically relevance for the activation of JNK by MLK2 (Nagata et al., 1998). Overexpression of MLK3 also activates p38 MAPK through MKK6, but fails to activate ERK MAPK (Tibbles et al., 1996).

Germinal center kinase (GCK), a mammalian homolog of Ste20, was identified as a protein kinase that is differentially expressed in germinal center B lymphocytes, but not in B cells from lymphoid follicles (Katz et al., 1994). Overexpression of GCK activates the JNK pathway, but
fails to activate p38 MAPK or ERK MAPK (Katz et al., 1994). The activation of JNK by GCK is inhibited by kinase dead MLK3, but not by kinase dead MEKK1 (Tibbles et al., 1996), suggesting MLK3 functions downstream of GCK in vivo. Recently, a GCK-like protein kinase (GLK) was identified that activates the JNK pathway without activating either p38 or ERK MAPK pathways (Diener et al., 1997). Unlike GCK, the activation of JNK by GLK is inhibited by kinase dead MEKK1 suggesting these highly related enzymes use different Ste11 homologs (Diener et al., 1997).

TAK1, or TGFβ-activated kinase, was identified in a genetic screen to find mammalian proteins that rescue Ste11 defects in S. cerevisiae (Yamaguchi et al., 1995). Overexpression of TAK1 activates both the JNK and the p38 MAPK pathways (Moriguchi et al., 1996; Shirakabe et al., 1997), but fails to activate ERK MAPK. Yeast two-hybrid analysis identified TAB1, a TAK1 binding protein, that binds to TAK1 and regulates its enzymatic activity (Shibuya et al., 1996). While the function of these proteins has not been determined in mice, studies on homologs of TAK1 and TAB1 in X. laevis suggest a role for these proteins for expression of ventral mesoderm during embryogenesis (Shibuya et al., 1998).

Hematopoietic protein kinase 1 (HPK1), a serine/threonine kinase with similarity to Ste20, is expressed predominantly in hematopoietic cells, including early progenitor cells (Hu et al., 1996; Kiefer et al., 1996). Overexpression of HPK1 activates the JNK pathway, and fails to activate p38 MAPK or ERK MAPK (Hu et al., 1996; Kiefer et al., 1996; Wang et al., 1997). HPK1 directly phosphorylates and activates MEKK1 providing a pathway for JNK activation (Hu et al., 1996). Catalytically inactive mutants of MLK3, MEKK1 and TAK1 prevent the activation of JNK by HPK1 (Hu et al., 1996; Kiefer et al., 1996; Wang et al., 1997a), suggesting linear cascades from HPK1 to JNK involving three distinct Ste11 homologs. The ability of TAK1, a known activator of the p38 MAPK pathway, to inhibit HPK1 is confusing as HPK1 fails to activate p38 MAPK. These differences may reflect cell-specific signal transduction pathways, or may exemplify the problems associated with over-expression studies and the use of dominant negative proteins.
The p21-activated kinases; PAK1, PAK2 and PAK3, contain CRIB domains and are effectors for GTP-bound Cdc42 and Rac1 (Manser et al., 1995; Manser et al., 1994; Martin et al., 1995; Teo et al., 1995). These serine/threonine kinases are activated directly by binding to GTP-bound Cdc42 or Rac. Over-expression of PAK1, PAK2 or PAK3 has been shown to activate both p38 MAPK and JNK pathways (Bagrodia et al., 1995; Brown et al., 1996; Frost et al., 1996; Zhang et al., 1995), the latter in a MEKK1-independent fashion (Fanger et al., 1997).

Several Ste20-like serine/threonine kinases are known that fail to activate any MAPK pathways. Two highly related enzymes Krs-1 and Krs-2 are yet to be tested for their ability to activate p38, JNK or ERK MAPK (Taylor et al., 1996). However, these enzymes are activated by stress stimuli such as extreme heat shock or arsenite implying a role upstream of JNK or p38 MAPK (Taylor et al., 1996). Another enzyme known as SOK, or Ste20/oxidant stress response kinase-1, is activated specifically by oxidative stresses such as reactive oxygen intermediates (Pombo et al., 1996). SOK is related to lymphocyte-oriented kinase (LOK) and both enzymes are unable to activate ERK, JNK or p38 MAPK pathways (Kuramochi et al., 1997; Pombo et al., 1996). Another Ste20 homolog that is highly related to SOK is Mst3 (Schinkmann and Blenis, 1997). Mst3 is an unusual kinase as it uses either ATP or GTP and manganese as co-factors for the phosphotransferase activity. The over-expression of Mst3 fails to activate p38α, p38γ, JNK, ERK1 or ERK2 (Schinkmann and Blenis, 1997). The failure of these Ste20 homologs to activate known MAPK pathways suggests the existence of novel MAPK family members, or a need to analyze other isoforms of known MAPK as substrates.

ERK5, or Big MAPK 1 (BMK1), is the least understood MAPK pathway (Lee et al., 1995; Zhou et al., 1995), and was identified in a yeast two-hybrid screen using MEK5 as bait (Zhou et al., 1995). ERK5 is activated by dual phosphorylation on tyrosine and threonine residues on a TEY motif within its activation loop by MEK5 (Kato et al., 1997). ERK5 is activated by oxidative stress possibly downstream of c-Src, and in some instances requires calcium release for activation (Abe et al., 1996, 1997). Serum and activated mutants of Ras, but not constitutively active mutants of Raf1, activate ERK5 (English et al., 1998). Overexpression of constitutively
active MEK5 activates ERK5 and leads to MEF2C phosphorylation in vivo (Kato et al., 1997). The phosphorylation of MEF2C is inhibited by a kinase dead form of ERK5 implying that MEF2C is an in vivo substrate of ERK5 (Kato et al., 1997).

1.5 The Stress-Activated Protein Kinases: JNK and p38 MAPK

1.5.1. Overview of the genes encoding JNK - The c-Jun N-terminal kinase (JNK) family of MAP kinases is encoded by three distinct genes: JNK1/SAPKγ, JNK2/SAPKα and JNK3/SAPKβ. The JNK1 and JNK2 genes are ubiquitously expressed. The JNK3 gene has a more restricted expression pattern and is found predominantly in the brain, heart and testis. These genes encode a total of 10 distinct JNK enzymes by differential splicing (Fig. 1.5) (Gupta et al., 1996). The JNK1 and JNK2 gene each encode 4 isoforms, whereas the JNK3 gene only encodes 2 isoforms. These genes encode both 46 and 55 kDa isoforms of JNK (Figure 1.4), that are formed by differential splicing at the 3'-end of the mRNA. The additional transcripts encoding α and β isoforms of JNK1 or JNK2 are generated by internal splicing within the kinase domain between sub-domains IX and X. The JNK3 gene does not appear to undergo this second splicing event.

1.5.2. Activators and Substrates of JNK - The immediate upstream activators and some downstream substrates of the JNK signalling pathway are fairly well characterized (Fig. 1.6). The JNK family of kinases is regulated by dual phosphorylation on both threonine and tyrosine residues within a TPY motif on their activation loop in sub-domain VIII. This phosphorylation event is regulated by two dual-specificity kinases known as MKK4 and MKK7. MKK4, also known as SAPK/ERK Kinase 1 (SEK1), was initially identified as a component of the JNK signalling pathway based on its homology with X-MEK2, a MKK from X. laevis (Lin et al., 1995). MKK4 can activate both p38 MAPK and JNK in vitro (Lin et al., 1995), whereas MKK7
**Figure 1.5 - Alignment of the Isoforms of JNK.** The predicted amino acid structure of human JNK1, JNK2 and JNK3. Splice variants are shown only where they differ from JNK1, JNK2 or JNK3 respectively. Where residues are identical to JNK1α1 they are indicated by periods. Gaps were introduced to optimize the alignment and are indicated by dashes.
Figure 1.6 - Activators and Substrates of the JNK pathway.
is a specific activator of JNK (Foltz et al., 1998; Tournier et al., 1997). These kinases are in turn presumably activated by several mammalian homologues of Ste11 (Fig. 1.4).

The JNK protein kinases phosphorylate and interact with a number of substrates that are mainly transcription factors including c-Jun and JunD, ATF-2, Elk-1 and Sap-1a. The phosphorylation of these transcription factors by JNK increases their transcriptional activity. c-Jun, a transcription factor that heterodimerizes with c-Fos to form AP-1, is phosphorylated on Ser-63 and Ser-73 within its N-terminus. JNK interacts with a 16 amino acid motif N-terminal to these phosphorylation sites in c-Jun known as the delta domain (Adler et al., 1994; Dai et al., 1995). This motif is believed to repress c-Jun function as it is deleted in v-Jun, the viral oncogenic form of c-Jun. v-Jun is not phosphorylated even though it contains the corresponding serine residues of c-Jun, demonstrating the importance of the delta domain to direct JNK to its substrate in vivo. However, v-Jun is transcriptionally active suggesting another role for the delta domain. Consistent with this notion, inactive JNK bound to c-Jun through the delta domain targets c-Jun for ubiquitinylation (Fuchs et al., 1996). In this model, phosphorylation by JNK not only increases its transcriptional activity, but also its half-life by preventing its ubiquitinylation. The delta domain interacts with a region between sub-domain IX and X near the catalytic pocket of JNK (Kallunki et al., 1996), and implies a functional role for the internal splicing event that generates the α and β isoforms of JNK (Gupta et al., 1996). While all isoforms phosphorylate the transcription factors c-Jun and ATF-2 in in vitro kinase assays, their interaction with these substrates differs (Gupta et al., 1996). The beta isoforms of JNK1 interact with both c-Jun and ATF-2 better than its alpha isoforms. The alpha isoforms of JNK2 interacted with c-Jun better than its beta isoforms, however the opposite is found for ATF-2. It is important to note that the β isoform of JNK1 contains the same splice variant as the α isoform of JNK2 (Fig. 1.5), likely explaining their preferential interactions with c-Jun, and suggesting a rationale for a change in their nomenclature. While the in vivo relevance of these studies is not clear, the interaction between JNK and its substrates must be important for in vivo substrate selection.
JNK has recently been shown to phosphorylate the anti-apoptotic protein Bcl-2 and the transcription factor p53. Bcl-2 and p53 are phosphorylated on the same sites *in vivo*, as are phosphorylated by JNK *in vitro* (Hu et al., 1997; Maundrell et al., 1997; Milne et al., 1995). However, whether these phosphorylation events are mediated by JNK *in vivo* has yet to be determined. The ability of JNK to phosphorylate p53 is supported by the findings that JNK and p53 are constitutively associated *in vivo*, and the phosphorylation of p53 by JNK stabilizes p53 by preventing its ubiquitylation, in a similar manner as seen with c-Jun (Fuchs et al., 1998; Fuchs et al., 1998).

1.5.3. Overview of different p38 genes - The p38 MAPK family is a group of serine/threonine kinases encoded by four genes; p38α/CSBP/RK/SAPK2a, p38β/SAPK2b, p38γ/ERK6/SAPK3, and p38δ/SAPK4 (Fig. 1.7). The p38α and p38δ genes are expressed ubiquitously. The p38β gene is highly expressed in the brain, and to a lower degree in most other tissues. The p38γ gene is specifically expressed in skeletal muscle. The p38α gene has two splice variants in humans known as CSBP1/p38α1 and CSBP2/p38α2 (Lee et al., 1994). These transcripts are generated by internal splicing of two different exons, without the addition or loss of an exon, as seen with the generation of the alpha and beta isoforms of JNK1 and JNK2. Mxi2 is potentially another splice variant of p38α, and is identical to p38α until sub-domain XI where it terminates after 16 unique amino acids (Zervos et al., 1995). The p38β gene also has two splice variants; p38β1 and p38β2, that are generated by the addition of a small exon between subdomain V and VI (Kumar et al., 1997; Stein et al., 1997). Splice variants encoding other functional kinases from p38γ or p38δ have not yet been characterized. However, two transcripts of p38δ with premature stop codons are known, but the significance of these splice variants is unknown *in vivo* (Stein et al., 1997).

1.5.4. A p38 MAPK inhibitor SB 203580 - In a drug screen at Smith-Kline Beecham, a compound that blocked the production of TNF and IL-1 by monocytic cells after treatment with
Figure 1.7 - Alignment of the Isoforms of p38. The predicted amino acid structure of human p38α, p38β, p38γ and p38δ. The splice variants p38α2 and p38δ2 are only indicated where they differ from p38α and p38β respectively. Where residues are identical to p38α they are indicated by periods. Gaps were introduced to optimize the alignment and are indicated by dashes.
endotoxic lipopolysaccharide was identified. These drugs belong to a class of pyridinyl imidazoles and are exemplified by SB 202190 and SB 203580 (Fig. 1.8). The target of these compounds is CSBP1/p38α1 and CSBP2/p38α2 MAPK (Lee et al., 1994). The drugs inhibit p38α and p38β, but not p38γ and p38δ, by acting as competitive inhibitors of p38 MAPK (Young et al., 1997). SB 203580 binds to the ATP-binding pocket of p38 MAPK, and inhibits the enzyme in vivo with an IC₅₀ around 1 μM (Lee et al., 1994; Young et al., 1997). The crystal structure of p38 MAPK bound to SB 203580 demonstrates the binding of the drug to the ATP-binding pocket (Fig. 1.9) (Pav et al., 1997; Tong et al., 1997). These crystal structures reveal specific amino acids in the ATP-binding pocket involved in drug interaction. Mutation of three amino acids in p38α (Thr₁₀⁶, His₁₀⁷, Leu₁₀₈) to the corresponding residues in p38γ (Met, Pro, Phe) converts p38α MAPK into an SB 203580-resistant kinase without affecting its substrate specificity (Gum et al., 1998).

These drugs were not only useful for the identification of p38 MAPK, but also have defined most of our knowledge of the substrate specificity and function of p38 MAPK. These drugs may inhibit Cyclooxygenase-2 and some isoforms of JNK at 10-fold or 40-fold higher concentrations respectively than required to ablate p38 MAPK activity.

1.5.5. Activators and Substrates of p38 MAPK - The four p38 genes encode protein kinases with a characteristic TGY motif in their activation loop. Similar to JNK and ERK MAPK, these enzymes require dual phosphorylation on both threonine and tyrosine residues to be activated (Fig. 1.10). This phosphorylation event is mediated by the dual specificity kinases MKK3 or MKK6, and at least in vitro by MKK4. MKK3 and MKK6 have many splice variants that differ in their length and their activity, with the longest isoforms of each being the most active. These two enzymes activate both p38α or p38δ MAPK equally, whereas MKK6 was more effective at activating p38β and p38γ MAPK (Cuenda et al., 1997; Han et al., 1996; Jiang et al., 1996; Jiang et al., 1997; Wang et al., 1997b). Several kinases have been suggested to act upstream of MKK3 and MKK6 including TAK1, MEKK5/ASK1 and MLK3 (Fig. 1.4).
Figure 1.8 - Structure of SB203580 - The p38 MAPK inhibitor.
Figure 1.9 - Crystal Structure of p38 MAPK bound to ATP or SB 203580. The crystal structure of the ATP-binding pocket was resolved at 2.0 Å bound to either ATP (left), or the p38 MAPK inhibitor SB203580 (right). This figure was adapted from Tong et al. 1997.
Figure 1.10 - Activators and Substrates of p38 MAPK.
Downstream targets for p38α and p38β MAPK are fairly well established, however substrates for p38γ and p38δ are unknown. The p38 MAPK cascade was initially described as a pathway leading to the phosphorylation of Hsp25 and was thought to act through MAPKAP kinase-2 (Freshney et al., 1994). MAPKAP kinase-2, and the related kinase MAPKAP kinase-3, were proven to be substrates for p38α and p38β using SB 203580 (Clifton et al., 1996; McLaughlin et al., 1996). The phosphorylation of MAPKAP kinase-2 and MAPKAP kinase-3 by p38 MAPK occurs on identical residues observed in vivo, and activates these enzymes (Ben-Levy et al., 1995). Recently, p38-regulated and activated kinase (PRAK), or MAPKAP kinase-4, was also identified as a substrate for p38α and p38β MAPK (New et al., 1998). These three enzymes regulate the phosphorylation of Hsp25, an actin capping protein, providing a link between p38 MAPK activity and regulation of the actin cytoskeleton. These isoforms of p38 also regulate the activity of MAPKAP kinase-5 and Mnk1. Mnk1 phosphorylates the RNA capping protein, eIF-4e, and may explain how p38 MAPK activity regulates the translation of several proteins including TNFα (Waskiewicz et al., 1997).

As with the JNK pathway, the p38 MAPK pathway also regulates several transcription factors. The CREB family of transcription factors including activating transcription factor (ATF)-1, and CREB is regulated by p38α or p38β, whereas ATF-2 is regulated by JNK and p38 MAPK. However, a study analyzing the E-selectin promoter with protein cross-linkers found that JNK was solely bound to c-Jun and ATF-2 was solely complexed with p38 MAPK, suggesting that p38 MAPK solely regulates ATF-2 in vivo (Read et al., 1997). ATF-1 and CREB are also regulated by the p38 MAPK pathway, and by a cAMP-responsive pathway (Tan et al., 1996). The regulation of ATF-1 and CREB is complicated as IL-3, an activator of p38 MAPK, activates ATF-1 and CREB through p38 MAPK-independent mechanisms, whereas ceremide activates ATF-1 and CREB solely through p38 MAPK-dependent pathways (Scheid et al., 1999). The differences likely reflect discrete substrate specificities of p38α and p38β MAPK, or cell-specific signalling cassettes implying the existence of discrete pools of ATF-1 and CREB that respond to different stimuli.
The p38 MAPK pathway regulates the C/EBP and the Ets families of transcription factors. CHOP/GADD, a member of the C/EBP family of transcription factors, is phosphorylated by p38 MAPK on sites that, when phosphorylated, prevent adipocyte differentiation (Wang and Ron, 1996). The p38 MAPK pathway also regulates Elk1 and Sap-1a, two Ets family members. These proteins are ternary complex factors for the c-fos promoter, and depending on the extracellular stimuli may be regulated by either p38 MAPK, JNK, or ERK MAPK pathways. Recently, the p38 MAPK and the ERK5 MAPK pathway were shown to regulate myocyte-enhanced factor (MEF) 2C, a transcription factor involved in c-Jun transcription. Together, these results provide a mechanism whereby p38 MAPK might regulate AP-1 activity through transcriptional regulation of c-Fos and c-Jun.

1.5.6. Stimuli upstream of p38 MAPK and JNK - The stress-activated protein kinases were initially characterized as enzymes activated by physical, chemical or environmental stresses. These stress stimuli include protein synthesis inhibitors such as anisomycin; DNA damaging agents such as cis-platinum, etoposide, or 1-β-D-arabinofuranosylcytosine; UV irradiation; γ-irradiation; hyperosmolarity; heat shock; ischemia; reperfusion; alkalinization or acidification of cells; or free radicals such as hydrogen peroxide. These stimuli are very diverse in nature and the mode of JNK or p38 MAPK activation is not obvious. Other more conventional receptor-mediated activators of the stress-activated protein kinase cascades include the pro-inflammatory cytokines including IL-1 and TNFα, the canonical growth factors such as EGF or PDGF, and the hematopoietic growth factors GM-CSF, IL-3 and SLF. Ligation of receptors that regulate the immune system such as the receptor for the Fc fragment of immunoglobulin G or E, the B-cell receptor for antigen, the T-cell receptor, MHC class 1, Fas and CD40 also induce both JNK and p38 MAPK activation. These stimuli are all initiated by receptor dimerization or oligomerization at the cell surface. The sheer diversity of receptors that engage these kinases argues for many diverse biological roles for signalling by these enzymes.
The mechanism of JNK or p38 MAPK activation by stress stimuli is under investigation. UV irradiation was believed to activate the stress-activated protein kinases through DNA damage, however JNK is activated by UV irradiation in enucleated cells demonstrating that DNA damage is not required for a cell to respond to UV irradiation (Devary et al., 1993). UV irradiation and hyperosmolarity aggregate receptors on the cell surface, thereby activating JNK (Rosette and Karin, 1996). Pre-treatment of cells with either antibodies against the TNFα or IL-1 receptors to prevent receptor dimerization, or with TNFα or IL-1 to downregulate cell surface expression of their receptors, ablates the activation of JNK by UV irradiation (Rosette and Karin, 1996). Therefore the signal induced by UV irradiation or osmotic shock should vary between cells depending on the expression of different cell-surface receptors. Similarly, UV irradiation-induced apoptosis requires cross-linking of Fas on the cell surface (Rehemtulla et al., 1997). Thus, UV irradiation and hyperosmolarity induced JNK and p38 MAPK activation are, at least in part, receptor mediated events.

Protein synthesis inhibitors also activate JNK and p38 MAPK through a mechanism that is presently being elucidated. Translational elongation of proteins involves the sequential binding of aminoacyl-tRNA, transferring of the peptidyl group to the nascent peptide, and translocation of the ribosome. Anisomycin targets the 3'-end of the 28S ribosomal RNA that is directly involved in these three steps and only activates JNK or p38 MAPK in the presence of transcriptionally active ribosomes (Iordanov et al., 1997). Antibiotics and ribotoxic agents that target the 28S ribosomal RNA also activate the JNK pathway in cells with transcriptionally active ribosomes (Iordanov et al., 1997). Together these findings indicate that anisomycin targets the 28S ribosomal RNA to activate the stress-activated protein kinase pathways, and likely explains why cycloheximide, a protein synthesis inhibitor that prevents ribosomal translocation, weakly activates the JNK or p38 MAPK pathway (Shu et al., 1996; Zinck et al., 1995). UV irradiation also damages the 28S ribosomal RNA, indicating that UV irradiation has at least two pathways that activate the stress-activated protein kinase pathways (Iordanov et al., 1998).
The activation of JNK and p38 MAPK by heat shock is poorly understood, and appears to require mitochondria and cytoplasmic proteins. Intact cells are not required for activation of JNK as heat shock of cell lysates greatly increases JNK activity (Adler et al., 1995). The over-expression of Hsp70, a chaperone protein involved in protein folding, or the addition of Hsp70 to crude lysates, abrogates JNK and p38 MAPK activation by heat shock (Gabai et al., 1997; Mosser et al., 1997). Conversely, immunoprecipitation of Hsp70 from cell lysates before heat shock enhances JNK and p38 activation (Gabai et al., 1997). Hsp70 maintains protein conformation during heat-shock, and prevents the conversion of procaspases into active caspases after heat shock (Buzzard et al., 1998; Mosser et al., 1997). Hsp70 may prevent the release of Cytochrome C from mitochondria, and therefore heat shock may reflect a sub-lethal activation of caspases leading to the activation of JNK and p38 MAPK. In support of this notion, cells lacking mitochondria fail to activate the stress-activated protein kinases after heat shock (Adler et al., 1995).

Reactive oxygen species potently activate the stress-activated protein kinase pathways. TNFα, hydrogen peroxide and UV irradiation all activate JNK through a mechanism requiring reactive oxygen species and are prevented by antioxidants such as N-acetylcysteine (Adler et al., 1995; Gotoh and Cooper, 1998). Interestingly, TNFα and hydrogen peroxide dimerize and activate ASK1 in an anti-oxidant sensitive mechanism (Gotoh and Cooper, 1998). The dimerization of ASK1 is likely regulated by its association with thioredoxin. Thioredoxin interacts with the N-terminus of ASK1 inhibiting its kinase activity, and N-acetylcysteine maintains the association of ASK1 with thioredoxin (Saitoh et al., 1998). Together, these studies suggest that reactive oxygen species alter the affinity of thioredoxin for ASK1 promoting its activation, and thereby activating the JNK and p38 MAPK pathways.

DNA damaging agents are potent activators of the stress-activated protein kinase pathways, but their mode of action is poorly characterized. 1-β-D-arabinofurosylcytosine or cis-platinum activate the JNK and p38 MAPK pathway downstream of the non-receptor tyrosine kinase c-Abl, as fibroblasts lacking c-Abl fail to activate JNK or p38 MAPK after these treatments (Kharbanda et
al., 1995; Pandey et al., 1996). In both instances, ectopic expression of c-Abl restored activation of JNK or p38 MAPK by these chemicals. The DNA alkylating agent methyl methanesulphonate (MMS) differs in its mechanism of JNK and p38 MAPK activation. MMS activates the stress-activated protein kinases normally in the absence of c-Abl, however this chemical requires the tyrosine kinase c-Src to activate JNK (Liu et al., 1996a).

Receptors involved in immune regulation, including the B cell antigen receptor (BCR), the T cell receptor (TCR), or the Fc receptors (FcR) for IgG or IgE, contain immunotyrosine activation motifs and ligation of these receptors activates several members of the Src tyrosine kinase family, the ZAP70/Syk protein tyrosine kinase family, and the Tec protein tyrosine kinase family. These receptors also activate the stress-activated protein kinases, but how these tyrosine kinases regulate upstream activators of the stress-activated protein kinases is not completely determined. In T cells, ligation of CD3 and CD28 synergizes to activate the stress-activated protein kinases (Salmon et al., 1997; Su et al., 1994). Signalling from Syk, but not Lck, synergizes with Rac1 to activate JNK (Jacinto et al., 1998). CD28 recruits PI-3 kinase that is required to activate JNK and p38 MAPK by these receptors. The requirement for PI-3 kinase may exist on several levels as the Tec family tyrosine kinases, and all known exchange factors for Rac/Cdc42 contain PH domains. In support of this notion, cells lacking Btk, a Tec family kinase, are deficient in JNK activation downstream of the FcR for IgG and IgE, BCR, SLF and heterotrimeric G-proteins signalling through Gq subunits (Bence et al., 1997; Kawakami et al., 1997, 1998; Jiang et al., 1998). Btk is required to maintain a sustained calcium flux, that may be required to activate calcineurin (Avraham et al., 1998; Fluckiger et al., 1998). In this instance, Btk may be activating JNK and p38 MAPK through the calcium-sensitive non-receptor tyrosine kinase Pyk2 (Tokiwa et al., 1996; Yu et al., 1996), or the calcium/calmodulin dependent protein kinase 4 (Enslen et al., 1996). The activation of JNK by the FcR for IgE, or by co-stimulation with CD3 and CD28, also uses a PKC-dependent pathway. The broad-specificity PKC inhibitor, Roche 3, partially blocks the activation of JNK in mast cells, and completely blocks the activation of JNK in mast cells lacking Btk after ligation of the FcR (Kawakami et al., 1998). In T cells, PKCØ and calcineurin synergize to activate the JNK
pathway (Avraham et al., 1998). These receptors may share a common mechanism for activating the stress-activated protein kinases that is yet to be elucidated.

1.6 Biological Roles of the Stress-Activated Protein Kinases

1.6.1. Role of Stress-Activated Protein Kinases in Yeast. Yeast must sense and respond to osmotic changes to grow and survive. The budding yeast, *S. cerevisiae*, has evolved two distinct sensors to respond to hyperosmolarity, one involves the histidine kinase receptor Sln1p, and the other involves the transmembrane receptor Sho1p. Both sensors activate the Ste7 homolog polymyxin B-sensitive kinase 2 (PBS2), and Hog1, or high osmolarity glycerol-1 (Maeda et al., 1995; Posas and Saito, 1997). Hog1, a homolog of mammalian p38 MAPK, was the first stress-activated protein kinase to be identified in yeast, and its activity is required for the growth of yeast in hyperosmotic media (Brewster et al., 1993). The genome of *S. cerevisiae* is known, and lacks a gene encoding a homolog of mammalian JNK (Hunter and Plowman, 1997). However, both mammalian p38 MAPK and JNK can complement the loss of Hog1, and allow the growth of yeast on hyperosmolarity medium (Han et al., 1994; Kumar et al., 1995). Complementation requires phosphorylation of these enzymes and kinase activity (Galcheva-Gargova et al., 1994; Kumar et al., 1995). Yeast lacking Pbs2 and Hog1 are not complemented by JNK or p38 MAPK alone, indicating that Pbs2 is upstream of p38 MAPK in yeast (Galcheva-Gargova et al., 1994; Kumar et al., 1995). Interestingly, only p38α1 MAPK, but not p38α2 MAPK, is able to complement the Hog1-deficiency in yeast (Kumar et al., 1995). The inability of p38α2 MAPK to rescue Hog1-deficient yeast was unexpected, and is believed to reflect greater activity of p38α2 MAPK leading to cell cycle arrest (Kumar et al., 1995).

The fission yeast *S. pombe* also express a homolog of mammalian p38 MAPK, Spc1 (Shiozaki and Russell, 1995). Yeast lacking Spc1 (Spc1−) were identified as suppressors of lethality due to loss of protein phosphatase 2C. Spc1− are sensitive to hyperosmotic medium and undergo a G2/M cell cycle arrest when grown on limiting nutrients. Expression cloning identified
Spc1 by its ability to rescue Spc1\(^{-}\) mutants grown on hyperosmotic medium. Another mutant Spc2\(^{-}\) was also identified in the same screen as Spc1\(^{-}\), and was rescued by Wis1, a MAPK kinase (Shiozaki and Russell, 1995). Yeast lacking Spc1\(^{-}\) and Spc2\(^{-}\) are phenotypically identical, suggesting these mutations lay on a single pathway (Degols et al., 1996; Shiozaki and Russell, 1995). Furthermore, over-expression of Wis1 is lethal in wildtype fission yeast, but not in Spc1\(^{-}\) yeast. In support of the genetic evidence, Wis1 directly phosphorylates and activates Spc1 \textit{in vitro} (Shiozaki and Russell, 1995). The over-expression of the tyrosine phosphatases Pypl and Pyp2 induces a similar G2/M cell cycle arrest as seen in the Spc1\(^{-}\) mutants in hyperosmotic medium (Shiozaki and Russell, 1995). The cell cycle arrest is exacerbated in high osmolarity, and does not occur on the Spc1\(^{-}\) background. Yeast lacking Pypl exhibit an increased phosphorylation of Spc1, and the overexpression of Pyp1 leads to the dephosphorylation of Spc1 (Shiozaki and Russell, 1995). Yeast lacking both Pypl and Pyp2 fail to proliferate, probably due to excess Spc1 activity as yeast lacking Pyp1, Pyp2 and Spc1 are viable (Degols et al., 1996; Shiozaki and Russell, 1995). As seen for p38\(\alpha\text{A}\) MAPK in \textit{S. cerevisiae}, excess Spc1 activity inhibits proliferation indicating that p38 MAPK activity can function to either promote or inhibit cell cycle progression in yeast. In summary, Wis1, Pypl and Pyp2 regulate Spc1 activity, and the activation of Spc1 is critical for growth during nutrient deficiency and hyperosmolarity.

1.6.2. Role of Stress-Activated Protein Kinases in \textit{Drosophila}. Components of the JNK and p38 MAPK signalling cascades are conserved in mammals and insects. The best established model for genetic analysis of the JNK signal transduction cascade is dorsal closure in \textit{D. melanogaster}. Dorsal closure occurs at mid-embryogenesis and at this time, the dorsal region of the embryo is covered by amnioserosa. The embryonic ectoderm, which is located ventral to the amnioserosa, moves upward and completely covers the embryo. This process does not involve cell division or cell recruitment, but instead the ectodermal cells change shape and move coordinately as an epithelial sheet to surround the embryo (Glise et al., 1995). \textit{Hemipterous} was identified by saturation mutagenesis looking for lethal mutations that blocked the morphogenetic
process of dorsal closure. Hemipterous is located on the X-chromosome and encodes dHep, a Drosophila MAPK kinase (Glise et al., 1995). A loss of dHep results in second generation homozygous recessive lethality as maternal gene products from heterozygous parents is sufficient to get homozygous null flies through embryogenesis. The role of dHep in dorsal closure was confirmed by reintroducing dHep under constitutive or inducible promoters to rescue the mutant flies (Glise et al., 1995). M KK7, and to a much lesser extent M KK4, complements the genetic defect in dHep null flies, indicating that M KK7 and dHep are orthologs (Holland et al., 1997). Drosophila M KK4 (DMKK4) was recently identified, but its role in dorsal closure has not been addressed (Han et al., 1998).

The Drosophila homolog of JNK (DJNK) was also identified in mutants that fail to undergo dorsal closure. In fact, two distinct mutations in the gene encoding DJNK, basket (bsk), are known and lead to embryonic lethality. Bskl has a point mutation between sub-domain IX and X that prevents interaction with substrate, while bsk2 has a nonsense mutation introducing a premature stop codon in DJNK (Riesgo-Escovar et al., 1996; Sluss and Davis, 1997). dHep phosphorylates and activates DJNK, which in turn phosphorylates DJun. Genetic disruption of DJun also results in a dorsal closure defect that is rescued by over-expressing a constitutively active mutant of DJun (Riesgo-Escovar et al., 1996; Sluss and Davis, 1997). Taken together, dHep, DJNK and DJun mediate dorsal closure, and this provides genetic evidence that DJNK phosphorylates DJun in vivo.

Loss of function mutations of DFos also result in embryonic lethality due to a failure in dorsal closure (Riesgo-Escovar and Hafen, 1997). As with mammalian c-Jun and c-Fos, DJun and DFos heterodimerize to form a functional AP-1 transcription factor. AP1 activity is required to induce the expression of decapentaplegic (Dpp), a member of the TGFβ family (Riesgo-Escovar and Hafen, 1997). Expression of Dpp is abolished in the leading edge cells of embryos lacking Hep, Bsk, DJun or DFos, and ectopic expression of dpp rescues the dorsal closure defect in bsk mutants (Glise and Noselli, 1997; Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997; Sluss and Davis, 1997). Furthermore, a constitutively active mutant receptor for
Dpp rescues embryos lacking DJNK or DJun (Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). Therefore, JNK signalling is required to induce Dpp expression for dorsal closure during Drosophila embryogenesis.

The JNK and the p38 MAPK pathways have also been implicated in Drosophila immunity. As in mammalian systems, insect cells respond to bacterial lipopolysaccharide (LPS) in response to bacterial infection. LPS induces the expression of a number of antimicrobial gene products including attacin and cecropin (Han et al., 1998). These proteins are secreted into the hemolymph and function synergistically to lyse invading microorganisms. Insect cells treated with LPS activate DJNK as well as two isoforms of p38 MAPK, Dp38a and Dp38b (Han et al., 1998). The role of DJNK in insect immune responses has not been determined due to embryonic lethality. However, SB 203580 also inhibits Dp38a and Dp38b activity and allows analysis of the function of p38 MAPK in Drosophila immunity. Surprisingly, inhibition of p38 MAPK greatly enhances LPS-induced production of message encoding attacin, and to a lesser degree cecropin (Han et al., 1998). Therefore, p38 MAPK activity appears to attenuate the insect immune response.

1.6.3. Role of Stress-Activated Protein Kinases in Embryogenesis and Hematopoiesis. The generation of mice lacking the genes encoding enzymes in the stress-activated protein kinase pathways has enhanced our understanding of their biological functions. Mice lacking MKK4 die in utero between day 12.5 and 14.5 (Yang et al., 1997b) due to an inability to undergo normal hepatogenesis (Ganiatsas et al., 1998). Livers of embryos lacking MKK4 are approximately normal size with a reduced total number of cells, that led to hemorrhaging during development (Ganiatsas et al., 1998). As hematopoiesis in yolk sac progenitors is normal in day 10.5 embryos, these embryos likely die of anemia from blood loss. Apoptosis in hepatocytes of MKK4 null embryos was increased about 2-fold indicating an anti-apoptotic function for MKK4 in liver organogenesis (Ganiatsas et al., 1998). Mice with a targetted disruption of c-Jun have a similar defect in liver organogenesis (Hilberg et al., 1993; Johnson et al., 1993). Interestingly, mice deficient in c-Met or HGF die in utero with similar phenotypes as
the mice lacking MKK4 or c-Jun (Schmidt et al., 1995). Cross-linking of the met oncogene, the receptor for hepatocyte growth factor (HGF), activates the JNK pathway suggesting a critical role of JNK downstream of c-Met (Rodrigues et al., 1997). Taken together, these studies indicate that MKK4 regulates c-Jun transactivation in vivo, providing genetic evidence to support biochemical findings. Mice lacking JNK1 or JNK2 exhibit defects in cytokine production by T helper subclasses of CD4^+ T cells (Dong et al., 1998; Yang et al., 1998), but otherwise are phenotypically normal, likely reflecting the functional redundancy of these enzymes. Genetic disruption of the JNK3 results in a defect in the apoptosis of neuronal cells after treatment with glutamate receptor agonists (Yang et al., 1997a). The genetic disruption of the genes encoding all stress-activated protein kinases is undoubtedly in progress, but has not as yet been described.

Chimeric mice produced from MKK4-deficient embryonic stem cells and RAG2-deficient blastocysts have allowed the generation and analysis of T and B lymphocytes lacking MKK4. Rag2-deficient mice have a block of CD4^-CD8^-TCR^- thymocyte progenitors to CD4^+CD8^-TCR^- immature thymocytes, and therefore fail to generate single or double positive T lymphocytes. MKK4-deficient blastocyst chimeric mice exhibit a profound block in the production of double positive T lymphocytes compared to wildtype MKK4 blastocyst chimeric mice (Nishina et al., 1997). Loss of MKK4 sensitizes thymocytes to apoptosis induced by ligation of CD3 or Fas (Nishina et al., 1997). MKK4-deficient thymocytes are also defective in production of IL-2, and proliferation after ligation of CD28 and CD3 (Nishina et al., 1997). In contrast, disruption of MKK4 by targetting either sub-domain VI, or the activation loop fails to reproduce the phenotypes of mice targetted in exon 2 outside the catalytic domain (Ganiatsas et al., 1998; Nishina et al., 1997; Yang et al., 1997b). Furthermore, thymocytes derived from transgenic mice expressing a dominant negative mutant of MKK4 undergo Fas-induced apoptosis normally (Alberola-Ila et al., 1998). The reason for these differences is not clear, but may represent other genetic differences between the ES cells used to generate the chimeric mice, or the expression of truncated forms of MKK4 in ES cells targetting the kinase domain. The MKK4-deficient blastocyst chimeric mice that were targetted in the catalytic domain display lymphadenopathy and plasmacytosis between 2
and 6 months of age (Swat et al., 1998). These T and B lymphocytes are polyclonal in nature, and their accumulation only occurred in peripheral lymphoid tissues, suggesting a role for MKK4 activity in the removal of T and B lymphocytes.

1.6.4. Role of Stress-Activated Protein Kinases in Cytokine Production and Inflammation. - Gene expression is regulated transcriptionally and post-transcriptionally. Post-transcriptional regulation includes mRNA processing, mRNA turnover, and translation. p38 MAPK was identified as the target of the pyridine imidazole compound, SB 202190, a compound that blocks the production of IL-1 and TNFα in monocytes treated with LPS (Lee et al., 1994). Pyridine imidazole compounds inhibit the production of TNFα at the translational level, while the mRNA encoding TNFα is unaffected (Young et al., 1993). Translation is inhibited by preventing the entry of mRNA encoding TNFα into the polysomal compartment, and the initiation of protein translation (Prichett et al., 1995). Translational initiation is known to be regulated by eIF-4e, that is sequestered in unstimulated cells in a complex with PHAS-1, another substrate for p38 MAPK (Jiang et al., 1996; Wang et al., 1997b). Upon phosphorylation of PHAS-1, eIF-4e is released and initiates translation of specific mRNA (Lin et al., 1994). Mnkl, a kinase activated by p38 MAPK and ERK MAPK, phosphorylates eIF-4e and thereby the activation of the p38 MAPK pathway may co-ordinately derepress eIF-4E by phosphorylating PHAS-1, and activate eIF-4e through Mnkl (Waskiewicz et al., 1997). The role of p38 MAPK in TNFα production is cell-type dependent. In MC/9 mast cells, the JNK pathway, but not ERK or p38 MAPK, is required for TNFα production after ligation of the Fc receptor for IgE (Ishizuka et al., 1997). However, ligation of the Fc receptor for IgE requires the ERK MAPK pathway, but not JNK or p38 MAPK activity, to produce TNFα in CPII or RBL cells (Csonga et al., 1998; Zhang et al., 1997). Despite the proposed roles of ERK or JNK in the production of TNFα, p38 MAPK regulates the production of TNFα in vivo. The lethality of LPS results from vascular failure associated with excess production of TNFα. Inhibitors of p38 MAPK reduce serum TNFα levels, and prevent the death of mice that have been injected with normally lethal doses of LPS (Badger et al., 1996).
The inhibition of p38 MAPK also antagonizes the biological actions of IL-1 and TNFα, two pro-inflammatory cytokines that activate JNK and p38 MAPK. Cells treated with these cytokines may express IL-6, IL-8, Cyclooxygenase-2, or several metalloproteinases. The p38 MAPK pathway is required for the production of IL-6, but not IL-8, in human umbilical vascular endothelial cells or gingival fibroblasts (Ridley et al., 1997). In contrast, the expression of IL-8 is regulated by p38 MAPK in peripheral blood mononuclear cells and THP-1 cells after hyperosmotic shock (Shapiro and Dinarello, 1995). This finding demonstrates that the function of p38 MAPK in cytokine production is cell-type dependent. In an animal model of adjuvant-induced arthritis, the production of IL-6 is prevented by SB 203580, demonstrating the importance of p38 MAPK activity for the production of IL-6 in vivo (Badger et al., 1996). The p38 MAPK inhibitor prevents the translation of mRNA encoding IL-6 without affecting the message, similar to IL-1 or TNFα (Ridley et al., 1997). These transcripts all contain AU-rich elements in their 3'-UTR, suggesting these elements may regulate translation through a p38 MAPK-dependent mechanism.

Besides cytokines, cells treated with IL-1 or TNFα release arachidonic acid (AA) which is required for the biosynthesis of prostaglandins (PG), leukotrienes (LT), and lipoxins (LX). The production of PG is regulated by Cyclooxygenase (COX) -1 and COX-2. Inhibition of p38 MAPK prevents IL-1 induced production of PGE2 and PGF2α at 0.1 μM (Badger et al., 1996). SB 203580 was derived from a series of compounds that inhibit COX and 5-Lipoxygenase, the enzyme required for LT production, and directly inhibits COX activity at 10 μM (Ridley et al., 1997). The p38 MAPK inhibitor also prevents the expression of message encoding COX-2 at 0.1 μM, a concentration that specifically inhibits p38 MAPK (Ridley et al., 1997). AA is produced by specific cleavage of phosphatidylcholine by cytosolic phospholipase A2. This enzyme is phosphorylated, and possibly activated, by p38 MAPK suggesting that SB 203580 not only inhibits production of prostanoids by blocking transcription of COX-2, but also by inhibiting the biosynthesis of PG, LT and LX by preventing AA release (Borsch-Haubold et al., 1997; Kramer et al., 1996).
The p38 MAPK inhibitor reduces many symptoms of inflammation and arthritis, that in large part are due to the actions of TNFα and IL-1. In murine models of collagen-induced or adjuvant-induced arthritis, the p38 MAPK inhibitor reduces footpad inflammation and serum amyloid levels (Badger et al., 1996). Inhibition of p38 MAPK also prevents the loss of bone mineral density and bone mineral content associated with adjuvant-induced arthritis, possibly by preventing the activation of osteoclasts by IL-1 and TNFα (Badger et al., 1996). Therefore, the p38 MAPK pathway regulates many different inflammatory conditions.

The infiltration of leukocytes into sites of inflammation is regulated by the upregulation of adhesion molecules such as E-selectin on endothelial cells after tissue injury. Mice lacking ATF-2 fail to express E-selectin, indicating the importance of ATF-2 for transcription of this protein (Reimold et al., 1996). ATF-2 is regulated by JNK and p38 MAPK in vivo and in vitro (Gupta et al., 1995; Li et al., 1996; Wang et al., 1997). Cells expressing constitutively active MEKK1, or treated with TNFα, upregulate E-selectin in both JNK and p38 MAPK-dependent fashions (Min and Pober, 1997; Read et al., 1997). UV cross-linking studies using the E-selectin promoter indicate that both ATF-2 and c-Jun constitutively bind to elements in the E-selectin promoter. These studies also indicate that c-Jun interacts solely with JNK, and surprisingly that ATF-2 interacts solely with p38 MAPK (Read et al., 1997). Therefore, the E-selectin promoter provides an example of how two MAPK pathways co-ordinate to regulate gene expression.

Besides the production of TNFα, p38 MAPK activity is required for the production of Interferon-γ (IFNγ). Transgenic mice expressing dominant negative p38 MAPK exhibit reduced expression of IFNγ and impaired Th1 responses (Rincon et al., 1998). Similarly, mice overexpressing dominant active MKK6 show an increased production of IFNγ and a predominant Th1 response (Rincon et al., 1998). Mice lacking JNK2 also fail to produce IFNγ normally and exhibit a defect in Th1 responses (Yang et al., 1998). JNK1 is also involved in cytokine production as CD4+ T cells derived from mice lacking JNK1 produce high levels of Th2 cytokines, even when differentiated to the Th1 subset (Dong et al., 1998). A failure in IFN-γ production may also reflect a defect in IL-12 production, as IL-12 induces the expression of...
interferon-γ (IFNγ) in T cells. However, the production of IFNγ was found to be regulated directly by p38 MAPK activity as the production of IFNγ was completely inhibited by SB 203580 in the presence of exogenous IL-12 (R. Salmon, unpublished observations). Inhibition of p38 MAPK activity also prevents the production of IL-12 by splenic antigen presenting cells (R. Salmon, unpublished observations). These findings demonstrate the importance of p38 MAPK and JNK activity in regulating not only cytokine production, but also the differentiation of CD4+ T cells in mice.

In T cells, the ligation of CD3 and CD28 synergizes for the activation of the stress-activated protein kinases and the production of IL-2. T lymphocytes lacking MKK4 fail to produce normal levels of IL-2, indicating the importance of the JNK pathway in regulating IL-2 production in vivo (Nishina et al., 1997). The IL-2 promoter contains AP-1 sites, providing p38 MAPK and JNK responsive elements. The expression and transcriptional activation of c-Jun is directly regulated by JNK, and indirectly regulated by p38 MAPK through MEF2C. Consistent with this notion, inhibition of p38 MAPK activity by SB 203580 or by dominant negative MKK6 inhibits the production of IL-2 at the promoter level (Matsuda et al., 1998). The JNK pathway also regulates the production of IL-2 by stabilizing the mRNA transcript through two cis-acting elements (Chen et al., 1998). In support of this notion, the IL-2 message is specifically stabilized by MEKK1 or M KK7, but not by constitutively active MKK6 or ERK (Chen et al., 1998). The increase in mRNA stability and transcription mediated by JNK and p38 MAPK likely explains the synergistic production of IL-2 after treatment with CD3 and CD28.

1.6.5. Role of Stress-Activated Protein Kinases in Proliferation and Tumorigenesis - The stress-activated protein kinases are required for the proliferation of yeast grown in hyperosmotic media. Similarly, these kinases regulate the proliferation and tumorigenesis of mammalian cells. DNA synthesis of hematopoietic cells grown in IL-3, SLF, IL-4, IL-7 or IL-2 is blocked by the p38 MAPK inhibitor (Crawley et al., 1997, I. Foltz, unpublished data). p38 MAPK may regulate mitogenesis through MAPKAP kinase 2, an Hsp25 kinase.
Hsp25 is an actin capping protein, and regulates the formation of F-actin from G-actin monomers (Lavoie et al., 1995). A role for the actin cytoskeleton in mitogenesis is not surprising, and is supported by the ability of cytochalsin D to block cell cycle progression (Sampath and Pollard, 1991). However, an SB 203580-resistant isoform of p38α MAPK restores MAPKAP kinase-2 activity in the presence of SB 203580, but fails to restore growth factor induced DNA synthesis in hematopoietic cells (I. Foltz, unpublished data). Therefore, either p38α MAPK and p38β MAPK exhibit different substrate specificities in vivo, or another target for SB 203580 is required for DNA synthesis.

The activation of the p38 MAPK pathway can also inhibit proliferation as cells over-expressing MKK3 and p38 MAPK fail to express cyclin D1 or progress through cell cycle. Similarly, the inhibition of p38 MAPK by SB 203580 increases the expression of cyclin D1 in CHO cells (Lavoie et al., 1996). The kinase activity of p38 MAPK also correlates with the arrest of cells in the M phase of the cell cycle. Nocodazole-treated cells arrest in M-phase due to the disruption of spindle fibers, and exhibit constitutive activation of p38 MAPK, but not JNK or ERK MAPK (Takenaka et al., 1998). The inhibition of p38 MAPK activity allows the cells to proceed through into the G1 phase of cell cycle, supporting the notion that p38 MAPK negatively regulates progression through cell cycle (Takenaka et al., 1998).

A role for the JNK pathway in mitogenesis is expected as ES cells lacking c-Jun are unable to proliferate unless they overexpress the middle T antigen of SV40 (Johnson et al., 1993). Proliferation induced by EGF is also inhibited by dominant negative c-Jun and by anti-sense oligonucleotides against JNK1 and JNK2; however, the basal proliferation rates are unaffected. In contrast, inhibition of MEK1 prevents basal proliferation, but not EGF-induced proliferation, suggesting discrete roles for ERK and JNK in basal and growth-factor-induced proliferation respectively (Bost et al., 1997). Similarly, IL-3 induced proliferation is prevented by over-expression of M3/6 phosphatase, a JNK-specific phosphatase, that indirectly implicates JNK with mitogenesis (Smith et al., 1997). These findings support a critical role for JNK and c-Jun in proliferation.
Transformation and tumorigenesis require the stress-activated protein kinase pathways. Activated Ras potently induces the expression of c-Jun, and the over-expression of a dominant negative c-Jun inhibits Ras-induced transformation. Furthermore, activated Ras is unable to cause a loss of contact inhibition, anchorage independence, or tumorigenicity in fibroblasts lacking the expression of c-Jun (Johnson et al., 1996c). Another potent transforming protein, v-Met, constitutively activates the JNK pathway, and JNK activity is required for transformation (Rodrigues et al., 1997). Transformation of cells by the v-Crk oncogene also implicates the activation of JNK with transformation. Co-expression of v-Crk and the guanine nucleotide exchange factor C3G potentiates JNK activation, and enhances both the growth rate and anchorage independent growth of NIH 3T3 cells in a JNK-dependent fashion, supporting a role for JNK activity in transformation (Tanaka et al., 1997). The transformation of pre-B cells expressing the oncogene Bcr-Abl also requires the JNK pathway. Cells transformed by Bcr-Abl exhibit constitutive JNK and p38 MAPK activity, and the transformation is reversible by ectopic expression of dominant negative c-Jun. Enforced expression of JNK-inhibitor protein 1 (JIP1) also potently inhibits Bcr-Abl induced transformation (Dickens et al., 1997). JIP1 was initially described as a cytoplasmic inhibitor of the JNK pathway that acts by preventing nuclear translocation of JNK (Dickens et al., 1997). Primary T cell leukemias, or T lymphocytes transformed with HTLV-1, also exhibit constitutive activation of the JNK pathway. Ectopic expression of Tax-1, the transactivator of HTLV-1, only activates the JNK pathway in transformed T cells, and simply expressing Tax-1 is insufficient to activate JNK (Xu et al., 1996b). Another virus that transforms human cells is Kaposi’s sarcoma-associated herpesvirus (KSHV). Transformation and tumorigenicity is induced by a constitutively active G-protein coupled receptor (ORF 74) that activates both the JNK and the p38 MAPK pathways, but not the ERK MAPK pathway, correlating the activation of these kinases with transformation (Bais et al., 1998). In contrast, MKK4 may act as a tumor suppressor based on loss of function mutations in two cell lines derived from pancreatic and lung carcinomas (Teng et al., 1997). Despite a role in transformation, the JNK pathway also regulates apoptosis, and perhaps a loss of MKK4 improves
the survival of transformed cells. Together, these findings support a role for JNK signalling in promoting the transformation of mammalian cells by oncogenes and viruses.

1.6.6. Role of Stress-Activated Protein Kinases in Apoptosis - Apoptosis is the process of programmed cell death, and cells undergoing apoptosis are characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation. Apoptosis removes unwanted cells without initiating an inflammatory response, and is critical for embryogenesis, organogenesis and for the regulation of the hematopoietic system. Many stimuli that induce apoptosis also activate JNK and p38 MAPK. However, the role of these enzymes in apoptosis is not straightforward.

JNK and p38 MAPK were first implicated as effectors of neuronal apoptosis. Apoptosis induced by nerve growth factor is accompanied by an increase in JNK and p38 MAPK activity, and a decrease in ERK MAPK activity (Xia et al., 1995). Neuronal apoptosis is also induced by constitutive MEKK1 activity in a JNK-dependent fashion, suggesting the JNK pathway is pro-apoptotic. Constitutive activation of p38 MAPK also weakly induces apoptosis in neuronal cells (Xia et al., 1995). Mice lacking JNK3 provides genetic evidence supporting a pro-apoptotic role of JNK activity in neuronal tissue (Yang et al., 1997). These mice are resistant to apoptosis of hippocampal neurons induced by the glutamate agonist kainic acid, but still undergo apoptosis normally during development indicating that JNK activity is not required for all neuronal apoptosis (Yang et al., 1997). Consistent with these findings, the stimulation of cultured neurons with kainic acid activates JNK and p38 MAPK, and induces apoptosis that is prevented by the p38 MAPK inhibitor (Kawasaki et al., 1997; Schwarzschild et al., 1997). Neuronal apoptosis is also prevented by insulin through the inhibition of p38 MAPK activity (Heidenreich and Kummer, 1996). Furthermore, the removal of insulin or NGF induces apoptosis in a p38 MAPK-dependent manner (Kummer et al., 1997). Neuronal apoptosis correlates with the induction of c-Jun and AP-1 activity (Herdegen et al., 1997), perhaps explaining how both enzymes can mediate neuronal apoptosis as both JNK and p38 MAPK regulate AP1 activity (Han et al., 1997a; Hazzalin et al., 1996; Price et al., 1996). An understanding of the role of JNK signalling in neuronal apoptosis is
important for the treatment of stroke patients. Ischemia induces neuronal apoptosis, and potently activates the JNK pathway. A recently identified inhibitor of the JNK pathway prevents apoptosis of cultured rat embryonic motoneurons after ischemia, providing evidence that inhibiting the JNK pathway may prevent the apoptosis of neurons in stroke patients (Maroney et al., 1998).

The TNF receptor superfamily encodes proteins that directly influence apoptosis and activate the stress-activated protein kinases. The prototypical death receptor Fas, when cross-linked by Fas ligand, initiates a signal that rapidly induces apoptosis. The cytoplasmic domain of Fas associates directly with both FADD, or Fas-associated death domain, and DAXX (Medema et al., 1997; Yang et al., 1997c). FADD and DAXX are adaptor proteins that recruit procaspase-8 and ASK1 to Fas (Chang et al., 1998; Ichijo et al., 1997). Signalling through Fas induces two phases of JNK and p38 MAPK activity (Salmon et al., 1997; Toyoshima et al., 1997). The early activation is caspase independent, and likely lies downstream of ASK1 (Toyoshima et al., 1997). The second phase is caspase-dependent and is caused by the process of apoptosis (Juo et al., 1997). Cross-linking of Fas cleaves procaspase-8 to produce active caspase-8, which activates a cascade of proteases and effectively sentences a cell to death. Several proteins have been identified as substrates of the caspases including Raf-1, FAK, MEKK1 and PAK. The cleavage of Raf-1 and FAK inactivates their respective signalling pathways in cells, effectively removing anti-apoptotic signalling pathways (Widmann et al., 1998). However during apoptosis, the activation of the JNK and p38 MAPK results from the cleavage of MEKK1, and maybe PAK2, to form constitutively active kinases (Cardone et al., 1997; Rudel and Bokoch, 1997). The expression of dominant negative PAK2 prevents the activation of JNK by Fas without affecting apoptosis, demonstrating that JNK activity is not required for Fas-induced apoptosis (Rudel et al., 1998). The activation of caspases cleaves MEKK1 (Deak et al., 1998), and this cleavage product is critical for apoptosis after genotoxic stress and anoikis, the apoptosis that occurs after adherent cells lose their integrin-mediated contacts with the extracellular matrix (Cardone et al., 1997; Widmann et al., 1998). Constitutively active MEKK1 potently induces both apoptosis and the JNK pathway; however, co-expression of dominant negative mutants of JNK or c-Jun fails to prevent this
apoptosis, suggesting the JNK pathway is not critical to this apoptotic event (Johnson et al., 1996b). Similarly, apoptosis induced by Fas is not prevented by inhibiting JNK or p38 MAPK activity (Lenczowski et al., 1997; Salmon et al., 1997). In contrast, ceramide or Fas-induced apoptosis is prevented by over-expressing dominant negative mutants of Ras, Rac1, p38 MAPK or JNK in Jurkat cells (Brenner et al., 1997). The apoptotic mechanisms may differ between cells and may partially explain the conflicting data on the role of JNK and p38 pathways in apoptosis.

The pro-inflammatory cytokine TNFα also causes the activation of JNK and p38 MAPK pathways as well as apoptosis. TNFα weakly induces apoptosis and pre-treatment of the cells with protein synthesis inhibitors is required for effectively cell killing. These inhibitors prevent the induction of anti-apoptotic genes by the transcription factor NF-kB and maybe by transcription factors downstream of JNK or p38 MAPK. Apoptosis induced by TNFα occurs in the absence of JNK or p38 MAPK activity. Cells lacking TRAF2 fail to activate JNK or p38 MAPK, but still die by apoptosis in the presence of TNFα (Lee et al., 1997; Liu et al., 1996; Yeh et al., 1997). In fact, cells lacking TRAF2 are more sensitive to TNFα-induced apoptosis, suggesting a role for JNK in the prevention of apoptosis (Yeh et al., 1997). Similarly, TNFα-induced apoptosis is potentiated by inhibition of JNK or p38 MAPK activity by dominant negative mutants of MKK4, MKK6, or the p38 MAPK inhibitor (Roulston et al., 1998). Apoptosis and activation of the stress-activated protein kinases is also split as dominant negative FADD blocks apoptosis without affecting the activation of JNK or p38 MAPK (Natoli et al., 1997). Therefore, the activation of p38 and JNK by TNFα appears to be cytoprotective in some instances.

Other stimuli such as heat shock cause concomitant activation of JNK and p38 MAPK and induction of apoptosis. In fact, cells selected by survival after heat shock are unable to activate JNK (Zanke et al., 1996). The overexpression of dominant negative MKK4 also makes cells resistant to heat shock induced-apoptosis (Zanke et al., 1996). Therefore in this instance, JNK activity promotes apoptosis. Heat shock potently drives the expression of heat shock proteins (Hsp) including Hsp70 (Wu et al., 1985). Cells expressing high levels of endogenous Hsp70 fail to activate JNK or p38 MAPK after heat shock, and fail to undergo heat shock induced apoptosis.
(Buzzard et al., 1998; Gabai et al., 1997; Meriin et al., 1998; Mosser et al., 1997). However the ectopic expression of Hsp70 does not prevent JNK or p38 MAPK activation by heat shock, even though it protects cells from heat shock-induced apoptosis (Buzzard et al., 1998; Mosser et al., 1997). These findings are contradictory, and suggest that JNK or p38 MAPK activity may provide a permissive signal for apoptosis only in the proper cellular context.

Activation of JNK and p38 MAPK often correlates with apoptosis, and while their activation is not a death sentence, it may provide a permissive signal for apoptosis. These pathways either directly phosphorylate proteins that modulate apoptosis, or indirectly regulate the expression of proteins that regulate apoptosis. The anti-apoptotic protein Bcl-2 is a substrate for JNK, but not p38 or ERK MAPK (Maundrell et al., 1997). Over-expression of a constitutively active mutant of Rac1 induces the phosphorylation of Bcl-2 on the identical sites that are phosphorylated by JNK \textit{in vitro}. The constitutively active mutants of Rac1 or Cdc42 are strong inducers of apoptosis in a JNK-dependent fashion (Chuang et al., 1997). Bcl-2 is believed to inhibit caspases, and prevent the release of cytochrome C from mitochondria. The phosphorylation of Bcl-2 by JNK may prevent the inhibition of caspases by Bcl-2, or prevent its interaction with pro-apoptotic Bcl-2 family members, thereby promoting apoptosis. A role for phosphorylation of Bcl-2 was suggested in a mutant strain of WEHI-231 cells, that is unusual as it fails to undergo apoptosis after treatment with anti-IgM antibodies. The resistance to IgM-induced apoptosis was due to a deletion in Bcl-2 that removed the putative JNK phosphorylation sites (Chang et al., 1997). Together, these findings suggest a role for JNK signalling in Bcl-2 dependent apoptotic pathways.

The tumor suppressor p53 is also phosphorylated by JNK on a site that is known to be phosphorylated in intact cells (Hu et al., 1997; Milne et al., 1995). p53 constitutively associates with JNK (Hu et al., 1997), and the association of inactive JNK with p53 promotes its ubiquitinylation (Fuchs et al., 1998a). In the absence of JNK activity, p53 and Mdm2 are constitutively associated, with Mdm2 also targeting p53 for ubiquitinylation and destruction (Fuchs et al., 1998b). The phosphorylation of p53 by JNK dissociates Mdm2 and p53, thereby

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stabilizing p53 and increasing its transcriptional activity (Fuchs et al., 1998b). The importance of p53 phosphorylation in apoptosis is unclear. However, the co-transfection of p53 and constitutively active MEKK1 into cells lacking p53 potentiates apoptosis compared to cells transfected with MEKK1 alone, suggesting a role for JNK in the promotion of p53-dependent apoptosis (Fuchs et al., 1998b).

The stress-activated protein kinases may also regulate apoptosis by upregulating pro-apoptotic proteins. The expression of Fas ligand (FasL) is increased by DNA damaging agents such as etoposide, an activator of JNK and p38 MAPK pathways (Kasibhatla et al., 1998). The ectopic expression of NFkB, or c-Fos and c-Jun is sufficient to drive the FasL promoter through kB or AP-1 sites respectively (Kasibhatla et al., 1998). Therefore, JNK or p38 MAPK activity may induce expression of FasL. Consistent with this notion, over-expression of constitutively active MEKK1 induces the expression of Fas ligand through the AP-1 site in its promoter, and dominant negative c-Jun or ATF-2 prevents the expression of FasL (Faris et al., 1998). The expression of Fas ligand provides a mechanism by which the stress-activated protein kinases regulate gene expression to promote apoptosis.

The JNK and p38 signalling pathways also prevent apoptosis in primary tissues. Mice lacking MKK4 die in utero of a failure in liver organogenesis that is due to the increased apoptosis of hepatocytes (Ganiatsas et al., 1998). Furthermore, T lymphocytes lacking MKK4 are more sensitive to apoptosis induced by crosslinking of CD3 or Fas (Nishina et al., 1997a). These reports suggest a role for MKK4 in the prevention of apoptosis in vivo. Mice lacking MKK4 also exhibit splenomegaly and lymphadenopathy that becomes evident with age. The cells represent a polyclonal expansion of both B and T-cell pools, suggesting a failure in the apoptosis of peripheral lymphocytes. In contrast, the inhibition of p38 MAPK has no effect on activation-induced cell death in T lymphocytes treated with anti-CD3 antibodies, or the prevention of apoptosis by CD28 (Salmon et al., 1997). UV irradiation potently induces apoptosis, and cells lacking c-Fos or c-Jun are more sensitive to UV-irradiation induced apoptosis (Haas and Kaina, 1995; Schreiber et al., 1998).
Together, these reports demonstrate that the JNK and p38 MAPK pathways serve a cytoprotective function in normal cells.

Therefore it appears the stress-activated protein kinases may provide pro-apoptotic, or anti-apoptotic signals in a cell-dependent and a stimulus-dependent fashion. These observations may be explained in part by the presence of other signals regulating apoptosis. For example, hematopoietic growth factors activate the stress-activated protein kinases (Foltz et al., 1997; Foltz and Schrader, 1997; Nagata et al., 1997a,b). These cytokines function to prevent apoptosis, further demonstrating that activation of JNK and p38 MAPK is not simply a signal for apoptosis. However, these cytokines also activate PI-3 kinase, AKT and the ERK MAPK pathways, and perhaps either JNK or p38 MAPK activity would provide an apoptotic stimulus in the absence of these anti-apoptotic pathways.
CHAPTER 2 - MATERIALS AND METHODS

Methods Used In Chapter 3 -

Antibodies and reagents. The anti-p38 MAP kinase anti-serum used for immunoprecipitation was raised against full-length CSBP2 as described (Lee et al., 1994). The anti-MAPKAP kinase-2 antibody (06-534) and anti-phosphotyrosine antibody 4G10 (05-321) were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-p38 MAP kinase antibody (sc-535) used for western blotting, the anti-p90rsk antibody (sc-231), and the truncated ATF-2 (1-96) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-p38 MAP kinase specific antibody (9211S) was from New England Biolabs (Beverly, MA), the recombinant murine Hsp25 was from StressGen Biotechnologies (Victoria, BC), the myelin basic protein (MBP) was from Sigma (St. Louis, MO), and the recombinant murine CSF-1 was from R&D Systems (Windsor, ON). The monoclonal antibody 5A1, specific for CSF-1, was provided by H. Ziltener (BRC, Vancouver). The antibody 2.4G2 (anti-FcR) that binds to and blocks the Fcγ receptor II and III without inducing signalling was purchased from Pharmingen (San Diego, CA), and the polyclonal rabbit anti-rat Ig antibody (anti-Ig) used to crosslink the anti-FcR antibodies on the cell surface to induce FcR mediated signalling was a gift of Dr. H. Ziltener (Vancouver, BC). RPMI 1640 was purchased from Canadian Life Technologies (Burlington, ON) and fetal calf serum (FCS) was purchased from Intergen (Purchase, NY). Recombinant SLF was provided by James Wieler and synthetic cytokines were provided by I. Clark-Lewis (BRC, Vancouver).

Cell culture and stimulation conditions. RPMI 1640 was supplemented with 10% FCS, 10 μM 2-mercaptoethanol (2-Me) and 100 Units of Penicillin/Streptomycin prior to using for cell culture. Primary mast cells were derived by culturing bone marrow cells from (C57BL/6 x DBA/2) F1 hybrid mice in RPMI 1640 supplemented with 2% WEHI-3B conditioned medium as a source of IL-3 and 3% X063-mIL4 conditioned medium as a source of IL-4 for 3 weeks as
described (Welham et al., 1992). The factor-dependent hematopoietic cell lines MC/9 and Ba/F3 were passaged in RPMI 1640 supplemented with 2% WEHI-3B conditioned medium. FD-5/13R and FD-MACII cells were passaged in RPMI 1640 supplemented with 2% L-cell conditioned medium (a source of CSF-1) as previously described (Welham et al., 1994, Orchansky et al., 1998). HU-3 cells were cultured in RPMI 1640 supplemented with 3% gibbon IL-3 conditioned medium.

Prior to stimulation, cells were cultured overnight in 0.2% IL-3 conditioned medium, washed 3 times with phosphate-buffered saline (PBS) and then incubated at 10^7 cells/mL in serum-free medium buffered with 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) pH 7.2, for 1 hr. Cells were stimulated with the following doses of recombinant or synthetic growth factors: IL-4 - 10 ng/mL; GM-CSF - 10 ug/mL; IL-3 - 10 ug/mL; TNFα - 50 ng/mL. For FcR cross-linking, MC/9 mast cells were initially incubated with anti-FcR for 10 min and then were incubated for a further 10 min either untreated or with the addition of anti-Ig to cross-link the α-FcR antibody. All stimulations had been shown in preliminary experiments to give maximal levels of tyrosine phosphorylation of the respective receptors.

**Immunoprecipitation conditions.** Cells extracts were prepared in solubilization buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40 (NP-40), 1 mM sodium molybdate, 200 mM sodium orthovanadate, 1 mM sodium fluoride, 50 mM β-glycerol phosphate, 10 µg/mL aprotinin, 10 µg/mL soybean trypsin inhibitor, 0.7 µg/mL pepstatin, 2 µg/mL leupeptin, and 40 µg/mL phenylmethylsulfonyl fluoride). To monitor the levels of tyrosine phosphorylation in the control and the treated samples, a portion of each cell lysate was routinely resolved by SDS-PAGE and immunoblotted with the anti-phosphotyrosine antibody 4G10 (0.1 µg/mL). The remaining lysate was subjected to immunoprecipitation and the precipitate was analysed by immunoblotting or kinase assays.
Protein-kinase assays. p38 MAP kinase activity was measured using an immune complex kinase assay with a truncated form of ATF-2 as a substrate. The cell lysate was mixed with an anti-p38 MAP kinase anti-serum and 20 μL packed volume of protein-A Sepharose beads. After 2 hrs the beads were washed extensively with solubilization buffer and once with kinase assay buffer (25 mM HEPES pH 7.2, 25 mM magnesium chloride, 2 mM dithiothreitol, 0.5 mM sodium vanadate, and 25 μM ATP). The kinase reaction was initiated by the addition of 20 μL of kinase assay buffer containing 2 μg of ATF-2 and 10 μCi of [γ-32P]ATP, and stopped after 20 min at 30°C by the addition of SDS-PAGE sample buffer.

To determine the effect of the p38 MAP kinase inhibitor SB 203580 on the in vivo kinase activation of p38 or ERK MAP kinases, we investigated the effects of SB 203580 on activation of their respective putative downstream targets MAPKAP kinase-2 and p90rsk. We incubated 10^7 cells/mL with or without 1 μM SB 203580 for 20 min prior to stimulation. To assay in vivo activation of p38 MAP kinase, MAPKAP kinase-2 was precipitated using 5 μg of anti-MAPKAP kinase-2 and 20 μL packed volume of protein-G Sepharose. The kinase assay was performed as described above, except that 5 μg of recombinant murine Hsp25 was used as substrate. To assay in vivo activation of the ERK MAP kinase, p90rsk was immunoprecipitated from aliquots of the same cell lysate using 5 μg of anti-p90rsk antibody bound to 20 μL of packed protein-G Sepharose. The kinase assay was initiated by the addition of 20 μL of kinase assay buffer (20 mM HEPES, 5 mM magnesium chloride, 1 mM EGTA, 5 mM mercaptoethanol, and 2 mM sodium vanadate) containing 3 μg of MBP and 10 μCi of [γ-32P]ATP, and stopped after 10 min at 30°C by the addition of SDS-PAGE sample buffer. In all kinase assays the phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

DNA synthesis - DNA synthesis was assayed by incorporation of 3H-thymidine. Cells were cultured in Terasaki plates at 200 cells per well with indicated doses of growth factors and SB 203580. After 24 or 48 hrs, cells were pulsed with 3H-thymidine for 4 hrs and then frozen to lyse.
the cells. The lysate was harvested onto glass filters and rinsed once with both water and ethanol. The amount of $^3$H-thymidine incorporated into DNA was determined by scintillation counting.

**Methods used in Chapter 4 -**

**Preparation of GST-c-Jun** - The N-terminus of c-Jun (1-169) fused to glutathione S-transferase was expressed in bacteria and purified by affinity chromatography on glutathione Sepharose 4B beads (Pharmacia Biotech Inc., CA) accordingly to the manufacturers instructions. Briefly, a 5 mL culture of *E. coli* strain UT5600 containing the plasmid encoding GST-c-Jun was grown in 2xYT broth containing 100 μg/mL ampicillin overnight. This culture was used to inoculate a 1 L culture of 2xYT broth containing 100 μg/mL ampicillin the next day and incubated at 37°C until the culture reaches an OD 600 of 0.5-0.7. The culture was then placed at 4°C for 30 min followed by the addition of isopropylthiogalactoside to a final concentration of 0.1 mM. The induced culture was then incubated at 26°C for another 4 hrs. The cells were pelleted at 4k rpm for 10 min and the supernatant was discarded. The bacterial pellet was resuspended in 10 mL of resuspension buffer (5 mM Tris, pH 7.5, 150 mM NaCl, 5 mM 2-Me, 10 μg/mL of soybean trypsin inhibitor and 40 μg/mL of PMSF). The bacteria were then treated with 50 μL of 10 mg/mL lysozyme and incubated on ice for 30 min. The remaining bacteria were lysed with successive freeze/thaw cycles between a dry ice/ethanol bath and 37°C waterbath (the lysate was aliquoted into 2-3 tubes of approximately 4 mL each to speed the process). The lysate was then incubated with 50 μL of 1 M MgCl₂ and 50 μL of 10 mg/mL DNAaseI until the solution no longer appeared viscous. To improve the yield of the GST-c-Jun, 1 mL of 10% (v/v) NP-40 was added to the preparation and incubated at 4°C for 30 min. The lysate was then centrifuged at 15k rpm for 30 min. The supernatant was retained and frozen in 1 mL aliquots at -70°C. Aliquots were subsequently thawed and incubated with 250 μL of a 50% slurry of glutathione Sepharose beads in a 15 mL Bio-Rad column at 4°C for 2 hrs. The supernatant was then eluted from the column and rinsed 3 times with ice-cold 1x PBS containing 0.5 % NP-40, 100 mM NaCl and 5 mM 2-Me.
GST-c-Jun was then eluted from the glutathione beads in 5 aliquots (150 μL) of elution buffer (20 mM Glutathione, 150 mM Tris, pH 7.5, and 5 mM 2-Me). The eluates were analyzed by Coomassie Blue staining after SDS-PAGE to determine yield.

**Antibodies.** The antibodies specific for isoforms of JNK1 (sc-474) or JNK2 (sc-827) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-MKK4 specific antibody (9151S) was purchased from New England Biolabs (Beverly, MA).

**Protein kinase assays.** The kinase activity of SAPK was measured using an *in vitro* kinase assay with GST-c-Jun as a substrate. The cell lysate was mixed with GST-c-Jun and 20 μL packed volume of glutathione Sepharose beads. After 2 hrs the beads were washed extensively with solubilization buffer and once with kinase assay buffer (25 mM HEPES pH 7.2, 25 mM magnesium chloride, 2 mM dithiothreitol, 50 mM β-glycerol phosphate and 0.5 mM sodium vanadate). The kinase reaction was initiated by the addition of 20 μL of kinase assay buffer containing 10 μCi of [γ-32P]ATP, and stopped after 20 min at 30°C by the addition of SDS-PAGE sample buffer.

For in-gel kinase assays, the cell lysate was mixed with an antibody specific for either JNK1 or JNK2 and protein A-sepharose beads for 2 hours. The beads were washed extensively in solubilization buffer and the immunoprecipitated proteins were eluted by boiling in SDS-sample buffer. The samples were analysed by SDS-PAGE using a 10% separating gel containing 1 mg of bacterially-expressed GST-c-Jun. The gel was then sequentially washed twice in buffer A (20% isopropanol and 50 mM Tris, pH 8) for 30 min, twice in buffer B (50 mM Tris, pH 8 and 5 mM 2-mercaptoethanol) for 30 min, once in buffer C (6 M guanidine HCl, 50 mM Tris, pH 8 and 5 mM 2-Me) for 1 hour, several times in cold buffer D (0.05% (v/v) Tween-20, 50 mM Tris, pH 8 and 5 mM 2-Me) over 15-18 hours, and once in kinase buffer (40 mM HEPES, pH 7.2, 2 mM DTT, 15 mM magnesium chloride, 1 mM manganese chloride, 0.3 mM vanadate and 0.1 mM EGTA) for 30 min. The kinase assay was initiated by the addition of 20 mL of kinase buffer containing 100 μCi
of [γ-32P]ATP and incubated for 1 hr. The gel was then rinsed with buffer E (5% trichloroacetic acid and 1% sodium pyrophosphate) repeatedly until radioactivity was undetectable by geiger counter. The gel was then dried using a gel dryer and phosphorylated GST-c-Jun was detected using autoradiography.

Methods used in Chapter 5 -

Molecular Cloning of MKK7. An Expressed-Sequence Tag (EST) clone (aa019720) containing the 3’ end (464 bp) of MKK7 was identified by screening the EST database (University of Washington-Merck EST project) with a degenerate oligonucleotide sequence based on a sequence conserved in other MKKs [a.a. F(x)₆F(x)₆CLxK(x)₄R(x)₈H]. We used oligonucleotide primers based on this MKK7 sequence to amplify the 5’ end of the cDNA from a human fetal kidney RACE cDNA library using Vent DNA polymerase for polymerase chain reaction (PCR). PCR products were randomly subcloned into pBSKS and blue-white colony screening, white colonies were selected. All white colonies were transferred onto nitrocellulose and Southern blotted with the EST fragment that had been radiolabelled by random priming. We used this technique to obtain a further 861 bp of MKK7α that contained an in-frame stop codon as well as a splice variant of MKK7 (hMKK7β). The cDNA encoding full length MKK7α was cloned from at least 2 separate PCR reactions and was sequenced on both strands. We used degenerate primers based on the human sequence to amplify the murine cDNA from Ba/F3 hematopoietic cell cDNA. The EST database was screened to identify a third splice variant of murine MKK7 (mMKK7γ). Published databases were screened for MKKs related to MKK7 using the National Center for Biotechnology Information Advanced BLAST search program.

Nucleotide sequence accession numbers. The nucleotide sequences presented in this thesis have been submitted to GenBank under the following accession numbers: hMKK7α - AF013588; hMKK7β - AF013589; mMKK7α - AF022112 and mMKK7γ - AF022113. The MKK from C.
C. elegans (cMKK7) was previously submitted to GenBank as part of the *C. elegans* genome sequencing project (Wilson et al., 1994) and was given the accession number U38377 (gene K08A8.1).

**Preparation of Competent *E. coli*** - A stab culture of *E. coli* (DH5α or UT5600 strains) was incubated in 5 mL of 2xYT or LB broth at 37°C overnight. This culture was used to inoculate 1 L of medium the next morning. The cells were incubated at 37°C until the optical density (OD) 550 reached 0.3 to 0.4 (2 to 3 hrs). The cells were chilled at 4°C for 45 min and then pelleted at 4k rpm for 10 min at 4°C (Sorvall GS3 rotor). The cells were resuspended in cold 100 mM CaCl2 and left on ice for 30 min. The cells were then pelleted as before and resuspended in 10 mL of cold 50 mM CaCl2, and left on ice for 4 to 24 hrs. The cells were dispensed in 250 μL aliquots and were frozen in a dry ice/ethanol bath after the addition of 1 mL of autoclaved 75% glycerol.

**Transformation of *E. coli*** - An aliquot of heat-shock competent *E. coli* was thawed slowly on ice for 30 min. The desired plasmid (1 μL) or ligation mixture (5 μL) was added to 100 μL of competent cells and left on ice for 45 min. The cell/DNA mixture was heat-shocked at 42°C for 1 min and 35 sec and then placed on ice for 2 min. Generally 2xYT broth (1 mL) was added to the heat-shocked cells and the cells were allowed to recover for 30 min at 37°C. The cells were pelleted at 4 k rpm for 30 sec and the supernatant was removed. The cells were resuspended in 200 μL of 2xYT and were plated onto either 2xYT or LB plates containing Ampicillin (40 μg/mL).

**Purification of plasmid DNA** - For the purification of DNA from small cultures (1 mL) of *E. coli* containing plasmids smaller than 10 kbp the TELT method was typically used. The cells were pelleted at 14k rpm for 1 min at room temperature and the supernatant was discarded. The cells were resuspended in 100 μL of TELT buffer (2.5 M LiCl, 50 mM Tris, pH 8.0, 4% (v/v) Triton X-100, 62.5 mM EDTA, pH 8.0) and an equal aliquot of TE (50 mM Tris, pH 8.0, 1 mM EDTA)-saturated Phenol:Chloroform:isoamylalcohol (50:49:1 ratio). This mixture was vortexed
vigorously for 1 min and then centrifuged at 14k rpm for 1 min. The upper phase containing the plasmid DNA was removed and added to 300 μL of 100% ethanol. The DNA was precipitated at -70°C for 10 min and then pelleted at 14k rpm for 10 min at 4°C. The ethanol was removed and the pellet was rinsed with 70% ethanol before the pellet was resuspended in 20 μL of TE containing 10 μg/mL RNAase A.

The STET method was used for plasmids larger than 10 kbp. The cells were pelleted at 14k rpm for 1 min and the supernatant was discarded. The cells were resuspended in 200 μL of STET buffer (8% Sucrose, 20 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% Triton X-100) and boiled for 1 min. The mixture was centrifuged at 14k rpm for 5 min at 4°C and the pellet was removed from the bottom of the tube with a toothpick. A 350 μL aliquot of TE and then Phenol/Chloroform was added to the lysate and mixed vigorously. The mixture was centrifuged at 14k rpm for 5 min at 4°C and the upper phase (550 μL) containing the plasmid DNA was transferred to a new eppendorf tube. An aliquot (350 μL) of chloroform was added to the upper phase and vortexed vigorously. The mixture was centrifuged as before and the upper phase (500 μL) was removed and added to 1 mL of 100% ethanol in a clean eppendorf tube. The DNA was precipitated at -70°C for 10 min and then was pelleted at 14k rpm for 10 min at 4°C. The ethanol was removed and the pellet was rinsed with 70% ethanol before the pellet was resuspended in 20 μL of TE containing 10 μg/mL RNAase A.

For medium scale purifications (100 mL cultures) of plasmid DNA to be used for DNA sequencing, the Qiagen method was used. The bacterial pellet was resuspended in one volume (4 mL for Q100 column; 10 mL for Q500 column) of Buffer P1 (50 mM Tris, pH 8.0, 10 mM EDTA, 100 μg/mL RNAase A). One volume of Buffer P2 (200 mM NaOH, 1% SDS) was added to the cells, mixed gently and the mixture was left at room temperature for 1 min. One volume of chilled Buffer P3 (3 M potassium acetate, pH 5.5) was added to the bacterial lysate and mixed gently. The insoluble material was removed by centrifugation at 4k rpm for 10 min at room temperature. The supernatant was filtered through a cheese cloth onto an equilibrated Q100 or
Q500 column and was eluted by gravity. The resin in the column was rinsed twice with Buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol) and the bound plasmid DNA was then eluted with 5 mL of Buffer QF (1.25 M NaCl, 50 mM Tris, pH 8.5, 15% ethanol). An aliquot (3.5 mL) of isopropyl alcohol was added to the eluant and the DNA was left to precipitate at room temperature for 30 min. The DNA was pelleted at 4k rpm for 10 min at room temperature and the DNA pellet was rinsed with 70% ethanol twice before the pellet was resuspended in 100 µL of TE. The concentration of the DNA was determined by measuring the absorbance at 260 nm (where 1 OD is approximately equal to 50 µg/mL plasmid DNA).

The cesium chloride method was used for large scale purifications (1 L cultures) of plasmid DNA to be used for transient and stable transfections. Briefly, the bacterial pellet was resuspended in 10 mL of Buffer 1 (25 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose, 5 mg/mL lysozyme). The cells were lysed by the addition of 20 mL of Buffer 2 (0.2 N NaOH, 1% SDS) and left on ice for 10 min. The lysate was neutralized by the addition of 15 mL of ice cold Buffer 3 (3 M potassium/ 5 M acetate) and left on ice for 10 min. The insoluble material was removed by centrifugation at 4k rpm for 10 min at room temperature. The supernatant was filtered with a cheese cloth and the DNA was precipitated by the addition of 0.6 times total volume of isopropyl alcohol. After 10 min at room temperature the DNA was pelleted by centrifugation at 4k rpm for 10 min at room temperature. The supernatant was discarded and the pellet was dried upside down. The pellet was resuspended in 8 mL of TE and then mixed with 8.8 g of cesium chloride and 400 µL of ethidium bromide. The solution was transferred to ultracentrifuge tubes and centrifuged at 60k rpm for 4 hrs at 15°C. The DNA was eluted from the tubes and was ultracentrifuged as before. The lower DNA band was removed from the ultracentrifuge tube using a 21 gauge needle into a 50 mL conical tube. The eluant was diluted up to 7.5 mL with TE. The ethidium bromide was repeatedly extracted (5-6 times) with an equal volume of TE-saturated butanol until the TE (lower phase) appeared colourless. The TE containing the plasmid DNA was diluted to 10 mL with TE and then 200 µL of 5 M NaCl and 20 mL of 100% ethanol were added to precipitate the DNA. The DNA was pelleted at 4k rpm for 10 min at room temperature and the DNA pellet was
rinsed with 70% ethanol twice before the pellet was resuspended in 500 μL of TE. The concentration of the DNA was determined by taking an OD 260 as above.

**Restriction analysis of plasmid DNA** - Restriction analysis was used routinely for subcloning of DNA fragments. Typically about 1-2 μg of DNA in a total volume of 17 μL of water was used for each digestion. Then 2 μL of the appropriate 10 x Reaction buffer and 1 μL of the Restriction enzyme were added to the DNA solution. The reaction was generally incubated at 37°C for 1-2 hrs (1 Unit of restriction enzyme cuts 1 μg of DNA in an hr). The reactions were stopped by the addition of 5 μL of 5x Stop buffer (0.13% bromophenol blue, 0.13% xylene cyanol, 15% glycerol). Reaction products were analyzed by separation through an agarose gel by electrophoresis.

**Ligations** - After digesting the vector and desired insert with the appropriate restriction enzyme, the DNA fragments were run out on an agarose gel. The gels were stained with ethidium bromide and visualized with low UV light. The DNA fragments were excised from the gel and the DNA was extracted from the agarose using the Qiagen gel extraction kit. Briefly the gel was solublized in 600 μL of QX1 buffer at 55°C for 10 min. The mixture was then applied to a Qiagen gel extraction column and spun at 14k rpm for 1 min. The eluate was discarded and the column was rinsed with 750 μL of Buffer PE by centrifugation as before. The eluate was discarded and the column was re-centrifuged as before to remove all the remaining wash buffer. The column was then routinely left for 5 min to allow the column to dry before eluting the bound DNA with 25 μL of elution buffer (10 mM Tris, pH 8.5). A sample of the vector and the insert were then routinely analyzed on an agarose gel as before to determine the relative quantities of vector and insert. The ligations were then set up with a 5:1 ratio of insert to vector. The DNA mixture was brought up to a total volume of 8 μL and the reaction was initiated after addition of 1 μL of 10x Ligase buffer by the addition of 1 μL of T4 DNA Ligase. The reaction was left at 16°C for either 1 hr (sticky
overhang ligation) or 4 hrs (blunt ended ligation). The ligation was then transformed into competent bacteria by heat-shock as described above.

**Blue/White colony selection** - PCR products or other blunt ended DNA fragments were typically sub-cloned into the multiple cloning region of the pBluescript phagemid (pBSKS). The multiple cloning region of pBSKS is within the coding region of the α-subunit of β-galactosidase and ligation of an insert into pBSKS will disrupt the reading frame of this enzyme. All transformed *E. coli* strain DH5α were plated onto 2xYT plates containing Amp and coated with 50 μL of 2% (w/v) Bluo-Gal (a compound related to galactose and conjugated to a blue chromophore) and 10 μL of 200 mg/mL Isopropyl-thiogalactoside (IPTG). The DH5α strain of *E. coli* only lack the α-subunit of β-galactosidase and cannot cleave galactose unless transformed with pBSKS encoding an intact α-subunit (α-complementation). β-galactosidase cleaves Bluo-gal to release the normally colorless chromophore and as a result the *E. coli* colonies that lack insert turn blue. Colonies failing to change color were mini-prepped and analyzed for the presence of the proper insert.

**Oligonucleotide primers** - Many oligonucleotide primers were used for the cloning and sequencing of MKK7. The sequences of the oligonucleotides involved in the RACE-PCR, RT-PCR and epitope tagging are detailed below:

**IF111:** (anti-sense): 5'- GAT GTC ATA GTC CGG CTT -3'

**IF113:** (anti-sense): 5'- TCA CGG CCG GTC TTC GCC ATG ACA TCC T -3'

**IF115:** (anti-sense): 5'- TGA GCT CCA TGG CGA TGA AGA -3'

**IF117:** (anti-sense): 5'- TGC CCA TGA GCT CCA T -3'

**IF122:** (sense): 5'- ATC GGA TCC CAG CGC TAC CAG GCA GA -3'

**IF129:** (anti-sense): 5'- TAC TCT AGA TCA GTC TTC GCC ATG ACA TC -3'

**IF134:** (sense): 5'- CAT GGA TCC GGG AAA ATG GCG GCG T -3'
API (sense): 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC -3'
AP2 (sense): 5'-ACT CAC TAT AGG GCT CGA GCG GC -3'

The underlined nucleotides represent the presence of a Restriction site introduced into the sequence to facilitate sub-cloning.

cDNA constructs. Human MKK7 was amplified by PCR using oligonucleotides IF134 and IF129 to allow subcloning into the BamHI and XbaI sites in the pEFBOS-Nmyc3 vector to produce pEFBOS-Nmyc3-MKK7. Oligonucleotide IF134 introduces an in-frame BamHI site at the 5'-end and oligonucleotide IF129 introduces a XbaI site at the 3'-end of the cDNA encoding MKK7. Similarly human MKK7 was amplified by PCR using oligonucleotides IF134 and IF113 to allow subcloning into the BamHI and EagI sites in the pEBG vector to produce pEBG-MKK7. Oligonucleotide IF113 introduces an EagI site in the cDNA of MKK7, and a partial EagI digest of the PCR product was required for subcloning into pEBG as the cDNA encoding MKK7 contains an EagI site within its open reading frame. The pEBG vector was produced by digesting pEBG-SEK1 with BamHI to release the cDNA encoding SEK1 followed by ligating the empty vector together. The vectors encoding pEBG-JNK1 and pEBG-SEK1 were the gifts of Dr. L. Zon. The mammalian expression vector encoding Flag-tagged CSBP2/p38 MAPK was provided by Dr. Peter Young. The vectors encoding constitutively active RasK61, RacV12 and Cdc42V12 were received from Dr. R. Kay, Dr. F. McCormick and Dr. R. Cerione respectively.

Polymerase Chain Reaction (PCR) - PCR was used extensively for the cloning of MKK7 from a RACE-cDNA library, to introduce restriction sites to facilitate sub-cloning and to analyze expression of mRNA encoding MKK7 from several cells and cell lines. A PCR reaction mixture included 1x ThermoPol Buffer, 200-250 μM of dNTP, 75 μM of each the sense and anti-sense oligonucleotides, and the DNA template in a 50 μL reaction volume. All PCR reactions were heated to 94°C for 5 min in a Perkin Elmer DNA Thermal Cycler and initiated by the addition of 1
μL of Vent DNA Polymerase. Reactions were carried out either for thirty cycles if cloning from cDNA derived from total RNA or ten cycles if amplifying the PCR products from plasmid DNA. One cycle involved heating the reaction up to 94°C for 45 seconds, cooling the reaction to 50-55°C for 45 seconds to allow the oligonucleotides to anneal, and then heating the reaction to 72°C for 1 min to allow elongation of the PCR products. All PCR products were visualized after separation on agarose gels by staining with ethidium bromide.

**Southern blot analysis** - Southern blotting was used to screen white colonies derived from 5'-RACE PCR products for the presence of an insert encoding MKK7. White colonies picked and streaked onto 2xYT plates. The colonies were allowed to grow for several hours and then were replica plated onto nitrocellulose. The nitrocellulose was soaked for 15 min in denaturation buffer (0.5 M NaOH, 1.5 M NaCl), dried and then soaked for 15 min in neutralization buffer (0.5 M Tris, pH 7, 1.5 M NaCl). The nitrocellulose was then baked at 80°C for 1 hr. The nitrocellulose was then soaked in wash buffer (0.1% SDS and 2x SSC) and the remnants of the bacterial colonies were scraped off the nitrocellulose. The nitrocellulose was then blocked in pre-hybridization buffer (33% deionized formamide, 40 mM sodium phosphate, pH 6, 4x SSC (20x Stock: 3 M NaCl, 0.3 M Sodium citrate, pH 7.0), 4 x Denhardt’s solution (50x Stock: 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), 0.8% glycine and 125 μg/mL herring sperm DNA). The membrane was left to block for 20 min and then placed in hybridization buffer (33% deionized formamide, 25 mM sodium phosphate, pH 6, 4x SSC, 10% Dextran Sulphate, 100 μg/mL herring sperm DNA) with radioactive probe at around 1 million cpm/mL for 1 hr to over-night in a 42°C hybridization oven. The probe was removed and the nitrocellulose was rinsed 3 times in wash buffer (0.1% SDS, 2x SSC) at 42°C. The temperature was raised slowly from 42°C to 60°C in 3 degree increments. The radioactivity present on the membrane was analyzed at each increment and washing continued until radioactivity was no longer detectable on the membrane using a Geiger counter (typically around 55°C). The membrane was then wrapped in saran wrap and exposed to autoradiographic film at -70°C in a film cassette with intensifying screens.
Probes were routinely prepared as follows using approximately 200 ng of DNA for each Klenow reaction. The DNA was brought up to 27 μL in water and then boiled for ten min. The DNA was then immediately placed onto ice followed by the addition of 15-18 μL of oligo mix lacking dCTP (10 μL of 1 M HEPES, pH 6.6, 0.5 μL of 1 M MgCl2, 0.5 μL of 0.1 M DTT, 2 μL of 2.5 mM dNTP, 3 μL of 5 mg/mL random hexamers and 2 μL of 10 mg/mL BSA), 2 μL of Klenow DNA Polymerase (10-20 Units) and 5 μL of 32P-dCTP (50 μCi). The reaction was incubated for 1-4 hours at room temperature before stopping by addition of 130 μL of 1x SET (10 mM Tris, pH 7.5, 5 mM EDTA and 1% SDS) and 20 μL of 0.25% bromophenol blue. The radiolabelled probe was separated from unincorporated radioactivity over a Sephadex G-50 column and the eluate was boiled for 5 min before addition to the hybridization solution at the desired concentration.

**Northern Blot Analysis** - A Northern blot of mRNA from multiple human tissues was purchased from ClonTech Biotechnology (San Diego, CA). The blot was hybridized with a probe from the 5' end of MKK7 [1-660 bp] that was radiolabelled by random priming as above. The probe was derived from pEFBOS-Nmyc3-MKK7 by restriction digestion with BamHI and NcoI. Hybridization and washing were performed according to manufacturer's specifications and the mRNA encoding MKK7 was visualized using autoradiography.

**Preparation of GST-JNK1** - The prokaryotic expression vector for GST-JNK1 was produced from pEBG-JNK1 by restriction digestion with BamHI and NotI followed by subcloning the cDNA encoding JNK1 into the in-frame BamHI and NotI sites in the polylinker of pGEX4T-3. Both GST-JNK1 and GST-c-Jun were expressed in *E. coli* strain UT5600 and were purified by affinity chromatography on glutathione Sepharose beads as described in Chapter 4.

**Purification of total RNA from mammalian cells** - Typically 1 x 10^6 cells were centrifuged at 14k rpm for 1 min and the supernatant was discarded. For adherent cells a 6 cm
dish at 50% confluency was used. The cells were then resuspended in 1 mL of Trizol (Gibco BRL, Grand Island, NY). If the Trizol appeared cloudy then 500 μL of the mixture was transferred to a new eppendorf and another 500 μL of Trizol was added. The mixture was diluted with 200 μL of chloroform and vortexed for 1 min. The samples were centrifuged at 14k rpm for 10 min at 4°C. The top aqueous phase (350 μL) was transferred to a new eppendorf tube and the RNA was precipitated with 500 μL of isopropyl alcohol. This was generally left at -20°C for one hr to overnight. The RNA was then pelleted by centrifugation for 10 min at 14k rpm and 4°C. The pellet was rinsed once with 70% ethanol and resuspended in 25 μL of distilled water. The concentration of the RNA was determined by taking an absorbance at 260 nm (where 1 OD is approximately equal to 40 μg/mL of total RNA).

Reverse transcription - To investigate the expression of mRNA encoding MKK7, total RNA from various cell lines was reverse transcribed using an anti-sense oligonucleotide present in both human and murine MKK7 for subsequent RT-PCR (Table 5.4). Briefly, 1.5 μg of total RNA was primed with the oligonucleotide IF113 at a final concentration of 20 pmol/μL in a 10 μL reaction volume. The mixture was heated to 70°C for 10 min and chilled on ice to allow the oligonucleotides to anneal. The following reagents were added to the RT reaction (4 μL of First strand synthesis buffer (250 mM Tris-HCl, pH 8.3, 375 mM potassium chloride, and 15 mM magnesium chloride), 2 μL of 0.1 M dithiothreitol, 1 μL containing dTTP, dATP, dCTP and dGTP at 10 mM, and 1 μL of Moloney Murine Leukemia Virus-Reverse Transcriptase) and the resulting mixture was incubated at 37°C for 1 hour.

Conditions for Transient Transfections in adherent cells - HeLa cells were transiently transfected using SuperFect reagent (Qiagen, CA) according to manufacturer’s recommendations. Briefly, HeLa cells were plated in a 100 mm dish, such that the cells would be at 50-80% confluency the next day. The desired combination of DNA (10 μg total) was combined and brought up to 300 μL in serum-free, antibiotic-free medium DMEM. The DNA solution was then
mixed with 60 µL of SuperFect Transfection Reagent and incubated for 5 to 10 min at room temperature to allow complexes to form. During this time the cells were rinsed once with 1x PBS. Then 3 mL of DMEM containing 10% FCS and antibiotics was combined with the DNA mixture, and immediately transferred to the cells. The cells were incubated with the DNA complexes for 2 hrs at 37°C before the supernatant was replaced with normal growth medium. Often the cells from a single transfection were split into several smaller dishes and used to analyze over-expressed proteins with several different stimuli. The cells were harvested 20-24 hrs post-transfection.

Conditions for Transient Transfections in non-adherent cell lines - Transient transfection of Ba/F3 cells was routinely performed to analyze the activation of exogenously expressed MKK7 by hematopoietic growth factors. Ba/F3 cells (1-1.5 x 10^7/sample) were transiently transfected with 15-25 µg of DNA using electroporation (300 V, 960 µF) in 400 µL of serum-free medium containing 10 µg/mL of DEAE dextran (Pharmacia Biotech Inc., CA). After electroporation, the cells were placed in 20 mL of room temperature RPMI 1640 containing 2% IL-3 conditioned medium and incubated for 20 min at room temperature before returing the cells to the 37°C incubator. The cells were harvested 20-24 hrs post-transfection.

Conditions for Stable Transfections in non-adherent cell lines - Ba/F3 clones that stably expressed MKK7 were generated by co-electroporation of a vector encoding myc-tagged MKK7 (pEFBOS-mycMKK7) and a vector encoding a puromycin resistance gene (pGK-puro). Cells were plated in 96 well microtiter plates at 1.5 x 10^4 cells/well and selected in 1 µg/mL puromycin (Calbiochem, CA). After two weeks, we observed 90 wells with drug-resistant clones. We selected 3 wells at random and recloned cells by limit dilution. Clones arising from each of these wells expressed readily detectable levels of myc-tagged MKK7 as determined by immunoblotting lysates with an anti-Myc monoclonal antibody 9E10 (Santa Cruz, CA).

Antigen preparation for immunization of rabbits - Peptides corresponding to the N-
terminus and the C-terminus of MKK7 were synthesized as C-terminal fusions with the T-cell epitope from Tetanus Toxin (Valmori et al., 1992). The sequence of the N-terminal peptide was QYIKANSKFIGITELKKSSLEQKLSRLAEKLKQENREARR and the C-terminal peptide was QYIKANSKFIGITELKKKDVMAKTESPRSTGVLSQP. The New Zealand White rabbits were initially injected with 250 µg of peptide in complete Freund's Adjuvant. The second injection (167 µg peptide) and all subsequent injections (83 µg peptide) were prepared with incomplete Freund's adjuvant. The appropriate dose of antigen was dissolved in 1 mL of PBS and added to 2 mL of Freund's adjuvant. The adjuvant was emulsified through 18-gauge needles on glass syringes and left at 4°C overnight. The rabbits were injected intramuscularly for the initial injection and subcutaneous for all subsequent injections. The rabbits received two injections before the first ear bleed and then the rabbits were either injected or bled every second week. The N-terminal peptide or the C-terminal peptide was injected into rabbits IF3/IF4 or IF5/IF6 respectively.

**ELISA Protocol** - The antigen (Ag) was dissolved at 10 µg/ml in 0.5% sodium azide in PBS and was stored at 4°C. Antigen was coated onto non-sterile 96 well microtitre plates at 50 µL of Ag to each well of the plate using a multichannel pipetter. All samples were analyzed in triplicate ie. coating 3 lanes per sample. The ELISA trays were stored in a tupperware container with damp paper towels covering the bottom of the container. The plates were incubated at room temperature from 2 to 24 hrs to ensure efficient binding of the antigen to the plate. The Ag was removed from the wells, and the wells were blocked with 0.5% skim milk powder dissolved in 1x PBS (about 1 L per plate to be washed). Briefly the milk solution was added to the tupperware container until it covered the ELISA plate (ensuring all the wells are filled). The milk solution was removed and the plate was dried on a paper towel. The block and dry steps were repeated two more times and the ELISA plate was air dried upside down on a clean paper towel. The antibody samples were typical diluted 1:100 for first bleeds and 1:200 for all subsequent bleeds. The antiserum was diluted in 0.5% milk in PBS and antibodies were incubated in the wells for 1 to 24 hrs in a tupperware container. Generally the antibodies were diluted in 1:2 steps. Every column of the plate contained
50 μL of 0.5% milk except the first column to which 100 μL of the appropriate dilution of antiserum in 0.5% milk in PBS was added. A Socorex pipettor was used to transfer 50 μL from the first well to the second well, from the second well to the third well, etc. leaving the last column as a control containing 0.5% milk alone. The primary antibody was removed from the wells and the plate was rinsed three times in 0.5% milk in 1x PBS as before. A secondary Horseradish peroxidase (POD)-coupled Goat anti-Rabbit antibody (DAKO) was used for detection of primary antibody bound to the ELISA plate. The POD antibody was diluted 1:2000 in 0.5% milk in PBS and 50 μl was added to each well. The secondary antibody was incubated for 1 to 2 hours at room temperature in the tupperware container. The secondary antibody was removed from the wells and the plate was rinsed three times in 0.5% milk in PBS followed by three washes with distilled water (dH2O). The citrate buffer was prepared by dissolving 1.29 grams of citric acid and 1.37 grams of Na2PO4•2H2O into 100 mL of dH2O. The substrate solution (1 mg/mL ABTS, 0.006% hydrogen peroxide in citrate buffer) was freshly prepared before use. The substrate solution (50 μL) was added to each well and the plate was incubated for 30 min at room temperature. The absorbance of each well was determined with an ELISA plate reader using dual mode between OD 405 and OD 490.

Antibodies. The anti-MKK4 polyclonal antibody (sc-964) used for immunoprecipitation and kinase assays was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The M2 (α-Flag) monoclonal antibody was obtained from Intersciences (Markham, ON). Rabbit polyclonal antibodies recognizing MKK7 were raised against peptides corresponding to the N-terminus (a.a. 4-26) and the C-terminus (a.a. 394-413) of MKK7. Anti-MKK7 antibodies were purified from serum of rabbits IF4 and IF6 using protein A-Sepharose. Immunoblotting procedures were performed as described previously (Duronio et al., 1992; Welham et al., 1994; Welham and Schrader, 1992).

Cell Culture Conditions. The factor-dependent hematopoietic cell lines MC/9 and Ba/F3 were
passaged in RPMI 1640 supplemented with 2% WEHI-3B conditioned medium. HeLa cells were
grown in DMEM supplemented with 10% FCS and 10 μM 2-mercaptoethanol. The B-cell
lymphoma cell line WEHI-231 was cultured in RPMI 1640 supplemented with 10% FCS and 10
μM 2-mercaptoethanol.

**Stimulation and Immunoprecipitation Conditions.** Prior to stimulation, Ba/F3 or MC/9
cells were cultured in 0.2% WEHI-3B conditioned medium for 16 to 20 hrs. Cells were washed
and equilibrated in serum-free RPMI 1640 buffered with 10 mM HEPES (N-[2-
hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH 7.2 prior to stimulation. Cells were
stimulated with 0.2 M NaCl, 50 μg/mL anisomycin, 100 ng/mL TNF-α, 100 ng/mL EGF, or
optimal doses of synthetic IL-3 or synthetic IL-4 (10 μg/mL) for the indicated times. For FcR
cross-linking, MC/9 mast cells were initially incubated with anti-FcR for 10 min and then were
incubated for a further 10 min either untreated or with the addition of anti-Ig to cross-link the anti-
FcR antibody. For UV irradiation, HeLa and Ba/F3 cells were exposed for 20 min in 60 mm
dishes containing 1 mL of serum-free medium from a 30 W UV light. HeLa cells were heat
shocked by incubating for 20 min at 42°C. After stimulation, cells were lysed in solubilization
buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 200 mM LiCl, 5 mM EDTA, 1% (v/v) Nonidet P-40,
1 mM sodium molybdate, 50 mM sodium orthovanadate, 1 mM sodium fluoride, 50 mM β-
glycerol phosphate, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 0.7 μg/mL pepstatin,
2 μg/mL leupeptin, and 40 μg/mL phenylmethylsulfonyl fluoride). Samples for subsequent assays
were normalized for total protein using a Pierce protein assay or for expression of transfected
proteins by assaying co-transfected β-galactosidase activity. Proteins tagged with GST were
affinity precipitated by incubating cellular extracts with 20 μL of a 50% slurry of glutathione-
Sepharose beads for 1 hr at 4°C. Endogenous MKK7 was immunoprecipitated using the rabbit
polyclonal antibody raised against the N-terminus of MKK7 and endogenous MKK4 was
immunoprecipitated with an α-MKK4 antibody. Flag-tagged p38 MAPK was immunoprecipitated
using the M2 antibody and protein G-Sepharose.
MKK7 Kinase Assays. Beads with associated kinases were washed extensively with solubilization buffer and once with kinase assay buffer (25 mM HEPES pH 7.2, 25 mM magnesium chloride, 2 mM dithiothreitol, 50 mM β-glycerol phosphate and 0.5 mM sodium vanadate). MKK7 activity was measured directly by the ability of MKK7 to phosphorylate 1 μg of GST-JNK1 in the presence of an excess of free GST protein to prevent the substrate from binding to the beads. MKK7 activity was also assessed indirectly by first incubating MKK7 with 1 μg of GST-JNK1 in the presence of 50 μM unlabelled ATP for 30 min and then removing an aliquot and determining the activity of GST-JNK1 in an in vitro kinase reaction using 1 μg of GST-c-Jun as substrate. All kinase reactions were initiated by the addition of kinase assay buffer containing the appropriate substrate and 10 μCi of [γ-32P]ATP, and stopped after 20 min by the addition of SDS-sample buffer. For p38 MAP kinase assays, Flag-tagged p38 MAPK was immunoprecipitated using α-Flag monoclonal antibodies and protein-G Sepharose beads. The beads were washed and kinase activity was assessed as described above. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography. Densitometry was performed using an HSI scanning densitometer.
CHAPTER 3 - Activation of p38 MAPK in hematopoietic cells.

3.1 Introduction

Our interest in p38 MAPK began with the work of Dr. Melanie Welham and her studies on tyrosine phosphoproteins induced by interleukin-3 and Steel locus factor in primary bone marrow mast cells. Using 2-dimensional gel electrophoresis and the monoclonal antibody 4G10, she observed phosphoproteins unique to each stimulus, and several phosphoproteins induced by both growth factors (Fig. 3.1). Phosphoproteins unique to each cytokine were observed at the predicted electrophoretic mobility of their respective receptors. Interestingly both stimuli induced phosphoproteins at 70 kDa (p70), 55 kDa (p55), 46 kDa (p46), 44 kDa (p44), 42 kDa (p42) and 38 kDa (p38). Subsequent work identified p70 as the tyrosine phosphatases SHP1 and SHP2, p55 and p46 as different isoforms of Shc, and p44 and p42 as the MAP kinases, ERK1 and ERK2. However the tyrosine phosphorylated p38 kDa protein remained to be identified.

Several independent groups identified murine p38, a new MAPK family member, as the mammalian homolog of HOG1 of S. cerevisiae. Dr. Peter Young and Dr. John Lee at Smith-Kline Beecham also identified CSBP2, the human p38 MAPK, as the target for a pyridinyl imidazole compound that inhibits IL-1 and TNFα production. They demonstrated that p38 MAPK ran at the same electrophoretic mobility and isoelectric point as the p38 phosphoprotein observed by Dr. Welham.

Hypothesis:

(1) The tyrosine phosphoprotein p38 was p38 MAPK, and that its activity would be regulated by both IL-3 and SLF in normal bone marrow mast cells.

(2) The activity of p38 MAPK is required to activate MAPKAP kinase-2, an enzyme previously demonstrated to be activated by IL-3 and GM-CSF.

(3) The activity of p38 MAPK is required for some biological functions of these cytokines.
Figure 3.1 - Analysis of phosphoproteins induced by IL-3 and SLF using 2D SDS-PAGE. Murine bone marrow-derived mast cells were lysed after being treated with SLF or IL-3 for the indicated times. Whole cell extracts were analyzed using 2 dimensional SDS-PAGE, and the proteins were immunoblotted with an anti-phosphotyrosine antibody 4G10. The position of the phosphoprotein putatively corresponding to p38 MAPK is indicated by arrowheads. This figure was adapted from Welham et al., 1992.
Objectives:

(1) We initiated a collaboration with Dr. Young to determine if p38 MAPK was indeed the same p38 phosphoprotein. Dr. Young provided us with antisera that recognized full-length p38 MAPK, that would be used to immunoprecipitate p38 and determine if it was tyrosine phosphorylated and activated by IL-3 and GM-CSF.

(2) Dr. Young also provided us with the specific inhibitor of p38 MAPK, SB 203580. We would use this inhibitor to determine if MAPKAP kinase-2 was indeed a substrate of p38 MAPK or the ERK MAPK, which were previously identified as activators of MAPKAP kinase-2.

(3) Hematopoietic growth factors are required for the proliferation, differentiation and the prevention of apoptosis in hematopoietic cells. We would test the role of p38 MAPK in these biological functions using the p38 MAPK inhibitor to acutely deprive a cell of p38 MAPK activity.

3.2 Results

3.2.1 Identification of p38 MAPK - an unknown phosphoprotein. To determine whether p38 MAP kinase was involved in responses to IL-3 or SLF, we stimulated primary bone-marrow derived mast cells with these factors, immunoprecipitated p38 MAP kinase and assessed its tyrosine phosphorylation by immunoblotting with the anti-phosphotyrosine specific antibody 4G10. As expected, both SLF and IL-3 induced tyrosine phosphorylation of p38 MAP kinase (Fig. 3.2).

3.2.2 Activation of p38 MAPK by IL-3, GM-CSF or SLF but not IL-4 or IL-13. To demonstrate that the tyrosine phosphorylation of p38 MAP kinase in cells treated with hematopoietic growth factors correlated with increased kinase activity, we immunoprecipitated p38 MAP kinase from either untreated MC/9 cells, or cells treated with saturating doses of GM-CSF, SLF or 0.2 M NaCl. We then assessed the activity of p38 MAP kinase in an in vitro kinase assay using a truncated form of ATF-2 as substrate (Fig. 3.3). p38 MAP kinase immunoprecipitated
Figure 3.2 - IL-3 or SLF, but not IL-4, induce tyrosine phosphorylation of p38 MAPK in bone marrow-derived mast cells. Cells were incubated at 37 °C in RPMI 1640 for 1 hr and then left untreated (CON), or treated with saturating doses of IL-3 (IL-3), IL-4 (IL-4), or SLF (SLF) for the indicated times. Immunoprecipitated p38 MAP kinase was analyzed after SDS-PAGE by immunoblotting (IB), initially with the anti-phosphotyrosine antibody 4G10 (4G10), and subsequently with an anti-p38 MAP kinase antibody (anti-p38) to quantitate loading. The position of p38 MAPK is indicated by an arrow.
Figure 3.3 - Activation of p38 MAPK by hematopoietic growth factors in MC/9 cells. Cells were incubated in RPMI 1640 at 37 °C for 1 hr. Equal numbers of cells were then left untreated (CON), or stimulated with saturating doses of IL-4 (IL-4), murine GM-CSF (GM) or SLF (SLF) or 0.2 M NaCl (NaCl) for the indicated times. The cells were lysed and p38 MAP kinase was immunoprecipitated with anti-p38 MAP kinase anti-serum. The relative kinase activity was assessed by an immune complex kinase assay using a truncated form of ATF-2 as substrate. SB 203580 (10 µM) was included in one sample (SB) to inhibit the in vitro kinase activity of p38 MAP kinase. The reaction products were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylation of ATF-2 was assessed by autoradiography (Top). The membrane was immunoblotted (IB) initially with the anti-phosphotyrosine antibody 4G10 (4G10) to assess tyrosine phosphorylation of p38 MAP kinase (Middle) and with an anti-p38 MAP kinase antibody (anti-p38) to assess loading (Bottom).
from cells stimulated with GM-CSF or SLF exhibited increased levels of kinase activity, which correlated with the levels of tyrosine phosphorylation of the enzyme. The activity of p38 MAP kinase was completely abolished by the inclusion of SB 203580 during the *in vitro* assay (Fig. 3.3). To examine the kinetics of SLF and GM-CSF induced activation of p38 MAP kinase in MC/9 cells, we used an antibody specific for the activated form of p38 MAP kinase. This antibody recognizes p38 MAP kinase when phosphorylated on the tyrosine of the TGY activation motif. The activation of p38 MAP kinase is dependent on dual phosphorylation on both threonine and tyrosine residues as previous demonstrated (Doza et al., 1995; Raingeaud et al., 1995). Treatment with growth factors resulted in a rapid increase in the levels of tyrosine phosphorylation of p38 MAP kinase (Fig. 3.4). Cells treated with SLF exhibited detectable phosphorylation of p38 MAP kinase as early as 2 minutes and maximal phosphorylation of p38 MAP kinase between 5 and 10 minutes. Cells treated with GM-CSF had maximal phosphorylation of p38 MAP kinase at around 10 minutes. In both instances the phosphorylation of p38 MAP kinase was transient and returned to almost basal levels by 30 minutes.

It was of particular interest to investigate the effect of IL-4 as we had previously shown that it failed to activate ERK MAP kinases (Welham et al., 1992). IL-4 failed to induce tyrosine phosphorylation of p38 MAP kinase in primary mast cells (Fig. 3.2), in MC/9 (Fig. 3.3) or in FD-5/13R (Fig. 3.5). As predicted from these results, IL-4 also failed to stimulate enzymatic activity of p38 MAP kinase (Fig. 3.3). Interestingly, treatment with IL-4 resulted in a small but reproducible reduction in both the levels of tyrosine phosphorylation and the enzymatic activity of p38 MAP kinase (Fig. 3.3) compared with untreated cells. The related cytokine IL-13 also failed to induce tyrosine phosphorylation of p38 MAPK (Fig. 3.5). Thus, both IL-4 and IL-13 fail to activate either p38 MAPK or ERK MAPK (Welham et al., 1995).

### 3.2.3 Activation of p38 MAPK by CSF-1 in FD-MACII cells

To test the effects of CSF-1 or GM-CSF on the activity of p38 MAP kinase, we used murine factor-dependent cell lines that respond to these factors (Welham et al., 1992; Welham et al., 1994). Stimulation of the
Figure 3.4 - Kinetics of tyrosine phosphorylation of p38 MAP kinase by GM-CSF or SLF. After 1 hr in RPMI 1640 at 37 °C, MC/9 cells were left untreated, or were stimulated with saturating doses of GM-CSF or SLF for the indicated times. The cells were lysed and the NP-40 soluble fraction was analysed by SDS-PAGE followed by immunoblotting using an antibody specific for activated p38 MAP kinase. Levels of activation of p38 MAP kinase as detected by this antibody were quantitated using densitometry.
Figure 3.5 - IL-13 fails to activate p38 MAP Kinase in FD5/IL13R cells. Cells were incubated in RPMI 1640 at 37 °C for 1 hr. Equal numbers of cells were then left untreated (CON), or stimulated with saturating doses of IL-4 (IL-4), IL-13 (IL-13) or 0.2 M NaCl (NaCl) for 10 min. The cells were lysed and the cellular lysate was resolved by SDS-PAGE and transferred onto nitrocellulose. The membrane was immunoblotted (IB) initially with the anti-phospho-p38 antibody (anti-phospho-p38) to assess tyrosine phosphorylation of p38 MAP kinase (right). The membrane was also immunoblotted with 4G10 to demonstrate the cells responded to IL-4 and IL-13 (left).
macrophage-like FD-MACII cells with CSF-1 resulted in rapid tyrosine phosphorylation of p38 MAP kinase (Fig. 3.6). This effect was seen following treatment with either recombinant murine CSF-1 or a source of natural murine CSF-1, L-cell conditioned medium. The tyrosine phosphorylation of p38 MAP kinase was abrogated by the presence of a monoclonal antibody that neutralizes CSF-1 activity (Fig. 3.6), demonstrating that the induced phosphorylation of p38 MAP kinase was due to CSF-1 and not, for example, to contamination by endotoxin or to osmotic stress.

3.2.4 Activation of p38 MAPK by cross-linking of the Fc Receptor for Immunoglobulin G. Besides hematopoietic growth factors, MC/9 mast cells also respond to ligation of the Fc receptor for Immunoglobulin G (FcR). We used two approaches to determine if p38 MAPK was tyrosine phosphorylated after cross-linking of the FcR. The first involved the use of antigen•antibody complexes, and the second involved cross-linking an anti-FcR antibody bound to FcR on the cell-surface with a secondary antibody against α-FcR (α-Ig). We found that treating MC/9 cells with either KLH (Ag) or an antibody to KLH (Ab) failed to induce tyrosine phosphorylation of p38 MAPK. However, tyrosine phosphorylation of p38 MAPK was observed after the cells were treated with Ag•Ab complexes (Fig. 3.7). Signalling through the FcγRII and FcγRIII can be specifically prevented with 2.4G2 (α-FcR), a monoclonal antibody that binds to these FcR without signalling (Unkeless, 1979). Pretreatment of the MC/9 cells with α-FcR blocked the ability of Ag•Ab complexes to tyrosine phosphorylate p38 MAPK, indicating the complexes were specifically signalling through the FcR to activate p38 MAPK. Importantly, cells treated with either α-FcR or α-Ig alone failed to induce tyrosine phosphorylation of p38 MAPK. However, cells pretreated with α-FcR followed by treatment with α-Ig to cross-link α-FcR bound to the cell surface also induced the tyrosine phosphorylation of p38 MAPK (Fig. 3.7).

3.2.5 Hematopoietic growth factors activate MAPKAP kinase-2. MAPKAP kinase-2 has been reported to be a substrate of p38 MAP kinase (Freshney et al., 1994; Raingeaud et al.,
Figure 3.6 - Colony Stimulating Factor (CSF)-1 induces tyrosine phosphorylation of p38 MAPK in FDMACII cells. Cells were incubated in RPMI 1640 for 1 hr prior to stimulation, and then treated with CSF-1 (CSF-1), L-cell conditioned medium (LCCM) as a source of natural CSF-1, a neutralizing antibody (5A1) against CSF-1 (α-CSF-1), or with indicated combinations of these for 5 min. Immunoprecipitated p38 MAP kinase was analyzed after SDS-PAGE by immunoblotting (IB) with the anti-phosphotyrosine antibody 4G10 (4G10), and subsequently with an anti-p38 MAP kinase antibody (anti-p38) to quantify loading.
Figure 3.7 - Cross-linking of the FcR for IgG induces tyrosine phosphorylation of p38 MAPK in MC/9 mast cells. MC/9 mast cells were pre-treated with α-FcR (α-FcR) for 10 min as indicated, and then incubated for 10 min in medium alone, or with a rabbit α-rat immunoglobulin antibody (α-Ig). MC/9 cells were also pre-treated for 10 min with an anti-keyhole limpet hemocyanin (KLH) antibody (Ab), and then incubated with or without KLH (Ag) for 10 min as indicated. Cellular lysates were immunoprecipitated with antibodies recognizing p38 MAPK. Immunoprecipitated p38 MAP kinase was analyzed after SDS-PAGE by immunoblotting (IB) with the anti-phosphotyrosine antibody 4G10 (4G10), and subsequently with an anti-p38 MAP kinase antibody (anti-p38) to quantitate loading.
1995), and to be activated in cells stimulated with GM-CSF or IL-3 (Ahlers et al., 1994). To determine whether the activation of MAPKAP kinase-2 by hematopoietic growth factors was due to activation of p38 MAP kinase, we immunoprecipitated MAPKAP kinase-2 from MC/9 cells that had been stimulated with IL-4, GM-CSF, SLF or 0.2 M NaCl and assessed its activity in an in vitro kinase assay using recombinant murine Hsp25 as substrate. As shown in the previous report (Ahlers et al., 1994), treatment with GM-CSF resulted in activation of MAPKAP kinase-2 (Fig. 3.8). SLF also induced strong activation of MAPKAP kinase-2 (Fig. 3.8). However, consistent with our finding that IL-4 failed to stimulate the enzymatic activity of p38 MAP kinase, IL-4 failed to induce activation of MAPKAP kinase-2 (Fig. 3.8). Indeed, treatment with IL-4 reduced the activity of MAPKAP kinase-2 to below the levels seen in untreated cells (Fig. 3.8), consistent with the reduction in p38 MAP kinase activity seen in IL-4 treated cells (Fig. 3.3). IL-4 was active on these cells as demonstrated by the induced tyrosine phosphorylation of a protein known to be IRS-2, or p170 (Fig. 3.8) (Welham et al., 1997). Thus the ability of hematopoietic growth factors to activate MAPKAP kinase-2 correlated with their ability to activate p38 MAP kinase.

3.2.6 GM-CSF or SLF activate MAPKAP kinase-2 via p38 MAPK. MAPKAP kinase-2 has been reported to be activated by members of the ERK MAP kinase family (Stokoe et al., 1992). GM-CSF, IL-3 and SLF induce activation of both ERK (Welham et al., 1992) and p38 MAP kinases (Fig. 3.2 and 3.3). To investigate which of these kinases was responsible for the activation of MAPKAP kinase-2 by these hematopoietic growth factors, we used SB 203580, a specific inhibitor of p38 MAP kinase activity (Cuenda et al., 1995; Lee et al., 1994). Pre-treatment of cells for 20 minutes with 1 μM SB 203580 abrogated the ability of IL-3 (Fig. 3.9), GM-CSF (Fig. 3.10) or SLF (Fig. 3.11) to induce activation of MAPKAP kinase-2. In in vitro experiments, the enzymatic activity of ERK-2 was not inhibited by concentrations of SB 203580 that abolished the enzymatic activity of p38 MAP kinase (Cuenda et al., 1995). To confirm that SB 203580 did not affect the activity of the ERK MAP kinases in vivo, we stimulated cells with GM-CSF or SLF and investigated the effects of the compound on the activation of p90rsk, which
Figure 3.8 - Activation of MAPKAP kinase-2 by hemopoietic growth factors. MC/9 cells were incubated at 37°C in RPMI 1640 for 1 hr prior to simulation. Cells were then left untreated (CON), or treated with maximal doses of IL-4 (IL-4), GM-CSF (GM-CSF), SLF (SLF) or 0.2 M NaCl (NaCl) for the indicated times. Cells were lysed and the majority of the lysate was used for a MAPKAP kinase-2 immune complex kinase assay with Hsp25 as a substrate. Autoradiograph of Hsp25 phosphorylation after SDS-PAGE (right). Immunoblotting of the proteins in the remaining cell lysate with an anti-phosphotyrosine antibody (4G10) after SDS-PAGE (left).
Figure 3.9 - IL-3 activates MAPKAP kinase-2 via p38 MAPK. Ba/F3 cells were incubated at 37°C in RPMI 1640 with or without 1 μM SB 203580 (SB 203580) for 20 minutes prior to stimulation. The cells were then left untreated, or were stimulated with saturating doses of IL-3 (IL-3) for the indicated times. MAPKAP kinase-2 was immunoprecipitated and its activity was assessed in immune complex kinase assays using recombinant murine Hsp25 as substrate. Reaction products were resolved by SDS-PAGE and the levels of phosphorylation of Hsp25 was assessed by autoradiography.
Figure 3.10 - GM-CSF activates MAPKAP kinase-2 via p38 MAPK. MC/9 cells were incubated at 37°C in RPMI 1640 with or without 1 μM SB 203580 (SB 203580) for 20 min prior to stimulation. The cells were then left untreated, or were stimulated with a saturating dose of GM-CSF (GM-CSF) for 10 min. MAPKAP kinase-2 or p90\textsuperscript{rsk} was immunoprecipitated from aliquots of cell lysates and their activities assessed in immune complex kinase assays using recombinant murine Hsp25 or MBP as the respective substrates. Reaction products were resolved by SDS-PAGE and the levels of phosphorylation of Hsp25 or MBP were assessed by autoradiography.

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Figure 3.11 - SLF activates MAPKAP kinase-2 via p38 MAPK. MC/9 cells were incubated at 37°C in RPMI 1640 with or without 1 μM SB 203580 (SB 203580) for 20 min prior to stimulation. The cells were then left untreated, or were stimulated with a saturating dose of SLF (SLF) for 5 min. MAPKAP kinase-2 or p90<rsk was immunoprecipitated from aliquots of cell lysates and their activities assessed in immune complex kinase assays using recombinant murine Hsp25 or MBP as the respective substrates. Reaction products were resolved by SDS-PAGE and the levels of phosphorylation of Hsp25 or MBP were assessed by autoradiography.
is known to be a downstream target of the ERK MAP kinases (Blenis, 1993). As shown in Fig. 3.10 and 3.11, pretreatment of cells with SB 203580 did not affect the activation of p90rsk by GM-CSF or SLF, implying that the ERK MAP kinase pathway was not affected by SB 203580 and that p38 MAP kinase did not activate p90rsk.

3.2.7 *p38α is sufficient for activation of MAPKAP kinase-2.* Two different p38 MAPK genes, p38α and p38β, encode protein kinases that are sensitive to inhibition by SB 203580 (Gum et al., 1998; Jiang et al., 1997; Kumar et al., 1997; Lee et al., 1994; Li et al., 1996). A mutant of p38α MAPK was produced that was resistant to inhibition by SB 203580 (SBR-p38) (Gum et al., 1998). We made a stable line of Ba/F3 cells that expressed about 1/3 as much mutant p38α MAPK compared to endogenous p38α MAPK (Fig. 3.12, bottom). To test if SB 203580-resistant p38α MAPK was sufficient to restore MAPKAP kinase-2 activity in vivo, we stimulated both wild-type and SB 203580-resistant p38α MAPK-expressing Ba/F3 cells with 0.2 M NaCl for 15 minutes. This expression of p38α MAPK was sufficient to completely restore the activation of MAPKAP kinase-2 after hyperosmotic shock (Fig. 3.12, top).

3.2.8 *Role of p38 MAPK in DNA synthesis.* To evaluate the physiological significance of the activation of p38 by these growth factors, we utilized SB 203580 to inhibit p38 MAPK activity. A series of experiments employing two factor-dependent hematopoietic cell lines, MC/9 and Ba/F3, indicated that SB 203580 inhibited cell growth. As shown in Fig. 3.13, MC/9 cells that were cultured with titrated amounts of SB 203580 in the presence of IL-3 exhibited a dose-dependent inhibition of ³H-thymidine incorporation with an IC₅₀ of 3-5 μM. Experiments using the IL-3 dependent myeloid cell-line Ba/F3, grown in the presence of IL-3, showed a similar inhibition of DNA synthesis (Fig. 3.14). We then examined the proliferation of Ba/F3 cells stably over-expressing the SB 203580-resistant form of p38α MAPK. However, the SB 203580-resistant mutant of p38α MAPK was unable to rescue DNA synthesis in the presence of SB 203580 (Fig. 3.14).
Figure 3.12 - p38α MAPK is sufficient for activation of MAPKAP kinase-2. Ba/F3 cells stably transfected with either the empty vector (WT-p38) or a mutant isoform of p38α (SBR-p38) were incubated at 37°C in RPMI 1640 with or without 1 μM SB 203580 (SB 203580) for 20 min prior to stimulation. The cells were then left untreated, or were stimulated with 0.2 M NaCl (NaCl) for 15 min as indicated. MAPKAP kinase-2 was immunoprecipitated and its activity was assessed in immune complex kinase assays using recombinant murine Hsp25 as substrate. (Top) The phosphorylation of Hsp25 was assessed by autoradiography. (Bottom) The relative level of expression of SBR-p38 and endogenous p38 are indicated.
Figure 3.13 - p38 MAPK activity is required for DNA synthesis in hematopoietic cells. $^3$H-thymidine incorporation in MC/9 cells was measured at 48 hours, and is shown plotted against the concentrations of SB 203580. Triplicate 15 μL cultures contained $10^3$ cells in medium with or without saturating IL-3, 10% FCS and the indicated concentrations of SB 203580 diluted in culture media. Mean values of cpm in cultures lacking SB 203580 were 2,969 cpm in the absence of IL-3 and 19,750 cpm in the presence of IL-3.
**Figure 3.14** - p38α MAPK fails to rescue the inhibition of DNA synthesis in hematopoietic cells. $^3$H-Thymidine incorporation in Ba/F3 cells was measured at 24 hours, and is shown plotted against the concentrations of SB 203580. Triplicate 15 µL cultures contained $10^3$ cells in medium with or without saturating IL-3, 10% FCS and the indicated concentrations of SB 203580.
3.3 Discussion

These experiments demonstrate that a series of hematopoietic growth factors which regulate the normal development and function of cells of the immune system stimulate tyrosine phosphorylation and enzymatic activation of p38 MAP kinase. This was the case for both those hematopoietic growth factors that signal through tyrosine kinase receptors, namely SLF (Fig. 3.2 and 3.3) and CSF-1 (Fig. 3.6), as well as those that signal through receptors of the hematopoietin receptor superfamily, namely IL-3 (Fig. 3.2) and GM-CSF (Fig. 3.3). Ligation of the Fc receptor for Immunoglobulin G, a receptor containing immunotyrosine activation motifs, also induces the tyrosine phosphorylation of p38 MAPK (Fig. 3.7). The observed correlation of tyrosine phosphorylation and activation of p38 MAP kinase (Fig. 3.3) is consistent with other evidence that phosphorylation of the tyrosine of the TGY activation motif of p38 MAP kinase is required for enzymatic activity (Doza et al., 1995; Raingeaud et al., 1995). The activation of p38 MAP kinase by GM-CSF and SLF in hematopoietic cells was rapid and transient (Fig. 3.4), and of the same order as that induced by hyperosmotic stress (Fig. 3.3). This contrasts with reports that stimulation of HeLa cells with epidermal growth factor activated p38 MAP kinase only weakly (Raingeaud et al., 1995) and that nerve growth factor (NGF) failed completely to activate p38 MAP kinase (RK) in PC-12 cells (Rouse et al., 1994). This may reflect differences in the cell types used in these studies. It should be noted that p38 MAP kinase was activated by hematopoietic growth factors in both cell lines and in mast cells from primary cultures.

Given that MAPKAP kinase-2 is known to be an in vivo substrate of p38 MAP kinase (Freshney et al., 1994; Rouse et al., 1994), our observations that p38 MAP kinase is activated in response to hematopoietic growth factors are consistent with the earlier report that IL-3 and GM-CSF activate MAPKAP kinase 2 (Ahlers et al., 1994). Our results confirm this observation and extend it by demonstrating that treatment of cells with SLF also induces MAPKAP kinase-2 activity (Fig. 3.8). In that the specific inhibitor of p38 MAP kinase, SB 203580, completely abrogated MAPKAP kinase-2 activation by IL-3 (Fig. 3.9), GM-CSF (Fig. 3.10) and SLF (Fig.
our data also extend current knowledge by indicating that the activation of MAPKAP kinase-2 by GM-CSF or SLF depended on p38 MAP kinase activity. Thus, despite the fact that treatment of cells with GM-CSF or SLF activates both ERK (Welham et al., 1992) and p38 MAP kinases (Fig. 3.3), SB 203580 specifically inhibited the in vivo activation of MAPKAP kinase-2 but not of p90\textsuperscript{rsk} (Fig. 3.10 and 3.11). These data demonstrate that SB 203580 fails to inhibit the activity of ERK MAP kinases in vivo, consistent with in vitro data that SB 203580 fails to inhibit ERK-2 or p90\textsuperscript{rsk} (Cuenda et al., 1995). These results are also consistent with experiments in which in vivo activation of ERK MAP kinases did not result in in vivo activation of MAPKAP kinase-2 (Rouse et al., 1994).

Despite the fact that both ERK and p38 MAP kinases can activate MAPKAP kinase-2 in vitro (Freshney et al., 1994; Rouse et al., 1994; Stokoe et al., 1992), and both are activated in cells treated with GM-CSF or SLF, p38 MAP kinase activity was essential for activation of MAPKAP kinase-2 in vivo (Fig. 3.10 and 3.11). This evidence that MAPKAP kinase-2 is activated by p38 MAP kinase and not by ERK MAP kinase, suggests differences in molecular localization of these enzymes. Another possibility is that inactive p38 MAP kinase has a greater affinity for MAPKAP kinase-2 than the ERK MAP kinases, and that, in vivo, MAPKAP kinase-2 is complexed with inactive p38 MAP kinase, and not with ERK MAP kinases. In keeping with this notion, p38 MAP kinase has been shown to form a complex in vivo with human MAPKAP kinase-3, a close homologue of MAPKAP kinase-2 (70% amino acid identity) (McLaughlin et al., 1996).

The failure of SB 203580 to inhibit the in vivo activation of p90\textsuperscript{rsk} in the same cells in which it inhibited the in vivo activation of MAPKAP kinase-2 (Fig. 3.10 and 3.11) is consistent with the notion that p90\textsuperscript{rsk} is activated only by the ERK MAP kinases and not by p38 MAP kinase. These results confirm the suggestion from unpublished in vitro experiments that p38 MAP kinase (RK) was unable to activate p90\textsuperscript{rsk} (Rouse et al., 1994).

It should be noted that the anti-MAPKAP kinase-2 antibody that we used was raised against a 16 amino-acid peptide from mouse MAPKAP kinase-2. This peptide differs by only two closely spaced, conservative amino acid substitutions from the corresponding peptide of human
MAPKAP kinase-3. If there is a murine homologue of human MAPKAP kinase-3, we would probably have precipitated it with this antibody. In the human, both MAPKAP kinase-2 and MAPKAP kinase-3 are activated by p38 MAP kinase (CSBP) (McLaughlin et al., 1996). Moreover both enzymes act as Hsp25 kinases in vitro, although whether both phosphorylate Hsp25 in vivo is unclear.

The functional significance of activation of the p38 MAP kinase pathway by growth factors and its relationship to actions of growth factors such as promotion of cell-cycle progression or suppression of apoptosis is unclear. Two possible roles for p38 MAP kinase relate to the regulation of actin polymerization and the activation of transcription factors. Actin polymerization appears to be regulated by phosphorylation of Hsp25, which lowers the affinity of its interaction with the barbed ends of filamentous (F) actin, thus allowing polymerization and the accumulation of F-actin (Lavoie et al., 1995). Growth factors and serum induce phosphorylation of Hsp25 on the same residues that are phosphorylated in response to stress (Landry et al., 1992; Saklatvala et al., 1991), consistent with the involvement of the same enzyme, most likely MAPKAP kinase-2 or MAPKAP kinase-3. Our results with hematopoietic growth factors demonstrate that inhibition of p38 MAP kinase completely blocks activation of MAPKAP kinase-2 (Fig. 3.10 and 3.11), suggesting that growth factor stimulated phosphorylation of Hsp25 reflects activation of p38 MAP kinase. Furthermore, the over-expression of an SB 203580-resistant isoform of p38α MAPK was sufficient to restore MAPKAP kinase-2 activation (Fig. 3.12). The role of actin polymerization in growth factor action is unclear although cell growth is inhibited by cytochalasin D (Lavoie et al., 1995) which, like unphosphorylated Hsp25, prevents elongation of F-actin (Sampath and Pollard, 1991). Over-expression of wild-type Hsp25, but not mutant Hsp25 that is unable to become phosphorylated, renders cells relatively resistant to the growth inhibitory effect of cytochalasin D (Lavoie et al., 1995). This implies a requirement for Hsp25 phosphorylation and F-actin accumulation for cell cycle progression. Our observations that cells treated with SB 203580 underwent a rapid and reversible change in cellular morphology (F. Lee, unpublished data) were also consistent with the notion that p38 MAP kinase regulated actin polymerization.
Transcription factors regulating cell growth may be regulated by the p38 MAP kinase pathway through several mechanisms. In vivo activation of p38 MAP kinase increases the transcriptional activity of Elk-1 (Raingeaud et al., 1996), implying a role for p38 MAP kinase in the transcriptional regulation of proteins such as c-Fos. Interestingly p38β, but not p38α MAP kinase, has been shown to phosphorylate and activate ATF-2 in vivo (Jiang et al., 1996), suggesting differential regulation of transcription factors by the p38 MAP kinase family members. Further experiments will be required to determine whether all family members are activated by hematopoietic growth factors. Recently p38 MAP kinase has also been shown to regulate the phosphorylation of CREB and ATF-1 through MAPKAP kinase 2 (Tan et al., 1996). In addition, p38 MAP kinase has been shown to regulate the production of IL-1, TNFα, IL-6 and GM-CSF (Beyaert et al., 1996; Lee et al., 1994), which are all encoded by mRNAs containing AU-rich motifs. It is possible that the production of the transcription factors c-Myc, c-Fos and c-Jun, which are translated from mRNA containing the same AU-rich motifs characteristic of cytokine mRNA (Chen and Shyu, 1995), may also be regulated in a p38 MAP kinase-dependent fashion.

The notion that activation of the p38 MAP kinase pathway has a critical role in cell cycle progression is suggested by our observations that the specific p38 MAP kinase inhibitor SB 203580 inhibited DNA synthesis (Fig. 3.13 and 3.14). However the over-expression of an SB 203580-resistant isoform of p38α MAPK was unable to rescue DNA synthesis in Ba/F3 cells (Fig. 3.14), even though MAPKAP kinase-2 activation was restored, suggesting that MAPKAP kinase activity was not sufficient to rescue DNA synthesis. However it is possible that MAPKAPK2 activity is not completely restored under normal cell culture conditions, and such possibilities require further investigation. These findings also suggest that there may be differences in the substrate specificity of p38α and p38β in vivo, or that the inhibition of DNA synthesis was due to a non-specific effect of SB 203580. The correct interpretation of these data requires the analysis of an identical SB 203580-resistant mutant of p38β MAPK. It will be important to develop precise genetic tools to explore the role of p38 MAP kinase in growth factor action and cell cycle progression in mammalian cells.
A recent report implicated p38 MAP kinase in the induction of apoptosis (Xia et al., 1995). Withdrawal of nerve growth factor from PC-12 cells led to activation of p38 MAP kinase and the c-Jun N-terminal kinases (JNK), followed by apoptosis. Over-expression of a constitutively active MKK3, which at least in some cells activates p38 MAP kinase (Raingeaud et al., 1995; Xia et al., 1995), promoted apoptosis, whereas over-expression of a dominant negative MKK3 inhibited apoptosis (Xia et al., 1995). However, these results do not discriminate between roles for p38 MAP kinase and JNK, as over-expression of a dominant negative MKK3 appears to block activation of both of these kinases (Raingeaud et al., 1995). In contrast, our results show that p38 MAP kinase is activated by stimulation by growth factors (Fig. 3.2 and 3.3), not by their withdrawal. Moreover, in that hematopoietic growth factors suppress apoptosis (Williams et al., 1990), our findings indicate that, at least in hematopoietic cells, activation of p38 MAP kinase correlates with the suppression of apoptosis rather than its induction. Finally, treatment of cells with concentrations of SB 203580 that we have shown to inhibit p38 MAP kinase activity in vivo (Fig. 3.8) failed to inhibit the apoptosis induced by withdrawal of hematopoietic growth factors (I. Foltz, unpublished data).

Interleukin-4 and IL-13 are notable in being the only growth factors we investigated that failed to induce tyrosine phosphorylation (Fig. 3.2, 3.3 and 3.4) or activation of p38 MAP kinase (Fig. 3.3). The inability of IL-4 to activate p38 MAP kinase accounts for its failure to activate MAPKAP kinase-2 (Fig. 3.8) and correlates with its failure to activate Ras (Duronio et al., 1992; Satoh et al., 1991), and ERK MAP kinase (Welham et al., 1992; Welham et al., 1994). Further experiments will be required to determine whether the activation of p38 MAP kinase by growth factors in hematopoietic cells is Ras dependent. Interestingly, IL-4 consistently reduced the state of tyrosine phosphorylation and enzymatic activity of p38 MAP kinase to levels below those observed in control cells (Fig. 3.3). Likewise, treatment of cells with IL-4 reduced the in vivo activity of MAPKAP kinase-2 to levels below those in control cells (Fig. 3.8). The basis for the inhibition of the p38 MAP kinase pathway by IL-4 is under further investigation.
In conclusion, these experiments demonstrate that p38 MAP kinase participates in the responses to hematopoietic growth factors that interact with two structurally distinct classes of receptor. They demonstrate that p38 MAP kinase is not only involved in responses to stresses, but also in the action of growth factors that regulate the development and function of hematopoietic cells.
CHAPTER 4 - Activation of JNK in Hematopoietic Cells.

4.1 Introduction

While working to characterize the hematopoietic growth factors IL-3, GM-CSF and SLF as activators of the p38 MAPK pathway, it became apparent that the activation of p38 MAPK strongly correlated with the activation of the JNK pathway. Stimuli such as IL-1, TNFα, and UV light were characterized as activators of both p38 MAPK and JNK. In fact, a stimulus capable of separating the activation of p38 MAPK and JNK had not been described as yet. Previous unpublished data in our laboratory conducted by James Wieler had established that IL-3, GM-CSF and SLF induced mRNAs encoding c-Jun. However, IL-4 was unable to induce the expression of c-Jun. JNK had been identified as the relevant kinase for increasing c-Jun transactivation through N-terminal phosphorylation, thereby increasing AP-1 activity. The promoter of c-Jun contains AP-1 sites and therefore the literature suggested the induction of c-Jun was indicative of JNK activity.

Hypothesis:
(1) IL-3, GM-CSF and SLF would increase the activity of JNK1 and JNK2.
(2) These cytokines would induce the phosphorylation of MKK4.
(3) IL-4 would fail to activate either isoform of JNK or induce the phosphorylation of MKK4.

Objectives:
(1) The transcription factor c-Jun had been described as having affinity for JNK through the delta peptide. We used GST-c-Jun to affinity precipitate JNK and perform in vitro kinase assays to determine the effect of cytokine stimulation on the activation of JNK. We also intended to identify commercial antibodies that could be used for immunoprecipitation of JNK for use in gel kinase assays, or immune-complex kinase assays.
At this time the only known activator of JNK was the dual specificity kinase, MKK4. There were no antibodies commercially available to immunoprecipitate MKK4 for direct analysis of kinase activity. However, New England Biolabs had just started producing antibodies that specifically recognized MKK4 when phosphorylated on an activating threonine residue. We decided to use this approach to correlate the activation of JNK with the activation state of MKK4.

Stimuli capable of separating the activation of p38 MAPK and JNK had not yet been described, and as such we did not expect IL-4 to be able to induce JNK activation. Both in gel kinase assays and immune complex kinase assays of cell extracts from cells treated with IL-4 would be conducted to determine if IL-4 could activate JNK or its activator MKK4.

4.2 Results

4.2.1 Activation of JNK1 and JNK2 by Hematopoietic Growth Factors with the Exception of IL-4 or IL-13. To determine whether JNK was involved in responses to IL-3, GM-CSF or SLF, we stimulated MC/9 mast cells with these factors and immunoprecipitated JNK, using antibodies that precipitated both the 46 and 55 kDa isoforms of either JNK1 or JNK2. The precipitate was subjected to SDS-PAGE followed by renaturation and JNK activity was assessed using an "in gel" kinase assay. We found that stimulation with GM-CSF, IL-3 or SLF induced activation of both the 46 and 55 kDa isoforms of JNK1 (Fig. 4.1). It was of particular interest to investigate the effect of IL-4 on JNK1 activity, as our laboratory had previously shown that IL-4 failed to activate Ras (Duronio et al., 1992; Welham et al., 1994), ERK-1/2 MAP kinases (Welham et al., 1992; Welham et al., 1994) or p38 MAP kinase (Foltz et al., 1997). Unlike the other hematopoietins, IL-4 and IL-13 failed to activate either JNK1 (Fig. 4.1 and 4.2) or JNK2 (Fig. 4.2 and 4.3). We also examined the activation of JNK2, and found that GM-CSF or SLF induced the activation of both 46 and 55 kDa isoforms of JNK2 (Fig. 4.3).

To examine the kinetics of the activation of JNK induced by treatment of MC/9 cells with SLF, IL-3 and GM-CSF, we used GST-c-Jun to preferentially immunoprecipitate activated JNK
Figure 4.1 - Activation of JNK1 by hematopoietic growth factors with the exception of IL-4 in MC/9 cells. Cells were incubated at 37°C in RPMI 1640 for 1 hr and then left untreated (CON), or treated with saturating doses of IL-3 (IL-3), IL-4 (IL-4), GM-CSF (GM), SLF (SLF) or 0.2 M NaCl (NaCl) for the indicated times. Immunoprecipitated JNK1 was analyzed using an in gel kinase assay after SDS-PAGE with a separating gel containing GST-c-jun. The phosphorylation of c-Jun was assessed by autoradiography and the presence of 46 and 55 kDa splice variants of JNK1 are indicated.
Figure 4.2 - IL-13 fails to activate JNK1 in FD5/IL13R cells. FD5 cells stably expressing the IL-13Rα chain (FD5/IL13R) were incubated at 37°C in RPMI 1640 for 1 hr and then left untreated (CON), or treated with saturating doses of IL-4 (IL-4), IL-13 (IL-13) or 0.2 M NaCl (NaCl) for 10 min. JNK was affinity precipitated (AP) using GST-c-Jun, and JNK activity was analyzed in a kinase assay with GST-c-Jun as substrate. The phosphorylation of c-Jun was assessed by autoradiography.
Figure 4.3 - Activation of JNK2 by GM-CSF and SLF, but not IL-4, in MC/9 cells. Cells were incubated at 37°C in RPMI 1640 for 1 hr and then left untreated (CON), or treated with saturating doses of IL-4 (IL-4), GM-CSF (GM), SLF (SLF) or 0.2 M NaCl (NaCl) for the indicated times. Immunoprecipitated JNK2 was analyzed after SDS-PAGE with a separating gel containing GST-c-Jun, using an in gel kinase assay. The phosphorylation of c-Jun was assessed by autoradiography and the presence of 46 and 55 kDa splice variants of JNK2 are indicated.
(Adler et al., 1994; Dai et al., 1995; Kallunki et al., 1996). Treatment with SLF or GM-CSF resulted in a rapid increase in the activation of JNK (Fig. 4.4). Cells treated with SLF exhibited detectable activation of JNK after 2 minutes, with maximal activation of JNK occurring around 10 minutes (Fig. 4.4). In cells treated with GM-CSF the activation of JNK again peaked at around 10 minutes (Fig. 4.4). In both instances the activation of JNK was transient and was returning to basal levels by 30 minutes (Fig. 4.4).

4.2.2 Threonine Phosphorylation of MKK4 is induced by GM-CSF or SLF, but not IL-4. At the time these experiments were done, MKK4 was the only established upstream activator of JNK (Derijard et al., 1995; Lin et al., 1995; Sanchez et al., 1994; Yan et al., 1994). Therefore we determined whether there was a correlation between activation of JNK by hematopoietic growth factors and activation of MKK4. We immunoblotted cell extracts from MC/9 cells that had been stimulated with GM-CSF, IL-4 or SLF (Fig. 4.5, top), or GM-CSF, SLF or 0.2 M NaCl (Fig. 4.5, bottom) with an antibody that specifically recognized the activated forms of human and murine MKK4. It was evident (Fig. 4.5, bottom) that stimulation of cells with GM-CSF, SLF or NaCl induced rapid phosphorylation of MKK4. This phosphorylation was transient and was returning to basal levels by 10 minutes. On shorter exposures, it was evident that GM-CSF was a weaker activator of MKK4 than SLF (Fig. 4.5, top). These data indicate the activation of MKK4 preceded the activation of JNK, consistent with a role in activation of JNK by these stimuli. In keeping with our finding that IL-4 failed to stimulate the enzymatic activity of JNK, IL-4 failed to induce phosphorylation of MKK4 (Fig. 4.5). The activation of JNK in all cases correlated with activation of MKK4.

4.3 Discussion

These experiments utilizing MC/9 mast cells demonstrate that a series of hematopoietic growth factors which regulate the normal development and function of cells of the immune system
Figure 4.4 - Kinetics of JNK activation by GM-CSF or SLF in MC/9 cells. After 1 hr in RPMI 1640 at 37°C, MC/9 cells were left untreated, or treated with saturating doses of GM-CSF (GM-CSF), SLF (SLF), or 0.2 M NaCl (NaCl) as a positive control for the indicated times in minutes. Cell extracts were prepared and GST-c-Jun was used as both affinity reagent and substrate for an in vitro kinase assay. The phosphorylation of c-Jun was assessed by autoradiography (left). The fold activation of JNK in cells treated with SLF or GM-CSF compared to untreated cells was quantitated using densitometry (right).
Figure 4.5 - Threonine phosphorylation of M KK4 by SLF or GM-CSF, but not IL-4. MC/9 cells were incubated at 37°C in RPMI 1640 for 1 hr prior to simulation. Cells were then left untreated (CON), or (Top) treated with maximal doses of IL-4 (IL-4), GM-CSF (GM-CSF) or SLF (SLF) or (Bottom) treated with maximal doses of GM-CSF (GM-CSF), SLF (SLF) or 0.2 M NaCl (NaCl) for the indicated times. After lysis, cell extracts were analysed by SDS-PAGE, and were immunoblotted with an antibody that specifically recognizes the activated form of SEK1/MKK4.
activate both JNK1 (Fig. 4.1) and JNK2 (Fig. 4.3). One of these hematopoietic growth factors, SLF, signals through a tyrosine kinase receptor, while the others, IL-3 and GM-CSF, signal through receptors of the hematopoietin receptor superfamily (Fantl et al., 1993; Taniguchi, 1995). Cells treated with GM-CSF, IL-3 or SLF exhibited increased activity of 46 and 55 kDa proteins corresponding to different splice variants of JNK1 (Fig. 4.1). Similarly, cells treated with GM-CSF or SLF activated both 46 and 55 kDa splice variants of JNK2 (Fig. 4.3). Together our results indicate that IL-3 (Fig. 4.1), GM-CSF (Fig. 4.1 and 4.3) and SLF (Fig. 4.1 and 4.3) stimulate the activation of 46 and 55 kDa isoforms of both JNK1 and JNK2 in MC/9 cells.

The kinetics of JNK activation in MC/9 cells treated with GM-CSF or SLF resembled that observed for activation of ERK (Welham et al., 1992; Welham et al., 1994) and p38 MAPK (Foltz et al., 1997) by these factors. The activation of JNK by SLF showed similar kinetics to that induced by GM-CSF (Fig. 4.4). In all cases the activation of JNK following stimulation with these factors was rapid and transient (Fig. 4.4). We also used an antibody specific for the activated form of MKK4 to analyze the ability of these growth stimuli to activate MKK4, the only known upstream activator of JNK (Derijard et al., 1995; Lin et al., 1995; Sanchez et al., 1994; Yan et al., 1994). The phosphorylation of MKK4 induced by treatment of cells with SLF, GM-CSF or 0.2 M NaCl was evident at 5 minutes and was declining by 10 minutes when levels of JNK activity were peaking. However, the phosphorylation of MKK4 by SLF was greater than that observed when cells were treated with GM-CSF. The earlier kinetics of phosphorylation of MKK4, compared with activation of JNK, are consistent with MKK4 being upstream of JNK in the cellular response to these stimuli. These results suggest that MKK4 is involved in the activation of JNK in response to SLF and GM-CSF. There have been reports of other activators of JNK (Moriguchi et al., 1995), but these proteins had not been characterized at the time these experiments were done.

Interleukin-4 and IL-13 were unique among the hematopoietins that we examined in this study in failing to increase the activation of JNK1 (Fig. 4.1) or JNK2 (Fig. 4.3). As noted, the inability of IL-4 to activate JNK correlated with its inability to induce phosphorylation of MKK4.
(Fig. 4.5). This failure of IL-4 to activate JNK correlates with its inability to activate either the ERK (Welham et al., 1992; Welham et al., 1994) or the p38 MAP kinase pathway (Foltz et al., 1997). Interleukin-4 is also notable for its inability to activate Ras (Duronio et al., 1992; Satoh et al., 1991). We hypothesize that hematopoietic growth factors activate p38 MAPK and JNK in a Ras-dependent fashion. Indeed, other growth factors activate JNK through Ras-dependent mechanisms as dominant inhibitory mutants of Ras have been shown to prevent the activation of JNK in response to EGF and NGF (Minden et al., 1994). Furthermore, JNK activity was increased by the expression of constitutively active mutants of Ras (Minden et al., 1995). However, dominant inhibitory mutants of Cdc42 or Rac1 prevent JNK activation by Ras (Minden et al., 1995), and constitutively active mutants of Cdc42 or Rac1 increased JNK activity (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995). These findings suggest that Ras was mediating JNK activation through either Cdc42 or Rac1. Observations that the activation of JNK by TNFα is not inhibited by over-expression of dominant-negative Ras however suggest that there may also be other Ras-independent pathways of JNK activation (Minden et al., 1995). Further studies using dominant inhibitory mutants of Ras and the Rho family of small GTPases will be required to elucidate the hierarchy of proteins leading to JNK activation by hematopoietic growth factors.

Hematopoietic growth factors promote cell cycle progression in cells of the immune system. The functional significance of the activation of JNK1 and JNK2 by hematopoietic growth factors, for example in the promotion of cell cycle progression, is unknown. Clearly the best argument for a role of JNK in cell cycle progression is their ability to phosphorylate and activate the transcription factors including c-Jun, ATF-2 and Elk-1 (Cavigelli et al., 1995; Derijard et al., 1994; Gupta et al., 1995; Kyriakis et al., 1995; Minden et al., 1994). JNK may have an obligate role in cell cycle regulation as JNK are the only known kinases capable of phosphorylating c-Jun on serines 63 and 73 and thereby increasing its transcriptional activity (Derijard et al., 1994; Kyriakis et al., 1995; Minden et al., 1994). The observation that c-Jun is required for the
transformation of fibroblasts by activated Ras implies a role for JNK in the Ras-mediated control of growth (Johnson et al., 1996).

Hematopoietic growth factors not only provide growth signals that promote growth of hematopoietic cells, but also signals that suppress apoptosis. In some cells activation of JNK correlates not with suppression of apoptosis but with its induction (Chen et al., 1996; Xia et al., 1995). It is possible that activation of the JNK pathway in the absence of other signals such as ERK activity results in a pro-apoptotic signal, whereas the integrated activation of ERK, JNK and p38 MAPK pathways, stimulated by factors such as GM-CSF, IL-3 and SLF, delivers a signal for the suppression of apoptosis. However, our data indicates that the activation of ERK, JNK or p38 MAPK pathways is not essential for the suppression of apoptosis as IL-4, which we have shown fails to activate any of these kinases, nevertheless suppresses the apoptosis of many hematopoietic cells, including the factor dependent cell line MC/9 used in these experiments.

In conclusion, we have demonstrated that JNK1 and JNK2 are activated in response to IL-3, GM-CSF and SLF, but not IL-4. Furthermore our results suggest that SAPK activation is mediated by MKK4, as both SLF and GM-CSF, but not IL-4, activated MKK4 prior to activation of JNK. Importantly, our results demonstrate that JNK activity is involved not only in response to stress, but also in signaling by hematopoietic growth factors that in some cases, such as SLF, regulate the normal growth and development of the hematopoietic system.
CHAPTER 5 - MKK7 - A Specific Activator of JNK.

5.1 Introduction

The activation of JNK by GM-CSF or SLF followed very similar kinetics. However, the induction of threonine phosphorylation of MKK4 by these cytokines was much more pronounced with SLF than with GM-CSF or NaCl. These findings suggested the existence of another JNK kinase that was regulated by GM-CSF. Several other lines of evidence pointed to the existence of another MKK capable of activating the JNK family. Moriguchi et al. identified at least one JNK kinase activity in addition to the activity of MKK4 in rat fibroblastic 3Y1 cells stimulated by hyperosmotic shock (Moriguchi et al., 1995). Similarly Meier et al. identified two JNK kinase activities operationally termed SKK4 and SKK5 in KB cells, a human oral carcinoma cell line, following treatment with hyperosmolarity or anisomycin (Meier et al., 1996). The generation of embryonic stem (ES) cells in which the MKK4 gene was disrupted provided conclusive evidence for the existence of another JNK kinase. ES cells lacking MKK4 still exhibited increased JNK activity following hyperosmotic shock or UV irradiation, but failed to exhibit JNK activation after treatment with anisomycin or interleukin-1 (Meier et al., 1996; Nishina et al., 1997; Yang et al., 1997). Together these studies suggested the existence of another JNK kinase.

Hypothesis:
(1) MC/9 cells express a novel MKK that acts as a direct activator of JNK.
(2) The unknown MKK is activated by GM-CSF, IL-3, and NaCl.

Objectives:
(1) Screen the expressed sequence tags (EST) database to identify a novel MKK. The sequences of the known MKK were aligned, and a consensus sequence at the C-terminus of these enzymes
was determined (Fig. 5.1). The resulting peptide was converted to a degenerate oligonucleotide (Fig. 5.2), and the oligonucleotide was used to screen the EST database.

(2) Determine the expression pattern of any unknown MKK that was identified.

(3) Characterize the enzymes for their substrate specificity and upstream activators.

5.2 Results

5.2.1 Cloning strategy to discover unknown MKK. We screened the EST (expressed sequence tags) database using a degenerate oligonucleotide sequence based on the amino acids conserved in the C-terminus of all previously characterized mitogen-activated protein kinase kinases (Fig. 5.1) (Lin et al., 1995). We identified 7 clones that encoded known MKK family members and one clone (aa019720) that contained a novel human sequence (Table 5.2). This clone contained 464 base pairs (bp) of coding region and approximately 1200 bp of 3' untranslated region. We used 5'-RACE (Rapid Amplification of cDNA Ends) to isolate overlapping 5'-fragments of this novel cDNA from a human fetal kidney RACE cDNA library (Fig. 5.2). We amplified several over-lapping clones of MKK7 using a fully nested PCR with the adaptor primers AP1/AP2 and primers IF113/IF111 or IF117/IF115 for the first or second/third screens respectively (Fig. 5.3). Using this technique we cloned a further 861 bp of human MKK7 (hMKK7α) that contained an in-frame stop codon. When theoretical translation was initiated at the first methionine, this cDNA encoded a 419 amino acid protein (Fig. 5.4) that contained a putative protein kinase domain. We also isolated a splice variant of human MKK7 (MKK7β) that contained an additional 126 base pairs encoding an insert of 42 amino acids (Fig. 5.5). We screened the yeast and invertebrate databases for other MKK related to MKK7 and although we failed to identify a yeast homolog, we did find an ortholog of MKK7 in D. melanogaster (dHep - 69% identity) (Glise et al., 1995). In C. elegans we identified an ortholog with 54% identity we termed cMKK7 (Wilson et al., 1994). We compared the catalytic domains of MKK7 with dHep and cMKK7 (Fig. 5.6) or all other known human MKK (Fig. 5.7). MKK7 was orthologous with
Alignment of MKK:

MKK4:  FSPSFINFVNLCLTKDESKRPKYKELLKHPF
MKK3:  ...E.VD.TAQ...R.NPAE.MS.L..ME...
MEK1:  ..LE.QD...K..I.NPAE.ADL.Q.MV.A.
PBS2:  ..SDAQD...S...Q.I.ER...T.AA.T...W

Consensus:  F.......F...CL.K....R.......L...H..

Nucleotide Sequence:

TT[TC].(.)16 TT[TC].(.)7 TG[TC].T....AA[AG].(.)11 G.(.)15 T.(.)5 CA

Figure 5.1 - Designing a probe to screen the EST database. The C-termini of the primary structures of human MKK4, MKK3, MEK2, MEK1 and S. cerevisiae PBS2 were aligned to determine a consensus motif to identify novel MKK. The amino acid motif was back-translated into degenerate nucleotides according to the human genetic code (Table 5.1). The resulting nucleotide sequence was used to screen the EST database.

Table 5.1 - The Genetic Code.

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<td>CCA - P</td>
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<td>ACT - T</td>
<td>AAT - N</td>
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<td>ACA - T</td>
<td>AAA - K</td>
</tr>
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<td>ACG - T</td>
<td>AAG - K</td>
</tr>
<tr>
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<td>GCT - A</td>
<td>GAT - D</td>
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<td>GCC - A</td>
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</tr>
<tr>
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<td>GCA - A</td>
<td>GAA - E</td>
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<td></td>
<td>GTG - V</td>
<td>GCG - A</td>
<td>GAG - E</td>
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</table>
Table 5.2 - Analysis of Clones derived from the EST database.

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</tr>
</thead>
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<td>aa008333</td>
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<td>Human</td>
</tr>
<tr>
<td>aa019720</td>
<td>Novel - MKK7</td>
<td>Human</td>
</tr>
<tr>
<td>n98702</td>
<td>MKK4</td>
<td>Human</td>
</tr>
<tr>
<td>r11022</td>
<td>MEK2</td>
<td>Human</td>
</tr>
<tr>
<td>r51239</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rircr2291a</td>
<td>Novel</td>
<td>Rice</td>
</tr>
<tr>
<td>t23364</td>
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<td>-</td>
</tr>
<tr>
<td>aa071840</td>
<td>M KK4</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

ATG Step 1: Nested PCR

EST clone: aa019720

Probe

Step 2: Blunt end sub-cloning of PCR products into pBSKS

Step 3: Blue/White Colony Selection

Step 4: Colony Blot with Specific Probe from EST Clone

Step 5: Sequence Putative Positive Clones

Figure 5.2- Strategy for cloning full length MKK7.
Figure 5.3- Amplification of fragments of MKK7 using 5'-RACE PCR. A human fetal kidney cDNA library was used to amplify MKK7. The adapter primers AP1 and AP2 were used for all PCR amplifications as depicted in Figure 5.2. A fully nested PCR using either the primers IF113 and IF111, or the primers IF117 and IF115, was used for the primary screen, or the secondary and tertiary screens respectively. The positive clones found in these screens are depicted to scale with respect to the full length human MKK7α, and the EST clone aa019720.
Figure 5.4 - Nucleotide and primary amino acid sequence of human MKK7. The nucleotides of the cDNA encoding human MKK7α. The initiating methionine is underlined and stop codons are shown in bold. The primary amino acid sequence is shown below the nucleotide sequence as predicted from the human codon usage chart (Table 5.1).
Figure 5.5 - Alignment of primary structures of human and murine isoforms of MKK7. Predicted amino acid sequence of human MKK7α (hMKK7α) and MKK7β (hMKK7β) and of the N-termini of murine MKK7α (mMKK7α) and MKK7γ (mMKK7γ). Where residues are identical with those of hMKK7α, they are indicated by periods. hMKK7β and mMKK7γ are shown only where they differ from MKK7α. Gaps were introduced to optimize the alignment and are indicated by dashes.
Figure 5.6 - Alignment of human MKK7 with its orthologs in C. elegans and D. melanogaster. Predicted amino acid sequence of human MKK7α (hMKK7), D. melanogaster MKK7 (dHep) and C. elegans MKK7 (cMKK7). Where residues are identical with those of hMKK7, they are indicated by periods. Gaps were introduced to optimize the alignment and are indicated by dashes.
### II

| MKK7 | DLENLGEMSGTGCQVVKMRFRKTGHVIAV/KMRSGNKENKRILMDLDVV/LKSHDCPFY |
| MKK6 |  | .PTML.RAY.V.E..HVPS.QIM..RI.ATV.S.Q..L..ISMTVR...F |
| MKK3 |  | VTTLS.RAY.V.E.V.HAQS.TIM..RI.ATV.S.Q..L..INMTRT. |
| MEK5 |  | IRYRTLH.N.TG..TAYHVPS.KIL...VIIIDTL.IQ..Q.MSE.BI.-.YKC.SS. |

### III

| MKK7 | IVQCFGTFITNTDVFIAMELMGT-CAEKLKKRMQGPIPERILGKMTVAIVKALYYL |
| MKK4 |  |  | FYALFREG.CW.C-SS.FYVYSVLDDV.E...I.L.T...NH. |

### VI

| MKK7 | IVQCFTFITNTDVFIAMELMGT-CAEKLKK-RMQGPIPERILKMTVAIVKALYYL.. |
| MKK4 |  | .NLK.I...RS.N..Q..I...D..RP..-.S |
| MKK6 |  | HSKLS...........V.INAL.V.M....Y...V...ID..KP...N-E |
| MEK3 |  | HSKLS...........V.INKE.HV..M....Y...V...MD..KP...N-E |
| MEK5 |  | WS-LKIL........M.VNLTR...V.TQ.N.I...Y-V.TN........SGEQ |
| MEK2 |  | R...QIM........VNSR.E...V.Q.I..M.NSF-V.TRS........LQGTH |
| MEK1 |  | R...KIM........VNSR.E...V.Q.I..M.NSF-V.TRS........LQGTH |

### VII

| MKK7 | PTKPDYDIRADVWSLGISLVELATGQFPYKNCKT--(--FSGDFQSFVKDCLTKDHR |
| MKK4 |  |  | ASRQG..V..S....T.Y....R...PKNNS- |
| MKK6 |  |  | LNQKG.SVKS.I.....TIMI...IIIL.....DSGW- |
| MEK3 |  |  | LNQKG.NVKS.V.....TIMI.M.IR...ESWG- |
| MEK5 |  |  | .GIHSFM.IQ- |
| MEK2 |  |  | ----SVQS.I..M.L......V.RY.IPPPAKELEAIMGRFVVDGEGEPHSISPRP |
| MEK1 |  |  | ----SVQS.I..M.L......M.V.RY.IPPPAKELELMFNCQV----EGDAETFPRP |

### X

| MKK7 |  |  | --DFEVLTKVLOEPEPPLPGHMG--FSGDFQSFVKDCLTKDHR |
| MKK4 |  |  |  | --P.QQ.KQ.V.E.PS.Q..ADK--AE.VD.TSQ.K.NSK |
| MKK6 |  |  |  | --P.QQ.KQ.V.E.PS.Q..ADR--PE.VD.TAQ.R.NPA |
| MEK5 |  |  |  | --KMQVSLPLQL.QCIVD.DS.V.VGE--EP.VH.I.QL.QRPQ |
| MEK2 |  |  |  | RPPGRFVSCHGMDSPAMAI.L.DYIVNP.P.K.NG--TP.E.NK.I.NPA |
| MEK1 |  |  |  | RTPGRFVSCHGMDSPAMAI.LD.YIVNP.P.K.SG--LE.D.NK.I.NPA |

### XI

| MKK7 |  |  | KRPKYNKLEHSF |
| MKK4 |  |  |  | KE..K.P. |
| MKK6 |  |  |  | .T.PE.MQ.P. |
| MEK3 |  |  |  | E.MS.LE.M.P. |
| MEK5 |  |  |  | E..APEE.MG.P. |
| MEK2 |  |  |  | E.ADLKM.TN.T. |
| MEK1 |  |  |  | E.ADLKQ.MV.A. |

### Figure 5.7 - Alignment of the catalytic domains of known human MKK.

The predicted amino acid sequence of known human MKK. Where residues are identical with those of MKK7, they are indicated by periods. Gaps were introduced to optimize the alignment and are indicated by dashes.
dHep and cMKK7 and was more closely related to these than to the known human MKK (Fig. 5.8).

We used primers derived from the human sequence of MKK7 to amplify a 869 bp fragment of cDNA from the murine Ba/F3 hematopoietic cell line. Sequencing indicated that this cDNA encoded the N-terminus and most of the kinase domain of a murine homologue of MKK7 (mMKK7α). We screened the EST database for cDNA encoding the N-terminus and identified another splice variant of mMKK7 (mMKK7γ) that contained an additional 48 base pair exon or in-frame intron (Fig. 5.5). We have not yet identified a human homologue of mMKK7γ. These splice variants may encode alternative forms of MKK7 or may represent incompletely processed mRNA. By far the most highly represented form of MKK7 in a human fetal kidney cDNA library was MKK7α and therefore we chose this isoform for continued analysis of MKK7 function.

5.2.2 Expression of MKK7 in human and murine cells. To investigate expression of endogenous and exogenous MKK7 we immunoblotted lysates of MC/9 mast cells, Ba/F3 cells and a clone of Ba/F3 cells that we generated stably expressing MKK7 (Ba/F3-MKK7) with an antiserum raised against the N-terminus of MKK7. We identified an immunoreactive protein of 47 kDa, consistent with the size predicted from the cDNA of MKK7α or MKK7γ (Fig. 5.9). Thus Ba/F3 and MC/9 cells expressed the N-terminus predicted by translation of the cDNA encoding mMKK7α or mMKK7γ. We were unable to detect an immunoreactive protein at the size (53 kDa) predicted to correspond to the hMKK7β splice variant in murine cells. These experiments indicated that the levels of exogenous MKK7 expressed in Ba/F3-MKK7 cells were at least 30-fold greater than endogenous MKK7 (Fig. 5.9). This was interesting as the Ba/F3-MKK7 cells exhibited no obvious abnormalities in growth and remained dependent on IL-3 for growth and survival. Moreover, over-expression of human MKK7 did not appear to affect the expression of endogenous murine MKK7, as 5 x 10⁵ cells of both untransfected and Ba/F3-MKK7 cells expressed comparable levels of endogenous MKK7 (Fig. 5.9).
Figure 5.8 - Dendrogram of known human MKK and the orthologs of MKK7. A radial dendrogram (Fitsch method) depicting the phylogeny of the catalytic domains of MKK7, dHep, cMKK7 and other known human MKK. The percent identities within the kinase domain of other MKK with human MKK7α were: dHep - 69%; MKK4 - 56%; cMKK7 - 54%; MKK6 - 49%; MKK3 - 47%; MEK1/2 - 35% and MEK5 - 30%.
**Figure 5.9 - Expression of MKK7 in Ba/F3 and MC/9 cells.** Immunoblot of endogenous MKK7 in lysates of Ba/F3 and MC/9 hematopoietic cells and endogenous and exogenous MKK7 in lysates of a clone of Ba/F3 cells that expressed human myc-tagged MKK7. The indicated numbers of cells were lysed and proteins were separated by SDS-PAGE. An antiserum raised against the N-terminus of MKK7 (a.a.4-26) was used for immunoblotting. The position of endogenous MKK7 and myc tagged exogenous MKK7 are indicated by arrowheads.

<table>
<thead>
<tr>
<th>Cells x 10^4</th>
<th>Ba/F3</th>
<th>MC/9</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 x 10^4</td>
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<td></td>
</tr>
<tr>
<td>1 x 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 x 10^4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 50 kDa
- myc-MKK7
- MKK7
We used Northern blotting to investigate the expression of MKK7 in multiple human tissues. The probe contained the unique N-terminus of MKK7 and the 5'-end of the kinase domain which exhibits little sequence homology with other known MKK family members. The probe bound to a single transcript of around 4 kbp in all tissues tested, with highest levels of hybridization occurring with mRNA from skeletal muscle (Fig. 5.10). We also used RT-PCR to examine expression of MKK7 in a number of human and murine cells and confirmed the PCR products as being derived from MKK7 by sequencing and restriction analysis. MKK7 mRNA was present in all tested cell lines corresponding to a number of cell lineages (Table 5.3). Thus, MKK7 is widely expressed.

5.2.3 MKK7 specifically activates JNK, but not p38 MAPK. It has been previously shown that the transient co-expression of mitogen-activated protein kinases with the appropriate upstream MKK leads to in vivo activation of the MAPK. We investigated the substrate specificity of MKK7 by co-expression of MKK7 with either JNK1 or p38α MAP kinase. Co-expression of MKK7 with JNK1 in HeLa epithelial cells (Fig. 5.11) or Ba/F3 hematopoietic cells (data not shown) without any deliberate stimulation of the cells, resulted in easily detectable activation of JNK1. Stimulation of these co-transfected cells with hyperosmotic shock (0.2 M NaCl) or UV light resulted in even greater JNK1 activation (Fig. 5.11). These results suggested that MKK7 was upstream of JNK1 and was activated by stimulation of cells by UV light or hyperosmotic shock. In contrast, co-expression of p38α MAPK with MKK7 had no effect on activation of p38α MAPK (Fig. 5.12). Nor did co-expression of MKK7 with p38 MAPK result in increased activation of p38α MAPK when the transfected cells were stimulated by hyperosmotic shock or UV light (Fig. 5.12). Thus MKK7, unlike MKK4, specifically activated JNK1, but not p38α MAP kinase.

5.2.4 MKK7 is activated by TNF or multiple stress stimuli. The c-Jun N-terminal kinases have been shown to be activated in response to a number of different stimuli including UV
Figure 5.10 - Tissue Distribution of mRNA encoding M KK7. Northern blot of M KK7 mRNA using poly-(A)^+ mRNA isolated from multiple human tissues. A probe was prepared by random priming a 600 bp fragment from the 5'-end of M KK7.

Table 5.3 - Expression of mRNA encoding M KK7 in various cell lines as determined by RT-PCR.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Species</th>
<th>Cell Lineage</th>
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<tbody>
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<td>HeLa</td>
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<td>Epithelial (Cervical carcinoma)</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human</td>
<td>Hepatocarcinoma</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Human</td>
<td>Acute T cell leukemia</td>
</tr>
<tr>
<td>Daudi</td>
<td>Human</td>
<td>B lymphoma</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human</td>
<td>Embryonic kidney cell</td>
</tr>
<tr>
<td>U937</td>
<td>Human</td>
<td>Monocytic</td>
</tr>
<tr>
<td>129/J ES cells</td>
<td>Mouse</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>Ba/F3</td>
<td>Mouse</td>
<td>IL-3 dependent hematopoietic cell</td>
</tr>
<tr>
<td>MC/9</td>
<td>Mouse</td>
<td>IL-3 dependent mast cell</td>
</tr>
</tbody>
</table>
Figure 5.11 - Co-expression of MKK7 with JNK1 activates JNK1. HeLa cells were transfected with constructs encoding GST-JNK1 and either myc-tagged MKK7 or the empty vector pEF-BOS (pEF). Transfected cells were split into 3 plates and either left untreated (Con) or stimulated with 0.2 M NaCl (Na) or UV irradiation (UV) for 20 min. GST-JNK1 was affinity purified with glutathione-Sepharose and its kinase activity was determined using GST-c-Jun as substrate. The phosphorylation of c-Jun was visualized after SDS-PAGE using autoradiography.
Figure 5.12 - Co-expression of MKK7 with p38α MAPK activates p38α MAPK. HeLa cells were transfected with constructs encoding Flag-tagged CSBP2/p38α and either myc-tagged MKK7 or the empty vector pEF-BOS (pEF). Transfected cells were split into 3 plates and either left untreated (Con) or stimulated with 0.2 M NaCl (Na) or UV irradiation (UV) for 20 minutes. Flag-tagged p38α MAPK was immunoprecipitated with the M2 antibody and its activity determined using ATF-2 as substrate. The phosphorylation of ATF-2 was visualized after SDS-PAGE using autoradiography.
light, hyperosmotic shock, protein synthesis inhibitors, heat shock, the pro-inflammatory cytokines IL-1 and TNFα and hematopoietic growth factors (Foltz and Schrader, 1997; Raingeaud et al., 1995). We tested the ability of these stimuli to activate GST-MKK7 that had been transiently over-expressed in HeLa or Ba/F3 cells. GST or GST-MKK7 was purified from extracts of cells subjected to various stimuli, and its ability to phosphorylate GST-JNK1 was assessed in an in vitro kinase assay. The activity of MKK7 was increased by treatment of cells with hyperosmotic shock (11 fold), heat shock (7.5 fold), UV light (5.5 fold), or TNFα (3.5 fold), but not by EGF (Fig. 5.13, top). The ability of these stimuli to activate MKK7 and to in turn activate JNK1 in vitro was also assessed. We observed that the ability of MKK7 to activate JNK1 was increased in transfected cells treated with TNFα (4 fold), hyperosmotic shock (5 fold) or anisomycin (5 fold - 30 min; 6.5 fold - 45 min) (Fig. 5.13, bottom). Thus MKK7 was activated by all these known activators of JNK.

5.2.5 Activation of MKK7 by IL-3, but not IL-4, in Ba/F3 cells. We and others have previously demonstrated the activation of JNK in response to stimulation by the hematopoietic growth factor IL-3 in both Ba/F3 hematopoietic cells and MC/9 mast cells (Foltz and Schrader, 1997; Nagata et al., 1997; Terada et al., 1997). In contrast, the related cytokines IL-4 and IL-13 failed to activate JNK (Foltz and Schrader, 1997). We transiently expressed GST-MKK7 in Ba/F3 cells, subjected them to various stimuli and assayed the ability of MKK7, that was activated in vivo, to phosphorylate GST-JNK1 in vitro. As seen in Fig. 5.14, MKK7 was strongly activated in cells treated with IL-3 (5.5 fold), 0.2 M NaCl (4.5 fold) or UV light (3.5 fold). In contrast, MKK7 was not activated in cells treated with IL-4 (Fig. 5.14), correlating with its inability to activate JNK (Foltz and Schrader, 1997).

5.2.6 Activation of endogenous MKK4 and MKK7 by IL-3, but not IL-4. We next investigated the activation of endogenous MKK7 in response to physiological stimuli. In parallel we also investigated the activation of the known JNK activator MKK4 in order to identify
Figure 5.13 - Activation of MKK7 by various stimuli. Cells were transiently transfected with vectors encoding GST or GST-MKK7 and aliquots were stimulated as follows. (A) HeLa cells were either left untreated (Con), or stimulated with 100 ng/mL EGF (EGF) for 5 min, 0.2 M NaCl (Na) for 20 min, UV irradiation (UV) for 20 min, 100 ng/mL TNF-α (TNF) for 20 min or heat shocked (Heat) at 42 °C for 20 min. (B) HeLa cells were left untreated (Con), or stimulated with 100 ng/mL TNF-α (TNF) for 15 min, 50 µg/mL anisomycin (Aniso) for 30 or 45 min, or 0.2 M NaCl (Na) for 20 min. In all cases, transiently expressed proteins were affinity precipitated using glutathione Sepharose from samples of lysates that had been normalized for total protein using the Pierce assay. MKK7 activity was determined by assaying its ability to phosphorylate 1 µg of GST-JNK1, or by measuring its ability to activate JNK1 in vitro by incubating it with 1 µg of GST-JNK1 and 50 mM unlabelled ATP for 30 min and determining the ability of an aliquot of this reaction mixture to phosphorylate GST-c-Jun. Phosphorylated substrates were visualized after SDS-PAGE by autoradiography. The precipitated proteins were immunoblotted after SDS-PAGE with an anti-MKK7 antibody (a.a.4-26) to quantitate loading. The position of MKK7 is indicated by an arrowhead.
Figure 5.14 - Activation of MKK7 by IL-3, but not IL-4, in BaF3 cells. Cells were transiently transfected with vectors encoding GST or GST-MKK7 and aliquots were stimulated as follows. Ba/F3 cells were left untreated (Con), or stimulated with 10 μg/mL synthetic IL-3 (IL-3) for 5 min, 10 μg/mL synthetic IL-4 (IL-4) for 10 min, 0.2 M NaCl (Na) for 20 min or UV irradiation (UV) for 20 min. In all cases, transiently expressed proteins were affinity precipitated using glutathione Sepharose from samples of lysates that had been normalized for total protein using the Pierce assay. MKK7 activity was determined by assaying its ability to activate JNK1 \textit{in vitro} by incubating it with 1 μg of GST-JNK1 and 50 μM unlabelled ATP for 30 min and determining the ability of an aliquot of this reaction mixture to phosphorylate GST-c-Jun. Phosphorylated substrates were visualized after SDS-PAGE by autoradiography. The precipitated proteins were immunoblotted after SDS-PAGE with an anti-MKK7 antibody (a.a.4-26) to quantitate loading.
potential functional differences. The antibodies used for immune complex kinase assays specifically recognized MKK4 or MKK7 (Fig. 5.15). We then examined the ability of IL-3, IL-4, anisomycin and 0.2 M NaCl to activate MKK7 in Ba/F3 cells. Consistent with the results of experiments with transiently expressed MKK7, cells treated with IL-3 exhibited an increased activation (8 fold) of endogenous MKK7 (Fig. 5.16). Cells treated with IL-3 also exhibited an increase in MKK4 activity (3 fold). In contrast, cells treated with IL-4 failed to activate MKK7 (Fig. 5.16). Anisomycin and hyperosmotic shock also activated endogenous MKK7 (4 and 14 fold).

5.2.7 Activation of endogenous MKK4 and MKK7 by TNF. The pro-inflammatory cytokine TNFα is a well described activator of the JNK pathway. We examined the ability of TNFα to activate MKK4 and MKK7 in HeLa cells. As seen in Fig. 5.17, we observed an increase in the activity of both MKK4 (5 fold) and MKK7 (2.5 fold).

5.2.8 Activation of endogenous MKK4 and MKK7 by cross-linking the Fc receptor for IgG. Activation of JNK has also been shown to follow cross-linking of the receptor for the Fc fragment of Immunoglobulin G (FcR) on myeloid cells. The monoclonal antibody 2.4G2 (α-FcR) binds to both FcγRII and FcγRIII and when cross-linked by a secondary antibody (α-Ig) induces signalling through the FcR. As seen in Fig. 5.18, cells either left untreated or incubated with either α-FcR or α-Ig alone exhibited baseline activities of MKK4 or MKK7. However, when cells were pre-treated for 10 minutes with α-FcR and then incubated with α-Ig to aggregate the α-FcR/FcR complexes, an increase in both MKK4 (7 fold) and MKK7 (8 fold) activity was observed.

5.2.9 Constitutively active GTPases activate MKK4 and MKK7. Expression of constitutively active mutants of the Ras and Rho family of small GTPases activates the JNK family of protein kinases (Bagrodia et al., 1995; Coso et al., 1995; Derijard et al., 1994; Hibi et al., 1993;
Figure 5.15 - Specificity of MKK4 and MKK7 antibodies. A lysate of WEHI-231 cells was split and immunoprecipitated with antibodies recognizing either MKK4, the N-terminus of MKK7, or with an irrelevant Ab (Con). Aliquots of immunoprecipitates were analysed by SDS-PAGE and immunobotted with either an antibody recognizing MKK4 (left) or the C-terminus of MKK7 (right). A sample containing $5 \times 10^5$ cell equivalents of whole cell extract (WCE) was analysed in parallel. The position of MKK4 (2 isoforms) and MKK7 are indicated by arrowheads.
Figure 5.16 - Activation of endogenous MKK4 and MKK7 by IL-3, but not IL-4. Ba/F3 cells were left untreated (Con), or stimulated with 10 μg/mL synthetic IL-3 (IL-3) for 5 min, 10 μg/mL synthetic IL-4 (IL-4) for 10 min, 0.2 M NaCl (NaCl) for 20 min or 50 μg/mL anisomycin (Aniso) for 30 min. Cellular lysates were immunoprecipitated with antibodies recognizing MKK4 or MKK7. The activity of MKK4 or MKK7 was determined by incubating 1 μg of GST-JNK1 with 50 μM of unlabelled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.
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<tr>
<th></th>
<th>MKK4</th>
<th></th>
<th>MKK7</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 5.17 - TNFα activates endogenous MKK4 and MKK7 in HeLa cells. HeLa cells were left untreated (Con), or stimulated with 100 ng/mL TNF (TNF) for 5 or 10 min. Cellular lysates were immunoprecipitated with antibodies recognizing MKK4 or MKK7. The activity of MKK4 or MKK7 was determined by incubating 1 μg of GST-JNK1 with 50 μM of unlabelled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.
Figure 5.18 - Cross-linking of the Fc receptor for IgG activates MKK4 and MKK7 in MC/9 mast cells. MC/9 mast cells were pre-treated with α-FcR (α-FcR) for 10 min as indicated, and then incubated for 10 min in medium alone, or with a rabbit α-rat immunoglobulin antibody (α-Ig). Cellular lysates were immunoprecipitated with antibodies recognizing MKK4 or MKK7. The activity of MKK4 or MKK7 was determined by incubating 1 μg of GST-JNK1 with 50 μM of unlabelled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.
Minden et al., 1995). We confirmed that co-expression of GST-JNK1 and \( \text{Ras}^{K61} \), \( \text{Rac}^{V12} \) or \( \text{Cdc42}^{V12} \) activated the JNK pathway in HeLa cells (data not shown). We then co-expressed GST-MKK7 with each of these mutant GTPases, affinity-precipitated the GST-MKK7 and assessed its ability to activate GST-JNK1 \textit{in vitro}, using phosphorylation of GST-c-Jun as a readout of JNK1 activity. We found that co-expression of activated mutants of Ras, Rac or Cdc42 and MKK7 in HeLa cells resulted in marked activation of MKK7, demonstrating that this kinase could be activated by signals downstream of each of these GTPases (Fig. 5.19). Similar results were obtained when GST-MKK4 was co-expressed with these GTPases in HeLa cells (Fig. 5.19). We also performed similar experiments in the murine hematopoietic cell line Ba/F3. Co-expression of GST-JNK1 and the constitutively active mutants of Rac and Cdc42 resulted in activation of JNK1 in Ba/F3 cells; however co-expression of activated Ras with JNK1 failed to activate JNK1 (data not shown). In keeping with these findings, co-expression of MKK7 and Rac or Cdc42 in Ba/F3 cells activated MKK7; however the co-expression of activated Ras with MKK7 was not sufficient to activate MKK7 (Fig. 5.20).

5.3 Discussion

Our results indicate that human and murine MKK7 are highly conserved (99% identity, Fig. 5.5) and are most closely related to dHep, a MKK in \textit{Drosophila}, exhibiting 69% identity in the kinase domain (Fig. 5.6). MKK7 was less related to MKK4, MKK6 or MKK3 (~45-55% identity), the other mammalian MKK known to be activators of JNK and p38 MAPK, and was even less similar to MEK1 or MEK2 (~30-35% identity), the activators of ERK MAPK (Fig. 5.7 and 5.8). We conducted extensive searches of the yeast and invertebrate databases and although we failed to identify MKK7 in \textit{S. cerevisiae}, we did identify a highly related \textit{C. elegans} MKK (cMKK7) that exhibited 54% identity to MKK7 within the kinase domain (Fig. 5.6) (Wilson et al., 1994). A more detailed inspection of alignments revealed a series of residues or motifs that were conserved among dHep, MKK7 and cMKK7, that were not present in MKK3, MKK4 and
Figure 5.19 - Activation of MKK7 or MKK4 by the small GTPases Ras, Rac and Cdc42. HeLa cells were transiently co-transfected with a vector encoding either GST, GST-MKK7 or GST-MKK4 and pEF-BOS that encoded the constitutively active mutants of Ras (Ras), Rac (Rac), Cdc42 (Cdc42) or an empty vector as a control. In every case, a reporter construct encoding β-galactosidase in the same pEF-BOS vector was also co-transfected and β-galactosidase activity was used to normalize the lysates for the expression of transfected genes. Cells transfected with the empty vector and MKK7 were left unstimulated (Con), or stimulated with 0.2 M NaCl (Na) for 20 min as controls for the kinase assay. The activity of affinity purified MKK7 or MKK4 was determined by incubating 1 μg of GST-JNK1 with 50 μM of unlabelled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.
Figure 5.20 - Activation of MKK7 by the small GTPases Rac and Cdc42. Ba/F3 cells were transiently co-transfected with a vector encoding either GST or GST-MKK7 and pEF-BOS that encoded the constitutively active mutants of Ras (Ras), Rac (Rac) or Cdc42 (Cdc42) or an empty vector as a control. In every case, a reporter construct encoding β-galactosidase in the same pEF-BOS vector was also co-transfected and β-galactosidase activity was used to normalize the lysates for the expression of transfected genes. Cells transfected with the empty vector and MKK7 were left unstimulated (Con), or stimulated with 0.2 M NaCl (Na) for 20 min as controls for the kinase assay. The activity of affinity purified MKK7 was determined by incubating 1 μg of GST-JNK1 with 50 μM of unlabelled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.
MKK6, and vice versa. Particularly striking are differences in the activation loops, with MKK7, dHep, and cMKK7 being characterized by a basic amino acid (S[K/R]AKT), at the same position where in MKK3, MKK4, and MKK6 a hydrophobic residue (S[I/V]AKT) is found. Overall these patterns of conservation of specific motifs support the notion that MKK7 is orthologous with dHep and cMKK7, and is more distantly related to MKK4, MKK3 and MKK6 (Fig. 5.6 and 5.7). It will be interesting to determine the functional significance of the conserved residues that characterize MKK7, dHep and cMKK7, and distinguish them from MKK4, MKK3 and MKK6.

Tournier et al. have recently reported the cloning of two splice variants of murine MKK7 and the N-terminus of human MKK7α (Tournier et al., 1997). The amino acid sequence predicted from their murine MKK7α clone is identical to the sequence that we report here, with the exception that it lacks the N-terminus that was present in all of our predicted human and murine MKK7 splice variants. We were able to immunoblot endogenous MKK7 in lysates of Ba/F3 hematopoietic cells and MC/9 mast cells using an antiserum raised against the N-terminus of our human and murine MKK7 sequence (Fig. 5.9), and therefore we are confident that the form of MKK7 predicted from our human and murine cDNA is indeed expressed in murine cells.

While this work was being completed, a number of articles describing the cloning of MKK7 were published (Holland et al., 1997; Lawler et al., 1997; Lu et al., 1997; Moriguchi et al., 1997; Wu et al., 1997; Yao et al., 1997). Holland et al. identified two isoforms of murine MKK7, one corresponding to murine MKK7α and the other having a unique N-terminus (Holland et al., 1997). Moriguchi et al. cloned murine MKK7γ and identified two MKK7 isoforms using an antiserum raised against full length murine MKK7γ (Moriguchi et al., 1997). Based on electrophoretic mobility, we believe that the larger isoform could represent the murine equivalent of our human MKK7β (Fig. 5.5). Lu et al. have identified another isoform of human MKK7 that has the same N-terminus and kinase domain as our MKK7 isoforms but contains 70 amino acids that are not found in any of the MKK7 isoforms reported to date (Lu et al., 1997).

Based on sequence identities among the known MKKs (Fig. 5.7), we investigated the ability of MKK7 to activate JNK or p38 MAPK and showed that in co-transfection assays MKK7
acted upstream of JNK1 (Fig. 5.11), but not p38 MAPK (Fig. 5.12). The identification of JNK1 as an *in vitro* substrate for MKK7 permitted us to investigate the ability of a range of stimuli to activate transiently expressed MKK7. In HeLa cells, MKK7 was strongly activated by hyperosmotic shock, UV light, anisomycin, heat shock and to a lesser extent TNFα (Fig. 5.13). We observed no detectable activation of MKK7 in HeLa cells treated with EGF (Fig. 5.13, top). These results are similar to that seen by Holland et al. with PDGF in NIH 3T3 cells (Holland et al., 1997). In Ba/F3 hematopoietic cells, hyperosmotic shock and UV light also activated MKK7 (Fig. 5.14). Our observations that stimulation of Ba/F3 cells with IL-3 increased the activity of MKK7 (Fig. 5.14 and 5.16) correlates with recent observations that JNK was activated by a range of hematopoietic growth factors including IL-3, GM-CSF, G-CSF, EPO or SLF (Foltz and Schrader, 1997; Nagata et al., 1997; Rausch and Marshall, 1997; Terada et al., 1997). Moreover, our observation that IL-4 failed to activate MKK7 (Fig. 4c, 5c) correlates with the inability of IL-4 to activate the Ras, ERK, p38 or JNK MAP kinase pathways in hematopoietic cells (Duronio et al., 1992; Foltz et al., 1997; Foltz and Schrader, 1997; Satoh et al., 1991; Welham and Schrader, 1992).

Co-expression of activated mutants of Ras, Rac or Cdc42 with MKK7 in HeLa cells resulted in readily detectable activation of MKK7 and MKK4 (Fig. 5.19). This is consistent with previous work which demonstrated that the Ras and Rho family of small GTPases are capable of activating JNK (Bagrodia et al., 1995; Coso et al., 1995; Derijard et al., 1994; Hibi et al., 1993; Minden et al., 1995). Slightly different results were obtained in Ba/F3 cells where MKK7 was activated by co-expression of activated Rac or Cdc42, but not Ras (Fig. 5.20). The inability of Ras to activate MKK7 was not surprising as a constitutively active Ras was insufficient to activate JNK in Ba/F3 cells, although IL-3-induced JNK activation was blocked by expression of a dominant-negative mutant of Ras (Terada et al., 1997). Taken together, these results support the notion that Ras is necessary but not sufficient for JNK activation. Our evidence that IL-3 increased MKK7 activity (Fig. 5.14 and 5.16), but that Ras alone failed to increase MKK7 activity (Fig. 5.20), is also consistent with data on activation of JNK by G-CSF, which is structurally related to
IL-3 and acts through a similar receptor. These experiments showed that activation of JNK in cells stimulated with G-CSF depended upon both an intact Ras signalling pathway, as well as a specific tyrosine residue in the G-CSF receptor (Rausch and Marshall, 1997). Together these data suggest that activation of MKK7 in response to IL-3 involves both activation of the Ras pathway and an as yet unknown signal.

The receptor for the Fc fragment of Immunoglobulin G (FcR) has many important roles in the immune system (Takai, 1996). These include the phagocytosis of Ig-coated particles by macrophages and neutrophils, the antibody-dependent cell mediated cytotoxicity by NK cells, the down-regulation of signalling through the BCR, and the release of TNFα and other mediators by macrophages and mast cells. Our observation that ligation of FcR results in the activation of both endogenous MKK4 and MKK7 in MC/9 mast cells (Fig. 5.18) supports previous findings that signalling through the FcR activates JNK in bone-marrow derived macrophages (Rose et al., 1997). Recent studies have shown that signalling through the JNK pathway is required for the production of TNFα in MC/9 mast cells (Ishizuka et al., 1997). As signalling through the FcR activates JNK through MKK4 and MKK7, it will be important to determine their individual roles in the production of TNFα in mast cells.

The existence of multiple activators of JNK which are responsive to different stimuli was established by the fact that MKK4-deficient ES cells still exhibited activation of JNK in response to hyperosmolarity and UV light, but not heat shock or anisomycin (Derijard et al., 1995; Lin et al., 1995). In that MKK7 is activated by all of the above stimuli (Fig. 5.13, 5.14 and 5.16), and MKK7 mRNA is present in ES cells (Table 5.3), the failure of MKK4-deficient ES cells to activate JNK in response to heat shock or anisomycin is paradoxical. It is possible that, although MKK7 is expressed in ES cells, it is not activated by heat or anisomycin because of cell-specific differences in upstream activators. This notion is supported by a recent report that in KB and U937 cells TNFα activates MKK7, but not MKK4 (Lawler et al., 1997; Moriguchi et al., 1997), whereas in HeLa cells we observed that TNFα activates both MKK7 and MKK4 (Fig. 5.17), an observation recently reported by Wu et al. (Wu et al., 1997). MKK4 has also been recently
reported to be activated in normal bone marrow-derived macrophages treated with TNFα, supporting the notion that these kinases will be regulated differently depending on their cellular context (Winston et al., 1997). Furthermore, Wu et al. have reported that ASK1 and GCK activate MKK7 in preference to MKK4, whereas MEKK1 and MEKK2 activate both MKK4 and MKK7 to comparable levels (Wu et al., 1997).

The functional significance of the activation of MKK7 is unclear but its activation by physiological stimuli such as IL-3 (Fig. 5.14 and 5.16), by ligation of immunoregulators such as FcR (Fig. 5.18), CD40, BCR, CD3 (Foltz et al., 1998, M. Luckach and R. Salmon, unpublished observations), and by the GTPases Ras, Rac and Cdc42 (Fig. 5.19 and 5.20) suggests that the role of MKK7 will not be confined to stress responses. The existence of an ortholog of MKK7 in *C. elegans* and our failure to identify an ortholog in *S. cerevisiae* suggest that MKK7 arose in evolution during the transition from single cellular to multicellular organisms. The notion that MKK7 is involved in processes important for multicellular organisms, such as embryonic development, chemotaxis or apoptosis, is in keeping with evidence that mutations in dHep and Bsk, the *Drosophila* homologue of JNK1, result in a similar failure of epithelial cell movement and dorsal closure (Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996). Holland et al. have demonstrated that MKK7 is able to partially complement a deficiency of dHep in *Drosophila* (Holland et al., 1997). This demonstrates that MKK7 is highly conserved functionally and suggests that it may play a role in embryological development in mammals. The embryonic lethality resulting from disruption of the MKK4 gene in mice also points to the importance of normal JNK signalling during embryonic development (Ganiatsas et al., 1998; Nishina et al., 1997; Yang et al., 1997), and indicates that MKK4 and MKK7 have discrete physiological functions. Future work detailing the function of individual activators of JNK is likely to reveal roles in multiple aspects of development and other physiological processes, including hematopoiesis and immune responses.
CHAPTER 6 - Conclusion

The stress-activated protein kinases, p38 MAPK and JNK, are activated by diverse stimuli acting on a cell. When I began working in this field, these kinases were known to be activated by cellular insults including hyperosmotic shock, UV irradiation, heat shock and the pro-inflammatory cytokines TNFα and IL-1. In contrast, growth factors such as EGF and PDGF failed to activate these enzymes to comparable levels. We had some biochemical evidence that a protein with similar isoelectric point and electrophoretic mobility as p38 MAPK was tyrosine phosphorylated by the hematopoietic growth factors, IL-3 and SLF (Welham and Schrader, 1992), and we hypothesized this unknown phosphoprotein was p38 MAPK. I demonstrated that p38 MAPK was indeed tyrosine phosphorylated and activated by these growth factors. Furthermore, we demonstrated that p38 MAPK activity was required for the activation of MAPKAP kinase-2 by these cytokines. Previous work in our laboratory indicated that IL-4 was unable to activate ERK MAPK or Ras (Duronio et al., 1992; Welham et al., 1992; Welham et al., 1994), and we were interested to examine the ability of IL-4 to activate p38 MAPK. I found that IL-4 was unable to activate p38 MAPK, or MAPKAP kinase-2, consistent with the notion that MAPKAP kinase-2 was a substrate of p38 MAPK in vivo.

Growth factors are important not only for the proliferation, but also for the survival of hematopoietic cells. We hypothesized that p38 MAPK would regulate some aspect of cytokine action. Indeed, we demonstrated using the inhibitor, SB 203580, that the activity of p38 MAPK was required for DNA synthesis. However, an SB 203580-resistant mutant of p38α MAPK failed to restore DNA synthesis, suggesting that p38α MAPK was not sufficient for DNA synthesis. The activation of MAPKAP kinase-2 was completely restored by the SB 203580-resistant mutant of p38α MAPK after hyperosmotic shock, suggesting that MAPKAP kinase-2 activity was not required for DNA synthesis. However it is not clear if p38α MAPK activity was completely restored under normal cell culture conditions. Since SB 203580 also inhibits p38β MAPK, these data suggest a role for p38β MAPK in DNA synthesis, or perhaps another target of this inhibitor.
When I began working in the stress-activated protein kinase field, all stimuli that activated p38 MAPK also activated JNK. I hypothesized that the hematopoietic growth factors that activated p38 MAPK would also activate JNK. Indeed, I demonstrated that IL-3, GM-CSF or SLF activated both 45 and 55 kDa isoforms of JNK1 and JNK2. Consistent with the failure of IL-4 to activate p38 MAPK, I also hypothesized that IL-4 would fail to activate JNK. The inability of IL-4 to activate Ras also supported the notion that IL-4 would fail to activate JNK as growth factors such as EGF activated JNK in a Ras-dependent fashion. Indeed, I found that IL-4 failed to activate JNK in hematopoietic cells. Together, these results indicated that IL-4 was unique among cytokines that we examined in its failure to activate any MAPK family member, or the small GTPase Ras. These findings lead to many important biochemical questions, including what is the role of Ras for the activation of p38 MAPK, do these hematopoietic growth factors activate Rac or Cdc42, and what are the biological implications of IL-4 failing to activate Ras or any MAP kinases?

The JNK family of protein kinases was known to be regulated by a single MAPK kinase, MKK4, at the time I was studying the activation of JNK by hematopoietic growth factors. Therefore, we presumed the activation of JNK by hematopoietic growth factors was through MKK4. Consistent with this hypothesis, we detected an increase in the threonine phosphorylation of MKK4 after stimulation with GM-CSF or SLF. However, the phosphorylation was greater with SLF than with GM-CSF or NaCl despite the fact that these stimuli activate JNK to a similar extent. We hypothesized this finding reflected the existence of another unidentified JNK kinase in MC/9 mast cells that was activated in cells after treatment with GM-CSF or NaCl. Several independent reports supported the existence of another JNK kinase (Meier et al., 1996; Moriguchi et al., 1995; Nishina et al., 1997a; Yang et al., 1997b). We screened the expressed sequence tags database and identified a novel JNK kinase, MKK7, with a great degree of homology to MKK4. We demonstrated this enzyme was activated by NaCl and IL-3, a cytokine that signals through a receptor that is shared with GM-CSF. Furthermore, MKK7 was expressed in MC/9 mast cells,
implicating MKK7 as the molecule we hypothesized to be activated after cells were treated with hyperosmolarity or GM-CSF.

My demonstration that JNK and p38 MAPK were activated by hematopoietic growth factors provided the first evidence that these enzymes were strongly activated by growth factors, in contrast to previous findings with EGF or PDGF. The activation of both of these enzymes by GM-CSF and SLF were on the same order of magnitude as that seen with hyperosmotic shock. Since then several physiologically relevant stimuli have been identified as activators of JNK and p38 MAPK including the hematopoietic growth factors Erythropoietin and Thrombopoietin, and receptors involved in the regulation of the immune system including the Fc receptors for IgG or IgE, the B cell antigen receptor and CD3, a component of the T cell antigen receptor. These findings are consistent with the notion that these enzymes have important physiological functions beyond simply a response to stress, and are supported by proposed roles for these kinases in cytokine production, apoptosis, proliferation, tumorigenesis, embryogenesis and organogenesis.

However, the specific functions of these enzymes in mammalian cells, and indeed in the whole organism is poorly understood. Most of our understanding of the function of these enzymes are inferred through the use of over-expressed dominant negative mutants that act to sequester either upstream activators or downstream effectors of these kinases. This approach is useful as it can potentially tell us something about enzyme functions, but it has inherent problems as one never really knows if the dominant negative specifically inhibits the intended pathway. The ability to use both dominant negative proteins and a specific inhibitor has allowed a rapid determination of the functions of p38α MAPK and p38β MAPK. However, the inhibitor also has inherent problems as unknown targets for the compound might confound the interpretation of data. The production of new p38 MAPK mutants that are resistant to SB 203580, but retain their substrate specificity, will hopefully provide more plausible data on the biological function of these proteins and maybe differentiate between functions of p38α MAPK and p38β MAPK isoforms \textit{in vivo}. 

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Genetic analysis of the function of these enzymes is certainly a major focus of the future research in this field. Mice lacking MKK4 provided the first evidence for the non-redundant functions for MKK4 and MKK7, as cells lacking MKK4 were still able to activate JNK (Nishina et al., 1997). However, these mice die in utero, and as such did not provide that much information on the role of JNK kinase activity in vivo. A better understanding of the role of MKK4 in adult mice will likely require tissue specific or conditional disruption of this gene using the Cre recombinase. Mice lacking JNK and p38 MAPK are also being generated. As there are three JNK genes and four p38 MAPK genes, the determination of in vivo function may require the disruption of multiple genes. However, mice with the JNK1, the JNK2 or the JNK3 gene disrupted had phenotypes, indicating that these enzymes are not entirely redundant at least in some tissues (Yang et al., 1997a; Yang et al., 1998a, Dong et al., 1998).

The field is moving rapidly, and as our understanding grows, better experiments are being devised to address these fundamental questions. Future work may proceed to generate mice expressing SB 203580-resistant isoforms of both p38α MAPK and p38β MAPK under endogenous promoters. As the mechanism of action of this drug is very well established, these mice would address issues such as other targets of the compound. Most importantly, ES cells derived from these mice could then be used to introduce mutations into other kinases to confer sensitivity to SB 203580 (Gum et al., 1998). These projects are now feasible, and potentially provide a valuable tool to determine the in vivo function of any number of kinases.
Bibliography


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