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IDENTIFICATION, INTERACTIONS AND SPECIFICITY OF A NOVEL MAP KINASE KINASE, MKK7

by

Pamela M. Holland

A dissertation submitted in partial fulfillment of the requirements for the degree of

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Approved by _____
Chairperson of Supervisory Committee

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Program Authorized to Offer Degree Department of Biochemistry

Date 3-16-99

Doctoral Dissertation

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University of Washington

Abstract

IDENTIFICATION, INTERACTIONS AND SPECIFICITY OF A NOVEL MAP KINASE KINASE, MKK7

by Pamela M. Holland

Chairperson of the Supervisory Committee: Jonathan A. Cooper
Department of Biochemistry

The mitogen activated protein kinase (MAP kinase) cascade has emerged as an evolutionarily conserved element in the transduction of a wide variety of extracellular signals. In mammals, at least three distinct pathways have been identified. These include ERKs, activated by growth factors and other mitogenic stimuli; JNKs, activated by stressful stimuli such as cytokines, UV radiation and protein synthesis inhibitors; and p38 MAP kinases, which are also responsive to stress-related stimuli. Although the existence of distinct MAP kinase pathways has been suggested to provide a basis for signaling specificity, it remains unclear how this might be achieved at a molecular level. One shared characteristic of MAP kinases is their requirement for phosphorylation on both threonine and tyrosine within a TXY motif for activation. These reactions are catalyzed by a family of kinases termed MAP kinase kinases (MKKs). Multiple MKKs have been shown to share specificity for a particular MAP kinase.

In order to further understand the activation, localization and substrate specificity of MKKs, a yeast two-hybrid library screen with MKK1 was performed. Results from this screen identified a novel MKK, termed MKK7. MKK7 is activated by stresses, including exposure of cells to osmotic shock or inflammatory cytokines such as interleukin-1. MKK7 directly phosphorylates and activates the MAP kinase JNK. Several stress-activated protein kinases are capable of activating MKK7, indicating that MKK7 may

function in multiple stress-mediated signaling pathways. MKK7 is a murine homolog of *Drosophila* Hemipterous (Hep) and can functionally rescue Hep deficient flies. Loss of function of Hep in *Drosophila* inhibits dorsal closure, a morphogenetic movement during late embryogenesis.

The human homolog of a *Drosophila* coiled-coil protein, BicaudalD (hBicD) associates specifically with MKK7, and this association is dependent on hBicD phosphorylation. In *Drosophila* BicD functions to properly localize developmental factors within the oocyte and the developing embryo. One role of hBicD may be to localize MKK7, or components of an MKK7 signaling pathway, to discrete locations within the cell. The properties of MKK7 demonstrate it may be important for normal development and for mediating stress responses, and these events may be partially regulated by its precise cellular localization.

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GLOSSARY

Anoikis. A process of apoptosis in adherent cells triggered by loss of intergrin mediated contacts with extra-cellular matrix.

Apoptosis. Programmed cell death; a specific suicide process in animal cells that occurs normally during development, or may be induced, for example by DNA damage that exceeds the capacity of repair mechanisms.

Dual-specificity kinase. A protein kinase that is capable of phosphorylating both serine/threonine and tyrosine residues in proteins. e.g.: MKK family members.

Kinase. An enzyme that adds the γ phosphate group from ATP to a substrate.

Morphogen. A molecule whose local concentration gradient determines the expression pattern of other molecules.

Phosphatase. An enzyme that removes a phosphate group from a substrate by hydrolysis.

Transgene. A cloned gene that is introduced and stably incorporated into an organism and is passed on to successive generations.

LIST OF ABBREVIATIONS

AKAP. A-kinase anchoring protein

ASK. apoptosis stimulating kinase

ATF2. activating transcription factor 2

BCR. B-cell receptor

BSA. bovine serum albumin

cDNA. complementary DNA

CRIB. Cdc42, Rac GTP-ase binding motif

DNA. deoxyribonucleic acid

ERK. extracellular signal regulated kinase

GCK. germinal center kinase

GST. glutathione-S-transferase

GTP. guanosine 5' triphosphate

HA. hemagglutinin

HGK. HPK/GCK like kinase

HOG. hyperosmolarity glycerol

HPK. human progenitor kinase

HSP. heat shock protein

ICE. interleukin converting enzyme

IL-1. interleukin-1

IL-2. interleukin-2

IL-8. interleukin-8

IP. immunoprecipitation

JIP. JNK interacting protein

JNK. Jun-NH_2 terminal kinase

JNKK. Jun-NH_2 terminal kinase kinase

JNKK2. Jun-NH_2 terminal kinase kinase 2

KHS. kinase homologous to SPS/STE20

MAP. mitogen activated protein

MAP-2. microtubule associated protein-2

MAPKAP. MAP kinase activated protein

MAPK. mitogen activated protein kinase

MAPKK. mitogen activated protein kinase kinase, also known as MKK, MEK

MAPKKK. mitogen activated protein kinase kinase kinase, also known as MEKK

MAPKKKK. mitogen activated protein kinase kinase kinase kinase

MEK. MAP kinase or ERK kinase, also known as MKK, MAPKK

MEKK. MAP kinase or ERK kinase kinase, also known as MAPKKK

MEKKK. MEKK kinase, also known as MAPKKKK

MHC. major histocompatibility complex

MIA. microtubule-interfering agent

MLK. mixed lineage kinase

MKK. MAP kinase kinase, also known as MAPKK, MEK

MKKIP. MKK interacting protein

mRNA. messenger RNA

MKP. MAP kinase phosphatase

MT. myc-tagged

MTOC. microtubule organizing center

Mxi. Max interactor

NES. nucelar export signal

NGF.nerve growth-factor

NFκB. nuclear factor κB

NIK. Nck interacting kinase

NIK. NFκB inducing kinase

PAGE. polyacrylamide gel electrophoresis

PAK. p21 activated kinase

PBS. phosphate buffered saline

PCR. polymerase chain reaction

PDGF. platelet derived growth-factor

PIPES. 1,4-piperazinediethanesulfonic acid

PMA. phorbol 12-myristate 13-acetate

POSH. plethora of SH3 domain containing protein

RACE. rapid amplification of cDNA ends

ROS. reactive oxygen species

RT-PCR. reverse transcriptase-polymerase chain reaction

SAPK. stress-activated protein kinase

SDS. sodium dodecyl sulfate

SEK. SAPK or ERK kinase

SH2. Src-homology 2

SH3. Src-homology 3

SPRK. SH3-domain containing proline-rich kinase

TAB. TAK activator binding protein

TAK. TGF-β activated kinase

TAO1. thousand and one amino acid kinase

TCF. ternary complex factor

TCR. T-cell receptor

TNF. tumor necrosis factor

TNFR. tumor necrosis factor receptor

TRAF. TNF receptor associated factor

UTR. untranslated region

UV. ultraviolet radiation

WB. Western blot

PREFACE

At the time this project was initiated a question existed concerning how signaling specificity was achieved by the MAPK cascade in mammals. Data from numerous labs employing a variety of cell types suggested that components of the pathway could be activated in response to a multiple extracellular stimuli and result in diverse cellular events. How could multiple signals converge on one set of proteins and result in different outputs? The existence of multiple parallel protein kinase cascades in lower eukaryotes implied strongly that related pathways would be found in mammalian cells. Indeed the discovery of MAPK cascades that are primarily activated in response to stresses has increased our understanding of how mammalian signaling specifity is achieved. Yet there is still considerable overlap in the stimuli that activate related MAPK cascades, suggesting that additional means of providing for specificity must exist. As previsously, knowledge obtained from lower eukaryotes as to how MAPK cascades are architecturally set up in cells may provide answers. The idea of secondary proteins which act to scaffold or properly localize components of a pathway to a particular cellular compartment or structure is yet another example of how incoming signals may be organized to result in specific cellular events. Our identification of a new MAP kinase kinase activated by stress and the identification of a new interacting protein which may serve to localize MKK7 to the cytoskeleton provide examples of ways in which cells coordinate intracellular signals. The existence of multiple activators for distinct targets allows for greater signal integration and perhaps fine tuning. Additionally, the subcellular distribution of and compartmentalization of signlaing pathways or components will also contribute to mediating specificity. Our understanding of how related pathways respond to different stimuli in a coordinated manner that regulates cellular metabolism is still naive. Undoubtedly there is far greater complexity that exists in intracellular communication that with advancements in technology and means to analyze cellular systems will slowly emerge.

ACKNOWLEDGMENTS

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I also wish to acknowledge the support and encouragement of all my friends and family, particularly during the loss of my brother Peter, my father Leo, and my uncle Cecil. They have all in their own ways kept me in touch with what is important in life.

Much of this work could not have been done without the help of many collaborators over the years who have maintained a continued interest in my project, even after the "official" collaboration was finished. This includes Jean Campbell, Stephane Noselli, Jerry Saklatvala, Michael Kracht, Jim Hopper and Pepe Alberola-Ila. Although Yukiko Gotoh was not really a collaborator --particularly in light of the fact that her student was cloning the same gene--her unwavering interest and support, as well as her friendship, has continued to be an inspiration.

Thanks also to the many individuals who have provided helpful discussions as well as provided numerous reagents, including Natalie Ahn, Roger Perlmutter, Irma Sanchez, Alan

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Special thanks to my advisor Jonathan Cooper for much scientific input and support, and members of my graduate committee, Richard Palmiter, Ed Krebs, Bob Eisenman and Susan Parkhurst. Finally, special thanks to my 'other' advisor and all around best friend, for his continued love and support, El Profesor, Jonathan Graves.

DEDICATION

The author wishes to dedicate this dissertation to the memory of her father, Leonard Holland (Sept. 29, 1924-May 17, 1997). He always believed an education was the best investment you could make (it's the only thing he ever gave me money for!) and would have been happy to see the completion of this undertaking.

INTRODUCTION

A MODEL FOR SIGNAL-TRANSDUCTION PATHWAYS:

A central question in biology is how signals from the cell surface can modulate intracellular processes. Changes in cell behavior are induced by a diverse set of extracellular signaling molecules, including hormones, growth-factors, and cytokines. Many of these factors exert their effects by binding to cell surface receptors, which act to mediate signals into the intracellular machinery. Events initiated from environmental signals control the activity and abundance of proteins crucial to virtually every cellular function. These events may vary from cell growth or migration to differentiation to apoptosis.

The flow of information from the cell surface into the cell interior involves the coordinated activities of specific enzymes and protein-protein interactions. These target enzymes are often called effectors, because changes in their activity or structure cause changes in other enzymes that ultimately lead to the cellular response. The target enzymes which respond to signals from cell surface receptors are largely comprised of families of tyrosine and serine/threonine kinases. In fact, many growth-factor receptors are themselves tyrosine kinases, and ligand binding to such receptors can induce their tyrosine kinase activity. Other receptors have binding sites for protein kinases that are exposed when receptors are activated. This results in the recruitment of additional protein kinases, leading to the activation of multiple protein kinases in a sequential fashion.

One of the primary features of a signal-transduction pathway involves protein phosphorylation. Phosphorylation alters the charge and sometimes the local conformation of a protein, generally changing its activity. It is a rapid, reversible event,

therefore an attractive means of rapidly activating or inhibiting a protein. The successive activation of protein kinases by phosphorylation results in a kinase cascade, which allows for mutliple regulatory steps. Each kinase within the cascade can itself be regulated. This includes feedback within a particular cascade as well as via crosstalk with related pathways.

Extensive research into proteins that make up signal-transduction pathways stemmed from the fact that components of these pathways were found to be cellular derivatives of known oncogenes, which when overexpressed in cells could lead to cellular transformation. Most oncogenes derive from genes whose products act along normal cellular growth-controlling pathways. These include growth-factors, receptors, intracellular signal transducers, nuclear transcription factors and cell-cycle-control proteins. The identification of the Ras oncogene as a GTP-binding protein that could regulate mitogenic and developmental signals within cells was an important step in understanding the biochemical basis of signaling pathways. The realization that cellular Ras might be activated by growth-factors was brought about by findings that microinjection of Ras antibodies could block DNA synthesis induced by receptor and non-receptor tyrosine kinases (Feramisco *et al.*, 1984; Mulcahy *et al.*, 1985). In response to growth-factor stimulation, Ras was found to accumulate in an active, GTP-bound state. These breakthroughs placed Ras downstream of growth-factor receptors as a potential effector in receptor signal-transduction pathways.

It is now well established that Ras activation results in the activation of a family of intracellular kinases known as the mitogen-activated protein kinases (MAP kinases), one class of which are referred to as the extracellular signal regulated kinases (ERKs). Activation of ERKs results a variety of events, one of which is the transcription of immediate-early genes required for proliferative responses. The active Ras-GTP complex initiates the sequential activation of three downstream kinases: Raf protein kinase, MAP kinase kinase 1 and ERK MAP kinase (Fig. 1). MAP kinase pathways in general are made up of a three component cascade consisting of a serine/threonine kinase (a MAP kinase kinase kinase, MKKK, or MEKK) which phosphorylates and activates a dual specificity kinase (a MAP kinase kinase, MKK, MAPKK, or MEK), which in turn phosphorylates and activates another serine/threonine kinase (MAP kinase). The Ras-MAP kinase cascade that leads to activation of the ERKs is the best characterized mammalian

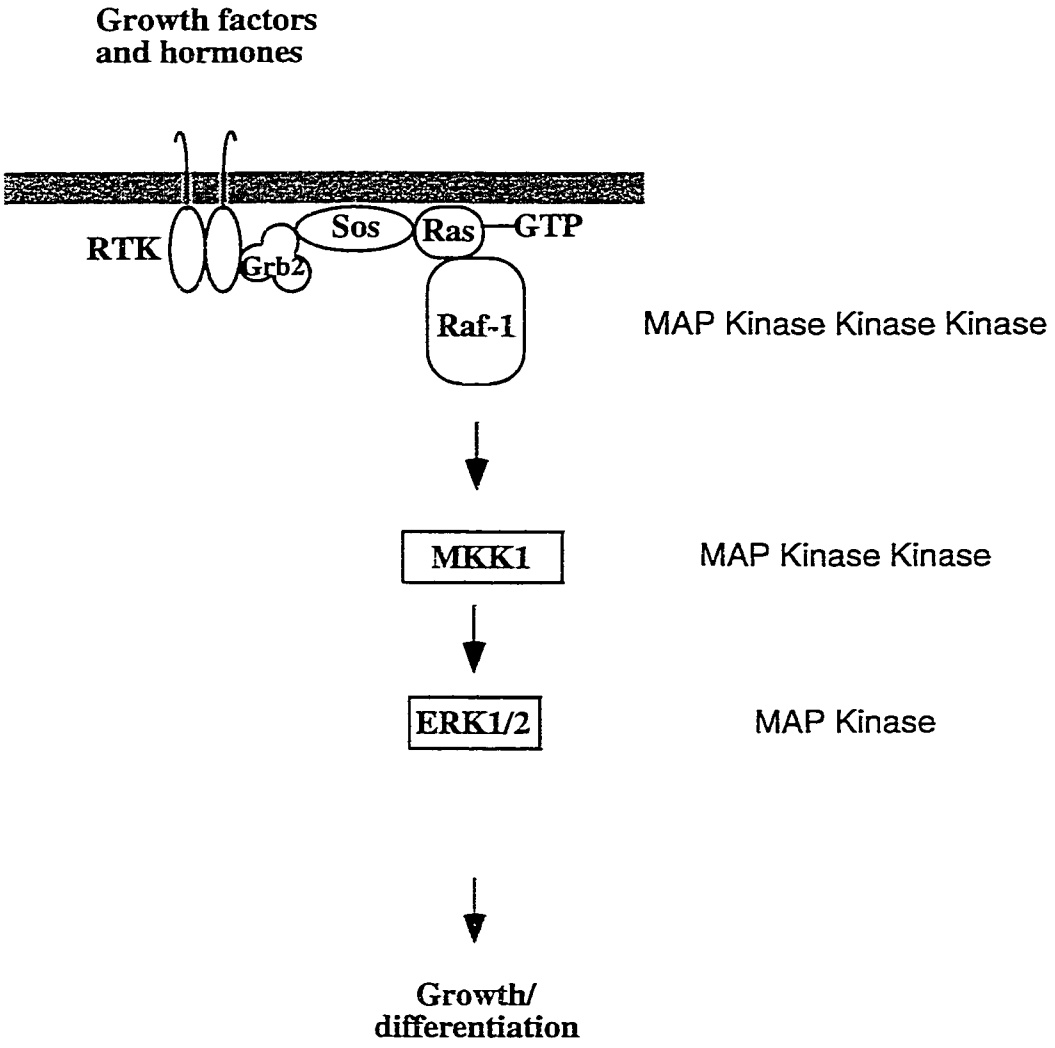


Fig. 1. The "classical" Ras-MAP kinase cascade. Illustrated is an example of the sequential activation of kinases within a cascade. Activation of a growth-factor receptor tyrosine kinase by binding of its cognate ligand creates binding sites for the adaptor protein Grb2 on its cytoplasmic domain. Grb2 also binds the Ras exchange factor Sos, thereby coupling active GTP-Ras to the receptor. Active Ras can activate the MAP kinase kinase kinase, Raf-1, which in turn activates MKK1, which in turn activates ERK1/2 MAP kinases. Although not diagrammed here, Ras may also be activated by coupling to various other receptors, resulting in the activation of Raf-1, MKK1 and ERK.

MAP kinase pathway. An understanding of the components in this pathway has served as a point from which to trace signal-transduction back through a protein kinase cascade and Ras to the plasma membrane, and forward to the regulation of various processes such as transcription in the nucleus.

The importance of signaling pathways in regulating metabolic processes is perhaps best exemplified by their high degree of evolutionary conservation. The MAP kinase pathway has been highly conserved during eukaryotic evolution. Genetic analysis of signal-transduction pathways in *S. pombe* and *S. cerevisiae*, as well as *Drosophila* and *C. elegans*, have revealed that homologous pathways function in the cellular differentiative pathways and in response to external stimuli. In *S. cerevisiae*, at least four distinct signal-transduction pathways exist, each stimulating unrelated physiological responses (Fig. 2) (Herskowitz, 1995). For example, response to mating pheromones produced by the opposite mating type via a G-protein βγ subunit can sequentially activate four protein kinases: STE20, STE11, STE7 and FUS3. STE7 is structurally similar to MKK1, and is phosphorylated in response to pheromone. The FUS3 MAP kinase is activated by STE7 phosphorylation of the analogous threonine and tyrosine residues involved in vertebrate ERK MAP kinase stimulation. The protein STE5 is capable of binding STE11, STE7 and FUS3, thereby providing a scaffold and localizing components of the mating response pathway (Choi *et al.*, 1994). Several of the kinases in this pathway are utilized for filamentous growth as well as mating responses. Both events require the protein kinases STE20, STE11 and STE7, but a distinct MAP kinase other than FUS3 is utilized for pseudohyphal growth (Madhani *et al.*, 1997). Instead, the KSS1 MAP kinase is activated, resulting in a filamentation/invasion response rather than a mating response.

Another pathway mediated by the yeast protein kinase C isozyme, PKC1, regulates cell-wall construction by activation of BCK1 (related to STE11), and MKK1 and MKK2, a redundant pair of yeast kinases related to STE7, which function upstream of MPK1 (related to FUS3, KSS1 and ERK MAP kinase). A third pathway which responds to conditions of high osmolarity in the environment involves activation of PBS2 (HOG4), an MKK1 relative, which subsequently activates HOG1 (high osmolarity glycerol-1), an ERK MAP kinase relative. Components upstream of PBS2 include SLN1, a histidine kinase, its response regulator SSK1, and a second osmosensor, SHO1 (Maeda *et al.*, 1995). SHO1 contains four transmembrane segments and can bind a proline rich region on

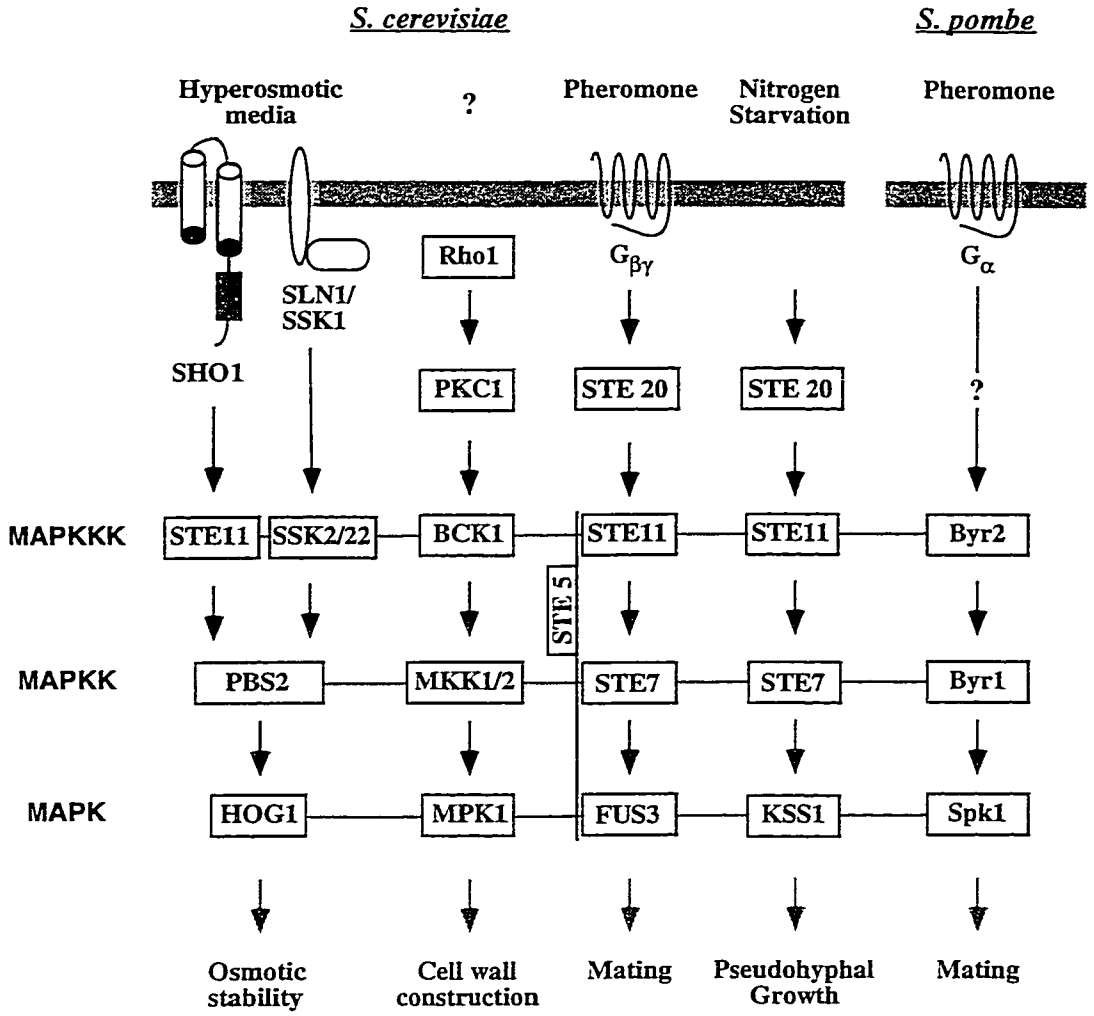


Fig. 2. Homologous MAP kinase pathways in yeast. Environmental signals can activate independent conserved MAP kinase pathways, each with a specific MAPKKK, MAPKK and MAPK (described in the text). Activation of each distinct pathway leads to a specific cellular response. Question marks indicate that specific signals or mechanisms of activation have not been fully described.

PBS2 directly via its C-terminal SH3 domain (not shown in Fig. 2). In *S. pombe* a G-protein α-subunit is the mating-response effector, which is coupled to the downstream components Byr2, Byr1 and Spk1, which correspond to MKK1 kinase, MKK1, and MAP kinase homologs, respectively.

A relatively high degree of specificity is maintained among these related pathways. For example, *S. cerevisiae* lacking HOG1 no longer respond to osmotic stress but retain their ability to respond to mating pheromone (Brewster *et al.*, 1993). This suggests that elimination of a protein kinase in one pathway does not compromise the function of another. However, in a yeast strain lacking the MAP kinase FUS3, the MAP kinase KSS1 is able to fill in and restore the mating response, resulting in a loss of signaling specificity (Madhani *et al.*, 1997). This occurs only in the absence of FUS3, and may be due to the fact that all upstream components are shared. In other studies, both the *S. pombe* MAPKKK, Byr2, and its substrate, the MAPKK, Byr1, are required for the rescue of a *S. cerevisiae* STE11 deletion. Similarly, rescue of Byr2 or Byr1 defects requires co-expression of both MKK1 and Raf-1 (Hughes *et al.*, 1993). Thus, the degree of specificty appears to be related to the substrate specificity of kinases within each pathway.

In addition, studies in R7 photoreceptor development and terminal cell-fate development in *Drosophila*, and studies in vulval development in *C. elegans*, have demonstrated the presence of elements of the MAP kinase pathway which may be activated in response to signals from receptor protein tyrosine kinases (Sundaram and Han, 1996; Wassarman *et al.*, 1995). As a result of these observations, the MAP kinase pathway appears to play a critical role in the transduction of diverse receptor-generated signals from the membrane to the cytoplasm and nucleus.

THE ERK MAP KINASE PATHWAY:

In mammalian cells, three MAP kinase pathways have now been identified and characterized. These are the ERK, SAPK and p38 MAP kinase pathways. ERK's were the first mammalian MAP kinases to be identified. Two isoforms, p44 ERK1 and p42 ERK2 have been studies most. As previously stated, the activation of ERKs in a pathway downstream of growth-factor receptors and Ras leads to the activation of multiple substrates. ERKs have been implicated in the regulation of cytosolic phospholipase A_2.

phosphorylation of cytoskeletal components such as microtubule associated protein 2 (MAP-2), and phosphorylation of the transcription factors c-Myc, c-Jun and p62 TCF/Elk-1 (Robinson and Cobb, 1997).

Activation of p42/p44 ERK MAP kinase requires phosphorylation on both threonine and tyrosine residues within a conserved TEY motif in kinase subdomain VIII. Sequence alignment of ERK with other kinases such as PKA and RSK illustrates the presence of regulatory phosphorylation sites between the invariant DFG and A/SPE residues in these subdomains. Phosphorylation of sites within this region is a common mechanism for activation of kinases, and it is often referred to as the activation loop (Johnson *et al.*, 1996). The kinase known as MAP kinase kinase 1 (MKK1) is capable of phosphorylating both threonine and tyrosine residues resulting in the activation of ERK MAP kinase (Robinson and Cobb, 1997). MKK1 belongs to a class of dual specificity kinases that can phosphorylate both tyrosine and serine or threonine residues in proteins. The structural basis for the dual specificity of MAP kinase kinase is not well understood, and a detailed structural characterization of any member of this class of kinases has yet to be described. MKK1 displays a high degree of specificity for ERK MAP kinase, no other substrates have been identified. MKK1 does not recognize denatured ERK MAP kinase or peptides derived from ERK MAP kinase as a substrate (Seger *et al.*, 1992). Two isoforms of MKK have been purified from cells in culture (MKK1, 45 kDa; MKK2, 46 kDa), and three peaks of activity have been identified in rabbit muscle (Matsuda *et al.*, 1992; Seger *et al.*, 1992).

Initial studies indicated that MKK1 is itself activated by phosphorylation, suggesting the existence of a MKK1 kinase. The first such kinase to be identified was the product of the c-Raf proto-oncogene, Raf-1 (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Confirmation of Raf-1 function as an MKK1 activator was provided by studies in which Raf-1 immunoprecipitated from serum-stimulated cells could activate MKK1, and the ability of baculovirus-expressed Raf-1 to associate with MKK1 *in vitro* (Howe *et al.*, 1992; Dent *et al.*, 1992; Kyriakis *et al.*, 1992). The finding that Raf-1 could bind to active GTP-Ras and thereby recruit Raf-1 to the plasma membrane for activation provided the final link in a signaling cascade from Ras to MKK1 and ERK MAP kinase (Dent and Sturgill, 1994; Irie *et al.*, 1994; Leevers *et al.*, 1994; Stokoe *et al.*, 1994; Vojtek *et al.*, 1993). The precise nature of activation of Raf-1 remains unclear, however, it is

likely to involve the 14-3-3 proteins and other unidentified protein kinases (Irie *et al.*, 1994).

Later studies established that A-Raf and B-Raf, kinases related to Raf-1, can also function as MKK activators (Lange-Carter and Johnson, 1994; Vaillancourt *et al.*, 1994; Wu *et al.*, 1996). A-Raf has been shown to be specific for activating MKK1 but not MKK2. Raf-1 and A-Raf are ubiquitously expressed, whereas B-Raf is predominantly expressed in neuronal tissues and testes. Other evidence suggests that isoforms of Raf may not be the only activators of MKK1. Another serine/threonine kinase, the product of the c-Mos proto-oncogene, a kinase previously identified as a component of cytostatic factor (CSF) and whose expression is restricted to germ cells, has also been shown to function as an MKK1 activator (Nebreda *et al.*, 1993). Activated c-Mos fusion protein phosphorylates and re-activates purified, phosphatase-inactivated, rabbit muscle MKK1 *in vitro* (Posada *et al.*, 1993), and injection of activated c-mos fusion protein into *Xenopus* oocytes can activate ERK MAP kinase via MKK1. Another kinase termed MEK kinase 1 (MEKK1) was cloned on the basis of its homology to the Byr2 and STE11 proteins in *S. pombe* and *S. cerevisiae*, respectively (Lange-Carter *et al.*, 1993). Although MEKK1 was described as a MKK1 kinase, activation of MKK1 by MEKK1 is weak. Thus, MEKK1 may function predominantly to regulate proteins distinct from the ERK MAP kinase pathway. It is of interest to note that several activators for MKK1 exist, suggesting that MKK1 may function to integrate signals from multiple sources. In contrast, the substrate specificity for MKK1 is restricted to only ERK1 and ERK2.

THE STRESS-ACTIVATED MAP KINASES:

The existence of multiple parallel protein kinase cascades in lower eukaryotes suggested that additional pathways would be identified in vertebrates. The discovery of proteins related to ERK MAP kinase led the way to the elucidation of new signaling pathways that responded strongly to stressful stimuli and weakly to mitogenic stimuli. This was in contrast to activation of the Ras-MAP kinase pathway by mitogens, which resulted in a proliferative response. Activation of these new MAP kinases by stressful stimuli results in growth arrest, apoptosis or activation of immune cells. The first mammalian ERK MAP kinase relative to be identified was the 54 kDa polypeptide activated in rat liver by injection of cycloheximide (Kyriakis and Avruch, 1990). Like the ERK

MAP kinases, the p54 kinase shared a requirement for concomitant phosphorylation on both a threonine and tyrosine residue for activity, and was susceptible to protein phosphatases 1 or 2A (Kyriakis *et al.*, 1991). However, the phosphatase-inactivated p54 kinase could not be reactivated by active MKK1, which could readily activate ERK MAP kinase. This suggested that p54 was also activated by dual specificity phosphorylation, but by a kinase distinct from MKK1.

Independently of the work referred to above, Karin's laboratory was studying a 46 kDa kinase that was activated by UV radiation, bound to the amino-terminal of c-Jun, and phosphorylated this protein on serines 63 and 73 (Hibi *et al.*, 1993). Like Karin's p46 kinase, the p54 kinase was also found to be a potent c-Jun kinase (Kyriakis and Avruch, 1990; Pulverer *et al.*, 1991), and was preferentially activated by cellular stresses and inflammatory cytokines. These characteristics led to the naming of SAPK (stress-activated protein kinase) and JNK (c-Jun N-terminal kinase), names which are used interchangeably. This molecular cloning revealed a family of protein kinases encoded by at least three genes (SAPKα, SAPKγ and SAPKβ, or *jnk2, jnk1* and *jnk3*, respectively), with alternative splicing into as many as 12 isoforms (D'Erijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sluss *et al.*, 1994). All three genes are expressed as 46 kDa and 54 kDa protein kinases due to differential processing of the 3' coding region of the corresponding mRNA (Gupta *et al.*, 1996). SAPKα/JNK2 and SAPKβ/JNK1 are expressed ubiquitiously, whereas SAPKγ/JNK3 expression is restricted to brain, heart, and testis. SAPKα and SAPKβ have been shown to differ in substrate specificity *in vitro*. The SAPK/JNKs share only 40-45% sequence identity with the ERKs but contain the motif TPY at sites of regulatory phosphorylation in catalytic subdomain VIII (D'Erijard *et al.*, 1994; Sluss *et al.*, 1994). This corresponds to the TEY phosphorylation loop motif in ERKs (D'Erijard *et al.*, 1994; Kyriakis *et al.*, 1994). Like the ERKs, the SAPK/JNKs are proline-directed kinases, preferring substrates containing a serine/threonine proline sequence.

A second signaling mechanism activated by inflammation and environmental stress was uncovered with the identification of a p38 MAP kinase. This kinase was first purified as a macrophage polypeptide that became tyrosine phosphorylated *in situ* in response to bacterial lipopolysaccharide (Han *et al.*, 1994). The p38 MAP kinase is most closely related to *S. cerevisiae* HOG1 and *Xenopus* Mpk2 (82% identity). Like the SAPK/JNKs, p38 is activated by osmotic stress and appears to be weakly activated by

phorbol esters (Han *et al.*, 1994). The p38 kinase was also identified as part of a protein kinase cascade activated by IL-1β or physiologic stress, which culminates in MAPKAP kinase 2 activation and Hsp25/27 phosphorylation (Freshney *et al.*, 1994; Rouse *et al.*, 1994). Although ERK2 MAP kinase can also phosphorylate and activate MAPKAP kinase 2 *in vitro*, ERK2 is not significantly activated by stress. Activated p38 is also capable of increasing reporter gene expression mediated by the transcription factors ATF2 and Elk-1 (Raingeaud *et al.*, 1996). An isoform of p38 known as Mxi2 was also identified through a yest two-hybrid screen for proteins that interacted with Max (Zervos *et al.*, 1995). Max is a basic helix-loop-helix protein that binds the product of the immediate early gene c-myc and is essential for its DNA binding and trans activating activity (Blackwood and Eisenman, 1991). Mxi2 is a splice variant of p38, and both p38 and Mxi2 have been shown to phosphorylate Max (Zervos *et al.*, 1995). Like ERKs and SAPK/JNKs, p38 is also activated by dual phosphorylation on threonine and tyrosine residues within a TXY (TGY for p38) motif in catalytic subdomain VIII (Freshney *et al.*, 1994; Raingeaud *et al.*, 1995; Rouse *et al.*, 1994). HOG1 also exhibits the motif TGY within its activation loop.

Although more is known about the SAPK and p38 stress-activated pathways, additional less characterized MAP kinases have also been identified. The MAP kinase, ERK3, has been shown to be constitutively nuclear and lacks the dual phosphorylation motif (Cheng *et al.*, 1996) present in other MAP kinases. ERK5 also defines a new signal-transduction pathway (Zhou *et al.*, 1995). It has an unusually long C-terminal domain predicted to target ERK5 to the cytoskeleton, and is also referred to as BMK (big MAP kinase) (Lee *et al.*, 1995). The most recent MAP kinase to be identified is ERK7, a 61 kDa protein with a long C-terminal tail (Abe *et al.*, 1999). Although ERK7 contains the signature TEY phosphorylation motif of ERKs, ERK7 appears to be constitutively active and is not activated by either mitogenic or stressful stimuli. ERK7 may be regulated by interactions involving the C-terminal tail. These discoveries have led to the grouping of subfamilies of MAP kinases on the basis of sequence similarity, mechanisms of upstream regulation, and sensitivity to activation by different MAP kinase kinases. Except for ERK3 other MAP kinases appear to be both nuclear and cytoplasmic. Translocation of a subpopulation of active MAP kinases to the nucleus provides them with access to substrates such as transcription factors. In transfection experiments with mutant ERK2, stimulus-dependent nuclear translocation required neither kinase activity nor phosphorylation sites (Chen *et al.*, 1992; Lenormand *et al.*, 1993). In activated cells half

of the ERKs are bound to the cytokeleton, (Reszka *et al.*, 1995) suggesting they also function outside the nucleus to contribute to proliferative responses by regulating cytoskeletal reorganization.

THE STRESS-ACTIVATED MAP KINASE KINASES:

The identification of multiple MAP kinases in mammalian cells coupled with the conserved architecture of these cascades led to the search for additional MAP kinase activators. Since the SAPK/JNK and p38 MAP kinases had been demonstrated to be activated by osmotic stress in mammalian cells, a homolog of the *S. cerevisiae* HOG1 MAP kinase PBS2, also activated by osmotic stress, seemed a possible candidate. Degenerate PCR approaches based on sequence similarity to PBS2 led to the identification of MKK3 and MKK4 as activators of p38 and SAPK/JNK, respectively (D'Erijard *et al.*, 1995). More recently, MKK6 was also identified as a specific activator of p38 (Han *et al.*, 1996; Matsuda *et al.*, 1995; Moriguchi *et al.*, 1996). The independent cloning of murine and human homologs to a novel *Xenopus* MAP kinase kinase (XMEK2) led to the identification of SEK1 (SAPK/ERK kinase) and JNKK1 (JNK kinase 1), which corresponded to MKK4 (Lin *et al.*, 1995; Sanchez *et al.*, 1994). MKK4/SEK1 shares 45% identity with MKK1/2 and is a strong activator of SAPK/JNK, with no ERK activating activity. Overexpression of kinase-negative forms of MKK4/SEK1 were shown to block SAPK/JNK activation without blocking ERK activation.

Both MKK3 and MKK4/SEK1 are structurally related to MKK1. The identification of MKK3 and MKK4/SEK1 initially defined new signaling cascades that coupled cellular stress agonists to p38 and SAPK/JNK and the transcription factor c-Jun. However, analysis of extracts from 3Y1 fibroblasts exposed to hyperosmolarity were shown to contain at least four chromatographically distinguishable SAPK-activating factors, only one of which co-purified with MKK4/SEK1 immunoreactivity (Meier *et al.*, 1996; Moriguchi *et al.*, 1995). This suggested that additional specific activators of SAPK/JNK would be identified. The identification of MKK7 corresponds to one of these SAPK-activating activities. Both MKK4 and MKK7 have been shown to be potent activators of SAPK in response to stressful stimuli. Although the p38 and SAPK/JNKs may both be activated by some overlapping stimuli, their mechanisms of activation at the level of MAP kinase kinases appear distinct. It was initially proposed that MKK4 could

activate both SAPK/JNK and p38 (D'Erijard *et al.*, 1995). Targeted disruption of the MKK4 gene in mice has no effect on p38 activation, although SAPK/JNK activation is compromised (Yang *et al.*, 1997). This suggests that MKK4 may not be a physiological activator of p38, although it readily phosphorylates p38 *in vitro*. To date it appears that SAPK/JNK isoforms are activated by MKK4 and MKK7 whereas p38 isoforms are activated by MKK3 and MKK6.

Intriguing new data regarding the activation of SAPK by MKK4 and MKK7 has recently emerged. Cohen and colleagues have demonstrated that MKK4 and MKK7 may act synergistically to fully activate SAPK *in vitro* (Lawler *et al.*, 1998). Based on phospho-amino acid analysis, MKK4 was shown to predominantly phosphorylate tyrosine, whereas MKK7 predominantly phosphorylated the threonine residue within the TPY motif in the activation loop of SAPK. This suggests that other MAP kinases may also be coordinately regulated, for example, p38 by MKK3 and MKK6, and provides another model whereby extracellular signals may be integrated. Alternatively, because components used in these experiments are highly purified, they may not represent events in a physiological system, in which multiple proteins contribute to the activation response. No evidence for such a model has been previously identified by genetic analyses of MAP kinase pathways in lower organsims.

UPSTREAM OF MAP KINASE KINASES:

A complex picture has emerged of the components immediately upstream of MKK4 and MKK3/MKK6 (Fig.3). There are now reports of at least seven MAPKKK's that have been shown to phosphorylate and activate MKK4 *in vitro*. The protein kinase MEKK1, originally identified as an activator of MKK1 (Lange-Carter *et al.*, 1993), is also capable of activating MKK4 (Lin *et al.*, 1995; Yan *et al.*, 1994) and MKK7 (P. Holland Ch. 2). MEKK1 has also been demonstrated to be a caspase target that is cleaved and activated during anoikis by removal of the N-terminal inhibitory domain (Cardone *et al.*, 1997). There are currently four MEKK proteins that have been identified. MEKK2 and MEKK3 may be functionally related, as their catalytic domains are 96% identical. MEKK1 and MEKK4 are roughly 50% identical with MEKK2, MEKK3 and each other. The amino-terminal domains are unrelated, suggesting differential regulation among the MEKKs. MEKK1 and MEKK4 can bind to the low molecular weight GTP-binding

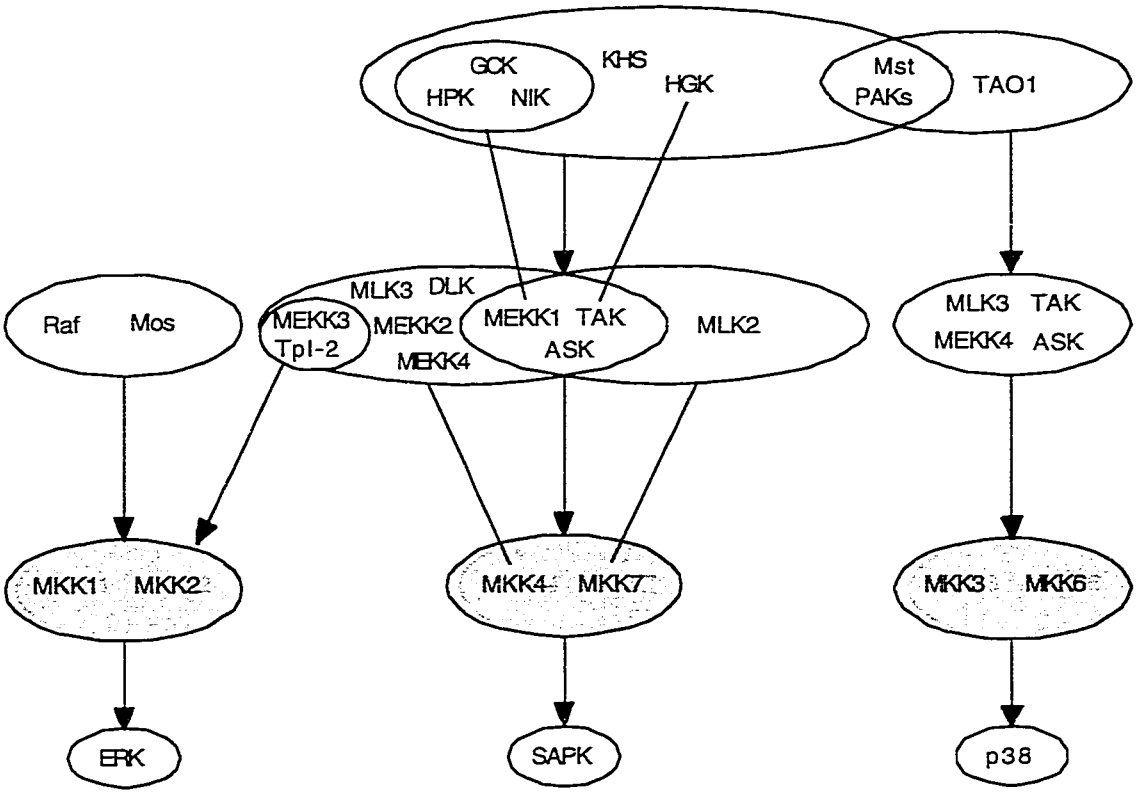


Fig.3. Activators of mammalian MAP kinases. A current model illustrating the branching and convergence of identified kinases that control the activity of members of the MAP kinase pathways. A line inside the bubble indicates that the upstream kinases signal through the particular substrate. An arrow to the bubble indicates that the upstream kinases signal through both substrates. Diagram has been simplified so as not to include activation by the Ras/Rac GTPases. See text for details.

proteins Rac and Cdc42, whereas MEKK2 and MEKK3 do not (Fanger *et al.*, 1997). There also exists some overlap in the stimuli capable of activating various MEKKs. Growth-factors such as EGF can activate MEKK1 but this is dependent on Ras-GTP (Lange-Carter *et al.*, 1993). TNF is also able to activate MEKK1 and MEKK2, and EGF can also activate MEKK2. Co-expression of MEKK2 or MEKK3 with MKK4 in COS cells results in activation of MKK4 (Deacon and Blank, 1997). Consistent with this, others have demonstrated that MEKK3 activates ERK and SAPK pathways via MKK1 and MKK4, respectively, but not the p38 pathway (Ellinger-Ziegelbauer *et al.*, 1997). In contrast, the human homolog of murine MEKK4, called MTK1 mediates activation of both SAPK and p38 in response to environmental stress. Using a yeast two-hybrid screen, three related proteins with similarity to GADD45 (growth arrest and DNA damage induced protein) that bound to the N-terminal domain of MTK1 were identified (Takekawa and Saito, 1998). The GADD45-like genes are induced by environmental stresses including MMS (methyl methanesulfonate), UV and γ irradiation. Expression of the GADD45-like genes induced p38 and SAPK activation and induced apoptosis, and this could be partially suppressed by co-expression of a dominant negative MTK1 mutant protein. Regulation of the MEKK family members is seemingly complex, and suggests that the same MEKK's can activate multiple MAP kinases. The means by which these signaling events are coordinated is currently an area of intense research.

The mixed lineage kinases (MLKs) are a group of kinases that are characterized by their catalytic domains that have features of both tyrosine and serine/threonine protein kinases. However, only serine/threonine kinase activity has been demonstrated for any member of this family. The family currently consists of MLK1, MLK2/MST, MLK3 (SPRK, PTK1) and DLK/MUK, and these kinases typically have several protein binding motifs. For example, the MLKs contain an SH3 domain at the amino terminus, proline-rich regions at the carboxy terminus and also have leucine/isoleucine-zipper motifs near the carboxyl terminus. MLK3 and DLK/MUK also contain modified CRIB domains, but DLK does not appear to bind Rac/Cdc42 (Lamarche *et al.*, 1996). MLK3 and DLK/MUK can also phosphorylate and activate MKK4/SEK1 (Hirai *et al.*, 1996; Rana *et al.*, 1996). MLK3 has also been shown to activate p38 via MKK3 and MKK6 (Tibbles *et al.*, 1996). Other reports have demonstrated that MLK2 can phosphorylate and activate MKK7 directly (Cuenda and Dorow, 1998; Hirai *et al.*, 1998) (Ch. 2). Two independent labs have proposed that activation of SAPK by MLK2 occurs

selectively via MKK7, whereas MEKK1 induced SAPK activation occurs via MKK4 (Cuenda and Dorow, 1998; Hirai *et al.*, 1998). This provides a model of differential regulation of the SAPK activators MKK4 and MKK7, but more work is needed to confirm this. In addition to being an inducer of apoptosis, MLK2 has also been shown to localize to microtubules with active SAPK/JNK and bind to kinesin proteins (Nagata *et al.*, 1998).

The Tpl-2 proto-oncogene, which has homology to *S. cerevisiae* STE11, is another MAPKKK shown to activate MKK1 as well as MKK4/SEK1 (Salmeron *et al.*, 1996). Expression of either full-length Tpl-2 or Tpl-2ΔC (an oncogenically active form) without any stimulus led to activation of MKK4/SEK1 in COS cells (Salmeron *et al.*, 1996). Finally, TAK1 and ASK1, two other MAPKKKs distantly related to MEKK1 (approximately 30% amino acid identity), also function to activate MKK4/SEK1 (Ichijo *et al.*, 1997; Yamaguchi *et al.*, 1995). Interestingly, TAK1 and ASK1 are also capable of activating the MKK3/MKK6-p38 pathway (Moriguchi *et al.*, 1996). We have also observed strong activation of MKK7 in response to transiently expressed TAK1 or ASK1. Co-expression of another protein, TAB1, with TAK1 enhances the activity of TAK1 in mammalian cells (Shibuya *et al.*, 1996). TAB1 is a 55 kDa protein with no marked sequence similarity to known proteins or binding motifs, which has been identified in a two-hybrid screen with TAK1. Kinase activity of TAK1 is stimulated in response to TGFβ and bone morphogenetic protein. Activation of ASK1 occurs by treatment of cells with TNFα and can lead to apoptotic cell death. Recently, ASK1 has been shown to function as a mediator of TRAF2-induced SAPK activation (Nishitoh *et al.*, 1998). The TNF receptor associated-factor 2 (TRAF2) binds the TNF receptor and mediates effector functions. TNF-induced activation of SAPK was previously shown to occur through a noncytotoxic TRAF2 dependent pathway, independent of a pathway resulting in TNF type 1 receptor induced apoptosis (Natoli *et al.*, 1997). ASK1 interacts with and is activated by TRAF2. A truncated form of TRAF2, which inhibits SAPK activation by TNF, blocks TNF-induced ASK1 activation. Other reports have suggested that the Fas receptor-associated protein, Daxx, also interacts with ASK1, resulting in ASK1 and JNK activation (Chang *et al.*, 1998). Clearly, multiple signals, some of which may be overlapping, activate a variety of MAPKKKs, resulting in the activation of JNK/SAPK and p38. These findings suggest that MKKs may function as targets for convergent regulation by a diverse group of upstream activators.

Many of the MAP kinase kinase kinases that can activate SAPK contain N-terminal regulatory domains. The identification of a full-length form of MEKK1, which contains a large N-terminal regulatory region, suggests that this may be involved in its activity (Xu *et al.*, 1996). Caspase-specific cleavage of MEKK1 results in the removal of this N-terminal domain and results in its constitutive activation (Cardone *et al.*, 1997). Recently, thioredoxin (Trx) was identified as a physiological inhibitor of ASK1 (Saitoh *et al.*, 1998). Only the reduced form of Trx associates with the N-terminal portion of ASK1 and keeps ASK1 kinase activity inactive. Upon treatment of cells with TNF or reactive oxygen species (ROS) such as hydrogen peroxide, Trx is oxidized and released from ASK1, which is activated. Thus, reduced Trx acts as a negative regulator in the TNF and ROS-mediated activation of ASK1. With TAK1, the deletion of 20 amino acids from the N-terminus of TAK1 results in its constitutive activation. However, the binding of TAB1 to the N-terminal region of TAK1 results in enhanced TAK1 activity. (Shibuya *et al.*, 1996). This suggests that the N-terminal domain may engage the catalytic domain to inhibit kinase activity. It is proposed that TAB1 may function to expose the catalytic domain by binding at the N-terminus, resulting in an activating conformational change of TAK1. In these examples the N-terminal region of the protein serves an inhibitory role. More work is required to address the significance of these bindings interactions *in vivo*. In addition to binding proteins, yet more kinases have been discovered that may activate MAP kinase kinase kinases.

FURTHER UPSTREAM:

The discovery of a direct interaction between RasGTP and Raf-1 provided the first example of a small GTPase that regulates a broad range of cellular functions as a proximal component of a protein kinase cascade. Several reports have demonstrated a role for the small GTP-binding proteins, Rac and Cdc42, but not Rho in regulating the JNK/SAPK and p38 MAP kinase cascades in COS and NIH3T3 cells (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995). Other reports have stated that Cdc42 and all Rho proteins (RhoA, RhoB, RhoC), but not Rac or Ras, induce activation of JNK/SAPK in the human kidney epithelial cell line 293T (Teramoto *et al.*, 1996). These findings suggest that small GTP-binding proteins signal to JNK/SAPK in a cell-type specific manner and may utilize independent signaling routes. Subsequently, specific effector mutants of Rac that distinguish the effects of Rac in mediating actin polymerization and

signaling to the SAPK pathway were described (Joneson *et al.*, 1996; Lamarche *et al.*, 1996). Rac mutants with a substitution at position F37 no longer induce cytoskeletal changes but still activate JNK/SAPK, whereas mutants with a substitution at position Y40 no longer interact with p65PAK, and are unable to activate JNK/SAPK; these fors are still capable of inducing actin polymerization. Substitution at position Y40 prevents the interaction of both Rac and Cdc42 with CRIB motif-containing proteins (Lamarche *et al.*, 1996). These data suggested that the effects of Rac on JNK/SAPK activation and cytoskeletal regulation were mediated by distinct non-overlapping pathways. It has been proposed that the effects of Rac and Cdc42 are mediated by PAKs (p21-activated kinase), a family of protein kinases with homology to *S. cerevisiae* STE20. Some PAKs (PAK1,2,3) contain a Rac1/Cdc42 binding domain (CRIB domain) within their N-terminal segment, and were originally identified based on their ability to be activated by GTP-Rac and GTP-Cdc42. Co-transfection of a truncation-mutant of PAK65, which can still bind Rac1/Cdc42 but lacks the catalytic domain, can block Rac1/Cdc42-induced activation of JNK/SAPK (Minden *et al.*, 1995). In addition, constitutively active mutants of PAK1 or PAK3 activate JNK/SAPK and p38 on co-transfection and when added to cell-free extracts of *Xenopus* oocytes (Bagrodia *et al.*, 1995; Zhang *et al.*, 1995). Yet, although p65PAK and other PAK homologs have been implicated in being upstream of JNK/SAPK, there is still no direct experimental evidence that a PAK activates a MAPKKK. The overexpression of a PAK regulatory domain would be expected to block Rac mediated JNK activation by titrating out any available Rac and Cdc42, whether a PAK played a direct role or not (Minden *et al.*, 1995). The MLKs have also been proposed to be candidate physiological Rac/Cdc42 effectors resulting in SAPK activation, because some MLKs contain CRIB domains. The recent identification of a new Rac (but not Cdc42) target, POSH (plethora of SH3 domains), a four SH3 domain-containing adaptor protein, suggests that activation of SAPK by MLKs is independent of Rac, and that PAKs dont activate SAPK (Tapon *et al.*, 1998). In this study, a Rac mutant (L61F37ARac) that no longer interacts with MLK2 or MLK3 was still able to activate SAPK. Similarly, expression of an activated mutant of PAK (L107F) had no effect on SAPK activation. Microinjection of POSH into Swiss 3T3 cells can stimulate nuclear translocation of NFκB and induce cell death. Co-transfection studies indicate that POSH can activate SAPK. SAPK activation and apoptotic responses induced by POSH both require the two N-terminal SH3 domains and the Rac-binding

domain. POSH is proposed to act as a scaffolding protein that triggers the formation of signaling complexes downstream of Rac.

Parallel to the PAKs lies another growing family of serine threonine kinases with homology to *S. Cerevisiae* STE20. These STE20-related kinases are distinct from the PAKs in that they do not contain CRIB domains. One of these kinases, GCK (germinal center kinase), was identified originally as a kinase expressed preferentially in the germinal center B cells, but it is also expressed in many other tissues including brain, lung and placenta (Katz *et al.*, 1994). More recently, GCK was shown to interact *in vivo* with the TNFR1 (Type 1 TNF receptor) signal transducer, TNFR-associated factor-2 (TRAF2), and with MEKK1, and thereby couple TRAF2 to the SAPKs (Yuasa *et al.*, 1998). Another STE20-related kinase, HPK1 (hematopoietic progenitor kinase) has also been shown to activate SAPK by binding and phosphorylating MEKK1 directly (Hu *et al.*, 1996). Others suggest that HPK1 activates SAPK by binding and phosphorylating MLK3 directly (Kiefer *et al.*, 1996). HPK1 is expressed predominantly in hematopoietic cells, including progenitor cells that ultimately undergo lineage determinations. A third STE20 homolog that has been demonstrated to bind MEKK1 and thereby activate SAPK is NIK (Nck-interacting kinase) (Su *et al.*, 1997). NIK is thought to bind MEKK1 via a C-terminal regulatory domain. Interestingly, both GCK and HPK1 contain homologous C-terminal regulatory domains, which were shown to bind MEKK1. This may represent a subgroup of STE20-related kinases that contain functional and structural similarity.

Separate from GCK, HPK1 and NIK are the STE20 homologs KHS and HGK (Tung and Blenis, 1997; Yao *et al.*, 1999). KHS (kinase homologous to SPS1/STE20) was identified in a screen of human cDNA libraries for STE20 homologs and found to activate SAPK via MKK4. KHS, as well as HPK1, have been shown to bind the first SH3 domains of c-Crk and CRKL, respectively (Oehrl *et al.*, 1998). HGK, (HPK/GCK-like kinase), is the newest member of STE20 homologs identified. Despite the relatedness of HGK to HPK and GCK, it does not activate MEKK1, but rather activates TAK1. This is the first evidence of a kinase that specifically activates TAK1. Another STE20-like kinase recently characterized is Mst1 (not to be confused with MLK2/MST). Like MEKK1, Mst1 may be specifically cleaved by caspases during apoptosis and this cleavage results in its activation (Graves *et al.*, 1998). Active Mst1 appears to activate both the SAPK and p38 pathways. It has been proposed that Mst1

activation by caspase mediated cleavage functions as part of a feedback loop that serves to amplify the apoptotic response. It is not known whether Mst1 also binds to MEKK1. SOK-1 (STE20/oxidant stress response kinase-1), is another STE20-related kinase that is activated by oxidant stress (Pombo *et al.*, 1996). SOK-1 is activated by autophosphorylation and is markedly inhibited by its non-catalytic C-terminal region. SOK-1 is activated 3- to 7-fold by reactive oxygen intermediates but is not activated by growth-factors, alkylating agents, cytokines or environmental stresses including heatshock and osmolar stress. Although these data place SOK-1 on a stress response pathway, SOK-1 does not activate either of the stress-activated MAP kinase cascades (p38 and SAPKs). Since SOK-1 does not activate any of the known MAP kinase cascades, its activation defines a novel stress response pathway that is likely to include a unique stress-activated MAP kinase cascade. Finally, a STE20-related kinase that appears specific for activation of the p38 pathway has also been described. This kinase, called TAO1, for its thousand and one amino acids, activates MKK3, but not MKK4 or MKK6 (Hutchison *et al.*, 1998).

INACTIVATION OF MAP KINASE PATHWAYS:

As described, MAP kinase pathways can be activated by numerous means and mediate numerous cellular events. Protein phosphorylation plays a central role in regulating these processes. Target proteins may be phosphorylated at specific sites, and these phosphates are removed by specific phosphatases. Cells contain specific tyrosine phosphatases as well as serine/threonine and dual specificity phosphatases. It is becoming apparent that the activities of phosphatases are regulated in a sophisticated manner by a combination of targeting and regulatory subunits and by specific inhibitors (Cohen, 1997; Hunter, 1995). Interestingly, many microbial toxins are inhibitors of phosphatases, emphasizing the importance of phosphatases in physiological systems. These inhibitors are useful tools in evaluating phosphatase function *in vivo*.

Several of the components of MAP kinase pathways are subject to regulation by phosphatases. The serine/threonine protein phosphatases PP1 and PP2A can dephosphorylate and inactivate the MAP kinase kinase kinase, Raf-1, MKK1, and ERK MAP kinase *in vitro* (Charbonneau and Tonks, 1992; Fischer *et al.*, 1990). Whether or not these are the physiologically relevant phosphatases is not known. In the case of the MAP kinases, dual specificty phosphatases capable of de-phosphorylating both threonine and

tyrosine residues have been identified. These are collectively called MAP kinase phosphatases (MKP's). MKP's are the products of immediate early genes induced by divergent stimuli such as serum, growth-factors, UV, and DNA-alkylating agents (Liu *et al.*, 1995; Misra-Press *et al.*, 1995; Sun *et al.*, 1993). The prototypic dual-specificity phosphatase (VH1) was first identified in vaccinia, however multiple human (CL100, PAC1, VH3), murine (MKP1), yeast (Yop51, MSG5) and *Drosophila* (*puc*) homologs have also been isolated. Early investigations proposed a role for MKP's as inactivators of ERK MAP kinase following serum stimulation (Sun *et al.*, 1993; Sun *et al.*, 1994). However, subsequent studies have demonstrated that growth factor-induced transcription of MKP1 and MKP2 did not correspond temporally with the kinetics of ERK inactivation (Misra-Press *et al.*, 1995; Wu *et al.*, 1994). This has suggested that although MKP1 may play a role in attenuating the ERK response at later time points, distinct mechanisms are likely to be responsible for the rapid inactivation of ERK. One possiblity is that both serine/threonine and tyrosine phosphatases are involved. More recent studies have implicated MKP's in the regulation of SAPK activity (Hirsch and Stork, 1997). UV irradiation-induced and MEKK1-induced SAPK activity can be blocked by expression of MKP1 and MKP2 (Hirsch and Stork, 1997). Interestingly, a *Drosophila* homolog of MKP1, encoded by the gene *puckered* (*puc*), has been shown to be required for proper dorsal closure (Martin Blanco *et al.*, 1998). Mutations of *puc* lead to cytoskeletal defects reminiscent of those observed in *basket* (*bsk*), the *Drosophila* SAPK homolog. Absence of Puc results in the hyperactivation of Bsk, and overexpression of Puc mimics loss of function mutants of Bsk. Puc expression itself is regulated by activation of the Bsk/SAPK pathway. Thus, Puc participates in a negative feedback loop that regulates Bsk activity during dorsal closure. These results provide an elegant demonstration of the feedback mechanism of a phosphatase on a specific MAP kinase in coordinating the fine tuning of cellular behavior.

Comparatively less progress has been made in the field of characterizing protein phosphatases relative to protein kinases. It is estimated that humans could have as many as 1000 phosphatase genes, which is much less than the estimated number of protein kinases (Hunter, 1995). This suggests that phosphatases will have a broader specificity compared to protein kinases. Although many serine/threonine and tyrosine phosphatases have been identified, their specificities within cells remains elusive.

CROSS-TALK:

The coordinated responses of cells to extracellular signals undoubtedly involves multiple signaling systems. One difficulty in deciphering the actions of protein kinases within MAP kinase pathways is that the consequences of activating a single cascade depend on the activation states of numerous other pathways in the cell. The hierarchical organization of MAP kinase cascades makes them particularly good targets for crosstalk. In this manner, multiple physiological processes, including cytoskeletal rearrangements, cellular metabolism, and cell cycle progression may be coordinated to ensure that cells commit to an appropriate functional outcome. The distribution of an extracellular signal begins at the level of the receptors themselves, where, for example, tyrosine kinase receptors bind multiple effectors through modular binding domains. The increasing numbers of kinases found to be capable of activating one or more MAP kinase kinases suggests that within these steps of the cascade, signals may be converging onto a subset of proteins. The association of signaling components into relatively stable complexes, either by scaffolding or precise cellular localization, is likely to play an important role in maintaining signal specificity by physically restricting access of signaling molecules to potential substrates. Ultimately the integration of multiple signaling pathways occurs at the level of substrates, where combinatorial inputs or sequential phosphorylation events introduce specificity required for the appropriate response. The study of these integrated actions will be the challenge ahead.

BIOLOGICAL ROLES OF MAP KINASES IN MAMMALIAN CELLS:

ERKs are activated by mitogens in all cells and appear to be an essential shared element of mitogenic signaling. For example, prolonged activation and nuclear retention of ERK is required for transcription of cyclin D1, suggesting a mechanism for ERK-mediated enhancement of the cell cycle (Lavoie *et al.*, 1996). Alternatively, prolonged activation and nuclear retention of ERK has also been implicated in differentiation of PC12 cells. It has been proposed that sustained activation of ERK leads to differentiation, whereas transient activation does not. Similarly, there have been observations that transient activation of SAPK enhances cell growth, while persistent activation induces apoptosis (Chen *et al.*, 1996; Guo *et al.*, 1998). The expression of constitutively active components of the ERK MAP kinase pathway leads to cell

transformation, establishing a role for ERK activity in neoplasia. Indeed, constitutively active ERK and MKK1 have been found in renal carcinomas, as well as other tumors (Oka *et al.*, 1995). This implies that the duration and magnitude of SAPK activation may be major determinants of cell fate, and both temporal and spatial expression of MAP kinases are likely to play important roles in regulating biological outputs.

A major role for the SAPK pathway is phosphorylation and activation of the transcription factor c-Jun, which participates in activator protein-1 (AP-1)-mediated gene regulation (Hibi *et al.*, 1993). SAPK also phosphorylates the transcriptional activation domains of ATF2 and JunD, and the Ets domain transcription factors, Elk-1 and Sap-1 (Whitmarsh and Davis, 1996). Similarly, p38 MAP kinase phosphorylates and activates the transcription factor ATF2 and has been shown to cooperate with ERK MAP kinase in the regulation of the serum response element (SRE) by phosphorylation of the ternary complex factor (TCF) (Hazzalin *et al.*, 1996; Price *et al.*, 1996). These results suggest that, in addition to a role in stress responses, these pathways may also contribute to mitogenesis. In support of this idea, the SAPKs are responsive to co-mitogenic stimuli in lymphocytes and p38 activity has been shown to be required for IL-2 induced proliferation in T cells (Berberich *et al.*, 1996; Crawley *et al.*, 1997; Su *et al.*, 1994). CD95/Fas-mediated SAPK activation has also been implicated in the induction of either growth arrest or proliferation, depending on signals via the B cell receptor (BCR) (Rathmell *et al.*, 1996).

Recent evidence suggests that the SAPK and p38 MAP kinase pathways may also play a role in regulating apoptosis. Apoptosis, or programmed cell death, is an acitve process that is fundamental to the development and homeostasis of multicellular organsims (Jacobson *et al.*, 1997). During developmental stages, apoptosis is critical in the removal of excess cells. Apoptosis is also a mechanism used in the thymus to eliminate self-reactive T lymphocytes and avoid autoimmunity. It is characterized by dramatic morphological alterations, particularly membrane blebbing, cell shrinkage, chromosome condensation and DNA fragmentation. Apoptosis can be triggered by a wide variety of cellular stresses, including DNA damage, UV radiation, ionizing radiation, heat shock and oxidative stress, as well as by extracellular stimuli acting via cell surface receptors. Regardless of the stimulus, most cells undergoing apoptosis exhibit a similar series of changes, suggesting that apoptotic signals ultimately converge to engage a common execution pathway leading to cell death. Significant advances have been made in

understanding the molecular mechanism of the execution phase of apoptosis. Central to the cell death machinery are a family of cysteine proteases, homologs of the *C. elegans* gene *ced-3*, which become activated in a proteolytic cascade and cleave specific substrates. These proteases, or caspases, are constitutively expressed in cells as inactive precursors.

The mechanism of activation of caspases is best understood for the Fas receptor. Fas (CD95/APO-1) is a transmembrane receptor belonging to the tumor necrosis factor (TNF) receptor family (Itoh *et al.*, 1991; Oehm *et al.*, 1992). Crosslinking Fas/CD95 with agonistic antibodies or Fas ligand results in rapid apoptosis in many cell types (Nagata, 1997). Although the role of Fas/CD95 in many tissues is not clear, Fas-induced apoptosis has been shown to play an important role in the development of the lymphoid system and the maturation of the immune response.

Although a pivotal role has been established for caspases in apoptosis, the identity of their targets is still mostly unknown. Some caspase substrates include poly (ADP-ribose) polymerase (PARP), the U1 small ribonucleoprotein, lamin A, DNA dependent protein kinase, and D4-GDI, a GDP dissociation inhibitor for Rho family GTPases (Casciola-Rosen *et al.*, 1995; Lazebnik *et al.*, 1994; Na *et al.*, 1996; Takahashi *et al.*, 1996; Tewari *et al.*, 1995). Interestingly, protein kinases which function in stress-activated pathways are being identified as caspase substrates. These include PAK2, MEKK1 and Mst1, and caspase mediated proteolysis results in activation of these kinases (Cardone *et al.*, 1997; Graves *et al.*, 1998; Rudel and Bokoch, 1997). These findings raise the possibility that the SAPK and p38 pathways may play a role in regulating apoptosis.

Late and sustained activation of both the SAPK and p38 pathways has been observed to correlate with apoptosis induced by CD95/Fas cross linking in human T and B lymphoma cells, NGF withdrawal of PC12 pheochromatocytoma cells, and BCR ligation in human B lymphoma cells (Graves *et al.*, 1996; Juo *et al.*, 1997; Xia *et al.*, 1995). Despite this, however, the precise role of SAPK activation in this process is unclear. Other reports suggest that inhibition of the SAPK signaling pathway does not block Fas-mediated killing of Jurkat T cells (Chen *et al.*, 1996; Lenczowski *et al.*, 1997). In contrast, in the human myeloid leukemia cell line U937, SAPK anti-sense oligonucleotides inhibit apoptosis, and SAPK signaling has been found to activate caspases known to mediate cell death (Seimiya *et al.*, 1997). In neuroblastoma cells, Fas-mediated apoptosis requires the

SAPK pathway (Goillot *et al.*, 1997). These differences may reflect the properties of different cell types and the possibility of SAPK-dependent and SAPK-independent apoptotic pathways (Chen *et al.*, 1996). Alternatively, it is possible that SAPK activation may provide a protective signal, as suggested by recent studies of MEKK1$^{-/-}$ embryonic stem cells (Yujiri *et al.*, 1998). Loss of MEKK1 expression results in increased apoptosis of cells in response to hyperosmolarity and microtubule disruption. As apoptosis is an extreme case of stress, it is possible that SAPK activation may occur in response to the stress of apoptosis.

Independent of apoptosis, a role for SAPK activation during inflammatory responses is also emerging. Although the inflammatory cytokine, TNF, is a potent activator of SAPK, in most cases it does not cause apoptosis unless cells are first treated with cycloheximide or Actinomycin D. IL-1 is also a potent activator of SAPK, and this is predominantly mediated by MKK7 (Finch *et al.*, 1997; Moriguchi *et al.*, 1997). The MAP kinase p38 has also been shown to be important in mediating inflammatory signals, and it likely that SAPK and p38 work in concert (Whitmarsh and Davis, 1996). Activation of both SAPK and p38 are necessary for IL-1 induced synthesis of IL-8 (Cohen, 1997; Holtmann *et al.*, 1999; Shapiro and Dinarello, 1995). IL-8 functions in recruiting leukocytes at sites of acute inflammation.

The generation of SAPK (*jnk1* and *jnk2*)-deficient mice has also established a role for this pathway in T cell differentiation. Activation of TH cells by antigen-presenting cells leads to their differentiation into TH1 or TH2 effector cells, which mediate inflammatory or humoral responses, respectively. T cells from JNK1$^{-/-}$ mice hyperproliferate, exhibit decreased activation-induced cell death, and preferentially differentiate into TH2 cells (Dong *et al.*, 1998). JNK2-deficient T cells also exhibit decreased JNK activity in TH1 cells but not TH2 cells, leading to an impairment in TH1 differentiation (Yang *et al.*, 1998). Thus, both JNK1 and JNK2 are involved in regulating the decision of TH cells to differentiate into TH1 or TH2 effector cells, influencing the type of immune response that is initiated.

Targeted disruption of the *jnk3* gene gives a distinct phenotype. JNK3 expression is restricted to brain, heart and testis, with the highest level of expression in the hippocampus. Loss of JNK3 protein results in a defect in hippocampal neuronal cell death

in response to excitotoxic stress, with no other developmental defects (Yang *et al.*, 1997). In contrast, mice that are deficient for the SAPK activator MKK4, do not live beyond E14.5 (Ganiatsas *et al.*, 1998; Yang *et al.*, 1997). Analysis of embryos from E11.5-E12.5 demonstrates abnormal liver development, including a reduced liver bud, abnormal histology and severe hemorrhage (Ganiatsas *et al.*, 1998). This phenotype is reminiscent of that observed in c-Jun deficient mice (Hilberg *et al.*, 1993; Johnson *et al.*, 1993; Roffler-Tarlov *et al.*, 1996). Thus, the targeted disruptions of signaling proteins in mice are beginning to demonstrate critical roles for these pathways in mammalian development. Developmental roles for SAPK pathways are also provided in *Drosophila*, in which MAP kinase pathways regulate eye development, immune responses and morphogenetic processes such as dorsal closure (Ip and Davis, 1998).

The increasing number of MAP kinase pathway components and their overlapping specificities *in vitro* has made it increasingly difficult to identify the physiological roles and substrates for individual kinases. Combinations of strategies will be required to understand the complexities of these signaling events. One strategy involves overexpressing dominant-negative and constitutively-active forms of kinases in cells. Overexpression studies have proven valuable but must be interpreted with caution. The generation of targeted disruptions in mice of various MAP kinase pathway components is also proving important in identifying physiological roles for these enzymes (Ip and Davis, 1998). However, this process is lengthy, and the degree of redundancy observed for some components suggests that double or triple knockout mice will be required. For example, the fact that loss of any one of the *jnk* genes results in viable mice suggests that some redundancy exists. This is in contrast to loss of a specific activator of JNK (MKK4/SEK1), which is an embryonic lethal. Another approach involves the use of transgenic mice, in which an activated or inactive form of a kinase may be overexpressed in a specific tissue by using a tissue-specific promoter. This may be favorable to a whole animal knockout, as transgenic animals are rarely embryonic lethals. However, concerns related to redundancy and overexpression artefacts must still be considered. In addition to biochemical characterization and use of specific mutants to address the functions of MAP kinase pathways, cell permeable inhibitors of MAP kinase pathway components have recently provided another means study specific components within a cascade. These inhibitors include PD098059, a selective inhibitor of MKK1 and to a lesser degree MKK2, and SB203580, which blocks the activity of p38 (Cohen, 1997). These specific inhibitors

have helped reveal that several proteins are targets for both ERK and p38 pathways (Waskiewicz *et al.*, 1997; Zinck *et al.*, 1995). They also present potential roles for therapeutic targets in regulating physiological processes.

In summary, the sequential protein kinase cascades that make up MAP kinase pathways impinge on a number of cellular functions. The continued identification of additional MAP kinase signaling pathways brings increasing complexity. Biochemical studies in mammalian cells indicate that these pathways are far from distinct and non-overlapping, as they appear to be in yeast-based genetic studies. In contrast, kinases at each level of a cascade (MAPKKKK, MAPKKK, MAPKK, MAPK) have multiple substrates and multiple activators. At present, it is not clear whether these are truly signal or cell-type specific. The broad number of cellular events in which activation of the SAPK pathway has been implicated may be in agreement with the large number of upstream activators currently known to activate SAPK. Our studies on the identification and characterization of MKK7 as a direct activator of SAPK suggest it will contribute to coordinating proper responses of SAPK activation. This signaling specificity may be achieved by differential activation from upstream signals, and/or by sequestering components of the pathway to distinct cellular locations. The focus of this thesis pertains to the characterization of MKK7, and its role in regulating the specificity of SAPK activation.

CHAPTER 1: PROTEIN-PROTEIN INTERACTIONS INVOLVING MKK1

INTRODUCTION:

In yeast, genetic analyses have demonstrated that multiple distinct MAP kinase pathways exist, with a single MAP kinase kinase kinase upstream of each MAP kinase kinase. In mammals, however, the situation appears more complex. The existence of multiple protein kinases upstream of MKK1/2 suggested that MKK1/2 might represent a significant integration point within the MAP kinase pathway. Although SAPK and p38 MAP kinases had been identified at the time this study was initiated, many of the upstream activators in these pathways had not. In addition, significant overlap had been shown to exist between stimuli that activated the ERK and SAPK MAP kinases. For example, treatment of some cells with mitogens or phorbol esters could lead to SAPK as well as ERK activation (D'Erijard *et al.*, 1994; Kyriakis *et al.*, 1994; Su *et al.*, 1994). Conversely, both ERK and SAPK were shown to respond to heat shock and stress in some cells (Kyriakis *et al.*, 1994). One possible explanation for these observations was that a considerable degree of crosstalk might exist between upstream components of these MAP kinases. Therefore, a better understanding of the regulation and function of the individual components within the MAP kinase pathway was required. We investigated interactions involving MKK1 using the yeast two-hybrid screen to search for novel proteins. By examining protein-protein interactions involving MKK1 and identifying associated proteins, we hoped to find components that were necessary for the physiological regulation and function of MKK1.

The yeast two-hybrid screen has proven to be a powerful method of identifying proteins that interact with a specific target protein of interest. To detect protein-protein interactions with this method, two-hybrid fusion proteins are generated; one to a

DNA binding domain, the second to an activation domain. Individually, these hybrids are unable to activate transcription. Formation of a complex between the fusion proteins of interest results in transactivation which can be detected by the use of reporter constructs (Vojtek and Hollenberg, 1995). One of the first applications of this technique to protein-protein interactions with signal-transduction pathways was the identification of the Ras-Raf interaction (Vojtek *et al.*, 1993). This screen has revealed several interactions between components of MAP kinase cascades, and has served to highlight the importance of protein-protein interactions within signaling cascades.

RESULTS:

TWO-HYBRID SCREEN WITH MKK1:

In collaboration with Dr. Jean Campbell in the laboratory of Dr. Edwin Krebs, University of Washington, a yeast two-hybrid library screen was performed using full-length human wild-type MKK1 as bait. This library screen has been successfully used in the past to identify interacting proteins for substrates and regulators. Two hybrid plasmids were co-expressed in an *S. cerevisiae* strain containing the yeast *HIS3* gene and bacterial *lacZ* gene as reporter constructs (Fig. 4). Both reporter genes contain binding sites for the LexA protein. One hybrid plasmid consisted of a fusion between MKK1 (the protein of interest), and the LexA DNA binding domain. The second plasmid was a fusion between a nuclear localized VP16 acidic activation domain and either a second protein of interest, or a cDNA library selected for screening. Transactivation of the *HIS3* and *lacZ* reporter constructs is dependent on formation of a complex between the LexA and VP16 fusion proteins. Therefore, a putative interaction between the two proteins of interest is detectable by yeast which are histidine prototrophs and produce detectable amounts of β-galactosidase.

To determine whether interacting partners of MKK1 could be identified in a two-hybrid screen, we tested the ability of MKK1 to interact with ERK2 MAP kinase and Raf-1, two proteins known to interact with MKK1. Yeast strains transformed with the VP16-Raf-1 (provided by Anne Vojtek) and LexA MKK1 or LexA MKK1 K97A vectors (MKK1, MKK1 K97A provided by Dr. Rony Seger) grew on His^- plates and turned blue

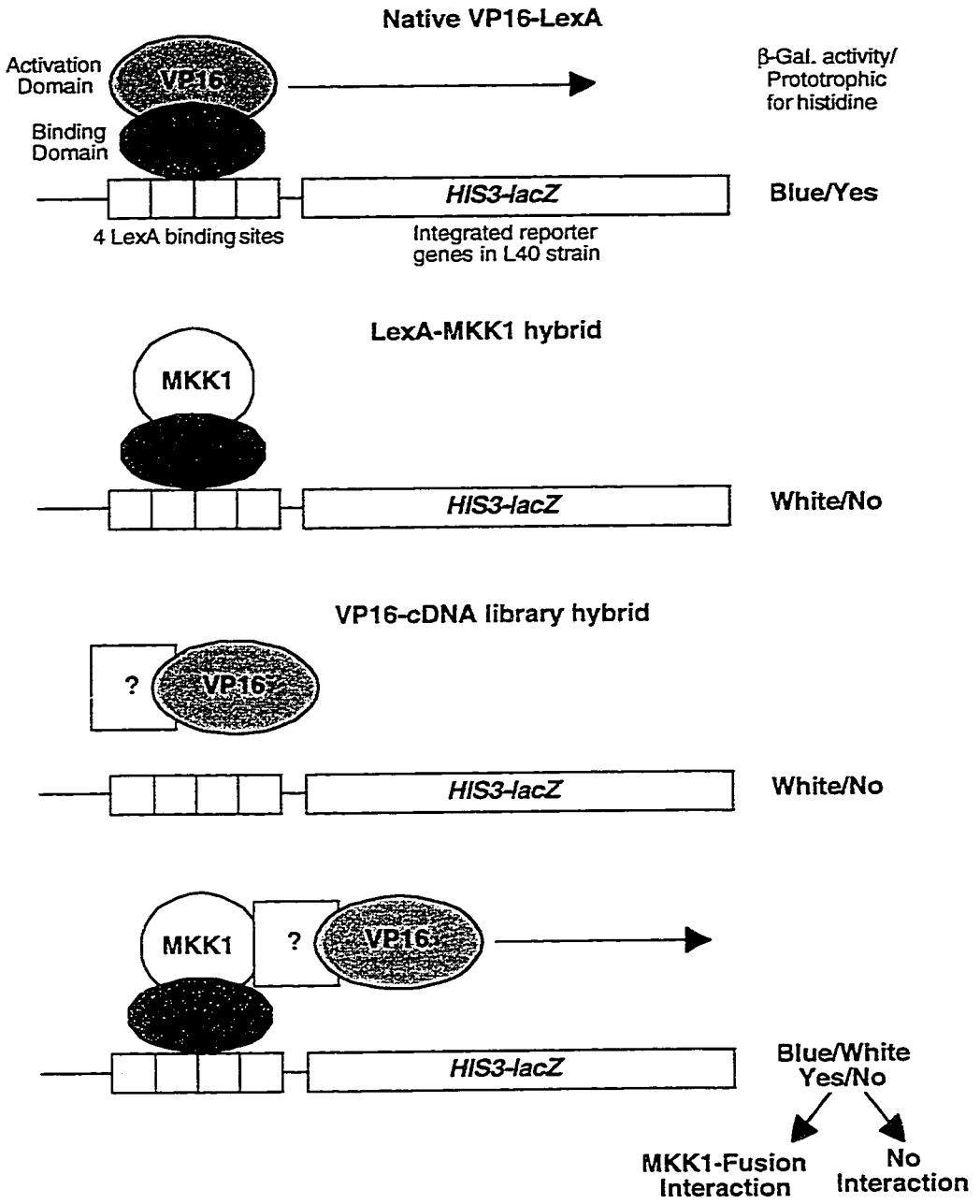


Fig. 4. A yeast two-hybrid screen with MKK1. MKK1 was fused to the LexA DNA binding domain. A mouse embryo cDNA library was fused to the VP16 acidic activation domain. Transactivation of the *HIS3* and *lacZ* reporter constructs is indicative of an interaction between MKK1 and an unknown protein from the VP16 library hybrid.

Table 1. Interaction of MKK1 and hybrid fusion proteins. Wild-type MKK1, kinase-inactive MKK1 (K97A), and wild-type full-length ERK MAPK proteins were fused to the LexA DNA binding domain. Wild-type Raf and MKK1 proteins were fused to the nuclear localized VP16 acidic activation domain. Co-expression of yeast with LexA MKK1 or LexA MKK1 K97A with VP16 Raf, and LexA ERK MAPK with VP16 MKK1, allowed for growth on histidine$^-$ plates and turned blue in a β-galactosidase filter assay. Co-expression of yeast with LexA MKK1, LexA MKK1 K97A, or LexA ERK MAPK with the control VP16 vector is not able to activate the *HIS3* and *lacZ* reporter constructs.

pLexA	pVP16	Growth on His$^-$	β-gal
MKK1	empty	-	-
MKK1 K97A	empty	-	-
MKK1	Raf-1	+	+++
MKK1 K97A	Raf-1	+	+++
ERK2	MKK1	+	+++
ERK2	empty	-	-

when tested for β-galactosidase activity, indicative of transactivation of both reporter constructs (Table 1). Co-expression of LexA ERK2 MAPK and VP16 MKK1 fusions also activated the *HIS3* and *LacZ* reporter genes. Alternatively, yeast transformed with the control VP16 expression vector and either LexA MKK1, LexA MKK1 K97A or LexA ERK2 MAPK were unable to grow in the absence of histidine and remained white in a filter assay for β-galactosidase activity. These results demonstrated that the two-hybrid system was capable of detecting associations with proteins known to bind MKK1, and was not able to activate transcription of the reporter genes alone.

To identify other proteins that interact with MKK1, the LexA MKK1 vector was co-transformed with an embryonic day 9.5/10.5 mouse cDNA library fused to the VP16 vector (developed by Stan Hollenberg). This library was generated by random primed cDNA synthesis and size-selected to have insert sizes in the range of 350 to 700 nucleotides. This size limitation may enhance the ability to identify individual protein binding domains. From approximately 1.2×10^6 screened transformants, 93 colonies were isolated. Of these, 72 colonies were able to grow in the absence of histidine, and 63 were also blue when tested for β-galactosidase activity by filter assay (Fig. 5). These 63 clones were subsequently segregated by replica-plating and tested for the presence of non-specific interactions by yeast mating assays. Results of mating assays led to the sequencing of 14 positive clones. Two clones, MKKIP68 and MKKIP82 (MKKIP stands for MKK1 interacting protein) are murine homologs of the original bait, human MKK1, suggesting that MKK1 interacts with itself. Another clone, MKKIP85a, has 44% identity to *Xenopus* MAPKK (XMEK2), suggesting that this was a novel murine homolog. The remaining clones either coded for proteins on the non-coding strand, contained no homologs in the database or were determined to encode proteins which interacted non-specifically with MKK1, and were not pursued.

Clones MKKIP68 and MKKIP82 represented independent fusions to the VP16 acidic activation domain. The region of homology between MKKIP68, MKKIP82 and MKK1 encompassed approximately the last 100 amino acid residues of MKK1, and regions of overlapping sequence were identical (Fig. 6) In contrast, MKKIP85a, the putative novel isoform, shared only 31% identity with MKK1 over the region cloned (Fig. 7A), but demonstrated higher homology to a region in XMEK2 (Fig. 7B).

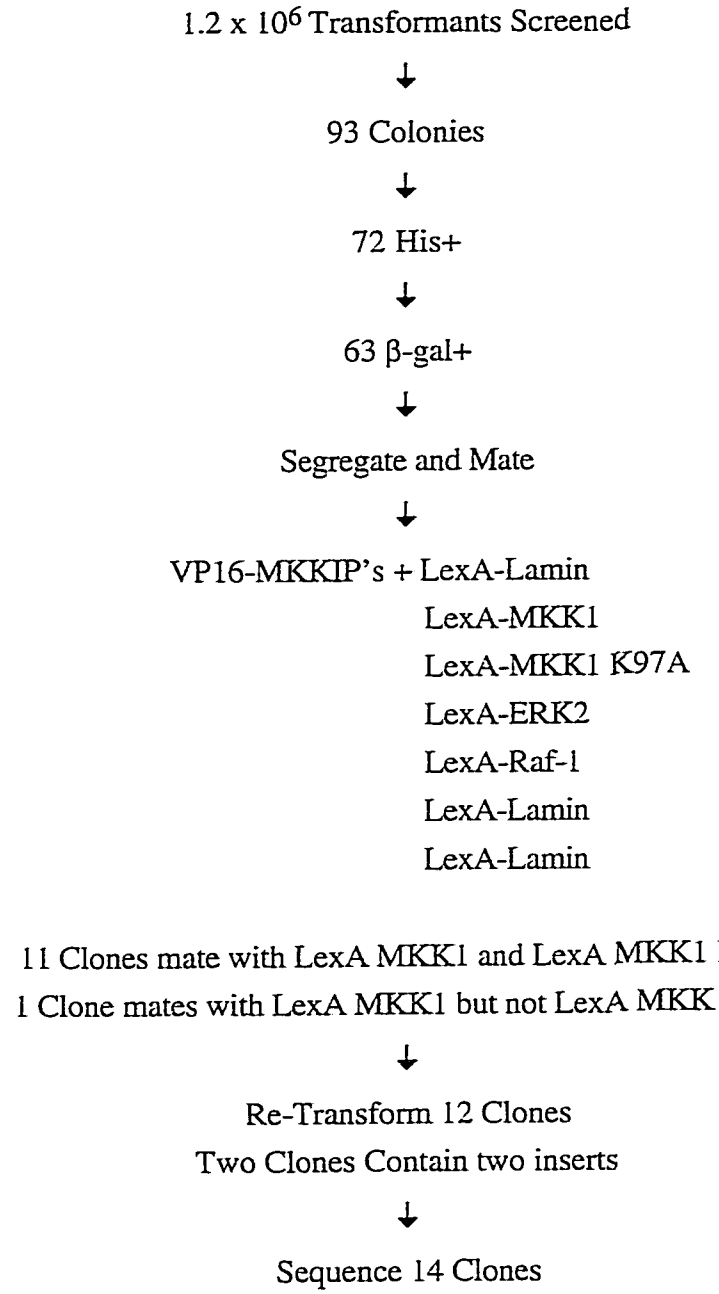


Fig. 5. Summary of MKK1 two-hybrid library screen results.

```
          1                                                            50
  mkk1a   MPKKKPTPIQ LNPAPDGSAV NGTSSAETNL EALQKKLEEL ELDEQQRKRL
mkkip82   .......... .......... .......... .......... ..........
mkkip68   .......... .......... .......... .......... ..........

          51                                                          100
  mkk1a   EAFLTQKQKV GELKDDDFEK ISELGAGNGG VVFKVSHKPS GLVMARKLIH
mkkip82   .......... .......... .......... .......... ..........
mkkip68   .......... .......... .......... .......... ..........

          101                                                         150
  mkk1a   LEIKPAIRNQ IIRELQVLHE CNSPYIVGFY GAFYSDGEIS ICMEHMDGGS
mkkip82   .......... .......... .......... .......... ..........
mkkip68   .......... .......... .......... .......... ..........

          151                                                         200
  mkk1a   LDQVLKKAGR IPEQILGKVS IAVIKGLTYL REKHKIMHRD VKPSNILVNS
mkkip82   .......... .......... .......... .......... ..........
mkkip68   .......... .......... .......... .......... ..........

          201                                                         250
  mkk1a   RGEIKLCDFG VSGQLIDSMA NSFVGTRSYM SPERLQGTHY SVQSDIWSMG
mkkip82   .......... .......... .......... .......... ..........
mkkip68   .......... .......... .......... .......... ..........

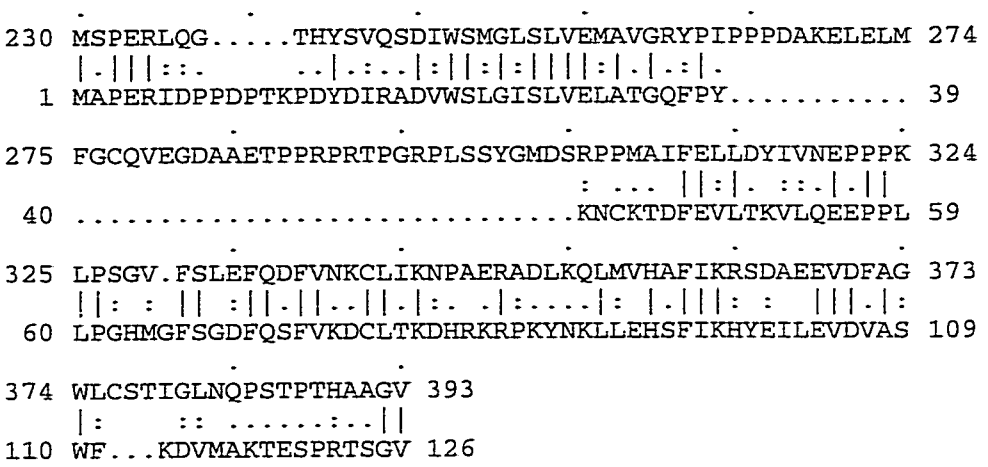
          251                                                         300
  mkk1a   LSLVEMAVGR YPIPPPDAKE LELMFGCQVE GDAAETPPRP RTPGRPLSSY
mkkip82   .......... .......... .......... GRSPKHHPGP RTPGRPLSSY
mkkip68   .......... .......... .......... ..RPLTPPXP RTPGRPLSSY

          301                                                         350
  mkk1a   GMDSRPPMAI FELLDYIVNE PPPKLPSGVF SLEFQDFVNK CLIKNPAERA
mkkip82   GMDSRPPMAI FELLDYIVNE PPPKLPSGVF SLEFQDFVNK CLIKNPAERA
mkkip68   GMDSRPPMAI FELLDYIVNX PPPKLPSGVF SLEFQDFVNK CLIKNPAERA

          351                                                         400
  mkk1a   DLKQLMVHAF IKRSDAEEVD FAGWLCSTIG LNQPSTPTHA AGV.......
mkkip82   DLKQLMVHAF IKRSDAEEVD FAGWLCSTIG LNQPSTPTHA AS*AX.....
mkkip68   DLKQLMVHAF IKRSDAEEVD FAGWLCSTIG LNQPSTPTHA ASI*AFRKQP
```

Fig. 6. Sequence alignment of MKK1 with clones MKKIP68 and MKKIP82. Alignment was determined by the BESTFIT program (WGCG). Sequence overlap covers catalytic subdomains IX through XI.

A.



B.

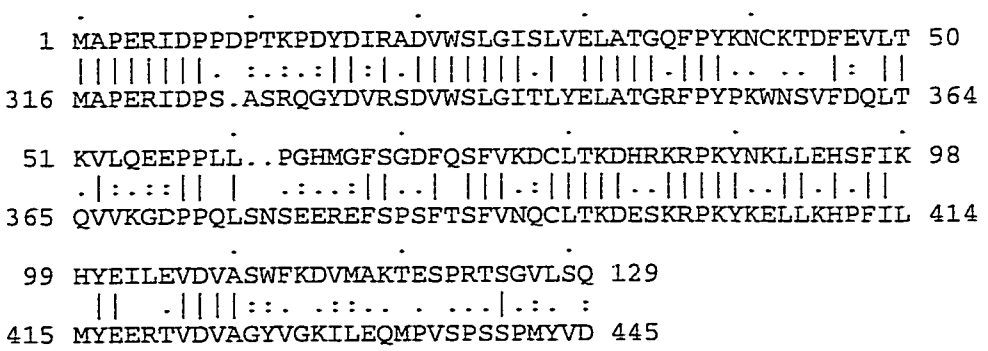


Fig. 7. A. Alignment between putative novel homolog MKKIP85a (1-126) and bait MKK1 (230-393). Alignment was created using the BESTFIT program (WGCG). Gaps are inserted to maximize the number of matches. Vertical lines denote identical residues. Dots represent conserved residues. The two share 38% identity and 59% similarity over the region cloned. B. Alignment of cloned fragment MKKIP85a (1-129) and its nearest relative *Xenopus* XMEK2 (316-445). Sequences are 48% identical and 61% similar.

PRELIMINARY ANALYSIS OF THE ASSOCIATION OF MKK1 WITH MKK1:

We initially sought to characterize the nature of the interaction between MKK1 and MKK1 by using the yeast two-hybrid system. To identify the MKK1 interacting domain associated with the C-terminus, a series of MKK1 deletion constructs fused to the VP16 activation domain (provided by Mingzi Chen) were tested for their ability to interact with LexA MKK1 based on the transcriptional activation of the *HIS3* and *lacZ* reporter constructs (Fig. 8). Results indicated that the N-terminus is not likely to be involved in this interaction, as neither of the N-terminal constructs were able to activate the reporter genes (Table 2). An alternative possibility, which was not tested during these preliminary studies, is that the expression levels of the various N-terminal constructs in yeast were not sufficient for interaction.

To address the question of whether the phosphorylation state of MKK1 had an effect on MKK1-MKK1 binding, we transformed yeast with full-length wild-type, kinase-inactive (K97A), or constitutively-active MKK1 (S218E, S222E) (provided by Rony Seger) fused to either the LexA or the VP16 vector. In a two-hybrid test, full-length wild-type MKK1 was capable of interacting equally well with kinase-inactive and constitutively-active MKK1, and slightly better with wild-type MKK1 (Table 3). Two kinase-inactive forms of MKK1 generated a much weaker interaction than the wild-types, and kinase-inactive (K97A) with constitutively-active (S218E, S222E) gave the weakest interaction. The constitutively-active form interacted only slightly better with itself than with wild-type MKK1, and very weakly with kinase-inactive MKK1.

To determine if the C-terminus was sufficient for an MKK1-MKK1 interaction, C-terminal constructs of MKK1 were subcloned into the LexA and VP16 vectors (Table 4). These transformants were not able to grow in the absence of histidine, and remained white in a β-gal filter assay, suggesting that the MKK1-MKK1 association requires more than just the C-terminus. Equal expression of these constructs by anti-LexA and anti-VP16 Westerns was confirmed (data not shown).

Finally, *in vitro* binding studies were performed in order to demonstrate the MKK1-MKK1 interaction. A GST-MKKIP82 fusion protein bound to glutathione agarose beads was incubated with a mixed pool of active and inactive MKK purified from rabbit

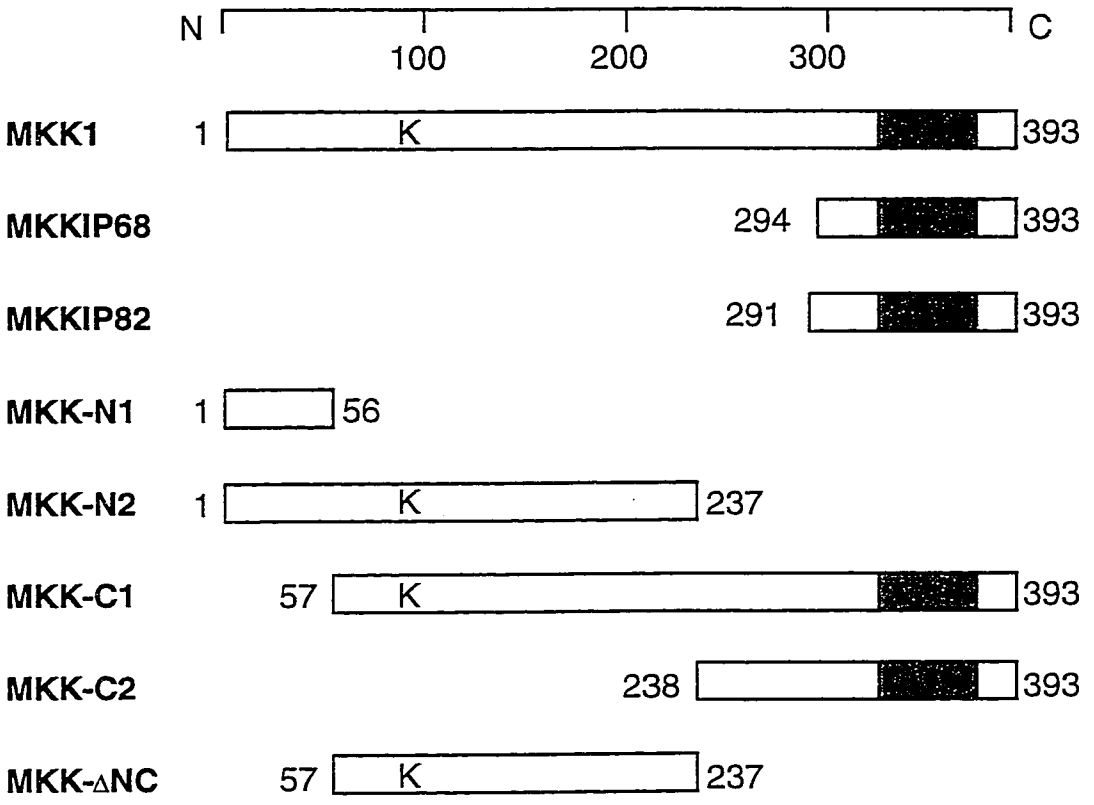


Fig. 8. MKK1 deletion constructs. K denotes lysine 97, required for ATP binding. Shaded regions denote proline rich regions.

Table 2. Interactions of MKK1 deletion constructs in a two-hybrid test. MKK1 was fused to the LexA DNA binding domain. Various MKK1 deletion constructs, or MKK1 C-terminal fragments represented by clones MKKIP68, MKKIP82 or MKKIP85a, were fused to the VP16 acidic activation domain.

pLexA	pVP16	Growth on His^-	β-gal
MKK1	MKKIP68	+	+++
MKK1	MKKIP82	+	+++
MKK1	MKKIP85a	+	+++
MKK1	MKK1 N1	-	-
MKK1	MKK1 N2	-	-
MKK1	MKK1 C1	-/+	+
MKK1	MKK1 C2	+	+++
MKK1	MKK1 ΔNC	-	-

Table 3. Interactions of wild-type and MKK1 mutants in a two-hybrid test. Associations between wild-type and wild-type MKK1, and active with active MKK1 (S-E 218/222) appear stronger than those between kinase-inactive and kinase-inactive MKK1. Reciprocal fusions (ie-subcloning of proteins into opposite vectors) has no effect (data not shown).

pLexA	pVP16	Growth on His^-	β-gal
MKK1	empty	-	-
MKK1	MKK1	+	+++
MKK1	MKK1 K97A	+	++
MKK1	MKK1 S218,222E	+	++
MKK1 K97A	MKK1 K97A	-/+	+
MKK1 K97A	MKK1 S218,222E	-	-/+
MKK1 S218,222E	MKK1 S218,222E	+	+++

Table 4. Interactions between MKK1 C-terminal regions in a two-hybrid test. MKKIP82 contains residues 291-393. MKKIP68 contains residues 294-393. The association of MKK1 with MKK1 requires a region within the catalytic domain in addition to the C-terminus for association.

pLexA	pVP16	Growth on His^-	β-gal
MKKIP82	empty	-	-
MKKIP82	MKK1	+	+++
MKKIP82	MKKIP85a	-	-
MKKIP82	MKKIP68	-	-
MKKIP82	MKKIP82	-	-/+
MKKIP82	MKK1 N1	-	-
MKKIP82	MKK1 N2	-	-
MKKIP82	MKK1 C1	+	+++
MKKIP82	MKK1 C2	-	-
MKKIP82	MKK1 ΔNC	-	-

muscle (provided by Lee Graves & Amy Jensen). We were unable to detect an association of these proteins as determined by anti-GST or anti-MKK1 Westerns (data not shown). From these studies, we could not rule out the possibility that another protein is also required for the MKK1-MKK1 interaction. Additional further studies to address MKK1 interactions involving MKK1 and MKK7, as well as MKK7 dimerization, are described in Chapter 5.

FULL-LENGTH CLONING OF THE NOVEL MKK, MKK85a:

PRIMARY LIBRARY SCREEN:

To isolate a full-length cDNA clone, a mouse day 16 embryo λ-phage cDNA library (Novagen) was screened, using the MKKIP85a fragment as a probe. PCR analysis of the isolated clones showed a range of insert sizes, from 2.5 to 3 kb, with the majority of sequence corresponding to the 3'-untranslated region. The longest clones were sequenced using a probe-specific primer and identified approximately 75 additional residues 5' of the probe sequence, as well as the 3' stop codon (8 additional 3' residues), comprising a total of 194 residues (Fig. 9). This novel MKK1 isoform was referred to as MKK85a, and the full-length form of MKK85a was eventually named MKK7. The sequence obtained contained all of the residues normally conserved in serine/threonine kinases, and encompassed catalytic subdomains VIb through XI (Hanks *et al.*, 1988). Of significant note were the presence of serine and threonine residues in kinase subdomains VII and VIII of MKK85a. Serine residues in this region are known to be phosphorylation sites required for the activation of MKK1 (Alessi *et al.*, 1994; Mansour *et al.*, 1994; Seger *et al.*, 1994; Yan and Templeton, 1994; Zheng and Guan, 1994). It was also of interest to note that MKK85a did not contain the C-terminal consensus MAPK phosphorylation site at Thr 386, (PXS/TP), which was present in all vertebrate MKK members identified to date, and was postulated to be involved in feedback regulation (Marshall, 1994). This reflected possible differences in the regulation of MKK1 and MKK85a. Another MAPK phosphorylation site at Thr 292 (PRTP), and a potential site for phosphorylation by $p34^{cdc2}$ at Thr 286 (ETPPR), were also absent in MKK85a. Comparison of the sequence with other known members of the MKK family by using the PILEUP program (WGCG) showed that MKK85a was a member of a subclass of kinases including the *Xenopus* MKK XMEK2, and 2 yeast kinases, PBS2 (*S. cerevisiae*) and WIS1 (*S. pombe*) (Fig. 10).

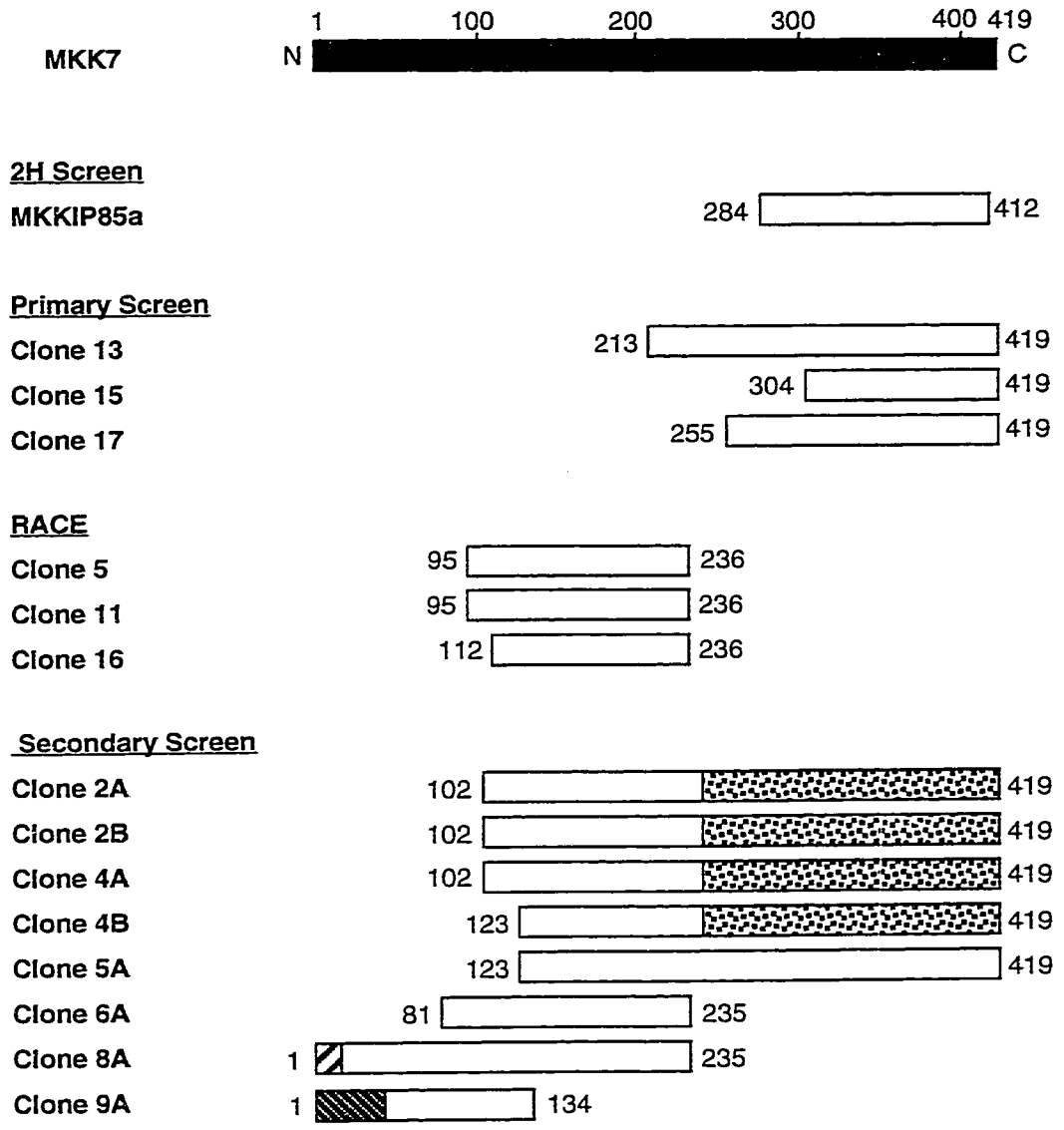


Fig. 9. Regions of overlap among MKK7 clones. The stippled areas in clones 2A, 2B, 4A and 4B represent regions in those clones not sequenced. The hatched areas in clones 8A and 9A represent variations in sequence, corresponding to MKK7b and MKK7a, respectively.

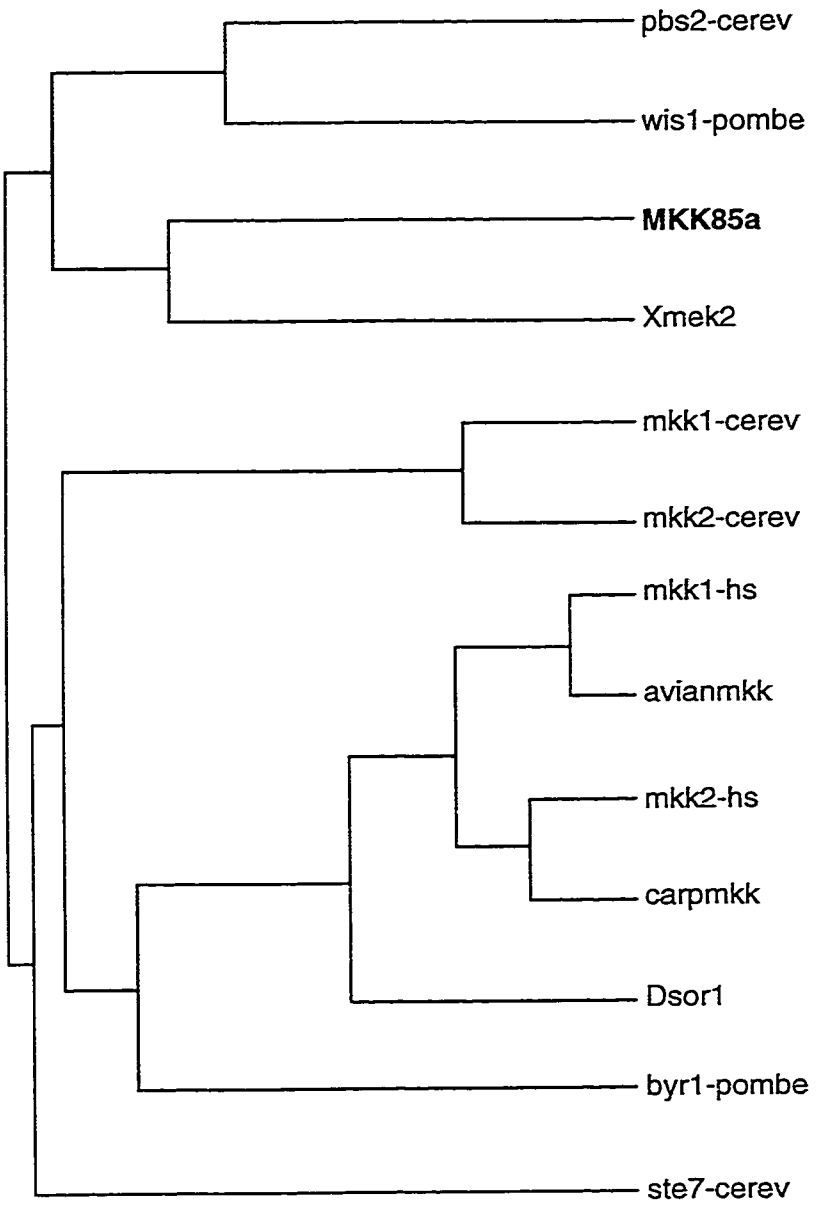


Fig. 10. Comparison of MKK85a with other MKKs. The comparison was created by the PILEUP program (WGCG) using a pair wise alignment comprising 194 residues of MKK85a sequence. The identity of the kinases with MKK85a was calculated with the BESTFIT program: pbs2-cerev: 41%, wis-1 pombe: 44%, Xmek2: 59%, mkk1-cerev: 44%, mkk2-cerev: 43%, mkk1-hs: 46%, avianmkk: 46%, mkk2-hs: 46%, carpmkk: 48%, Dsor1: 45%, byr1-pombe: 43%, ste7-cerev: 37%.

MKK85a was most closely related to XMEK2 (58% identity), with slightly less identity to PBS2 (41%) and WIS1 (43%). Sequence information to this point indicated that MKK85a was only distantly related to vertebrate MKK1, and would likely have a different substrate specificity.

RACE PCR CLONING:

To obtain further sequence within the open reading frame of MKK85a, a RACE (Rapid Amplification of cDNA Ends) PCR approach was used (Innis *et al.*, 1990). The RACE protocol generates cDNA's by using PCR to amplify copies of the region between a single point in the transcript and a 3' or 5' end. From a region of known sequence, a primer oriented in the 5' direction was generated for reverse transcription from a mouse brain RNA template. A homopolymer was then appended as an adapter sequence using terminal transferase to tail the first strand reaction products. PCR amplification was performed using a nested gene specific primer and a primer which would hybridize to the appended tail (adapter primer). PCR products were purified, subcloned and analyzed by DNA sequencing. This molecular cloning approach yielded an additional 141 amino acids of 5' sequence within the open reading frame (Fig. 9). This region of sequence encompassed all of the catalytic subdomains present in protein kinases, but did not appear to encode the full-length cDNA.

SECONDARY LIBRARY SCREEN:

In a continued attempt to obtain a full-length cDNA of the novel MKK1 isoform MKK85a (MKK7), a second cDNA library was screened. Using the most N-terminal region of sequence available as a probe (residues 101-237 of full-length MKK7), a mouse brain cDNA library (Stratagene) was screened. The sequencing of eight clones finally yielded two clones containing initiator methionine residues and upstream, in-frame stop codons, but with diverging 5' coding sequence (Fig. 9). This indicated that alternative splice forms of the novel MKK1 isoform were present. The novel isoform MKK85a was ultimately named MKK7, and the differing splice forms were MKK7a and MKK7b, respectively (Fig. 11). MKK7a is encodes a 419 amino acid protein with a predicted molecular weight of 48 kDa. MKK7b is encoded by 391 amino acids. For expression studies, a full-length MKK7a was generated using an overlap PCR strategy (Innis *et al.*, 1990), and sequence of the forward and reverse strands was confirmed. This clone was

```
   1  MAASSLEQKLSRLEAKLKQENREARRRIDLNLDISPQRPR   40
                                    MLTPFMPLVFNS   12

  41  PTLQLPLANDGGSRSPSSESSPQHPTPPTRPRHMLGLPST   80
      PA

  81  LFTPRSMESIEIDQKLQEIMKQTGYLTIGGQRYQAEINDL   120

 121  ENLGEMGSGTCGQVWKMRFRKTGHIIAVKQMRRSGNKEEN   160

 161  KRILMDLDVVLKSHDCPYIVQCFGTFITNTDVFIAMELMG   200

 201  TCAEKLKKRMQGPIPERILGKMTVAIVKALYYLKEKHGVI   240

 241  HRDVKPSNILLDERGQIKLCDFGISGRLVDSKAKTRSAGC   280

 281  AAYMAPERIDPPDPTKPDYDIRADVWSLGISLVELATGQF   320

 321  PYKNCKTDFEVLTKVLQEEPPLLPGHMGFSGDFQSFVKDC   360

 361  LTKDHRKRPKYNKLLEHSFIKHYEILEVDVASWFKDVMAK   400

 401  TESPRTSGVLSQHHLPFFR  419
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Fig. 11. The amino acid sequences of MKK7a and MKK7b. MKK7b starts at position 29 of MKK7a and is 391 amino acids long. Except at those residues noted, MKK7a and MKK7b are identical. GenBank Accesion numbers: U74463 (MKK7a) and U74464 (MKK7b).

used for all the studies presented here, and will hereby be referred to as MKK7 unless otherwise indicated. To confirm that the full MKK7 sequence was present in tissues, standard RT-PCR was performed using mouse brain and kidney RNA templates (data not shown).

DISCUSSION:

The yeast two-hybrid screen showed that human MKK1 can bind specifically to the C-terminus of mouse MKK1 or MKK7. Experiments with fragments of MKK1 suggest that the central region of MKK1 (the kinase domain) binds to the C-terminus. One possibility is that our observations reflect an intra-molecular association within MKK1. According to such a model, this interaction would reflect an association between the catalytic domain and a C-terminal regulatory domain within a single molecule. A second possibility is that MKK1 can homodimerize. Additionally, the identification of MKK7 as a binding partner suggested that MKK1 could also heterodimerize. One model could be that heterodimerization among various MKKs could serve as a means to activate or inhibit different pathways in response to certain stimuli. This could serve as a method of cross-regulation and integration among related pathways.

Initial attempts to address whether the dimerization of MKK1 affected its activity suggested that this may not be the case. Purification of active MKK1 from rabbit muscle had revealed three peaks of activity. It was possible that one of these peaks corresponded to a dimeric form of MKK1 (L. Graves, R. Seger, personal communication). However, in our two-hybrid experiments to look at the phosphorylation requirements of MKK1 for dimerization, we saw only subtle differences in the ability of active and inactive forms of MKK1 to dimerize. These differences may have been due to unequal expression levels of the different mutants and not to their activation states.

Studies to map the region of MKK1 required for dimerization demonstrated that the N-terminal region is not necessary, but a region within the catalytic domain in addition to the C-terminus is required. Association between just the C-terminus with the C-terminus was not detected. Since a region in addition to the C-terminus but not in the N-terminus is also required for this interaction, we cannot determine whether this is an inter-

or an intra-molecular association. More recent studies addressing the dimerization of MKK7 as well as the heterodimerization of MKK1 and MKK7 are presented in Chapter 6.

At least two ERK MAP kinase phosphorylation sites exist within the C-terminal region of MKK1. Both Thr 292 and Thr 386 have been demonstrated by mass spectrometry and by site-directed mutagenesis to be phosphorylated by ERK MAP kinase *in vitro* (Mansour *et al.*, 1994). It has been proposed that phosphorylation of MKK1 by ERK could be one means of feedback regulation of the pathway, as ERK can also phosphorylate Raf-1 (Anderson *et al.*, 1991; Lee *et al.*, 1992). Phosphorylation of these sites by ERK does not significantly affect the phosphorylation or activation of MKK1 by Raf (Mansour *et al.*, 1994). Both of these phosphorylation sites are contained in the C-terminal MKK1 clones identified in the two-hybrid screen. In addition, the sequence just proximal to Thr 292, PPRP, falls within the consensus sequence found to mediate the interaction of several proteins with SH3 domains (Ren *et al.*, 1993; Yu *et al.*, 1994). It is possible that under conditions in which MKK1 is in a dimerized form involving the C-terminus, accessibility to either the ERK MAP kinase phosphorylation sites or to the proline rich sequence could be blocked. This could influence the association of MKK1 with other signaling intermediates or affect the ability of MKK1 to form complexes with other proteins.

Although the mouse embryo library originally screened in the two-hybrid screen contained the MKK1 activator Raf-1 (Vojtek *et al.*, 1993), this protein was not detected in our screen. In addition, ERK MAP kinase, the only known substrate for MKK1, also was not detected in our screen. This is possibly due to the fact that a complete representation of the library was not screened, due to poor transformation efficiencies. Since the associations of MKK1 with Raf-1 or ERK are believed to be relatively transient in cells, it is also possible that unstable associations are more difficult to identify. A report of a two-hybrid screen with MKK5 used catalytically inactive forms of MKK5, (S311A, T315A) and K195M, to identify the MAP kinase relative, ERK5 (Zhou *et al.*, 1995). Although a screen with wild-type MKK5 was also performed and wild-type MKK5 binds to ERK5, no ERK5 isolates were identified from the screen using wild-type MKK5. A two-hybrid screen with MKK4 lacking the first 40 amino acids revealed an interaction between MKK4 and actin binding protein 280 (ABP 280) (Marti *et al.*, 1997). These data suggest that the characteristics of the bait can influence the types of proteins identified, and

that dominant-negative mutants may enhance interactions with upstream- or downstream-signaling molecules. Guan and colleagues also performed a two-hybrid screen with wild-type MKK1 as well as a phosphorylation site mutant (S218A, S222A) (Wu *et al.*, 1996). No clones were isolated when the wild-type MKK1 was used as bait, but all three members of the Raf family (A-Raf, B-Raf, c-Raf) were identified as positive clones with MKK1 S218A, S222A. Thus, the use of an inactive form of MKK1, such as MKK1 (S218A, S222A), as bait in a two-hybrid screen may stabilize the interaction between a kinase (Raf-1) and its substrate (MKK1). In an independent screen for MKK1-interacting proteins, MKK1 was also found to associate with itself (Hans Schaeffer, personal communication). This study also used wild-type MKK1 as bait and revealed a novel protein, MP1, which has been demonstrated to bind MKK1 and ERK1 specifically and is thought to function in the formation of a scaffolding complex (Schaeffer *et al.*, 1998).

The initial sequence information of clone MKKIP85a from the yeast two-hybrid screen suggested that this was a novel member of the growing MAP kinase kinase family. Cloning of the full-length sequence of this kinase, called MKK7, confirmed this hypothesis. The remaining chapters focus on studies concerning the functions, regulation and interactions of the newly identified MKK7. It should be noted that several other investigators concurrently reported the identification of MKK7 (Foltz *et al.*, 1998; Lawler *et al.*, 1997; Moriguchi *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997).

MATERIALS AND METHODS:

DNA CONSTRUCTS:

The plasmids, pBTM116, pVP16 (Hollenberg *et al.*, 1995; Vojtek *et al.*, 1993), pLexA-lamin (Bartel *et al.*, 1993) have been described. To generate pLexA-MKK1, pLexA-MKK1 K97A and pLexA-MKK1 (S218E, S222E), the open reading frames of each human MKK1 construct (gifts of Rony Seger) were PCR-amplified using the following oligonucleotide primers:

Sense: 5'-GACTAGGATCCAAATGCCCAAGAAGAAGCC-3'

Antisense: 5'-TGCATGCATGTTCTTCATCCTTTGTCACAGGTG-

Each resulting PCR product was subsequently digested with BamHI and NsiI, and ligated into pBTM116 digested with BamHI and PstI. To generate pVP16-MKK1, pVP16-MKK1 K97A and pVP16-MKK1 (S218E, S222E), each construct was amplified with the above sense oligonucleotide and the following antisense oligonucleotide 5'-GACTAGGATCCGTTCTTCATCCTTTGTCACAGGTG-3'. Resulting products were digested with BamHI and ligated into the BamHI site of VP16. To generate pLexA-ERK2, rat ERK2 (gift of Melanie Cobb) was PCR-amplified using the sequence 5'-TGACTAGGATCCGTATGGCGGCGGCGGCG-3' for the sense oligonucleotide and 5'-TATAAATGCATATTAAGATCTGTATCCTGG-3' for the antisense oligonucleotide. The product was digested with BamHI and NsiI and subcloned into pBTM116 as described for MKK1 above. To generate pGEX3X MKKIP82, MKKIP82 was cut out of VP16 at BamHI and EcoRI sites, and cloned into the BamHI and EcoRI sites of pGEX 3x.

YEAST TWO-HYBRID TRANSFORMATION AND SCREEN:

The yeast transformations were carried out as previously described (Vojtek *et al.*, 1993). For the screen (also previously described), the *Saccharomyces cerevisiae* strain L40 was transformed with LexA-MKK1 and a VP16 fusion of a mouse embryo cDNA library at E9.5-E10.5 stages of development. Fourteen clones from 1.2 x 10^6 transformants were sequenced. Clone MKKIP85a represented a putative novel MKK C-terminal fragment of 128 amino acids.

β-GALACTOSIDASE ASSAYS:

Yeast transformants to be analyzed were grown 2-3 days as patches on plates with the appropriate markers. A dry nitrocellulose filter with proper orientation marks was overlaid on the yeast. Once yeast were transferred to filters, the latter were placed in liquid nitrogen for 10 seconds to break open the yeast. After removal from liquid nitrogen, the filters were placed colony side up onto a #1 Whatman filter circle previously soaked in 2 ml of Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and 30 µl X-Gal (50 mg/ml). Filters were covered, placed at 30 °C and allowed to develop. Strong interactions were visible within 30 minutes.

LIBRARY SCREENING AND RACE CLONING:

The primary and secondary library screens (16-day mouse embryo cDNA library in λSHlox™ vector, Novagen, and Lambda ZAP II mouse brain cDNA library, Stratagene, respectively) were carried out exactly according to the manufacturer's protocols. The probe for the primary library screen was the full-length MKKIP85a sequence originally isolated. The probe for the secondary screen was from a 408 base pair PCR template product generated with the following primers:

Sense: 5'-GCTGCAGGAGATCATGAA-3'

Anti sense: 5'-CCATGCTTCTCCTTCAGATAGTACAGT-3'

Probes were generated using a single primer in the presence of [α-^{32}P]-dCTP. Unincorporated nucleotides were removed by ethanol precipitation. To obtain sequence of the 3' end, clones in the λSHlox™ vector were sequenced with a vector specific primer, SP6, as well as the following MKK85a specific primer:

5'-CACTATGAGATACTCGAGGTG-3'

For RACE PCR cloning, the Marathon cDNA Amplification Kit (Clontech) was utilized. For use with the manufacturer's adapter primers AP1 and AP2, the following MKK85a specific external and nested oligonucleotides were generated:

Sense: 5'-GAGCTTGATCTGGCCCCGCTC-3'

Antisense: 5'-GGAATTCGCCATGCTTCTCCTTCAGATAGTA-3'.

Full-length MKK7a was generated by overlap PCR, combining two separate PCR products with overlapping sequence into one longer product (Innis *et al.*, 1990). The two overlapping ("inside" primers) contained the sequence, 5'-AGCTGCAGGAGATCATGAAGCAGACAGG-3' and its complement . The forward and reverse full-length primers were as follows:

Sense: 5'-CGCGGATCCCGGGGAAGATGGCGGCGTCCTCCCTG-3'

Antisense: 5'-CGGGATCCCATGAGGCTACCTGAAGAAGGGC-3'.

Confirmation that the MKK7 sequence was present in tissues was determined by standard RT-PCR using mouse brain and kidney RNA templates and the above forward and reverse oligonucleotide primers. Sequence of forward and reverse strands was confirmed by automated sequencing with a Model 377 ABI sequencer.

CHAPTER 2: CHARACTERIZATION OF MKK7

INTRODUCTION:

The identification and cloning of MAP kinase kinase 1 (MKK1) defined a new family of mammalian dual specificity kinases that phosphorylate and activate MAP kinase family members on threonine and tyrosine residues (Crews *et al.*, 1992; Nakielny *et al.*, 1992; Rossomando *et al.*, 1992; Seger *et al.*, 1992). This family of enzymes share several characteristics that suggest they have a unique and highly dedicated relationship to their cognate MAP kinases. To date, no substrate other than the appropriate MAP kinase has been identified for any MAP kinase kinase. Although *in vivo* specificities of MKK's have not been fully defined, their *in vitro* specificities suggest that each acts in a single MAP kinase pathway, and that MKK's are likely to be targets for convergent regulation by a diverse group of upstream activators. MKK1 and MKK2 phosphorylate and activate ERK1 and ERK2 MAP kinase, MKK3 and MKK6 activate p38 MAP kinase, and MKK4 activates SAPK.

Whereas activation of ERK occurs primarily in response to mitogenic signals, both p38 and SAPK are activated in response to a variety of cellular stresses and inflammatory cytokines (D'Erijard *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). Specific activators for p38, MKK3 and MKK6 have been described. For SAPK, MKK4 has been identified as a direct activator. However, biochemical fractionation studies suggested that additional SAPK activators stimulated by cellular stresses existed (Meier *et al.*, 1996; Moriguchi *et al.*, 1995). In addition, studies with $MKK4^{-/-}$ cells indicated that there were MKK4-dependent and MKK4-independent signaling pathways leading to SAPK activation.

An important question concerns the fact that multiple mammalian MKKs function upstream of specific MAP kinases. It is unclear whether this reflects functional redundancy within the MAP kinase pathways, whether the MKKs are functionally distinct, or whether they serve to integrate signals from distinct activators or stimuli. Upstream of the MKKs the situation is even more complicated. A diverse group of MAPKKKs have been shown to have the ability to activate multiple MKKs (Fig. 3). For example, MEKK4, TAK1, MLK3 and ASK1 have all been demonstrated to activate both the SAPK and p38 pathways (Fanger *et al.*, 1997; Ichijo *et al.*, 1997; Takekawa *et al.*, 1997; Tibbles *et al.*, 1996). Many of the mammalian MKKKs are activated by a family of MKKKKs that are homologous to the yeast STE20 kinase (Kyriakis and Avruch, 1996). Finally, members of the Ras and Rho families of small GTP-binding proteins including Ras, Rac, Cdc42 and Rho have also been shown to play an important role in the activation of MAP kinase pathways (Vojtek and Cooper, 1995). How all of these upstream signals are organized or converge on one or more MAP kinase kinases is not well understood.

From a yeast two-hybrid screen using MKK1 as bait, a new MAP kinase kinase family member, MKK7 was identified. The full-length MKK7a encodes a 419 amino acid protein with a predicted molecular weight of 48 kDa (Fig 11). MKK7b has a different sequence in the amino terminus, and is encoded by 391 amino acids. We presume that MKK7a and MKK7b are alternatively spliced isoforms. Both MKK7a and MKK7b contained upstream in-frame stop codons, suggesting that they are full-length clones. MKK7a/b contain all the residues conserved among dual specificity kinases. This chapter describes the structural, biochemical and biological characterization of MKK7. We demonstrate that MKK7 is a major activator of SAPK. Substrate specificity of MKK7 has been examined. Extracellular stimuli as well as direct upstream activators of MKK7 have been identified *in vitro* and *in vivo*. The use of site-directed mutants that alter the activation state of MKK7 have been utilized in transient cell expression assays. Information from these studies has provided a foundation on which to further address the biological function of MKK7 in cells.

RESULTS:

STRUCTURAL CHARACTERIZATION OF MKK7:

A comparison of the deduced amino acid sequence of MKK7 with the GenBank database reveals homology to the mammalian MAP kinase kinases MKK4, MKK6 and MKK3 (44%, 44% and 45%, respectively). Across species, it was noted that MKK7 was most closely related to the *Drosophila* MAP kinase kinase *hemipterous* (*hep, Dhep*), sharing 56% amino acid identity over the full-length sequence and 71% identity within the catalytic domain (Fig. 12). Notably, alignment of the conserved activation loop within subdomains VII and VIII of the mammalian MAP kinase kinases and Hep indicated that MKK7 and Dhep contain an additional serine residue at position 277 (Fig. 13). This residue is absent in MKK1, MKK2 and MKK5, and replaced by an aspartic acid residue in MKK3, MKK4 and MKK6. This residue is also absent in other yeast and *Drosophila* MAP kinase kinases. This serine residue may serve as an additional regulatory phosphorylation site in MKK7 and Hep, which could distinguish its upstream activators from those of other MAP kinase kinases. In addition, both MKK7a and MKK7b contain stretches of proline rich sequences in their shared N-terminal region, which could serve as SH3-domain binding motifs (Ren *et al.*, 1993).

It is of interest to note that MKK7a, but not MKK7b, contains a predicted nuclear export sequence (NES) in its N-terminus (Fig. 14). The presence of a leucine rich NES has recently been established in several other proteins, including MKK1 and PKI of cAMP dependent protein kinase (Fukuda *et al.*, 1996; Wen *et al.*, 1995). This leucine rich sequence is also present in Hep, but absent in MKK4 (Glise *et al.*, 1995; Lin *et al.*, 1995). In MKK1, the NES has been demonstrated to be required for its cytoplasmic localization and may be required for the proper regulated activation of MAP kinase. The predicted NES in MKK7a may serve to differentially localize it from MKK7b or from MKK4.

TISSUE DISTRIBUTION OF MKK7:

To examine the tissue distribution of MKK7, we used Northern blot analysis (Clontech), and a C-terminal probe common to MKK7a and MKK7b. A major transcript of approximately 4 kb was present in all tissues analyzed, and was more abundant in skeletal muscle, heart, brain, and testis than in spleen, kidney, lung or liver (Fig. 15). Additional weaker transcripts of 7 and 9 kb were also noted in all tissues. These may represent alternative processing of transcripts from a single gene or other closely related protein kinases. The abundant short 2 kb transcript found in testis could

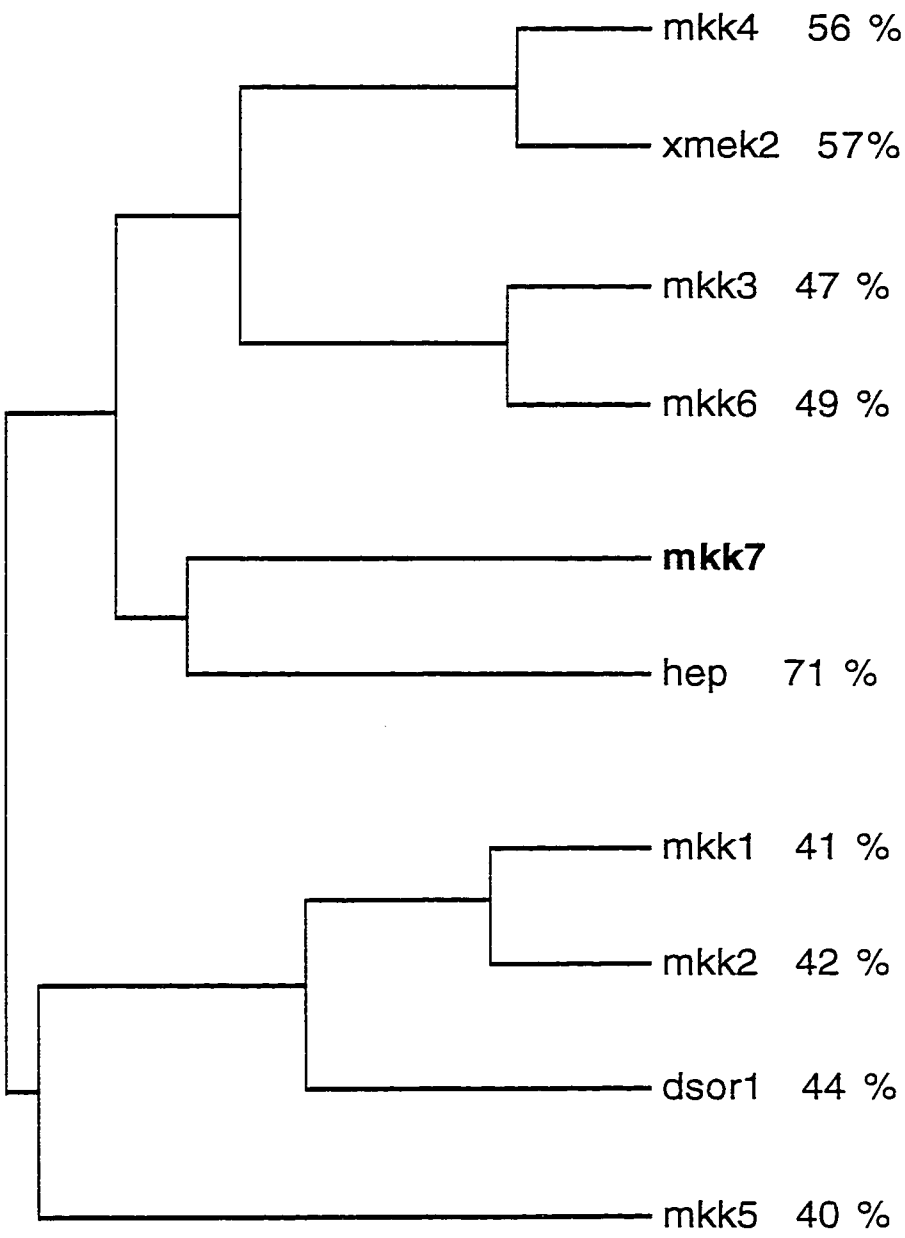


Fig. 12. Comparison of the catalytic domains of *Drosophila* and vertebrate MKKs. Comparison was created by the PILEUP program using a pair wise alignment. Percent identity of the catalytic domains was calculated using BESTFIT.

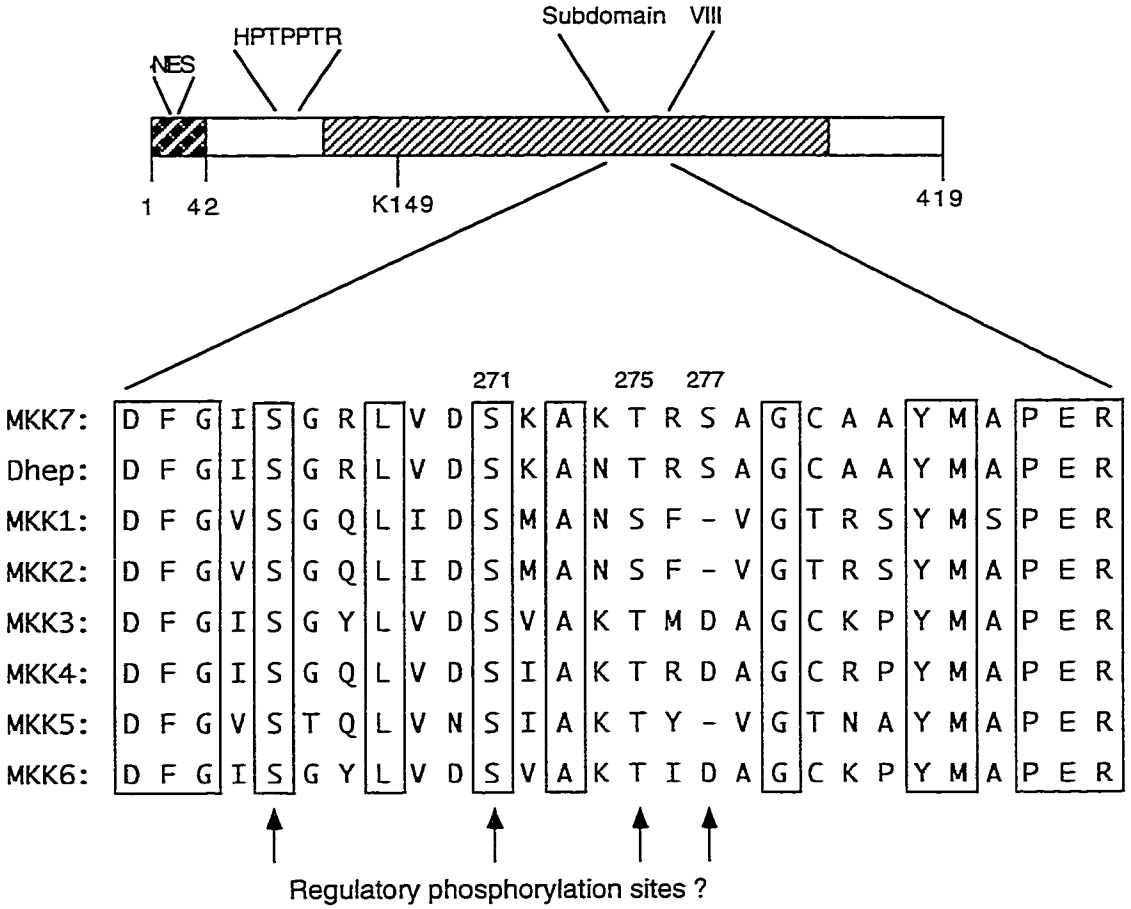


Fig. 13. Alignment of the activation loops of vertebrate MKKs and Hep. Residues within catalytic subdomain VIII conserved among all of the kinases are boxed. Proposed regulatory phosphorylation sites are indicated by arrows. Only MKK7 and Hep contain a phsophorylatable residue at position of 277. Residue numbers 271, 275 and 277 correspond to MKK7. The upper cartoon of MKK7 indicates the proline-rich region in the N-terminus, as well as the putative NES sequence.

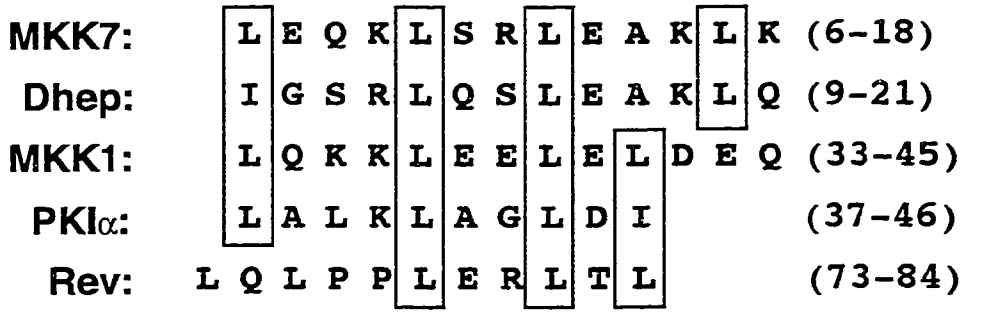


Fig. 14. Comparison of the predicted NES of MKK7 with others. Presented are the NES regions of Dhep (Glise *et al.*, 1995), MKK1 (Fukuda *et al.*, 1996), PKIα, (Wen *et al.*, 1995) and HIV-Rev (Meyer *et al.*, 1996). Conserved hydrophobic leucine and isoleucine residues are boxed. All sequences are located within the amino terminal portion of the protein.

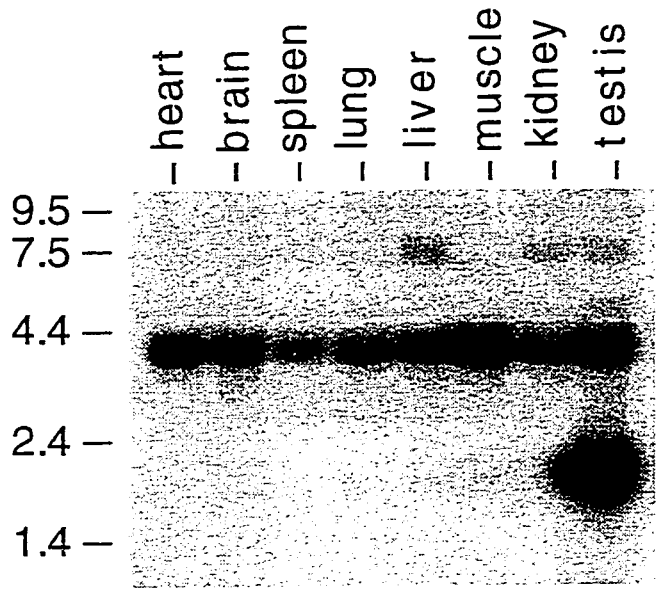


Fig. 15. Expression of MKK7 in adult mouse tissues. A Northern blot (Clontech) was probed with a 128 amino acid C-terminal fragment of MKK7. The position of RNA size markers in kilobases is illustrated.

represent a germ-cell specific transcript. The related MAP kinase kinase, MKK4, is also expressed in all tissues analyzed, and is predominantly in brain and skeletal muscle (D'Erijard *et al.*, 1995; Yan *et al.*, 1994).

MKK7 BINDS SAPK BUT NOT p38 OR ERK2:

Based on the sequence homology of MKK7 to other mammalian MAP kinase kinases, we tested whether MKK7 might associate with any known MAP kinases. We used a yeast two-hybrid assay to examine the ability of MKK7 to associate with either JNK1, p38 or ERK2 MAP kinases. A yeast strain containing the *HIS3* gene as an integrated reporter construct was transformed with wild-type full-length MKK7 fused to the LexA DNA-binding domain of the vector pBTM116 and either JNK1, p38 or ERK2 MAP kinase fused to the acidic activation domain of pVP16. Only the yeast expressing MKK7 and SAPK were able to grow in the absence of histidine (Table 5). This assay demonstrated that MKK7 and JNK1 could form a complex in yeast but did not show whether the complex required additional unidentified proteins. To test whether the association between MKK7 and JNK1 could be detected *in vitro*, we used an *in vitro* association assay. This assay has been previously used to identify specific interactions between proteins (Vojtek *et al.*, 1993; Waskiewicz *et al.*, 1997). In this assay, bacterially expressed GST or GST-MKK7 coupled to glutathione Sepharose beads was incubated with [^{35}S]-Met labeled, *in vitro* translated JNK1, p38 or ERK2 MAP kinase. Following incubation, samples were washed and analyzed by SDS-PAGE and autoradiography. We observed an interaction between GST-MKK7 and JNK1, but not p38 or ERK2 MAP kinase (Fig. 16). This assay demonstrated that the interaction between MKK7 and JNK1 could be detected *in vitro* as well as in yeast. Neither assay rules out the possibility that the association could be indirect, as a bridging protein present in yeast as well as in rabbit reticulocyte lysate could be required for the interaction.

MKK7 IS ACTIVATED BY STRESSES AND ACTIVATES SAPK/JNK:

To investigate the substrate specificity and activation of MKK7, we expressed epitope-tagged MKK7 in NIH3T3 cells and measured its activity *in vitro*. Cells were transiently transfected with either myc-tagged wild-type MKK7 or vector alone. Forty-eight hours after transfection, cells were left untreated or stimulated with either PDGF, anisomycin or NaCl. Immunoprecipitated MKK7 was then assayed for its ability

Table 5. MKK7 Interacts with JNK1 in a Yeast Two-Hybrid Assay. The L40 reporter strain was transformed with pLexA-MKK7 or pLexA-Lamin and the indicated pVP16 plasmids. Individual transformants were streaked to synthetic medium plates lacking histidine and incubated at 30 °C for 3 days. A + symbol indicates the ability of yeast to grow on plates lacking histidine.

	pVP16 Derivative			
pLexA Derivative	vector	p38	JNK1	ERK2
pLexA MKK7	–	–	+	–
pLexA Lamin	–	–	–	–

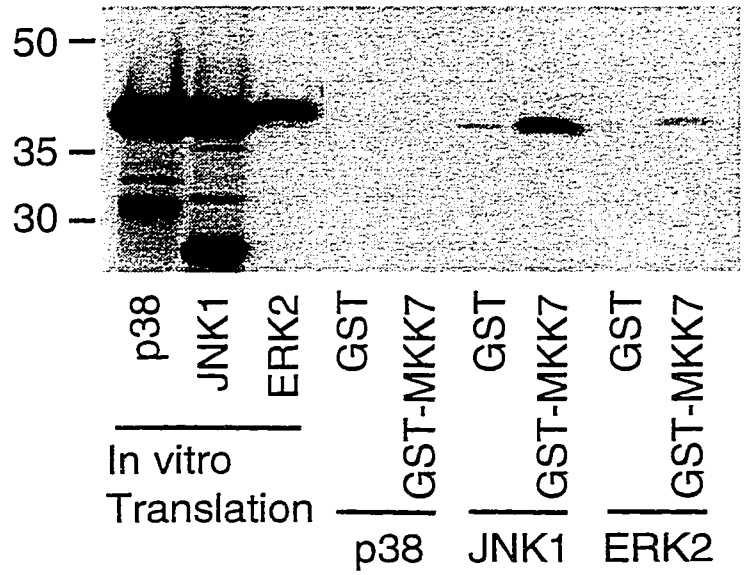


Fig. 16. MKK7 and JNK1 Associate *in vitro*. Recombinant GST-MKK7 or GST alone bound to glutathione sepharose was incubated at 4 °C for 1 hour with either [^{35}S]-Met labeled *in vitro* translated p38, JNK1 or ERK2 (Promega TNT Kit). Samples were washed and analyzed by SDS-PAGE and autoradiography. p38, JNK1 and ERK2 lanes to the left indicate the amount of input translation product.

to phosphorylate the substrate proteins, His-SAPKα, GST-p38 or a catalytically inactive mutant of ERK2, His-ERK2 K52R, (Fig. 17). Parallel experiments were performed using HA-tagged MKK4. Figure 17A demonstrates that MKK7 and MKK4 were able to phosphorylate SAPKα better than p38 or ERK2 K52R. Phosphate incorporation into p38 was one quarter to one eighth of incorporation into SAPKα, and phosphorylation of ERK2 K52R was barely detectable. Since MKK7 and MKK4 both phosphorylate SAPKα and p38, these results suggest that like MKK4, MKK7 might also function in a pathway upstream of SAPKα and p38, but is unlikely to regulate ERK. Both MKK7 and MKK4 were expressed and immunoprecipitated, as judged by Western blotting (Fig. 17B).

Consistent with their abilities to phosphorylate stress-activated kinases, MKK4 and MKK7 were activated in stressed cells. Both kinases were activated by osmotic stress and by anisomycin, and were not activated by a mitogen, PDGF. MKK7 was consistently activated more by osmotic stress than by anisomycin, whereas MKK4 was activated equally (Fig. 17A). This may be indicative of differences in upstream activators.

To determine whether phosphorylation of SAPKα and p38 by MKK7 occurred at the physiological sites, we measured SAPKα and p38 activities. Both MKK7 and MKK4 were able to efficiently activate SAPKα, assayed with GST c-Jun as a substrate, and weakly activate p38, assayed with GST ATF2 as a substrate (Fig. 18). In summary, these results indicated that MKK7 can strongly activate SAPKα and weakly activate p38 *in vitro*. MKK7 is itself activated by extracellular stressors, particularly in response to osmotic shock, but not by mitogens such as PDGF. We have also observed activation of MKK7 in response to UV (data not shown).

MKK7 IS THE MAJOR SAPK ACTIVATOR IN RESPONSE TO OSMOTIC SHOCK IN NIH3T3 CELLS:

To address whether endogenous MKK7 is regulated by stresses, osmotically shocked untransfected NIH3T3 cell lysates were chromatographed sequentially on MonoQ and MonoS columns. Fractions obtained were assayed for SAPK-activating activity using either His-SAPK and GST c-Jun or GST c-Jun alone as substrates. A major peak of SAPK stimulating activity was observed in fractions 29-32 (Fig. 19). To test whether these fractions contained MKK7, MKK4/SEK1 or both enzymes, samples of the

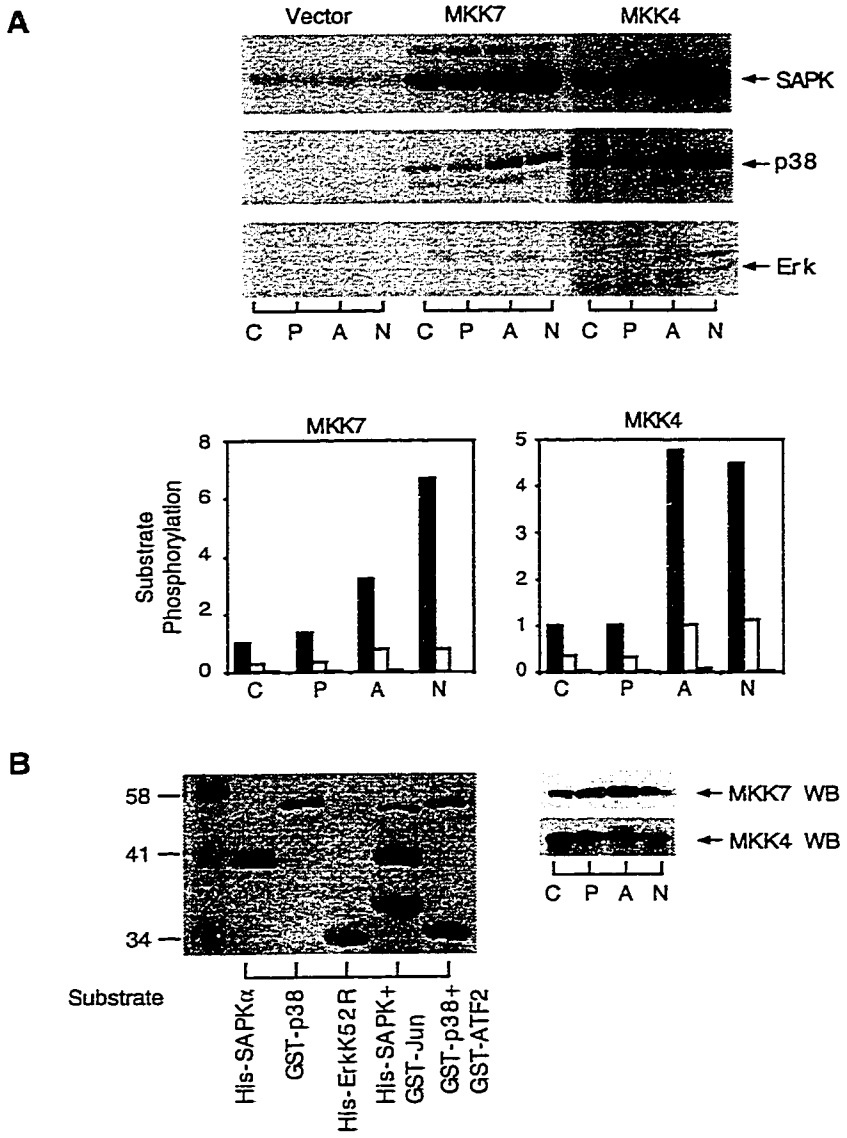


Fig. 17. MKK7 phosphorylates SAPK in response to stress. NIH3T3 cells were transfected with empty vector, pCS3MT-MKK7 or pSRαHA-SEK1 (MKK4) onto 100 mm plates. Cells were treated with either PDGF (P), anisomycin (A), NaCl (N), or left untreated (C). Immunoprecipitated MKK7 and MKK4 activity was measured using the indicated substrates. (A) Phosphorylation of SAPKα and p38 by MKK7 and MKK4. Immune complexes were incubated with either His-SAPKα, GST-p38 or His-ERK2 K52R (see Materials and Methods). ^{32}P-labeled proteins are indicated. Substrate phosphorylation is expressed as fold increase with respect to MKK7 transfected cells without stimulus (control). (B) Coomassie stained SDS-PAGE indicating equal substrate input into *in vitro* kinase assays, and MKK7 and MKK4 immunoblots probed with α-MKK7 (3936) and α-HA (12CA5).

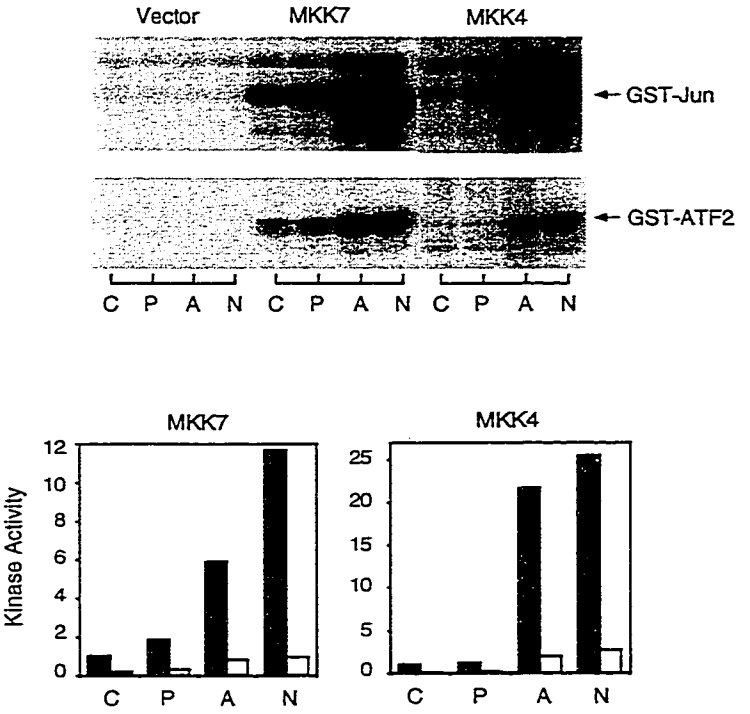


Fig. 18. Activation of SAPKα and p38 by MKK7 and MKK4. Cells were transfected and treated as in Fig. 17. Immune complexes were incubated with either His-SAPKα or GST-p38, followed by addition of 2 μg of GST c-Jun to the SAPK reactions, and 2 μg of GST ATF2 to the p38 reactions. ^{32}P-labeled proteins are indicated. Quantitation was as described for Fig. 17.

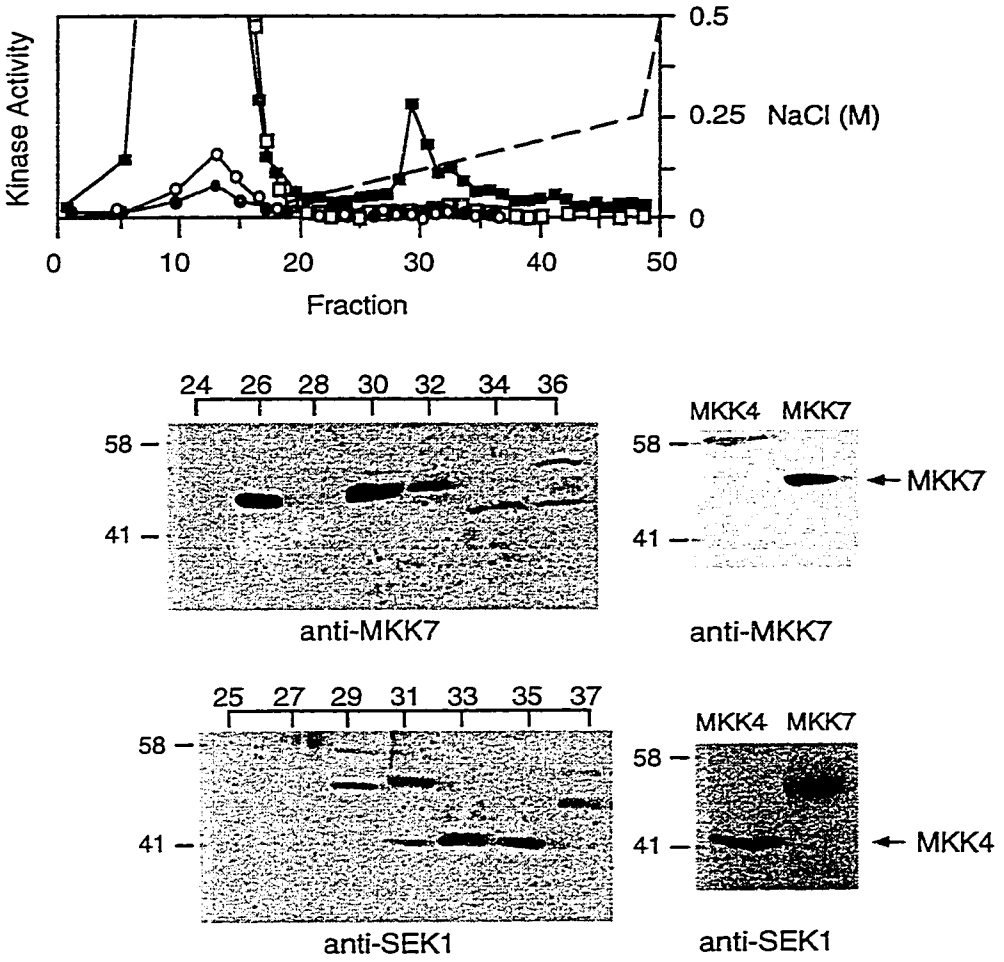


Fig. 19. MKK7 activates SAPK in cells. Activity profile from Mono S fractionation of lysates of NaCl-stimulated (■, □) and unstimulated (●, ○) NIH3T3 cells. Fractions were assayed for GST c-Jun phosphorylation in the presence (■, ●) or absence (□, ○) of His-SAPKα. Activity in flow-through fractions 5-20 is His-SAPKα independent and due to cellular SAPK's (data not shown). Activity in fractions 25-41 is His-SAPKα dependent and due to SAPK activators. Fractions were concentrated, run on SDS-PAGE and immunoblotted with anti-MKK7 (3936) or anti-MKK4 (Santa Cruz). The higher mobility species in fraction 26 represents a cross-reacting band. To test the specificity of the antibodies, in vitro translated pCS3-MKK7 and pcDNA3-MKK4 (Promega TNT kit) were immunoblotted with anti-MKK7 and anti-MKK4. Note that anti-MKK4 recognizes MKK7 as well as MKK4.

fractions were analyzed by immunoblotting with antibodies raised to MKK7 and MKK4/SEK1. Immunoblots of the stimulated fractions showed an MKK7 immunoreactive band of the correct molecular weight in fractions 30-32, indicating that MKK7 was present in the fractions containing the major peak of SAPK activating activity. An MKK4/SEK1 immunoreactive band corresponded to a second smaller peak of SAPK activating activity in fractions 33-35. No SAPK stimulating activity was observed in unstimulated cells (Fig. 19). Western blot analysis of MKK7 and MKK4 in the right hand panels demonstrates that the endogenous immunoreactive bands in the indicated fractions migrate at the correct molecular weights. The MKK7 antibody was specific, whereas the anti-SEK1 antibody also appeared to recognize MKK7. These data indicate that endogenous MKK7 is a cellular JNK/SAPK activator chromatographically distinguishable from MKK4.

ACTIVATION OF SAPK BY IL-1 IN RABBIT LIVER IS MEDIATED BY MKK7:

As part of a collaborative effort with Michael Kracht at the Medical School Hannover, we examined whether the inflammatory cytokine, IL-1, could activate MKK7 in rabbit liver. IL-1 had been previously demonstrated to activate all three types of MAP kinases in a variety of cells in culture (Freshney *et al.*, 1994; Guesdon *et al.*, 1993; Raingeaud *et al.*, 1995). The liver is a physiological target for IL-1 in the acute phase response. In rabbit liver, IL-1 was shown to activate SAPKα rapidly following injection, but did not significantly activate ERK or p38 MAP kinases (Kracht *et al.*, 1994). To address whehter MKK7 was the physiological SAPK activator in rabbit liver following IL-1 injection, cytosolic liver extracts from IL-1 or vehicle injected rabbits were applied to a column of S Sepharose which was eluted with a salt gradient. The fractions were assayed for an activator of SAPK using recombinant GST-SAPK and GST-c-Jun as substrates in a coupled assay (Fig. 20). Fractions from IL-1-treated animals showed an increase in a peak of SAPK activating activity compared with controls. Material from unstimulated and IL-1-stimulated rabbit livers was taken through three purification steps. The purification did not achieve homogeneity. A single active peak was chromatographed on Superose 12, and tested for its ability to react with antibodies raised against known MAP kinase kinases.

Antisera to MKK3 and MKK4 did not immunoprecipitate or immunodeplete the activator, or recognize the activity as determined by Western blot. However, two

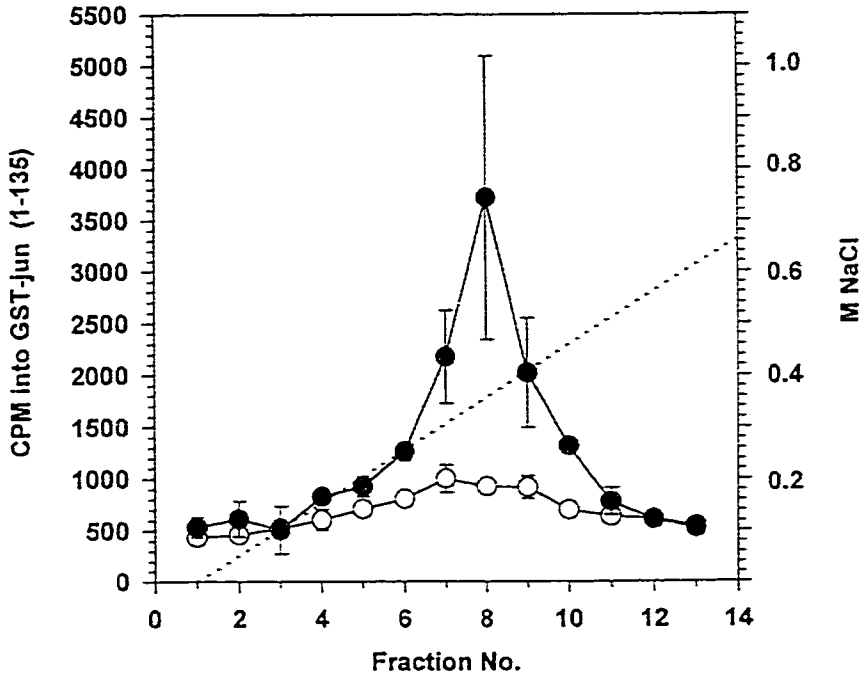


Fig. 20. Cation exchange chromatography of hepatic activator of SAPK. Liver cytosol of control (○), or IL-1 stimulated (●) rabbits was chromatographed on Fast Flow S Sepharose at pH 6.0 and eluted with a salt gradient. Fractions from IL-1 treated animals showed an increase in a peak of SAPK activator compared to controls. Activation in four experiments varied between two- and six-fold.

antisera raised against MKK7 immunoprecipitated the activitor (Fig. 21). Antibody 3936 was raised to a GST fusion of the C-terminal 128 amino acids originally identified in our two-hybrid screen. Antibody 2125 was raised to a C-terminal synthetic peptide of MKK7. Antiserum 2125 strongly precipitated the SAPK activator from the fractions of S Resource chromatography, the fourth purification step (Fig. 21).

Neither non-immune nor pre-immune sera precipitated activity and no activated SAPK was detected if recombinant GST-SAPK was omitted. These data demonstrated that a SAPK activating activity induced by IL-1 in rabbit liver is recognized by antisera to MKK7 but not other stress-activated MAPKKs (MKK3, MKK4). Since the activity was not purified to homogeneity and no amino acid sequence was determined, we cannot rule out the possibility that this activity corresponded to an unidentified relative of MKK7. However, as judged by immunoprecipitation, substrate preference and size on gel filtration (data not shown), this activity is likely to be MKK7.

MUTATION OF PHOSPHORYLATION SITES IN MKK7 BLOCKS ACTIVATION BY STRESSES:

Previous studies have shown that many protein kinases contain key regulatory phosphorylation sites within kinase subdomains VII and VIII (Marshall, 1994; Seger *et al.*, 1992). Among MAPKK family members, this region is highly conserved, suggesting that all MAPKK's are activated by phosphorylation within this loop. Mutation of serine residues within this region to alanine renders the mutated residues unable to be phosphorylated. This approach has revealed phosphorylation sites that are required for activation of MAP kinase kinases. MKK1 contains at least two critical serine residues in this region which have been shown to be phosphorylated by Raf-1 (Alessi *et al.*, 1994; Seger *et al.*, 1994; Zheng and Guan, 1994). MKK4 is also known be activated by phosphorylation on analogous threonine and serine residues; mutations of these residues to alanine results in a catalytically inactive kinase which is not activatable by upstream elements (Sanchez *et al.*, 1994). MKK7 and Hep contain an additional serine residue (S277) within the activation loop that is not present in other MKK's (Fig. 13). To test whether S277 might also be a regulatory phosphorylation site, we generated mutants in which only those residues analogous to phosphorylation sites in other MKK's were mutated to alanine (S271A, T275A, called S2A), or where S277 was also mutated (S271A,

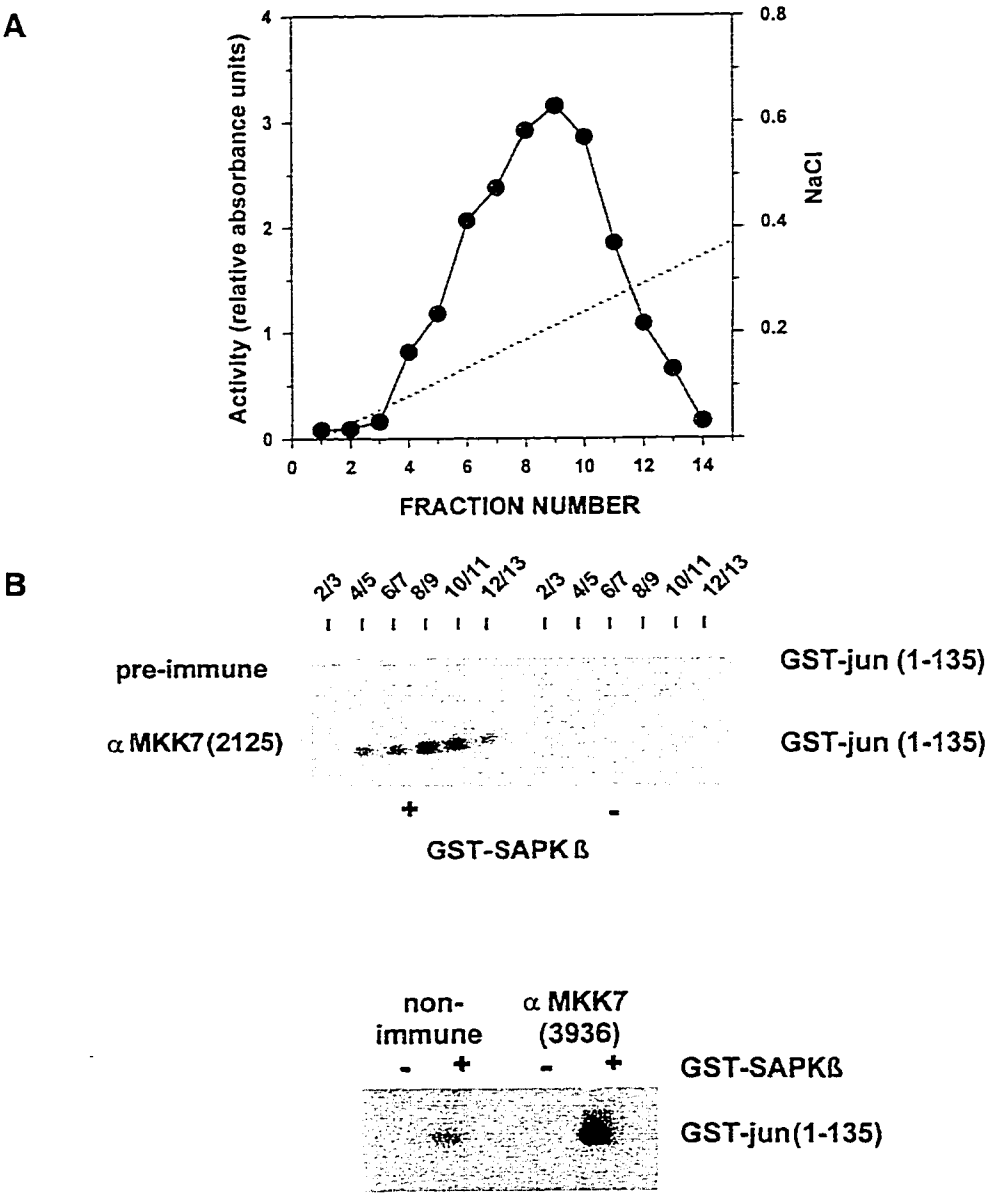


Fig. 21. MKK7 Antibodies Immunoprecipitate the SAPK Activator. (A) Chromatography of the SAPK activator from Fig. 20 on S Resource column which was eluted with a salt gradient. Fractions were assayed for an activator of SAPK (Materials and Methods), and absorbance units were obtained by densitometry of an autoradiograph. (B) Anti-MKK7 antibody 2125 precipitates the SAPK activator. Pairs of active fractions from (A) were treated with protein A agarose coated with antiserum 2125 or pre-immune serum, and the beads were incubated with (+) or without (-) GST-SAPKβ (25 μg/ml) in buffer containing ATP for 1 h at room temperature. GST c-Jun and [γ-^{32}P]-ATP were added for a further 20 min. Phosphorylation of GST c-Jun (1-135) was detected by autoradiography after SDS-PAGE. (C) Anti-MKK7 antibody 3936 precipitates SAPK activator from a pool of fractions 2-13 from S Resource.

T275A, S277A, called S3A). These mutants were transiently overexpressed in NIH3T3 cells and the immunoprecipitated proteins were analyzed for their ability to activate SAPKα *in vitro* using GST c-Jun as a substrate. Figure 22 demonstrates that the S2A double mutant was still capable of being activated in response to NaCl, although its basal activity was significantly decreased compared to wild-type MKK7. However, the S3A triple mutant was inactive. Therefore, all three putative phosphorylation sites appear necessary for the activation of MKK7. Mutation of these residues to aspartic or glutamic acid, negatively charged residues thought to mimic the effect of phosphorylation, has been shown to result in an activated MAP kinase kinase (Mansour *et al.*, 1994; Seger *et al.*, 1994). A corresponding S3E triple mutant (S271E, T275E, S277E) had increased basal activity relative to wild-type MKK7, suggesting that replacement of the putative phosphorylation sites with acidic residues causes partial activation. Curiously, the S3E mutant could still be further activated in response to NaCl (Fig. 22). It is possible that additional phosphorylation sites within or outside of the activation loop may exist, or that other modifications in MKK7 must occur for full activation to take place.

A second activating mutation has been identified for MKK1 which increases its basal activity over 400 fold (Mansour *et al.*, 1994). Removal of residues 32-51 in the N-terminus of MKK1 in addition to substituting glutamic or aspartic acid for the serine residues results in a fully constitutively activated kinase. We were interested to determine whether removal of an analogous N-terminal regulatory region in MKK7 in the context of S3E would increase its activity. Mutants of MKK7 S3E lacking the first 10 (Δ10-S3E) or 22 (Δ22-S3E) amino acids were generated and expressed in 293T cells. Deletion of these residues in MKK7 corresponds to the equivalent region removed in MKK1. Cells were stimulated by osmotic shock or left untreated and immunoprecipitated JNK1 was assayed for its activity using GST c-Jun as a substrate. In cells transfected with either Δ10-S3E or Δ22-S3E, JNK1 activity was equal to that from cells transfected with wildtype MKK7 (Fig 23). Activity of these mutants was increased by osmotic shock, indicating that they could still be activated further. These data suggest that the region in the N-terminus of MKK7 may not be an analogous region to the regulatory region in MKK1.

MKK7 POTENTIATES SAPK ACTIVITY:

We also investigated whether MKK7 could activate SAPK/JNK *in vivo* by

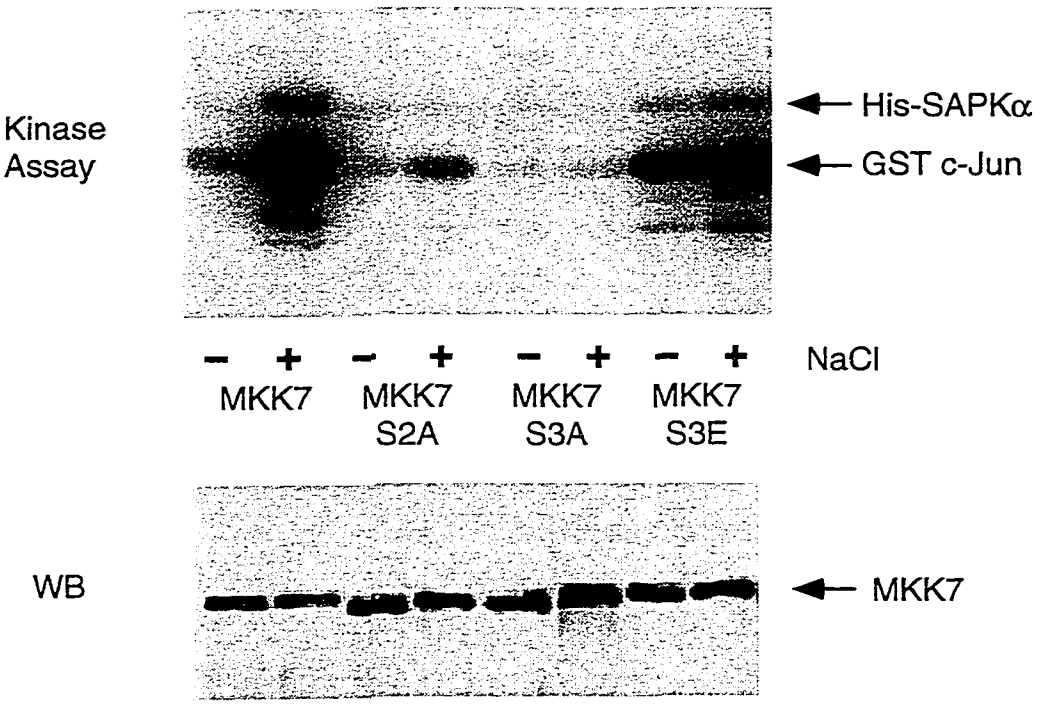


Fig. 22. Mutations at phosphorylation sites affect MKK7 activity. NIH3T3 cells were transfected with either pCS3MT-MKK7, MKK7 S2A (S271A, T275A), MKK7 S3A (S212A, T275A, S277A), or MKK7 S3E (S212E, T275E, S277E). Cells were treated with 0.4 M NaCl for 30 min or left untreated. Immunoprecipitated MKK7 activity was measured using His-SAPKα and GST c-Jun as substrates in a coupled kinase assay as in Fig. 18. Expression of MKK7 was analyzed by immunoblotting with anti-MKK7 (3936).

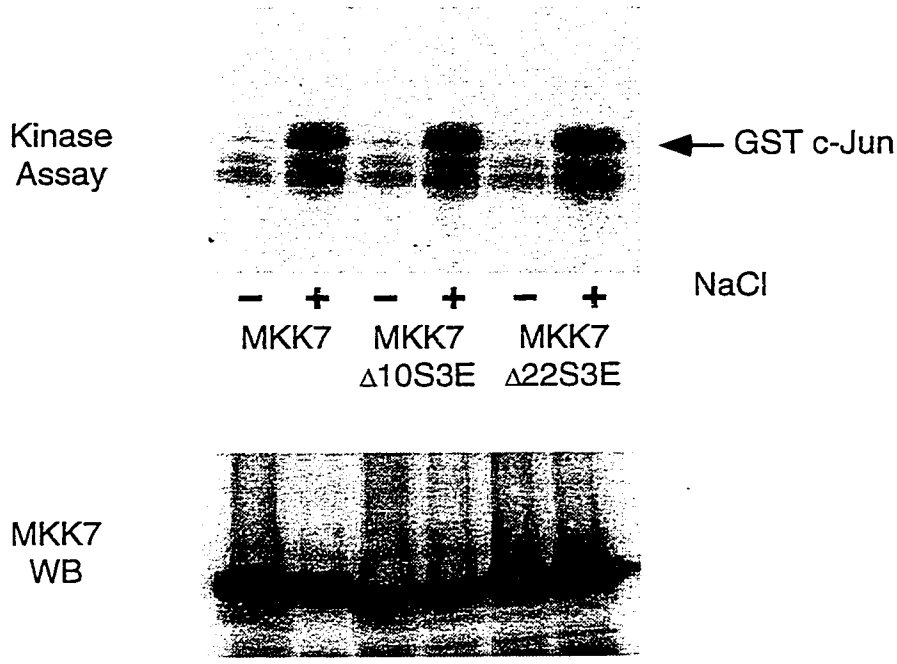


Fig. 23. Deletion of the N-terminus of MKK7 does not increase activity. 293T cells were transfected with either pCS3MT MKK7, MKK7 Δ10-S3E, or MKK7 Δ22-S3E. Cells were treated with 0.4 M NaCl for 30 min or left untreated. JNK1 was immunoprecipitated (αJNK1, Santa Cruz), and its activity was measured using GST c-Jun as a substrate. MKK7 expression was analyzed by immunoblotting with anti-myc (9E10).

performing transient co-transfection assays in NIH3T3 cells. Cells transfected with epitope-tagged JNK1 alone, or JNK1 with wild-type or inactive MKK7 (S3A), were treated with anisomycin or left unstimulated. JNK1 activity was measured in immune complex assays, using GST c-Jun as a substrate. In cells co-transfected with the S3A mutant, JNK1 activity was equal to cells transfected with JNK1 alone (Fig. 24). It is of interest to note that although the S3A mutant lacks kinase activity, it did not inhibit JNK1 stimulation by anisomycin. A similar result was observed with a K149M mutant of MKK7 (data not shown). Therefore, unlike inactive mutants of MKK4/SEK1 (Rana *et al.*, 1996; Sanchez *et al.*, 1994; Yan *et al.*, 1994) inactive mutants of MKK7 are not dominant inhibitors of JNK1 activation. It is possible that MKK4 may interact with different upstream activators from MKK7 and that those molecules are able to activate JNK1 via MKK4 when the MKK7 pathway is blocked.

SUBCELLULAR DISTRIBUTION OF MKK7:

We examined the subcellular localization of MKK7 by immunofluorescence analysis. NIH3T3 cells and HeLa cells were transfected with epitope-tagged wild-type MKK7, MKK7 S3A, or MKK7 S3E, and were either left untreated, or exposed to osmotic shock or anisomycin. These studies demonstrated that MKK7 was distributed in both the cytoplasm and the nucleus (Fig. 25). The distribution of either active or inactive forms of MKK7 was equivalent to that of the wild-type (data not shown). Similarly, exposure of the cells to osmotic shock or anisomycin did not cause any marked changes in the distribution of MKK7 (data not shown). This suggests that the distribution of MKK7 may not be regulated by its activation state.

EFFECTS OF RAC MUTANTS ON MKK7 ACTIVATION:

Members of the Rho subfamily of GTP-binding proteins have been shown to activate SAPK and p38 in various cells. Several reports have demonstrated a role for Rac and Cdc42, but not Rho, in regulating SAPK and p38 in COS and NIH3T3 cells (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995). Other reports have stated that Cdc42 and all Rho proteins (RhoA, RhoB, RhoC), but not Rac or Ras, could induce activation of SAPK in the human kidney epithelial cell line 293T (Teramoto *et al.*, 1996). We were interested to see whether activated Rac and Cdc42 might increase MKK7 activity. We found that co-transfection of MKK7 with activated Rac or Cdc42 in NIH3T3 cells

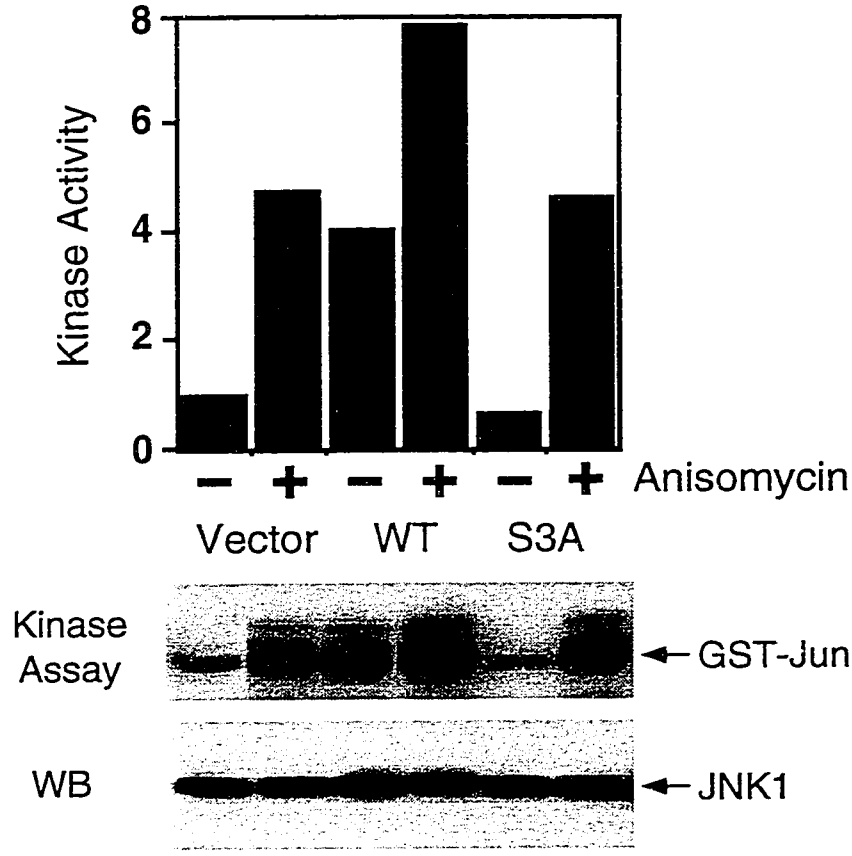


Fig. 24. MKK7 Potentiates SAPK Activity. NIH3T3 cells were co-transfected with pCS3MT-JNK1 (SAPKγ) and either wild type pCS3MT-MKK7), MKK7 S3A (S271A, T275A, S277A), or empty vector. Cells were treated with anisomycin (10 μg/ml; 20 min) or left untreated. Immunoprecipitated JNK1 activity was measured using GST c-Jun as a substrate. JNK1 expression was analyzed by immunoblotting with anti-JNK1 (Santa Cruz). GST c-Jun phosphorylation was quantitated with a phosphorimager and is expressed as fold increase with respect to wild type JNK1 transfected cells without stimulus. Immunoprecipitated MKK7 alone has no phosphorylating activity on GST c-Jun (data not shown).

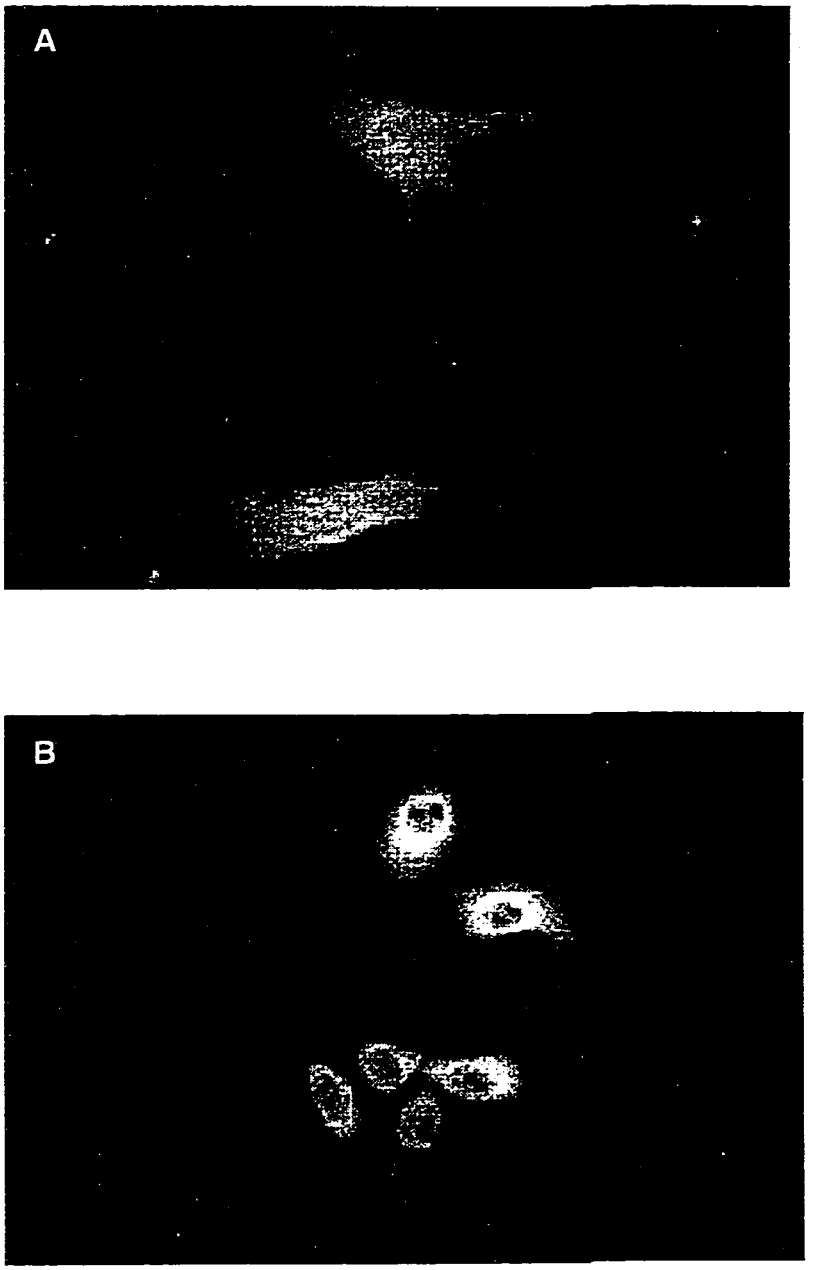


Fig. 25. Subcellular distribution of MKK7. pCS3MT MKK7, MKK7 S3A, or MKK7 S3E was transfected into NIH 3T3 (A) or HeLa (B) cells, and detected by immunofluorescence analysis using anti-Myc (9E10) primary antibody and a FITC conjugated secondary antibody.

significantly increased MKK7 activity, and co-transfection of MKK7 with dominant-negative versions of Rac (V12N17) or Cdc42 (N17) partially blocked NaCl-induced MKK7 activation. These data suggested that MKK7 lies in a pathway downstream of Rac1 and Cdc42 (Fig. 26). Although suppression MKK7 by dominant-negative Rac and Cdc42 was significant, it was not complete, suggesting that alternate, Rac/Cdc42-independent pathways may also regulate MKK7.

Subsequent to the identification of Rac as an activator of SAPK, two papers described specifc effector mutants of Rac that distinguish the effects of Rac on mediating actin polymerization and signaling to the SAPK pathway (Joneson *et al.*, 1996; Lamarche *et al.*, 1996). Rac mutants with a substitution at position F37 could no longer induce cytoskeletal changes but still activated SAPK, whereas mutants with a substitution at position Y40 no longer interacted with p65PAK, and were unable to activate SAPK, but were still capable of inducing actin polymerization. Substitution at position Y40 prevents the interaction of both Rac and Cdc42 with CRIB motif-containing proteins, (Lamarche *et al.*, 1996). This result suggested that the effects of Rac on SAPK activation and cytoskeletal regulation are mediated by distinct non-overlapping pathways.

We tested whether effector mutants of Rac that no longer induced actin polymerization (RacV12L37) or no longer induced SAPK activation (Racv12H40) could still signal to MKK7. Figure 27 indicates that co-expression of RacV12L37 could activate MKK7, although not to the extent of RacV12, whereas RacV12H40 only slightly increased MKK7 activity. These results are consistent with previous results which demonstrate stimulation of JNK activity by RacV12L37 but not RacV12H40. The failure of MKK7 to be fully activated by RacV12H40 suggests that MKK7 may also be downstream of PAK.

MKK7 RESPONDS TO MULTIPLE ACTIVATORS:

Several kinases including the MEKK's, MLK's, PAK's, Tpl-2, ASK1 and TAK1, have been reported to activate the SAPK pathway in various cell types (Blank *et al.*, 1996; Hirai *et al.*, 1996; Ichijo *et al.*, 1997; Pombo *et al.*, 1995; Rana *et al.*, 1996; Salmeron *et al.*, 1996; Teramoto *et al.*, 1996; Yan *et al.*, 1994). We examined whether these kinases were also activators of MKK7 in transient co-transfection assays. Co-expression of MKK7 with either ASK1 or with TAK1 and TAB1 in NIH3T3 cells resulted in a significant increase in MKK7 activity, as judged by assaying immunoprecipitated

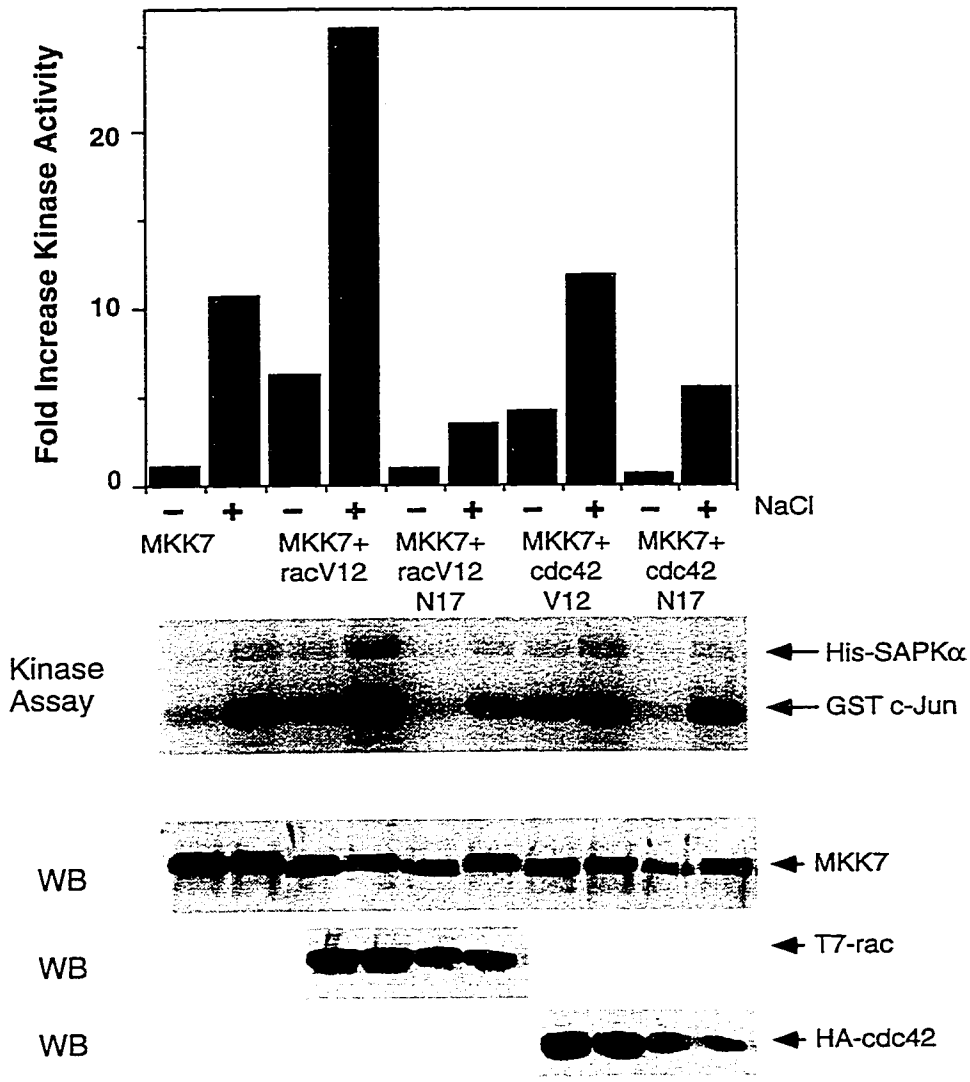


Fig. 26. Effects of Rac and Cdc42 on MKK7 activity. NIH3T3 cells were co-transfected with wild-type pCS3MT-MKK7 and either pCGT-T7 RacV12, RacV12N17, pXJ40 HA Cdc42V12, Cdc42N17 or empty vector. Cells were treated with 0.4 M NaCl for 30 min or left untreated. Immunoprecipitated MKK7 activity was measured using His-SAPKα and GST c-Jun as substrates in a coupled kinase assay as in Fig. 18. Expression of proteins was analyzed by immunoblotting with anti-MKK7 (3936), anti-T7 (Novagen) for Rac constructs, and anti-HA (12CA5) for Cdc42 constructs.

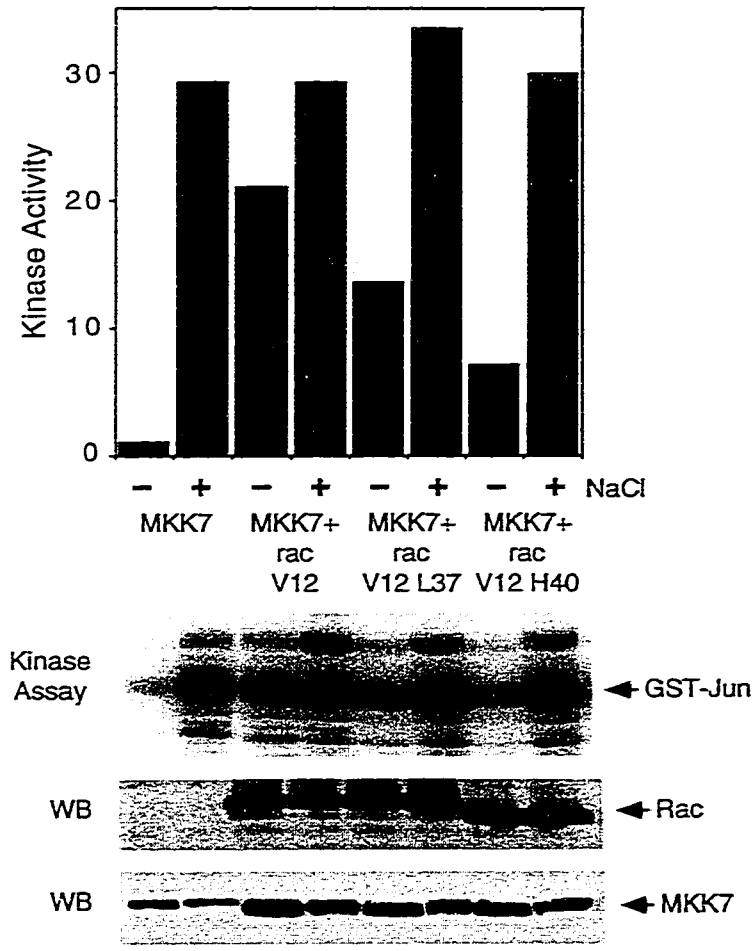


Fig. 27. Effect of Rac1 Mutants on MKK7 activity. NIH3T3 cells were co-transfected with pCS3MT-MKK7 and either pCGT T7 RacV12, RacV12L37, RacV12H40, or empty vector. Cells were treated with NaCl (0.4 M; 30 min) and MKK7 was immunoprecipitated and assayed for activity as in Fig. 18. Expression of Rac and immunoprecipitated MKK7 was determined by immunoblotting with anti-T7 (Novagen) and anti-MKK7 (3936), respectively. GST c-Jun phosphorylation is expressed as fold increase with respect to MKK7 transfected cells without stimulus.

MKK7 *in vitro* using SAPKα and GST c-Jun as substrates. This activation was greater than that observed with co-expression of activated mutants of Rac or Cdc42 (Fig. 28). In a separate experiment, NIH3T3 cells were co-transfected with either MLK2, a catalytically active or dominant negative form of MEKK1, and MKK7 (Fig. 29). MLK2 was also transfected with a phosphorylation mutant of MKK7 (S3A). Analysis of immunoprecipitated MKK7 activity as judged by SAPK phosphorylation indicated that both MLK2 and active MEKK1 were capable of activating MKK7 in the absence of stimulus. The S3A mutant not activated by MLK2, and a dominant negative form of MEKK1 had no effect. To date, all of the MAPKKKs demonstrated to activate SAPK that we have tested are capable of activating MKK7. The comparable abilities of these kinases to selectively activate MKK7 or MKK4 was not examined in these experiments. This suggests that MKK7 may receive inputs from a variety of stimuli.

DISCUSSION:

Our cloning and characterization studies of MKK7 have established this new kinase as an important physiological regulator of SAPK. Concurrently, at least seven investigators have reported on the cloning and characterization of MKK7 (JNKK2, SEK2) (Foltz *et al.*, 1998; Holland *et al.*, 1997; Lawler *et al.*, 1997; Moriguchi *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997). Four mouse and four human clones have been reported that differ by four amino acids (I145V, H382R, I385T, H413P); in which the first residue refers to the mouse sequence, and the second residue refers to the human sequence. Two of the mouse sequences reported start at the methionine corresponding to Met 100 in our sequence (Tournier *et al.*, 1997; Yao *et al.*, 1997). The third mouse isolate has the same start as our sequence, but the sequence diverges at position 42, and contains a 17 amino acid insert (Moriguchi *et al.*, 1997). Interestingly, position 42 is the point at which the divergent N-terminal sequence between MKK7a and MKK7b comes back into alignment. This suggests that at least three mouse isoforms may exist, and position 42 in our sequence may correspond to a site at which multiple exon splicing occurs. Except for one 33 amino-acid extension at the C-terminus (Moriguchi *et al.*, 1997), all the reported sequences share the same stop codon. Recently, the gene that encodes murine MKK7 was cloned, and demonstrates that six isoforms of MKK7 are

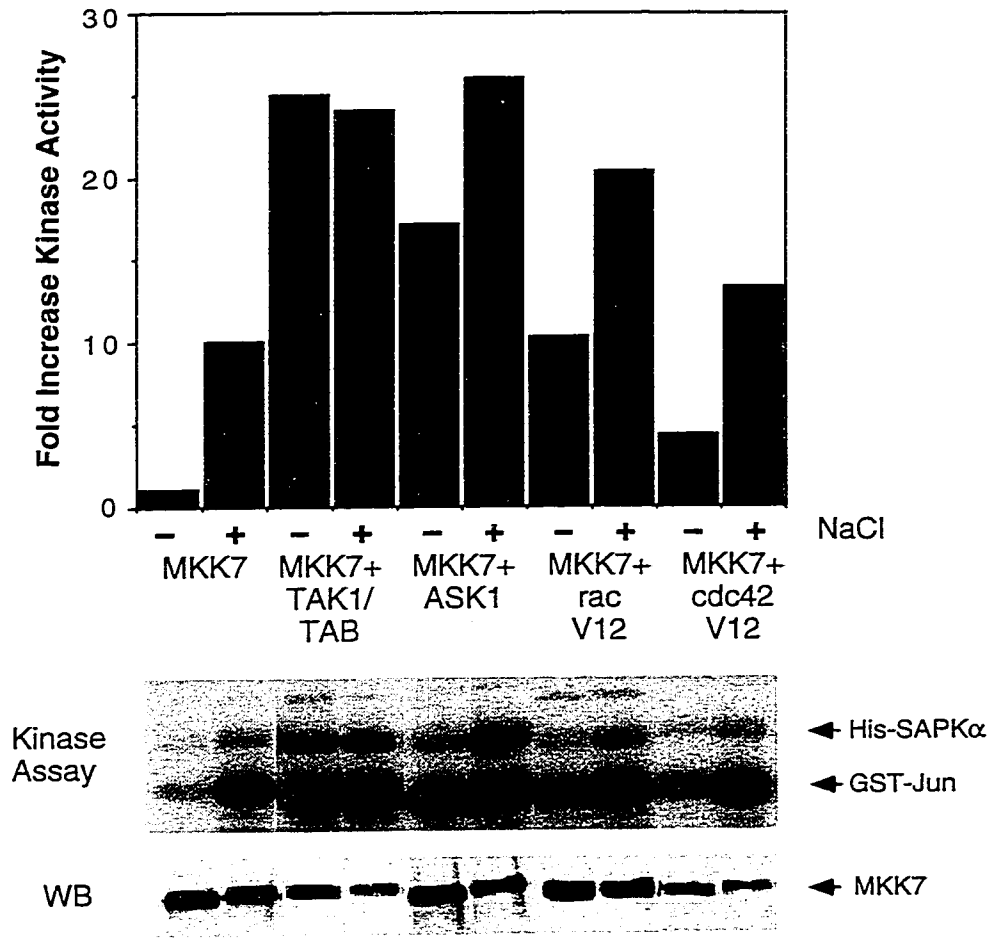


Fig. 28. MKK7 has multiple activators. NIH3T3 cells were transfected with pCS3MT MKK7 and either pEF TAK1 + pEF TAB1, pcDNA ASK1, pCGT T7 RacV12, pXJ40 HA Cdc42V12 or empty vector. Cells were treated with NaCl (0.4 M; 30 min) and MKK7 was immunoprecipitated and assayed for activity as in Fig. 18. Expression of immunoprecipitated MKK7 was determined by immunoblotting with anti-MKK7 (3936). GST c-Jun phosphorylation is expressed as fold increase with respect to MKK7 transfected cells without stimulus.

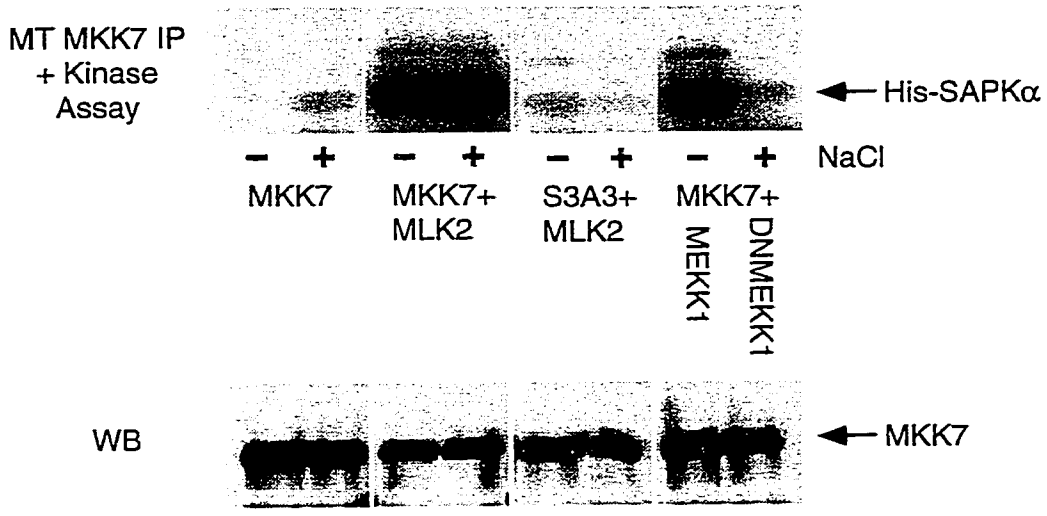


Fig. 29. MEKK1 and MLK2 can activate MKK7. NIH3T3 cells were transfected with pCS3MT MKK7 and either pRK5 MLK2, pCMV HA MEKK1, DN MEKK1, or empty vector. MKK7 S3A3 was also co-transfected with MLK2. Cells were treated with NaCl (0.4 M; 30 min) or left untreated as indicated, and MKK7 was immunoprecipitated and assayed for activity using His-SAPKα as a substrate. Expression of immunoprecipitated MKK7 was determined by immunoblotting with anti-MKK7 (3936).

created by alternative splicing to generate a group of kinases with three different N-termini (α, β and γ isoforms), and two different N-termini (1 and 2 isoforms) (Tournier *et al.*, 1999). MKK7a corresponds to the β1 isoform, which contains an N-terminal extension (amino acids 1-72) reported to bind SAPK (Tournier *et al.*, 1999).

By sequence homology, MKK7 is most similiar to the enzyme in *Drosophila,* Hemipterous, sharing 56% amino acid identity. The most closely related mammalian MAP kinase kinase to MKK7 is MKK4, sharing 44% identity overall and 56% identity within the catalytic domain. Both MKK7 and MKK4 have been demonstrated to be potent activators of SAPK in response to overlapping stimuli and have a similar tissue distribution. Although the activities of MKK7 and MKK4 appear similar, the two kinases may also play independent roles .

Close analysis of the sequence of MKK7 and MKK4 reveals structural differences between these kinases. The proline-rich region located in the N-terminal portion of both MKK7a and MKK7b, which is absent in MKK4, could be involved in specific interactions with SH3 domains contained within upstream regulatory proteins. Two candidates are mixed lineage kinase (MLK) family members, MLK3/SPRK (Rana *et al.*, 1996) and MLK2/MST (Dorow *et al.*, 1995; Katoh *et al.*, 1995). In addition, MKK7 and Hep contain an additional serine residue (S277) within the activation loop that is not present in other MKK's (Fig. 13). We have demonstrated that all three putative phosphorylation sites are necessary for the full activation of MKK7. It is possible that this will reflect a means of regulation that is different from MKK4.

In fibroblasts, MKK7 is activated by environmental stress and not by mitogens. Specifically, MKK7 appears slightly more responsive to osmotic shock than to anisomycin. In addition, column fractionation studies have identified MKK7 as one of the major SAPK activating kinases in osmotically shocked NIH3T3 cells, as well as in IL-1-stimulated rabbit liver. These findings are consistent with those of others, in which treatment of HeLa cells, KB cells, U937 cells and 293 cells with inflammatory cytokines such as TNF or IL-1, or by osmotic shock with sorbitol or NaCl, results in a stronger activation of MKK7 compared to MKK4 (Lawler *et al.*, 1997; Moriguchi *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997). MKK4 has been shown to be more responsive than MKK7 to treatment by UV irradiation, heat shock and anisomycin. Although both MKK4 and

MKK7 respond to overlapping stimuli, the sensitivity with respect to the degree of activation seems varied.

Activation of MKK7 leads to the phosphorylation and activation of SAPK. Although the MKK7 phosphorylation site mutant (S3A) lacks activity, it is interesting that it does not inhibit JNK activation by anisomycin. This suggests that stimulus-induced activators upstream of MKK4 and MKK7 may be selective for either MKK4 or MKK7, and activation of SAPK via a parallel pathway cannot be fully blocked in this manner. Our studies using the S3E mutant in which the phosphorylation sites have been substituted with negatively charged amino acids demonstrates that mutating these residues is not sufficient to fully activate MKK7. The S3E MKK7 mutant has an increased basal activity relative to wild-type, but may still be activated further by external stimuli. The decreased maximal activity of the S3E mutant compared to wild-type can be explained by the fact that a glutamic acid residue is an inadequate mimic of a phosphorylated serine residue. The ability of the S3E mutant to be further activated by stresses suggests the existence of additional activating phosphorylation sites or phosphorylation independent activation mechanisms.

Removal of either 10 or 22 residues from the N-terminus did not increase the basal activity of the S3E mutant. Removal of the analogous residues (32-51) in MKK1 deletes an α-helical motif that is implicated in regulating kinase activity (Mansour *et al.*, 1994). This region also contains the sequence (residues 32-44) that has been demonstrated to function as a nuclear export sequence (NES) (Fukuda *et al.*, 1996). Although this has not been demonstrated, it is possible that removal of the NES translocates the activated MKK1 to the nucleus, thereby leading to sustained ERK activation. Since MKK7 also contains a leucine-rich sequence in its N-terminus (residues 6-18) that aligns with the NES of MKK1, we were interested to see if removal of this motif would increase MKK7 basal activity. We observed no increase in MKK7 activity compared to the full-length S3E when we removed either 10 or 22 residues from the N-terminus. One possibility is that the sequence in MKK7 is not a functional NES. Immunolocalization studies of various MKK7 mutants suggest that this may be the case, as wild-type MKK7 is partially nuclear (Fig. 25), and the Δ10 and Δ22 mutants do not show an increase in the nuclear compartment relative to wild-type (data not shown). The full-length S3A and S3E mutants also have equivalent distributions. Another possibility is that removal of the sequence N-terminal to

the predicted NES (residues 1-5) alters the folding conformation in this region of the protein. This may influence whether this region is exposed or buried within the structure. Mutants of MKK1 in which the highest activity (400-fold) was observed retained the first 31 residues of the sequence and only removed residues 32-51. In contrast, the basal activities of two mutants with deletions spanning residues 1-32 and 1-52 were one-tenth that of the wild-type enzyme (Mansour *et al.*, 1994). Data presented in Chapter 4 suggest that the N-terminus of MKK7 may be involved in binding other proteins.

Although the substrate specificity of MKK7 seems to be restricted to SAPK, the specificity of activators upstream of MKK7 appears to be broad and overlapping. It is apparent that in addition to Rac and Cdc42, MKK7 may be activated by many different MAP kinase kinase kinases. Co-expression of constitutively active mutants of Rac and Cdc42 in NIH3T3 cells activates MKK7, and dominant negative Rac and Cdc42 mutants can suppress NaCl induced activation of MKK7. However, the incomplete block of NaCl induced MKK7 activation by these mutants suggests that MKK7 may be partially independent of Rac and Cdc42.

Additional upstream activators include multiple MEKK's, multiple MLK's and multiple kinases with homology to STE20. Many of these have been implicated in mediating apoptosis or activating immune responses, suggesting a role for MKK7 in these events. All of these have been demonstrated to activate SAPK, either through MKK7, MKK4 or both. So far, all of the MAP kinase kinase kinases that we have tested, including MEKK1, ASK1, TAK1 and MLK2, are capable of activating MKK7. In addition, MST1, a MAP kinase kinase kinase kinase demonstrated to be a caspase target, can also activate MKK7 (Graves *et al.*, 1998). At present it is not clear whether these are truly signal- or cell-type specific. How all these upstream kinases are regulated, how they regulate MKK7 and MKK4, and the physiological relevance of their activation of SAPK, as well as why there are so many potential inputs into the SAPK pathway, are the subject of intense investigation. Because many of these studies rely on transient transfection analysis, it may be difficult to determine which of these kinases will emerge as endogenous regulators of the SAPK pathway. Adding complexity to the interpretation of component members of this pathway is the growing number of SAPK genes being identified (Gupta *et al.*, 1996). Currently ten SAPK isoforms corresponding to alternatively spliced forms from three genes have been identified in human brain by molecular cloning. Some work

has suggested that the individual SAPK isoforms selectively target specific transcription factors *in vivo* (Gupta *et al.*, 1996). The presence of multiple SAPK's suggests there may be additional MKK's, and possibly MKKK's regulating these pathways.

Since the identification of MKK7, numerous reports have emerged providing physiological roles for MKK7, and differences between MKK7 and MKK4. Many of these demonstrate that activation of SAPK by different environmental stimuli occurs selectively through different MKK's. Studies from targeted disruptions of the MKK4/SEK1 gene in mice have demonstrated that activation of SAPK in MKK4/SEK1$^{-/-}$ cells still occurs in response to osmotic shock and UV irradiation but not in response to anisomycin or heat shock (Nishina *et al.*, 1997; Yang *et al.*, 1997). This is consistent with our data from overexpression studies in which MKK7 activation was repeatedly stronger in response to osmotic shock than to anisomycin. The use of RAG-2-deficient blastocyst complementation in mice has shown that MKK4/SEK1 deficient embryonic stem cells are capable of SAPK activation and are not impaired in B and T lymphocyte development (Swat *et al.*, 1998). This suggests that MKK7, or another as yet unidentified kinase, is sufficient to mediate these responses. Similarly, expression of a catalytically inactive MKK4 as a transgene targeted to the thymus in mice does not interfere with normal T cell development (Alberola-Ila *et al.*, 1998). In these studies, SAPK activation by Fas was blocked, but SAPK activation by phorbol ester and ionomycin treatment was unaffected. In contrast, it has been demonstrated that MKK7 but not MKK4/SEK1 is required for Fas mediated SAPK activation in Jurkat cells (Toyoshima *et al.*, 1997).

Both MKK7-SAPK and MKK6-p38 signaling pathways have been implicated in contributing to IL-2 gene expression in T lymphocytes (Chen *et al.*, 1998; Matsuda *et al.*, 1998). The JNKK2/MKK7-SAPK pathway has been shown to stabilize IL-2 mRNA synthesis and turnover. Cyclosporin A is an immunosuppresive drug that specifically inhibits the Ca^{++}-sensitive phosphatase calcineurin and thereby blocks the Ca^{++} dependent increase in SAPK or p38 activities (Schaad *et al.*, 1996; Werlen *et al.*, 1998). Treatment of T cells with CsA leads to a decreased half-life of IL-2 mRNA, whereas expression of an activated JNKK2/MKK7 construct increases the IL-2 mRNA half-life.

Other studies have demonstrated a role for MKK7 in the onset of cardiac hypertrophy and cell death in response to hemodynamic overload and ischemia/reperfusion

injury (Wang *et al.*, 1998). Infection of cultured rat myocytes with an activated MKK7 mutant leads to specific activation of SAPK and is associated with classic features of hypertrophy, including an increase in cell size, elevated expression of atrial natriuretic factor and induction of sarcomere organization. These data suggest that sustained activation of the SAPK pathway by MKK7 may contribute to the development of cardiac hypertrophy.

A role for MKK7-mediated SAPK activation in response to Bruton's tyrosine kinase (Btk) activation in mast cells has also been demonstrated (Kawakami *et al.*, 1998). Stimulation of mast cells through the high-affinity IgE receptor (FcεRI) induces degranulation, lipid mediator release and cytokine secretion, leading to allergic responses. FceRI crosslinking can induce the activation of Btk as well as SAPK in mast cells (Kawakami *et al.*, 1997). FcεRI-induced SAPK activation is enhanced by overexpression of MKK7. In mast cells lacking Btk, derived from Btk-knockout mice, the ability of MKK7 to activate SAPK is reduced. These data suggest that MKK7 can be activated upon FcεRI crosslinking in mast cells and that this activation may be mediated by Btk. This suggests a role for the SAPK pathway in mast cell immune responses.

Finally, MKK7 has also been shown to be involved in IL-8 synthesis (Shapiro and Dinarello, 1995). IL-8 is a prototype chemokine induced by IL-1 or TNF which functions in recruiting leukocytes at sites of acute inflammation (Krause *et al.*, 1998). Studies using the specific p38 MAP kinase inhibitor, SB 203580, have also demonstrated a role for p38 in IL-1-induced IL-8 synthesis (Cohen, 1997; Holtmann *et al.*, 1999; Shapiro and Dinarello, 1995). Overexpression of an activated mutant of MKK7 (MKK7 S3E) is sufficient to induce IL-8 synthesis and transcription from a minimal IL-8 promoter. Therefore, maximal expression of IL-8 requires input from pathways which activate NFκB and p38, in addition to MKK7. This is one example of an integration of signals involving cooperation between the MKK7 and p38 pathways, as well as NFκB.

In summary, we have identified a new member of the dual specificity family of MAP kinase kinases, MKK7. MKK7 is activated by a variety of extracellular stresses, including osmotic shock and inflammatory cytokines such as IL-1. MKK7 binds to and activates SAPK in response to these environmental stimuli. Co-expression with the GTP-

binding proteins, Rac or Cdc42, or with MAP kinase kinase kinases such as MLK2, MEKK1 and ASK1 also results in the activation of MKK7.

MKK7 is most closely related to the *Drosophila* MAP kinase kinase, Hep, and to the mammalian MAP kinase kinase, MKK4. In biochemical studies, MKK7 and MKK4 have overlapping stimuli and both are potent SAPK activators, suggesting a redundancy in SAPK activation mechanisms. In *Drosophila*, Hep has been shown to be a mediator of dorsal closure, a morphogenetic process in late embryogenesis. To further address the biological roles of MKK7 and MKK4 we investigated their functional similarity to Hep by substituting MKK4 or MKK7 into *hep* mutant flies. The following chapter provides evidence to suggest that despite the biochemical similarities between MKK7 and MKK4, they may also function independently in regulating distinct biological processes.

MATERIALS AND METHODS:

DNA CONSTRUCTS:

pBTM116 LexA MKK7, and LexA lamin have been described (Ch. 1). pVP16 JNK1, pVP16 ERK2 and pVP16 p38JNK1 were gifts from A. Waskiewicz. (p54γ), p38 (gifts from R. Davis), and ERK2 cDNAs were sublconed into the pCS2 expression vector (D. Turner, R. Rupp and H. Weintraub) as PCR-amplified BamHI fragments. MKK7 and JNK1 were subcloned into the BglII site of pCS3-MT and pCS2 (D. Turner, R. Rupp and H. Weintraub) as PCR-amplified BamHI fragments. MKK7 mutants were generated as described by the Quik-Change Site Directed Mutagenesis Kit (Stratagene). All mutations were confirmed by automated sequencing with a Model 377 ABI sequencer. To generate Δ10-MKK7 S3E and Δ22-MKK7 S3E, a common 3' primer the following 5' primers were used to PCR amplify MKK7 S3E:

Δ10: 5'-CGGGATCCTGTCCCGCCTGGAAGCCAAGCTG-3'

Δ22: 5'-CGGGATCCGTGAGGCCCGCAGGAGGATCGAC-3'

ΔMKK7 PCR products were digested with BamHI and cloned into the BglII site of pCS3MT vector. pSRαHA-SEK1, pET16bHis-SAPKα, GST p38, and GST

c-Jun (1-79), were generous gifts from Y. Gotoh. His-SAPKα and His-ERK2 K52R (M. Cobb) were purified using the pET system for protein purification as described by the manufacturer (Novagen). GST-MKK7, GST-ATF2, GST-p38 and GST c-Jun (1-79) were bacterially expressed and purified following standard procedures (Ausubel, 1992). pCGT-RacV12, pCGT-RacV12L37 and pCGT-RacV12H40 were gifts from L. Van Aelst. pCGT-RacV12N17 was generated using the Quik Change Site Directed Mutagenesis kit (Stratagene). pXJ40 HA-Cdc42V12 and pXJ40 HA-Cdc42N17 were gifts from P. Rodriguez Viciana. pcDNA ASK1, pEF TAK1, pEF TAB1, pCMV HA MEKK1, and pCMV MEKK1 DN were generous gifts from Y. Gotoh. pRK5 MT MLK2 was a gift from A. Hall.

GENERATION OF ANTIBODIES:

Antiserum 3936 is a rabbit polyclonal antibody raised against a GST fusion of a C-terminal 128 amino acid fragment of MKK7. Antiserum 2125 is a rabbit polyclonal antibody raised against the following C-terminal MKK7 peptide sequence: NH_2-C-RTSGVLSQHHLPFFR.

IN VITRO BINDING ASSAYS:

CS2-p38, CS2-JNK1, and pTM1-ERK2 were *in vitro* translated with [^{35}S]-Met using the TNT Coupled Reticulocyte Lysate System (Promega). Bacterially expressed GST fusion proteins were purified according to standard protocols (Ausubel, 1992), and remained coupled to glutathione-Sepharose (Pharmacia). Protein concentrations were estimated by Coomassie blue staining of SDS-polyacrylamide gels and comparison with standards. For association studies, GST or GST-MKK7 bound to glutathione Sepharose was incubated with either [^{35}S]-p38, [^{35}S]-JNK1 or [^{35}S]-ERK2 in a volume of 500 µl containing 1% Triton X-100, 10 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, 0.1% b-mercaptoethanol, 1% aprotinin, and 1mM phenylmethylsulfonylfluoride (PMSF) for 1 hour at 4 °C. Beads were then washed four times with buffer and resuspended in 30 µl of Laemmli sample buffer. Proteins were separated on SDS-PAGE and analyzed by autoradiography.

CELL CULTURE AND STIMULATION:

NIH3T3 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Plasmid DNA was transfected with the Lipofectamine method (GIBCO-BRL), and cells were harvested 48 hours after transfection. NIH 3T3 cells were serum-starved for 18 hours before stimulation. Total amount of plasmid DNA was 5 μg for 60 mm plates and 12 μg for 100 mm plates and was adjusted with pCS2 vector DNA. 293T and HeLa cells were transfected with calcium phosphate, and cells were harvested 48 hours after transfection. Cells were treated with either PDGF (P, 5 μg/ml; 20 min), anisomycin (A, 10 μg/ml; 20 min), NaCl (N, 0.4 M; 30 min) or left untreated.

IMMUNOASSAYS:

For immunoprecipitations, cells were left untreated or stimulated with various agents, washed with PBS, and lysed on ice in a buffer containing 1% Triton X-100, 10 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, 0.1% β-mercaptoethanol, 1% aprotinin, and 1mM phenylmethylsulfonylfluoride (PMSF). For myc-tagged JNK1 or myc-tagged MKK7 immunoprecipitations, protein was immunoprecipitated from the cleared lysates by incubation with 9E10 antibody for 1 hour at 4 °C. Immunocomplexes were recovered using a mixture of Pansorbin coated with goat-α-mouse IgG. For HA-SEK1 immunoprecipitations, the specific antibody 12CA5 was used, and complexes were recovered with Protein A Sepharose beads (Sigma). Complexes were washed three times with lysis buffer and once with 10mM Pipes (pH 7.0), 0.1 M NaCl, and 1% aprotinin, and resuspended in 10 μl of kinase reaction buffer containing 25 mM Pipes (pH 7.4), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4, 2.5 μCi [γ-^{32}P]ATP, 100 μM unlabeled ATP and 2 μg of the indicated substrate. Reactions were incubated for 30 min at 30 °C. For coupled in vitro kinase assays, 2 μg of the second substrate (GST c-Jun or GST ATF2) was added after the 30 min incubation, and samples were then incubated 30 min at 20 °C. A lower temperature was used for the second incubation to minimize autophosphorylation activity. Reactions were terminated by addition of Laemmli sample buffer. Proteins were separated by SDS-PAGE and analyzed by autoradiography. The relative levels of substrate phosphorylation were determined by phosphorimage analysis.

NIH3T3 CELL CHROMATOGRAPHY:

NaCl stimulated (0.4 M, 30 min) or unstimulated cells (10 x 10 cm dishes) were harvested as described (Ahn and Krebs, 1990) except that the lysis buffer contained 25 mM Tris, pH 7.3, 10 mM β-glycerophosphate, 1.5 mM EDTA, 1.5 mM EGTA, 1 mM Na_3VO_4, 1mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM DTT, and 200 nM microcystin. 100,000 x g lysates from cells were applied to a MonoS column after passing over a MonoQ column. Chromatography conditions were as described (Rouse *et al.*, 1994), except that the salt gradient was 30 ml. Fractions were assayed for kinase activity, and analyzed by SDS-PAGE and autoradiography. Phosphorylated GST c-Jun was quantitated with a Phosphorimager.

RABBIT IL-1 STIMULATION:

Female Dutch rabbits were sedated and injected via an ear vein with IL-1α (5μg/kg) or vehicle. After 4 min. a rapidly lethal anaesthetic was administered and the livers were removed and placed on ice: removal took about 4 min. Livers were frozen in liquid nitrogen and stored at -70 °C.

RABBIT LIVER LYSIS AND CHROMATOGRAPHY:

Frozen livers were thawed and homogenized using a Polytron (Kinematica, Switzerland) in lysis buffer containing 20 mM Tris, pH 7.4, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μM E64, and 1 μM pepstatin. Five ml lysis buffer was used for each gram of liver. Lysates were centrifuged at 20,000 x g, 4 °C for 1 h. 1 M MES pH 6.0 was added to a final concentration of 20 mM and the sample was filtered (0.8 μ M). For chromatography, a 2 ml Fast Flow S Sepharose column was equilibrated in buffer C (20 mM MES pH 6.0, 20 mM b-glycerophosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT) at 4 °C. One hundred mg of soluble protein was loaded onto the column. A 40 ml gradient of 0-1.2 M NaCl in buffer C was run. Two ml fractions were collected and assayed for an activator of SAPK.

SAPK ACTIVATOR PURIFICATION:

A 50 ml FFS Sepharose column (flow rate 60 cm/h) was equilibrated in buffer C and approximately 5 g of liver cytosol protein was loaded. The column was

washed in buffer C containing 0.3 M NaCl, and the activity was eluted in buffer C containing 0.6 M NaCl. Tris pH 8.5 (1M) was added to the eluate to a final concentration of 20 mM and pH 7.4. This was applied to a 10 ml phenyl Sepharose column (flow rate 60 cm/h) equilibrated in lysis buffer containing 0.6 M NaCl. The column was washed with lysis buffer and the active material was eluted in lysis buffer containing 1% CHAPS. Tris pH 8.5 (1M) was added to 50 mM final concentration and the sample was passed through a 1 ml Q Resource colum equilibrated in buffer D (20 mM Tris, pH 8.5, 10 mM NaF, 20 mM β-glycerophosphate, 0.2 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT). The material passing through was dialysed overnight at 4 °C into buffer E (20 mM Hepes pH 7.5, 10 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 0.05% Brij 35) and was loaded on a S Resource column equilibrated in buffer E at 4 °C. This was eluted at 1 ml/min with a 20 ml gradient from 0 to 0.5 M NaCl in buffer E. One-ml fractions were collected.

WESTERN BLOTTING:

Lysates of total cellular protein or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the corresponding rabbit antiserum or mouse monoclonal antibody. For MKK7 Westerns, α3936 was used. For 9E10 immunoprecipitated JNK1 Westerns, a specific JNK1 antibody was used (Santa Cruz). For MKK4 Westerns, anti-MKK4 antibody was used (Santa Cruz). For pCGT-Rac Westerns, an anti-T7 antibody was used (Novagen). For pXJ40 HA Cdc42 Westerns, anti-HA (12CA5) antibody was used. Immunoblots were visualized by enhanced chemiluminescence detection (Amersham) using horseradish peroxidase coupled secondary antibodies (Bio-Rad).

IMMUNOFLUORESCENCE MICROSCOPY:

NIH 3T3 cells and HeLa cells were transfected on glass cover slips. After stimulation, cells were washed once in PBS and fixed for 10 min in 3.7% formaldehyde in PBS. Cells were then permeabilized for 5 min with 0.2% Triton X-100 in PBS. After blocking in PBS containing 5% BSA (bovine serum albumin) for 60 min, the coverslips were incubated with anti-myc (9E10) primary antibody for 1 hr. Immune complexes were detected with flourescein (FITC) conjugated anti-mouse immunoglobulin (Ig) (Jackson Immunoresearch) in 2% BSA in PBS. Cover slips were mounted in Vectashield (Vector

Laboratories, Inc.) and cells were viewed on a laser scanning confocal microscope (Bio-Rad). All procedures were performed at room temperature.

CHAPTER 3: MKK7 AND DROSOPHILA DORSAL CLOSURE

INTRODUCTION:

The genetic analysis of development in *Drosophila* and *Caenorhabditis elegans* has revealed the importance of protein kinase signal-transduction pathways for regulating cell-fate specification and migration (Perrimon and Desplan, 1994; Sundaram and Han, 1996). During *Drosophila* embryogenesis, inductive signals from the follicle cells at the embryonic termini are received by the tyrosine kinase, torso, and induce the formation of embryonic terminal structures (Lu *et al.*, 1993). Ventral signals from the embryo are received by the EGF receptor homolog, DER, and lead to the establishment of ventral cell fates (Clifford and Schupbach, 1992; Raz and Shilo, 1993). During *C. elegans* development, signals from the vulval precursor cell are received in the hypodermis by the tyrosine kinase receptor, *let-23*, and induce the development of the vulva (Sundaram and Han, 1996). This triggers a highly conserved, signal-transduction cascade involving a Grb2 like adaptor protein (SEM-5), a Ras protein (LET-60), a Raf protein (LIN-45), a MAP kinase kinase (LET-537/MEK2) and a MAP kinase (SUR-1/MPK-1). In all these examples, tyrosine kinase receptor-mediated signals are relayed by a MAP kinase cascade, containing a MAP kinase kinase kinase, a MAP kinase kinase and a MAP kinase that are activated sequentially by direct phosphorylation. The homologous MAP kinase cascade in mammalian cells, which includes the p42 and p44 ERKs, is similarly activated by extracellular mitogenic and inductive signals, and dominant-negative and antisense experiments indicate that this cascade is required for mitogenic and differentiation responses.

The first indications that some developmental processes in *Drosophila* may require another MAP kinase cascade were the discoveries that the *Drosophila hemipterous*

(*hep*) and *basket* (*bsk*) genes encoded a MAP kinase kinase and a MAP kinase, respectively (Glise *et al.*, 1995; Sluss *et al.*, 1996). Loss of the function of either protein inhibits dorsal closure, a morphogenetic movement during late embryogenesis. The process of dorsal closure establishes the dorsal ectoderm by stretching the lateral ectoderm over the amnioserosa (Fig. 30). This movement is accompanied by epithelial and epidermal cell elongation in the absence of either cell proliferation or cell rearrangement. Dorsal closure can be divided into a three step process consisting of initiation, spreading and suture phases (Noselli, 1998). These processes are derived from the fact that some dorsal closure mutants selectively affect one or more phases. Initiation involves the dorsoventral elongation of the single row of ectodermal cells of the leading edge which border the amnioserosa (Fig. 30). During the spreading phase, a stretching of more lateral cells occurs, and a characteristic dorsalward movement is maintained. Dorsal closure is complete when the leading-edge cells have met and fused at the dorsal midline. The closure of the embryo starts in the anterior and posterior-most regions and progresses towards the center in a synchronous fashion.

To date, numerous genes have been identified that when mutated result in defects in dorsal closure, or a 'dorsal open' phenotype. These genes may be categorized into one of three classes based on their structure and function. One class consists of membrane-associated proteins, including genes which function in cell adhesion and cell junctions. These proteins are likely required for maintaining cell shape and the integrity of epithelial cell sheets. Some of the mutants in this class include *zipper* (*zip*), a non-muscle myosin heavy chain (Young *et al.*, 1993), *myospheroid* (*mys*) and *scab*, integrin β and α subunits, respectively (MacKrell *et al.*; 1988, Stark *et al.*, 1997), and *coracle* (*cor*), a membrane skeletal 4-1 protein homolog (Fehon *et al.*, 1994). A second class of genes consists of signaling molecules that comprise a conserved SAPK signaling cascade. These include *Drosophila* homologs of mammalian Rac, Cdc42 and RhoA (*Drac, Dcdc42, DrhoA*), NIK (*misshapen*), MKK7 (*hep*), SAPK (*bsk*), the transcription factors Jun (*DJun*) and Fos (*kayak*) (Riesgo Escovar and Hafen, 1997a) and the SAPK phosphatase MKP1 (*puckered*) (Noselli, 1998; Su *et al.*, 1998) (Fig. 31). The activation of this SAPK-signaling pathway is restricted to leading-edge cells, and is important in leading-edge specification. The elongation and differentiation of leading-edge cells during the initiation phase of dorsal closure is accompanied by specific patterns of protein accumulation, including asymmetric localization of fasciclin III (Martinez Arias and Bate, 1993), non-

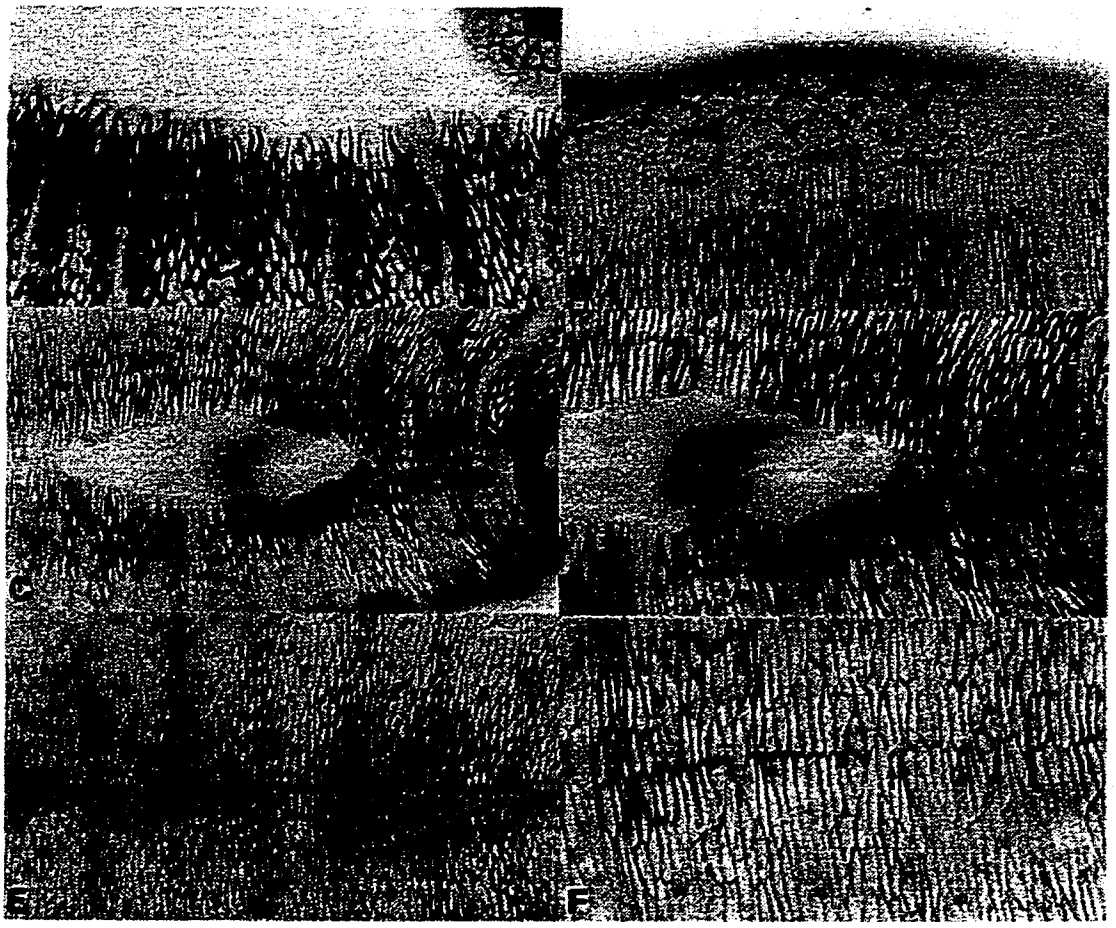


Fig. 30. Dorsal Closure. Except for panel B, all embryos are stained with α-Fasciclin III. Dorsal is on top and anterior to the left. (A) Dorsal lateral epidermis during stage 13. Cells of the amnioserosa are devoid of staining. The leading-edge cells lack Fasciclin III along the perimeter facing the amnioserosa. (B) Same as A, but stained with α-spectrin. The leading-edge cells are the columnar cells facing the large cells of the amnioserosa. (C) Dorsal view at stage 15. Closure proceeds from both ends of the embryo. (D) Detail of panel C. (E) and (F) Leading-edge cells meet at the dorsal midline, cells intersperse at the segment boundary (arrows in F). Photo from (Martinez Arias and Bate, 1993).

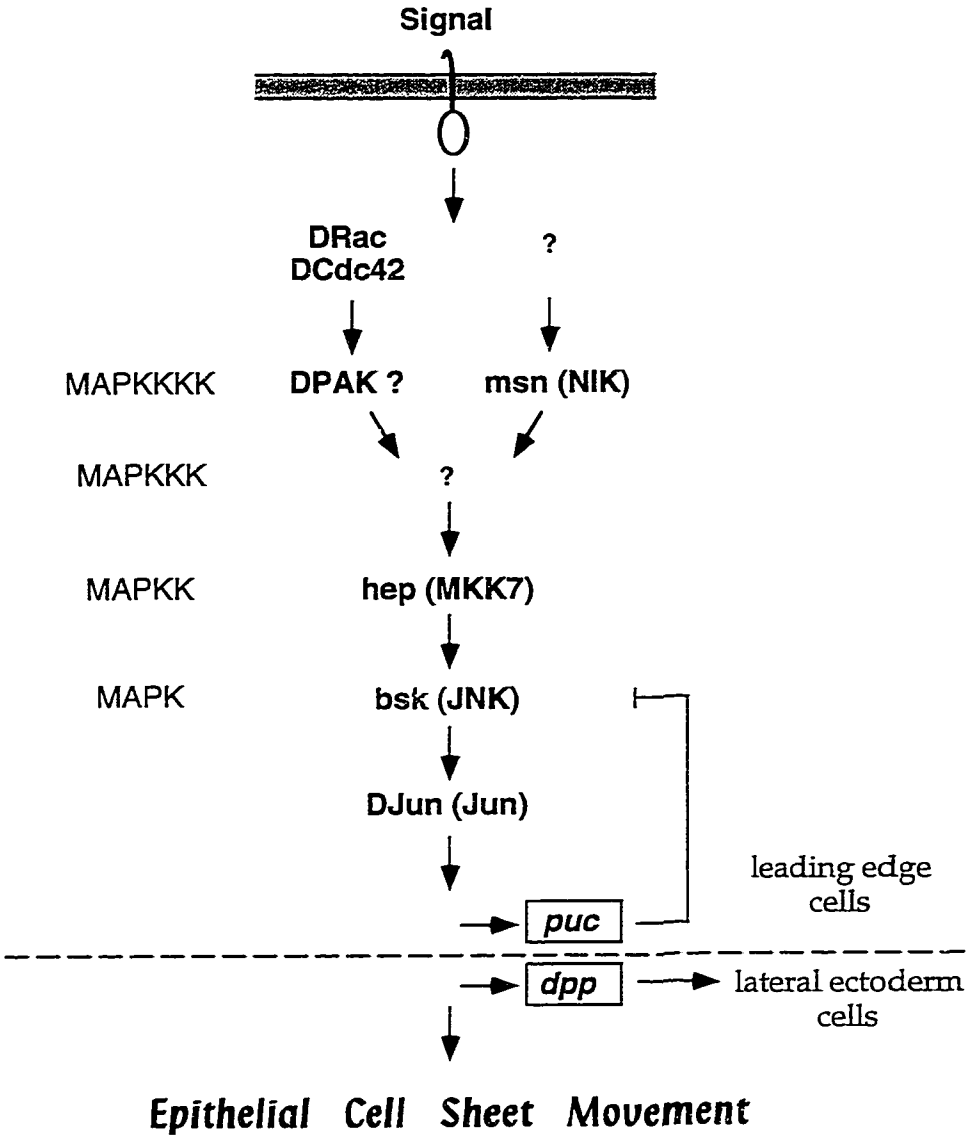


Fig. 31. Dorsal closure signaling pathway. The cascade leading to activation of SAPK in the leading-edge cells. Inactivation of the pathway is regulated by negative feedback from the MKP-1 phosphatase, Puckered. Activation of the pathway also induces expression of DPP, which can diffuse to the lateral ectoderm and establish a gradient, resulting in concerted cell migration.

muscle-myosin heavy chain (Young *et al.*, 1993), the skeletal protein, *coracle* (Fehon *et al.*, 1994), DPAK, and other cellular components phosphorylated on tyrosine (Harden *et al.*, 1996). It is possible that activation of the SAPK pathway in the leading-edge cells either directly or indirectly regulates this class of cytoskeletal proteins. Activation of the SAPK pathway in leading-edge cells also results in the expression of DPP (*decapentaplegic*), which can subsequently diffuse in the neighboring tissues to establish a gradient in the lateral ectoderm (Glise and Noselli, 1997; Hou *et al.*, 1997; Riesgo Escovar and Hafen, 1997b). DPP encodes a secreted molecule of the transforming growth-factor-β (TGF–β) superfamily, and is a component of the third class of dorsal closure genes. These are comprised of genes participating in DPP signaling and include *thick veins* (*tkv*) and *punt*, which are Type I and II TGF–β receptors, and *schnurri*, a nuclear zinc-finger protein homolog (Arora *et al.*, 1995; Grieder *et al.*, 1995; Staehling Hampton *et al.*, 1995). DPP signaling-mediated by the SAPK pathway from the leading-edge cells to the lateral ectoderm is an important step in the spreading phase of dorsal closure.

The identification of Hep as a MAP kinase kinase required for proper dorsal closure by Stephane Noselli was the first demonstration that a signaling-kinase functioned in this process (Glise *et al.*, 1995). The striking sequence similarity between Hep and MKK7 prompted us to investigate whether Hep and MKK7 might be functionally related. We subsequently initiated a collaboration with Stephane Noselli at the CNRS in Toulouse, France, to determine if MKK7 could functionally replace Hep.

RESULTS:

MKK7 RESCUES HEP MUTANT FLIES:

The degree of sequence conservation between the MKK7 and *Drosophila* Hep proteins (Fig. 12) suggested potential functional homology. MKK7 and Hep share 56% amino acid identity over the full-length sequence and 71% identity within the catalytic domain. A demonstration of functional homology for a kinase had been established by complementation in yeast, but never in a multicellular organism. We therefore employed a heterologous complementation assay in *Drosophila*, by examining three related, vertebrate MKK proteins in parallel. We analyzed MKK7, MKK4 and XMEK2. MKK7 is closely

related to MKK4, and both are potent SAPK activators. XMEK2 is the *Xenopus* homolog of MKK4, sharing 88% amino acid identity. Transgenic flies expressing MKK7, XMEK2 or MKK4/SEK1 under the control of a *ubiquitin* promoter were constructed (UBMKK7, UBXMEK2 and UBSEK1, respectively), and the ability of these elements to rescue *hep*-associated lethality of nine different alleles was tested. Specificity was also tested by using a mutation in a different *Drosophila* MAPKK, *Dsor1* (Tsuda *et al.*, 1993) (Table 6). Dsor1 protein is not required for dorsal closure, but is required for other events during development. UB*hep* rescued all the *hep* alleles tested, leading to the formation of normal adult flies. Rescue was specific, because flies mutant for *Dsor1* were not rescued. Expression of MKK7 rescued 42% and 62%, respectively, of flies carrying two lethal *hep* alleles (*rh1* and *rh99*, respecetively). MKK7 was quantitatively more efficient than XMEK2 and MKK4/SEK1 to rescue viability of *hep* alleles (Table 6), confirming the relatedness of MKK7 and Hep. Although comparable rescue of two weak alleles, *hep rh1* and *hep rh99*, was observed for UBMKK7 and UB*hep*, other stronger alleles were either weakly or not rescued. Adult males rescued by MKK7 showed defects in the dorsal thorax (Fig. 32) and in the development and rotation of the anal plate and genital arches, more posteriorly (not shown). However, some fully fertile and viable males with normal posterior structures were obtained, indicating complete rescue. This spectrum of defects was very reminiscent of those displayed by a hypomorphic, viable *hep* allele (Glise *et al.*, 1995) (Fig. 32). XMEK2 and MKK4/SEK1 both confer a lower level of rescue, indicating that they were less competent than MKK7 in the replacement of Hep function in the fly (Table 6). These levels of rescue activity could reflect species-specific requirements for upstream components of the pathway as opposed to the protein kinase alone. This phenomenon was originally observed in a yeast heterologous complementation assay using mammalian MKK1, in which complete rescue required the co-expression of MKK1 and its activator Raf-1 (Hughes *et al.*, 1993). A specific kinase that activates Hep in *Drosophila* has not been identified.

In summary, MKK7 was able to rescue *hep* mutations partially, but significantly as compared to related XMEK2 and MKK4, suggesting that MKK7 and Hep have some conserved functions. Although MKK7 and MKK4 have similar biochemical properties, the ability of MKK7 to complement a mutation in a specific developmental pathway in *Drosophila* in which MKK4 cannot, provides a hint that at an organismal level, these kinases perform independent tasks. Recent data from targeted gene disruptions of

Table 6. Rescue of *hep* Mutations by UB Transgenes. Summary of the rescue of *hep* lethality by UBhep, UBMKK7, UBSEK1 and UBXMEK2 transgenes. Left column represents different *hep* alleles and numbers indicate percent of rescued males. Control is a control transgene.

hep alleles	Control	HEP	MKK7	MKK4/SEK1	XMEK2
hep r39	0	48	5	0	0
hep r45	0	55	1	0	0
hep r51	0	33	0	0	0
hep r75	0	66	0	0	0
hep rh1	0	100	42	2	6
hep rh8	0	65	1	0	0
hep rh19	0	100	1	0	0
hep rh31	0	43	0	0	0
hep rh99	0	96	62	14	15
Dsor1 rl	0	0	0	0	0

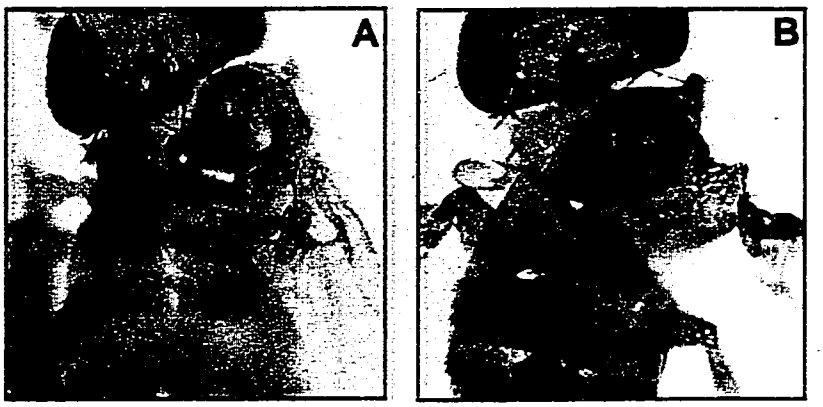


Fig. 32. Comparison of defects observed in hep^{rh99} rescues. Alleles rescued with UBHEP (A) and UB MKK7 (B) are shown. Both males showed similar defects in the dorsal thorax.

MKK4 in the mouse also support the idea that MKK7 and MKK4 have distinct as well as overlapping functions (Nishina *et al.*, 1997; Yang *et al.*, 1997). The partial rescue activity of MKK7 in Hep mutant flies could be indicative of evolutionary divergences at the level of MKK7/Hep activators or targets.

DISCUSSION:

We have provided evidence that the participation of the SAPK cascade in embryonic development is likely to be conserved in mammals, since we identified a mammalian Hep homolog, MKK7, that can replace the function of Hep in *Drosophila* dorsal closure. This implies that interactions with upstream activators and downstream effectors required for modulation of morphogenetic changes in the embryonic ectoderm have been conserved throughout evolution. Indeed, increasing data from numerous labs are providing insights into the close relationship between *Drosophila* and the vertebrate SAPK stress-activated pathway. The small GTPases, Rac and Cdc42, which regulate the actin cytoskeleton in mammalian cells, also participate as elements in the dorsal closure signaling pathway. Although studies with various Rac1/Cdc42 effector mutants in mammalian cells establish that JNK/SAPK activation and regulation of the cytoskeleton occur via independent downstream signaling events, studies in *Drosophila* suggest that these events are linked. Several lines of evidence support this. Both DRacA and DCdc42 regulate the cytoskeleton in developmental processes. Expression of a dominant-negative transgene of *Drosophila* RacA or Cdc42 (*N17DRacA*, *N17DCdc42*, respectively) during embryonic development causes a high frequency of dorsal closure defects, due to disruption of cell shape changes in the lateral epidermis (Harden *et al.*, 1995; Riesgo Escovar *et al.*, 1996). Interestingly, in N17DRacA embryos the cytoskeletal proteins actin and non-muscle myosin heavy chain fail to accumulate in the leading-edge cells (Harden *et al.*, 1995). In addition, Hep mutations can strongly suppress the effects of overexpressed dominant-activated forms of DRacA and DCdc42, supporting the idea that they act upstream of Hep and Bsk. A *Drosophila* homolog of PAK, DPAK, is a potential downstream effector for DRacA and DCdc42; DPAK binds to GTP-bound forms of both DRacA and DCdc42 (Harden *et al.*, 1996). DPAK expression is elevated in the leading-

edge of epidermal cells and colocalizes with dynamic actin structures, properly localizing it for an involvement in dorsal closure.

More recently, a STE20-related kinase, Misshapen (Msn) was demonstrated to function upstream of Hep and Bsk to stimulate dorsal closure (Su *et al.*, 1998). The mammalian homolog of Misshapen has been identified as NIK (Nck-interacting kinase), which is a MAP kinase kinase kinase kinase, similar to GCK (germinal center kinase) (Su *et al.*, 1997). [It should be noted that Nck-Interacting Kinase, NIK, is unrelated to NFκB Inducing Kinase, NIK (Malinin *et al.*, 1997).] In 293 cells, NIK has been demonstrated to associate with the SH3 domains of the adaptor protein Nck. NIK is thought to act upstream of the MAP kinase kinase kinase, MEKK1, as NIK and MEKK1 can directly associate, and a dominant-negative MEKK1 can block NIK induced SAPK activation. Misshapen is thought to function coordinately with DRac to activate a downstream MAP kinase kinase kinase, as DRac and Misshapen do not interact directly in an overlay assay using GTP-Rac or in a yeast two-hybrid assay (Su *et al.*, 1998). However, co-expression of N17Rac was able to suppress Misshapen-induced SAPK activation in 293 cells. It is possible that DRac and Misshapen will function in parallel and converge on the activation of Bsk. This is consistent with the existence of multiple, parallel upstream activators for mammalian MKK7, including Rac/Cdc42, HPK1, GCK, MST1 and others (Graves *et al.*, 1998; Hu *et al.*, 1996; Katz *et al.*, 1994; Kiefer *et al.*, 1996; Pombo *et al.*, 1996). It will be of interest to determine which MAP kinase kinase kinase functions in the dorsal closure pathway to transduce signals from DRac/DCdc42 and Misshapen to Hep.

In contrast to studies on vertebrate signaling pathways, the methodologies used to study signaling molecules in *Drosophila* rely on genetic techniques rather than biochemical approaches. The genetic approach is based on observations that mutations that have related mutant phenotypes most likely correspond to genes whose protein products function in the same biochemical pathway. Thus, although the rescue of *hep* mutant flies by MKK7 was incomplete, it suggests that both molecules function in a homologous signaling pathway. Evidence in support of this is provided by the conservation of homologous substrates as well as upstream activators. We cannot rule out the possibility that another as of yet undiscovered mammalian MKK would rescue *hep* mutant flies more efficiently. Incomplete rescue may also reflect divergence in evolution. Sequential

molecules in a signaling pathway may co-evolve, so replacing a series of molecules in the pathway may allow for a more complete rescue than replacing only one.

The process of dorsal closure provides interesting parallels to cellular processes in vertebrate cells, most notably those of wound healing and cell invasiveness. All of these events consist of a migration of epithelial cell sheets with free edges, in contrast to other epithelial movements like neurulation. During wound healing, cell matrix attachment is dependent on TGF-β and Jun/AP-1 activitites (Martin, 1997; Mauviel *et al.*, 1996). These function to regulate the activity of metalloproteases like type IV collagenase which degrades components of the extracellular matrix (Mauviel *et al.*, 1996). In fact, remodeling of the basement membrane following expression of a dominant negative collagen IV has been demonstrated to induce dorsal closure defects (Borchiellini *et al.*, 1996). An important question to address in the future concerns whether cytoskeletal proteins required for proper dorsal closure play a regulatory role, and whether they are to linked to the SAPK- or DPP-signaling pathways. Data presented in the following chapter provide evidence to suggest that MKK7 may associate with cytoskeletal proteins directly.

MATERIALS AND METHODS:

GENETICS:

Genetic markers and balancer chromosomes have been described (Lindsley, 1992). Novel *hep* alleles were obtained by imprecise excision of a P element from the hep1 stock, as previously described (Glise *et al.*, 1995).

The ability of either MKK7, XMEK2, or SEK1 to rescue hep zygotic lethality was tested as follows: hep / FM6 ; ry506/ry506 females were mated to w/Y ; p{UB-X}/TM3 or w/Y ; p{UB-X}/p{UB-X} males. X represents either hep, MKK7, XMEK2 or SEK1 cDNAs. Rescue activity was calculated as the percentage of hep / Y ; UB-X / + (rescued) males, as compared to FM6 / Y ; UB-X / + (control) males. For each cross, two independent lines were tested, and showed similar results. At least 50 control males were counted in each experiment.

UB TRANSGENE CONSTRUCTS:

The construction of UBhep is described in (Glise *et al.*, 1995). To construct UBMKK7, UBXMEK2, and UBSEK1, a NotI fragment containing a ubiquitin promoter-cDNA_X-hsp 70 3' UTR was cloned into the pCaSpeR4 transformation vector (Thummel and Pirotta, 1992), where X represents either MKK7, XMEK2 or SEK1 cDNAs. The size and origin of the restriction fragments containing the coding region for MKK7, XMEK2, and SEK1 are as follows : 1.3 kb MKK7a EcoRI fragment from pCR-II; 1.8 kb EcoRI fragment from a pXM-XMEK2 vector (a gift of Dr. L. Zon), (Tsai *et al.*, 1989); 2.28 kb XhoI fragment from a pXM-SEK1 vector (a gift of Dr. L. Zon). P-element-mediated germ-line transformation followed standard protocols (Rubin and Spradling, 1983).

CHAPTER 4: A NOVEL MKK7 INTERACTING PROTEIN

INTRODUCTION:

Our previous studies identified MKK7 and established that this kinase functions downstream of multiple activators, including the MAP kinase kinase kinases MEKK1, MLK2 and ASK1, in a pathway leading to the activation of SAPK. The fact that both MKK4 and MKK7 have been shown to function in these pathways raises questions concerning the degree of redundancy between these two SAPK activators. One possibility, supported by our data and the data of others, is that MKK4 and MKK7 are responsive to different stimuli. Data from MKK4 knockout mice demonstrate that in the absence of MKK4, activation of SAPK in response to anisomycin and heat shock is undetectable, whereas activation of SAPK in response to osmotic shock and UV irradiation remains unaffected (Nishina *et al.*, 1997; Yang *et al.*, 1997). There are also reports showing the selective activation of MKK7 in response to MLK2 and preferential activation of MKK4 in response to MEKK1 in some cells (Cuenda and Dorow, 1998; Hirai *et al.*, 1998). Another possibility is that through localization to different cellular compartments and/or interaction with distinct proteins, MKK4 and MKK7 make a differential contribution to the activation and function of the SAPK pathway. The importance of proteins that serve this function, called scaffolding or anchor proteins, is becoming apparent (Whitmarsh and Davis, 1998). Scaffolding proteins can make a contribution to specificity by localizing components of a cascade to different subcellular compartments, and organizing signaling proteins relative to upstream activators and downstream targets. For example, the A-kinase anchoring proteins (AKAP's) function to maintain the protein kinase A (PKA) holoenzyme at precise intracellular sites (Pawson and Scott, 1997). AKAP's bind the regulatory subunit dimer of PKA via a conserved amphipathic helix and tether individual PKA-AKAP complexes via a

special targeting region to specific subcellular structures. Anchoring ensures that PKA is exposed to localized changes in cAMP and is compartmentalized with substrates.

In *S. cerevisiae*, at least two proteins have been demonstrated to function as scaffolding proteins for MAP kinase pathways. Scaffolding proteins bind several signaling molecules to create multi-enzyme complexes. The protein STE5 is made up of docking sites for the MAP kinase kinase kinase, STE11, the MAP kinase kinase, STE7, and the MAP kinase homolog, FUS3 (Choi *et al.*, 1994). Activation of the pathway via a G-protein coupled receptor and the STE20 kinase is required for mating. STE5 also provides a physical link to STE4 (a G-protein β subunit) without which the cascade cannot be initiated. The assembling of successive members within the cascade favors tight regulation of this pathway by ensuring that signals pass from one kinase to the next, thus preventing crosstalk between components that function in parallel pathways. In addition to the direct association between STE7 and FUS3 through STE5, these two kinases may interact directly in the absence of STE5 through an N-terminal peptide motif in STE7 (Bardwell *et al.*, 1996). This interaction may increase the fidelity of the pathway and allow the MAP kinase kinase, STE7, to serve as a cytoplasmic anchor for the MAP kinase. Thus, both the direct interactions between pathway components and the presence of a scaffolding protein contribute to the specificity.

The protein PBS2, in addition to acting as a MAP kinase kinase, appears to also serve a scaffolding function by interacting with SHO1, a transmembrane osmosensor, STE11, a MAP kinase kinase kinase, and the HOG1 MAP kinase (Posas and Saito, 1997). Activation of PBS2 in this osmoregulatory pathway is mediated by the STE11 MAP kinase kinase kinase, which is alternatively found as an integral component of the mating pheromone response pathway. Formation of a multiprotein complex with PBS2 as a scaffold restricts STE11 to activating only PBS2, and thereby prevents crosstalk with components of the mating response pathway. The examples of STE5 and PBS2 show that scaffolding proteins can assemble alternative signaling complexes using some of the same proteins.

Mammalian proteins thought to be important in creating scaffolding complexes for protein kinases have also been identified. A two-hybrid screen with SAPK was used to identify the protein JIP-1 (JNK interacting protein-1) (Dickens *et al.*, 1997).

JIP-1 contains an N-terminal JNK binding domain and an SH3 domain. JIP-1 was originally described as an inhibitor of JNK because of its capacity to retain JNK in the cytoplasm and thereby inhibit JNK-gene regulated expression. More recently JIP-1 has been demonstrated to also interact with HPK1, MLK3 and MKK7 by co-immunoprecipitation experiments (Whitmarsh *et al.*, 1998). Co-expression of JIP-1 was shown to enhance JNK activation by MKK7 and not MKK4, suggesting JIP-1 selectively scaffolds MKK7, JNK and the upstream activators MLK3 and HPK1.

In another screen for interacting proteins with MKK1, Weber and colleagues identified a protein called MP1 (MEK partner 1) which binds specifically to MKK1 and ERK1 and facilitates their activation (Schaeffer *et al.*, 1998). MP1 binds a proline-rich sequence present in MKK1 and MKK2 between subdomains IX and X, which is absent in other mammalian MKK's. It is proposed that MP1 functions as an adaptor to enhance the efficiency of the ERK1 MAP kinase cascade by localizing MKK1 and ERK1 to one another. MP1 efficiently binds ERK1 in the absence of MKK1. MP1 preferentially binds inactive MKK1 and is selective for ERK1 over ERK2, thus favoring the activation of ERK1 by MKK1.

Taken together, these examples demonstrate that the assembly of signaling proteins into complexes may influence where and when protein kinases are activated in the cell. The role of scaffold, anchoring or adaptor proteins, in combination with direct interactions between pathway components, thereby aid in the specificity of signal-transduction. Given the overlapping biochemical properties observed for MKK4 and MKK7, we were interested in identifying proteins that might influence MKK7 specificity. These proteins might be novel specific activators or substrates of MKK7, or proteins which function to bring MKK7 in proximity to specific activators. Our results from *Drosophila* studies demonstrating that MKK7 could functionally substitute for Hep to a significantly greater degree than MKK4, had already suggested that at some level these kinases would have distinct functions. The identification of novel MKK7 interacting proteins might provide us with information into the differential regulation between MKK7 and MKK4.

In a screen for novel MKK7 interacting proteins we identified a human homolog of *Drosophila* Bicaudal-D (BicD). BicD is a 90 kDa protein with structural similarity to the tail end of myosin heavy chain (Suter *et al.*, 1989; Wharton and Struhl,

1989). Studies from dominant and recessive BicD mutations suggest that BicD may function to properly localize developmental factors. In addition, the phosphorylation state of BicD may also be important for its function (Suter and Steward, 1991). Our identification of a human homolog of BicD as a protein that binds to MKK7 raises the possibility that human BicD may function to localize MKK7.

RESULTS:

TWO-HYBRID SCREEN WITH MKK7:

In a search to identify novel MKK7 interacting-proteins, a LexA MKK7 fusion protein was constructed for use in a yeast two-hybrid screen (performed by Jim Hopper, while on sabbatical from University of PA). To test whether full-length MKK7 would interact with proteins in a two-hybrid assay, we tested the ability of LexA MKK7 to interact with its substrate SAPK fused to VP16 (Table 5, Chapter 2). Yeast strains transformed with LexA MKK7 and VP16 SAPK grew on His^- plates and turned blue when tested for β-galactosidase activity, indicative of an association between the two proteins. Two independent libraries were selected for screening: a mouse embryonic day 9.5-10.5 cDNA library, and a human HeLa S3 cell cDNA library (Clontech). From the mouse embryo library screen, four positive clones were sequenced. Two independent clones had a high degree of homology to chicken filamin over a region of roughly 100 amino acids. The overall primary structure of filamin resembles that of human actin-binding protein (Barry *et al.*, 1993) and is made up of 24 repeating units. Filamin forms dimers in solution and binds to and cross-links actin. The third clone showed high similarity to cytoplasmic linker protein-170 (CLIP-170). CLIP-170, also referred to as restin, is an intermediate filament-associated protein that links endocytic vesicles to microtubules (Pierre *et al.*, 1992). It is highly expressed in Reed Sternberg cells, the tumoral cells diagnostic for Hodgkin's Disease (Bilbe *et al.*, 1992). Structurally, CLIP-170/restin contains a large coiled-coil alpha-helical domain similar to those found in myosins. The region of CLIP-170/restin that binds MKK7 corresponds to the central alpha-helical region made up of heptad repeats. The remaining clone was determined to be a non-specific interaction. Ten clones that were sequenced from the HeLa cell library screen all contained an identical sequence, which showed high homology to the *Drosophila* protein BicaudalD (BicD)

(Suter *et al.*, 1989; Wharton and Struhl, 1989). The predicted BicD protein contains several extended amphipathic alpha-helices with similarity to myosin heavy chain tails, kinesin and paramyosin. Similar to CLIP-170/restin, BicD is predicted to form a coiled-coil structure, and the region of BicD to which MKK7 bound corresponds to an alpha-helical region made up of heptad repeats. The fact that all the clones identified in this screen appeared to represent large cytoskeletal proteins made up of helical repeats was of interest. However, it also raised the concern that these associations might be occurring in a non-specific manner.

To further address whether the association between MKK7 and a putative novel human BicD homolog represents a specific interaction, we tested whether other MAP kinase kinases could bind to the BicD-like isolate. A GST fusion of the BicD-like isolate was generated and co-expressed in 293T cells with either MKK7, MKK4, MKK3 or MKK6. In co-immunoprecipitation experiments, only MKK7 was able to associate with the GST-BicD-like isolate (Fig. 33). These data suggested that the ability of MKK7 to bind the BicD-like isolate was not due to non-specific interactions. Because the clone corresponding to BicD represented a novel human protein and appeared to bind MKK7 in a specific manner, we decided to pursue this clone for further study.

ISOLATION OF FULL-LENGTH H-BICD:

Prior to a library screening to obtain full-length human BicD cDNA, we observed that a human homolog to *Drosophila* BicD had recently been submitted into the GenBank database (Baens and Marynen, 1997). In a hybridization selection procedure with cosmids derived from the short arm of human chromosome 12, Baens and Marynen isolated a cDNA fragment of human BicD. The sequence information was used to determine the complete coding sequence of human Bicd (hBicD), as well as a partial mouse BicD1 cDNA. Although the percent sequence identity between *Drosophila* and human BicD is relatively low (41%), the predicted secondary structure appears to be conserved. Sequence similarity is highest within the amphipathic helices and the leucine zipper motif, and the conserved order of these domains suggests a related biological function. Although these investigators had no full-length cDNA, we were able to obtain several plasmids, each of which contained partial sequence to hBicD. Using an overlap PCR strategy (Innis *et al.*, 1990), a full-length hBicD cDNA was generated (Fig. 34). To confirm that this new

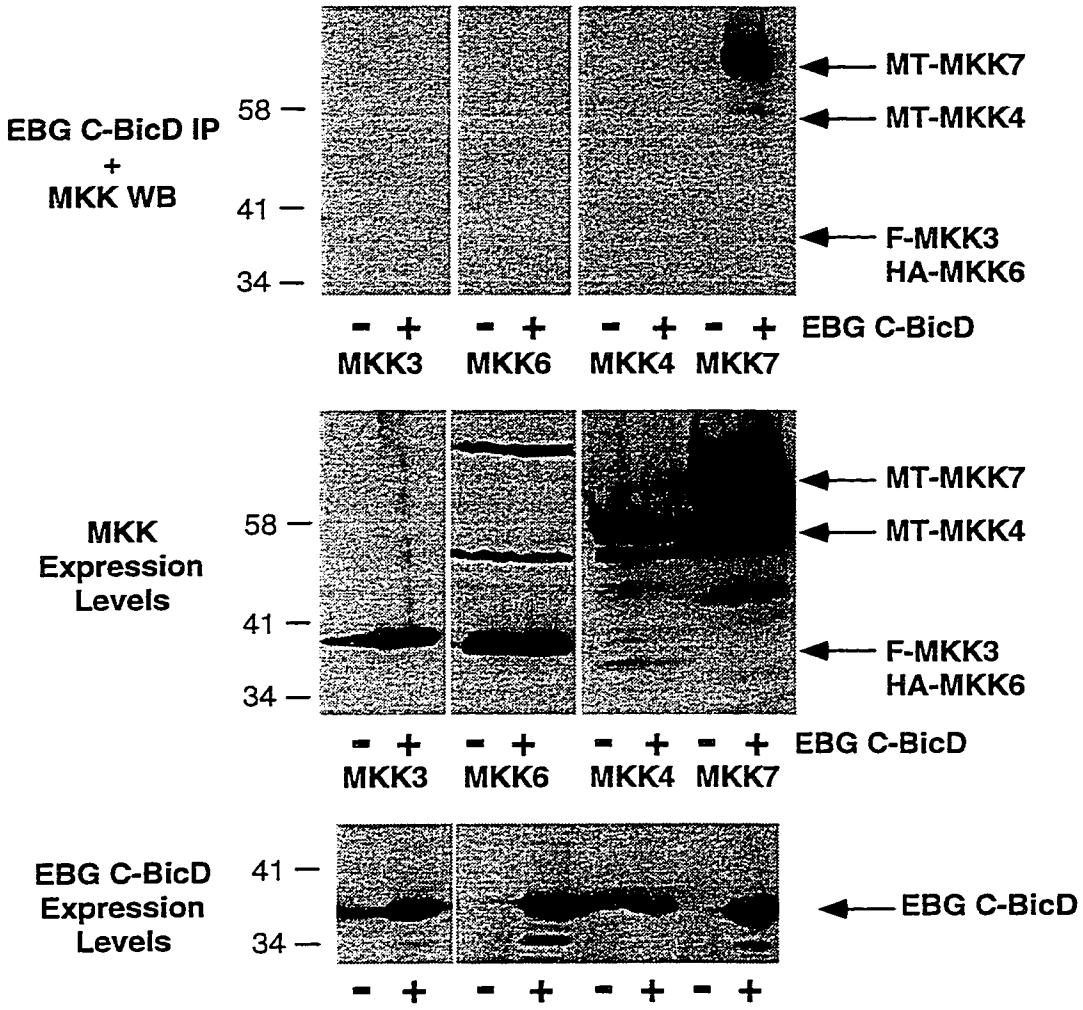


Fig. 33. MKK7 immunoprecipitates with the C-terminal fragment of BicD. 293T cells were co-transfected with pEBG vector or pEBG C-BicD and either pRSV F MKK3, pME HA MKK6, pCS3MT MKK4 or pCS3MT MKK7. pEBG or pEBG C-BicD was immunoprecipitated from cells using glutathione Sepharose (Pharmacia) and evaluated by Western blot analysis with either anti-Flag antibody, anti-HA antibody (12CA5), or anti-myc antibody (9E10). These antibodies were used to monitor expression of MKK's in the total lysates. Expression of pEBG C-BicD was determined by Western blotting with anti-GST antibody.

full-length clone produced the expected protein, we translated full-length hBicD using [^{35}S]-Met *in vitro*. Figure 35 demonstrates that this clone generated a 90 kDa protein, corresponding to the expected molecular weight. To determine whether this protein bound MKK7, a GST fusion of MKK7 or GST alone generated in *E. coli* was coupled to glutathione beads and incubated with [^{35}S]-Met-labeled hBicD. hBicD was able to bind GST MKK7 but not GST alone (Fig. 35). This indicated that MKK7 could bind to full-length hBicD *in vitro*.

HBICD IMMUNOPRECIPITATES WITH MKK7 FROM 293T CELLS BUT NOT WITH OTHER MKKS:

We examined whether full-length hBicD and MKK7 could interact in cells. We generated a T7 epitope-tagged hBicD expression construct and co-expressed T7 hBicD with either F-MKK3, MT-MKK4, HA-MKK6 or MT-MKK7 in 293T cells. Immunoprecipitated hBicD was analyzed by Western blotting for the presence of any asoociated proteins. The results in Fig. 36 demonstrate that MKK7 is capable of binding T7 hBicD in cells. No other MKKs bound to T7 hBicD, suggesting that the association between MKK7 and hBicD is specific.

THE N-TERMINUS OF MKK7 IS REQUIRED FOR HBICD BINDING:

To determine which region of MKK7 was involved in binding hBicD, a series of MKK7 deletion constructs was utilized in co-immunoprecipitation experiments. Deletion constructs of MKK7 lacking the first 10, 22, 75 or 100 residues from the amino terminus (Δ10-MKK7, Δ22-MKK7, Δ75-MKK7, Δ100-MKK7) were co-transfected with a GST fusion of the region of hBicD originally identified (GST C-hBicD) in 293T cells. Wild-type MKK7 was able to immunoprecipitate with GST C-hBicD, as judged by immunoprecipitaion with glutathione Sepharose followed by an anti-myc (9E10) MKK7 Western blot. However, removal of even ten residues from the N-terminus of MKK7 resulted in a dramatic reduction of the ability of MKK7 to immunoprecipitate with GST C-hBicD (Fig.37). All of the deletion constructs showed an equally weak ability to co-immunoprecipitate with GST C-hBicD. Similar results were observed when full-length hBicD was tested for its ability to bind the different ΔN-MKK7 mutants. This demonstrated that the N-terminus of MKK7 is important for its ability to bind hBicD. Co-expression of an N-terminal fragment of MKK7 with hBicD to determine whether the

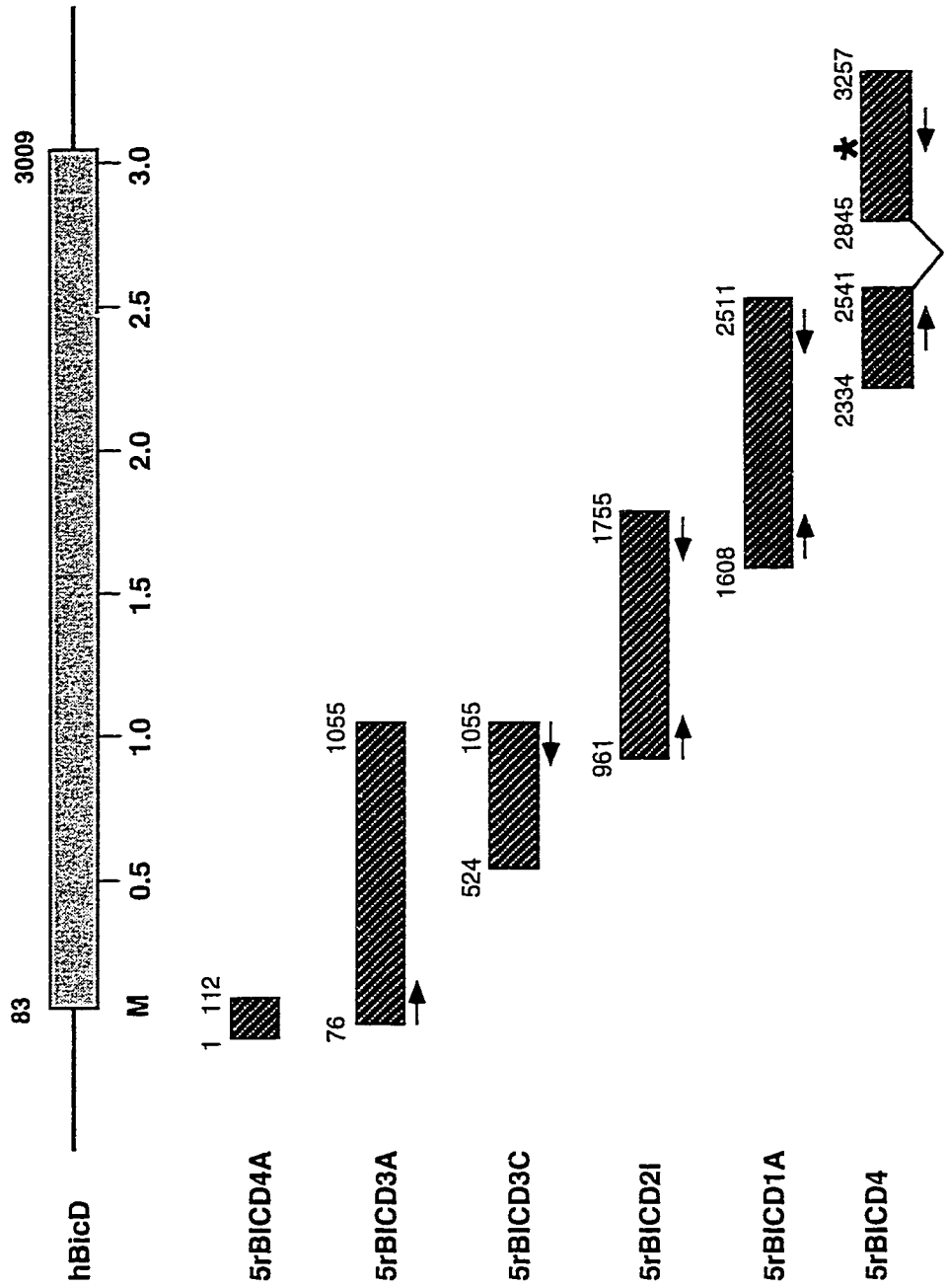


Fig. 34. Full length human BicD was generated using an overlap PCR strategy. Illustrated are six plasmid cDNA's encoding the sequence of hBicD. Numbers correspond to the region of nucleotide sequence in each plasmid. Arrows denote regions where primers were made. Plasmid 5rBICD4 corresponds to the most abundant splice form of hBicD, in which the region between nucleotide 2541 and 2845 has been spliced out. The * denotes the stop codon.

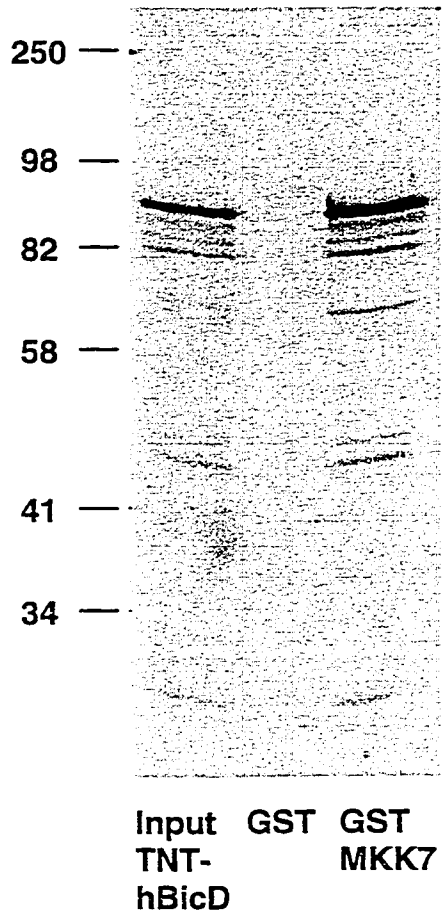


Fig. 35. hBicD and MKK7 associate *in vitro*. Recombinant GST-MKK7 or GST alone bound to glutathione Sepharose was incubated at 4 °C for 1 hour with [^{35}S]-Met labeled *in vitro* translated pT7 Blue hBicD. Samples were washed and analyzed by SDS-PAGE and autoradiography. Input TNT-hBicD lane to the left indicates the amount of input translation product.

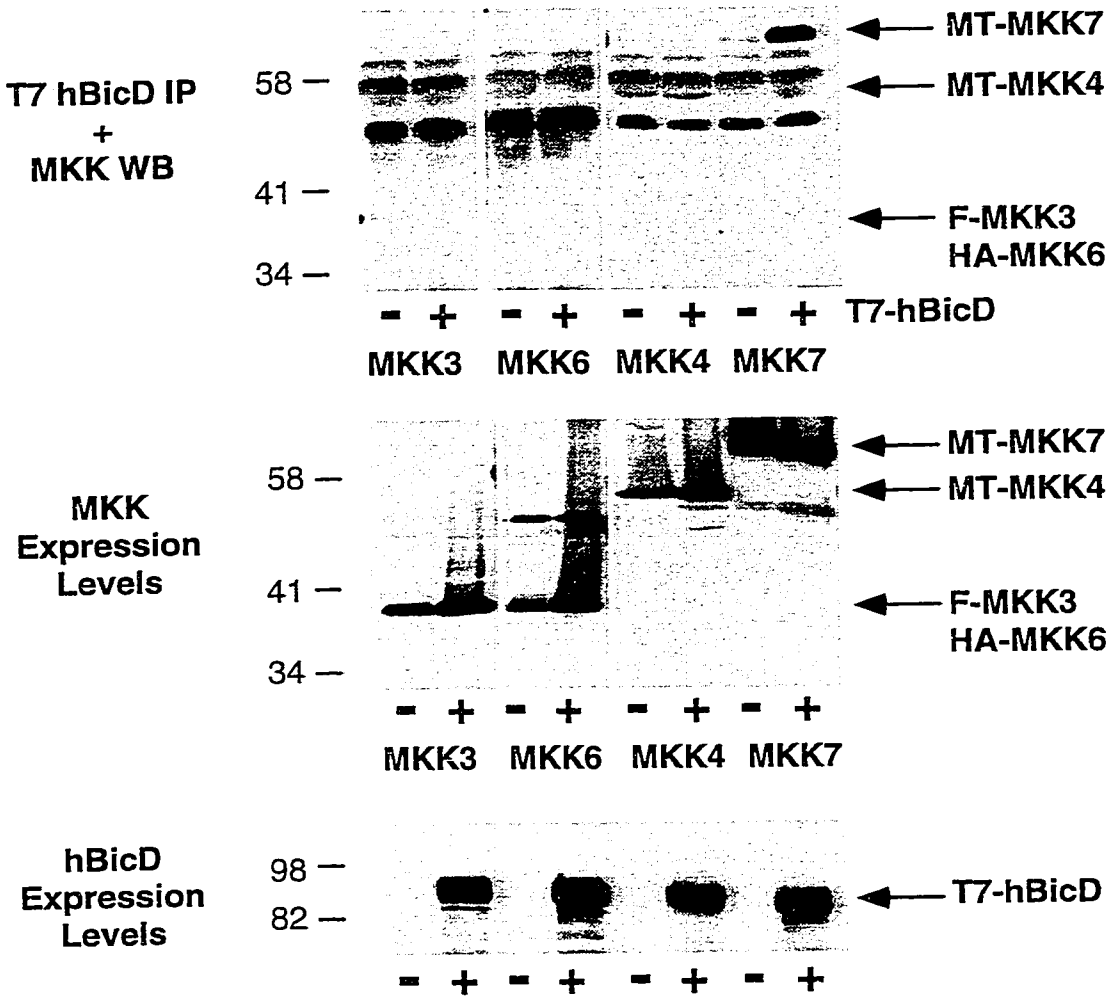


Fig. 36. MKK7 immunoprecipitates with full-length hBicD. 293T cells were co-transfected with pCGT T7 vector or pCGT T7-hBicD and either pRSV F MKK3, pME HA MKK6, pCS3MT MKK4 or pCS3MT MKK7. pCGT T7 or pCGT T7-hBicD was immunoprecipitated from cells using anti-T7 antibody (Novagen) and evaluated by Western blot analysis with either anti-Flag antibody, anti-HA antibody (12CA5), or anti-myc antibody (9E10). These antibodies were used to monitor expression of MKKs in the total lysates. Expression of pCGT T7-hBicD was determined by Western blotting with anti-T7 antibody.

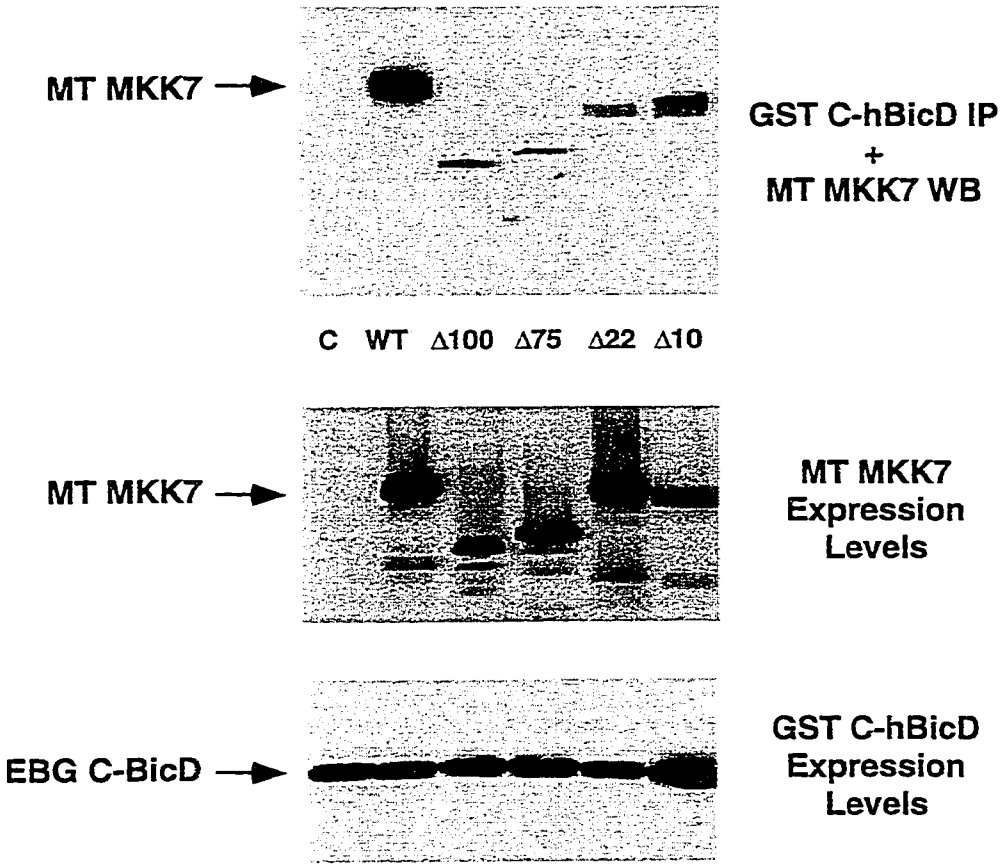


Fig. 37. The N-terminus of MKK7 is required for hBicD binding. 293T cells were co-transfected with pEBG C-BicD and either wild-type pCS3MT MKK7, Δ100-MKK7, Δ75-MKK7, Δ22-MKK7, Δ10-MKK7 or empty vector. pEBG C-BicD was immunoprecipitated from cells using glutathione Sepharose (Pharmacia) and evaluated by Western blot analysis with anti-myc antibody (9E10). Expression levels of MKK7 and the N-terminal deletion mutants was determined by Western blotting with anti-myc antibody (9E10). Expression of pEBG C-BicD was determined by Western blotting with anti-GST antibody.

N-terminus alone is sufficient for binding has not been performed.

Although the N-terminal 22 amino acids of MKK7 are important for associating with hBicD, removal of this region does not influence MKK7 activity (Fig 38). SAPK was immunoprecipitated from 293T cells expressing either wild-type MKK7, Δ10-MKK7, Δ22-MKK7, Δ75-MKK7 or Δ100-MKK7, and its activity was assayed using GST c-Jun as a substrate. Expression of either wild-type MKK7, Δ10-MKK7 or Δ22-MKK7 all increased the activity of SAPK when compared to expressing SAPK alone (Fig. 38). The Δ75-MKK7 or Δ100-MKK7 mutants were not able to activate SAPK, demonstrating that removal of 75 or 100 N-terminal residues in MKK7 interferes with SAPK activation. Davis and colleagues have recently reported that the N-terminal 73 amino acids of MKK7 are required for binding to SAPK (Tournier *et al.*, 1999), suggesting that binding of MKK7 to SAPK is important for full activation.

CO-EXPRESSION OF HBICD DOES NOT AFFECT MKK7 ACTIVITY:

We were interested in addressing whether the expression of hBicD affected the ability of MKK7 to be activated in cells. Although the region of MKK7 necessary for binding hBicD is not required for MKK7 catalytic activity, it is possible that the binding of hBicD to MKK7 may affect its conformational state, thereby influencing its ability to be activated. MT-MKK7 was co-expressed with either a catalytically active form of MEKK1 (C-MEKK) or MLK2, two known activators of MKK7, in the presence or absence of T7 hBicD. MKK7 was immunoprecipitated and assayed for activity in a kinase assay using bacterially-expressed, kinase-inactive, KR GST SAPK as a substrate. MKK7 was equally able to phosphorylate KR GST SAPK either in the presence or absence of T7 hBicD (Fig. 39). This suggests that the ability of MKK7 to activate SAPK is independent of hBicD binding, as co-expression of hBicD does not affect its ability to be activated.

CO-EXPRESSION OF AN MKK7 ACTIVATOR INFLUENCES MKK7-HBICD BINDING:

We demonstrated that the presence of T7 hBicD had no influence on the ability of MKK7 to activate SAPK in response to activators such as MLK2 or MEKK1. However, when we analyzed the ability of MKK7 to bind to T7 hBicD in the presence or absence of these activators we noticed a dramatic increase in the amount of MKK7-hBicD

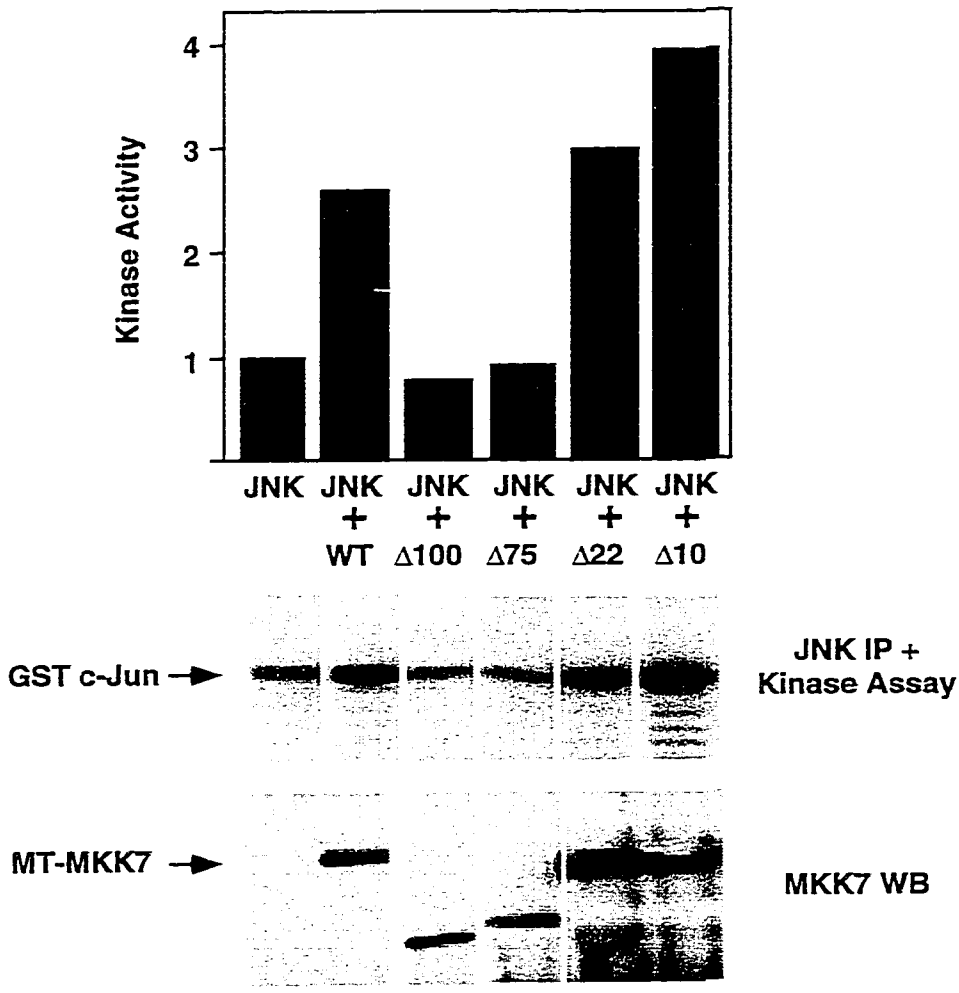


Fig. 38. Effects of MKK7 N-terminal deletions on its activity. 293T cells were co-transfected with pCS3MT JNK1 and either wild type pCS3MT MKK7, Δ100-MKK7, Δ75-MKK7, Δ22-MKK7, Δ10-MKK7 or empty vector. JNK1 was immunoprecipitated with anti-JNK1 antibody (Santa Cruz) and assayed for activity using GST c-Jun as a substrate. Substrate phosphorylation is expressed as fold increase with respect to JNK1 transfected cells alone (control). Expression levels of MKK7 and the N-terminal deletion mutants was determined by Western blotting with anti-myc antibody (9E10).

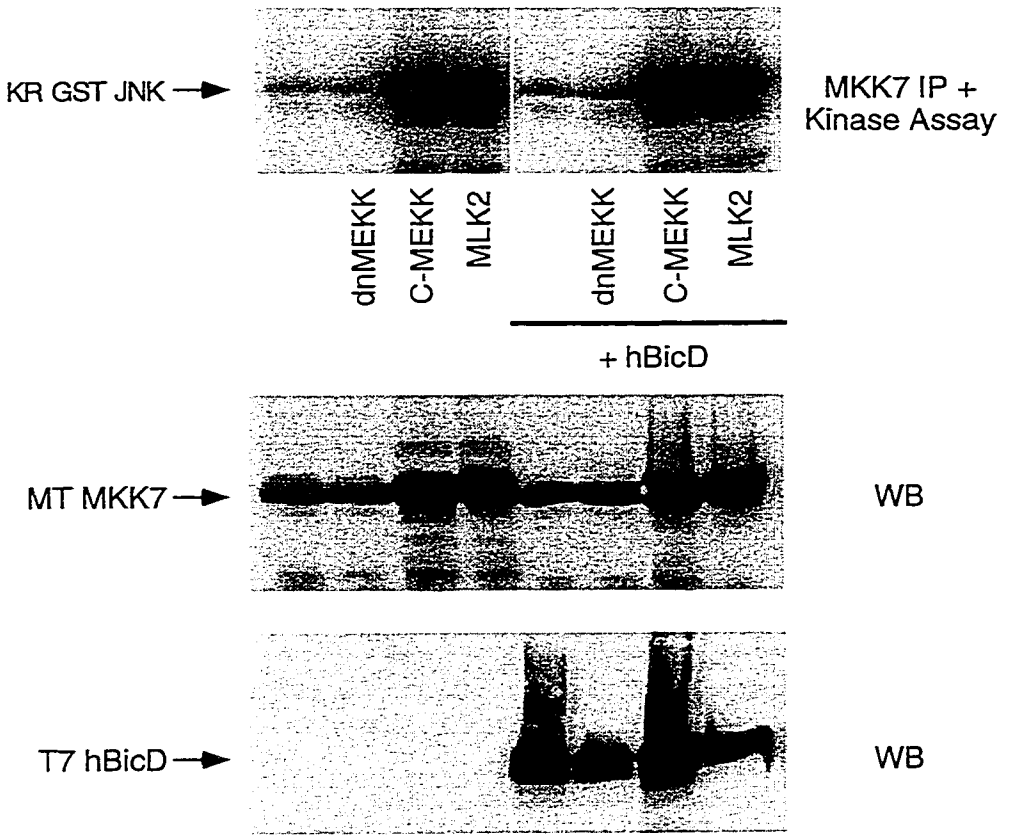


Fig. 39. Over-expression of hBicD does not affect MKK7 activity. 293T cells were co-transfected with wild-type pCS3MT MKK7 and either pRK5 MLK2, pCMV C-MEKK, pCMV dn-MEKK or empty vector, in the presence or absence of pCGT T7 hBicD. MKK7 was immunoprecipitated with anti-myc antibody (9E10) and assayed for activity using KR GST JNK as a substrate. Expression of MKK7 and hBicD in total lysates was monitored by Western blotting with anti-myc (9E10) and anti-T7 antibody, respectively.

binding when either MLK2 or MEKK1 was co-expressed (Figs. 40, 41). Because MEKK1 and MLK2 can bind to and activate MKK7 directly, this immediately suggested that the phosphorylation state of MKK7 or hBicD, or perhaps both, could influence the ability of these proteins to interact. More importantly, it implied that the association between MKK7 and hBicD might be regulated by phosphorylation. The co-immunoprecipitation studies, which originally demonstrated that BicD could bind MKK7 but not other MKKs, were performed in the absence of an activator such as MLK2 or MKK7. To confirm that the interaction between hBicD and MKK7 was still specific in the presence of MLK2, MKK4 was re-examined for its ability to bind hBicD in a co-immunoprecipitation assay in the presence of MLK2. Figure 42 shows that in the presence of MLK2, no detectable MKK4 was observed binding to hBicD, whereas MKK7 binds hBicD strongly in the presence of MLK2, and comparatively less in the absence of MLK2. As discussed below, not all MKK7/SAPK activators increase the association between MKK7 and hBicD (Fig. 47).

ACTIVATION STATE OF MKK7 DOES NOT INFLUENCE MKK7-HBICD BINDING:

We subsequently addressed whether the binding of a phosphorylation-site mutant of MKK7 (MKK7 S3A) or a kinase-inactive mutant (MKK7 K149M) could be affected by the presence or absence of MLK2. T7 hBicD was co-expressed with either wild-type MKK7, MKK7 S3A or MKK7 K149M in the presence or absence of MLK2 in 293T cells. Immunoprecipitated T7 hBicD was analyzed by Western blotting for the presence of MKK7 (Fig. 43). We observed no real differences in the ability of wild-type MKK7 or the S3A or K149M mutants to bind hBicD as long as MLK2 was present. Comparatively little binding was seen in all cases in the absence of MLK2. Since altering the activation state of MKK7 had no effect on its ability to bind hBicD, this result suggested that the phosphorylation state of hBicD might be what was important for regulating this interaction.

HBICD IS PHOSPHORYLATED:

Studies from *Drosophila* have indicated that BicD exists in multiple phosphorylation states, and that phosphorylation is required for the proper localization of BicD during oogenesis (Suter and Steward, 1991). A closer examination of Western blots of hBicD showed that T7 hBicD could undergo a mobility shift (Fig. 44). T7 hBicD runs

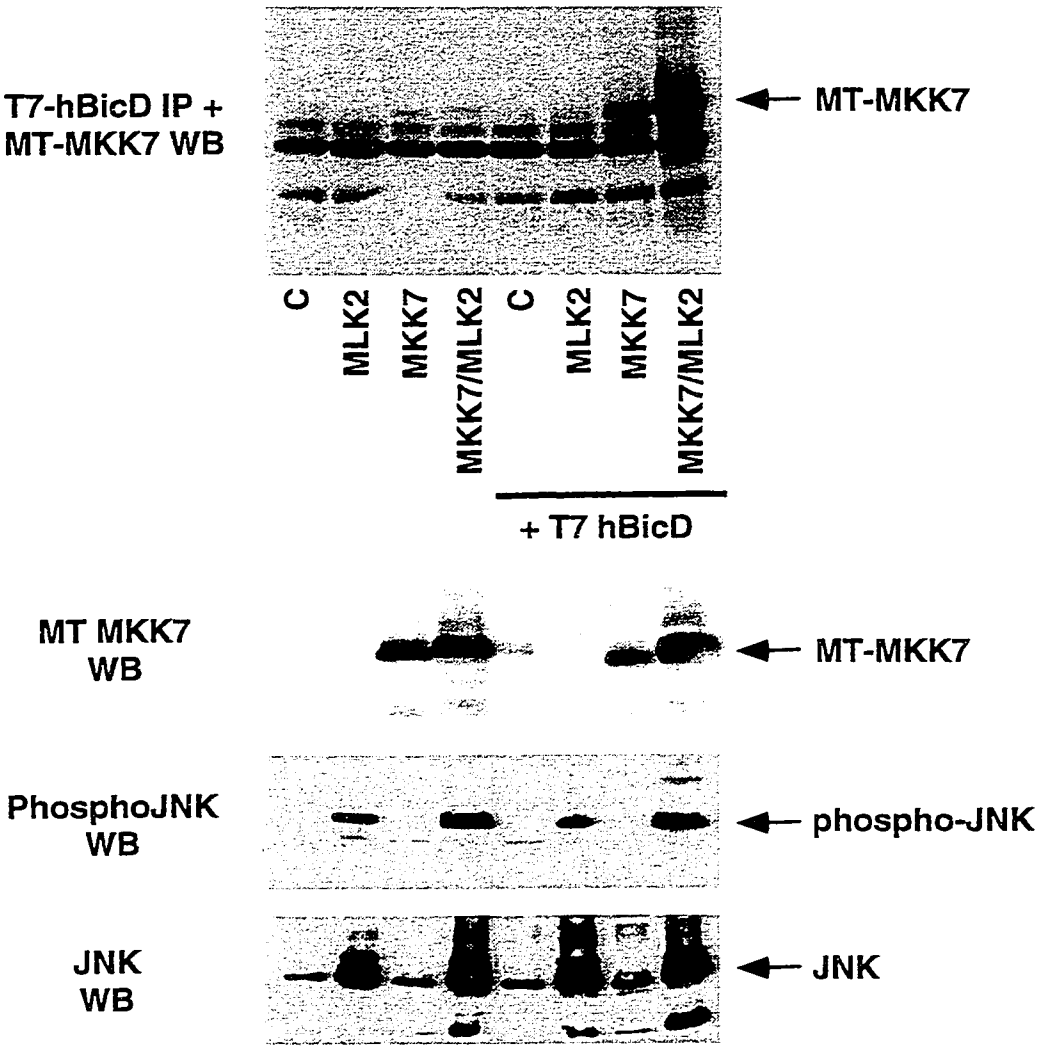


Fig. 40. Co-expression of MLK2 increases MKK7-hBicD binding. 293T cells were co-transfected with either pCS3MT MKK7, pRK5 MLK2 or both together, in the presence or absence of pCGT T7 hBicD. hBicD was immunoprecipitated with anti-T7 antibody and analyzed for the presence of MKK7 by Western blotting with anti-myc (9E10) antibody. Expression of MKK7 and JNK in total lysates was monitored by Western blotting with anti-myc (9E10) and anti-JNK1 antibody, respectively. Activation of MKK7 was analyzed by Western blotting for phospho JNK1 using a phospho-specific JNK1 antibody (NEB).

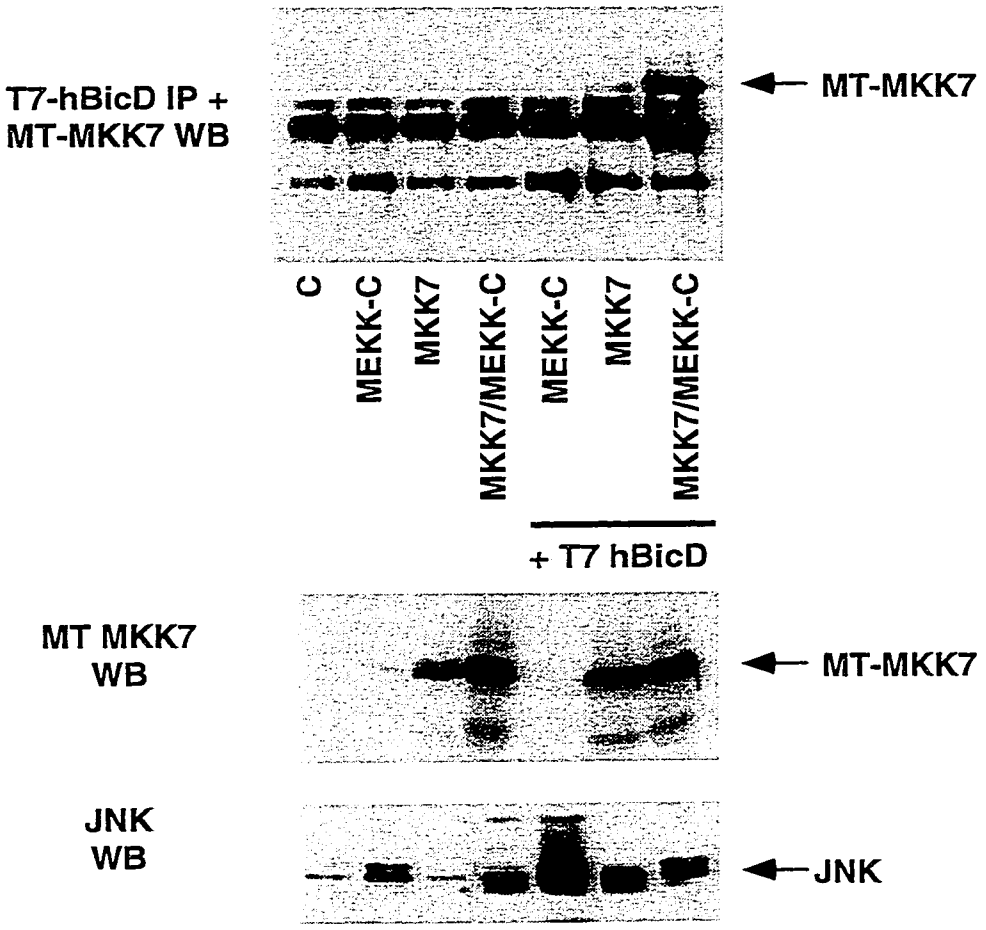


Fig. 41. Co-expression of MEKK1 increases MKK7 hBicD binding. 293T cells were co-transfected with either pCS3MT MKK7, pCMV HA MEKK1 or both together, in the presence or absence of pCGT T7 hBicD. hBicD was immunoprecipitated with anti-T7 antibody and analyzed for the presence of MKK7 by Western blotting with anti-myc (9E10) antibody. Expression of MKK7 and JNK in total lysates was monitored by Western blotting with anti-myc (9E10) and anti-JNK1 antibody, respectively.

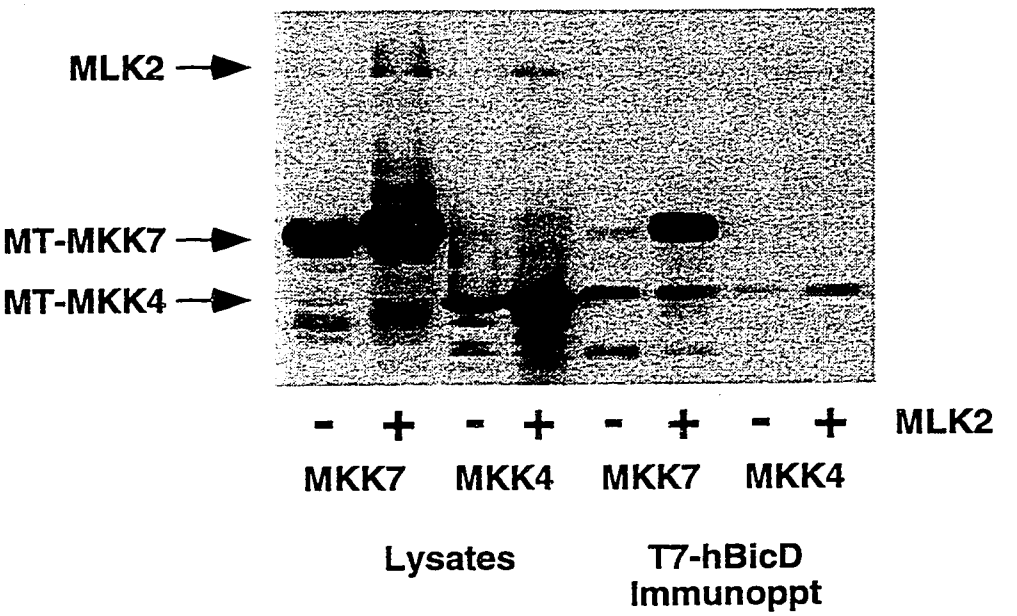


Fig. 42. MKK4 does not bind hBicD in the presence of MLK2. 293T cells were co-transfected with pCGT T7 hBicD and either pCS3MT MKK7 or pCS3MT MKK4 in the presence or absence of pRK5 MLK2. hBicD was immunoprecipitated with anti-T7 antibody and analyzed for the presence of MKK7 or MKK4 by Western blotting with anti-myc antibody (9E10). Expression of MKK7 and MKK4 in total lysates was monitored by Western blotting with anti-myc (9E10).

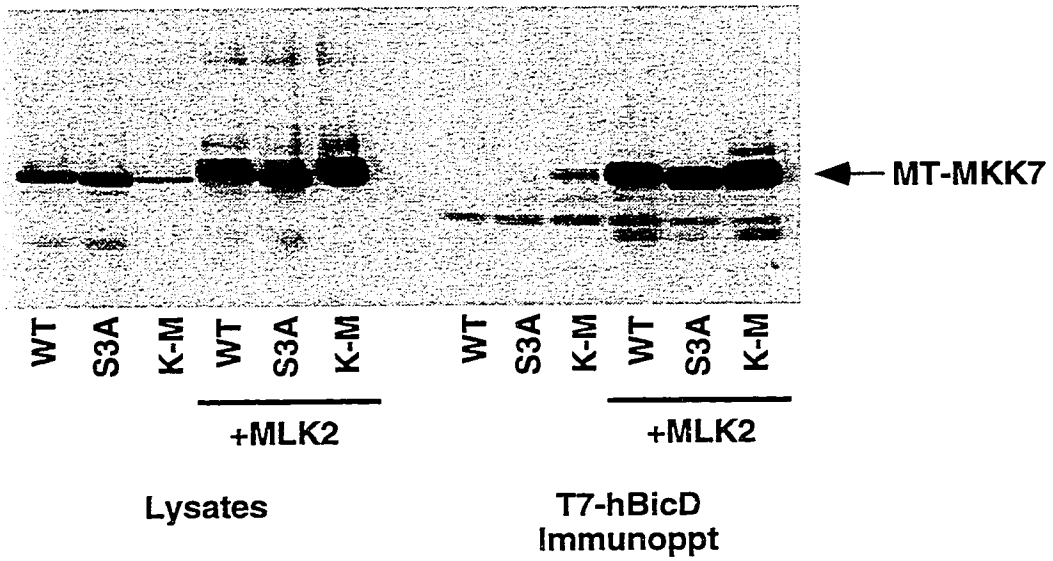


Fig. 43. Activation mutants of MKK7 can bind to hBicD. 293T cells were co-transfected with pCGT T7 hBicD and either pCS3MT MKK7 (wild-type), MKK7 (S3A), or MKK7 (K149M) in the presence of absence of pRK5 MLK2. hBicD was immunoprecipitated with anti-T7 antibody and analyzed for the presence of wild-type or mutant MKK7 by Western blotting with anti-myc antibody (9E10). Expression of MKK7 in total lysates was monitored by Western blotting with anti-myc (9E10).

as three different species. When MLK2 or MEKK1 are co-expressed the relative abundance of the slowest migrating form of hBicD is significantly increased. This has been demonstrated to be indicative of a change in phosphorylation state, for example with ERK MAP kinases (Posada and Cooper, 1992). This altered mobility is not observed when only MKK7 is co-expressed with hBicD.

We addressed whether the altered mobility of hBicD observed under conditions in which MLK2 or MEKK1 was co-expressed was due to a change in phosphorylation state. T7 hBicD was immunoprecipitated from 293T cells, which were also expressing MLK2 or vector alone, and subjected to phosphatase assays. Immunoprecipitated T7hBicD was incubated with either alkaline phosphatase, λ-phosphatase 1, or left untreated, and then analyzed by Western blotting. Both alkaline phosphatase and λ-phosphatase 1 are capable of dephosphorylating serine, threonine and tyrosine residues. Figure 45 shows that the majority of T7 hBicD immunoprecipitated from cells over-expressing MLK2 was in the slower migrating band (uppermost band). Treatment of those immunoprecipitates with either alkaline phosphatase or λ-phosphatase 1 resulted in a collapse of the slower migrating band into the faster migrating band (lower band). This result demonstrated that the altered mobility shift in hBicD observed when MLK2 was co-expressed was the result of phosphorylation. Treatment of phosphorylated hBicd with either alkaline phosphatase or λ-phosphatase 1 restores hBicD to its predominantly de-phosphorylated state. None of these experiments address the question of what kinase is phosphorylating hBicD.

We were interested to determine whether the phosphorylation of hBicD was a result of SAPK activation, either directly or indirectly. To address this, we co-expressed hBicD with the MAP kinase phosphatase MKP-1 or a catalytically inactive mutant, MKP1 C-S, in the presence or absence of MLK2. MKP-1 has been shown to effectively dephosphorylate and inactivate SAPK (Hirsch and Stork, 1997). If SAPK was capable of directly phosphorylating hBicD, then co-expression of the wild-type phosphatase but not the mutant should block MLK2 induced hBicD phosphorylation. Figure 46 demonstrates that in Western blots of hBicD expressing lysates from 293T cells, co-expression of MKP1 was not able to block hBicD phosphorylation. This result demonstrated that SAPK was unlikely to be the kinase which phosphorylates hBicD.

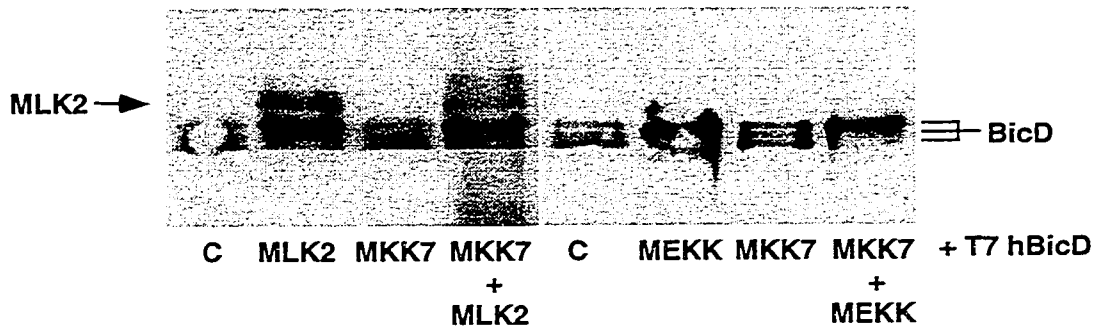


Fig. 44. hBicD undergoes a mobility shift in the presence of MLK2 or MEKK1. 293T cells were co-transfected with pCGT T7 hBicD and either pRK5 MLK2, pCMV HA MEKK1, or pCS3MT MKK7 in the presence or absence of MLK2 or MEKK1. Total lysates were analyzed by Western blotting with anti-T7 antibody to evaluate the pattern of T7 hBicD mobility.

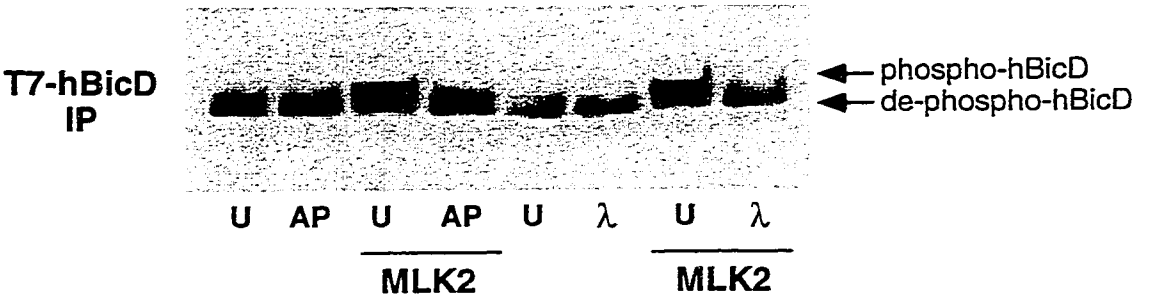


Fig. 45. hBicD can be de-phosphorylated by phosphatases. 293T cells were co-transfected with pCGT T7 hBicD in the presence or absence of pRK5 MLK2. hBicD was immunoprecipitated with anti-T7 antibody and phosphatase assays were conducted. hBicD immunoprecipitates were either untreated (U), treated with 10 U alkaline phosphatase (AP) for 1 hour at 37 °C, or treated with 400 U λ-PPase (λ) for 1 hour at 30 °C, and analyzed by Western blotting with anti-T7 antibody.

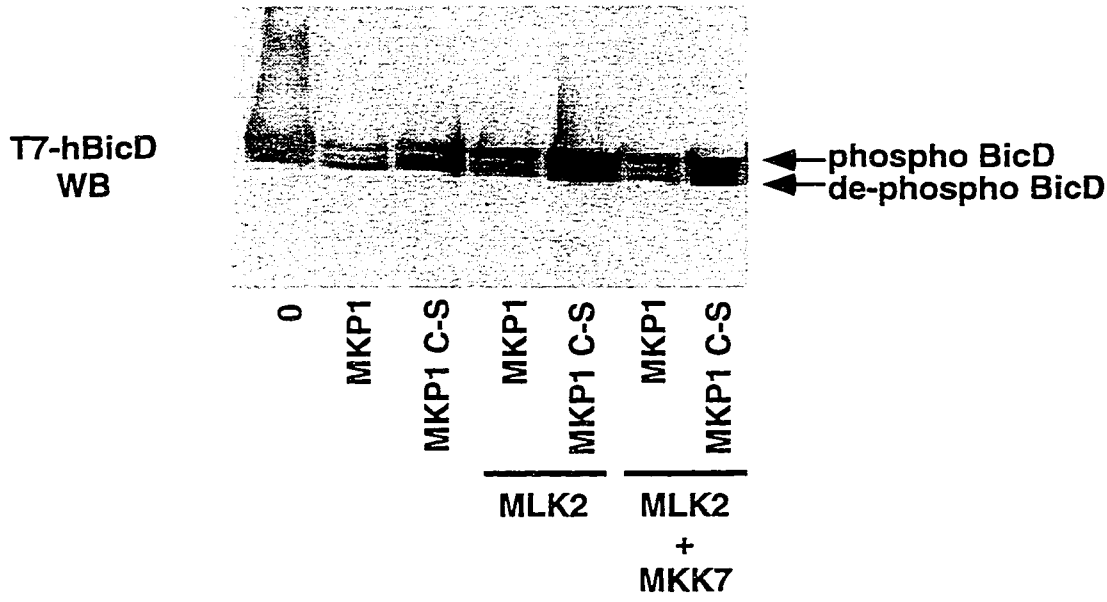


Fig. 46. Co-expression of MKP1 phosphatase does not affect hBicD mobility. 293T cells were co-transfected with pCGT T7 hBicD and either pCEP4 MKP1 or pCEP4 MKP1 C-S in the presence or absence of pRK5 MLK2 or pRK5 MLK2 + pCS3MT MKK7. Total lysates were analyzed by Western blotting with anti-T7 antibody to evaluate the pattern of T7 hBicD mobility.

HBICD PHOSPHORYLATION IS INDUCED BY A SUBSET OF SAPK ACTIVATORS:

We also investigated whether external stimuli or co-expression of SAPK activating molecules other than MEKK1 or MLK2 could also induce hBicD phosphorylation. Cells over-expressing T7 hBicD were treated with anisomycin, NaCl or two doses of nocodazole. Nocodazole is a microtubule-disrupting agent which has been demonstrated to activate SAPK (Wang *et al.*, 1998). Although ansiomycin and NaCl induced activation of SAPK, as judged by anti-phospho SAPK Western blotting, they did not induce hBicD association or phosphorylation (Fig. 47). On the other hand, 10 μM nocodazole was a poor activator of SAPK but induced detectable MKK7-hBicD association. Similar to results observed with co-expression of MLK2 or MEKK1, co-expression of ASK1 with hBicD also increased the MKK7-hBicD association. These data indicated that hBicD phosphorylation might be catalyzed by a SAPK/MKK7 independent pathway, for example via p38/MKK3/MKK6, and thereby lead to the association of hBicD with MKK7.

DISCUSSION:

In a two-hybrid screen to identify novel MKK7 interating proteins we identified a protein fragment homologous to the *Drosophila* BicaudalD gene. The *Drosophila* BicaudalD (BicD) gene encodes a 782 amino acid protein with a predicted mass of 89 kDa and similarity to the rod region of myosin heavy chain, kinesin and other intermediate filament proteins (McLachlan and Karn, 1983; Parry *et al.*, 1977; Steinert and Roop, 1988). The common attribute of these proteins is an extended coiled-coil structure built with a characteristic heptad repeat pattern. The prominent feature of this repeat is a strong bias toward hydrophobic residues in positions 1 and 4 of every 7 amino acids that form the core of an alpha-helical coiled-coil. Within the second heptad repeat of BicD a leucine zipper motif has also been identified. These motifs are proposed to support dimerization of closely related proteins (Buckland and Wild, 1989).

Genetic studies in *Drosophila* have established that BicD is involved in

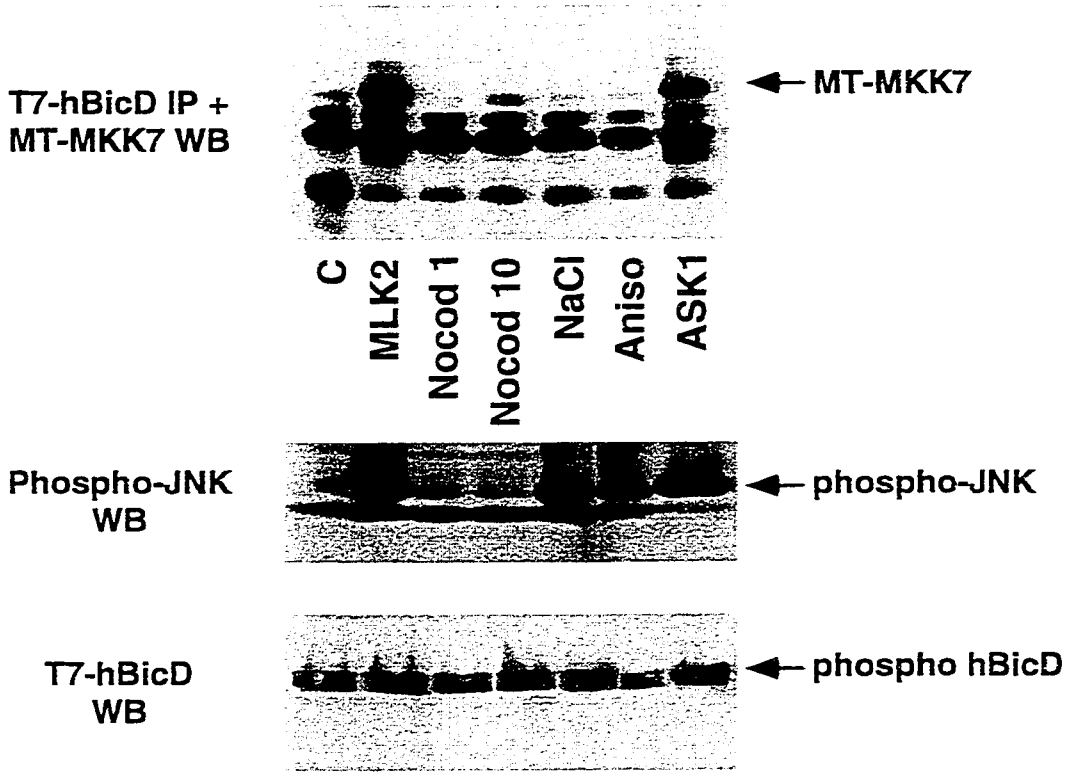


Fig. 47. MKK7-hBicD binding is influenced by a subset of SAPK activators. 293T cells were co-transfected with pCGT T7 hBicD, pCS3MT MKK7, pCS2 JNK1, and either pRK5 MLK2, pcDNA ASK1, or empty vector. Cells without MLK2 or ASK1 were treated with either 1 μM nocodazole, 10 μM nocodazole, NaCl, or anisomycin. hBicD was immunoprecipitated with anti-T7 antibody and analyzed for the presence of wild-type or mutant MKK7 by Western blotting with anti-myc antibody (9E10). Activation of SAPK/JNK was analyzed by Western blotting for phospho JNK1 using a phospho-specific JNK1 antibody (NEB). To evaluate the pattern of T7 hBicD mobility, total lysates were analyzed by Western blotting with anti-T7 antibody.

several different processes during oogenesis and embryonic development. Oogenesis in *Drosophila* begins in the germarium , where division of a founder cystoblast gives rise to a cluster of 16 sister germ cells. The 16-cell cyst becomes surrounded by somatic follicle cells as it descends the germarium. Together, the germ cells and the follicle cells build a sphere shaped egg chamber in which the oocyte is always found at the posterior end in contact with the posterior-most follicle cells. Cytokinesis is not completed during the four divisions that generate these cells, which are consequently connected by cytoplasmic bridges that persist throughout development of the oocyte. A microtubule organizing center (MTOC) appears in one of these cells and extends microtubules through the ring canals into the other 15 interconnected germarial cells. Accumulation of specific mRNAs and proteins in the MTOC-containing cell result in its oocyte specification. Increased actin accumulation is also observed in the ring canals most proximal to the pro-oocyte. Recessive mutations at the *(BicD)* locus disrupt the formation and maintenance of the microtubule cytoskeleton and block oocyte differentiation (Theurkauf *et al.*, 1993). None of the 16 nurse cells develops into a mature oocyte. In addition, flies fed the microtubule depolymerizing agent colchicine exhibit a similar phenotype to that observed in a *BicD* loss of function mutant (Koch and Spitzer, 1983). In *BicD R26* or *BicD PA66* mutants, microtubules appear disperse throughout the germline cells. While BicD protein is expressed in both mutants at the same stages as in the wild-type, its distribution is altered. The R26 protein is expressed at higher levels than wild-type in the pro-oocyte, whereas the PA66 protein does not accumulate at all in the pro-oocyte or oocyte as wild-type protein does (Suter and Steward, 1991). The recessive, loss-of-function phenotype of *R26* is caused by an in-frame deletion of 12 nucelotides generating a mutant protein that lacks three amino acids within the third heptad repeat. It is possible that this could disrupt specific protein-protein interactions. In the *PA66* allele, an Ala to Val substitution occurs within a Ser/Ala rich sequence in the first heptad repeat. This mutation has been demonstrated to interfere with phosphorylation of the BicD protein, which is required for its accumulation in the pro-oocyte (Suter and Steward, 1991). A *PA66* suppressor mutation restores phosphorylation and allows for normal differentiation of oocytes.

Dominant *BicD* mutations have demonstrated a role for BicD in the establishment of anterior and posterior polarity in the early embryo (Wharton and Struhl, 1989). Mothers with either of two dominant *BicD* mutations, $BicD^{1}$ or $BicD^{2}$, produce bicaudal embryos in which the head, thoracic and anterior abdominal segments are replaced

by a symmetrical set of the remaining abdominal segments and terminalia. Cytoplasmic transplantation experiments indicate that the morphogen, Nanos, is present at both poles of embryos from *BicD* mutant mothers, while it is only at the posterior pole in embryos from wild-type mothers (Lehmann and Nusslein, 1986). Nanos is thought to be an RNAse that controls the expression of maternal *hunchback* (*hb*). The absence of hb protein is necessary and sufficient for the expression of a second segmentation gene, *knirps* (*kni*), which is required for abdominal segmentation. The mislocalization of *nanos* RNA as a result of the dominant BicD mutation leads to an expansion of the region in which *knirps* is expressed, resulting in a duplication of abdominal structures. It is proposed that BicD protein acts as a scaffold to which *nanos* RNA is selectively attached, presumably by the activities of other factors that are sequestered at the posterior pole. The proteins encoded by $BicD^{1}$ or $BicD^{2}$ bear single amino acid substitutions of isoleucine for phenylalanine in the fifth heptad repeat, and lysine for glutamic acid in the second heptad repeat, respectively. The residue corresponding to the $BicD^{1}$ mutation is conserved in both human BicD homologs, which suggests a possible site for mutagenesis in the generation of hBicD mutants. Thus, studies from dominant and recessive BicD mutations suggest that BicD may function to properly localize developmental factors and that the phosphorylation state of BicD may also be important for its function. In addition, BicD may be associated with the cytoskeleton.

It is possible that in *Drosophila* there is no link between Hep, the MKK7 homolog, and BicD. Hep is thought not to be required for embryonic patterning, since *hep* embryos develop normally until late gastrulation, when dorsal closure takes place (Glise *et al.*, 1995). Similarly, expression of hBicD protein is not detectable during late gastrulation (Wharton and Struhl, 1989). However, other reports have indicated that the expression of two different BicD transcripts persists during embryogenesis (Suter *et al.*, 1989). A 4.4 kb transcript, which is initially maternally supplied, degraded, and reappears around 8 hr after egg laying, is detected in all stages of the *Drosophila* life cycle examined, including adult males. A 5.7 kb transcript is found in late embryos, pupae and adult males. Whether these transcripts make functional protein has not been determined. Interestingly, the STE20-related protein Misshapen (*msn*), which functions in the dorsal closure pathway upstream of Hep, is essential for oogenesis (Treisman *et al.*, 1997). The enhancer traps inserted into *msn* are expressed predominantly in the border cells of the ovarian follicle, which migrate from the anterior tip of the egg chamber through the nurse cells to the oocyte. Germline

clones mutant for *msn* either fail to lay eggs or lay eggs that fail to develop. It is possible that Misshapen protein may function in part of a signaling pathway in *Drosophila* oocytes.

The fragment of BicD identified in our screen corresponds to the fifth heptad repeat of human BicD (hBicD). In this region our clone has 95% amino acid identity to hBicD, and 100% amino acid identity to a recently identified second BicD homolog, KIAA0699 (Ishikawa *et al.*, 1998) (Fig. 48). Both human homologs encode a roughly 840 amino acid protein with a predicted coiled-coil structure, and appear to be ubiquitously expressed. Northern blot analysis with a partial mouse cDNA homolog of hBicD indicates that it is also expressed during mouse embryonic development. The two human homologs map to different chromosomes (hBicD on chromosome 12, KIAA0699 on chromosome 9), and thus are independent genes.

Our studies suggest that the binding of MKK7 to hBicD is specific, since MKK3, MKK4 and MKK6 were unable to bind either the full-length hBicD or the fragment corresponding to the fifth heptad repeat. We propose that the other heptad repeats in hBicD are involved in mediating other protein-protein interactions. Even under conditions in which hBicD is phosphorylated and the binding of MKK7 is enhanced, MKK4 did not bind to hBicD. The region in MKK7 important in binding to hBicD is contained within the N-terminus. As previously mentioned, this region contains a sequence resembling a nuclear export sequence (NES). This sequence in MKK7 has not been demonstrated to function as a nuclear export signal, but may be relevant in its ability to bind hBidD. An analysis of the N-terminal sequence of other MKK's indicates that only MKK1 and MKK2 (and Hep) also contain a leucine rich sequence. It is possible that another feature within the N-terminus is important, since we have not directly mutated the leucines in MKK7 and assayed for hBicD binding. It will be of interest to determine whether MKK1 binds hBicD, as this has not been examined.

The overexpression of a subset of SAPK activators with hBicD and MKK7 greatly enhanced the hBicD-MKK7 association. This increased association corresponded to an increase in the amount of phosphorylated hBicD. We do not believe the phosphorylation state of MKK7 regulates its association with hBicD, because catalytically inactive mutants or mutants that could no longer be phosphorylated bound equally well to hBicD. Thus it is possible that the kinase that phosphorylates hBicD lies upstream of

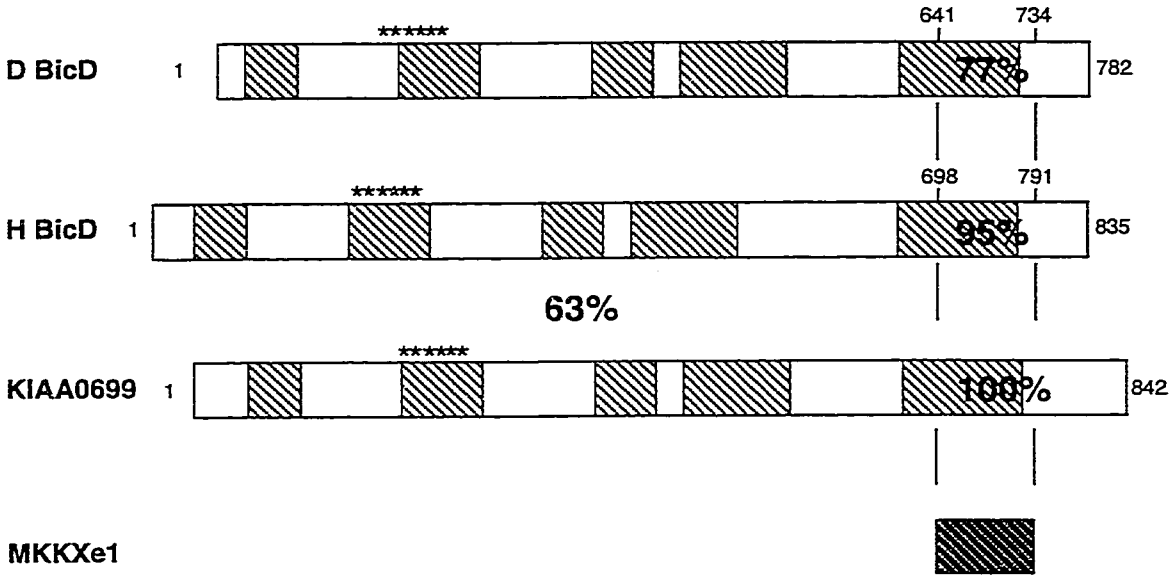


Fig. 48. Alignment of *Drosophila* and human BicD homologs. The clone identified in the two hybrid screen, MKKXe1, corresponds to the fifth heptad repeat. Hatched boxes indicate regions of heptad repeats. Stars denote leucine zipper motifs. MKKXe1 has 77%, 95%, and 100% a.a. identity to the regions of D BicD, H BicD and KIAA0699, respectively. Overall, H BicD and KIAA0699 are 63% identical to each other, and 41% and 39% identical to D BicD, respectively.

MKK7. These data are also consistent with our studies in co-expressing the SAPK phosphatase MKP1. Over-expression of the wild-type phosphatase or a catalytically-inactive mutant with hBicD and MLK2 had no significant effects on MLK2's ability to mediate hBicD phosphorylation. It will be important to determine whether MLK2, ASK1 or MEKK1 are capable of phosphorylating hBicD directly, and whether they may bind to hBicD directly. One possibility is that the phosphorylation of hBicD by a kinase like MLK2, ASK1 or MEKK1 serves to recruit MKK7 to hBicD. This could lead to activation of MKK7 by the MKKK, followed by activation of SAPK. The observation that hBicD phosphorylation induces MKK7 binding indicates that the interaction between the two proteins is regulated. This is an important characteristic of a scaffolding protein that functions to contribute to the regulation of signaling pathways.

In *S. cerevisiae*, genetic analyses have recently demonstrated that a coiled-coil protein, Spa2, interacts with components of two MAP kinase pathways, and may be involved in polarized morphogenesis (Buehrer and Errede, 1997; Sheu *et al.*, 1998). Activation of the mating pathway by pheromone stimulates the formation of projections oriented toward the gradient of mating pheromone secreted by a mating partner. Proper projection formation requires cell wall synthesis, which is controlled by the cell integrity pathway. Polarized growth during budding, pseudohyphal growth or mating involves the polarized organization of the actin cytoskeleton, the coordinated function of many polarity proteins, and the regulation of signal-transduction cascades. Activation of the mating pathway leads to the transcription of STE12-dependent genes, which results in the activation of the cell integrity pathway (Fig. 2). It has been proposed that the two pathways may be linked by a series of cytoskeletal binding proteins. The actin cytoskeleton appears as distinct structures during polarized cell growth, and many components that influence cell polarity localize to sites of polarized growth. The proteins Spa2p, Bud6p, Pea2p and Bni1p, localize to tips of buds and mating projections. Spa2p is a large coiled-coil protein (150 kDa) that interacts with other proteins to form a large multi-protein complex. Bud6p and Pea2p are smaller coiled-coil proteins; Bud6p interacts with actin as well as Spa2p. Bni1 is a member of the formin family of proteins that participate in cell polarization, cytokinesis, and vertebrate limb formation. Bni1 forms complexes with Cdc42p, actin, profilin and Bud6p (Evangelista *et al.*, 1997). Spa2p and Bud6p also interact directly with components of the mating and cell integrity pathways. Spa2p interacts strongly with MKK1/MKK2 and STE7 (*S. cerevisiae* MAP kinase kinases), and weak

interactions have been detected for both Sap2p and Bud6p with STE11. The precise mechansism that coordinates these two MAP kinase pathways is unclear. However, genetic analyses suggest that Spa2p plays an important role in mediating these events, and may function to properly localize signaling components to the actin cytoskeleton during polarized cell growth. This provides an interesting example of how a coiled-coil protein like hBicD may function to localize signaling-proteins in mammalian cells.

We proposed that the phosphorylation of hBicD, which is induced by kinases such as MLK2, ASK1 and MEKK1, regulates its interaction with MKK7. All of the stimuli which lead to hBicD phosphorylation may link signaling events to the cytoskeleton. For example, microinjection studies in Swiss 3T3 cells have shown that MLK2 and activated SAPK co-localize to punctate structures along microtubules (Nagata *et al.*, 1998). The protein KIF3X, a new member of the kinesin family of superproteins, was identified in a yeast two-hybrid screen with MLK2, and also shown to localize to punctate structures along microtubules. This raises the possibility that microtubule motors and the SAPK cascade may be coordinately controlled. MLK2 has also been shown to be an inducer of apoptosis (Nagata *et al.*, 1998). Microinjection of MLK2 into Swiss 3T3 cells results in cytoplasmic shrinkage and nuclear condensation, characterisitcs of cells undergoing apoptosis. This can be blocked by treatment of cells with the protease inhibtor ZVAD-fmk just after microinjection.

The MAP kinase kinase kinase, ASK1, which becomes activated in cells treated with TNF and is implicated in stress and cytokine induced apoptosis, has also been shown to be activated by treatment of cells with microtubule interfering agents (MIA's) (Wang *et al.*, 1998). MIA's interfere with the dynamic process of microtubule assembly. These effects include an arrest of cells at the G2/M phase of the cell cycle and initiation of apoptosis (Jordan and Wilson, 1998; Wahl *et al.*, 1996). It has been proposed that cell cycle arrest may not be sufficient to induce apoptosis and that additional phosphoregulatory pathways are required. Treatment of a variety of cell types with taxol, vinblastine, vincristine, nocodazole and colchicine (all MIA's), induced ASK1-mediated SAPK activation. This could be blocked by expression of a dominant-inhibitory ASK1 mutant (Wang *et al.*, 1998). The mechanism by which microtubule disruption leads to ASK1 activation has not been established. In addition, the subcellular localization of ASK1 under different conditions has not been studied.

Recent studies of MEKK1-targeted gene disruptions in mice indicate that MEKK1-mediated SAPK activitiy may also be stimulated by the MIA, nocodazole (Yujiri *et al.*, 1998). The loss of MEKK1 expression in embryonic stem cells results in an increased apoptotic response of cells to microtubule disruption, cold shock and mild osmotic shock. Notably, in the absence of MEKK1, cells treated with nocodazole can no longer activate SAPK. All of these stresses involve a change in cell shape, and it has been suggested that MEKK1 activates SAPK under conditions in which cells are undergoing shape changes. A role for MEKK1 in apoptosis was first identified in cells undergoing anoikis (Cardone *et al.*, 1997). Anoikis refers to the loss of integrin-mediated contacts with extracellular matrix in epithelial, endothelial and muscle cells. The loss of cell-matrix contact can induce caspases that cleave MEKK1 into a catalytically active form and thereby promote apoptosis. Likewise, this process can influence changes in cell shape. Full-length, inactive MEKK1 has been shown to localize to coarse cytoplasmic particles, which have not been identified (Deak *et al.*, 1998). Immunostaining of the cleaved active form of MEKK1 shows a fine reticular pattern. This is reminiscent to our preliminary observations of the localization of hBicD. Unphosphorylated hBicD appears to localize to discrete circular structures which may represent large protein complexes of hBicD. Phosphorylated hBicD appears to be more dispersed through the cell and may be organized radially within cells (data not shown). More studies are needed to address the precise localization patterns of phosphorylated and un-phosphorylated hBicD in cells.

The mechanisms of how ASK1 or MEKK1 receive signals from microtubule disruptions or cell shape changes is not understood. Although MLK2 localizes to microtubules, an increase in MLK2 activity in response to MIAs has not yet been demonstrated. Nevertheless, these observations suggest that coordination between cytoskeletal changes and signaling pathways may be linked. At this time it is not clear where the signal originates. In other words, the flow of information may go from cytoskeletal components to signaling proteins, or vice versa. One possibility is that one or more components within a target signaling pathway may bind, either directly or via an associated protein, to a cytoskeletal component (Fig. 49). This would place some of the relevant kinases in proximity to their signal. A near proximity would allow for rapid responses to changes in cytoskeletal dynamics, such as loss of microtubule or actin filament integrity, or a requirement for cell migration. Only MLK2 has been demonstrated

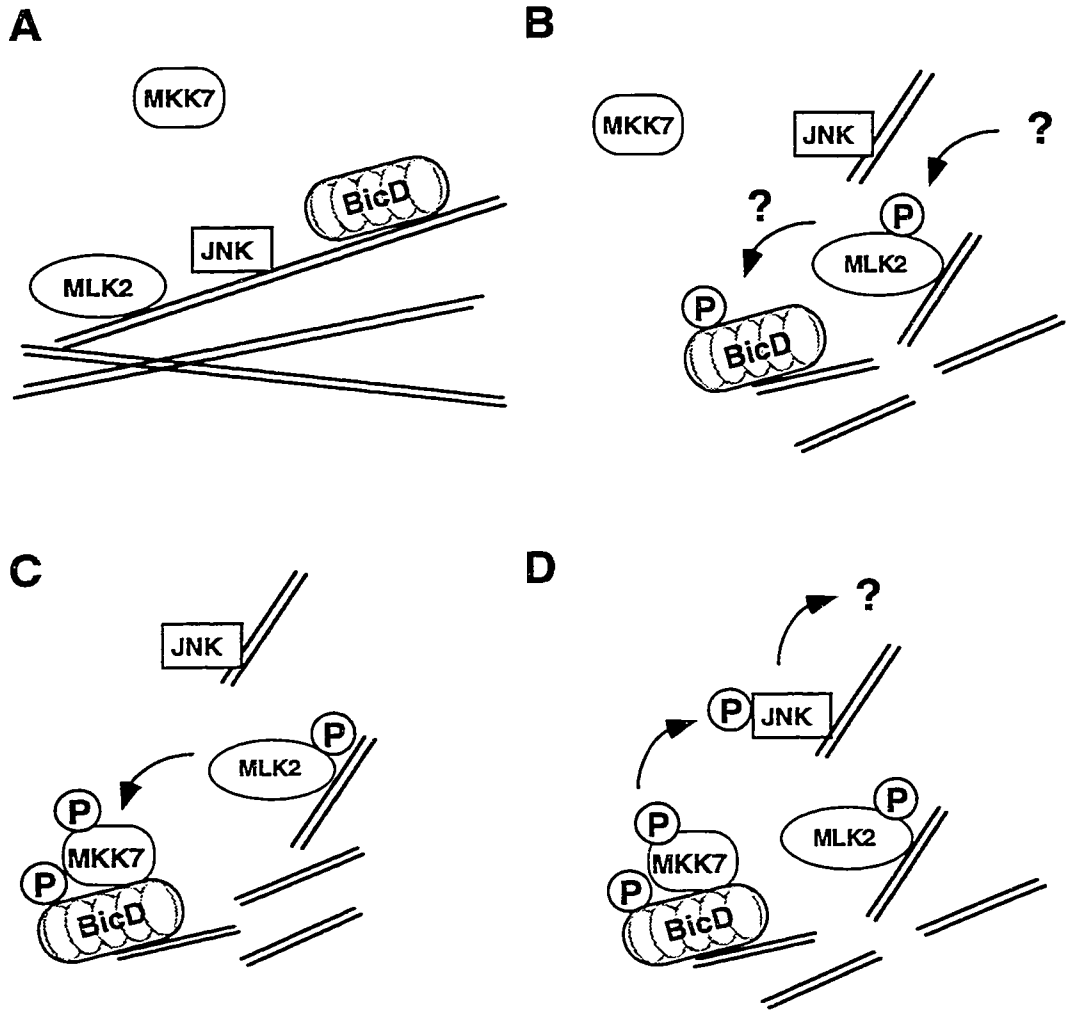


Fig. 49. A model for MKK7 binding to hBicD. (A) hBicD is localized to a cytoskeletal component such as microtubules. MLK2 and JNK may also localize there. MKK7 is distributed in the cytoplasm. (B) In response to a stressful stimulus (depolymerization of microtubules?), MLK2 becomes activated and induces the phosphorylation of hBicD. (C) Phosphorylated hBicD creates a binding site for MKK7, which brings MKK7 in proximity to MLK2 and allows MKK7 to be activated. (D) Activated MKK7 can now activate JNK, which can then activate its substrate and lead to signal transduction.

to localize to microtubules with activated JNK. The subcellular distributions for ASK1 have not been examined. This provides a model of how hBicD might function in mammalian cells.

In addition to being correlated with cytoskeletal changes, MLK2, ASK1 and MEKK1 are all inducers of apoptosis, and can activate MKK7. Our results indicate that expression of MEKK1, MLK2 or ASK1 in 293T cells can stimulate hBicD phosphorylation, enhance the association between hBicD and MKK7, and induce SAPK activity. We have observed that treating cells with nocodazole produces similar effects. Nocodazole is a microtubule disrupting agent that can also induce apoptosis. In contrast, treatment of cells with anisomycin results in SAPK activation, but no phosphorylation of hBicD or binding of hBicD to MKK7 is detected. This raises the possibility that the targeting of MKK7 to hBicD when hBicD is phosphorylated occurs only under specific circumstances. These could be any circumstances that directly affect cytoskeletal or microtubule integrity. One circumstance is in the leading edge of migrating cells; an extreme circumstance is during an apoptotic response. Apoptosis may be induced by microtubule targeted compounds, but whether activation of the SAPK pathway acts as an apoptotic sensor or contributes to the apoptotic phenotype is not known. More studies are needed to address the link between microtubule or actin filament disruption, apoptosis and the SAPK pathway.

MATERIALS AND METHODS:

TWO-HYBRID SCREEN WITH MKK7:

The two-hybrid screen method has been previously described (Ch. 1). To look for novel MKK7-interacting proteins, a two-hybrid screen was performed using wild-type MKK7 as bait. Two independent libraries were screened: a mouse embryonic day 9.5-10.5 cDNA library in the vector VP16 (previously used for MKK1 screen, described in Ch. 1) and a HeLa S3 cell cDNA library (Clontech), with an average insert size of 1.5 kb, cloned into the pGAD GH vector. Since full-length MKK7 has weak trans-activating activity, library transformants were plated on media containing 5 mM 3-aminotriazole.

GENERATION OF FULL-LENGTH HBICD:

A human HeLa cell cDNA library (Clontech) was used in a yeast two-hybrid library screen with wild-type MKK7 as bait. Ten clones (MKKXe1-MKKXe10) sequenced represented a 94 amino acid fragment with 75% identity to the *Drosophila* bicaudalD (BicD) protein. More recently, a human BicD homolog was sequenced (Baens and Marynen, 1997). We obtained six cDNA plasmids containing fragments of the open reading frame of human BicD. Using an overlap PCR strategy, we generated a full-length hBicD cDNA encoding a protein of 835 amino acids. The hBicD protein used in some of these studies contained mutations at the following positions: Q64R, K284R, I289M, I631V. These mutations were corrected using the Quik Change Site-Directed Mutagenesis Kit (Stratagene) to generate the wild-type protein. The plasmids obtained from P. Marynen contained the following regions of coding sequence in the vector pGEM3Z: 5rBICD4A: nucleotides 1-112; 5rBICD3A: nucleotides 76-112; 5rBICD3C: nucleotides 524-1055; 5rBICD2I: nucleotides 961-1755; 5rBICD1A: nucleotides 1608-2511; and 3rBICD4: nucleotides 2334-2541 spliced to 2845-3257. The following oligonucleotide primers were generated for overlap PCR from plasmids 5rBICD3A, 5rBICD2I, 5rBICD1A, and 3rBICD4 (Innis *et al.*, 1990).

BD01: 5'-GTGAAACTGAATGGAGACTATCGGACTCCC-3' position: 961-991

BD02: 5'-GGTGACTCTGCTCTGCCTATAGTAATCCAG-3' position: 1714-1684

BD03: 5'-CTGGATTACTATAGGCAGAGCAGAGTCACC-3' position: 1684-1714

BD04: 5'-GCCTTTGCTGCGTCGGGACTGCTCATGGTC-3' position: 2508-2479

BD05: 5'-GGGAGTCCGATAGTCTCCATTCAGTTTCAC-3' position: 991-961

BD09: 5'-GCTCTAGAGGGGCTATGGCCGCAGAAGAGGTATTG-3' 78-104

BD07: 5'-GACCATGAGCAGTCCCGACGCAGCAAAGGC-3' position: 2479-2508

BD08: 5'-CGGGATCCTCGTCCACAGGAGATGAAGACTAGGGGTG-3' 3030-3001

Individual regions were PCR-amplified using Pfu DNA polymerase (Stratagene). Three successive overlaps were generated from the following plasmids: 1.

5rBICD1A + 5rBICD2I 2. (product of 1) + 3rBICD4 3. (product of 2) + 5rBICD3A. The full-length PCR-amplified clone was subcloned into the pT7 Blue3 vector (Novagen) and tested by in vitro translation using the Promega TNT kit as described by the manufacturer. hBicD was subsequently cloned into the XbaI and BamHI sites of pCGT-T7 vector for mammalian expression and sequenced using an ABI373 sequencer. This vector contains an N-terminal T7 epitope tag corresponding to residues 1-11 of T7 gene 10 (MASMTGGQQMG). pCGT T7 hBicD was used for all mammalian expression studies.

OTHER CONSTRUCTS:

To make pEBG-MKKXe1 (C-terminal fragment of hBicD), the following oligonucleotides were used to PCR amplify pGAD GH MKKXe1. PCR product was subsequently digested with BamHI and subcloned into the BamHI site of pEBG 3X vector.

Sense: 5'-CGGGATCCGAGCCGAGGTGGCCCTTGCCAAC-3'

Antisense: 5'-CGGGATCCCGGTCAGCGCCAGCTTCTGCTG-3'

The pCS3MT Δ10-MKK7 and Δ22-MKK7 constructs have been described (Ch. 2) To make pCS3MT Δ75-MKK7 and Δ100-MKK7, a common MKK7 3' primer and the following 5' primers were used to PCR amplify MKK7:

Δ75: 5'-CGGGATCCACATGCTGGGGCTCCCATCAACC-3'

Δ100: 5'-CGGGATCCCGGAGATCATGAAGCAGACAGGGT-3'

PCR products were digested with BamHI and cloned into the BglII site of pCS3MT vector. Other constructs: pRK5 MT-MLK2 was a generous gift from A. Hall. pSRα HA MKK6, pCMV HA MEKK1 C and dnKn MEKK1 were gifts from Y. Gotoh. pRSV F-MKK3 was from R. Davis. GST KR-SAPK/JNK was a gift from D. Templeton. pCEP4 MKP1 and pCEP4 MKP1 C-S were gifts from C. Franklin. GST-MKK7, GST-C BicD, GST-KR-SAPK/JNK and GST c-Jun (1-79) were bacterially-expressed and purified following standard procedures (Ausubel, 1992). *In vitro* binding assays were performed as described in Ch. 2.

CELL CULTURE AND STIMULATION:

293T cells were cultured in . Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Plasmid DNA was transfected using $CaPO_4$ and cells were harvested two days after transfection. Total amount of DNA was kept constant and adjusted with pCS3 vector DNA. Cells were treated with NaCl or anisomycin as described in Ch. 2. Cells were treated with 1 or 10 μM nocodazole for 90 min at 37 °C.

IMMUNOASSAYS:

For immunoprecipitations, cells were washed with cold PBS and lysed on ice in a buffer containing 50 mM Hepes, pH 7.6, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM $MgCl_2$, 100 mM NaF, 20 mM β-glycerophosphate, 0.5 mM PMSF, 1 mM DTT, 0.1 mM Na_3VO_4 and 10 μg/ml leupeptin. T7-hBicd and MT-MKK7 were immunoprecipitated for 1 h at 4 °C with anti-T7 (Novagen) and anti-Myc (9E10), respectively. Immune complexes were recovered using Protein A Sepharose beads (Sigma) coated with goat α-mouse IgG. Complexes were washed three times in lysis buffer and once with wash buffer containing 20 mM Tris pH 7.5, 25 mM β - glycerophosphate, 2 mM EGTA, 2 mM DTT and 1 mM Na_3VO_4, and either mixed with sample buffer or subjected to kinase or phosphatase assays. For kinase assays, complexes were incubated in 15 μl of assay buffer with 2.5 μCi [γ-^{32}P] ATP, 100 μM unlabeled ATP, and 2 μg of GST KR JNK as substrate. Reactions were performed at 30 °C for 30 minutes, and stopped by the addition of sample buffer. Protein kinase activities were visualized by SDS-PAGE and autoradiography. For phosphatase assays, T7-hBicD immunoprecipitated complexes were isolated as previously described. Following antibody incubation, beads were washed three times in lysis buffer with no vanadate, and once with either CIP buffer containing 100 mM NaCl, 50 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$ and 1 mM DTT, or with λ-PPase buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT and 0.01% Brij 35. For CIP phosphatase assays complexes were incubated in 15 μl of assay buffer with or without 10 Units of CIP (NEB) for 1 hour at 37 °C. For λ-PPase assays, complexes were incubated in 15 μl of assay buffer supplemented with 2 mM $MnCl_2$ with or without 400 Units of λ-PPase (NEB) for 1 hour at 30 °C. Reactions were stopped by the addition of sample buffer and visualized by SDS-PAGE and Western blot analysis.

WESTERN BLOTTING:

Lysates of total cellular protein or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the corresponding rabbit antiserum or mouse monoclonal antibody. For MT-MKK7 and MT-MKK4 Westerns, anti-Myc (9E10) antibody was used. For F-MKK3 and HA-MKK6 Westerns, anti-M2 Flag (Kodak Scientific Imaging) and anti-HA (12CA5) antibodies were used, respectively. For pCGT-T7 hBicD Westerns, an anti-T7 antibody was used (Novagen). For phospho-JNK Westerns, a phospho-specific anti-JNK antibody (NEB) was used. Immunoblots were visualized by enhanced chemiluminescence detection (Amersham) using horseradish peroxidase coupled secondary antibodies (Bio-Rad).

CHAPTER 5: DIMERIZATION OF MKK7

INTRODUCTION:

A screen to look for interacting partners of MKK1 led us to the identification of a novel MAP kinase kinase, MKK7. Clones corresponding to the C-terminal 100 amino acids of MKK1 were also identified in this screen. These results suggested that MKK1 was capable of forming dimers, and that MKK1 and MKK7 might heterodimerize. Results from yeast two-hybrid assays indicated that the C-terminus of MKK1 was necessary but not sufficient for mediating the MKK1 dimerization. Fusions of the C-terminal 100 amino acids of MKK1 to VP16 and LexA were shown not to interact, as determined by β-galactosidase assays and growth on a selective marker (Table 4). Although the N-terminus of MKK1 was dispensable, some region within the catalytic domain in addition to the C-terminus of MKK1 was needed for an association (Table 2). Furthermore, the activation state of MKK1 did not greatly influence its dimerization ability (Table 3). Although interactions between activated mutants of MKK1 appeared stronger than interactions between kinase-inactive mutants, the difference was not significant. These results suggested that dimerization of MKK1 did not play an important role in regulating its activity.

The studies presented here demonstrate that MKK7 is capable of dimerizing in cells. MKK7 also associates with MKK1, but not MKK4. Consistent with our two-hybrid results addressing the activation state of MKK1 and its ability to dimerize, we were not able to demonstrate any relationship between the dimerization and activation state of MKK7. Our data suggest that the dimerization of MKK7 may not be important for regulating its activity.

RESULTS:

MKK7 DIMERIZES IN CELLS:

To address whether MKK7 could associate in cells, we utilized two differentially-tagged MKK7 mammalian expression constructs. Full-length wild-type MKK7 was fused to GST (pEBG MKK7) and co-expressed in 293T cells with Myc-tagged fusions of either full-length MKK7, or a series of MKK7 N-terminal deletion constructs. The GST-MKK7 fusion protein was immunoprecipitated from cells with glutatione Sepharose and analyzed for the presence of Myc-tagged MKK7 by Western blotting with anti-myc antibody. Figure 50 indicates that Myc-tagged MKK7 was able to co-immunoprecipitate with GST MKK7. Deletions of up to 100 N-terminal amino acids of MKK7 did not significantly decrease its ability to dimerize. This is consistent with results observed for MKK1 dimerization, in which the N-terminus was not required for dimerization (Khokhlatchev *et al.*, 1998).

MKK7 ASSOCIATES WITH MKK1 BUT NOT MKK4 IN CELLS:

We were interested in determining whether MKK7 and MKK1 might also heterodimerize in cells. An interaction between full-length MKK7 and full-length MKK1 had not previously been demonstrated. 293T cells were co-transfected with GST MKK7 and either Myc-tagged MKK1, MKK4 or MKK7. GST MKK7 was immunoprecipitated with glutathione Sepharose and analyzed for co-immunoprecipitating proteins by Western blotting with anti-myc antibody. As shown in Figure 50, Myc-tagged MKK7 co-immunoprecipitated with GST MKK7. Myc-tagged MKK1 was also able to bind to GST MKK7, although less binding was observed than with Myc-tagged MKK7 (Fig. 51). Myc-tagged MKK4 was not able to bind. These results confirmed our original observations that MKK1 and MKK7 could heterodimerize. The fact that MKK4 did not bind MKK7 suggested that this may be specific. Nevertheless, the heterodimerization between MKK7 and MKK1 was significantly weaker than the homodimerization of MKK7. It is possible that MKK7 preferentially is in a homodimerized state, but may be induced to heterodimerize with MKK1.

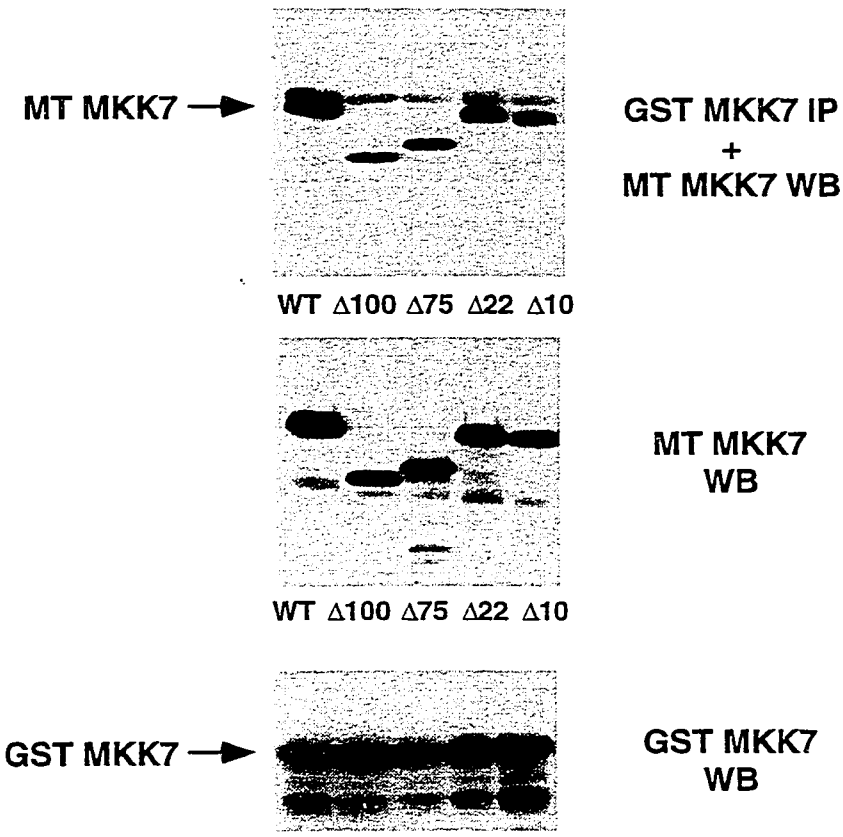


Fig. 50. MKK7 dimerizes in cells. 293T cells were co-transfected with pEBG MKK7 and either pCS3MT MKK7, MKK7 Δ100, MKK7 Δ75, MKK7 Δ22, or MKK7 Δ10 N-terminal deletions. GST MKK7 was immunoprecipitated using glutathione sepharose and analyzed by Western blotting with anti-myc antibody (9E10) for the presence of co-immunoprecipitated proteins. Expression of Myc-tagged proteins was determined by Western blotting with anti-myc antibody (9E10). Expression of GST MKK7 was determined by Western blotting with anti-GST antibody.

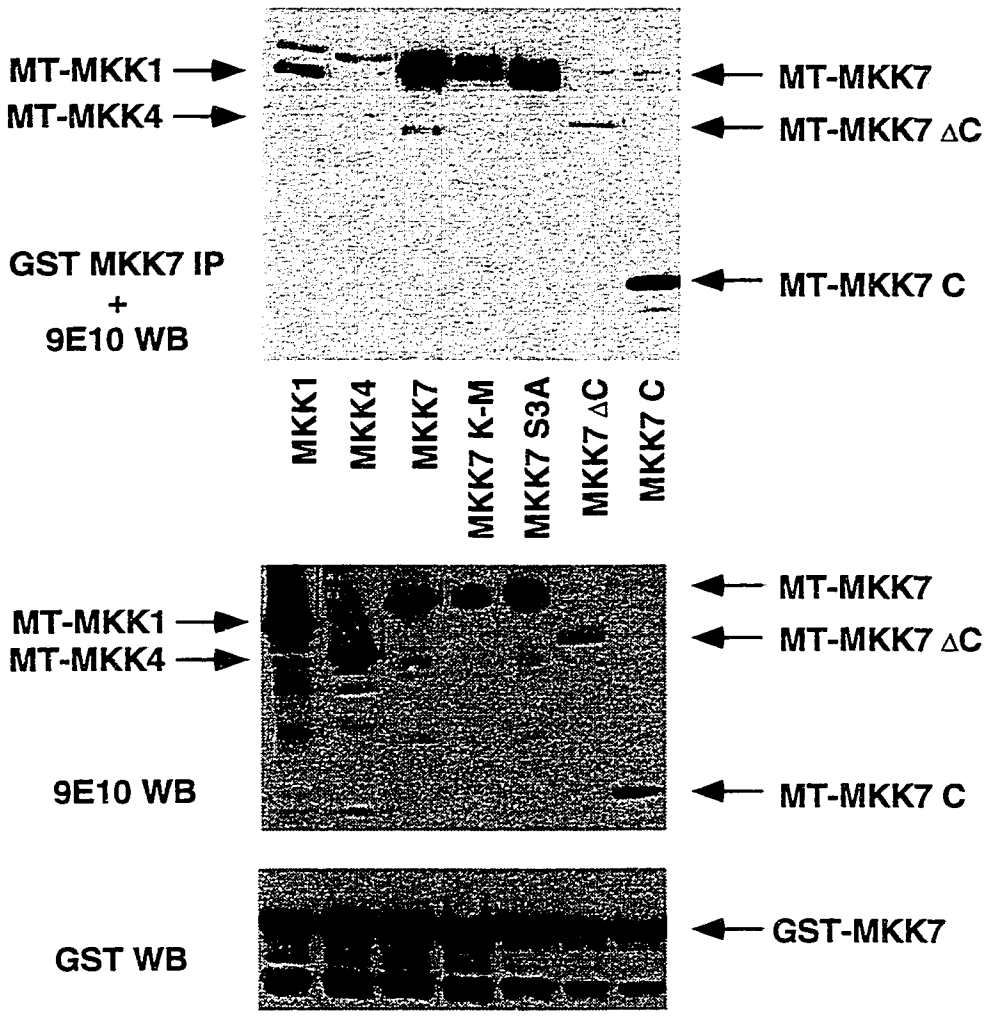


Fig. 51. MKK7 binds to MKK1 but not MKK4. 293T cells were co-transfected with pEBG MKK7 and either pCS3MT MKK7, MKK1, MKK4, MKK7 K149M, MKK7 S3A, MKK7 MKK7 ΔC (1-322), or MKK7 C (286-416). GST MKK7 was immunoprecipitated using glutathione Sepharose and analyzed by Western blotting with anti-myc antibody (9E10) for the presence of co-immunoprecipitated proteins. Expression of Myc-tagged proteins was determined by Western blotting with anti-myc antibody (9E10). Expression of GST MKK7 was determined by Western blotting with anti-GST antibody.

We also addressed whether the activation state of MKK7 affected its dimerization, and whether the C-terminus was important for dimerization. GST MKK7 was co-transfected with either a Myc-tagged kinase-inactive MKK7 (K149M) or a Myc-tagged phosphorylation site mutant of MKK7 (S3A). A Myc-tagged construct of MKK7 lacking the C-terminal 97 amino acids (MKK7 ΔC, 1-322) or a construct of just the MKK7 C-terminus (MKK7 C, 286-416) was also co-transfected with GST MKK7. Analysis of the immunoprecipitated GST MKK7 by Western blotting with anti-myc antibody indicated that both MKK7 K-M and MKK7 S3A could bind to GST MKK7 in cells (Fig. 51). The association between MKK7 K149M and GST MKK7 appeared weaker than that seen with wild-type MKK7 or MKK7 S3A, however, it was still greater than that observed between MKK1 and MKK7. Similar results were observed with MKK1 in yeast two-hybrid assays, in which MKK1 K97A and MKK1 showed a slightly weaker interaction than MKK1 and MKK1. It is possible that mutation of the lysine residue within the ATP binding pocket disrupts the folding of each MAPKK, thereby diminishing the ability of these mutants to dimerize. A Myc-tagged fusion of the C-terminal portion of MKK7 also co-immunoprecipitated with GST MKK7. In contrast, the MKK7 ΔC construct was barely detectable in the co-immunoprecipitate. This indicated that the C-terminus of MKK7 was necessary for dimerization. Like the results obtained with MKK1 in two-hybrid assays, the activation state of MK7 did not signficantly alter its dimerization state, and the C-terminus was necessary for the dimerization.

EXPRESSION OF THE C-TERMINUS OF MKK7 DOES NOT AFFECT ACTIVITY:

Our results indicated that inactive mutants of MKK7 were capable of dimerizing, and that the C-terminus of MKK7 was necessary for dimerization. We were interested in determining whether the C-terminus alone was able to influence MKK7 activity. For example, over-expression of a C-terminal fragment might interfere with dimerization of full-length molecules. If dimerization of MKK7 were a requirement for its activation, then the presence of C-terminal constructs would serve to reduce dimerization between full-length molecules and thereby decrease MKK7 activity. To test this, Myc-tagged MKK7 was expressed in 293T cells in the presence or absence of Myc-tagged MKK7 C. Cells were stimulated with NaCl or left untreated, and immunoprecipitated MKK7 was assayed for activity using kinase-inactive GST JNK as a substrate. Figure 52 demonstrates that expressing MKK7 C along with full-length MKK7 did not affect MKK7

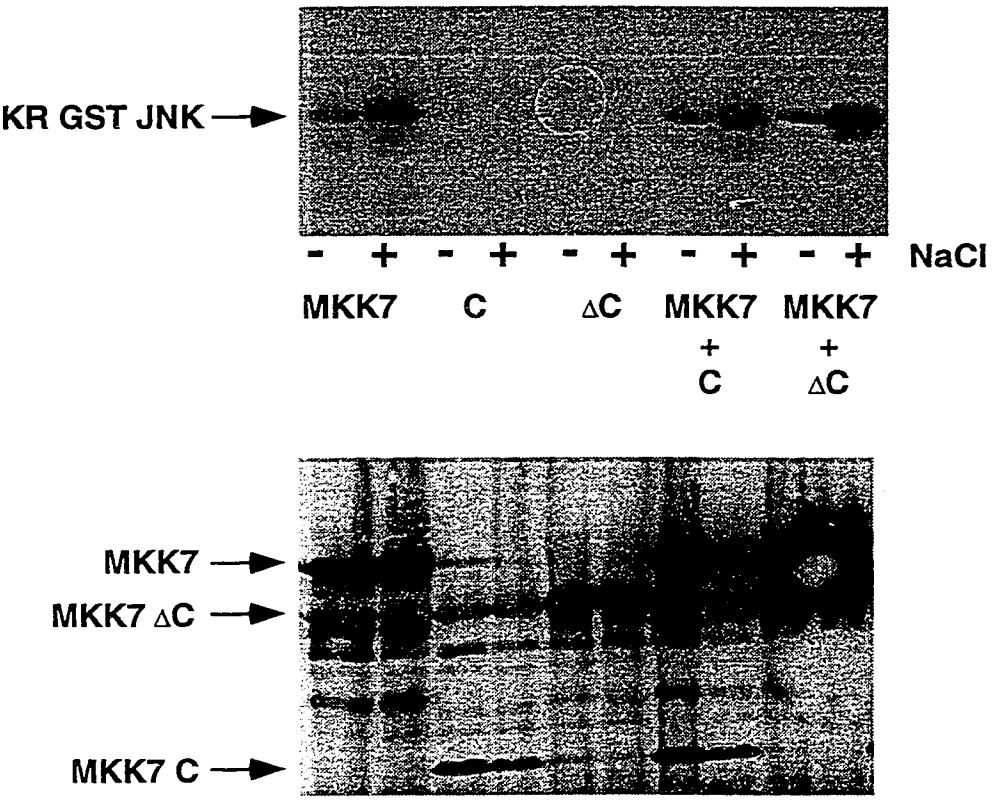


Fig. 52. Expression of the MKK7 C-terminus does not affect MKK7 activity. 293T cells were co-transfected with pCS3MT MKK7 in the presence or absence of pCS3MT MKK7 C (286-416), or pCS3MT MKK7 ΔC (1-322). Cells were stimulated with 0.4M NaCl for 30 min or left untreated. pCS3MT MKK7 was immunoprecipitated with anti-myc (9E10) antibody, and assayed for activity using KR GST JNK/SAPK as a substrate. Expression of MKK7 constructs was determined by Western blotting with anti-myc antibody (9E10).

activity. Similarly, co-expression of MKK7 ΔC along with full-length MKK7 also had no effect on MKK7 activity. Our results suggest that MKK7 may dimerize in cells, but we have been unable to demonstrate that dimerization affects MKK7 activity. We cannot rule out the possibility that MKK7 is preferentially dimerized in an inactive state, and that dissociation is required for activation. To address this would require a dimerized mutant of MKK7 that was incapable of dissociating upon activation. Although our data from two-hybrid assays and co-transfection experiments indicate that dimerization may occur, the physiological significance of these interactions is not clear.

DISCUSSION:

The dimerization of molecules such as MKK1 and MKK7 has not been extensively characterized. To our knowledge this is the first demonstration of MKK7 dimerization and MKK7-MKK1 heterodimerization in cells. Regions within the C-terminus of MKK7 as well as a region in the catalytic domain, are required for dimerization. Our preliminary analyses have suggested that dimerization of MKK7 or MKK1 may not be important for activation, but a comprehensive analysis of this has not been performed. Dimerization may have alternate roles, for example, in subcellular localization, or in regulating the access to other molecules.

Other components of MAP kinase pathways have also been demonstrated to dimerize, and in several cases dimerization is an important mechanism of regulating activation. The MAP kinase ERK2 has been demonstrated to form dimers which are dependent on at least one ERK2 molecule being phosphorylated (Khokhlatchev *et al.*, 1998). Based on the crystal structure of ERK2 and mutagenesis studies, the dimer interface has been mapped to leucine residues (Leu 333,336,341,344) within in the C-terminus and a histidine residue (His 176) within the activation loop. Mutation of two or four leucine residues in combination with His 176 is sufficient to block dimerization. The phosphorylation of ERK2 induces its dimerization, which is required for its nuclear translocation. Disruption of dimerization by mutagenesis reduces the ability of ERK2 to accumulate in the nucleus, thereby diminishing its access to downstream nuclear targets. In this manner, the formation of dimers upon activation contributes to the mechanism of

action. This is an example in which phosphorylation regulates dimerization and dimerization affects localization.

MKK7 also contains C-terminal leucine residues (Leu 308, 312, 315), which may be important for its dimerization. Whether or not MKK7 would localize differently in a dimerized or monomeric state is not known. Studies from our lab and those of others indicate that populations of MKK7 in stimulated and unstimulated cells are both nuclear and cytoplasmic (Fig. 25) (Tournier *et al.*, 1999). It is possible that one of these populations may be preferentially monomeric and the other dimeric, however, this has not been addressed. Generation of dimerization mutants or constitutive dimers of MKK7 would be useful for such studies.

Our data indicate that unphosphorylated or inactive mutants of MKK7 are also capable of dimerizing with wild-type. However, we did not directly test whether dimerization would occur with two unphosphorylated molecules of MKK7. In two-hybrid assays with MKK1, the binding of two kinase-inactive molecules of MKK1 (MKK1 K97A + MKK1 K97A) was weaker than with wild-type or activated mutants, yet it was still detectable (Table 3). Since these assays utilize over-expressed proteins, it may not reflect the dimerization characteristics of these molecules *in vivo*. It will be important to address whether or not two phosphorylation site mutants of MKK7 dimerize, and whether or not endogenous MKK7 (or MKK1) are detectable in a dimerized state.

The Raf-1 MAPKKK may also be activated by synthetic dimerization using either a gyrase B-coumermycin system or the FKBP-FK1012A system (Farrar *et al.*, 1996; Luo *et al.*, 1996). Although the precise mechanism of activation of Raf-1 by oligomerization is not clear, it is thought that membrane localization of Raf-1 may promote clustering, leading to activation. Proper localization would increase the local concentration and thus promote dimerization. Membrane recruitment of Raf-1 by active Ras has also been shown to activate Raf-1 (Moodie and Wolfman, 1994). It is not clear whether dimerization promotes activation of Raf-1, or whether activation promotes dimerization.

The stress-activated MAPKKK, ASK1, is another kinase thought to be activated by dimerization (Gotoh and Cooper, 1998). Chemical crosslinking of ASK1 following cell stimulation indicates that activated ASK1 is dimerized. Induced dimerization

of ASK1 using the gyrase B-coumermycin system also results in ASK1 activation. However, it is still not clear whether dimerization precedes activation, or vice versa.

The fact that components upstream as well as downstream of MKKs have been shown to dimerize suggests that dimerization of MKKs may also be important. For upstream components such as Raf-1 and ASK1, dimerization appears to correlate with activation. It is possible that dimerization creates binding sites for upstream activators, or allows for trans-phosphorylation of subunits, as has been observed for receptor tyrosine kinases. Alternatively, phosphorylation may induce conformational changes which serve to promote dimerization. For the MAP kinase ERK2, phosphorylation is known to induce dimerization and this affects ERK2 localization. More work is required to determine whether this will serve as a common mechanism for other kinases, and what the biological roles for dimerization might be. The ability of an active subunit to complex with either an active or inactive subunit to dimerize may serve to fine tune the time course of activation, the threshold for activation, and the efficacy of regulation of downstream substrates, as has been suggested for ERK2 (Khokhlatchev *et al.*, 1998). Similarly, the heterodimerization of two related kinases may also function in controlling the signal output. Thus, differential activation of dimeric substrates by dimeric kinases may be a mechanism for generating distinct response thresholds.

MATERIALS AND METHODS:

DNA CONSTRUCTS:

pEBG 3X MKK7 was generated by digesting MKK7 as a BamHI-BamHI fragment from pGEX 3X MKK7 and subcloning into the BamHI site of pEBG 3X. To make pCS3MT MKK1, MKK1 was digested from pBTM116 LexA MKK1 with BamHI and EcoRI, and subcloned into the BglII and EcoRI sites of pCS3MT. pCS3 MT MKK7-C corresponds to the C-terminal fragment originally isolated from the MKK1 two-hybrid screen (MKKIP85a, 286-416). MKKIP85a was PCR-amplified as a BamHI-BamHI fragment and subcloned into the BglII site of pCS3MT. To make pCS3MT MKK7 ΔC (1-322), a common MKK7 5' primer and the following 3' primer were used to PCR amplify MKK7:

5'-CGCGGATCCGTCCTGTTGCCAGCTCCACCAG-3'

This PCR fragment was digested with BamHI and cloned into the BglII site of pCS3MT. All other plasmids and constructs have been previously described (Ch. 2, Ch. 4).

CELL CULTURE AND STIMULATION:

293T cells were maintained and stimulated as described in Ch. 4.

IMMUNOASSAYS:

Assays were performed as described in Ch. 4.

WESTERN BLOTTING:

Procedures for Western blotting and antibodies used have been described in Ch. 2 and Ch. 4.

CHAPTER 6: PERSPECTIVES

Over the past few years, much progress has been made in elucidating the mechanisms of signal-transduction of MAP kinase pathways. In particular, the identification of mammalian stress-activated MAP kinase pathways, and of numerous components that feed into them, has uncovered a complex network of interactions that participate in regulating diverse cellular responses. The large number of MAP kinase kinase kinases, STE20 homologs and small GTPases that have been implicated in the SAPK-signaling pathway has led to confusion concerning the definition of the upstream elements of the pathway that are relevant to particular extracellular stimuli. It is not clear whether this represents redundancy amongst the known signaling pathways or whether additional stress-activated pathways exist.

This study has identified several protein-protein interactions that may have important regulatory and functional implications for the SAPK pathway. The identification of MKK7 as a component of the SAPK pathway has increased the complexity and the possibility of redundancy among activators of SAPK. A major question concerns the relative contributions of MKK7 and MKK4 to activation of SAPK in response to different stimuli. The use of transgenic mice expressing dominant-negative forms of MKK7 and MKK4, either alone or in combination, should provide insight into the requirements of MKK7 and MKK4 in SAPK activation (Alberola-Ila, *et al.*, 1998; work in progress). In addition, the identification of additional MKK7 binding proteins that may function to provide crosstalk or localize MKK7 in the cell, may ultimately provide a better understanding of the means by which the specificity of SAPK activation is achieved.

The recent identification of six distinct alternatively spliced isoforms of MKK7 in mice suggests that significant functional heterogeneity may exist within the

MKK7 subfamily of kinases (Tournier *et al.*, 1999). This is in contrast to MKK4, in which only two possible alternative splice forms have been described (D'Erijard *et al.*, 1995; Sanchez *et al.*, 1994). Comparative studies indicate that these MKK7 isoforms differ in the extent of activation in response to different upstream components of the SAPK signaling pathway. Two MKK7 isoforms are lacking the first 90 N-terminal amino acids. Our studies and those of others have demonstrated that the first 70 amino acids of MKK7 are necessary for efficient activation of SAPK as well as to mediate binding to hBicD. Therefore, it is possible that some of these isoforms may signal to different substrates. In addition, the dimerization of different MKK7 isoforms with one another might also contribute to their distinct physiological roles.

The identification of MKK7 in a two-hybrid screen using MKK1 as bait raises several important issues. One concerns the possibility that MKK7 might homo- and hetero-dimerize with other MKK's. In this respect it is interesting that although MKK7 interacts with MKK1, we have been unable to detect any interaction between MKK7 and MKK4. The interaction between MKK7 and MKK1 may provide a basis for crosstalk between components of the SAPK and the ERK pathways. Alternatively, these observations may reflect a mechanism by which coordinate regulation of the SAPK and ERK pathway is achieved in response to certain stimuli..

Our dimerization experiments have not addressed whether endogenous MKK7 is found as a dimer. One method whereby this question could be addressed is by gel filtration techniques. Analysis of lysates from stimulated and unstimulated cells by gel filtration may resolve MKK7 into monomeric and multimeric forms. To more directly address the phosphorylation state of these forms, pre-treatment of lysates with serine/threonine phosphatases could also be performed. Gel filtration could also be used to characterize the activity of MKK7 by analyzing fractions from stimulated cells for SAPK activating activity. By comparing the amount of MKK7 in each fraction to the amount of MKK7 activity in each fraction, a rough estimate of the activity of MKK7 in monomeric and multimeric states could be obtained.

In addition to further characterizing the effects of phosphorylation or activation state of MKK7 on dimerization, the construction of a dimerization mutant might also be of value. Since the C-terminus of MKK7 is known to be important in mediating the

dimerization, this region could be mutagenized to generate a library of MKK7 C-terminal random mutants. Mutants no longer capable of dimerizing but still capable of binding SAPK/JNK could be screened in yeast two-hybrid assays. Dimerization mutants of ERK2 MAP kinase have activity comparable to wild-type, so based on common dimerization domains within the proteins, one might predict the same for MKK7 (Khokhlatchev *et al.*, 1998). This mutant screening procedure has been successfully used to identify mutants of Ras-GTP which no longer bind to Raf-1, but still bind to other effectors (Winkler *et al.*, 1997).

One role of dimerization may be to affect the localization of MKK7. Localization of a kinase at sites of action is one determinant of its function. It is possible that the dimerization of MKK7 could influence its ability to bind hBicD, or the scaffolding protein JIP-1, for example (Whitmarsh *et al.*, 1998). The region of MKK7 required for JIP-1 binding has not been identified. Loss-of-binding to regulatory proteins could alter the availability of MKK7 to specific activators or pools of substrates within the cell. Pools of SAPK and ERK have been identified in nuclear and cytoplasmic fractions, and are probably activated at different sites within the cell (Khokhlatchev *et al.*, 1998; Nagata *et al.*, 1998; Reszka *et al.*, 1995). Immunofluorescence analyses of dimerization mutants is one method by which this could be addressed. Although we have not been able to demonstrate any functional consequences of MKK7 dimerization, it is possible that dimerization may influence the substrate specificity, activation, or localization of MKK7.

The identification of hBicD as a binding partner for MKK7 proposes a model in which the association of MKK7 to hBicD serves to localize MKK7 within the cell and make it available for activation by a subset of upstream activators. The precise subcellular distribution of hBicD within cells, as well as the co-localization of hBicD and MKK7 in cells, has yet to be demonstrated. Preliminary studies examining the localization of unphosphorylated hBicD over-expressed in cells indicate that it is found primarily in discrete circular structures. These may represent large oligomeric complexes of hBicD. In contrast, phosphorylated hBicD (hBicD co-expressed with MLK2) appears to be more dispersed through the cell. The generation of antibodies directed against hBicD are currently underway, and should aid in defining the localization of endogenous hBicD, as well as the ability of MKK7 to co-localize to hBicD.

Our observations indicate that the interaction MKK7 and hBicD appears to be regulated by the activity of upstream components of the SAPK pathway such as MLK2 and MEKK1, which can mediate hBicD phosphorylation. This raises the possiblity that MLK2 or MEKK1 may phosphorylate hBicD directly or may bind to hBicD directly. The heptad repeats in hBicD may mediate such protein-protein interactions. Determining whether kinases such as MLK2 or MEKK1 also bind hBicD at various heptad repeats may be helpful in determining whether these kinases directly phosphorylate hBicD. Alternatively, a two-hybrid screen with either full-length or individual heptad repeats of hBicD might reveal associations with other candidate hBicD kinases. One dominant BicD mutation in *Drosophila* (*BicD'*) contains a single amino acid substitution of isoleucine for phenylalanine in the fifth heptad repeat. This residue is conserved among the human BicD homologs and is present in the fragment originally identified in the MKK7 two-hybrid screen. It has been demonstrated that the *BicD'* protein product is mislocalized in *Drosophila*, resulting in the bicaudal phenotype (Wharton and Struhl, 1989). It is possible that this residue is important for the association between MKK7 and hBicD, and mutation of this site in hBicD might also result in mislocalization.

Studies from *Drosophila* mutants suggest hBicD is localized to microtubules. Microtubule-associated pools of both mammalian SAPK and ERK have been previously demonstrated, suggesting they may be activated and possess substrates at that site (Nagata *et al.*, 1998; Reszka *et al.*, 1995). However, it is currently not known which cytoskeletal component hBicD or MKK7 may localize to, if any. It is possible that hBicD may localize to the actin cytoskeleton or to intermediate filaments. Microtubules are known to overlap with actin stress fibers at the cell periphery and this may be a point of convergence (Huang *et al.*, 1999). Cell fractionation studies as well as immunofluorescence studies would be useful in addressing this. Soluble, cytoskeletal and nuclear matrix/intermediate filament fractions may be prepared and probed with antibodies against MKK7 and hBicD, as well as antibodies against tubulin, actin and vimentin. These antibodies may also be used in immunofluorescence analyses. Extraction procedures which isolate the cytoskeleton may also be used to eliminate nuclear and cytosolic pools of MKK7, leaving only a cytoskeleton bound portion intact. These approaches have been useful in identifying microtubule-associated fractions of ERK2 (Reszka *et al.*, 1997; Reszka *et al.*, 1995). Similarly, analysis of hBicD or MKK7 immunostaining in cells treated with cytochalasinD or colchicine may be informative in identifying the cytoskeletal

components with which they associate. These agents should specifically disrupt the pattern of the actin or microtubule cytoskeleton, respectively, without disrupting the pattern of the other.

A better understanding of the links between the SAPK-signaling pathway and the regulation of cytoskeletal dynamics is required. It is important to note that the relationship between the cytoskeleton and MAPK pathways is unclear. Although evidence exists to suggest that MAPK pathways might function to regulate cytoskeletal organization, it has also been suggested that MAPK pathways might be regulated by changes in the cytoskeleton (Yujiri *et al.*, 1998). It is intriguing to speculate that during conditions in which cytoskeletal rearrangement takes place, signals could originate from microtubules or the actin cytoskeleton and be mediated by components of a signaling pathway. Changes in cell shape, cell movement, migration, or apoptotic stress signals all induce dynamic events involving the assembly and breakdown of cytoskeletal components at sites of active growth or movement. This model provides a role for the activation of the SAPK pathway in the maintenance of cellular functions as well as under conditions of extreme stress.

A major goal for the future will be to define the physiologically relevant pathways that lead from receptors to the activation of SAPK. Understanding these processes requires not only the knowledge of specific signaling molecules, but also the three dimensional arichitecture of the pathway *in vivo*. The arrangement of signaling molecules within the cell, and the possible role of scaffolding proteins, are likely to be important in understanding the mechanisms by which specificity is achieved within the SAPK signaling pathway.

BIBLIOGRAPHY

Abe, M., Wen-Liang, K., Hershenson, M., and Rich Rosner, M. (1999). Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. Molecular and Cellular Biology *19*, 1301-12.

Ahn, N. G., and Krebs, E. G. (1990). Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3 cells. Activation of serine peptide kinase activity by myelin basic protein kinases in vitro. Journal of Biological Chemistry *265*, 11495-501.

Alberola-Ila, J., Levin, S. D., Barton, G., Forbush, K., Zon, L. I., and Perlmutter, R. M. (1998). Analysis of the role of MKK-4/Sek-1 in T cell development and apoptosis. International Immunology *10*, 1077-82.

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO Journal *13*, 1610-9.

Anderson, N. G., Li, P., Marsden, L. A., Williams, N., Roberts, T. M., and Sturgill, T. W. (1991). Raf-1 is a potential substrate for mitogen-activated protein kinase in vivo. Biochemical Journal *277*, 573-6.

Arora, K., Dai, H., Kazuko, S. G., Jamal, J., O'Connor, M. B., Letsou, A., and Warrior, R. (1995). The Drosophila schnurri gene acts in the Dpp/TGF beta signaling pathway and encodes a transcription factor homologous to the human MBP family. Cell *81*, 781-90.

Ausubel, F. M. (1992). Current protocols in molecular biology (New York: J. Wiley).

Baens, M., and Marynen, P. (1997). A human homologue (BICD1) of the Drosophila bicaudal-D gene. Genomics *45*, 601-6.

Bagrodia, S., D'Erijard, B., Davis, R. J., and Cerione, R. A. (1995). Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. Journal of Biological Chemistry *270*, 27995-8.

Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R., and Thorner, J. (1996). Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. Molecular and Cellular Biology *16*, 3637-50.

Barry, C. P., Xie, J., Lemmon, V., and Young, A. P. (1993). Molecular characterization of a multi-promoter gene encoding a chicken filamin protein. Journal of Biological Chemistry *268*, 25577-86.

Bartel, P., Chien, C., Sternglanz, R., and Fields, S. (1993). Elimination of false positives that arose in using the two-hybrid system. Biotechniques *14*, 920-24.

Berberich, I., Shu, G., Siebelt, F., Woodgett, J. R., Kyriakis, J. M., and Clark, E. A. (1996). Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. EMBO Journal *15*, 92-101.

Bilbe, G., Delabie, J., Bruggen, J., Richener, H., Asselbergs, F. A., Cerletti, N., Sorg, C., Odink, K., Tarcsay, L., Wiesendanger, W., and et al. (1992). Restin: a novel intermediate filament-associated protein highly expressed in the Reed-Sternberg cells of Hodgkin's disease. EMBO Journal *11*, 2103-13.

Blackwood, E. M., and Eisenman, R. N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-7.

Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996). Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. Journal of Biological Chemistry *271*, 5361-8.

Borchiellini, C., Coulon, J., and Le, P.-Y. (1996). The function of type IV collagen during Drosophila muscle development. Mechanisms of Development *58*, 179-91.

Brewster, J. L., de, V.-T., Dwyer, N. D., Winter, E., and Gustín, M. C. (1993). An osmosensing signal transduction pathway in yeast. Science *259*, 1760-3.

Buckland, R., and Wild, F. (1989). Leucine zipper motif extends. Nature *338*, 547.

Buehrer, B. M., and Errede, B. (1997). Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in Saccharomyces cerevisiae. Molecular and Cellular Biology *17*, 6517-25.

Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997). The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell *90*, 315-23.

Casciola-Rosen, L. A., Anhalt, G. J., and Rosen, A. (1995). DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. Journal of Experimental Medicine *182*, 1625-34.

Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998). Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. Science *281*, 1860-3.

Charbonneau, H., and Tonks, N. K. (1992). 1002 protein phosphatases? Annual Review of Cell Biology *8*, 463-93.

Chen, C. Y., Del, G.-K. -. F., Wu, Z., and Karin, M. (1998). Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. Science *280*, 1945-9.

Chen, R. H., Sarnecki, C., and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Molecular and Cellular Biology *12*, 915-27.

Chen, Y. R., Meyer, C. F., and Tan, T. H. (1996). Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. Journal of Biological Chemistry *271*, 631-4.

Chen, Y. R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. Journal of Biological Chemistry *271*, 31929-36.

Cheng, M., Boulton, T. G., and Cobb, M. H. (1996). ERK3 is a constitutively nuclear protein kinase. Journal of Biological Chemistry *271*, 8951-8.

Choi, K. Y., Satterberg, B., Lyons, D. M., and Elion, E. A. (1994). Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in S. cerevisiae. Cell *78*, 499-512.

Clifford, R., and Schupbach, T. (1992). The torpedo (DER) receptor tyrosine kinase is required at multiple times during Drosophila embryogenesis. Development *115*, 853-72.

Cohen, P. (1997). The search for physiological substrates of MAP kinases and SAP kinases in mammalian cells. Trends in Cell Biology *7*, 353-61.

Cohen, P. T. (1997). Novel protein serine/threonine phosphatases: variety is the spice of life. Trends in Biochemical Sciences *22*, 245-51.

Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137-46.

Crawley, J. B., Rawlinson, L., Lali, F. V., Page, T. H., Saklatvala, J., and Foxwell, B. M. (1997). T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. Journal of Biological Chemistry *272*, 15023-7.

Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992). The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science *258*, 478-80.

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

D'Erijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025-37.

D'Erijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science *267*, 682-5.

Deacon, K., and Blank, J. L. (1997). Characterization of the mitogen-activated protein kinase kinase 4 (MKK4)/c-Jun NH2-terminal kinase 1 and MKK3/p38 pathways regulated by MEK kinases 2 and 3. MEK kinase 3 activates MKK3 but does not cause activation of p38 kinase in vivo. Journal of Biological Chemistry *272*, 14489-96.

Deak, J. C., Cross, J. V., Lewis, M., Qian, Y., Parrott, L. A., Distelhorst, C. W., and Templeton, D. J. (1998). Fas-induced proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. Proceedings of the National Academy of Sciences of the United States of America *95*, 5595-600.

Dent, P., Haser, W., Haystead, T. A., Vincent, L. A., Roberts, T. M., and Sturgill, T. W. (1992). Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science *257*, 1404-7.

Dent, P., and Sturgill, T. W. (1994). Activation of (His)6-Raf-1 in vitro by partially purified plasma membranes from v-Ras-transformed and serum-stimulated fibroblasts. Proceedings of the National Academy of Sciences of the United States of America *91*, 9544-8.

Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997). A cytoplasmic inhibitor of the JNK signal transduction pathway. Science *277*, 693-6.

Dong, C., Yang, D., Wysk, M., Whitmarsh, A., Davis, R., and Flavell, R. (1998). Defective T cell differentiation in the absence of Jnk1. Science *282*, 2092-95.

Dorow, D. S., Devereux, L., Tu, G. F., Price, G., Nicholl, J. K., Sutherland, G. R., and Simpson, R. J. (1995). Complete nucleotide sequence, expression, and chromosomal localisation of human mixed-lineage kinase 2. European Journal of Biochemistry *234*, 492-500.

Ellinger-Ziegelbauer, H., Brown, K., Kelly, K., and Siebenlist, U. (1997). Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein Kinase/ERK kinase kinase 3 (MEKK) derivative. Journal of Biological Chemistry *272*, 2668-74.

Evangelista, M., Blundell, K., Longtine, M. S., Chow, C. J., Adames, N., Pringle, J. R., Peter, M., and Boone, C. (1997). Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis. Science *276*, 118-22.

Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B., and Johnson, G. L. (1997). MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? Current Opinion in Genetics and Development *7*, 67-74.

Fanger, G. R., Johnson, N. L., and Johnson, G. L. (1997). MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. EMBO Journal *16*, 4961-72.

Farrar, M. A., Alberol, I., and Perlmutter, R. M. (1996). Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature *383*, 178-81.

Fehon, R. G., Dawson, I. A., and Artavanis Tsakonas, S. (1994). A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. Development *120*, 545-57.

Feramisco, J. R., Gross, M., Kamata, T., Rosenberg, M., and Sweet, R. W. (1984). Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells. Cell *38*, 109-17.

Finch, A., Holland, P., Cooper, J., Saklatvala, J., and Kracht, M. (1997). Selective activation of JNK/SAPK by interleukin-1 in rabbit liver is mediated by MKK7. FEBS Letters *418*, 144-8.

Fischer, E. H., Tonks, N. K., Charbonneau, H., Cicirelli, M. F., Cool, D. E., Diltz, C. D., Krebs, E. G., and Walsh, K. A. (1990). Protein tyrosine phosphatases: a novel family of enzymes involved in transmembrane signalling. Advances in Second Messenger and Phosphoprotein Research *24*, 273-9.

Foltz, I. N., Gerl, R. E., Wieler, J. S., Luckach, M., Salmon, R. A., and Schrader, J. W. (1998). Human mitogen-activated protein kinase kinase 7 (MKK7) is a highly conserved c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activated by environmental stresses and physiological stimuli. Journal of Biological Chemistry *273*, 9344-51.

Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell *78*, 1039-49.

Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996). Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. Journal of Biological Chemistry *271*, 20024-8.

Ganiatsas, S., Kwee, L., Fujiwara, Y., Perkins, A., Ikeda, T., Labow, M. A., and Zon, L. I. (1998). SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proceedings of the National Academy of Sciences of the United States of America *95*, 6881-6.

Glise, B., Bourbon, H., and Noselli, S. (1995). hemipterous encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. Cell *83*, 451-61.

Glise, B., and Noselli, S. (1997). Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in Drosophila morphogenesis. Genes and Development *11*, 1738-47.

Goillot, E., Raingeaud, J., Ranger, A., Tepper, R. I., Davis, R. J., Harlow, E., and Sanchez, I. (1997). Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proceedings of the National Academy of Sciences of the United States of America *94*, 3302-7.

Gotoh, Y., and Cooper, J. A. (1998). Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction. Journal of Biological Chemistry *273*, 17477-82.

Graves, J. D., Draves, K. E., Craxton, A., Saklatvala, J., Krebs, E. G., and Clark, E. A. (1996). Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. Proceedings of the National Academy of Sciences of the United States of America *93*, 13814-8.

Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998). Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. EMBO Journal *17*, 2224-34.

Grieder, N. C., Nellen, D., Burke, R., Basler, K., and Affolter, M. (1995). Schnurri is required for Drosophila Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. Cell *81*, 791-800.

Guesdon, F., Freshney, N., Waller, R. J., Rawlinson, L., and Saklatvala, J. (1993). Interleukin 1 and tumor necrosis factor stimulate two novel protein kinases that phosphorylate the heat shock protein hsp27 and beta-casein. Journal of Biological Chemistry *268*, 4236-43.

Guo, Y. L., Baysal, K., Kang, B., Yang, L. J., and Williamson, J. R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. Journal of Biological Chemistry *273*, 4027-34.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., D'Erijard, B., and Davis, R. J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO Journal *15*, 2760-70.

Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science *265*, 808-11.

Han, J., Lee, J. D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R. J. (1996). Characterization of the structure and function of a novel MAP kinase kinase (MKK6). Journal of Biological Chemistry *271*, 2886-91.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42-52.

Harden, N., Lee, J., Loh, H. Y., Ong, Y. M., Tan, I., Leung, T., Manser, E., and Lim, L. (1996). A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. Molecular and Cellular Biology *16*, 1896-908.

Harden, N., Loh, H. Y., Chia, W., and Lim, L. (1995). A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in Drosophila. Development *121*, 903-14.

Hazzalin, C. A., Cano, E., Cuenda, A., Barratt, M. J., Cohen, P., and Mahadevan, L. C. (1996). p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. Current Biology *6*, 1028-31.

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more. Cell *80*, 187-97.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes and Development *7*, 2135-48.

Hilberg, F., Aguzzi, A., Howells, N., and Wagner, E. F. (1993). c-jun is essential for normal mouse development and hepatogenesis. Nature *365*, 179-81.

Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. (1996). Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. Oncogene *12*, 641-50.

Hirai, S., Noda, K., Moriguchi, T., Nishida, E., Yamashita, A., Deyama, T., Fukuyama, K., and Ohno, S. (1998). Differential activation of two JNK activators, MKK7 and SEK1, by MKN28-derived nonreceptor serine/threonine kinase/mixed lineage kinase 2. Journal of Biological Chemistry *273*, 7406-12.

Hirsch, D. D., and Stork, P. J. (1997). Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. Journal of Biological Chemistry *272*, 4568-75.

Holland, P. M., Suzanne, M., Campbell, J. S., Noselli, S., and Cooper, J. A. (1997). MKK7 is A stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. Journal of Biological Chemistry *272*, 24994-8.

Hollenberg, S. M., Cheng, P. F., and Weintraub, H. (1995). Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. Molecular and Cellular Biology *15*, 3813-22.

Holtmann, H., Winzen, R., Holland, P., Eickemeier, S., Hoffmann, E., Wallach, D., Malinin, N., Resch, K., and Kracht, M. (1999). Induction of interleukin-8 synthesis integrates effects on transcription and RNA degradation from at least three different cytokine/stress-activated signal transduction pathways. Manuscript in preparation.

Hou, X. S., Goldstein, E. S., and Perrimon, N. (1997). Drosophila Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. Genes and Development *11*, 1728-37.

Howe, L. R., Leevers, S. J., G'Omez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. Cell *71*, 335-42.

Hu, M. C., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T. H. (1996). Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes and Development *10*, 2251-64.

Huang, J.-D., Brady, S., Richards, B., Stenoien, D., Resau, J., Copeland, N., and Jenkins, N. (1999). Direct interaction of microtubule- and actin-based transport motors. Nature, 267-70.

Hughes, D. A., Ashworth, A., and Marshall, C. J. (1993). Complementation of byr1 in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase. Nature *364*, 349-52.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell *80*, 225-36.

Hutchison, M., Berman, K. S., and Cobb, M. H. (1998). Isolation of TAO1, a protein kinase that activates MEKs in stress-activated protein kinase cascades. Journal of Biological Chemistry *273*, 28625-32.

Ichijo, H., Nishida, E., Irie, K., ten, D.-P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90-4.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (1990). PCR protocols: a guide to methods and applications, M. A. Innis, ed. (San Diego: Academic Press).

Ip, Y. T., and Davis, R. J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Current Opinion in Cell Biology *10*, 205-19.

Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1994). Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. Science *265*, 1716-9.

Ishikawa, K., Nagase, T., Suyama, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998). Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Research *5*, 169-76.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233-43.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-54.

Johnson, L. N., Noble, M. E., and Owen, D. J. (1996). Active and inactive protein kinases: structural basis for regulation. Cell *85*, 149-58.

Johnson, R. S., van, L.-B., Papaioannou, V. E., and Spiegelman, B. M. (1993). A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. Genes and Development *7*, 1309-17.

Joneson, T., McDonough, M., Bar, S.-D., and Van, A.-L. (1996). RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science *274*, 1374-6.

Jordan, M. A., and Wilson, L. (1998). Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Current Opinion in Cell Biology *10*, 123-30.

Juo, P., Kuo, C. J., Reynolds, S. E., Konz, R. F., Raingeaud, J., Davis, R. J., Biemann, H. P., and Blenis, J. (1997). Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. Molecular and Cellular Biology *17*, 24-35.

Katoh, M., Hirai, M., Sugimura, T., and Terada, M. (1995). Cloning and characterization of MST, a novel (putative) serine/threonine kinase with SH3 domain. Oncogene *10*, 1447-51.

Katz, P., Whalen, G., and Kehrl, J. H. (1994). Differential expression of a novel protein kinase in human B lymphocytes. Preferential localization in the germinal center. Journal of Biological Chemistry *269*, 16802-9.

Kawakami, Y., Hartman, S. E., Holland, P. M., Cooper, J. A., and Kawakami, T. (1998). Multiple signaling pathways for the activation of JNK in mast cells: involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7. Journal of Immunology *161*, 1795-802.

Kawakami, Y., Miura, T., Bissonnette, R., Hata, D., Khan, W. N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S. E., Yao, L., Alt, F. W., and Kawakami, T. (1997). Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proceedings of the National Academy of Sciences of the United States of America *94*, 3938-42.

Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell *93*, 605-15.

Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996). HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. EMBO Journal *15*, 7013-25.

Koch, E. A., and Spitzer, R. H. (1983). Multiple effects of colchicine on oogenesis in Drosophila: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. Cell and Tissue Research *228*, 21-32.

Kracht, M., Truong, O., Totty, N. F., Shiroo, M., and Saklatvala, J. (1994). Interleukin 1 alpha activates two forms of p54 alpha mitogen-activated protein kinase in rabbit liver. Journal of Experimental Medicine *180*, 2017-25.

Krause, A., Holtmann, H., Eickemeier, S., Winzen, R., Szamel, M., Resch, K., Saklatvala, J., and Kracht, M. (1998). Stress-activated protein kinase/Jun N-terminal kinase is required for interleukin (IL)-1-induced IL-6 and IL-8 gene expression in the human epidermal carcinoma cell line KB. Journal of Biological Chemistry *273*, 23681-9.

Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. Nature *358*, 417-21.

Kyriakis, J. M., and Avruch, J. (1990). pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. Journal of Biological Chemistry *265*, 17355-63.

Kyriakis, J. M., and Avruch, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. Journal of Biological Chemistry *271*, 24313-6.

Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature *369*, 156-60.

Kyriakis, J. M., Brautigan, D. L., Ingebritsen, T. S., and Avruch, J. (1991). pp54 microtubule-associated protein-2 kinase requires both tyrosine and serine/threonine phosphorylation for activity. Journal of Biological Chemistry *266*, 10043-6.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell *87*, 519-29.

Lange-Carter, C. A., and Johnson, G. L. (1994). Ras-dependent growth factor regulation of MEK kinase in PC12 cells. Science *265*, 1458-61.

Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science *260*, 315-9.

Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyss'egur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. Journal of Biological Chemistry *271*, 20608-16.

Lawler, S., Cuenda, A., Goedert, M., and Cohen, P. (1997). SKK4, a novel activator of stress-activated protein kinase-1 (SAPK1/JNK). FEBS Letters *414*, 153-8.

Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998). Synergistic activation of SAPK/JNK1 by two MAP kinase kinases in vitro. Current Biology *8*, 1387-90.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature *371*, 346-7.

Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., and et al. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature *372*, 739-46.

Lee, J. D., Ulevitch, R. J., and Han, J. (1995). Primary structure of BMK1: a new mammalian map kinase. Biochemical and Biophysical Research Communications *213*, 715-24.

Lee, R. M., Cobb, M. H., and Blackshear, P. J. (1992). Evidence that extracellular signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. Journal of Biological Chemistry *267*, 1088-92.

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

Lehmann, R., and Nusslein, V.-C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. Cell *47*, 141-52.

Lenczowski, J. M., Dominguez, L., Eder, A. M., King, L. B., Zacharchuk, C. M., and Ashwell, J. D. (1997). Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. Molecular and Cellular Biology *17*, 170-81.

Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., and Pouyss'egur, J. (1993). Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. Journal of Cell Biology *122*, 1079-88.

Lin, A., Minden, A., Martinetto, H., Claret, F. X., Lange, C.-C., Mercurio, F., Johnson, G. L., and Karin, M. (1995). Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science *268*, 286-90.

Lindsley, D. L., Zimm, G. G. (1992). The genome of Drosophila melanogaster, D. L. Lindsley, ed. (San Diego: Academic Press).

Liu, Y., Gorospe, M., Yang, C., and Holbrook, N. J. (1995). Role of mitogen-activated protein kinase phosphatase during the cellular response to genotoxic stress. Inhibition of c-Jun N-terminal kinase activity and AP-1-dependent gene activation. Journal of Biological Chemistry *270*, 8377-80.

Lu, X., Perkins, L. A., and Perrimon, N. (1993). The torso pathway in Drosophila: a model system to study receptor tyrosine kinase signal transduction. Development Supplement *1993*, 47-56.

Luo, Z., Tzivion, G., Belshaw, P. J., Vavvas, D., Marshall, M., and Avruch, J. (1996). Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature *383*, 181-5.

MacKrell, A. J., Blumberg, B., Haynes, S. R., and Fessler, J. H. (1988). The lethal myospheroid gene of Drosophila encodes a membrane protein homologous to vertebrate integrin beta subunits. Proceedings of the National Academy of Sciences of the United States of America *85*, 2633-7.

Madhani, H. D., Styles, C. A., and Fink, G. R. (1997). MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell *91*, 673-84.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science *269*, 554-8.

Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997). MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature *385*, 540-4.

Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande, W.-G. F., and Ahn, N. G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. Science *265*, 966-70.

Mansour, S. J., Resing, K. A., Candi, J. M., Hermann, A. S., Gloor, J. W., Herskind, K. R., Wartmann, M., Davis, R. J., and Ahn, N. G. (1994). Mitogen-activated protein (MAP) kinase phosphorylation of MAP kinase kinase: determination of phosphorylation sites by mass spectrometry and site-directed mutagenesis. Journal of Biochemistry *116*, 304-14.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Current Opinion in Genetics and Development *4*, 82-9.

Marti, A., Luo, Z., Cunningham, C., Ohta, Y., Hartwig, J., Stossel, T. P., Kyriakis, J. M., and Avruch, J. (1997). Actin-binding protein-280 binds the stress-activated protein kinase (SAPK) activator SEK-1 and is required for tumor necrosis factor-alpha activation of SAPK in melanoma cells. Journal of Biological Chemistry *272*, 2620-8.

Martin Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M., and Martinez Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes and Development *12*, 557-70.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. Science *276*, 75-81.

Martinez Arias, A., and Bate, M. (1993). The Development of Drosophila melanogaster, Volume 2, A. Martinez Arias, ed. (Plainview, N.Y.: Cold Spring Harbor Laboratory Press).

Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y., and Nishida, E. (1995). Activation of protein kinase cascades by osmotic shock. Journal of Biological Chemistry *270*, 12781-6.

Matsuda, S., Kosako, H., Takenaka, K., Moriyama, K., Sakai, H., Akiyama, T., Gotoh, Y., and Nishida, E. (1992). Xenopus MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade. EMBO Journal *11*, 973-82.

Matsuda, S., Moriguchi, T., Koyasu, S., and Nishida, E. (1998). T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. Journal of Biological Chemistry *273*, 12378-82.

Mauviel, A., Chung, K. Y., Agarwal, A., Tamai, K., and Uitto, J. (1996). Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-beta in fibroblasts and keratinocytes. Journal of Biological Chemistry *271*, 10917-23.

McLachlan, A. D., and Karn, J. (1983). Periodic features in the amino acid sequence of nematode myosin rod. Journal of Molecular Biology *164*, 605-26.

Meier, R., Rouse, J., Cuenda, A., Nebreda, A. R., and Cohen, P. (1996). Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. European Journal of Biochemistry *236*, 796-805.

Meyer, B. E., Meinkoth, J. L., and Malim, M. H. (1996). Nuclear transport of human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. Journal of Virology *70*, 2350-9.

Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell *81*, 1147-57.

Misra-Press, A., Rim, C. S., Yao, H., Roberson, M. S., and Stork, P. J. (1995). A novel mitogen-activated protein kinase phosphatase. Structure, expression, and regulation. Journal of Biological Chemistry *270*, 14587-96.

Moodie, S. A., and Wolfman, A. (1994). The 3Rs of life: Ras, Raf and growth regulation. Trends in Genetics *10*, 44-8.

Moriguchi, T., Kawasaki, H., Matsuda, S., Gotoh, Y., and Nishida, E. (1995). Evidence for multiple activators for stress-activated protein kinase/c-Jun amino-terminal kinases. Existence of novel activators. Journal of Biological Chemistry *270*, 12969-72.

Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996). A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. Journal of Biological Chemistry *271*, 13675-9.

Moriguchi, T., Toyoshima, F., Masuyama, N., Hanafusa, H., Gotoh, Y., and Nishida, E. (1997). A novel SAPK/JNK kinase, MKK7, stimulated by TNFalpha and cellular stresses. EMBO Journal *16*, 7045-53.

Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985). Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature *313*, 241-3.

Na, S., Chuang, T. H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., and Danley, D. E. (1996). D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. Journal of Biological Chemistry *271*, 11209-13.

Nagata, K., Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N., and Hall, A. (1998). The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3. EMBO Journal *17*, 149-58.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-65.

Nakielny, S., Cohen, P., Wu, J., and Sturgill, T. (1992). MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. EMBO Journal *11*, 2123-9.

Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Levrero, M. (1997). Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science *275*, 200-3.

Nebreda, A. R., Hill, C., Gomez, N., Cohen, P., and Hunt, T. (1993). The protein kinase mos activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo. FEBS Letters *333*, 183-7.

Nishina, H., Fischer, K. D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E. A., Bernstein, A., Mak, T. W., Woodgett, J. R., and Penninger, J. M. (1997). Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. Nature *385*, 350-3.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998). ASK1 is essential for JNK/SAPK activation by TRAF2. Molecular Cell *2*, 389-95.

Noselli, S. (1998). JNK signaling and morphogenesis in Drosophila. Trends in Genetics *14*, 33-8.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li, W.-M., Richards, S., Dhein, J., Trauth, B. C., and et al. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. Journal of Biological Chemistry *267*, 10709-15.

Oehrl, W., Kardinal, C., Ruf, S., Adermann, K., Groffen, J., Feng, G. S., Blenis, J., Tan, T. H., and Feller, S. M. (1998). The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene *17*, 1893-901.

Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y., Kawaichi, M., Kohno, M., and Yoshida, O. (1995). Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. Cancer Research *55*, 4182-7.

Parry, D. A., Crewther, W. G., Fraser, R. D., and MacRae, T. P. (1977). Structure of alpha-keratin: structural implication of the amino acid sequences of the type I and type II chain segments. Journal of Molecular Biology *113*, 449-54.

Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075-80.

Perrimon, N., and Desplan, C. (1994). Signal transduction in the early Drosophila embryo: when genetics meets biochemistry. Trends in Biochemical Sciences *19*, 509-13.

Pierre, P., Scheel, J., Rickard, J. E., and Kreis, T. E. (1992). CLIP-170 links endocytic vesicles to microtubules. Cell *70*, 887-900.

Pombo, C. M., Bonventre, J. V., Molnar, A., Kyriakis, J., and Force, T. (1996). Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway. EMBO Journal *15*, 4537-46.

Pombo, C. M., Kehrl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T., and Kyriakis, J. M. (1995). Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. Nature *377*, 750-4.

Posada, J., and Cooper, J. A. (1992). Requirements for phosphorylation of MAP kinase during meiosis in Xenopus oocytes. Science *255*, 212-5.

Posada, J., Yew, N., Ahn, N. G., Vande, W.-G. F., and Cooper, J. A. (1993). Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro. Molecular and Cellular Biology *13*, 2546-53.

Posas, F., and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science *276*, 1702-5.

Price, M. A., Hill, C., and Treisman, R. (1996). Integration of growth factor signals at the c-fos serum response element. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences *351*, 551-9.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991). Phosphorylation of c-jun mediated by MAP kinases. Nature *353*, 670-4.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. Journal of Biological Chemistry *270*, 7420-6.

Raingeaud, J., Whitmarsh, A. J., Barrett, T., D'Erijard, B., and Davis, R. J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Molecular and Cellular Biology *16*, 1247-55.

Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L., Kyriakis, J. M., and Avruch, J. (1996). The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. Journal of Biological Chemistry *271*, 19025-8.

Rathmell, J. C., Townsend, S. E., Xu, J. C., Flavell, R. A., and Goodnow, C. C. (1996). Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. Cell *87*, 319-29.

Raz, E., and Shilo, B. Z. (1993). Establishment of ventral cell fates in the Drosophila embryonic ectoderm requires DER, the EGF receptor homolog. Genes and Development *7*, 1937-48.

Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157-61.

Reszka, A. A., Bulinski, J. C., Krebs, E. G., and Fischer, E. H. (1997). Mitogen-activated protein kinase/extracellular signal-regulated kinase 2 regulates cytoskeletal organization and chemotaxis via catalytic and microtubule-specific interactions. Molecular Biology of the Cell *8*, 1219-32.

Reszka, A. A., Seger, R., Diltz, C. D., Krebs, E. G., and Fischer, E. H. (1995). Association of mitogen-activated protein kinase with the microtubule cytoskeleton. Proceedings of the National Academy of Sciences of the United States of America *92*, 8881-5.

Riesgo Escovar, J. R., and Hafen, E. (1997a). Common and distinct roles of DFos and DJun during Drosophila development. Science *278*, 669-72.

Riesgo Escovar, J. R., and Hafen, E. (1997b). Drosophila Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. Genes and Development *11*, 1717-27.

Riesgo Escovar, J. R., Jenni, M., Fritz, A., and Hafen, E. (1996). The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. Genes and Development *10*, 2759-68.

Robinson, M. J., and Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. Current Opinion in Cell Biology *9*, 180-6.

Roffler-Tarlov, S., Brown, J. J., Tarlov, E., Stolarov, J., Chapman, D. L., Alexiou, M., and Papaioannou, V. E. (1996). Programmed cell death in the absence of c-Fos and c-Jun. Development *122*, 1-9.

Rossomando, A., Wu, J., Weber, M. J., and Sturgill, T. W. (1992). The phorbol ester-dependent activator of the mitogen-activated protein kinase p42mapk is a kinase with

specificity for the threonine and tyrosine regulatory sites. Proceedings of the National Academy of Sciences of the United States of America *89*, 5221-5.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso, L.-A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell *78*, 1027-37.

Rubin, G. M., and Spradling, A. C. (1983). Vectors for P element-mediated gene transfer in Drosophila. Nucleic Acids Research *11*, 6341-51.

Rudel, T., and Bokoch, G. M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science *276*, 1571-4.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO Journal *17*, 2596-606.

Salmeron, A., Ahmad, T. B., Carlile, G. W., Pappin, D., Narsimhan, R. P., and Ley, S. C. (1996). Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO Journal *15*, 817-26.

Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994). Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature *372*, 794-8.

Schaad, N. C., De, C.-E., Nef, S., Hegi, S., Hinrichsen, R., Martone, M. E., Ellisman, M. H., Sikkink, R., Rusnak, F., Sygush, J., and Nef, P. (1996). Direct modulation of calmodulin targets by the neuronal calcium sensor NCS-1. Proceedings of the National Academy of Sciences of the United States of America *93*, 9253-8.

Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L. S., Krauss, A., and Weber, M. J. (1998). MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science *281*, 1668-71.

Seger, R., Seger, D., Lozeman, F. J., Ahn, N. G., Graves, L. M., Campbell, J. S., Ericsson, L., Harrylock, M., Jensen, A. M., and Krebs, E. G. (1992). Human T-cell mitogen-activated protein kinase kinases are related to yeast signal transduction kinases. Journal of Biological Chemistry *267*, 25628-31.

Seger, R., Seger, D., Reszka, A. A., Munar, E. S., Eldar, F.-H., Dobrowolska, G., Jensen, A. M., Campbell, J. S., Fischer, E. H., and Krebs, E. G. (1994). Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. Journal of Biological Chemistry *269*, 25699-709.

Seimiya, H., Mashima, T., Toho, M., and Tsuruo, T. (1997). c-Jun NH2-terminal kinase-mediated activation of interleukin-1beta converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. Journal of Biological Chemistry *272*, 4631-6.

Shapiro, L., and Dinarello, C. A. (1995). Osmotic regulation of cytokine synthesis in vitro. Proceedings of the National Academy of Sciences of the United States of America *92*, 12230-4.

Sheu, Y. J., Santos, B., Fortin, N., Costigan, C., and Snyder, M. (1998). Spa2p interacts with cell polarity proteins and signaling components involved in yeast cell morphogenesis. Molecular and Cellular Biology *18*, 4053-69.

Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E., and Matsumoto, K. (1996). TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science *272*, 1179-82.

Sluss, H. K., Barrett, T., D'Erijard, B., and Davis, R. J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Molecular and Cellular Biology *14*, 8376-84.

Sluss, H. K., Han, Z., Barrett, T., Davis, R. J., and Ip, Y. T. (1996). A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes and Development *10*, 2745-58.

Staehling Hampton, K., Laughon, A. S., and Hoffmann, F. M. (1995). A Drosophila protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for dpp signaling. Development *121*, 3393-403.

Stark, K. A., Yee, G. H., Roote, C. E., Williams, E. L., Zusman, S., and Hynes, R. O. (1997). A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during Drosophila development. Development *124*, 4583-94.

Steinert, P. M., and Roop, D. R. (1988). Molecular and cellular biology of intermediate filaments. Annual Review of Biochemistry *57*, 593-625.

Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science *264*, 1463-7.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben, N.-Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727-36.

Su, Y. C., Han, J., Xu, S., Cobb, M., and Skolnik, E. Y. (1997). NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. EMBO Journal *16*, 1279-90.

Su, Y. C., Treisman, J. E., and Skolnik, E. Y. (1998). The Drosophila Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. Genes and Development *12*, 2371-80.

Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell *75*, 487-93.

Sun, H., Tonks, N. K., and Bar, S.-D. (1994). Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. Science *266*, 285-8.

Sundaram, M., and Han, M. (1996). Control and integration of cell signaling pathways during C. elegans vulval development. Bioessays *18*, 473-80.

Suter, B., Romberg, L. M., and Steward, R. (1989). Bicaudal-D, a Drosophila gene involved in developmental asymmetry: localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. Genes and Development *3*, 1957-68.

Suter, B., and Steward, R. (1991). Requirement for phosphorylation and localization of the Bicaudal-D protein in Drosophila oocyte differentiation. Cell *67*, 917-26.

Swat, W., Fujikawa, K., Ganiatsas, S., Yang, D., Xavier, R. J., Harris, N. L., Davidson, L., Ferrini, R., Davis, R. J., Labow, M. A., Flavell, R. A., Zon, L. I., and Alt, F. W. (1998). SEK1/MKK4 is required for maintenance of a normal peripheral lymphoid compartment but not for lymphocyte development. Immunity *8*, 625-34.

Takahashi, A., Musy, P. Y., Martins, L. M., Poirier, G. G., Moyer, R. W., and Earnshaw, W. C. (1996). CrmA/SPI-2 inhibition of an endogenous ICE-related protease responsible for lamin A cleavage and apoptotic nuclear fragmentation. Journal of Biological Chemistry *271*, 32487-90.

Takekawa, M., Posas, F., and Saito, H. (1997). A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. EMBO Journal *16*, 4973-82.

Takekawa, M., and Saito, H. (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell *95*, 521-30.

Tapon, N., Nagata, K., Lamarche, N., and Hall, A. (1998). A new rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-kappaB signalling pathways. EMBO Journal *17*, 1395-404.

Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J. S. (1996). Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. Journal of Biological Chemistry *271*, 27225-8.

Teramoto, H., Crespo, P., Coso, O. A., Igishi, T., Xu, N., and Gutkind, J. S. (1996). The small GTP-binding protein rho activates c-Jun N-terminal kinases/stress-activated protein kinases in human kidney 293T cells. Evidence for a Pak-independent signaling pathway. Journal of Biological Chemistry *271*, 25731-4.

Tewari, M., Beidler, D. R., and Dixit, V. M. (1995). CrmA-inhibitable cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein during Fas- and tumor necrosis factor-induced apoptosis. Journal of Biological Chemistry *270*, 18738-41.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N., and Jongens, T. A. (1993). A central role for microtubules in the differentiation of Drosophila oocytes. Development *118*, 1169-80.

Thummel, C. S., and Pirotta, V. (1992). New pCaSpeR P element vectors. Drosophila Information Service *71*, 150.

Tibbles, L. A., Ing, Y. L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J. R., and Lassam, N. J. (1996). MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO Journal *15*, 7026-35.

Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997). Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proceedings of the National Academy of Sciences of the United States of America *94*, 7337-42.

Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1999). The MKK7 gene enocdes a group of NH2-terminal kinase kinases. Molecular and Cellular Biology *19*, 1569-81.

Toyoshima, F., Moriguchi, T., and Nishida, E. (1997). Fas induces cytoplasmic apoptotic responses and activation of the MKK7-JNK/SAPK and MKK6-p38 pathways independent of CPP32-like proteases. Journal of Cell Biology *139*, 1005-15.

Treisman, J. E., Ito, N., and Rubin, G. M. (1997). misshapen encodes a protein kinase involved in cell shape control in Drosophila. Gene *186*, 119-25.

Tsai, S. F., Martin, D. I., Zon, L. I., D'Andrea, A. D., Wong, G. G., and Orkin, S. H. (1989). Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature *339*, 446-51.

Tsuda, L., Inoue, Y. H., Yoo, M. A., Mizuno, M., Hata, M., Lim, Y. M., Adachi, Y.-T., Ryo, H., Masamune, Y., and Nishida, Y. (1993). A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila. Cell *72*, 407-14.

Tung, R. M., and Blenis, J. (1997). A novel human SPS1/STE20 homologue, KHS, activates Jun N-terminal kinase. Oncogene *14*, 653-9.

Vaillancourt, R. R., Gardner, A. M., and Johnson, G. L. (1994). B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP. Molecular and Cellular Biology *14*, 6522-30.

Vojtek, A. B., and Cooper, J. A. (1995). Rho family members: activators of MAP kinase cascades. Cell *82*, 527-9.

Vojtek, A. B., and Hollenberg, S. M. (1995). Ras-Raf interaction: two-hybrid analysis. Methods in Enzymology *255*, 331-42.

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

Wahl, A. F., Donaldson, K. L., Fairchild, C., Lee, F. Y., Foster, S. A., Demers, G. W., and Galloway, D. A. (1996). Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. Nature Medicine *2*, 72-9.

Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998). Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. Journal of Biological Chemistry *273*, 4928-36.

Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., and Chien, K. R. (1998). Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. Journal of Biological Chemistry *273*, 5423-6.

Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. (1997). Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO Journal *16*, 1909-20.

Wassarman, D. A., Therrien, M., and Rubin, G. M. (1995). The Ras signaling pathway in Drosophila. Current Opinion in Genetics and Development *5*, 44-50.

Wen, W., Taylor, S. S., and Meinkoth, J. L. (1995). The expression and intracellular distribution of the heat-stable protein kinase inhibitor is cell cycle regulated. Journal of Biological Chemistry *270*, 2041-6.

Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998). Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes. EMBO Journal *17*, 3101-11.

Wharton, R. P., and Struhl, G. (1989). Structure of the Drosophila BicaudalD protein and its role in localizing the the posterior determinant nanos. Cell *59*, 881-92.

Whitmarsh, A., and Davis, R. J. (1998). Structural organization of MAP kinase signaling modules by scaffold proteins in yeast and mammals. Trends in Biochemical Sciences *23*, 481-85.

Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998). A mammalian scaffold complex that selectively mediates MAP kinase activation. Science *281*, 1671-4.

Whitmarsh, A. J., and Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. Journal of Molecular Medicine *74*, 589-607.

Winkler, D. G., Johnson, J. C., Cooper, J. A., and Vojtek, A. B. (1997). Identification and characterization of mutations in Ha-Ras that selectively decrease binding to cRaf-1. Journal of Biological Chemistry *272*, 24402-9.

Wu, J., Lau, L. F., and Sturgill, T. W. (1994). Rapid deactivation of MAP kinase in PC12 cells occurs independently of induction of phosphatase MKP-1. FEBS Letters *353*, 9-12.

Wu, X., Noh, S. J., Zhou, G., Dixon, J. E., and Guan, K. L. (1996). Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. Journal of Biological Chemistry *271*, 3265-71.

Wu, Z., Wu, J., Jacinto, E., and Karin, M. (1997). Molecular cloning and characterization of human JNKK2, a novel Jun NH2-terminal kinase-specific kinase. Molecular and Cellular Biology *17*, 7407-16.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326-31.

Xu, S., Robbins, D. J., Christerson, L. B., English, J. M., Vanderbilt, C. A., and Cobb, M. H. (1996). Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain. Proceedings of the National Academy of Sciences of the United States of America *93*, 5291-5.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science *270*, 2008-11.

Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature *372*, 798-800.

Yan, M., and Templeton, D. J. (1994). Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. Journal of Biological Chemistry *269*, 19067-73.

Yang, D., Tournier, C., Wysk, M., Lu, H. T., Xu, J., Davis, R. J., and Flavell, R. A. (1997). Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. Proceedings of the National Academy of Sciences of the United States of America *94*, 3004-9.

Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., Rinc'on, M., and Flavell, R. A. (1998). Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity *9*, 575-85.

Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rinc'on, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature *389*, 865-70.

Yao, Z., Diener, K., Wang, X. S., Zukowski, M., Matsumoto, G., Zhou, G., Mo, R., Sasaki, T., Nishina, H., Hui, C. C., Tan, T. H., Woodgett, J. P., and Penninger, J. M. (1997). Activation of stress-activated protein kinases/c-Jun N-terminal protein kinases (SAPKs/JNKs) by a novel mitogen-activated protein kinase kinase. Journal of Biological Chemistry *272*, 32378-83.

Yao, Z., Zhou, G., Wang, X. X., Brown, A., Diener, K., Gan, H., and Tan, T.-H. (1999). A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. Journal of Biological Chemistry *274*, 2118-25.

Young, P. E., Richman, A. M., Ketchum, A. S., and Kiehart, D. P. (1993). Morphogenesis in Drosophila requires nonmuscle myosin heavy chain function. Genes and Development *7*, 29-41.

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell *76*, 933-45.

Yuasa, T., Ohno, S., Kehrl, J. H., and Kyriakis, J. M. (1998). Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38. Journal of Biological Chemistry *273*, 22681-92.

Yujiri, T., Sather, S., Fanger, G., and Johnson, G. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science *282*, 1911-1914.

Zervos, A. S., Faccio, L., Gatto, J. P., Kyriakis, J. M., and Brent, R. (1995). Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein. Proceedings of the National Academy of Sciences of the United States of America *92*, 10531-4.

Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. Journal of Biological Chemistry *270*, 23934-6.

Zheng, C. F., and Guan, K. L. (1994). Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO Journal *13*, 1123-31.

Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995). Components of a new human protein kinase signal transduction pathway. Journal of Biological Chemistry *270*, 12665-9.

Zinck, R., Cahill, M. A., Kracht, M., Sachsenmaier, C., Hipskind, R. A., and Nordheim, A. (1995). Protein synthesis inhibitors reveal differential regulation of mitogen-activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. Molecular and Cellular Biology *15*, 4930-8.

VITA

Pamela M. Holland

University of Washington

Pamela Holland was born in Viña del Mar, Chile, May 30, 1963. Her family moved to the United States in 1970. She obtained a Bachelor's degree in Animal Physiology from the University of California at San Diego in June, 1985. After working in a neuro-endocrinology lab at the Salk Institute for Biological Studies in La Jolla, CA, she relocated to Northern California and worked at Cetus Corporation. During that time she worked predominantly on PCR research applications, and studied the biochemical properties of thermostable DNA polymerases. This led to the development of a PCR based assay now used for detection of specific DNA sequences (TaqMan™). In 1992 she began her graduate studies in the Biochemistry department at the University of Washington, and obtained a National Science Foundation Pre-Doctoral Fellowship award. Listed below are some publications related to TaqMan, as well as to work presented in this dissertation.

Holtmann, H., Winzen, R., Holland, P., Eickemeier, S., Hoffman, E. Wallach, D., Malinin, N., Resch, K., Cooper, J. & Kracht, M. (1999) Induction of interleukin-8 synthesis integrates effects on transcription and RNA degradation from at least three different cytokine/stress-activated signal transduction pathways. Manuscript in preparation.

Kawakami, Y., Hartman, S.E., Holland, P.M., Cooper, J.A. & Kawakami, T.(1998) Multiple Signaling Pathways for the Activation of JNK in Mast Cells: Involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7. *J. Immunol.*, 161 ,1795-802.

Finch, A., Holland, P., Cooper, J., Saklatvala, J. & Kracht, M. (1997) Selective activation of JNK/SAPK by interleukin-1 in rabbit liver is mediated by MKK7. *FEBS Letters*, 418, 144-148.

Holland, P.M., Suzanne, M., Campbell, J.S., Noselli, S. & Cooper, J.A. (1997) MKK7 Is A Stress-activated Mitogen-activated Protein Kinase Kinase Functionally Related to *hemipterous*. *J. Biol. Chem.*, 40, 24994-24998.

Holland, P.M. & Cooper, J.A. (1997) Identification and Characterization of MKKX, a Novel Mammalian MAP Kinase Kinase. NATO ASI Series, Vol. H102, Interacting Protein Domains, Their Role in Signal Energy Transduction; Ed. Ludwig Heilmeyer, Springer Verlag Berlin, Heidelberg.

Holland, P.M., Abramson, R.D., Watson, R. & Gelfand, D.H. (1991) Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5'-3' Exonuclease Activity of Thermus aquaticus DNA Polymerase, *Proc. Natl. Acad. Sci., USA*, 88, 7276-7280.

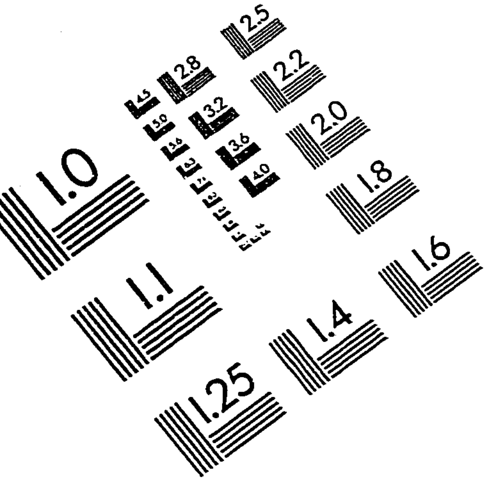
PATENTS:

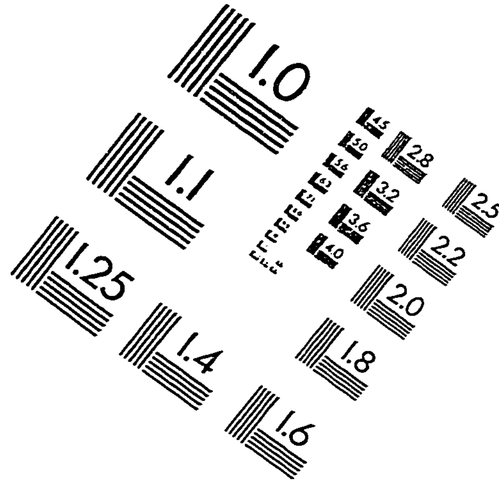
Gelfand, D.H., Holland, P.M., Saiki, R.K. & Watson, R., U.S. Patent No. 5,804,375. Reaction Mixtures for Detection of Target Nucleic Acids; Filed April 1995, Accepted September, 1998.

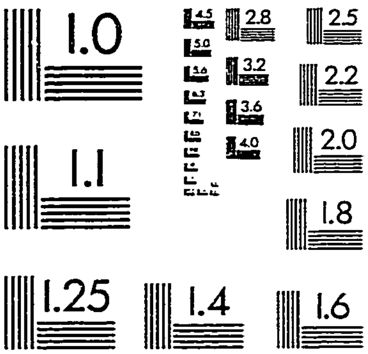
Gelfand, D.H., Holland, P.M., Saiki, R.K. & Watson, R., U.S. Patent No. 5,487,972. Nucleic Acid Detection by the 5'-3' Exonuclease Activity of Polymerases Acting on Adjacently Hybridized Oligonucleotides; Filed January 1993, Accepted January, 1996.

Gelfand, D.H., Holland, P.M., Saiki, R.K. & Watson, R., U.S. Patent No. 5,210,015. Homogeneous Assay System Using the Nuclease Activity of a Nucleic Acid Polymerase; Filed August 1990, Accepted April, 1993.

IMAGE EVALUATION TEST TARGET (QA-3)







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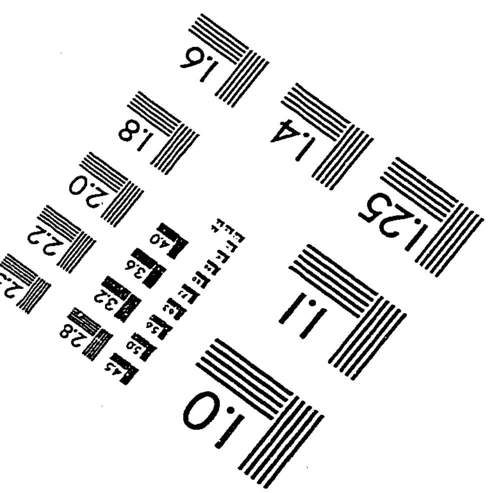

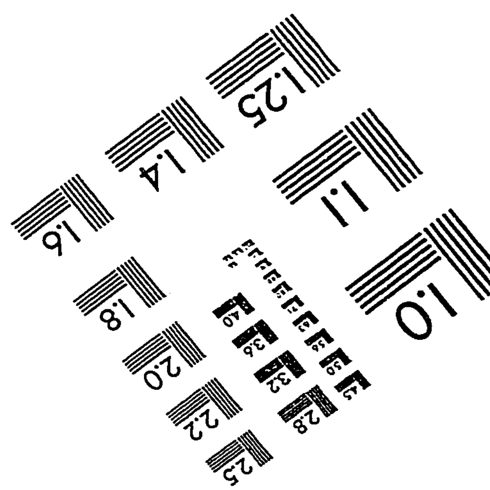

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989