

Heat Shock-Induced Signal Transduction in Hematopoietic Cells

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Abstract

Heat shock is a cellular stress that induces a characteristic set of signalling events, many of which are highly conserved throughout evolution. This includes the increased synthesis of a set of protein chaperones known as heat shock proteins (hsps) which facilitates survival during harsh environmental conditions. In eukaryotes, heat shock also leads to the stimulation of signalling pathways that negatively regulate translation via phosphorylation of the eukaryotic initiation factor 2 α and also activate protein kinases of the MAPK superfamily. In mammals, the JNK and p38 MAPK signalling cascades have been found to be activated in response to numerous forms of environmental stress, including heat shock, however the mechanisms governing their activation in response to these stresses are poorly understood.

In this work, the activation status of the JNK signalling cascade was investigated in a variety of cell types following heat shock treatment. Surprisingly, we have found that the degree of JNK activation that occurs in response to heat shock varies markedly in different murine cell types. Thus, while heat shock induced strong activation in macrophages and mast cells, a dramatically reduced activation was noted in T and B lymphocytes. Despite the lack of heat-induced JNK activation in murine lymphocytes, they could respond to heat shock in terms of the induction of the heat shock protein response which includes phosphorylation of the heat shock factor 1 (HSF1) and subsequent upregulation of the heat shock protein, Hsp70. In addition, we found that heat shock in murine lymphocytes, as in other cells, led to the phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α). These findings suggest that JNK signalling may be dispensable for the activation of the heat shock protein response and translational inhibition mediated by eIF2 α .

To help understand the mechanistic basis for the altered JNK signalling response in murine lymphocytes, the potential involvement of various proximal signalling events in the activation of JNK during heat shock was investigated. It was found that both of the JNK kinases, MKK4 and MKK7 were activated during heat shock in most cells although not in murine lymphocytes. We also discovered that, unlike all other cell types tested, murine lymphocytes failed to activate JNK in response to the ribosomal toxin anisomycin, suggesting that there may be a common mechanistic link between the effects of this compound and heat shock on JNK activation.

To address the possible biological significance of attenuated JNK signalling in murine lymphocytes in response to heat shock, we compared the induction of apoptosis in these cells with non-lymphoid cells. These studies indicated that murine lymphoid cells were, in fact, more susceptible to undergoing apoptosis as compared to non-lymphoid cells during heat shock. Thus, our evidence suggests that JNK activation is not likely to be the main factor influencing the progression of apoptosis in cells exposed to heat shock. We speculate that the attenuation of JNK signalling in murine lymphocytes during heat shock is due to the possible negative influence of this pathway on aspects of lymphocyte function during this form of stress or others that mimic its effects.

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Abbreviations

APC	antigen presenting cell
ASK1	apoptosis signalling kinase1
BCR	B cell receptor
cAMP	cyclic adenosine monophosphate
CSF	colony-stimulating factor
DAG	diacylglycerol
DLK	dual leucine zipper kinase
DNA	deoxyribonucleic acid
DRM	detergent-resistant membrane
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FACS	fluorescence activated cell sorter
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GCK	germinal centre kinase
GDP	guanosine diphosphate
GDS	guanine nucleotide dissociation stimulator
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLK	GCK-like kinase
GM-CSF	granulocyte/macrophage colony-stimulating factor
GRF	guanine nucleotide releasing factor
GRP	guanine nucleotide releasing protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPK	hematopoietic progenitor kinase
HRP	horseradish peroxidase
IL	interleukin
IPTG	isopropyl- β -D-thiogalactopyranoside
IRES	internal ribosomal entry site
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MEK	MAP/Erk kinase
MEKK	MEK kinase
MKK	MAP kinase kinase
MLK	mixed lineage kinase
mRNA	messenger ribonucleic acid
NF- κ B	nuclear factor kappa B
NP-40	nonidet P-40
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PdBu	phorbol-12,13-dibutyrate
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI	phosphatidylinositol
PI-3K	phosphatidylinositol-3 kinase
PAK	p21 activated kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
RA	Ras association
RBD	Ras binding domain
RPMI	Roswell Park Memorial Institute
TAK	TGF- β -activated kinase
TAO	thousand and one
TBS-N	Tris-buffered sodium chloride solution with Nonidet P-40
TCR	T cell receptor
WEHI	Walter and Eliza Hall Institute

Preface

Thesis Format

This thesis is organized into five chapters. Chapter one is an introduction that contains background information on the signalling systems and molecules that have been studied in this work. Chapter two outlines the experimental techniques and various reagents that have been used and has been organized into two sections – one for each chapter of experimental results. Chapters three and four include the experimental results obtained during this study along with a discussion of the relevant data. Chapter five contains an overall summary and discussion of the results as well as a discussion of the future experimental directions that could extend the findings presented here.

Publications obtained during the course of this thesis:

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Defining the involvement of p38 α MAP kinase in the production of anti and pro-inflammatory cytokines using an SB203580-resistant form of the kinase. *J. Biol. Chem.* (in press).

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CHAPTER I Introduction

1.1 Influence of heat stress from a biochemical perspective

Adaptation to hostile environments is a feature that is essential to survival of all organisms. One ubiquitous environmental parameter affecting all forms of life is temperature. Temperature has influenced the very evolution of protein structure itself so that within a given organism, the proteome has been optimized to function within the thermal confines of their particular habitat. As will be discussed in more detail later, the cell has evolved a number of biochemical processes that aid the cell in adaptation to changes in temperature. Although cellular responses have evolved to cope with decreases and with increases in environmental temperature, this discussion will focus on aspects involved with the latter – commonly known as heat shock. Drawing upon knowledge of bacteria and yeast, we will give a brief overview of how heat can influence biochemical processes and then provide a more detailed discussion of the heat-responsive mammalian signalling pathways that were the focus of study in this thesis.

Temperature has a profound affect on protein structure. High temperatures can disrupt intramolecular interactions within a polypeptide leading to loss of structure and hence function. Exposure of hydrophobic domains normally kept buried within the protein during thermal stress leads to localized unfolding and can result in the formation of protein aggregates within the cell (Pfeil, 1981; Tsai et al., 1998). Beyond a certain threshold, heat causes a complete denaturation of protein structure (Dill and Shortle, 1991). Given its susceptibility to heat, it is important for protein structure to possess a certain amount of inherent thermo-stability. This need, on the other hand, is balanced by the requirement for sufficient flexibility in protein structure in order to allow the allosteric

changes essential for protein function (Fields, 2001). Flexibility in structure, together with specific protein-binding chaperones (to be discussed later), is also important in allowing proteins to be translocated across various membranes within the cell during the sorting of proteins to designated organelles (Kang et al., 1990; McClellan et al., 1998).

The fine balance between stability and flexibility in protein structure is attributed to the specific folding patterns adopted by a protein. The folding arrangements are themselves governed by the primary amino acid sequence of a particular polypeptide. The colonization of organisms in the extreme ends of thermal environments on earth from polar seas to geothermal vents indicates the diverse range in structural stability that can be attained in proteins. Surprisingly, only subtle changes in amino acid sequence are necessary to mediate the increased thermostability of proteins found in thermophilic species. It is believed that these small number of amino acid substitutions contributes to the overall enhanced protein thermostability by increasing the number of salt bridges, hydrogen bonds and influencing protein-protein and protein-solvent interactions (Ladenstein and Antranikian, 1998; Scandurra et al., 1998).

Regardless of the temperature range for which the proteins of a particular organism have adapted to, the danger of increased temperature remains a common threat to survival. As a consequence, all organisms have evolved a suite of biochemical mechanisms to detect and respond to these thermal perturbations in order to facilitate survival during these transient periods of stress. What will follow is a brief overview of some of the signalling mechanisms that have been uncovered in bacteria and yeast that allow for the sensing of thermal stress and the regulation of appropriate responses in these organisms.

1.2 Heat-induced signalling responses in bacteria and yeast

As unicellular organisms living in environments subject to extreme fluctuations in temperatures, bacteria and yeast have developed a number of biochemical responses that have facilitated their survival in the face of this form of stress. Many responses to heat shock involve the triggering of kinase signalling cascades that regulate transcription. Other responses are independent of kinases. The initiation of many heat-responsive signalling cascades remains unknown however, in some cases experimental evidence has provided strong clues to their probable nature. Given the profound influence of temperature on protein stability, the induction of conformational changes in a sensory protein by heat is one obvious way for a cell to sense heat and trigger signalling. Indeed there is evidence for sensory molecules of this nature. However, heat can also influence the structure of other cellular components such as lipid membranes, RNA and DNA and there is evidence that temperature-induced perturbations in these molecules can also trigger biochemical responses.

One example of the role of thermo-sensitive proteins in the regulation of heat shock responses comes from the studies of bacterial membrane receptors which are perhaps more well-known for their role in chemotactic responses. For example, the Tar1 aspartate chemoreceptor mediates bacterial chemotaxis via stimulating the activity of the CheA histidine kinase, which in turn regulates aspects of flagellar motility through activation of CheY or CheB (Nara et al., 1991). The activation of this signalling response, which is an example of the characteristic bacterial two-component signalling systems (West and Stock, 2001), allows Tar1 to function as a sensor for temperature changes in the environment. This allows bacteria to modify their swimming patterns in response to thermal stress in their environment.

In the yeast *Saccharomyces cerevisiae* (which will be the yeast species discussed hereafter unless otherwise indicated), genetic screens to identify molecules critical for survival in response to heat shock have uncovered several classes of integral membrane proteins that seem to be important for this response. These include Sho1 (Maeda et al., 1995) and Sln1 (Ota and Varshavsky, 1993) two membrane proteins that function in yeast osmoregulation and Wsc1 (Verna et al., 1997) which is required for cell wall integrity. Each of these distinct membrane proteins couple to signalling cascades that are analogous to the MAPK cascades found in mammals (Gustin et al., 1998). To date, homologues of either, Sln1, Sho1 or Wsc1 have not been found in mammalian cells. In the case of Sho1 and Wsc1, it is believed that heat stress may mimic the effects of osmotic stress or mechanical stress on the cell wall or the cell membrane and thus lead to the triggering of the signalling cascades coupled to these sensory proteins (Gustin et al., 1998). The functional consequences of triggering these cascades are to adapt the cell to thermal stress. In the case of Wsc1, signalling activates genes important in cell wall biosynthesis to strengthen this structure - thus preventing cell lysis under heat shock conditions (Philip and Levin, 2001). The Sho1 response has been more commonly studied in the response to high osmolarity whereby activation of the mammalian p38 MAPK homolog, Hog1, mediates an increase in glycerol production which helps to restore the osmotic gradient in the cell (Brewster et al., 1993). In addition to its role in osmoregulation, glycerol can also serve as a protein stabilising agent. Thus it can be speculated that the activation of Hog1 and glycerol production may have an additional function in the protection of proteins during heat stress conditions. However, as discussed below, there are additional pathways that have evolved in yeast to respond to the challenge of heat on protein stability.

One of the most well-studied heat shock responses in organisms involves the induction of the specialized protein chaperones such as heat shock proteins (hsps). The induction of hsps during stressful events is conserved throughout all kingdoms of life (Feder and Hofmann, 1999). These proteins serve an important function in the cell during periods of stress by binding to damaged proteins and preventing their aggregation and also facilitate their repair through refolding reactions. The hsp chaperones induced in response to stress are related to a diverse set of protein chaperones in the cell that maintain protein homeostasis in the cell driving the necessary folding reactions, protecting native proteins from aggregating prior to adopting their fully folded state and associating with other proteins to help regulate their activities (Hartl and Hayer-Hartl, 2002).

The fundamental processes involved in the activation of hsp transcription are essentially the same in all organisms. This involves the heat-induced derepression of a transcription factor that regulates the synthesis of hsp genes essential for cell survival during periods of heat stress. The ability of this transcription factor to modulate its activity in direct response to heat stress, allows it to essentially function as the cell's internal thermometer. In the case of bacteria, the sigma factor, σ^{32} , also known as RpoH, is responsible for the induction of various hsps such as DnaK - the homolog of mammalian Hsp70 (Bukau, 1993). In a manner analogous to the proposed mode of induction of the homologous transcription factors in yeast and mammals, RpoH activity is believed to be triggered when hsps such as DnaK, which are bound to RpoH and suppress its activity, are sequestered away by binding to the unfolded proteins that accumulate during heat shock (Hurme and Rhen, 1998). Freed of its repression by these hsps, RpoH can then associate with transcriptional machinery and promote transcription of various hsp genes (see Fig. 1.1). Recently, the mechanism of transcriptional activation of another sigma

factor important for heat shock-induced responses was uncovered (Walsh et al., 2003). This concerns the sigma factor, σ^E , also known as RhoE, which is activated downstream of a set of proteases that function in the periplasmic space of bacteria. In this system, RhoE activity is repressed by the inner membrane RseA and RseB proteins. This repression is lost when RseA and RseB are cleaved by the DegS protease (with contribution by YaeL protease). Although the involvement of DegS in this process had been known for some time from genetic studies, its mode of activation was unclear.

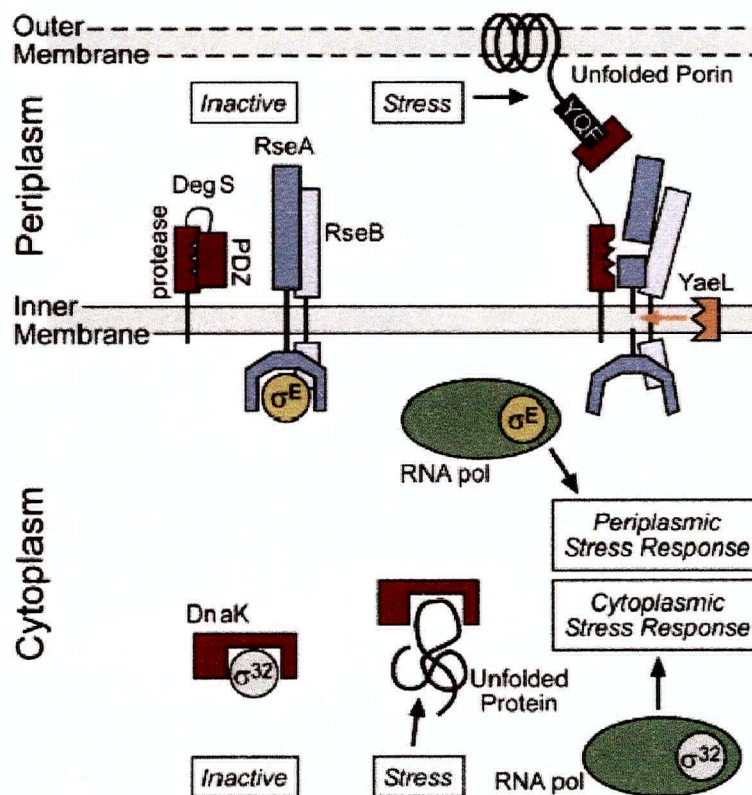


Figure 1.1 – Heat stress causes the activation of RpoH and RhoE sigma factors in bacteria. The illustration above outlines how heat stress leads to the activation of these bacterial sigma factors via two divergent mechanisms (Walsh et al., 2003). See text for details.

The mechanism uncovered by Walsh et al, reveals that a PDZ domain which normally represses DegS proteolytic activity, binds to the carboxy terminus of outer membrane porins (OMPs) when these domains become exposed during heat stress. The binding of the DegS PDZ domain to OMPs derepresses DegS proteolytic function and results in the cleavage of the RhoE repressors, RheA and RheB, allowing RhoE-mediated transcriptional events to occur (Walsh et al., 2003). Interestingly, a DegS-related protease has been shown to exist in mammals. This molecule, known as HtrA2, resides in the outer mitochondrial space and may play a role in transmitting stress responses in this compartment analogous to the role of DegS in the bacterial periplasm (Gray et al., 2000; Savopoulos et al., 2000).

In yeast, and all other eukaryotes, activation of hsp production is regulated by the transcription factor HSF1 (Sorger and Pelham, 1988). Through a mechanism analogous to that which activates the sigma factor RpoH, yeast HSF1 activity is believed to be triggered when associated hsps are titrated away as they bind to unfolded proteins generated by heat shock - thus allowing HSF1 to adopt an active conformation (Zou et al., 1998). HSF1 activation involves a transition from a repressed monomeric form to a trimeric form capable of site-specific DNA binding and transactivation abilities (Westwood et al., 1991). Further discussion of HSF1 will follow in our review of signalling events that participate in the mammalian response to heat shock. Studies in yeast have also indicated an equally important role for the activities of the zinc-finger transcription factors Msn2 and Msn4 in mediating stress-induced transcriptional events (Zahringer et al., 2000). It was shown that these transcription factors regulate a distinct set of genes apart from those activated by HSF1 (although there is some overlap) (Grably et al., 2002). One of the most important responses regulated by Msn2 and Msn4 involves the activation of biosynthetic pathways that mediate trehalose production in cells. Trehalose

is a disaccharide that has been shown to have remarkable properties in preventing protein aggregation in yeast and facilitating survival of yeast spores during harsh conditions (Ribeiro et al., 1997). Currently the pathway that activates Msn2 and Msn4 is not known however, it has been shown that the Ras-cAMP pathway antagonizes Msn2/Msn4-induced transcriptional events (Smith et al., 1998).

As already mentioned, proteins are not the only biological macromolecules that can be affected by heat stress. Studies from bacteria have provided some fascinating examples of how temperature can directly influence translation via its affect on RNA structure. Thus, it has been found that several bacterial mRNA species can, in essence, function as thermosensors due to the effects of secondary structure found in their 5 prime UTRs (Hurme and Rhen, 1998; Narberhaus, 2002). This property is mediated by the formation of stem-loop structures which can obstruct access of the ribosome to proper recognition sequences required for translation initiation. Elevation in temperature destabilizes the secondary structure and promotes translation of the mRNAs. This mode of regulation, for instance, has been shown to account for mRNA translation of a variety of bacterial heat shock proteins as well as their transcriptional regulator sigma factor, RpoH (Hurme and Rhen, 1998). Bypassing the need for transcription to rapidly produce proteins needed in response to stress may be one reason why this mechanism has evolved. It is not known whether a similar mode of regulation operates in eukaryotes.

Another thermosensitive biomolecule is DNA, the topology of which has been shown to be modulated by temperature. Changes in DNA supercoiling can affect the activity of certain promoters (Dorman et al., 1990). It is possible that such changes can influence the accessibility of various transcription factors and accessory molecules to particular

promoters, making these promoters, and the genes they regulate, temperature responsive.

The final example of temperature-responsive biological molecules we will discuss involves the role of lipid membrane structures in the cell. Studies in yeast have shown that modifying the ratio of saturated to unsaturated fatty acids in the cell membrane can influence the temperature at which heat shock proteins are induced (Horvath et al., 1998). Influencing membrane fluidity in thylakoids from cyanobacteria was also shown to directly affect the threshold of heat shock protein induction in response to thermal stress (Carratu et al., 1996). The conclusion drawn from these studies was that changes in membrane fluidity during heat stress can directly regulate signalling pathways such as those that regulate Hsp production.

1.3 Heat-responsive pathways in mammalian cells

The examples provided above highlight the multiplicity of sensors and signalling responses that can be activated by heat shock in bacteria and yeast. As unicellular organisms that often face harsh environments, their development of a diversity of signalling processes to sense and respond to noxious conditions was probably critical for their survival. Interestingly, many of the same signalling systems in these lower organisms can be found in mammalian cells, which are not likely to be exposed to the same diversity and magnitude of stresses. The most conserved among these signalling pathways are the MAPK signalling cascades. As in yeast, five distinct MAPKs have been identified in mammals. However, the complexity of MAPK pathways has grown tremendously in mammals with the coupling of the core signalling module, MAPKKK-MAPKK-MAPK, to a diverse array of receptors, GTPases, adaptor molecules and

scaffolds. In many cases, molecules critical for signalling responses in yeast have not been found in mammalian cells. Whether alternative signalling strategies have been evolved in mammals or whether their mammalian homologs still await discovery cannot be said. What follows is an overview of some of the major mammalian signalling molecules known to be activated in response to heat stress. A more detailed explanation of the JNK signalling pathway is provided since it was the primary focus of this work. However, we also provide background on the related p38 MAPK stress kinase pathway as well as a review of the mammalian HSF1-hsp signalling axis and the regulation of translation initiation via eIF2 α .

1.3.1 The cJun N-terminal kinase (JNK) signalling cascade

1.3.1.1 General introduction

The mammalian cJun N-terminal kinase (JNK) is a member of the mitogen activated protein kinase (MAPK) superfamily which also includes the p38 MAPK kinases (to be discussed later) and the extracellular regulated kinases (ERKs). Among the three closely related kinase subfamilies, JNK and p38 MAPK are commonly thought to be activated by environmental stresses while the ERK subfamily is often associated with proliferative stimuli. The mammalian JNK and p38 MAPK signalling cascades have been shown to be activated in response to all the common forms of environmental stress including oxidative, hyperosmotic, UV, ribosomal inhibitors, heavy metals, and of course heat stress (Derijard et al., 1994; Hibi et al., 1993; Jordanov et al., 1997; Raingeaud et al., 1995). Nevertheless, in mammals these signalling cascades also play a critical role in transmitting signals from a great number of physiological stimuli. In the hematopoietic

system for example, JNK and p38 MAPK have been found to be activated by proinflammatory cytokines, hematopoietic growth factors and ligation of the TCR, BCR and stimulation of Fc receptors (Avraham et al., 1998; Chen et al., 1996; Foltz et al., 1998; Ishiai et al., 1999; Rose et al., 1997; Su et al., 1994).

The hierarchical nature of signalling components in MAPK cascades has been remarkably conserved throughout evolution and thus molecules homologous to those found in the yeast pheromone and hyperosmotic stress signalling cascades can also be found in the mammalian MAPK signalling cascades. This conservation is most apparent in those molecules in the core MAPK-MAPKK-MAPKKK modules. The basic mechanism common to these pathways involves the serial activation of one set of kinases by those that function upstream, culminating in the phosphorylation of various protein substrates including transcription factors that mediate transcriptional responses as well as non-transcription factor targets which can modulate various biological processes.

In mammals, multiple isoforms of each MAPK family members exist. In the case of JNK, three separate genes have been identified, *jnk1/sapky*, *jnk2/sapk α* and *jnk3/sapk β* . Splicing of *jnk* mRNA generates at least 10 distinct JNK isoforms (Kyriakis and Avruch, 2001). Differential splicing at the 3' end of JNK mRNA leads to JNK proteins of either 46 or 54 kDa in size. Additional splicing occurs within the kinase domain of JNK1 and JNK2 mRNA. Thus, four distinct transcripts can be produced from each of the *jnk1* and *jnk2* genes, and at least two alternate transcripts can be produced from the *jnk3* gene. There is some evidence that the various JNK isoforms can differ in their substrate specificity (Gupta et al., 1996). Expression profiles indicate that JNK1 and JNK2 proteins are ubiquitous, while expression of JNK3 is restricted to the brain and testes. Like other MAPKs, JNK is activated by phosphorylation on threonine and tyrosine in the T-X-Y

motif found on the activation loop of the kinase (Kyriakis and Avruch, 1990; Kyriakis et al., 1991).

1.3.1.2 Signalling components of the JNK cascade

1.3.1.2.1 Signalling molecules functioning upstream of JNK

Phosphorylation of JNK is mediated by two distinct kinases, MKK4 and MKK7. These are known as dual-specificity kinases in that they can phosphorylate either threonine or tyrosine residues. These kinases are each encoded for by single genes, although

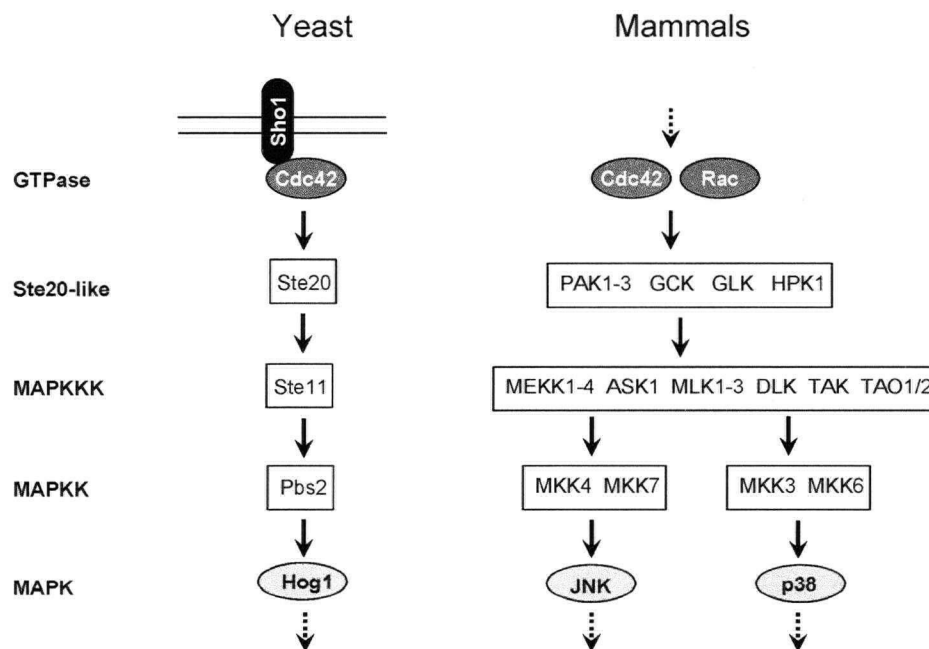


Figure 1.2 Signalling molecules of the mammalian JNK and p38 MAPK cascade and homologous molecules from the *Saccharomyces cerevisiae* Hog1 pathway. The core signalling module of mammalian stress activated kinases is shown. Omitted are the numerous downstream transcription factors and protein substrates of the MAPKs and in the case of the mammalian pathway the diverse numbers of receptor types that couple to this pathway along with various adaptor molecules. See page viii for abbreviations.

splicing events lead to the generation of multiple isoforms. The kinases responsible for phosphorylation and activation of MKK4 and MKK7 are known as the MAPKKKs. A large number of MAPKKKs have been identified (at least 12) including MEKK1-4, ASK1, TAK1, MLK1-3, DLK, and TAO1-2 (reviewed by (Kyriakis and Avruch, 2001). Many of these MAPKKKs have been shown to couple directly to small GTPase such as Ras, Rac and Cdc42 via a specialized Ras or Cdc42/Rac interacting binding (CRIB) domain. However, they can also themselves be activated by phosphorylation by another class of kinases related to yeast Ste20 that can also couple to GTPases. These include the GCK and PAK serine/threonine kinases. The large number of signalling molecules in the top tiers of the JNK cascade presumably allows for the integration of multiple, distinct proximal stimuli. One study has shown that overlapping sets of MAPKKKs are required for JNK activation by some stress responses, while other stresses utilize specific MAPKKKs upstream of JNK (Chen et al., 2002a).

What are the signalling events upstream of MAPKKKs and Ste20-related kinases that lead to their activation? As mentioned before, both classes of kinases can couple to small GTPases Ras, Rac and Cdc42. Overexpression of dominant active versions of these GTPases activates JNK, while overexpression of dominant negative versions can prevent JNK activation by certain stimuli (Brenner et al., 1997; Coso et al., 1995; Minden et al., 1995; Teramoto et al., 1996). Signalling to the MAPKKKs and Ste20-related kinases can also occur independent of GTPases, instead relying on adaptor molecules that link to various receptor systems. For example, the JNK agonists TNF and IL-1 utilize TRAF2 and TRAF6 adaptor molecules respectively, to couple to their distinct receptors. Knockout studies and deletion of the domains that mediate receptor interaction in TRAFs have shown that they are essential for JNK activation in response to both IL-1 and TNF (Naito et al., 1999; Yeh et al., 1997).

1.3.1.2.2 Scaffold molecules for the JNK cascade

Studies in yeast had shown that the function of pheromone signalling depended not only on essential kinases in the pathway but also on a scaffold molecule Ste 5, which has the ability to bind multiple members within the pathway simultaneously (Choi et al., 1994; Kranz et al., 1994; Marcus et al., 1994). Pbs2, the yeast homolog of MKK4, has a dual function serving both as a kinase in the hyperosmolarity response, and as a scaffold for other kinases in this signalling cascade including Hog1, the p38 MAPK homolog (Park et al., 2003). Thus, scaffold proteins serve an important function in facilitating the efficiency and specificity of signaling responses by bringing together various pathway members into distinct signalling complexes. Like its yeast homolog Pbs2, mammalian MKK4 has also been shown to possess a limited amount of scaffold activity in that it recruits the MAPKKK, MEKK1 into a signalling complex (Xia et al., 1998). However, other scaffold proteins in mammalian MAPK signalling systems were not identified until relatively recently. Thus, yeast-two hybrid experiments have identified a family of JNK interacting proteins (JIP1-3) which have been shown to selectively bind other signalling molecules including MKK7, and the MAPKKK MLK3 and the Ste20-related kinase HPK1 (Dickens et al., 1997; Ito et al., 1999; Kelkar et al., 2003; Kelkar et al., 2000; Xia et al., 1998; Yasuda et al., 1999a). Expression of JIP2 and JIP3 is localized to the brain, while JIP1 is more ubiquitously expressed. In addition to the JIP family, JNK scaffold function has also been proposed for the 280-kDa actin binding protein-280 (ABP-20) and the G-protein coupled receptor kinase substrate beta-arrestin2 (Marti et al., 1997; McDonald et al., 2000). The ability of various JNK scaffolds to assemble only a subset of components of the JNK signalling cascade, suggests that these scaffolds may link JNK activation to specific upstream stimuli. The regulated expression of scaffold members could also

influence specificity in JNK signalling by sequestering and thereby limiting the activation of different pathway members in response to certain stimuli.

1.3.1.2.3 Negative regulation of JNK signalling - phosphatases

Activation of the JNK pathway is counterbalanced by the action of phosphatases. The influence of JNK phosphatase activity mediates the rapid decline in the kinetics of JNK kinase activity noted after many stimuli. Inhibition of the basal level of JNK phosphatase activity in a cell via okadaic acid, an inhibitor of PP1 and PP2A serine/threonine phosphatases, or sodium vanadate, a general tyrosine phosphatase inhibitor, results in strong JNK activity (Cano et al., 1994). This indicates the importance of JNK phosphatases in controlling activation of the JNK pathway under basal conditions. A number of dual specificity phosphatases have been proposed to function as JNK phosphatases by virtue of their selective inactivation of this pathway upon overexpression. These include M3/6, MKP5, MKP7 and MKP1 (Muda et al., 1996; Tanoue et al., 1999; Tanoue et al., 2001). The regulation of JNK phosphatase activity is poorly understood. There is some evidence that specific stimuli can result in the elevated expression of certain phosphatases and thereby repress JNK activity (Byon et al., 2001; Chen et al., 2002a; Ishibashi et al., 1994). Studies have also suggested that recruitment of phosphatases to specific JNK scaffolds can serve to regulate the activity of this pathway (Willoughby et al., 2003). Importantly, one study has proposed that phosphatase activity can be negatively affected by heat shock and that this mechanism may account for JNK activation during this form of stress (Meriin et al., 1999). Experiments that were conducted in this study involved testing the validity of this model.

1.3.1.2.4 Substrates of JNK

Like other members of the MAP kinase family, JNK regulates the activity of numerous transcription factors. JNK was first identified as an activity that phosphorylated cJun on two serine residues within its N-terminal transactivation domain (Derijard et al., 1994; Hibi et al., 1993; Kyriakis and Avruch, 1990; Kyriakis et al., 1991). Since then JNK has been shown to also phosphorylate other transcription factors including JunD, ATF2, Elk1, Sap1a, NFAT4, NFATc1 and p53 (Chow et al., 2000; Chow et al., 1997; Chung et al., 2000; Fuchs et al., 1998; Gupta et al., 1995; Srivastava et al., 1999; Wang and Friedman, 2000). Jun family members can homodimerize or form heterodimers with either Fos or ATF2 to form the AP-1 transcriptional complex. Transcription of a variety of stress-responsive genes such as those encoding IL-1, IL-2, CD40, CD30, TNF and Jun itself, can be activated by AP-1-mediated transcription. Phosphorylation of NFAT4 by JNK has been shown to prevent its nuclear localization and thus functions as a negative regulator of this transcription factor (Chow et al., 1997). The functional significance of JNK phosphorylation of the p53 tumour suppressor is not fully known. One study has suggested that JNK-mediated p53 phosphorylation can stabilize and prevent ubiquitin-mediated degradation of this transcription factor (Fuchs et al., 1998). JNK is likely to have other cellular substrates in addition to transcription factors. Recent studies have shown that the anti-apoptotic molecule Bcl-2 and the related pro-apoptotic BH-3 only proteins, Bim and Bmf, may be phosphorylated by JNK (Deng et al., 2001; Lei and Davis, 2003). However, the significance of this has yet to be fully explored.

1.3.1.3 Biological functions of the JNK signalling pathway

1.3.1.3.1 Role of JNK in development

Studies from animals deficient in components of the JNK signalling pathway have revealed an importance for this pathway in developmental processes. While deletion of either *jnk1*, *jnk2*, or *jnk3* alone in mice does not markedly affect normal development, ablation of both *jnk1* and *jnk2* - which are the only JNK isoforms expressed in the majority of tissues - resulted in death in utero (Kuan et al., 1999; Sabapathy et al., 1999). These results indicate a degree of functional redundancy amongst the different JNK isoforms. In contrast, deletion of either of the two upstream JNK kinases MKK4 or MKK7 resulted in a lethal phenotype (Nishina et al., 1997; Tournier et al., 2001; Yang et al., 1997a). In the case of deletion of *mkk4*, one report indicated that the embryonic lethality was likely to be caused by an increase in the levels of apoptosis in the liver (Ganiatsas et al., 1998). The *mkk7* deficient mice have not been fully characterized with respect to the nature of the development abnormalities. The fact that neither MKK4 nor MKK7 can compensate for the absence of the other during development, indicates that these molecules have non-overlapping functions. It may be the case that MKK4 and MKK7 have unique sets of substrates other than JNK. Alternatively, it is possible that during development various tissues may express only one of these two JNK activators so that ablation of this gene cannot be compensated for by the other JNK kinase.

Studies in *Drosophila* have shown that the JNK signalling pathway is required in these organisms for normal embryonic development. In embryos deficient in JNK signalling components, the normal movement of epithelial cells across the exposed dorsal surface

is impaired, leaving an open hole in the dorsal epidermis – and generates the “dorsal open” phenotype. Genetic ablation of the *Drosophila* homologues of Rac, CDC42, MLK, MKK4, MKK7 and JNK (dRac, dCDC42, slipper, dMKK4, hemipterous, and basket respectively) all result in a dorsal open phenotype (Gallo and Johnson, 2002; Sluss et al., 1996). The loss of JNK in the nematode *C.elegans* has a surprisingly subtle phenotype, involving only a slight perturbation in the normal sinusoidal wave-like pattern of locomotion noted in these animals (Villanueva et al., 2001). The variation noted in phenotypes across different species lacking JNK or other components in this pathway presumably reflects the multiple roles for JNK in various cellular processes.

1.3.1.3.2 Role of JNK in the immune system

Much interest has been focused on the role of JNK in the immune system since it has been found that this pathway is activated in response to a vast array of immunomodulatory receptors, including those specific for the TCR, BCR, FcR and those specific to many hematopoietic growth factors and proinflammatory cytokines. While previous studies in T cells defective in either JNK1 or JNK2 have reported defects in the differentiation of CD4⁺ cells into the TH1 subset (Dong et al., 1998; Yang et al., 1998) Reconstitution of Rag1 knockout mice with *jnk1/2* deficient ES cells revealed that T and B cell development can occur normally in the absence of JNK protein expression (Dong et al., 2000). Analysis of *jnk1/2* deficient T cells from these animals indicated an increased proliferative capacity of these cells over wildtype T cells following TCR stimulation suggesting that JNK may negatively regulate growth in T cells. Furthermore, JNK null naive T cells, when induced to differentiate into effector cells in the presence of wildtype APCs and IL-2, showed an increased production of TH2 cytokines such as IL-4, IL-5, IL-10 and IL-13 indicating that JNK normally plays a role in

suppressing TH2 cell development. These data correlate with the observations made in the analysis of T cells deficient in either JNK1 or JNK2 which were found to preferentially differentiate into TH2 effector cells.

Studies of MKK4 deficient T cells revealed a role for this kinase in the protection of double positive thymocytes from Fas-mediated apoptosis (Nishina et al., 1997). Thus, the JNK pathway may promote survival of this population of T cells although others failed to observe this effect (Swat et al., 1998). A thorough analysis of cell function in B cells lacking jnk1/2 has not yet been described.

1.3.1.3.3 Role of JNK in apoptosis

A great number of stimuli that can potentiate the process of apoptosis have also been shown to cause the activation of JNK. These include cell stimulation by death receptor agonists such as TNF and Fas and a wide variety of environmental stresses which include among others UV, oxidative and osmotic stress and heat shock (Adler et al., 1996; Chan et al., 1997; Mendelson et al., 1996; Natoli et al., 1997; Rosette and Karin, 1996; Zanke et al., 1996a). In the case of JNK3, which is predominantly expressed in the brain, studies of knockout mice indicated that this particular JNK isoform was required for neuronal apoptosis induced by kainic acid - a glutamate receptor agonist (Yang et al., 1997b). The investigation of JNK activity in the role of apoptosis has recently been augmented by the development of JNK1 and JNK2 double-deficient mice – the two isoforms of JNK which are expressed in the majority of tissues. Mice lacking jnk1 and jnk2 are embryonic lethal however, fibroblast cultures can be prepared from embryos (Kuan et al., 1999). Fibroblasts obtained from jnk1/jnk2 knockout mice are devoid of all

JNK protein since the remaining JNK isoform, JNK3, is not expressed in this cell type. The results from the limited analysis of these cells thus far have indicated that JNK is required for apoptosis in response to UV, anisomycin and DNA-damaging agents, but is not needed for apoptosis induced by Fas (Tournier et al., 2000). Identical findings have been reported in the fibroblasts obtained from mkk4/mkk7 double knockout animals (Tournier et al., 2001). Thus, it may be the case that JNK is specifically required for induction of the so-called "intrinsic" or "mitochondria-mediated" form of apoptosis while not affecting the "extrinsic" or "receptor-mediated" form. A more detailed analysis will be required to establish this paradigm. Whether JNK is required for apoptosis initiated in response to heat shock in these cells has not yet been reported.

In the case of UV irradiation, JNK was required to cause cytochrome c release and subsequent caspase activation - indicating a role for JNK upstream of mitochondria related events. It has been shown that cytochrome c release from mitochondria during apoptosis requires the presence of either Bax or Bak - two members of the pro-apoptotic Bcl-2 related family (Shen et al., 2001). Although the precise mechanism remains unclear, Bax and Bak are believed to antagonize the function of Bcl-2 and Bcl-XL which leads to increased mitochondrial membrane permeability and cytochrome c efflux. Bax and Bak, can themselves, be modulated by a class of proteins termed BH3-only proteins (which possess only one of the three Bcl-2 homology domains found in Bcl-2 family members). How JNK activity influences the function of these processes has not been fully investigated. A recent report has suggested that JNK can phosphorylate the BH3-only proteins Bim and Bmf which may affect their localization and the subsequent promotion of apoptosis via association with Bax and Bak in the mitochondrial outer membrane (Lei and Davis, 2003). Whether JNK phosphorylation is a key event in modulating the function of each of the ten different BH3-only proteins identified thus far

remains to be determined. It is also unclear whether the requirement of JNK in fibroblasts for the apoptotic stimuli tested also applies to other cell types. Given that analysis of the *jnk1/jnk2* deficient compound mutant mice showed that *jnk* deficiency could lead to both increased and decreased rates of apoptosis within different regions of the brain (Kuan et al., 1999), it seems likely that other tissues may have different requirements for JNK activity during apoptotic processes.

1.3.1.3.4 Role of JNK in cell survival

The studies described above using *jnk1/2* deficient murine embryonic fibroblasts have shown convincing data that JNK is required for apoptosis initiated by a subset of apoptotic stimuli. However, evidence from other studies has suggested that JNK signalling may also have anti-apoptotic functions. For example, in other experiments involving murine embryonic fibroblasts, cells that were deficient in either *jnk1* or *jnk2* were found to be more susceptible to apoptosis than wildtype cells following exposure to osmotic stress or TNF indicating a protective function of JNK under these stress conditions (Hochedlinger et al., 2002). Further evidence for a pro-survival function of JNK is evident in observations of particular regions of the brains of *jnk1/ jnk2* deficient embryos. Thus although decreased rates of apoptosis were observed in some areas (hindbrain), in others (forebrain) the lack of JNK1 and JNK2 proteins correlated with increased rates of apoptosis (Kuan et al., 1999; Sabapathy et al., 1999). Thus, it seems that the specific cellular context is important in determining the outcome of JNK signalling. This is supported by studies involving *mkk4*-deficient embryos, where it seems that the lack of MKK4 renders these embryos extremely susceptible to apoptosis in the liver (Ganiatsas et al., 1998; Nishina et al., 1999). A similar phenotype was

reported in mice deficient in *cjun*, suggesting that the MKK4-JNK-cJun axis is critical for survival of hepatocytes during development (Hilberg et al., 1993).

1.3.2 The p38 MAPK signalling pathway

The mammalian p38 MAPK is most similar to the Hog1 protein from the yeast *Saccharomyces cerevisiae* which functions in the hyperosmolarity response. In these yeast, activation of Hog1 results in the production of intracellular glycerol that helps to reduce the osmotic gradient faced by the cell (Albertyn et al., 1994). In mammals, the p38 MAPK cascade is activated by many of the same types of environmental stresses and physiological stimuli that have been shown to potentiate JNK activity (Raingeaud et al., 1995). However, despite the shared overlap in activating stimuli, p38 MAPK mediates distinct processes apart from other MAPKs. These include a role in cytoskeletal remodeling, and distinct transcriptional and translational regulatory effects.

Mammalian p38 MAPKs are encoded by four distinct genes termed p38 α , p38 β , p38 γ and p38 δ . Expression of p38 α and p38 δ MAPK isoforms is ubiquitous while the p38 β and p38 γ MAPK isoforms are more restricted in their tissue distribution (Jiang et al., 1997a). As a MAPK superfamily member, the p38 MAPK signal transduction cascade involves the same system of upstream kinase networks involved in JNK and ERK signalling. In this regard, p38 MAPK is activated by two distinct dual specificity kinases, MKK3 and MKK6 (Han et al., 1996; Han et al., 1997; Raingeaud et al., 1996). These phosphorylate the threonine and tyrosine residue in the T-G-Y motif within the activation loop leading to kinase activity. Upstream of MKK3 and MKK6, a large amount of overlap in signalling molecules occurs with kinases shown to be involved in the JNK signalling

cascade. The dual involvement of these upstream kinases as well as the GTPases and adapter molecules that lie above in both the JNK and p38 MAPK signalling pathways, is suggested by the observation that the majority of proximal signals that activate JNK result in the concomitant activation of p38 MAPK.

Substrates of the p38 MAPK also show some overlap with the JNK cascade in that ATF2, a component of the AP1 transcriptional complex, is also a target of p38 MAPK (Jiang et al., 1996). The influence of p38 MAPK on AP1 transcriptional activity allows for the regulation of various AP1-responsive genes. These include TNF and IL-1, two proinflammatory cytokines which were shown to be induced in response to endotoxin via p38 MAPK-dependent signalling (Lee et al., 1994). The involvement of p38 MAPK in various signalling pathways has been demonstrated via the use of a relatively specific inhibitor SB203580 which binds within the ATP pocket of p38 α and p38 β MAPK isoforms. Another transcription factor target of the p38 MAPK pathway is CREB homologous protein (CHOP) (Wang and Ron, 1996). CHOP mediates the repression of genes downstream of cAMP but can also function to activate other stress-induced genes. Importantly, CHOP can induce the process of growth arrest in cells following exposure to genotoxic agents thus shutting down DNA synthesis so that repair processes can be initiated. In addition to transcription factors, substrates of p38 MAPK include various protein kinases. Thus, MAPKAP-K2, MAPKAP-K3 and PRAK are a class of serine/threonine kinases, the activity of which is directly regulated by phosphorylation by the p38 α and p38 β MAPK isoforms (Ben-Levy et al., 1995; McLaughlin et al., 1996; New et al., 1998). MAPKAP2/3 can in turn phosphorylate the small heat shock protein Hsp27 resulting in changes in its oligomeric properties and its association with the actin cytoskeleton. This redistribution of Hsp27 to the cytoskeleton is believed to mediate the reorganization of F-actin into stress fibers and affect cell motility (Huot et al., 1997). An

additional role of the p38 MAPK pathway concerns its affect on protein translation. In this regard, activation of the p38 MAPK pathway by various stimuli results in the phosphorylation of the p38 MAPK substrate PHAS-I, a negative regulator of the eIF-4F translation initiation complex (Vries et al., 1997). Phosphorylation of PHAS-I inhibits its binding to the eIF-4F complex, and thus promotes translation. Another influence of p38 MAPK signalling on translation is via phosphorylation of MNK1/2. Activation of this kinase by p38 MAPK results in MNK1/2-mediated phosphorylation of eIF-4E (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). Phosphorylation of eIF-4E increases its affinity for the 5' cap structure of various mRNAs thus resulting in an overall enhancement of the translation process.

1.3.3 The heat shock protein stress response

As mentioned previously, the activation of heat shock protein synthesis during periods of stress is a response that occurs in all organisms. This is due to the fact that proper protein structure cannot be maintained beyond certain thresholds in temperature. Elevated temperatures unfold domains within proteins that often expose hydrophobic stretches which tend to promote aggregation of proteins within the cell. Aside from the resultant loss of function of the proteins within these aggregates, these structures themselves have cytotoxic effects and are believed to account for the neurotoxicity observed in the human diseases Alzheimer's and Huntington's (Cowan et al., 2003; Liu, 1998; Liu et al., 2000; Merienne et al., 2003; Nishitoh et al., 2002; Yasuda et al., 1999b). The mechanism which has evolved to deal with unfolded proteins involves the increased production of protein chaperones, also known as heat shock proteins. These proteins along with a diverse class of related chaperones have an essential role in the cell in

promoting the folding of nascent polypeptides, the stabilization and regulation of mature proteins and the repair or degradation of damaged proteins (Hartl and Hayer-Hartl, 2002).

Heat shock proteins that are upregulated by stress in mammalian cells include those of the Hsp25, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 molecular weight families. Of these, Hsp70 is the most studied and perhaps has the most essential role in the response to thermal stress. Overexpression of Hsp70 alone in cells is sufficient to protect cells from normally lethal doses of heat and other forms of stress (Mosser et al., 1997; Park et al., 2000). This phenomenon, known as thermotolerance, is attributed to the actions of Hsp70 and other chaperones in the prevention of excessive stress-induced protein damage. In the case of mammalian Hsp70 as with its bacterial homologue DnaK, this protective effect stems from the ability of these chaperones to recognize short stretches of hydrophobic amino acids that are exposed in unfolded polypeptides (Fourie et al., 1994; Rudiger et al., 1997). Shielding of hydrophobic patches in its substrates prevent protein-protein aggregation and stabilizes protein structure. In addition, Hsp70, along with other inducible hsps, have ATPase activity, which together with regulatory cofactors, allows them to facilitate the refolding of their protein substrates. In the case of Hsp70, binding to unfolded substrates is stabilized by its association with Hsp40, which maintains Hsp70 in an ADP-bound state that allows the peptide binding cleft of Hsp70 to "clamp down" on its substrate (Laufen et al., 1999). Exchange of ADP for ATP which can be promoted by the binding of additional cofactors opens this clamp and releases the substrate. Through multiple rounds of substrate binding and release, Hsp70 can eventually refold a protein into its correct conformation.

The induction of Hsp70 and other inducible hsp is regulated by the transcription factor HSF1. Monomeric HSF1 resides in the cytoplasm of unstressed cells complexed with Hsp70 and Hsp90 chaperones (Zou et al., 1998). During periods of heat stress or other proteotoxic stresses, HSF1 forms a trimeric structure, undergoes a significant degree of phosphorylation and accumulates in the nucleus at characteristic sites known as stress-granules (Cotto et al., 1997; Holmberg et al., 2000). These events result in the potentiation of HSF1 transcriptional activity leading to the increased synthesis of critical hsp such as Hsp70. In animals lacking HSF1, heat shock fails to induce Hsp70 production and the phenomenon of thermotolerance is lost (McMillan et al., 1998). Thus, despite the presence of two additional HSF family members in mammals - HSF2 and HSF4 - it seems that HSF1 is the main regulator of stress-inducible Hsp synthesis in cells. The regulation of HSF1 activity is controlled at multiple levels. Conversion of HSF1 monomers to a trimer complex is thought to be due to the loss of monomer bound Hsp70 and Hsp90 as these hsp associate with the rising number of unfolded proteins that accumulate in the cytosol during heat shock. This mode of activation is similar to that proposed for bacterial HSF-like sigma factor RpoH. The ability of HSF1 to form trimers during stress has also been shown to occur in the absence of hsp by heating purified HSF1 in vitro (Larson et al., 1988; Zhong et al., 1998). Regardless of the mechanism involved, HSF1 remains one of the only known mammalian proteins identified thus far that can function as a thermosensor. Phosphorylation of HSF1 has been shown to play a role in modulating its activity (Bonner et al., 2000; Chu et al., 1998; Hietakangas et al., 2003; Park and Liu, 2000; Xavier et al., 2000). This feature allows the heat shock response to be modulated by other signalling pathways in the cell. The regulation of HSF1 by phosphorylation is likely to be a complex process. This molecule is heavily phosphorylated in resting cells and undergoes multiple additional phosphorylation events in response to cell stress. The kinases and phosphatases involved in this process are

largely unknown. However, there is some evidence to suggest that phosphorylation of HSF1 can be mediated by ERK, GSK-3 and JNK which can negatively regulate HSF1 transcriptional activity (Dai et al., 2000; Kim et al., 1997; Xavier et al., 2000).

In addition to the roles of hsp's in general protein homeostasis and damage repair, it has been shown that these chaperones are critical for the function of various signalling proteins. The best known chaperone for a role in this regard is Hsp90. In particular, this chaperone is involved in regulating the activity of steroid hormone receptors and various protein kinases. Studies aimed at determining the involvement of Hsp90 in various chaperone functions have been fostered by the availability of a specific inhibitor for this chaperone called geldanamycin (Stebbins et al., 1997). Hsp90 has been found to play a role in the stabilization of several large multi-domain proteins. It is believed that these larger multi-domain molecules require Hsp90 to influence conformational changes required for proper activity as well as ensuring stability of these molecules in the absence of substrate or ligand interaction. In this regard, Hsp90 has been shown to interact with diverse signalling molecules including Raf1, Akt, v-src, Wee1, cdk4 and the receptors for the steroid hormones progesterone and glucocorticoid (Aligue et al., 1994; Sato et al., 2000; Stancato et al., 1993; Stepanova et al., 1996; Young et al., 2001). In addition to Hsp90, other hsp's have been shown to directly influence signalling pathways. For example the elevated levels of Hsp70 that accumulate in cells following stress exposure, has recently been shown to have inhibitory action on the JNK pathway. One group has reported that Hsp70 associates directly with JNK without affecting the activity of its upstream kinase MKK4 (Park et al., 2001). Subsequently, this same group also reported an inhibitory association of Hsp70 with ASK-1, a kinase upstream of MKK4 (Park et al., 2002). Thus, it is not clear whether Hsp70 inhibits JNK signalling by targeting specific JNK pathway members, or whether it has a more general effect

through simultaneously suppressing multiple kinases in this pathway or those of entirely unrelated signalling cascades.

Another heat shock protein that is intimately associated with signalling pathways is the small heat shock protein hsp25. Hsp25 is a substrate of MAPKAP-K2 and MAPKAP-K3 which are themselves activated by the stress responsive p38 MAPK pathway. Hsp25, which exists as oligomeric structures in cells has a role in modulating actin cytoskeletal dynamics. Phosphorylation of Hsp25 during stress leads to a stabilization of actin filaments thus protecting cytoskeletal integrity in the cell (Lavoie et al., 1995). Regulation of hsp activity via phosphorylation may also be a feature common to other hsps however we are unaware of any studies which have attempted to investigate this in detail.

In addition to the host of heat shock chaperones that function in the heat shock response, there are an addition class of specialized chaperones that function in the endoplasmic reticulum (ER) to mediate the folding and stabilization requirements of various proteins that reside in this cellular compartment. Disruption of protein folding in the ER induces a response known as the unfolded protein response (UPR). Two key ER membrane integral proteins, PERK (Harding et al., 1999) and IRE1 (Nikawa and Yamashita, 1992) play important roles in mediating the cellular response to unfolded proteins in the ER. The activation of PERK leads to this molecule phosphorylating the translational initiation factor eIF2 alpha (to be discussed later) which serves to shut down general protein synthesis. Activation of IRE1 results in the activation of the cytoplasmic RNase component of this molecule which leads to non canonical splicing of the mRNA transcript of XBP (Lee et al., 2002). Translation of this modified XBP transcript produces a transcription factor that upregulates various protein chaperones that aid in the repair and stabilization of damaged proteins in the ER. Chaperones that have prominent roles

in the ER include calnexin, BiP/GRP78, GRP94, protein disulphide isomerase and Hsp47 (Hampton, 2000). Heat shock temperatures that activate the cytosolic HSF1 response does not cause marked induction of ER chaperones. Instead, inhibitors of glycosylation (tunicamycin), ER calcium influx (thapsigargin) and reducing agents activate ER chaperone production. Recently, it has been shown that the induction of the UPR is an important requirement for the genesis of plasma cells during B cell differentiation (Iwakoshi et al., 2003). This suggests that normal physiological processes are intimately connected to the status of protein folding in the ER.

1.3.4 Eukaryotic initiation factor eIF2 α

The deleterious consequences of heat shock on proteins, especially nascent proteins, which have yet to attain their fully-folded, stable structure, has lead to the formation of stress-responsive signalling pathways that modulate protein synthesis. In eukaryotes, protein synthesis involves a myriad of factors that function at the discrete stages of this process which are known as: initiation, elongation and termination (Rhoads, 1999). While a multitude of growth related signals positively regulate protein synthesis at the elongation stage, during periods of stress it is the initiation phase that is the target of signalling pathways which inhibit this process. The initiation phase of protein synthesis involves the assembly of a ternary complex containing the 40 S ribosomal subunit together with a GTP-bound heterotrimeric GTPase, eukaryotic initiation factor 2 (eIF2), which is itself coupled to the initiating methionyl-tRNA. These molecules then associate with an initiation complex known as eIF-4F which binds to the 7-methyl guanosine cap at the 5' end of mRNAs. After recognition of the initiating AUG in the mRNA, and GTP hydrolysis of eIF2, a new round of initiation can begin. This requires the exchange of

GDP to GTP on eIF2 a process which is catalyzed by the exchange factor eIF2B. In response to stress stimuli, the alpha subunit of eIF2 (eIF2 α) is phosphorylated by a number of kinases. This event has been shown to prevent eIF2B dissociation from GDP-bound eIF2 which inhibits the initiation of a new round of synthesis thus effectively shutting down protein synthesis.

Mammalian kinases that have been shown to be capable of phosphorylating eIF2 α include the interferon and dsRNA-inducible PKR, the endoplasmic reticulum membrane kinase PERK, the heme regulated kinase HRI, and a kinase induced by amino acid starvation GCN2 (Rhoads, 1999). Phosphorylation of eIF2 α by these kinases allows the cell to downregulate protein synthesis in response to a diverse array of cellular stresses. PERK autoactivation during ER stress and PKR activation by double stranded viral RNA for example, are two examples of distinct stress-induced events that both culminate with inhibition of translation initiation. Studies from knockout mice have indicated that PKR and PERK are dispensable for eIF2 α phosphorylation in response to heat stress (Kimball et al., 2001). A role for the HRI as a heat-responsive eIF2 α kinase was shown in mouse erythroid progenitors however, the lack of expression of HRI in other tissues indicates that another kinase, perhaps GCN2, may be the main heat-inducible eIF2 α kinase in the majority of cell types. Analysis of knockout mice lacking the various eIF2 α kinases would help determine which particular kinase played a role in regulating eIF2 α phosphorylation during heat shock.

CHAPTER 2 Materials and Methods

2.1 Methods used for Chapter 3

Reagents. Sodium m-arsenite (Sigma) was dissolved in water and used at 200 μ M to stimulate cells.

cDNA constructs. The cDNA encoding an amino terminal fragment of cJun (AA 1-169) was cloned into the bacterial expression vector pGEX4T-1 for generation of recombinant GST-cJun protein used as a JNK substrate in kinase assays.

Antibodies. The following description lists the source of the antibodies used in this chapter and their applications. For JNK1 immunoprecipitation (IP) kinase assays, the goat polyclonal JNK1 (sc-474G) was used for immunoprecipitations while a rabbit polyclonal (sc-474R) was used for immunoblots of the IP fractions – both from Santa Cruz Biotechnology. Phospho-specific antibodies to JNK (9251) and p38 MAPK (9211) were purchased from Cell Signaling Technology. A rabbit polyclonal p38 MAPK antibody from Santa Cruz Biotechnology (sc-435) was used as a loading control for phospho-p38 MAPK blots while the corresponding loading control antibody for phospho JNK was from Cell Signaling Technology (9252). Antibodies against heat shock proteins Hsp70 (SPA-810) and Hsc70 (SPA-815) were purchased from StressGen Biotechnologies. The rabbit polyclonal anti-HSF1 antibody was kindly provided as a gift by Dr. R. Morimoto (Northwestern U). The anti-phospho eIF2 α (9721) antibody was from Cell Signalling and an anti actin monoclonal (AC-15) was purchased from Sigma.

Preparation of recombinant proteins. The pGEX4T-1 bacterial expression construct encoding GST-c-Jun[AA 1-169] was expressed in *Escherichia coli* (UT5600 strain). A 50 mL overnight culture of LB containing 100 µg/mL ampicillin was used to seed 1 L of the same culture medium the following day. This culture was grown at 37°C until the density reached OD₆₀₀ 0.5-0.8. Following this, the culture was placed at 4°C for 20 minutes prior to the addition of 0.1 mM IPTG to induce expression of GST-cJun. Cultures were induced for 4 hours at 26°C and were then pelleted and resuspended in 10 mL of resuspension buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol, 10 µg/mL soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride). This suspension was then treated with 50 µl of 10 mg/mL lysozyme on ice for 30 minutes followed by the addition of NP-40 to 1% final concentration to facilitate cell lysis. The suspension was then sonicated to shear bacterial DNA and then centrifuged at 14 000 rpm for 10 minutes. Aliquots from the supernatant were added to glutathione sepharose 4B beads and rotated at 4 °C for 1 hour. The beads were washed extensively in resuspension buffer and then eluted from beads using elution buffer (20 mM glutathione, 150 mM Tris HCl, pH 7.5). The eluates were analyzed by Coomassie Blue staining after SDS PAGE to determine yield.

Preparation of cell lysates. Cellular lysates were prepared using solubilization buffer (50 mM Tris, pH 7.5, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P40 (NP-40), 1 mM sodium molybdate, 50 mM sodium orthovanadate, 1 mM sodium fluoride, 50 mM β-glycerol phosphate, 10 µg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride). Protein quantitation of lysates was determined using the BCA method (Pierce). For anti-phospho p38 and anti-phospho JNK blots, cells were lysed directly in 1 X SDS sample buffer and sonicated briefly to shear DNA prior to loading on SDS PAGE gels.

Western Blotting. SDS PAGE gels were transferred to nitrocellulose (0.2 μ M) using a semi-dry electrotransfer apparatus. Membranes were blocked in 1X TBS with 0.05% NP-40 (TBSN) containing 5% skim milk powder. Blots were incubated with primary antibodies overnight in TBSN and developed with HRP-conjugated secondary antibodies (DAKO) using enhanced chemoluminescence reagents (Amersham).

Cell lines. Murine hematopoietic cell lines MC/9, WEHI-231, A20, CTLL and EL4 were cultured in RPMI 1640 supplemented with 10% FCS, 10 μ M 2-mercaptoethanol, 1 mM sodium pyruvate and 20 mM HEPES. Additional media supplements were added for the IL-3-dependent MC/9 cell line which included 2% WEHI-3B conditioned media as a source of IL-3 and for the IL-2 dependent CTLL cell line, 3% XO63 conditioned media was added to cultures as a source of IL-2. NIH 3T3 cells were grown in DMEM with 10% calf serum (Invitrogen).

Primary cell cultures. Primary T and B cell cultures were obtained from single cell suspensions of spleen from Balb/c mice. For T cell cultures, dispersed cells were cultured at 2×10^6 /ml in a tissue-culture treated flask for 48hr with 5 μ g/mL Con A (Sigma) in RPMI 1640 and 3% XO63 conditioned media as a source of IL-2. Following this, non-adherent cells were removed, washed twice in serum-free RPMI 1640 and then cultured in RPMI 1640 with IL-2 for an additional 4 to 8 days prior to use in experiments. To obtain B cell cultures, adherent cells were first removed by culture on a tissue-culture treated flask for 1 hour. Suspension cells were then removed and cultured at 2×10^6 /ml in a tissue-culture treated flask for 48 hours in media supplemented with 15 μ g/mL LPS. Suspension cells were then removed for use in experiments. Mast cells were prepared from bone marrow cultures from BALB/c mice. Briefly, femurs were flushed with PBS

and cultured on tissue-culture treated plates in RPMI 1640 supplemented with 2% WEHI-3B conditioned media as a source of IL-3. Suspension cells were removed occasionally and replated to exclude adherent cells from the culture. Following 4 weeks of culture in IL-3, mast cell cultures were used in experiments. Macrophages were obtained from BALB/c bone marrow cultures by first culturing them for 48 hours on tissue-culture treated plates in RPMI 1640 supplemented with 20% L-cell conditioned media as a source of CSF-1. Non-adherent cells were then plated on 60 mm tissue-culture treated dishes and allowed to differentiate in the presence of CSF-1 for an additional 5-8 days prior to use in experiments. Primary embryonic fibroblasts were prepared from day 15 BLACK6 embryos. Briefly, heads were removed and the body was cut into small pieces and then dispersed into single cell suspension using a metal screen. Fibroblasts obtained from cell suspensions were cultured in DMEM with 10% FCS on tissue-culture treated dishes and used for experiments when cultures reached subconfluence.

Heat shock treatment. Heat shock was performed by floating parafilm-wrapped dishes of cells in a 43°C re-circulating water bath. The water bath temperature was monitored by an internal thermostat and separate thermometers and found to be accurate to +/- 0.1 °C. Control plates were incubated in a 37°C re-circulating water bath.

JNK kinase assays. Cellular lysates (250-500 µg) were incubated with 1 µg anti-JNK1 antibody and 20 µl settled bead volume of protein G Sepharose for 2 hours with rotation at 4°C. JNK1 immunoprecipitates were washed extensively with solubilization buffer and once with kinase assay buffer (25 mM HEPES pH 7.2, 25 mM magnesium chloride, 2 mM dithiothreitol, 50 mM β-glycerol phosphate and 0.5 mM sodium vanadate). The kinase assay was initiated by the addition of kinase assay buffer including 3 µg of

recombinant GST-cJun [AA 1-169] and 10 μ Ci of [γ -³²P]ATP, and stopped after 20 minutes by the addition of SDS-sample buffer. To visualize the extent of GST-cJun phosphorylation samples were the run on SDS-PAGE, transferred to nitrocellulose and exposed to film.

For quantitation of JNK kinase activity, ³²P incorporation into GST-c-Jun was measured using a PhosphorImager (Molecular Dynamics).

2.2 Methods used for Chapter 4

Reagents. The ribosomal toxin anisomycin and the protein kinase C inhibitor bisindolymaleimide were dissolved in dimethylsulfoxide (DMSO). The calcium ionophore, ionomycin, was prepared in water and the diacylglycerol analogue and PKC agonist, phorbol 12-myristate 13-acetate (PMA), was prepared in DMSO. The anti-oxidant, free radical scavenger, N-acetyl cysteine was prepared in cell culture media. The protein synthesis inhibitor cycloheximide was prepared in water. All chemicals were obtained from CalBiochem.

cDNA constructs. The pEBG mammalian expression vector (a gift from Dr. L. Zon) was used to express GST fusion proteins of MKK4 and MKK7 in cell lines. The cDNA for JNK3 was subcloned into the prokaryotic expression vector pGEX4T-3 via restriction digest with *Bam* HI and *Not* I in order to generate recombinant GST-JNK3 for endogenous MKK4 linked kinase assays. Dominant negative TRAF2 and TRAF6 cDNAs were kindly provided by Tularik Inc. in mammalian expression constructs as amino terminal FLAG-tagged fusions. The cDNA for human Bcl-2 was kindly provided by Dr. S. Cory.

Antibodies. The following description lists the source of the antibodies used in this chapter and their applications. The phosphorylation status of exogenous GST-MKK4 and GST-MKK7 was detected with the respective phospho-specific antibodies P-MKK4 (9151) and P-MKK7 (test sera provided as a gift) both from Cell Signalling Technologies. Expression levels of exogenous GST-MKKs were detected with an anti-GST rabbit polyclonal antibody (A-5800) purchased from Molecular Probes, Inc. Endogenous MKK4 was immunoprecipitated with a rabbit polyclonal antibody (sc-964) from Santa Cruz Biotechnology and fractions of these immunoprecipitates were analyzed for MKK4 loading amounts using the same antibody. For apoptosis studies, the antibody recognizing both the intact and cleaved form of caspase 3 was from StressGen Biotechnologies (APP-103). Intact and cleaved forms of caspase 9 were detected with the rabbit polyclonal from Cell Signaling Technology (9504). Cytochrome c was detected using a mouse monoclonal antibody from Pharmingen (556433). The anti-human Bcl-2 monoclonal antibody (05-341) was from Upstate Biotechnology.

Cell lines. WEHI-231 B cells overexpressing human Bcl-2 were generated by retroviral infection. A clone was selected on the basis of high Bcl-2 expression via Western blotting.

MKK4/MKK7 phosphorylation assays. MC/9 or WEHI-231 cells (2×10^7 /sample) were transiently transfected with 20 μ g of the mammalian GST expression vector pEBG-MKK4, pEBG-MKK7 or pEBG empty vector using electroporation (300 V, 975 microfarads) in 500 μ L of serum-free RPMI medium. Cells were then replated in RPMI with serum and allowed to recover 10-20 hr prior use in experiments. After stimulation with heat or other cell stresses, the cells were lysed in solubilization buffer and equal

amounts of protein were incubated with 20 μ l settled bead volume of glutathione Sepharose 4B (Pharmacia). Following rotation for 1 hour at 4°C, beads were washed 3 times in 500 μ l solubilization buffer and then boiled in SDS sample buffer. Samples were then run using SDS PAGE and subsequent blots were probed with anti-phospho MKK4/MKK7 antibodies. Blots were then stripped and re-probed with an anti-GST antibody to ensure equal amounts of expressed proteins were being analysed.

Endogenous MKK4 linked kinase assay. To determine endogenous MKK4 kinase activity, cells were lysed in Buffer A (20 mM Tris pH 7.4, 10% glycerol, 1% Triton X-100, 137 mM NaCl, 25 mM β -glycerol phosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF and 10 μ g/mL soya bean trypsin inhibitor). Lysates (500-1000 μ g) were then immunoprecipitated with (1 μ g) anti-MKK4 antibody using protein A sepharose beads (Pharmacia) for 2 hours at 4°C. Immunoprecipitates were then washed twice in Buffer A, twice in Buffer B (500 mM LiCl, 100 mM Tris pH 7.6, 0.1 % Triton X-100, 1 mM DTT) and once in Buffer C (20 mM MOPS pH 7.2, 2 mM EGTA, 1 mM DTT, 0.1 % Triton X-100, 25 mM MgCl₂, 25 mM β -glycerol phosphate). Immunoprecipitates were then incubated in 30 μ l of Buffer C containing 1 μ g of the MKK4 substrate GST-JNK3 with 50 μ M unlabeled ATP for 20 min at 30°C. To assess the extent of MKK4-induced GST-JNK3 activity, a 15 μ l aliquot from this reaction was added to 15 μ l of Buffer C containing 1 μ g GST-cJun and 10 μ Ci of [γ -³²P]ATP. This reaction was allowed to proceed for 20 min at 30°C and then stopped by the addition of SDS-sample buffer. An aliquot of this reaction was then run out using SDS PAGE, transferred to nitrocellulose and exposed to film. To ensure equal amounts of endogenous MKK4 was present in these assays, Western blots were performed on immunoprecipitates using an anti-MKK4 antibody.

Quantitation of Apoptosis. Apoptosis was measured using FITC-Annexin V and PI reagents following the manufacturer's protocol (BD Biosciences).

Cytochrome c release. For the analysis of cytochrome c release, cytosolic and membrane fractions were prepared as described by Ekert et al (Ekert et al., 2001). Briefly, cell pellets from $5-10 \times 10^6$ cells were lysed in 100 μ l digitonin lysis buffer (0.025% digitonin in 250 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris, pH 7.4, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM PMSF) and incubated on ice for 10 min. Lysates were then centrifuged for 2 min at 14 000 rpm and the supernatant was removed (cytosolic fraction). The remaining pellet was washed once in digitonin lysis buffer and then resuspended in 100 μ l of radioimmune precipitation assay lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5), lysis was allowed to proceed for a further 30 min, and cellular debris was removed by centrifugation for 10 min at 14 000 rpm. The supernatant comprising the membrane fraction was retained.

CHAPTER III

Identification of an atypical JNK signalling response in murine lymphocytes in response to heat shock

3.1 Introduction

The activation of JNK signalling is a common response noted in cells when exposed to various environmental challenges. These include diverse stresses such as UV irradiation, hyperosmolarity, reactive oxygen species, mechanical stress, ischemia and heat shock. The role of JNK in mediating the cellular response in the face of such insults is largely unknown. In the case of heat shock, stress kinase activation has been observed in yeast suggesting that this is an ancient highly conserved response to stressful environments (Degols et al., 1996; Winkler et al., 2002).

In addition to stress kinase activation, other signalling pathways and stress responses are initiated following heat shock exposure. A better understood cellular response that occurs upon exposure to elevated temperatures and other noxious stimuli is the induction of heat shock protein chaperones (hsps). These molecular chaperones function primarily in the stabilization, protection and refolding of proteins in the cell. The hsp family of proteins is one of the most highly conserved groups in evolution, with homologues being found in all organisms (Feder and Hofmann, 1999). The susceptibility of proper protein structure and function to heat shock has necessitated the development of cellular responses designed to minimize damage to the proteome of organisms via thermal stress. While hsps function to protect and repair the existing pool of mature proteins, cessation of mRNA translation by the cell ensures that no new proteins are made during these unfavourable conditions. Translation inhibition during stress is

mediated by the phosphorylation of eIF2 α – a subunit of the eIF2 heterotrimeric G protein that mediates the process of initiation of peptide chain elongation during cap-dependent mRNA translation.

Thus, a variety of distinct signalling systems can be activated in the cell during the response to heat shock. However, it is unknown how many of these responses are initiated and whether signalling cross-talk plays a role amongst the various pathways. Here we have made a surprising discovery that one of these heat-induced responses, the activation of JNK, is severely impaired in murine lymphoid cell types. Normal JNK signalling responses could be activated in these cells by cell stresses other than heat including hyperosmotic and oxidative stresses. In addition, we found that other signalling responses activated by heat including HSF1 hyperphosphorylation, Hsp70 induction and eIF2 α phosphorylation were not impaired in murine lymphocytes. These results suggest JNK signalling induced by heat involves discrete proximal signals that are not involved in the activation of other heat-induced signalling events.

3.2 Results

3.2.1 Analysis of JNK activity in a variety of murine cell types in response to heat shock

Heat shock is one of a number of cell stresses which has been shown to strongly activate the JNK family of stress-activated kinases (Hu et al., 1997; Kyriakis et al., 1994; Nagata and Todokoro, 1999; Zanke et al., 1996b; Zhang et al., 1996). During an investigation involving the analysis of stress-induced JNK activity in various murine cell types, we observed dramatic cell type-dependent differences in the levels of JNK activity following exposure to heat shock (Fig. 3.1). Thus, while strong JNK kinase activity was detected in 3T3 murine fibroblasts and MC/9 mast cells, murine lymphoid cell lines that we tested – including the pre-B cell line WEHI-231, the more mature B-cell line A20, the thymoma-line EL4 and the IL-2- dependent cytotoxic T lymphocyte line CTLL, exhibited strikingly weak activation of JNK in response to heat. The defective activation of JNK in response to heat in these cell lines was selective in that a robust activation of JNK in response to hyperosmolarity (0.4M NaCl) and oxidative stress (treatment with sodium arsenite) could be induced. These results suggested that the signalling mechanisms engaged during the heat shock response which lead to JNK activation were somehow differentially modulated in these murine lymphoid cell lines. Loading controls showed that the differences in kinase activity could not be attributed to loss of JNK protein levels. In addition, we could also rule out that the property of cell adherence during growth was a factor since MC/9 mast cells, which, like lymphocytes, grow in suspension, showed a robust activation of JNK in response to heat shock.

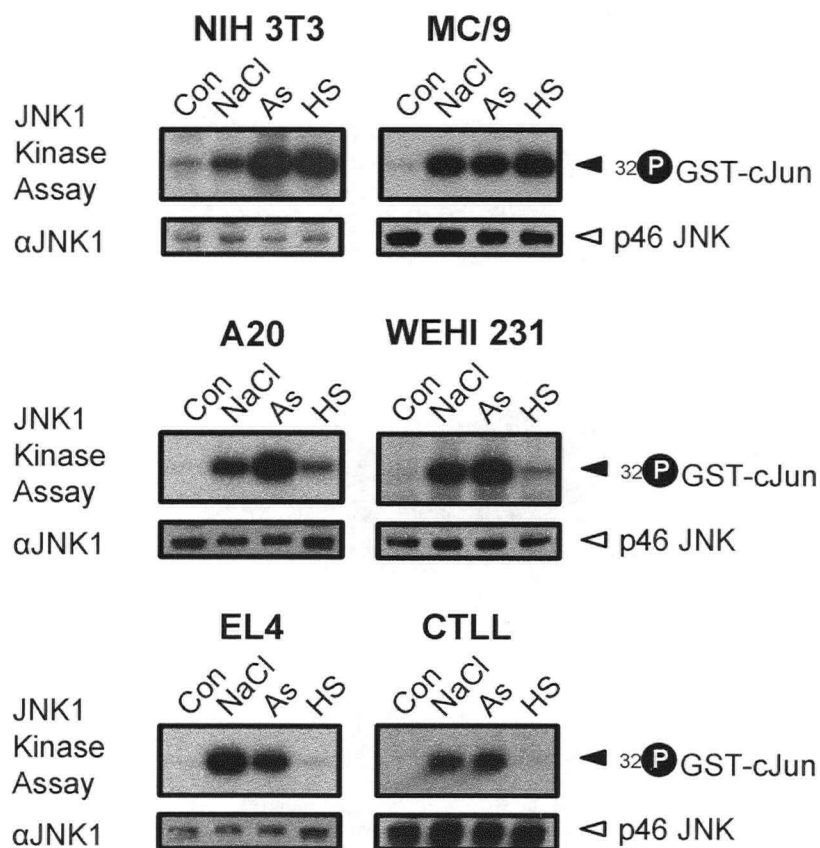


Figure 3.1 Comparison of JNK activation in response to cell stresses in various murine cell lines. The indicated murine cell lines were tested for JNK activity using JNK1 immunoprecipitation kinase assays following exposure to the following stresses: no treatment (Con), osmotic shock induced by 0.4M sodium chloride for 10 min (NaCl), oxidative stress induced by 200 μ M sodium arsenite for 60 min (As) and heat shock at 43°C for 60 min (HS). The relative amount of immunoprecipitated JNK1 kinase is shown in the anti-JNK immunoblots displayed in the panel below each kinase assay.

3.2.2 Characterization of heat-induced JNK phosphorylation in bone marrow-derived macrophages

Our initial investigation into the activation of JNK via heat shock utilized heat shock conditions derived from previously published studies (1 hr at 43°C). We wanted to

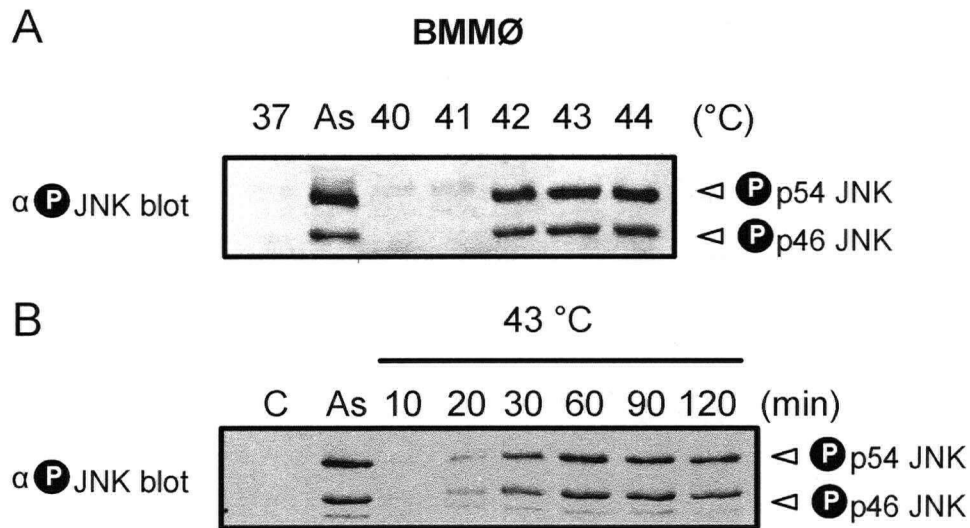


Figure 3.2 JNK phosphorylation in bone marrow-derived macrophages (BMMØ) in response to heat shock. (A) Cells were incubated at the indicated temperatures for 1 hour or 200 μ M sodium arsenite (As) prior to preparation of lysates for analysis by Western blot using an anti-phospho JNK antibody. **(B)** Cells were incubated at 43°C for the time indicated prior to analysis of JNK phosphorylation as described above.

ensure that these conditions were in fact optimal for inducing JNK activity via heat shock so we characterized the temperature dependence and kinetics of JNK activity in response to heat for ourselves using primary bone marrow-derived macrophages (BMMØ). In these experiments, JNK activation was measured indirectly using an antibody that recognizes the phosphorylation of a threonine and tyrosine residue in the activation loop of this kinase. Phosphorylation of these two residues has been shown to be essential for JNK kinase activity (Jiang et al., 1997b; Kyriakis et al., 1991). As shown in Figure 3.2, one hour incubations of BMMØ cells at a range of temperatures indicated a drastic increase in JNK phosphorylation at 42°C which seemed to be maximal as a further increase was not seen at either 43°C or 44°C. As a positive control for JNK activity, cells were treated with the oxidative stressing agent sodium arsenite (As). In a subsequent experiment, we tested the extent of JNK phosphorylation in a time-

dependent manner upon exposure to 43°C. These results indicated that maximal JNK phosphorylation was achieved after 60 minutes of incubation at this temperature. The results of our analysis of the temperature dependence and kinetics of JNK phosphorylation in BMMØ cells indicated that the heat shock conditions we have used in our survey of various cell types lies within the optimal range required for efficient JNK activation.

3.2.3 Analysis of phosphorylation status of JNK and p38 isoforms in murine lymphocytes in response to heat shock

Using two model cell lines that we had previously analyzed (Fig 3.1) for JNK1 kinase activity - MC/9 murine mast cells and WEHI-231 B cells – we set out to examine the phosphorylation profiles of JNK in these cells in response to heat shock. This experiment allowed us to rule out the possibility that alternate isoforms of JNK that could have been excluded from the JNK1 immunoprecipitation kinase assay could be activated in murine lymphocytes during heat shock. As shown in Figure 3.3, very little JNK phosphorylation could be detected in WEHI-231 B cells with heat while a strong signal was noted in MC/9 mast cells. Given that the phosphorylation-specific antibody used will recognize the conserved activation motif in all isoforms of JNK, we can conclude from these studies that heat does not lead to a significant extent in phosphorylation of any of the JNK isoforms expressed in WEHI-231 B cells. Interestingly, the analysis of total JNK protein levels in heat shocked WEHI-231 cells indicated that the p54 kDa isoform of JNK may undergo a cleavage event as we noted an additional ~52 kDa band appearing at the latter stages of the heat shock. This may be due to caspase-mediated cleavage as a recent report has indicated that p54 JNK may be a target for caspase 3 (Enomoto et al., 2003).

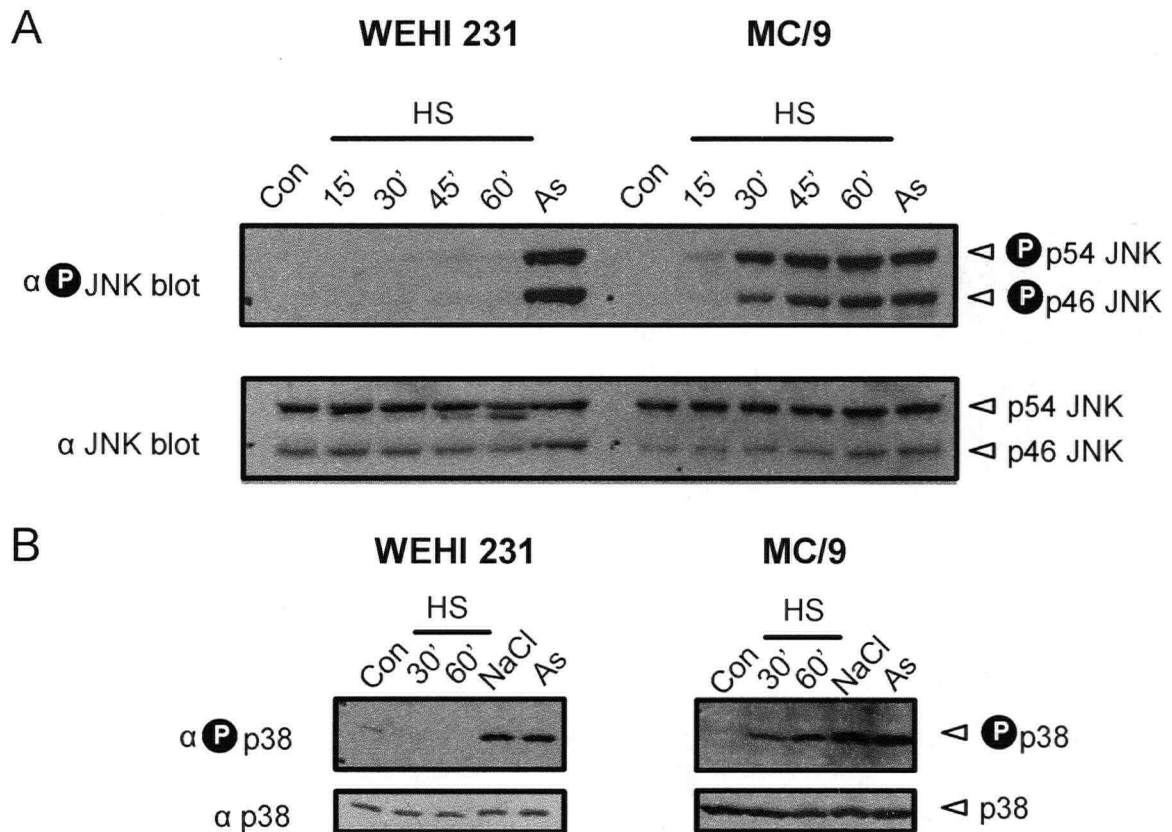


Figure 3.3 Phosphorylation status of JNK and p38 MAPK in response to heat in WEHI-231 B cells and MC/9 mast cells. Lysates of stimulated MC/9 mast cells and WEHI-231 B cells were prepared and analysed via Western blot using antibodies specific for the phosphorylated form of JNK (**A**) or p38 MAPK (**B**). The levels of total JNK or p38 are shown below each phospho blot. Stimuli used for anti-phospho JNK blots included 43°C for the times indicated and 200 μ M sodium arsenite for 60 min (As). Stimuli used for anti-phospho p38 MAPK analysis included 43°C for the times indicated, 200 μ M sodium arsenite for 60 min (As) and osmotic stress using 0.4 M NaCl for 10 min (NaCl).

Using the analogous phospho-specific antibody against p38 MAPK, we showed that, like JNK, phosphorylation of this related stress-activated kinase was also lacking in response to heat shock in the WEHI-231 B cell line. Given that JNK and p38 MAPK can be activated in concert by a variety of upstream signalling molecules, these results suggest

that heat shock involves the activation of one or more of these shared signalling intermediates.

3.2.4 Quantitation of JNK activation in response to heat shock in two murine cell lines – WEHI-231 B cells versus MC/9 mast cells

In further experiments using MC/9 mast cells and WEHI-231 B cells, we set out to quantify the magnitude of JNK kinase activation in these two cell lines in response to heat shock using PhosphorImager analysis of JNK1 immunoprecipitation kinase assays. Sodium arsenite treatment was used as a positive control in these experiments since we had found that both murine lymphoid and non-lymphoid cells could activate JNK equally well in response to this stimuli. In a set of five independent experiments, we found that while heat and sodium arsenite led to comparable fold induction of JNK activity in MC/9 mast cells, levels of JNK kinase activity induced by heat shock in WEHI-231 B cells were on average 20 fold lower than those induced by sodium arsenite (see Fig. 3.4).

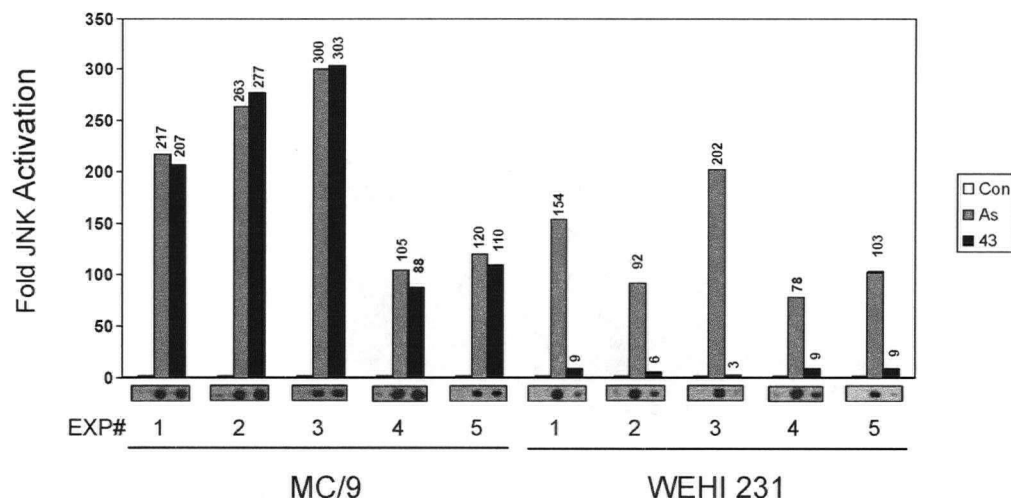


Figure 3.4 Quantitation of JNK kinase activity in WEHI-231 B cells and MC/9 mast cells. JNK1 immunoprecipitation kinase assays were used in a series of five independent experiments to quantitate the differences in JNK activation between MC/9 mast cells and WEHI-231 B cells. The fold activation of JNK was determined via quantitation of radiolabel incorporated into the JNK substrate GST-cJun using PhosphorImager analysis. Stimuli used included 200 μ M sodium arsenite for 60 min (As) and heat shock at 43°C for 60 min (Heat).

3.2.5 Analysis of JNK activation in primary murine hematopoietic cells

The above experiments indicated to us that a specific deficit in heat-induced JNK signalling was present in this particular subset of lymphoid cell lines. We were interested to find out whether this phenomenon was merely a characteristic intrinsic to these select murine lymphoid cell lines or reflected a broader trend characteristic of lymphoid cells in general. In order to address this question, we decided to repeat the above experiments comparing cell types derived from primary sources. We isolated a variety of primary cell types from murine tissues including embryonic fibroblasts, macrophages, mast cells and T and B lymphocytes. When these cultures were compared for their ability to activate JNK in response to osmotic, oxidative and heat stress treatments, we discovered that the T and B lymphocyte cultures failed to exhibit the robust JNK activity noted in the

other cell types examined in response to heat but had a normal response to oxidative or osmotic stress. The results of these studies suggest that both the select group of murine lymphoid cell lines we tested and primary murine T and B lymphocytes likely share a common adaptation in JNK signalling that accounts for their reduced activation of this kinase in response to heat shock.

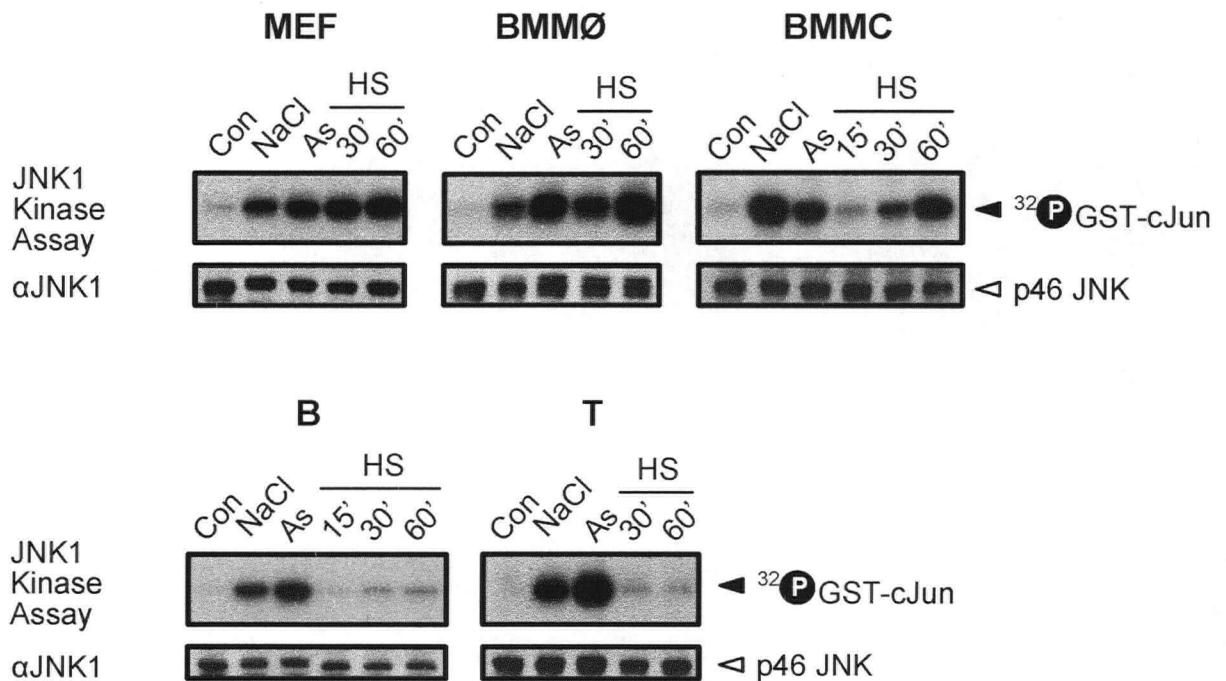


Figure 3.5 Primary murine lymphocytes display reduced JNK activity in response to heat shock. Primary cell cultures including murine embryonic fibroblasts (MEF), bone marrow-derived macrophages (BMMØ), bone marrow-derived mast cells (BMMC), and T and B lymphocytes were tested for JNK activity in response to cell stresses using JNK1 immunoprecipitation kinase assays. Stress stimuli included: no treatment (Con), 0.4 M sodium chloride for 20 min (NaCl), 200 μM sodium arsenite for 60 min (As) and 43°C for the times indicated. JNK1 immunoblots were performed on immunoprecipitates to quantitate loading (lower panels).

3.2.6 Heat shock can activate JNK in human T lymphocytes

While we were unable to find another study that had analyzed heat-induced JNK activity in murine lymphocytes, we found that in the Jurkat human T cell leukemia cell line, it had been previously reported that heat shock could induce strong JNK activity (Brenner et al., 1997). We decided to repeat these experiments for ourselves as well as determine the status of JNK activation in response to heat shock in primary human T cells obtained from OKT3-stimulated peripheral blood leukocytes. As shown in Figure 3.6, we found that JNK phosphorylation was induced in response to heat shock in both Jurkat T cells as well as in OKT3-stimulated primary human T cell cultures. JNK activation was noted in heat shock stimulation experiments using primary human T cells derived from multiple donors. The reason for the difference in JNK response between murine and human lymphoid cells remains unclear at this point and will be crucial in the understanding of the mechanisms that dictate JNK responsiveness during heat shock stimulation. One possibility that exists is that the Jurkat T cells, as well as activated peripheral human T lymphocytes, may have upregulated or downregulated a critical molecule involved in heat-induced JNK signalling. It is possible that naïve human T cells may behave differently in terms of heat-induced JNK activation.

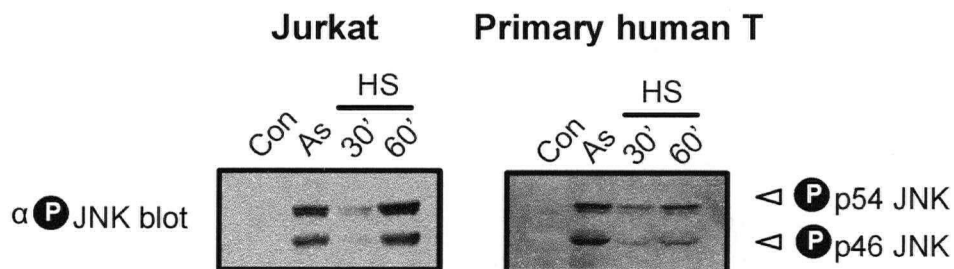


Figure 3.6 Jurkat human T cells and activated primary human T cells can activate JNK in response to heat shock. Jurkat human T cells (left panel) and primary human T cells obtained from cultures of OKT3-stimulated peripheral blood (right panel) were tested for phosphorylation of JNK in response to 43°C (HS) for the times indicated or 200 μM sodium arsenite for 60 min (As).

3.2.7 Normal activation of the heat shock response pathway in murine lymphocytes

Collectively, our results demonstrated that there was a pronounced deficit in heat-induced activation of JNK in B and T murine lymphocytes, that did not reflect a general impairment in all signalling pathways leading to JNK activation as responses to osmotic or oxidative stress were comparable to those in non-lymphoid cells. Thus, there appeared to be a selective deficit in the mechanisms specific to heat stress-induced activation of JNK. These could include mechanisms that sensed heat stress or that couples the sensor to the kinase cascade that activates JNK. To examine the former possibility we investigated the ability of murine lymphocytes to activate other types of responses to heat stress.

We first tested whether heat-induced hyperphosphorylation of the transcription factor HSF1 could be detected in murine lymphocytes. This was monitored by the observed shift in electrophoretic mobility on SDS-PAGE using immunoblotting with an HSF1-specific antibody (Sistonen et al., 1994). We observed that, as in primary murine fibroblasts, heat treatment of primary murine T and B lymphocytes induced a time-dependent shift in the mobility of HSF1 (Fig. 3.7A). Following this, we investigated the heat-induced upregulation of Hsp70 – which is mediated by HSF1. We observed that primary lymphocytes responded to heat with strong induction of synthesis of Hsp70 (Fig. 3.7 B). The triggering of both HSF1 hyperphosphorylation and the subsequent induction of Hsp70 in murine lymphocytes indicated that these cells did not exhibit a global inability to sense heat stress and that the threshold for induction of these responses was similar to that found in non-lymphoid cells.

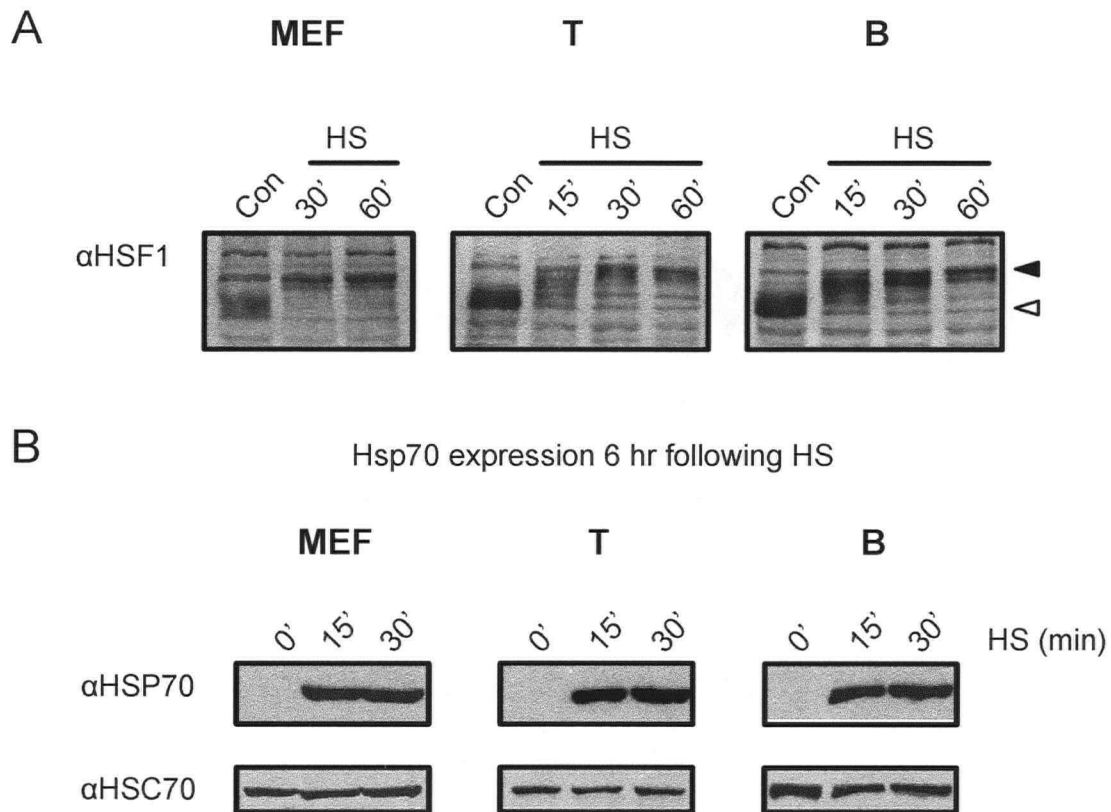


Figure 3.7 Heat shock leads to HSF1 activation and HSP70 induction in primary lymphocytes. (A) The phosphorylation status of HSF1 was monitored in murine embryonic fibroblasts (MEF) and T and B lymphocytes following exposure to heat for the indicated times. A mobility shift in HSF1 due to increased phosphorylation was detected by Western blotting using HSF1 antisera (black arrow indicates the position of hyperphosphorylated HSF1 over basal HSF1, white arrow). (B) Induction of HSP70 protein levels was determined by Western blot in murine embryonic fibroblasts (MEF) and T and B lymphocytes 6 hours following initial exposures to 43°C for the indicated times (min). The levels of the constitutively-expressed heat shock protein HSC70 are shown in the Western blot of these same lysates in the lower panel.

3.2.8 Comparable phosphorylation of eIF2 α in response to heat in both murine lymphoid and non-lymphoid cells

An additional signalling system that is triggered in cells in response to various forms of stress culminates in the phosphorylation of eIF2 α . Phosphorylation of eIF2 α results in the cessation of the initiation events involved in cap-dependent mRNA translation via its inhibitory association with eIF2B an exchange factor required for catalyzing each new round of initiation. During heat shock, shutting down translation helps to prevent the accumulation of unfolded and aggregated proteins made in the cell during these unfavourable conditions. We tested to see if this signalling response was functional in murine lymphocytes in response to heat shock. As seen in Figure 3.8, both WEHI-231 murine B lymphocytes and MC/9 murine mast cells showed a rapid heat-induced phosphorylation of eIF2 α . These results indicate that eIF2 α phosphorylation, like HSF1 and HSP70 induction, can occur in the absence of significant JNK activity.

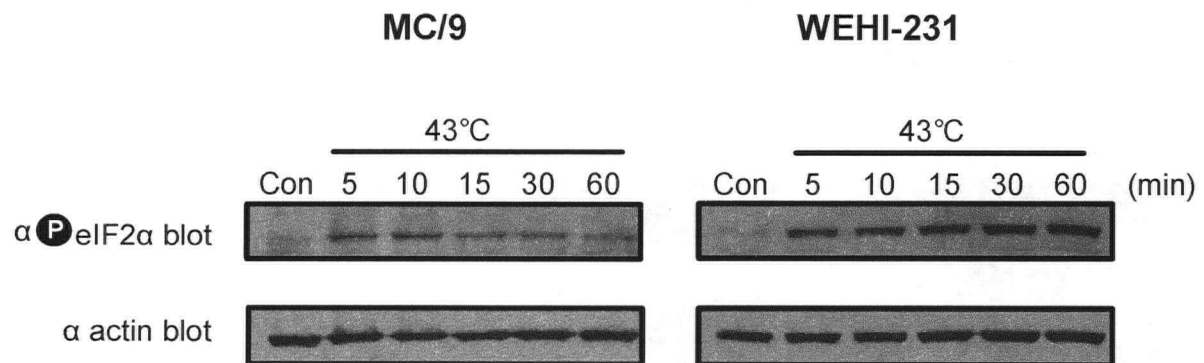


Figure 3.8 Analysis of eIF2 α phosphorylation in non-lymphoid versus lymphoid murine cells in response to heat shock. MC/9 murine mast cells and WEHI-231 murine B cells were heat shocked at 43°C for the times indicated (in minutes) and lysates were then prepared for Western blot analysis using an anti phospho-eIF2 α specific antibody (upper panel). The same blot was then stripped and re-probed with an antibody specific for actin in order to ensure equal amounts of protein were present in each lane (lower panel).

3.3 Discussion of Chapter III

In this chapter we have shown that murine lymphocytes fail to respond to heat shock treatment with the strong activation of JNK noted in other cell types we examined. Since previous reports in a variety of different cell types (although none have focused specifically on murine lymphocytes) seemed to indicate that JNK activation by heat shock was ubiquitous, the atypical response in murine lymphocytes was intriguing as it suggested that a potentially novel form of regulation of this pathway existed in these cells. We followed up our initial observations with the examination of this feature in primary cell cultures and an investigation of alternative heat-induced signalling responses in various hematopoietic cell types.

Our findings showed that the initial observation of this feature in murine lymphoid cell lines was consistent with a lack in JNK activation in primary murine lymphocyte cell cultures following heat shock. Thus, we cannot attribute the lack in JNK responsiveness to properties that may have arisen in these immortalized cell lines during their continual passaging in culture. Analysis of heat shocked lymphocytes for the phosphorylation of another stress-activated MAPK, p38 MAPK, showed that, like JNK, this molecule was also not significantly phosphorylated. This finding was not a surprise since the majority of stress stimuli that have been shown to activate JNK also lead to activation of p38 MAPK. This suggests that mechanistically, heat shock is similar to many other stress stimuli in that it involves the initiation of proximal signalling events that converge on both the JNK and p38 MAPK signalling cascades. Further clues as to the mechanism responsible for stress kinase activation by heat shock came from the observation that osmotic stress as well as oxidative stress could strongly activate JNK in murine lymphocytes. Thus, these two stress stimuli seem to not require the same set of factors in cells that are needed in

order to cause robust activation of JNK via heat shock. Indeed, previous work has shown that heat shock utilizes signalling mechanisms that are distinct from other stresses such as UV irradiation (Adler et al., 1995).

Interestingly, we discovered that human primary lymphocytes differed from murine lymphocytes in that strong JNK activation could be induced in response to heat shock. This finding indicates that the basis for the differential JNK activation by heat shock may not be a feature intrinsic to cells of lymphoid origin but may instead rely on the expression of a species specific molecule that is important for JNK activation. However, it is also possible that the conditions used to stimulate and propagate human peripheral T cells may have altered the heat-induced JNK signalling capacity in these cells. This could come about by either upregulating or downregulating the expression of a key molecule that modulates heat-induced signalling. Thus, it is possible that naive human lymphocytes may behave differently in terms of JNK activation following heat shock and this is one aspect that remains to be tested. The elucidation of the signalling molecules required for efficient JNK signalling in response to heat shock will facilitate the understanding of how JNK responses may potentially be modulated in a cell context-dependent manner.

Our analysis of HSF1 activation and Hsp70 induction in murine lymphocytes showed that these cells were capable of initiating this subset of heat-induced signalling responses despite the concurrent deficit in JNK signalling. From these results, we can conclude that the deficit in JNK activation found in murine lymphocytes in response to heat shock is not due to a general resistance or increased tolerance to sensing and initiating heat-induced signal transduction. In fact, the data from previous reports indicate that primary murine T lymphocytes may have a lower threshold for the activation of HSF1 as

compared to other cell types (Gothard et al., 2003; Ostberg et al., 2002). Furthermore, the finding that HSF1 activation can proceed normally in murine lymphocytes without the necessity for potent JNK activation, suggests that JNK has little influence on the initiation of HSF1 activation. One study has indicated that JNK-mediated phosphorylation of HSF1 is important in downregulating HSF1 activity by accelerating its removal from transcriptional complexes in the nucleus known as "stress granules". Thus, it is possible that reduced JNK signalling in murine lymphocytes may prolong HSF1 activity and thereby result in a greater accumulation of inducible heat shock proteins such as Hsp70 which could confer greater protection and enhanced recovery of thermally-damaged proteins in these cells. A more definitive answer to the involvement of the JNK pathway in mediating aspects of the heat shock protein response could be obtained from analysis of the recently developed JNK1/JNK2 double knock out embryonic fibroblasts (Dong et al., 2000).

As it has recently been shown that Hsp70 can directly inhibit JNK activity (Gabai et al., 1997; Park et al., 2001; Yaglom et al., 1999) we considered whether this could account for our observations in murine lymphocytes. We believe it unlikely to be the case that Hsp70-mediated inhibition of JNK activity explains why murine lymphocytes show reduced JNK activation during heat shock due to the following observations. First, previous studies have shown that high levels of Hsp70 achieved in response to stress are required to block JNK activation. Thus, we would expect to find high basal levels of Hsp70 in murine lymphocytes however, this was not the case. The other piece of evidence against an Hsp70-mediated mode of JNK inhibition in murine lymphocytes is that we can achieve perfectly normal activation of JNK by other stimuli (Figs. 3.1, 3.3). According to the findings of previous reports, it would be expected that all stimuli tested - not just heat shock - would fail to activate JNK under a scenario where Hsp70 mediates

inhibition of JNK (Volloch et al., 1999; Yaglom et al., 1999). While we feel confident that we can exclude a role for Hsp70 in the reduced activation of JNK observed in heat-shocked murine lymphocytes, we cannot rule out the possibility that other chaperones may play a role in mediating JNK activity in these cells.

As with the induction of the HSF1 – Hsp70 response by heat, the heat-induced phosphorylation of eIF2 α occurred normally in murine lymphocytes. Thus, it is likely that JNK activity is not a requirement for the inhibition of translation in the cell during periods of stress. Analysis of the kinetics of eIF2 α phosphorylation in response to heat shock indicated that maximal phosphorylation occurred within 5 minutes (Fig 3.8). Given that the initiation of JNK phosphorylation noted in non-lymphoid cells during heat shock was not detected until 15 minutes, the temporal separation of JNK and eIF2 α signalling events provides further evidence that these two pathways are mutually exclusive. Although a number of kinases including PERK, HRI, GCN2 and PKR have been shown to induce the phosphorylation of eIF2 α , it is not known which kinase is responsible for this activity during heat shock.

CHAPTER IV

Analysis of potential mechanisms regulating JNK signalling in response to heat shock

4.1 Introduction

The mechanisms involved in the activation of the JNK signalling cascade in mammals in response to stress remain ill-defined. While this area of research is not as prominent in the mammalian cell system, a significant amount of work has been done to explore the mechanistic details of stress signalling in yeast. From the significant homology that exists between mammalian and yeast MAPK signalling components, it is likely that the mechanisms found to govern activation of MAPK signalling pathways in yeast may also apply to mammalian cells. In yeast, the mechanisms of activation of related stress kinase MAPKs in response to heat shock have been analyzed in both of the common yeast models - *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). In *S. cerevisiae*, recent experimental evidence is available for the mechanism of activation of Hog1 during heat stress. Although this kinase was, until recently, thought to be activated exclusively by hyperosmotic stress, at least one study has shown that it plays an important role during the response to heat stress. Thus, similar to the mechanism utilized in response to osmotic stress, it was proposed that the Sho1 membrane-bound osmosensor can respond to heat stress and trigger the activation of signalling events culminating in Hog1 activation (Winkler et al., 2002). In *S. pombe*, the relevant stress-activated MAPK is known as Spc1 (also Sty1). Spc1 has been shown to be activated by a diverse array of stresses including heat shock. In this system, the full cascade of kinases required to activate Spc1 is not known but has been shown to involve the dual specificity MAPKK, Wis1. One report has suggested that Spc1

activation during heat shock can be accounted for by the inactivation of its phosphatases (Nguyen and Shiozaki, 1999), however another report disputes this finding (Shiozaki et al., 1998).

In mammalian cells, the elucidation of the mechanisms accounting for stress-induced JNK activation is complicated by the vast number of MAPKKs in this pathway. Gene knockout studies have indicated that MEKK1 (Yujiri et al., 1998) and ASK1 (Hidenori Ichijo pers. comm.) are not required for JNK activation by heat shock however, many more potentially redundant kinases are found at this level in the signalling cascade. Surprisingly, one of the few studies published on the analysis of MAPKK activation during heat shock showed that in the Hc9 rat myogenic cell line, MKK4 was not phosphorylated in response to heat shock despite the strong activation of JNK (Meriin et al., 1999). These authors concluded that JNK activation during heat shock was the result of inactivation of a JNK-specific phosphatase. A study from our own lab indicated that JNK activation in HeLa cells via heat shock was accompanied by a concomitant increase in the kinase activity of the JNK activator MKK7 (Foltz et al., 1998). However, the findings of Meriin et al have prevailed as the currently accepted model relating to the mechanism of heat-induced JNK activity despite the relatively scant investigation of MKK4 (and no investigation of MKK7) activity that was carried out in this study.

JNK activity has been shown to influence a wide variety of processes in the cell including inflammation, transformation, apoptosis and cell survival. How these varied cellular outcomes are achieved may rely on a number of factors such as the strength of signal, the duration of signal and the availability of particular substrates within the cell. Recently, a significant amount of insight into the function of JNK has been obtained from studies using embryonic fibroblasts genetically deficient in *jnk1* and *jnk2* – the only *jnk*

genes known to be normally expressed in this cell type. These studies have indicated an absolute requirement for JNK1 and JNK2 expression in the release of cytochrome c and the induction apoptosis in these cells in response to UV stress, DNA-damaging agents and the ribotoxic compound anisomycin (Tournier et al., 2000). The requirement of JNK to mediate apoptosis in response to heat shock was not examined. It is not known if a similar requirement for JNK exists in other cell types in response to various apoptotic stimuli.

Our findings outlined in the previous chapter indicated that the activation of JNK signalling during the heat shock response could be influenced by cell type or cell status-specific factors. This conclusion was drawn from the finding that murine lymphocytes lacked the robust activation of JNK that was noted in a variety of other cell types we studied. The objectives of the experiments outlined in this chapter include among others, an understanding of the potential mechanisms that influence JNK signalling in response to heat shock. We also investigate how the markedly different JNK activation profiles found in murine lymphocytes versus murine mast cells following heat shock affects the induction of apoptosis.

4.2 Results

4.2.1 Analysis of potential involvement of density dependence, reactive oxygen species and PKC in JNK activation induced by heat shock in MC/9 mast cells

The signalling mechanisms involved in JNK activation downstream of environmental stresses remain ill-defined. To understand what potential factors could be mediating the impaired JNK activation in murine lymphocytes during heat shock, necessitated an investigation into the mechanisms involved in heat-induced JNK activation in other cells. For these experiments we used MC/9 murine mast cells and tested what potential factors were required in order to mediate JNK activation during heat shock stimulation.

One potential mechanism we considered that could account for JNK signalling during heat stress was the production of heat-induced secreted factors that could stimulate an autocrine JNK signal. A characteristic of autocrine-mediated signalling is that it is influenced by cell density, that is, a higher concentration of autocrine factors would be present in the media of denser cell cultures and would lead to a stronger stimulation of signalling as compared to more dilute cell cultures. We tested heat-induced JNK phosphorylation in MC/9 cultures stimulated at a low density ($2 \times 10^6/\text{mL}$) or high density ($20 \times 10^6/\text{mL}$) to see whether this could affect signalling intensity. As shown in Figure 4.1A we did not see any difference in the ability of heat shock to activate JNK phosphorylation at either of these dilutions suggesting autocrine factors do not play a role in this process. In other experiments (data not shown), we treated cells with media harvested from dense cell cultures exposed to heat shock to test if there were autocrine factors present in this media that could stimulate JNK activity. We failed to see JNK activation in cells treated with the heat-conditioned media again suggesting that heat does not cause JNK activation via autocrine activation mechanisms.

Another potential mechanism we considered that could lead to JNK activation during heat shock was the production of reactive oxygen species (ROS). Generation of ROS has been shown to be required for JNK activation by a number of stimuli including the toxic compounds cadmium chloride and sodium arsenite. Evidence also exists for ROS production downstream of the JNK agonists TNF, IL-1 and LPS (Lo et al., 1996). To test for a possible role of ROS in heat-induced JNK activation, we treated cells with the free radical scavenger N-acetyl cysteine (NAC) prior to heat shock treatment. As shown in Figure 4.1B, NAC treatment could block JNK activation induced by the oxidative stress agent, sodium arsenite, but it had no effect on JNK activation caused by heat shock treatment or hyperosmotic stress which is not known to depend on ROS for activation of JNK.

Our investigation of possible mechanisms involved in JNK activation via heat shock was extended to determine a potential involvement of upstream signalling molecules known to be linked to JNK activation. One way in which these types of experiments can be achieved is through the use of inhibitory compounds that can specifically target a given molecule in the pathway. While specificity for a target can never be absolutely guaranteed, information obtained from these sorts of studies can nevertheless provide clues to the mechanistic basis of signaling pathways. PKC is a molecule which has been shown to be upstream of JNK activation in response to physiological stimuli such as TCR cross-linking and also certain stress stimuli. To investigate a role of PKC in JNK activation during heat shock we treated cells with bisindolymaleimide – an inhibitor of conventional PKC isoforms. As shown in Figure 4.1C, JNK activation stimulated by PMA

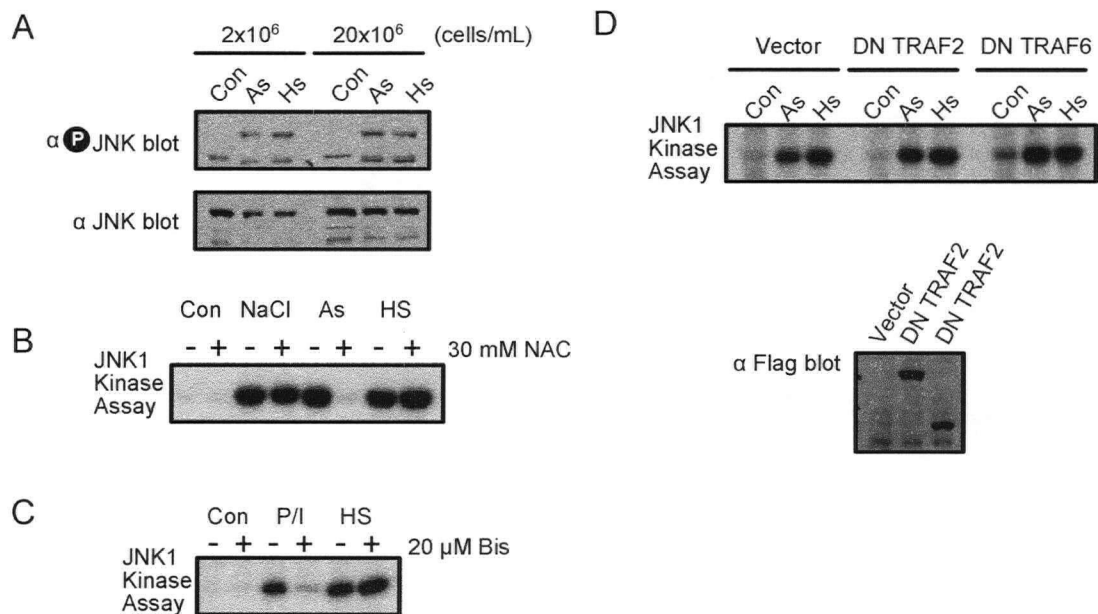


Figure 4.1 Testing for the involvement of various factors in heat shock-induced JNK activation in MC/9 murine mast cells. (A) Cells were heat shocked (HS) at 43°C, for 1hr, or stimulated with 200μM sodium arsenite (As) for 1hr at the indicated cell densities, followed by Western blot analysis of cell lysates probing for phospho JNK immunoreactivity (lower panels show JNK loading). (B) Cells were pretreated with or without 30mM N-acetyl cysteine (NAC) for 30 minutes and then stimulated with 0.4M NaCl for 10 minutes, sodium arsenite (As) or heat shocked (HS) followed by performing JNK1 kinase assays. (C) Cells were pretreated with or without 20μM of the PKC inhibitor bisindolymaleimide (Bis) for 45 minutes and then stimulated with 1 ng/mL PMA and 1 μg/mL ionomycin (P/I) for 10 minutes or 43°C (HS) for 1 hour followed by performing JNK1 kinase assays. (D) Cells were electroporated with cDNAs for HA-tagged JNK together with dominant negative (DN) FLAG-tagged TRAF2 or TRAF6 constructs. Cells were then stimulated 10 hours later with sodium arsenite (As) or heat shock (HS) and HA-tagged JNK activity was assayed following anti-HA immunoprecipitation. Lower panels indicate the expression of FLAG-tagged dominant negative TRAF molecules in these cells.

and ionomycin, which mimics signalling by the TCR, was severely abrogated by treatment with the PKC inhibitor however, heat-induced JNK was not. From these studies we can rule out the involvement of PKC isoforms that signal downstream of PMA and ionomycin in the mechanism accounting for heat-induced JNK activity. An additional molecule we decided to investigate for involvement in heat-induced JNK activation was a class of adaptor molecules known as the TNF receptor associated factors (TRAFs). As their name implies, these adaptors are important for TNF-induced signalling but they

also function in IL-1 signalling. Stimulation of TNF and IL-1 receptors in cells leads to robust JNK activation which is mediated by TRAF2 and TRAF6 respectively. Our interest in these molecules was heightened by a report that indicated TRAF involvement in relaying endoplasmic reticulum protein stress signals to the JNK pathway (Urano et al., 2000). Although heat shock does not trigger the activation of the ER stress response, we surmised that potential signals generated by protein stress in the cytoplasmic compartment might also use these adapters to trigger JNK activation. To test for the potential involvement of TRAF2 and TRAF6 in the activation of JNK triggered by heat stress, we overexpressed dominant negative forms of these adapter molecules in cells along with HA-tagged JNK1. Truncation of an amino terminal effector domain of TRAFs converts these molecules into dominant negative proteins (Rothe et al., 1995). As shown in Figure 4.1D, expression of DN TRAFs did not prevent JNK activation by heat shock. Thus, we suggest that JNK activation by heat shock is independent of TRAF function. TRAF molecules serve as a link between signalling events initiated via receptor activation and the JNK pathway most likely via interaction with MAPKKK family members such as MEKK1. An alternative link from receptor signalling to JNK is via the small GTPase Rac. Coupling of Rac activation to Ste20-related kinases such as PAK or via interaction with MEKKs allows for JNK activation downstream of stimuli that potentiate Rac activity. To test for the role of Rac in heat shock signalling, we measured Rac activity in MC/9 cells during heat shock using a pull-down assay with GST-CRIB. While we could detect Rac activation in response to steel factor stimulation in MC/9 cells, we failed to see activation in response to heat at multiple time points (data not shown). Overall, our studies in MC/9 mast cells suggest that heat shock can stimulate JNK activity independently of receptor signalling events that involve recruitment of TRAFs or Rac. We also rule out the involvement of ROS generation and activation of typical PKC isoforms in the mechanism of heat-induced JNK activity.

4.2.2 The JNK kinases MKK4 and MKK7 are phosphorylated in response to heat shock

Continuing with our analysis of the mechanism of JNK activation via heat shock, we set up assay systems to define the involvement of the JNK kinases MKK4 and MKK7 in heat-induced signalling. Although numerous studies have documented JNK activation in response to heat shock we could only find one study that had attempted to characterize the involvement of a JNK kinase during heat shock signalling. Surprisingly, this study showed that in a rat myogenic cell line H9c2, heat-induced JNK phosphorylation was not accompanied by a concomitant activating phosphorylation of the JNK kinase MKK4. (Meriin et al., 1999). The investigators in this study suggested that JNK activation during heat shock was the result of the inactivation of phosphatases specific for JNK. From previous studies conducted in our lab, we had evidence that MKK7 could be activated by heat shock so we decided to conduct a more thorough examination of the role of JNK kinase activation during heat shock. In these experiments, we used a phosphorylation-specific antibody against MKK4 as an indirect measure of its activation in response to heat and other stresses. However, due to a high degree of background cross-reactivity we could not reliably analyze endogenous MKK4 phosphorylation in our cells. Instead, we used a methodology whereby we transiently transfected cells with a mammalian expression vector encoding GST-MKK4 which allowed us to pull down and detect the phosphorylation status of this molecule following stress treatment. Using this assay in MC/9 murine mast cells, we discovered that MKK4 was indeed phosphorylated in response to heat shock and this occurred in a time and temperature dependent manner. We repeated these experiments using the human HeLa cell line (as well as 3T3 murine fibroblasts – data not shown) and we found that heat shock of these cells also led to MKK4 phosphorylation indicating this result to be consistent among different cell types.

Analysis of JNK activity in these cells (lower panels Figure 4.2) correlated with the extent of MKK4 phosphorylation observed in each case. These results suggested that the mechanism of heat-induced JNK is dependent on the increased activation of MKK4.

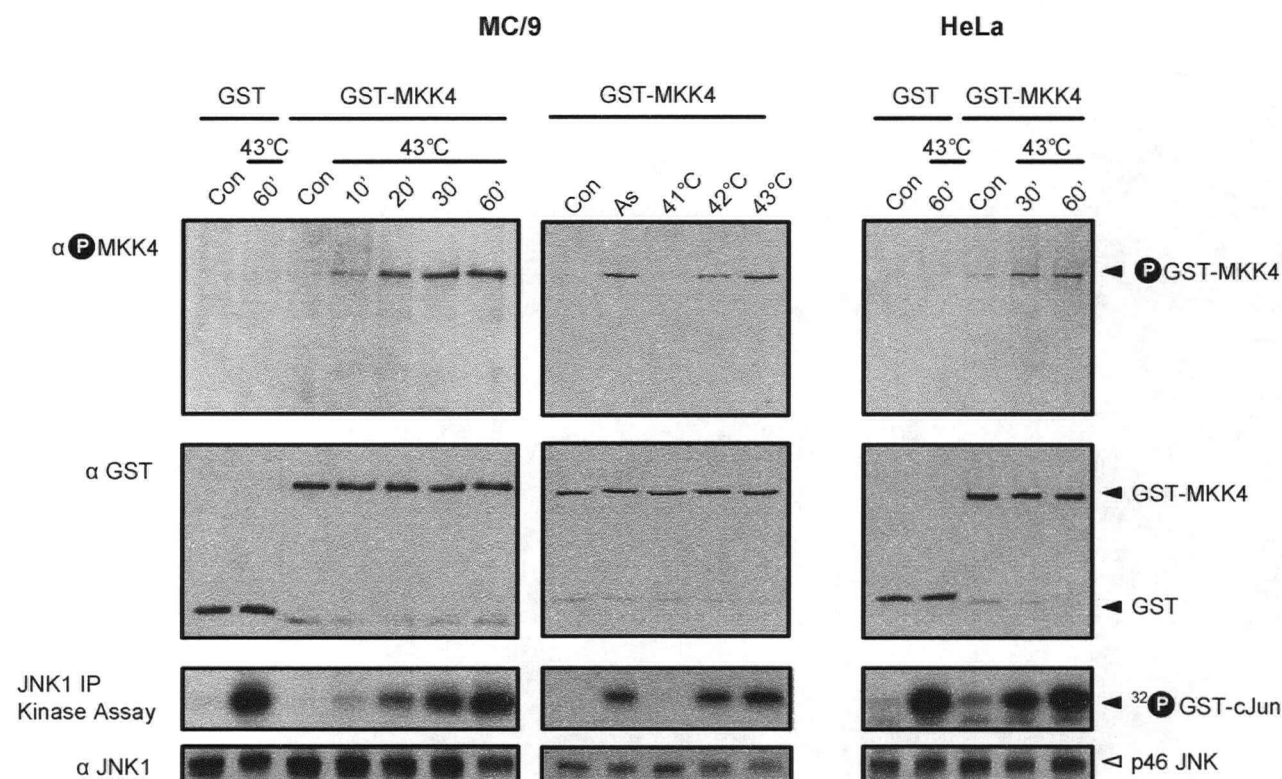


Figure 4.2 Heat shock induces phosphorylation of the JNK activator MKK4. MC/9 murine mast cells (left panels) or human HeLa cells transfected with DNA constructs for either GST or GST-MKK4 were subjected to heat stress as indicated. GST-MKK4 transfected MC/9 cells were also exposed to a range in temperature for 1 hour or 200 μ M sodium arsenite for 1 hour. Transiently-expressed proteins were then precipitated with glutathione sepharose beads, SDS PAGE was performed and the phosphorylation status of GST-MKK4 was determined by Western blotting with an anti-phospho MKK4 - specific antibody. The same blots were then stripped and reprobed with an anti-GST antibody to ensure equivalent amounts of transfected protein were precipitated and analyzed in these assays. A fraction from the same lysates used in the above experiments were then tested for JNK activity using JNK1 immunoprecipitation kinase assays. The relative levels of JNK1 immunoprecipitated in each experiment are shown in the immunoblots in the bottom most panels.

4.2.3 Lack of JNK activity in murine lymphoid B cell WEHI-231 during heat shock correlates with the lack of activation of both MKK4 and MKK7

Having established this assay system we wanted to determine whether the deficit in JNK activation noted in WEHI-231 B cells was a consequence of a lack in heat-induced activation of its upstream kinase or, among other possibilities, could be due to a direct suppression of JNK. In addition to MKK4, we also studied the phosphorylation of the other known JNK kinase, MKK7, using a phospho-specific antibody for this molecule. A comparison of MC/9 mast cells and WEHI-231 B cells transfected with either GST-MKK4 or GST-MKK7 indicated that the lack of potent JNK activity in WEHI-231 B cells was accompanied by a similar lack of substantial phosphorylation of both GST-MKK4 and GST-MKK7. Figure 4.3A shows that both cell types respond equally well to osmotic and oxidative stresses with the phosphorylation of exogenous MKK4 and MKK7. We followed up these studies with an analysis of the activation status of endogenous MKK4 using a linked kinase assay. In this assay, we immunoprecipitated endogenous MKK4 from MC/9 mast cells and WEHI-231 B cells and incubated these immunoprecipitates with recombinant GST-JNK3 *in vitro* in the presence of unlabelled ATP. Aliquots of this kinase reaction were then incubated with the JNK substrate GST-cJun along with radiolabelled ATP to assess the extent of MKK4-induced GST-JNK3 activity. In correlation with our analysis of MKK4 phosphorylation, we found that endogenous MKK4 kinase activity could be induced by heat in MC/9 mast cells but not in WEHI-231 B cells (Fig 4.3B). Thus, our results indicate that heat shock causes the activation of both MKK4 and MKK7 which results in JNK activation. The observed lack of JNK activity in heat shocked murine lymphocytes is a result of the impaired activation of the JNK kinases MKK4 and MKK7.

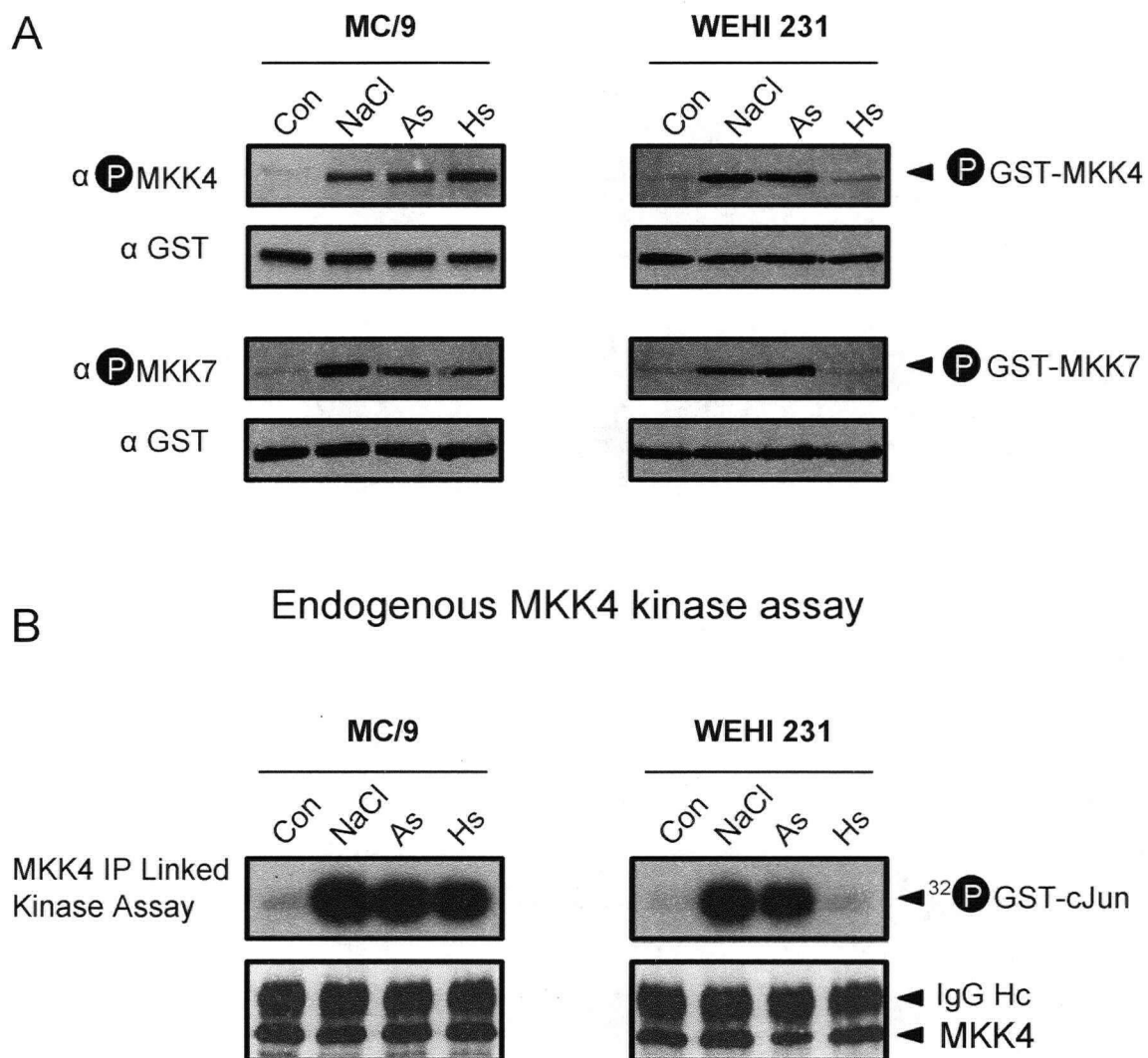


Figure 4.3 Heat shock fails to cause MKK4 phosphorylation in the lymphocyte B cell line WEHI-231. (A) MC/9 mast cells and WEHI-231 B cells transfected with GST-MKK4 or GST-MKK7 were either left untreated (Con), or stimulated with 0.4 M sodium chloride for 10 min (NaCl), 200 μ M sodium arsenite for 60 min (As) or 43°C for 60 min (Hs). Transiently-transfected GST-MKK4, GST-MKK7 was precipitated from lysates with glutathione sepharose, SDS PAGE performed and then the phosphorylation status of these molecules was determined by blotting with anti-phospho MKK4 and anti-phospho MKK7-specific antibodies. The levels of GST-MKK4 and GST-MKK7 precipitated were determined by stripping the blots and reprobing with an anti-GST antibody. (B) Endogenous MKK4 kinase activity was performed in MC/9 mast cells and WEHI-231 B cells using a linked kinase assay. MKK4-induced GST-JNK3 activation was assayed using GST-cJun as a substrate. Western blots of the MKK4 immunoprecipitates were done to ensure equal levels of MKK4 were immunoprecipitated in this assay (lower panel).

4.2.4 Murine lymphocytes fail to display JNK activation following exposure to the ribotoxic compound anisomycin

Another interesting observation we have made during the course of these studies was that the ribosomal toxin, and strong JNK agonist, anisomycin, like heat shock, failed to activate JNK in murine lymphocytes. In Figure 4.4 we compared JNK phosphorylation induced by various stimuli in WEHI-231 murine B cells versus MC/9 murine mast cells and also extended this analysis to primary murine cells as well as primary human T cells. The pattern of JNK activation in response to anisomycin in these cells matched that seen in response to heat shock. That is, both murine lymphoid cell lines and primary murine T cells which fail to activate JNK in response to heat, also fail to activate JNK in response to anisomycin. Also, as shown in chapter 3, cultured human T cells that were capable of JNK activation in response to heat shock also activated JNK in response to anisomycin. From these results we suggest that heat shock and anisomycin may induce JNK activation through a similar mechanism that is somehow impaired in murine lymphocytes. One common link might be that the ribosomal impairment induced by anisomycin, like heat shock, generates increased levels of misfolded proteins that in normal cells, but not murine lymphocytes, can initiating a signalling response leading to JNK activation.

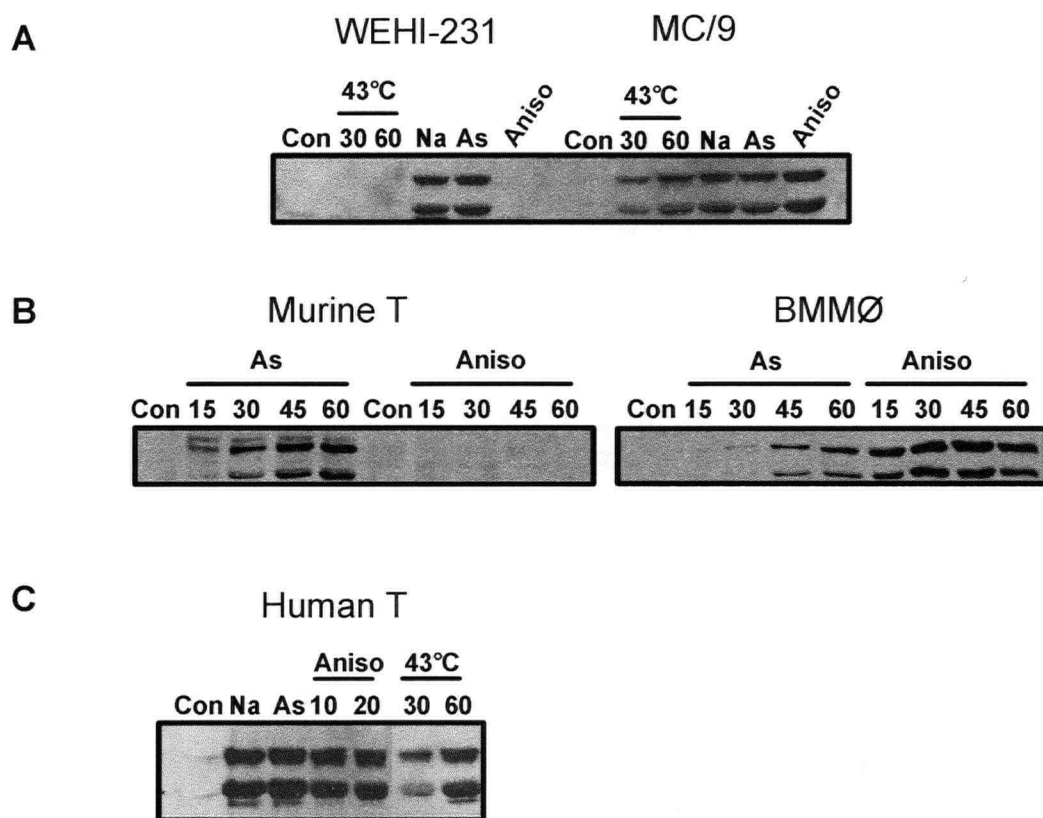


Figure 4.4 Anisomycin fails to cause JNK phosphorylation in murine lymphocytes. (A) WEHI-231 murine B cells and MC/9 murine mast cells were tested for JNK phosphorylation in response to 43°C for 30 for 60 minutes, 0.4 M NaCl for 10 minutes (Na), 200 µM sodium arsenite for 60 minutes (As) or 10 µg/mL anisomycin for 60 minutes (Aniso). (B) Primary murine T cells or murine bone marrow derived macrophages (BMMØ) were tested for JNK phosphorylation following exposure to 200 µM sodium arsenite or 10 µg/mL anisomycin for the times indicated (in minutes). (C) Human T cells derived from OKT3-stimulated peripheral blood were tested for JNK phosphorylation following treatment with 0.4M NaCl for 10 minutes (Na), 200 µM sodium arsenite for 60 minutes (As), 10 or 20 µg/mL anisomycin for 60 minutes (Aniso) or 43°C for 30 for 60 minutes.

4.2.5 Enhanced apoptotic response in WEHI-231 B cells over MC/9 mast cells as a result of heat shock

One of the potential consequences of heat stress on the cell is the induction of apoptosis. It is believed that this response occurs once a certain threshold of heat-induced cellular damage has been surpassed from which the cell cannot recover. The apoptotic response to heat, similar to many forms of environmental stress, has been shown to involve cytochrome c release, the activation of caspase 9 and caspase 3 leading to the subsequent cleavage of caspase substrates such as PARP (Li et al., 2000). Several studies have indicated a role for JNK in the apoptotic response of cells following stress treatment. Most notably, in murine embryonic fibroblasts genetically deficient in jnk1 and jnk2 (the only isoforms expressed in these cells) it was shown that cytochrome c release and cell death in response to UV required jnk1 and jnk2 (Tournier et al., 2000). It was of interest to us to determine whether the relatively low level of JNK activity noted in WEHI-231 B cells in response to heat shock would confer a survival advantage over MC/9 mast cells where we note strong JNK activity after exposure to heat stress. For these studies, we analyzed biochemical markers of apoptosis including cytochrome c release, caspase 9 and caspase 3 activation in addition to performing quantitative analysis of apoptosis via annexin V staining using flow cytometry. A comparison of cytochrome c release and proteolysis of caspases assessed via Western blotting, suggested a greater degree of apoptosis occurred in WEHI-231 B cells in response to heat shock (Fig. 4.5). In support of this, we found that over 19% of WEHI-231 cells became apoptotic with heat treatment versus 7% in MC/9 mast cells as determined by annexin V assay. Hence, a significant extent of apoptosis can occur in heat shocked WEHI-231 B cells despite the relatively weak JNK response. These results

suggest JNK activity is likely not a major influence in the initiation of apoptosis in response to heat shock in these cells.

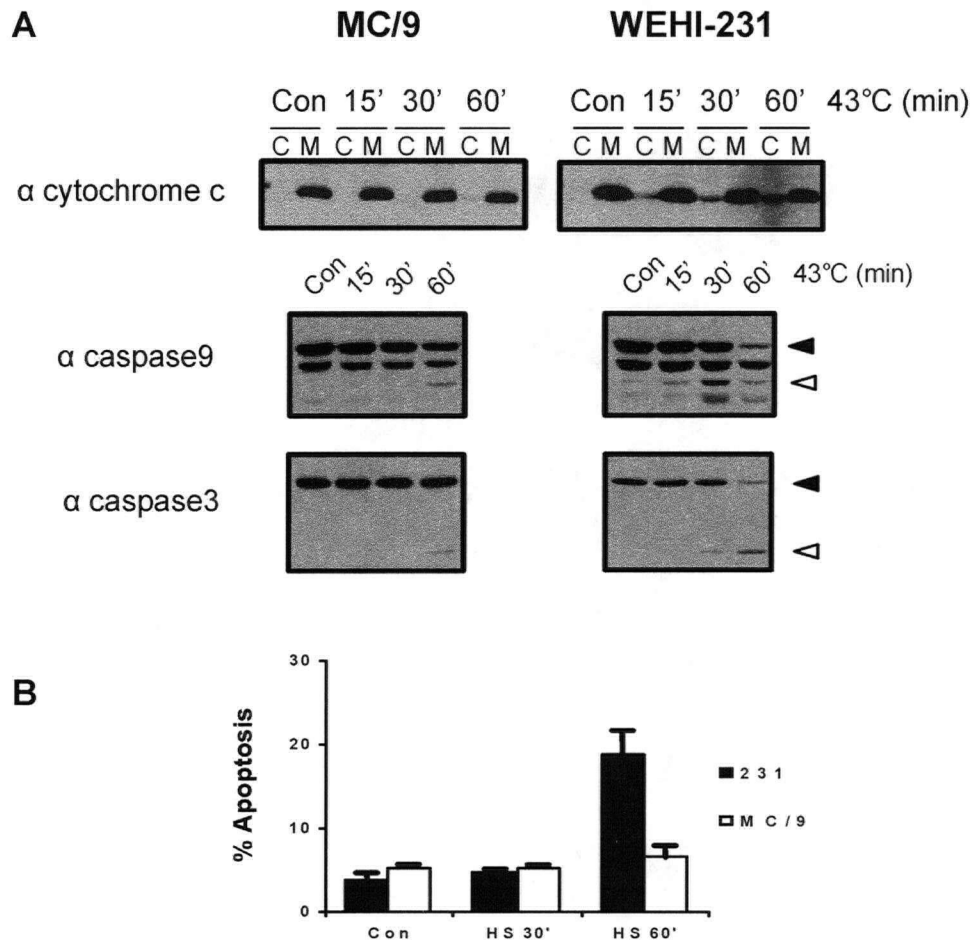
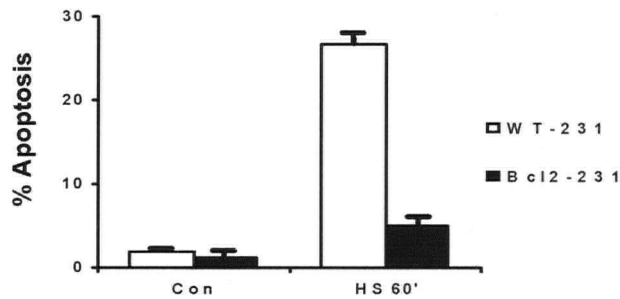


Figure 4.5 Increased heat-induced apoptosis in WEHI-231 B cells over MC/9 mast cells. (A) MC/9 mast cells and WEHI-231 B cells were compared for the extent of cytochrome c release in response to 43°C heat shock for the indicated times. Western blotting was used to detect the release of membrane localized cytochrome c (M) into the cytosolic fraction (C) (upper panels). Lysates from these two cell lines were also compared for the processing of caspase 9 and caspase 3. Black arrows denote the position of the full length caspases while white arrows indicate cleavage products. (B) Flow cytometry analysis of heat shocked MC/9 mast cells and WEHI-231 B cells was used to quantitate the number of cells positive for the early apoptotic marker annexin V. Shown is a graph representative of three separate experiments.

4.2.6 Bcl-2-overexpression significantly inhibits heat-induced apoptosis in WEHI-231 B cells but does not alter JNK activation status

Given the enhanced sensitivity of WEHI-231 B cells to apoptosis induced by heat, we were concerned that this property alone could account for the reduced JNK activity in these cells. We considered that the higher levels of apoptosis in these cells might also be associated with a general decline in the operation of signalling cascades in these cells. To help rule out this possibility, we made use of a Bcl-2-overexpressing WEHI-231 B cell line. Overexpression of Bcl-2 has been shown to prevent the induction of apoptosis that occurs upon exposure of cells to various noxious stimuli or in response to the removal of essential growth factors (Vaux et al., 1988). In agreement with the known effects of Bcl-2 overexpression, we found that the Bcl-2-WEHI-231 cell line used in our experiments showed a dramatic resistance to heat-induced apoptosis as determined via annexin V assays (Fig. 4.6A). However, when we measured the extent of JNK phosphorylation in these cells, despite their resistance to apoptosis, it was not restored to levels comparable with those seen in other cell types (Fig. 4.6B). These results suggest that the properties that mediate the enhanced susceptibility of WEHI-231 B cells to heat-induced apoptosis do not account for the reduced JNK signalling noted in these cells following heat shock.

A



B

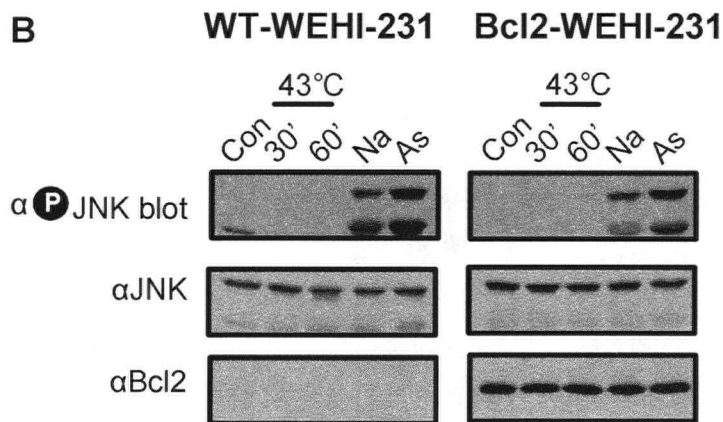


Figure 4.6 Resistance of WEHI-231 B cells to apoptosis using Bcl-2-WEHI-231 B cell line does not restore JNK activation in response to heat. (A) Wildtype or Bcl-2 overexpressing WEHI-231 B cells were compared for the extent of apoptosis in response to heat shock using annexin V staining. This experiment is representative of three independent experiments. (B) The same two cell lines were compared for JNK activation in response to heat shock for the times indicated or 0.4 M NaCl for 10 minutes (Na) or 200 μM sodium arsenite for 60 minutes (As) using anti-phospho JNK Western Blotting. The same blot was reprobed for JNK loading and an antibody against human Bcl-2 to detect exogenous Bcl-2 expressed in the WEHI-231 Bcl-2 cell line.

4.2.7 Lack of JNK activity in heat shocked WEHI-231 B cells is not a result of heat-induced suppression of the JNK pathway

Another possibility we considered that might account for the altered JNK signalling in murine lymphoid cells was the induction of a factor(s) that could actively suppress the JNK pathway in response to heat. This could include, for example, a phosphatase or chaperone whose activity or expression level could be elevated as a result of heat shock. To rule out this possibility, we determined whether it was possible to activate JNK phosphorylation in WEHI-231 Bcl-2 cells with osmotic shock whilst these cells were undergoing heat shock exposure. Our results show that JNK phosphorylation can be achieved in these cells even during the course of a heat shock indicating the pathway is not dominantly suppressed by factors induced by heat shock exposure. To further support these results, we pre-treated wildtype WEHI-231 B cells with the protein synthesis inhibitor cycloheximide prior to heat shock of these cells. These experiments were designed to prevent the synthesis of any JNK-inhibitory molecules that might be actively suppressing the JNK pathway in these cells during heat shock. As shown in Figure 4.7B we did not observe an increase in JNK activity in WEHI-231 cells in response to heat shock if protein synthesis was inhibited, which is in agreement with the findings from the previous experiment that the JNK pathway is not actively suppressed in these cells during heat shock.

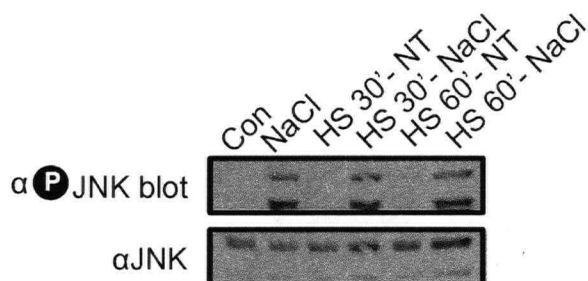
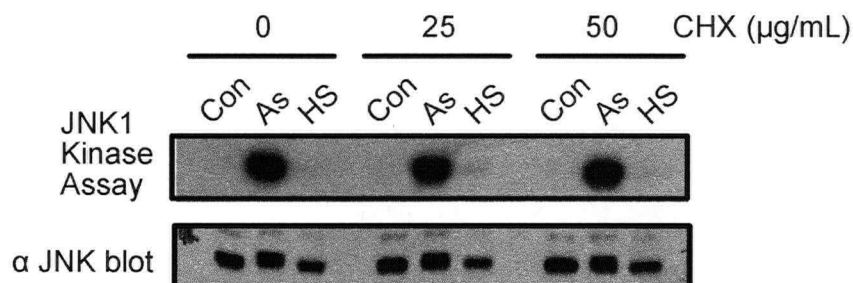
A**Bcl2-WEHI-231****B****WT-WEHI-231**

Figure 4.7 Heat shock does not lead to a suppression of the JNK pathway in WEHI-231 B cells. (A) WEHI-231 B cells overexpressing Bcl-2 were tested for JNK phosphorylation in response to 0.4 M NaCl treatment 10 minutes. Cells were either stimulated with NaCl while at 37°C or while at 43°C after 30 or 60 minutes of prior heat shock. NT indicates cells were not treated with NaCl during heat shock. The blot was then stripped and reprobed to indicate the levels of total JNK present in these lysates. (B) Wildtype WEHI-231 murine B cells were pre-incubated with different concentrations of the protein synthesis inhibitor cycloheximide (CHX) for 45 minutes prior to a 1 hour heat shock at 43°C (HS) or treatment with 200 μM sodium arsenite for 1 hour (As). Lysates were then prepared and JNK activity was determined using a JNK1 kinase assay. The levels of JNK1 protein in the assay was determined by blotting with an anti-JNK1 antibody.

4.3 Discussion of Chapter IV

The mechanisms involved in the activation of stress signalling pathways in mammalian cells are poorly understood. While there is good evidence linking stress kinase activation to receptor-mediated events initiated in response to various protein ligands, the delineation of such a circuit in response to environmental stresses has not been achieved. In yeast and bacteria a variety of integral membrane proteins can serve as sensory proteins for a diverse array of stresses. Many of these are part of two component signalling systems which involve histidine kinases and response regulators (often a transcription factor). In mammals, a more complex network of receptors, adaptors, scaffolding molecules and multi-tiered kinase cascades have evolved, allowing the cell to respond appropriately to a much wider range of signalling inputs. The involvement of various components of these mammalian signalling networks in transmitting stress signals remains largely undefined.

In this chapter we tested for the possibility that JNK activation might involve events initiated at or in close proximity to the plasma membrane. First, we tested whether heat shock might induce the production of an autocrine factor that could stimulate receptors on the cell surface and transduce a stress signal. Our data showed that this was unlikely to be the case given the density-independence of JNK activation during heat shock and the failure of heat shock-conditioned media to stimulate JNK when added to cells. We also found that the activation of the Rac GTPase, which can activate JNK upon overexpression and which is activated by stimulation of various receptors upstream of JNK, was not affected by heat shock. In addition, we investigated the involvement of the TRAF2 and TRAF6 adaptor molecules which couple receptor activation induced by TNF and IL-1 ligand binding to stress kinase activation. Using dominant negative TRAF

constructs, our results precluded the involvement of these adaptors as necessary for the activation of JNK in response to heat shock. Finally, we undertook an investigation into the role of PKC in mediating JNK activation induced by heat as this molecule has been shown to couple several receptor-initiated events to JNK activation and can be conveniently inhibited with the use of a pharmacological agent. We found no effect of inhibition of PKC function on the activation of JNK during heat shock in MC/9 cells suggesting that PKC (at least the conventional isoforms) does not play a role in transmitting heat-induced signals to this pathway. While there are admittedly many other signalling systems that could be initiated at the plasma membrane in response to stress it was beyond the scope of this study to pursue an investigation of all these possibilities. One study has demonstrated that JNK activation can be achieved upon heating cell extracts indicating membrane events are not required and that cytosolic factors are responsible for heat sensing and JNK activation (Adler et al., 1995). However, it should be noted that these findings have not been reported by any other groups and that we were unable to achieve these same results in our own experiments. Thus, it remains debatable as to whether cytosolic factors are sufficient to mediate JNK activation during heat shock or whether membrane-bound sensors are also required. A recent study investigating heat shock signalling in yeast has indicated a role for the membrane-bound osmosensor Sho1 in the activation of the yeast stress kinase Hog1 during heat shock (Winkler et al., 2002). Currently it is not known whether homologues of Sho1 occur in mammalian cells.

Significant insight into the mechanism of JNK activation during heat shock was obtained from our analysis of MKK4 and MKK7 activation. Previously, it had been shown that in the H9c2 rat myogenic cell line heat shock did not activate MKK4 despite a strong induction of JNK activity (Meriin et al., 1999). Thus, the mechanism that was thought to

account for JNK activation during heat shock was believed to be a result of the inhibition of JNK-specific phosphatases. Our results indicated that in both MC/9 and HeLa cells, heat shock led to a robust stimulation of MKK4 phosphorylation. Thus, inhibition of JNK-phosphatases alone cannot account for JNK activity during heat shock. Importantly, we did not observe MKK4 or MKK7 activation in WEHI-231 B cells during heat shock. This indicates that a deficit in heat-induced signalling events in murine lymphocytes occurs at the level of MKK4/MKK7 or their upstream activators.

How heat shock may cause the activation of kinases upstream of JNK is not known. As mentioned above, one possibility is that a signalling cascade initiated by heat-induced activation of a membrane-bound receptor could lead to JNK activation. The validation of this hypothesis remains an important priority. However, it is also a possibility that the activation of cytosolic kinases upstream of JNK during heat shock occurs without the need of a receptor at the membrane. Given this model, we believe it possible that two separate mechanisms could account for the activation of the JNK pathway during heat shock. One possibility, involves the inactivation of specific phosphatases which regulate MKK4, MKK7 or a particular kinase(s) which functions upstream in the pathway. The inactivation of a phosphatase specific for a given MAPKKK for example would lead to the serial activation of MKK4 and MKK7 and subsequent JNK activation. One important question in this model concerns the mechanism whereby heat can specifically target a given phosphatase or set of phosphatases that regulate the JNK pathway. In accordance with this model we would have to suggest that the lack of JNK activity we note in murine lymphocytes is a result of the relative insensitivity of the complementary phosphatases in these cells. It is possible that murine lymphocytes express different isoforms of the phosphatases that regulate upstream JNK kinases which are inherently less susceptible to heat-inactivation or that the phosphatases in these cells may be

stabilized by chaperone interactions not present in other cells. In addition, it may be possible that signalling events not present in murine lymphocytes can negatively regulate phosphatase activity. Evidence for this mode of phosphatase regulation has been shown in the case of the tyrosine phosphatases HePTP and PTP-SL which can be negatively regulated by PKA signalling (Blanco-Aparicio et al., 1999; Shen et al., 2001). We find it unlikely that increases in cellular phosphatase activity in murine lymphocytes during heat shock mediates JNK suppression. We showed that JNK signalling was not actively suppressed in WEHI-231 B cells during heat shock since we achieved normal activation of JNK when these cells were challenged with osmotic stress during the course of an ongoing heat shock. We also showed that inhibition of protein synthesis, which would prevent the increased production of phosphatases during heat shock, did not alter JNK activity in heat-shocked WEHI-231 B cells.

The second possibility we have considered that could account for JNK activation during heat shock, is via the direct activation of a kinase that signals upstream of MKK4 and MKK7. This could include a MAPKKK family member or a Ste20-related kinase such as PAK. In this mechanism, we would propose that the influence of heat would be to induce conformational changes in the kinase itself that would lead to its activation as opposed to the previous model whereby kinase activation is achieved by loss of negative regulation by its associated phosphatase. In this model, we would propose that murine lymphocytes would lack the expression of such a molecule. Given the abundance of signalling molecules at the MAPKKK level and beyond, the identification of a molecule critical for JNK activation during heat shock may prove a daunting task. Evidence from knockout studies thus far have determined that MEKK1 (Yujiri et al., 1998) and ASK-1 (Hidenori Ichijo – pers. comm.) are not required for heat-induced JNK activity. It is possible that there is a high level of redundancy in MAPKKK function and thus targeting

of multiple kinases at this level would be required in order to reveal their roles in stress signalling. Indeed, recent studies using siRNA for MAPKKKs in insect cells indicated that silencing of multiple MAPKKKs was required in order to prevent JNK activation in response to the osmotic stress agent sorbitol (Chen et al., 2002b). The requirement of individual or multiple MAPKKKs for JNK activation in response to heat shock was not tested in these studies.

Our finding that the ribotoxic compound anisomycin was similar to heat in terms of its JNK activation profile in the cells used in our study may indicate a common mechanism of stress kinase activation by these two stresses. Interestingly, one report has indicated that anisomycin can negatively affect phosphatase activity in the cell (Theodosiou and Ashworth, 2002). Anisomycin has been shown to bind directly to the 28S ribosomal subunit which results in the impairment of the peptidyl transfer event during translation. However, it seems that it is not simply a matter of inhibition of protein synthesis that leads to JNK activation since not all compounds that can inhibit translation also activate JNK. One possibility is that anisomycin treatment can lead to the generation of improperly translated and thus unfolded peptides within the cytosol. In this way, anisomycin may cause the same stress signal as heat shock - a rise in the number of unfolded proteins in the cell. Thus, it is possible that both heat and anisomycin use a common signaling intermediate required for efficient JNK activation in the response to these two stresses.

Finally, our analysis of the biological consequences of heat shock on murine lymphoid and non-lymphoid cells has indicated that JNK activation does not play a role in the initiation of apoptotic events in response to this form of stress. Indeed, we found that WEHI-231 B cells had higher levels of apoptosis as compared to MC/9 mast cells

following a 1 hour heat shock (Fig. 4.5). This suggests that factors other than JNK activation mediate apoptosis in WEHI-231 B cells during heat stress. One possibility is that differences in expression levels of molecules regulating apoptosis in WEHI-231 B cells and MC/9 mast cells such as Bcl-2 or Bcl-XL, caspases and inhibitors of apoptosis proteins (IAPs) could account for the differences in apoptotic susceptibility in these two cell lines. Alternatively, JNK activation may have a direct positive effect on cell survival during heat stress. Recent evidence suggests that JNK-mediated Bcl-2 phosphorylation during growth factor withdrawal enhances the pro-survival function of this molecule (Deng et al., 2001). In yeast, the deletion of the stress kinase homologue Spc1 is required for the survival of cells exposed to heat shock and other stresses (Degols et al., 1996). However, excessive activation of yeast stress kinases by deletion of relevant phosphatases has a negative impact on cell survival (Winkler et al., 2002). Thus, the duration of JNK activation and perhaps also the magnitude seem to be important factors in determining the outcome of JNK signalling in terms of apoptosis. The positive influence of JNK activity on cell survival during stress differs from the findings of Tournier et al, where analysis of JNK-deficient murine embryonic fibroblasts (MEFs) indicated that JNK was required for apoptosis in response to UV stress. It is possible that JNK may be required for certain forms of apoptosis while not required for others. It is also possible that JNK-mediated apoptosis may be a cell-type dependent phenomenon.

CHAPTER V General conclusions and future directions

The cellular response to heat shock involves the triggering of various signalling cascades many of which are highly conserved throughout evolution. The activation of heat shock protein synthesis for example, is a universally conserved response in all organisms following exposure to heat and other protein damaging stresses. In some organisms, including mammals, heat shock also triggers a kinase cascade which culminates in activation of a class of stress-activated MAPK family members known as JNK and p38 MAPK. In this thesis we have studied the activation of these kinases, with emphasis on the JNK pathway, following heat shock in a variety of different murine hematopoietic cell types. As a result of these studies, we have discovered a marked variation in the activation of JNK that was associated with the cell type examined. Pursuant to these observations, we have undertaken an investigation aimed at understanding the potential mechanisms that contribute to JNK activation during heat shock.

The basis for the studies carried out in this work stemmed from our observation of the dramatically reduced JNK activation found in murine lymphocytes following heat shock stimulation. We discovered a similar deficit in the heat-induced activation of the related stress kinase p38 in these cells. This was in dramatic contrast to the robust heat-induced activation of these kinases we observed in our own studies of other hematopoietic cell types such as macrophages and mast cells, and to that of numerous other cell types previously reported in the literature. Our finding that other forms of stress such as hyperosmotic or oxidative stress had the ability to potentiate JNK activity in murine lymphocytes comparable to other cell types indicated that JNK activation could proceed

normally in murine lymphocytes and that heat shock likely required the loss or gain of additional factors that influence JNK activity in murine lymphocytes.

From our analysis of the induction of HSF1 hyperphosphorylation and Hsp70 synthesis in murine lymphocytes, we were able to conclude that these processes can occur in the absence of a strong JNK signal suggesting that initiation of HSF1 activity can function independently of JNK. Our analysis of another heat-induced signalling response – the phosphorylation of eIF2 α - indicated that this event occurs normally in both murine lymphoid and non-lymphoid cells alike. This finding along with the much more rapid induction of eIF2 α phosphorylation as compared to JNK during heat shock, suggests that the triggering of these two stress-responsive pathways occurs in a mutually exclusive fashion.

The focus of our efforts outlined in chapter 4 was to define more clearly, the mechanism responsible for JNK activation during heat shock. From this information we hoped to obtain a better understanding of the mechanisms that could account for the lack of JNK activation in heat-shocked murine lymphocytes. We conducted experiments to determine whether signalling molecules known to associate with membrane proximal events had any influence on JNK activity caused by heat shock. From our limited analysis we could not conclude whether signalling events initiated by membrane-bound receptors during heat shock are critical for JNK activation. Evidence exists in yeast that membrane-bound sensors can regulate stress signalling however it is not known whether similar molecules exist in mammalian cells. It is possible that membrane receptors known to be involved in other aspects of JNK signalling in mammals may have retained properties that can also allow them to function as thermosensors analogous to those found in lower organisms

(e.g. yeast Sho1). Defining the critical sequence requirement for this function may allow candidate molecules to be discovered in mammals.

In addition to the data shown in chapter four, numerous experiments were conducted in an attempt to define the basis for the differential activation of JNK in lymphoid and myeloid cells in response to heat shock. One possibility we considered was that heat shock might lead to a differential induction of ceramide in lymphoid and myeloid cells. As ceramide has previously been shown to potentiate JNK activity (Verheij et al., 1996) and has been shown to be produced in response to heat shock (Chang et al., 1995), it was possible that a differential induction of ceramide between these two cell types might explain the difference in JNK activity during heat shock. To address this possibility, we conducted several sets of experiments. First, we measured the production of ceramide produced by MC/9 mast cells and WEHI-231 B cells during heat shock using a radioactive assay involving tritiated palmitate – a ceramide precursor. Although we were able to detect increases in ceramide produced during heat shock, no significant differences were observed between cell types. In addition to these studies, we utilized two different compounds to inhibit ceramide generation. This included Fumonisin B1, an inhibitor of ceramide synthase and desipramine which has been shown to interfere with sphingomyelinase, an enzyme that produces ceramide as a byproduct from the breakdown of sphingomyelin. We found that neither of these inhibitors could interfere with the activation of JNK caused by heat shock in MC/9 mast cells suggesting that JNK activation during heat shock was not a result of ceramide generation.

Additional experiments were conducted in order to determine whether the signalling pathways that are induced by heat shock could be mimicked by increasing the relative amount of unfolded proteins in the cell. We considered the possibility that murine

lymphocytes may be more tolerant to the induction of JNK signalling that might occur in response to a rise in unfolded proteins. One experimental technique that was tried was to overexpress a GFP-tagged polyglutamine repeat protein in cells. Constructs containing multiple glutamine repeats designed to mimic the effects of expanded polyglutamine stretches in the Huntington protein have been shown to lead to the formation of insoluble aggregates when expressed in cells and could also lead to JNK activation (Liu, 1998; Merienne et al., 2003; Yasuda et al., 1999b). However, when expressed in MC/9 mast cells, a GFP fusion protein with a stretch of 82 polyglutamines failed to cause JNK activation despite the ability of this protein to form large aggregates within the cell. These results suggested that expanded polyglutamines may not result in JNK activation in all cell types and raised the possibility that JNK activation caused by heat shock might be a result of the unfolding of specific proteins - perhaps only those involved in JNK regulation. However, further studies, perhaps using other models of unfolded proteins will be required in order to establish whether this is the case.

Our studies have clearly shown that JNK activation during heat shock is mediated by activation of its direct upstream kinases MKK4 and MKK7. This finding suggests that the current model accounting for heat-induced JNK activation which is based on the inactivation of its dedicated phosphatases requires modification. Like JNK, we found that MKK4 and MKK7 were not activated by heat shock in murine lymphocytes.

We have proposed two models to account for how upstream signalling is initiated during heat shock and how murine lymphocytes differ in the triggering of these events. These models are exclusive of a mechanism involving a critical membrane bound component. However, it is important to note that our evidence at this point does not allow us to exclude a role for membrane-bound sensors hence the following mechanisms we

describe should be regarded only as possible alternative explanations. The first mechanism involves the heat-induced inactivation of phosphatases operational on JNK signalling components at the level of MKK4, MKK7 or kinases upstream of these. We suggest that murine lymphocytes possess a cohort of phosphatases specific for these upstream kinases that are not as susceptible to this form of inactivation. This proposed model is outlined in Figure 5.1 as model 1. The validity of this hypothesis could be tested by the development of assays to measure the activity of the relevant phosphatases involved in cell extracts. The use of phospho peptide substrates with sequences matching those found in MKK4, MKK7 and various MAPKKs may allow for a more specific measurement of the appropriate phosphatase activities in cell extracts. Alternatively, with the use of antibodies specific for candidate JNK pathway phosphatases a more direct assessment of their activity could be made.

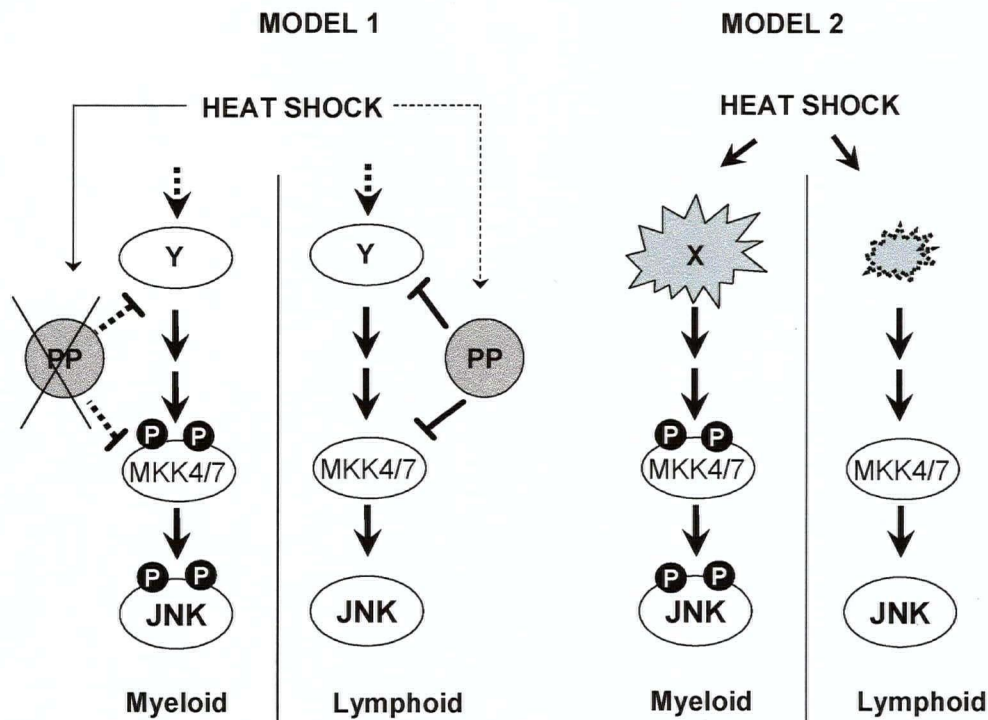


Figure 5.1 – Proposed models accounting for differential JNK activation in murine lymphoid and myeloid cells in response to heat shock. See text for explanations.

As an alternative to a model which involves inhibition of phosphatases during heat shock, we propose that specific kinase(s) upstream of JNK may become directly activated during heat shock as a result of conformational changes during this form of stress (Model 2 – Figure 5.1). Precedence for this autonomous mode of regulation has been shown for the MAPKKK, ASK-1 which can be activated independently of upstream signals during redox stress via release of its association with thiorodoxin - a negative regulator of its kinase activity. ASK1 deficient cells do not have a defect in heat-induced JNK activity (Hidenori Ichijo – pers. comm.) however, we propose that an alternative molecule upstream of JNK may have heat-responsive autocatalytic behaviour and that in murine lymphocytes the expression of this molecule would be diminished or absent.

Identifying the cascade of molecules that are activated upstream of MKK4 and MKK7 during heat shock is a prime objective. One approach which has been successfully used in studying the requirements of MAPKKs in stress signalling in insect cells involves the use of siRNA. This approach could be used in these and other cell types to determine which molecules are required in order to activate JNK during heat shock. Another way to identify kinases with a role in this pathway could be achieved using in-gel kinase assays with recombinant MKK4 or MKK7 as substrates combined with standard fractionation/purification techniques of cell extracts from heat shocked cells. A proteomics approach to isolate proteins induced in serine or threonine phosphorylation as a result of heat shock could also identify previously unknown candidate molecules in this signalling pathway.

An additional technique that could be used to identify signalling molecules involved in JNK activation in response to heat shock would be via the use of cell lines containing a readout system for JNK activation. For example, cell lines could be engineered so that GFP expression was made to be responsive to JNK activation for example, with the use of an AP-1 responsive promoter driving GFP expression. Using this system, cDNA libraries made from either murine lymphoid or myeloid cells could be transfected into GFP reporter cell lines and tested for GFP expression following heat shock. Lymphoid GFP reporter cell lines could be transfected with myeloid cDNA libraries to screen for cells which have restored JNK activation (GFP positive) upon heat shock. Alternatively, the introduction of lymphoid cDNA libraries into myeloid reporter cell lines could be used to identify lymphoid-specific molecules that may suppress JNK activation during heat shock. This same procedure could be used to identify molecules that are involved in the signalling response to anisomycin. If similar molecules were identified in this screening

approach it would strengthen the hypothesis that heat shock and anisomycin induce JNK signalling via common mechanisms.

On the basis of the strong activation of JNK in human T cells following heat shock, we cannot conclude that the factors that mediate the reduced JNK activity we note in murine lymphocytes are those that are intrinsic to all mammalian lymphocytes. Instead, it is possible that alternative factors such as those that might vary depending on the activation status the cell could contribute to JNK responsiveness during heat shock. Thus, it will be of interest to determine whether the activation of JNK in human cells in response to heat shock could be modified by altering the conditions used to stimulate and propagate these cells. It will also be important to determine the activation profile of JNK in naïve primary human T cells. An investigation of various hematopoietic progenitor cells would allow us to determine whether these cells begin as those capable of activating JNK in response to heat shock or whether this feature is only acquired later on in lineage commitment. These studies may help to identify contributing factors that influence JNK responsiveness.

One of the most obvious questions arising from these studies is the potential biological significance of the signalling peculiarity observed in murine lymphocytes. Can this difference in JNK signalling affect aspects of cellular function of lymphocytes in vivo? From our analysis of heat-induced apoptosis in WEHI-231 B cells we have found that the lower level of JNK activity in these cells does not allow them to better tolerate the harmful effects of heat shock. Thus, it is possible that this adaptation in JNK signalling is not strictly related to aspects mediating cell survival during heat stress. While the magnitude of heat shock we have used in our studies may be encountered by some forms of life it is highly unlikely for mammals to achieve body temperatures in this range.

For example the maximum temperature that can be reached during fever in humans is close to 40°C which we have found is not high enough to cause measurable JNK activity in the cells used in our study. One possibility is that during episodes of fever, the combined effect of a rise in temperature in conjunction with the elevated rates of protein production such as immunoglobulin synthesis in B cells and cytokine secretion in T cells could lower the threshold for activation of heat shock responses in these cells. There is evidence to suggest that a prolonged activation of JNK in cells is detrimental and thus it would be advantageous to suppress this activation to allow lymphocytes to function during these conditions. There could also be other negative consequences of JNK activity in these cells apart from its proposed pro-apoptotic function. This could include its influence on the rate at which secreted factors could be made by lymphocytes during fever, their proliferative capacity or other effector functions such as their homing to sites within the body. A final influence of JNK activity on lymphocyte function that we consider is the possible negative effects of JNK on aspects of heat shock protein synthesis in these cells. Lymphocytes may require higher rates of transcription of heat shock genes during episodes of fever in order to cope with the increased protein chaperoning demands placed on these cells as a result of elevated rates of protein synthesis. As mentioned previously JNK may have a negative affect on the transcriptional activity of HSF1 thus the suppression of JNK activity under these conditions would allow for more efficient production of the required chaperones. A comparison of these various effector responses of lymphocytes in wildtype and JNK deficient cells would help to clarify the role of JNK in mediating these processes.

Paramount to an understanding of the influence of JNK signalling during heat shock will be to define the substrates that it regulates during this form of stress. This would include an analysis of the transcriptional events regulated by JNK as well as the influence of

JNK phosphorylation on protein substrates with other functions. To identify only those targets specifically affected by JNK activity during heat shock, experiments would involve a comparison of wildtype cells versus JNK null cells. In this manner, a comparison of gene expression profiles (e.g. via microarrays) between these two cell types following heat shock would establish the various transcriptional responses mediated specifically by JNK during heat shock. To identify the various transcription factors phosphorylated by JNK and various other substrates during heat shock, a proteomics approach could be taken. Thus, a comparison of the differential phosphorylation events induced during heat shock in wildtype and JNK null cells would allow the identification of substrates specifically targeted by JNK.

References

- Adler, V., Polotskaya, A., Kim, J., Dolan, L., Davis, R., Pincus, M. and Ronai, Z. (1996) Dose rate and mode of exposure are key factors in JNK activation by UV irradiation. *Carcinogenesis*, **17**, 2073-2076.
- Adler, V., Schaffer, A., Kim, J., Dolan, L. and Ronai, Z. (1995) UV irradiation and heat shock mediate JNK activation via alternate pathways. *J Biol Chem*, **270**, 26071-26077.
- Albertyn, J., Hohmann, S., Thevelein, J.M. and Prior, B.A. (1994) GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol*, **14**, 4135-4144.
- Aligue, R., Akhavan-Niak, H. and Russell, P. (1994) A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *Embo J*, **13**, 6099-6106.
- Avraham, A., Jung, S., Samuels, Y., Seger, R. and Ben-Neriah, Y. (1998) Co-stimulation-dependent activation of a JNK-kinase in T lymphocytes. *Eur J Immunol*, **28**, 2320-2330.
- Ben-Levy, R., Leighton, I.A., Doza, Y.N., Attwood, P., Morrice, N., Marshall, C.J. and Cohen, P. (1995) Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. *Embo J*, **14**, 5920-5930.
- Blanco-Aparicio, C., Torres, J. and Pulido, R. (1999) A novel regulatory mechanism of MAP kinases activation and nuclear translocation mediated by PKA and the PTP-SL tyrosine phosphatase. *J Cell Biol*, **147**, 1129-1136.
- Bonner, J.J., Carlson, T., Fackenthal, D.L., Paddock, D., Storey, K. and Lea, K. (2000) Complex regulation of the yeast heat shock transcription factor. *Mol Biol Cell*, **11**, 1739-1751.
- Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F. and Gulbins, E. (1997) Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J Biol Chem*, **272**, 22173-22181.
- Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science*, **259**, 1760-1763.
- Bukau, B. (1993) Regulation of the *Escherichia coli* heat-shock response. *Mol Microbiol*, **9**, 671-680.

- Byon, J.C., Dadke, S.S., Rulli, S., Kusari, A.B. and Kusari, J. (2001) Insulin regulates MAP kinase phosphatase-1 induction in Hirc B cells via activation of both extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK). *Mol Cell Biochem*, **218**, 131-138.
- Cano, E., Hazzalin, C.A. and Mahadevan, L.C. (1994) Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. *Mol Cell Biol*, **14**, 7352-7362.
- Carratu, L., Franceschelli, S., Pardini, C.L., Kobayashi, G.S., Horvath, I., Vigh, L. and Maresca, B. (1996) Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. *Proc Natl Acad Sci U S A*, **93**, 3870-3875.
- Chan, E.D., Winston, B.W., Jarpe, M.B., Wynes, M.W. and Riches, D.W. (1997) Preferential activation of the p46 isoform of JNK/SAPK in mouse macrophages by TNF alpha. *Proc Natl Acad Sci U S A*, **94**, 13169-13174.
- Chang, Y., Abe, A. and Shayman, J.A. (1995) Ceramide formation during heat shock: a potential mediator of alpha B-crystallin transcription. *Proc Natl Acad Sci U S A*, **92**, 12275-12279.
- Chen, P., Li, J., Barnes, J., Kokkonen, G.C., Lee, J.C. and Liu, Y. (2002a) Restraint of Proinflammatory Cytokine Biosynthesis by Mitogen-Activated Protein Kinase Phosphatase-1 in Lipopolysaccharide-Stimulated Macrophages. *J Immunol*, **169**, 6408-6416.
- Chen, W., White, M.A. and Cobb, M.H. (2002b) Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway. *J Biol Chem*, **277**, 49105-49110.
- 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. *J Biol Chem*, **271**, 31929-31936.
- 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.
- Chow, C.W., Dong, C., Flavell, R.A. and Davis, R.J. (2000) c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. *Mol Cell Biol*, **20**, 5227-5234.
- Chow, C.W., Rincon, M., Cavanagh, J., Dickens, M. and Davis, R.J. (1997) Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science*, **278**, 1638-1641.

- Chu, B., Zhong, R., Soncin, F., Stevenson, M.A. and Calderwood, S.K. (1998) Transcriptional Activity of Heat Shock Factor 1 at 37 °C Is Repressed through Phosphorylation on Two Distinct Serine Residues by Glycogen Synthase Kinase 3 α and Protein Kinases Calpha and Czeta. *J. Biol. Chem.*, **273**, 18640-18646.
- Chung, K.C., Kim, S.M., Rhang, S., Lau, L.F., Gomes, I. and Ahn, Y.S. (2000) Expression of immediate early gene pip92 during anisomycin-induced cell death is mediated by the JNK- and p38-dependent activation of Elk1. *Eur J Biochem*, **267**, 4676-4684.
- 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**, 1137-1146.
- Cotto, J., Fox, S. and Morimoto, R. (1997) HSF1 granules: a novel stress-induced nuclear compartment of human cells. *J Cell Sci*, **110** (Pt 23), 2925-2934.
- Cowan, K.J., Diamond, M.I. and Welch, W.J. (2003) Polyglutamine protein aggregation and toxicity are linked to the cellular stress response. *Hum. Mol. Genet.*, **12**, 1377-1391.
- Dai, R., Frejtag, W., He, B., Zhang, Y. and Mivechi, N.F. (2000) c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. *J Biol Chem*, **275**, 18210-18218.
- Degols, G., Shiozaki, K. and Russell, P. (1996) Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Mol Cell Biol*, **16**, 2870-2877.
- Deng, X., Xiao, L., Lang, W., Gao, F., Ruvolo, P. and May, W.S., Jr. (2001) Novel role for JNK as a stress-activated Bcl2 kinase. *J Biol Chem*, **276**, 23681-23688.
- Derijard, 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**, 1025-1037.
- 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**, 693-696.
- Dill, K.A. and Shortle, D. (1991) Denatured states of proteins. *Annu Rev Biochem*, **60**, 795-825.
- Dong, C., Yang, D.D., Tournier, C., Whitmarsh, A.J., Xu, J., Davis, R.J. and Flavell, R.A. (2000) JNK is required for effector T-cell function but not for T-cell activation. *Nature*, **405**, 91-94.

- Dong, C., Yang, D.D., Wysk, M., Whitmarsh, A.J., Davis, R.J. and Flavell, R.A. (1998) Defective T cell differentiation in the absence of Jnk1. *Science*, **282**, 2092-2095.
- Dorman, C.J., Bhriain, N.N. and Higgins, C.F. (1990) DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. *Nature*, **344**, 789-792.
- Ekert, P.G., Silke, J., Hawkins, C.J., Verhagen, A.M. and Vaux, D.L. (2001) DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J Cell Biol*, **152**, 483-490.
- Enomoto, A., Suzuki, N., Morita, A., Ito, M., Liu, C.Q., Matsumoto, Y., Yoshioka, K., Shiba, T. and Hosoi, Y. (2003) Caspase-mediated cleavage of JNK during stress-induced apoptosis. *Biochem Biophys Res Commun*, **306**, 837-842.
- Feder, M.E. and Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol*, **61**, 243-282.
- Fields, P.A. (2001) Review: Protein function at thermal extremes: balancing stability and flexibility. *Comp Biochem Physiol A Mol Integr Physiol*, **129**, 417-431.
- 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. *J Biol Chem*, **273**, 9344-9351.
- Fourie, A.M., Sambrook, J.F. and Gething, M.J. (1994) Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J Biol Chem*, **269**, 30470-30478.
- Fuchs, S.Y., Adler, V., Buschmann, T., Yin, Z., Wu, X., Jones, S.N. and Ronai, Z. (1998) JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev*, **12**, 2658-2663.
- Fukunaga, R. and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *Embo J*, **16**, 1921-1933.
- Gabai, V.L., Meriin, A.B., Mosser, D.D., Caron, A.W., Rits, S., Shifrin, V.I. and Sherman, M.Y. (1997) Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J Biol Chem*, **272**, 18033-18037.
- Gallo, K.A. and Johnson, G.L. (2002) Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol*, **3**, 663-672.

- 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. *Proc Natl Acad Sci U S A*, **95**, 6881-6886.
- Gothard, L.Q., Ruffner, M.E., Woodward, J.G., Park-Sarge, O.K. and Sarge, K.D. (2003) Lowered Temperature Set Point for Activation of the Cellular Stress Response in T-lymphocytes. *J Biol Chem*, **278**, 9322-9326.
- Grably, M.R., Stanhill, A., Tell, O. and Engelberg, D. (2002) HSF and Msn2/4p can exclusively or cooperatively activate the yeast HSP104 gene. *Mol Microbiol*, **44**, 21-35.
- Gray, C.W., Ward, R.V., Karran, E., Turconi, S., Rowles, A., Viglienghi, D., Southan, C., Barton, A., Fantom, K.G., West, A., Savopoulos, J., Hassan, N.J., Clinkenbeard, H., Hanning, C., Amegadzie, B., Davis, J.B., Dingwall, C., Livi, G.P. and Creasy, C.L. (2000) Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *Eur J Biochem*, **267**, 5699-5710.
- Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Derijard, B. and Davis, R.J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo J*, **15**, 2760-2770.
- Gupta, S., Campbell, D., Derijard, B. and Davis, R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, **267**, 389-393.
- Gustin, M.C., Albertyn, J., Alexander, M. and Davenport, K. (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev*, **62**, 1264-1300.
- Hampton, R.Y. (2000) ER stress response: getting the UPR hand on misfolded proteins. *Curr Biol*, **10**, R518-521.
- 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). *J Biol Chem*, **271**, 2886-2891.
- Han, J., Wang, X., Jiang, Y., Ulevitch, R.J. and Lin, S. (1997) Identification and characterization of a predominant isoform of human MKK3. *FEBS Lett*, **403**, 19-22.
- Harding, H.P., Zhang, Y. and Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*, **397**, 271-274.
- Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, **295**, 1852-1858.

- 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 Dev*, **7**, 2135-2148.
- Hietakangas, V., Ahlskog, J.K., Jakobsson, A.M., Hellesuo, M., Sahlberg, N.M., Holmberg, C.I., Mikhailov, A., Palvimo, J.J., Pirkkala, L. and Sistonen, L. (2003) Phosphorylation of Serine 303 Is a Prerequisite for the Stress-Inducible SUMO Modification of Heat Shock Factor 1. *Mol. Cell. Biol.*, **23**, 2953-2968.
- Hilberg, F., Aguzzi, A., Howells, N. and Wagner, E.F. (1993) c-jun is essential for normal mouse development and hepatogenesis. *Nature*, **365**, 179-181.
- Hochedlinger, K., Wagner, E.F. and Sabapathy, K. (2002) Differential effects of JNK1 and JNK2 on signal specific induction of apoptosis. *Oncogene*, **21**, 2441-2445.
- Holmberg, C.I., Illman, S.A., Kallio, M., Mikhailov, A. and Sistonen, L. (2000) Formation of nuclear HSF1 granules varies depending on stress stimuli. *Cell Stress Chaperones*, **5**, 219-228.
- Horvath, I., Glatz, A., Varvasovszki, V., Torok, Z., Pali, T., Balogh, G., Kovacs, E., Nadasdi, L., Benko, S., Joo, F. and Vigh, L. (1998) Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of hsp17 as a "fluidity gene". *Proc Natl Acad Sci U S A*, **95**, 3513-3518.
- Hu, Y., Metzler, B. and Xu, Q. (1997) Discordant activation of stress-activated protein kinases or c-Jun NH2-terminal protein kinases in tissues of heat-stressed mice. *J Biol Chem*, **272**, 9113-9119.
- Huot, J., Houle, F., Marceau, F. and Landry, J. (1997) Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res*, **80**, 383-392.
- Hurme, R. and Rhen, M. (1998) Temperature sensing in bacterial gene regulation--what it all boils down to. *Mol Microbiol*, **30**, 1-6.
- Iordanov, M.S., Pribnow, D., Magun, J.L., Dinh, T.H., Pearson, J.A., Chen, S.L. and Magun, B.E. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol Cell Biol*, **17**, 3373-3381.
- Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A.C. and Kurosaki, T. (1999) BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity*, **10**, 117-125.

- Ishibashi, T., Bottaro, D., Michieli, P., Kelley, C. and Aaronson, S. (1994) A novel dual specificity phosphatase induced by serum stimulation and heat shock. *J. Biol. Chem.*, **269**, 29897-29902.
- Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T. and Yamamoto, K.I. (1999) JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. *Mol Cell Biol*, **19**, 7539-7548.
- Iwakoshi, N.N., Lee, A.H., Vallabhajosyula, P., Otipoby, K.L., Rajewsky, K. and Glimcher, L.H. (2003) Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol*, **4**, 321-329.
- Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J.A., Lin, S. and Han, J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem*, **271**, 17920-17926.
- Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R.J. and Han, J. (1997a) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J Biol Chem*, **272**, 30122-30128.
- Jiang, Y., Li, Z., Schwarz, E.M., Lin, A., Guan, K., Ulevitch, R.J. and Han, J. (1997b) Structure-function studies of p38 mitogen-activated protein kinase. Loop 12 influences substrate specificity and autophosphorylation, but not upstream kinase selection. *J Biol Chem*, **272**, 11096-11102.
- Kang, P.J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A. and Pfanner, N. (1990) Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature*, **348**, 137-143.
- Kelkar, N., Delmotte, M.H., Weston, C.R., Barrett, T., Sheppard, B.J., Flavell, R.A. and Davis, R.J. (2003) Morphogenesis of the telencephalic commissure requires scaffold protein JNK-interacting protein 3 (JIP3). *Proc Natl Acad Sci U S A*.
- Kelkar, N., Gupta, S., Dickens, M. and Davis, R.J. (2000) Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Mol Cell Biol*, **20**, 1030-1043.
- Kim, J., Nueda, A., Meng, Y.H., Dynan, W.S. and Mivechi, N.F. (1997) Analysis of the phosphorylation of human heat shock transcription factor-1 by MAP kinase family members. *J Cell Biochem*, **67**, 43-54.
- Kimball, S.R., Clemens, M.J., Tilleray, V.J., Wek, R.C., Horetsky, R.L. and Jefferson, L.S. (2001) The double-stranded RNA-activated protein kinase PKR is dispensable for regulation of translation initiation in response to either calcium mobilization from the endoplasmic reticulum or essential amino acid starvation. *Biochem Biophys Res Commun*, **280**, 293-300.

- Kranz, J.E., Satterberg, B. and Elion, E.A. (1994) The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. *Genes Dev*, **8**, 313-327.
- Kuan, C.Y., Yang, D.D., Samanta Roy, D.R., Davis, R.J., Rakic, P. and Flavell, R.A. (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron*, **22**, 667-676.
- 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. *J Biol Chem*, **265**, 17355-17363.
- Kyriakis, J.M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev*, **81**, 807-869.
- 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**, 156-160.
- 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. *J Biol Chem*, **266**, 10043-10046.
- Ladenstein, R. and Antranikian, G. (1998) Proteins from hyperthermophiles: stability and enzymatic catalysis close to the boiling point of water. *Adv Biochem Eng Biotechnol*, **61**, 37-85.
- Larson, J.S., Schuetz, T.J. and Kingston, R.E. (1988) Activation in vitro of sequence-specific DNA binding by a human regulatory factor. *Nature*, **335**, 372-375.
- Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J. and Bukau, B. (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc Natl Acad Sci U S A*, **96**, 5452-5457.
- Lavoie, J.N., Lambert, H., Hickey, E., Weber, L.A. and Landry, J. (1995) Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol*, **15**, 505-516.
- 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**, 739-746.
- Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K. and Kaufman, R.J. (2002) IRE1-mediated unconventional mRNA

- splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.*, **16**, 452-466.
- Lei, K. and Davis, R.J. (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *PNAS*, **100**, 2432-2437.
- Li, C.-Y., Lee, J.-S., Ko, Y.-G., Kim, J.-I. and Seo, J.-S. (2000) Heat Shock Protein 70 Inhibits Apoptosis Downstream of Cytochrome c Release and Upstream of Caspase-3 Activation. *J. Biol. Chem.*, **275**, 25665-25671.
- Liu, Y.F. (1998) Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. *J Biol Chem*, **273**, 28873-28877.
- Liu, Y.F., Dorow, D. and Marshall, J. (2000) Activation of MLK2-mediated signaling cascades by polyglutamine-expanded huntingtin. *J Biol Chem*, **275**, 19035-19040.
- Lo, Y.Y.C., Wong, J.M.S. and Cruz, T.F. (1996) Reactive Oxygen Species Mediate Cytokine Activation of c-Jun NH2-terminal Kinases. *J. Biol. Chem.*, **271**, 15703-15707.
- Maeda, T., Takekawa, M. and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science*, **269**, 554-558.
- Marcus, S., Polverino, A., Barr, M. and Wigler, M. (1994) Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module. *Proc Natl Acad Sci U S A*, **91**, 7762-7766.
- 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- α activation of SAPK in melanoma cells. *J Biol Chem*, **272**, 2620-2628.
- McClellan, A.J., Endres, J.B., Vogel, J.P., Palazzi, D., Rose, M.D. and Brodsky, J.L. (1998) Specific molecular chaperone interactions and an ATP-dependent conformational change are required during posttranslational protein translocation into the yeast ER. *Mol Biol Cell*, **9**, 3533-3545.
- McDonald, P.H., Chow, C.-W., Miller, W.E., Laporte, S.A., Field, M.E., Lin, F.-T., Davis, R.J. and Lefkowitz, R.J. (2000) β -Arrestin 2: A Receptor-Regulated MAPK Scaffold for the Activation of JNK3. *Science*, **290**, 1574-1577.
- McLaughlin, M.M., Kumar, S., McDonnell, P.C., Van Horn, S., Lee, J.C., Livi, G.P. and Young, P.R. (1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J Biol Chem*, **271**, 8488-8492.

- McMillan, D.R., Xiao, X., Shao, L., Graves, K. and Benjamin, I.J. (1998) Targeted Disruption of Heat Shock Transcription Factor 1 Abolishes Thermotolerance and Protection against Heat-inducible Apoptosis. *J. Biol. Chem.*, **273**, 7523-7528.
- Mendelson, K.G., Contois, L.R., Tevosian, S.G., Davis, R.J. and Paulson, K.E. (1996) Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. *Proc Natl Acad Sci U S A*, **93**, 12908-12913.
- Merienne, K., Helmlinger, D., Perkin, G.R., Devys, D. and Trottier, Y. (2003) Polyglutamine Expansion Induces a Protein-damaging Stress Connecting Heat Shock Protein 70 to the JNK Pathway. *J. Biol. Chem.*, **278**, 16957-16967.
- Meriin, A.B., Yaglom, J.A., Gabai, V.L., Zon, L., Ganiatsas, S., Mosser, D.D. and Sherman, M.Y. (1999) Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. *Mol Cell Biol*, **19**, 2547-2555.
- 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**, 1147-1157.
- Mosser, D., Caron, A., Bourget, L., Denis-Larose, C. and Massie, B. (1997) Role of the human heat shock protein hsp70 in protection against stress- induced apoptosis. *Mol. Cell. Biol.*, **17**, 5317-5327.
- Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A. and Arkinstall, S. (1996) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J Biol Chem*, **271**, 27205-27208.
- Nagata, Y. and Todokoro, K. (1999) Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. *Blood*, **94**, 853-863.
- Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T. and Inoue, J. (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells*, **4**, 353-362.
- Nara, T., Lee, L. and Imae, Y. (1991) Thermosensing ability of Trg and Tap chemoreceptors in *Escherichia coli*. *J Bacteriol*, **173**, 1120-1124.
- Narberhaus, F. (2002) mRNA-mediated detection of environmental conditions. *Arch Microbiol*, **178**, 404-410.

- Natoli, G., Costanzo, A., Ianni, A., Templeton, D.J., Woodgett, J.R., Balsano, C. and Levvero, M. (1997) Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. *Science*, **275**, 200-203.
- New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L.J., Kato, Y., Parry, G.C. and Han, J. (1998) PRAK, a novel protein kinase regulated by the p38 MAP kinase. *Embo J*, **17**, 3372-3384.
- Nguyen, A.N. and Shiozaki, K. (1999) Heat shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. *Genes Dev.*, **13**, 1653-1663.
- Nikawa, J. and Yamashita, S. (1992) IRE1 encodes a putative protein kinase containing a membrane-spanning domain and is required for inositol phototrophy in *Saccharomyces cerevisiae*. *Mol Microbiol*, **6**, 1441-1446.
- 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**, 350-353.
- Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J.L., Furlonger, K., Paige, C., Hui, C., Fischer, K.D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J.R. and Penninger, J.M. (1999) Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development*, **126**, 505-516.
- Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A. and Ichijo, H. (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev*, **16**, 1345-1355.
- Ostberg, J.R., Kaplan, K.C. and Repasky, E.A. (2002) Induction of stress proteins in a panel of mouse tissues by fever-range whole body hyperthermia. *Int J Hyperthermia*, **18**, 552-562.
- Ota, I.M. and Varshavsky, A. (1993) A yeast protein similar to bacterial two-component regulators. *Science*, **262**, 566-569.
- Park, H.-S., Cho, S.-G., Kim, C.K., Hwang, H.S., Noh, K.T., Kim, M.-S., Huh, S.-H., Kim, M.J., Ryoo, K., Kim, E.K., Kang, W.J., Lee, J.-S., Seo, J.-S., Ko, Y.-G., Kim, S. and Choi, E.-J. (2002) Heat Shock Protein Hsp72 Is a Negative Regulator of Apoptosis Signal-Regulating Kinase 1. *Mol. Cell. Biol.*, **22**, 7721-7730.
- Park, H.S., Lee, J.S., Huh, S.H., Seo, J.S. and Choi, E.J. (2001) Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. *Embo J*, **20**, 446-456.

- Park, J. and Liu, A.Y. (2000) Pervanadate induces the hyperphosphorylation but not the activation of human heat shock factor 1. *J Cell Physiol*, **185**, 348-357.
- Park, K.C., Kim, D.S., Choi, H.O., Kim, K.H., Chung, J.H., Eun, H.C., Lee, J.S. and Seo, J.S. (2000) Overexpression of HSP70 prevents ultraviolet B-induced apoptosis of a human melanoma cell line. *Arch Dermatol Res*, **292**, 482-487.
- Park, S.H., Zarrinpar, A. and Lim, W.A. (2003) Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science*, **299**, 1061-1064.
- Pfeil, W. (1981) The problem of the stability globular proteins. *Mol Cell Biochem*, **40**, 3-28.
- Philip, B. and Levin, D.E. (2001) Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. *Mol Cell Biol*, **21**, 271-280.
- 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. *J Biol Chem*, **270**, 7420-7426.
- Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. and Davis, R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol*, **16**, 1247-1255.
- Rhoads, R.E. (1999) Signal transduction pathways that regulate eukaryotic protein synthesis. *J Biol Chem*, **274**, 30337-30340.
- Ribeiro, M.J., Reinders, A., Boller, T., Wiemken, A. and De Virgilio, C. (1997) Trehalose synthesis is important for the acquisition of thermotolerance in *Schizosaccharomyces pombe*. *Mol Microbiol*, **25**, 571-581.
- Rose, D.M., Winston, B.W., Chan, E.D., Riches, D.W., Gerwins, P., Johnson, G.L. and Henson, P.M. (1997) Fc gamma receptor cross-linking activates p42, p38, and JNK/SAPK mitogen-activated protein kinases in murine macrophages: role for p42MAPK in Fc gamma receptor-stimulated TNF-alpha synthesis. *J Immunol*, **158**, 3433-3438.
- Rosette, C. and Karin, M. (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science*, **274**, 1194-1197.
- Rothe, M., Sarma, V., Dixit, V.M. and Goeddel, D.V. (1995) TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science*, **269**, 1424-1427.
- Rudiger, S., Buchberger, A. and Bukau, B. (1997) Interaction of Hsp70 chaperones with substrates. *Nat Struct Biol*, **4**, 342-349.

- Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E.F. (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev*, **89**, 115-124.
- Sato, S., Fujita, N. and Tsuruo, T. (2000) Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A*, **97**, 10832-10837.
- Savopoulos, J.W., Carter, P.S., Turconi, S., Pettman, G.R., Karran, E.H., Gray, C.W., Ward, R.V., Jenkins, O. and Creasy, C.L. (2000) Expression, purification, and functional analysis of the human serine protease HtrA2. *Protein Expr Purif*, **19**, 227-234.
- Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L. and Engel, P.C. (1998) Protein thermostability in extremophiles. *Biochimie*, **80**, 933-941.
- Shen, Y., Luche, R., Wei, B., Gordon, M.L., Diltz, C.D. and Tonks, N.K. (2001) Activation of the Jnk signaling pathway by a dual-specificity phosphatase, JSP-1. *Proc Natl Acad Sci U S A*, **98**, 13613-13618.
- Shiozaki, K., Shiozaki, M. and Russell, P. (1998) Heat stress activates fission yeast Spc1/Sty1 MAPK by a MEKK-independent mechanism. *Mol Biol Cell*, **9**, 1339-1349.
- Sistonen, L., Sarge, K.D. and Morimoto, R.I. (1994) Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. *Mol Cell Biol*, **14**, 2087-2099.
- 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 Dev*, **10**, 2745-2758.
- Smith, A., Ward, M.P. and Garrett, S. (1998) Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. *Embo J*, **17**, 3556-3564.
- Sorger, P.K. and Pelham, H.R. (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell*, **54**, 855-864.
- Srivastava, S., Weitzmann, M.N., Cenci, S., Ross, F.P., Adler, S. and Pacifici, R. (1999) Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD. *J Clin Invest*, **104**, 503-513.
- Stancato, L.F., Chow, Y.H., Hutchison, K.A., Perdew, G.H., Jove, R. and Pratt, W.B. (1993) Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J Biol Chem*, **268**, 21711-21716.

- Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U. and Pavletich, N.P. (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell*, **89**, 239-250.
- Stepanova, L., Leng, X., Parker, S.B. and Harper, J.W. (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes Dev*, **10**, 1491-1502.
- Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M. and Ben-Neriah, Y. (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell*, **77**, 727-736.
- 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**, 625-634.
- Tanoue, T., Moriguchi, T. and Nishida, E. (1999) Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem*, **274**, 19949-19956.
- Tanoue, T., Yamamoto, T., Maeda, R. and Nishida, E. (2001) A Novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 alpha and beta MAPKs. *J Biol Chem*, **276**, 26629-26639.
- 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. *J Biol Chem*, **271**, 27225-27228.
- Theodosiou, A. and Ashworth, A. (2002) Differential effects of stress stimuli on a JNK-inactivating phosphatase. *Oncogene*, **21**, 2387-2397.
- Tournier, C., Dong, C., Turner, T.K., Jones, S.N., Flavell, R.A. and Davis, R.J. (2001) MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev*, **15**, 1419-1426.
- Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A. and Davis, R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*, **288**, 870-874.
- Tsai, A.M., van Zanten, J.H. and Betenbaugh, M.J. (1998) I. Study of protein aggregation due to heat denaturation: A structural approach using circular dichroism spectroscopy, nuclear magnetic resonance, and static light scattering. *Biotechnol Bioeng*, **59**, 273-280.

- Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P. and Ron, D. (2000) Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1. *Science*, **287**, 664-666.
- Vaux, D.L., Cory, S. and Adams, J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, **335**, 440-442.
- Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z. and Kolesnick, R.N. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature*, **380**, 75-79.
- Verna, J., Lodder, A., Lee, K., Vagts, A. and Ballester, R. (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*, **94**, 13804-13809.
- Villanueva, A., Lozano, J., Morales, A., Lin, X., Deng, X., Hengartner, M.O. and Kolesnick, R.N. (2001) jkk-1 and mek-1 regulate body movement coordination and response to heavy metals through jnk-1 in *Caenorhabditis elegans*. *Embo J*, **20**, 5114-5128.
- Volloch, V., Gabai, V.L., Rits, S. and Sherman, M.Y. (1999) ATPase activity of the heat shock protein hsp72 is dispensable for its effects on dephosphorylation of stress kinase JNK and on heat-induced apoptosis. *FEBS Lett*, **461**, 73-76.
- Vries, R.G.J., Flynn, A., Patel, J.C., Wang, X., Denton, R.M. and Proud, C.G. (1997) Heat Shock Increases the Association of Binding Protein-1 with Initiation Factor 4E. *J. Biol. Chem.*, **272**, 32779-32784.
- Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A. and Sauer, R.T. (2003) OMP Peptide Signals Initiate the Envelope-Stress Response by Activating DegS Protease via Relief of Inhibition Mediated by Its PDZ Domain. *Cell*, **113**, 61-71.
- Wang, J. and Friedman, E. (2000) Downregulation of p53 by sustained JNK activation during apoptosis. *Mol Carcinog*, **29**, 179-188.
- Wang, X.Z. and Ron, D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science*, **272**, 1347-1349.
- 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 J*, **16**, 1909-1920.
- West, A.H. and Stock, A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci*, **26**, 369-376.

- Westwood, J.T., Clos, J. and Wu, C. (1991) Stress-induced oligomerization and chromosomal relocation of heat-shock factor. *Nature*, **353**, 822-827.
- Willoughby, E.A., Perkins, G.R., Collins, M.K. and Whitmarsh, A.J. (2003) The JNK-interacting Protein-1 Scaffold Protein Targets MAPK Phosphatase-7 to Dephosphorylate JNK. *J. Biol. Chem.*, **278**, 10731-10736.
- Winkler, A., Arkind, C., Mattison, C.P., Burkholder, A., Knoche, K. and Ota, I. (2002) Heat Stress Activates the Yeast High-Osmolarity Glycerol Mitogen-Activated Protein Kinase Pathway, and Protein Tyrosine Phosphatases Are Essential under Heat Stress. *Eukaryotic Cell*, **1**, 163-173.
- Xavier, I.J., Mercier, P.A., McLoughlin, C.M., Ali, A., Woodgett, J.R. and Ovsenek, N. (2000) Glycogen Synthase Kinase 3 β Negatively Regulates Both DNA-binding and Transcriptional Activities of Heat Shock Factor 1. *J. Biol. Chem.*, **275**, 29147-29152.
- Xia, Y., Wu, Z., Su, B., Murray, B. and Karin, M. (1998) JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. *Genes Dev*, **12**, 3369-3381.
- Yaglom, J.A., Gabai, V.L., Meriin, A.B., Mosser, D.D. and Sherman, M.Y. (1999) The function of HSP72 in suppression of c-Jun N-terminal kinase activation can be dissociated from its role in prevention of protein damage. *J Biol Chem*, **274**, 20223-20228.
- Yang, D., Tournier, C., Wysk, M., Lu, H.T., Xu, J., Davis, R.J. and Flavell, R.A. (1997a) Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. *Proc Natl Acad Sci U S A*, **94**, 3004-3009.
- Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M. and Flavell, R.A. (1998) Differentiation of CD4⁺ T cells to Th1 cells requires MAP kinase JNK2. *Immunity*, **9**, 575-585.
- Yang, D.D., Kuan, C.Y., Whitmarsh, A.J., Rincon, M., Zheng, T.S., Davis, R.J., Rakic, P. and Flavell, R.A. (1997b) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature*, **389**, 865-870.
- Yasuda, J., Whitmarsh, A.J., Cavanagh, J., Sharma, M. and Davis, R.J. (1999a) The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol*, **19**, 7245-7254.
- Yasuda, S., Inoue, K., Hirabayashi, M., Higashiyama, H., Yamamoto, Y., Fuyuhiko, H., Komure, O., Tanaka, F., Sobue, G., Tsuchiya, K., Hamada, K., Sasaki, H., Takeda, K., Ichijo, H. and Kakizuka, A. (1999b) Triggering of neuronal cell death

- by accumulation of activated SEK1 on nuclear polyglutamine aggregations in PML bodies. *Genes Cells*, **4**, 743-756.
- Yeh, W.C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J.L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Goeddel, D.V. and Mak, T.W. (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity*, **7**, 715-725.
- Young, J.C., Moarefi, I. and Hartl, F.U. (2001) Hsp90: a specialized but essential protein-folding tool. *J Cell Biol*, **154**, 267-273.
- Yujiri, T., Sather, S., Fanger, G.R. and Johnson, G.L. (1998) Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science*, **282**, 1911-1914.
- Zahringer, H., Thevelein, J.M. and Nwaka, S. (2000) Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. *Mol Microbiol*, **35**, 397-406.
- Zanke, B.W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L.A., Zon, L., Kyriakis, J., Liu, F.F. and Woodgett, J.R. (1996a) The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol*, **6**, 606-613.
- Zanke, B.W., Rubie, E.A., Winnett, E., Chan, J., Randall, S., Parsons, M., Boudreau, K., McInnis, M., Yan, M., Templeton, D.J. and Woodgett, J.R. (1996b) Mammalian mitogen-activated protein kinase pathways are regulated through formation of specific kinase-activator complexes. *J Biol Chem*, **271**, 29876-29881.
- Zhang, P., Miller, B.S., Rosenzweig, S.A. and Bhat, N.R. (1996) Activation of C-jun N-terminal kinase/stress-activated protein kinase in primary glial cultures. *J Neurosci Res*, **46**, 114-121.
- Zhong, M., Orosz, A. and Wu, C. (1998) Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. *Mol Cell*, **2**, 101-108.
- Zou, J., Guo, Y., Guettouche, T., Smith, D.F. and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell*, **94**, 471-480.