Control of subcellular distribution of the MAP kinase phosphatase, MKP-2

A thesis presented by

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Abstract

Mitogen-activated protein (MAP) kinases are a group of serine/threonine kinases that play a key role in the transduction of signals from the cell membrane to the nucleus. MAP kinase activation is controlled by phosphorylation upon a T-X-Y (activation) motif. Inactivation of MAP kinases is achieved by dephosphorylation of the activation motif by a family of enzymes termed the MAP kinase phosphatases (MKPs). The MAP kinases are able to move from compartment to compartment whereas the phosphatases are predominantly restricted to either the cytosol or the nucleus. In this thesis the substrate specificity and the factors controlling the localisation of MKP-2 have been studied.

Human MKP-2 was cloned from human umbilical vein endothelial cell cDNA library using PCR. A transient transfection system and epitope-tagged MAP kinases were then utilised to study the substrate specificity of human MKP-2 towards the MAP kinase family. MKP-2 was able to reverse JNK activation in COS7 cells, but unable to reduce either ERK or p38 MAPK activation. Creation of a catalytically inactive MKP-2 by mutation of the cysteine residue critical for phosphatase activity yielded a protein with no phosphatase activity that is able to act in a dominant negative fashion by preventing the activity of wild type MKP-2 towards JNK.

Sequence analysis followed by mutational studies indicated two sequences within MKP-2 that are critical for subcellular localisation. Expression of single and double nuclear localisation sequence (NLS) mutants, defined NLS1 and NLS2 as novel localisation sequences, each individually able to direct MKP-2 to the nucleus. Loss of both NLS resulted in the cytosolic accumulation of MKP-2.

The ability of nuclear export sequences derived from a cytosolic dual specificity phosphatase (DSP) to alter the locale of MKP-2 was also assessed. Addition of single nuclear export sequence (NES) proved insufficient to change the locale of MKP-2, suggesting that a single NES is unable to compete with two NLS.

Publications

Papers: Robinson, C.J.M., Sloss, C.M. Plevin, R. (2001) Inactivation of JNK activity by mitogen-activated protein kinase phosphatase-2 in EAhy926 endothelial cells is dependent upon agonist specific JNK translocation to the nucleus. *Cellular Signalling* **13**, 29-41.

Abstracts: "The inactivation of Mitogen-activated protein kinases by MAP kinase phosphatase 2 (MKP-2) a nuclear dual specificity phosphatase". Sloss, C.M., Robinson, C.J.M., and Plevin R. Poster communication. The 11th International conference of second messengers & phosphoproteins. Melbourne. Australia. April 22-26 2001.

"Localisation of MKP-2 is controlled by two NLS". Sloss, C.M., Cadalbert, L. and Plevin R. Signalling The Future, University of Liverpool. U.K. September 3-6 2002.

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Abbreviations

Ala	Alanine residue
AP-1	Activating protein-1
BMK	Big MAP kinase
Ca2+	Ionic calcium
cAMP	Cyclic adenosine mono phosphate
CDK	Cyclin-dependent kinases
cDNA	Complementary DNA
cGDP	Cyclic guanine di-phosphate
cGMP	Cyclic guanine mono-phosphate
cGTP	Cyclic guanine tri-phosphate
CH2	Cdc25 homology domains
CHO	Chinese hamster ovary cells
CI	Catalytically Inactive
COX	Cyclo-oxygenase
$cPLA_2$	Phospholipase-A-2
CSAID	Cytokine suppressive anti inflammatory drug
CSBP	CSAID binding protein
DAG	Diacylglycerol
DNA	Deoxy ribonucleic acid
DSP	Dual-specificity phosphatase
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
ES	Embryonic stem cells
GPCR	G-protein coupled receptor
GRK	GPCR kinase
GSK	Glycogen synthase kinase
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
IP_3	Inositol tri-phosphate
JNK	c-Jun N-terminal kinase
KSR	Kinase supressor of Raf
LDL	Low density lipoprotein
Leu	Leucine residue
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAPKAP	MAP kinase activated proteins
MAPKAPK2	MAP kinase activated protein-kinase-2
MBP	Myelin basic protein
MEF	Mouse embryonic fibroblasts
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MEKKK	MAP/ERK kinase kinase kinase
MKK	MAP kinase kinase

MKKK	MAP kinase kinase kinase
MKP	MAP kinase phosphatase
MP-1	MEK partner 1
mRNA	message RNA
NES	Nuclear export sequence
NGF	Nerve growth factor
NLS	Nuclear localisation sequence
NO	Nitric oxide
OA	Okadaic Acid
PCR	Polymerase chain reaction
PFK-2	Phosphofructo-2-kinase
PI-3-K	phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PP	Protein phosphatase (ser/thr)
PTP	Protein tyrosine phosphatase (tyr)
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
Ser	Serine residue
STRE	Stress response element
Thr	Threonine residue
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA response element
Tyr	Tyrosine residue
UV	Ultra violet
VEGF	Vascular endothelial growth factor

Chapter 1

General Introduction

1.1 Introduction

One of the major factors controlling cellular function is the binding of various agonists to the cell surface receptors. As well as co-ordinating a response, there are two main methods of passing the signal from the cell surface to the nucleus. Firstly, the generation of second messengers such as cyclic-AMP and Ca^{2+} and secondly the use of kinase cascades. Ultimately, the modulation of cell growth and function is controlled by the altered expression of genes within the nucleus. Therefore, the activation and deactivation of transcription factors is likely to be the important factor controlling these changes in the nucleus.

1.2 The Cell Cycle

Several basic cellular processes are required for the survival of multicellular organisms. Cell growth and cell differentiation are required to form the myriad of tissues, vessels and glands required for multicellular life. Cell death is required to dispose of damaged and malfunctioning cells or those cells that have fulfilled their purpose. A process known as the cell cycle controls all the decisions of growth, differentiation and death.

The course of the cell cycle can be classified into four distinct phases named G1, S, G2 and M. G1 is a gap phase prior to DNA replication, during S phase new DNA is synthesised. G2 is a second organisational phase prior to M, which is the mitotic phase. This mitotic phase can also be subdivided into eight distinct stages. Cells that are not actively dividing are either terminally differentiated and cannot re-enter the cell cycle or are held in a state of temporary quiescence prior to G1 called G0 (Doree & Galas, 1994).

The control of the cell cycle takes the form of various protein complexes that are activated in a specific order and trigger events such as DNA replication, nuclear envelope breakdown, spindle formation and chromosome segregation. These protein complexes are under strict control of signal transduction pathways and feedback loops that ensure each event is performed correctly and in the proper sequence (Hartwell & Weinert, 1989). The proteins responsible predominantly belong to one family termed the cyclin-dependent kinases (CDKs). CDKs are serine/threonine

kinases and require association with cyclins for activation. There are three groups of cyclins and CDKs all with the same nomenclature, the G1, S-phase and M-phase cyclins or CDKs, (reviewed in Doree & Galas, 1994). The activity of the CDKs is strictly controlled on a number of levels by several differing mechanisms including both activating and inhibitory phosphorylation, interaction with a number of low molecular weight protein inhibitors and degradation of the cyclin subunits (Morgan, 1997; Morgan, 1995). This strict control ensures that the correct cyclins are expressed and bind to the correct CDKs at specific times in the cell cycle. The absolute requirement of specific cyclins by the cell to progress is used by certain "checkpoints" in the cell cycle. There are checkpoints at several parts of the cell cycle, which prevent the initiation of a particular cell cycle event until the successful and accurate completion of all the prior stages. There are two main checkpoints, one at the transition between G1 and S and the other at the transition between G2 and M, which allow cell division to be arrested if a certain process has not been finished or the genomic material has become damaged and requires repair. Cells with damage to their DNA rapidly produce a protein called p53 (Kuerbitz *et al.*, 1992). p53 acts at both the G1/S and G2/M checkpoints either by arresting the growth of the cell to allow repair or causing apoptosis. Apoptosis is the process that allows multicellular organisms to selectively eliminate damaged or malignant cells that threaten the survival of the organism. All mammalian cells possess the ability to 'self destruct' by the activation of the apoptosis pathways. The activation of this death machinery can be triggered in one of two ways, either rapidly, (less than 8 hours) by activation of the extracellular cell death receptors leading to caspase activation and cell death (Grutter, 2000; Krammer, 2000), or slowly (8 hours to 2 days) via the Bcl-2 family of proteins which along with loss of mitochondrial integrity lead to cell death (Hengartner, 2000; Vander Heiden *et al.*, 2000). Apoptosis is tightly controlled via the cell cycle to prevent faulty genetic material from being passed to daughter generations. Only one functional copy of the p53 cell cycle checkpoint gene is required for proper function and loss of both from a cell can result in the loss of cell cycle control. Lack of cell cycle control coupled with other genetic damage can result in an impaired ability to trigger apoptosis and can lead to neoplastic growth. Indeed more than half of all human cancers harbour p53 mutations. The checkpoint

processes are so sensitive that a single double-stranded break anywhere in the genome will prevent the cell from passing the second checkpoint and entering mitosis (Sandell & Zakian, 1993).

As described above, the cell cycle allows the control of many processes including growth differentiation and death. The nucleus must make the decision as to which process the organism requires. To make this decision the nucleus requires information in the form of signals from the extracellular environment. There are a multitude of receptors, channels and other sensory mechanisms that can be activated by extracellular stimuli. Signals from this sensory apparatus must be transmitted from the membrane, across the cytoplasm and into the nucleus to allow changes in gene expression, leading to the correct physiological response.

There are two main methods of passing the signal from cell surface to the nucleus. Firstly, the generation of second messengers, and secondly the initiation of multiple phosphorylation cascades often referred to as kinase cascades. These two processes are not completely isolated from each other and many studies have shown the activation of kinases by second messengers and conversely the involvement in second messenger production by many protein kinases (see below). There are three major classes of second messengers, the cyclic nucleotides, c-AMP and cGMP, the lipid-based messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) and lastly, calcium ions (Ca^{2+}). Multiple families and signalling complexes make up the kinase cascades. A few of the best characterised are aptly named the protein kinase A, B and C families. The PKC family is made up of many isoforms which have been shown to be involved in the signal transduction of a wide range of biological responses including differentiation, proliferation and changes in cell morphology (Toker, 1998). The activity of some PKCs is dependent on Ca^{2+} however, all are activated by the lipid second messenger DAG (second messenger to kinase cascade activation) (Le Good *et al.*, 1998). The PKB family members have been shown to be involved in growth factor signalling and oncogenesis. PKB is activated by upstream kinases known as PDKs and also by the enzyme PI-3 kinase. PI-3-kinase is involved in the phosphorylation of phosphatidylinositol and its derivative phosphoinositides to create PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, both of which have been linked with the

activation of PKB and PDK1 (Vanhaesebroeck & Alessi, 2000). PKB has a number of cellular substrates including GSK and PFK-2 which are involved in insulin signalling and 4E-BP which is involved in mRNA elongation. Many substrates remain unidentified but the PKB family has been shown to be involved in many of the key cellular processes including, gene transcription, protein synthesis, cell survival and glucose metabolism (for review see (Kandel & Hay, 1999).

Among the plethora of proteins that are involved in kinase signalling within mammalian cells, one group of enzyme families has been the focus of extensive study, the mitogen-activated protein kinases (MAP kinase).

1.3 The MAP Kinase Pathway and The Three Kinase Cascade.

Historically, the MAP kinases were identified when several groups discovered that proteins of around 40-50KDa in size became tyrosine phosphorylated in response to many differing extracellular stimuli. Ray and Sturgill demonstrated that a 42KDa serine/threonine protein kinase, termed mitogen-activated protein kinase or MAPK, is phosphorylated on both tyrosine and threonine in response to insulin in 3T3-L1 cells (Ray & Sturgill, 1988; Ray & Sturgill, 1988). It was soon realised that the same protein was phosphorylated in response to many differing stimuli including growth factors, phorbol esters and viral transformation, and that this phosphorylation was required for MAPK activity (Rossomando *et al.*, 1989). The cDNA encoding MAPK was soon isolated and the protein renamed ERK for extracellular signal regulated kinase (Boulton *et al.*, 1990). Sequence alignment showed ERK to be closely related to a family of protein kinases found in yeast including Fus3 and Kss1, demonstrating the close homology between mammalian and yeast MAP kinases.

Since the cloning of the first ERK, 11 more mammalian MAP kinase family members have been identified. These 12 MAP kinases can be separated into five families based on sequence homology and function, and are termed ERK1/2, c-Jun N-terminal kinase (JNK), p38-MAPK, ERK3/4 and big MAP kinase (BMK)/ERK5 respectively.

Studies to identify the mechanism of activation of these MAP kinase families led to the discovery of a sequential pathway involving three distinct families of kinases. This activation module is termed the three-kinase cascade. See Figure 1.1.

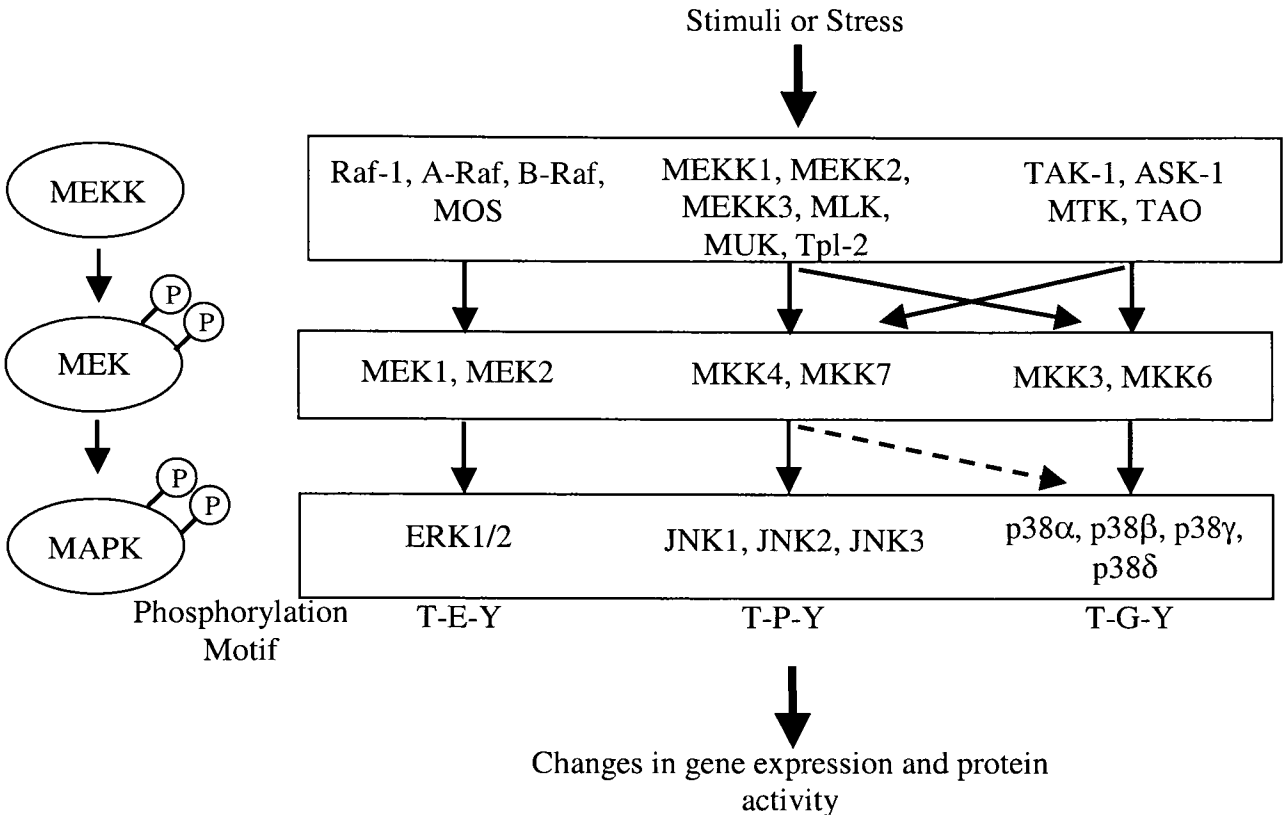

Figure 1.1
General model of the three-kinase cascade leading to activation of the MAP kinases. The activation motif for each MAP kinase is shown at the bottom of the figure. (Adapted from Obata et al., 2000).

Previously undescribed abbreviations: ASK, Apoptosis stimulating kinase. MLK, Mixed lineage kinase. MTK, MAP three kinase-1. MUK, MAPK upstream kinase. TAK-1, TGF-b activated kinase. TAO, Two thousand and one amino acid kinase. TPL-2, Tumour progression locus-2.

Although activated by many differing cellular conditions, the MAP kinases are all activated by a sequential phosphorylation and activation of two upstream protein kinases (for review see (Widmann *et al.*, 1999). Briefly, the MAP kinases are activated by phosphorylation within their activation loop by a family of dual kinases known as the MAP kinase kinases (MKK) or MAP/ERK kinases (MEK) (Zheng & Guan, 1994). These MEKs are themselves activated by dual phosphorylation by a family of upstream kinases termed MAP kinase kinase kinases (MKKK) or MAP/ERK kinase kinases (MEKK) (Blank *et al.*, 1996; Kyriakis *et al.*, 1992; Lin *et al.*, 1995; Zheng & Guan, 1994). Activation of the MKKKs is both stimuli and cell type-dependent but can be via phosphorylation by another upstream kinase or interaction with small GTP binding proteins from the Rho or Ras family (Van Aelst & D'Souza-Schorey, 1997; Vojtek *et al.*, 1993).

In mammalian cells, 12 members of the MAP kinase family, 7 members of the MEK family and 14 members of the MEKK family have been identified to date (for details see (Widmann *et al.*, 1999). As there are multiple members of each component of the three-kinase cascade, the maintenance of signal fidelity and specificity is essential to ensure the correct cellular response. The MEKs are the smallest group within the cascade and have high specificity for their substrate MAP kinases, and thus there is little chance of signal deviation within this part of the cascade. The MEKKs on the other hand are more diverse in both their structure and their substrate specificity with most being able to activate 2 or more of the MEKs (see figure 1.1). This variation allows the cascade the flexibility to integrate many differing extracellular stimuli but also raises the question of signal fidelity. Recent evidence suggests that scaffold proteins may hold specific members of the three kinase cascade within close proximity to each other to allow not only specific signalling but also extremely rapid conveyance of the signal. Several candidate proteins have been identified, including MEK partner-1 (MP-1) and kinase supressor of Raf (KSR) for the ERK cascade and JNK interacting protein-1 (JIP-1) for the JNK cascade. (Reviewed in Garrington & Johnson, 1999; Schaeffer & Weber, 1999). In the case of MEKK1, an MKKK upstream of JNK, the kinase itself may act as the scaffold by binding MKK4 and JNK as well as fulfilling its role as the activating kinase (Xu & Cobb, 1997).

Most experimental studies on the MAP kinases to date have focused on the ERK1/2, JNK and p38 pathways and these will be discussed in detail later, however important cellular roles are becoming apparent for the other MAP kinase families.

BMK/ERK5 is activated in response to epidermal growth factor (EGF), oxidative stress, hyperosmolarity and the presence of serum (Abe *et al.*, 1996; Kato *et al.*, 1997; Kato *et al.*, 1998). These studies have as yet failed to clearly identify either a cellular role for BMK/ERK5 or fully elucidate the upstream kinases and downstream substrates. They do however, indicate a possible role in cellular proliferation via phosphorylation of the transcription factor MEF2C and also suggest that H-Ras may lead to phosphorylation of c-Myc via BMK/ERK5. As the name 'big MAP kinase' suggests BMK/ERK5 is much larger than previously cloned MAP kinases, at 816 amino acids it contains a 400 amino acid extension to the C- terminal tail the function of which is yet to be defined (Zhou *et al.*, 1995).

ERK4 is also responsive to growth factors, it can be activated by NGF and EGF via a Ras-dependent mechanism (Peng *et al.*, 1996). Any individual characteristics of ERK4 have yet to be identified. Current work indicates ERK4 may be a splice variant or pseudo-gene of ERK1.

ERK3 has been cloned in both rat and human and has been found to be constitutively nuclear irrespective of stimuli. No substrates have yet been identified for ERK3 but many of the ERK1/2 substrates have been discounted suggesting a very differing role for ERK3 from ERK1/2. ERK3 is expressed ubiquitously in human tissues with particularly high levels in muscle (Boulton *et al.*, 1991; Gonzalez *et al.*, 1993; Zhu *et al.*, 1994).

1.3.1 ERK Pathway

As outlined above, the first pathway to be characterised in detail was the pathway leading to the activation of extracellular signal regulated kinase (ERK) subgroup of the MAP kinases. To date, five kinases termed ERKs (ERK1-5) have been defined, although this nomenclature is misleading as they are part of separate subfamilies. The 3-residue activation motif within the activation loop is the same in all ERKs but, as outlined above, sequence differences and large C-terminal extensions suggest separate roles for differing ERKs.

The ERK 1/2 pathway was first characterised by its ability to phosphorylate myelin basic protein (MBP) and microtubule-associated protein (MAP). The phosphorylation and activation of these proteins is mediated by stimulation by many extracellular signalling molecules including nerve growth factor and insulin (Boulton *et al.*, 1991). ERK 1 and 2 are isoforms, 44 and 42 KDa in size respectively. There are several differing proteins at each level of the three-kinase cascade that activate ERK1/2. At the level of the MKKK related proteins there is: Raf-1, A-Raf, B-Raf, MOS, MEKK1, MEKK2 and MEKK3.All these proteins are able to activate the two proteins at the MEK level, MEK1 and MEK2. MEK1 and 2 are both highly specific for ERK1/2, no other substrates for either MEK1 or MEK2 have been identified (Crews *et al.*, 1992; Kosako *et al.*, 1992).

Each of the MAP kinases have a specific sequence containing a threonine and a tyrosine upon which they are phosphorylated and activated by their upstream MEK. The central amino acid in this T-X-Y motif is different for all the MAP kinase sub-families and probably aids in the identification of the MAP kinases by specific upstream MEKs. In the case of ERK1/2, and all the ERKs, the spacer amino acid is glutamate making the activation motif, T-E-Y.

ERK1/2 is a proline directed kinase and is specific for a Leu-Ser/Thr-Pro motif on its substrates. Although most of the well characterised substrates of ERK1/2 are transcription factors, many additional substrates of ERK1/2 are cytoplasmic proteins including the S6 kinase $pp90^{rsk}$, phospholipase A_2 and several microtubule-associated proteins (Lin *et al.*, 1993; Sturgill *et al.*, 1988). Interestingly other cytosolic targets of ERK1/2 include Raf-1, MEK1, β-arrestin and some GPCR kinases (GRKs). All of these proteins can form part of the upstream mechanisms that activates ERK1/2, thus providing a negative feedback mechanism for the control of ERK1/2 activity (Lin *et al.*, 1999; Pitcher *et al.*, 1999).

The effect of ERK1/2 activation on cell function is varied, and is associated with cell proliferation, growth inhibition, differentiation, cell cycle regulation and in certain cell types mediation of apoptosis. For example, in PC12 cells ERK activity prevents cell death (Xia *et al.*, 1995), whereas in B-cells activation of ERK is thought to be required for apoptosis (Sakata *et al.*, 1995).

1.3.2 JNK Pathway:

In 1991 a new subfamily of MAP kinase was identified. JNK was originally cloned and named as p54, which was isolated from cyclohexamide treated rats and termed a MAP kinase homologue due to its 40-45% homology to ERK. Three genes have been found to encode JNK MAP kinases, JNK1 JNK2 and JNK3. JNK1 and 2 are ubiquitously expressed whereas JNK-3 is only expressed in the brain, heart and testis. Transcripts from each of the genes encode several splice variants and to date 10 JNK isoforms have been found (Gupta *et al.*, 1996). Generally, little difference has been found between the activities of the isoforms. JNK1 does show a slightly higher affinity for binding to c-Jun than JNK2 although their regulation appears similar (Hibi *et al.*, 1993; Su *et al.*, 1994).

Originally JNKs were distinguished from ERK1/2 by two characteristics. The JNKs were found to be activated by cellular stress and cytokines rather than mitogens and secondly, their substrate profile was distinct in that they phosphorylated c-Jun at the N-terminal activation sites rather than the C-terminal inhibitory sites which are phosphorylated by the ERKs (Pulverer *et al.*, 1991). Phosphorylation of any one of three sites at the carboxy terminus of c-Jun was found to have an inhibitory effect on its activity whereas phosphorylation ser-63 and –73, within an area termed the NH_2 terminus activation domain, stimulates transcriptional activity. These studies led to the naming of this new family the c-Jun N-terminal kinases or JNK MAP kinases.

The JNK pathway is the better characterised of the two stress-activated MAP kinase families. Members of the JNK family are activated by many diverse stimuli including both chemical and physical stress as well as growth factors, UV irradiation, proinflammatory cytokines, and ischaemia/reperfusion (Bogoyevitch *et al.*, 1996; Kallunki *et al.*, 1994; Matsuda *et al.*, 1995; Minden *et al.*, 1994). Similar to the ERKs, JNK is proline-directed and requires dual phosphorylation on both Threonine and Tyrosine for maximal activity. The dual phosphorylation site present in the JNK activation loop is Thr-Pro-Tyr (Derijard *et al.*, 1994; Hibi *et al.*, 1993; Kyriakis *et al.*, 1994).

The protein c-Jun is a component of the transcription factor AP-1 (Angel & Karin, 1991; Karin, 1995), which is a dimer incorporating two of several different possible proteins. Formation and activation of AP-1 leads to the transcriptional activation of genes via the TPA response element (TRE) (Angel & Karin, 1991). Thus the phosphorylation of c-Jun by JNK is significant in the regulation of gene transcription. Along with AP-1 the JNKs phosphorylate and activate several other transcription factors most of which contribute to the activation of AP-1 including JunB, JunD and ATF2 (Ip & Davis, 1998). Although heavily involved in control of gene transcription, it has also recently been discovered that transcription factors are not the only substrate of the JNKs. JNK can also translocate to the mitochondria and phosphorylate the anti-apoptotic protein Bcl-X_L (Kharbanda *et al.*, 2000).

The upstream activating proteins of the JNK pathways have been intensely studied and similar to the ERK cascade only two MEKs have been identified that are able to phosphorylate the T-P-Y motif within JNK. These are MKK4 and MKK7 (Derijard *et al.*, 1995; Lin *et al.*, 1995; Sanchez *et al.*, 1994). Similar to the ERK pathway the JNK cascade has a diverse range of MEKK proteins that can lead to JNK activation. Although there are overlaps in specificity, the MEKKs of the JNK pathway differ from those of the ERK pathway. These include MEKK-1, 2, 3, and 4, TAK-1, MUK-1, Tpl-2, SPRK, ASK-1 and MST, all of which have been shown to activate MKK4 and MKK7 (Widmann *et al.*, 1999). The exact physiological function and consequence of JNK activation by each of these MEKKs is yet to be elucidated. Recently however, knockout experiments in embryonic stem (ES) cells have shown that MEKK1 is critical in JNK activation by serum, lysophosphatidic (Yujiri *et al.*, 1999; Yujiri *et al.*, 1998) and various pro-inflammatory stimuli (Xia *et al.*, 2000) but not involved in JNK's response to UV irradiation, oxidative stress or anisomycin (Minamino *et al.*, 1999). This suggests a possible role for MEKK1 in linking receptor activated signalling to JNK rather than stress signals arising within the cytoplasm or nucleus.

The exact role of JNK within the cell is yet to be fully understood. JNK signalling has been shown to be involved in apoptosis, cell survival and tumour development (reviewed in Davis, 2000). The cellular outcome of JNK activation will be discussed in more detail in section 1.3.4.

1.3.3 p38 MAPK Pathway

The other main homologue of the MAP kinase family, activated by stress, and the focus of extensive study is the p38 MAPK family. p38 MAPK was originally identified as the mammalian homologue of the yeast protein HOG1 (high osmolarity glycerol responsive) which is activated in response to hyperosmotic shock (Brewster *et al.*, 1993). Human p38 was identified in a protein screen as a cytokine suppressive anti inflammatory drug (CSAID) binding protein (CSBP) which regulates the synthesis of IL-1 and TNFα from stimulated monocytes (Lee *et al.*, 1994).

The p38 MAPK subfamily consists of four major classes of protein: p38α, p38β, p38γ and p38δ. Of these, p38α and p38β both have a splice variant, giving rise to a total of six distinct p38 MAPK isoforms. All isoforms show high homology to each other, their closest relation is the above mentioned prokaryotic MAP kinase found in yeast named HOG1. Despite the high homology clear functional differences between the isoforms have been identified. This may be, in part, due to their different patterns of expression. Analysis of tissues indicates α, β and γ isoforms are widely expressed whereas δ is limited to skeletal muscle. Also, analysis of inflammatory cell lines indicates differences in p38 MAPK isoform expression in T-cells, neutrophils, macrophages and endothelial cells (Hale *et al.*, 1999; Hu *et al.*, 1999; Wang *et al.*, 1997). It has also been found that the differing isoforms can have varying substrate specificity *i.e.* p38α-MAPK and p38β-MAPK but not p38γ-MAPK and p38δ-MAPK are able to phosphorylate MAPKAPK2 and MEF2C, whereas all four p38 homologues are able to activate ATF2 (Yang *et al.*, 1999).

Many of the protein kinases responsible for the activation of p38 MAPK have been identified but as yet very little work has been done to examine their interactions in detail. Activation of p38 MAPK requires the same dual phosphorylation as ERK and JNK but the motif within the activation loop of p38 MAPK contains a glycine to give T-G-Y. Upstream at the MEK family level, similar to JNK and ERK1/2, there are two proteins able to activate p38 MAPK, MKK3 and MKK6 (Raingeaud *et al.*, 1996). Recent work has shown that MKK3 is able to selectively activate only the p38α, γ and δ MAPK isoforms whereas MKK6 is able to activate all (Enslen *et al.*, 1998; Hale *et al.*, 1999; Wang *et al.*, 1997). Clearly defining the activation of MKK3

and MKK6 is complex due to the potential cross talk between the MAP kinase family members at the MEKK level. MEKK-4, TAK-1, ASK-1 and TAO all appear to be effective activators of the p38 pathway. It must be noted, however, that most of these MEKKs are also able to phosphorylate the JNK kinases MKK4 and MKK7 and that many of the MEKKs upstream of JNK can also activate p38 MAPK (for review see (Obata *et al.*, 2000; Widmann *et al.*, 1999). This cross talk is likely to result in the potential for both JNK and p38 MAPK to be activated simultaneously via certain MEKKs. On the contrary however, activation of other specific MEKKs will likely allow activation of JNK or p38 individually.

Collectively p38 MAPK isoforms are activated in response to stress stimuli, in a manner similar to JNK. However, p38 MAPK proteins are also activated by ligand binding to the cell surface. There are several proteins that can induce a response including certain cytokines (interleukin-1 and tumour necrosis factor-α) and G-protein coupled receptors (Whitmarsh & Davis, 1996). The substrates of p38 MAPK include the MAP kinase activated proteins MAPKAP1 and p90 as well as several transcriptional control factors, including ATF1 and 2, CHOP, ELK-1 and NFκB (Obata *et al.*, 2000; Raingeaud *et al.*, 1995; Raingeaud *et al.*, 1996).

Elucidating the cellular roles of p38 MAPK was facilitated by the identification of a specific p38 MAPK inhibitor : Smithkline Beecham (SB)-203580. SB-203580 is a pyridinyl-imidazole compound. All bi- and tri-aryl imidazole compounds inhibit p38-MAPK activity by competing with ATP for the nucleotide-binding site on p38 MAPK. Various variations of the basic structure of these compounds have been used to find inhibitors specific for the p38 MAPK isotypes (Wilson *et al.*, 1997). Use of the SB-203580 compound has helped to identify many roles for p38 MAPK including IL-2 and IL-7 stimulated cell proliferation (Crawley *et al.*, 1997) and a major role in cell apoptosis (Brenner *et al.*, 1997).

1.3.4 Functions of the MAP kinases.

Although a great deal of study has been carried out to characterise the components of the MAP kinase pathways and elucidate their organisation, localisation and substrates, much more remains to be learned about the biological functions of the MAP kinases. As described above the MAP kinases are able to phosphorylate a number of targets within the cell including nuclear, cytosolic, membrane bound and mitochondrial proteins. Phosphorylation of proteins can have many varied effects including increasing or decreasing activity, stabilisation and extension of the half-life and also the targeting of a protein for degradation.

Associating a specific cellular function with the activity of a single transduction pathway is made more difficult as a number of pathways are probably activated in response to a single stimulatory event, and therefore multiple pathways are likely to contribute to any given response. The situation is further complicated by the tendency for MAP kinase function to vary depending on cell type and environmental conditions. This is exemplified by the factors controlling cell death. In PC12 cells JNK is thought to trigger apoptosis while ERK is proposed to prevent cell death (Xia *et al.*, 1995). Whereas in B-cells JNK activity rescues the cells from apoptosis and activation of ERK is thought to cause apoptosis (Sakata *et al.*, 1995). As well as the same MAP kinase having differing roles in different cell types there is also evidence of differing MAP kinases fulfilling an identical functional role in some cells. For example, both p38 MAPK and ERK are able to phosphorylate the kinase Mnk1. Mnk1 is activated in response to both phorbol esters and stress factors and these effects can be blocked by inhibitors of the ERK and p38 MAPK pathway. Mnk1 is responsible for the activation of eIF-4E, which plays critical role in protein synthesis machinery recruitment to mRNA (Waskiewicz *et al.*, 1997). Similarly, both p38 MAPK and ERK are able to phosphorylate and activate the transcription factor Elk-1 (Price *et al.*, 1996).

Crosstalk between MAP kinase pathways further complicates the signalling process. There is evidence of MAP kinase activity resulting in the modulation of other MAP kinase families. For example, in HepG2 cells, p38 MAPK activity

reduces LDL receptor expression by inactivating the ERK pathway (Singh *et al.*, 1999), whilst in primary bovine aortic endothelial cells ERK activation is necessary for JNK activation in response to VEGF (Pedram *et al.*, 1998).

With this kind of pathway crosstalk and convergence being a major part of nearly every signalling response the most effective way to isolate the function of a protein within these cascades is to remove that proteins activity and analyse the effects. There are four main ways of achieving this, firstly the design and use of highly specific inhibitor compounds. Secondly, the addition of dominant negative versions of the protein, thirdly the expression of antisense RNA to prevent protein expression and lastly, probably the most effective method of removing a protein from a cell, is to remove or damage the area of the genome which encodes that protein thus preventing its production. In particular mice have been used with this technique as they have a similar physiology to humans and reproduce rapidly. The creation of transgenic mice lacking specific proteins has a number of uses. Firstly, the role of a protein in growth and development can be assessed and secondly, tissue and cell type specific roles can be identified that would not be easily elucidated by the use of either inhibitors or dominant negatives *in vitro*. Many studies have also used the addition of constitutively active proteins or inducible systems, but these do not remove the possibility of pathway crosstalk leading to the final cellular response and must be viewed with care.

Knockout mice have been used in the study of ERK signalling at a number of points in the cascade. A transgenic mouse lacking ERK1 was created and the mice are predominantly phenotypically normal in that they are viable, fertile and of normal weight and form. The only abnormality so far discovered is a reduced rate of thymocyte proliferation and maturation (Pages *et al.*, 1999). Interestingly ERK2 activity does not increase in cells lacking ERK1, suggesting that it is not functioning in a compensatory role. To properly assess the function of ERK1/2 in this manner a knockout lacking ERK2 must also be created, and if a similar result is obtained, it may be of use to breed the two mutant mice to knockout both ERK1 and ERK2. The involvement of ERK in cardiac hypertrophy has also been studied using a transgenic mouse. This mouse however, did not lack ERK1 or 2, but instead carried a

constitutively active mutant form of the ERK activator, MEK1. Mice carrying this mutant MEK1 showed an increase in ERK but not p38 or JNK activity and an increase in cardiac function and hypertrophy (Bueno *et al.*, 2000).

As described above (section 1.3.1) the wide variety of stimuli that activate ERK1/2 and the diverse array of substrates indicate a role in the signalling processes controlling cell growth and differentiation. There are many studies that support this hypothesis. In fibroblasts a constitutively active MEK1 can induce proliferation (Brunet *et al.*, 1994). ERK activation can induce proliferation in PC12 cells and transformation in NIH3T3 cells (Cowley *et al.*, 1994). Also in PC12 cells activation of ERK by NGF or EGF is followed by differentiation into sympathetic neurones. This effect can also be induced using a constitutively active MEK mutant (Cowley *et al.*, 1994) and blocked using the ERK pathway inhibitor PD98059 (Pang *et al.*, 1995). ERK activity is also required for differentiation of fibroblasts into adipocytes in response to insulin (Sale *et al.*, 1995). Endothelial cell proliferation in response to VEGF is dependent on both ERK and JNK activation and interestingly the activation of JNK in this model is also dependent on ERK activity (Pedram *et al.*, 1998), providing further evidence of crosstalk between pathways.

The JNK pathway has been the focus of a great deal of interest, and separation of the functions of the various JNK isoforms has proved difficult. This is perhaps why these JNK isoforms have all been targeted for deletion in transgenic mice.

Mice lacking JNK1 develop normally and are fertile. Alterations have been noticed in the immune system, notably CD4+ T-cell activation leads to differentiation into only one of the T_H effector cells rather than the usual two subtypes (Dong *et al.*, 1998). Studies on JNK2 knockout mice have been contradictory, but in general indicate defects in T cell function including altered differentiation and resistance to apoptosis (Sabapathy *et al.*, 1999; Yang *et al.*, 1998). As JNK3 is predominantly expressed in neuronal tissue, analysis of JNK3 -/- mice has focused on brain function. Although these mice have no detectable JNK3 activity within the brain they are of normal size, fertile and healthy. However, knockout mice are less sensitive to kainate-induced seizures and the apoptotic damage caused by

these seizures, suggesting JNK3 may be involved in apoptosis in response to this kind of excitotoxic stress (Yang *et al.*, 1997).

Mice with disruptions to combinations of the JNK genes were analysed in two recent studies. JNK1/JNK3 and JNK2/JNK3 -/- mice develop normally. However, JNK1/JNK2 knockout mice have severe defects in brain development due to reduced apoptotic signalling and were embryonic lethal (Kuan *et al.*, 1999; Sabapathy *et al.*, 1999). This possibly suggests JNK1 and 2 are able to compensate for the loss of each other. Studies on the JNK1/JNK2 -/- mice ES cells have indicated a role for JNK in activation of effector caspases, including caspase-3 (Tournier *et al.*, 2000).

As JNK is activated by a variety of cell stresses, it has been proposed that JNK functions as a major component of the apoptosis process. Initial studies discovered JNK-dependent apoptosis in both human T cells and rat neuronal cells (PC12) (Wilson *et al.*, 1996; Xia *et al.*, 1995). Several cellular proteins with known roles in apoptosis are targets for JNK phosphorylation. These include p53 (Fuchs *et al.*, 1998) and c-Myc (Noguchi *et al.*, 1999), however neither have been shown to be indispensable for apoptosis so their roles remain unclear. The mechanism of JNK-dependent apoptosis is unclear but is unlikely to involve a single mechanism. On exposure to ionising radiation, JNK can translocate to the mitochondrion and phosphorylate Bcl-X_L, a protein involved in release of cytochrome-c and apoptosis (Kharbanda *et al.*, 2000). This may indeed be one mechanism by which JNK can control apoptosis, but mutant c-Jun, lacking JNK phosphorylation sites, interferes with the apoptotic response (Behrens *et al.*, 1999) suggesting a non-mitochondrial, AP-1-dependent transcription system is required. Similarly, mutant versions of both c-Jun and MKK4 (JNKK) are able to prevent apoptosis induced by ceramide, a major product of TNFα activation (Verheij *et al.*, 1996). The JNK1/JNK2 -/- ES cells mentioned above, are insensitive to Fas (death receptor) induced apoptosis and have defects in apoptosis in response to UV radiation, anisomycin and the DNA damaging agent methylmethanesulfonate (Tournier *et al.*, 2000).

Interestingly, a study using MKK4 knockout mice showed thymocytes from these mice to be more susceptible to apoptosis (Nishina *et al.*, 1997), suggesting a role in preventing apoptosis for the JNK pathway. This is supported by other studies that suggest sustained activation of JNK is required for apoptosis, and that transient

JNK signals may be protective or contribute to growth signalling (Chen & Tan, 2000). Several studies have also indicated a role for JNK signalling in neoplastic growth. Ras induced transformation of cells is dependent on activation of c-Jun (Johnson *et al.*, 1996) and similar to the effect on the apoptotic response, mutation of the JNK phosphorylation sites on c-Jun suppresses transformation by Ras (Behrens *et al.*, 2000).

Taken together these data do not demonstrate conclusively the involvement of JNK in all forms of apoptosis. This is not surprising as the pathways involved in mediating apoptosis probably varies in form from cell type to cell type. And thus, the role of JNK is also likely to vary between differing cell types. It is of interest however, that the knockouts tend to show immune system defects. JNK1 and 2 may play an important role in T-cell development and activation, and thus be critical to secondary immune function.

Through regulation of gene transcription, p38 MAPK has been implicated in the production of many signalling molecules including cytokines, nitric oxide (NO), catecholamines and arachidonate products like eicosanoids and prostaglandins.

Stimulation of p38 MAPK activity can enhance the activity of many transcription factors including ATF-1/2, CHOP, multiple members of the CREB family, NF-κB, ELK-1, ETS, MAX, MEF-2A, and MEF-2C. These activated transcription factors can then go on to enhance gene expression by binding to specific promoter regions. For example, the CREB family of proteins are able to bind to the cyclic AMP response element present in the promoter region of many genes and enhance transcription. Activated ATF-2 can bind to c-Jun (activated by JNK) to form a dimer capable of activating genes that have an AP-1 binding site. Also MEF-2C can increase c-Jun transcription thus further linking the requirement of both p38 MAPK and JNK activity for AP-1 promoter activity.

The metabolites of arachidonic acid are involved in both the stimulation and repression of inflammatory responses. Production of these metabolites can be influenced by p38 MAPK via its activation of phospholipase-A_2 or the increase in expression of COX-2. Thus, p38 MAPK can affect inflammatory responses by controlling both arichidonate release and prostaglandin production (Guan *et al.*,

1998; Hwang *et al.*, 1997; Waterman *et al.*, 1996). JNK is also thought to induce COX-2 (Herschman, 1996), further emphasising the overlap in MAP kinase function and cross talk between the pathways.

p38 MAPK is involved in NO signalling at two points. Firstly, p38 MAPK can enhance arginine-transporter activity which increases the amount of free arginine, a precursor of NO (Caivano, 1998). Secondly p38 MAPK can increase gene expression of the protein iNOS, an inducible form of nitric oxide synthetase (Guan *et al.*, 1997). Further evidence of a link between p38 MAPK and NO indicates that NO releasing compounds like sodium nitroprusside are able to enhance p38 MAPK activation (Jun *et al.*, 1999).

p38 MAPK is also involved in adrenergic signalling through at least two separate mechanisms . Firstly, p38 MAPK is activated in response to both α and β adrenoreceptor activity (Moule & Denton, 1998; Zhong & Minneman, 1999) and secondly, the downstream p38 MAPK substrate, MAPKAP-2, can modify tyrosine hydroxylase activity which in turn effects catecholamine biosynthesis (Thomas *et al.*, 1997).

Knockout studies on p38 MAPK led to the discovery that p38α MAPK is essential for development and knockout mice die during embryogenesis. Studies were therefore carried out in the knockout ES cells. In these cells there is a 95% loss in activation of MAPKAP kinase-2 and a 75% loss in IL-6 production in response to IL-1 (Allen *et al.*, 2000). Both the remaining MAPKAP kinase-2 activity and IL-6 production are insensitive to the p38 MAPK inhibitor (SB203058) suggesting that another pathway is responsible for the residual activity.

One area of intense research into the biological functions of MAP kinases that has encompassed all three of the main MAP kinase families at the level of the tissue rather than the cell is in the area of cardiac pathology. Expression of all of ERK, JNK and p38 MAPK has been detected in human heart tissue. Although a slight decrease in the actual amount of JNK was noted, the activity of both JNK and p38 MAPK is greatly increased in failing hearts, relative to healthy tissue. ERK expression levels and activity however, are the same in both healthy and failing heart tissues (Cook *et al.*, 1999). p38 MAPK activity has also been shown to be increased in rat hearts

subjected to global ischaemia. In this model, an increase in JNK activity is not noted until 10-20 minutes after reperfusion. (Bogoyevitch *et al.*, 1996). These and other studies suggest a role for JNK and p38 MAPK in the responses of cardiac tissue to damage.

The JNK and ERK pathways are also known to be involved in the hypertrophic response of cardiac tissue (Bogoyevitch *et al.*, 1996; Wang *et al.*, 1998). Use of the JNK scaffold protein, JIP, as an inhibitor of the JNK pathway reduces transcriptional activation of hypertrophic responsive genes in response to hypertrophy stimulating agonists (Finn *et al.*, 2001). And transgenic mice, with mutations causing an increase in ERK activity, show an increase in cardiac hypertrophy (Bueno *et al.*, 2000).

The studies described here give a rough outline of the main cellular processes that ERK, JNK and p38 are involved in. The original descriptions described ERK as being involved in growth, with JNK and p38 controlling stress responses and apoptosis. Although these ideas still hold true to some extent, it is now clear that it is not possible to delineate the three pathways using such simple criteria. The functional outcome of any stimuli is likely to involve multiple signalling pathways that react in a cell and stimuli specific manner. The exact cellular response is likely to rely on a fine balance between the combined activities of all the pathways following stimulation.

1.3.5 MAP Kinases and the Nucleus

As outlined above (section 1.3.4) one of the main substrates of all the MAP kinases are the transcription factors or their associated proteins. Although some transcription factors like NFκB are found in the cytosol many spend their entire life within the nucleus. As MAP kinases are predominantly activated in the cytosol, a translocation event must occur for phosphorylation and activation of nuclear substrates and DNA interaction to occur.

ERK has been shown to translocate to the nucleus in various cell types and after various stimulatory events (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). Interestingly, however the activators of ERK, MEK1 and MEK2, are localised solely in the cytosol (Lenormand *et al.*, 1993). This was found

to be due to the presence of a NES in the N-terminal tail of MEK1 (Fukuda *et al.*, 1996) (see section 1.6 for details on NES function). This proved to be a critical step in ERK signalling as inactive ERK is bound to MEK1 (and probably MEK2) and held in the cytosol by the NES. For nuclear translocation to occur, ERK must be released from MEK1, and this release is concomitant to the activating tyrosine phosphorylation (Adachi *et al.*, 1999; Rubinfeld *et al.*, 1999).

The mechanism of translocation of ERK to the nucleus has only recently started to become clear. Many of the early studies noticed that catalytically inactive ERK or mutants lacking the threonine and tyrosine required for activation were still able to undergo stimulus-dependent translocation to the nucleus. This suggests that nuclear accumulation of ERK is independent of its kinase activity, and thus either an upstream component of the pathway or a separate stimulus-dependent process is responsible for translocation of ERK. Using microinjection techniques, Khokhlatchev *et al*, 1998 discovered that inactive or mutant ERK will translocate to the nucleus only in the presence of active ERK. This is due to the ability of the active ERK to form dimers with either another active ERK protein or an inactive molecule of ERK (Khokhlatchev *et al.*, 1998). The same study also revealed that the other main MAP kinase family members, JNK and p38, are able to form dimers but that there was no cross binding between family groups. Adachi *et al* confirmed dimer transport using various inhibitors of nuclear import and ERK mutants. They discovered that active ERK can pass into the nucleus as a monomer by passive diffusion but that the dimer of active ERK is translocated by an active transport mechanism (Adachi *et al.*, 1999). Once within the nucleus an anchoring protein retains ERK, this anchoring protein is synthesised in response to ERK activation and has a very short half-life (~1 hour) (Lenormand *et al.*, 1998). The removal of ERK from the nucleus presumably follows the removal of the translocating stimulus and thus the breakdown of the nuclear anchors. Transport of ERK from the nucleus to the cytosol is facilitated by the upstream kinase MEK1, which shuttles into the nucleus, binds the inactive ERK and the whole complex is transported to the cytosol via the NES on MEK1.

Most of the translocation studies have focused on the ERK family but as JNK and p38 are able to form dimers of active protein similar to ERK, it is reasonable to assume that the nuclear translocation of the rest of the MAP kinases will function in an analogous manner to ERK. In general, p38 has been reported in both the cytosol and the nucleus although 3 cases of distinct nuclear accumulation have been reported after hyperosmotic stimulation (Raingeaud *et al.*, 1996), after ischaemic stress adaptation (Maulik *et al.*, 1998) and after high glucose stimulation in neuroblastoma cells (Cheng & Feldman, 1998). JNK also can be found in both cytosol and the nucleus, and similar to p38 high glucose causes nuclear translocation of JNK in neuroblastoma cells (Cheng & Feldman, 1998). Additionally stimulation with H_2O_2 in endothelial cells has been shown to lead to nuclear accumulation of JNK (Robinson *et al.*, 2001).

1.4 Phosphatases and the Cell

The activity of any cellular protein, which is subject to modification by phosphorylation, is a fine balance between the activity of upstream kinases and the activity of specific phosphatases. Protein phosphatases can be separated into two superfamilies based on substrate specificity. Firstly there are the protein phosphatases (PPs) which are specific for hydrolysis of phospho-serine and threonine. Secondly there are the protein tyrosine phosphatases (PTPs) which, as the name suggests, are able to hydrolyse phospho-tyrosine residues.

There are several other phosphatase families within the cell including, lipid-phosphatases and nucleic acid phosphatases. To aid in the clarity of this work these shall not be discussed further.

1.4.1 Serine/Threonine protein phosphatases (PPs)

The human genome encodes roughly 20 times fewer ser/thr phosphatases than ser/thr kinases, this suggests individual PPs have broad substrate specificity and are able to reverse the effects of multiple ser/thr kinases. However, this broad substrate system would be of little use in regulating the diverse and complex kinase cascades involved in cell signalling. Unlike the kinases that have evolved as differing monomeric proteins around the catalytic kinase machinery, the PPs have evolved as a

small family of catalytic subunits that are able to interact with a diverse range of regulatory subunits. These regulatory subunits impart substrate binding, substrate specificity and even unique localisations upon the catalytic subunits.

There are four major classifications of ser/thr phosphatase enzymes in the mamalian cell. Three show high degrees of structural homology and thus are likely to be part of the same evolutionary gene family: PP1, PP2A and PP2B (calcineurin). These protein phosphatases are complexed *in vivo* to regulatory subunits. These subunits increase the activity of the PPs and direct their activity e.g. when bound to glycogen, PP1 shows increased activity towards glycogen synthase (Tonks, 1990). The other major ser/thr phosphatase group (PP2C) however has only been isolated as a monomeric protein (Cohen, 1989). The four families of ser/thr phosphatases can be identified functionally and experimentally by their sensitivity to differing inhibitors and inhibitor concentrations. Okadaic Acid (OA) inhibits PP1, PP2A and PP2B. PP2A is most sensitive (K_I 0.2nM), followed by PP1 (K_I 2nM), and PP2B requires micromolar concentrations of OA for an inhibitory effect to be seen (K_I 10μM). PP2C is insensitive to (Cohen *et al.*, 1990; Shenolikar & Nairn, 1991).

There are over 100 regulatory subunits that have been shown to interact with the PP1, PP2A and PP2B catalytic subunits. Each of the regulatory proteins can be grouped into one of three categories based on its interaction with the catalytic subunit. Firstly the activity-modulators which includes inhibitors as well as activators of PP activity. Secondly, the targeting proteins which impart substrate specificity on the PPs and lastly the substrate group of regulatory proteins, which whilst also being a substrate for PPs can enhance specificity and/or activity of the catalytic subunit (Bollen, 2001; Janssens & Goris, 2001).

The PPs have been shown to have many diverse roles within the cell. PP1 is involved in the control of glycogen metabolism, calcium transport and protein synthesis (discussed in Wera & Hemmings, 1995). PP2A also has many cellular roles not least of which is in the control of the cell cycle, PP2A is able to prevent the cell passing the G_2/M transition by maintaining certain CDKs in a non phosphorylated state. (Janssens & Goris, 2001). Additionally PP2A has also been shown to have roles in inflammatory signalling by inactivating both JNK and IκB kinase (a key regulator of NFκB signalling) (DiDonato *et al.*, 1997) and in control of gene

expression by dephosphorylation of transcription factors (Wera & Hemmings, 1995). PP2B is also known as calcineurin, and as the name suggests is associated with Ca^{2+} signalling and is in fact dependent on Ca^{2+} for its activity. PP2B has been shown to have roles in many calcium-dependent processes including T cell activation leading to increased IL-2 expression in response to Ca^{2+} (Jain *et al.*, 1993) and also inhibition of PP2B can protect cells from calcium-induced apoptosis (Wera & Hemmings, 1995). As mentioned above PP2C differs from the rest of the PPs in that it is monomeric. Many of the substrates of PP2C remain to be identified but work using yeast strains has suggested a role for PP2C in control of osmoregulation via the regulation of the MAP kinases (Gaits *et al.*, 1997) and in mammalian systems PP2C has been implicated in Ca^{2+} signalling in the brain (Fukunaga *et al.*, 1993).

1.4.2 Protein Tyrosine Phosphatases (PTPs)

The protein tyrosine phosphatases (PTPases) were not the focus of intensive study until the 1980s when it was discovered that they were linked to control of cell proliferation, normal and neoplastic cell growth.

Two major classes of PTPases have been discovered: the receptor-like PTPases and the non-receptor PTPases. As the names suggest, the receptor like PTPases are membrane associated and the non-receptor PTPases are cytosolic. The relative activity of the phosphatases in relation to the kinases that they oppose can be up to two thousand fold greater. This level of activity must be tightly regulated to stop uncontrolled de-phosphorylation, but at the same time, be sensitive enough to allow signal transduction to control normal cell growth (Fischer *et al.*, 1991). The mechanisms of phosphatase control were first studied using PTP1B, a non-receptor PTP isolated from the soluble fraction of human placenta. (Tonks *et al.*, 1988). PTP1B is highly specific for phospho-tyrosine but non-specific in respect of the substrate protein. Inhibition with standard phosphatase inhibitors (zinc, molybdate, orthovanadate) appears to be non-competitive in nature, which suggests that PTP1B contains an inhibitory site that is separate from its catalytic domain.

Analysis of the PTPases, both receptor and non-receptor families, led to the discovery of a highly conserved catalytic motif: [I/V]HCxxGxxR[S/T]G. This was used to scan nucleotide databases for other novel PTPases, leading to the discovery

of VH1 from the vaccinia genome. VH1 protein was found to be able to dephosphorylate not only the tyrosine on raytide and myelin basic protein but also the phospho-serine on casein and thus was termed a dual specificity phosphatase (DSP). Site-directed mutagenesis was used to alter the conserved cysteine in the catalytic domain. This cysteine has been shown previously to be essential for PTPase activity. (Guan & Dixon, 1990). Interestingly, the removal of the critical cysteine leads not only to the loss of PTPase activity, but also the phospho-serine/threonine activity as well. This suggests that both serine and tyrosine activity require the same catalytic mechanism. This mechanism involves the formation of a phospho-thiol intermediate between the substrate and the cysteine in the catalytic domain prior to hydrolysis and release of the phosphate group (Guan *et al.*, 1991).

1.5 The Control of MAP kinases by Phosphatases.

Given that all three MAP kinase pathways either alone or in tandem can play important roles in cellular function, the termination of the MAP kinase signals is clearly important. As MAP kinases are activated by dual phosphorylation within their activation loop on both a tyrosine and threonine residue and both are required for activity it is possible for either PPs or PTPs to inactivate the MAP kinases. Indeed, studies have shown that the serine/threonine phosphatase PP2A is able to inactivate ERK (Alessi *et al.*, 1995) and that the PTPases: PTP-SL and PTP-STEP are also able to bind and inactivate ERK (Pulido *et al.*, 1998). PP2A is able to decrease AP-1 activity, possibly via inactivation of JNK, this hypothesis is further supported by the co-precipitation of the PP2A regulatory subunit, PP2A-Aα, with JNK (Shanley *et al.*, 2001). However, the precise role these proteins play in the control of the MAP kinase cascades and the possibility of homologues that play a role in the control the p38 and JNK pathways is yet to be elucidated.

Studies assessing PP and PTP mediated dephosphorylation in regard to the MAP kinases appear to have received less focus due to the significant and rapid progress in the understanding of the role played by DSPs in the control of MAP kinase activity. As mentioned above VH-1 was the first DSP cloned and thus considered the prototypic member of the DSP family. Studies to elucidate the structure and function and the search for similar proteins led to the discovery of an

entirely new family of proteins that appear to function solely as MAP kinase phosphatases.

These so called MAP kinase phosphatases or MKPs all have a highly conserved catalytic domain that contains the consensus PTPase motif. The catalytic domain is located in the C-terminal domain of all MKPs. Centred around the standard PTPase motif is an area of high homology present in all DSPs cloned to date. This area is now used to identify DSP activity from protein sequence alone: Dx_{26}(V/L)x(V/I)HcxAG(I/V)SRSxT(I/V)xxAY(L/I)M.

The N-terminus of the MKP family is less well conserved and shows considerable variance between members. The N-terminal region is believed to be involved in substrate binding and specificity. All MKPs also have two short stretches of sequence that contain residues conserved with areas flanking the active site of the phosphatase Cdc25. These areas have been designated Cdc25 homology domains (CH2).

Since the discovery of the first MKP, the family has been growing at a rate of faster than one new MKP cloned per year. Each has its own substrate specificity and expression pattern (see Table 1.1).

1.5.1 MKP-1

By far the best understood and studied of the MKP family is MKP-1. MKP-1 was first identified as the PTPase, CL100, in a study designed to isolate human genes that are inducible by oxidative stress (Keyse & Emslie, 1992). The cDNA of CL100 was found to be homologous to the archetypal DSP, VH1, encoded by vaccina virus (Guan *et al.*, 1991). However, unlike VH1 which has broad substrate specificity, when CL100 or its mouse homologue 3CH134 were expressed and purified from bacteria they were found to be highly specific for the 42KDa MAP kinase, now known as ERK2 (Alessi *et al.*, 1993; Charles *et al.*, 1993). The gene locus of CL100 maps to human chromosome 5q34, which is an area of chromosome 5 often associated with malignancies. Deletions of this region were found in leukaemias and translocations have been associated with lymphoma (Emslie *et al.*, 1994). Later

Phosphatase	Other Names	Specificity	Locale	Tissue Distribution	References
B23	hVH-3, DUSP5	Active towards ERK others not tested	Nuclear	Pancreas, Brain, heart, lung, liver, skeletal muscle, kidney and placenta	(Ishibashi *et al.*, 1994)
M3/6	hVH-5, DUSP8	JNK only	Cytosol or nucleus Depends on cell type	Brain, heart, skeletal muscle and lung	(Martell *et al.*, 1995; Theodosiou *et al.*, 1996)
MKP-1	hVH-1, CL100, DUSP1	p38>JNK>ERK	Nuclear	High levels detected in lung, liver, pancreas and placenta. Lower in brain and kidney.	(Charles *et al.*, 1993; Keyse & Emslie, 1992; Kwak *et al.*, 1994; Sun *et al.*, 1993)
MKP-2	hVH-2, TYP1, DUSP4	JNK=ERK>p38	Nuclear	Ubiquitous in all human tissues tested	(Chu *et al.*, 1996; Guan & Butch, 1995; Misra-Press *et al.*, 1995)
MKP-3	Pyst-1, rVH6, DUSP6	Solely ERK	Cytosolic	heart, pancreas, lung and kidney	(Camps *et al.*, 1998; Groom *et al.*, 1996; Mourey *et al.*, 1996; Muda *et al.*, 1996)
MKP-4	Pst3, DUSP9	ERK low activity for p38 and JNK	Cytosolic with some punctate staining	Limited and low. Kidney, placenta and fetal liver	(Camps *et al.*, 1998; Muda *et al.*, 1997)
MKP-5	DUSP10	p38=JNK>ERK	Equal between cytosol and nucleus	Heart, lung, skeletal muscle and kidney. Less in brain spleen or testis.	(Tanoue *et al.*, 1999; Theodosiou *et al.*, 1999)
MKP-6		ERK=JNK=p38	N.D but as MKP6 associates with a membrane bound receptor (CD28), likely to be cytosolic	Present in low levels in all tissues tested.	(Marti *et al.*, 2001)
MKP-7		JNK>p38>>ERK can differentiate between p38 and JNK isoforms	Cytosol but shuttles into nucleus	Brain, kidney, intestine, testis, thymus, spleen, bone marrow	(Masuda *et al.*, 2001; Tanoue *et al.*, 2001)
MKP-M		JNK	Cytosolic	High levels in kidney, heart and testis. Low everywhere else tested	(Matsuguchi *et al.*, 2001)
MKP-X	Pyst-2, B59, DUSP7	N.D	N.D	N.D	(Groom *et al.*, 1996; Muda *et al.*, 1996)
PAC-1	DUSP2	ERK=p38>JNK	Nuclear	High levels in spleen and heart, low everywhere else tested except brain	(Rohan *et al.*, 1993; Ward *et al.*, 1994)
VHR		ERK>>JNK=p38	Nuclear	Highest levels in liver. Easily detectable in all tissues tested.	(Alonso *et al.*, 2001; Ishibashi *et al.*, 1992)
VHX		Active towards ERK others not tested	Cytosolic	Detectable in thymus and some immortalised cell lines.	(Alonso *et al.*, 2002)

Table 1.1. The MAP kinase phosphatases.
Summary of all MKPs identified to date, indicating the differing specificity's, subcellular and tissue distributions. N.D. indicates data has not yet been determined

studies identifying other members of the MKP family led to the renaming of CL100 to MAP kinase phosphatase 1 (MKP-1).

MKP-1 is the typical nuclear MKP and along with the other nuclear MKPs is an immediate early gene. Analysis of MKP-1 expression found high levels of MKP-1 mRNA in the lung, liver, placenta and pancreas with lower levels detected in heart and skeletal muscle (Kwak *et al.*, 1994). MKP-1 was originally identified as being specific for ERK2 both *in vitro* and *in vivo* (Charles *et al.*, 1993; Sun *et al.*, 1993). However, more recent studies have shown that MKP-1 can in fact inactivate certain JNK and p38 isoforms (Chu *et al.*, 1996; Groom *et al.*, 1996; Lin *et al.*, 1995; Raingeaud *et al.*, 1995). A more in depth study using yeast two hybrid analysis to detect binding between MKP-1 and members of the MAP kinase superfamily showed MKP-1 to be able to distinguish between MAP kinases of the same family. MKP-1 showed interaction with ERK1 and 2, also p38α but not p38γ or p38δ MAPK (Slack *et al.*, 2001). Another study used co–precipitation and *in vitro* kinase assays to show MKP-1 able to inactivate p38α and p38β but not p38γ or p38δ MAPK (Tanoue *et al.*, 2001).

The roles of MKP-1 have been looked at in many differing cell types and situations. Many of these studies focusing on the cell as a whole or particular cellular function have confirmed the substrate specificity of MKP-1. For example, in monocytes and macrophages chemotaxis to a site of inflammation is dependent on ERK activity. This chemotaxis is inhibited by hypoxic conditions via the upregulation of MKP-1 mRNA transcription and subsequent MKP-1 translation leading to inactivation of ERK (Grimshaw & Balkwill, 2001). In tumour cells the chemotherapeutic drug cisplatin is able to induce apoptosis but the trans-isomer of the same drug, transplatin, is ineffective. This was discovered to be caused by the ability if transplatin to induce MKP-1 mRNA production, thus inactivating JNK and preventing apoptosis. Cisplatin on the other hand is unable to upregulate MKP-1 leading to strong JNK activation and apoptosis (Sanchez-Perez *et al.*, 2000). Lastly, MKP-1 expression is enhanced by calcineurin leading to inactivation of p38 in cardiac myocytes (Lim *et al.*, 2001).

There are many cellular processes or responses that lead to either the activation or abrogation of the activity of the MAP kinase pathways. The balance of MKP-1 to

MAPK activity appears to be critical as many factors and processes lead to an increase or decrease in MKP-1 mRNA and protein levels. Glucocorticoids inhibit inflammation by inactivating ERK and possibly JNK through increased MKP-1 expression. (Kassel *et al.*, 2001). Cyclohexamide activates $cPLA_2$ via the ERK pathway by preventing the translation of MKP-1 allowing sustained ERK activity (Lin & Hsu, 2000).

These studies show, generally, that MKP-1 activity is controlled at the transcriptional level. Several studies show that ERK and JNK can induce MKP-1 expression suggesting a simple feedback system to prevent over activation of the MAPK (Bokemeyer *et al.*, 1998; Bokemeyer *et al.*, 2000). This hypothesis is supported by work showing that ERK inactivation is dependent on *de novo* protein synthesis, presumably MKP-1 (Sun *et al.*, 1993). These effects however, appear to be cell-type specific with activation of ERK leading to reduced MKP-1 expression in fibroblasts (Bokemeyer *et al.*, 1996) and inactivation of ERK proceeding despite the presence of protein synthesis inhibitors (Alessi *et al.*, 1995; Wu *et al.*, 1994). In several cell types it appears that other mechanisms exist for induction of MKP-1. In fibroblasts MKP-1 expression is dependent on calcium rather than MAPK activity. (Scimeca *et al.*, 1997) and in macrophages MKP-1 expression appears to be dependent on PKC activity (Valledor *et al.*, 1999). Interestingly, another mechanism of MKP-1 control was discovered in fibroblasts by Brondello *et al.* 1999. They found that ERK can phosphorylate MKP-1 and that this phosphorylation prevented the targeting of MKP-1 for degredation via the ubiquitin proteosomal pathway. Thus, phosphorylation increases the half-life MKP-1 within the cell (Brondello *et al.*, 1999).

All of the above studies, and many others, indicate that MKP-1 does not have a single clearly defined role within the cell. *In vivo* MKP-1 can inactivate all three of the MAPK family members. In some cell types this is appears to be a simple negative feedback process but in others a complex system of cross talk between signalling pathways utilises changes in MKP-1 levels and activity. It must be noted however, that as much as MKP-1 appears to play a significant role in many cellular processes generation a mouse deficient in MKP-1 did not appear to affect

development. The mouse is phenotypically normal and studies using MEFs isolated from the deficient mice appear to have no changes in either the kinase kinetics or the growth of the cells (Dorfman *et al.*, 1996). This suggests that one or more of the other MKPs may be able to compensate for the loss of MKP-1. As MKP-1 and MKP-2 have a similar gene structure and expression pattern it is likely they share a common ancestral gene (Zhang *et al.*, 2001). This may suggest that MKP-2 would be a viable candidate for compensating for the loss of MKP-1.

1.5.2 MKP-2

Of the three most studied DSPs MKP-1, MKP-2 and MKP-3, MKP-2 is the least well characterised. Its localisation, specificity and tissue distribution have been identified, however regulation of expression and control of MKP-2 activity by various stimuli remains largely unknown.

MKP-2 is a nuclear DSP and similar to the typical nuclear MKP, MKP-1, it is expressed from an immediate early gene. MKP-2 was cloned almost simultaneously from rat and human (Guan & Butch, 1995; Misra-Press *et al.*, 1995). The structural organisation of the MKP-2 gene is very similar to that of MKP-1. The intron and exon sizes and splicing patterns of the two genes are nearly identical suggesting that they are both derived from the same ancestral gene (Zhang *et al.*, 2001). Interestingly Guan *et al* found MKP-2 is expressed in most rat tissues analysed including brain spleen and testes, with noticeably larger amounts in the heart and lung. In contrast to these studies Misra-Press *et al* find MKP-2 to be highest in human placenta and pancreas with no MKP-2 detected in the lung. Another group subsequently cloned MKP-2 from a human T-cell cDNA library and show MKP-2 to be expressed to varying degrees in every human tissue tested. The highest expression is found in the prostate, testes, stomach and pancreas (Chu *et al.*, 1996). The tissue types that MKP-2 appears in most abundantly all have a high proportion of epithelial cells. This may suggest that MKP-2 has an important role in epithelial cell function.

All groups did however agree on the activity of MKP-2, showing it to be specific for the ERK members of the MAP kinase family. Although Chu *et al*, in a more detailed study, not only showed MKP-2 to be able to inactivate ERK, in

transfected HELA and NIH3T3 cells, but also JNK. MKP-2 is not able to inactivate the p38 MAPK isoforms (Chu *et al.*, 1996).

Apart from the above basic characterisation there is little published work analysing MKP-2. As the nuclear MKPs are immediate early genes, what little has been done has focused mainly on the mechanisms of inducing MKP-2 expression by various stimuli. As shown above MKP-1 expression is thought in many cases to be part of a feedback loop to prevent over activity of the MAP kinases. Brondello *et al* 1999, tested this theory for both MKP-1 and MKP-2 in CCL39 cells. In this cell type, MKP-2 expression is upregulated by serum stimulation and this increase is dependent on ERK activation, thus supporting the negative feedback hypothesis (Brondello *et al.*, 1999). A study to look at the upregulation of genes by the oncogenic gene v-Jun (a constitutively active form of c-Jun) found increased levels of MKP-2. As c-Jun is a target for activation by the MAP kinases, this may still represent part of a negative feed back loop system (Fu *et al.*, 2000).

Many of the tissues that MKP-2 is expressed in to a high level are hormone responsive tissues i.e. prostate, testes and pancreas (Chu *et al.*, 1996). Zhang *et al* (2001) discovered that the signalling processes involved in release of FSH and LH from pituitary cells in response to gonadotrophin releasing hormone (GnRH) involved activation of MAPK and expression of MKP-2. However, in these cells the signals involved in the stimulation of MKP-2 expression proved to be very complex. MKP-2 expression is dependent upon activation of both JNK and ERK pathways, PKC is required for the activation of ERK but not of JNK. Also the expression of MKP-2 is dependent on the presence of both intracellular and extracellular calcium. (Zhang *et al.*, 2001).

These studies led on to the important discovery of a response element upstream of the MKP-2 gene that is required for MKP-2 expression in response to GnRH and possibly many other factors. This MKP-2 GnRH response element (MGRE) contains a putative STRE element which is known to bind the transcription factors Sp1, Sp2 and Egr-1. Egr-1 has been identified as part of the MGRE activating complex and appears to have a similar expression profile to MKP-2. (Zhang *et al.*, 2001) . Egr-1 is induced by a broad range of stimuli (Gashler & Sukhatme, 1995) and may be

involved in transcriptional activation of MKP-2 by many cellular factors not just GnRH.

The MKP-2 gene locus has been shown to be missing or damaged in several cancerous disease states suggesting MKP-2 acts as a tumour suppressor. Interestingly, contradicting a role as a tumour suppressor, MKP-2 has also been shown to have an increased level of expression in hepatomas (Yokoyama *et al.*, 1997) and pancreatic tumour cells (Yip-Schneider *et al.*, 2001). It appears that depending on the cell type and other mutations present MKP-2 may be involved in cell immortalisation in both its absence and its presence. Removal of MKP-2 may allow over activation of ERK leading to uncontrolled cell growth. Conversely, chronic mitogenic stimulation of ERK leading to massive induction of MKP-2 could suppress JNK activation thus preventing growth arrest and apoptosis in malignant cells. This further exemplifies the differing roles played by the MAP kinases in various cells.

This work indicates MKP-2 is involved in feedback control of JNK and ERK but not p38 MAPK in a number of situations. Although able to inactivate more than one of the MAP kinase family members, there is yet to be any evidence of pathway cross talk as seen for MKP-1. Like MKP-1 however, MKP-2 also appears to have a prominent role in maintenance of controlled cell growth.

1.5.3 MKP-3

MKP-3 was cloned from a human brain cDNA library using an EST closely related to the human MKP-1 (Groom *et al.*, 1996) and cloned from a rat brain cDNA library using RT-PCR (Muda *et al.*, 1996). mRNA for MKP-3 has been found in most human tissues except the peripheral blood leukocytes. Expression of MKP-3 in human tissue is highest in the heart and pancreas, whereas in rat it is highest in the lung and kidney. As MKP-3 differed from the rest of the MKP family in all but sequence homology, it was assumed that MKP-3 depicted a novel branch of the MKP family. MKP-3 differs from the previously identified MKPs in that it is not stress inducible, is only slightly inducible by serum and is constitutively expressed in fibroblasts. This indicates that unlike MKP-1 and 2, MKP-3 is not encoded by an

immediate early gene. All the previously cloned MKPs had been found to be strictly nuclear and thought to inactivate the MAP kinases after translocation to the nucleus. MKP-3 however, is localised solely in the cytoplasm opening up a new aspect of MAP kinase control (Groom *et al.*, 1996; Muda *et al.*, 1996). Studies of MKP-3 specificity revealed that MKP-3 is highly specific for the inactivation of ERK but unable to inactivate either JNK or p38 in both mammalian cells (Groom *et al.*, 1996; Muda *et al.*, 1996; Muda *et al.*, 1996) and in Drosophila (Kim *et al.*, 2002).

MKP-3 is the first of the MKP family to have its crystal structure elucidated. The structure of the catalytic domain adopts a PTPase fold with a shallow active site. The binding of MKP-3 substrate ERK causes a conformational change that increases phosphatase activity (Stewart *et al.*, 1999). This activation by substrate binding is common to many MKPs and is discussed in detail in section 1.5.6.

As MKP-3 is not an immediate early gene, research has been focusing less on the induction of expression of MKP-3 and more on the structure and interaction of MKP-3 with the MAP kinases. Examples of MKP-3 interaction with the MAP kinases can be found in section 1.5.6. Induction of MKP-3 expression, however, has been noted in some cell types in response to certain mitogenic stimuli or growth. In PC12 cells, MKP-3 mRNA levels are strongly induced by NGF but little effect is seen with other mitogens and stress factors (Camps *et al.*, 1998; Mourey *et al.*, 1996). In P19 cells and MM14 muscle cells, MKP-3 mRNA is strongly induced at several stages of differentiation. This induction follows closely the activation of ERK, again suggesting a negative feedback loop function for the MKP family. (Mourey *et al.*, 1996; Reffas & Schlegel, 2000). These limited studies may suggest a role for MKP-3 in differentiation although the precise mechanics remains unclear.

Functionally, MKP-3 has been shown in CHO cells to inactivate ERK preventing $cPLA_2$ activation and release of arachidonic acid (Hirabayashi *et al.*, 1998). This study used transfected MKP-3 and so although providing strong evidence for MKP-3 specificity *in vivo*, the role of MKP-3 in arachidonic acid release must be viewed with caution as the expressed levels of MKP-3 are likely to far exceed normal physiological levels.

In summary, MKP-3 represents a prototypical member of a new sub family of MKPs. This new family is cytosolic and not expressed from immediate early genes. There is some evidence of the negative feedback role seen for many of the other phosphatases. Also MKP-3 appears to be involved in the process of differentiation, although a lot more work is required before the full functional role of MKP-3 will become clear.

1.5.4 Other MKPs

Other than the three MKPs described above, eleven others have been cloned to date PAC-1, MKP-4, MKP-5, MKP-6, MKP-7, MKP-M, MKP-X, B23, M3/6, VHR and VHX.

The search for proteins encoded by immediate early genes in periphery blood T cells, which are expressed in response to the mitogenic agents, phytohemagglutinin and phorbol 12-myristate 13-acetate (PMA), led to the identification and cloning of PAC-1. PAC-1 is a 314 amino acid, nuclear, dual specificity phosphatase that is expressed predominantly in T-cells and other hematopoietic cells (Rohan *et al.*, 1993). PAC-1 is specific for the inactivation of ERK1/2 and p38 MAPK with very little activity towards JNK (Ward *et al.*, 1994).

MKP-4 was cloned from a human placental cDNA library. MKP-4 encodes a protein of 384 amino acids, which is predominantly able to inactivate ERK with lower activity towards JNK and p38 MAPK at relatively high doses. Interestingly, MKP-4 shows very limited tissue distribution being detectable in only placenta, kidney and foetal liver. Although predominantly cytosolic in several cell types, MKP-4 also shows punctate staining within the nucleus and co-localises with PML to nuclear bodies. The gene locus of MKP-4 was found to be Xq28 on the long arm of Chromosome X. Unusually for an MKP, this region of chromosome X does not appear to be involved in any cell growth diseases (Muda *et al.*, 1997). To date little further work has been done to further characterise MKP-4.

M3/6 was isolated from a human foetal brain cDNA library, after first being partially identified during a search for novel DSPs from a human genomic library. The M3/6 gene encodes a protein of 625 amino acids. M3/6 has a longer C-terminal tail than the other MKPs and within this sequence there is a putative PEST sequence. PEST sequences target proteins for rapid degradation reducing their active half-life within the cell (Martell *et al.*, 1995). M3/6 is abundantly expressed in the brain and also the heart, skeletal muscle and lung. The genetic locus of M3/6 was isolated to three different locations on chromosomes 10 and 11 suggesting multiple copies within the human genome. M3/6 is able to inactivate JNK but not ERK or p38 MAPK and is found in both the nucleus and the cytoplasm (Theodosiou *et al.*, 1996). Similar to MKP-1, M3/6 is phosphorylated in stimulated cells, yet unlike MKP-1, this phosphorylation does not stabilise the protein or increase its half-life (Johnson *et al.*, 2000). M3/6 is thermolabile and becomes insoluble upon heat shock but has also been found to interact with certain heat shock proteins, suggesting a role in JNK downregulation during protein damaging stress. (Palacios *et al.*, 2001).

B23 was cloned from a human mammary epithelial cDNA library using the PTPase catalytic domain as probe. B23 is 397 amino acid long and has highest homology with the nuclear MKPs, MKP-1 and PAC-1 and accordingly has a putative NLS. The expression of B23 is highest in brain and pancreas with detectable levels in many other tissues including heart, lung, liver, skeletal muscle, kidney and placenta. B23 is able to efficiently dephosphorylate ERK but as there has been no comparison with other MAP kinases, its specificity can not be confirmed. Rapid induction with both serum and stress factors like heat shock indicates that B23 is probably an immediate early gene (Ishibashi *et al.*, 1994).

MKP-M was cloned from a mouse macrophage cDNA library and encodes a protein of 677 amino acids. MKP-M shares high homology with M3/6 and has the same long C-Terminal tail containing a PEST sequence. Three other splice variant forms of MKP-M mRNA have been discovered in macrophages but preliminary data suggests that only one of them is stable enough to encode a protein and that this protein is unlikely to be an active phosphatase. MKP-M is specific for JNK over

ERK or p38 and is localised in the cytosol. Similar to M3/6, MKP-M contains a motif similar to the delta domain, which is involved in JNK binding. Also similar to MKP-1, MKP-M expression is not induced by the kinase it is best able to dephosphorylate perhaps indicating a potential role in cross cascade control. Although able to inactivate only JNK with any great effect, MKP-M expression appears to be dependent on p38 MAPK activity, this may explain why MKP-M is strongly induced by LPS but not cytokines (Matsuguchi *et al.*, 2001). MKP-M appears to play a very similar role to M3/6, perhaps fulfilling the same function but in immune cells rather than the brain.

MKP-X was isolated at the same time as MKP-3 by two separate groups due to its high homology to MKP-3 (Groom *et al.*, 1996; Muda *et al.*, 1996). No further study has been carried out on this phosphatase other than the identification of its chromosomal localisation as 3p21 (Smith *et al.*, 1997).

MKP-7 was cloned from a human foetal brain cDNA library using probes from the MKP-5 sequence and rapid amplification of cDNA ends (RACE) PCR. MKP-7 is 665 amino acids long and bears greatest resemblance to M3/6 and MKP-M with the characteristic extended C-terminus. MKP-7 is able to co-precipitate JNK and p38 MAPK but not ERK, and an inactive mutant of MKP-7 caused an increase in magnitude and length of the JNK signal suggesting MKP-7 is specific for JNK *in vivo*. To further analyse the specificity, MKP-7 binding was tested against the three varying isoforms of JNK: JNK1, JNK2 and JNK3. MKP-7 is able to co-precipitate JNK2 and JNK3 but not JNK1. Also similar to both MKP-1 and MKP-5, MKP-7 is able to bind and inactivate p38α and p38β but not p38γ or p38δ MAPK (Tanoue *et al.*, 2001).

Analysis of the sequence revealed the presence of both a kinase interacting motif (KIM) and a delta like domain, along with both NLS and NES sequences. When the subcellular locale of MKP-7 was assessed it was found to be cytosolic but the addition of the nuclear export inhibitor LMB sequestered MKP-7 in the nucleus. This indicates MKP-7 must be travelling into the nucleus, probably via its NLS, but being immediately transported out by the NES (Masuda *et al.*, 2001). This may allow

MKP-7 to act as a chaperone, 'grabbing' active MAP kinases from the nucleus, deactivating and transporting them back to the cytosol before releasing them.

MKP-5 was isolated using yeast two hybrid analysis of a human liver cDNA library with inactive p38 MAPK as bait. The MKP-5 cDNA encodes a protein 482 amino acids in length with all the hallmarks of an MKP. As well as the expected catalytic domain and N-terminal binding region, MKP-5 also contains 150 amino acids on the N terminus that matches no other MKP or indeed has any homology to any known protein or distinct structural motif. The expression of MKP-5 is detectable in heart, lung, skeletal muscle and kidney with significantly lower amounts in brain spleen and testis. The MKP-5 gene localises to human chromosome 1q32, which is deleted in some renal collecting duct carcinomas and certain breast tumours.

MKP-5 is able to inactivate p38 MAPK and JNK to a much greater extent than ERK and this specificity is paralleled in the ability of MKP-5 to co-precipitate with both p38 MAPK and JNK but not ERK. Unlike the other MKPs, which show strong catalytic activation upon substrate binding, MKP-5 increases less than 2-fold in the presence of JNK and p38 MAPK. MKP-5 mRNA is induced by certain stress stimuli and there is some evidence of this requiring p38 MAPK activation and not JNK. This may again hint towards a negative feed back role for the MKPs. (Tanoue *et al.*, 1999; Theodosiou *et al.*, 1999).

MKP-6 is the first MKP to be shown to interact with anything other that the MAP kinases. MKP-6 was cloned from a yeast two-hybrid study using the cytoplasmic tail of the CD28 receptor as bait. MKP-6 is able to bind to the cytoplasmic tail of the CD28 receptor both *in vitro* and *in vivo*. MKP-6 is able to inactivate ERK, JNK and p38 MAPK without preference. Signalling from the CD28 receptor to the nucleus has been shown to involve the MAP kinase cascades, to test for MKP-6 involvement a dominant negative MKP-6 mutant was created. This mutant is able to enhance both ERK and JNK activation in response to CD28 activation suggesting a specific role for MKP-6 in inactivating ERK and JNK *in vivo* (Marti *et al.*, 2001). The reason for MKP-6 to CD28 interaction remains unclear but

the receptor may act as an anchor keeping MKP-6 in close proximity to possible sites of MAPK activation.

VHR was the first MKP cloned. It was identified as the first mammalian homologue of the vaccinia virus DSP, VH-1 (Ishibashi *et al.*, 1992) and thus was named VHR for VH-1 related phosphatase. VHR is specific for both ERK and JNK depending on the cell type but has no affinity for p38 MAPK (Alonso *et al.*, 2001; Todd *et al.*, 1999). Unlike the rest of the MKP family VHR lacks the N-terminal non-catalytic region responsible for substrate binding and is thus a lot smaller than most of the MKPs at only 185 amino acids (Ishibashi *et al.*, 1992). Interestingly, VHR is very similar to the latest MKP to be cloned, VHX. The VH nomenclature was retained due to the similarity of VHX to both VH1 and VHR. VHX, VHR and VH1 are smaller than most MKPs and are missing most of the standard N-terminal binding region. Despite this, VHX is still able to efficiently inactivate ERK although no comparison with JNK or p38 has been made (Alonso *et al.*, 2002). This discovery of a second MKP with very similar size and structure to VHR suggests the existence of yet another subfamily of MKPs.

VHR is located solely in the nucleus and constitutively expressed suggesting that possibly this new VH-1 like subfamily may play a role in restriction of ERK, and JNK basal activity. Perhaps preventing excessive MAP kinase activity in the time between MAP kinase activation and the expression of more specific DSPs from the immediate early genes.

MAP kinase phosphatases do not account for all of the dual specificity phosphatases expressed in the eukaryotic cell. Many more are being discovered with roles other than the inactivation of the MAP kinases. A family of DSPs has been recently found that are smaller than the MKPs. They have been termed the low molecular weight DSPs or LMW-DSPs. To date 5 LMW-DSPs have been cloned and all but one are solely expressed in the testis. They are all lacking the N-terminal regions of the MKPs, similar to the VH like MKPs. The LMW-DSPs do not have any affinity for any of the MAP kinases (Aoki *et al.*, 2001). A substrate or function for this new family of DSPs is yet to be identified.

1.5.5 The role of MKP in Cancer

MKPs are negative regulators of the MAP kinase cascades and as such are assumed to be involved in carcinogenesis by regulating cell proliferation and apoptosis. With this in mind, many of the MKPs cloned to date have had their gene locus analysed and assessed for involvement in a disease state:

The gene locus of the human orthologue of MKP-1 maps to human chromosome 5q34 which is an area of chromosome 5 often associated with malignancies. Deletions of this area have been found in leukaemias and translocations have been associated with lymphoma (Emslie *et al.*, 1994).

The MKP-7 gene is localised to markers in the 12p12 region of the human genome. Although there is no direct evidence linking MKP-7 with any disease state this area of the genome is known to be damaged or deleted in acute lymphoblastic leukaemia, acute and chronic myeloid leukaemia and myelodysplastic syndrome, a disease which often leads to leukaemia. Interestingly, MKP-7 gene expression has been detected in the bone marrow (Masuda *et al.*, 2001), indicating a possible involvement of MKP-7 in disease states of this tissue.

The MKP-5 gene was found at human chromosome 1q32. This area of the genome has been found to be deleted in renal collecting duct carcinomas and in some breast tumours. Although again there is no direct evidence of MKP-5 involvement in these, malignancies expression has been detected in the kidney. (Theodosiou *et al.*, 1999).

The functional copy of the gene encoding human M3/6 was found on human chromosome 11p15.5, another copy discovered at 10q11 was found to be an intron-less pseudo-gene. The loss of the region of chromosome 11 to which M3/6 maps has been associated with a number of tumours, including non-small-cell lung cancer, breast cancer, rhabdomyosarcoma, Wilm's tumour, bladder and testicular cancer (Nesbit *et al.*, 1997).

The short arm of human chromosome 3p and more specifically the band 3p21 which encodes MKP-X are frequently deleted in a number of differing tumour types, including lung cancer, gastric cancer, cervical cancer and renal cell carcinoma (Smith *et al.*, 1997).

Similarly the chromosomal locations of MKP-3 (12q22-q23) and MKP-2 (8p11-p12) are thought to encode genes involved in pancreatic and prostate cancers respectively (Furukawa *et al.*, 1998; Smith *et al.*, 1997).

Data has also been published indicating that MKP-1 may be involved in tumour development by overexpression as well as damage to the gene locus. MKP-1 levels are elevated in the pre-invasive stages of prostate cancer and may be involved in the transformation process. In more advanced prostate tumours, the only cells to survive apoptosis induction by androgen ablation are the sub-populations of cells that are overexpressing MKP-1, suggesting MKP-1 inhibits apoptosis in human prostate tumours, possibly through inactivation of the JNK pathway (Magi-Galluzzi *et al.*, 1997).

The literature described above suggests that it is likely MKPs function as tumour suppressors and that, at least some, if not all members of the MKP family are likely to be proto-oncogenes. As research progresses, it is likely that aberrant cell growth and malfunction of MKP expression or activity will be found to be strongly associated. This obviously indicates a group of proteins that could either be used themselves as therapeutics or targeted for therapeutic intervention. Research into the structure and function of the MKPs and their interactions with the MAP kinases will, no doubt, be highly intensive in the coming years.

1.5.6 Control of MKP specificity and interaction with MAPK.

As described above each member of the MKP family has some degree of substrate specificity. This ranges from MKP-1 that can inactivate all three MAP kinase families and yet is able to distinguish between certain JNK and p38 isoforms to MKP-3, which is highly specific for ERK and cannot inactivate p38 or JNK even at concentrations far exceeding normal physiological levels. The interaction between MKP and MAPK is obviously of great interest in elucidating the mechanism of specificity. Both MKP-1 and MKP-3 have been shown to bind to their MAP kinases regardless of phosphorylation state of the substrate. Wild type MKP-3 is able to co-precipitate its substrate ERK, whereas even though ERK is also a substrate of MKP-1 only the catalytically inactive form of MKP-1 is able to co-precipitate ERK

(Groom *et al.*, 1996; Sun *et al.*, 1993). This may suggest MKP-3 forms a long-term complex with ERK but that MKP-1 is free before and after dephosphorylation takes place.

In all MKPs the catalytic domain is in the C-terminal half of the protein. This area surrounding the DSP consensus sequence is highly homologous among all members of the MKP family. The N-terminal region of the protein however has a high level of divergence both in protein sequence and in length but does however contain two areas with homology to the CH2 domains of the phosphatase Cdc25 (Keyse, 1995). It is this divergent N-terminal region and the CH2 domains of the MKPs that is thought to control substrate binding and specificity. To test this, Muda *et al* analysed the interaction of ERK with MKP-3. As expected full length MKP-3 can bind and precipitate ERK1 and ERK2, but not p38 MAPK or JNK. They then created a chimera of the catalytic area of M3/6, a JNK specific MKP, with the N terminal region of MKP-3. The MKP-3-M3/6 chimera can also precipitate only ERK1 and not JNK or p38 MAPK similar to wild type MKP-3. This experimental evidence supported the hypothesis that MKP substrate binding and specificity is controlled by the N-terminal region of the proteins (Muda *et al.*, 1998).

Interestingly, a following study discovered that the phosphatase activity of MKP-3 can be increased 30 fold in the presence of ERK. There is no increase in activity in the presence of JNK or p38 MAPK suggesting that substrate binding is required for enhanced activity. The same study also used several MKP-3 and ERK mutants to discover that the enhanced phosphatase activity requires the N-terminal section of MKP-3 and is not dependent on ERK activity (Camps *et al.*, 1998). This led to several groups looking at catalytic activation of differing members of the MKP family by their substrate MAP kinases. MKP-X activation mimicked its substrate specificity with large activation by ERK, slight activation by JNK and no detectable effect by p38 MAPK (Dowd *et al.*, 1998). p38α MAPK but not ERK2 activated MKP-7. MKP-1 and MKP-4 are activated by binding of p38 (Camps *et al.*, 1998; Hutter *et al.*, 2000) and MKP-2 is activated preferentially by ERK and JNK (Chen *et al.*, 2001).

The mechanism of activation of MKPs by MAPK was studied using MKP-3 and ERK. It was discovered that in the absence of ERK, the asparagine residue that acts as the general acid to donate a proton to the leaving group during the hydrolysis reaction is positioned away from the phosphatase active site. Upon ERK binding, a conformational change moves the asparagine into the active site thus increasing activity of the phosphatase (Farooq *et al.*, 2001; Zhou & Zhang, 1999). Although the MAP kinases can exist as dimers the maximal activation of MKP-3 is achieved when the ratio of ERK to MKP-3 is 1:1 suggesting a 1:1 binding stoichiometry. Several areas of sequence within MKP-3 were also identified that are required for ERK/MKP-3 binding. The first is a KIM sequence that is present on every ERK interacting protein. The second is a 17 amino acid stretch that is present on all cytosolic MKPs. Although mutation of these sequences affected the ability of MKP-3 to bind ERK they did not affect the ability of ERK to activate MKP-3. A separate sequence in the C terminal catalytic region of MKP-3 was found to be critical in MKP-3 activation (Zhou *et al.*, 2001). Also mutation of a conserved aspartic acid (so called *sevenmaker* mutation) residue in both p38 an ERK, leads to a reduction in the ability of the kinase to bind to the phosphatases and consequently reduces the activation of MKP-3 and MKP-1 by mutant ERK and p38 MAPK respectively (Camps *et al.*, 1998; Chu *et al.*, 1996; Hutter *et al.*, 2000).

Along with specific transcriptional induction, subcellular localisation and possible phosphorylation, binding and catalytic activation of the MKP family by the MAP kinases is likely to be a major factor in controlling specificity of the MKPs.

1.6 Cellular Control of Nuclear or Cytoplasmic Localisation

The discovery of the cell nucleus in the 17th century by Leeuwenhoek started a area of study that has shaped all forms of modern biochemistry. Possession of a nucleus also has many implications for the cell as it effectively separates the genetic information, the DNA, from the protein synthesis machinery in the cytosol. This separation is achieved by a double lipid membrane known as the nuclear envelope. As gene transcription into RNA and translation into protein occur in two different compartments, specific transport events must occur. mRNA must move to the cytoplasm to be translated into protein and proteins required for gene transcription,

DNA replication and a host of other nuclear processes must travel from the cytoplasm into the nucleus.

In terms of signal transduction, any signal that a cell receives whether it is a stress, growth factor or hormonal signal must be transduced to the nucleus and cross the nuclear membrane. Passing of a signal over the nuclear membrane usually involves a protein translocation event.

All active and passive movement of molecules between the nucleus and the cytosol occurs through the nuclear pore complex (NPC). The NPC is a large protein complex containing at least 50-100 distinct proteins with a combined mass of over 100 MDa. The NPC as the name suggests has a pore like structure allowing ions and molecules smaller than 40-45 KDa to diffuse freely in and out of the nucleus. Molecules bigger than 45 KDa must be targeted to the nucleus or the cytosol. For molecules of this size transport is active rather than passive and is both ATP and temperature-dependent allowing for transport against the concentration gradient. (Doye & Hurt, 1997; Izaurralde *et al.*, 1999).

Molecules are targeted for transport into the nucleus by the presence of a nuclear localisation sequence (NLS). This NLS sequence is recognised by members of the importin family which then act as carriers to transport the substrate protein through the NPC, for review see (Gorlich, 1998). Briefly, importin-α recognises and binds the NLS bearing substrate. Importin-β then binds the importin-α/substrate complex and mediates its transport through the NPC. The exact details of this translocation process are still not fully understood. Once on the nuclear side of the membrane, Ran-GTP, a small GTPase, binds to importin-β causing dissociation of the complex and release of the NLS bearing substrate into the nucleoplasm. Importin-α and -β are then recycled back to the cytoplasm to allow further rounds of nuclear import.

The first NLS to be characterised was the nuclear targeting sequence of SV40 large T antigen, PKKKRKV (Kalderon *et al.*, 1984). This sequence is still used as a guide when searching protein sequence for NLS although identifying an NLS from

sequence alone can be difficult due to the lack of a definite consensus sequence. With recent advances in protein modelling leading to secondary and tertiary structure determination from the primary protein sequence, putative NLS identification has become somewhat easier. This is due to the absolute requirement of the NLS to be exposed on the cell surface to enable its interaction with the importin family of proteins. So any NLS-like sequences that are entirely shielded in the interior of the protein can potentially be discounted.

It has been proposed that NLS sequences can be split into two groups. The first exemplified by the SV40 T antigen NLS are the monopartite or one-cluster NLS. These NLS are generally less than 12 amino acids long and contain a high proportion of basic amino acids. The second are the bipartite where two small basic motifs are separated by a spacer of roughly 10 amino acids. The prototypic bipartite NLS was discovered on nucleoplasmin (Robbins *et al.*, 1991). In bipartite NLS both regions are required for proper function of the NLS for review (Dingwall & Laskey, 1991; Jans & Hubner, 1996).

Although these categorisations can suggest the presence of an NLS sequence within a protein, experimental verification is required. A number of NLS, that appear to exactly match what loose consensus there is, have proven to be non-functional. Whereas sequences with no homology to the T-antigen or nucleoplasmin style NLS have proven to be powerful directors of nuclear localisation. Verification of NLS activity usually takes on one or more of three forms of experiment. Firstly, deletion experiments where sections of protein sequence are removed and localisation analysed. Secondly, the reverse experiment where the sequence in question is added to a cytosolic protein, usually β-Galactosidase, and its ability to direct the protein to the nucleus analysed. The last method of NLS analysis is point mutation where one or two amino acids are changed to non-basic residues, usually alanine, and localisation assessed. This last method assumes that the NLS is one of the more common basic mono or bipartite NLS and not a neutrally charged NLS (Makkerh *et al.*, 1996).

An experiment to analyse the C-terminal region of the C/EBP transcription factor family for NLS activity, by Williams *et al*, used all three of the above methods to identify an NLS (Williams *et al.*, 1997). Deletion mutants allowed the

identification of a 21aa region required for nuclear localisation. Addition of this sequence to β-Galactosidase did not shift the protein fully into the nucleus suggesting other sequences must play role in the NLS. Addition of larger and larger areas of C/EBP sequence to the β-Galactosidase indicated the requirement for 4 non-basic, residues outside of the predicted bipartite NLS sequence, for nuclear localisation. The importance of these amino acids however was shown to be minimal when point mutations were used to analyse the effect of changing the basic residues at each end of the bipartite NLS to alanine. This study is a good example of how all three methods can be used to analyse putative NLS and also indicates the limits of the second method, as there is a risk of artefactual results due to the presence of the β-Galactosidase sequence.

Certain small areas or single amino acids within the NLS sequences have often been found to be absolutely required for NLS function. Substitution of these often basic amino acids for a neutral counterpart leads to complete loss of function of the NLS and cytosolic localisation of the protein in question. Notably in Thogoto virus and Influenza-A virus mutation of two arginines and a lysine to alanine resulted in cytosolic localisation of the nucleoprotein (Weber *et al.*, 1998).

For the cell to function the genetic information must be passed from the nucleus to the protein translating machinery in the cytosol. This requires the movement of very large RNA molecules across the NPC to the cytosol. In a manner analogous to the NLS system, RNA and many other molecules targeted to the cytosol either contain a NES or are chaperoned by proteins that do. These NES are short motifs rich in leucine/isoleucine or other hydrophobic residues. The prototypical NES was discovered in the protein kinase-A inhibitor PKI, and took the form LxLxLxxL where 'x' can be any amino acid. Similar to NLS, NES are variable in that other hydrophobic residues can replace the leucines and spacing between the critical residues can vary. This variance along with the abundance of leucine and other hydrophobic residues in standard protein sequences makes the identification NES from sequence data alone very difficult.

NES function is very similar to NLS in that it is an active transport mechanism that requires interaction with several proteins, reviewed in Macara, 2001. Briefly the

NES binds to a member of the exportin family prior to transport. On their own the NES and exportins have low affinity for each other, but binding of Ran-GTP stabilises the complex allowing for export through the NPC. Once in the cytosol, hydrolysis of the Ran-GTP destabilises the complex releasing the NES bearing cargo.

One would have thought, that in the interests of efficiency that importins and exportins would overlap their functions allowing for an extra round of transport for the same energy cost to the cell, but research has shown their functions to be mutually exclusive. One of the major exportins able to bind the classic leucine rich NES identified to date is Crm1. An inhibitor of NES to Crm1 binding called leptomycin-B has been identified and has subsequently allowed the conformation of the existence of a functional NES in many proteins.

1.7 Aims

The characterisation of the mechanisms regulating MAP kinase signalling as a consequence of various stimulatory and stress conditions is an important issue from both biological and clinical perspectives. From a biological view, this will enhance our understanding of how the cell controls a decision making process that allows the choice between cell growth, death and differentiation to be made based on the cellular environment. From a clinical view, understanding the MAP kinase model will allow the understanding of many disease states not least of which is cancer, and also hopefully understanding the control of the MAP kinases may indicate cellular targets for development of novel pharmaceuticals. The review of the literature indicates that a balance between the activity of the upstream kinases and the activity of the phosphatases able to inactivate the MAP kinases controls the activity of the MAP kinases. The emerging family of MAP kinase specific phosphatases, the MKPs, obviously plays an important role in the inactivation of the MAP kinases. As has been described, the MAP kinases can be found in different cellular compartments and are able to phosphorylate many differing targets in both the cytosol and the nucleus, Appropriately, the MKPs are present in both the nuclear and cytosolic compartments. With a couple of exceptions the MKPs tend to be restricted to either the cytosol or the nucleus and with 12 MKPs, each with differing substrate specificity, all aspects of MAP kinase control are likely covered. To date, the effect of the MKPs on MAP kinase signal transduction has been characterised in a number of cell types and under various conditions. Perhaps the most common theme to be apparent in the literature is that of a feedback mechanism where kinase activation leads to increased MKP expression and greater half-life within the cell. Although the general function of MKPs within the cell is reasonably well understood the exact mechanism that controls their subcellular distribution has yet to be fully analysed.

The aim of this thesis was to clone human MKP-2, confirm its substrate specificity and analyse the mechanism of localisation control. This will hopefully allow the generation of a cytosolic version of MKP-2, and both nuclear and cytosolic dominant negative MKP-2s. These mutants could be used as a tool in the study of nuclear and cytosolic activity of the JNK and ERK MAP kinases. From such studies

the respective roles of the nuclear and cytosolic activity of these kinases could be elucidated.

These investigations have been carried out predominantly in the COS7 and HEK293 cell lines, due to their ease of transfection and ease of immunostaining respectively.

Chapter 2

Materials and Methods

2.1 General Reagents

All materials used were of the highest commercial grade available and were obtained from Sigma Chemical Company, Poole, Dorset, UK unless otherwise stated.

Amersham International Plc, Aylesbury Buckinghamshire, UK.

ECL Detection reagents.

Biorad Laboratories. Hertfordshire, U.K.

SDS-PAGE molecular weight markers.

Boehringer Mannheim. East Sussex, U.K.

BSA (Albumin, Fraction V).

Clontech Laboratories Ltd, Basingstoke, UK.

Advantage® Ultrapure dNTP mix.

Costar, Buckinghamshire, UK.

Nitrocellulose membranes, Tissue culture plasticware.

Gibco Life Technologies Ltd, Paisley, Scotland.

Growth media, antibiotics and sera, LipofectAMINE™, PLUS Reagent.

NEN Dupont, Hertfordshire , U.K.

[γ-^{32}P]-ATP (3000Ci/mmol)

Qiagen Ltd, Dorking, Surrey, UK.

Endofree Plasmid-MAXI kit.

Whatmann, Kent, UK.

3MM paper.

2.2 Antibodies

Amersham International Plc, Aylesbury, Buckinghamshire, UK.

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG.

Santa Cruz Biotechnology Inc. CA, USA.

Mouse monoclonal anti-c-Myc (9E10) (cat no. sc-40), rabbit polyclonal anti-HA (cat no. sc805), rabbit polyclonal anti-MKP-2 (cat no. sc728

Sigma Chemical Company, Poole, Dorset, UK

Fluorescein Isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, mouse monoclonal anti FLAG.

2.3 Plasmids

GST tagged truncated N-terminus of c-Jun5-89 was a kind gift of J.R. Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada).

MYC tagged WT-ERK2 was gifted by Melanie Cobb (The University of Texas).

The haemeaglutinin tagged JNK1 and p38 constructs were a kind gift of J.L. Blank (University of Leicester, School of Medicine).

The human umbilical vein endothelial cell line cDNA library was kindly supplied by J. Allen (IBLS University of Glasgow).

2.4 Cell Culture

2.4.1 COS-7 (Monkey Kidney Transformed)

All cell culture was performed in a class 2-flow hood. Cos-7 cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) (v/v), 27mg/ml Glutamine and penicillin/streptomycin (250units/ml and 25mg/ml respectively) at 37°C in humidified air with 5% CO2. Cells were sub-cultured using 0.25% trypsin (w/v) in PBS (137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 anhydrous, 0.9mM CaCl, 0.5mM MgCl, pH7.4). Cells were rendered quiescent by replacing growth media with media lacking serum for 24 hours prior to stimulation. Cultures were maintained to a maximum passage number of 35.

2.4.2 HEK293 (Human Epithelial Kidney)

HEK293 cells were maintained in α-Modified Eagles Medium (αMEM) supplemented with 10% Foetal Calf Serum (FCS) (v/v) and penicillin/streptomycin (250units/ml and 25mg/ml respectively) at 37°C in humidified air with 5% CO2. Cells were sub-cultured using 0.25% Trypsin (w/v) in PBS. Cells were rendered quiescent by replacing growth media with media lacking serum for 24 hours prior to stimulation.

2.4.3 Transfection of COS7 cells using DEAE Dextran.

COS7 cells were seeded into 100mm dishes to give 50-60% confluency the following day. 5-20μg of DNA for transfection was diluted into 500μl of sterile PBS. 100μl of sterile 10mg/ml DEAE dextran was then added. The growth medium was then replaced by 5mls of transfection medium (Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% Nu Serum (v/v) and 100μM chloroquine.

The DNA/DEAE solution was then dropped evenly over the plate. Plates were incubated at 37°C in humidified air with 5% CO_2 for 3 hours. After 3 hours cells should contain inclusion bodies (bright spots under phase contrast microscopy). Cells were then shocked in 10% dimethyl sulphoxide (DMSO) (v/v) in PBS for 1 minute, cells were then washed twice in PBS before replacing the normal growth medium. Cells were assayed 48 hours later. Transfection efficiency was assessed as described in section 2.4.5 and found to be roughly 1-2% of total cell number. As the concentration of DNA can affect transfection efficiency all reactions were normalised to a standard DNA concentration using the appropriate empty vector.

2.4.4 Transfection of HEK293 cells

HEK cells for transfection were grown to 70 – 80% confluency in a six well plate and transfected using the Lipofectamine and Lipofectamine-Plus Reagents (Gibco Life Technologies). 2μg of DNA for transfection was mixed with 7μl of Plus Reagent in 100μl of serum free, antibiotic free growth medium and left for 15 min to complex. During the incubation 4μl of Lipofectamine was mixed with 100μl serum free, antibiotic free growth medium. The two solutions were then mixed and incubated at room temperature for 15 min. The growth medium of cells was replaced

with 0.8ml of transfection medium containing 10% FCS (v/v) and 27mg/ml Glutamine (no antibiotics). The transfection cocktail (200µl) was dropped evenly over the cells, and cells were incubated at 37°C for 4.5 hours. After this time the transfection medium was replaced by ordinary growth medium. Cells were assayed 48 hours later. Transfection efficiency was assessed as described in section 2.4.5 and found to be roughly 3-5% of total cell number. As the concentration of DNA can affect transfection efficiency all reactions were normalised to 2µg of DNA using the appropriate empty vector.

2.4.5 Assessment of transfection efficiency

Cells were transfected with appropriate amount of β-Galactosidase as described in sections 2.4.3 and 2.4.4. Cells were washed twice in PBS and fixed for 20 minutes in 0.2% (v/v) gluteraldehyde in PBS at room temperature. The fixative was removed by washing twice in PBS. Cells were then covered in 2ml X-Gal stain (1 mg/ml X-Gal in 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, 1 M $MgCl_2$ in PBS) and incubated at 37°C for 4-16 hours. Cells containing β-Galactosidase stain dark blue and were counted by microscopy.

2.5 Detection and analysis of proteins

2.5.1 Preparation of samples for SDS-PAGE and immunoblotting

Cells were rendered quiescent and stimulated for the required time before being rinsed twice in ice cold PBS and lysed by the addition of hot (~85°C) Laemmli sodium dodecyl sulphate (SDS) sample buffer (63mM Tris HCl pH 6.8, 2mM $Na_4P_2O_7$, 5mM EDTA. 10% Glycerol (v/v), 2% SDS (w/v) and 0.007% (w/v) bromophenol blue to which was added 50 mM dithiothreitol (DTT) immediately before use). Cells were scraped from wells on ice and lysates drawn repeatedly through a 16-gauge needle attached to a 1ml syringe to shear chromosomal DNA. Samples were transferred to a microfuge tube, the lid pierced and boiled for 5

minutes to denature proteins and stored at -20°C until required for SDS-polyacrylamide gel electrophoresis.

2.5.2 Preparation of nuclear extracts

Cells were rendered quiescent and stimulated for the required time before being washed twice in ice cold PBS. Cells were scraped from wells on ice into 500µl of ice cold PBS. Cells were recovered by centrifugation for 5 min at 13000 X g. Supernatant was discarded before cells were resuspended in 400µl of low salt buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, 0.5mg/ml leupeptin, 0.5mg/ml aprotinin pH7.9) and incubation on ice for 15 minutes. 25µl of 10 % NP-40 (w/v) was then added followed by brief vortex. Cytosolic extracts were collected after centrifugation. The nuclear pellet was then extracted in 50µl of high salt buffer (20mM HEPES, 25% glycerol (v/v), 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF, 0.5mg/ml leupeptin, 0.5mg/ml aprotinin pH7.9) at 4°C with shaking for 15 minutes. The final extract was obtained by disruption of nuclei on ice in a bath sonicator for 2 X 30 seconds followed by centrifugation at 13000 X g for 15 minutes at 4°C. Supernatants were removed to sterile eppendorfs and 5µl removed for determination of protein content as described in section 2.5.13. Samples were stored at -80°C until use.

2.5.3 SDS-PAGE

Resolving gels were prepared containing the appropriate amount of acrylamide:N,N'-methylene-bis-acrylamide (37.5:1), 0.375M Tris pH 8.8 and 0.1% (w/v) SDS. The gel solution was prepared and polymerisation was initiated by the addition of 0.05% (w/v) ammonium persulfate (APS) and 0.05% N, N, N, N', N'-tetramethylethylenediamine (TEMED) (v/v). Solution was mixed and poured into the assembled glass plates, leaving sufficient space at the top for the stacking gel. Gels were overlaid with 100 µl 0.1%SDS (w/v) and allowed to set for 45 minutes.

Once set the 0.1% SDS was poured off and the gel surface rinsed with water to remove excess polyacrylamide. The stacking polyacrylamide gels were made up as follows: 5% acrylamide (w/v), 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.05%

(w/v) ammonium persulphate and 0.1% (v/v) TEMED. The stacker was added directly to the surface of the resolving gel and an appropriate teflon spacer comb inserted into the stacker. The stacker was allowed to polymerise for 10 minutes before the comb was removed and the gel mounted into a Hoeffer Series SE600™ or a BioRad Mini-Protean II™ electrophoresis tank. Both the inner and outer reservoirs were filled with electrophoresis running buffer (24.8mM Tris, 191.8mM glycine, 0.1% (w/v) SDS). Standardised protein samples (2.5.1) were loaded into the wells of the stacker gel and run concurrently with a pre-stained SDS protein marker of known molecular weight. An electrical current of 115V was passed between the electrodes from a Hoeffer P250/2.5 amp DC power supply until the loading dye bands had passed out of the resolving gel.

2.5.4 Fixing and drying cells

Gels containing [^{32}P] labelled proteins were placed in fixer (20%(v/v) methanol and 10%(v/v) acetic acid) for at least 30 minutes. The gels were then placed between cellophane sheets in a drying frame and dried for 60 minutes at 80°C in a Hoeffer Eazy-Breeze™ gel dryer. The dry gel was then exposed to Kodak X-OMAT LS x-ray film for 4-24 hours at -20°C in a metal exposure cassette and developed using a KODAK M35-M X-OMAT processor.

2.5.5 Western blotting

Resolved proteins on gels were transferred to nitrocellulose filters by electrophoresis blotting (Towbin *et al.*, 1992). The gel was placed into a transfer cassette against a nitrocellulose sheet, between Whatmann 3MM paper and two porous foam sheets. The cassettes were then placed into a cooled Hoeffer TE Series Transfor™ or BioRad Mini Trans-Blot™ electrophoresis tank containing electrophoresis blotting buffer (25mM Tris, 192mM Glycine and 20%(v/v) methanol). The presence of SDS in the resolving gel would confer a negative charge on the proteins so the cassette was orientated with the nitrocellulose towards the anode.

2.5.6 Immunological detection of proteins

Nitrocellulose membranes from Western Blotting were washed in NaTT buffer (150mM NaCl, 20mM Tris, 0.2%(v/v) polyoxyethylene-sorbitan monolaurate (Tween 20) (pH7.4). The areas of Nitrocellulose unbound to protein were then blocked using 3% Bovine Serum Albumin (BSA) (w/v) in NaTT incubated at room temperature for 3 hours on a platform shaker. The 3% BSA was then replaced by 0.2% BSA (w/v) in NaTT, to this the primary antibody was added and incubated overnight at room temperature on a platform shaker. The nitrocellulose was then washed every 15 minutes for 1.5 hours in NaTT to remove all excess antibody. The secondary antibody (horseradish peroxidase-conjugate IgG directed against the primary antibody) was added in 0.2% BSA (w/v) in NaTT for 2 hours. Again the membranes were washed in NaTT for 1.5 hours to remove excess antibody. Immunoreactive proteins were then detected using ECL chemiluminescence. The nitrocellulose was washed with ECL for 90 seconds and mounted into an exposure cassette. Blots were exposed to Kodak X-OMAT LS x-ray for 1-5 minutes and developed using a KODAK M35-M X-OMAT processor.

2.5.7 Immunostaining of slides for indirect immunofluorescence analysis

Cells were seeded onto No. 0 glass cover slips and grown to ~80% confluency. Cells were then quiesced in serum free medium for 24 hours before stimulation for the appropriate time. Cells were washed three times in PBS and fixed in -20°C methanol for 20 minutes at room temperature. The methanol was removed by washing three times with PBS before addition of 1% (w/v) BSA in PBS for 30 minutes at room temperature. The coverslips were then placed face-down onto 100µl of primary antibody solution for 1 hour. The antibody was diluted to the required concentration using PBS. The cover slips were again washed in PBS then placed over 100µl of secondary antibody solution as before. The cells were then washed a final time in PBS before being mounted face-down onto slides. Immunostaining could then be observed using a Nikon Eclipse E600 fluorescent microscope.

2.5.8 JNK activity assays

Cells were rendered quiescent and stimulated for the required time before being lysed in solubilisation buffer (20mM HEPES, pH 7.7, 50mM NaCl, 1% Triton X-100 (w/v), 0.1mM EDTA, 0.2mM PMSF 0.5mg/ml Leupeptin, 0.5mg/ml aprotinin). Samples were centrifuged to remove insoluble material and the protein concentration of the supernatant assessed as described in section 2.5.13. Equal amounts of protein were then mixed with 2μg of anti-HA-Antibody pre-coupled to Protein-A Sepharose beads (Sigma) and mixed for at least 3 hours at 4°C. The beads were then pelleted by centrifugation and washed twice in lysis buffer and once in kinase buffer (25mM HEPES, 20mM $MgCl_2$, 5mM β-Glycerophosphate, 0.1mM Na_3VO_4, 2mM DTT, pH7.6). The imunoprecipitates were resuspended in 25μl of kinase buffer and the reaction initiated by the addition of 1μCi [γ^{32}P] ATP, 150μM ATP and 1μg GST-c-Jun_{5-89} to a total volume of 30μl. The reaction was agitated at 30°C for 30 minutes before being terminated by the addition of 10μl of 4X Laemmli sample buffer. The samples were boiled for five minutes and resolved by 11% SDS/PAGE. Gels were fixed and dried and the incorporation of [γ^{32}P] into the substrate detected by autoradiography and quantified through scanning densitometry.

2.5.9 ERK activity assays

Cells were rendered quiescent and stimulated for the required time before being lysed in solubilisation buffer (20mM HEPES, pH 7.6, 50mM NaCl, 1% Triton X-100 (w/v), 0.1mM EDTA, 0.2mM PMSF 0.5mg/ml Leupeptin, 0.5mg/ml aprotinin Samples were centrifuged to remove insoluble material and the protein concentration of the supernatant assessed as described in section 2.5.13. Equal amounts of protein were then mixed with 2μg of MYC-Antibody coupled to Protein-G Sepharose beads and mixed for at least 2 hours at 4°C. The beads were then pelleted by centrifugation and washed twice in lysis buffer and once in kinase buffer (25mM HEPES, 20mM $MgCl_2$, 5mM β-Glycerophosphate, 0.1mM Na_3VO_4, 2mM DTT, pH7.6). The beads were resuspended in 25μl of kinase buffer and the reaction initiated by the addition of 1μCi [γ^{32}P] ATP, 150μM ATP and 1μg/tube myelin basic protein (MBP) to a total

volume of 30µl. The reaction was agitated at 37°C for 15 minutes before being terminated by the addition of 10µl of 4X Laemmli sample buffer. The samples were boiled for five minutes and resolved by 15% SDS/PAGE. Gels were fixed and dried and the incorporation of [γ^{32}P] into the substrate detected by autoradiography and quantified through scanning densitometry.

2.5.10 p38 MAPK kinase assays

Cells were rendered quiescent and stimulated for the required time before being lysed in solubilisation buffer (20mM Tris, 137mM NaCl, 2mM EDTA, 10% Glycerol (v/v), 1% Triton-X-100 (v/v), 0.2mM PMSF 0.5mg/ml Leupeptin, 0.5mg/ml aprotinin). Samples were centrifuged to remove insoluble material and the protein concentration of the supernatant assessed as described in section 2.5.13. Equal amounts of protein were then mixed with 2µg of FLAG-Antibody coupled to Protein-G Sepharose beads and mixed for at least 2 hours at 4°C. The beads were then pelleted by centrifugation and washed twice in lysis buffer and once in kinase buffer (25mM HEPES, 20mM $MgCl_2$, 5mM β-Glycerophosphate, 0.1mM Na_3VO_4, 2mM DTT, pH7.6). The beads were resuspended in 25µl of kinase buffer and the reaction initiated by the addition of 1µCi [γ^{32}P] ATP, 25µM ATP and 2µg GST-ATF-2 to a total volume of 30µl. The reaction was agitated at 30°C for 15 minutes before being terminated by the addition of 10µl of 4X Laemmli sample buffer. The samples were boiled for five minutes and resolved by 11% SDS/PAGE. Gels were fixed and dried and the incorporation of [γ^{32}P] into the substrate detected by autoradiography and quantified through scanning densitometry.

2.5.11 Synthesis of GST-fusion proteins

A single colony of BL21 E. coli containing the appropriate GST-fusion protein vector was used to inoculate 5ml of LB media containing the appropriate antibiotic. The culture was grown overnight at 37°C in an orbital shaker. This culture was then used to inoculate 500ml of LB media containing the appropriate antibiotic which was grown at 37°C in an orbital shaker until an OD_{600} of 1.0 had been reached. Expression of fusion proteins was induced by addition of 100µM isopropyl

β-D-thiogalactopyranoside (IPTG). The culture was then incubated at 30°C for 4 hours in an orbital shaker. Bacteria were harvested by centrifugation (6000 X g for 10 min) and stored -20°C until required for purification.

2.5.12 Purification of GST-fusion proteins

Bacterial cells were resuspended in 30ml lysis buffer (25 mM Tris base pH 8.0, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg aprotinin) before being frozen in dry ice/methanol and thawed slowly in cold water. Further disruption of the bacterial cells was promoted by the use of a probe sonicator. 1.5ml of 20% (v/v) Triton X-100 was then added to give a final concentration of 1%(v/v) and the cells lysed by gentle rotation for 30minutes at 4°C. Following centrifugation (10000 x g for 15 minutes at 4°C) the supernatant was collected and added to a slurry (1:1) of glutathione (GSH)-sepharose beads in lysis buffer, and incubated for 1 hour at 4°C with gentle rotation. Beads were then washed twice in lysis buffer to remove unbound protein.

The fusion protein bound to the beads was eluted using 10mM reduced glutathione and the protein concentration assessed (Section 2.5.13). The result of this assay was used to calculate the volume of eluate to provide sufficient substrate per assay.

Sequential purification of the substrate was confirmed by SDS-PAGE of samples taken at various stages during the purification procedure, followed by staining of the gel with Coomassie Blue to visualise proteins.

2.5.13 Protein concentration assay.

The concentration of proteins in aqueous solution was ascertained using the BioRad protein assay. The BioRad protein assay solution was prepared by diluting the stock 1:5 in ddH_2O. A standard curve of protein concentrations was created using BSA, usually 0, 5, 10, and 20μg. 5μl of the ~200μl total of each unknown sample was used for assay. To each of the standards and the unknown samples 1ml of the prepared BioRad solution was added. These solutions were mixed and then incubated at room temperature for 10min. Solutions were then transferred to a suitable cuvette

and the absorbance at 595nm was obtained. Unknown protein concentrations were calculated using the standard curve.

2.5.14 Scanning densitometry

Western blots and autoradiographs from kinase assays were scanned on a Tiny 1200dpi scanner using photosuite™ software. Captured images were then assessed using Scion Image for Windows software.

2.5.15 Statistical analysis

Statistical tests were carried out using Graph-pad Prism software.

2.6 Molecular Biology

2.6.1 General bacterial culture

JM109 and XL1-Blue strains of E-Coli were grown in broth cultures containing LB media (5g/L NaCl, 10g/L Tryptone and 5g/L Yeast Extract. pH 7). Solid Media was prepared by the addition of 1.5% (w/v) Agar to LB Media prior to sterilisation.

2.6.2 Preparation of plasmid DNA from small scale cultures (MINIPREP).

A single colony of either JM109 or XL1-Blue E. Coli containing the appropriate vector was used to inoculate 5ml of LB. The culture was grown at 37°C overnight before bacteria from 1.5ml of culture were harvested by centrifugation at 13000 X g for 2 min. The supernatant was removed and the pellet resuspended in 100µl of solution I (50mM Glucose, 25mM Tris.Cl pH-8.0, 10mM EDTA pH-8.0). 200µl of solution II (0.2M NaOH and 0.1% SDS(w/v)) was then added to lyse the cells. 300µl of solution III (5M potassium acetate and 11.5% glacial acetic acid (v/v)) was then added to neutralise the solution. Solution III is 3M with respect to potassium and 5M with respect to acetate. The tubes were incubated on ice for 5 minutes to precipitate out the chromosomal DNA and proteins, and this was then removed by centrifugation at 13000 X g for 5 minutes. The supernatant was

transferred to a fresh tube and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to remove any protein contaminants. The samples were again centrifuged at 13000 X g for 2 minutes and the supernatant was transferred to a fresh tube. This process was repeated using an equal volume of chloroform to remove any residual phenol. The volume of sample was ascertained and two volumes of room temperature ethanol used to precipitate the plasmid DNA. The DNA was collected by centrifugation at 13000 X g for 10 minutes and the supernatant removed, the tubes were allowed to air dry on the bench before the DNA was resuspended in an appropriate amount of sterile distilled water.

2.6.3 Restriction enzyme digests

The reactions contained roughly 1μg of plasmid DNA. To this was added 2μl of the appropriate 10X buffer and 2 units of restriction enzyme. The volume was made up to 20μl with sterile distilled water and the reaction incubated for at least 1 hour at 37°C. For complete digestion for cloning purposes the reactions were incubated overnight at 37°C.

2.6.4 PCR reactions

PCR reactions for cloning and site-directed mutagenesis were carried out in a Techne Genius™ PCR machine. All custom primers were obtained from Gibco Life Technologies. The PFU turbo and appropriate buffers (Clontech) were used for all PCR and mutagenesis reactions.

2.6.5 Analysis of DNA by Agarose gel electrophoresis

The size of DNA fragments from digests and PCR reactions was analysed by Agarose gel electrophoresis. An appropriate amount of molecular grade Agarose (Sigma) was melted in 50ml of TBE (45 mM Tris base, 45mM boric acid, 1 mM EDTA pH 8.0) using a microwave. This solution is then poured into a horizontal gel kit with appropriate combs and allowed to set for 30 minutes. Once set the combs are removed and the reservoir filled with TBE. Between 0.5 and 1μg of DNA fragment

were mixed with loading buffer (50% Glycerol (v/v), 100mM EDTA, pH 8.0, 1% SDS (w/v), 0.1% Bromophenol Blue (w/v), 0.1% Xylene Cyanol (w/v)) are then loaded into wells along side a standard ladder of known molecular weight. Gels were run at 8 V/cm for 1-2 hours depending on fragment size. DNA bands were visualised using ethidium bromide and a UV trans-illuminator.

2.6.6 Ligation of cohesive DNA termini

The two fragments of DNA to be annealed were mixed in a 1:1 ratio. To the DNA, 1 weiss unit of T4-DNA-Ligase (Promega) and 1µl of the appropriate X10 buffer is added. The reaction is made up to 10µl with sterile distilled H_2O and incubated overnight at 4°C. 1µl of this reaction was used for transformation into competent E. Coli.

2.6.7 Screening of bacterial colonies.

Several colonies of each clone to be screened were inoculated into 5ml of L-broth media containing the appropriate antibiotic and grown overnight at 37°C. Plasmid DNA was isolated from these cultures as described in section 2.6.2. Purified plasmid was then digested with appropriate restriction enzymes (section 2.6.3) to confirm the presence of the correct clone and the size of DNA fragments obtained was assessed by agarose gel electrophoresis (section 2.6.5).

Chapter 3

Cloning and characterisation of human MAP kinase phosphatase 2 (MKP-2)

3.1 Introduction

The MAP kinase family has been shown to play a major role in the control of many critical cellular processes including apoptosis, differentiation and proliferation (Lewis *et al.*, 1998). They exert their effects on the cell by the phosphorylation of a multitude of cellular proteins including transcription factors, which subsequently alter levels of gene expression. The activity of the MAP kinases is controlled by reversible phosphorylation of the conserved threonine and tyrosine within the MAP kinase tri-peptide activation motif (Tyr-X-Thr). In mammalian cells, inactivation of the MAP kinases is accomplished primarily by a family of dual specificity phosphatases that are able to dephosphorylate both the phosphotyrosine and phosphothreonine required for MAP kinase activity(for review see (Camps *et al.*, 2000)). To date 14 MAP kinase phosphatases (MKP) have been identified and these can be separated in to two main groups according to their subcellular distribution and patterns of transcriptional regulation (Camps *et al.*, 2000). The first group includes MKP-1, MKP-2, PAC1 and B23, which are localised predominantly in the nuclear compartment. This group are encoded by immediate early genes and thought to play a role in feedback control of the MAP kinases within the nucleus. The second group includes MKP-3, MKP-X, MKP-4 and MKP-5, which are localised in the cytosol of the cell.

The aims of this chapter were firstly to clone human MKP-2 from a cDNA library. Secondly to describe the characterisation of kinase assay systems in which the substrate specificity of the MKP-2 clone could be tested. Thirdly to describe the generation of a catalytically inactive form of MKP-2 and analyse its ability to prevent the MKP-2 mediated dephosphorylation of the three main MAP kinase family members.

3.2 Results

3.2.1 Cloning of Human MKP-2 cDNA

Human MKP-2 was cloned using the Polymerase Chain Reaction (PCR). The template used was a human umbilical vein endothelial cell line cDNA library (HUVEC). PCR primers were designed based on the published human MKP-2 sequence (NCBI accession No. U21108). The sequence of the primers and their distribution relative to the published sequence are shown in Table 3.1 and Figure 3.1. Various combinations of these primers were used to amplify areas of the library that contained, at least in part, the MKP-2 cDNA. Further refinement of the PCR conditions lead to the amplification of several MKP-2 fragments which were cloned together using restriction enzymes to create a cDNA fragment of 1184 base pairs (Fig. 3.2), corresponding to the full coding region of human MKP-2.

The primers designed to amplify the full coding region of MKP-2, F1 and R4 incorporated a *HindIII* and *BamHI* (table 3.1 bold text) restriction site respectively for directional subcloning. In addition these primers were given a six nucleotide tail to aid restriction enzyme binding.

The PCR temperature cycles used to amplify the final target sequence are described in Table 3.2. *HindIII* and *BamHI* restriction enzymes were used to prepare the mammalian expression vector, pcDNA3 (Stratagene) and the amplified PCR product. The vector and insert were then ligated together to generate the pcDNA3-WT-MKP-2 expression vector (WT-MKP-2). Clones were screened by restriction digestion to ensure an insert of the correct size was present. Appropriate clones were then fully sequenced to ensure the validity of the product and also ascertain whether the PCR reactions and enzymatic cloning had incorporated any errors into the MKP-2 sequence. The full MKP-2 sequence obtained and the predicted translational product are shown in figure 3.3. The predicted length of the protein is 394 amino acids with a molecular mass of 42.9KDa. Interestingly the sequence was not an identical match of the U21108 sequence but did match a second MKP-2 sequence published by a separate group (Chu *et al.*, 1996) (GenBank accession No. U48807).

Primer Name	Sequence – 5′ to 3′
F1	TTT TTT **AGG CTT** ATG GTG ACG ATG GAG GAG CT
F2	ACC GAC ATC TGC CTG CTC AA
F3	GAC ATC AGC TCC TGG TTC AT
F4	GTT AAG CAG CGC CGC AGC AT
R1	ATG GCT GCC AGC GCC TTG GTT
R2	AGT CCT TCA CCG CAT CGA TG
R3	TGA AGA CGA ACT GCG AGG TG
R4	AAA AAA **GGA TCC** CTC TAA CAG CTG GGA GAG GT

Table 3.1.

Sequence of primers used to amplify MKP-2 fragments from the human umbilical vein cDNA library. Restriction enzyme sites used for directional subcloning are shown in bold.

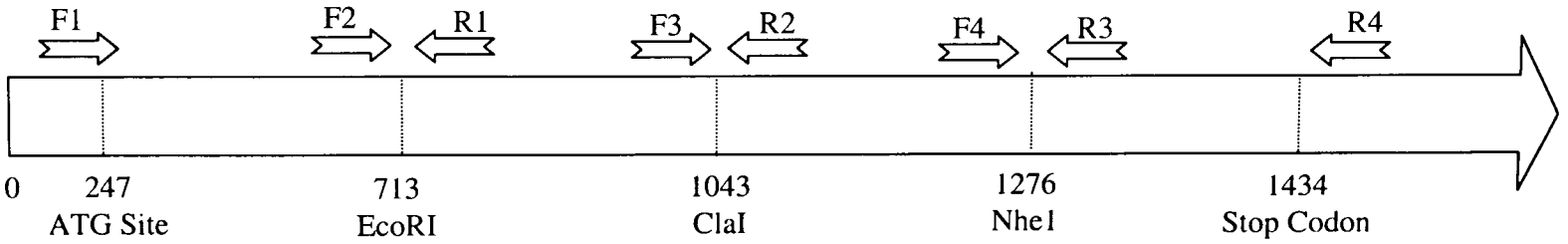

Figure 3.1 Schematic showing location of cloning primers relative to human MKP-2 sequence (U21108)

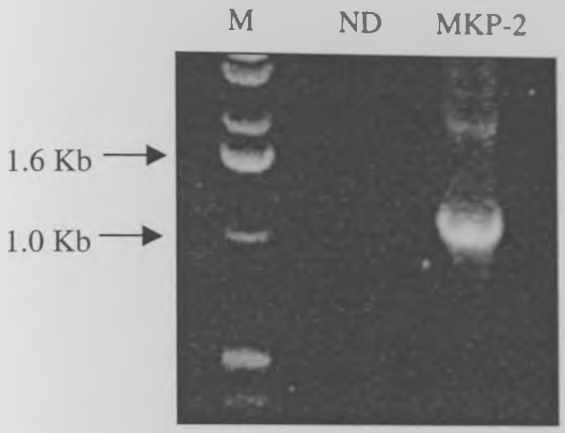

Figure 3.2. Agarose gel analysis of MKP-2 cDNA digest.
Restriction enzyme digest of cloned MKP-2 using *HindIII* and *BamHI* (MKP-2). 1Kb Molecular weight marker (M). No DNA control (ND). Digestion products were analysed on 1% agarose gel.

	Temp(°C)	Time	No of Cycles
Step 1	95	5min	1
Step 2	95	30sec	35
Step 3	60	1min	
Step 4	72	1min 30sec	
Step 5	72	5min	1

Table 3.2.

Temperature cycles used to amplify full-length human MKP-2 cDNA from the human umbilical vein cDNA library.

ATGGTGACGATGGAGGAGCTGCGGGAGATGGACTGCAGTGTGCTC
M V T M E E L R E M D C S V L
AAAAGGCTGATGAACCGGGACGAGAATGGCGGCGGCGCGGGCGGC
K R L M N R D E N G G G A G G
AGCGGCAGCCACGGCACCCTGGGGCTGCCGAGCGGCGGCAAGTGC
S G S H G T L G L P S G G K C
CTGCTGCTGGACTGCAGACCGTTCCTGGCGCACAGCGCGGGCTAC
L L L D C R P F L A H S A G Y
ATCCTAGGTTCGGTCAACGTGCGCTGTAACACCATCGTGCGGCGG
I L G S V N V R C N T I V R R
CGGGCTAAGGGCTCCGTGAGCCTGGAGCAGATCCTGCCCGCCGAG
R A K G S V S L E Q I L P A E
GAGGAGGTACGCGCCCGCTTGCGCTCCGGCCTCTACTCGGCGGTC
E E V R A R L R S G L Y S A V
ATCGTCTACGACGAGGGCAGCCCGCGCGCCGAGAGCCTCCGCGAG
I V Y D E G S P R A E S L R E
GACAGCACCGTGTCGCTGGTGGTGCAGGCGCTGCGCCGCAACGCC
D S T V S L V V Q A L R R N A
GAGCGCACCGACATCTGCCTGCTCAAAGGCGGCTATGAGAGGTTT
E R T D I C L L K G G Y E R F
TCCTCCGAGTACCCAGAATTCTGTTCTAAAACCAAGGCCCTGGCA
S S E Y P E F C S K T K A L A
GCCATCCCACCCCCGGTTCCCCCCAGCGCCACAGAGCCCTTGGAC
A I P P P V P P S A T E P L D
CTGGGCTGCAGCTCCTGTGGGACCCCACTACACGACCAGGGGGGT
L G C S S C G T P L H D Q G G
CCTGTGGAGATCCTTCCCTTCCTCTACCTCGGCAGTGCCTACCAT
P V E I L P F L Y L G S A Y H
GCTGCCCGGAGAGACATGCTGGACGCCCTGGGCATCACGGCTCTG
A A R R D M L D A L G I T A L
TTGAATGTCTCCTCGGACTGCCCAAACCACTTTGAAGGACACTAT
L N V S S D C P N H F E G H Y
CAGTACAAGTGCATCCCAGTGGAAGATAACCACAAGGCCGACATC
Q Y K C I P V E D N H K A D I
AGCTCCTGGTTCATGGAAGCCATAGAGTACATCGATGCCGTGAAG
S S W F M E A I E Y I D A V K
GACTGCCGTGGGCGCGTGCTGGTGCACTGCCAGGCGGGCATCTCG
D C R G R V L V H C Q A G I S
CGGTCGGCCACCATCTGCCTGGCCTACCTGATGATGAAGAAACGG
R S A T I C L A Y L M M K K R
GTGAGGCTGGAGGAGGCCTTCGAGTTCGTTAAGCAGCGCCGCAGC
V R L E E A F E F V K Q R R S
ATCATCTCGCCCAACTTCAGCTTCATGGGACAGCTGCTGCAGTTC
I I S P N F S F M G Q L L Q F
GAGTCCCAGGTGCTGGCCACGTCCTGTGCTGCGGAGGCTGCTAGC
E S Q V L A T S C A A E A A S

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CCCTCGGGACCCCTGCGGGAGCGGGGCAAGACCCCCGCCACCCCC
P  S  G  P  L  R  E  R  G  K  T  P  A  T  P
ACCTCGCAGTTCGTCTTCAGCTTTCCGGTCTCCGTGGGCGTGCAC
T  S  Q  F  V  F  S  F  P  V  S  V  G  V  H
TCGGCCCCCAGCAGCCTGCCCTACCTGCACAGCCCCATCACCACC
S  A  P  S  S  L  P  Y  L  H  S  P  I  T  T
TCTCCCAGCTGTTAG
S  P  S  C  *
```

Figure 3.3.

Sequence of cloned human MKP-2 with predicted amino acid sequence. Bold text indicates translation initiation codon and '*' indicates translation stop codon.

3.2.2 Characterisation of human MKP-2 clone

The cloned WT-MKP-2 cDNA was transfected into COS7 cells (as outlined in section 2.4.3) and the presence of the protein analysed by Western blotting with a commercial MKP-2 antibody. As can be seen in Figure 3.4 an increasing amount of MKP-2 cDNA transfected into the cells leads to a direct increase in the amount of MKP-2 protein present within the cell. The Western blot also shows the protein detected was the predicted size of ~43 KDa (Fig. 3.4).

Having identified the cloned protein was indeed human MKP-2 the next step was to characterise its phosphatase activity towards the three members of the MAP kinase family JNK, ERK and p38-MAPK. The phosphatase activity and substrate specificity was assessed using an indirect assay; the activity was quantified by the phosphatases ability to reduce the activity of agonist stimulated kinases.

Before the activity of MKP-2 could be assessed the individual kinase assay systems had to be analysed and optimised. This was done by transient transfection of kinase cDNA into COS7 cells. To ensure the fidelity of the *in vitro* stage of the assays the kinases were isolated from the cellular extracts by immunoprecipitation with antibodies directed towards epitope tags. The JNK1, p38-MAPK and ERK2 cDNAs are tagged with haemeaglutinin (HA), FLAG, and c-MYC respectively. HA-JNK1 activity was assessed by the *in vitro* phosphorylation of the purified JNK substrate GST-c-$Jun_{(5\text{-}89)}$. FLAG-p38-MAPK activity was measured by its ability to phosphorylate purified GST-ATF2 and MYC-ERK-2 by the phosphorylation of myelin basic protein (MBP).

As can be seen in figure 3.5 transient transfection of increasing amounts of HA-JNK1 cDNA lead to a direct increase in agonist stimulated HA-JNK1 activity following stimulation with 0.5M sorbitol. The activity was maximal at 50 fold with 7.5μg of cDNA however transfection of HA-JNK1 cDNA amounts of 5μg and above lead to deterioration in the viability of the cells and an increase in cell death, as assessed by microscopy. For subsequent experiments only 2μg of HA-JNK1 was used.

Osmotic stress-induced by 0.5M sorbitol was also used to monitor the activity of increasing amounts of p38-MAPK in COS7 cells. As can be seen in figure 3.6

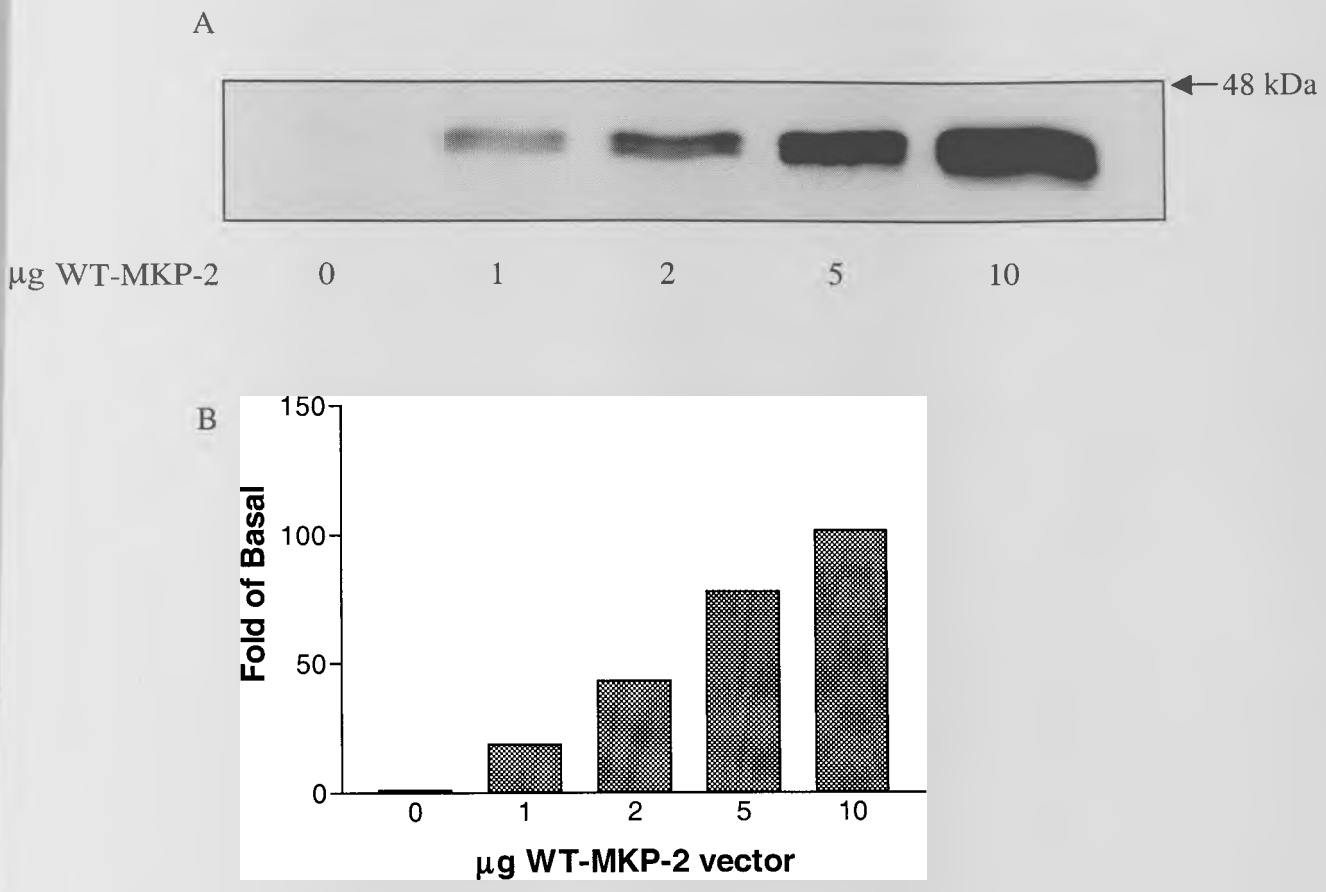
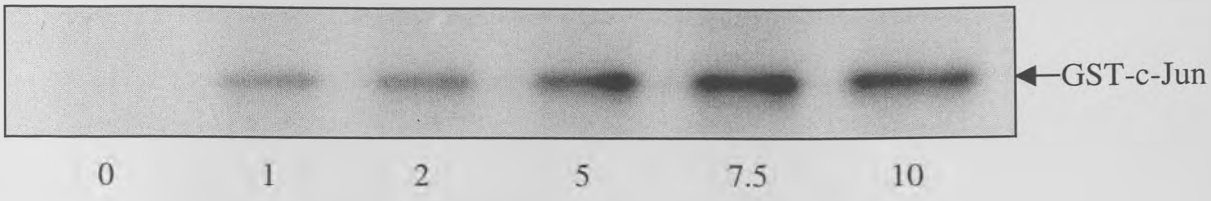


Figure 3.4 Immunodetection of WT-MKP-2
COS7 cells were transfected with increasing amounts of the WT-MKP-2. (A) Cell extracts were analysed by Western blotting using an anti-MKP-2 antibody as described in section 2.5.5. (B) Densitometric evaluation of WT-MKP-2 levels by scanning densitometry. Blots are indicative of at least 2 experiments.

A



B

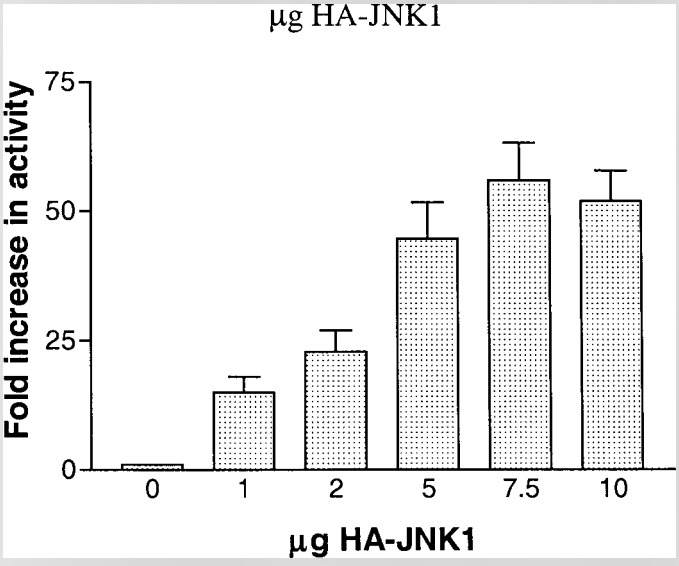
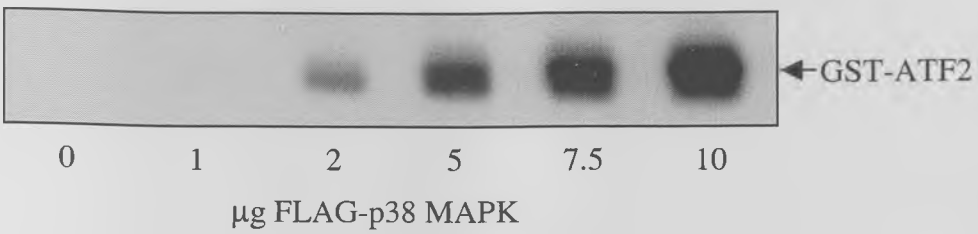


Figure 3.5 Effect of increasing amounts of HA-JNK1 cDNA on kinase activity in COS7 cells.

COS7 cells were transfected with either vector (0) or increasing amounts of HA-JNK1. Cells were stimulated with 0.5M Sorbitol for 60 mins. (A) Activity was assessed by *in vitro* kinase assay using GST-c-$Jun_{(5\text{-}89)}$ as substrate as described in Section 2.5.8. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as fold increase in activity relative to control cells. Mean ± s.e.m. (n= 3).

A



B

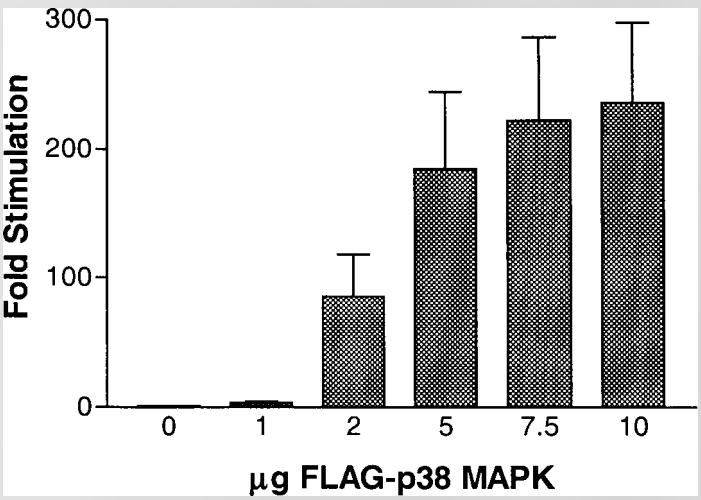


Figure 3.6 Effect of increasing amounts of FLAG-p38-MAPK cDNA on kinase activity in COS7 cells.
COS7 cells were transfected with either vector (0) or increasing amounts of FLAG-p38-MAPK. Cells were stimulated using osmotic stress via 0.5M sorbitol for 60 minutes. (A) Activity was assessed by *in vitro* kinase assay using GST-ATF2 as substrate as described in Section 2.5.10. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of FLAG-p38-MAPK activity by scanning densitometry expressed as fold of basal levels. Mean ± s.e.m. (n= 3).

activity of p38-MAPK increased in direct correlation with amount of transfected p38- MAPK cDNA. No maximal value was obtained for p38-MAPK activity as transfection of more than 10μg of cDNA leads to a decrease in transfection efficiency. Transfection efficiency was assessed by β-Galactosidase staining.

Transfection of COS7 cells with MYC-ERK2 cDNA lead to an increase in serum stimulated ERK2 activity (Fig. 3.7). Activity was close to maximal at 2μg of cDNA at 18 fold of basal and only increased to 21 fold by transfection of up to10 μg of cDNA. The small change in activity between the varying amounts of cDNA suggests that the amount of ERK protein expressed may be flooding the assay system by either binding up all the activating MEK1 and 2 or phosphorylating all the available MBP. For subsequent experiments 1μg of MYC-ERK2 cDNA was used.

With conformation that each of the kinase assay systems were robust it was still necessary to identify time points with sufficient active phosphorylated kinases that could be used to assess the phosphatase activity of MKP-2. Each of the kinases activation profile was assessed. In transiently transfected cells the stress kinases HA-JNK1 and FLAG-p38-MAPK were stimulated by using osmotic stress in the form of 0.5M sorbitol. JNK activity was found to be maximal at just under 2.5 fold of basal within 60 minutes (Fig. 3.8) and this response was sustained for at least 2 hours. FLAG-p38-MAPK activity was maximal at 10 minutes at 25 fold of basal and was maintained for at least 60 minutes soon after which cell viability was compromised (Fig. 3.9). Stimulation of cells with 20% serum (v/v) gave a three-fold increase in MYC-ERK2 activity maximal at 15 minutes decreasing to basal over the next two hours (Fig. 3.10). Although the MAP kinase isoforms are rapidly activated in response to extracellular and stress stimuli, longer time courses were used in subsequent experiments to ensure that the MAP kinases would have sufficient time to translocate to the nucleus.

To assess the specificity of MKP-2 towards the MAP kinases, both HA-JNK1 and MKP-2 were transfected into COS7 cells. Activity of MKP-2 was assessed indirectly by monitoring the reduction of HA-JNK1 activity in the presence of increasing amounts of WT-MKP-2. Transfection of increasing amounts of WT-MKP-2 lead to a concentration-dependent reduction in HA-JNK1 activity (Fig. 3.11)

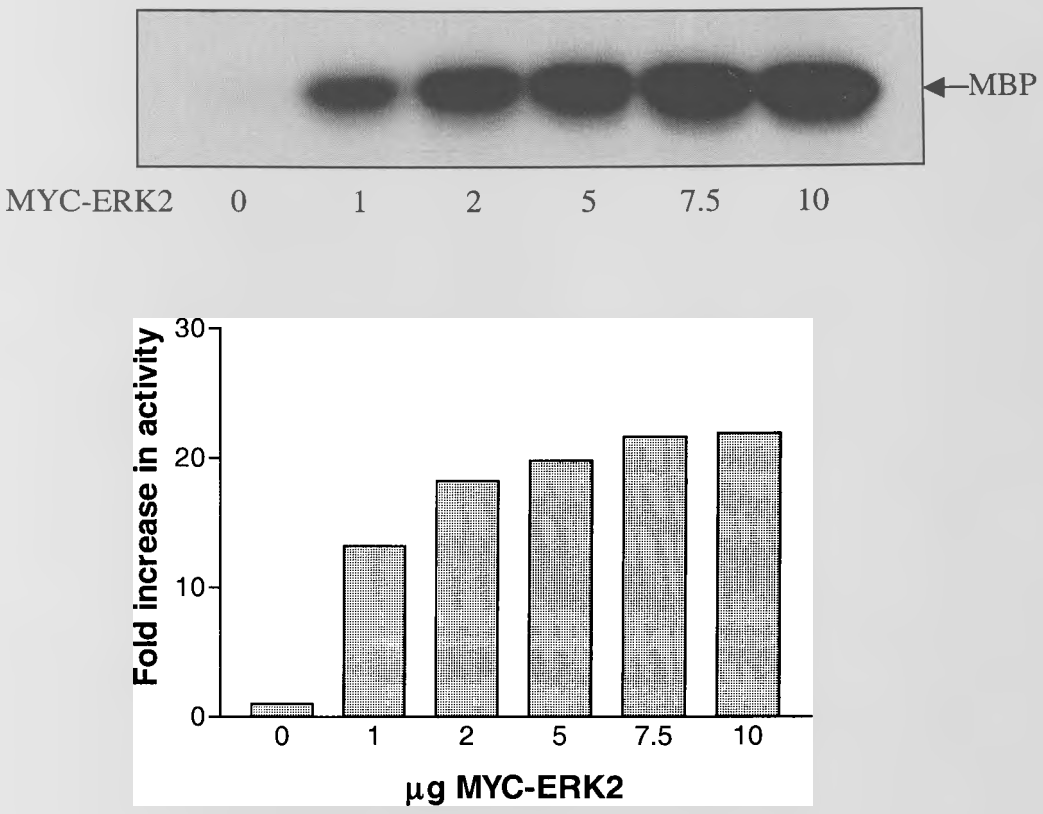


Figure 3.7. Effect of increasing amounts of MYC-ERK2 cDNA on kinase activity in COS7 cells.
COS7 cells were transfected with either vector (0) or increasing amounts of MYC-ERK2 . Cells were stimulated with 20% FCS for 15 minutes. (A) Activity was assessed by *in vitro* kinase assay using MBP as substrate as described in Section 2.5.9. Autoradiographs are indicative of at least 2 experiments. (B) Semi-quantitative evaluation of MYC-ERK2 activity by scanning densitometry expressed as fold of basal levels (n= 2).

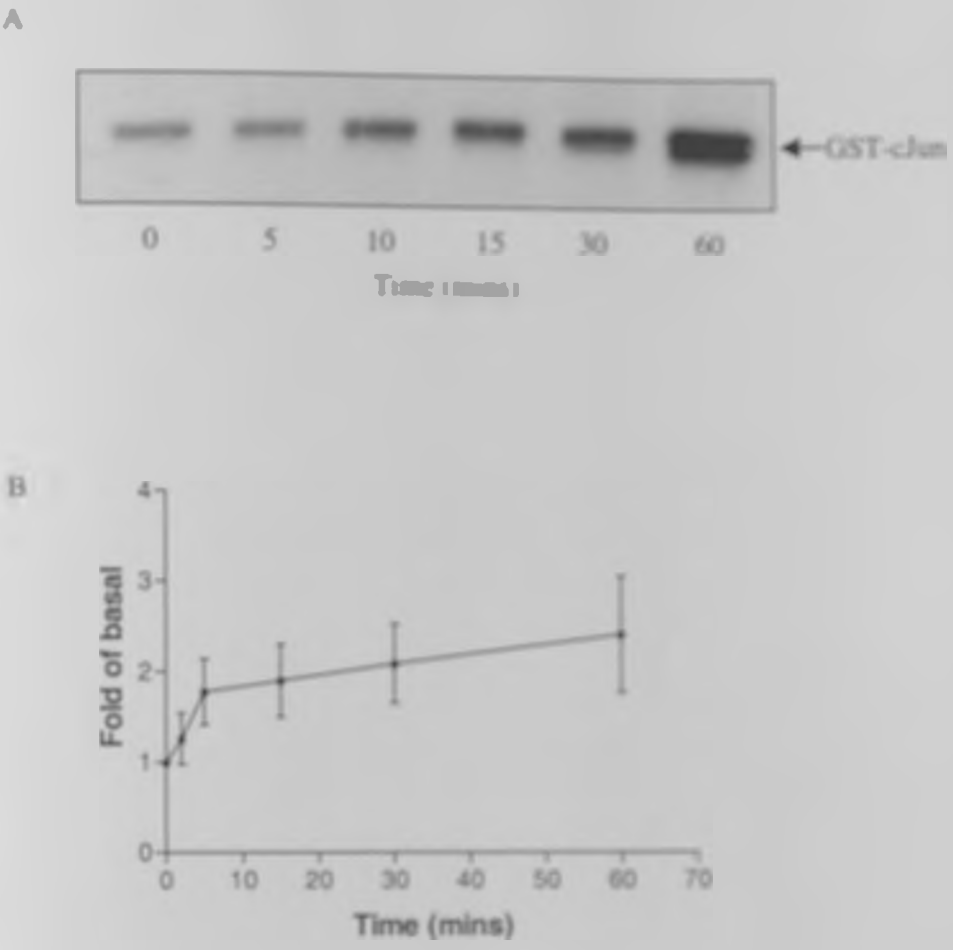
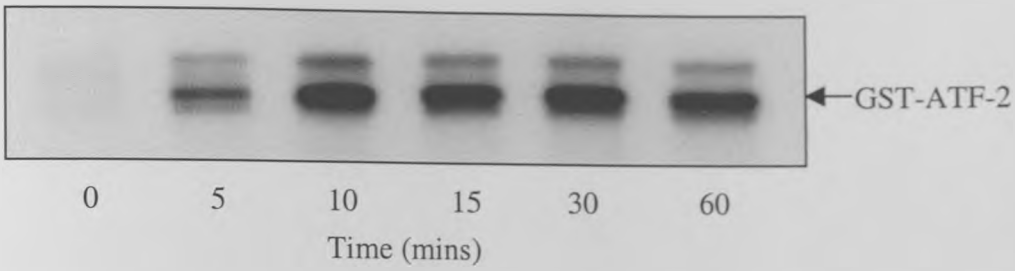


Figure 3.8. Time course of osmotic stress induced HA-JNK1 activation in COS7 cells.

COS7 cells were transfected with 2μg HA-JNK1 and stimulated with 0.5M sorbitol for the times indicated. (A) Activity was assessed by an *in vitro* kinase assay using GST-c-Jun_{1-89} as described in Section 2.5.8. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as fold of basal levels. Mean ± s.e.m. (n= 3).

A



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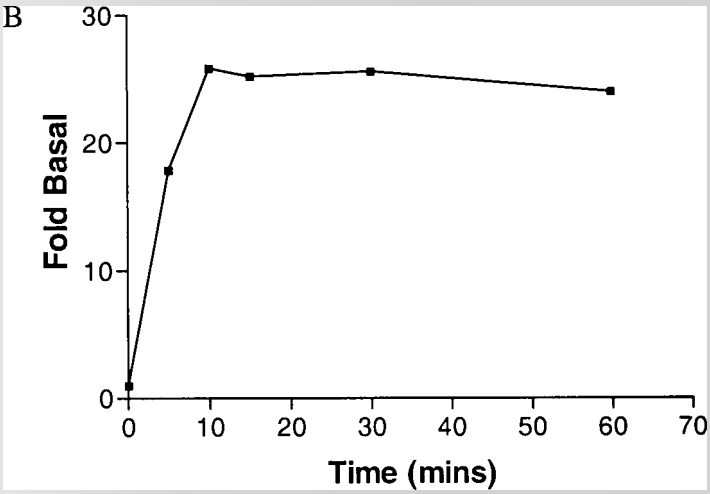
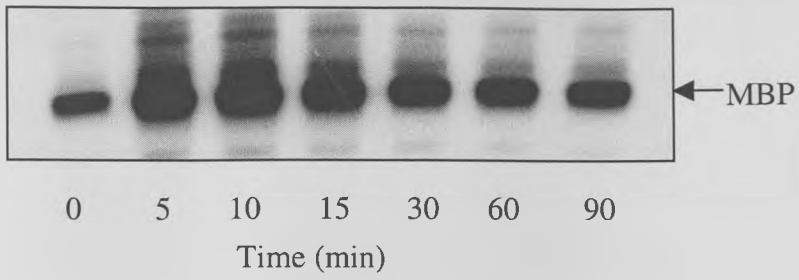


Figure 3.9. Time course of osmotic stress-induced FLAG-p38 activation in COS7 cells.

COS7 cells were transfected with 2μg FLAG-p38 and stimulated with 0.5M sorbitol for the times indicated. (A) Activity was assessed by *in vitro* kinase assay using GST-ATF2 as substrate as described in Section 2.5.10. Autoradiographs are indicative of at least 2 experiments. (B) Semi-quantitative evaluation of FLAG-p38 activity by scanning densitometry expressed as fold of basal levels. (Mean of n= 2).

A



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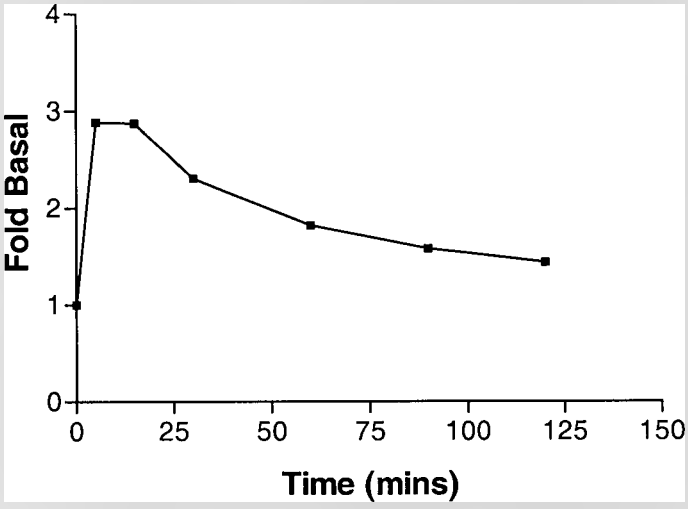

Figure 3.10. Time course of serum stimulated MYC-ERK2 activation in COS7 cells.

COS7 cells were transfected with 1μg MYC-ERK2 and stimulated with 20% FCS for times indicated. (A) Activity was assessed by *in vitro* kinase assay using MBP as substrate as described in Section 2.5.9. Autoradiographs are indicative of at least 2 experiments. (B) Semi-quantitative evaluation of MYC-ERK2 activity by scanning densitometry expressed as fold of basal levels. (n= 2).

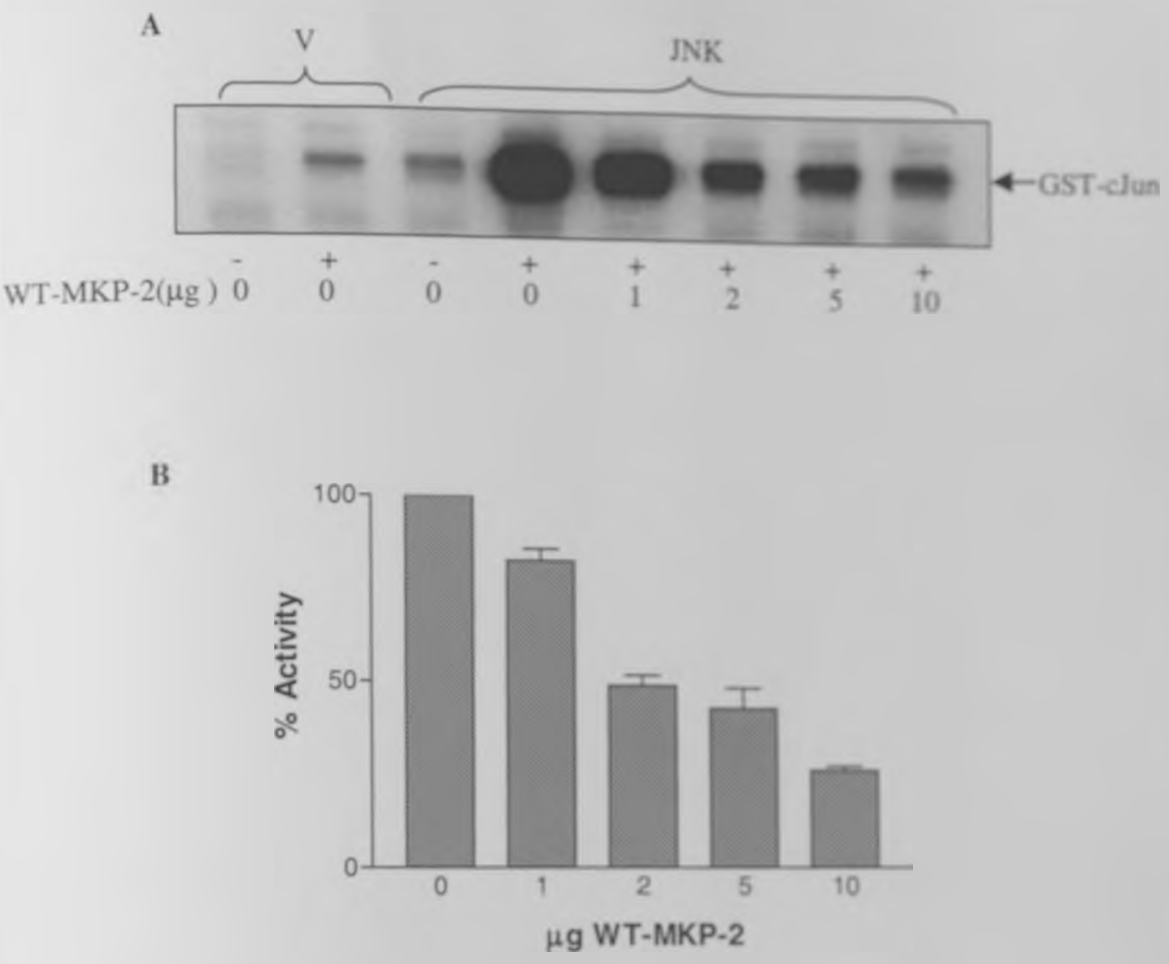

Figure 3.11 Inactivation of HA-JNK-1 by WT-MKP-2 in COS7 cells. COS7 cells were transiently transfected with either vector (V) or 2μg of HA-JNK-1 (JNK) and increasing amounts of WT-MKP-2. (A)Cells were stimulated for 60min with vehicle (-) or 0.5M sorbitol (+). Activity was assessed by *in vitro* kinase assay using GST-c-Jun$_{(1-79)}$ as substrate as described in Section 2.5.8. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as a percentage of HA-JNK-1 activity relative to positive control (0). Mean ± s.e.m. (n= 3).

suggesting that WT-MKP-2 is able to bind and dephosphorylate JNK1. MKP-2 phosphatase activity was also tested against p38-MAPK. Increasing amounts of MKP-2 was transfected into COS7 cells along with FLAG-p38-MAPK. However in the case of p38-MAPK, MKP-2 was unable to reduce p38-MAPK activity even at relatively high concentrations (Fig. 3.12). Similar experiments were used to identify whether ERK2 is a substrate of MKP-2. MYC-ERK2 was transfected along with WT-MKP-2 into COS7 cells and ERK activity measured (Fig. 3.13). Increasing amounts of MKP-2 failed to reduce MYC-ERK2 activity, suggesting MKP-2 is also unable to dephosphorylate ERK2. In all of the above experiments to test the specificity of MKP-2 it must be noted the low level of activity shown in the reactions that should contain no epitope-tagged kinase (V lanes). This indicates that the kinase assay system used is not completely faithfull. Contaminating proteins from the cell lysate may be able to bind to the antibody or the carrier beads, or perhaps the substrate itself is incorporating radioactive phosphate in the absence of a kinase enzyme. The data was analysed with this limitation of the assay system in mind.

These data suggest that in COS7 cells WT-MKP-2 is specific for the JNK family of MAP kinases but not p38-MAPK or ERK. This data differs from that of previous studies (Guan & Butch, 1995; Misra-Press *et al.*, 1995), which suggest MKP-2 is able to dephosphorylate JNK and ERK equally. To ascertain if this was due to the subcellular distribution of either p38 MAPK or ERK differing from the nuclear locale of MKP-2 their distribution was assessed. Epitope-tagged MAP kinases were transfected into COS7 cells and their locale elucidated using FITC conjugated secondary antibodies and indirect immunoflourescence. To define the nucleus within the cells the DNA stain 4,6-Diamidino-2-Phenylindole (DAPI) was used. As DAPI excites and emits at a lower wavelength than the FITC used to identify the MAP kinases, it is possible to visualise both the DAPI and FITC staining within the same field of cells. As expected, HA-JNK1 was present in the nuclear compartment of COS7 cells, and this locale was unchanged in quiescent or osmotically challenged cells (Fig. 3.14). MYC-ERK2 was found to be cytosolic in resting cells (Fig. 3.15), and stimulation with serum for up to 8 hours failed to induce nuclear translocation. Preliminary data showed other stimuli including PMA and cisplatin are also unable to induce nuclear translocation of ERK in this cell type .

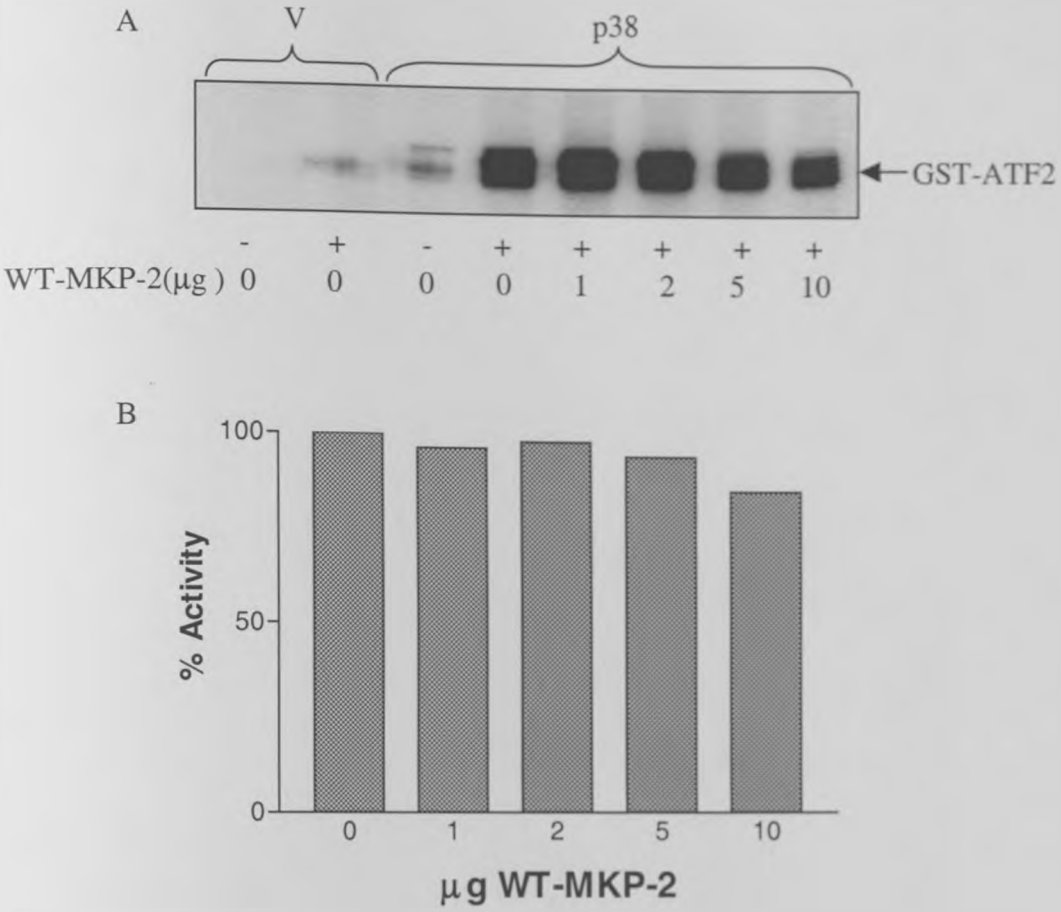

Figure 3.12 Inactivation of FLAG-p38-MAPK by WT-MKP-2 in COS7 cells.

COS7 cells were transiently transfected with either vector (V) or 2μg of FLAG-p38 MAPK (p38) and increasing amounts of WT-MKP-2. (A)Cells were stimulated for 60min with vehicle (-) or 0.5M sorbitol (+) activity was assessed by *in vitro* kinase assay using GST-ATF2 as substrate as described in section 2.5.10. Autoradiographs are indicative of at least 2 experiments. (B) Semi-quantitative analysis of FLAG-p38-MAPK activity by scanning densitometry expressed as percentage reduction in activity. Mean ± s.e.m. (n= 2).

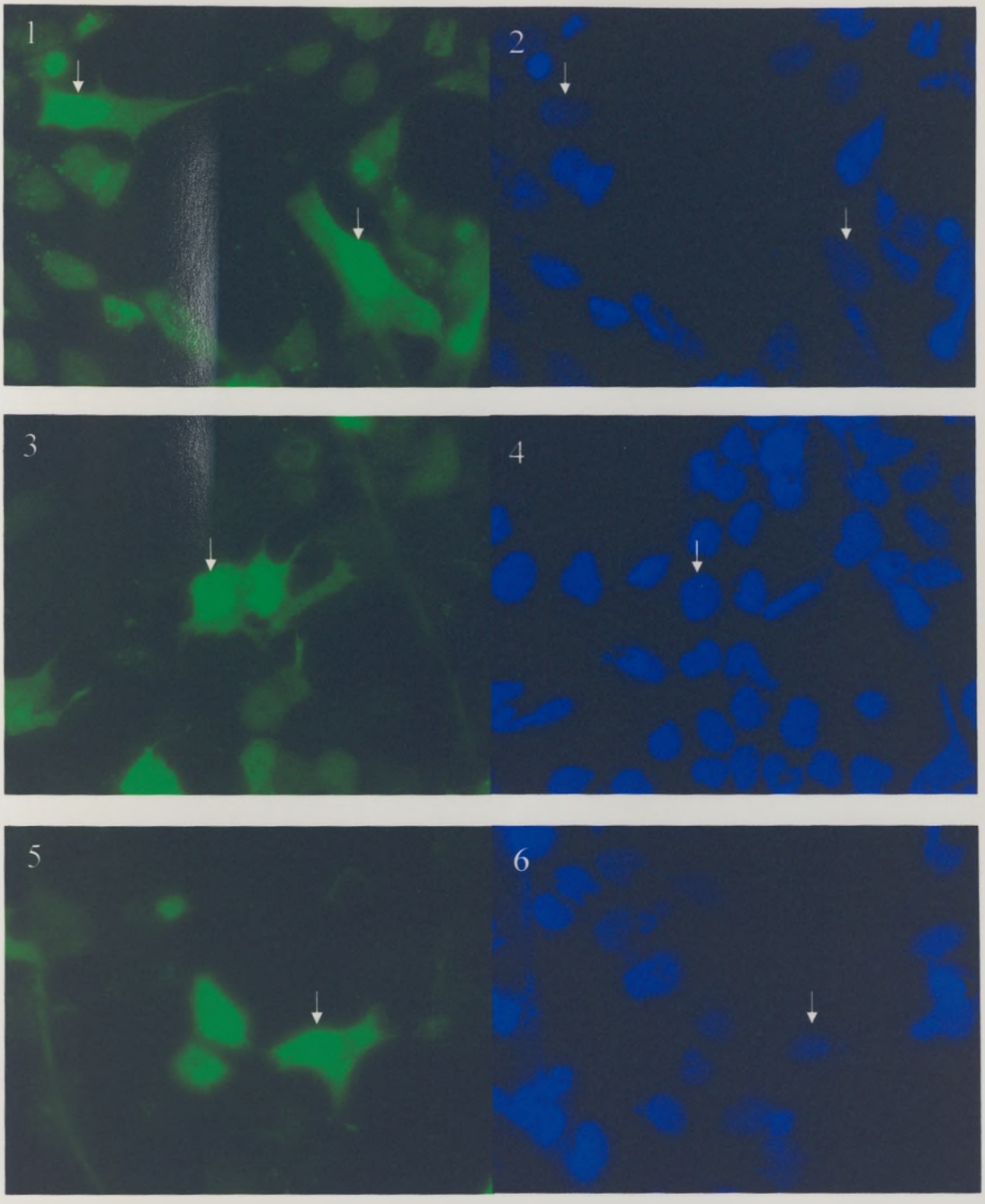

Figure 3.14. Subcellular distribution of HA-JNK1 in COS 7 cells.
COS7 cells were transiently transfected with 2μg of HA-JNK and either left unstimulated (1&2), or stimulated with 0.5M sorbitol for 30 min (3&4) and 60 min (5&6). Cells were the fixed and stained as described in section 2.5.7 using anti-HA (1,3&5) and DAPI (2,4&6). Arrows indicate same nuclei in each field. Magnification X40.

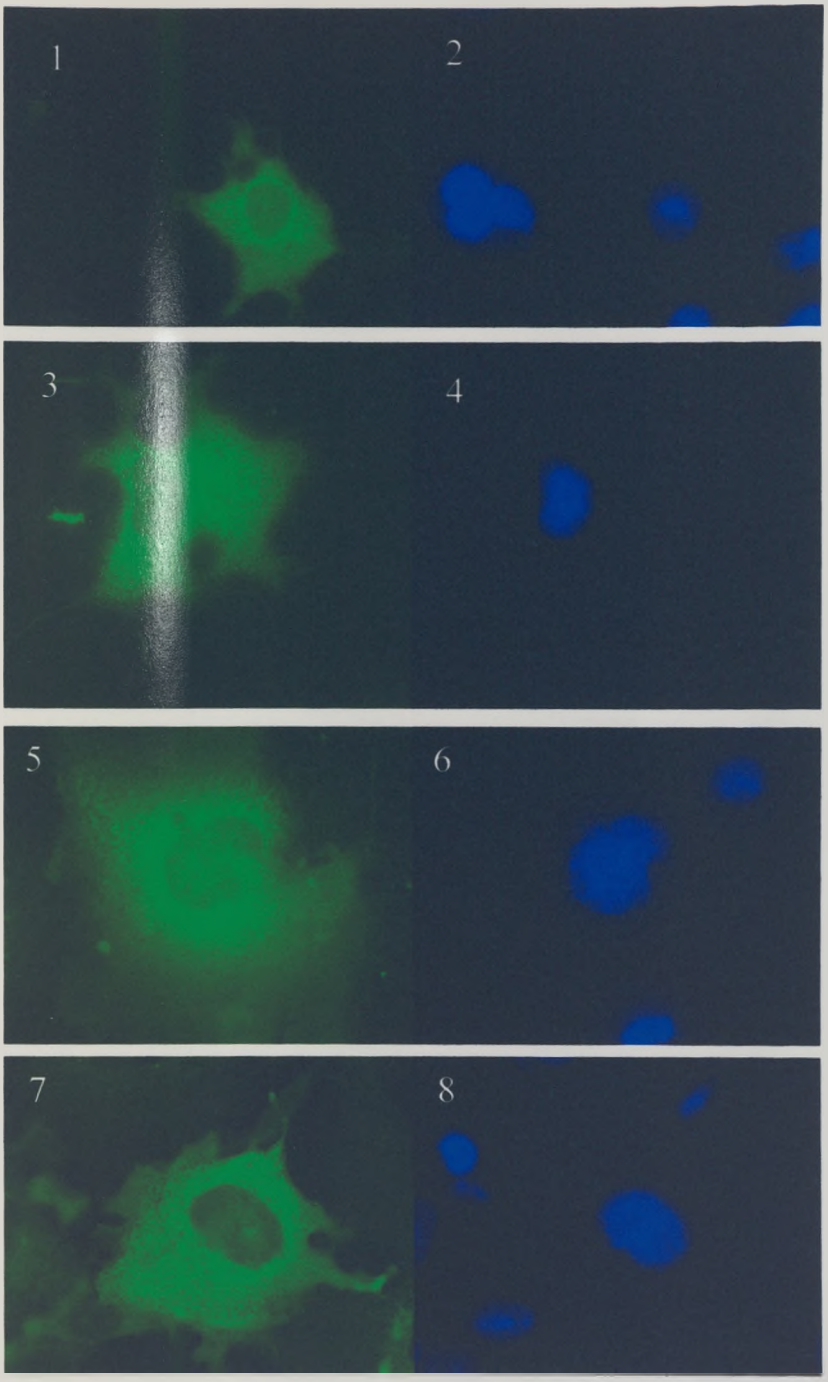

Figure 3.15. Subcellular distribution of MYC-ERK2 in COS 7 cells. COS7 cells were transiently transfected with 1μg of MYC-ERK2 and either left unstimulated (1&2) or stimulated with 20% FCS for 15 min (3&4), 30 min (5&6) or 8 hours (7&8). Cells were the fixed and stained as described in section 2.5.7 using anti-MYC (1,3,5&7) and DAPI (2.4,6&8).. Magnification X60.

(Sloss and Plevin, unpublished data). p38-MAPK was also located predominantly in the cytosol (fig 3.16) and again, osmotic stress was unable to alter its distribution.

3.2.3 Creation of a catalytically inactive MKP-2.

All protein phosphatases contain, within their active site, a cysteine residue that is critical for phosphatase activity. To confirm the location of this critical cysteine in the newly cloned human MKP-2 the putative amino acid (Cys279) was changed by site-directed mutagenesis to serine.

The mutagenesis was carried out using the Quick-Change Site-directed Mutagenesis Kit (Stratagene) as described in the manufacturers instructions.

The pcDNA3-WT-MKP-2 plasmid was used as template to create pcDNA3-CI-MKP-2 (CI-MKP-2). The primers CImutFor and CImutRev spanning the region coding for the cysteine were designed to incorporate a sequence change that would lead to the translation of a serine residue rather than a cysteine (Table 3.3).

The mutation of the cysteine to serine also created a *DdeI* restriction site, which was used to screen for the CI-MKP-2 constructs. Digestion of both the WT and CI-MKP-2 with *BamHI* and *HindIII* released the expected 1.2Kb full MKP-2 cDNA (Fig. 3.17 lanes 2 and 5) addition of *DdeI* to this digestion lead to the cleavage of the CI-MKP-2 but not the WT-MKP-2 (lanes 4 and 7). Clones of the correct size that contained the *DdeI* restriction site were confirmed by sequence analysis.

To confirm the CI-MKP-2 vector was still expressing the full-length protein the plasmid was transfected into COS7 cells and the lysates analysed by Western blotting using the MKP-2 antibody (Fig. 3.18). As expected the protein was still reactive with the antibody and migrated at the correct ~43Kda size.

The CI-MKP-2 phosphatase activity was then tested, as before against HA-JNK1, to assess if the amino acid change had indeed removed the phosphatase activity of the protein. COS7 cells were transfected with kinase cDNA and increasing amounts of CI-MKP-2. The CI-MKP-2 was unable to attenuate activation of the JNK1 by osmotic stress (Fig. 3.19). To test whether CI-MKP-2 was able to act in a dominant negative fashion, its ability to reverse the effects of WT-MKP-2 was

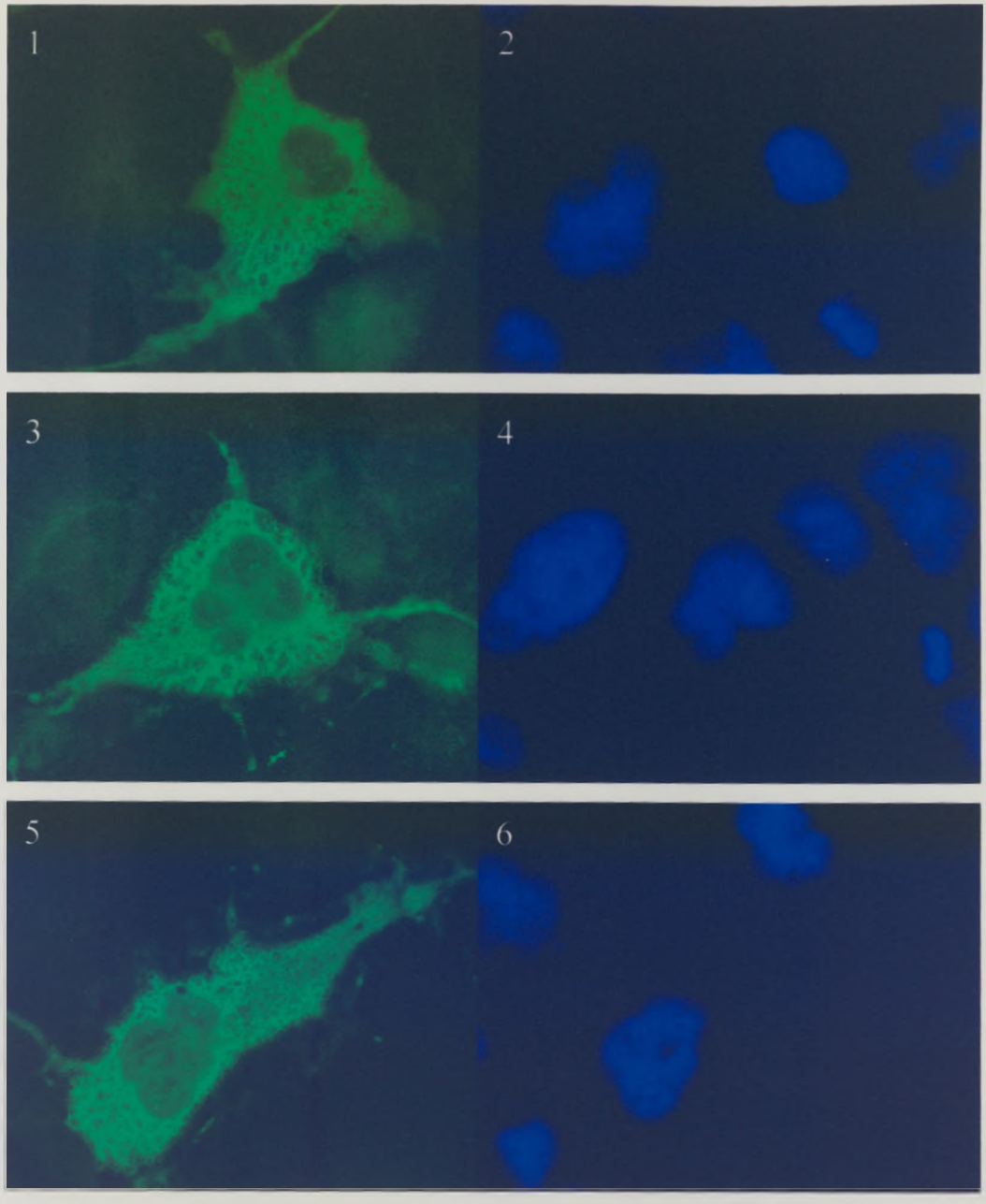

Figure 3.16 Subcellular distribution of FLAG-p38 MAPK in COS 7 cells.

COS7 cells were transiently transfected with 2μg of FLAG-p38 MAPK and either left unstimulated (1&2) or stimulated with 0.5M Sorbitol for 30 min (3&4) or 60 min (5&6). Cells were the fixed and stained as described in section 2.5.7 using anti-p38 MAPK (1,3 &5) and DAPI (2,4&6). Magnification X60.

Primer Name	Sequence- 5'-3'
ClmutFor	CGT GCT GGT GCA CT**C** **T**CA GGC GGG CAT CTC
ClmutRev	GAG ATG CCC GCC TG**A** **G**AG TGC ACC AGC ACG

Table 3.3

Primer sequences of mutagenesis primers used to mutate the putative critical cysteine (aa279) to serine. Amino acids depicted in bold differ from the published human MKP-2 sequence. (Genbank U48807).

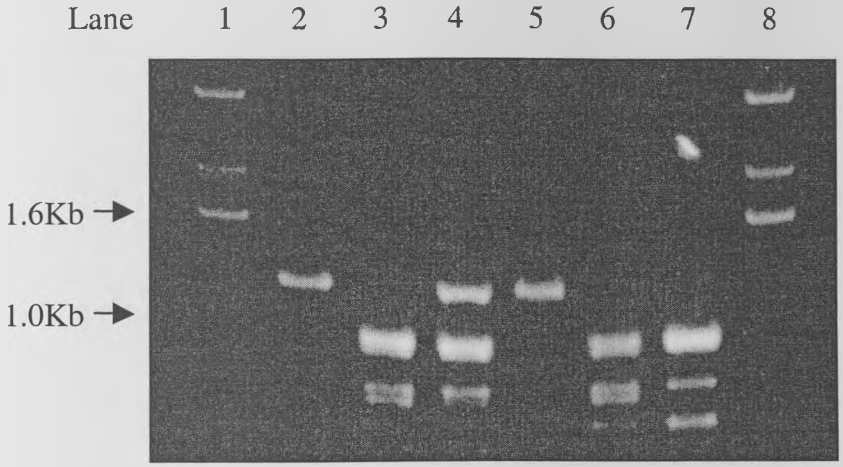

Figure 3.17 Restriction analysis of WT and CI-MKP-2. WT and CI-MKP-2 were digested with HindIII, BamHI and DdeI before size separation on 1% Agarose Gel. DNA was stained with ethidium bromide and visualised under UV light.

Lanes 2-4 WT-MKP-2
Lane1: 1Kb Marker
Lane2: BamHI and HindIII
Lane3: DdeI
Lane4: BamHI,HindIII and DdeI

Lanes 5-7 CI-MKP-2
Lane5: BamHI and HindIII
Lane6: DdeI
Lane7: BamHI,HindIII and DdeI
Lane8: 1Kb Marker

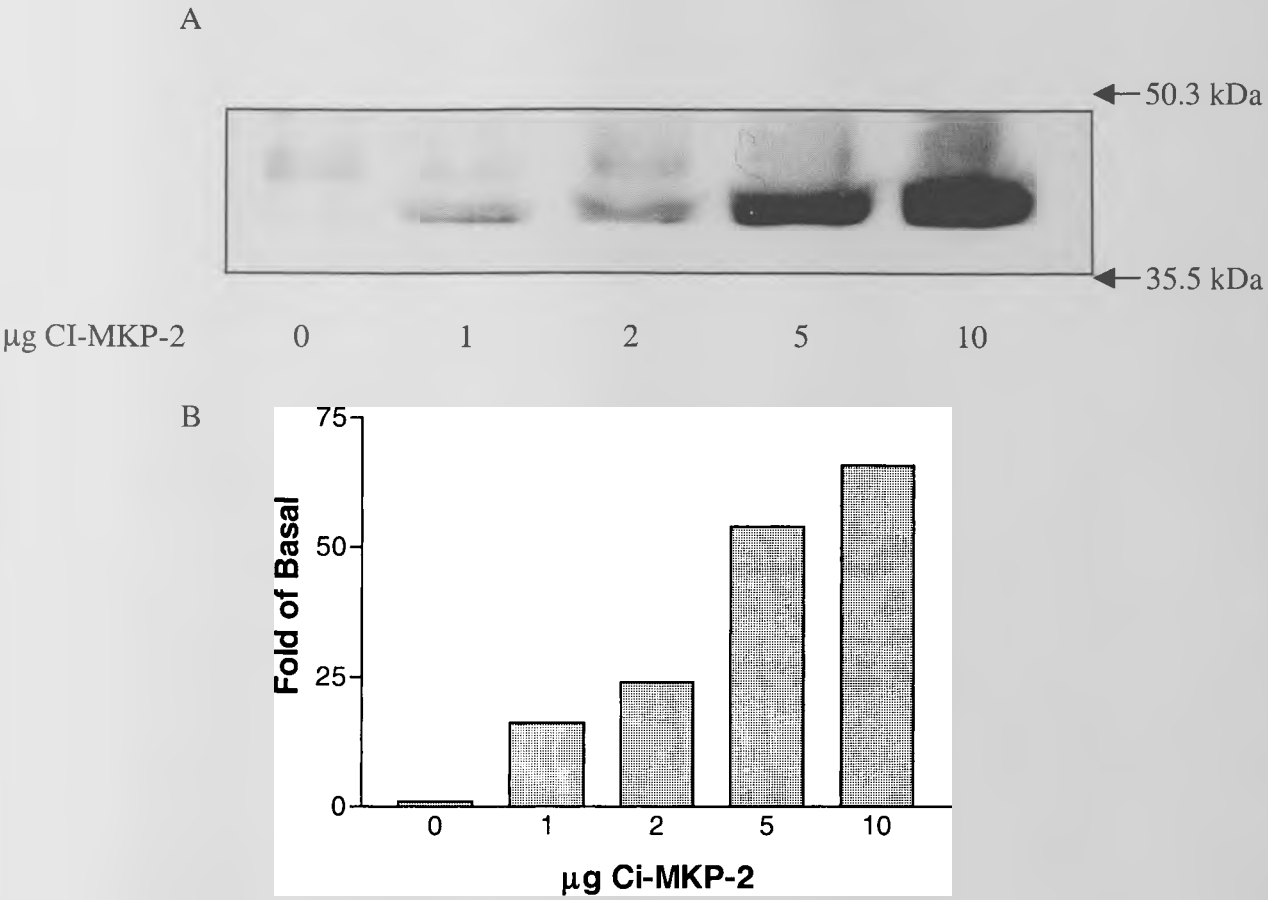

Figure 3.18. Immunodetection of CI-MKP-2.
COS7 cells were transfected with increasing amounts of the CI-MKP-2. (A) Cell extracts were analysed by Western blotting using an anti-MKP-2 antibody as described in section 2.5.5. (B) Densitometric evaluation of CI-MKP-2 levels by scanning densitometry. Blots are indicative of at least 2 experiments.

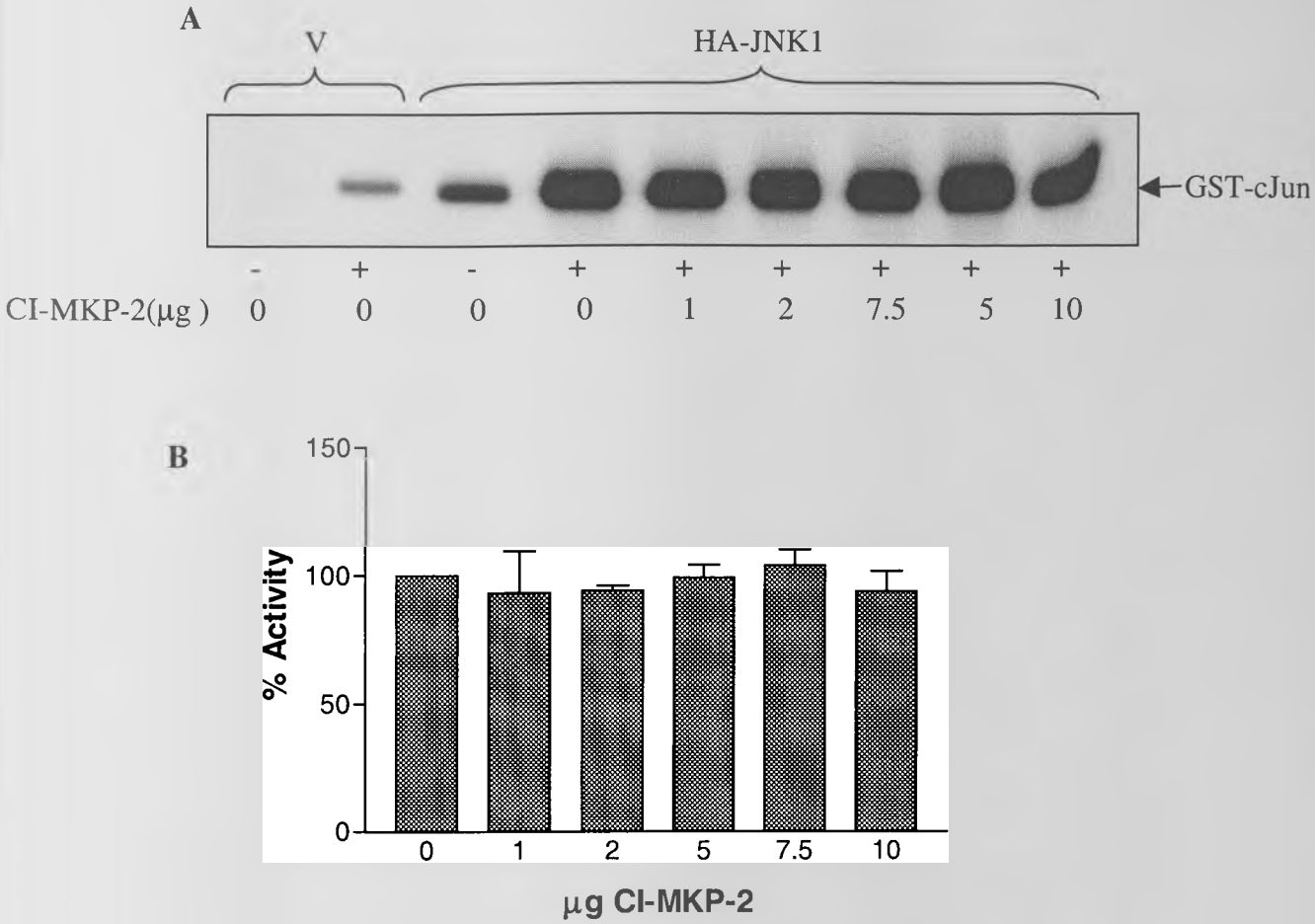

Figure 3.19. CI-MKP-2 is inactive towards HA-JNK-1.
COS7 cells were transiently transfected with either vector (V) or 2μg of HA-JNK-1 (JNK) and increasing amounts of CI-MKP-2. (A) Cells were stimulated for 60min with vehicle (-) or 0.5M sorbitol (+) activity was assessed by *in vitro* kinase assay using GST-c-Jun$_{(5\text{-}89)}$ as substrate. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as a percentage of HA-JNK-1 activity relative to positive control (0). Mean ± s.e.m. (n= 3).

tested. This was achieved by transfection of HA-JNK1, WT-MKP-2 and increasing amounts of CI-MKP-2 into COS7 cells. As can be seen in Figure 3.20 WT-MKP-2 is able to reduce JNK activity back to basal levels. The addition of increasing amounts of CI-MKP-2 lead to a concentration-dependant recovery of HA-JNK-1 activity.

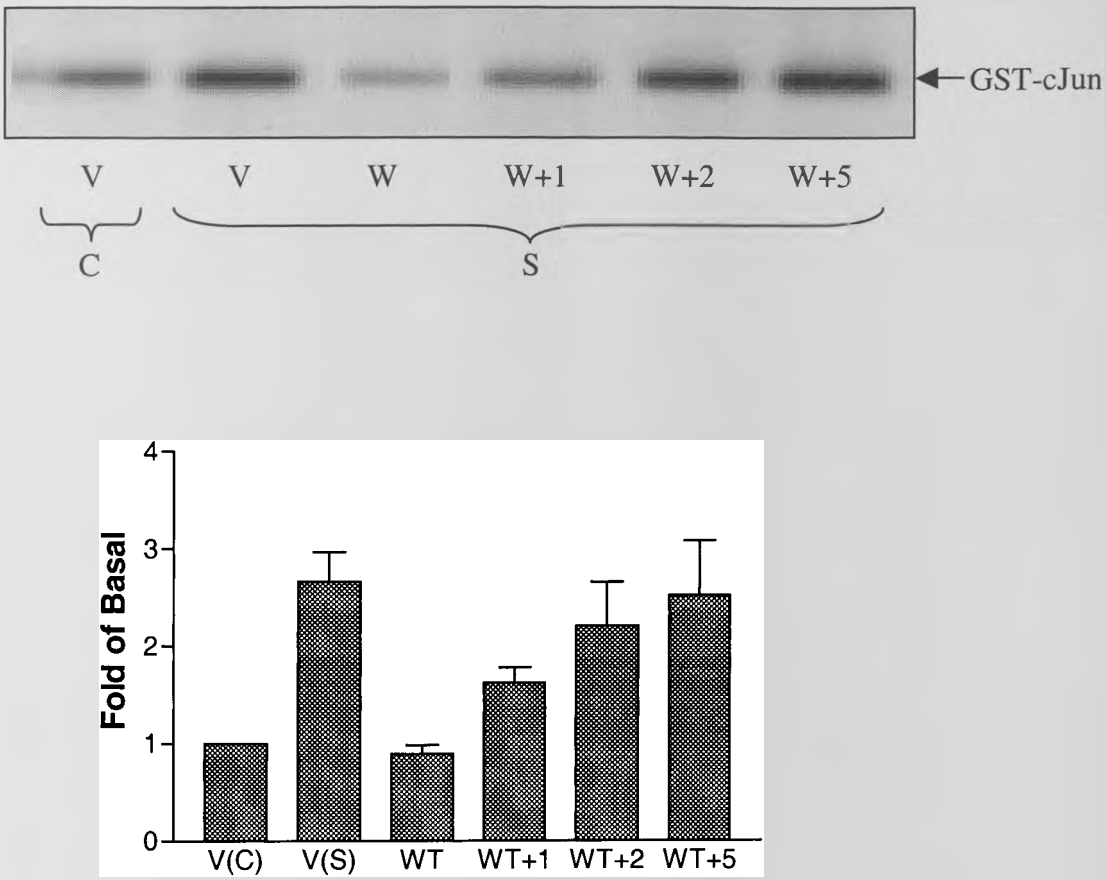

Figure 3.20. Dominant negative effect of CI-MKP-2 on inhibition of HA-JNK1 by WT-MKP-2.

COS7 cells were transfected with 1μg HA-JNK1 and either 5μg of vector (V), 5μg of WT-MKP-2 (W) or increasing amounts of CI-MKP-2 (+). Cells were stimulated with either vehicle (C) or 0.5M Sorbitol for 60 minutes (S). (A) Activity was assessed by *in vitro* kinase assay using GST-c-$Jun_{(5\text{-}89)}$ as substrate as described in Section 2.5.8. Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as fold of basal levels. Mean ± s.e.m. (n= 3).

3.3 Discussion

This chapter describes the cloning and characterisation of the human form of the nuclear MAP kinase phosphatase MKP-2. This enzyme was previously cloned and identified nearly simultaneously by two groups (Guan & Butch, 1995; Misra-Press *et al.*, 1995) and named HVH2 and MKP-2, although recently the name MKP-2 has become the more commonly used of the two. The two differing published sequences are 99% homologous whether this is due to an error in the sequencing of the original isolated sequence or indicates silent polymorphisms is unclear. Guan et al revealed that MKP-2 mRNA was present in large amounts in both the pancreas and placenta of rat tissue (Guan & Butch, 1995). Misra-Press, also using northern blot analysis, identified MKP-2 in a wide range of tissues and immortalised cell lines including brain, heart, lung and PC12 cells (Misra-Press *et al.*, 1995).

Using this information an umbilical vein library was chosen as a hopeful source of the human MKP-2 cDNA. PCR amplification revealed presence of the 3' end of the MKP-2 cDNA. Attempts to amplify of the entire length of MKP-2 failed, but it was possible to utilise the presence of several naturally occurring restriction enzyme sites within the MKP-2 sequence (Fig. 3.1) to build a full-length (1184bp) cDNA from several PCR products.

The early findings of this chapter provide strong evidence that the final cDNA product did indeed encode full length human MKP-2. Not only does the expressed protein migrate at the correct size it is also highly reactive with the commercial MKP-2 antibody and sequence analysis shows the cDNA to be 100% homologous with one of the previously identified MKP-2s (Chu *et al.*, 1996).

In order to assess the substrate specificity of MKP-2 it was necessary to develop a system in which the activity of the three MAP kinase family members could be accurately measured. As antibodies directed to the phosphorylated active forms of the three main MAP kinases are poor in quality and show multiple non-specific bands when used for Western blotting, it was decided to use an assay that measured the activity of the kinases by phosphorylation of one of their respective substrates: ATF2 for p38-MAPK, c-Jun for JNK and MBP for ERK. As many of the cellular substrates of the MAP kinases can be phosphorylated by more than one kinase it was necessary to ensure the fidelity of the system. This was done by using

epitope-tagged MAP kinases: The JNK1, p38-MAPK and ERK2 cDNAs are tagged with haemaglutinin (HA), FLAG, and c-MYC respectively. This allowed the selective immunoprecipitation of the kinases from the cellular extracts before the phosphorylation reaction is carried out. COS7 cells were chosen for the assay system as they are fast growing, easily transfected and give high protein expression from the transiently transfected plasmids.

Transfection of large amounts of p38-MAPK and JNK led to an increase in cell shedding from culture vessel and a reduction in the protein recovered from the cells. As mentioned above this may be due to the non-physiological levels of SAPK protein within the cell activating the apoptotic response. Both JNK and p38-MAPK have been shown to be involved in the programmed cell death response (De Zutter & Davis, 2001; Johnson *et al.*, 1996; Stefanis *et al.*, 1996; Tournier *et al.*, 2000; Xia *et al.*, 1995).

Activation of the MAP kinases was achieved using simple agonists that have long been known to activate this group of pathways: Osmotic stress along with many other types of environmental stress has been shown to activate both the JNK and p38-MAPK pathways (Kyriakis & Avruch, 1996). Serum and other mitogens are able to activate the ERK members of MAP kinase family (Camps *et al.*, 2000).

It must be noted that non-stimulated cells transfected with HA-JNK-1 (Fig. 3.8) still show large amounts of JNK activity which, when used as a basal value reduces the apparent increase in JNK activity over time.

The experiments shown here suggest the specificity of MKP-2 to be towards the JNK group of MAP kinases but not p38-MAPK or ERK. Both the original MKP-2 cloning papers quote MKP-2 as being able to dephosphorylate ERK (Guan & Butch, 1995; Misra-Press *et al.*, 1995). Several other groups have published results in agreement with those shown here that MKP-2 can also inactivate JNK (King *et al.*, 1995; Ward *et al.*, 1994) and in more recent studies, it has been confirmed that MKP-2 is unable to bind and dephosphorylate p38-MAPK (Chen *et al.*, 2001; Chu *et al.*, 1996). To assess why MKP-2 was unable to inactivate ERK in this system, the locale of all three kinases was assessed. As described JNK is predominantly nuclear, whereas p38 MAPK and ERK are both cytosolic. As the locale of p38-MAPK and ERK is unchanged by osmotic stress or serum respectively, this could explain the

inablility of MKP-2 to inactivate these kinases. The reasons for the lack of translocation of MAP kinases in this system is unclear. It may be due to the non-physiological expression levels of the kinases swamping the mechanism of nuclear import. Or indeed, may be due to the presence of the epitope tags on all three kinases. It has been previously shown that homo-dimerisation of the MAP kinases is an important step in nuclear translocation (Adachi *et al.*, 1999; Khokhlatchev *et al.*, 1998). Perhaps the presence of the epitope tags interferes with the ability of the kinases to dimerise, and thus preventing nuclear accumulation. Even though the differing locale of MKP-2 and p38-MAPK may explain the lack of interaction in this system. In the face of evidence produced by many groups, it seems likely that p38-MAPK is not a physiological substrate of MKP-2. ERK on the other hand, has been shown to interact with MKP-2 and it is probable that the limits of this system are giving a false indication of the inability of MKP-2 to dephosphorylate ERK.

Analysis of the MKP-2 protein sequence indicated the presence of a DSP consensus sequence centred around the cysteine at Cys279. All PTPases including DSPs have been shown to require the presence of an essential cysteine within their catalytic site for phosphatase activity (Guan & Dixon, 1991; Zhou *et al.*, 1994). The sulphur present within the cysteine is utilised to form a covalent thiol-phosphate intermediate with the substrate protein (Denu *et al.*, 1995; Guan & Dixon, 1991). Several studies have shown that mutation of this essential cysteine to serine will remove all DSP activity. In Pmp1, a yeast MKP, mutation of cysteine 158 lead to a total loss in activity (Sugiura *et al.*, 1998) and in MKP-1 mutation of cysteine 258 affects both activity and binding (Sun *et al.*, 1993). To assess if Cysteine 279 of MKP-2 was indeed this essential cysteine it was changed to serine. The experimental evidence shown here indicates that Cysteine 279 is indeed essential for phosphatase activity, as CI-MKP-2 is unable to inactivate HA-JNK1 (Fig. 3.19).

Many catalytically inactive proteins have been shown to be able to act as dominant negative mutants. Usually via their terminal binding to either an activating or substrate protein. CI-MKP-2 shows dominant negative activity in its ability to reverse the inhibiton of HA-JNK1 by WT-MKP-2. The mechanism of this action is likely to involve the ability of CI-MKP-1 to remain bound to JNK but not to dephosphorylate the activation motif. WT-MKP-2 is therefore unable to access the

activation motif and the JNK molecule remains active. This type of terminal binding has been shown in the interaction of several mutant DSPs with their respective MAP kinases. The CI mutant of MKP-7 causes an increase in length of the JNK signal suggesting dominant negative properties (Tanoue *et al.*, 2001), and in fibroblast cells transfected with a CI mutant of MKP-3, ERK is unable to translocate to the nucleus as it is terminally bound to the MKP-3 in the cytosol (Brunet *et al.*, 1999). Additionally, CI-MKP-3 is able to inhibit the activation of WT-MKP-3 by ERK suggesting these CI mutants are competing for MAP kinase binding (Camps *et al.*, 1998).

Chapter 4

Mutation and analysis of the nuclear localisation sequences within MKP-2

4.1 Introduction

Having confirmed the substrate specificity of human MKP-2. The next step was to elucidate the structure function relationships of MKP-2 in particular the mechanism controlling subcellular locale.

The MKPs have varying cellular localisations and these obviously have a great bearing on the phosphatase's ability to interact with their substrate kinases. There are several MKPs in both the cytoplasm and the nucleus that presumably cover all aspects of MAP kinase inactivation. The nuclear MKPs include MKP-2 which attenuates the activity of either or both of JNK and ERK (Chu *et al.*, 1996). MKP-1 can bind and inactivate p38 (Hutter *et al.*, 2000) and PAC-1 is specific for ERK and p38 (Chu *et al.*, 1996). In the cytosol PYST1 is highly specific for inactivation of ERK (Camps *et al.*, 1998), MKP-4 appears to inactivate all three MAPK kinase isoforms without preference (Muda *et al.*, 1997) and MKP-5 inactivates p38 and JNK but not ERK (Tanoue *et al.*, 1999). Two MKPs have been discovered that are not restricted to either nucleus or cytosol. M3/6 is found in both compartments and is able to inactivate both p38 and JNK MAP kinases (Muda *et al.*, 1996), and the recently cloned DSP, MKP-7, has been found to shuttle between the nucleus and the cytosol. Possibly filling two roles firstly as a phosphatase and secondly as a chaperone or carrier molecule by transporting the inactivated MAP kinases out of the nucleus (Masuda *et al.*, 2001).

The subcellular distribution of these proteins is thought to be controlled, at least in part, by the presence of short hydrophilic stretches of amino acid sequence known as nuclear localisation signals (NLS) and short stretches of hydrophobic sequence known as nuclear export sequences (NES). NLS and NES have been shown to be critical in controlling the subcellular localisation of many different cellular proteins (for review see (Gorlich, 1998). and section 1.6)

Many of the MKPs identified so far have been shown to have areas of protein sequence that putatively could be NES or NLS sequences. The paper describing the original cloning of the nuclear phosphatase MKP-2 describes the presence of two putative NLS sequences (Guan & Butch, 1995). MKP-7 contains both putative NES and NLS and is found in both the cytoplasm and the nucleus (Masuda *et al.*, 2001).

Similarly PYST1 contains two putative NES sequences thought to be responsible for its cytoplasmic locale.

The aim of the experiments in this chapter was to elucidate the areas of MKP-2 sequence that were responsible for the proteins' nuclear localisation. This was done by disruption of the putative NLS sequences using site-directed mutagenesis and subsequent analysis of locale using indirect immunofluorescence and Western blotting. The effects of changes in protein sequence and localisation on MKP-2 activity towards JNK were also investigated. To further investigate the roles of NES in controlling MKP locale, MKP-2 mutants were generated that contained the NES of PYST1 and again the locale assessed by indirect immunofluorescence.

4.2 Results

4.2.1 Localisation of MKP-2

Due to problems with the staining of MKPs within the COS7 cell line it was decided to assess the localisation of the cloned human MKP-2 within the human embryonic kidney immortalised cell line HEK293. Transfection of the COS7 cells using DEAE dextran appeared to alter the cell morphology making identification of cellular organelles difficult. HEK293 cells are an epithelial cell in origin and require a different transfection technique than that of the COS7 cells. This is described in section 2.4.4.

To assess the localisation of both the wild type and the catalytically inactive MKP-2 the cDNAs were transfected into HEK293 cells. As expected the WT-MKP-2 was localised within the nucleus (Fig 4.1). Mutation of the catalytic site to generate the CI-MKP-2 had no effect on the localisation (Fig 4.2) suggesting that the phosphatase activity of the protein does not play a role in the nuclear import or retention of the molecule.

4.2.3 Disruption of nuclear localisation sequences.

MKP-2 contains two putative NLS that are thought to be responsible for its subcellular distribution (Fig 4.3). Although no definitive consensus sequence for an NLS has been found they tend to be identified by short sections of basic sequence (usually arginine and lysines). Within these areas it has been found that the removal or change of the basic arginine or lysine residues to a non-charged group like alanine can disrupt the ability of the sequence to direct the protein to the nucleus. Analysis of the MKP-2 sequence using the PSORTII website (http://psort.nibb.ac.jp) only identified one NLS. This was the bipartite NLS starting at K298. The other NLS like sequence starting at R74, although not matching the strict consensus used by PSORTII, has a structure very similar to that of a monopartite NLS. This putative NLS closest to the N terminus from R74 to K78 has been designated NLS1 and the C-terminal bipartite NLS between K298 and R318, NLS2.

To assess the involvement of the two putative NLS in the localisation of MKP-2 the NLS sequences were disrupted by site-directed mutagenesis. The primers shown in

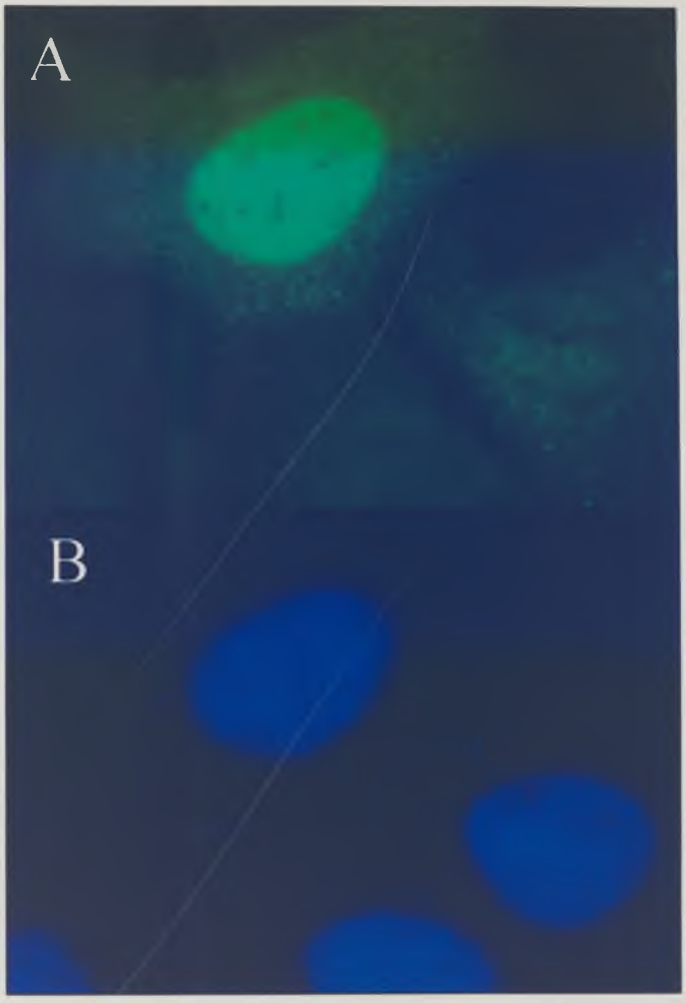

Figure 4.1 Subcellular location of WT-MKP-2 in HEK293 cells.
HEK293 cells were transiently transfected with 1μg of WT-MKP-2. Cells were fixed and stained as described in Section 2.5.7. (A) FITC staining against MKP-2. (B) DAPI stain. Magnification X100.

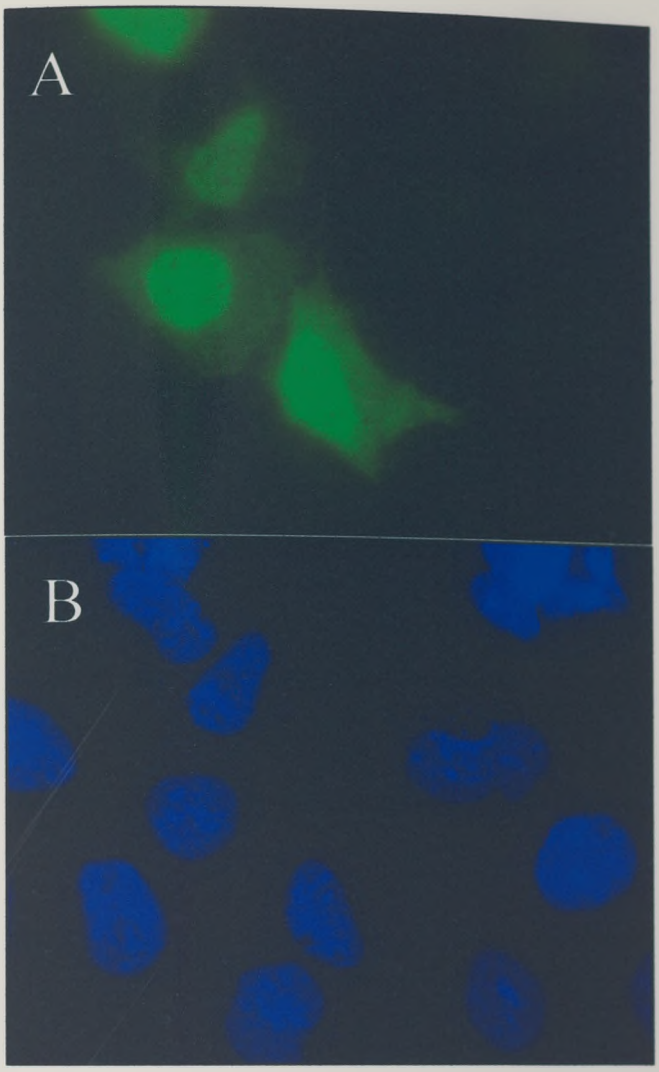

Figure 4.2 Subcellular location of CI-MKP-2 in HEK293 cells. HEK293 cells were transiently transfected with 1μg of CI-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X60.

```
120 ATGGTGACGATGGAGGAGCTGCGGGAGATGGACTGCAGTGTGCTC
    M  V  T  M  E  E  L  R  E  M  D  C  S  V  L

165 AAAAGGCTGATGAACCGGGACGAGAATGGCGGCGGCGCGGGCGGC
    K  R  L  M  N  R  D  E  N  G  G  G  A  G  G

210 AGCGGCAGCCACGGCACCCTGGGGCTGCCGAGCGGCGGCAAGTGC
    S  G  S  H  G  T  L  G  L  P  S  G  G  K  C

255 CTGCTGCTGGACTGCAGACCGTTCCTGGCGCACAGCGCGGGCTAC
    L  L  L  D  C  R  P  F  L  A  H  S  A  G  Y

300 ATCCTAGGTTCGGTCAACGTGCGCTGTAACACCATCGTGCGGCGG
    I  L  G  S  V  N  V  R  C  N  T  I  V  R  R

345 CGGGCTAAGGGCTCCGTGAGCCTGGAGCAGATCCTGCCCGCCGAG
    R  A  K  G  S  V  S  L  E  Q  I  L  P  A  E

390 GAGGAGGTACGCGCCCGCTTGCGCTCCGGCCTCTACTCGGCGGTC
    E  E  V  R  A  R  L  R  S  G  L  Y  S  A  V

435 ATCGTCTACGACGAGGGCAGCCCGCGCGCCGAGAGCCTCCGCGAG
    I  V  Y  D  E  G  S  P  R  A  E  S  L  R  E

480 GACAGCACCGTGTCGCTGGTGGTGCAGGCGCTGCGCCGCAACGCC
    D  S  T  V  S  L  V  V  Q  A  L  R  R  N  A

525 GAGCGCACCGACATCTGCCTGCTCAAAGGCGGCTATGAGAGGTTT
    E  R  T  D  I  C  L  L  K  G  G  Y  E  R  F

570 TCCTCCGAGTACCCAGAATTCTGTTCTAAAACCAAGGCCCTGGCA
    S  S  E  Y  P  E  F  C  S  K  T  K  A  L  A

615 GCCATCCCACCCCCGGTTCCCCCCAGCGCCACAGAGCCCTTGGAC
    A  I  P  P  P  V  P  P  S  A  T  E  P  L  D

660 CTGGGCTGCAGCTCCTGTGGGACCCCACTACACGACCAGGGGGGT
    L  G  C  S  S  C  G  T  P  L  H  D  Q  G  G

705 CCTGTGGAGATCCTTCCCTTCCTCTACCTCGGCAGTGCCTACCAT
    P  V  E  I  L  P  F  L  Y  L  G  S  A  Y  H

750 GCTGCCCGGAGAGACATGCTGGACGCCCTGGGCATCACGGCTCTG
    A  A  R  R  D  M  L  D  A  L  G  I  T  A  L

795 TTGAATGTCTCCTCGGACTGCCCAAACCACTTTGAAGGACACTAT
    L  N  V  S  S  D  C  P  N  H  F  E  G  H  Y

840 CAGTACAAGTGCATCCCAGTGGAAGATAACCACAAGGCCGACATC
    Q  Y  K  C  I  P  V  E  D  N  H  K  A  D  I
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```
885 AGCTCCTGGTTCATGGAAGCCATAGAGTACATCGATGCCGTGAAG
    S  S  W  F  M  E  A  I  E  Y  I  D  A  V  K

930 GACTGCCGTGGGCGCGTGCTGGTGCACTGCCAGGCGGGCATCTCG
    D  C  R  G  R  V  L  V  H  C  Q  A  G  I  S

975 CGGTCGGCCACCATCTGCCTGGCCTACCTGATGATGAAGAAACGG
    R  S  A  T  I  C  L  A  Y  L  M  M  K  K  R

1020 GTGAGGCTGGAGGAGGCCTTCGAGTTCGTTAAGCAGCGCCGCAGC
     V  R  L  E  E  A  F  E  F  V  K  Q  R  R  S

1065 ATCATCTCGCCCAACTTCAGCTTCATGGGACAGCTGCTGCAGTTC
     I  I  S  P  N  F  S  F  M  G  Q  L  L  Q  F

1110 GAGTCCCAGGTGCTGGCCACGTCCTGTGCTGCGGAGGCTGCTAGC
     E  S  Q  V  L  A  T  S  C  A  A  E  A  A  S

1155 CCCTCGGGACCCCTGCGGGAGCGGGGCAAGACCCCCGCCACCCCC
     P  S  G  P  L  R  E  R  G  K  T  P  A  T  P

1200 ACCTCGCAGTTCGTCTTCAGCTTTCCGGTCTCCGTGGGCGTGCAC
     T  S  Q  F  V  F  S  F  P  V  S  V  G  V  H

1245 TCGGCCCCCAGCAGCCTGCCCTACCTGCACAGCCCCATCACCACC
     S  A  P  S  S  L  P  Y  L  H  S  P  I  T  T

1290 TCTCCCAGCTGTTAG 1304
     S  P  S  C  *
```

Figure 4.3
cDNA(open boxes) and protein(grey boxes) NLS sequences relative to the entire MKP-2 sequence.

figure 4.4 were used to disrupt the NLS1 by changing R74, R75 and R76 to alanine. WT-MKP-2 was used as cDNA template and the resulting mutant clone was called NLS1-MKP-2. Similarly the primers shown in figure 4.5 were used to mutate WT-MKP-2 to create NLS2-MKP-2 by changing K298, K299 and R300 to alanine. Changes to sequences and fidelity of PCR products were confirmed by sequencing (data not shown).

To assess the mutations had not affected the expression or primary structure of the proteins, they were analysed by Western blotting. As can be seen in figure 4.6 both NLS1-MKP-2 and NLS2-MKP-2 were still reactive with the commercial MKP-2 antibody and migrated at the expected size of 43KDa.

To assess the localisation of NLS1-MKP-2, the cDNA was transfected into HEK293 cells and the localisation assessed as described in section 2.5.7. NLS1-MKP-2 is localised within the nucleus (Fig 4.7) suggesting that disruption of NLS1 is not sufficient to alter the localisation of the protein. Similarly NLS2-MKP-2 is also localised within the nucleus (Fig. 4.8). These results taken together suggest that either removal of either NLS can be compensated for by the presence of the other or that these sequences are not solely responsible for the localisation of MKP-2
To assess if each of the NLS sites is able to compensate for the loss of the other a mutant MKP-2 lacking both NLS was created using site-directed mutagenesis to disrupt the NLS2 sequence on the NLS1-MKP-2 mutant. This new double NLS mutant was designated DNLM-MKP-2. The expression and primary structure of DNLM was assessed as before by Western blot. As can be seen in figure 4.9, DNLM-MKP-2 is still expressed to high levels in COS7 cells and reacts with the MKP-2 antibody at the expected size of 43KDa. The localisation of DNLM-MKP-2 was elucidated as before in HEK293 cells and as can be seen in figure 4.10 DNLM-MKP-2 is localised in the cytosol. Due to the high non-specific staining of the MKP-2 antibody and the probable presence of endogenous WT-MKP-2 within the nucleus it was difficult to ascertain whether the DNLM-MKP-2 was localised entirely within the cytosol or whether a small amount remained in the nucleus. To test this, a crude nuclear fraction from cells transfected with WT-MKP-2 and the MKP-2 mutants were isolated as described in section 2.5.2 and the nuclear and cytosolic extracts

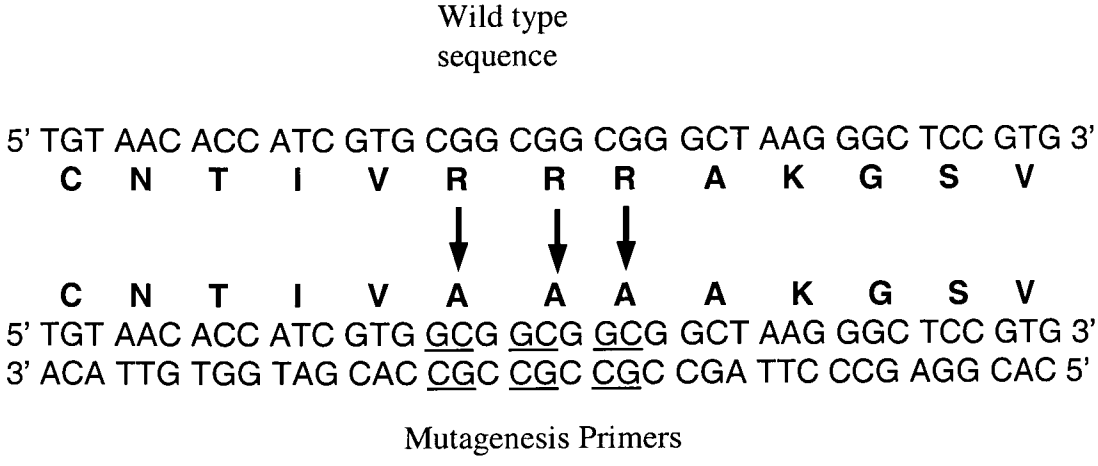

Fig 4.4 Disruption of NLS1 to create NLS1-MKP-2.
cDNA and protein(bold) sequences showing both wild type MKP-2 (upper) and NLS1-MKP-2(lower) and the primer sequences used to create the mutations. Underlined bases indicate areas of sequence that differ from the wild type MKP-2

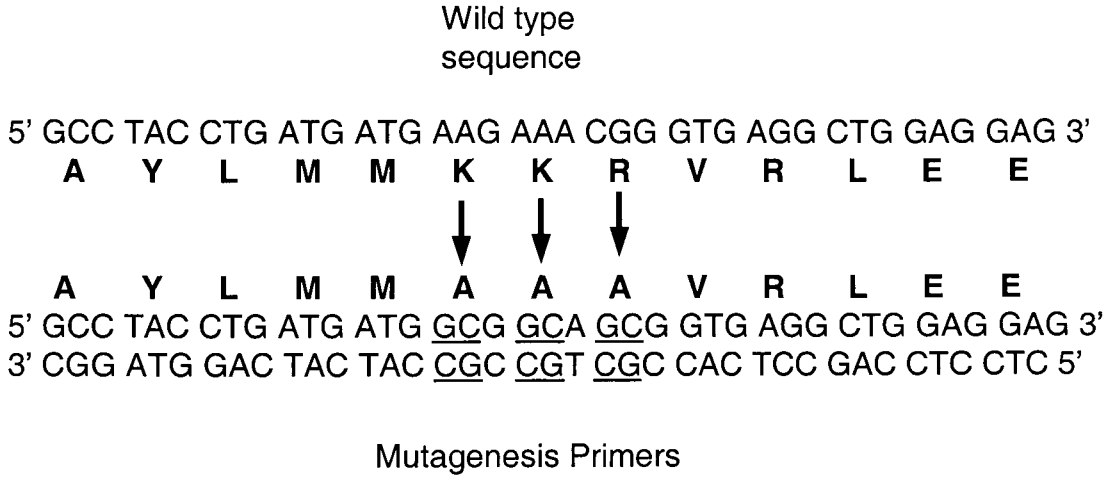

Fig 4.5 Disruption of NLS2 to create NLS2-MKP-2.
cDNA and protein(bold) sequences showing both wild type MKP-2 (upper) and NLS2-MKP-2(lower) and the primer sequences used to create the mutations. Underlined bases indicate areas of sequence that differ from the wild type MKP-2

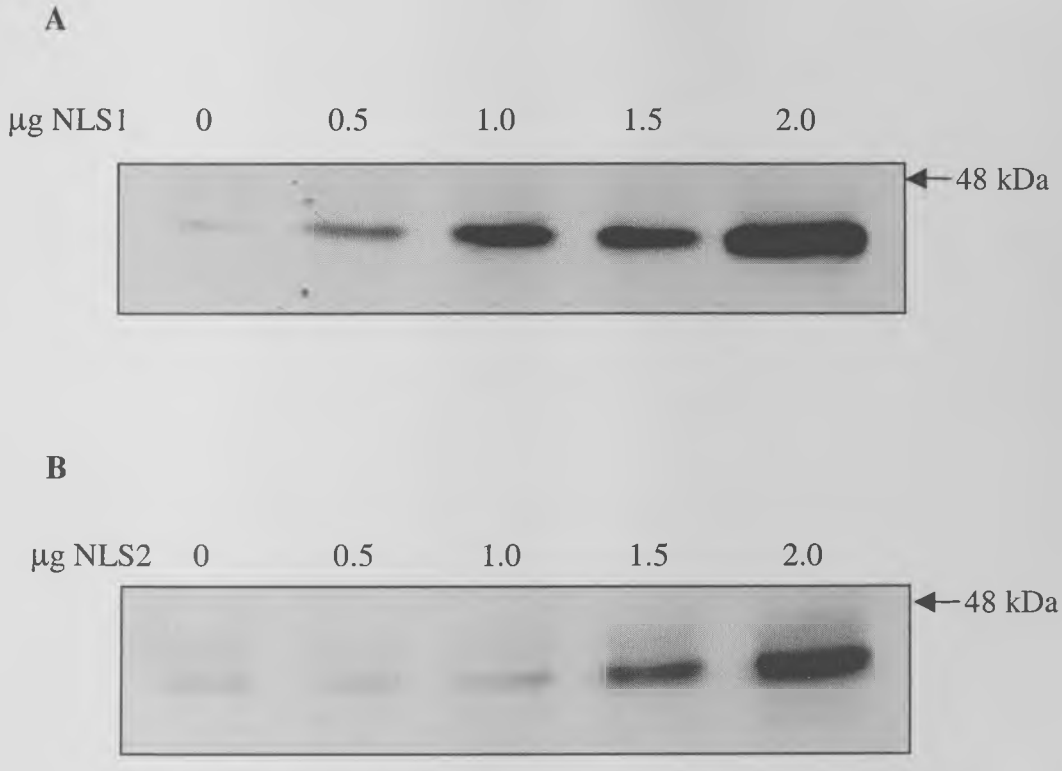

Figure 4.6. Immunodetection of NLS1-MKP-2 and NLS2-MKP-2.
HEK293 cells were transfected with increasing amounts of NLS1- and NLS2-MKP-2 cDNA. Cell extracts were analysed by Western blotting using an anti MKP-2 antibody as described in section 2.5.5. (A) NLS1. (B) NLS2. Blots are indicative of at least 2 experiments.

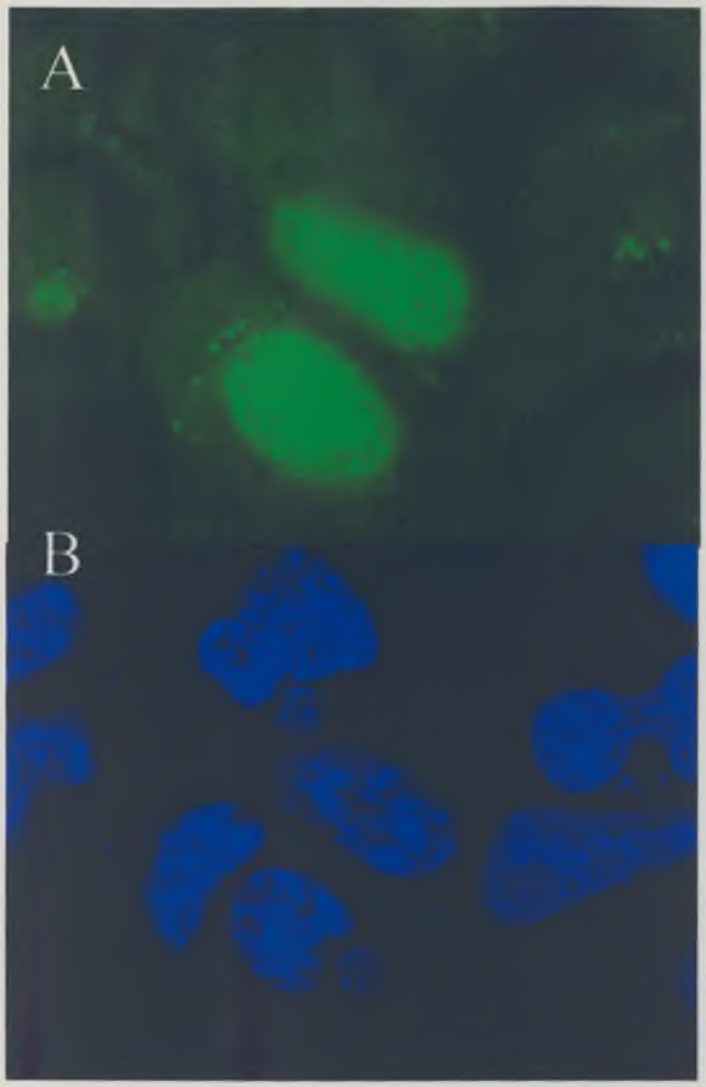

Figure 4.7 Subcellular localisation of NLS1-MKP-2 in HEK293 cells. HEK293 cells were transiently transfected with 1μg of NLS1-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X100.

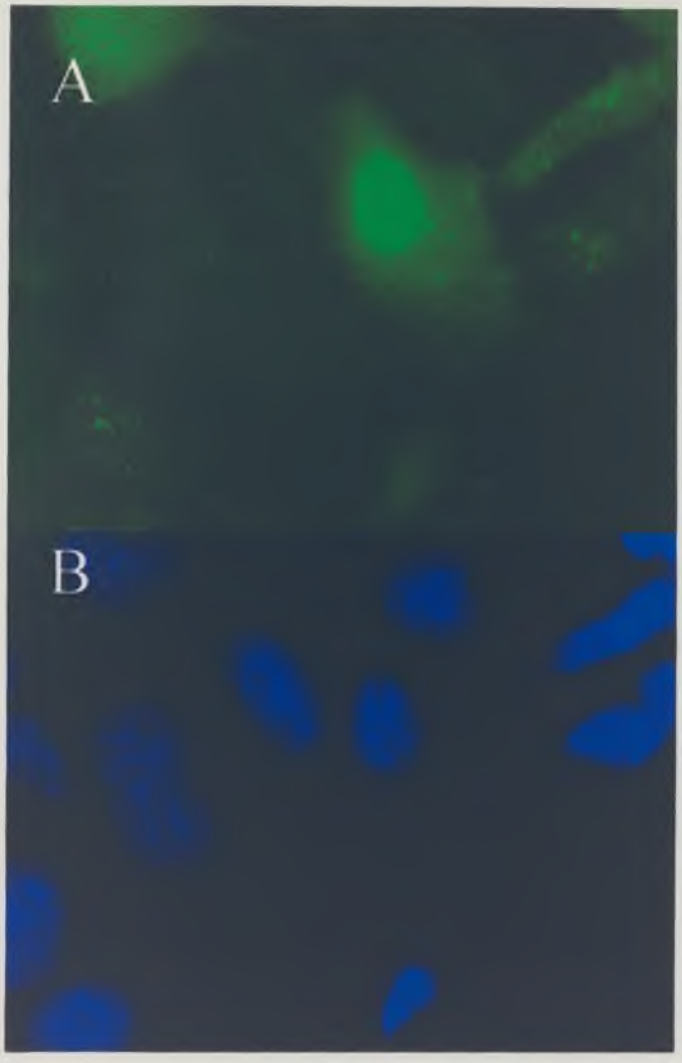

Figure 4.8 Subcellular localisation of NLS2-MKP-2 in HEK293 cells. HEK293 cells were transiently transfected with 1μg of NLS2-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X60.

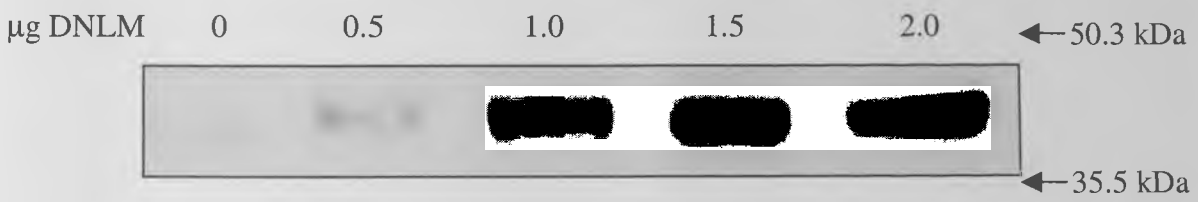

Figure 4.9. Immunodetection of DNLM-MKP-2.
HEK293 cells were transfected with increasing amounts of the DNLM-MKP-2. Cell extracts were analysed by Western blotting using an anti MKP-2 antibody as described in section 2.5.5. Blots are indicative of at least 2 experiments.

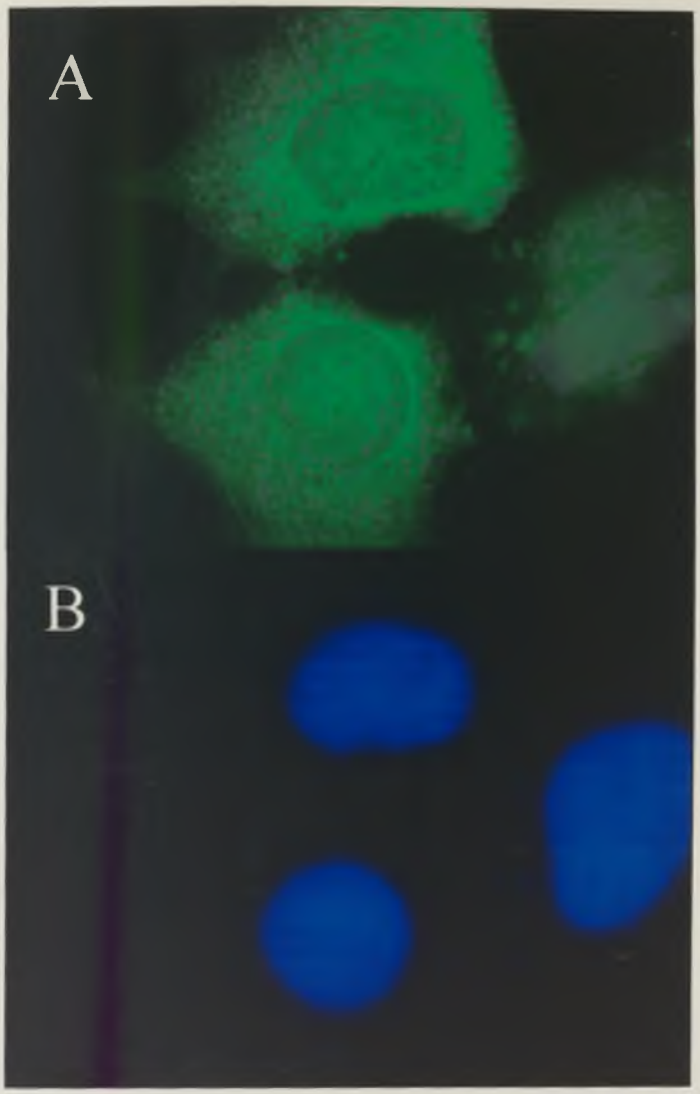

Figure 4.10 Subcellular location of DNLM-MKP-2 in HEK293 cells. HEK293 cells were transiently transfected with 1μg of DNLM-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X100

analysed for the presence of MKP-2 by Western blotting. As can be seen in figure 4.11 the WT-MKP-2 is localised predominantly within the nucleus. The NLS1 and NLS2-MKP-2 are also principally localised within the nucleus. DNLM-MKP-2 however was localised predominantly within the cytosol thus supporting the data in figure 4.10. Some contamination of the cytosolic extracts was observed suggesting the preparation of extracts was imperfect.

The next stage was to elucidate what effect, if any, the sequence changes had on the activity of the MKP-2 mutants. The activity was assessed as before by transient transfection of both HA-tagged JNK-1 and the MKP-2 mutant in question, as the localisation work had been carried out in HEK293 cells these were also used for the assessment of MKP-2 mutant activity. As shown in fig 4.12 both NLS1 and NLS2 are still effective in inactivating JNK by reducing its activation from 12 fold of basal down to 2 and 5 fold respectively. DNLM however is unable to inactivate HA-JNK-1.

4.2.4 Addition of NES to MKP-2

Having identified two NLS that control the nuclear locale of MKP-2 it was decided to attempt to obtain a cytosolic MKP-2 by a second method, the addition of the putative NES sequences from PYST1 into the sequence of MKP-2. This mutant could then be used to analyse the relative strengths of the NLS and NES and gauge their importance in the control of MKP locale. Additionally this method would hopefully generate a cytosolic mutant that would have unaltered MAP kinase binding properties.

PYST1 contains two putative NES termed NES1 and NES2 (fig 4.13). Similar to NLS, NES do not have a well-defined consensus sequence that can be used to specifically identify a NES. Most of the NES discovered to date are similar to the NES characterised on PKI in that they are short sequences containing four or five hydrophobic residues, usually leucines. The putative NES within the sequence of PYST1 had to be identified 'by eye' as there are no computer analysis programs with the capability to distinguish a distinct pattern of leucines involved in NES activity. As both NLS and NES function via their interaction with other proteins the putative

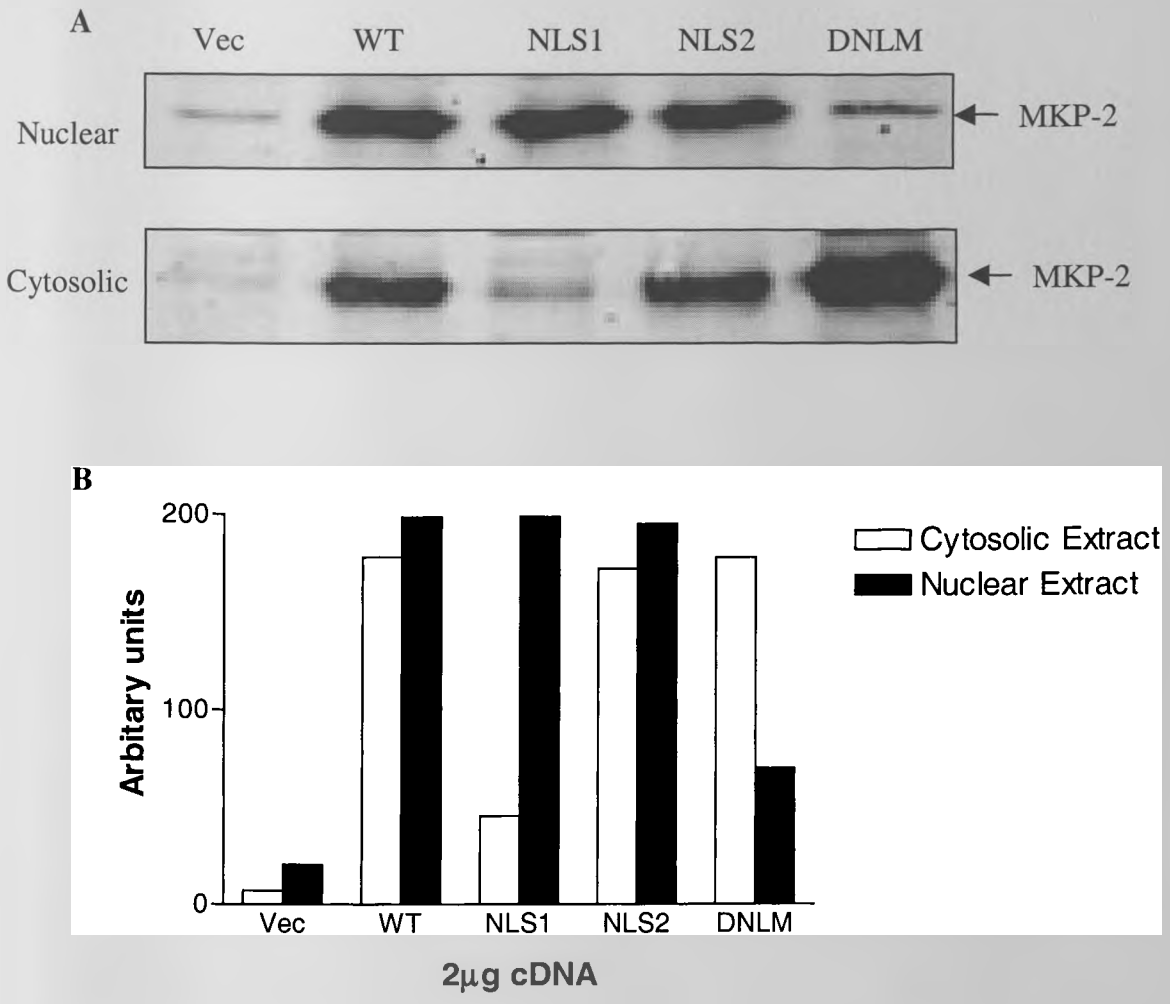

Figure 4.11. Western blot analysis of nucleocytoplasmic localisation of WT and mutant MKP-2s.

HEK 293 cells were transiently transfected with 2µg of WT, NLS1, NLS2 and DNLM MKP-2. Nuclear and cytosolic proteins were separated as described in section 2.5.2 and 5µg of extracts were used for Western blotting with an anti-MKP-2 antibody as described in section 2.5.5. A. Nuclear extracts and Cytosolic Extracts. C & D. Semi-quantitative evaluation of nuclear and cytosolic levels by scanning densitometry.

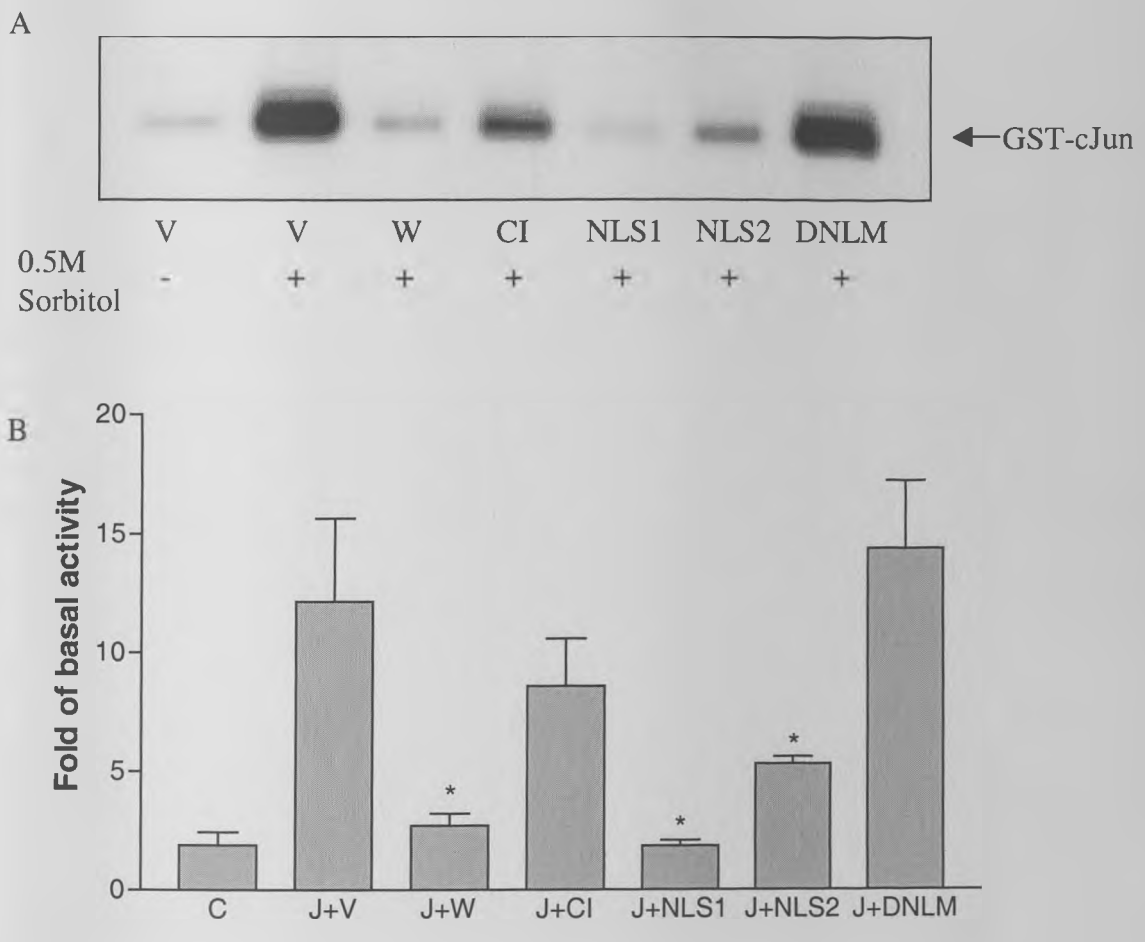

Figure 4.12 Inactivation of HA-JNK-1 in HEK293 cells by the MKP-2 localisation mutants.

HEK293 cell were transiently transfected with 0.1μg of HA-JNK-1 (J) and 1μg of either vector (V), WT-MKP-2 (W), CI-MKP-2 (CI), NLS1-MKP-2 (NLS1), NLS2-MKP-2 (NLS2) or DNLM-MKP-2 (DNLM). Cells were stimulated with vehicle (-) or 0.5M sorbitol (+) for 60 min and then the JNK Activity was assessed by *in vitro* kinase assay using GST-c-$Jun_{(5-89)}$ as substrate as described in Section 2.5.8. (A) Autoradiographs are indicative of at least 3 experiments. (B) Semi-quantitative analysis of HA-JNK-1 activity by scanning densitometry expressed as fold of basal levels. Mean ± s.e.m. (n= 3). "* " indicates $P<0.05$ vs J+V.


MKP-2 MVTMEELREMDCSVLKRLMNRDENGGGAGGSGSHGTLGLPSGGKCLLLDCRPFLAHSAGY 60
PYST1 --MIDTLRPVPFASEMAISKTVAWLNEQLELGNE---------RLLLMDCRPQELYESSH 49
MKP-2 ILGSVNVRCN-TIVRRRAKGSVSLEQILPAEEEVRARLRSGLYSAVIVYDEGSP-RAESL 118
PYST1 IESAINVAIPGIMLRRLQKGNLPVRALFTRGEDRDRFTRRCGTDTVVLYDESSSDWNENT 109
MKP-2 REDSTVSLVVQALRRNAERTDICLLKGGYERFSSEYPEFCSK--TKALAAIPPPVPPSAT 176
PYST1 GGESLLGLLLKKLKD--EGCRAFYLEGGFSKFQAEFSLHCETNLDGSCSSSSPPLPVLGL 167
NES1
NES2
MKP-2 EPLDLG----------CSSCGTPLHDQGG--PVEILPFLYLGSAYHAARRDM 216
PYST1 GGLRISSDSSSDIESDLDRDPNSATDSDGSPLSNSQPSFPVEILPFLYLGCAKDSTNLDV 227
MKP-2 LDALGITALLNVSSDCPNHFE--GHYQYKCIPVEDNHKADISSWFMEAIEYIDAVKDCRG 274
PYST1 LEEFGIKYILNVTPNLPNLFENAGEFKYKQIPISDHWSQNLSQFFPEAISFIDEARGKNC 287
MKP-2 RVLVHCQAGISRSATICLAYLMMKKRVRLEEAFEFVKQRRSIISPNFSFMGQLLQFESQV 334
PYST1 GVLVHCLAGISRSVTVTVAYLMQKLNLSMNDAYDIVKMKKSNISPNFNFMGQLLDFERTL 347
MKP-2 LATSCAAEAASPSGPLRERGKTPATPTSQFVFSFPVSVGVHSAPSSLPYLHSPITTSPSC 394
PYST1 GLSSPCDN----------RVPAQQLYFTTPSNQNVYQVDSLQST---------- 381

Fig 4.13 Alignment of MKP-2 and PYST1 protein sequences.
Boxed areas indicate putative NES of PYST1. Arrows indicate residues of MKP-2 that were changed to leucine to create the NES-MKP-2 mutants.

NES were added to the areas of MKP-2 that corresponded to similar areas of PYST1 (fig 4.13). Due to the similarity in size and function of these two MKPs this would hopefully lead to the mutant NES on MKP-2 being presented in a position favourable for interaction with the nuclear export machinery.

Mutagenesis primers were designed to change certain residues within MKP-2 to leucine (fig 4.14 and 4.15), it was also possible to utilise the presence of native leucines within the sequence of MKP-2 to minimise the disruption to the native sequence and yet still hopefully incorporate a functional NES. Mutagenesis was carried out as above with the Quickchange™ reagents from Stratagene using WT-MKP-2 cDNA as a template. The putative NES lying towards the N-terminus (from aa114 to aa122) of PYST1 was incorporated into amino acids 127 to 134 in MKP-2 to create NES1-MKP-2 (see figure 4.14). Similarly, the NES from PYST1 closest to the C-terminus (aa162 to aa170) was incorporated into amino acids 164 to 167 of MKP-2 to create NES2-MKP-2 (see figure 4.15). Changes to sequences and fidelity of PCR products were confirmed by sequencing (data not shown).

To ensure the changes had not altered the primary structure of the MKP-2 NES1-MKP-2 and NES2-MKP-2 were expressed in HEK293 cells and the lysates analysed by Western blotting. As expected both mutants still reacted with the MKP-2 antibody and migrated at the expected size of ~43KDa (Fig 4.16).

Next, the subcellular distribution of the NES-MKP-2 mutants was assessed. As before the mutants were expressed in HEK293 cells and stained using FITC conjugated secondary antibodies and DAPI, to counter stain the nucleus. Both NES1-MKP-2 and NES2-MKP-2 were located in the nucleus (Fig 4.17 and 4.18).

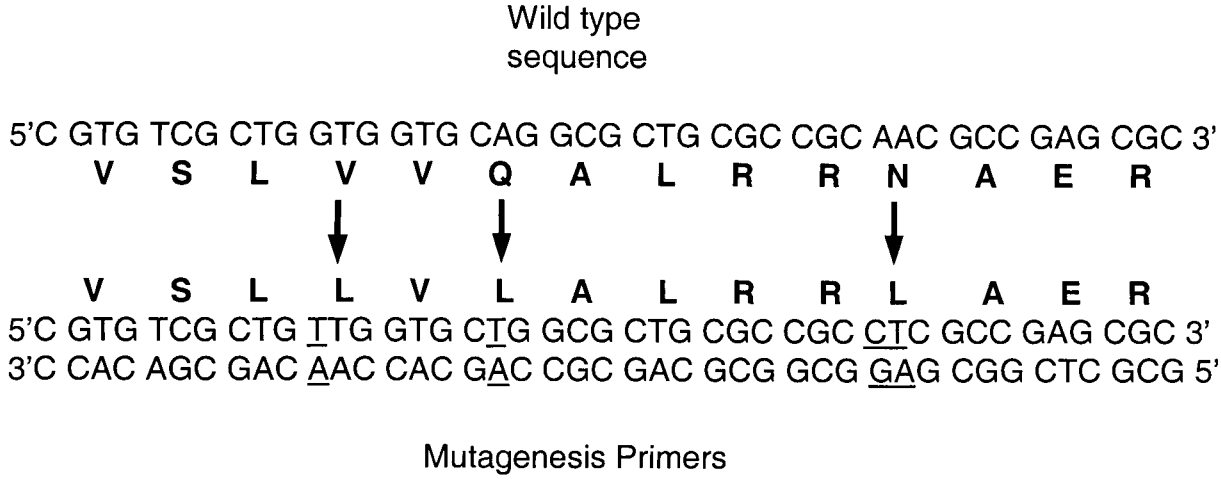

Fig 4.14 Mutagenesis of WT-MKP-2 to create NES1-MKP-2.
cDNA and protein (bold) sequences showing both wild type MKP-2 (upper) and NES1-MKP-2(lower) and the primer sequences used to create the mutations. Underlined bases indicate areas of sequence that differ from the wild type MKP-2

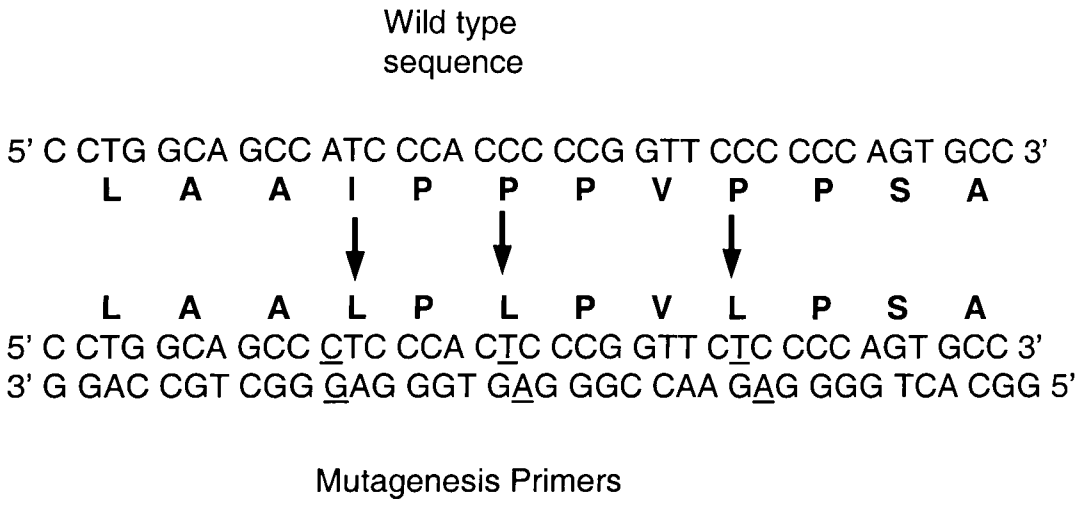

Fig 4.15 Mutagenesis of WT-MKP-2 to create NES2-MKP-2.
cDNA and protein (bold) sequences showing both wild type MKP-2 (upper) and NES2-MKP-2(lower) and the primer sequences used to create the mutations. Underlined bases indicate areas of sequence that differ from the wild type MKP-2

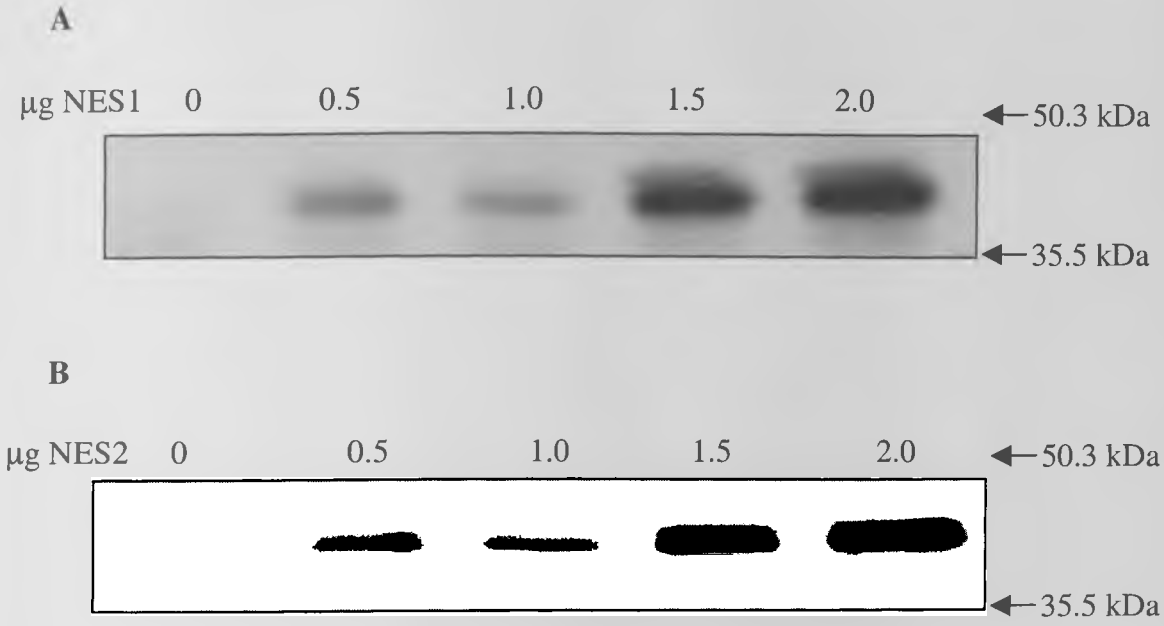

Figure 4.16. Immunodetection of NES1-MKP-2 and NES2-MKP-2.
HEK293 cells were transfected with increasing amounts of NES1- and NES2-MKP-2 cDNA. Cell extracts were analysed by Western blotting using an anti MKP-2 antibody as described in section 2.5.5. (A) NES1. (B) NES2. Blots are indicative of at least 2 experiments.

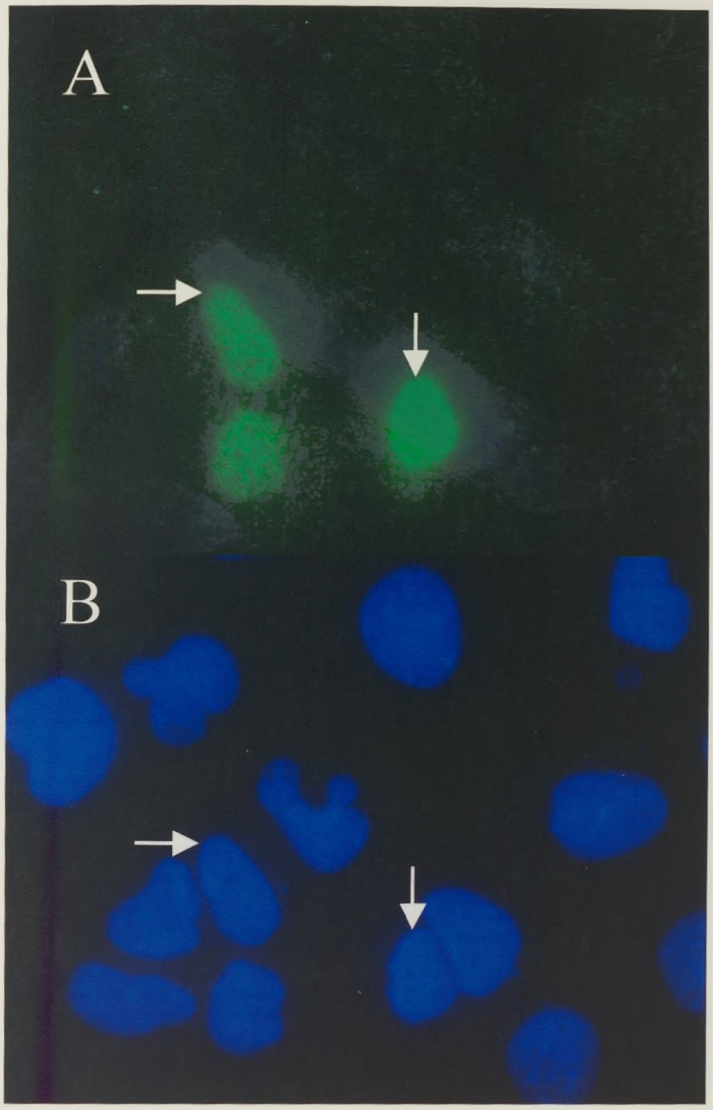

Figure 4.17 Subcellular location of NES1-MKP-2 in HEK293 cells. HEK293 cells were transiently transfected with 1μg of NES1-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X60. Arrows indicate same nuclei in both fields.

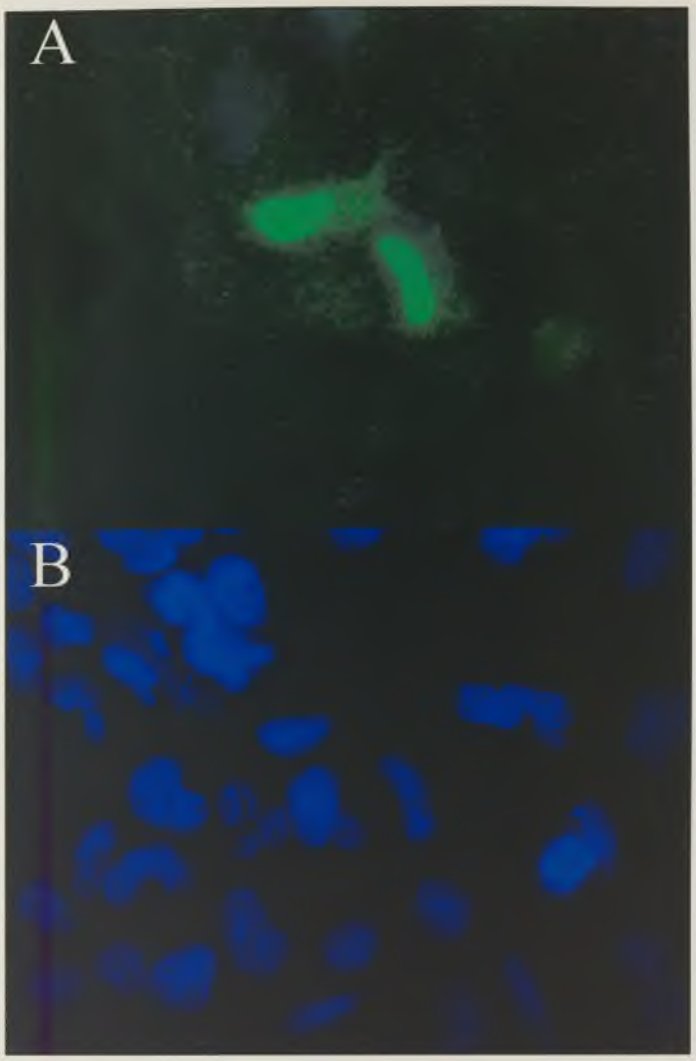

Figure 4.18 Subcellular location of NES2-MKP-2 in HEK293 cells.
HEK293 cells were transiently transfected with 1μg of NES2-MKP-2. Cells were fixed and stained as described in Section 2.5.7. A. FITC staining against MKP-2. B. DAPI stain. Magnification X40.

4.3 Discussion

This chapter describes the analysis of the localisation of MKP-2 and the sequences controlling this localisation. Analysis of WT-MKP-2 and CI-MKP-2 localisation showed them to be nuclear in HEK293 cells. The nuclear locale of the CI-MKP-2 indicates that the phosphatase activity of MKP-2 is not required for maintenance of its localisation. MKP-2 has been previously described, by other groups, as a nuclear DSP and this localisation was thought to be controlled by two NLS sequences (Guan & Butch, 1995). The first of these, NLS1, sequence roughly matched the consensus sequence for an SV40 like monopartite NLS. This type of NLS is characterised by a group of 4 or 5 hydrophobic amino acids, usually Lysines and Arginines, within a six amino acid stretch. (Christophe *et al.*, 2000). The NLS1 of MKP-2 is **RRR**AK. The alanine present in the sequence is probably the reason the search algorithm, used by the PSORTII website to find NLS, does not recognising this sequence as a potential NLS. The second NLS of MKP-2, NLS2 (**KKR**VRLEEAFEFV**KQRR**), is recognised as a bipartite NLS by PSORTII as it matches the archetypal bipartite NLS discovered in nucleoplasmin (**KR**PAATKKAGQA**KKKK**) (Robbins *et al.*, 1991). Proof of NLS function can be achieved in one of two ways. Firstly by adding the sequence in question to a cytosolic protein which is too large to enter the nucleus by passive diffusion, and then assessing whether this cytosolic protein becomes nuclear. Secondly by disruption of the putative NLS and assessing if the mutant protein becomes cytosolic. Disruption of NLS usually involves either deletion of the entire NLS sequence or point mutation of the positive residues within the NLS to alanine or another non-charged amino acid.

The point mutation method was chosen to disrupt the NLS of MKP-2 as it is less likely than the deletion method to cause a conformational change in the protein that may affect protein integrity or activity. Several previous studies have used point mutation to disrupt NLS. Kleinschmidt *et al* showed that mutation of a specific lysine to threonine in either part of a putative bipartite NLS led to cytoplasmic accumulation of the Xenopus histone binding protein N1. Mutation of other lysines within the two ends of the bipartite NLS had little or no effect suggesting that certain positive residues within the NLS play a critical role in NLS function (Kleinschmidt

& Seiter, 1988). The study that identified the archetypal bipartite sequence in nucleoplasmin also indicated the requirement for positive charge at both the N- and C- terminal areas of a bipartite NLS (Robbins *et al.*, 1991). Replacement of an N-terminal arginine with the positive residue lysine had no effect on NLS function, but changing the same arginine to the negatively charged residue asparagine resulted in loss of NLS function. Mutation of the C-terminal end of the NLS showed that again one specific residue was more important than the surrounding positive residues (Robbins *et al.*, 1991). More recently, a study to analyse a putative NLS in avian sarcoma virus showed mutation of single lysines and arginines to alanine was sufficient to prevent NLS function (Kukolj *et al.*, 1998). In summary of the above group's work and many others not mentioned here, basic type NLS require a positive charge and certain residues within their sequence are more critical than others. Similarly, bipartite NLS must be positively charged at both ends and also have residues which appear to have greater importance to function than others. These residues, which are critical for function, do not appear to have a fixed location within the NLS sequence. The tertiary shape and charge of the NLS presented to the carrier molecules is probably of more importance that the exact sequence, thus allowing for the large amount of variability seen between differing proteins NLS.

Using this information it was decided that in order to disrupt the NLS of MKP-2 that the first three arginine residues of NLS1 would be targeted for mutation to alanine in order to reduce the positive charge of the area and also hopefully disrupt a residue critical for NLS function. For NLS2 the N terminal end was chosen for mutagenesis. As either pair of positive residues: K298-K299 or K299-R300, would theoretically be able to function as the N terminal end of the NLS it was decided to change all three residues to alanine.

Disruption of NLS1 or NLS2 had no effect on the localisation of MKP-2 suggesting one of two possibilities; firstly that neither NLS is involved in nuclear targeting of MKP-2 and that localisation is controlled by some unidentified sequence. Secondly that both NLS are functional and are able to compensate for the loss of the other. The later hypothesis was proven to be the case by the creation of the DNLM-MKP-2 clone, which lacked both NLS1 and NLS2 and was found to be localised within the cytosol of transfected cells. These findings are in contrast with

recent data published by Chen *et al* (2001) during the final stages of this work. Chen *et al* (2001) suggest that the same mutations used here to create NLS1-MKP-2 do have an effect on the localisation of MKP-2. They report that in HeLa cells NLS1-MKP-2 expressed at high levels is no longer tightly localised to the nucleus. They do however, report the difference in localisation is dependent on expression level of the mutant MKP-2 suggesting that lower level expression of the mutant results in nuclear localisation (Chen *et al.*, 2001). As the group do not state the amounts of MKP-2 expressed within the cells it is impossible to compare to the work shown here. However during staining experiments it was noted that some cells staining more brightly than others did have an even distribution of staining throughout the cytosol and nucleus. As this phenomenon has been observed in experiments staining for many differing proteins, including WT and mutant MKP-2, and often appears in cells with an irregular and unhealthy appearance. It was assumed that either the nuclear envelope was 'leaking' in cells in the latter stages of apoptosis or that the antibodies were not being 'washed' from damaged cells efficiently. It is conceivable that the harsh mechanisms used during transfection and the artificial modulation of the MAPK pathways may, in some cells, lead to cell death, poor staining, and the misinterpretation of the localisation of proteins. The separation of the cell into its cytosolic and nuclear components further indicates the cytosolic location of the DNLM-MKP-2 mutant. It is clear that there is some contamination of the cytosolic fraction by nuclear proteins, hence the presence of WT-MKP-2 in the cytosolic fraction. This could be due to lysis of some nuclei during collection of the cytosolic extract. Or possibly, in agreement with Chen *et al* (2001), the high level of expression from the vector results in 'leakage' of the expressed proteins into the cytosol. Either way, the DNLM-MKP-2 mutant is still chiefly found in the cytosol with very little nuclear staining, unlike the other mutants which all show strong staining in the nucleus suggesting the DNLM-MKP-2 mutant is indeed predominantly cytosolic.

To further analyse the changes of sequence effects on MKP-2 the phosphatase activity of the MKP-2 mutants was assessed using stress-induced JNK1 activity. As before WT-MKP-2 could effectively reduce JNK1 activity, and the CI-MKP-2 had

little effect. NLS1-MKP-2 retained much of its ability to inactivate JNK1 which agrees with the data shown by Chen *et al* that suggests the NLS1 area of MKP-2 is involved in the interaction of MKP-2 with p38 and ERK but not JNK (Chen *et al.*, 2001). NLS2-MKP-2 although still able to significantly reduce JNK1 activity was not as effective as WT or NLS1-MKP-2. This may suggest that JNK interaction with MKP-2 is not controlled by the N terminal region, as with many of the other MAPKs and MKPs, but instead interaction may be mediated by sequences within the C-terminal catalytic area of MKP-2.

To analyse the differing binding properties and phosphatase activity displayed by the various mutants, GST-fusion proteins of WT and all mutant variants of MKP-2 were created. This was done using PCR to add restriction enzyme sites that allowed the in frame directional subcloning of the MKP-2s into the GST containing pGex4T3 vector (pharmacia). Sequencing confirmed the in-frame cloning of both the WT- and CI-MKP-2 fusion proteins. Expression and purification of these proteins from bacteria proved difficult, levels of expression were low with high levels of contaminating proteins. This may be due to a number of reasons including toxicity of the construct or possibly mutation within the expression machinery of the vector. Other groups have utilised this same style of experiment to assess MKP interaction with the MAP kinases and they utilised a mammalian expression system to isolate GST fused MKPs. As this is a much more expensive process than bacterial expression, it may suggest they also had problems with a bacterial system (Hutter *et al.*, 2000). Because of this difficulty the analysis of mutant binding and activity *in vitro* could not be assessed.

To further characterise the function of NLS and NES sequences in control of MKP locale, the putative NES of PYST-1 were added to WT-MKP-2 in an attempt to alter its subcellular distribution. As mentioned above the lack of a definitive consensus sequence for NES identification makes isolating putative NES by sequence analysis alone very difficult. Similar to NLS proof of NES function can be obtained in two ways. One method involves disrupting the NES by mutagenesis or deletion resulting in altered distribution of the protein. Or secondly, by changing the locale of another protein from the nucleus to the cytosol by addition of the putative NES. Although by sequence alone NES are more difficult to identify than NLS, the

presence of a functional NES can be detected using the NES inhibitor, Leptomycin-B (see section 1.7).

Analysis of the sequence of the cytosolic MKP, PYST1 revealed two putative NES, NES1 and NES2. Both these NES were similar to the NES previously described on PKI (**L**x**L**x**L**xx**L**) and HIV1-REV (**L**x**L**xx**L**xx**L**x**L**) (Wen *et al.*, 1995). NES1 takes the form **LL**x**LLL**xx**L**, whereas NES2 is **L**xx**L**x**L**xx**L**. Addition of these NES, individually, to WT-MKP-2 failed to alter the locale of MKP-2. This may be due to the either of the single NES being able to 'overpower' the two native NLS.

Previous evidence has suggested that in proteins containing both NES and NLS, the NES activity is more 'effective' than NLS leading to predominantly cytosolic locale. Addition of both an NES and an NLS to a GFP tagged reporter protein was used to assess the competition between these signals in *sacheromyces cerevisiae* . The reporter protein was located in the cytosol indicating the rate of nuclear export is higher than the import rate (Stade *et al.*, 1997). Also, LIM-kinase-1 contains both NES and NLS and yet appears predominantly cytosolic until either the addition of leptomycin-B or disruption of NES by mutagenesis renders it nuclear (Yang & Mizuno, 1999). Similarly MKP-7 contains both NLS and NES and staining shows MKP-7 to be cytosolic. Again, addition of leptomycin-B induces nuclear accumulation of the protein. As leptomycin-B inhibits nuclear export of proteins, these leptomycin-B sensitive proteins must be present in the nucleus. This suggests that proteins bearing both NLS and NES shuttle in and out of the nucleus but the 'stronger' NES give the impression of a predominantly cytosolic locale.

Chapter 5

General Discussion

5.1 General Discussion

As discussed earlier it is obvious that the localisation and ability to change localisation is critical to the function of the MAP kinases. *Ipso facto* the localisation of the MKPs is also critical for MAP kinase control. Studies to date have mainly focused on either the activity and specificity's of the various MKPs or the factors controlling their transcription and translation. An obvious avenue of research was the analysis of the mechanisms involved in the control of MKP localisation. To elucidate this MKP-2 and MKP-3 were chosen for study as they are clearly defined as nuclear and cytosolic respectively.

The data presented here serves to confirm the specificity of human MKP-2 towards the JNK members of the MAP kinase superfamily. Verification of the ability of MKP-2 to inactivate the ERK family of MAP kinases could not be achieved in this system as ERK was constitutively cytosolic in the cell types used. To address this problem either a stimulus, which induces nuclear accumulation of ERK, or a differing cell type with defined translocation of ERK could be used. Previous studies have shown clear nuclear accumulation of ERK in response to serum in fibroblasts (Brunet *et al.*, 1999; Lenormand *et al.*, 1998), this cell type could perhaps be used to confirm MKP-2 activity towards ERK in similar manner to the co-transfection system used here.

The co-transfection techniques used to show the specificity of MKP-2 have been used before by other groups with great success and so a similar approach was utilised (Chu *et al.*, 1996). The limitations of this approach must however be noted in that the physiological concentrations at which these proteins naturally interact are far lower than those achieved in a transient transfection system. This may lead to protein-protein interactions that would not normally be seen and thus false indications of specificity. The data obtained was analysed with these limitations in mind, but as all the data attained supported previous indications of MKP-2 specificity it was assumed to be accurate.

This thesis also identifies two regions within the sequence of MKP-2 that together function to direct the nuclear localisation of MKP-2. As described in section 1.6 control of subcellular locale can be mediated by short areas of protein sequence,

namely NLS and NES. Analysis of the MKP-2 sequence, both by one of the original cloning groups and this study indicated the presence of two NLS sequences within MKP-2 and two NES within MKP-3. As described in this thesis either of the NLS within MKP-2 is sufficient to direct nuclear localisation. During the preparation of this work another group studying the interaction of MKP-2 with the MAP kinases also identified NLS1 as a sequence involved in the targeting of MKP-2 to the nucleus. The identification of NLS2 however, represents the discovery of a novel sequence involved in the control of MKP2 localisation.

Addition of the individual NES sequences believed to be responsible for the cytosolic locale of MKP-3 failed to alter the locale of MKP-2. However, the addition of both NES concomitantly to MKP-2 may be able to override the NLS control of locale. As mentioned above, previous studies have suggested that NES are 'stronger' than NLS and thus proteins with both tend to show a cytosolic distribution. Any protein that cycles via the NES machinery however, can be easily identified with the NES inhibitor Leptomycin-B. Full analysis of the relative importance and strengths of the NLS of MKP-2 will likely require the creation of mutants that include the addition of NES concomitantly to the disruption of NLS and the use of leptomycin-B.

The main priority of this study was to create a cytosolic MKP-2 mutant that could be used as a tool in analysis of cytosolic versus nuclear signalling of the JNK and ERK pathway. Although successful in the creation of a cytosolic MKP-2 mutant, its use for further study may be limited. As mentioned above the mutation used to create NLS1-MKP-2 may also have an effect on substrate interaction and thus may affect the mutants ability to act as a cytosolic anchor for JNK and ERK signalling (Chen *et al.*, 2001). To further this line of study it would require the generation of epitope-tagged versions of all MKP-2 mutants and analysis of their ability to bind and co-precipitate substrates *in vitro*. This work was started but isolation of acceptably pure GST tagged WT- and CI-MKP-2 proved difficult. The reason for this is unclear although may have been due to the toxicity of these proteins at high levels in the expressing bacteria. Other groups also looking at MKP to MAP kinase interaction have utilised similar binding experiments. Isolation of MKPs by these

groups was achieved from mammalian cells. This may suggest that they also had trouble with the more widely used bacterial expression systems (Hutter *et al.*, 2000). Isolation of GST tagged MKP-2 is being attempted from an mammalian system.

When this study was started the DSPs with both nuclear and cytosolic locale had yet to be identified and characterised. In hindsight, creation of localisation mutants of M3/6 or MKP-7 may make a more useful tool to study the differences in cytosolic and nuclear MAP kinase signalling. Nishida *et al* cloned MKP-7, which interestingly, contained both NLS and NES sequences. The NLS sequence of MKP-7 was similar to the NLS of MKP-2; the NES however was located on the C-terminal tail that is not present on MKP-2. This unfortunately makes it impossible to accurately add the NES of MKP-7 to MKP-2 in an attempt to create a cytosolic or shuttling protein. MKP-7 may still make a useful tool for studying the functions of the MKP family in both nuclear and cytosolic cellular compartments and also assessing the relative strengths of NLS and NES sequences. MKP-7 may represent an ancestral protein that serves a general function in MAP kinase inactivation over the whole cell. The VHR like DSPs may also be ancestors of the MKP- family as they lack of the N terminal regulatory domain suggesting these phosphatases are not restricted to dephosphorylating only the MAP kinases and may function to maintain several families of dual phosphorylated kinases in a basal state. Indeed the original DSP cloned from vaccinia virus, VH1, was able to dephosphorylate many substrates other than the MAP kinases.

One of the main areas of current MKP study that will continue to attract interest in the future is the mechanism of control and substrate specificity. These problems are starting to unravel with the discovery of cellular processes that can effect the activity and half-life of MKPs *in vivo*. However there still remains a lot to be elucidated, it is unclear as yet whether this phenomenon of increased half-life of MKP-1 by ERK phosphorylation is MKP-1 specific or is common to all MKPs. Studies that generate data relating to the control of the activity of the MKPs may lead to the discovery of novel therapeutics in cancer treatment and prevention.

Another area, which requires further research, is into the promoter systems involved in MKP expression. The inducible expression of MKPs by MAP kinases

has been shown to play a role in a possible negative feedback system. This system appears to involve cross-cascade activation of MKPs as ERK can stimulate MKP-2 expression but the data in this thesis and elsewhere suggests MKP-2 is best able to target JNK. If this system is employed by all MAP kinases it may allow one specific group of MAP kinases to inhibit the others, preventing the transmission of conflicting signals. The roles of the MKPs in this process may again prove a viable target for therapeutic intervention although other regulatory pathways have also been implicated in the control of the expression of MKPs, including increased intracellular Ca^{2+} (Cook *et al.*, 1997), and PKA activity (Pursiheimo *et al.*, 2002).

The exact mechanism of increased MKP expression is not yet fully understood and as yet only the promoter regions of MKP-1, PAC1 and MKP-2 have been looked at in detail. These studies identified several areas involved in increasing MKP expression including two CRE sites and an E box in MKP-1 an AP2 site in PAC1 and a GnRH responsive element in MKP-2. This may indicate differing roles for the MKPs in the cell response to various transcription increasing stimuli. Clearly for a full understanding of MKP function the differences and similarities between the control elements of all MKPs must be studied in detail.

Many of the factors controlling MKP protein level do not act upon the genetic promoter machinery but on the mRNA itself. This type of control is dependent on the mRNA levels and thus on cell type and stimuli. The tissue distribution of the MKPs is wide and varied and this may prevent the ability to generalise MKP function based on promoter structure and substrate specificity. It may indeed be necessary to isolate the functions of each individual MKP in every cell or tissue type separately.

Tyrosine and serine/threonine phosphorylation is well documented to alter protein behaviour in many ways. In the future it would be interesting to study the phosphorylation state of MKP-2 under varying conditions for a number of reasons. Firstly to identify if indeed MKP-2 is stabilised in a manner similar to MKP-1 and whether activity or activation by MAP kinase binding are changed by an altered phosphorylation state. Additionally, the activity localisation signals can also be affected by adjacent phosphorylation although this is more likely to be of interest in

proteins whose locale is variable between cytosol and nucleus, like M3/6 and MKP-7.

The MKPs are still an emerging family and as such require a great deal of further research to fully determine their role in cellular signalling. These studies have outlined a novel mechanism for control of MKP locale and hopefully will pave the way for extended research into the mechanisms of MKP locale and possibly lead to the creation of proteins that will be useful analytical tools in the continued elucidation of the MAP kinase signalling pathways.

Chapter 6

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6.1 References

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