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INTERACTIONS AMONG THE MITOGEN-ACTIVATED PROTEIN KINASE CASCADES AND THE IDENTIFICATION OF A NOVEL cdc2-RELATED PROTEIN KINASE

by

Susan Randall

A thesis submitted in conformity with the requirements for the Degree of Master of Science, Graduate Department of Medical Biophysics, in the University of Toronto

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Interactions among the mitogen-activated protein kinase cascades and the identification of a novel cdc2-related protein kinase.

Susan Randall
Master of Science, 1997
Department of Medical Biophysics
University of Toronto

ABSTRACT

Signal transduction is the means by which cells recognize and respond to changes in their external environment through the modification of cytoplasmic and nuclear events. In several cases, transduction of the signal to the nucleus of the cell occurs through cascades of protein kinases.

In mammalian cells, structurally homologous mitogen-activated protein kinase (MAPK) cascades regulate diverse biological functions. This raises the question of how specificity is achieved in signalling via MAPKs. With the yeast two hybrid system, we reveal that the three known MAPK pathways, ERK, SAPK, and p38, do not require scaffolding proteins to maintain signalling specificity and that crosstalk among them does not readily occur in vivo.

The cloning of a novel 67 kD cytoplasmic serine/threonine kinase is also described. This kinase, termed NKIATRE, is the third member of a unique family of kinases that are highly related to the MAPKs and the cyclin-dependent kinases.
ACKNOWLEDGEMENTS

The work accomplished in this thesis would not have been possible without the support of many people. Firstly, I would like to thank the members of my supervisory committee, Dr. Brent Zanke, Dr. Jim Woodgett, Dr. Dwayne Barber, and the late Dr. Ron Buick, for not only their support but their guidance. I would also like to thank both Kim Boudreau and Phyllis Billia for helping me to master many of the laboratory techniques I have learned and for motivating me to attend many more exercise classes than I would have on my own. To the remainder of my friends, my parents, my sister, and especially Gabe, thanks for believing in me and putting up with me!
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<p>| Ac  | acetate                           |
| ADP | adenosine diphosphate            |
| ATP | adenosine triphosphate           |
| ATF | activating transcription factor   |
| BMK | big mitogen-activated protein kinase |
| cAMP| cyclic adenosine monophosphate   |
| CAK | CDK activating kinase             |
| cdc | cell division cycle              |
| CDK | cyclin-dependent kinase           |
| CDI | cyclin-dependent kinase inhibitor |
| cDNA| complimentary DNA                |
| cGMP| cyclic guanosine monophosphate   |
| CKI | cyclin-dependent kinase inhibitor |
| dCTP| deoxycytidine triphosphate       |
| DEPC| diethyl pyrocarbonate            |
| <em>Drosophila</em> | <em>Drosophila melanogaster</em>         |
| <em>Dictyostelium</em> | <em>Dictyostelium discoideum</em>       |
| Dig | down-regulator of invasive growth |
| DNA | deoxyribonucleic acid            |
| dNTP| deoxyribonucleoside triphosphates|
| E. coli. | <em>Escherichia coli</em>              |
| EDTA| ethylenediaminetetraacetate      |
| EGF | epidermal growth factor          |
| EGFR| epidermal growth factor receptor |
| ERK | extracellular signal-regulated kinase |
| G0  | quiescent state                  |
| G1  | first gap phase                  |
| G2  | second gap phase                 |
| GCK | germinal centre kinase           |
| GDP | guanosine diphosphate            |
| GST | glutathione-S-transferase        |
| GTP | guanosine triphosphate           |
| HA  | hemagglutinin                    |
| HOG | high osmolarity glycerol response|
| HPK | hematopoietic progenitor kinase  |
| IL  | interleukin                      |
| IPTG| isopropylthio-β-D-galactoside    |
| JNK | c-Jun NH2-terminal kinase        |
| LPS | lipopolysaccharide               |
| M phase | mitosis                        |
| MAP | microtubule-associated protein  |
| MAPK| mitogen-activated protein kinase |</p>
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MAPKAPK</td>
<td>MAP kinase-activated protein kinase</td>
</tr>
<tr>
<td>MAT</td>
<td>menage a trois</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<td>MEKK</td>
<td>MEK kinase</td>
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<tr>
<td>MKH</td>
<td>MAP kinase homologue</td>
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<tr>
<td>M KK</td>
<td>MAP kinase kinase</td>
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<tr>
<td>MLK</td>
<td>mixed lineage kinase</td>
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<tr>
<td>MUK</td>
<td>MAPK upstream kinase</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenylgalactoside</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAK</td>
<td>p21 activated protein kinase</td>
</tr>
<tr>
<td>PBS2</td>
<td>polymyxin B sensitivity 2</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PKA-Cα</td>
<td>type α cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPTK</td>
<td>receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>Rst</td>
<td>regulator of sterile twelve</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SD</td>
<td>synthetic deficient</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEK</td>
<td>SAPK/ERK kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3 domain</td>
</tr>
<tr>
<td>SHO</td>
<td>synthetic, high osmolarity sensitive</td>
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<tr>
<td>SLN</td>
<td>synthetic lethal of N-end rule</td>
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<td>SOS</td>
<td>son of sevenless</td>
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<td>S phase</td>
<td>DNA synthesis phase</td>
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<tr>
<td>S. pombe</td>
<td><em>Schizosaccharomyces pombe</em></td>
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<td>SSC</td>
<td>sodium sodium citrate</td>
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<td>SSK</td>
<td>suppressor of sensor kinase</td>
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<tr>
<td>STE</td>
<td>sterile</td>
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<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
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<tr>
<td>TE</td>
<td>tris-EDTA</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract peptone dextrose</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>°C</td>
<td>degree celsius</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>kD</td>
<td>kilodalton</td>
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<td>M</td>
<td>molar</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>microgram</td>
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<td>μl</td>
<td>microlitre</td>
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<td>micromolar</td>
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<td>μmol</td>
<td>micromole</td>
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CHAPTER 1

Literature Review
1.1 INTRODUCTION

As unicellular organisms evolved into more complex multicellular forms, the ability of the cell to respond to extracellular stimuli became increasingly important to many biological processes. The ability to coordinate growth and differentiation of single cells into multicellular organisms requires the input of many growth factors, growth hormones, and growth inhibitors. In addition, exposure of cells to alterations in their external environment requires the ability to sense these changing conditions and respond to them appropriately. Cells must therefore be capable of recognizing a multitude of stimuli and translating these into coordinated signals that evoke appropriate changes in cytoplasmic and nuclear events.

The mechanism by which cells translate external stimuli to cytoplasmic and nuclear changes is known as signal transduction. Many signal transduction pathways are composed of kinase cascades which involve series of interacting proteins regulated by phosphorylation events. Kinases transfer the terminal phosphate group of ATP to serine, threonine, or tyrosine residues within their substrates. This change in phosphorylation leads to a corresponding change in the substrate's activation state. Therefore, within these cascades, the protein catalyzing each step is activated or inhibited by the product of the preceding step. Not only do kinase cascades serve to amplify the original signal but they enable the effects of that signal to be rapidly reversed by dephosphorylation through the action of phosphatases.
Many different families of kinases exist but two that have been studied in extensive detail are the MAPKs and the CDKs. In the literature review of this thesis, these kinases will be discussed in terms of their structures and functions. Further, the signalling pathways that they comprise, in addition to their involvement in cellular processes, will be highlighted.

1.2 KINASES

1.2.1 Conserved Features

The catalytic domain of a kinase imparts its enzymatic activity and functions to bind and orientate the phosphate donor (either GTP or ATP), to bind and orientate the protein substrate, and to transfer the γ-phosphate from the ATP or GTP to the acceptor hydroxyl of a serine, threonine, or tyrosine residue on the protein substrate (reviewed in Hanks and Hunter, 1995). An alignment of 65 protein kinases by Hanks et al. revealed that an overall similarity exists among the catalytic domains (Hanks et al., 1988). The catalytic domains are not conserved uniformly but consist of regions of high conservation interspersed with regions of low conservation. Within these 250 to 300 amino acids units are 12 major conserved subdomains. These are numbered sequentially from subdomain I through subdomain XI, with two subdomains VIA and VIB. These subdomains contain characteristic patterns of conserved residues and are defined as regions never interrupted by large amino acid insertions.
Twelve residues within the kinase domain are either invariant or nearly invariant. Using the PKA-Cα catalytic subunit as an example (since it was the first kinase structure to be solved), these residues include Gly50 and Gly52 in subdomain I (within the motif GXGXXGXV), Lys72 in subdomain II, Glu91 in subdomain III, Asp166 and Asn171 in subdomain VIB, Asp184 and Gly186 in subdomain VII (within the DFG motif), Glu208 in subdomain VIII (within the APE motif), Asp220 and Gly225 in subdomain IX, and Arg280 in subdomain XI (Hanks et al., 1988; Hanks and Quinn, 1991). Because these residues are highly conserved, they are believed to be involved in either the binding of ATP or the phosphotransfer reaction (reviewed in Hanks and Hunter, 1995).

Within the kinase superfamily two main subdivisions exist. These include the serine/threonine specific protein kinases and the tyrosine specific protein kinases. The amino acid residues described above are conserved among all kinases; however, other short sequences are found in either the serine/threonine specific kinases or the tyrosine specific kinases (Hanks et al., 1988; Hanks and Quinn, 1991). The first of these sequences lies between the two conserved Asp and Asn residues of subdomain VI. A strong indicator of serine/threonine specific kinase activity is the consensus sequence DLKPEN, whereas DLRAAN or DLAARN indicates tyrosine specificity. A second sequence that indicates the specificity of the kinase can be found in Subdomain VIII. This region is found immediately amino terminal to the conserved APE sequence and has the consensus PIVK/RWT/M for tyrosine specific kinases and
1.2.2 Three Dimensional Structure

Because of the homology among the various kinase domains, it is believed that they all fold into similar three-dimensional structures that phosphorylate their substrates by a common mechanism. The first three-dimensional structure of a kinase to be solved was that of mouse PKA-Cα with a substrate analog inhibitor (Knighton et al., 1991a; Knighton et al., 1991b). Later, the structures of PKA-Cα with the peptide inhibitor and either MgATP or MnAMP-PNP (a MgATP analog) were solved (Zheng et al., 1993; Bossemeyer et al., 1993). The structures of other kinases including ERK2 and CDK2 have been solved (Zhang F et al., 1994; De Bondt et al., 1993). As a result of these studies, precise functional roles have been assigned to the highly conserved kinase domain residues (reviewed in Hanks and Hunter, 1995). These studies also suggest a two-lobed organization of the kinase domain with a deep cleft between the two lobes where catalysis occurs. Within the smaller amino-terminal lobe are located subdomains I-IV, which contain most of the residues necessary for MgATP binding. Within the larger carboxy-terminal lobe are subdomains VIA-XI that contain the residues associated with peptide binding and catalysis. Subdomain V residues span both lobes.
1.3 MITOGEN-ACTIVATED PROTEIN KINASE SIGNALLING

When cells are exposed to environmental change, adaptive signalling pathways may become stimulated. Often such signal transduction pathways utilize the MAPK family of enzymes. These enzymes have been implicated in regulating several important biological functions and several MAPK isoforms have been assigned to specific signal transduction pathways. Currently four pathways have been described in yeast that are involved in the regulation of osmotic stability, mating, cell wall integrity, and sporulation. In mammalian cells five distinct pathways have also been described that are involved in the regulation of growth and differentiation, osmotic stability and the immune response, and the response to "stress" stimuli. Clearly, the MAPKs represent an evolutionarily conserved group of enzymes that play an essential role in diverse intracellular signalling processes from yeast to vertebrates.

Three protein kinases are central to MAPK signalling and form a sequential protein kinase cascade. Although the specific kinases differ from one MAPK pathway to the next, they each contain a MAPKKK, a MAPKK, and a MAPK. The MAPKKKs are a group of serine/threonine specific protein kinases that can phosphorylate and activate the MAPKKs upon appropriate stimulation. The MAPKKs; however, are a unique class of dual specificity kinases. They activate their substrates, the MAPKs, by phosphorylation on both threonine and tyrosine residues found within a characteristic Thr-X-Tyr motif of subdomain VIII. The MAPKs, like the MAPKKKs, are
serine/threonine specific protein kinases.

Due to the nature of the work presented within this thesis, the remainder of the discussion of MAPK signalling will focus on the MAPK homologues that have been assigned to specific cellular responses. While other members of the MAPK family have been identified, including ERK3, p63MAPK, and p97MAPK, their roles in cell signalling have yet to be identified (Boulton et al., 1991; Gonzalez et al., 1992; Zhu et al., 1994). Based on the existence of multiple MAPK homologues in both mammalian cells and lower eukaryotes, there may be many more members of this family that are involved in the regulation of other important biological functions.

1.3.1 The FUS3/KSS1 and ERK1/ERK2 Pathways

Mating within the yeast S. cerevisiae involves a pheromone-induced MAPK signal transduction pathway (Figure 1A) (reviewed in Herskowitz et al., 1995). Binding of the pheromone to a seven transmembrane receptor (STE2 or STE3) triggers the dissociation of a trimeric G protein into its Ga (Gpa1) and Gβγ (STE4 and STE18) subunits. Free Gβγ activates cdc42 and STE20 which in turn activate the MAPKKK homologue STE11. STE5, a Zn²⁺ finger domain containing protein, functions to tether STE11, STE7 (a MAPKK homologue), and FUS3/KSS1 (a MAPK homologue) into a multikinase complex, allowing for the efficient and sequential activation of STE7 and FUS3/KSS1. Among the MAPK cascades, the yeast mating pheromone pathway is the only
Figure 1: The FUS3/KSS1 and ERK signalling pathways. (A) Binding of pheromone to G protein coupled receptors on the surface of the yeast cell leads to activation of the FUS3/KSS1 kinase cascade. (B) Activation of the ERK pathway by growth factor binding to RPTKs. Double lines represent plasma membranes and single lines represent nuclear membranes. Arrows indicate activation.
one described to date that utilises a binding protein, STE5, to ensure protein kinase specificity in the presence of multiple MAPK pathways.

FUS3 and KSS1, which are functionally redundant MAP kinases, activate many substrates involved in mating. For example, the protein Far1 which mediates pheromone induced G1 arrest is activated by FUS3/KSS1 (Elion et al., 1993; Peter et al., 1993). In addition, FUS3/KSS1 activates the transcription factor STE12 by phosphorylating two recently identified proteins, Rst1 and Rst2 (also known as Dig1 and Dig2), that directly inhibit STE12 (Tedford et al., 1997; Cook et al, 1996).

Interestingly, the mating pheromone pathway also mediates the invasive growth response initiated when the same yeast are exposed to nutrient limitation (reviewed in Herskowitz et al., 1995). Therefore, a single MAPK pathway mediates two developmental programs. The invasive growth pathway also depends upon many of the same components, including STE20, cdc42, STE11, STE7, KSS1, Rst1, Rst2, and STE12 (Roberts and Fink, 1994; Tedford et al., 1997). Unlike the mating pathway, STE5 is not required for the invasive growth response and it has therefore been suggested that an analogous protein might be involved in mediating this signal or that STE5 functions to effectively isolate the kinase components thereby preventing their interactions with other pathways (Yashar et al., 1995).

In mammalian cells, a homologous pathway exists that is involved in the regulation of cell growth and differentiation. The first members of this
pathway to be identified were the MAPKs, ERK1 and ERK2 (also referred to as RSK kinase, MAP-2 kinase, MBP kinase, and MAPK), based on their capacity to activate RSK, a kinase initially thought to be responsible for the phosphorylation of ribosomal protein S6 (Chung et al., 1991). Cloning of the two ERKs reveals that they are most homologous to the yeast kinases FUS3 and KSS1, sharing 55% identity at the amino acid level (Boulton et al., 1990; Boulton et al., 1991). ERK1, which encodes a protein with a molecular size of 43 kD, is 90% identical to ERK2 which encodes a protein with a molecular weight of 41 kD. The ERKs are activated during meiotic maturation and upon exposure to various stimuli including EGF, insulin, nerve growth factor, platelet derived growth factor, insulin-like growth factor, and phorbol esters (Sanghera et al., 1990; Ahn et al., 1990; Hoshi et al., 1988; Gomez et al., 1990; Ray and Sturgill, 1987).

ERK1 and ERK2 are activated by MEK (also referred to as M KK), a unique kinase that possesses dual specificity for the regulatory threonine and tyrosine residues found within their subdomain VIII TEY motifs (Nakielny et al., 1992). Cloning of the cDNAs for two distinct MEKs, MEK1 and MEK2, reveals that they encode proteins with predicted molecular weights of 42 kD and 44 kD respectively (Ashworth et al., 1992; Crews et al., 1992; Zheng and Guan, 1993). MEK1 and MEK2 are approximately 80% identical and are most homologous to the S. cerevisiae MAPKK STE7. In addition, MEK is also dependent on serine/threonine phosphorylation for its activation (Gomez and
The ERK pathway can become activated through receptors coupled to heterotrimeric G proteins, through receptors linked to protein kinase C, through receptors coupled to cytoplasmic tyrosine kinases, and through receptor protein tyrosine kinases. Of these mechanisms, the most information exists regarding ERK activation via receptor protein tyrosine kinases (reviewed in Blenis et al., 1993).

One of the most extensively studied mechanisms of RPTK coupling to the ERK pathway involves the binding of an adaptor protein termed Grb2, via its SH2 domain, to a phosphotyrosine on the receptor (Figure 1B) (reviewed in Schlessinger and Bar-Sagi, 1994). Typically, ligand binding induces receptor dimerization which leads to trans-phosphorylation of sites outside of the catalytic domain. Grb2 binds to a phosphotyrosine on the receptor via its SH2 domain while its two SH3 domains interact with the proline-rich binding sites on the guanine nucleotide exchange factor SOS (Lowenstein et al., 1992; Egan et al., 1993). SOS catalyzes the dissociation of GDP from Ras thereby initiating the loading of GTP and the subsequent activation of Ras (Buday and Downward, 1993). GTP-bound Ras binds to the amino-terminus of Raf thus recruiting Raf to the membrane where it becomes activated (Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Raf can then activate MEK1/MEK2 by phosphorylation on two serine residues (Kyriakis et al., 1992; Yan and Templeton, 1994; Alessi et al., 1994). In turn MEK1/MEK2 activates
ERK1/ERK2 by phosphorylating the threonine and tyrosine residues within the consensus TEY (Nakielny et al., 1992). Following activation, ERK1/ERK2 can phosphorylate cytoplasmic substrates such as cytosolic phospholipase A2 and other protein serine/threonine kinases such as RSK which can then enter the cell's nucleus and activate specific transcription factors (Lin et al., 1993; Sturgill et al., 1988). In addition, activated ERK1/ERK2 can enter the nucleus and phosphorylate transcription factors such as Elk1 (Marais et al., 1993).

### 1.3.2 The SAPK Pathway

SAPK (also known as JNK) was first purified in active form from the livers of cycloheximide treated rats as a 54 kD protein that could phosphorylate MAP-2 on serine/threonine residues (Kyriakis and Avruch, 1990). Subsequent cloning of the cDNA for SAPK revealed the existence of three distinct genes that were subject to differential splicing and could generate up to eight proteins of 46 kD and 54 kD (Kyriakis et al., 1994). The three genes, termed α, β, and γ, share 85-92% identity with each other, and approximately 43% identity with ERK1 and the yeast MAPKs, FUS3 and KSS1.

Like the MAPKs, the SAPKs are serine/threonine specific kinases that are dependent on threonine and tyrosine phosphorylation for activation (Kyriakis et al., 1991). In contrast to the ERKs which contain a TEY motif as the site of activating phosphorylation, the SAPKs contain a TPY motif,
suggesting a distinct regulating kinase. In support of this notion, the SAPKs, unlike the ERKs, are poorly activated by mitogens and phorbol esters (Kyriakis et al., 1994). Further, the SAPKs appear to be involved with a stress-activated signalling pathway since their activity is greatly enhanced in the presence of agents like TNF-α, IL-1, anisomycin, cycloheximide, heat shock, UV irradiation, sodium arsenite, muscarinic acetylcholine receptors, and ischemia-reperfusion (Figure 2) (Kyriakis et al., 1994; Derijard et al., 1994; Bird et al., 1994; Coso et al., 1995a; Pombo et al., 1994).

SEK1 (also referred to as MKK4 and JNKK) is the physiologic activator of SAPK (Sanchez et al., 1994). As expected, this kinase is most homologous to the MAPKKs, with the highest homology of 48% to a yeast MAPKK, PBS2 (see section 1.3.3). In addition, SEK potently activates the SAPKs in vitro and in vivo without interfering with the ERK pathway (Sanchez et al., 1994). Once activated by SEK, SAPK can phosphorylate the transcription factors c-Jun and ATF2 within their amino-terminal transactivation domains on two serine or threonine residues respectively (Pulverer et al., 1991; Gupta et al., 1995).

SEK is activated by the serine/threonine kinase MEKK1, which was originally thought to be the activator of MEK (Lange-Carter et al., 1993). Expression of MEKK1 at physiological levels is incapable of activating the ERK pathway but can still stimulate the SAPKs, suggesting that it is a physiological SEK kinase and not a physiological MEK kinase (Yan et al., 1994, Minden et al., 1994). Other MAPKKK family members or STE11 homologues,
Figure 2: The SAPK signalling pathway. Through an unclear mechanism stressful agonists in the cell's external environment initiate the SAPK cascade. Dashed arrows represent an undetermined set of events and solid arrows represent direct activation. The double line indicates the plasma membrane and the single line represents the nuclear membrane.
including MUK, Tpl-2, and MLK3, activate the SAPK pathway (Hirai et al., 1996; Salmeron et al., 1996; Tibbles et al., 1996). While it remains unclear how MUK and Tpl-2 are regulated, two STE20 homologues, HPK1 and GCK, activate the SAPK pathway via MLK3 (Kiefer et al., 1996; Tibbles et al., 1996). The PAK family of protein kinases are also STE20 homologues that are activated by the small GTP-binding proteins Rac1 and cdc42 (Bagrodia et al., 1995). Rac1 and cdc42 can regulate the activity of the SAPK pathway and recently human PAK1 has been suggested to act upstream of MEKK (Coso et al., 1995; Brown et al., 1996). However, a direct interaction between PAK and MEKK has yet to be been demonstrated.

1.3.3 The HOG1 and p38 Pathways

An osmosensing signal transduction pathway in yeast was first described when mutagenized yeast cells were screened for the failure to grow on high-osmolarity medium (Brewster et al., 1993). From these mutants, two different yeast genes were isolated that are required for restoring the osmotic gradient across the cell membrane in response to exposure to external osmolarity. The product of the first of these genes, termed HOG1, was demonstrated to become rapidly tyrosine phosphorylated by the product of the second gene, PBS2, in response to increases in extracellular osmolarity. Sequence comparisons of HOG1 and PBS2 indicated that HOG1 was most similar to the MAPK family members and that PBS2 was most homologous to the MAPKKs.
Recent work on this yeast MAP kinase cascade has suggested its regulation by a two-component osmosensor composed of a transmembrane sensor histidine kinase termed SLN1 and a cytoplasmic response regulator termed SSK1 (Figure 3A) (Maeda et al., 1994). Under normal salinity conditions the SLN1 histidine kinase represses the activation of the PBS2-HOG1 kinase cascade through phosphorylation of SSK1. In the presence of high-osmolarity media however, the histidine kinase is inactive, allowing the unphosphorylated SSK1 to activate the PBS2-HOG1 kinase cascade. Further work has identified two MAPKKKs, SSK2 and SSK22, that are under the control of the SLN1-SSK1 two-component osmosensor and that activate PBS2 (Maeda et al., 1995). PBS2 is also activated by the interaction of its proline-rich amino terminal motif with the SH3 domain of a putative transmembrane osmosensor termed SH01 (Maeda et al., 1995).

A mammalian homologue of HOG1 was first identified from studies involving LPS, an activator of cells of the immune and inflammatory systems (Han et al., 1994). When mammalian prolymphocytes are exposed to LPS, a protein kinase cascade is initiated resulting in the tyrosine phosphorylation of a 38 kD protein, termed p38. Sequence analysis of this clone indicates that it shares homology with HOG1 (52% identity) and the other mammalian MAPKs (46-49% identity). p38 also complements a yeast HOG1 mutation and becomes tyrosine phosphorylated upon exposure of cells to increased osmolarity.

Like the other MAPKs, both HOG1 and p38 possess threonine and
Figure 3: The HOG and p38 signalling pathways. (A) High osmolarity leads to activation of the HOG pathway in yeast through two possible osmosensors. (B) Through an unclear mechanism stressful agonists in the cell's external environment activate the p38 pathway. Double lines represent plasma membranes and single lines represent nuclear membranes. Dashed arrows represent an unclear series of events, solid arrows indicate direct activation.
tyrosine residues in subdomain VIII which must be phosphorylated for enzymatic activity (Raingeaud et al., 1995). p38 and HOG1 have a TGY sequence while the ERKs and the SAPKs have TEY and TPY sequences respectively. This suggests that p38 and HOG1 form a distinct subset of the MAPK family that is regulated by different upstream kinases and under differing conditions.

As described, p38 was first identified in mammalian cells from studies involving LPS; however, further work has shown that p38 activation can be observed under a multitude of stimuli including TNF-α, IL-1, high osmolarity, UV irradiation, heat shock, and arsenite (Rouse et al., 1994; Raingeaud et al., 1995). While many of these agonists overlap with the corresponding SAPK profile, it appears that p38 has a unique role in the inflammatory response based on its specific inhibition by a class of pyridinyl imidazoles that block LPS-induced TNF and IL-1β production (Lee et al., 1994).

To further support the hypothesis that p38 is a member of a distinct MAPK signalling pathway in mammalian cells, several new MAPKKs have been implicated in its activation (Figure 3B). For example, MKK3 and MKK4, which activate p38 in vitro, were cloned as the human homologues of PBS2 (Derijard et al., 1995). Human MKK3b, an alternatively spliced form of MKK3, was cloned by complementation of a yeast PBS2 deletion mutant with cDNA libraries in p38-dependent manner and shown to be activated by osmotic shock (Moriguchi et al., 1996a). In addition, another mammalian MAPKK
termed MKK6, and MKK3, are p38 activators in vivo (Han et al., 1996; Raingeaud et al., 1996). While little information currently exists regarding the upstream activators of MKK3 or MKK6, in vitro and in vivo studies have shown that the MAPKKK TAK1 can work as a direct activator for both (Moriguchi et al., 1996b). Similarly, it has recently been demonstrated that MLK3 can activate both MKK3 and MKK6 (Tibbles et al., 1996). Of the multiple in vitro substrates of p38, only MAPKAPK2, MAPKAPK3, and the transcription factors ATF2 and CHOP have been suggested to play a role in vivo (Freshney et al., 1994; Rouse et al, 1994; McLaughlin et al., 1996; Raingeaud et al., 1996; Wang and Ron, 1996). However, new evidence suggests that ATF2 is not likely a physiologic p38 substrate (Cuenda et al., 1997).

A new member of the p38 family, termed p38β, was isolated (Jiang et al., 1996). p38β is slightly larger than p38, the product of a separate gene, and is approximately 74% identical to p38. Like p38, p38β contains the TGY dual phosphorylation site which is required for its kinase activity and is also activated by pro-inflammatory cytokines and environmental stress. p38β is preferentially activated by MKK6 when co-expressed in cultured cells whereas p38 is activated nearly equally by MKK3, MKK4, and MKK6. Further, in vitro and in vivo experiments show a much stronger substrate preference of p38β for ATF2 than seen with p38. Lastly, like p38, p38β is inhibited by the anti-inflammatory pyridinyl imidazole drug implying that it too is involved in the anti-inflammatory response.
1.3.4 The ERK5 Signalling Pathway

Two components of a new human protein kinase signal transduction pathway, ERK5 (also known as BMK1) and MEK5, were recently identified (Zhou et al., 1995; Lee JD et al., 1995; English et al., 1995). MEK5 which is most closely related to MEK1 (48% identical) has two alternative splice forms, the larger of which, MEK5α, contains an extra 23 amino acids thought to associate with the actin cytoskeleton (English et al., 1995). In addition, an amino terminal sequence, present in both isoforms, may be important in coupling GTPase signalling molecules to this cascade (Zhou et al., 1995).

ERK5 and BMK1, which are 98% identical, are believed to be differentially spliced products of the same gene (Lee JD et al., 1995). ERK5, which is 50% identical to FUS3, encodes an 815 amino acid protein with a predicted molecular weight of 98kD. ERK5 is therefore approximately twice the size of all known MAP kinases, with an extra 400 amino acid carboxy-terminal domain. Within this domain lie two proline-rich regions that are believed to be involved in targeting the kinase to the cytoskeleton and regulating its kinase activity (Zhou et al., 1995). Like ERK1, ERK5 also has a TEY motif; however, the extra carboxy-terminal domain and the unique loop between subdomain VII and VIII suggest that it is involved in a previously unknown pathway in mammalian cells.

Consistent with the belief that these two kinases compose a distinct signalling cascade are data that show the inability of MEK5 to phosphorylate,
in vitro, any of the other known mammalian MAPKs (English et al., 1995). Further, in vitro and in vivo binding assays demonstrate the ability of ERK5 to bind MEK5 but not MEK1 or MEK2 (Zhou et al., 1995). Lastly, studies to define ERK5 regulation have shown it to be stimulated upon exposure of cells to sorbitol and H₂O₂ but not to agonists that are strong activators of ERK1/2, raising the possibility that it is a redox-sensitive kinase (Abe et al., 1996).

1.3.5 The SAPK3/ERK6 Signalling Pathway

The most recent member of the MAPK family to be described was cloned independently from both rat and human cDNA libraries and termed SAPK3 and ERK6 respectively (Mertens et al., 1996; Lechner et al., 1996). Sequence comparison reveals that this kinase shares approximately 60% identity with p38, 45% identity with SAPK, and 40% identity with the ERKs. Like p38, ERK6 also contains a TGY motif in the activation domain and is activated by many of the same stimuli that activate both p38 and SAPK (Cuenda et al., 1997).

Experiments to examine the role of ERK6 in cell signalling reveal that it is activated, both in vitro and in vivo, by MKK6, and not MKK1, MKK4, or MKK3, (Cuenda et al., 1997). Additional experiments demonstrate that the substrate specificity of ERK6, in vitro, is similar to p38; however, significant distinctions exist. For example, ERK6 is far less effective at activating the MAPKAPKs and, unlike p38, is able to phosphorylate ATF2 on all residues
believed to be critical for its in vivo activation (Cuenda et al., 1997). In addition, ERK6 is not inhibited by one of the p38 inhibiting drugs described in section 1.3.3 (Cuenda et al., 1997). These findings suggest that ERK6 may be responsible for mediating some of the functions previously attributed to either SAPK or p38.

1.3.6 Crosstalk

Although the previously described pathways seem physiologically distinct, a substantial amount of interaction, or crosstalk, occurs among them. In support of this belief, activation of the ERK, SAPK, and p38 pathways is not mutually exclusive, since the stimuli of one these kinases can partially activate the other two (Kyriakis et al., 1994; Raingeaud et al., 1994). However, when a broad range of agonists are analyzed by comparing their efficiencies of activation of the MAPKs, it is clear that there is discrimination between the ERKs and SAPK (Kyriakis et al., 1994).

It has been demonstrated in vitro and with in vivo overexpression studies that the MAPKKs from one pathway can activate the MAPKs of a homologous pathway. For example, SEK can phosphorylate both p38 and SAPK in vitro and co-transfection assays show that both MKK3 and MKK6 can phosphorylate p38 (Derijard et al., 1995; Han et al, 1996). As demonstrated in the past, these experiments can lead to false associations between members of different pathways. For example, MEKK was originally described as the
activator of MEK and not as the activator of SEK (Lange-Carter et al., 1993). With the increasing numbers of kinases being described that are involved with MAPK signalling, it will be important to determine how these pathways are regulated such that only a specific subset of kinases become activated.

1.4 THE EUKARYOTIC CELL CYCLE

The eukaryotic cell cycle is the process by which a single cell gives rise to two genetically identical daughter cells (reviewed in Norbury and Nurse, 1992). This cycle is composed of four main phases: G1, S, G2, and M (Figure 4). Proliferating cells must progress sequentially through all four stages of the cell cycle from G1 to S to G2 and finally to M. DNA synthesis occurs during S phase and mitosis occurs during M phase. The gaps between the M phase and S phase (G1) and the S phase and M phase (G2), enable the cell to prepare for DNA synthesis and cell division respectively. In addition to these four stages, a non-proliferative, quiescent state also exists, termed G0. While cells in this phase are not actively dividing, they are not terminally differentiated and therefore retain the ability to re-enter the proliferative cell cycle.

The initial progression of cells from G0 or G1 into the S phase requires the presence of growth factors specific for the cell type. However, once the cells reach a restriction point in late G1, they are committed to completing the cell cycle, even in the absence of the growth factors that initiated the progression (Pardee, 1974).
Figure 4: The mammalian cell cycle. A schematic of the stages of the cell cycle showing the points of action of the various cyclin-CDK complexes and the CKIs. R point = restriction point (serum independence).
For the accurate transmission of genetic information from mother cell to daughter, the order and timing of events within the cycle are critical. Failing to repair DNA damage, entering mitosis with unreplicated DNA, or commencing anaphase before correct alignment of the chromosomes, leads to dead, mutant, or aneuploid cells. Therefore, to ensure that the cell cycle is properly maintained, biochemical pathways, known as checkpoints, exist that prevent the initiation of new cell cycle events until the accurate completion of others.

1.4.1 Cell Cycle Regulation by CDKs

Progression of the cell cycle is governed by a group of CDKs that regulate cell cycle checkpoints in response to environmental or intracellular conditions such as the presence of damaged or unreplicated DNA. In yeast, a single CDK, either cdc2 in *S. pombe* or CDC28 in *S. cerevisiae*, is responsible for the major transitions of the cell cycle. The genes encoding these kinases were identified by temperature-sensitive mutated forms which could arrest cells at specific points within the cell cycle, namely at the G1/S and the G2/M transitions (Nurse and Bissett, 1981; Hartwell et al., 1973; Reed, 1980; Piggott et al., 1982). A human homologue to cdc2 was cloned by its ability to complement an *S. pombe* temperature-sensitive CDC28* mutant strain (Lee and Nurse, 1987). Unlike the yeast cdc2, mammalian cdc2 controls only the G2/M transition since the G1/S transition is regulated by different cdc2 homologues,
namely CDK2 and CDK4 (Fang and Newport, 1991). In mammalian cells, multiple CDKs exist whose activities are regulated by their phosphorylation and dephosphorylation, by the binding of cyclins, and by the activity of cyclin-dependent kinase inhibitors.

1.4.2 Cyclins as Activators of CDKs

Cyclins are a family of structurally related proteins that function to bind and activate CDK catalytic subunits in a regulatory fashion. Homology between the different members of the cyclin family is not extensive and is mainly confined to a stretch of 100 amino acids termed the cyclin box (Nugent et al., 1991). The cyclin box lies in the middle of the 400 amino acid cyclin sequence and is involved in CDK binding and activation (Kobayashi et al., 1992).

Based on the crystal structures of both an inactive monomeric CDK and an active cyclin-CDK complex, it is believed that cyclins activate CDKs by altering the conformation of the catalytic domain (De Bondt et al., 1993; Jeffrey et al., 1995). Specifically, activation of the CDK through cyclin binding is thought to result from two structural changes. First, binding of a cyclin changes the conformation of the amino-terminal lobe of the CDK, thereby reorienting the bound ATP and facilitating phosphotransfer to the CDK substrate. Second, cyclin binding displaces a region in the middle of the CDK, termed the T loop, such that it no longer obscures the substrate binding cleft
To date, cyclins can be classified into two different families depending on how they are degraded within the cell (reviewed in Pines, 1996). The G1 cyclins, D and E, are short lived proteins that have half lives of approximately 30 minutes. The instability of these cyclins is conferred by PEST amino acid sequences that are carboxy-terminal to the cyclin box; however, the biochemical basis of this proteolysis has not yet been elucidated. Because of their instability, the levels of the G1 cyclins are determined by their rates of transcription. In contrast, the mitotic cyclins or G2 cyclins, A and B, are stable throughout the cell cycle except during mitosis when they are rapidly degraded in a ubiquitin-dependent manner (Glotzer et al., 1991). This instability is dependent on a region located amino-terminal to the cyclin box, termed the destruction box (Glotzer et al., 1991). Because CDKs are stable throughout the cell cycle, inactivation of a cyclin-CDK complex occurs mainly through the cell specific degradation of the cyclin partner.

Cyclins also play an important role in targeting their partner kinase to specific parts of the cell. For example, cyclin A is a nuclear protein whereas cyclin B is a cytoplasmic protein (Pines and Hunter, 1991). Because most cyclin-CDK complexes recognize the same basic consensus sequence \textit{in vitro}, K/R-S/T-P-X-K/R, the targeting of these complexes to different locations in the cell would likely restrict the substrates available to them (reviewed in Pines, 1995).
1.4.3 Cyclin-CDK Complexes Throughout the Cell Cycle

In mammalian cells CDKs complex with various cyclins throughout the stages of the cell cycle (Figure 4). The first cyclin-CDK complex to form once cells are released from quiescence is cyclin D with either CDK4 or CDK6, depending on the cell type (Matsushime et al., 1992; Meyerson and Harlow, 1994). The formation of this complex is highly dependent on the presence of growth factors since cyclin D levels drop immediately when growth factors are removed (Matsushime et al., 1991). Microinjection of anti-cyclin D1 antibodies into normal fibroblasts during G1 prevents cells from entering S phase, indicating that this CDK-cyclin complex functions in middle to late G1 and is important for the initiation of S phase (Baldin et al., 1993).

The second CDK-cyclin complex to become activated after release from quiescence is the G1 cyclin, cyclin E, and CDK2 (Dulic et al., 1992; Koff et al., 1992). This complex plays a crucial role in the progression from G1 to S phase and regulates different aspects of G1 than the cyclin D-CDK4/6 complex (Reznitzky and Reed, 1995).

Following cyclin E degradation in S phase, CDK2 associates with cyclin A (Pagano et al., 1992). This complex is required for DNA replication since cyclin A antibodies or antisense cyclin A cDNA will inhibit DNA synthesis when micro-injected into cells (Pagano et al., 1992). Cyclin A and CDK2 also localize to subnuclear replication foci, further suggesting a role of this cyclin-CDK complex in DNA replication (Cardoso et al., 1993).
Entry into mitosis is triggered by formation of the Maturation Promoting Factor which is composed of cdc2 and cyclin B (reviewed in King et al., 1994). In addition, cyclin A also plays an essential role in the initiation of mitosis by forming a complex with cdc2 (Pagano et al., 1992). Experiments with Drosophila cells that are mutant in both cyclin A and cyclin B suggest that the two cyclins act synergistically to initiate mitosis but that once in mitosis the cyclin B-cdc2 complex plays a more central role in its progression (Knoblich and Lehner, 1993). Following destruction of cyclin B at the metaphase-anaphase transition, cdc2 becomes inactivated and the cells exit from mitosis (reviewed in King et al., 1994).

While the complexes of cyclins A, B, D, and E with their respective CDKs clearly have a function in the regulation of the cell cycle, it is believed that other cyclin-CDK complexes exist that have other functions than participating directly or exclusively in cell cycle control. For example, the cyclin H-CDK7 complex forms part of the general transcription factor TFIIH and is also part of CAK (see section 1.4.4.1) (Shiekhattar et al., 1995). Another complex, composed of CDK8 and cyclin C, is also involved in mammalian transcription (Tassan et al., 1995b). In addition, two other cyclins have been identified, cyclins F and G, but their cell cycle role, if any, remains to be demonstrated (Bai et al., 1994; Okamoto and Beach, 1994).
1.4.4 Modulation of Cyclin-CDK Activity

1.4.4.1 Activation by Phosphorylation

Complete CDK activation requires not only cyclin binding but also its phosphorylation at a conserved threonine residue within the T loop. In cdc2 this threonine corresponds to amino acid 161, whereas in CDK2 this corresponds to amino acid 160 (Solomon et al., 1992; Gu et al., 1992). While cyclin binding is thought to facilitate phosphorylation of this threonine residue, its phosphorylation is also predicted to strengthen cyclin binding.

The enzyme responsible for phosphorylating Thr160 in cdc2 and Thr161 in CDK2 was originally named CAK, but it has since been realized that, like its substrates, it is also a cyclin-CDK complex composed of cyclin H, CDK7, and an assembly factor termed MAT1 (Fisher and Morgan, 1994; Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Tassan et al., 1995a). In addition to activating both cdc2 and CDK2 complexes, CAK has also been demonstrated to activate cyclin D-CDK4 (Matsuoka et al., 1994). This suggests that a single CAK might be responsible for activating the major CDKs involved in cell cycle control.

Throughout the cell cycle, phosphorylation of CDK residue Thr160/161 parallels cyclin binding. Changes in CDK phosphorylation at this site are probably not due to changes in CAK activity but rather in the ability of cyclin binding to stimulate CDK phosphorylation, since CAK activity does not change in a cell cycle-dependent manner (Matsuoka et al., 1994).
1.4.4.2 Inhibition by Phosphorylation

In mammalian cells cyclin-CDK inactivation is regulated by cyclin degradation, dephosphorylation of Thr160/161, and by phosphorylation of two amino acid residues near the amino terminus of the CDK (Norbury et al., 1991; Gu et al., 1992). These two residues, Thr14 and Tyr15 in both cdc2 and CDK2, are found within the ATP binding site buried beneath the T loop (De Bondt et al., 1993). Phosphorylation of Tyr15 is dependent on cyclin binding and is carried out by the Weel kinase (Parker et al., 1991; McGowan and Russell, 1993). In yeast, phosphorylation occurs only on Tyr15 and is carried out by the redundant Wee1 and Mik1 kinases (Lundgren et al., 1991). In animal cells, Thr14 is phosphorylated by an unidentified membrane-bound kinase activity which is distinct from that of the Wee1 kinase (Kornbluth et al., 1994).

In yeast, dephosphorylation of Tyr15, and hence activation of the cyclin-CDK complex, is accomplished by the product of the cdc25 gene (Russell and Nurse, 1986). In mammalian cells at least three members of this family exist which appear to interact with different cyclin-CDK complexes. cdc25A is phosphorylated and activated by cyclin E-CDK2 at the initiation of DNA synthesis. Following its activation, cdc25A is thought to dephosphorylate and activate one of the CDKs functioning early in the cell cycle, most likely CDK2 in complex with cyclin A (Hoffman et al., 1994; Jinno et al., 1994). At the onset of mitosis cdc25C is activated and dephosphorylates Thr14 and Tyr15 in cdc2, leading to activation of the cyclin B-cdc2 complexes (Hoffman et al.,
A positive feedback loop is initiated since cyclin B-cdc2 activates more cdc25C such that the activation of cyclinB-cdc2 occurs rapidly and irreversibly (Hoffmann et al., 1993). The point of action of cdc25B in the cell cycle is unclear.

1.4.4.3 CDK Inhibitors

Cyclin-CDK complexes are also regulated by a diverse family of proteins, known as CKIs or CDIs, that bind to and inactivate the cyclin-CDK complexes. Two families of CKIs have been isolated from animal cells. The first family is composed of p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}, while the second family is comprised of p21\textsuperscript{CIP1/WAF1}, p27\textsuperscript{KIP1}, and p57\textsuperscript{KIP2}. Together, these proteins play an important role in mediating extracellular negative signals that arrest the cell at various points throughout its cycle (Figure 4).

The INK4 family members, p15, p16, p18, and p19, bind only to CDK4 and CDK6, apparently competing for cyclin D binding sites, thereby arresting cells in G1 (Hannon and Beach, 1994; Guan et al., 1994; Hirai et al., 1995 Serrano et al., 1993). The extracellular signals regulating the activities of p16, p18, and p19 have yet to be defined; however, it is known that inhibition by p16 and p18 occurs in an Rb-dependent fashion (Serrano et al., 1993; Guan et al., 1994). p15 mediates cell cycle arrest induced by the negative growth factor TGF\(\beta\) (Hannon and Beach, 1994).

The second family of CKIs have a preference for CDK2- and CDK4-cyclin
complexes. The first member of this family to be described, p21, binds and inhibits a variety of cyclin-CDK complexes including cyclin D-CDK4 and CDK2 with either cyclin E or A (Harper et al., 1993). p21 transcription is activated by p53, suggesting that increased p21 synthesis occurs when DNA is damaged in G1 (El Deiry et al., 1993). This would prevent the cells from entering S phase by inhibiting both cyclin D-CDK4 and cyclin E-CDK2 complexes.

p27, a cyclin E-CDK2 inhibitor, blocks cells in late G1 in response to TGFβ or contact inhibition (Polyak et al., 1994). However, p27 is competitively bound by cyclin D-CDK4 complexes suggesting that when p27 is sequestered by cyclin D-CDK4 it relieves its inhibition of cyclin E-CDK2 (Polyak et al., 1994). p27 also mediates cAMP induced G1 arrest by binding to cyclin D-CDK4 complexes and preventing their activation by CAK (Kato et al., 1994).

p57 which potently inhibits cyclin E-CDK2, cyclin D-CDK4, and cyclin A-CDK2, is thought to be involved in the decision to exit the cell cycle since most cells expressing p57 are terminally differentiated (Lee MH et al., 1995; Matsuoka et al., 1995).

1.5 cdc2-RELATED PROTEIN KINASES

A series of cdc2-related protein kinases exist that have greater than 40% amino acid identity with cdc2. Unlike the CDKs, these kinases have not been demonstrated to associate with a cyclin or to posses activities that fluctuate in a cell cycle-dependent fashion. Therefore, they have been named after their
amino acid sequences corresponding to the characteristic PSTAIRE motif, found in cdc2 and CDK2, which is involved in cyclin binding.

To date, seven cdc2-related kinases have been described whose roles, if any, in the cell cycle have yet to be determined. Within this group are PITALRE, PISSLRE, PITAIRE, PITSLRE, PCTAIRE, KKIALRE, and KKIAMRE. The majority of these kinases were initially isolated by PCR based techniques in an attempt to identify other kinases involved in the cell cycle (Grana et al., 1994a; Grana et al., 1994b; Lapidot-Lifson et al., 1992; Bunnell et al., 1990; Okuda et al., 1992; Meyerson et al., 1992; Taglienti et al., 1996). Because of the limited information that exists regarding these kinases and their potential functions, the remainder of this discussion will be limited to KKIALRE and KKIAMRE whose sequences most closely resemble each other and a novel protein kinase, NKIATRE, which forms the subject of this thesis.

1.5.1 KKIALRE and KKIAMRE

KKIALRE and KKIAMRE were isolated by cDNA library screening using degenerate oligonucleotides corresponding to conserved regions of cdc2 and the MAPKs respectively (Meyerson et al., 1992; Taglienti et al., 1996). Both KKIALRE and KKIAMRE, over their total sequences, share approximately 40% amino acid identity with cdc2 and approximately 34% amino acid identity with the ERKs (Bestfit program, Wisconsin Genetics Computer Group). In contrast, KKIALRE and KKIAMRE share 50% amino
acid identity with each other (Bestfit program, Wisconsin Genetics Computer Group). Both KKIALRE and KKIAMRE contain the amino acid residues described in section 1.4.4 that are necessary for activation as CDKs. In contrast, both KKIALRE and KKIAMRE contain the motif Thr-X-Tyr in subdomain VIII of their catalytic domains that is characteristic of the members of the MAPK family (see section 1.3). Further, this sequence, Thr-Asp-Tyr, is the same in both KKIALRE and KKIAMRE, and is unique to the MAPKs, suggesting that as MAPKs, these may be part of novel signalling pathways.

KKIALRE is expressed in human ovary and kidney, moderately in the brain and lung, and at a low level in placenta, spleen, prostate, and duodenum (Taglienti et al., 1996). KKIAMRE is expressed in human testis and kidney with a lower level detected in brain and lung (Taglienti et al., 1996). KKIAMRE and KKIALRE have a similar tissue distribution with the exception that KKIAMRE is found in the testis while KKIALRE is expressed in the ovary. The differential expression of these two kinases would suggest that they may play a specialized role in meiosis or the production of germ cells.

c-Myc and the EGFR are in vitro substrates for both KKIAMRE and KKIALRE (Taglienti et al., 1996). In addition, both KKIALRE and KKIAMRE can become activated in COS cells stimulated with EGF but this activation does not require phosphorylation of the Thr and Tyr residues in the Thr-Asp-Tyr motif (Taglienti et al., 1996). MEK1 is also able to phosphorylate KKIAMRE in vitro but this is 50-fold weaker than the phosphorylation of
ERK2 by MEK1 (Taglienti et al., 1996). Together, these data indicate that KKIAMRE and KKIALRE may not function as either MAPKs or CDKs and therefore represent a new subfamily of proline-directed protein kinases.

1.6 SUMMARY

The mechanism by which cells alter their cytoplasmic and nuclear events, in response to extracellular signals, is a complex process that often involves the input of many different signalling molecules such as protein kinases. This introduction has reviewed two important classes of kinases that are involved in regulating diverse biological functions. The MAPKs enable cells to grow and differentiate and adapt to multiple changes in their external environment, whereas the CDKs regulate progression of the cell cycle. In addition, two members of a subfamily of cdc2-related protein kinases, KKIALRE and KKIAMRE, have been introduced. These two kinases are unique since they contain conserved features characteristic of both the MAPKs and the CDKs.

Chapter 2 of this thesis describes experiments using the yeast two hybrid approach that demonstrates that the three known MAPK pathways, ERK, SAPK, and p38, do not require scaffolding proteins to maintain signalling specificity and that crosstalk among them does not readily occur in vivo. In chapter 3, the search and discovery of a novel serine/threonine specific protein kinase, NKIATRE, is described. This kinase appears to be the third member
of a novel group of kinases that includes KKIALRE and KKIAMRE.
CHAPTER 2

Examination of In Vivo Protein Interactions Among the Three Known Mitogen-Activated Protein Kinase Pathways

Parts of this chapter have been previously published in:
"Mammalian Mitogen-activated Protein Kinase Pathways Are Regulated through Formation of Specific Kinase-Activator Complexes"
Zanke BW, Rubie EA, Winnett E, Chan J, Randall S, Parsons M, Boudreau K, McInnis M, Yan M, Templeton DJ, and Woodgett JR
2.1 INTRODUCTION

Structurally similar MAPK pathways have been identified in both yeast and mammalian cells. While these pathways regulate diverse biological events, they each contain a conserved kinase cascade consisting of a MAPKKK, a MAPKK, and a MAPK. Of the MAPK pathways in mammalian cells, the most highly characterized involves the sequential kinase cascade of Raf (a MAPKKK), MEK (a MAPKK), and ERK (a MAPK), upon stimulation of cells with growth factors (see section 1.3.1). Two other MAPK pathways that have also been studied in detail are the SAPK and p38 kinase cascades (see sections 1.3.2 and 1.3.3). Unlike the ERK pathway, when cells are exposed to stressful agonists including UV irradiation, heat shock, osmotic shock, TNFα, and IL-1, the SAPK pathway becomes activated. This pathway is typically composed of MEKK, SEK, and SAPK, although other MAPKKKs have been demonstrated to activate SEK. Similarly, when cells are exposed to UV irradiation, TNFα, IL-1, and osmotic shock, the p38 pathway becomes activated. Although not as clearly defined as the ERK and SAPK pathways, p38 is activated by both M KK3 and M KK6, which are both activated by TAK1 and MLK3.

Although it may seem that these three pathways are physiologically distinct, data obtained to date has been less than conclusive. In vitro experiments and in vivo overexpression studies have lead to false conclusions in the past regarding interactions between molecules. For example, MEKK was originally described as the activator of MEK both in vitro, and when
expressed at high levels in COS-7 cells (Lange-Carter et al., 1993). However, at physiologic levels MEKK specifically activates the SAPK pathway via activation of SEK (Yan et al., 1994; Minden et al., 1994). It has been demonstrated that activation of these three pathways is not mutually exclusive since TNFα and heat shock can partially activate the ERK cascade and EGF can partially activate the SAPK pathway (Kyriakis et al., 1994). Further, it has been shown that SEK can phosphorylate both p38 and SAPK in vitro (Derijard et al., 1995). Therefore, it would appear that a significant amount of crosstalk could occur between the different MAPK pathways.

To maintain signalling specificity in the presence of multiple kinases with high structural homology, mechanisms must exist that prevent interactions between members of the different MAPK cascades. In yeast cells, STE5 functions as a scaffolding protein that brings together KSS1/FUS3, STE20, and STE11, thereby preventing the activation of any other kinases (Choi et al., 1994). Because no STE5 homologue has been identified in mammalian cells, we decided to investigate how the specificity of MAPK signalling is maintained. Further, we have chosen to examine the extent of crosstalk that occurs between these three homologous signalling pathways.

With the yeast two hybrid system, a yeast based genetic assay that detects protein-protein interactions in vivo, we have evaluated the physical interactions between the MAPKs, ERK, SAPK, and p38, and the MAPKKs, MEK, SEK, MKK3, and MKK6. Here we describe that the ERK, SAPK, and
p38 signalling pathways do not require specific scaffolding proteins to maintain signalling specificity. Further, these experiments, in combination with other data by Zanke et al., indicate that the mammalian MAPK pathways are distinct signalling cascades and that crosstalk does not readily occur in vivo (Zanke et al., 1996).

2.2 MATERIALS AND METHODS

2.2.1 The Yeast Two Hybrid System

The two hybrid system is a yeast based genetic assay that detects protein-protein interactions in vivo. The assay works by reconstituting the function of the transcription factor GAL4 which can be can be separated into a DNA-binding domain and a transcriptional activation domain. Normally both domains are located in the same protein; however, separated components can be assembled in vivo to give a functional activator (Ma and Ptashne, 1988; Brent and Ptashne, 1985). In the Matchmaker™ Two Hybrid System (Clontech, Palo Alto CA, USA) the gene for one protein is cloned into the vector pAS1-CYH2, enabling the generation of a hybrid with the GAL4 DNA-binding domain. The gene for a putative interacting protein is cloned into the vector pACTII, enabling the generation of a hybrid protein with the GAL4 activation domain.

Following construction of the two plasmids, they are cotransformed into the yeast strain Y190 which contains the lacZ and HIS3 reporter genes under
the control of upstream GAL4 binding sites. Because the vector pAS1-CYH2 carries the TRP1 gene and the vector pACTII carries the LEU2 gene, transformant yeast can be selected by growth in the absence of tryptophan and leucine. If the two hybrid proteins interact with each other, the GAL4 DNA-binding domain and the GAL4 activation domain will be brought into close proximity, allowing reconstitution of the transcription factor function and expression of the two reporter genes (Figure 5).

To identify transformant colonies positive for expression of the lacZ gene, β-galactosidase assays are performed (Figure 6). With this assay, the yeast will develop a blue colour in the presence of X-gal due to expression of the enzyme β-galactosidase from the lacZ gene. Similarly, expression of the HIS3 gene can be detected by the ability of transformant yeast to grow in the absence of histidine.

### 2.2.2 Plasmid DNAs

The complete coding sequences of SAPKα, p38AGF (a kinase-inactive version of p38 which has the two sites of activating phosphorylation mutated from threonine and tyrosine to alanine and phenylalanine respectively), and ERK1, were cloned into the multiple cloning site of vector pAS1-CYH2 such that in-frame fusion proteins could be generated (Figure 7). SAPKα was cloned into the NcoI site while p38AGF and ERK1 were cloned into the NcoI and BamHI sites. Because of difficulties in growing yeast cultures expressing
Figure 5: The basis of the yeast two hybrid system. (A) The GAL4 DNA-binding domain (BD) hybrid binds to the GAL1 upstream activating sequence (UAS) but cannot activate transcription. (B) The hybrid of protein Y and the GAL4 activation domain (AD) cannot localize to the UAS and activate transcription. (C) Interaction between the two proteins, X and Y, leads to reconstitution of the GAL4 transcription factor and expression of the reporter genes lacZ and HIS3.
Figure 6: Flow Diagram of the yeast two hybrid system. The interaction between two known proteins, X and Y, is assayed using the yeast two hybrid System as described in the text. If the two proteins interact, all colonies are expected to turn blue in a β-galactosidase filter assay.
Figure 7: Functional map of pAS1-CYH2. The cloning vector provided in the yeast two hybrid kit for construction and expression of GAL4 DNA-binding domain (BD) fusion proteins. ERK, SAPK, and p38 were cloned into this vector as described in the text.
wild type p38, the kinase-inactive version was used in these experiments. The coding sequences for SEK1, MEK, MKK3, and MKK6 were cloned into the multicloning site of vector pACTII, also ensuring that in-frame fusion proteins would be generated (Figure 8). SEK1 was cloned into the NcoI and XhoI sites, MEK was cloned into the EcoRI and XhoI sites, MKK3 was cloned into the NcoI and SmaI sites, and MKK6 was cloned into the EcoRI and the XhoI (with a blunt end due to fill-in by Klenow) sites of pACTII. Plasmid DNA was prepared from bacterial cells for use in the transformations using either the Qiagen system (Qiagen, Studio City CA, USA) or by alkali lysis and polyethylene glycol purification (Sambrook et al., 1989).

2.2.3 Transformation of Yeast

Competent yeast cells were prepared by inoculating 20 ml of YPD broth with a single colony of the yeast strain Y190. The culture was grown overnight at 30°C with shaking. The following day, enough of the culture was added to 300 ml of YPD to give an OD$_{600}$ of approximately 1.0. This culture was grown for three additional hours, as before. The cells were then spun at 3000 rpm for 5 minutes, and washed in 50 ml of sterile water. Following, the cells were resuspended in 1.5 ml of 1X LiAc/TE (100 mM LiAc pH 7.5, 10 mM Tris pH 7.5, 1 mM EDTA) and left at room temperature for 10 minutes. For cotransformations, 1-10 μg of a given MAPK-pAS1-CYH2 vector was combined with 1-10 μg of a MAPKK-pACTII vector and 100 μg of salmon sperm carrier
Figure 8: Functional map of pACTII. The cloning vector provided in the yeast two hybrid kit for construction and expression of GAL4 activation domain fusion (AD) proteins. MEK, SEK, MKK3, and MKK6 were cloned into this vector as described in the text.
DNA. 100 µl of competent cells was added to each sample, followed by 600 µl of PEG/LiAc solution (50% PEG 4000, 10 mM Tris pH 7.5, 1 mM EDTA, 100 mM LiAc). The mixtures were then incubated at 30°C for 30 minutes with shaking. Following, 70 µl of dimethyl sulfoxide was added to each transformation reaction, and mixed gently. The cells were then heat shocked for 15 minutes at 42°C and chilled on ice. The reactions were spun for 5 seconds at full speed in a microcentrifuge, and resuspended in 500 µl of TE (10 mM Tris pH 7.5, 1 mM EDTA). Transformed cells were spread on SD plates lacking leucine and tryptophan, to select for the pACTII and pAS1-CYH2 vectors respectively, and grown at 30°C for 3-4 days.

2.2.4 Liquid Culture β-galactosidase Assays

Five ml of liquid media (SD lacking tryptophan and leucine) was inoculated with an entire yeast colony, and the culture was incubated overnight at 30°C with shaking. The following morning 4 ml of YPD was inoculated with 1 ml of overnight culture, and this was incubated as described until the OD₆₀₀ reached 0.5-0.8. Each culture was vortexed to disperse any cell clumps, and the OD₆₀₀ was recorded. One and a half ml of culture was spun at 14 000 rpm for 30 seconds, and the pellet was washed and resuspended in 300 µl of Z buffer (16.1 g/l Na₂HPO₄·7H₂O, 5.5 g/l NaH₂PO₄·H₂O, 0.75 g/l KCl, 0.246 g/l MgSO₄·7H₂O, pH 7.0), thereby concentrating the cells 5-fold. One hundred µl of cells was transferred to a fresh tube and placed in liquid
nitrogen for 1 minute. The cells were then thawed at 37°C for 1 minute. To each reaction tube, 700 μl of Z buffer with β-mercaptoethanol (100 ml of Z buffer to 0.27 ml of β-mercaptoethanol) was added, in addition a blank was set up with 100 μl of Z buffer. A timed incubation of each lysate and 160 μl of ONPG (4 mg/ml ONPG in Z buffer) was performed. The tubes were incubated at 30°C until a yellow colour developed. At this point, 400 μl of a 1 M Na₂CO₃ solution was added to the reaction. Once all reactions were complete, the tubes were spun at 14 000 rpm to pellet cell debris, and the supernatant was transferred to a cuvette. The degree of colour intensity was determined by measuring the OD₄₂₀ of the samples. To compare different samples, β-galactosidase units (where 1 unit is the amount of enzyme which hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose) were calculated using the following formula:

\[
\text{β-galactosidase units} = 1000 \times \frac{\text{OD}_{420}}{(t \times V \times \text{OD}_{600})}
\]

where: \( t \) = elapsed time
\( V = 0.1 \text{ ml} \times \text{concentration factor} \)

2.2.5 Filter Assays for β-galactosidase Activity

Once the transformant yeast colonies growing on the SD plates lacking leucine and tryptophan were 1-2 mm in diameter, a nylon filter was placed over top of the agar and slowly removed such that the majority of the yeast colonies adhered. These filters were submerged in liquid nitrogen for 10
seconds and then thawed at room temperature. Once thawed, the nylon filters were placed inside the lid of a petri dish on top of Whatman filter paper that had been presoaked in 2 ml of Z buffer and X-gal solution (100 ml Z buffer, 270 µl β-mercaptoethanol, 1.67 ml of a 20 mg/ml X-gal stock in dimethylformamide). The dishes were then covered and sealed with parafilm so that the buffer did not evaporate. Following, the filters were incubated at 30°C for up to 30 hours so that the blue colour could develop.

2.3 RESULTS

2.3.1 β-galactosidase Liquid Culture Assays

To examine the protein-protein interactions occurring between members of the ERK, SAPK, and p38 pathways, four separate trials of β-galactosidase liquid culture assays were performed on yeast cotransformed with each combination of a MAPK-pAS1-CYH2 vector and a MAPKK-pACTII vector (Figure 9). Since an interaction was seen with the ERK hybrid and the pACTII empty vector, we can not conclude that any of the interactions seen with the ERK hybrid were not artifactual. However, no interactions were seen with any of the remaining controls implying a true interaction between SEK and SAPK, SEK and p38, and MKK6 and p38. Further, no interactions were detected between MEK and SAPK, MEK and p38, MKK3 and SAPK, or MKK3 and p38.
Figure 9: β-galactosidase liquid culture assays. Each bar represents the interaction in β-galactosidase units of a given MAPK and MAPKK. The combination of MAPK (SAPK, p38, or ERK) and MAPKK (SEK, MEK, M KK3, or M KK6) tested is indicated beneath each bar. Those bars marked with MAPK-C or MAPKK-C represent controls that assay for interactions between the given kinase and the opposite empty vector. The pAS1-CYH2 and pACTII bars are the results of control transformations with empty vector. For further description see text.
To examine the interactions between components of the p38 and SAPK pathways further, yeast cotransformed with members of these pathways were analyzed by the β-galactosidase filter assays. The experiment was repeated five times, and the results indicate that SAPK interacts with SEK but not with M KK3 or M KK6 and that p38 interacts with M KK3, M KK6, and SEK (Figure 10). No interactions were seen in the controls which were cotransformed with either a MAPK-pAS1-CYH2 and empty pACTII or a MAPKK-pACTII and empty pAS1-CYH2 (data not shown).

**2.4 DISCUSSION**

Within a cell multiple signalling pathways exist that regulate diverse physiological functions. Because these cascades utilize many kinases with seemingly similar sequences and structures, mechanisms must exist which allow the components of a distinct cascade to phosphorylate and activate very specific targets. In the yeast mating pathway, the protein STE5 functions to bind together and effectively isolates the three kinase components KSS1/FUS3, STE20, and STE11 from all other kinases that might interfere with this signalling pathway. While STE5 appears to be effective at compartmentalising these kinases, to date no homologous scaffolding proteins have been found in mammalian cells. This would indicate that another mechanism might be responsible for maintaining the specificity required for signalling within higher
**Figure 10:** β-galactosidase filter assays. The association of the MAPKs, p38 and SAPK with the MAPKKs, MKK3, MKK6, and SEK is presented. (A) The yeast were streaked uniformly on SD agar plates, lacking tryptophan and leucine, and were grown for approximately 48 hours at 30°C. (B) A filter lift of the yeast colonies was assayed for β-galactosidase activity as described in section 2.2.5. Those yeast which turned blue demonstrated an interaction between the respective MAPK and MAPKK.
eukaryotic cells. Because it is unlikely that a yeast cell would contain the appropriate scaffolding proteins that could effectively bind the kinase components of a signalling cascade from a higher eukaryote, the yeast two hybrid system was exploited to examine how members of the mammalian MAPK pathways might maintain specificity. The results of these experiments suggest that scaffolding proteins are not involved in maintaining the specificity of a given pathway, but that another mechanism, possibly protein-protein interactions, may be largely responsible.

Not only has this yeast work been integral in examining how the different MAPK cascades maintain their functional distinctiveness, but it has helped to further clarify the degree of crosstalk that occurs between the different pathways. Previous work has shown that activation of the ERK pathway, the SAPK pathway, and the p38 pathway are not mutually exclusive and that a significant overlap in the agonist profiles of SAPK and p38 exists (see section 1.3.6). Crosstalk among the different pathways has also been noted when in vitro kinase assays or transient transfection over-expression studies are performed (see section 1.3.6).

The results of these yeast two hybrid experiments combined with other work from our group demonstrates that crosstalk does not readily occur in vivo under physiologic conditions (Zanke et al., 1996). For example, we have shown that SAPK and p38 are differentially activated by a variety of stress stimuli and that these results are not only agonist- but cell type-dependent. Further,
we have demonstrated MEKK over-expression does not result in p38 activation and that SEK coprecipitates from cells with SAPK but not with p38. These data support the hypothesis that SEK does not activate p38. Other coimmunoprecipitation experiments have shown that MEK will coprecipitate with ERK but not with SAPK or p38. A dominant-negative SEK mutant also impairs SAPK activation but not p38 activation when transfected into cells prior to stimulation with anisomycin. Similarly a dominant-negative MKK3 impairs the activation of p38 but not SAPK when transfected into cells prior to stimulation with either sorbitol or anisomycin. Lastly, a dominant-negative version of MKK6 prevents p38 activation in response to hyperosmolar stress in COS cells, but has no effect on the activation of SAPK.

While the results of the yeast two hybrid work is generally consistent with the aforementioned data, a few minor discrepancies exist. Firstly, p38 was shown to interact with both SEK and MKK6 by the liquid culture assays and SEK, MKK6, and MKK3 by the filter assays. In contrast, the results of our group demonstrates that p38 interacts with both MKK3 and MKK6 but not SEK (Zanke et al., 1996). Because X-gal is $10^5$ fold more sensitive than ONPG, a transient or weak interaction would not be quantifiable by the liquid culture assay. Therefore, by combining all of this information one could conclude that SEK and SAPK are components of one distinct signalling pathway, MEK and ERK are members of a second, and that p38 with either MKK3 or MKK6 are members of a third. Clearly, more work will be critical to further elucidating
the role of MKK3 and/or MKK6 in p38 signalling.
CHAPTER 3

The Cloning of a Serine/threonine Specific Kinase with Homology to the Cyclin-Dependent Kinases and the Mitogen-Activated Protein Kinases
3.1 INTRODUCTION

The regulation of cellular activities following exposure to differing environmental stimuli is a complex process that includes the phosphorylation and dephosphorylation of many members of multiple signalling pathways. One such signalling pathway that has been studied extensively is the MAPK cascade (see section 1.3). Homologues of this pathway exist in both lower and higher eukaryotes that regulate physiologically distinct processes. For example, in yeast, the FUS3/KSS1 pathway is involved in mating, the HOG1 pathway is involved in osmoregulation, the SMK1 pathway is involved in sporulation, and the MPK1 pathway is involved in cell wall biosynthesis (see sections 1.3.1 and 1.3.3) (Krisak et al., 1994; Mazzoni et al., 1993). In mammalian cells other homologues of the MAPK-family exist. The ERKs are involved in growth and differentiation and the SAPKs and the p38 family are involved in the stress response (see sections 1.3.1, 1.3.2, and 1.3.3).

Just as changes in the cell’s phenotype resulting from exposure to mitogenic and stressful stimuli are regulated by phosphorylation and dephosphorylation events, cell division is also regulated by similar mechanisms (see section 1.4). The cell cycle is regulated, in part, by a family of protein kinases termed cyclin dependent kinases, of which eight have been identified to date. cdc2 plays a critical role in the transition from G2 to mitosis while CDK2, CDK3, CDK4, and CDK6 are believed to be involved in the progression from G1 to S phase (see section 1.4.3). CDK5 has been implicated in neuronal
development and CDK7 and CDK8 appear to be involved in cell transcription (see section 1.4.3). In addition, a series of cdc2-related protein kinases exists. This subgroup includes PITSLRE, PCTAIRE, PITAIRE, PITALRE, PISSLRE, KKIAMRE, and KKIALRE (see section 1.5). The association of these kinases with any cyclins or their involvement with cell cycle progression has yet to be determined.

With the goal of isolating a novel MAPK member, we performed PCR on cDNAs from a rat jejunum library with degenerate oligonucleotide primers designed to regions specific to known MAPK family members. A product of this reaction was used to screen a rat jejunum cDNA library and a rat brain library, and in combination with the RACE technique, we isolated a cDNA for a novel protein kinase. This cDNA encodes a rat serine/threonine specific kinase that is related to the MAPK family, but most closely resembles KKIAMRE and KKIALRE, members of the cdc2-related protein kinase family. This kinase has been named NKIATRE based on its sequence corresponding to the PSTAIRE motif characteristic of CDKs.

3.2 MATERIALS AND METHODS

3.2.1 PCR Amplification and Library Screening

To clone a novel MAPK, an alignment of 20 sequences of MAPK homologues from various species was created. Two regions that were highly conserved among these kinases were selected and compared to the
corresponding sequences found within the other families of kinases as aligned by Hanks and Quinn (Hanks and Quinn, 1991). Because of their restriction to the members of the MAPK family, the degenerate oligonucleotide primers SRSP and SRAP were designed to the sequences TLREIK in subdomain III and YVA/VTRW in subdomain VIII respectively (Figure 11, Table 1). Although both of these primers were designed to be used for the PCR amplification, NKIATRE was isolated by use of SRAP and the M13/pUC Forward amplification primer (Gibco BRL, Gaithersburg MD, USA), a specific primer that recognizes a sequence flanking the cDNA insert site within the phage.

To obtain a satisfactory product by PCR, it is necessary to optimize the reaction conditions for any particular combination of template and primers. Primer concentrations are important since limiting concentrations will result in low yields and excessive amounts will result in contamination due to binding at non-target sequences. Because the levels of dNTPs and oligonucleotides are the major contributors of phosphate groups to the reaction, any change in their concentration will affect the levels of available Mg$^{2+}$. Therefore, it is important to optimize not only the primer concentration but the Mg$^{2+}$ concentration as well.

NKIATRE was isolated from the pooled products of four slightly different PCR amplifications. Each of these reactions contained 1 ug of cDNA isolated from a rat jejunum cDNA library (Stratagene, La Jolla CA, USA) as described by Sambrook et al. (Sambrook et al., 1989). Conditions for the first
Figure 11: Design of the degenerate oligonucleotide primers. To create the degenerate primers SRAP and SRSP, two regions conserved within the mammalian MAPK family members were selected. Primer SRSP was designed corresponding to the amino acid sequence TLREIK within subdomain III of the catalytic domain and primer SRAP was designed corresponding to the sequence YVATRW in subdomain VIII. Nucleotide sequence of the primers can be seen in Table 1. Abbreviations are as follows: cer = S. cerevisiae, alb = Candida albicans, hum = human, mou = mouse, bov = bovine, and dct = Dictyostelium.
<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>SEQUENCE 5'-3'</th>
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</thead>
<tbody>
<tr>
<td>Forward_M13/LacZ</td>
<td>GCC AGG GTT TTC CCA GTC ACG A</td>
</tr>
<tr>
<td>Reverse M13/LacZ</td>
<td>GAG CGG ATAA CAA TTT CAC ACA GG</td>
</tr>
<tr>
<td>MKH-1</td>
<td>ATC AAG CCT CCT CTC ACT CT</td>
</tr>
<tr>
<td>MKH-2</td>
<td>CCA CCG AGA TAT AAA GCC TG</td>
</tr>
<tr>
<td>MKH-3</td>
<td>GGT GGT TCT TCC TCA AGT TC</td>
</tr>
<tr>
<td>MKH-4</td>
<td>TGT CTT CTT CAG GTC TGG GA</td>
</tr>
<tr>
<td>MKH-5</td>
<td>GGA CTA GTC TTA ATG GCC ACT ATC CGC</td>
</tr>
<tr>
<td>MKH-6</td>
<td>CTT TCC TGT CGC ATT TGG</td>
</tr>
<tr>
<td>MKH-7</td>
<td>TCC CAG ACC TGA AGA AGA CA</td>
</tr>
<tr>
<td>MKH-8</td>
<td>CAG GCT TTA TAT CTC GGT GG</td>
</tr>
<tr>
<td>MKH-9</td>
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<tr>
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<td>3CL</td>
<td>ATG AAG CTT TGC ATT CTG ACC TGT TCG</td>
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Table 1: Primers. The nucleotide sequences of the primers used for cloning and sequencing NKIATRE.
50 µl reaction was 10 µmol primer SRAP, 20 µmol M13/pUC Forward amplification primer, 2 mM MgCl₂, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 0.1 U/µl Taq DNA polymerase (Promega, Madison WI, USA). The other three reactions differed by having 10 µmol of each primer and MgCl₂ concentrations of either 1.5 mM, 2.0 mM, or 2.5 mM. Before the addition of Taq DNA polymerase each reaction was heated to 94°C for 3 minutes. For 30 cycles the reaction mixtures were denatured at 94°C for 1 minute, allowed to anneal at 57°C for 1 minute and 30 seconds, and extended at 72°C for 2 minutes. Before a 4°C soak, an additional cycle was performed as above with a 5 minute primer extension at 72°C.

After all cycles were complete, the four reactions were pooled and 4 U of DNA polymerase I Klenow fragment (Amersham, Amersham UK) were added to create blunt termini by incubation at 37°C for 30 minutes. A 0.5 kb product was purified from an agarose gel using the Geneclean II kit (Bio 101, La Jolla CA, USA) and cloned into the SmaI site of the pUC18 plasmid.

Several products of the PCR amplification were analyzed by DNA sequencing, as described in section 3.2.2. These sequences were compared to those found in the GenBank database using the Blast program (Wisconsin Genetics Computer Group). One unique product was selected to screen the original rat jejunum cDNA library and a second, rat brain cDNA library (Stratagene, La Jolla CA, USA).

For library screening, plating cultures were prepared by inoculating LB
broth, supplemented with 0.2% maltose and 10 mM MgSO₄, with a single colony of the bacterial strain XL1 Blue. Cultures were grown overnight at 30°C to ensure that the cells would not overgrow. The following day the cells were spun down for 10 minutes at 2000 rpm and resuspended in 10 mM MgSO₄ so that the OD₆₀₀ = 0.5. To 6 ml of the cells, 5x10⁵ pfu of the appropriate library were added and the solution was gently mixed and incubated at 37°C for 15 minutes. To each of 10, 150 mm, NZY plates, 600 µl of the phage and bacteria mixture with 8 ml of top agar was poured. The plates were incubated at 37°C until the bacteria cells were completely lysed (approximately 12 hours).

The phage DNA from the lysed bacteria cells was transferred to Hybond-N nylon membranes (Amersham, Amersham UK) for 2 minutes. Duplicate filters were allowed to transfer for 5 minutes. After transfer, the filters were denatured by submersion for 2 minutes in a solution of 1.5 M NaCl and 0.5 M NaOH, and neutralized by submersion for 5 minutes in a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 8). The filters were then briefly rinsed in 2x SSC and allowed to dry at room temperature. DNA was UV crosslinked to the filters using the UV Stratalinker (Stratagene, La Jolla CA, USA) and the agar plates were stored at 4°C.

For screening, the filters were pre-hybridized in a solution of 50% formamide, 5x SSC, 5x Denhardt’s reagent, 20 mM Na₂PO₄, and 0.2 mg/ml salmon sperm DNA for two hours at 42°C. Hybridization was carried out
overnight at 42°C in a solution of 50% formamide, 5x SSC, 5x Denhardt’s reagent, 20 mM Na₂PO₄, 10% dextran sulphate, 0.2 mg/ml salmon sperm DNA, and 1x10⁶ cpm/ml of probe. To make the probe, 25 ng of the PCR product was radiolabelled with 50 μCi of [α-³²P]dCTP using the Multiprime DNA labelling system (Amersham, Amersham UK) and unincorporated label was removed by gel filtration through 7 cm of Sephadex G-50. Following hybridization, the filters were washed at room temperature in 2x SSC, and 0.1% SDS until the wash buffer radioactivity could no longer be detected by a Geiger counter. This was followed by two 20 minute washes at 55°C in 0.1x SSC and 0.1% SDS. Filters were exposed to film overnight at -70°C with an intensifying screen.

Purification of the hybridizing plaques was performed by secondary, tertiary, and quaternary screens, as described above. Excision of the cDNA inserts from the purified phage followed the manufacturer’s lambda ZAP II automatic excision protocol (Stratagene, La Jolla CA, USA).

### 3.2.2 DNA Sequence Analysis

Plasmid DNA was purified for sequencing with the Wizard Minipreps DNA Purification System (Promega, Madison WI, USA) and approximately 0.2 ug was sequenced with either [α-³⁵S]dATP and the AmpliCycle Sequencing Kit (Perkin Elmer, Branchburg NJ, USA) or [α-³³P]ddNTPs and the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Amersham UK). DNA sequence was read manually following exposure of the
gels to film at room temperature for 24 - 48 hours.

Sequencing primers used included the Forward M13(lacZ) Primer and the Reverse M13(LacZ) Primer (Perkin Elmer, Branchburg NJ, USA) (Table 1). In addition, twelve primers, MKH1-12, were constructed to allow overlapping sequence determination from the 5' and 3' ends of the clones (Table 1).

3.2.3 Amplification of 5' cDNA End

Amplification of the 5' end of the cDNA was accomplished with the 5' RACE System (Gibco BRL, Gaithersburg MD, USA). This technique allows for the amplification of nucleic acid sequences from an mRNA template between a defined internal site and unknown sequences at the 5' end (Figure 12). The general strategy of this protocol is as follows: first strand cDNA synthesis is primed using a gene-specific oligonucleotide which permits conversion of the specific RNA and maximizes the potential for complete extension to the 5' end. The cDNAs are then tailed with TdT which adds homopolymeric tails to the 3' ends. Following, the target cDNA is amplified by PCR using a nested gene specific primer which anneals 5' to the first gene-specific primer and an anchor primer that permits amplification from the homopolymeric tail.

To amplify the 5' end of NKIATRE, rat poly(A)*RNA (see section 3.2.5 for preparation of poly(A)*RNA) was used as a template and sequencing primer MKH4 was used to prime first strand cDNA synthesis (Table 1). PCR amplification of the NKIATRE cDNA utilised MKH8 (Table 1) as the nested
Figure 12: Flow diagram of the 5' RACE procedure. (A) Gene specific primer (GSP) 1 is annealed to mRNA. (B) First strand cDNA synthesis is performed with reverse transcriptase. (C) mRNA template is degraded with RNase H. (D) cDNA is purified and tailed with dCTP by terminal transferase. (E) The cDNA is PCR amplified with GSP2, a second primer that recognizes a sequence 5' to GSP1, and an anchor primer that recognizes the tailed region. To further increase specificity a second PCR amplification can be performed with the anchor primer and GSP3, a third primer that recognizes a sequence 5' to GSP2.
gene specific primer in a 50 µl reaction volume as described in the manufacturer's manual. For 35 cycles the reaction mixture was denatured at 94°C for 1 minute, allowed to anneal at 60°C for 30 seconds, and extended at 72°C for 2 minutes. One final cycle was performed as above with a 10 minute extension at 72°C.

To further enrich the PCR product for the specific cDNA, 1 µl of the PCR reaction was re-amplified under the outlined conditions with primer MKH5 as a second nested gene specific primer (Table 1). Following amplification, 1 U of DNA polymerase I Klenow fragment (Amersham, Amersham UK) was added to the reaction to create blunt termini by incubation at 37°C for 30 minutes. Products were cloned into the SmaI site of pBluescript KS and analyzed by sequencing with the Forward M13/lacZ primer and the Reverse M13/LacZ primer (Perkin Elmer, Branchburg NJ, USA) as previously described.

3.2.4 Southern Blot Analysis

For Southern blot analysis, 10 µg of rat genomic DNA was digested overnight at 37°C with either EcoRI, BamHI or HindIII. For the cross species Southern blot, 10 µg of either rat, pig, mouse, human or Drosophila genomic DNA was digested overnight at 37°C with EcoRI. Each of these digests were fractionated overnight by electrophoresis on a 1.0% agarose gel. Following electrophoresis, the gel was incubated with shaking for 15 min at room temperature in 0.1 M HCl, followed by 30 min in a solution of 0.5 M NaOH, 1.5 M NaCl, and then for 45 min in a solution of 1 M Tris pH 7.0, 1.5 M NaCl.
The genomic DNA was transferred to nylon membrane by capillary transfer (Sambrook et al., 1989). For all blots, the 1.3 kb EcoRI fragment (nucleotides 1-1372) of the rat jejunum cDNA was radiolabelled with [α-32P]dCTP, as described in section 3.2.1, and used as the probe. Prehybridization, hybridization, and wash conditions were performed as previously described for the library screens (section 3.2.1). Filters were visualized by Phosphor Imager (Molecular Dynamics) using the Image Quant software (Molecular Dynamics).

### 3.2.5 Northern Blot Analysis

Total cellular RNA was prepared using the TRIzol Reagent (Gibco BRL, Gaithersburg MD, USA) and from this poly(A)+RNA was prepared using Oligo (dT) cellulose (Boehringer Mannheim Biochemica, Laval PQ, Canada) as follows. 1 g of Oligo (dT) cellulose was suspended in 20 ml of loading buffer (400 mM NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS) and mixed for 20 minutes at room temperature. Oligo (dT) cellulose was added to each sample of total RNA (1 g of cellulose binds 10 mg of total RNA), and each mixture was gently rotated at room temperature overnight. All samples were then spun for 5 minutes at 2000 rpm, washed 2-3 times with 20 ml of loading buffer, and then 2-3 times with 20 ml of wash buffer (100 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% SDS). Cellulose samples were then resuspended in 10 ml of wash buffer and poured into columns (Bio Rad, Hercules California, USA). Columns were packed with 10 ml of wash buffer.
and then poly(A)+RNA was eluted with five 1 ml volumes of elution buffer pre-
warmed to 37°C (1 mM Tris pH 7.4, 1 mM EDTA, 0.2% SDS). Poly(A)+RNA
was precipitated overnight at -20°C by the addition of 30 μg tRNA, 0.6 ml 3 M
sodium acetate, and 12 ml 99% ethanol. Pellets were air dried and
resuspended in DEPC-treated water.

Ten μg of poly(A)+RNA from intestine, brain, muscle, lung, spleen, heart,
liver, and thymus were used to generate a rat tissue blot. RNA was
fractionated overnight by electrophoresis on a 1.2% formaldehyde agarose gel
with buffer recirculation. The gel was then washed thoroughly in DEPC
treated water and the RNA was transferred to nylon membrane by capillary
transfer (Sambrook et al., 1989). Like the Southern blots, the Northern blot
was probed with the 1.3 kb EcoRI fragment of the rat jejunum cDNA,
radiolabelled with [α-32P]dCTP, as described in section 3.2.1. To control for the
level of RNA in each lane, a rat β-actin probe was also used. Prehybridization,
hybridization, and wash conditions were carried out as described in section
3.2.1. Exposure of the filter and visualization of the image was as described
in section 3.2.4.

3.2.6 Prokaryotic Expression of NKIATRE

To express and purify NKIATRE from prokaryotic cells, the vector
pGEX-1λT was utilised (Pharmacia, Picataway NJ, USA). This vector contains
the sequence for GST just upstream of the multiple cloning site, allowing for
the generation of a GST fusion protein. To create an in-frame GST-NKIATRE fusion, the rat jejunum cDNA was digested with the restriction endonuclease EcoRI and the 1.38 kb fragment containing the 5' end of the cDNA was purified and cloned into the EcoRI site of the prokaryotic expression vector pGEX-1λT.

Fusion proteins were expressed in the *E. Coli*. strain BL21(DE3)pLysS, which carries the plasmid pLysS, that codes for T7 lysozyme, an enzyme that lowers the basal expression of target genes (Novagen, Madison WI, USA). Induction of protein expression in large bacterial cultures was accomplished with the addition of 1 mM IPTG. Bacteria were then lysed by gentle sonication in 50 mM Tris pH 8.0, 10 mM EDTA, 2.5% Nonidet P40, 50 mM NaCl, and fusion proteins were affinity purified with glutathione-Sepharose. Following, proteins were eluted with 3 mg/ml glutathione in 50 mM Tris pH 8.0 and dialysed against phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄) at 4°C. Protein integrity was confirmed by 0.25% Coomassie Brilliant Blue staining of SDS-PAGE products.

3.2.7 Preparation for Eukaryotic Expression

To obtain a complete cDNA for NKIATRE, the rat intestine clone and the rat brain clone #2 were digested with the enzyme BglII and the 1.3 kb and 2.0 kb bands were purified from an agarose gel utilizing the GeneClean II kit
(Bio 101, La Jolla CA, USA). Following ligation of the two products, PCR was used to generate the complete cDNA with approximately 100 ng of ligated DNA and 2 µl each of the 20 µM primers 5Cl and 3Cl (Table 1). Conditions for a total reaction volume of 50 µl were 2 mM MgCl₂, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 0.1 U/µl Taq DNA polymerase (Promega, Madison WI, USA). Before addition of the Taq DNA polymerase, the reaction was heated to 94°C for 3 minutes. For 30 cycles the reaction mixture was denatured at 94°C for 1 minute, allowed to anneal at 57°C for 1 minute and 30 seconds, and extended at 72°C for 2 minutes. Before a 4°C soak, an additional cycle was performed as above with a 5 minute primer extension at 72°C. After the reaction was complete, 4 U of DNA polymerase I Klenow fragment (Amersham, Amersham UK) were added to create blunt termini by incubation at 37°C for 30 minutes. Following, the reaction was purified from an agarose gel by the Geneclean II kit (Bio 101, La Jolla CA, USA), and cloned into the SmaI site of pBluescript KS.

To allow for immunoprecipitation from eukaryotic cells, NKIATRE was tagged with three HA repeats and ten histidine residues by subcloning into the vector pBluescript-3HA-10His. pBluescript-3HA-10His is a pBluescript derivative with the tags at the 3' end of the multiple cloning site. The complete NKIATRE cDNA was cut out of pBluescript KS with XhoI and HindIII (sites that were engineered into the PCR primers) and cloned into pBluescript-3HA-10His digested with the same enzymes, creating NKIATRE-
pBluescript-3HA-10His.

To subclone the complete, tagged, cDNA into the eukaryotic expression vector, pcDNA3 (Invitrogen, Carlsbad CA, USA), NKIATRE-pBluescript-3HA-10His was cut with XhoI and XbaI. Following, the fragment containing the cDNA and tags was ligated to pcDNA3 digested with the same enzymes and the integrity of the construct was validated by diagnostic restriction endonuclease digestions.

### 3.2.8 In Vitro Transcription and Translation

To linearize the vectors for in vitro transcription and translation, NKIATRE-pcDNA3 and control pcDNA3 were digested with the enzyme XbaI which cuts to the 3' side of NKIATRE. The DNA was purified by phenol/chloroform extraction and ethanol precipitation, and was resuspended in DEPC treated water. Transcription and translation were coupled using the TNT T7 Coupled Wheat Germ Extract System (Promega, Madison WI, USA), and 40 μCi of $^{35}$S-methionine. Following transcription and translation, the products were separated by SDS-PAGE and analyzed using a Phosphor Imager (Molecular Dynamics) and the Image Quant Software (Molecular Dynamics).

### 3.3 RESULTS

#### 3.3.1 Isolation of a Rat cDNA

To isolate a novel MAPK, degenerate oligonucleotide primers were
designed to two regions specific for sequences within the kinase catalytic domain of the MAPK family members. PCR amplification using the antisense degenerate primer, SRAP, and the M13/puc18 Forward primer generated a product of approximately 500 bp from a template of rat jejunum cDNAs (Figure 13). Sequence analysis of 44 of these products led to the discovery a single clone that was a putative MAP kinase.

To obtain the complete cDNA, the rat jejunum cDNA library and a rat brain cDNA library were screened with the PCR product. Three clones were isolated from the jejunum library which upon sequence analysis were shown to be identical. From the brain library two additional positive phage were isolated, each representing independent clones. Criteria for a full length cDNA included the presence of a consensus translation initiation sequence of GCCGCCA/GCCAUGG at a potential initiator methionine codon, and the presence of an upstream stop codon in the same reading frame as the initiation codon (Kozak, 1987; Kozak, 1989). Sequencing of all three clones demonstrated that none were complete by these criteria. The jejunum clone was truncated with a premature stop codon after amino acid 457 and the putative start codon was within 15 nucleotides from the 5' end of the clone. Although both brain clones contained the 3' end of the coding region, they were both missing the 5' end. Clone #2 commenced at amino acid 95 and clone #6 commenced at amino acid 62 (Figure 14). Of note, clone #2 contained a sequence of unknown origin, that had no homology to any known kinase, immediately upstream of the codon
Figure 13: PCR amplification. The products of PCR amplification from rat jejunal cDNAs with combinations of primers SRAP (A), SRSP (S), M13/puc18 Reverse (R), and M13/puc18 Forward (F) are shown above. Mg$^{2+}$ concentrations are indicated above each set of reactions. The first three sets of reactions had 10µmol of each primer indicated. The fourth set of reactions, labelled 20:10, had a Mg$^{2+}$ concentration of 2mM and contained 20µmol of primer R or F and 10µmol of primer A or S. A positive control (+) reaction had 10µmol each of the primers R and F. A negative control (-) reaction had 10µmol each of the primers R and F but no template DNA. See text for more detailed description of conditions. Size markers indicated are in kb.
for amino acid 95. By combining all of this data, it is apparent that the deduced amino acid sequence of the complete clone encodes a protein of 505 amino acids (Figure 15), with a predicted molecular weight of 67 kD.

3.3.2 Amplification of the 5' cDNA End

To determine if the putative initiation codon was the true start of translation, based on the previously outlined criteria, a 5' RACE was performed on rat brain poly(A)+RNA. The initial PCR amplification with the anchor primer and primer MKH8 generated a product of approximately 700 bp. To increase specificity, re-amplification of a small sample of this product with the internal primer MKH5 and the anchor primer, resulted in a product of approximately 400 bp. As expected from the distance between the two internal primer sites on the clones, the size of the product was approximately 300 bp shorter (Figure 16).

Sequence analysis of the cloned products revealed an upstream stop codon forty-two nucleotides from the putative initiation methionine. Comparison of the sequence surrounding this methionine codon to the Kozak consensus sequence, demonstrates that seven of the thirteen nucleotides are conserved at this site, including the purine at position -3 and the guanine at position +4 (Figure 17). Of the thirteen nucleotides, the purine at position -3 and the guanine at position +4 have been determined to be the most highly conserved nucleotides in all eukaryotic mRNAs (Kozak, 1986). Further, by considering only these two positions one can predict the relative strength of the
Figure 14: Sequence alignment of the cDNA clones. The amino acid sequences for the three cDNA clones are presented. Asterisks represent in frame stop codons.
Figure 15: Sequence of NKIATRE. The nucleotide and deduced protein sequence of NKIATRE is presented.
Figure 16: Products of the 5' RACE procedure. (A) First round PCR amplification. Lane 1: 5' end of NKIATRE amplified with primer MKH8 and the anchor primer. Lane 2: Control PCR using only primer MKH8. Lane 3: Control PCR using only the anchor primer. Lane 4: Control PCR with primer MKH8 and the anchor primer using untailed template. (B) Second round PCR amplification. Lane 1: 5' end of NKIATRE amplified with primer MKH5 and the anchor primer. Lane 2: Control PCR with no template. For more detailed descriptions of PCR conditions see text. Size markers indicated are in bp.
Figure 17: Analysis of NKIATRE as a serine/threonine specific kinase. The twelve conserved amino acid residues characteristic of the kinase catalytic domain and the two sequences that denote serine/threonine specificity are presented in bold font and are underlined with double lines. The twelve kinase domains are also indicated with italic bold font above the corresponding regions. The Kozak consensus sequence for the start of translation is highlighted and presented above the corresponding sequence of NKIATRE that is in bold font and underlined with a single line. In-frame stop codons are denoted by asterisks.
3.3.3 Sequence Analysis

Protein kinases share a core catalytic domain with various structural motifs being conserved (see section 1.2.1). These features can be used not only to help identify novel kinases, but to further classify them as serine/threonine specific or tyrosine specific. Analysis of the sequence of NKIATRE reveals that it contains all twelve of the invariant or nearly invariant residues that are characteristic of the kinase catalytic domain, and that it most closely resembles a serine/threonine specific kinase.

The 12 residues that are conserved within the kinase catalytic domain and their positions within NKIATRE are as follows: Gly11 and Gly13 within subdomain I (GXGXXGXXV motif), Lys33 in subdomain II, Glu50 in subdomain III, Asp125 and Asn130 in subdomain VIB, Asp143 and Gly145 in subdomain VII (DFG motif), Glu170 in subdomain VIII (APE motif), Asp183 and Gly188 in subdomain IX, and Arg274 in subdomain XI (Figure 17).

Strong indicators of the specificity of the kinase can be found within two short motifs in subdomain VI and subdomain VIII (Figure 17). In subdomain VI, the consensus DLKPEN represents serine/threonine specificity whereas DLRAAN or DLAARN represents tyrosine specificity. NKIATRE contains the sequence DIKPEN (residues 125-130), which conforms most closely to the serine/threonine specific kinase consensus sequence. In subdomain VIII, the
sequence GT/SXXY/FX is found to be conserved in serine/threonine specific kinases, whereas PI/VK/RWT/M is characteristic of the tyrosine specific kinases. NKIATRE contains the sequence ATRWYR (residues 162-167) which again is most closely related to the serine/threonine specific kinases.

Comparison of NKIATRE with all protein kinases reveals that it is most homologous to the cdc2-related protein kinases KKIAMRE and KKIALRE. Within the kinase domain, at the amino acid level, NKIATRE shares 60% identity to KKIAMRE, 50% identity to KKIALRE, 33% identity to rat ERK1, 32% identity to rat ERK2, 34% identity to mouse p38, and 33% identity to rat SAPKα (Figures 18 and 19).

NKIATRE has conserved residues characteristic of both the MAPKs and the CDKs. In subdomain VIII are located Thr158 and Tyr161 which correspond to the sites of activating phosphorylation for the MAPKs. Thr158 also corresponds to a critical site of activating phosphorylation for the CDKs. In subdomain I of the CDKs are also located Thr14 and Tyr15 which are involved in regulating CDK activity. While Tyr15 is conserved in NKIATRE, the residue Thr14 is analogous to a serine. In addition, a motif search revealed no protein-protein interaction domains within NKIATRE.

3.3.4 Southern Blot Analysis

To examine if NKIATRE was a component of the rat genome, the 1.3 kb
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| 241 | P38 | T---TDH---KL---RLV-TPGAELKLKISSESA.RN-IQS-AQPMKN-ANV-IG... |
| 241 | CONSENSUS | PLFPG-SDIDQ-L---IL---LG---F---F---Y---LP----FP... |
| 301 | NKIATRE | NGLLA-AHVC-QI-AC---SSSLH---H-D---TR..DFIEKFIPE-RA |
| 301 | KKIALRE | SYPL---LG---KGC-HM---TE-LTCEQL-H---ENIR-IE-LKHDKPTRK |
| 301 | KKIAMRE | SEVFI---AK-C-HI---D---PFCAEL-H-D---QMDGFAERSQ-EQ-QL |
| 301 | CDC2 | DENG---S---I---A---SGKM---N---ND---LDNZIKM.. |
| 301 | CDK2 | DEDG---S---Q---H---N---SAKA---A---QD---VTKPVPH--RL |
| 301 | ERK1 | SDS---L---DR---T-N-N---TVEE---A---EQQY-PT---EPVAEP-TF |
| 301 | ERK2 | ADSK---L---D---T-N-H---EVEQ---A---EQQY-PS---EPIAEAP-KF |
| 301 | SAPK | PSESERDKITQ---R---S---VI-D---SVDE---R---ITVW-PA~AEAPPQI---DA |
| 301 | P38 | ANPL-V---E---V---SD---TAQA---A-A---AQYH--PD---EPVAD.P-DQ |
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| 361 | ERK2 | D-EELDLPKERL-ELI-EERTAFQPAERS ..................................
| 361 | SAPK | Q-EERBHAIEE-W-ELI-KEVMDEERKSNVKDSQPSDAVSSKATPSQSSSINNIMST |
| 361 | P38 | S-ESRDLLEIE-SIT-DVYESFVPPDQEMESES.. ..................................
| 361 | CONSENSUS | -L---K--- ..........................................................
<p>| 421 | NKIATRE | VIKAKKGKGDVPDLKKTESEGHEHRQQGTAEDTHPTSLDRKPSVSELT ................. |
| 421 | KKIALRE | NI.......................................................... |
| 421 | KKIAMRE | KIKGSKIDGEKAEGKNRNASCLHDSRTSHNKIVPSSTLKDCNVSVDHTRPSVAIPP |
| 421 | CDC2 | .......................................................... |
| 421 | CDK2 | .......................................................... |
| 421 | ERK1 | .......................................................... |
| 421 | ERK2 | .......................................................... |
| 421 | SAPK | EHTLASDHTSLDASTGPLGCR ........................................ |
| 421 | P38 | .......................................................... |
| 421 | CONSENSUS | .......................................................... |</p>
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**Figure 18:** Multiple Sequence Alignment. The deduced sequence of NKIATRE is compared with KKIALRE, KKiAMRE, cdc2, CDK2, ERK1, ERK2, SAPK, and p38 using the pileup program (Wisconsin Genetics Computer Group). Dashes indicate residues identical to the consensus and periods represent gaps introduced into the sequences to optimize the alignment.
Figure 19: Dendogram. NKIATRE is compared to other serine/threonine kinases using the pileup program (Wisconsin Genetics Computer Group). The identity of the kinases with NKIATRE was calculated using the bestfit program (Wisconsin Genetics Computer Group). Total refers to the percent identity over the entire sequence of the kinase whereas kinase refers to the percent identity within the catalytic domain.
fragment of the rat jejunum cDNA clone was used to screen rat genomic DNA digested with three separate restriction endonucleases (Figure 20). Results of Southern blotting revealed that three bands hybridized to EcoRI digested DNA with approximate sizes of 7.5 kb, 5.6 kb, and 4.7 kb. Two bands of approximately 5.4 kb and 4.0 kb hybridized in BamHI digests, whereas only two bands of 9.4 kb, and 4.7 kb were seen in the HindIII digested DNA. This would suggest that NKIATRE is a single copy gene.

Results of cross species Southern blot analysis with the above described probe demonstrate that NKIATRE hybridizes to EcoRI digested genomic DNA from rat, pig, mouse, human, and hamster (data not shown) (Figure 21). No hybridizing bands were seen in EcoRI digested DNA from either Drosophila, Dictyostelium (data not shown) or S. cerevisiae (data not shown).

3.3.5 Northern Blot Analysis

To determine the tissue distribution of NKIATRE, a Northern blot of poly(A)⁺mRNA from rat intestine, brain, muscle, lung, spleen, heart, liver, and thymus was probed with the 1.3 kb EcoRI fragment of the rat jejunum cDNA (Figure 22). Northern blotting revealed bands of approximately 7 kb in brain, muscle, and heart, bands of approximately 4.4 kb in intestine, brain, muscle, lung, and heart, bands of approximately 2.5 kb in brain, muscle and heart, and a band of approximately 2.2 kb in muscle tissue. No expression of NKIATRE was seen in rat spleen, liver, or thymus (not shown) tissue. Quality of
Figure 20: Southern blot of rat genomic DNA for NKIATRE. Rat genomic DNA was digested overnight with either EcoRI, BamHI, or HindIII and was probed with the 1.3 kb EcoRI fragment of the rat jejunum cDNA.
Figure 21: Cross species Southern blot for NKIATRE. Rat, pig, mouse, human, and *Drosophila* genomic DNAs were digested with EcoRI and probed with the 1.3 kb EcoRI fragment of the rat jejunum cDNA.
Figure 22: Expression of NKIA TRE in rat tissues. (A) Expression of NKIA TRE was examined by Northern blot analysis of poly(A)'mRNA from rat tissues with the 1.3 kb EcoRI rat jejunum fragment as a probe. (B) The same blot probed for β-actin.
poly(A)⁺ mRNA was determined by probing for rat β-actin.

3.3.6 Prokaryotic Expression of NKIATRE

Expression of the GST-NKIATRE fusion protein from five independent *E. coli* colonies yielded three clones with the NKIATRE cDNA in the correct orientation. As seen in figure 23, a fusion protein of approximately 85 kD was isolated whereas only GST was isolated from the other two clones.

3.3.7 *In Vitro* Transcription and Translation

Translation of full length NKIATRE is predicted to yield a protein product of 67 kD. As seen in figure 24, a product of 67 kD is generated following in vitro transcription and translation of the NKIATRE cDNA. In addition, a smaller product of approximately 50 kD is present which is likely the result of degradation or internal initiation/termination. No protein was seen in the negative control reaction which utilised pcDNA3 DNA as a template.

3.4 DISCUSSION

In an attempt to isolate a novel MAPK we have cloned a cDNA that is most homologous to KKIALRE and KKIAMRE, members of the cdc2-related protein kinase family. Before cloning the full cDNA for NKIATRE, we confirmed by Southern blotting that the product of the initial PCR
Figure 23: Prokaryotic expression of GST-NKIATRE fusion protein. The 1.38kb EcoR1 fragment from the rat jejunum cDNA library was expressed as a GST fusion protein in bacterial cells upon induction by IPTG. Fusion proteins were purified with glutathione-Sepharose and analyzed by SDS-PAGE. Lanes 1-2: Fusion protein resulting from NKIATRE cloned in the 3'-5' direction. Lanes 3-5: GST-NKIATRE fusion protein. Lane C: GST.
Figure 24: *In vitro* transcription and translation of NKIATRE. Coupled in vitro transcription and translation of NKIATRE was accomplished with a T7 wheat germ extract system. Labelled protein products were visualized by Phosphor Imager following SDS-PAGE, as described in the text.
amplification was a component of the rat genome and not an artifact of the reaction. Further, by cross species Southern blots we investigated whether NKIATRE homologues exist in other mammals and lower eukaryotes. From these results it does not appear that an NKIATRE homologue exists in lower eukaryotes, although it remains possible that evolutionary changes have resulted in sequence divergence that can not be readily detected by high stringency screening.

The tissue distribution of NKIATRE, as shown by the Northern blot, suggests that the expression of NKIATRE is restricted to "non-proliferative" tissues. This would imply a discrete role in their biology, perhaps by playing a role in suppressing the cell cycle and therefore proliferation. Further, the multiple bands seen in several of the lanes on the Northern blot suggest the existence of differentially spliced products.

While little is known about the function of NKIATRE, we have determined from sequence analysis and searches of the Genbank database that NKIATRE likely represents a single domain intracellular serine/threonine specific kinase. Motif searches have identified possible phosphorylation sites for other kinases but most of these sites are very short amino acid stretches that probably play no role in the biological function of this kinase. The role of NKIATRE in cell signalling will require further study.

Sequence comparison of NKIATRE to the MAPK family members indicates that it is most homologous to ERK2. However, if NKIATRE is a
MAPK, it will define a new subgroup of this family based on its sequence within the site of activating phosphorylation. As described in chapter 1, the MAPKs are unique in their ability to become activated by dual phosphorylation on both a threonine and tyrosine residue. To date the members of this family have been subdivided based on the amino acid separating these two sites of phosphorylation. The ERKs contain the sequence TEY, p38 has TGY, and the SAPKs have TPY. NKIATRE contains the sequence TDY. Because these sequences suggest different upstream activators, NKIATRE may be part of a novel MAPK signalling cascade. However, to be considered a member of the MAPK family NKIATRE would have to become activated by phosphorylation on both threonine 158 and tyrosine 160 in the presence of the appropriate agonist and mutation of these sites must render the protein kinase inactive.

NKIATRE is more likely a member of the cdc2-related protein kinase family and appears to be a third member of a new subgroup that includes KKIAMRE and KKIALRE. Because these kinases are highly homologous and share unique sequences that are conserved in both CDKs and MAPKs, it is possible that they are involved in the regulation of similar cellular events. In support of this, comparison of the expression pattern of NKIATRE to that of KKIALRE and KKIAMRE reveals that they are highly similar. Over the tissues examined, NKIATRE is the only one that is expressed in either heart or muscle tissue. While the level of expression is only moderate in heart tissue, a relatively high level is seen in muscle. Therefore, it is possible that
NKIATRE may have an additional role, especially in muscle cells, perhaps by regulating their differentiation. As described in section 1.5.1, it has been hypothesized that KKIALRE and KKIAMRE are involved in the production of germ cells based upon their differential expression in ovary and testis. To investigate whether NKIATRE may be involved in similar cellular processes, it will be important to examine its expression in testis and ovaries and to compare these levels to that seen in brain and muscle.

One important question that remains to be answered is how these kinases are regulated. As discussed in section 1.5.1, both KKIAMRE and KKIALRE can be stimulated by EGF; however, this activation does not result in phosphorylation of the TDY motif. While it seems that this family is not regulated like the MAPKs upon exposure to EGF, it is possible that other stimuli will phosphorylate them within the TDY motif or that they are regulated by phosphorylation at other sites. The high sequence conservation of this family of enzymes to the MAPKs and the CDKs suggests the existence of an evolutionary relationship; however, it remains to be determined just how related their functions really are.

3.5 FUTURE WORK

As with KKIALRE and KKIAMRE, it will be necessary to resolve whether NKIATRE is a CDK or a MAPK. To determine if NKIATRE functions as a novel MAPK, external stimuli that elicit an increase in its kinase activity
will be sought. It will be critical to show that activation of the kinase upon such stimulation occurs through dual phosphorylation within the TDY motif. As mentioned above, both KKIALRE and KKIAMRE also contain the TDY motif; however, stimulation of the enzymatic activity of these two kinases upon exposure to EGF does not require dual phosphorylation as would be expected of a MAPK (Taglienti et al., 1996).

To examine the physiological significance of NKIATRE it will be important to identify upstream activators as well as downstream targets. The interaction of NKIATRE with any of the known MAPKKs could be assessed through in vitro kinase assays, co-immunoprecipitation experiments, or with the yeast two hybrid system. Based on the fact that NKIATRE contains an aspartic acid residue between the threonine and tyrosine, which differs from any other MAPK and suggests the existence of a novel upstream activator, it would be surprising if NKIATRE is phosphorylated well by a characterized MAPKK. Lastly, as with any other MAPK signalling pathway, it will be important to investigate what kinases or transcription factors lie downstream of NKIATRE. This can be accomplished through in vitro kinase assays with known MAPK substrates, co-immunoprecipitation experiments or with the yeast two hybrid system. If none of these experiments provide a clue as to the physiological significance of NKIATRE, the yeast two hybrid system could be used to screen a cDNA library for any proteins that will interact with it in vivo.
cdc2-related kinases may be reclassified as CDKs once it has been demonstrated that their kinase activities fluctuate in a cell cycle-dependent manner, or that they are capable of binding a cyclin. NKIATRE will be examined for these properties. However, neither KKIALRE nor KKIAMRE has been demonstrated to bind any known cyclin or to have kinase activity that fluctuates in a cell cycle-dependent manner. Although it is possible that the cyclin partners of KKIALRE and KKIAMRE have yet to be identified, by comparison, it is unlikely that NKIATRE functions as a CDK. Clearly, much work remains to elucidate the role of NKIATRE in either intracellular signalling or cell cycle control.
CHAPTER 4

References


Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera AG, Xiong Y, 1994. Growth suppression by p18, a p16\textsuperscript{INK4A/MTS1} and p14\textsuperscript{INK4B/MTS2}-related CDK6 inhibitor, correlates with wild-type pRb function. Genes and Dev. 8: 2939-2952.


