

The Mek1-Erk1 node:  
A place of convergence

by

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## ABSTRACT

At present, five mitogen-activated protein (MAP) kinase families have been identified in vertebrates. At least three of these MAP kinases have been shown to operate within highly conserved intracellular signalling modules composed of sequentially activating protein kinases. Cell surface receptors in response to extracellular stimuli invoke MAP kinase cascades to regulate gene expression within the nucleus. The best characterized MAP kinases are the extracellular signal-regulated kinases (Erks), the jun N-terminal kinases (Jnks) and the high osmolarity glycerol kinases (Hogs). Mitogenic signals that stimulate the G protein Ras activate the Raf→Mek→Erk→Rsk module. In turn, the Nik→Mekk1→Mkk4→Jnk and the Mkk3→Hog→MAPKAPK modules respond to environmental stress signals. Recent progress in MAP kinase signalling has revealed that MAPKs respond to a variety of environmental cues that promote cell growth, differentiation and death.

The experimental work described in this doctoral thesis was initiated six years ago as an independent study into the regulation of MAP kinase. At the time, purified MAP kinase was shown to be a prolyl-directed seryl/threonyl kinase that was regulated by phosphorylation on tyrosyl and threonyl residues. In addition, a partial cDNA sequence for a MAP kinase termed Erk1 was shown to be homologous to Fus3 (for fusion), a kinase that regulated the mating pathway in *Saccharomyces cerevisiae*. Results from genetic epistasis experiments revealed that Fus3 was positioned downstream in a cascade that involved several protein kinases. Consequently, we hypothesized that MAP kinase probably functioned as a key intermediary in the transmission of information from the plasma membrane to the nucleus.

To understand the function MAP kinase plays in regulating the flow of information from plasma membrane to the transcriptional machinery in the nucleus, a cDNA clone of Erk1 was isolated from a human liver library. The mRNA message encoded a 379-amino acid protein which was 12 residues longer (MAAAAAQGGGGG) than Erk1 described previously from rat and, therefore, represented the full-length sequence. By using recombinant Erk1 protein, a MAP kinase activator activity was purified from 1-MeAde-treated sea star oocytes. Immunoblotting with antipeptide antibodies directed against mouse Mek1 (MAP/Erk kinase) and yeast Ste7 (Sterile 7) revealed that the sea star activator protein was a Mek1 homologue. Unexpectedly, the purified activator was able to increase Erk1 MBP phosphotransferase activity without directly phosphorylating the enzyme. This contrasts results obtained by other researcher groups that showed direct phosphorylation by the protein kinase activator Mek1 leads to Erk1 activation. Therefore, the sea star Mek-like protein may activate human Erk1 by a different mechanism. The data presented here indicates that sea star Mek may activate human Erk1 via allosteric modulation.

MAP kinase family subgroups Erk, Jnk and Hog are activated by phosphorylation on threonyl and tyrosyl residues that are located within a tripeptide Thr-Xaa-Tyr (where the intervening residue Xaa codes for Glu in Erk1, Gly in Hog and Pro in Jnk) regulatory motif. Phosphorylation of these sites in MAP kinases is performed by one of two specific pairs of MAP kinase kinases. Erk1 and Erk2 are the only known substrates for Mek1 and Mek2. A similar narrow specificity has been observed for Jnks and Hogs by their immediate activators Mkk4/Mkk7 and Mkk3/Mkk6, respectively.

A systematic analysis of the TEY tripeptide sequence in Erk1 was undertaken to understand the importance each residue plays in the substrate specificity of mammalian Mek1. Constitutively active mouse Mek1 was used to phosphorylate a battery of



threonyl and tyrosyl phosphorylation site mutants. Mutation of either Thr-202 or Tyr-204 reduced the efficiency at which Mek1 was able to phosphorylate the Erk1 mutant proteins. In fact, serine substitution for Thr-202 was the only allele that retained MBP kinase activity. The intervening residue located between the regulatory threonyl and tyrosyl phosphorylation sites of MAP kinase may also serve as an important specificity determinant to prevent inappropriate cross-phosphorylation by MAP kinase kinases from parallel modules. The replacement of Erk1 TEY motif with the Jnk (TPY) and Hog (TGY) regulatory sites also reduced Mek1 specificity and markedly decreased Erk MBP phosphotransferase activity. Taken together, these results indicate that the TEY motif in Erk1 is an important consensus sequence for Mek1 recognition.

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## NOMENCLATURE AND ABBREVIATIONS

### 1. Measurements

°C	degrees celsius
Ci	Currie, $2.22 \times 10^{12}$ disintegrations per minute
g	gram
x g	times the force of gravity
h	hour
kDa	kilodalton
mA	milliamp
mg	milligram
ml	mlilitre
M	moles/litre
mmol	millimol
mM	millimolar
min	minute
nM	nanomole ( $10^{-9}$ mole).
nm	nanometre ( $10^{-9}$ metre)
sec	second
$\mu$	micrometre ( $10^{-6}$ metres)
$\mu\text{m}$	mircorgram ( $10^{-6}$ gram)
$\mu\text{l}$	microlitre ( $10^{-6}$ litre)
$\mu\text{M}$	micromole ( $10^{-6}$ mole)

## 2. Amino acids

Name	Three letter abbreviation	One letter symbol	Characteristics
Alanine	Ala	A	non-polar
Arginine	Arg	R	basic
Asparagine	Asn	N	polar
Aspartate	Asp	D	acidic
Cysteine	Cys	C	polar
Glutamate	Glu	E	acidic
Glutamine	Gln	Q	polar
Glycine	Gly	G	polar
Histidine	His	H	basic
Isoleucine	Iso	I	non-polar
Leucine	Leu	L	non-polar
Lysine	Lys	K	basic
Methionine	Met	M	nonpolar
Phenylalanine	Phe	F	non-polar
Proline	Pro	P	non-polar
Serine	Ser	S	polar
Threonine	Thr	T	polar
Tryptophan	Trp	W	basic (weak)
Tyrosine	Tyr	Y	polar (weak)
Valine	Val	V	non-polar

### 3 Nucleotides

Adenine	A
Cytosine	C
Guanine	G
Thymine	T
Deoxyadenosine 5'-triphosphate	dATP
Deoxycytosine 5'-triphosphate	dCTP
Deoxyguanine 5'-triphosphate	dGTP
Deoxythymidine 5'-triphosphate	dTTP

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## INTRODUCTION

### 1. HISTORICAL PERSPECTIVE

For more than twenty-five years, oncogenes have been the subject of intense investigation. The ability of these mutant genes to mediate unregulated proliferation resulting in tumour growth was, in part, the result of excessive protein phosphorylation within the cell (reviewed in Cantley *et al.*, 1991). In support of this notion, some oncogene products possess intrinsic enzymatic activities that control the catalysis of protein phosphorylation (reviewed in Bishop, 1991). Previously, it was demonstrated that phosphorylation cascades were essential for regulating a variety of metabolic activities in the cell and it was later postulated that similar signalling relay systems may modulate many other biological activities (reviewed in Krebs, 1983). The premise that the cellular counterparts of oncogenes, the proto-oncogenes, were key players in intracellular communication systems has been supported by a large body of genetic and biochemical research.

The vast majority of oncogene kinases, whether retrovirally-induced or naturally occurring in mammals, were found to encode a class of enzymes that transfer the  $\gamma$ -phosphate moiety of ATP onto the hydroxyl group of tyrosyl residues (Hanks *et al.*, 1988). This subfamily of kinases exist as membrane-spanning receptor or membrane-associated protein-tyrosyl kinases. The EGF-encoded receptor oncogene ErbB- and the v-Src-family kinases are two examples from the tyrosyl-directed family of protein kinases. Very few seryl/threonyl-directed protein kinases (for example Raf1, Mos, Pim1, Akt for AKR mouse thymoma (alias PKB) and Tpl2 (for tumour progression locus 2 alias Cot for cancer Qsaka thyroid) are known to be cancer-causing oncogenes. This is surprising given that more than 99.9% of phosphate ester-linkages in cells occurs on



serine and threonine (Cooper *et al.*, 1981). It is believed that persistent elevated levels of protein tyrosyl phosphorylation has very strong oncogenic potential. A possible explanation for the large number of oncogene-encoded tyrosyl kinases may be their location high upstream within signal transduction pathways. By and large, these enzymes direct signalling messages to a variety of regulatory elements within different cascades that exist downstream of them. In addition, the complex architectural design of many of these enzymes, such as critical autoinhibitory domains for example, may make them more susceptible to transforming mutations.

The fact that unrelated classes of oncogenes such as extracellular ligands, eg. Neu; membrane-spanning and -associated tyrosyl kinases, e.g. ErbB and Src family; guanine-nucleotide-binding (GTP-binding) proteins, e.g. Ras family proteins located at the cell membrane; cytoplasmic seryl/threonyl kinases, e.g. Raf1 and Tpl2; and transcription factors present in the nucleus, e.g. Jun and Fos that differed vastly in their structure and function were able to cause a similar transformation phenotype in a variety of immortalized cell types implied that they may activate similar signal transduction pathways. Several lines of evidence in different model systems indicate this to be the situation in the living cell. The relationship between each of these specialized signal transduction molecules has been the subject of intense research during the past two decades.

Some of our earliest insights into the relationship between these disparate cell signalling molecules as well as their position within cell signalling cascades occurred from studies into the regulation of Ras activity. In quiescent cells, Ras was shown to exist in an inactive GDP-bound state. However, certain point mutations could maintain Ras in a constitutively active GTP-bound state. Microinjection of activated GTP-Ras protein caused the transient transformation of a number of mammalian cell lines and induced

germinal vesicle breakdown (GVBD) in *Xenopus* oocytes (Stacey *et al.*, 1984; Feramisco *et al.*, 1984; Birchmeier *et al.*, 1985). Later, dominant inhibitory mutants of Ras were used to block growth in NIH 3T3 cells and differentiation in PC12 and 3T3-L1 cell lines (Cai *et al.*, 1990; Szeverényi *et al.*, 1990; Ogiso *et al.*, 1990 and Benito *et al.*, 1991). Furthermore, engagement of platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) cell surface receptors caused a rapid but transient increase in biologically active Ras-GTP complexes. Similarly, transformation of the activated forms of these receptors also elicited the same effect. These findings indicated that wild type Ras was a downstream transducer of these mitogens (Satoh *et al.*, 1990; Satoh *et al.*, 1990; Gibbs *et al.*, 1990).

In a manner similar to the control of Ras by membrane receptors, the seryl/threonyl kinase Raf was shown to be regulated by similar growth factors in many different mammalian cells (reviewed in Rapp, 1991). The ability of activated recombinant v-Raf to transform microinjected NIH 3T3 cells supports a role for Raf as an important intracellular messenger (Smith *et al.*, 1990; Kolch *et al.*, 1991). Several lines of evidence indicated that Raf acts downstream of Ras in growth factor signal transduction. The constitutively active form of Raf was able to reverse growth-factor arrest caused by overexpression of dominant negative Ras or microinjection of neutralizing Ras antibodies (Smith *et al.*, 1986; Huleihel *et al.*, 1986). Also, transfection of the kinase-inactive Raf1 as well as expression of the anti-sense RNA or N-terminal regulatory domain of this enzyme was sufficient to block cells transformed with activated Ras or ligand-activated receptors (Heideker *et al.*, 1990; Kolch *et al.*; Bruder *et al.*, 1992).

One of the first consequences of growth factor signal transduction in cells is the rapid increase in transcription of early-response genes like c-Fos, which was first

identified through its oncogenic counterpart v-Fos (Curran *et al.*, 1983; Greenberg and Ziff, 1994). In these studies, constitutively active Raf1 was shown to be capable of increasing c-Fos promoter transactivation. This clearly positioned Raf as an intermediary regulator since it was shown to act downstream of growth factor receptors and upstream of Fos gene expression (Jamal and Ziff, 1990).

Taken together these results placed several receptor tyrosine kinases (eg. PDGF and EGF) and their ligands, intracellular transducers (eg. Ras, Raf) and nuclear effectors (eg. Fos) as well as their oncogenic homologues (eg. v-Sis, v-ErbB, v-Ras, v-Raf and v-Fos) within the same signalling system. Until recently, little was known about the intervening steps following the engagement of receptor tyrosine kinases by their ligands and the ensuing mechanisms that activated cytoplasmic signal transducers and the consequent elevation of gene expression in the nucleus.

For those researchers seeking to understand the regulatory mechanisms that operate within the cell at a molecular level, several outstanding questions remained to be answered concerning each step in the signalling process from the cell membrane to the nucleus. What regulated the switch from tyrosyl phosphorylation at the cell membrane to seryl/threonyl phosphorylation within the cytoplasm? How does ligand-induced receptor stimulation activate transcription factors in the nucleus to increase gene expression? Using the combinatory approach of biochemical reconstitution and genetic disruption new signalling proteins were positioned within signalling pathways. In situations where genetic manipulations were not amenable in biochemical models or biochemical assays too difficult to develop for genetic systems, molecular biology provided the necessary tools to advance the research. Isolation of new genes by the yeast two-hybrid analysis or site-directed mutagenesis of known genes using recombinant DNA technology enabled biologists to uncover key molecular interactions and determine essential regulatory sites

within the individual signalling proteins. It has become apparent from the diverse research efforts of geneticists, biologists and biochemists involved in the study of such diverse organisms as yeast, flies, nematodes and mammals that the cell has the molecular equivalent to electronic circuitry or a cellular intranet (Egan and Weinberg, 1993; Pelech, 1996).

It has become evident from research conducted during the past few years that the prototypical MAP kinase defined by the extracellular-signal regulated kinases 1 and 2 (Erk1 and Erk2 ) isoforms operate within a specific cell signalling pathways. In addition, Erks were shown to be regulated by over 50 different stimuli in a variety of model systems (Pelech and Charest, 1995). More recently, several other MAP kinase family homologues have been discovered that function within distinct, non-overlapping signal transduction pathways (Pelech and Charest, 1995). In fact, as will be discussed later, the level at which MAP kinases operate within the signal transduction pathways (i.e. intermediary between the cell membrane and nucleus) means that these enzymes may act as critical convergent points or nodes through which the flow of information must traverse. The remainder of this review will discuss the MAP kinase superfamily

## 2. MAP KINASE SIGNALLING MODULE: DISCOVERY OF A SIGNAL TRANSDUCTION PARADIGM.

### 2.1 Lessons from studies in glycogen metabolism.

Cell biologists have been fascinated with the complexity and regulation of important biological processes such as cell growth, division, differentiation and death in response to specific stimuli. To uncover the molecular events that control essential cellular activities it has been necessary to define ways to assay changes observed in

response to external cues. Alterations in cellular protein phosphorylation in response to hormone treatment was one such approach, that in the middle of the 1950's, was demonstrated to be essential for regulating glycogen metabolism (reviewed in Krebs, 1983; Cohen, 1983). Phosphorylase is a key regulatory enzyme required for glycogen breakdown. The interconversion of phosphorylase between active and inactive states is a dynamic process involving enzyme phosphorylation-dephosphorylation reactions. Several groups exploited this hormone regulated phosphorylation event to successfully detect and purify the phosphorylase regulatory factor. After many years of experimental investigation, the first protein phosphorylation cascade activated as a consequence of hormone-dependent stimulation was shown to cause an increase in glycogen breakdown. This important final metabolic step in glycogen metabolism was the starting point to begin working backward in a step wise fashion toward the epinephrine receptor located at the cell surface. Both Drs. Edwin Krebs and Edmond Fisher received the Nobel Prize in Medicine in 1992 for their pioneering work in the area of protein phosphorylation research.

## 2.2 Dissection of a ubiquitous signalling pathway.

Insulin- and growth factor-dependent stimulation also cause dynamic changes in the phosphate content of many cellular proteins in a manner similar to that observed during studies into glycogenolysis (reviewed in Maller *et al.*, 1990; Pelech *et al.*, 1990). One consequence of cell exposure to polypeptide ligands such as insulin is a robust increase in protein synthesis (Krieg *et al.*, 1988). Phosphorylation of multiple seryl residues within the 36-kDa S6 protein of the 40S ribosome subunits was reported to be an essential regulatory step for modulating translation of specific mRNAs (Gressner and Wood, 1974; Halselbacher *et al.*, 1979; Bommer *et al.*, 1980; Kozma *et al.*, 1989). Subsequently, phosphorylation of 40S ribosomes *in vitro* identified S6 kinase activities in

cytosolic extracts obtained from serum- and ligand-induced activation of mammalian cells and progesterone-induced *Xenopus* oocyte maturation (Rosen *et al.*, 1981; Novak-Hofer and Thomas; 1985; Tabarini *et al.*, 1985; Erikson and Maller, 1986; Cobb, 1986; Blenis *et al.*, 1987; Pelech and Krebs, 1987). Subsequent purification and cloning experiments identified two distinct classes of S6 kinases. The 85- to 92-kDa ribosomal S6 kinase (Rsk) family isolated from *Xenopus* was demonstrated to be structurally unique from the 70-kDa S6 kinase isolated from mammals (Jones *et al.*, 1988; Bannerjee *et al.*, 1990). It is now widely accepted that the 70-kDa S6 kinase and not the Rsk kinases is responsible for modulating protein synthesis within cells (Chung *et al.*, 1992).

During this same period, several research groups initiated studies into the cellular regulation of insulin and growth factor signalling in mammalian cells as well as GVBD in maturing *Xenopus* and echinoderm oocytes (Sturgill and Ray, 1986; Ray and Sturgill, 1987; Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Hoshi *et al.*, 1998). A protein kinase activity, distinct from the S6 protein kinase described previously, was uncovered in insulin-treated murine fibroblast cells that phosphorylated the exogenous substrate microtubule-associated protein 2 (MAP2) (Ray and Sturgill, 1987; Hoshi *et al.*, 1988). Pelech and co-workers also described an enzyme with a similar molecular mass that was stimulated to phosphorylate the substrate myelin basic protein (MBP) *in vitro* in response to hormone-induced GVBD in immature frog and sea star oocytes (Cicirelli *et al.*, 1988; Pelech *et al.*, 1988). This novel enzyme termed MAP kinase (for mitogen-activated protein kinase) was shown to be identical to pp42, a protein that becomes transiently phosphorylated on tyrosyl residues in response to a variety of mitogens, including EGF, PDGF, 12-O-tetradecanoylphorbol-13-acetate (TPA), thrombin, insulin and oncogenically transformed cells (Rossomando *et al.*, 1989 and references 1-10 therein).

The fact that MAP kinase and S6 kinase were acutely regulated by insulin and that the time course of activation for MAP kinase preceded that of S6 kinase in 3T3-L1 cells led Drs. Sturgill and Maller and their colleagues to investigate the relationship between the two enzymes. The S6 kinase from *Xenopus* oocytes was inactivated *in vitro* by incubation with seryl/threonyl-specific phosphatases 1 and 2A. The dephospho-S6 kinase phosphotransferase activity was partially restored *in vitro* after preincubating the enzyme with insulin-stimulated MAP kinase that was partially purified from *Xenopus* oocytes (Sturgill *et al.*, 1988). Support for the notion of an insulin- and growth factor-regulated cascade involving MAP kinase and S6 kinase in the same pathway was demonstrated by other researcher groups using partially purified enzymes and reconstitution experiments (add-back) from fractionated cell extracts (Gregory *et al.*, 1989; Ahn and Krebs, 1990; Kyriakis and Avruch, 1990; Chung *et al.*, 1991a; Chung *et al.*, 1991b). However, MAP kinase was unable to activate the 70-kDa S6 kinase in similar experiments and therefore the two proteins were recognized to operate within distinct signalling pathways (Ballou *et al.*, 1991; Mukhopadhyay *et al.*, 1992). These MAP kinase enzymes were later termed Erk1 and Erk2.

Erk protein kinases are regulated in mammalian cells by many growth factors and their cognate receptor tyrosyl kinases (Pelech *et al.*, 1990; Cobb *et al.*, 1991). Activation of the monomeric guanine nucleotide-binding protein Ras is accomplished by engaging many of these same receptors (Downward *et al.*, 1990; Gibbs *et al.*, 1990; Satoh *et al.*, 1990; Burgering *et al.*, 1991). Leivers and Marshall (1992) were the first researchers to demonstrate that Ras and Erk kinases lie on the same signalling pathway. In the absence of growth factor activation, constitutively active Ras (Val-12) introduced into Swiss 3T3 cells by scrape loading activated MAP kinase within minutes. Furthermore, in a NIH-3T3 cell line overexpressing the insulin receptor, a dominant interfering Ras (Asn-17) blocked insulin-induced activation of Erk2 without affecting phosphatidylinositol-3-

kinase (PI3-kinase) activity in these same cells (de Vries-Smits *et al.*, 1992). In Rat-1 cells, the activity was inhibited by the same Ras mutant after PDGF treatment but remained active after exposure to phorbol ester (Leever and Marshall, 1992; de Vries-Smits *et al.*, 1992).

Concurrently, experiments using Swiss 3T3 and NIH-3T3 fibroblastic cells overexpressing activated forms of Raf1 led to the activation of Erk1 and Erk2 in a Ras- and receptor tyrosyl kinase-independent manner (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Dent *et al.*, 1992). As further support, Erk2 was stimulated by TPA and EGF in a COS-1 cell line overexpressing full-length Raf1, while Raf1 and Ras were shown to cooperate in the activation of Erk1 in coexpression experiments in baculovirus Sf9 insect cells (Howe *et al.*, 1992; Williams *et al.*, 1993). MAP kinases are also directly activated by regulatory kinases, since isoforms of this enzyme family isolated as recombinant proteins or present in unstimulated cells are activated with partially purified fractions from NGF- TPA- or EGF-stimulated cells (Gómez and Cohen, 1991; Ahn *et al.*, 1991; Adams and Parker, 1991). MAP kinase kinase isoforms Mek1 and Mek2 (MAPK Erk1 kinase) in turn are regulated by direct phosphorylation, since they become inactivated upon treatment with the protein-seryl/threonyl phosphatase PP2A (Gómez and Cohen, 1991; Matsuda *et al.*, 1992; Crew and Erikson, 1992). Erk1 MBP phosphotransferase activity and consequently that of dephosphorylated Meks was restored *in vitro* by direct phosphorylation and activation with immunoprecipitated Raf1 from transfected cell lines (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Dent *et al.*, 1992). Reconstitution experiments involving prokaryotic and eukaryotic expressed proteins supported the hypothesis that Raf-Mek-Erk operate within the same signal transduction pathway (MacDonald *et al.*, 1993).



Induction of neurite-like processes in rat adrenal pheochromocytoma PC12 cells in response to nerve growth factor (NGF) treatment has been shown in separate experiments to coincide with the stimulation of Ras, Raf, MAP kinase and Rsk activities (Hagag *et al.*, 1986; Blenis and Erickson, 1986; Gotoh *et al.*, 1990; Boulton *et al.*, 1991; Ohmichi *et al.*, 1992; Scimeca, *et al.*, 1992). Expression of dominant inhibitory mutant of Ras (Asp-17) blocked NGF-induced tyrosyl phosphorylation of Erk1 and Erk2, and also inhibited Raf1 hyperphosphorylation (previously used as a marker of Raf1 activation) and Rsk activation (Thomas *et al.*, 1992; Wood *et al.*, 1992). The MAP kinase activator activity detected in PC12 cells was also attenuated by the Ras inactive-mutant (Gómez and Cohen, 1991; Robbins *et al.*, 1992). Catalytically compromised mutants of Raf1, Mek1, and MAP kinase isoform, Erk2, also interfered with the neurological differentiation of PC12 cells (Wood and Roberts, 1993). However, overexpression of constitutively active truncated form of Raf1 in PC12 cells did not significantly stimulate MAP or Rsk kinase activities, and neither was Raf1 able to activate MAP kinase directly during *in vitro* reconstitution experiments (Wood *et al.*, 1992; Jaiswal *et al.*, 1994). These results implied that although Ras activates all three enzymes in an NGF-dependent fashion, Raf-1 and MAP/Rsk kinases may lie on separate signalling pathways. In light of these results, Jaiswal *et al.*, (1994 and 1996) later revealed that RafB is the principal mediator for Ras activation of the MAP kinase pathway in PC12 cells.

In *Xenopus* oocytes, microinjection of oncogenic Ras (Val-12) induced maturation that was slightly delayed from what is observed after progesterone treatment (Birchmeier *et al.*, 1985). As expected, activated Ras protein is able to stimulate quiescent oocytes to undergo GVBD and hence circumvent the need for receptor stimulation by triggering the activation of a protein termed REKS (for Ras p21-dependent Erk-kinase stimulator), along with Mkk1 (MAP kinase kinase 1), Erk2, and Rsk (Pomerance *et al.*, 1992; Shibuya *et al.*, 1992; Hattori *et al.*, 1992; Itoh *et al.*, 1993). REKS is a multimeric

protein-complex in which one of the proteins, a 98-kDa protein kinase was shown to be immunologically distinct from Raf1, Mos and Ste11 (Kuroda *et al.*, 1995). Partial purification of an identical complex from bovine brain cytosol revealed that the active kinase component is the 95-kDa brain-specific RafB (Yamamori *et al.*, 1995).

### 2.3 Lessons from *Saccharomyces cerevisiae* mating pathway

The first MAP kinase signal transduction pathway was elucidated from genetic epistasis experiments performed with budding yeast. At present, five distinct MAP kinase signal transduction pathways have been identified in *S. cerevisiae* (Herskowitz, 1995; Pelech and Charest, 1995). The conservation of the basic MAP kinase signalling module (consisting of a MAPKKK, MAPKK and MAPK) is a testament of its importance in transducing messages in response to environmental cues (Errede and Leven, 1993; Neiman, 1993). In yeast, highly related MAP protein kinase modules regulate such disparate biological activities as mating, growth, differentiation and homeostasis (Herskowitz, 1995; Pelech and Charest, 1995).

The MAP kinase pathway was first elucidated from genetic studies into the regulation of mating in haploid yeast (Herskovitz, 1995). Mating between two yeast strains through conjugation initiates cell cycle exit prior to DNA replication. Engagement of unique seven transmembrane receptors (Ste2 and Ste3 for sterile) with the specific mating pheromone ligand of the opposite cell type (MAT $\alpha$ -factor for Ste2 and MAT $\alpha$ -factor for Ste3) initiates this process (Jenness *et al.*, 1983; Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985; Hagen *et al.*, 1986). Binding of these small oligopeptide pheromones ( $\alpha$ -factor is a 12 residue lipoprotein;  $\alpha$ -factor is a 13 residue peptide) leads to the induction of a cascade of biological events that increases mating-specific functions including G1 cell cycle arrest before Start (the point at which a cell

commits itself to a further round of mitosis) and changes associated with the conjugation process (Bender and Sprague, 1986; Nakayama *et al.*, 1987; Sprague, 1991). The pheromones **a** and  $\alpha$  interaction with Ste3 and Ste2 promotes the exchange of GDP for GTP on the heterotrimeric guanylyl nucleotide-binding protein G  $\alpha$ -subunit (Gpa1) and subsequent dissociation of G $\beta$ - (Ste4) and G $\gamma$ - (Ste18) subunits from the receptor (Nakafuku *et al.*, 1987; Jang *et al.*, 1988; Whiteway *et al.*, 1989; Blumer and Thorner, 1990). The liberated G $\beta$ -subunit, in turn, forms a complex with Ste20 and activates the kinase by a poorly defined mechanism (Wu *et al.*, 1995; Leberer *et al.*, 1997). At present, Ste20 appears to be the most proximal kinase to the membrane receptor within the mating-pheromone-regulated MAP kinase pathway. Although the biochemical evidence is sketchy, genetic epistasis studies support a role for Ste20 in the sequential activation of the Ste11, Ste7 and the MAP kinases Fus3 and Kss1 within a non-branching pathway (Herskovitz, 1995). The non-kinase protein Ste5 may function to maintain fidelity within the module by sequestering Ste4, Ste11, Ste7, Fus3 and Kss1 into a large signalling particle (Choi *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994). Another possible function for this platform protein may be to localize the MAP kinase module and more specifically Ste11 in the vicinity of Ste20 at the cell surface.

### 3. REGULATION OF MAP KINASE

#### 3.1 Identification of MAP kinases Erk1 and Erk2.

The first purification of a MAP kinase to homogeneity was achieved from sea star oocytes induced to undergo GVBD (Sanghera *et al.*, 1990). Subsequently, the MAP kinase isoform Erk1 was purified from several mammalian sources (Boulton, *et al.*, 1991; Northwood *et al.*, 1991). The Erk2 isoform first detected by Ray and Sturgill (1988) in mouse 3T3 cells was purified from mammalian cell culture and mature *Xenopus* oocytes

(Gotoh *et al.*, 1991; Payne *et al.*, 1991; Barrett *et al.*, 1992). All three kinases display apparent relative molecular masses of 42-44 kDa by gel filtration and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in addition to exhibiting similar substrate preference for MAP2 or MBP proteins (Rossomondo *et al.*, 1991). Subsequent isolation of Erk cDNAs revealed a high degree of sequence homology. The predicted amino acid sequences for rat and human Erk1 and Erk2 are 85-90% identical (Boulton *et al.*, 1991; Owaki *et al.*, 1992; Gonzalez *et al.*, 1992; Charest *et al.*, 1993). There is also a high degree of conservation across species. Human full-length Erk1 is 96% identical to rat Erk1 (Marquardt and Stabel, 1992; Owaki *et al.*, 1992; Charest *et al.*, 1993), while the deduced protein sequence of Erk2 from human is 95% identical to *Xenopus* Erk2 (aliases Mpk1 and Xp42) (Boulton *et al.*, 1991; Posada *et al.*, 1991; Gotoh *et al.*, 1991). Representatives of the Erk MAP kinase subfamily have been isolated from all four eukaryotic kingdoms (Ferrell, 1996 and references therein).

### 3.2 Regulation of Erk phosphotransferase activity.

Erk MAP kinases are members of the seryl/threonyl family of protein kinases while themselves being subject to phosphorylation on tyrosine and threonine (Hanks *et al.*, 1988; Rossomondo *et al.*, 1989 and references therein; Anderson *et al.*, 1990; Rossomondo *et al.*, 1991). In cells labeled *in vivo* with  $^{32}\text{P}$ , Erk1 and Erk2 become phosphorylated on tyrosyl, seryl and threonyl residues (Ray and Sturgill, 1988; Robbins and Cobb, 1992). To maintain full enzymatic activity, however, Erk1 and Erk2 appear to require phosphorylation on threonyl and tyrosyl residues. Removal of either of these phosphoresidues with seryl/threonyl or tyrosyl specific phosphatases completely inactivates these enzymes (Sturgill *et al.*, 1988; Anderson *et al.*, 1990; Ahn *et al.*, 1991; Rossomondo *et al.*, 1991; Pollack *et al.*, 1991). Payne *et al.* (1991) identified the regulatory phosphorylation sites from  $^{32}\text{P}$ -labeled Erk2. Active Erk2 purified from EL4

mouse T cell line after stimulation with phorbol dibutyrate (PDB) and subsequently proteolytically treated with trypsin yielded a single phosphopeptide containing phosphorylated threonine and tyrosine. The phosphorylated residues mapped to two neighbouring sites, namely Thr-183 and Tyr-185 (equivalent to Thr-202 and Tyr-204 in Erk1), at positions within kinase subdomains VII and VIII (Hanks *et al.*, 1988; Payne *et al.*, 1991). It has become clear from 3D structural analysis of several seryl/threonyl protein kinases that phosphorylation of residues between subdomains VII and VIII can induce the necessary global conformational changes to increase enzyme activity (Johnson *et al.*, 1996; Canagarajah *et al.*, 1997). The characteristic phosphothreonine and phosphotyrosine motif is separated by an intervening amino acid TXY (where X is Glu-184 in Erk2), which is a hallmark of the superfamily of MAP kinases (Ferrell, 1996). The level of seryl phosphorylation appears to remain unaffected following growth factor stimulation (Robbins and Cobb, 1992).

Erks obtained from rat 1 HIRcB cells autophosphorylate on tyrosyl residues *in vitro* (Seger *et al.*, 1991; Scimeca *et al.*, 1991). These same kinases also display tyrosyl phosphorylation *in situ* when expressed in *Escherichia coli* as well as *in vitro* after their purification as fusion proteins (Seger *et al.*, 1991; Crews *et al.*, 1991; Wu *et al.*, 1991; Robbins *et al.*, 1993; Charest *et al.*, 1993). Since bacteria possess no intrinsic tyrosyl kinase activity, the Erk enzymes probably accumulate tyrosine by an autophosphorylation reaction (Wu *et al.*, 1991; Seger *et al.*, 1991; Charest *et al.*, 1993). This basal autophosphorylating activity on tyrosyl is concentration independent and thus occurs by an intramolecular reaction (Wu *et al.*, 1991; Robbins *et al.*, 1993). The small but detectable increase in tyrosyl autophosphorylation consequently leads to a nominal stimulation in Erk phosphotransferase activity toward exogenous substrates including MBP (Seger *et al.*, 1991; Crews *et al.*, 1991; Wu *et al.*, 1991; Rossomondo *et al.*, 1992; Robbins *et al.*, 1993; Charest *et al.*, 1993). Tyr-185 in Erk2 and Tyr-204 in Erk1 are the

sites of autophosphorylation (Rossomondo *et al.*, 1992b, Charest *et al.*, 1993). Only minor amounts of Thr-183 were detected from fusion proteins expressed in bacterial cells or during *in vitro* experiments and may explain the observed Erk activation. This self-catalyzed phosphate incorporation prompted several research groups to speculate that, in a manner similar to PKA, (cAMP-dependent protein kinase) autophosphorylation may play a significant role in Erk regulation (Knighton *et al.*, 1991; Seger *et al.*, 1991; Crews *et al.*, 1991; Wu *et al.*, 1991; Robbins and Cobb, 1992). However, catalytically compromised Erk2 when expressed in *Xenopus* oocytes or CCL39 hamster cells becomes phosphorylated at the regulatory phosphorylation sites after stimulation (Posada and Cooper, 1992; Her *et al.*, 1993). This supported earlier observations that Erks could be activated by a kinase activator without the need for autophosphorylation (Ahn *et al.*, 1991; Gómez and Cohen, 1991; L'Allemain *et al.*, 1992; Rossomondo *et al.*, 1992a; Alessandrini *et al.*, 1992).

### 3.3 Receptor-mediated stimulation of MAP kinases Erk1 and Erk2.

The ubiquitous distribution of the MAP kinase family proteins Erk1 and Erk2 may explain why these enzymes are activated by a bewildering array of extracellular stimuli (Pelech *et al.*, 1990; Boulton *et al.*, 1991; Cobb *et al.*, 1991; Megan and Cobb, 1997). Besides mitogens such as PDGF and NGF which activate tyrosyl specific cell surface receptors, the MAP kinase module is stimulated by a variety of other ligands. On the one hand, these include peptides (eg. angiotensin and bradykinin) that activate seven transmembrane spanning (7TM) receptors that are coupled to heterotrimeric G proteins. Interleukins, on the other hand, interact with their cognate single transmembrane (1TM) receptors which stimulate the activities of the Src (sarcoma) and Jak (Janus kinase) family of receptor-associated tyrosyl kinases. Steroid hormones (e.g. estradiol) which

have high affinity nuclear receptors also increase MAP kinase activity by a pathway that remains poorly understood (Migliaccio *et al.*, 1996).

### 3.4 Substrates of the Erk protein kinase.

The microtubule-associated protein 2 and myelin basic protein kinases were the substrates first utilized to detect activation of MAP kinases (Ray *et al.*, 1987; Hoshi *et al.*, 1988; Cicicelli *et al.*, 1988). Thr-97 in MBP was identified as the phosphorylation site (Sanghera *et al.*, 1990b; Erickson *et al.*, 1990). Synthetic peptides patterned after this site were used to determine the optimal substrate recognition sequence for the Erk family to be Pro-(basic/neutral)-(Ser/Thr)-Pro motif (Clark-Lewis *et al.*, 1991). This result was later confirmed using a random peptide expression library (Songyang *et al.*, 1996). A large number of proteins have been identified as *in vitro* substrates for Erk1 and Erk2 including metabolic enzymes acetyl-CoA carboxylase (Pelech *et al.*, 1991), tyrosine hydroxylase (Haycock *et al.*, 1992) and phospholipase A<sub>2</sub> (Lin *et al.*, 1993; Nemenoff *et al.*, 1993; Sa *et al.*, 1995); structural proteins caldesmon (Childs *et al.*, 1992; Adam and Hathaway, 1993) and tau (Drewes *et al.*, 1992; Ledesma *et al.*, 1992; Goedert *et al.*, 1992); signalling proteins EGF receptor (Northwood *et al.*, 1991; Thérout *et al.*, 1992), the guanine nucleotide exchange protein Sos (for son-of-sevenless) (Cherniack *et al.*, 1995; Waters *et al.*, 1995; Rozakis-Adcock *et al.*, 1995; László *et al.*, 1995; Porfir and McCormick, 1996; Corbalan-Garcia *et al.*, 1996; Klarlund *et al.*, 1996), Raf1 (Anderson *et al.*, 1991; Lee *et al.*, 1992) Mek1 (Saito *et al.*, 1994), Rks1-3 (Zhao *et al.*, 1996) and Mnk1-2 (Waskiesicz *et al.*, 1997; Fukugana and Hunter, 1997); transcription factors Tal1 (Cheng *et al.*, 1993) and Elk1 (Gille *et al.*, 1992; Janknecht *et al.*, 1993; Marais *et al.*, 1993); and finally the nuclear estradiol steroid hormone receptor (Kato *et al.*, 1995; Bunone *et al.*, 1996).

#### 4. MAP KINASE ACTIVATORS

##### 4.1 Identification of MAP kinase kinase isoforms Mek1 and Mek2.

A number of groups studying MAP kinase signal transduction worked backwards using recombinant Erk proteins as activatable substrates to purify MAP kinase kinases from a variety of model systems including *Xenopus* and sea star oocytes induced to undergo GVBD (Matsuda *et al.*, 1992; Charest *et al.*, unpublished data), insulin-treated rabbit skeletal muscle (Nakielnny *et al.*, 1992a; Wu *et al.*, 1992), as well as from phorbol ester- and EGF-stimulated human and murine cell lines (Seger *et al.*, 1992a; Shirakabe *et al.*, 1992; Crews and Erikson, 1992). Mek s exhibit apparent molecular masses of between 45-46-kDa as observed on SDS-PAGE. Partial protein sequence of Mek revealed that it contains sequence homology to MAP kinase kinases Ste7 (Sterile 7) and Byr1 (By pass of Ras1), two kinases that are regulated by pheromone-dependent MAP kinase signalling pathways in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kosako *et al.*, 1992; Nakielnny *et al.*, 1992b; Wu *et al.*, 1992; Crew and Erikson, 1992). The purified Mek proteins possess identical functional and immunological properties as those MAP kinase activators described in previous *in vitro* studies (Ahn *et al.*, 1991; Gómez and Cohen 1991; Matsuda *et al.*, 1992; L'Allemain *et al.*, 1992; Rossomondo *et al.*, 1992; Alessandrini *et al.*, 1992).

Several Mek cDNAs were obtained by using the polymerase chain reaction (PCR) technique and degenerate oligonucleotides based on peptide sequences obtained from purified proteins (Crew *et al.*, 1992; Ashworth *et al.*, 1992; Seger *et al.*, 1992b; Kosako *et al.*, 1993; Wu *et al.*, 1993a; Döring *et al.*, 1993; Zheng and Guan, 1993; Yashar *et al.*, 1993). Mammalian Mek1 is a 393 amino acid long polypeptide displaying an approximate molecular mass of 43-kDa (Crew *et al.*, 1992; Ashworth *et al.*, 1993;



Seger *et al.*, 1992b; Wu *et al.*, 1992a; Döring *et al.*, 1993; Zheng *et al.*, 1993). Sequence comparisons between Mek1 isolated from murine pre-B cells and human T cells revealed homologies of 97% and 91%, respectively, with the rat brain isoform. These same mammalian proteins are 30-40% homologous with yeast Ste7 and Byr1 (Crews *et al.*, 1992; Seger *et al.*, 1992b; Döring *et al.*, 1993). Recombinant Mek1 purified as a GST (glutathione-S-transferase) fusion protein phosphorylates Erk1 *in vitro* while its overexpression in COS cells causes a 3-fold greater activity after TPA treatment than non-transfected cells (Crew *et al.*, 1992; Seger *et al.*, 1992b).

Dr. Edwin Krieb's research team also obtained a second clone with a coding region identical to Mek1 except for a 78 nucleotide deletion located at position 471-584 in subdomain V (equivalent to 26 amino acids) (Seeger *et al.*, 1992b). This alternative mRNA transcript which exists in A431 cells might therefore explain the presence of 45- and 46-kDa Mek isoforms described at the protein level in these same cells (Seeger *et al.*, 1992a). What unique role this 45-kDa isoform performs in the cells awaits further investigation. Zheng and Guan (1993) isolated a second MAP kinase kinase family member, termed Mek2, from several human libraries. Human Mek1 and Mek2 are 80% identical at the amino acid level. Bacterially expressed GST-Mek2 incubated with serum-treated NIH-3T3 cell extract phosphorylated and activated recombinant human Erk1 *in vitro* (Zheng and Guan, 1993). Mek2 has also been cloned from rat (Otsu *et al.*, 1993; Largaespada *et al.*, 1993; Wu *et al.*, 1993b).

A 45-kDa MAP kinase activator similar to Mek1 (alias MAPKK in frog) was isolated from *Xenopus* and was shown to be expressed predominantly in ovarian tissue (Kosako *et al.*, 1993). Two other Meks distinct from Mek1 described during progesterone-induced oocyte maturation were cloned from frog (Yashar *et al.*, 1993). Mek2 is also expressed early in the developing central nervous system including brain,

spinal neurons and the eye (Yashar *et al.*, 1993). A developmentally-regulated MAP kinase with a similar expression pattern has been observed in tissue from the nervous system and, therefore, may define a new MAP kinase signal transduction pathway (Zaitsevskaya and Cooper, 1993). A third distinct MAP kinase kinase, Mkk3, (alias Mek3) mRNA transcript was shown to be expressed in the later stages of the developing frog zygote (Yashar *et al.*, 1993).

#### 4.2 Mek protein kinases as regulators of MAP kinase activity.

Mek1 and Mek2 are the only known physiological activators described for Erk1 and Erk2. These same enzymes are able to phosphorylate catalytically-compromised Erk1 and Erk2 mutants. However, active Mek2 displays a higher phosphotransferase activity toward both Erk isoforms than does Mek1 (Zheng and Guan, 1993). Furthermore, Mek1 and Mek2 exhibit a high degree of specificity toward these MAP kinase enzymes, since neither can phosphorylate denatured Erk proteins nor synthetic TEY peptides patterned after the physiologically relevant regulatory phosphorylation sites (TEY peptide: PEHDHTGFLTEYVAWATR WYR) (Seger *et al.*, 1992a). Also, these kinases possess a very narrow substrate specificity such that common and commercially available proteins (e.g. MBP, histones, casein) act as poor *in vitro* substrates (Seger *et al.*, 1992a).

MAP kinase activators that are capable of phosphorylating Erk enzymes also stimulate their phosphotransferase activity *in vitro* (Ahn *et al.*, 1991; Gómez and Cohen 1991; Matsuda *et al.*, 1992; L'Allemain *et al.*, 1992; Rossomondo *et al.*, 1992; Alessandrini *et al.*, 1992). To eliminate the possibility that these MAP kinase activators are cellular factors that stimulate Erks by enhancing their autophosphorylating activity, several research laboratories used kinase-inactive Erk1 and Erk2 to demonstrate that

these Meks were *bona fide* kinases. In fact, Meks activate Erks by phosphorylating them on the physiologically relevant sites both *in vitro* and *in vivo* (L'Allemain *et al.*, 1992; Rossomondo *et al.*, 1992a; Alessandrini *et al.*, 1992; Posada and Cooper, 1992; Her *et al.*, 1993). These combined results contrast earlier observations that MAP kinases act as points of integration for seryl/threonyl and tyrosyl signalling pathways (Anderson *et al.*, 1990). Because Meks are capable of phosphorylating both seryl/threonyl and tyrosyl residues, they are considered to be dual-specificity protein kinases. Mek protein kinases phosphorylate Erks on their regulatory sites in an ordered manner (Robbins and Cobb, 1992; Haystead *et al.*, 1992). Under conditions of limiting ATP concentrations *in vitro* Tyr-185 phosphorylation accumulates before the appearance of Thr-183 in Erk2 (Haystead *et al.*, 1992). Moreover, only the diphospho form of Erk2 appears to be fully active toward MBP. In fact, in NGF-treated PC12 cells, the dual phosphorylated form is coincident with Erk1 and Erk2 attaining maximal enzymatic activity (Robbins and Cobb, 1992).

#### 4.3 Mek/Erk protein complexes and distribution within the cell.

Spatial distribution of the Mek/Erk complexes has been the subject of intense research and debate. In quiescent cells, both Mek and Erk isoforms are located exclusively in cytoplasm (Chen *et al.*, 1992; Sanghera *et al.*, 1992; Seth *et al.*, 1992; Lenormand *et al.*, 1993; Zheng and Guan, 1994). After growth factor stimulation, the Erk isoforms undergo a massive redeployment and subsequently translocate from the cytoplasm into the nucleus where they are presumed to regulate gene expression (Chen *et al.*, 1992; Sanghera *et al.*, 1992; Seth *et al.*, 1992). However, the Mek activator remains localized within the cytoplasm (Lenormand *et al.*, 1993; Zheng and Guan, 1994). Recently, Nishida's laboratory discovered that a short amino acid sequence located in the amino-terminal region (residues 32-44) that is responsible for exporting the Mek protein

from the nucleus into the cytoplasm (Fukuda *et al.*, 1996). This nuclear export signal (NES) is related to the leucine-rich  $\alpha$ -helical conformation present in other NES sequence-containing proteins including the protein kinase inhibitor of cAMP-dependent protein kinase (PKA) and the human immunodeficiency virus, type I-coded Rev protein (Fakuda *et al.*, 1996). In addition, a 32 amino acid Erk-binding site peptide located at the very amino-terminal of the Mek protein and contiguous with the NES region, promotes the specific association between Mek and Erk proteins (Fukuda *et al.*, 1997). Mutations at any of the critical leucine residues in the NES region enabled Erk that was microinjected into the cytoplasm to diffuse passively into the nucleus. Hence, The MAP kinase-binding and NES sequences in Mek are thought to act cooperatively as a cytoplasmic anchoring region for Erk (Fukuda *et al.*, 1997). However, Jaaro *et al.*, (1997) recently showed that Mek does massively translocate to the nucleus along with Erk upon cell stimulation, but it is rapidly exported back to the cytoplasm. Therefore, the rapid exclusion of Mek from the nucleus by an active process probably explains the observed subcellular distribution after cell activation (Lenormand *et al.*, 1993; Zheng and Guan, 1994; Fukura *et al.*, 1997). The mechanisms that promotes the Erk/Mek translocation to the nucleus and the subsequent dissociation of the molecular complex remain to be established at the molecular level.

## 5 REGULATION OF MEK PROTEIN KINASE

### 5.1 Stimulation of Mek protein kinase activity.

The MAP kinase kinases are regulated by reversible protein phosphorylation. The seryl/threonyl-specific protein phosphatase 2A (PP2A) has been shown to inactivate impure Mek preparations but not with tyrosyl specific phosphatases like CD45 (Gómez and Cohen, 1991; Matsuda *et al.*, 1992; L'Allemain *et al.*, 1992; Rossomondo *et al.*,

1992). Mek phosphorylation occurs on serine and threonine residues (Gómez and Cohen, 1992; Matsuda *et al.*, 1993). However, stimulation of Mek enzyme activity is achieved by phosphorylation of two seryl residues located within the kinase activation loop. Ser-218 and Ser-222 phosphorylation sites are conserved in all isoforms of MAP kinase kinases (Figure 41). Exchange of either of the regulatory residues with a non-phosphorylatable amino acid like alanine inactivates the Mek enzyme catalytic activity (Zheng and Guan, 1994; Pagès *et al.*, 1994; Alessi *et al.*, 1994; Seger *et al.*, 1994; Gotoh *et al.*, 1994; Yan *et al.*, 1994). In contrast, substitution of one or both of the serine sites with acidic residue(s) (e.g. aspartic or glutamic acid) and/or deletion of the NES peptide causes Mek to become constitutively active (Zheng and Guan, 1994; Pagès *et al.*, 1994; Alessi *et al.*, 1994; Brunet *et al.*, 1994; Seger *et al.*, 1994; Gotoh *et al.*, 1994; Yan *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1994). Injection of transformed cells expressing activated forms of Mek into mice promoted the formation of solid tumours (Mansour *et al.*, 1994).

## 5.2 Mek/Erk signalling module.

The combination of Mek's narrow substrate specificity for the Erk protein kinase, the ability of these two enzymes to form stable protein complexes, and their dynamic protein distribution pattern within the cell, implies that these two cytoplasmic kinases act as a critical conversion point for cell signalling. Mek protein kinases appear to function as nodal points since these enzymes can be phosphorylated and activated by several upstream kinases including seryl/threonyl-specific proto-oncoproteins Raf1, RafB, Tpl2, Mos, and the budding yeast Ste11-related mammalian Mekk. Therefore, the tight regulation of the Mek/Erk module serves as an important convergence point for many inputs emanating from different ligands and their cognate receptors.

## 6 ACTIVATION OF THE ERK PROTEIN KINASE MODULE

### 6.1 The Raf Nodal point.

The Raf family of kinases which include Raf1 (74-kDa), RafA (68-kDa) and RafB (95-kDa), exhibit more than 70% amino acid sequence identity in their kinase catalytic domain (Storm *et al.*, 1990). Both RafA and RafB transcripts are highly expressed in just a few non-proliferating tissues such as kidney and brain, respectively, whereas Raf1 displays a more ubiquitous pattern of expression (Storm *et al.*, 1990). Raf protein kinases share a common architectural design composed of three conserved regions. The amino-terminal non-catalytic CR1 domain contains both the monomeric G protein Ras effector binding and the cysteine-rich zinc finger motif subdomains that have the potential to interact with cellular lipid cofactors. The CR2 is a subdomain rich in seryl and threonyl residues that may be phosphorylated. These phosphorylation events may be important for regulation of enzyme activity of Raf. These two regulatory subdomains control Raf kinase catalytic domain CR3 presumably by folding over and obstructing the active site. Truncation of this region renders the enzyme constitutively active provided that the enzyme is suitably phosphorylated. The precise mechanism involved in the activation of Raf has been difficult to ascertain, since the kinase is regulated by different cell surface receptors and each receptor regulates a set of unique downstream effectors. Furthermore, full enzyme activity may only be achieved through the regulation one or more critical molecular events including translocation, phosphorylation, complex oligomerization and lipid interaction (Hall, 1994; Morrison, 1994; Pelech and Charest, 1995; Gosh *et al.*, 1996; Marshall, 1996).

### 6.1.1 The Ras-Raf1 regulated pathway.

While the MAP kinase pathway was being dissected at the molecular level, other researchers were investigating the mechanisms related to the transduction of the signalling information across the cell's selectively permeable plasma membrane. It is now possible to trace the flow of information from engagement of transmembrane receptor activation with its specific ligand to activation of monomeric Ras GTP-binding protein and subsequent stimulation of cytoplasmic kinases such as MAP kinases and ultimately gene expression within the nucleus.

Ligand interactions (e.g. epidermal growth factor - EGF) with specific binding sites located on the extracellular portion of cell surface receptor kinases trigger a myriad of biochemical events just beneath the surface of the plasma membrane (Panayotou and Waterfield, 1993). In addition to the amino-terminal ligand binding domain, the protein-tyrosyl kinase receptor also possesses a hydrophobic transmembrane domain and a carboxy-terminal cytoplasmic catalytic domain (Fantle *et al.*, 1993). Placement of the ligand between the polypeptide receptor chains promotes the oligomerization of these proteins. Juxtaposition of receptors such as PDGF and EGF, allows the cytoplasmic kinase catalytic subdomains to promote phosphorylation on tyrosyl residues via an intermolecular autophosphorylation reaction (Heldin, 1995). The details of the mechanism has been confirmed recently from X-ray crystallographic studies (Hubbard *et al.*, 1994). These phosphorylated tyrosines are embedded within specific consensus recognition sequences that together serve as high affinity binding sites for tyrosyl-directed peptide binding domains like the Src homology domain 2 (SH2) (Panayotou and Waterfield, 1993). This domain is present in many receptor binding proteins including Grb2 (for growth receptor binding-protein 2), the tyrosyl kinase Src, PI 3-kinase (phosphatidyl-inositol-4,5-bis-phosphate 3-kinase), the Ras GTPase-activating protein

(GAP), and phospholipase C $\gamma$  (PLC $\gamma$ ). Many of these effectors can evoke more than one signalling pathway within the cell. In receptors for such mitogens as insulin, hepatocyte growth factor (HGF) and fibroblast growth factor receptor (FGF), autophosphorylation leads to the increase in catalytic activity and subsequent phosphorylation of other SH2-containing proteins like insulin receptor substrate-1 (IRS-1), Shc and the EGF receptor substrate 2 (FRS2) which can then serve as binding sites for tyrosyl-directed interacting proteins (Skolnik *et al.*, 1993; Koushara *et al.*, 1997).

On the cytoplasmic side of the plasma membrane, Grb2 indirectly binds to these receptors by interaction with the tyrosyl phosphorylated forms of these docking proteins. The two SH3 domains in Grb2 then bind the proline-rich region of the nucleotide exchange protein Sos. Although cellular GTP is more abundant than GDP, the dissociation of GDP from Ras is a rate-limiting step. Once recruited to the cell membrane, however, the guanine nucleotide exchange protein, Sos, stimulates the exchange of GDP for GTP in the G protein Ras (Shou *et al.*, 1992; Wei *et al.*, 1992; Martegani *et al.*, 1992; Botwell *et al.*, 1992; Chardin *et al.*, 1993). The intrinsic GTPase activity regulates the duration of the Ras effector signal. Upon ligand stimulation, conversion of Ras to the active conformation by Sos induces the mobilization of different cellular effectors.

The link that couples events that begin on the cytoplasmic side of the tyrosyl receptor complex to the seryl/threonyl phosphorylation signalling apparatus within the cytoplasm occurs at the interface between the Ras and Raf proteins. Precisely how Ras activation by receptor kinases stimulates Raf activity remains ill defined. Cell stimulation promotes complex formation between Ras and Raf proteins both *in vitro* and *in vivo* (Moodie *et al.*, 1993; Zhang *et al.*, 1993; Warne *et al.*, 1993; Vojtek *et al.*, 1993; Van Aelst *et al.*, 1993). Interaction of the amino-terminal domain of Raf1 (residues 52-



132) occurs with the effector binding domain of Ras (residues 26-48) in a GTP-dependent manner. The biologically inert Ras (Ala-38) effector domain mutant and the Ras (Asn-17) dominant negative mutant fail to interact with Raf kinase (Moodie *et al.*, 1993; Zhang *et al.*, 1993; Warne *et al.*, 1993; Vojtek *et al.*, 1993; Van Aelst *et al.*, 1993; Traverse *et al.*, 1993; Levers *et al.*, 1994; Stokoe *et al.*, 1994). It was postulated that the Ras-Raf interaction may lead to Raf translocation to the plasma membrane where it becomes activated by a mechanism that still remains poorly defined at the molecular level. It appears that Raf activation might require other elements besides protein-protein interaction with GTP-Ras (Traverse *et al.*, 1993; Zhang *et al.*, 1993). Direct translocation to the plasma membrane can in fact bypass the requirement for Ras. Incorporation of a CAAX isoprenylation signal motif [C, cysteine; A, aliphatic amino acid; X, any amino acid] followed by six polybasic lysine residues in the amino-terminal region of Raf was sufficient to target this enzyme to the inner surface of the plasma membrane. Membrane localization of the CAAX-Raf chimera permitted its constitutive stimulation in the absence of Ras activation (Levers *et al.*, 1994; Stokoe *et al.*, 1994). Hence, it appears that one important function for Ras during cell signalling is the recruitment of Raf enzyme within the vicinity of its activators located on the inner surface of the plasma membrane. This indicated that other factors located at the cell surface may be required for Raf1 activation.

Stokoe and McCormick (1997) devised an *in vitro* system to examine K-Ras activation of Raf1. Using reaction conditions that prohibited phosphorylation, farnesylated GTP $\gamma$ S-K-Ras alone or present in membrane preparations potently activated Raf1 in a fashion similar to purified membranes harbouring the activated mutant form of RasG12V. This is in contrast previous studies where other modification events, namely, seryl/threonyl phosphorylation, was implied to be required for Raf1 activation after translocation of this protein to the plasma membrane (Kolch *et al.*, 1993; Morrison *et al.*,

1993; Dent *et al.*, 1995; Jelinek *et al.*, 1996). In fact, addition of  $Mg^{2+}$ -ATP to H-RasG12V-activated Raf resulted in a rapid autophosphorylation and concomitant inactivation of Raf1 activity toward Mek1 (Stokoe and McCormick, 1997). Moreover, these data are consistent with earlier findings which demonstrated that an intact zinc finger was necessary for Ras-dependent Raf activation and that this interaction may be facilitated by the farnesyl moiety of Ras (Brtva *et al.*, 1995; Hu *et al.*, 1995; Luo *et al.*, 1997).

#### 6.1.2. cAMP-Raf1 regulated pathway.

Production of the second messenger cyclic adenosine 3', 5' monophosphate (cAMP) follows hormone stimulation of heterotrimeric  $G_s$  protein-linked by a seven membrane spanning receptor such as the  $\beta$  adrenergic receptor. The intracellular increase in cAMP is regulated by  $G_s\alpha$  subunit ( $G\alpha_s$ -GTP) activation of adenylyl cyclase. The cAMP subsequently causes the dissociation of the cAMP-dependent protein kinase (alias protein kinase A, PKA) catalytic subunits from the regulatory subunits. There are numerous reports of cell-type specific effects produced by cAMP. In thyroid cells, hepatocytes, epithelial and Swiss 3T3 cells, cAMP promotes cell division. In contrast, proliferation in cells of lymphoid, fibroblastic or neuronal origin as well as Ras- and Src-transformed cell lines are all inhibited by elevated cAMP levels (Hordijk *et al.*, 1993 and references therein). These results were obtained with the use of stable analog mimetics of cAMP (e.g. 8-bromo-cAMP, dibutyryl cAMP), factors that elevate intracellular cAMP levels through activation of adenylyl cyclase (e.g. forskolin), inhibition of phosphodiesterase (e.g. 3-isobutyl-1-methylxanthine, IBMX), or expression of constitutively active  $G_s\alpha$  subunit. Furthermore, these same agents also produce similar inhibitory effects on receptor protein-tyrosyl kinase-dependent activation (insulin, PDGF, EGF) and phorbol 12-myristate 13-acetate (PMA) activation of MAP kinases Erk1 and

Erk2 (Wu *et al.*, 1993c; Cook and McCormick, 1993; Graves *et al.*, 1993; Stevetson *et al.*, 1993; Burgering *et al.*, 1993; Hordjik *et al.*, 1993).

PKA has no direct inhibitory effect on either of the Erk isoforms or the immediate MAP kinase activators Mek1 and Mek2 (Graves *et al.*, 1993). The mechanism of cAMP-dependent inhibition of the MAP kinase pathway appears to occur at the level of Raf1 or above (Burgering *et al.*, 1993; Cook *et al.*, 1993; Graves *et al.*, 1993; Sevetson *et al.*, Wu *et al.*, 1993; Häfner *et al.*, 1994; Russell *et al.*, 1994; Mischak *et al.*, 1996). Raf1 phosphorylated by PKA displays a lower binding affinity for Ras protein (Wu *et al.*, 1993; Chuang *et al.*, 1994; Häfner *et al.*, 1994). Therefore, Raf1 lies at a crucial interface for two fundamental cell signalling pathways and has the task of sensing and integrating these opposing effects into an appropriate cell response. The inhibitory effect elicited by the increase in cAMP levels and consequently PKA activity on the MAP kinase pathway may be due to the phosphorylation of Ser-43, which is located near the Ras binding site in the CR1 regulatory region (Wu *et al.*, 1993). In fact, treatment of Rat1 cells with the pharmacological agent forskolin or direct phosphorylation with PKA increased Ser-43 phosphorylation which dramatically reduced Ras-Raf1 protein complex formation. However, other studies showed that PKA phosphorylation of a Raf1 Ser-43 to alanine mutant protein still caused a dramatic reduction in Raf interaction with Ras. This PKA-dependent inhibition of Raf activity was reversible by PKC $\alpha$  phosphorylation (Häfner *et al.*, 1994). These results imply that there may be other subdomains in Raf1 that are equally important for Ras interaction. Furthermore, a similar PKA-dependent inactivation also occurred in Raf mutant proteins that are unable to bind Ras due to a point mutation in the Ras-binding-site (Arg-89) or truncation of the regulatory amino-terminal (Häfner *et al.*, 1994; Mischak *et al.*, 1996). This inhibitory phosphorylation mapped to residue Ser-621 located within the CR3 kinase catalytic domain. The surrounding tryptic peptide sequence RSASEPSLHR

conformed to the minimal consensus sequence (RXXS where X is any amino acid) for PKA (Pearson and Kemp, 1991). Previously, Ser-621 was shown to be constitutively phosphorylated *in situ* and alteration of this residue by mutagenesis resulted in catalytic inactivation of Raf1 (Morrison *et al.*, 1993). Indeed, an intact Ser-621 is required for Raf activity, however, phosphorylation by PKA correlates with kinase inactivation which can be reversed by treatment with seryl/threonyl-specific protein phosphatases 1 and 2A (Mischak *et al.*, 1996, Sprengle *et al.*, 1997). Furthermore, autophosphorylation of Raf1 at Ser-621 may lead to its inactivation *in vivo* (Mischak *et al.*, 1996; Stokoe and McCormick, 1997).

#### 6.1.3 Rap1-RafB regulated pathway

In the rat adrenal medullary-derived pheochromocytoma PC12 cell line, elevated levels of cAMP stimulated by the NGF ligand causes increased gene expression and neuronal differentiation (Marshall, 1995). Consistent with these results, NGF activation of Erk protein kinase also requires cAMP-dependent activation of PKA to promote these same morphological changes (Frödin *et al.*, 1994; Young *et al.*, 1994; Pan *et al.*, 1994).

There has been much debate as to effectors involved in the regulation of neurite outgrowth formation by NGF. Initial studies supported a role for Ras and Raf1 in NGF activation of the MAP kinase pathway. Induction of neurite-like processes in PC 12 cells in response to NGF treatment has been shown in separate experiments to coincide apparently with the stimulation of Ras, Raf, Erk and Rsk catalytic activities (Hagag *et al.*, 1986; Blenis and Erickson, 1986; Gotoh *et al.*, 1990; Boulton *et al.*, 1991; Ohmichi *et al.*, 1992; Scimeca *et al.*, 1992). Expression of dominant inhibitory mutant Ras (Asp-17) attenuated NGF-induced phosphorylation and activation of Erk and Mek protein kinases (Gómez and Cohen, 1991; Robbins *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992).

Also, atalytically compromised forms of Raf, Mek and Erk also interfered with proper neurological differentiation of PC12 cells, whereas the constitutive-active forms promoted their differentiation (Robbins *et al.*, 1992; Wood *et al.*, 1992; Cowley *et al.*, 1992; Wood and Roberts, 1993). However, in a more rigorous experimental analysis of the involvement of Raf1 in PC12 cell signalling, overexpression of an activated form of Raf1 did not stimulate MAP kinase (Wood *et al.*, 1993). Moreover, partially purified Raf1 from NGF-treated cells failed to activate the Mek-Erk module (Jaiswal *et al.*, 1994). These results nullify the earlier studies that relied on changes in Raf1 phosphorylation state and retarded electrophoresis on SDS-PAGE as a measure of kinase activation. Therefore, a separate signalling pathway originating from the NGF receptor must converge at the Mek-Erk node.

RafB is also expressed in neuronal cells (Vaillancourt *et al.*, 1994). Similar to Raf1, growth factor activation of RafB and its subsequent interaction with Ras-GTP is inhibited by elevated cAMP levels present in the cell (Wu *et al.*, 1993; Moodie *et al.*, 1994; Vaillancourt *et al.*, 1994; Peraldi *et al.*, 1995). However, Erhardt *et al.* (1995) have argued that RafB displays no difference in sensitivity toward cAMP and that an additional signalling component may be required for the kinase in PC12 cells. The GTP-binding protein Rap1, which has the same effector domain structure as Ras, interacts with RafB in cell free extracts and stimulates the enzymes phosphotranferase activity toward the Mek (Ohtsuka *et al.*, 1996). The exchange of GDP for GTP is mediated by PKA phosphorylation of Rap1. Indeed, PC12 cells are able to differentially employ RafB and not Raf1 in a cAMP-PKA-dependent manner by increasing GTP-loading of Rap1, while inhibiting Ras effector function (Vossler *et al.*, 1997). Consequently, stimulation of Erk and Mek activities in differentiating neuronal cells requires cAMP-dependent activation of PKA, Rap1 and RafB.

#### 6.1.4 The Ksr1-Raf1 regulated pathway

In the nematode *Caenorhabditis elegans*, hyperactive Ras causes pluripotent precursor cells to assume vulval cell fates. Similarly, nonneuronal cells in the fruit fly *Drosophila melanogaster*, ectopic expression of activated Ras can induce nonneuronal cells to differentiate into specialized R7 photoreceptor cells. Ksr (kinase suppressor of Ras) was initially identified in genetic screens for suppressors of constitutively active forms of Ras in these genetic model systems (Therrien *et al.*, 1995; Sundaram and Han, 1995; Kornfeld *et al.*, 1995). The proteins are predicted to range in size from 95-kDa in *C. elegans*, 100-kDa in mouse and 115-kDa in *D. melanogaster*. The isolation of amino-terminal splice variants for human Ksr indicate that the mammalian gene may add another level of complexity in the regulation of the activity of this enzyme (Therrien *et al.*, 1995). Within the catalytic domain, Raf1 appears to be the closest relative of the Ksr protein. One unusual feature found in mammalian Ksr is the substitution of an arginyl residue in place of the highly conserved lysyl-amino acid in catalytic subdomain II that is essential for the phosphotransferase reaction (Therrien *et al.*, 1995). The amino-terminal region contains five conserved areas (CA1 to CA5) (Therrien *et al.*, 1995). CA1 is a conserved domain unique to Ksr. The CA2 domain is a prolyl-stretch that is reminiscent of an SH3-domain binding site. A cytseinyll-rich domain (CA3) is similar to the lipid-binding domain in PKC and to CR1 domain in Raf1 and thus may associate with lipid derivatives. CA4 is a region rich in seryl and threonyl residues (similar to CR2 in Raf1) that contains a MAP consensus phosphorylation site. Finally, CA5 domain in Ksr, like that of Raf CR3, encodes the kinase catalytic domain.

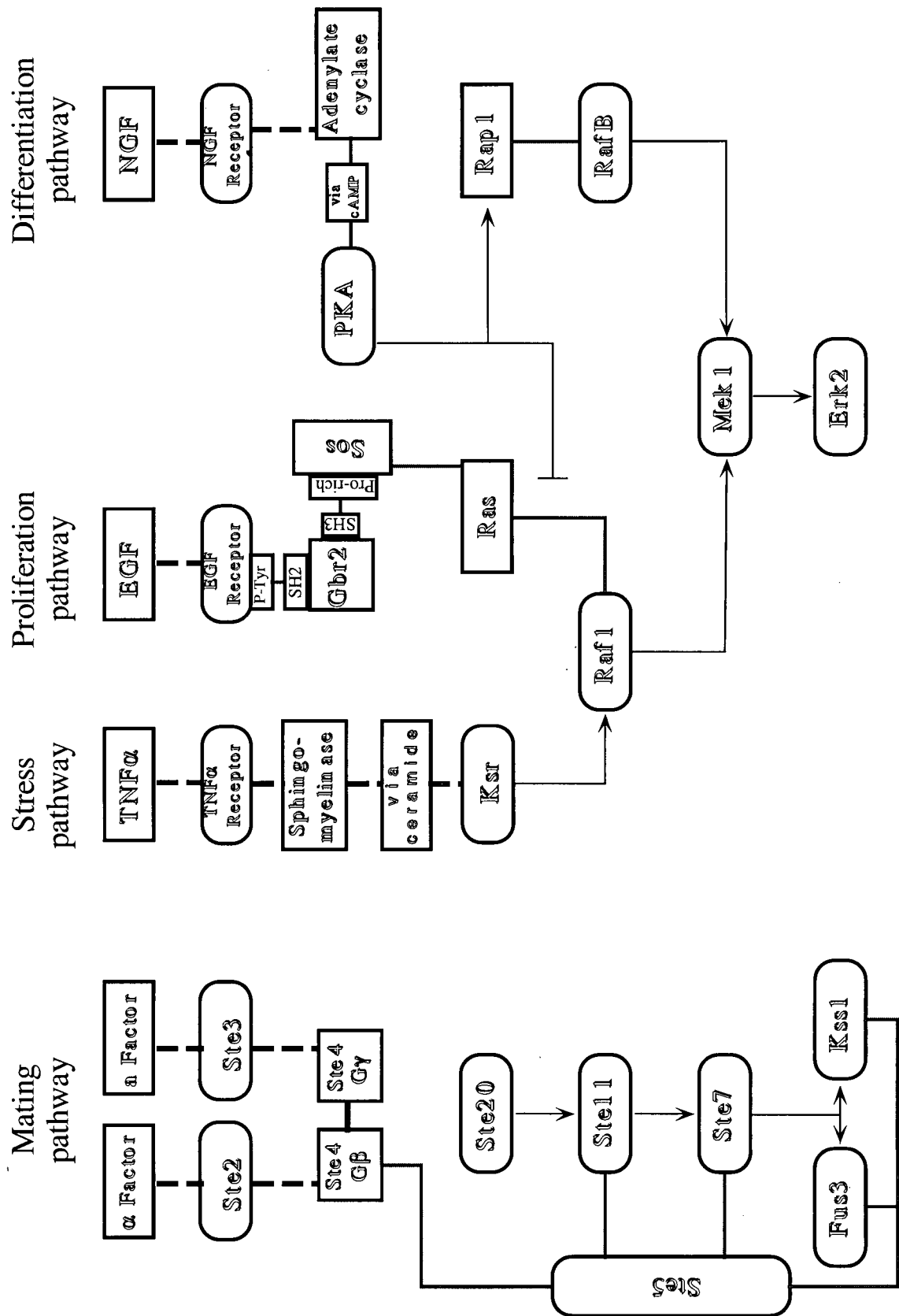
Recently, Ksr has been shown to be related to the ceramide-activated protein (CAP) kinase (Zhang *et al.*, 1997). CAP kinase is a membrane-bound enzyme that is activated in a variety of cell types by natural or synthetic analogues of ceramide (Mathias *et al.*,

1991). The cytokine  $\text{TNF}\alpha$ , which stimulates sphingomyelinase to release ceramide through hydrolysis of sphingomyelin, can also stimulate the activation of CAP kinase (Dressler *et al.*, 1992). Biochemical evidence indicates that the two kinases may be one and the same protein (Zhang *et al.*, 1997). Both enzymes display the same molecular mass after renaturation in SDS-PAGE (Lui *et al.*, 1994). In a manner similar to CAP kinase, Ksr autophosphorylation was stimulated after *in vivo* treatment of COS-7 Ksr overexpressing cells with  $\text{TNF}\alpha$  or after *in vitro* exposure of purified membranes to ceramide (Zhang *et al.*, 1997).

Raf1 was also shown to be activated in a sphingomyelinase-dependent manner in response to  $\text{TNF}\alpha$  receptor engagement (Belka *et al.*, 1995). Moreover,  $\text{TNF}\alpha$  or ceramide stimulation of endogenous CAP kinase in myeloid HL-60 cells, or transient overexpression of Ksr in COS-7 cells led to the complex formation and activation of Raf1 (Yao *et al.*, 1995; Zhang *et al.*, 1997). Ksr/CAPK phosphorylation of Raf1 on Thr-269 was prolyl-directed and led to the stimulation of its phosphotransferase activity toward Mek1 (Yao *et al.*, 1995; Zhang *et al.*, 1997). This regulatory phosphorylation site conforms to minimal substrate recognition motif Thr-Leu-Pro for CAPK that was identified using synthetic peptide substrates (Joseph *et al.*, 1993). Therefore, Raf1 is probably an important intermediary between engagement of the  $\text{TNF}\alpha$  receptor and activation of cytoplasmic effectors via the MAP kinase module (Raines *et al.*, 1993; Winston *et al.*, 1995). Complete details of the Erk MAP kinase pathway are summarized in Figure 1.

Figure 1: Regulation of the Mek-MAP kinase signalling module by distinct biological processes in mammals and in yeast *Saccharomyces cerevisiae*.





## 7. PARALLEL MAP KINASE MODULES IN MAMMALS

### 7.1 MAP kinase superfamily of proline-directed kinases.

Several research groups have identified MAP kinases that are distinct from the p42/p44 isoforms first detected in growth factor-treated quiescent ( $G_0$ ) mammalian cells and hormone-induced germinal vesical breakdown ( $G_2$ -M) in *Xenopus* or sea star oocytes (Pelech and Sanghera 1992; Mordret 1993). These MAP kinase isoforms have been identified by molecular cloning studies (Boulton *et al.*, 1991; González *et al.*, 1992; Zhu *et al.*, 1994, Lee *et al.*, 1995; Zhou *et al.*, 1995), biochemical analysis (Kariakis and Avruch, 1990; Adler *et al.*, 1992; Hibi *et al.*, 1993; Han *et al.*, 1993; Guesdon *et al.*, 1993; Minshull *et al.*, 1994 and Heider *et al.* 1994) or immunological detection (Boulton and Cobb, 1991; Sanghara *et al.*, 1992) and have been shown to range in size from between 38-110 kDa. Because so much attention has focused on delineating the Ras → Raf → Mek → MAP kinase regulatory pathway, the importance of these new isoforms in various cellular processes via signal transduction mechanisms has only now begun to be elucidated at the molecular level. It appears that each of these MAP kinase isoforms operate independently in cells within distinct signalling modules with their cognate MAP kinase kinase activators (Zanke *et al.*, 1996a). The notion of distinct MAP kinase pathway regulating unique cellular processes has been demonstrated widely with yeast (Errede and Levin, 1993; Ammerer, 1994; Herskovitz, 1995). Indeed, these recently identified MAP kinase isoforms are members of the MAP kinase superfamily of prolyl-directed, seryl/threonyl-specific kinases (Ferrell, 1996).

## 8.1 Identification of Jun protein kinase in the regulation of stress signalling.

A second Microtubule-associated protein 2 (MAP2 later called SAPK for stress-activated protein kinase or Jnk for Jun N-terminal kinase) kinase was detected in rat liver after intraperitoneal injection of the protein synthesis inhibitor cyclohexamide (Kyriakis and Avruch, 1990). Polypeptide misfolding caused by cycloheximide treatment stimulated a 54-kDa MAP2 kinase that was distinct from the insulin-treated p42 MAP kinase Erk2 originally observed from 3T3-L1 cells (Ray and Sturgill, 1987). Besides the obvious difference in molecular mass of the two proteins, both kinases had distinguishable substrate specificity. Although both Erk2 and MAP2 kinases are proline-directed seryl-threonyl kinases (Kyriakis and Avruch, 1990) that require threonyl and tyrosyl phosphorylation for activity (Kyriakis *et al.* 1991) only Erk2 was capable of phosphorylating and activating the *Xenopus* Rsk *in vitro*. Furthermore, Erk2 was activatable by ligands that regulate growth and differentiation, while MAP2 kinase was induced by chemical stress. This implied that MAP2 kinase may be involved in a unique signalling pathway (Kyriakis and Avruch, 1990).

During this same period, the c-Jun transcription factor was shown to be phosphorylated by several distinct kinase activities in response to stress factors (Pulverer *et al.*, 1991; Alvarez *et al.*, 1991; Alder *et al.*, 1992a; Baker *et al.*, 1992; Hibi *et al.*, 1993). However, upon closer scrutiny only a few of these protein kinase activities could phosphorylate c-Jun within its N-terminal activation domain. Also, a phorbol ester-stimulated proline-directed kinase was detected in the human leukemic cell line U937 (Adler *et al.*, 1992a). This c-Jun kinase was affinity purified using a glutathione S-transferase-c-Jun fusion protein linked to Sepharose beads and was shown to be a 67 kDa

protein (Adler *et al.*, 1992b). Hibi *et al.* (1993) also identified two protein kinases activated in response to UV and transforming oncogenes that were able to bind tightly to a GST-c-Jun affinity resin. The major and minor forms of the two proline-directed seryl/threonyl Jnks had molecular masses of 46 kDa and 55 kDa, respectively. Like Jnks, MAP2 kinase had been shown to be a c-Jun amino-terminal transcription factor phosphorylating kinase (Pulverer *et al.* 1991; Kyriakis *et al.*, 1994). Later cloning experiments would show Jnk and MAP2/SAP to be homologous proteins. Immunoblotting with Erk specific polyclonal antibodies revealed that Jnk/SAP kinases were novel MAP protein kinases that may function within distinct MAP kinase signalling module.

cDNAs have been isolated for human Jnk (Dérjard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994) and rat SAP protein kinases (Kyriakis *et al.*, 1994). Jnk1 and Jnk2 are closely related at the amino acid level (85-90% identity) with a predicted molecular masses of 44-kDa and 48-kDa, respectively. Similarly, rat SAP kinase  $\alpha$  and  $\beta$  isoforms display a high degree of conservation (88-90% identity) and are predicted to have a molecular mass of ~48-kDa. Kyriakis *et al.* (1994) also reported the isolation of additional shorter SAPK clones identical to SAPK $\beta$  cDNA. Divergence at the amino acid level was observed when one of the clones was identified as having a 5-nucleotide insertion at Ser-379 resulting in a reading frame shift and a premature stop. The forty residue truncation was replaced with the sequence AQVQQ-stop. Interestingly, these same authors reported that Jnk1 (Dérjard *et al.*, 1994) was identical to the partial SAP kinase  $\gamma$  amino acid sequence through the first 379 amino acids (Leu-379) after which it terminated with the sequence AQVQQ-stop observed with the shorter SAPK $\beta$ . Heterogeneity of mRNA transcripts as observed by Northern hybridization analysis, the variance in the carboxy-terminal sequence and the apparent lower predicted molecular mass of the translated protein product than the observed size of the purified 54-kDa on

SDS-PAGE led to further analysis of Jnk at the genomic level. Gupta *et al.* (1996) identified at least 10 isoforms of Jnk in adult human brain cDNA libraries. Analysis of the nucleotide sequence revealed that three genes: Jnk1, Jnk2 and Jnk3 yielded the multiple isoforms through alternative processing of their transcripts. The four isoforms of Jnk1 and Jnk2 are derived from splice variations in (i) the protein kinase subdomains IX and X and (ii) the carboxy-terminal domain. Two Jnk3 isoforms possess two unique carboxy-terminal sequence that are generated by differential processing of the mRNA. Although Jnk3 showed no variation within the protein kinase catalytic domains IX and X, this kinase did code for a unique amino-terminal sequence that was contiguous with the initiating methionine for Jnk1 and Jnk2. The shorter Jnk isoforms were recognized as 46- to 48-kDa proteins by Western blot analysis, while the larger isoform counterparts migrated in the range of 55- to 57-kDa consistent with these enzymes having longer amino- and carboxy-termini. It remains to be determined if these Jnk splice variants are differentially regulated in response to stimuli. To date, no homologues of the Jnk protein kinase family have been identified in yeast.

Sequence comparisons between the catalytic domains of the Jnk subfamily of protein kinases reveal that these enzymes share ~45% homology at the amino acid level with their Erk1 and Erk2 counterparts (Kyriakys *et al.*, 1994; Dérijard *et al.*, 1994). Moreover, Jnk isoforms display sequence conservation at sites that are essential for MAP kinase regulation (Ferrell, 1996). A dual phosphorylation site motif similar to the Thr-Glu-Tyr (TEY) site critical for Erk protein kinase activation, is present in Jnk protein kinases (Kyriakys *et al.*, 1994; Dérijard *et al.*, 1994). Replacement of these sites by site-directed mutagenesis with non-phosphorylatable residues prevented the activation of Jnk1 in UV irradiated cells (Dérijard *et al.*, 1994). However, the tripeptide motif in Jnk, i.e. Thr-Pro-Tyr (TPY), differs slightly from the Erk subfamily members (Kyriakys *et al.*, 1994; Dérijard *et al.*, 1994). The Thr-Xaa-Tyr regulatory sequence is located in a peptide

loop between kinase catalytic subdomains VII and VIII, a region that harbours the regulatory phosphorylation and activation domain for many known kinases (Johnson *et al.*, 1996). This linker loop structure, termed L12, in Erk2 when phosphorylated by Mek protein kinase is presumed to undergo critical changes in conformation that promote activation of the MAP kinase enzyme (Zhang *et al.*, 1994). In the case of Jnk protein kinases, the length of the L12 loop between the conserved DFG and the APE subdomains is four amino acids shorter than the comparable region in Erk (Farrell, 1996). It is hypothesized that the length of the linker loop structure in combination with the change in the intervening amino acid separating the regulatory phosphorylation sites, plays a crucial role in recognition by specific activators (Han *et al.*, 1994; Butch and Guan, 1996).

## 8.2 Stimulation of the Jun kinase pathway.

Jun kinases are activated primarily by stimuli that cause cellular stress, and as such may have a protective role by enabling cells to activate damage repair systems or trigger cell death by apoptosis. Jnk is stimulated in response to a variety of stresses, including environmental stresses such as short-wavelength U.V. radiation (Hibi *et al.*, 1993), fluctuations in osmolarity (Galcheva-Gargova *et al.*, 1994; Sluss *et al.*, 1994), (Kyriakis *et al.*, 1994) elevations in temperature (Kyriakis *et al.*, 1994), metabolic inhibitors like ribotoxic (Kyriakis and Avruch, 1990; Kyriakis *et al.*, 1994; Iordanov *et al.*, 1997), and genotoxic agents (Kharbanda *et al.*, 1995; Zanke *et al.*, 1996b; Liu *et al.*, 1996), pro-inflammatory cytokines IL-1 and TNF- $\alpha$  (Kyriakis *et al.*, 1994; Sluss *et al.*, 1994) as well as ischemia and reperfusion (Pombo *et al.*, 1994). In haemopoietic cells, Jun kinase display a modest activation by the tumour promoter TPA, while no activation of Jnk is observed in fibroblasts and epithelial cells (Pulverer *et al.*, 1991; Pulverer *et al.*, 1992; Adler *et al.*, 1992a; Adler *et al.*, 1992b; Sluss *et al.*, 1994). Moreover, Jnk

activation in some cell types may require the integration of inputs from two different stimuli (Su *et al.*, 1994). In T lymphocytes, co-stimulation by TPA and  $\text{Ca}^{2+}$  is necessary for maximal Jun protein kinase activation and IL-2 production (Su *et al.*, 1994).

### 8.3 Effectors of Jnk protein kinase signalling.

c-Jun is a component of the activator protein-1 (AP-1) transcription factor complex (reviewed in Angel and Karin, 1991). Heterodimerization between c-Jun and c-Fos bind specific promoter regions and regulate gene expression in an AP-1-dependent manner. The c-Jun transcription factor binds short, cis-acting DNA sequence known as a promoter binding site. The c-Jun transcription factor is composed of two domains; an amino-terminal trans-activation domain contiguous with a carboxy-terminal DNA-binding domain. Protein phosphorylation controls the transcriptional activity of c-Jun (Hunter and Karin, 1992; Karin and Smeal, 1992). Phosphorylation of two critical residues, Ser-63 and Ser-73, located within the amino-terminal transactivation domain promote transcription of AP-1 responsive genes (Smeal *et al.*, 1991; Smeal *et al.*, 1994). Both residues conform to the consensus sequence for MAP kinases: a phosphorylatable seryl/threonyl amino acid followed by prolyl residue at the P-1 position. The critical importance of Ser-73 phosphorylation in the regulation of c-Jun activity was elegantly demonstrated in experiments where the sequence surrounding this phosphoacceptor site was converted to a cAMP-dependent protein kinase consensus site (Smeal *et al.*, 1994). The transcriptional potential of this modified c-Jun was acutely regulated by signals that normally affected PKA. Indeed, both Erk and Jnk family members phosphorylated c-Jun activation sites *in vitro*. However, both Erk1 and Erk2 phosphorylated Ser-246, a site located in the carboxy-terminal DNA-binding domain that causes inhibition of gene expression (Alvarez *et al.*, 1991; Baker *et al.*, 1992; Minden *et al.*, 1994). In addition, the

Erk protein kinases could only phosphorylate the activating phosphorylation sites of the carboxy-terminal deleted c-Jun protein. As described earlier, Hibi *et al.* (1993) the Jnk protein kinases interacted specifically with GST-c-Jun chromatography resin and phosphorylated full-length c-Jun transcription factor on the two amino-terminal activating seryl sites. Like the Erk family of MAP kinases, the Jnk protein kinase translocated to the nucleus after cell stimulation with U.V. irradiation; where these enzymes are postulated to have an important role in the regulation of gene expression (Cavigelli *et al.*, 1995).

ATF-2 is also a target of protein phosphorylation and is linked to specific signal transduction pathways (Hunter and Karin, 1992). The transcriptional activation of the c-Jun promoter by ATF-2 homodimers or ATF-2/c-Jun heterodimers is induced *in vivo* by cellular stresses including genotoxic agents, and proinflammatory cytokines (Livingstone *et al.*, 1995 and references therein). These same stimuli also activate Jnk protein kinases (Sluss *et al.*, 1994; Kharbanda *et al.*, 1995). Phosphorylation has no effect on the DNA binding properties of ATF-2 in nuclear extracts (Gupta *et al.*, 1995). However, several putative MAP kinase consensus phosphorylation sites were localized in the amino-terminal transactivation domain (Livingstone *et al.*, 1995). Tryptic phosphopeptide mapping localized the Jnk phosphorylation sites within the amino-terminal transactivation domain (Gupta *et al.*, 1995). This was subsequently confirmed by replacement of Thr-69 and Thr-79 with alanines by *in vitro* mutagenesis that caused a significant reduction in phosphorylation of ATF-2 as well as transcriptional activation of the reporter genes ( $\beta$ -galactosidase or chloramphenicol acetyl-transferase) and c-Jun induction in cells (Livingstone *et al.*, 1995; Gupta *et al.*, 1995; van Dam *et al.*, 1995).

The inducible nuclear localization of Jnk protein kinases in murine FR3T3 cells is consistent with the function of these enzymes in the regulation of transcription factors



c-Jun and ATF-2 (Dérjard *et al.*, 1995). Further evidence for the role of Jun kinase in the stimulation of c-Jun and ATF-2 transcriptional activity derives from analysis of the enzyme's ability to complex with specific transcription factor regulatory domains. Jnk binds with high affinity to GST-c-Jun (Hibi *et al.*, 1993; Dérjard *et al.*, 1994; Kyriakis *et al.* 1994). Analysis of the Jnk/c-Jun interaction revealed that Jnk complexes to c-Jun more effectively than the truncated v-Jun oncoprotein despite the fact that both transcription factors retained the regulatory Ser-63 and Ser-73 phosphorylation sites (Adler *et al.*, 1992a; Hibi *et al.*, 1993). c-Jun phosphorylation by Jnk requires a small 30 amino acid region (the  $\delta$ -subdomain) located amino-terminal to the Ser-63 and Ser-73 (Adler *et al.*, 1992b; Hibi *et al.*, 1993; Dérjard *et al.*, 1994). A pseudo-binding  $\delta$ -domain peptide prevents Jnk interaction and phosphorylation of c-Jun (Adler *et al.*, 1994). Furthermore, Jun kinase phosphorylation of c-Jun promotes the dissociation of the complex (Hibi *et al.*, 1993). A similar Jun kinase docking site was identified in ATF-2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995).

A specificity-determining region that promotes efficient binding of Jnk to the transcription factor c-Jun has been located in subdomains IX and X of the kinase catalytic core (Kallunki *et al.*, 1994; Sluss *et al.*, 1994). This region contains alternative amino acid sequences or docking sites that mediate substrate specificity for the binding of the various Jnk isoforms to c-Jun (Gupta *et al.*, 1996). The high degree of substrate recognition was further demonstrated with JunB and JunD transcription factors. JunB binds effectively to Jun kinase but is a poor *in vitro* substrate because the transcription factor lacks the appropriate phosphorylation sites (Gupta *et al.*, 1996; Kallunki *et al.*, 1996). In contrast, JunD possesses the regulatory seryl residues but lacks the necessary docking sites to interact with Jun kinase and consequently is unable to be phosphorylated effectively (Gupta *et al.*, 1996; Kallunki *et al.*, 1996). Interestingly, heterodimeric complexes between c-Jun and JunB and JunD can promote Jnk to phosphorylate the JunD

transcription factor that lacks the docking site (Kallunki *et al.*, 1996). These new complexes may create another level of specificity and diversity in the regulation of gene expression by different extracellular signals.

## 9. REGULATION OF MKK4/MKK7 PROTEIN KINASES

### 9.1 Jun protein kinase activators.

The failure of Mek1 and Mek2 to stimulate Jnk protein kinase activity indicated that a unique Jun kinase kinase regulatory enzyme was required to phosphorylate and activate this enzyme (Kyriakis *et al.*, 1994). Previously, Yashar *et al.* (1993) isolated two MAP kinase kinase cDNAs from by PCR (XMek2 alias Mkk4, and XMek3). XMek2 and XMek3 were distinct from the previously identified MAP kinase kinase that was shown to be activated during *Xenopus* oocyte maturation and hence may define a new MAP kinase pathway in the frog. Interestingly, both XMek2 and XMek3 were similar to the yeast Hog1 (High osmolarity glycerol) MAP kinase kinase activator Pbs2 (Polymyxin B sensitive) from *S. cerevisiae* such that both XMek2 and XMek3 lacked the prolyl-rich insert region located between the catalytic subdomains IX and X. Moreover, activation of Jnk1 by osmotic shock in mammalian cells implied that the enzyme may be activated in a manner similar to Hog1 (Galcheva-Gargova *et al.*, 1994). Indeed, Jnk1 expression in a yeast strain carrying a null mutation in Hog1 (high osmolarity glycerol) complemented the defect under hyper-osmolar growth conditions, while Erk2 failed to rescue (Galcheva-Gargova *et al.*, 1994). Jnk kinase (Jnkk and more commonly termed Mkk4) from human and murine Sek1 (for Sapk/Erk kinase-1) share ~40% amino acid identity with Mek1 and Mek2 within the catalytic region (Sánchez *et al.*, 1994; Dérillard *et al.*, 1995; Lin *et al.*, 1995). The ubiquitously expressed 43-kDa kinase is most abundantly expressed in mammalian brain and skeletal muscle (Sánchez *et al.*, 1994; Dérillard *et al.*, 1995). This

may explain why this pathway is acted upon by a large number of stimulators including EGF in some cell types (Kyriakis *et al.*, 1994). Activation of Jnk1 by Mkk4 is achieved through the direct phosphorylation of Thr-183 and Tyr-185 regulatory sites (Lin *et al.*, 1994; Dérjard *et al.*, 1995).

Targeted disruption of the Mkk4 gene in embryonic stem cells (ES cells) by homologous recombination impaired the activation of Jnk following stimulation with known activators like anisomycin and heat shock (Nishina *et al.*, 1997; Yang *et al.*, 1997). Control experiments demonstrated that the Erk- and Hog-specific MAP kinase pathways remained unaffected by the impairment to Jnk signalling. This is the first genetic evidence in mammals that Mkk4 is required for activation of Jnk *in vivo*. Unexpectedly, however, U.V. irradiation treatment and changes in osmolarity retained the ability to fully activate the Jun kinases to normal levels (Nishina *et al.*, 1997). These data support previous results that demonstrated the existence of several Jun kinase kinases in protein extracts (Moriguchi *et al.*, 1995; Meier *et al.*, 1996). In fact, a new Jun kinase activator, termed Mkk7 (alias Jkk2), has been identified that may have compensated for the Mkk4 deletion in cells (Tournier *et al.*, 1997; Wu *et al.*, 1997; Moriguchi *et al.*, 1997). Mkk7 is the mammalian homologue of Hep (Hemipterous), the activator for *Drosophila* Jnk (Glise *et al.*, 1995; Sluss *et al.*, 1996).

## 10. ACTIVATION OF THE JNK PROTEIN KINASE MODULE

### 10.1 The Mek kinase (Mekk) node.

The first mammalian homologue of Ste11 (Sterile) and Byr2 (Bypass of Ras1 deficiency) kinases, the cognate activators of the budding yeast Mek1 kinases, Ste7 and Byr2, was isolated from a murine brain cDNA library (Lange-Carter *et al.*, 1993). This

protein kinase was denoted Mekk (Mek kinase), since the enzyme activated Mek in an EGF-dependent manner in cells transiently transfected with the Mekk1 cDNA (Lange-Carter *et al.*, 1993; Fanger *et al.*, 1997). The evolutionary conservation of Mekk1 was demonstrated when this enzyme was able to rescue the Ste11 homologue in the pheromone signalling pathway and Bck1 (Bypass of C kinase) gene product cell-wall lysis defect in *S. cerevisiae* (Blumer *et al.*, 1994). Further studies into the function of Mekk1 revealed that expression of the truncated form of Mekk1 in NIH 3T3 cells activated the Jnk protein kinase pathway (Yan *et al.*, 1994). This observation was corroborated in NGF- and EGF-treatment of PC12 and Hela cells, respectively (Minden *et al.*, 1994b). Jnk activation occurred through Mekk1 phosphorylation of Mkk4 on Ser-220 and Thr-224 residues; moreover, the location of these sites was equivalent to the regulatory sites (Ser-218 and Ser-222) identified in Mek (Yan *et al.*, 1994). Recently, the full-length Mekk1 from rat (94% homologous in the catalytic region) was shown to be a 196-kDa protein which possesses a larger than anticipated carboxy-terminal region (Xu *et al.*, 1996). Three additional Mekk cDNAs were subsequently isolated from murine brain (Blank *et al.*, 1996; Gerwins *et al.*, 1997).

The Mekk2, Mekk3 and Mekk4 isoforms can stimulate the Jnk protein kinase pathway in transient transfection experiments (Blank *et al.*, 1996; Gerwin *et al.*, 1997). The kinase catalytic domains of the smaller 70-kDa Mekk2 and Mekk3 enzymes are 94% conserved within their carboxy-terminal kinase (Blank *et al.*, 1996). The amino-terminal region, which are 65% homologous, may contain a modified bipartite nuclear localization signal (Blank *et al.*, 1996; Fanger *et al.*, 1997). No proximal activators have yet been identified for both Mekk2 and Mekk3 protein kinases. Although the larger 180-kDa Mekk4 is similar in size to Mekk1, both proteins share only a modest 55% conservation in their amino acid sequence (Gerwin *et al.*, 1997). A similar level of homology is observed between Mekk4, Mek2 and Mekk3. Mekk1 and Mekk4 contain a prolyl-rich

region and pleckstrin homology domain (PH) motifs located at the very amino terminus of the non-catalytic domain (Xu *et al.*, 1996; Gerwin *et al.*, 1997). A modified Cdc42/Rac1 interactive binding motif (CRIB motif consensus ISXPXXXXFXHXXHVG where X is any amino acid) also lies adjacent to the kinase catalytic domain in Mekk4, while no such motif was observed in the Mekk1 isoform (Gerwin *et al.*, 1997). However, both Mekk1 and Mekk4 associate specifically with GTP-bound forms of Rac and Cdc42 (Gerwin *et al.*, 1997). It is expected that these and other functional motifs would allow the Jnk protein kinase pathway to be regulated by a variety of unique regulatory inputs. Overexpression of the four Mekk isoforms in transient transfection assays in HEK293 cells stimulated the activation the Jnk signalling pathway (Blank *et al.*, 1996; Gerwins *et al.*, 1997). Recently, the human homologue of Mekk4, termed Mtk1 (MAP three kinase 1), was shown to be a minor mediator of Jnk1 activity in transient transfection assays using COS-7 and HeLa cell lines (Takekawa *et al.*, 1997). In contrast, dominant negative Mtk1 did not block Jnk activation in response to environmental stress (osmotic stress, UV irradiation, anisomycin) or exposure to the cytokine TNF- $\alpha$  (Takekawa *et al.*, 1997). In support of the notion that all four Mekk protein kinase isoforms regulated specific as well as overlapping signalling pathways is the distinct intracellular distribution of these enzymes; Mekk2 and Mekk4 appear associated primarily with the Golgi, while Mekk1 is both nuclear and cytoplasmic (Fanger *et al.*, 1997):

#### 10.2 The mixed lineage kinase (Mlk) node.

The Mlk protein kinases were first isolated using degenerate oligonucleotide primers directed against conserved amino acid sequences from a large number of protein kinases (Dorow *et al.*, 1993). A common feature of Mlk is that these enzymes possess amino acid residues that are homologous to both seryl/threonyl and tyrosyl families of protein kinases. In fact, the catalytic subdomains I through VII in Mlk are closely related

to sequences found in the Mekk family, whereas subdomains VIII through XI resemble sequences found in FGF and HER4 receptor tyrosyl kinases (Rana *et al.*, 1996). To date, two members of the Mlk family that are closely related to the Mekk protein kinases are Mlk3 (alias Ptk1 for protein tyrosine kinase 1 and Sprk for Src-homology 3 domain-containing proline-rich kinase), and Dlk (dual-leucine-zipper kinase alias Muk for MAP upstream kinase and Zpk for leucine-zipper protein kinase (Ing *et al.*, 1994; Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Holzman *et al.* 1994; Reddy and Pleasure, 1994; Hirai *et al.*, 1994). The mixed lineage kinase family possess several structural motifs within their carboxy terminal regulatory domain that play an essential role in promoting specific protein interactions. Juxtaposed to the catalytic domain are two contiguous  $\alpha$ -helical leucine/isoleucine zipper motifs followed by a basic region and a proline rich segment. Additionally, the Mlk3 and Dlk contain an amino-terminal SH3 domain and a CRIB domain with a modified consensus sequence (Burbelo *et al.*, 1995). These protein-protein interaction domains within the Mlk family of kinases add another level of complexity in that they may be activated by a variety of extracellular stimuli.

Until recently, the role these hybrid kinases played in signal transduction remained undefined at the molecular level. However, the high basal activity of these kinases when expressed in transfection experiments revealed that these Mekk-like proteins may be involved in the regulation of the c-Jun stress-activated protein kinase pathway. The dual leucine-bearing kinases Dlk and Muk caused the increase in Jnk phosphotransferase activity in COS and NIH3T3 cells (Hirai *et al.*, 1996; Fan *et al.*, 1996). Still, a catalytically compromised version of Dlk was only able to partially block Jnk activation by GTPase-deficient Cdc42 (Fan *et al.*, 1996). Therefore, these results indicate that the Rho family of G-proteins can use more than one signalling pathway to activate Jnk protein kinase such as the Mekk route. The Mlk3 family also links the Cdc42/Rac1 GTP-binding proteins to the Jnk signalling cascade. Furthermore, there is

evidence that Mlk3 is able to bind to Cdc42 and Rac1 *in vivo* through the CRIB motif. However, the proteins appeared to interact with much lower affinity than observed with other Rho-binding proteins (Teramoto *et al.*, 1996). Indeed, Mlk3 displayed less than a two-fold increase in its ability to stimulate Jnk phosphotransferase activity toward the substrate ATF2, indicating that other co-activators may be involved in the regulation of this enzyme. Whatever the mechanism of Mlk3 activation, signalling to Jnk was shown to be the result of direct phosphorylation of Mkk4 by this enzyme (Rana *et al.*, 1996). Mutation of the two regulatory phosphorylation sites in Mkk4 (Ser-220 and Thr-224) completely abolished the ability of Mlk3 to activate Jnk in transfected COS cells.

### 10.3 Orphan MAPK kinase kinase nodes.

Tpl2, a protein kinase closely related to budding yeast Ste11 and mammalian Mek1 was demonstrated to activate the Jnk signalling module in addition to activating the prototypical Erk pathway (Troppmair *et al.*, 1994; Patriotis *et al.*, 1994; Salmerón *et al.*, 1996; Ceci *et al.*, 1997). Both full-length and carboxy-terminal truncated forms of Tpl2 phosphorylate recombinant Mkk4 on regulatory sites (Salmerón *et al.*, 1996). Furthermore, transfection of intact and truncated forms of Tpl2 into COS-1 cells and Jurkat T cells caused the activation of the Jnk signal transduction cascade (Salmerón *et al.*, 1996; Ceci *et al.*, 1997). The extracellular signal that stimulates Tpl2 regulated activation of Jnk remains unknown. Ask1 (apoptosis signal-regulated kinase) a protein kinase distantly related to Raf1, Ksr1 and Tpl2 protein kinases may act as a key intermediary in cytokine- and stress-induced apoptosis (Ichijo *et al.*, 1997).

#### 10.4 The Rho monomeric G-protein node.

Morphological changes associated with rearrangements to the actin cytoskeleton depend on the coordinate activities of the monomeric Rho subfamily (Cdc42, Rac1, Rac2, RhoA, RhoB and RhoC) of Ras-related proteins (Votjek and Cooper, 1995; Nobes and Hall; 1995). In mammalian cells, Rho induces stress fibers at cell adhesion sites; Cdc42 regulates a distinct signalling pathway that mediates the formation of actin-containing projections termed filopodia; while membrane ruffling or lamellopodia is controlled by Rac (Pelech and Charest, 1995). Although the function of the Rho proteins is associated with the regulation of events at the cell surface, these same small GTP-binding proteins can activate specific cellular kinases.

Besides coordinating the organization of the actin cytoskeleton, Rho family G proteins are involved in the regulation of specific cytoplasmic and nuclear targets (Votjek and Cooper, 1995; Nobes and Hall; 1995). Activation of the Jnk signalling pathway can be mediated by Rac, Cdc42 and Rho. In transient transfection assays, Jnk phosphotransferase activity toward c-Jun was stimulated by activated forms of Cdc42 and Rac1 expressed in Hela, NIH3T3, and COS-7 cell lines (Coso *et al.*, 1995; Minden *et al.*, 1995). In addition, the Cdc42 guanine nucleotide exchange factor protein (GEF) Dbl stimulated Jnk activity. The guanine nucleotide dissociation inhibitors (GDI) RhoGDI and RhoGAP overexpression blocked the activation effect of oncogenic Dbl on the Jnk enzyme activity (Coso *et al.*, 1995). Furthermore, RhoA, B and C in addition to Cdc42 all induce the activation of Jnk in 293T human kidney epithelial cell line (Teramoto *et al.*, 1996). Therefore, all three Rho G-proteins can participate in the activation of the Jnk stress-activated pathway.



## 10.5 The Ste20-related kinase node

Ste20 is an essential gene that operates within the MAP kinase regulated pheromone response pathway in the budding yeast *S. cerevisiae* (Leberer *et al.*, 1992). The monomeric (Cdc42) and heterotrimeric G $\beta\gamma$ -subunits (Ste4 and Ste18, respectively) converge at the level of Ste20 located at the top of the Ste11  $\rightarrow$  Ste7  $\rightarrow$  Fus3/Kss1 MAP kinase pathway. Several Ste20-related mammalian homologues have been identified by sequence analysis of tryptic peptides from Cdc42/Rac affinity purified proteins or by PCR using degenerate oligonucleotide primers (Manser *et al.*, 1994; Katz *et al.*, 1994; Manser *et al.*, 1995; Martin *et al.*, 1995; Teo *et al.*, 1995; Bagrodia *et al.*, 1995; Creasy and Chernoff, 1995a; Creasy and Chernoff, 1995b; Brown *et al.*, 1996; Taylor *et al.*, 1996; Pombo *et al.*, 1996; Keifer *et al.*, 1996; Su *et al.*, 1997). These seryl/threonyl kinases form part of a rapidly expanding family of Ste20-related kinases that are linked to cognate MAP kinase signalling modules. The Ste20-related kinases display distinctive regulatory and structural features; and consequently these enzymes can be arranged into two classes based on enzyme topology: the p21-activated kinase family (Pak) and the germinal center kinase family (Gck).

### 10.5.1 Pak regulation of the Jnk protein kinase module.

The Pak kinase family consists of the original 68-kDa Pak1 ( $\alpha$ Pak) and the most recently identified 62-kDa Pak2 ( $\gamma$ Pak) and the 65-kDa Pak3 ( $\beta$ Pak) isoforms (Manser *et al.*, 1994; Manser *et al.*, 1995; Bagrodia *et al.*, 1995; Teo *et al.*, 1995; Martin *et al.*, 1995; Brown *et al.*, 1996). These proteins share >95% identity within their catalytic domains and ~70% with the kinase domain of yeast Ste 20 (Leberer *et al.*, 1992). Furthermore, Pak1 complements a Ste20 defect in *S. cerevisiae*, indicating that this enzyme may be functionally equivalent to the yeast counterpart (Brown *et al.*, 1996). All three Pak

isoforms have their kinase subdomain located at the carboxy-terminal side of the protein. The Pak enzymes are targeted by the Rho subfamily of small G-proteins Cdc42 and Rac1 in a GTP-dependent manner, but not by RhoA (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995). Interaction of the activated forms of Cdc42 or Rac1 with the conserved 8 amino acid CRIB binding located in the amino-terminal portion of the kinase induces autophosphorylation on seryl residues and activation of Pak phosphotransferase activity toward MBP. Although genetic epistasis experiments have positioned Ste20 as the immediate activator of Ste11 in budding yeast, there has been no direct evidence for Pak isoform directly regulating Mekk nor Mkk4 activity in mammalian cells (Su *et al.*, 1997; Fanger *et al.*, 1997). Therefore, it would appear that important accessory proteins may have been absent or expressed in such low abundance in the transient transfection assays that proper Pak/Mekk signalling was disrupted in these cells. Equally plausible is the possibility that the Pak subfamily of Ste-related kinases phosphorylate and activate distinct Mekk-like protein kinases. A potential candidate is the recently identified activator of the Jnk pathway, MAPKKK5 a seryl/threonyl kinase that differs from the four Mekk isoforms in that the catalytic region of these enzymes is centrally located between two presumptive amino- and carboxy-terminal regulatory domains (Wang *et al.*, 1996). Despite the lack of evidence of a proximal kinase effector, Pak1 and Pak3 were demonstrated to link Cdc42 and Rac1 G-proteins in the activation of Jnk protein kinase in transfected COS cells (Bagrodia *et al.*, 1995; Knauss *et al.*, 1995; Brown *et al.*, 1996). Similarly, constitutively activated forms of Pak1 and Pak3 induced Jnk activation in these same cells. Activation of Jnk phosphotransferase activity by Pak1 was also observed in cell-free extracts of *Xenopus* oocytes induced to undergo maturation with progesterone (Polverino *et al.*, 1995). Coexpression of Mekk1 and Pak3 in sf9 insect cells also lead to activation of Mekk1. However, Pak3 was unable to phosphorylate and activate Mekk1 *in vitro* (Siow *et al.*, 1997). This result was not surprising since Mekk protein kinases also

display some phosphotransferase activity in transient transfection assays in the absence of extracellular stimulation (Lange-Carter *et al.*, 1993; Fanger *et al.*, 1997). Most importantly, however, was the observation that the Pak subfamily of Ste20-related enzymes stimulated the Jnk signalling module without effecting the parallel Erk protein kinase cascade. Further work will be required to determine the intermediary kinases involved in Jnk activation by Pak.

#### 10.5.2 Gck regulation of the Jnk protein kinase module

Members of the Gck family of protein kinases, which have been cloned from several different mammalian sources are related to Sps1, a Ste20-like kinase that is expressed at specific stages during spore cell wall biosynthesis in *S. cerevisiae* (Krisak *et al.*, 1991; Friesen *et al.*, 1994). The topology of Gck differs from the Pak protein kinases in that the enzyme catalytic domain is positioned at the amino-terminal. Also in contrast to Pak enzymes, the Gck protein kinases lack the Cdc42/Rac1 CRIB motif. Two classes of Gck have been identified based on the carboxy-terminal region of the enzyme. Gck as well as the related kinases Nik (Nck interacting kinase), Hpk1 (hematopoietic progenitor kinase 1), Khs (kinase homologous to Sps/Ste20) and Glk (Gck-like kinase), all range in size from 100- to 140-kDa, contain a large regulatory domain that possesses none of the known protein interaction motifs (Katz *et al.*, 1994; Kiefer *et al.*, 1996; Su *et al.*, 1997; Tung and Blenis, 1997; Diener *et al.*, 1997). The second class of Gck protein kinases including Mst1 (mammalian sterile twenty-like alias Krs1 for kinase responsive to stress) and Sok1 (Ste-20/oxidant stress kinase) have molecular masses in the range of 50- to 56-kDa and consequently a smaller regulatory domain of unknown function (Creasy and Chernoff, 1995a; Taylor *et al.*, 1996; Pombo *et al.*, 1996).

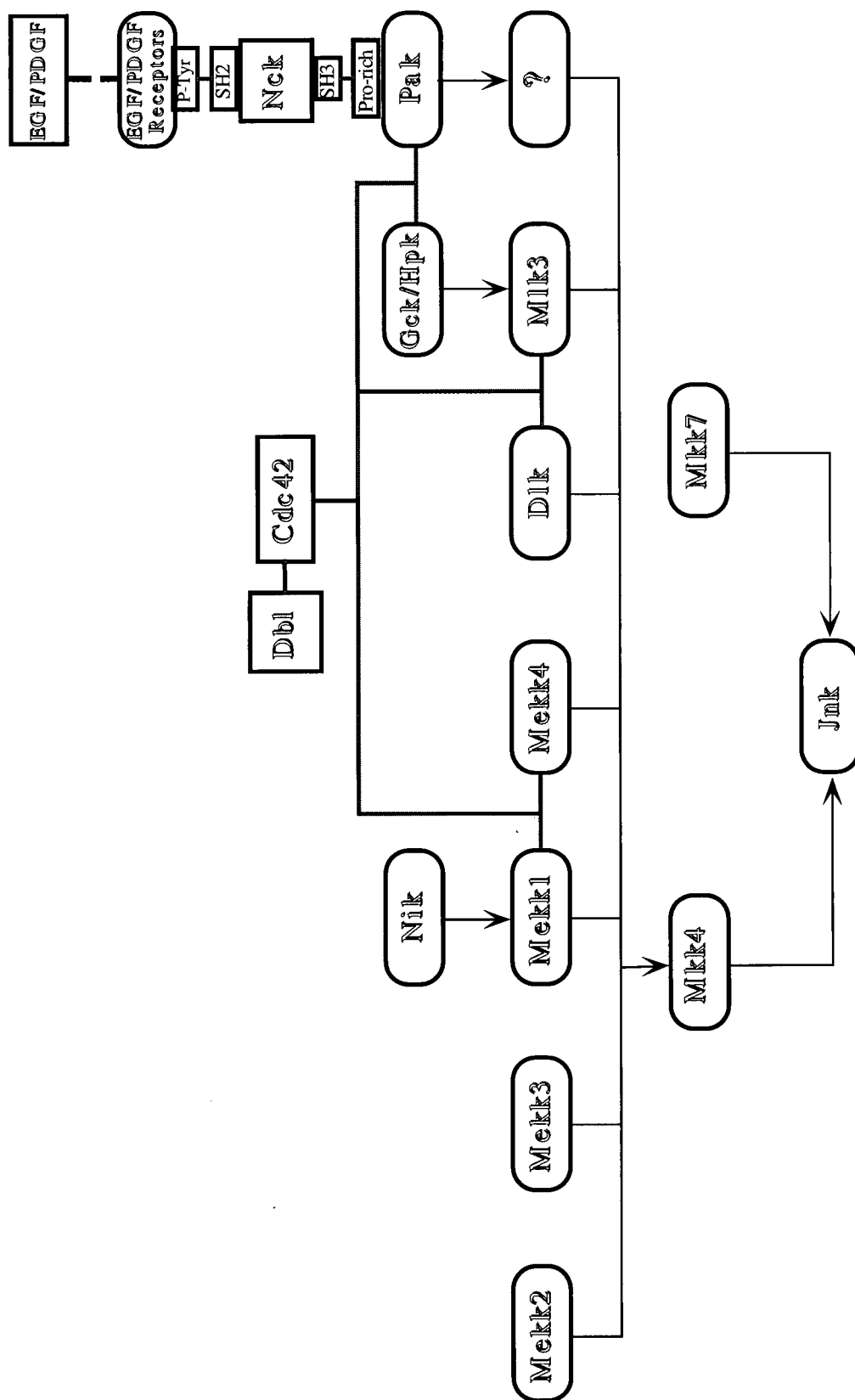
Sps1 regulates a specific MAP kinase, Smk1 during the yeast sporulation process. It might be expected that the Gck family of mammalian Ste20 kinases may regulate unique MAP kinase modules. Indeed, transient transfection of Gck protein kinase in COS cells was shown to operate within the Jnk stress-activated kinase cascade (Pombo *et al.*, 1995). In a manner similar to Jnk protein kinase, Gck was activated by TNF- $\alpha$  *in situ*. Overexpression of the Gck isoform Hpk1 and Khs in COS1 fibroblast cells also stimulated Jnk phosphotransferase activity toward c-Jun (Kiefer *et al.*, 1996; Hu *et al.*, 1996; Tung and Blenis, 1997). The kinase catalytic activity of Hpk1 was required for the activation of the Jnk pathway, whereas the expression of carboxy-terminal regulatory portion had no effect (Hu *et al.*, 1996). Mlk3 or a similar intermediary kinase may link Hpk1 to Jnk protein kinase. Hpk1 was able to directly phosphorylate Mlk3 *in vitro* (Kiefer *et al.*, 1996; Hu *et al.*, 1996). Furthermore, co-expression studies demonstrated that the two enzymes could physically associate via the amino-terminal SH3 domain of Mlk3 and two of four prolyl-rich motifs in Hpk1. Khs and Glk also contain polyprolyl helix structures that may interact with SH3 binding sites on proteins that have yet to be identified experimentally (Tung and Blenis, 1997; Diener *et al.*, 1997). The Jnk regulator Gck, which does not contain these prolyl-rich motifs was also capable of signalling to Jnk through Mlk3 (Pombo *et al.*, 1995; Tibbles *et al.*, 1996). It is possible that other sites in the carboxy-terminal domain may play essential roles in the regulation of cytoplasmic effectors. Mlk3 which has several protein interaction motifs co-immunoprecipitated with Mkk4 in co-transfection studies (Tibbles *et al.*, 1996). As mentioned previously, the mixed lineage kinase homologue, Sprk, phosphorylated and activated Mkk4, the Jnk activator (Rana *et al.*, 1996).

The yeast two-hybrid system was used to screen for proteins with prolyl-rich sequences that associated with SH3 domain of the adaptor protein Nck (Su *et al.*, 1997). Nik (Nck interacting kinase) is a 140-kDa seryl/threonyl kinase homologous to

Ste20/Sps1. Like Gck and Hpk1, Nik activated the Mekk  $\rightarrow$  Mkk4  $\rightarrow$  Jnk  $\rightarrow$  c-Jun module in transient overexpression assays. Although there was no evidence that Nik directly regulates Mekk *in vivo*, two pieces of indirect evidence support this notion. Catalytically-inactive mutants of Mekk inhibited stimulation of Jnk activity by Nik. Also, Mekk and Nik associate when co-expressed in 293 cells (Su *et al.*, 1997). Interaction between the two enzymes was mediated through the amino-terminal of Mekk and carboxy-terminal of Nik. Su *et al.* (1997) observed a strong sequence conservation (>70%) between the carboxy-terminal domains of Nik (see below) and a *C. elegans* gene whose function is unknown. This 325 amino acid domain is present in Gck and Hpk1, and consequently may be critical for the regulation of downstream effectors by the Gck family (Su *et al.*, 1997). In contrast, the related Mst1/Sok class of Gck protein kinases which possess a smaller carboxy-terminal tail are missing this 325 amino acid region. This may explain the inability of Sok1, Mst1 and Krs1 kinases to activate the Jnk pathway (Creasy and Chernoff, 1995; Taylor *et al.*, 1996; Pombo *et al.*, 1996; Su *et al.*, 1997). Therefore, these Sps1-like protein kinases may operate within distinct MAP kinase modules. At present, the mechanism of how Nck couples Nik to receptor or non-receptor tyrosyl activities that occur at the inner surface of the cell membrane are not well understood.

Recently, the SH3- SH2-adaptor protein Nck and Pak were shown to form a tight association *in vitro* and *in vivo* when co-expressed in L6 rat myoblast, Cos7 and 293T cell lines (Galisteo *et al.*, 1996; Bokoch *et al.*, 1996; Lu *et al.*, 1997). The interaction is mediated between the first prolyl-rich SH3-binding domain of Pak1 and the second SH3 domain of Nck (Galisteo *et al.*, 1996; Bokoch *et al.*, 1996). Furthermore, Pak1/Nck complexes are recruited to the inner face of the plasma membrane after cell stimulation with growth factors EGF and PDGF (Galisteo *et al.*, 1996; Bokoch *et al.*, 1996). Translocation to the membrane led to an increase in Pak1 phosphotransferase activity

Figure 2: Diverse proximal inputs regulate the Mkk4/Mkk7-Jnk stress signalling module.



(Galisteao *et al.*, 1995; Lu *et al.*, 1997). Therefore, the targeting of Pak to receptor tyrosyl kinases may lead to the activation of one or more of the MAP kinase modules. Figure 2 summarizes the various regulatory inputs that modulate Jnk activity.

## 11. HIGH OSMOLARITY MAP KINASE SIGNALLING PATHWAYS

### 11.1 The Hog MAP protein kinase pathway in *Saccharomyces cerevisiae*

In budding yeast, changes in environmental osmolarity activates a stress-related MAP kinase to increase the expression of genes critical for survival of the cell (Herskowitz, 1995). *S. cerevisiae* utilizes an osmosensor mechanism that is similar to the prokaryotic two-component system. This multistep phosphorelay system, Sln-Ypd-Ssk1, is coupled to a MAP kinase module distinct from the pheromone pathway. Under homeostatic conditions, the transmembrane histidyl-kinase osmosensor, Sln1, maintains Ssk1 (suppressor of sensor kinase) in an inactive state (Maeda *et al.*, 1994). Immediately following autophosphorylation of Sln1 receptor on a histidyl residue, the high energy phosphate is relayed to an aspartyl site located in the receiver domain of the same protein. The phosphate is shunted from the aspartyl residue on Sln to the inactivating aspartyl site on Ssk1 via histidine on the Ypd1 intermediary receiver protein. Elevated osmotic levels reduce Sln1 activity and consequently promote Ssk1 stimulation through reduced phosphorylation of the protein. Ssk1 promotes the production of intracellular glycerol by recruiting a MAP kinase module composed of two redundant Mek kinases, Ssk2 and Ssk22, the Mek homologue, Pbs2 (polymyxin B sensitive) and the MAP kinase, Hog1.



## 11.2 Identification of Hog protein kinase in the regulation of stress signalling.

Engagement of the CD14 glycosylphosphatidylinositol-anchored cell-surface glycoprotein with endotoxin (LPS for lipopolysaccharide) triggers the rapid tyrosyl phosphorylation and activation of the 42- and 44-kDa MAP kinase isoforms Erk2 and Erk1 (Weinstein *et al.*, 1992). However, the appearance of an additional 38-kDa tyrosyl phosphorylated protein was also observed in RAW264.7 monocytic-like and pre-B 70z/3 cell lines (Weinstein *et al.*, 1992; Han *et al.*, 1993). Purification and molecular cloning revealed that p38 was related to the *S. cerevisiae* gene, Hog1 (Han *et al.*, 1994). Ultimately, LPS stimulates the production and release of proinflammatory cytokines IL-1 and TNF- $\alpha$  from cells like monocytes and macrophages. LPS is an endotoxin associated with Gram-negative bacteria. Since this inflammatory stimulus can induce powerful immune responses which may lead to septic shock, compounds that can block this process could be useful therapeutically for many acute and chronic inflammatory diseases (Lee *et al.*, 1994). Pyridinyl-imidazole compounds termed CSAID (cytokine-suppressive anti-inflammatory drug) were shown to be powerful inhibitors in the biosynthesis of these proinflammatory compounds (Lee *et al.*, 1994 and references therein). Identification of the cellular targets of CSAIDs revealed that one of the CSAID binding proteins (Csbp) was a 38-kDa Hog1-related protein kinase (Lee *et al.*, 1993). The bicyclic pyridinyl imidazole inhibitors were shown to prevent Hog $\alpha$  activity by interfering with ATP access to the ATP binding pocket (Young *et al.*, 1997). Selectivity most likely occurs through interaction of CSAID with non conserved regions close to the ATP binding site. One consequence of cell exposure to IL-1 and TNF- $\alpha$  is the phosphorylation of small heat shock proteins, Hsp25 and Hsp27 (Kaur *et al.*, 1989; Saklatvala *et al.*, 1991). IL-1 and stress (arsenite and heat shock) stimulated a protein kinase pathway in human epithelial KB cells and *Xenopus laevis* oocytes involving a p38 homologue (p40 and Rk for Reactivating kinase, respectively) and MAPKAPK2 (mitogen-activated protein kinase-

activated protein kinase 2) that subsequently led to the phosphorylation of Hsp27 (Guesdon *et al.*, 1993; Freshney *et al.*, 1994; Rouse *et al.*, 1994). Since p38 can complement a Hog1 null mutant and become activated in response to changes in osmolarity, the mammalian isoform will be referred to as Hog $\alpha$ .

Hog $\alpha$  cDNAs have been isolated from mouse (p38), *Xenopus* (Mpk2), and human (Csbp1 and Csbp2) (Han *et al.*, 1994; Rouse *et al.*, 1994; Lee *et al.*, 1994). The two human Csbp isoforms were identical within the coding sequence except for a 75 nucleotide (25 amino acid polypeptide) mRNA splice variation in which there is a choice between two exons (Lee *et al.*, 1994). An second alternatively spliced variant of Hog $\alpha$ , Mxi2 (Max interactor 2), was isolated in a search for proteins that interact with the transcription factor Max in a two-hybrid screen (Zervos *et al.*, 1995). Although catalytic subdomain XI is replaced with a shorter 17 amino acid sequence which leads to an abrupt truncation of Mxi2 carboxy-terminus, the enzyme retained the ability to phosphorylate Max *in vitro*. Typically, human Hog $\alpha$  is synthesized as a 360-amino acid polypeptide chain (Lee *et al.*, 1994; Zervos *et al.*, 1995). The predicted molecular mass of Hog $\alpha$  is ~41-kDa; however, the activated form of the protein kinase appears to migrate faster under certain SDS-PAGE conditions.

To date, three new members of the Hog family of protein kinases have been isolated by using PCR amplification or by performing data base searches for cloned polypeptide sequences similar to Hog $\alpha$  in the expressed sequences tag (EST) database at the National Center for Biological Information. Once identified, the EST clones were then retrieved by DNA cloning. The largest of the Hog family is the 372-amino acid human  $\beta$ -isoform (p38 $\beta$ ) which is 74% identical with the Hog $\alpha$  (Jiang *et al.*, 1996). In contrast, Hog $\delta$  (alias Sapk4) displays 58% and 59% identity over its 365-amino acid length when compared to the  $\alpha$ - and  $\beta$ -isoforms (Goedert *et al.*, 1997). Finally, the 367-

amino acid human Hogγ is 63%, 62% and 64% identical with the α-, β- and δ- isoforms (Li *et al.*, 1996; Lechner, *et al.*, 1996; Mertens *et al.*, 1996). Interestingly, Hogγ tissue distribution is restricted to skeletal muscle whereas the other isoforms are expressed more ubiquitously, albeit at varying levels (Li *et al.*, 1996). Like the Erk and the Jnk family of protein kinases, the mammalian and yeast Hog proteins share the signature threonyl and tyrosyl phosphorylation site motif located between catalytic subdomains VII and VIII. Also, the activation lip that harbours the TGY motif is six residues shorter than observed in MAP kinases Erk1 and Erk2. Unexpectedly, only Hogα and β isoforms were inhibited by SB 202190 implying that there may be subtle differences at the amino acid level that enables certain isoforms of Hog protein to interact with pyridinyl imidazole compounds (Lee *et al.*, 1994; Jiang *et al.*, 1996)

### 11.3 Activation of Hog protein kinases

The four Hog isoforms become activated to varying degrees by the proinflammatory cytokines IL1 and TNF-α (Freshney *et al.*, 1994; Jiang *et al.*, 1996; Cuenda *et al.*, 1997; Goedert *et al.*, 1997). In addition, these same enzymes are also regulated in response to other environmental stresses such as hyperosmotic conditions (Han *et al.*, 1994; Jiang *et al.*, 1996; Cuenda *et al.*, 1997; Goedert *et al.*, 1997), U.V. irradiation (Dérjard *et al.*, 1995; Lin *et al.*, 1995), increases in temperature (Rouse *et al.*, 1994), ribotoxic agents (Jiang *et al.*, 1996; Cuenda *et al.*, 1997; Goedert *et al.*, 1997), thrombin or thromboxane activation of platelets through seven-transmembrane receptors linked to heterotrimeric G proteins (Kramer *et al.*, 1995; Saklatvala *et al.*, 1996). However, the Hogγ isoform may be involved in tissue-specific functions because of the protein's unique expression pattern. Indeed, C2C12 pre-muscle cell line stably expressing Hogγ (alias Erk6) were induced to undergo myoblast cell differentiation into myotubes (Lechner *et al.*, 1996). Many of the prototypic growth factors that are known

to activate the Erk protein kinases such as EGF and IGF-1 fail to robustly stimulate Hog isoforms. One exception is fibroblast growth factor (FGF) which activates the Hog signalling pathway in the neuroblastoma SK-N-MC cell line (Tan *et al.*, 1996).

#### 11.4 Effectors of Hog protein kinase signalling.

##### 11.4.1 Regulation of gene expression.

The amount of contribution made by the Hog isoforms in response to stress signalling is not well defined at present. To date, the amino terminal fragment of ATF2 has been shown to be a more useful *in vitro* substrate than MBP (Lee *et al.*, 1994; Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995). It is unclear, however, what role ATF phosphorylation by Hog has in the regulation of gene expression (Bayaert *et al.*, 1996; Hazzalin *et al.*, 1996; Iordinov *et al.*, 1997). To identify novel *in vivo* substrates for Hog, a human fetal brain cDNA library was screened by yeast two-hybrid analysis (Han *et al.*, 1997). The transcription factor, myocyte-enhancer factor 2C (MEF2C), was recovered using an inactive mutant of Hog $\alpha$  as a bait. MEF2C was the preferred substrate of Hog $\alpha$  whereas MAP kinase family members Erk2 and Jnk1 were less efficient (Han *et al.*, 1997). Stimulation of MEF2C transcriptional activity in LPS-treated RAW264.7 cells was reversible by incubation with the p38-specific inhibitor SB202190 or expression of dominant-negative Hog $\alpha$ . MEF2C transcriptional activity is regulated by Hog $\alpha$  phosphorylation of Thr-293 and Thr-300 located within the transactivation domain and a third site, Ser-387, located outside this region (Han *et al.*, 1997). Of critical importance is the presence of a MEF2C DNA-binding site in the c-Jun promoter region, which is LPS-inducible (Han *et al.*, 1995). LPS triggers a defence response in the innate immune system by inducing changes in the *de novo* synthesis of cytokine factors like IL-1 and TNF- $\alpha$  in monocytic cells (Lee *et al.*, 1994). Moreover, gene expression from many

cytokine promoter regions is regulated by the AP-1 transcription factor complex (Newell *et al.*, 1994; Han *et al.*, 1997). Hence, LPS-induced MEF2C phosphorylation may contribute to the regulation of the c-Jun expression and indirectly to cytokine production (Han *et al.*, 1997).

#### 11.4.2 Regulation of MAPKAPK2 activity

The mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) was first identified as an enzyme that was activated after phosphorylation by MAP kinase (Stokoe *et al.*, 1992). Originally purified from rabbit skeletal muscle, MAPKAPK2 was demonstrated to exist as a 45- and 55-kDa protein by gel electrophoresis (Stokoe *et al.*, 1992; Cano *et al.*, 1994; Cano *et al.*, 1996). The 55-kDa, 400 residue MAPKAPK2 protein contains a prolyl-rich amino-terminal domain followed by the catalytic domain and a putative nuclear translocation signal at the carboxy-terminal (Engel *et al.*, 1993; Stokoe *et al.*, 1993; Zu *et al.*, 1994). A second related 382-amino acid protein with a predicted molecular mass of 42 kDa probably corresponds to the smaller isoform (Sithanandam *et al.*, 1996; McLaughlin *et al.*, 1996). MAPKAPK3 (alias 3pK for chromosome 3p kinase) is 75% identical at the amino acid level to MAPKAPK2 and is also structurally similar. Furthermore, both MAPKAP kinases lie within the Hog signalling pathway.

In addition to Erk protein kinases, Hog $\alpha$  was shown to be an activator of MAPKAPK2 in stress-induced cells (Rouse *et al.*, 1994; Freshney *et al.*, 1994). Pyridinyl imidazole compounds such as SB 203580 directly inhibit Hog *in situ* and prevent stress activation of MAPKAPK2 (Cuenda *et al.*, 1995). It was initially demonstrated that MAPKAPK2 activation required phosphorylation on carboxy-terminal Thr-334 to become activated by Erk1 and Erk2 (Stokoe *et al.*, 1992). This implied that

the process of MAPKAPK2 activation is unique from that of other seryl/threonyl protein kinase enzymes that require phosphorylation within the catalytic domain (Johnson *et al.*, 1996). Indeed, a second MAP kinases consensus site at Thr-222 was identified in the L12 activation loop (Engel *et al.*, 1995; Ben-Levy *et al.*, 1995). In fact, constitutive activation of MAPKAPK2 can be achieved by replacement of Thr-222 and Thr-334 with glutamic acid mimetic residues or by deleting the A-helix motif and consequently Thr-334 in the carboxy-terminal region. Therefore, MAPKAPK2 may require two phosphorylation events to become fully-active: 1) phosphorylation at Thr-222 to promote local conformational changes for peptide substrate binding and; 2) phosphorylation at Thr-334 located in the A-helix motif to relieve inhibition from the hydrophobic pocket between ATP- and substrate-binding catalytic lobes (Engel *et al.*, 1995).

The consensus phosphorylation site sequence for MAPKAPK2 recognition is Hyd-X-Arg-X-X-Ser, where Hyd corresponds to a hydrophobic residue (Stokoe *et al.*, 1993; Clifton *et al.*, 1996). To date, only a few potential *in vivo* substrates have been identified for MAPKAPK2. Murine Hsp25 and human Hsp27 are phosphorylated in response to many of the stress stimuli that activate the Hog kinases (Pelech and Charest, 1995). Phosphorylation of heat shock proteins appears to initiate reconstruction of the actin microfilament network (Lavoie *et al.*, 1995). Studies using SB 203580 supported a role for Hog in regulating MAPKAPK2 phosphorylation of Hsp25 and Hsp27 at physiologically relevant sites (Cuenda *et al.*, 1995). Stress-induced stimulation of tyrosine hydroxylase (TH) has been noted in chromaffin cells (Sutherland *et al.*, 1993). Inhibition of the Hog signalling pathway demonstrated that MAPKAPK2 phosphorylation of Ser-19 modulates THs rate-limiting activity in the synthesis of catecholamine (Thomas *et al.*, 1997).

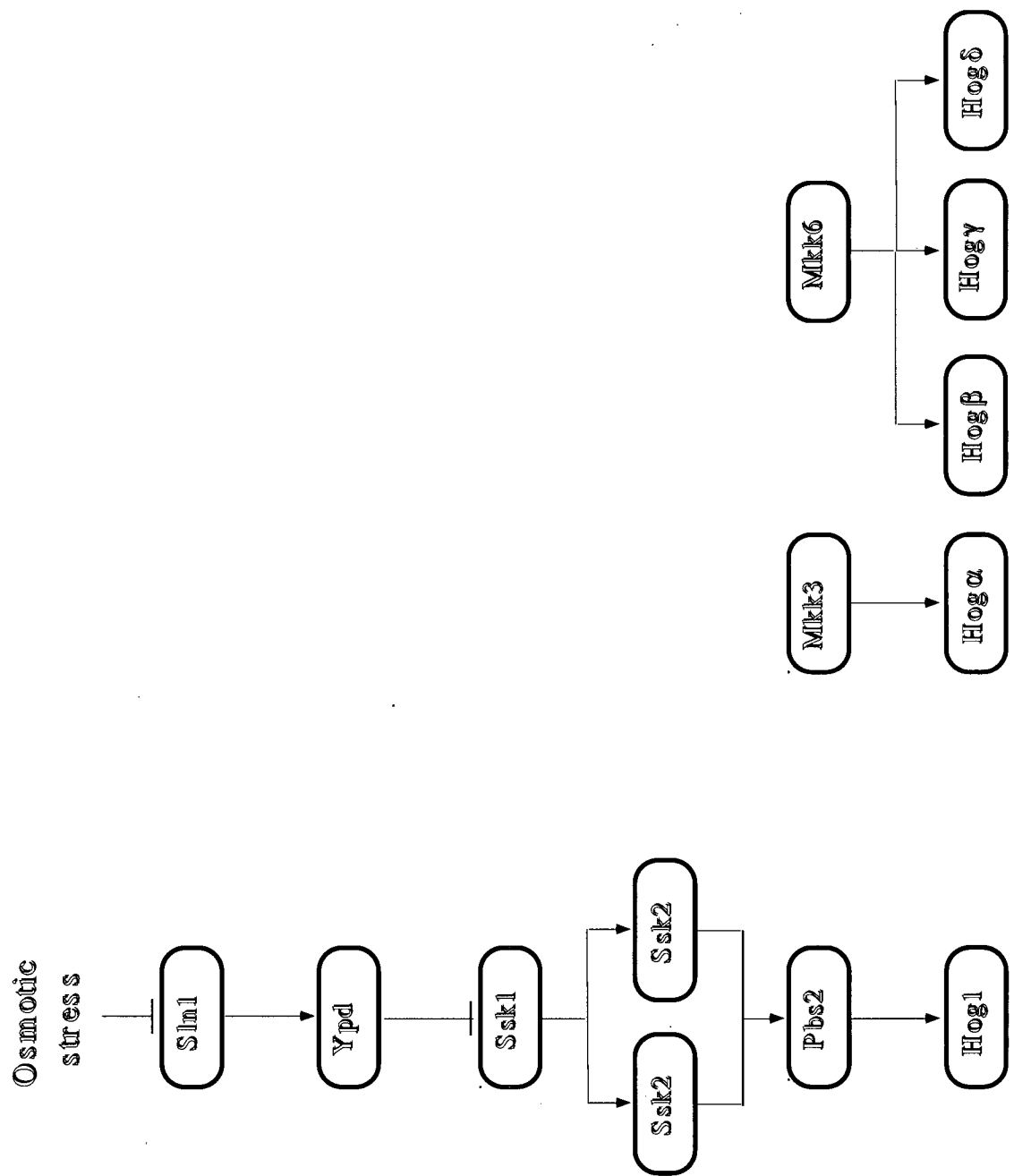
### 11.5 Hog protein kinase activators.

The ability of Hog and Jnk protein kinases to complement a mutation in Hog1 in budding yeast implied that a kinase similar to the Hog1 activator Pbs1 might regulate both enzymes. Indeed, the Jnk activator, Mkk4, was demonstrated *in vitro* to phosphorylate Hog $\alpha$  at the regulatory TGY motif (Dérjard *et al.*, 1995; Lin *et al.*, 1995). However, cotransfection studies with constitutively activated Mekk, a known activator of Mkk4, did not effectively activate Hog $\alpha$  in COS cells (Lin *et al.*, 1995). A separate protein kinase, namely Mkk3, appears to operate exclusively within the Hog signalling module. In fact, Mkk3 appears to activate only the Hog $\alpha$  isoform (Jiang *et al.*, 1996; Cuenda *et al.*, 1997; Goedert *et al.*, 1997). The identities of several other upstream activators have been investigated in PC12 and KB cells after treatment with cytokines or cellular stressors (Meier *et al.*, 1996). Several research groups have identified other novel MAP kinase kinases (Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud *et al.*, 1996; Cuenda *et al.*, 1996; Stein *et al.*, 1996; Moriguchi *et al.*, 1996). One such Hog protein kinase activator is Mkk6. The substrate specificity of Mkk6 (alias Mek6, Sapkk3) is similar to that of Mkk3 in that neither can enhance Erk or Jnk activity (Han *et al.*, 1996; Raingeaud *et al.*, 1996). However, one difference between Mkk3 and Mkk6 is that Mkk6 could phosphorylate the  $\beta$ ,  $\gamma$  and  $\delta$  isoforms of Hog in coexpression experiments (Jiang *et al.*, 1996; Cuenda *et al.*, 1997; Goedert *et al.*, 1997). These two closely related protein kinases (~80 identity between Mkk3 and Mkk6) are regulated by dual phosphorylation on conserved seryl and threonyl sites located in the activation loop.

To date only a couple of upstream activators have been demonstrated to communicate with the Mkk3/Hog signalling module. The murine homologue of Mekk4 was identified in functional complementation of osmosensitivity in budding yeast. Mtk1 is structurally similar to Ssk2 and Ssk22 and shares 98% identity with human Mekk4

Figure 3: Hog-dependent signalling modules in osmosensing in *Saccharomyces cerevisiae* and stress signalling in mammals.





within the kinase catalytic domain (Takekawa *et al.*, 1997). Furthermore, in coexpression studies, catalytically compromised Mtk1 was still able to mediate Hog $\alpha$  activation that was normally induced by changes in osmolarity (Takekawa *et al.*, 1997). The ability of Mtk1 to activate the Hog pathway depended on the activities of Mkk3 and Mkk6 enzymes. Another proposed activator of the Hog signal transduction pathway is the Rho family GTPases Cdc42 and Rac1 (Zhang *et al.*, 1995). However, recent results by the same research group reported that overexpression of Cdc42/Rac1 does not lead to activation of Hog in a Mkk3/Mkk6-dependent-manner but instead uses Mkk4 in the process (Han *et al.*, 1996). Figure 3 outlines the known players in the Hog stress-activated pathway in yeast and mammals.

## 12 ORPHAN MAP KINASE SIGNALLING MODULES

### 12.1 Erk3 signalling module

The 63-kDa Erk3 possesses many structural features that distinguish it from the other MAP kinase family members. In contrast to Erk, Jnk and Hog isoforms, the tyrosyl residue present in the signature Thr-Xaa-Tyr regulatory motif is absent in Erk3 (Boulton *et al.*, 1991). Also, the regulatory threonine is replaced by serine at residue 189 in Erk3 (equivalent to Thr-202 in Erk1) (Boulton *et al.*, 1991). The lack of tyrosine may explain the inability of Erk3 to phosphorylate any of the common MAP kinase substrates (Cheng *et al.*, 1996a). Recently, the crystal structure of the active form of Erk2 revealed that Tyr-185 may play a critical role in recognizing the prolyl residue in the P + 1 site of the substrate (Canagarajah *et al.*, 1997). In mammalian cells, Erk3 is a constitutively nuclear protein kinase which contrast the behavior of Erk, Jnk and Hog protein kinases which are located in the cytoplasm and the nucleus (Cheng *et al.*, 1996a).

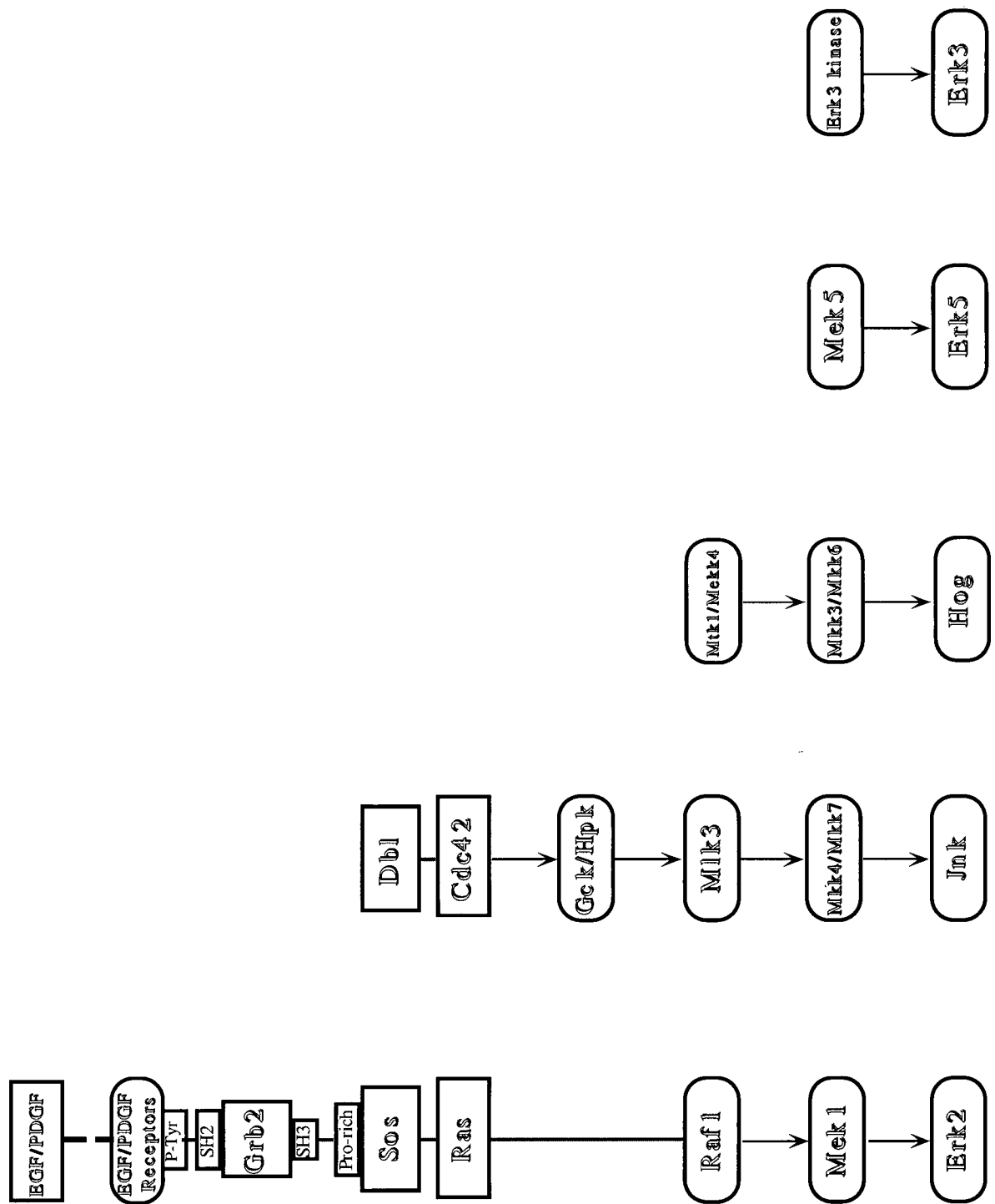
Rat Erk3 is most closely related to Erk1 and Erk2 MAP kinase family isoforms (Boulton *et al.*, 1991). The human homologue of Erk3 is almost identical (98%) to rat within the first two-thirds of the enzyme (Zhu *et al.*, 1994). Alternative splicing at the carboxy-terminal region incorporates a unique extension of 178 amino acids that generates a 97-kDa protein (Zhu *et al.*, 1994). Multiple Erk3-like genes have been reported to exist in mammalian cells (Boulton *et al.*, 1991). An Erk3-related protein kinase that is 72% identical to the rat isoform has been identified for humans (Gonzalez *et al.*, 1992).

An Erk3 protein kinase activator activity has been identified in rabbit muscle extracts and NGF-treated PC12 cells (Cheng *et al.*, 1996b). The Erk3 kinase was shown to bind tightly to the Erk3 catalytic domain. Erk3 kinase phosphorylation site was determined experimentally to be the regulatory Ser-189 (Cheng *et al.*, 1996b). The Erk3 kinase activator is present in both the cytosol and nuclear compartments of PC12 and 293 cells. In quiescent human fibroblasts, the HH1 kinase activity of the 97-kDa Erk3 isoform is activated by serum and phorbol ester treatment (Zhu *et al.*, 1994). Sauma and Friedman (1996) have reported that stable transfection of PKC  $\beta$ 1 into colon cancer cells activates Erk3 MBP phosphotransferase activity. However, in both these instances the degree of phosphorylation appeared to be very low.

## 12.2 Erk5 signalling module

The fifth member of the MAP kinase family is Erk5 (also termed Bmk1 for Big mitogen-activated kinase 1) (Zhou *et al.*, 1995; Lee *et al.*, 1995). Like Erk1 and Erk2 isoforms, Erk5 contains the canonical TEY phosphorylation motif. However, Erk5 contains a 400-amino acid carboxy-terminal regulatory/localization domain. Erk5 is

Figure 4: MAP kinase specific signalling modules in mammalian cells.



uniquely paired with a specific MAP kinase activator, Mek5 (Zhou *et al.*, 1995; English *et al.*, 1995). Alternative splicing at the amino-terminal domain of Mek5 may confer specific subcellular localization (Zhou *et al.*, 1995; English *et al.*, 1995). Erk5 was activated in response to H<sub>2</sub>O<sub>2</sub> and therefore may participate in a redox-sensitive pathway (Abe *et al.*, 1996). Src, which also is stimulated by reactive oxygen species, appeared to be required for activation of Erk5 in mouse fibroblast cells (Abe *et al.*, 1997). A summary of the five MAP kinase modules identified in mammals is depicted in Figure 4.

### 13 REGULATION OF MAP KINASE IN MATURING SEA STAR OOCYTES

#### 13.1 Identification of MAP kinase in sea star oocytes.

Echinoderm oocytes are naturally arrested in prophase of meiosis I. Consequently, the sea star oocyte is a useful model system for studying the cell cycle since the immature egg can be induced to mature synchronically from a quiescent state. Furthermore, a large number of cells may be easily obtained for biochemical and biological analysis. Treatment of sea star oocytes with the hormone 1-methyladenine (1-MeAde) stimulates ovulation in the female and resumption of oocyte maturation. Mature eggs from *pisaster ochraceous*, like other species of echinoderm, arrest at the G<sub>1</sub>-phase of meiosis II (also considered the pronuclear stage). A number of protein kinases become activated during the maturation process that phosphorylate the exogenous substrates histone H1 (HH1), MBP and S6 (Pelech and Krebs, 1987; Meijer *et al.*, 1987; Pelech *et al.*, 1988). This M-phase specific HH1 kinase which a component of maturation promoting factor (MPF) was subsequently identified as a homologue of Cdc2 the cell cycle regulator of the fission yeast *S. pombe* (Arion *et al.*, 1988; Labbé *et al.*, 1988). MBP kinase was purified to homogeneity from 1-MeAde-treated oocytes (Sanghera *et al.*, 1990). Microsequencing of tryptic peptide fragments revealed that the enzyme was a

member of the MAP kinase family (Posada *et al.*, 1991). Contrary to other MAP kinases, Mpk1 purified from sea star oocytes appeared not to require phosphorylation on threonine to become activated in maturing oocytes (Sanghera *et al.*, 1991). However, recent cloning of the Mpk1 cDNA revealed that the conserved threonyl and tyrosyl regulatory motifs are present in the sea star MAP kinase (Charest *et al.*, unpublished data). In addition, recombinant Mpk1 purified from bacteria was poorly activated *in vitro* by mouse Mek1 (Charest *et al.*, unpublished data). These results imply that sequence differences in Erk1 and Mpk1 protein kinases may confer substrate specificity for the upstream MAP kinase activators. Raf- or Mos-like kinases have yet to be unequivocally confirmed in echinoderms, although immunoblotting data supports the presence of Raf (Palaty and Pelech personal communication; Meijer, personal communication).

Although the identity of the 1-MeAde receptor at the cell surface of the sea star oocyte is unknown, it appears to activate heterotrimeric G proteins. Pretreatment of the oocytes with pertussis toxin, an inhibitor of G<sub>i</sub> class of proteins, prevented the 1-MeAde-induced maturation (Shilling *et al.*, 1989; Chiba *et al.*, 1992; Tademuda *et al.*, 1992). Microinjection of the  $\beta\gamma$ -subunit from bovine retina into immature sea star oocytes induced germinal vesicle breakdown (GVBD) with the same time course as observed with 1-MeAde treatment (Jaffe *et al.*, 1993). In contrast, microinjection of non-myristoylated G protein  $\alpha$  subunit blocked 1-MeAde-induced maturation of sea star oocytes (Jaffe *et al.*, 1993). It remains to be determined what effect heterotrimeric protein G<sub>i</sub>  $\beta\gamma$ - and  $\alpha$ -subunits have on activation of Mpk1 in sea star oocytes.

## HYPOTHESIS

1. MAP protein enzyme is activated in many cell types by a variety of stimuli (Pelech and Charest, (1995). Isolation of the cDNA will allow further characterization of the kinase at the protein level.
2. MAP protein kinase phosphorylates Rsk1 on regulatory sites that lead to its activation *in vivo* (Sturgill *et al.*, 1988). As MAP kinase is regulated by reversible protein phosphorylation, it is expected that the enzyme will be regulated by an upstream MAP kinase activator.
3. As a kinase which is tightly regulated by phosphorylation, it will be important to examine the importance of the regulatory phosphorylation sites (Sturgill *et al.*, 1988; Anderson *et al.*, 1991). Characterization of the regulatory phosphorylation sites in MAP kinase will aid in understanding the specificity of the consensus site recognition sequence for its upstream activator.



## **OBJECTIVES**

1. To clone human and sea star MAP kinase.
2. To express the cloned MAP kinase in prokaryotic cells.
3. To purify and characterize the direct activator of MAP kinase from sea star oocytes.
4. To clone the MAP kinase activator and express the protein in prokaryotic cells .
5. To determine the substrate specificity of the MAP kinase activator for MAP kinase by altering the regulatory phosphorylation sites by site-directed mutagenesis.

## MATERIALS AND METHODS

### 1. MATERIALS

A list of commonly used laboratory chemicals and their supplier(s) is summarized in Table 1. Although many of the chemicals are used for both molecular and biochemical technologies, only molecular biology grade reagents were used for the manipulation of DNA and RNA. This minimized degradation of the nucleic acids. As a second measure, all stock solutions used in molecular biology were sterilized by autoclaving for 20 min at 15 pound/square inch on liquid cycle. In those instances where a reagent could not be autoclaved the solutions were filter sterilized using a 0.2  $\mu\text{m}$  syringe filter or in the case of solutions over a 50 ml volume a self-contained 0.2  $\mu\text{m}$  membrane filtering unit. Also included in Table 1 are the miscellaneous reagents, consumables and photographic supplies required for many of the experimental protocols.

### 2. CELL MANIPULATIONS

#### 2.2. Oocyte isolation and cell culture

##### 2.2.1. Oocyte preparation

##### 2.2.1.1 Mechanical disruption

Gravid adult female sea stars (*Pisaster ochraceus*) were gathered between the months of March and July from the intertidal zone surrounding Vancouver, British Columbia. The animals were kept until needed in sea water tanks at the Department of Fisheries and Oceans located in West Vancouver, B. C. Immature sea star oocytes,

Table 1: Research materials and their commercial sources

A. Chemicals

Acetic acid (CH <sub>3</sub> COOH)	Fisher Scientific
Acetonitrile	Applied Biosystems
Acrylamide	Fisher Scientific/ICN
Adenosine 5'-triphosphate disodium salt	Sigma
Agarose	Gibco BRL
Agarose (low melting point)	BRL
Ammonium bicarbonate (NH <sub>4</sub> HCO <sub>3</sub> )	BDH
Ammonium hydroxide	Fisher Scientific
Ammonium persulphate	Fisher Scientific
Ammonium sulphate	Fisher Scientific
Bis-acrylamide	Fisher Scientific
N,N'-Methylene bis-acrylamide	Fisher Scientific
Boric acid	Amersham
Bovine serum albumin	Sigma
Brilliant Blue G	Sigma
5-Bromo-4-chloro-3-indoyl phosphate (BCIP)	Sigma
1-Butanol	Fisher Scientific
iso-butanol	Fisher Scientific
β-glycerolphosphate	ICN
β-methyl aspartic acid	Sigma
Calcium chloride (CaCl <sub>2</sub> )	Sigma
Citric acid	BDH
Chloroform	Fisher Scientific
Coomassie brilliant blue R	EM Science
Denatured alcohol	Fisher Scientific
2'-Deoxynucleoside 5'-triphosphates (dNTP kit)	Pharmacia
Diethyl pyrocarbonate	Sigma
N,N-dimethyl formamide (DMF)	Sigma/Fisher Scientific
Dimethyl sulfoxide (DMSO)	Fisher Scientific
Dithiothreitol (DTT)	BDH
Ethanolamine	Sigma
Ethidium bromide	Molecular Probes Inc.
Formaldehyde solution	Fisher Scientific/BDH
Gelatin	BioRad/Sigma
Glutathione	Sigma
Glycerol	Anachemia
Glycine	Sigma/Fisher Scientific
Guanidine thiocyanate	ICN
Hydrochloric acid	Fisher Scientific
N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES)	Sigma
N-Lauroyl sarcosine	Sigma
Liquid paraffin	BDH
Lithium chloride anhydrous	BDH/Sigma/Fisher Scientific
Magnesium acetate tetrahydrate	BDH
Magnesium sulphate (MgSO <sub>4</sub> ·7H <sub>2</sub> O)	Fisher Scientific
Magnesium chloride (MgCl <sub>2</sub> ·6H <sub>2</sub> O)	Fisher Scientific

Table continued...

Maltose	BDH
Manganous chloride ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ )	BDH
2-Mercaptoethanol	BioRad
Methanol	Fisher Scientific/BDH
1-Methyladenine	Sigma
DL-threo- $\beta$ -methylaspartic acid	Sigma
2-[N-Morpholino]ethanesulfonic acid (MES)	Sigma
3-[N-Morpholino]propanesulfonic acid (MOPS)	Sigma/ICN
Myelin basic protein (MBP)	Kinetek/Sigma
N-ethyl maleimide	Sigma
Ninhydrin	BDH
Nitric Acid ( $\text{HNO}_3$ )	Fisher Scientific
Nitro blue tetrazolium (NBT)	Sigma
Nonidet P-40	BDH
Petroleum ether (60-80°)	BDH
Phenyl phosphate disodium salt (phosphatase inhibitor)	ICN
Phenolsulfonphthalein (Phenol red dye)	Sigma
Delbucco's phosphate-buffered saline (PBS)	Gibco
Phosphoric acid	
Potassium acetate ( $\text{C}_2\text{H}_3\text{O}_2\text{K}$ )	BDH
Potassium chlorate (KCl)	Fisher Scientific
Potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ )	BDH
Potassium dihydrogen orthophosphate monobasic	BDH
Potassium hydroxide (KOH)	BDH
Potassium phosphate (dibasic)	Sigma
di-Potassium hydrogen orthophosphate 3-hydrate	BDH
Potassium dihydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ )	BDH
Ponceau S concentrate	Sigma
Propanol	Fisher Scientific
Pyridine	Fisher Scientific
Silver nitrate ( $\text{AgNO}_3$ )	Fisher Scientific
Sodium acetate (dibasic)	BDH
Sodium azide	Fisher Scientific
Sodium bicarbonate ( $\text{NaHCO}_3$ )	Fisher Scientific
Sodium borate	Fisher Scientific
Sodium carbonate - anhydrous ( $\text{Na}_2\text{CO}_3$ )	BDH
Sodium chloride	Fisher Scientific
tri-Sodium citrate	BDH
Sodium deoxycholate	BDH
Sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )	BDH
Sodium dodecyl sulfate (SDS)	Fisher Scientific
Sodium fluoride	BDH/Fisher Scientific
di-Sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ )	BDH
Sodium hydroxide	Fisher Scientific
Sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ )	Fisher Scientific
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Fisher Scientific
Tris hydroxymethyl aminomethane hydrochloride (Tris-HCl)	Fisher Scientific
Tris hydroxymethyl methylammonium chloride (Tris-HCl)	BDH

Table continued...

Tris hydroxymethyl methylamine  
Triton X-100  
Tween-20  
Urea  
Zinc Chloride

BDH/Fisher Scientific  
BDH/Fisher Scientific  
Fisher Scientific  
BioRad  
BDH

#### B. Miscellaneous reagents

Ampicillin (D[-]- $\alpha$ -Aminobenzylpenicillin)  
Bind silane  
Counting scintillant (biodegradable)  
DNA 1kb ladder  
DNA herring sperm  
GeneClean kit  
Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)  
PKI - cAMP-dependent protein kinase peptide inhibitor  
Prestained SDS-PAGE standards  
Random Primers 5'pd(N)6  
Repel silane  
RNase Guard  
RNasin  
Sephaglas Bandprep Kit

Sigma  
LKB  
Amersham  
Gibco BRL  
Boehringer Mannheim  
Bio 101  
Fisher Scientific/Promega  
Sigma  
Kinetek/BioRad  
Pharmacia  
Pharmacia  
Pharmacia  
Promega  
Pharmacia

#### C. Consumables

Centricon tubes (10 and 30)  
2070 Conical tubes (50 ml)  
2059 Culture tubes (14 ml)  
disPo/ Culture Tubes (13 x 100 mm)  
GeneAmp Reaction Tubes  
Hybond-N hybridization membrane  
Immobilon P (PVDF)  
3MM chromatography paper  
Membrane filter unit  
Micro centrifuge tubes (1.5 ml)  
Nitrocellulose transfer and immobilization membrane  
P81 phosphocellulose chromatography paper  
Pipet tips (1-200  $\mu$ l) and (1-1000  $\mu$ l)  
Syringe filter unit  
Tissue culture dishes (90-mm and 150-mm)

Amicon  
Falcon  
Falcon  
Baxter  
Perkin Elmer Cetus  
Amersham  
Millipore/Dupont  
Whatman  
Nalgene  
Elkey/Eppendorf  
Schleicher and Schuell  
Whatman  
National Scientific  
Nalgene  
Corning

#### D. Photographic supplies

Developer

Fixer

ISO 3000 Polaroid film 667

ISO 100 Polaroid film

Reflection NEF-Autoradiography film

X-OMAT AR Imaging film

Kodak

Kodak

Polaroid

Polaroid

DuPont

Kodak

which were naturally arrested at the G<sub>2</sub>/M border of meiosis I, were isolated and prepared as previously described (Meijer *et al.*, 1984). Ovaries were removed from the sea stars by excision and were kept in cold natural sea water (4°C). The oocytes were released by gently rupturing the ovarian sacks with a sharp instrument and forceps. Free oocytes filtered through a sieve to remove large debris (eg. connective tissue and gut) were combined into large 500 ml bottles for centrifugation at 1,000 rpm (125 g) for 5 min. Normally a yellowish upper layer of broken oocytes and follicle cells appears above the pink layer of packed oocytes which is discarded with the supernatant. The oocytes were carefully washed by resuspending the pellet several times in cold natural sea water to remove the remaining contaminants. Following several washes, one litre of oocytes was added to 3,000 ml of natural sea water containing 10 µM 1-methyladenine. After incubation at 14°C for approximately 80 min post-hormone treatment, 80-90% of the oocytes underwent germinal vesical breakdown (GVBD), indicating that they had completed maturation. Cell cycle progression was monitored using a Leitz Labovert FS microscope at 25X magnification. The mature oocytes were concentrated by centrifuging 500 ml volume at 1,000 rpm (125 g) for 5 min. A 40% oocyte homogenate was prepared in chilled homogenization buffer A containing 20 mM MOPS [pH 7.2], 0.25 mM DTT, phosphatase inhibitors (50 mM β-glycerolphosphate, 1 mM NaVO<sub>3</sub>, 5 µM β-methyl aspartic acid), and several broad specificity protease inhibitors that are described in Table 2 (5 mM EGTA, 2 mM EDTA, 1.0 mM PMSF and 1.0 mM benzamidine) by applying two 30 sec bursts at 18,000 rpm using a Brinkmann Polytron PT300. Following cell disruption, cellular organelles (mitochondria, endoplasmic reticulum etc.) were removed by centrifuging for 10 min at 9,000 rpm (12,000 g) with a Beckman J2 HS centrifuge and its companion JA10 rotor. The combined post mitochondrial supernatant was pooled on ice and subsequently centrifuged at 40,000 rpm (100,000 g) for 30 min at 40°C using a Sorvall ultracentrifuge and T647.5 rotor to separate the cell membranes. The pooled

Table 2: Specificity of protease inhibitors.

Aprotinin	Serine protease inhibitor	Sigma
Benzamidine	Peptidase inhibitor	ICN
Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Metalloprotease inhibitor (divalent cation-dependent)	Fisher Scientific
Ethylene diamine tetraacetic disodium salt (EDTA)	Metalloprotease inhibitor (divalent cation-dependent)	Fisher Scientific
Leupeptin	Serine and thiol proteases	Sigma/ICN
Phenyl methylsulphonyl fluoride	Serine and thiol proteases	Sigma
Soybean trypsin inhibitor	Trypsin and Factor Xa	Sigma



cytosolic supernatants were subsequently transferred to 50-ml conical tubes and frozen at -70°C.

#### 2.2.1.2 1-Methyladenine injection

A second technique employed for isolating sea star oocytes involved inducing their maturation *in vivo* by injecting the hormone directly into the live animal. This approach proved extremely convenient since fewer volunteers were required for harvesting the oocytes and more environmentally friendly, because the sea stars were returned live to the waters in the Greater Vancouver area. Spawning in live gravid adult females was carried out according to the protocol described by Meijer *et al.* (1984). A minimum of 1 ml of 0.14 mM 1-MeAde diluted in sea water was injected into each sea star arm. Sea stars began shedding usually within 90 min after the first injection; if not, then a second injection of 1-MeAde was given. Once the sea stars began spawning they were inverted over 250- or 400-ml plastic beakers filled with 20 ml of natural sea water. The nearly 100% mature oocytes were collected, concentrated by gentle centrifugation at 1,000 rpm (125 g) for 5 min finally resuspended to 40% (vol/vol) with homogenization buffer. All subsequent steps were executed as described above.

#### 2.2.2 Cell culture

The human hepatocellular carcinoma cell line Hep G2 and the epidermoid cell line A-431 were obtained from American Type Tissue Collection (Rockville, Maryland). The Hep G2 cells were maintained in Eagle's minimum essential medium (10% fetal bovine serum, Earle's salts, and 5 µM 2-mercaptoethanol) (Charest *et al.*, 1993). Hep G2 cells were grown in monolayers to a confluency of  $10^8$  (eight to ten 150 mm plates) before treating them with 100 µM insulin for 5 min at 37°C. The cells were scraped from

the plate with a rubber policeman and collected by centrifugation 3,000 rpm (~800 x g) using a Heraeus Biofuge 15 microcentrifuge. The cells were resuspended in 2 ml of homogenization buffer A and lysed by sonication using a Vibra-cell sonicator at setting 45. Using a Beckman TL100 Ultracentrifuge, the homogenate was clarified by centrifugation at 70,000 rpm (175,000 x g) for 30 min and stored at -70°C until needed. The A431 cells were maintained in Dulbecco's modified Eagle medium (5% fetal bovine serum, 5% fetal calf serum and 5 µM 2-mercaptoethanol).

### 3. BIOCHEMICAL TECHNIQUES

Table 3 outlines the kinases, phosphatases and proteases used during the course of this work. Several of the kinases and phosphatases were the generous gifts of many researchers.

#### 3.1 Determination of protein concentrations

The protein concentration was determined by the Bradford (1976) method. Ten or twenty microlitres of crude cytosolic extract or column fraction were mixed with 2.5 ml of Bradford reagent (100 mg/l Coomassie brilliant blue G-250) and was allowed to stand for 5 min before reading the colorimetric change at an optical density of 595 nm. Varying concentrations of BSA (1 mg/ml) were used as a standard. In the case of recombinant GST-fusion proteins, 20 µl of glutathione-GST beads were subjected to a 10% SDS-PAGE and visualized using Coomassie blue staining (see later). Varying concentrations of BSA electrophoresed side-by-side on the same gel were used as standards.

Table 3: Sources of kinases, phosphatases and proteases

Acid phosphatase	Sigma
CD45 protein tyrosine phosphatase from human spleen	Dr. Nicholas Tonks
$\alpha$ -Chymotrypsin	Sigma
Protein tyrosine phosphatase $\beta$ from human (HPTP $\beta$ )	Dr. Ken Harder
Lck from baculovirus infected Sf9 cells	Dr. Ruedi Abersold
Protein phosphatase 2A (PP2A)	Dr. David Brautigan
Lysozyme	Boehringer Mannheim
Trypsin sequencing grade	Sigma
Thrombin	Sigma

## 3.2 Column chromatography of sea star cytosolic extracts

### 3.2.1 Anion exchange chromatography

A list of the chromatography resins, their properties and their commercial sources is provided in Table 4. The most common analytical chromatography columns used during the course of this study are described below.

A protocol, used by all members of Dr. Steve Pelech's laboratory was created for routine fractionation and analysis of crude cytosolic extracts. This approach fulfilled two important purposes. First, the elution profiles of several different protein seryl/threonyl kinases have been well characterized after many years of biochemical analysis. Second, the activation of these kinases can be compared in such disparate model systems as echinoderms and mammalian cells. Approximately 1-2 mg of protein were applied at a flow rate of 0.8 ml/min to a Mono Q column (HR5/5) equilibrated with buffer B (20 mM MOPS [pH 7.2], 5 mM EGTA, 2.5 mM EDTA, 100  $\mu$ M NaVO<sub>3</sub>, 1 mM NaF, 25 mM  $\beta$ -glycerophosphate, 5  $\mu$ M  $\beta$ -methyl aspartic acid and 2 mM DTT). The column was eluted at the same flow rate using a 15 ml linear NaCl gradient (0-0.8 mM) (Figure 5A). Two hundred and fifty microlitre fractions were collected and analyzed for enzyme activity and/or immunodetection with specific antibodies.

### 3.2.2 Cation exchange chromatography.

Samples containing 1-2 mg of protein were diluted with 10 volumes of buffer C (20 mM MES [pH 6.5], 1mM EGTA, 1mM EDTA, 100  $\mu$ M NaVO<sub>3</sub>, 1 mM NaF, 25 mM  $\beta$ -glycerolphosphate, 5  $\mu$ M  $\beta$ -methyl aspartic acid and 2 mM DTT) and subsequently loaded at a flow rate of 0.8 ml/min onto a Mono S (HR5/5) column equilibrated with the

Table 4: Chromatography resins used for protein analysis and purification.

DEAE-cellulose	Fibrous anion exchanger	Sigma
Heparin-agarose	Cation exchanger	Sigma
Hydroxylapatite	Ionic differential surface binding	BioRad
Mono Q (HR5/5)	Strong anion exchanger	Pharmacia
Mono S (HR5/5)	Strong cation exchanger	Pharmacia
Phosphocellulose P11	Fibrous cation exchanger	Whatman
Polylysine-agarose	Anion exchange	Sigma
S-Sepharose	Strong cation exchanger	Pharmacia
Superose 12	Gel filtration	Pharmacia

same buffer. The column was developed with a 15 ml linear NaCl gradient (0-0.5 mM) and the eluate was collected over a range of 50 fractions at 300  $\mu$ l each (Figure 5B). A small sample from alternate fractions was tested for enzyme activity and/or Western blotting.

### 3.2.3 Gel filtration chromatography

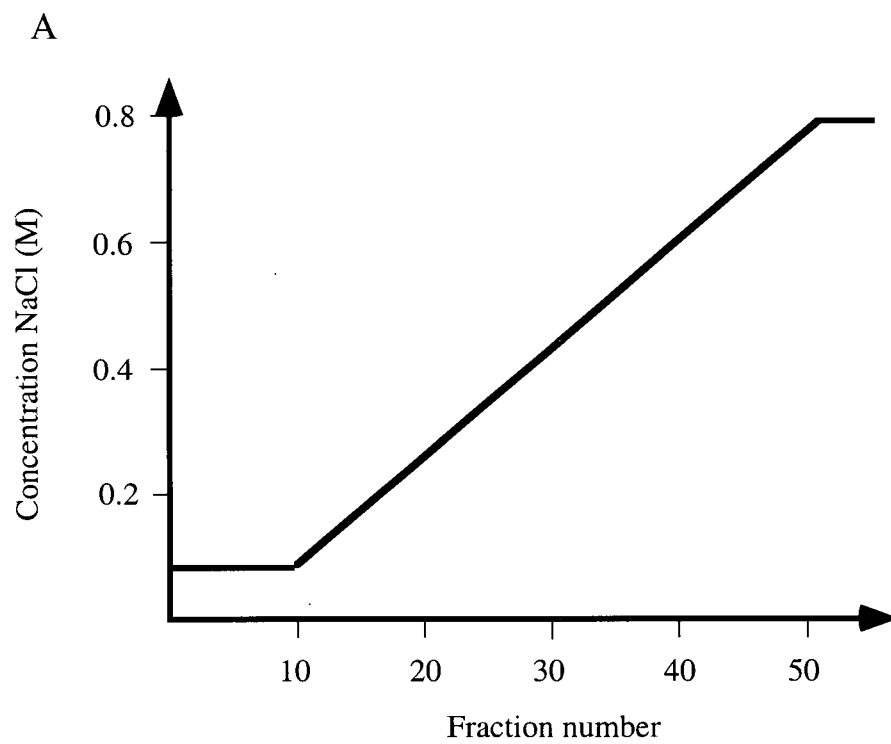
The Superose 12 column (HR10/30) equilibrated with buffer B containing 150 mM NaCl was loaded with 200  $\mu$ l of purified protein (approximately 1-2  $\mu$ g) at a flow rate of 0.25 ml/min. Once the void volume of 27.5 ml had eluted from the column, 200  $\mu$ l fractions were collected and assayed for enzyme activity. Protein molecular mass standards used for calibrating the column were: Blue Dextran, 443-kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 67 kDa; and  $\beta$ -lactoglobulin, 35 kDa.

Please note that all chromatography procedures described in this work were performed in a refrigeration unit at or near 4°C with a programmable Pharmacia Fast Protein Liquid Chromatography (FPLC) system.

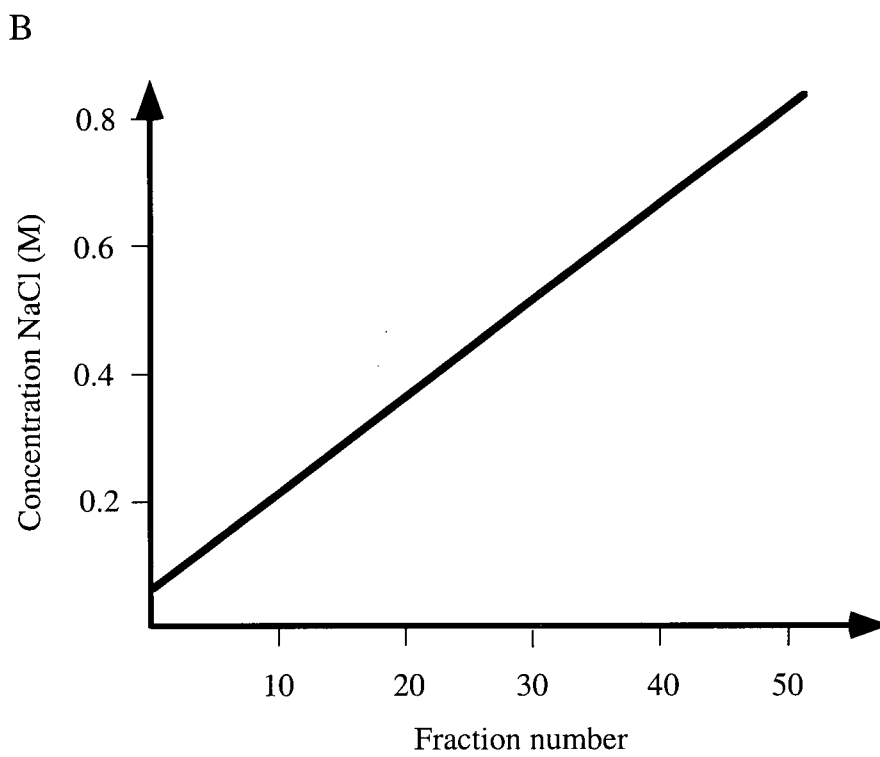
### 3.4. Purification of sea star MAP kinase activator-like protein

A schematic flow chart of the MAP kinase activator purification is outlined in Figure 6. Sea star cytosolic extract (100 ml; ~2 g of protein) prepared as described in Section 1 was thawed and diluted to 2,000 ml with buffer D (20 mM MOPS [pH 7.2], 12.5 mM  $\beta$ -glycerolphosphate, 10 mM EGTA, 2 mM EDTA, 100  $\mu$ M NaVO<sub>3</sub>, 1 mM NaF, 5  $\mu$ M  $\beta$ -methylaspartic acid, and 1 mM DDT). The diluted homogenate was applied to a DEAE-cellulose column (5 cm x 7 cm) and hydroxylapatite column (2.5 cm x 5 cm) linked in series. Hence, the flow through material from the DEAE-cellulose

Figure 5: Mono Q and Mono S column elution profiles. Panel A. The Mono Q column was developed with a 15 ml linear NaCl (0-0.8M) at a flow rate of 0.8 ml/min into 50-250  $\mu$ l fractions. Panel B. The Mono S column was developed with a 15 ml linear NaCl (0-0.8M) at a constant flow rate of 0.8 ml/min into 50-300  $\mu$ l fractions.



10





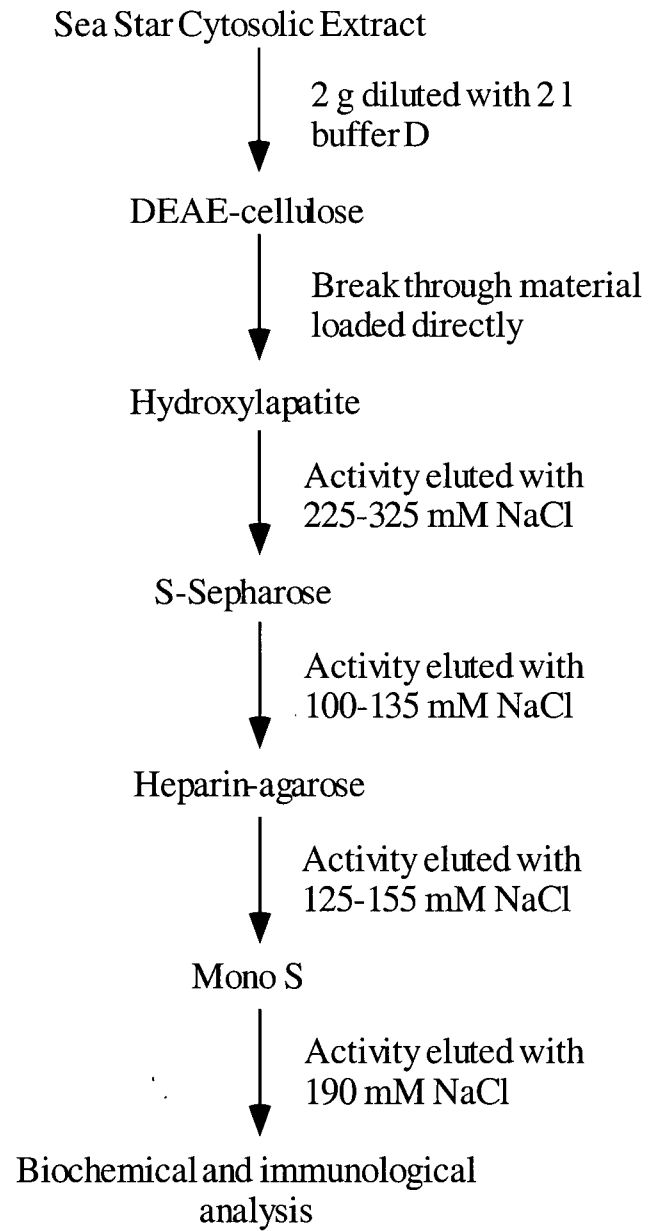
column was loaded directly onto the hydroxylapatite column. After washing the two columns with 300 ml of buffer D at 4 ml/min, the hydroxylapatite was uncoupled and developed separately with a 300 ml linear gradient of potassium phosphate (0-200 mM) at a flow rate of 1.5 ml/min. Four millilitre fractions were collected and tested for MAP kinase kinase activity using the MAP kinase activator assay (Section 3.5.2). The activator activity eluted between 25-60 mM NaCl.

The fractions that were able to increase recombinant GST-erk1 activity toward MBP from the hydroxylapatite column were combined and diluted to 1:4 with buffer D prior to loading an S-Sepharose column (2.5 cm x 5 cm) at 1.5 ml/min. The column was washed, at the same flow rate, with 90 ml of buffer D before collecting 4 ml fractions using a 300 ml linear 0-500 mM NaCl gradient in buffer D. Maximal activator activity was detected at a concentration of 100-135 mM NaCl.

The fractions from the S-Sepharose column were pooled, diluted 1:1 with buffer D, and applied to a phosphocellulose column (2.5 cm x 3 cm) that had been equilibrated in buffer D. After washing the column with 50 ml of buffer D, the MAP kinase activator was collected in 3 ml fractions from the phosphocellulose column that had been developed with a 195 ml linear 0-800 mM NaCl gradient in buffer D. The peak activity fractions eluted in the range of 225-325 mM NaCl.

The active MAP kinase activator fractions were combined, diluted to 1:3 with buffer D, and loaded at 1.5 ml/min onto a heparin-agarose column (2.5 cm x 3 cm). The column was washed with 50 ml of buffer D. The column was developed with a linear 0-200 mM NaCl gradient in buffer D. Three millilitre fractions were collected and the MAP kinase activator was eluted in the range of 125-155 mM NaCl.

Figure 6: Purification of sea star MAP kinase activator. The sea star MAP kinase activator was purified using a combination of ion exchange chromatographic resins. The concentration of salt required to elute the kinase activity is shown for each column.



The fractions containing activator activity were dialyzed against buffer E (20 mM MES [pH 6.5] and all the components of buffer D except the MOPS) for 2.5 h at 4°C. The dialysate was applied at 0.8 ml/min onto a Mono S column equilibrated with buffer E. Using the same flow rate, the column was washed with 3 ml of buffer E before application of a 15 ml linear 0-500 mM NaCl gradient in buffer E. The 300 µl fractions that were assayed for MAP kinase activator activity revealed that the enzyme eluted at a concentration of ~190 mM NaCl.

### 3.5. Protein kinase assays

#### 3.5.1 Single-step seryl/threonyl protein kinase reaction

The MAP kinase phosphotransferase activity was measured using the exogenous substrate MBP or peptides patterned after the MBP Thr-97 phosphorylation site (Clark-Lewis *et al.*, 1991). Details of the filter paper assay have been published (Pelech *et al.*, 1988). Briefly, the enzyme assays were carried out in a reaction volume of 25 µl: 5 µl column fraction or crude sea star extract, 15 µl phosphorylation buffer F (1 mg/ml MBP or 2 mM MBP peptides, 20 mM MOPS [pH 7.2], 30 mM β-glycerophosphate, 20 mM MgCl<sub>2</sub>, 5 mM EGTA, 2 mM EDTA, 0.5 mM NaVO<sub>3</sub>, 1 mM DTT and 500 nM PKI). The reaction was initiated by addition of 5 µl of 50 µM [γ-<sup>32</sup>P]ATP (~2,000 cpm/pmol) and was incubated at 30°C for 5 min. The reaction was terminated by application of 20 µl of the reaction to a 2 cm<sup>2</sup> P81 phosphocellulose paper. Once the unincorporated [γ-<sup>32</sup>P]ATP was removed from the filter papers by repeated washing (10-20) using 1% phosphoric acid, they were transferred to 6 ml scintillation vials containing 200 µl of scintillation fluid and counted in a Wallac 1410 Liquid Scintillation Counter.

### 3.5.2 MAP kinase activator assay

Figure 7 schematically outlines the two-step MAP kinase kinase assay. The principal of the assay was to assess MAPKK activity indirectly by phosphorylating and activating the fusion protein GST-Erk1 immobilized on glutathione-agarose beads. In turn, the activated GST-Erk1 then phosphorylated MBP as a substrate. The kinase reactions were performed according to the protocol described by Charest *et al.* (1993). Each 70  $\mu$ l reaction consisted of 20  $\mu$ l packed GST-Erk1 glutathione beads (1-2  $\mu$ g) combined with 40  $\mu$ l of column fraction and 10  $\mu$ l of phosphorylation buffer G (20 mM MOPS [pH 7.2], 10 mM  $\text{MgCl}_2$ , 10 mM  $\text{MnCl}_2$ , 2 mM NaF, 5  $\mu$ M  $\beta$ -methyl aspartic acid, 25 mM  $\beta$ -glycerolphosphate, 1 mM DTT, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml SBTI, 10  $\mu$ g/ml leupeptin and 50  $\mu$ M ATP). The kinase reactions commenced upon placing them at 30°C. At the completion of the 20 min incubation period, the supernatant was removed and the beads washed several times with chilled phosphorylation buffer G containing 0.1% Triton X-100 and 150 mM NaCl. The reaction was continued by addition of phosphorylation buffer H (20 mM MOPS [pH 7.2], 10 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , 2 mM NaF, 1 mM  $\text{NaVO}_3$   $\beta$ -methyl aspartic acid, 25 mM  $\beta$ -glycerolphosphate, 1 mg/ml MBP) and 50 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP (~2,000 cpm/pmol) in a final volume of 50  $\mu$ l. The incubation proceeded for 20 min at 30°C and was terminated by spotting 25  $\mu$ l of the reaction mixture onto a P81 phosphocellulose paper and analysed as described for the MAP kinase assay.

Figure 7: MAP kinase activator assay. The bacterially expressed protein erk1



linked to glutathione S-Sepharose beads through its glutathione-S-transferase

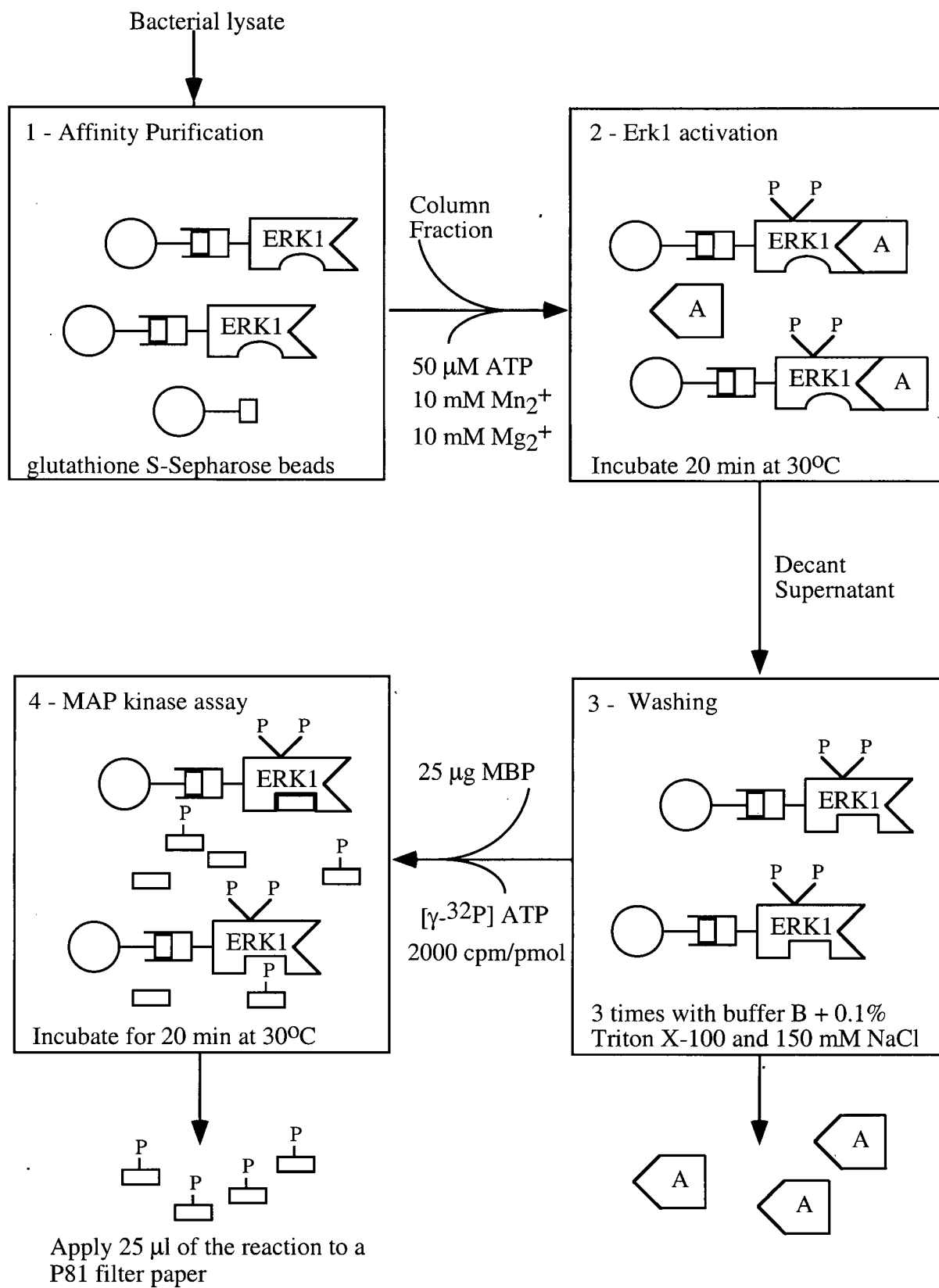
fusion protein —was used to assay for MAP kinase activator activity. The bound acti-

vator caused an increase in phosphorylation and consequently activation of Erk1 kinase

The activated Erk1 was then combined with its

*in vitro* substrate MBP and  $[\gamma^{32}\text{-P}]$  ATP . The phosphorylated MBP was

spotted onto a P81 chromatography paper and counted.



### 3.5.3 Autophosphorylation

#### 3.5.3.1 Recombinant proteins

Autophosphorylation experiments were performed with recombinant GST-fusion proteins linked to glutathione-agarose beads or thrombin-cleaved GST-fusion proteins (0.5-2.0  $\mu$ g), 50 mM [ $\gamma$ - $^{32}$ P]ATP (9,000 cpm/pmol) and phosphorylation buffer I (25 mM sodium  $\beta$ -glycerolphosphate, 20 mM MOPS [pH 7.2], 10 mM  $\text{MgCl}_2$  and/or 10 mM  $\text{MnCl}_2$ , 2 mM NaF, 1 mM DTT, 1 mM  $\text{NaVO}_3$  10  $\mu$ g of aprotinin per ml, 10  $\mu$ g of soybean trypsin inhibitor (SBTI) per ml and 5  $\mu$ g of leupeptin per ml) in a final reaction volume of 40  $\mu$ l for 30 min. The reaction was terminated by addition of 30  $\mu$ l of 5X concentration sodium dodecyl sulfate (SDS) sample buffer J (125 mM Tris-HCl [pH 6.8], 4% SDS, 0.01% bromophenol blue, 10 mM  $\beta$ -mercaptoethanol, and 20% glycerol) and boiling for 5 min (Laemmli, 1970).

#### 3.5.3.2 Purified sea star activator

Sea star MAPKK was tested for autophosphorylating activity by incubation of the most purified preparation of the activator (1-2  $\mu$ g of protein) with phosphorylation buffer H without  $\text{MnCl}_2$ . The 40  $\mu$ l reaction continued for 20 min at 30°C before addition of sample buffer E.



### 3.6 Protein phosphatase assays

#### 3.6.1 Protein tyrosyl phosphatase assays

Wild type GST-Erk1 protein (100  $\mu$ g) linked to glutathione-agarose beads was equilibrated by washing several times in dephosphorylation buffer K (25 mM Tris-HCl [pH 7.0] and 10 mM DTT) prior to being diluted 1:1 (vol/vol) in the same buffer containing 100 units of CD45 protein tyrosine phosphatase (see Table 3). The reaction was incubated for 60 min at 30°C. A time course of inactivation was performed by removing 40  $\mu$ l of the bead slurry and combining it with 1 mM of the protein tyrosine phosphatase inhibitor sodium orthovanadate. The beads were washed with buffer G and half of the material was used to assay MBP phosphotransferase activity (see Section 3.5.1) while the remainder of the beads were used for Western blotting analysis using the anti-phosphotyrosine antibody 4G10 (see Section 3.9.3).

#### 3.6.2 Protein seryl/threonyl phosphatase assays

The GST-Erk1 beads were equilibrated with dephosphorylation buffer L (20 mM MOPS [pH 7.2], 1 mM DTT, 1 mM EDTA and 0.1 mg/ml) before being resuspended in the same buffer containing ~4 U of human protein phosphatase 2A (1 U releases 1 nmol of phosphate/min from 15  $\mu$ M phosphorylase at 30°C). The time-course of inactivation proceeded for 60 min at 30°C. During this period 40  $\mu$ l of slurry was removed from the reaction at 10 min intervals and combined with 1  $\mu$ M okadaic acid to inhibit the phosphatase reaction. The beads from each measurement in the time-course were washed with buffer I before being assessed for MBP kinase activity (see Section 3.5.1) or immunoreactivity with 4G10 antibody (see Section 3.9.3).

Table 5: Polyclonal antibodies

Antibody	Peptide Antigen Location	Subdomain Region	Amino Acid Sequence	Reference
MAP Kinase R1	63-98 Rat	III	PFEHQTYCQRTLREIQIL LGFRHENVIGIRDILRAP	Boulton et al., 1990
MAP Kinase R2	337-367 Rat	CT	PFTFDMELDDLPKERLK ELIFQETARQFPGAPEAP	Boulton et al., 1990
MAP Kinase R3	8-32 Rat	NT	GGGGGEPRRTEGVGPG VPGEVEMVK	Boulton et al., 1990
MAP Kinase <i>mpk-I</i>	unknown Sea star	complete protein	N.A.	Sanghera et al., 1991
MAP Kinase GEGA	unknown Sea star	I	GLAYIGEGAYGMV	Posada et al., 1991
MAPKK-XI	335-356 <i>Xenopus</i>	XI	EFQDFVNKCLVKNPAER ADLKQ	Kosako et al., 1993
MEK-CT	360-378 Rat	CT	HSFIKQSELEEVDFAW LC	Kosako et al., 1993
STE7-VIII	434-446 <i>S. cerevisiae</i>	VIII	FVGTSTYMSPERIC	Teague et al., 1986
MEKK-CT	667-684 Rat	CT	CQDRPPSRELLKHPVFR	Lange-Carter et al., 1993
STE11-II	464-474 <i>S. cerevisiae</i>	II	HTGELMAVKQVC	Rhodes et al., 1990

### 3.7 Antibody production

Kinetic Pharmaceuticals Inc. provided many of the anti-peptide antibodies used during the course of this study including  $\alpha$ -MAPK,  $\alpha$ -MAPKK  $\alpha$ -Mekk and  $\alpha$ -Raf (Table 5). The short polypeptides used to generate the antibodies were synthesized by Dr. Ian Clark-Lewis' research group at the Biomedical Research Centre (University of British Columbia), Darryl Hardie at the University of Victoria or supplied by Upstate Biotechnology Incorporated (Lake Placid).

#### 3.7.1 Antigen preparation

Each synthetic peptide was conjugated to the carrier protein KLH (keyhole limpet hemocyanin) through their C- or N-terminal cysteine residues using the conjugating agent SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate). This usually created a peptide concentration of 5.3 mg/ml. Peptide dimerization through cysteine residues was reduced by first dissolving the lyophilized powder in PBS containing 4 M guanidine hydrochloride pH 7.5 and 5 mM DTT (see protocol described in Appendix 1 and 2). After removing the excess DTT by centrifuging through a Sephadex G10 spin-column at 1,000 rpm (600 x g) for 3 min, the carrier/peptide reaction mixture was allowed to couple with gentle stirring overnight. Peptides crosslinked to KLH are then stored at -70°C. The initial rabbit immunization involved injecting equal volumes of complete Freund's adjuvant with 250  $\mu$ g/ $\mu$ l of antigen diluted in PBS. However, incomplete Freund's adjuvant was used for subsequent injections of decreasing peptide antigen concentrations of 200  $\mu$ g/ $\mu$ l and 100  $\mu$ g/ $\mu$ l respectively. All rabbit immunizations were performed by trained animal technicians at the University of British Columbia's South Campus Animal Care Facility.

### 3.7.2 Antibody purification and quantitation

Appendix 3 provides a detailed description of the antibody purification procedure. Approximately 30 ml of blood was collected from the rabbit's ear and was stored at room temperature to allow partial clotting. The clot was then removed by centrifugation at 2,500 rpm ( $\sim 1300 \times g$ ) for 5 min using a Du Pont Sorvall RT 6000D bench-top centrifuge. The serum was centrifuged a second time to remove any remaining erythrocytes. Large impurities were removed by sequentially filtering the serum through a  $0.45 \mu\text{m}$  filter followed by a  $0.22 \mu\text{m}$  filter. The serum was added to 1-2 ml of thiol-linked Sepharose peptide affinity beads. The complex formation between peptide and antibody proceeded overnight at  $4^\circ\text{C}$  with continuous mixing using a vertical rotating wheel. After the incubation period was complete, an affinity column was prepared from the bead/serum mixture. The column was washed with one bed volume of PBS and subsequently eluted with 5 ml of 100 mM glycine [pH 2.5]. The 1 ml eluates were immediately neutralized with saturated Tris-base. The antibody titre was determined by the enzyme-linked immunosorbent assay (ELISA) using a Bio-Tek Instruments EL 312e Bio-Kinetics Reader.

### 3.7.3 Other antibody sources

All the monoclonal and several of the polyclonal antibodies used during the course of this study were generous gifts from other research laboratories or were purchased from commercial sources.

### 3.8 One dimensional gel electrophoresis

The analytical electrophoresis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) utilizes the discontinuous buffer system. The sample and stacking gel contain Tris-HCl [pH 6.8], upper and lower reservoirs contain Tris-glycine [pH 8.3], and resolving gel contains Tris-HCl [pH 8.8] described by Ornstein (1964) and Davis (1964) in combination with denaturing conditions (strongly anionic detergent SDS, reducing agent  $\beta$ -mercaptoethanol and heat) devised by Laemmli (1970). A Hoeffer gel electrophoresis system was used to cast 1.5 mm thick separating gels (0.25 M Tris-HCl [pH 8.8], 0.1% SDS). By varying the acrylamide concentration (9-12%) (see Appendix 5 for details) it was possible to analyze medium (40 kDa) or large (80 kDa) molecular mass proteins. A 15 well 4 % acrylamide stacking gel (0.25 M Tris-HCl [pH 6.8], 0.1% SDS) was used to load the samples prepared from 80  $\mu$ l of eluted column fraction or 20  $\mu$ l GST-fusion proteins linked to glutathione-agarose beads after they were boiled in 5X SDS sample buffer J for 5 min. The proteins were electrophoresed for 16 h at 10 mA in Tris-glycine electrophoresis buffer K (0.25 mM Tris-base, 250 mM glycine [pH 8.3], and 0.1% SDS). For rapid protein analysis, the BioRad mini-gel system was used routinely (summarized in Appendix 5). A maximum sample volume of 80  $\mu$ l was electrophoresed for 1.5 h at 400 V and 150 mA.

### 3.9 Protein visualization

#### 3.9.1 Coomassie blue staining

Coomassie blue staining was used primarily for detecting and quantitating the expression levels of recombinant GST-fusion proteins purified from *E. coli*. At the

completion of the electrophoresis, the polypeptides separated in the SDS-polyacrylamide resolving gel were simultaneously fixed with methanol and acetic acid and stained with Coomassie blue (methanol:glacial acetic acid:water [45:10:45 by volume] and 0.25% Coomassie Brilliant Blue R250 [wt./vol.]). The gels were immersed in 5 volumes of staining solution and placed on a rotating platform to assure complete saturation of the gel. Typically the immersion lasted for 10-20 min at room temperature. The protein bands were revealed by removing excess stain from the gel with destain buffer (10 % MeOH and 10 % glacial acetic acid [vol/vol]). The gel was preserved by drying it between two sheets of cellophane using a Bio Rad gel slab drier.

### 3.9.2 Silver staining

Silver staining technique involves the differential reduction of silver ions bound to the polypeptide amino acid side chains. This procedure is 100- to 1000-fold more sensitive than staining with Coomassie Brilliant Blue R250. Due to its detection capabilities (minimum capacity in the 0.1-1.0 ng range) the silver staining method was used for assessing the purity of the protein preparations. Appendix 6 outlines the protocol described by Merril *et al.* (1981). The proteins were separated through an SDS-polyacrylamide gel electrophoresis as described in Section 9 and sequentially fixed in methanol:acetic acid:water (40:10:50 by volume) then ethanol:acetic acid:water (10:5:85 by volume). To improve protein affinity for the silver reagent, the gels were placed in an oxidizing solution containing 3.4 mM  $K_2Cr_2O_7$  and 3.2 mM nitric acid prior to being incubated with a silver nitrate solution (0.2 %  $AgNO_3$  [wt/vol]). The fixed protein bands were revealed by reducing the bound  $AgNO_3$  in a developer solution (2.5% sodium carbonate [wt/vol] and 0.017% formaldehyde [vol/vol]). After the desired contrast was obtained, the reaction was quenched with a 5% acetic acid solution. The gel was preserved by drying as outlined in Section 10, i.

### 3.9.3 Western immunodetection

Western blotting is a very sensitive technique for detecting a specific protein that is present at low concentration in a crude sample of polypeptides. Proteins separated electrophoretically are transferred from the polyacrylamide gel to a solid support and probed with an antibody specific for antigenic sequences displayed within the polypeptide. Generally, Western analysis was performed routinely on recombinant GST-Erk1 and GST-Mek1 expressed in *E. coli*; MAP kinase activator and MBP/HH1 kinase purified from sea star oocytes. Appendix 7 summarizes the procedure used for protein gel transfer and Western immunoblotting. Briefly, the electrophoresed proteins were electroeluted onto nitrocellulose for 3 h at 400 mA in transfer buffer L (120 mM glycine, 20 mM Tris-base [pH 8.6], and 20% methanol by volume) using an LKB 2005 Transphor Power Supply. For total protein detection the blot was immersed in Ponceau S stain for 5 min and rinsed several times with water to reduce the background. Immunoreactive protein bands were revealed by Western blotting with polyclonal anti-peptide or full-length protein antibodies. The membrane was blocked for 2 h at ambient temperature in TBST (50 mM Tris-base, 150 mM NaCl, and 0.2% Tween-20) with 5% dry milk powder. After the blocking step, the membrane was washed twice with TBST for 10 min and incubated overnight with a 1/256 000 titre rabbit primary polyclonal anti-peptide antibody diluted 1:500 in TBST. The membrane was washed extensively in TBST then incubated with goat anti-rabbit alkaline phosphatase conjugated secondary antibody diluted 1:2500 in the same buffer. Trace amounts of secondary antibody was removed with successive washes with TBST followed by a final wash with TBS to remove the detergent. The immunoreactive protein bands were revealed by placing the membrane in alkaline phosphatase development solution (100  $\mu$ M NaHCO<sub>3</sub> [pH 9.8], 1 mM MgCl<sub>2</sub>, 0.03% NBT and 0.015% BCIP). The development continued until the desired colour intensity was obtained after which the membrane was placed in deionized water.

The apparent molecular masses of protein from Western blotting and SDS-polyacrylamide gels were estimated by comparison with Bio Rad or Kinetek prestained protein standards: phosphorylase b 100-kDa; BSA 66-kDa; ovalbumin 49-kDa; GST (glutathione-S-transferase) 27-kDa; SBTI (soybean trypsin inhibitor) 21-kDa; and lysozyme 17-kDa.

### 3.10 Phosphoamino acid analysis

The  $^{32}\text{P}$  radiolabeled protein samples were electrophoresed on an SDS-polyacrylamide gel as outlined in Section 3.8. The electrophoresed proteins were transferred to Immobilon P membrane in buffer L for 4 h at 500 mA. The membrane was autoradiographed and the appropriate bands attached to the solid support were excised for phosphoamino acid analysis. The radioactive membrane was minced into manageable pieces and placed into a reacti-vial (Pierce) for digestion with 6 N constant boiling HCl. Longer incubations were avoided due to the instability of the phosphate moiety on the phosphoserine and phosphothreonine (Posada and Cooper, 1992). The acid-hydrolyzed sample was transferred to an Eppendorf tube and lyophilized under vacuum using a Labconco Centrivap Concentrator. After successive washing and drying steps to remove the HCl, the free  $^{32}\text{P}$ -labelled phosphoamino acids were resuspended in electrophoresis buffer containing pyridine-acetic acid-water (1:10:189 by volume) and 1 ug each of phosphoserine, phosphothreonine and phosphotyrosine standards. Samples spotted onto an thin layer chromatography (TLC) cellulose plate were electrophoresed with cooling at 1,000 V. Ninhydrin (0.2% in ethanol) was used to reveal the migration positions of the standards while autoradiography revealed the phosphoamino acid content of the radiolabelled protein. A detailed protocol is outlined in Appendix 8.



### 3.11 Two-dimensional phosphopeptide mapping

Electrophoresis of  $^{32}\text{P}$ -labelled proteins was performed as described in Section 3.8. After identifying the bands by autoradiography, the radiolabelled proteins were excised from dried SDS polyacrylamide gels. The gel slice was reswollen in methanol:acetic acid:water (10:10:80 by volume) and washed in methanol:water (50:50 by volume) before being dessicated in a Labconco Centrивap Concentrator. The protein was digested in a 50 mM ammonium bicarbonate buffer [pH 8.0] containing 100  $\mu\text{g}$  of TPCK-treated (tolysulfonyl phenylalanyl chloromethyl ketone-treated) trypsin. Following the digestion period, the supernatant was vacuum dessicated. The tryptic peptides were washed several times with decreasing volumes of water interspersed with drying in the vacuum concentrator and finally resuspended in first dimension eletrophoresis buffer (see below) containing 0.5% phenol red before spotting on a TLC cellulose plate. First dimension electrophoresis was executed in acetic acid:pyridine:water (10:1:89 by volume) at 750 V and second dimension chromatography was performed in a solution containing 1-butanol:acetic acid:water:pyridine (10:3:12:15 by volume). The TLC cellulose plate was air dried before autoradiography. The 2-D phosphopeptide mapping protocol is detailed in Appendix 9.

## 4. MOLECULAR BIOLOGY TECHNIQUES

All molecular biology enzymes were available from commercial sources (Table 6). The Erk1 and Mek1 oligonucleotide primers used throughout this study were synthesized on a Applied Biosystems DNA Synthesizer model 300A.

Table 6: Sources of DNA and RNA modifying enzymes

AmpliTaq DNA polymerase	Perkin-Elmer Cetus
Phosphatase, alkaline	Boehringer Mannheim
DNA polymerase I large fragment (Klenow)	New England Biolabs
Polynucleotide kinase	Boehringer Mannheim
Superscript reverse transcriptase	BRL
Ribonuclease A	Pharmacia
RNasin	Promega
T4 DNA ligase	New England Biolabs
T7 DNA polymerase	Pharmacia
T7 sequencing kit	Pharmacia
Vent DNA polymerase	New England Biolabs

#### 4.1 RNA Isolation

Total cellular RNA was isolated according to the protocol described by Chomczynski and Sacchi (1987). The guanidinium thiocyanate-phenol-chloroform extraction is a rapid method for isolating undegraded RNA in high yield by eliminating the need for ultracentrifugation. All aqueous solution were treated with diethyl pyrocarbonate and autoclaved. All glassware, polypropylene microcentrifuge tubes and pipette tips, etc. were autoclaved. Individually wrapped sterile disposable plasticware was utilized whenever possible.

For RNA isolation, A431 cells were grown in monolayers to a confluency of  $10^8$ /per 150 mm plate. After washing the cells with ice-cold 1X PBS (lacking magnesium and calcium) they were removed from the cell culture plate by mechanical disruption with a rubber policeman and collected in a Falcon 2059 polypropylene tube by centrifugation at 3,000 rpm ( $\sim 800 \times g$ ) for 5 min at 4°C. The supernatant was aspirated before addition of 1 ml of guanidinium thiocyanate RNA extraction buffer M per 140-mm plate. The RNA was extracted from the cell homogenate by adding the following reagents sequentially with thorough mixing by inversion between each addition: 100  $\mu$ l of 2 M sodium acetate, 1.0 ml water-saturated phenol and 200  $\mu$ l of chloroform:isoamyl alcohol (49:1 by volume). The liquid suspension was shaken vigorously for 10 sec before storage at 4°C for 15 min. The aqueous layer containing the RNA was separated from the organic layer by centrifuging at 15,000 rpm ( $10,000 \times g$ ). After transferring the aqueous to a fresh tube it was combined with one volume of isopropanol and stored at -20°C for a minimum of 1 h. The precipitated RNA was sedimented at 15,000 rpm ( $10,000 \times g$ ) for 20 min. The pellet was then resuspended in a 300  $\mu$ l volume of buffer M, transferred to a microfuge tube and reprecipitated with 1 volume of isopropanol at -20°C for at least 1 h. The RNA suspension was sedimented at 15,000 rpm ( $10,000 \times g$ ) for

10 min at 4°C. The pellet was washed with 75% ethanol, vacuum dessicated and dissolved in 10 mM Tris-Cl [pH 7.4], 5 mM EDTA and 0.1 % SDS. The RNA preparation was stored by adding 0.1 volume of 3 M sodium acetate [pH 5.2] and 2.2 volumes of ice-cold absolute ethanol. A detailed protocol is outlined in Appendix 10.

#### 4.2 PCR amplification of a partial human Erk1 cDNA

A single stranded cDNA template was synthesized from A431 cell RNA. In a final volume of 20 µl, approximately 2.5 µg of total cellular RNA were combined with 1 mM nucleotide triphosphates, 20 units RNasin, and 100 pmol six base oligonucleotide random primers in PCR buffer N (50 mM KCl; 10 mM Tris-Cl, [pH 8.3] at 20°C; 1.5 mM Mg<sub>2</sub>Cl; 0.1% gelatin). The reaction was initiated by addition of 200 U of Superscript reverse transcriptase (10 min at 23°C; 45 min at 42°C; and 1 min 95°C). The specific coding and complementary oligonucleotides (sense strand of subdomain II, GTG GCT/C ATC AAG AAG ATC AGC CCC TTC GAG CAT; antisense strand of subdomain VII, CTC AGG GCT AGC AAT CCG GGC AAG GCC G/AAA G/ATC; and antisense strand of subdomain IX, GCA GCC CAC AGA CCA GAT GTC A/GAT GGA T/CTT GGT GTA) were synthesized based on the sequence published for rodent Erk1 (Boulton *et al.*, 1991) and modified for human codon preference described by Lathe (1985). The DNA/RNA hybrid was separated by heating the reverse transcriptase reaction at 95°C for 5 min before diluting the mixture with 100 µl of 1X PCR buffer N supplemented with 100 pmol of each paired coding and complementary oligonucleotides. The DNA was amplified using 10 U of ampliTaQ DNA and an automated programmable Perkin-Elmer Cetus thermal cycler. The cDNA was amplified using a two-step approach: the first three cycles occurred at low primer-annealing temperatures to provide more efficient priming (95°C for 30 sec, 37°C for 60 sec and 72°C for 120 sec) followed by 35 cycles at higher primer-annealing temperatures to allow increased specificity (95°C for 30 sec,

55°C for 60 sec and 72°C for 120 sec). Synthesis of complete blunt-end termini was ensured by subjecting the PCR mixture to a final elongation cycle (72°C for 5 min). An aliquot of the amplified DNA sample was analyzed by horizontal agarose gel electrophoresis and visualized by ethidium bromide staining (Appendix 11).

Bands displaying the appropriate size were excised and purified using a Sephaglas Bandprep™ Kit as described by Pharmacia's specifications. The purified fragment was then blunt-end ligated into the *Eco* RV restriction site of Stratagene Bluescript™ vector (Appendix 12). The ligation reaction (Appendix 13) was allowed to proceed for 24 h at 16°C before transforming the cDNA-vector into the competent *E. coli* strain XL.1 Blue (Appendix 14). Recombinant clones were selected using an  $\alpha$ -complementation system. In this method, the deletion mutant *E. coli* host strain constitutively expresses the 3' distal portion of the  $\beta$ -galactosidase (*lacZ*) gene while the plasmid vector expresses the 5' proximal portion of the same protein from an inducible operator region. Complementation occurs when the isopropylthio- $\beta$ -D-galactoside (IPTG) inducible plasmid-encoded fragment associates with the host-encoded fragment to form an active *lacZ* enzyme complex that is detectable in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal). As a consequence the  $lac^+$  bacteria form blue colonies. In the case of recombinant bacteria, a fragment of foreign DNA inserts into the multiple cloning site which is located within the *lacZ* gene of the plasmid vector. Inevitably, bacteria carrying plasmids with amino-terminal fragment insertions, results in the disruption of the *lacZ* enzyme, are usually unable to complement and as a result form white colonies. Positive clones were grown in 3 ml of 2 x YT bacterial growth medium (Appendix 15) which was ideal for promoting the amplification of the high copy number Bluescript™ plasmid. A small sample of the plasmid (2-4  $\mu$ g DNA) was isolated by the boiling lysis procedure adapted from Holmes and Quigley (1981) (Appendix 16) and the DNA prepared for sequencing using the Pharmacia LKB Deaza

<sup>T7</sup> Sequencing Mixes<sup>TM</sup>. The cDNAs templates were sequenced by the enzymatic dideoxy-mediated chain-termination method developed by Sanger *et al.* (1977) and electrophoresed on 6% vertical buffer-gradient polyacrylamide gel (Appendix 17).

#### 4.3 Cloning and sequencing a full-length human erk1 cDNA

The 400 base pair partial erk1 cDNA cloned by PCR was used to probe a human Hep G2 cDNA  $\lambda$  Zap library commercially available from Stragene Cloning Systems (Short *et al.*, 1988). The technique was modified from the work of Benton and Davis (1977).

##### 4.3.1 Preparation of plating bacteria

A single bacterial colony of XL.1 Blue strain of *E. coli* was inoculated into 50 ml of LB medium supplemented with 0.2% maltose in a sterile 250-ml Erlenmeyer flask. The culture was placed in a rotary shaker (220 cycles/min) preheated to 37°C. The addition of sugar to the medium improved the efficiency of  $\lambda$  bacteriophage infection, since expression of the  $\lambda$  receptor gene *lamB* is controlled by the maltose operon. The bacteria were sedimented at 7000 rpm (~4,000 g) for 10 min at room temperature. The supernatant was discarded and the cell pellet resuspended in 20 ml of sterile 0.01 MgSO<sub>4</sub> solution to an OD<sub>600</sub>=2 or  $\sim 1.6 \times 10^9$  cells/ml. The cells could be stored for up to 3 weeks at 4°C.

##### 4.3.2 *in situ* hybridization of bacteriophage $\lambda$ plaques

Since first being introduced in the late seventies (Sim *et al.*, 1979), *in situ* hybridization has been used widely in molecular biology to detect, isolate and purify

DNAs from complex cDNA and genomic DNA libraries consisting of hundreds of thousands recombinant bacteriophage.

#### 4.3.2.1 Plating

The titre of the Strategene human Hep G2 cDNA  $\lambda$  Zap library was determined by plating 1:10 serial dilutions onto plating bacteria (*E. coli* strain XL 1 Blue). Infection was performed by combining 200  $\mu$ l of plating bacteria ( $\sim 1.6 \times 10^9$  cells/ml) with 1  $\mu$ l of each of the bacteriophage serial dilutions. The infection was allowed to proceed at 37°C for 20 min. During this period hardened NZY top agarose (Appendix 15) was liquified by heating the bottle in a microwave. The hot liquified top agarose was cooled to 55°C in a warm water bath. At the completion of the infection period, 4-5 ml of molten NZY top agarose were added and the mixture was immediately poured and spread onto pre-heated NZY plates (the NZY plates were warmed to 37°C for at least 2 h with their lids left open slightly to allow the water to evaporate). After the plates were sufficiently dried, they were incubated overnight in a 37°C bacterial incubator.

#### 4.3.2.2 Immobilization of bacteriophage $\lambda$

The plates were incubated until the plaques reached a suitable size and density (the plaques usually attained a diameter of 1.5-2.0 mm before almost coming in contact with one another). The plates were then placed at 4°C for at least 1 h to allow the top agarose to harden.

The plates were numbered with a ethanol-resistant felt pen. Two nitrocellulose filters/Hybond<sup>+</sup> nylon membranes were numbered identically to their corresponding plates using a soft-lead pencil. In addition the filters/membranes were further identified

by using the letters 'A or B'. The filter/membrane carrying the same number was carefully placed on the surface of the top agarose. The first filter/membrane (A) was marked asymmetrically in at least three different locations by puncturing through the filter into the underlying agar with a 20G 1 1/2 gauge needle which was immersed in black India ink. The filter/membrane was removed after 30-60 sec with a pair of blunt-end forceps. A replica was created by placing a second filter marked B onto the agarose gel. To assure sufficient transfer of bacteriophage, the filter remained in contact with the plate for 1-2 min. Note that the excess ink left from the previous puncture also transferred to the filter/membrane.

#### 4.3.2.3 Fixation of bacteriophage DNA to nitrocellulose

The phage were lysed and the target bacteriophage DNA was denatured *in situ* by placing the filter/membrane plaque side up onto 2-3 Whatmann 3MM papers saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30-60 sec. The filter/membrane were then transferred to a 3MM paper saturated with neutralizing solution (0.5 M Tris-Cl [pH 7.0], 1.5 M NaCl) for 1-2 min. The filter/membrane were then placed on dry 3MM to dry at room temperature for 30-60 min. The DNA was fixed to nitrocellulose filters by baking for 30 min at 80°C or to Hybond<sup>+</sup> nylon membranes by cross-linking for 30 sec with U.V. light.

#### 4.3.2.4 Hybridization of immobilized $\lambda$ DNA with a <sup>32</sup>P-labeled probe.

The filter/membrane was rehydrated in prewashing solution (5X SSC see Appendix 18, 0.5% SDS and 1 mM EDTA [pH 8.0]) for 30 min with agitation using a rotating platform in a water bath maintained at 42°C. The solution was changed two times. The solution was decanted and the filters/membrane were washed with three



changes of 2X SSC for 15 min. The filters/membranes were transferred to heat-sealable bags containing a sufficient volume of prehybridization buffer [(50% formamide, 5X Denhardt's reagent (6X SSC, 0.5% SDS and 100 µg/ml denatured, fragmented salmon sperm DNA (see Appendix 18)] to cover their surface (230 µl per square centimeter of nitrocellulose filter or nylon membrane). The bag was sealed in a manner that removed any bubbles. The bag was submerged in water bath with a rotary platform for 1-2 h at 42°C. A <sup>32</sup>P radiolabeled probe was prepared with a specific activity equal to 10<sup>9</sup> cpm/ug of DNA (Appendix 19) and denatured by heating for 5 min at 100°C. The hybridization was performed by addition of 1-2 ng denatured DNA/ml radiolabeled probe to the heat-sealable bag. The bag was submerged in a 42°C rotary water bath. At the completion of the incubation period the filters were transferred to a plastic Tupperware container and excess radiolabel removed by washing 5 x 100 ml with 5X SSC at room temperature. The washing conditions were made increasingly more stringent by placing the filters in 2X SSC and raising the incubation temperatures in incremental stages (37°C, 42°C, 47°C and 52°C). The filters/membranes were dried on paper towel and immobilized, using adhesive tape, onto a piece of 3MM paper cut to the size of a film cassette. Using an <sup>35</sup>S radioactive ink pen, several asymmetric symbols were inscribed on the sheet of 3MM paper which facilitated the alignment of the X-ray film with the positive spots located on the filters/membranes. The filters/membranes were autoradiographed overnight. Autoradiographs from the two separate plate lifts were aligned and plugs from the potential double-positive Erk1 clones were excised and stored in 500 µl of SM buffer and 20 µl of chloroform. Secondary and tertiary screens were performed as described above to eliminate false double-positives and isolate a single pure λ bacteriophage recombinant plaque. The positive recombinant bacteriophage contains plasmid sequences that can be excised *in vivo* and converted to the Bluescript SK (M13-) plasmid vector (Short *et al.*, 1988). The putative positive Erk1 clones were rescued by the method described by Stratagene and sequenced using plasmid-based T3 and T7

bacteriophage-specific oligonucleotide primers (Appendix 17). After confirming that the inserts were *bona fide* Erk1 clones, the complete cDNA sequence was obtained in a step-wise fashion by using custom-designed oligonucleotide primers (Table 7) based on previous sequence results and walking along the cDNA in both directions.

#### 4.4 Sub-cloning human Erk1 into the pGEX-2T vector

Expression of Erk1 as a GST-fusion protein required that the cDNA be ligated into the polyclonal site situated downstream of the glutathione-S-transferase gene in the pGEX-2T vector (Figure 12). Toward this end, forward and reverse oligonucleotide primers were synthesized based on the 5' and 3' coding region of the original Erk1 26a $\beta$ -3 clone (Table 7). Several additional bases were included at the 5' ends of each of the primers. First, an additional two bases were placed immediately upstream of the ATG start codon of the Erk1 cDNA to assure that the Erk1 protein was in the correct reading frame. Second, *Eco* R1 restriction sites were placed 5' to the start and stop codons of the forward and reverse primers. And third, five additional bases were placed 5' to the *Eco* R1 restriction site to facilitate binding of the *Eco* R1 enzyme to the PCR amplified DNA fragment. The 26a $\beta$ -3 clone was linearized with *Eco* R1 (Appendix 12) before addition of 100-500 pg of it to the reaction cocktail containing specific coding and complementary oligonucleotides (Table 9) 1 mM nucleotide triphosphates, 20 units RNasin, PCR buffer J and 10 U of high-fidelity VentR<sup>®</sup> DNA polymerase in a final volume of 50  $\mu$ l. Twenty-five rounds of amplification (95°C for 30 sec, 55°C for 45 sec, and 72°C for 90 sec) were performed with a Perkin-Elmer Cetus thermo cycler. The DNA sample was electrophoresed on an horizontal agarose slab gel and visualized by staining with ethidium bromide (Appendix 11). The 1.2 kb. band was excised and purified using a Pharmacia Sephaglas Bandprep<sup>®</sup> Kit. The purified fragment was restriction digested with *Eco* R1 (Appendix 12) ligated in the pGEX-2T expression vector that was digested

Table 7: Erk1 sequencing oligonucleotide primers

Sequence location	Oligonucleotide orientation	Oligonucleotide DNA Sequence	Amino Acid Sequence
149-174	Reverse	GAG G TG GAG ATG GTG AAG	Glu-Val-Glu-Met-Val-Lys-
151-167	Forward	GAG GTG GAG ATG GTG AA	Glu-Val-Glu-Met-Val-Lys
214-231	Reverse	ATC GGC GAG GGC GCG TAC	Ile-Gly-Glu-Gly-Ala-Tyr
335-352	Forward	AGA TCC AGA TCC TGC TGC	Glu-Ile-Gln-Ile-Leu-Leu
443-459	Reverse	GTC CAG TCA GAG GTA GT	Leu-Met-Glu-Thr-Asp-Leu
483-500	Forward	GCT GAG CAA TGA CCA TAT	Ile-Ser-Asn-Asp-His-Ile
615-631	Forward	GAT TTG TGA TTT CGG CC	Ile-Cys-Asn-Phe-Gly
724-742	Reverse	ACT CCA AGG GCT ATA CCA	Gln-Ser-Lys-Gly-Tyr-Thr
724-742	Forward	ACT CCA AGG GCT ATA CCA	Gln-Ser-Lys-Gly-Tyr-Thr
916-934	Forward	CAG TCT CTG CCC TCC AAG	Gln-Ser-Leu-Pro-Ser-Lys
1086-1103	Reverse	TGA GCC AGT GGC CGA GGA	Glu-Pro-Val-Ala-Glu-Glu
1165-1182	Forward	TTC CAG GAG ACA GCA CGC	Phe- Gln-Glu-Thr-Ala-Arg
1205-1221	Reverse	CCC CCT AGC CCA GAC AG	Ala-Pro-stop-3'-untranslated
1396-1312	Forward	TTC TCC TCC CCA CCC GC	3'-untranslated region
1396-1313	Reverse	TCC TCC TCC CCA CCC GCC	3'-untranslated region
1476-1494	Reverse	CGC CCT TAC TCC CCC CAG	3'-untranslated region
1611-1629	Forward	CAT CTC ATT CAA ACC CCA	3'-untranslated region
1707-1725	Reverse	CCT GTC AAA GCT GTC ACT	3'-untranslated region

with the same enzyme (Appendix 13) and transformed into competent *E. coli* strain UT5600 (Appendix 14). A *Sma* I digest was performed to verify that the full-length Erk-1 cDNA was subcloned in the proper orientation.

#### 4.5 Chromosomal Assignment

The chromosomal localization of the human *erk1* gene was determined by probing nylon membrane panels of *Hind*III digested hamster-human hybrid cell line purchased from BIOS Corporation. The DNA blots were placed in a heat sealable plastic bag and prehybridized with pre-warmed BIOS Speed-Hyb Solution (6X SSC, 5X Denhardt's, 10% dextran sulfate, 1% SDS and 100 µg/ml denatured, fragmented salmon sperm DNA; Appendix 19) for 2 h in a rotator water bath set at 65°C. The partial human Erk1 cDNA clone A431-400-8 was radiolabeled by the random oligonucleotide primer method (Feinberg and Vogelstein 1983,1984) to a specific activity of  $10^8$ - $10^9$  cpm/ug DNA and added to the prehybridization solution. After hybridizing for 16 h at 65°C with agitation, the membranes were washed twice at low stringency (2X SSC, 0.5% SDS, room temperature) for 10 min, once at medium stringency (1X SSC, 1% SDS, 65°C) for 15 min and twice at high stringency (0.1% SSC, 1% SDS, 65°C) for 15 min. To prevent the blots from drying, they were wrapped in plastic before placing them in an imaging cassette for autoradiography.

#### 4.6. Primer Extension

The primer extension technique was used to measure the size of the 5' terminal region of the human Erk1 mRNA. A complementary oligonucleotide primer was patterned after a sequence located within 150 base pairs of the 5' terminus of the Erk1 26aβ-3 clone (Table 8). In this procedure the bacteriophage T4 polynucleotide kinase

was used to catalyze the 5' phosphorylation of the primer with [ $\gamma$ - $^{32}\text{P}$ ]ATP (Appendix 18). Once purified, 10  $\mu\text{l}$  of labelled primer (1 ng / $\mu\text{l}$ ) was combined with 20  $\mu\text{l}$  of total RNA from Hep G2 cells (50  $\mu\text{g}$ ), 6  $\mu\text{l}$  of 3 M sodium acetate and sufficient DEPC water to increase the volume to 50  $\mu\text{l}$  final. The nucleic acids were precipitated by addition of 2.5 volumes of 100% ethanol and followed by storage at  $-20^{\circ}\text{C}$  for 30 min. The RNA was recovered by centrifugation at 13,000 rpm ( $\sim 12,000\times g$ ) for 10 min in a microcentrifuge cooled to  $4^{\circ}\text{C}$ . The pellet was washed once with 70% ethanol, and recentrifuged. The supernatant was carefully removed by pipette and the residual ethanol allowed to evaporate from the pellet. Thirty microlitres of hybridization buffer O (40 mM PIPES [pH 6.4], 1 mM EDTA [pH 8.0], 0.4 NaCl and 80% formamide) were added to the precipitated nucleic acids and resuspended by repeatedly pipetting the liquid. To fully separate the nucleic acids, the hybridization mixture was heated to  $85^{\circ}\text{C}$  for 10 min. The hybridization reaction was immediately placed at the annealing temperature of  $30^{\circ}\text{C}$  for 16 h. The RNA/DNA hybrid was precipitated by addition of 170  $\mu\text{l}$  of 3 M sodium acetate, 500  $\mu\text{l}$  of 100% ethanol and storing at  $4^{\circ}\text{C}$  overnight. After centrifuging at  $4^{\circ}\text{C}$  for 15 min, the supernatant was removed and the pellet washed with 70% ethanol. The RNA/DNA hybrid was air dried before diluting the nucleic acids in 25  $\mu\text{l}$  of reverse transcriptase reaction buffer (2.5  $\mu\text{l}$  buffer N, 2  $\mu\text{l}$  100 mM each dNTPs, 2  $\mu\text{l}$  100 mM DTT, 1 $\mu\text{l}$  RNase inhibitor, 13  $\mu\text{l}$  deionized water). The reaction was initiated by addition of 2  $\mu\text{l}$  (400 U) of Superscript reverse transcriptase<sup>®</sup>. At the completion of a 2 hour incubation period at  $42^{\circ}\text{C}$ , the reaction was quenched by adding 1  $\mu\text{l}$  of 0.5 M EDTA. Ribonuclease A was added to the mixture to degrade the RNA template. The volume was increased with 150  $\mu\text{l}$  of TE buffer containing 150 mM NaCl prior to extracting the DNA with 200  $\mu\text{l}$  of phenol/chloroform solution. The upper aqueous phase was transferred to a fresh tube and the DNA precipitated with 500  $\mu\text{l}$  of 100 % ethanol for 1 h at  $0^{\circ}\text{C}$ . The DNA was recovered by centrifuging at  $4^{\circ}\text{C}$  for 15 min at 12,700 rpm ( $12,000\times g$ ), and washed with 70% ethanol. The pellet was air dried to remove any traces of residual

ethanol and then dissolved in 4  $\mu$ l of TE buffer and 6  $\mu$ l of Pharmacia loading buffer. The longest Erk1 cDNA clone, 26a $\beta$ -3, was also subjected to sequence analysis using the very same primer extension oligonucleotide primer. Both reactions were electrophoresed on DNA sequencing gels as outlined in Appendix 17. The difference in length observed between the primer extended fragment obtained from the RNA template to that of the Erk1 cDNA template was predicted to be the amount of 5' sequence missing from the 26a $\beta$ -3 clone.

#### 4.7 PCR Amplification of a murine Mek1 cDNAs

Total RNA from mouse liver was used to synthesize a single stranded cDNA template for PCR. One to two micrograms of total cellular RNA were combined with 20 units RNasin, 1 mM nucleotide triphosphates, 100 pmol mixture of six base random oligonucleotide primers and New England Biolabs PCR buffer N in a final reaction volume of twenty microliters. After addition of 200 U of Superscript reverse transcriptase, the mixture was subjected to annealing (10 min at 23°C), elongation (45 min at 42°C) and denaturation (3 min at 95°C) steps. Oligonucleotide primers specific for the coding and complementary DNA sequences (sense strand beginning with the ATG-initiation codon, ATG CCC AAG AAG AAG CCG ACG CCC ATC CAG CTG AAC; antisense strand beginning with the TGA stop codon, AAC CAG CCC AGC ACA CCA ACC CAC GCT GGC AGC ATC TGA) of the murine Mek1 gene product were synthesized from the published results of Crews *et al.* (1992). At the completion of the denaturation step, the reaction mixture was diluted with 75  $\mu$ l of 1X PCR buffer N containing 100 pmol of the forward and reverse oligonucleotide primers. Amplification of the Mek1 cDNA was performed by adding 10 U of high-fidelity Vent<sub>R</sub><sup>®</sup> DNA polymerase. The thermal cycler (Perkin-Elmer Cetus) was programmed for 30 rounds of amplification (95°C for 45 sec, 50°C for 90 sec and 74°C for 120 sec). The PCR

reactions were analyzed by electrophoresis using an 0.8% agarose slab gel. The bands were visualized by low-intensity U.V. light after staining the gel in ethidium bromide (Appendix 11).

The appropriate size bands were excised and purified using Pharmacia's Sephaglas Band Prep Kit<sup>TM</sup>. The purified DNA fragments were sequentially digested first with *Sma* I then *Eco* RI restriction endonucleases (Appendix 12) and ligated into the identical cloning sites of the pGEX-2T prokaryotic expression plasmid (Appendix 13). Recombinant clones were identified by restriction analysis of small-scale preparations of plasmid DNA (Appendix 16). The putative recombinant clones were sequenced by the Sanger DNA sequencing procedure (Appendix 17) using specific sequencing oligonucleotides (Table 8) to confirm the presence and fidelity of a Mek1 cDNA.

#### 4.8 Prokaryotic expression of recombinant GST-fusion proteins

The pGEX-2T vector, carrying a mutant of the *lacZ* inhibitor gene *lacI<sup>q</sup>* that overproduces *lac* promoter repressor protein, was used for the expression of GST-Erk1 and GST-Mek1 fusion proteins used during the course of this study (Müller-Hill *et al.*, 1968). The expression of recombinant proteins is more tightly controlled with *lacI<sup>q</sup>* thereby minimizing the spurious transcription of the *lac* operon and the consequent synthesis of potentially toxic foreign polypeptides. Erk1 and Mek1 fusions were expressed in the *E. coli* strain UT5600 generated by Elish *et al.* (1988). The unique feature of this strain for prokaryotic expression of recombinant proteins is that it has a mutation in the periplasmic protease. This reduced protein degradation during lysis and purification.

Table 8. Mek1 sequencing oligonucleotide primers

Sequence Location	Oligonucleotide Orientation	Oligonucleotide DNA Sequence	Amino Acid Sequence
197-214	Forward	TTT CTG ACG CAG AAG CAG	Phe-Leu-Thr-Gln-Lys-Gln
439-458	Reverse	TAC AGC GAC GGC GAG ATC	Tyr-Ser-Asp-Gly-Glu-Ile
575-592	Forward	TAT CTT CGG GAG AAG CAC	Tyr- Leu-Arg-Glu-Lys-His
663-682	Reverse	ATT TTG GGG TCA GCG GGC	Phe-Gly-Val-Ser-Gly-Gln
731-748	Forward	TCG CCT GAG AGA CTC CAG	Ser-Pro-Glu-Arg-Leu-Gln
879-898	Forward	GGA GAC GCA GCC GAA ACA	Gly-Asp-Ala-Ala-Glu-Trp



#### 4.8.1 Protein expression in bacterial culture

The purification of glutathione-S-transferase by glutathione-affinity chromatography was first described by Simons and Jagt (1977) and later adapted for the expression and purification of eukaryotic GST-fusion proteins in bacteria (Smith *et al.*, 1988). The protocol used for the isolation of milligram quantities of fusion protein was modified from Guan and Dixon (1991). Before performing a purification of the fusion protein, the recombinant Erk1 or Mek1 clone was transformed into competent UT5600 bacteria (Appendix 14) to insure they grow and express the highest levels of fusion protein. A volume of 2-50 ml of 2 x YT medium containing 100 µg/ml ampicillin was inoculated with a single colony of *E. coli* containing the recombinant pGEX plasmid. The culture was incubated at 37°C overnight in a Lab Line shaker/incubator with vigorous shaking (220-230 rpm). The culture was diluted 1:10 (vol/vol) in fresh 2 x YT medium supplemented with 100 µg/ml ampicillin. To insure proper aeration, the volume of the media did not exceed 20-25% of the capacity of the flask. The bacteria were grown at 37°C with shaking until the cell density reached an absorbance of 1-2 at OD<sub>600</sub>. The cells were then induced with 400 µM of IPTG for another 4-6 h.

#### 4.8.2 Preparation of bacterial cytoplasmic sonicates

After inducing bacterial expression of the GST-fusion proteins for the required amount of time, the culture was transferred to centrifuge bottles or tubes and the cells sedimented at 8,000 rpm (~11,300 x g) for 10 min at 4°C with a Beckman J2 HS centrifuge. The supernatant was discarded and the residual media drained from the pellet. The bottles/tubes were placed on ice. The pellet was resuspended by adding 50 µl of ice-cold buffer P (50 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], and 150 mM NaCl) per millilitre of culture. The peptidylglycan structure of the outer cell membrane

was disrupted by addition of 100 µg/ml of lysozyme. An increase in the viscosity of the liquid usually observed within 20 min was indicative of cell disruption. Triton X-100 was added to a final concentration of 1% to facilitate solubilization of the protein. The genomic and plasmid DNA was sheered by sonicating with a Vibra-cell sonicator for 10 sec bursts at a setting 45-60. Using a DuPont Sorvall® ODT Combi ultracentrifuge and its companion T865 rotor, the homogenate was clarified at 30,000 rpm (~81,900 x g) for 15 min at 4°C and the supernatant transferred to a fresh container. A battery of broad spectrum protease inhibitors (10 µg/ml each of aprotinin, soybean trypsin inhibitor and leupeptin see Table 2 for details) was added to the cell extract. At this point, the GST-fusion was immediately purified or the homogenate was stored at -70°C until needed.

#### 4.8.3 Purification of the fusion proteins

The glutathione-agarose beads were prepared by swelling them in buffer P containing 0.1% Triton X-100 and 1% bovine serum albumin. Prior to adding the beads to the bacterial homogenate, the beads were washed three times with buffer P. The supernatant was incubated with a volume of beads appropriate for the volume of cytoplasmic sonicate (1-2 ml bed volume per 50 ml of sonicate) for 30 min at 4°C on a rotating platform. The GST-fusion bound to the matrix was recovered by centrifugation at 1000 rpm (~200g) for 30-60 sec using a Sorvall RT600D centrifuge. After discarding the supernatant, the beads were washed with 10 bed volumes of buffer P. The beads were again recovered by centrifugation. The washing and centrifugation steps were repeated two more times. The protein concentration was determined by electrophoresing 10-20 µl of beads on a Bio-Rad SDS-PAGE mini-gel apparatus (Appendix 5) followed by staining with Coomassie blue (Section 10, ii). Small volumes of the beads were aliquotted into Eppendorf tubes and stored at -70°C.

#### 4.8.4 Elution of glutathione bound GST-fusion proteins

The GST-fusion protein bound to the glutathione matrix was washed once with 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3). Glutathione elution buffer (25 mM reduced glutathione in 1X PBS pH 7.0) was added to the equilibrated beads and the mixture incubated with rotation at 4°C for 1-2 h and sometimes overnight. The eluted GST-fusion protein was recovered by gentle centrifugation at 1,000 rpm (~200 x g) for 30-60 sec using a DuPont Sorvall RT 6000 bench-top centrifuge. The protein concentration was measured by the Bradford (1976) assay using bovine serum albumin as a protein standard (Section 3). Before performing any kinase reactions, the free-glutathione was removed by dialysis against 1X PBS with Spectrapor<sup>®</sup> membrane tubing (M.W. cutoff 12,000-14,000) for 1 h at 37°C. Aliquots (100-250 µl volume) were stored at -70°C.

#### 4.8.5 Thrombin cleavage of glutathione bound GST-fusion proteins

In preparation for thrombin cleavage, the GST-fusion protein matrix was first washed with 10 bed volumes of 1X PBS. The reaction was performed by addition of 500 µl of thrombin solution (1 cleavage unit per microlitre of 1X PBS) to an equivalent bed volume of bead matrix. The incubation was allowed to continue for 2-16 h at room temperature with rotation. At the completion of the reaction, the bead matrix was centrifuged at 1,000 rpm (~200 x g) for 2 min at 4°C with a Hereaus Biofuge 15. Protease inhibitors (10 µg/ml each leupeptin and SBTI see Table 2) were added to inhibit thrombin activity. Before storing the cleaved protein at -70°C, the concentration was determined by SDS-PAGE mini-gel electrophoresis (Appendix 5).

#### 4.9 Oligonucleotide-mediated mutagenesis

The *in vitro* site-directed mutagenesis technique is a powerful tool for analyzing the structural and functional properties of protein kinases for which the cDNA is available for study. Short oligonucleotide primers are synthesized with base substitutions, deletions or additions for one or more specific amino acid change(s) in the wild type protein. Using the original cDNA as a template, paired mutant and wild type primers were subjected to several rounds of amplification by the polymerase chain reaction. After purification and restriction digestion, the wild type portion of the cDNA was substituted with the corresponding mutagenized fragment. Sequence analysis of the subcloned region confirms that only the desired mutations are generated by the PCR procedure. Two different approaches described below were used to generate the mutant cDNA fragments.

##### 4.9.1 Mutagenesis by the megaprimer method

The two-step PCR megaprimer approach requires that the mutagenized fragment be synthesized in two separate PCR reactions (Sarkar and Sommer, 1990). In the first PCR reaction step, the mutant oligonucleotide primer in combination with a second template-based wild type primer are used to amplify a partial (300-500 base pair) mutagenized cDNA fragment. The purified mutagenized cDNA fragment is then paired with a second template-based wild type primer to synthesize a full-length or nearly full-length mutant fragment by PCR. A detailed protocol for *in vitro* mutagenesis by two-step PCR is outlined below. The sequence of the oligonucleotide primers is indicated in Table 9.

The template was prepared by digesting 1-2  $\mu$ g of recombinant pGEX-2T vector containing the Erk1 or Mek1 cDNA insert with the appropriate restriction enzyme

Table 9: Erk1 and Mek1 mutant oligonucleotide primers

A. Erk1 mutant oligonucleotide primers

Amino Acid Location	Amino Acid Change	Oligonucleotide Orientation	Oligonucleotide DNA Sequence and base change(s)	Amino Acid Sequence
Wild type	None	Forward	ATG GCG GCG GCG GCG GCT CAG GGG	Met-Ala-Ala-Ala-Ala-Ala-Gln-Gly
Wild type	None	Reverse	UUC CAG CCC GGA GAG CTG GAG GCC CCC	Phe-Gln-Pro-Gly-Val-Leu-Glu-Ala-Pro
71	K→A	Forward	ACT CGC GTG GCC ATC <b>GCG</b> AAG ATC AGC CCC TTC	Thr-Arg-Val-Ala-Ile- <b>Ala</b> -Lys-Ile-Ser-Pro-Phe
201*	Wild Type	Reverse	GAC CAC ACC GGC TTC CTG	Asp-His-Thr-Gly-Phe-Leu
202*	T→S	Forward	<b>TCT</b> GAG TAT GTG GCT ACG	<b>Ser</b> -Glu-Tyr-Val-Ala-Thr
202	T→E	Forward	CAC ACC GCC TTC CTG <b>GAG</b> GAG TAT GTG GCT ACG	His-Thr-Gly-Phe-Leu <b>Glu</b> -Glu-Tyr-Val-Ala-Thr
203*	Wild Type	Reverse	GCC TTC CTG ACG GAG	Gly-Phe-Leu-Thr-Glu
204*	Y→T	Forward	<b>ACT</b> GTG GCT ACG CGC TGG	<b>Thr</b> -Val-Ala-Thr-Arg
204	Y→E	Forward	GGC TTC CTG ACG GAG <b>GAG</b> GTG GCT ACG CGC TGG	Gly-Phe-Leu-Thr-Glu- <b>Glu</b> -Val-Thr-Arg-Trp
202*	T→Y	Reverse	CAC ACC GGC TTC CTG <b>TAT</b>	His-Thr-Gly-Phe-Leu- <b>Tyr</b>
204*	Y→T	Forward	GAG <b>ACT</b> GTG GCT ACG CGC	Glu- <b>Thr</b> -Val-Ala-Thr-Arg

202/204	T→E Y→E	Reverse	GGC TTC CTG <u>GAG</u> GAG <u>GAG</u> GTG GCT ACG CGC TGG	Gly-Phe-Leu- <u>Glu</u> -Glu- <u>Glu</u> -Val-Ala-Thr-Arg-Trp
201/202*	L→E T→E	Reverse	CAC ACC GGC TTC <u>GAG</u> <u>GAG</u>	His-Thr-Gly-Phe- <u>Glu</u> - <u>Glu</u>
204/205*	Y→E V→E	Forward	GAG <u>GAG</u> <u>GAG</u> GCT ACG CGC TGG	Glu-Glu-Glu-Ala-Thr- Arg-Trp

#### B. Mek mutant oligonucleotide primers

Wild type	None	Forward	ATG CCC AAG AAG AAG CCG ACG CC	Met-Pro-Lys-Lys-Lys-Pro- Thr-Pro
Wild type	None	Reverse	ACA CCA ACC CAC GCT GCC AGC ATC TGA	Thr-Pro-Thr-His-Ala-Ala- Ser-Ile-stop
97	K→A	Reverse	CTG GTT ATG GCT AGA <u>GCG</u> CTG ATC CAC CTG GAG	Leu-Val-Mat-Ala-Arg- <u>Ala</u> -Ile-His-Leu-Glu
218	S→E	Reverse	AGC GGG CAG CTA ATT GAC <u>GAG</u> ATG GCC AAC TCC TTC GTG	Ser-Gly-Gln-Leu-Ile-Asp- <u>Glu</u> -Met-Ala-Asn-Ser- Phe-Val
222	S→E	Forward	ATT GAC TCT ATG GCC AAC <u>GAG</u> TTC GTG GGC ACG AGA TCC	Ile-Asp-Ser-Met-Ala-Gln- <u>Glu</u> -Phe-Val-Gly-Thr- Arg-Ser

Note: those letters that are underlined and bolded indicate the base alteration and the corresponding amino acid substitution.

(Appendix 12). Opening the plasmid enabled the oligonucleotide primers to have better access to their template. After heat-inactivating the enzyme at 90°C, the PCR cocktail was prepared in a volume of 50 µl by mixing 250-500 ng of the digested recombinant vector with 2mM nucleotide triphosphates, 1X PCR buffer N, 100 pmol of each paired mutant and wild type primers (Table 9) and 10 U of high-fidelity VentR<sup>®</sup> DNA polymerase. The reaction mixture was subjected to thirty cycles of amplification (95°C for 45 sec, 55°C for 90 sec, and 74°C for 90 sec) using a Perkin-Elmer Cetus thermal cycler. The cDNA fragment was purified from an ethidium bromide stained horizontal agarose slab gel (Appendix 11) by a novel glass matrix system designed by Pharmacia. The second PCR amplification was executed by combining 200 µg of the purified mutant cDNA fragment with a second template-based primer using the same condition as described above except for the change in reaction conditions (95°C for 30 sec, 55°C for 30 sec and 74°C for 60 sec). The mutant cDNA fragment was gel purified (Appendix 11), restriction digested with the appropriate enzymes (Appendix 12), and ligated back into the wild type background (Appendix 13). The mutation(s) were confirmed by DNA sequencing (Appendix 17).

#### 4.9.2 Mutagenesis by the double-primer method

Several Erk1 and Mek1 mutants were generated by the single-step site-directed mutagenesis method. In this procedure, two contiguous (non-overlapping) coding and complementary mutant oligonucleotide primers are synthesized with a phosphate group located at their 3' terminus to facilitate PCR fragment ligation. A second pair of coding and complementary wild type oligonucleotide primers based on the 5' and 3' termini of the full-length cDNA are synthesized with vector specific restriction endonuclease sites for cloning purposes. Individual fragments of the mutant cDNA are created by performing two separate PCR reactions using paired combinations of 5' wild type coding and 3'

mutant complementary primers or 5' mutant coding and 3' wild type complementary primers. The complete Erk1 and Mek1 clones were reconstituted by digesting the plasmid vector and fragments with the specific restriction endonucleases (Appendix 12) and performing a three-way ligation (Appendix 13).



## RESULTS

### 1. ISOLATION OF A HUMAN ERK1 cDNA FROM HEP G2 CELLS

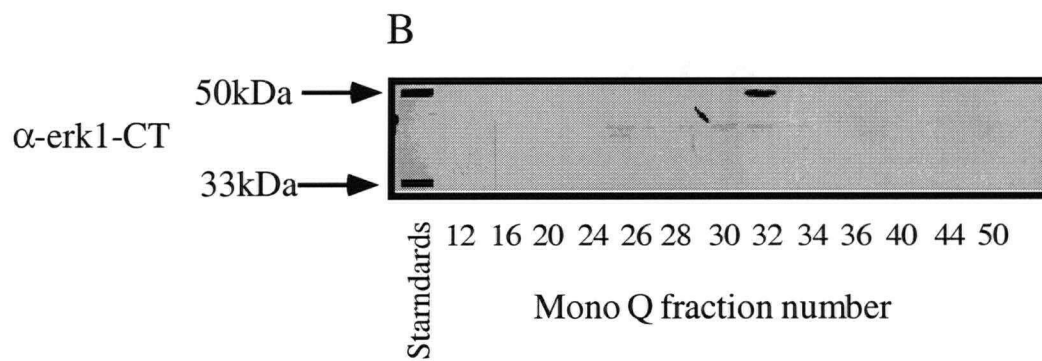
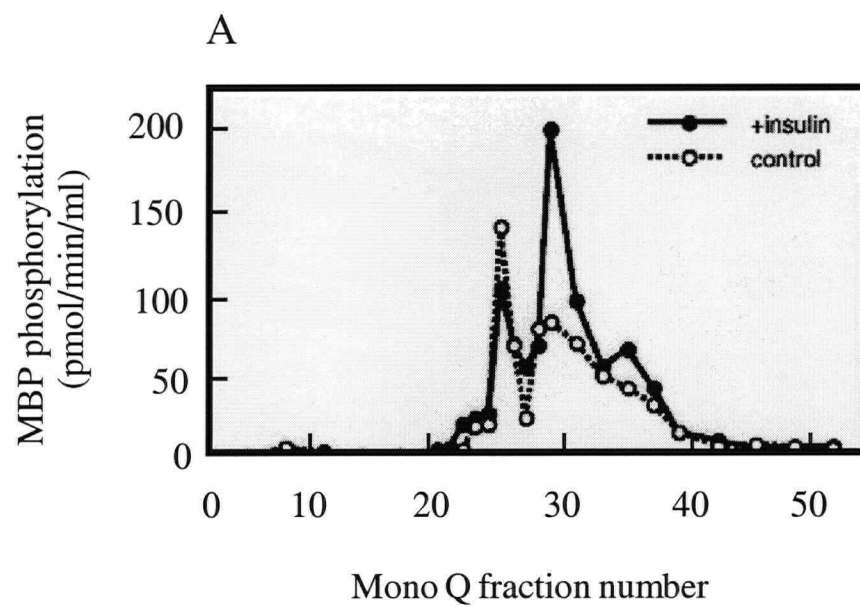
#### 1.1 Amplification of a partial cDNA encoding a human MAP kinase

Erk1 was originally purified as a 43-kDa protein from insulin-stimulated Rat 1 (HIRc B) cells that were transfected with the human insulin receptor (Boulton *et al.*, 1991). Sanghara *et al.* (1992) identified two proteins of equivalent molecular mass, 42-kDa (Erk2) and 44-kDa (Erk1), from human epidermoid A431 cell line that were immunoreactive with several MAP kinase antibodies. The only MAP kinase complementary DNA (cDNA) sequence that had been described in the literature was a partial Erk1 clone obtained from rat (Boulton *et al.*, 1990). Therefore, using the partial rat Erk1 sequence data, a strategy was devised to isolate the full-length human Erk1 homologue. The reverse transcriptase polymerase chain reaction (RT-PCR) technique and specific oligonucleotides were used to amplify a partial cDNA fragment from total A431 cellular RNA. Design of the primers was based on two criteria: (i) the sites for oligonucleotide design were chosen from within the kinase catalytic subdomains domain (i.e., II, VII and IX); and (ii) the codon selection of the base pairs adhered to the usage for *homo sapiens* (Hanks *et al.*, 1988; Lathe, 1985). Figure 9 shows the location of the single 5' primer (subdomain II) and the two 3' primers (subdomains VII and IX) used for amplification. RT-PCR performed with paired forward and reverse primers yielded two partial cDNA fragments of 400 (A431-400-3) and 500 (A431-500-8) base pairs in size which fell within the range expected for a product of equivalent distance in rat cDNA (Boulton *et al.*, 1990). Only two amino acid differences were observed between the predicted translational sequence of the PCR fragments and the published rat Erk1 sequence (data not shown).

## 1.2 Detection of a MAP kinase in human Hep G2 cells

At the time this work was initiated, MAP kinase was demonstrated to be activated in a very small number of cell types (Pelech et al., 1990). Therefore, to successfully screen a cDNA library for a full-length MAP kinase, it was essential to determine which cell line expressed the enzyme. The exogenous substrate MBP was used to assay for the presence of MAP kinase(s) in the transformed human hepatocellular carcinoma line Hep G2. The cells were grown to confluency ( $\sim 10^8$  cells) before fasting overnight in medium containing 0.5% calf serum. The quiescent ( $G_0$ ) cells were treated without or with 0.1 mM insulin for 5 min. Approximately 1.5 mg of the clarified control- and insulin-treated homogenates were applied to a Mono Q anion exchange column at a flow rate of 1 ml/min. In this system, MAP kinases Mpk1 from mature sea star oocytes and Erk2 from *Xenopus* oocytes eluted as broad peaks between 330 to 470 mM NaCl (Sanghera et al., 1990; Posada et al., 1991). After elution of the bound proteins with a linear 0-0.8 M NaCl gradient, odd numbered fractions were assayed for MAP enzyme activity in a 5 min MBP kinase assay. As can be seen in Figure 8A, a rather sharp peak of MBP kinase activity eluted between 395 to 450 mM NaCl (fractions 27 through 31, closed circles). This marked a two-fold increase in kinase activity when compared to untreated Hep G2 cells over the same fractionation range (open circles). The elution profile of the MBP kinase activity was similar to the behavior of MAP kinases that had been described in other studies (Sanghera et al., 1990; Posada et al., 1991). Because MBP may be a substrate for other kinases a small volume of each assayed fraction was analyzed by Western immunoblotting. The MBP kinase activity peak coincided with a band that was immunoreactive with rat Erk1-CT anti-peptide antibody (Figure 8B). Therefore, both enzymatic and immunological data support a role for MAP kinase activation following insulin treatment in Hep G2 cells. Hence, a cDNA library of this cell line would be useful for screening human Erk1.

Figure 8: Detection of MAP kinase activity in Hep G2 cells. (A) The hepatocarcinoma cell line Hep G2 was stimulated for 10 min with 100  $\mu$ M insulin (filled circles) or without insulin (open circles), lysed and 1.5 mg of the homogenate chromatographed on a Mono Q column as described in the Material and Methods. MAP kinase activity was assessed in a 25  $\mu$ l reaction by incubating 5  $\mu$ l of extract with  $\text{Mg}^{2+}$ •[ $\gamma$ - $^{32}\text{P}$ ]ATP (2000 pmol/cpm) and 1 mg/ml final of MBP substrate. After 5 min the reaction was terminated by applying 20 ml of the reaction onto a 2 cm X 2 cm Whatman filter paper. Activity is expressed in pmol/min/ml of the original column fraction. (B) The insulin-treated fractions from panel A were electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with Erk1-CT, a MAP kinase polyclonal antibody specific for the carboxy-terminal of the rat Erk1 protein (Boulton et al., 1991).



### 1.3 Cloning of a full-length cDNA encoding the human Erk1 protein

The partial 500-bp A431-500-8 clone isolated by RT-PCR was labelled with  $^{32}\text{P}$  and used to probe a  $\lambda$ ZAP bacteriophage cDNA library prepared from Hep G2 cell poly(A)<sup>+</sup> enriched mRNA that were cloned into the *Eco* R1 cloning site. Three sets of ten large plates containing a minimum of 10,000 plaques were 'lifted' twice with two sheets Hybond-N hybridization membranes. After incubating the membranes overnight at room temperature in the presence of the  $^{32}\text{P}$ -labelled partial human Erk1 probe, the first low-stringency screen (incremental washes at room temperature, 40°, 45°, 50° and 60° C using a solution of 0.1% SDS and 2 X SSC) of ~300,000 plaques identified 26 potential MAP kinase recombinants. However, following more stringent conditions during the secondary (incremental washes at room temperature, 40° and 45° using a solution of 0.1% SDS and 2 X SSC) and tertiary screens (62.5°C using a solution of 0.1% SDS and 0.1 X SSC) only 4 double-positive clones were enriched following these plaque purification steps. Three of the clones were rescued as Bluescript plasmids from the Lambda ZAP<sup>®</sup> II vector using the R408 interference resistant Helper phage according to Stratagenes specifications. The cDNA inserts were then characterized by *Eco* R1 restriction mapping. All three clones appeared to be identical, since they were isolated as single fragments ranging in size from 1.2-1.9 kbs after electrophoresis on an agarose gel. This was confirmed by limited sequence analysis from the Bluescript plamid T3 and T7 sequencing primers. One of the clones encoded a partial Erk1 sequence, while a second contained two poly(A)<sup>+</sup>-tails and some partial Erk1 coding sequence. The largest clone designated 26a $\beta$ -3 was sequenced completely since the 1.9 kb fragment was similar in size to the partial rat Erk1 sequence (Boulton *et al.*, 1990).

The complete sequence of 26a $\beta$ -3 clone yielded a cDNA insert that was 1,850 bp in length excluding the poly(A)<sup>+</sup> region. Figure 9 shows the complete cDNA and

Figure 9: Nucleotide and predicted amino acid sequence of human Erk1 cDNA from Hep G2 cells. The sequence is derived from the full-length cDNA clone identified using a partial Erk1 PCR probe as outlined in Materials and Methods. The putative methionine translational initiation sites are indicated by the numbers 1 and 2 whereas the RNA polyadenylation signal (AUAAA) is italicized and underlined. The regulatory phosphorylation sites are designated with an asterisk. The catalytic subdomains (Roman numerals) and their conserved sequences (bolded and underlined) adhere to the nomenclature of Hanks et al. (1988). The horizontal arrows situated above the protein sequence correspond to the location of the oligonucleotides used for PCR amplification (Boulton et al., 1991). The vertical arrows located above the protein sequence correspond to potential thrombin cleavage sites.

CGTTCCTCGGCGCCGCGGGGCCCCAGAGGGCAGCGGCAGCAACAGCAGCAGCAGCAGCAGCGGGAGTGGAGATGGCCGGCGGCGCGCT  
15 30 45 60 75 90

Q G G G G G E P R R T E G V G P G V P G E V E M V K G Q P F  
105 120 135 150 165 180

D V G P R Y T Q L Q Y I G E A Y G M V S S A Y D H V R K T  
195 210 225 240 255 270

R V A I K K I S P F E H Q T Y C Q R T L R E I Q I L L R F R  
285 300 315 330 345 360

H E N V I G I R D I L R A S T L E A M R D V Y I V Q D L M E  
375 390 405 420 435 450

T D L Y K L L K S Q Q L S N D H I C Y F L Y Q I L R G L K Y  
465 480 495 510 525 540

I H S A N V L H R D L K P S N L L S N T T C D L K I C D F G  
555 570 585 600 615 630

L A R I A D P E H D H T G F L T E Y V A T R W Y R A P E I M  
645 660 675 690 705 720

L N S K G Y T K S I D I W S V G C I L A E M L S N R P I F P  
735 750 765 780 795 810

G K H Y L D Q L N H I L G I L G S P S Q E D L N C I I N M K  
825 840 855 870 885 900

A R N Y L Q S L P S K K T K V A W A K L F P K S D S K A L D L  
915 930 945 960 975 990

L D R M L T F N P N K R I T V E E A L A H P Y L E Q Y Y D P  
1005 1020 1035 1050 1065 1080

T D E P V A E E P F T F A M E L D D L P K E R L K E L I F Q  
1095 1110 1125 1140 1155 1170

E T A R F Q P G V L E A P STOP  
1185 1200 1215 1230 1245 1260

GCCCTCTCCGCGCAGACTGTAGAAAAATGGACTGTGCCAGCCCGGACCTTGGCAGCCAGGCGGGGTGGAGCATGGGCCTGGCCA  
1275 1290 1305 1320 1335 1350

CCTCTCTCCTTTGTGTGAGGCCTCCAGCTTCAGGCAGGCCAAGGCCTTCTCTCCCCACCCGCCCTCCCCACGGGGCCTCGGGAGCTCAGG  
1365 1380 1395 1410 1425 1440

TGGCCCCAGTTCAATCTCCCGCTGTCTGCTGTCTGCGCCCTTACCTTCCCCAGCGTCCCAGTCTCTGGCAGTTCTGGAATGGAAGGTT  
1455 1470 1485 1500 1515 1530

CTGGCTGCCCCAAGCTGTGAAGGCAGAGGTGGAGGGTGGGGCGCTGAGTAGGGACTCAGGGCCATGCCTGCCCCCTCATCTCATT  
1545 1560 1575 1590 1605 1620

CAAACCCACCCCTAGTTTCCCTGAAGGAACATTCCTTAGTCTCAAGGGCTAGCATCCCTGAGGAGCCAGGCCGGCCGAATCCCCCTCCCT  
1635 1650 1665 1680 1695 1710

GTCAAAGCTGTCACTTCGCGTGCCTCGCTGCTTCTGTGTGTGGTGAGCAGAAGTGGAGCTGGGGGCGTGAGAGCCCGCGCCCTGTC  
1725 1740 1755 1770 1785 1800

CACCTCCCTGACCGTCTAATATATAATAATAGAGATGTGTCTATGGCTGAAAAAATAAAAAA  
1815 1830 1845 1860 1866

predicted amino acid sequence for human Erk1. A consensus polyadenylation signal (AUAAA) was located upstream of the poly(A)<sup>+</sup> tail. A single translational termination codon UAG was located at nucleotide 1212 of the cDNA. Two potential AUG translational initiation codons, that satisfy the minimum consensus sequence first defined by Kozak (1987), were identified at nucleotides 73 and 160, respectively (Figure 9 and 10A). Furthermore, the sequence surrounding both in-frame methionine start sites (GUGGAGAAUGG) is identical and thus both may serve to initiate translation.

The human Erk1, like the previously reported rat homologue, was published as a partial cDNA that lacked the initiating methionine (Boulton *et al.*, 1991; Owaki *et al.*, 1992; Gonzalez *et al.*, 1992). Furthermore, no stop signal was identified 5' to either of the putative methionine start sites in the human Erk1 cDNA isolated from Hep G2 cells (Figure 10A). The GC-rich nature of Erk1 5' prime sequence may have hampered the first-strand cDNA synthesis and consequently more sequence may perhaps exist. To verify that the 26a $\beta$ -3 clone contains the complete Erk1 sequence, the size of the 5' region preceding the first methionine was analyzed by primer extension. A 26-base reverse primer patterned after a sequence (nucleotides 148 to 172) located 147 base pairs from the 5' terminus. A complementary 185 base single-stranded cDNA fragment was synthesized by this method (Figure 10B). The primer extended fragment generated from the mRNA template was approximately 13 bases longer than what was predicted from the Erk1 cDNA clone. No heterogeneity was observed in the primer extended products.

If we assume that translation initiation begins at the first methionine (corresponding to nucleotide 73), then the full-length Erk1 cDNA clone is expected to encode a 379-amino acid polypeptide with a predicted molecular mass of 43-kDa (Figure 9). The highly conserved invariant residues (bold type) that are found to be essential for catalytic function in all protein kinases are also conserved within the catalytic domain of

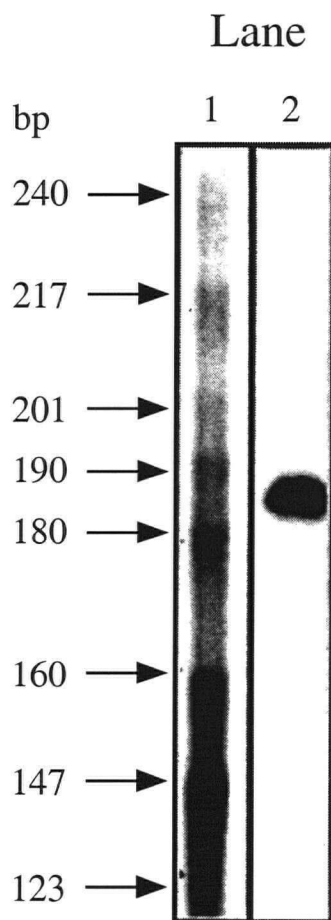


Figure 10: Primer extension analysis of the 5' terminal region of the human Erk1 mRNA. (A) Nucleotide and predicted amino acid sequence of the 5' region of human Erk1 clone 26a $\beta$ -3. The putative methionine initiation sites are numbered 1 and 2. (B) A complementary oligonucleotide primer was designed after a 5' sequence (amino acids 27-32) of the human Erk1 cDNA clone 26a $\beta$ -3. The primer was phosphorylated with [ $\gamma$ - $^{32}$ P]ATP at its 5' terminus using T4 polynucleotide kinase. After elongation with reverse transcriptase, the RNA/DNA hybrid was treated with ribonuclease A followed by separation on a DNA sequencing gel (lane 2). A 1 kb DNA standard from Gibco BRL was end-labelled with [ $\gamma$ - $^{32}$ P]ATP and electrophoresed on the same gel (lane 1).

A.

R	S	S	A	P	P	D	P	N	R	A	A	A	A	T	A
CGT	TCC	TCG	GCG	CCG	CCG	GGG	CCC	CAG	AGG	GCA	GCG	GCA	GCA	ACA	GCA
①															
A	A	A	A	A	G	V	E	<b>M</b>	A	A	A	A	A	Q	G
GCA	GCA	GCA	GCA	GCG	GGA	GTG	GAG	ATG	GCG	GCG	GCG	GCG	GCT	CAG	GGG
②															
G	G	G	G	E	P	R	R	T	E	G	V	G	P	G	V
GGC	GGG	GGC	GGG	GAG	CCC	CGT	AGA	ACC	GAG	GGG	GTC	GGC	CCG	GGG	GTC
②															
P	G	E	V	E	<b>M</b>	V	K	G	P						
CCG	GGG	GAG	GTG	GAG	ATG	GTG	AAG	GGG	CAG						

B.



human Erk1 (Hanks *et al.*, 1988). Alignment of the full-length cDNA clones for rat and human Erk1 revealed a high degree of identity (97%) throughout the entire protein sequence (Marquardt and Stabel, 1992; Charest *et al.*, 1993). Rat Erk1 is longer than its human homologue by a single alanine amino acid in the region of overlap. This residue is part of a poly-alanyl stretch located immediately following the initiating methionyl amino acid. MAP kinase isoform Erk2 from rat and human also features a string of six alanyl residues in its amino-terminal sequence (Boulton *et al.*, 1991; Gonzalez *et al.*, 1992). Interestingly, this poly-alanyl rich region of Erk1 is followed by a repeat of five glycyl residues (Figure 10).

The minimal catalytic unit of a functional protein kinase has been defined as a 28-kDa polypeptide chain comprising approximately 350 amino acid residues. This has been determined by truncation experiments and sequence alignment analysis of dozens of known enzymes (Hanks *et al.*, 1988 and references therein). In figure 11, protein sequence alignments of the catalytic domains (corresponding to amino acids 39 to 338 in PKA as outlined by Hanks *et al.*, 1988) were created for six human MAP kinase isoforms. Comparisons revealed that Erk1 is more closely related to Erk2 (90%) than to any other MAP kinase family member. Erk5 (53%) and Hog1 (49%) are the next MAP kinases most closely related to Erk1 while Erk3 (42%) and Jnk1 (41%) display the least homology. At first glance, the regions displaying the highest degree of amino acid conservation (subdomains I-II and VI-IX) are those regarded essential for enzyme catalysis. These residues are involved in binding ATP and participate in the phosphotransfer reaction in all kinases (Hanks *et al.*, 1988). The regulatory phosphorylation sites for Erk1, namely Thr-202 and Tyr-204, (equivalent to Thr-183 and Tyr-185 in Erk2) were mapped to an intermediate region between subdomains VII and VIII (Figure, 11), a location displaying little conservation among protein kinases (Hanks *et al.*, 1988). MAP kinase isoforms Erk5, Jnk1 and Hog1 possess comparable Thr and

Figure 11: Sequence comparison of several MAP kinase isoforms. Sequences from human Erk1 (Charest et al., 1993); Erk2 (Gonzalez et al., 1992); Erk3 (Gonzalez et al., 1992); Jnk1 (Dérjard et al., 1994); Hog $\alpha$  (Lee et al., 1994) and Erk5 (Zhou et al., 1995) were aligned using the BEST FIT program. To improve alignments spacing, dashed lines were introduced into the sequence. Residues that are identical or conserved in the MAP kinase isoforms are shown with an asterisk or a dot, respectively. Those residues in bold correspond to invariant or highly conserved residues and consequently define the eleven catalytic subdomains described for 62 of 65 unique kinase sequences by Hanks et al. (1988). Conserved amino acids are those residues with similar physical or structural properties: non-polar chain R groups (M, L, I, V, and C); neutral polarity R groups (A, G, S, T, and P); acidic and uncharged R groups (D, E, N, and Q); basic polar R groups (K, R, and H); and aromatic R groups (F, Y, and W).

ERK1	MA-----AAAAQGQGGGEPRTTEGVGPGVPEVEMVKGPFDV-----GPRYTQLQYI	48
ERK2	MA-----AAAAAGAGP-----EMVRGQVFDV-----GPRYTNLSTYI	31
ERK3	MA-----EKGDCIASVYG-----YDLGGRFVDFQPL	26
JNK1	MSRSKRDN-----FYSVEIGDSTFTVLKRYQNLKPI	32
HOG1	MSQERP-----TFYRQELNKTWEVPERYQNLSPV	30
ERK5	MAEPLKEEDGEDGSAEPAREGRTPHRCCLAK-----NLALLKARSFDTFDVGDEYEIETID	58
	<b>I</b> <b>II</b> <b>III</b> <b>IV</b>	
ERK1	GEGAYGMVSSAYDHRKTRVAIKKIS-PFEHQTYCQRTLREIKILLRFRHENVIGIRDILRAST-----LEAMRD	117
ERK2	GEGAYGMVCSAYDNVNKVRVAIKKIS-PFEHQTYCQRTLREIKILLRFRHENIIGINDIIRAPT-----IEQMKD	100
ERK3	GFGVNGLVLSAVDSRACRKVAVKKI--ALSDARSMKHALREIKIIRRLDHDNIVKVYEVLGPKGTDLQGELFKFSV	100
JNK1	GSGAQGIVCAAYDAILERNAIKKLSRPFQNTTHAKRAYRELVMKCVNHKNIIGLLNVFTPKS-----LEEFQD	103
HOG1	GSGAYGVSAAFDTKTGLRVAVKKLSRPFQSIHAKRTYRELRLKHKHENVIGLLDVFTPARS-----LEEFND	101
ERK5	GNGAYGVSSARRRLTGQQAIIKKIPNAFVDVTNAKRTLRELKILKHFHDNIIAIIKDILRPTVP-----YGEFKS	129
	* * * * *	
	<b>V</b> <b>VI</b> <b>VII</b>	
ERK1	VYIVQDLMETDLYKLLKS-QQLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLSNTTC-DLKICDFGLAR-I	190
ERK2	VYIVQDLMETDLYKLLKT-QHLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLNTTC-DLKICDFGLAR-V	173
ERK3	AYIVQEYMETDLARLLEQG-TLAEHAKLFMYQLLRGLKYIHSANVLHRDLKPSNIFISTEDLVLKIGDFGLARIV	174
JNK1	VYIVMELMDANLCQVIQ--MELDHERMSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVKSDC-TLKILEDFGLARTA	175
HOG1	VYLVTHLMGADLNNIVKC-QKLTDDHVQFLIYQILRGLKYIHSADIIHRDLKPSNLAVNEDC-ELKILDFGLARHT	174
ERK5	VYVLDLMESDLHQIIHSSQPLTLEHVRYFLYQLLRGLKYMHSQAQVIHRDLKPSNLLVNENC-ELKIGDFGMARGL	204
	* . . * . . *	
	<b>VIII</b> <b>IX</b> <b>X</b>	
ERK1	ADPEHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPKGHYLDQLNHILGILGSPSQ	266
ERK2	ADPDHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPKGHYLDQLNHILGILGSPSQ	249
ERK3	-DQHYSHKGYLSEGLVTKWYRSPRLLSNNYTKAIDMWAAGCILAEMLTGRMLFAGAHELEQMQLILET-----	243
JNK1	GTS-----FMTPYVVTYRYRAPEVILG-MGYKENVDLWSVGCIMGEMVCHKILFPGRDYIDQWNKVIEQLGTPCP	245
HOG1	DDE-----MTGYVATRWYRAPEIMLNMWHYNQTVDIWSVGCIMAELLTGRTLPDTHINQLQQIMRLTGTPPA	243
ERK5	CTSPAEHQYFMTEYVATRWYRAPEIMLSLHEYTQAI DLWSVGCIFGEMLARRQLFPKGNYVHQLQLIMMVLGTPSP	280
	. . . * . * . * . *	
	<b>XI</b>	
ERK1	EDLNCIINMKARNYLQSLPSKTKVAWAKLFP-KS-----DSKALDLLDRMLTFNPNKRITVEEALAHPY	329
ERK2	EDLNCIINLKARNYLLSLPHKNKVPWNRLFP-NA-----DSKALDLLDKMLTFNPNKRIEVEQALAHPY	312
ERK3	--IPVIREEDKDELLRVMPFSVSSSTWEVKRPLRKLLE-----VNSEIDFLEKILTFNPMDRLTAEMLQHPY	310
JNK1	EFMKKL-QPTVRTYVENRPKYAGYSFEKLFP-DVLFPPADSEHNKLLKASQARDLLSKMLVIDASKRISVDEALQHPY	319
HOG1	YLINRMPSEARNYIQLSTQMPKMNANFVFI-GA-----NPLAVDLLEKMLVLDSDKRITAAQALAHAY	306
ERK5	AVIQAVGAERVRAIYIQLSPPRQPVWETVYP-GA-----DRQALSLGRMLRFEPARSISAAAALRHPF	343
	* . . * . . *	
ERK1	LEQYYDPTDEPVAEPPF-TFAMELDDLPERLKLIFQETA-----RFQPGV-----	375
ERK2	LEQYYDPSDEPIAEAPF-KFDMELDDLPEKELKELIFEETA-----RFQPGY-----	358
ERK3	MSPYSCPEDEPTSQHPFRIED-EIDDIV-----LMAANQSQSLNWDTCSS--RYPVSL-----SSDLE--	365
JNK1	INVWYDPSEAEAPPKIPDKQLDEREHTIEEWKELIYKEVMDLEER-----	365
HOG1	FAQYHDPDDEPVAD-PY-DQSFESRDLLIDEWKSLEYDEVISF-----	347
ERK5	LAKYHDPDDEPDCAAPPF-DFAFDREALTRERIKEAIVAEIEDFHARREGIRQQIRFQPSLQPVASEPGCPDVEMPS	418
	* .	
ERK1	-----	
ERK2	-----	
ERK3	WRP-DRCQDASEVQRDPRGFGAL-AEDVQVD---PRKDSHSSSERFLEQSHSSMERAFEADYGRS--CDYKVGSPS	434
JNK1	-----TKNGVIRGQ-----	374
HOG1	-----	
ERK5	PWAPSGDCAMESPPPPAPPPCPGPAPDTIDLTLQPPPPVSEPAPPKDGAISDNTKAALKAALLKSLRSRLRDGPSA	494
	-----	
ERK1	-----	
ERK2	-----	
ERK3	YLD-----KLLWRD-----NKPHHYSEPK-----LILD-----LSHW	461
JNK1	-----	
HOG1	-----	
ERK5	PLEAPEPRKPVTAQERQREEREKRRRQERAKERERKERKERGAGASGGPSTDPLAGLVLSNDNRSLLERW	570

ERK1	-----	
ERK2	-----	
ERK3	KQ-----AAGAPPTATGLADTGAREDEPASLFLEIAQWVKSTQGAQSTPARPPTTPS-----	514
JNK1	-----PSPLAQV-----	381
HOG1	-----	
ERK5	TRMARPAAPALTSVPAPAPAPTPTPTPVQPTSPPPGPVAQPTGPPQPSAGSTSGPVPQPACPPPGPAPHPTGPPG	646
ERK1	-----	
ERK2	-----	
ERK3	-----AACLRPP-----PPGPGGRRRQ-----	532
JNK1	-----	
HOG1	-----VPPP-----	358
ERK5	PIFVPAPPQIATSTSLAAQSLVPPPGGLPGSSTPGVLPYFPPGLPPPDAGGAPQSSMSSESPDVNLVTQQLSKSQV	722
ERK1	-----	
ERK2	-----	
ERK3	-----PPVRPGR-----	539
JNK1	-----	
HOG1	-----LDQEEME-----	358
ERK5	EDPLPPVFSGTPKSGAGYGVGFLEEFLNQSFDMGVADGFPQDGQADSASLSASLLADWLEGHGMNPADIESLQR	798
ERK1	-----LEAP	379
ERK2	-----RS	360
ERK3	-VHLPRPEALHQARGPAGQ	557
JNK1	-----QQ	376
HOG1	-----S	359
ERK5	EIQMDSPMLLADLPDLQDP	817

Tyr phosphorylation motifs located in similar positions in the sequence. Interestingly, two distinguishing features emerge from the sequence comparison within the activation domain for MAP kinases. First, the intervening glutamyl residue in TEY of Erk1, Erk2 and Erk5 is replaced by glycyl and prolyl residues in Hog1 and Jnk1 respectively. Second, the number of amino acid separating subdomain VII and VIII vary from six, eight and twelve residues each for Hog1, Jnk1 and Erk1, respectively. Variations in the number and kinds of residues in this region may account for some of the specificity observed during activation of specific MAP kinase modules. Erk3 differs from other MAP kinases in that a seryl residue replaces the threonyl residue and the tyrosyl residue is completely absent. Erk3 and Erk5 have a unique structural feature that distinguishes them from other members of the MAP kinase family. Both kinases possess a large non-catalytic domain at their carboxy-terminal. It remains to be established whether this polypeptide extension plays a regulatory role similar to that observed with the amino-terminal of Raf1 and protein kinase C (PKC) enzymes. Both of these enzymes possess cystein-rich motifs. In the case of PKC, interaction of the cystein-rich fingers with membrane-associated lipid derivatives stimulates the enzymes phosphotransferase activity. The function of these same motifs in Raf1 activation may have a similar role in regulating Raf kinase activity..

## 2. CHARACTERIZATION OF RECOMBINANT HUMAN ERK1 PROTEIN

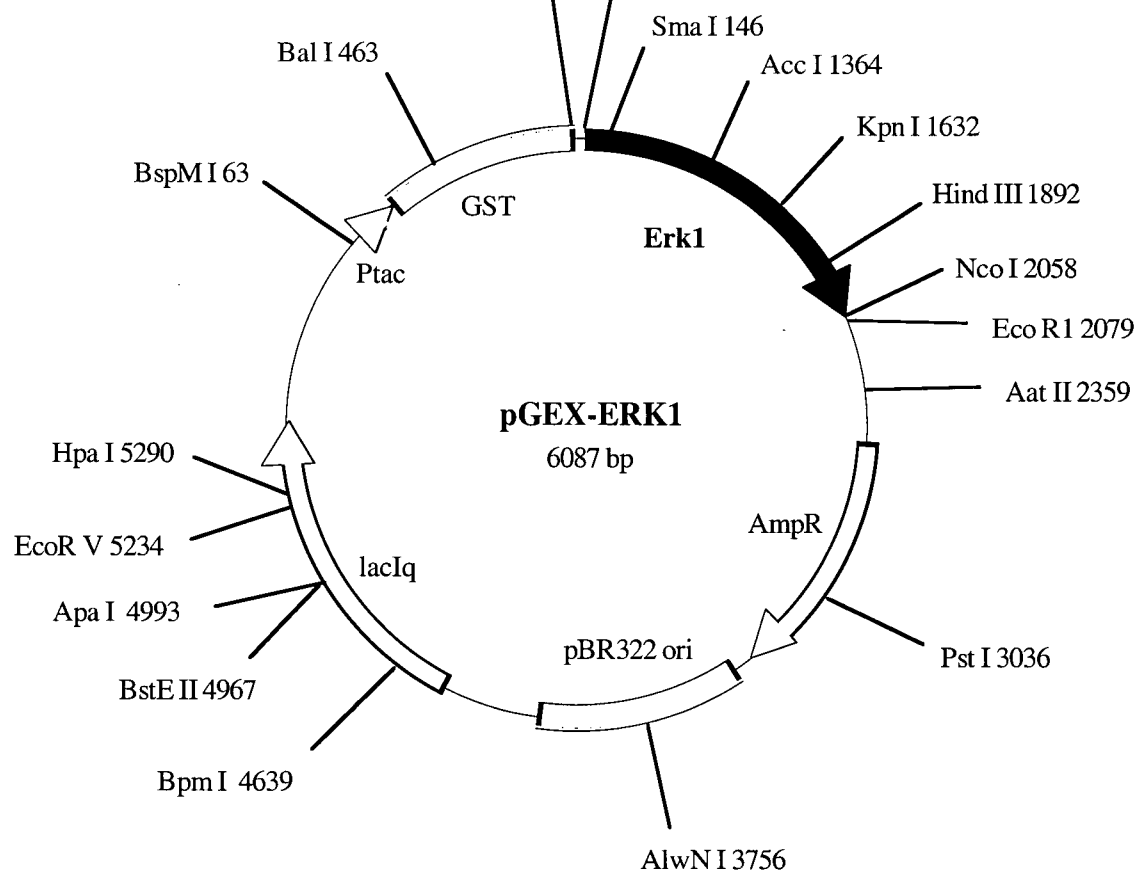
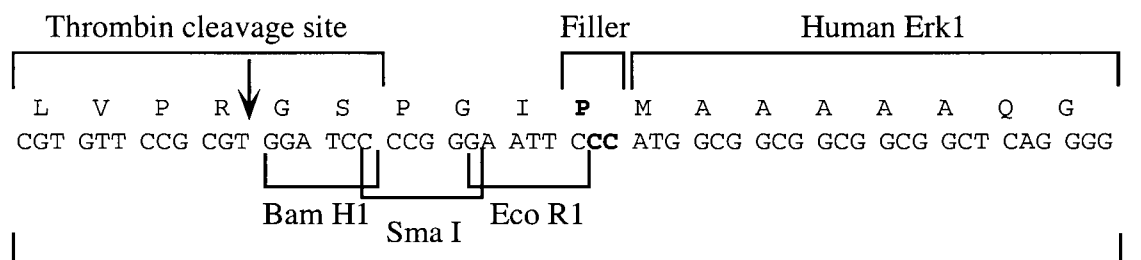
### 2.1 Expression of human Erk1 in *E. coli* as a recombinant GST-fusion protein

To express Erk1 protein in sufficient quantity for biochemical analysis, we used the *E. coli* pGEX-2T plasmid. This system was shown to be ideal for the expression and purification of GST-fusion proteins in bacteria since the recombinant protein can be affinity purified from the contaminating proteins by binding the glutathione-S-transferase fusion to the glutathione substrate that is cross-linked to Sepharose beads (Guan and Dixon, 1991). Oligonucleotides with *Eco* R1 sites were patterned after the 5' end and 3' end coding regions of the 26a $\beta$ -3 human Erk1 clone. PCR was performed under very stringent conditions to minimize mutations. In addition, VENT polymerase which has proof-reading abilities, was used during the PCR reaction to maintain sequence fidelity. As a cautionary measure, the amplified product was verified for mutations by DNA sequencing. After amplification, the 1.2 kb fragment was digested with *Eco* R1 and subcloned into the pGEX-2T vector (Figure 12). The proper orientation of the Erk1 cDNA was determined by restriction analysis with *Sma* I. Using the pGEX-2T prokaryotic expression vector, production of recombinant fusion proteins is tightly regulated by the LacZ promoter. The highest expression levels of GST-Erk1 were obtained by the addition of 80-100  $\mu$ M of isopropyl- $\beta$ -D-thiogalactopyranoside .

We first determined the ability of human Erk1 to be expressed as a recombinant fusion protein in *E. coli* by using a series of polyclonal antibodies directed against known human, rat Erk1 and Mpk1 peptide sequences as well as purified full-length sea star Mpk1. The glutathione-affinity purified GST-Erk1 (4  $\mu$ g), its Mono Q concentrated thrombin-cleaved Erk1 (1  $\mu$ g) and purified sea star Mpk1 (0.5  $\mu$ g) were electrophoresed



Figure 12: pGEX-2T Prokaryotic expression vector of the full-length human Erk1 cDNA cloned in-frame with the glutathione-S-transferase (GST) protein. Construction of the vector is described in Materials and Methods. The 3' end of the glutathione-S-transferase protein was fused in frame with the the 5' human Erk1 cDNA coding sequence by a two cytosine base linker (**bold**). As a result, a histidyl amino acid was exchanged for a prolyl residue (**bold**). This two base intervening linker was added to maintain the proper reading frame during protein translation. The thrombin cleavage site recognition sequence is indicated by a vertical arrow. The pBR322 origin of replication, ampicillin resistance gene, and the LacZ inhibitor protein gene, lacIq, are also presented.

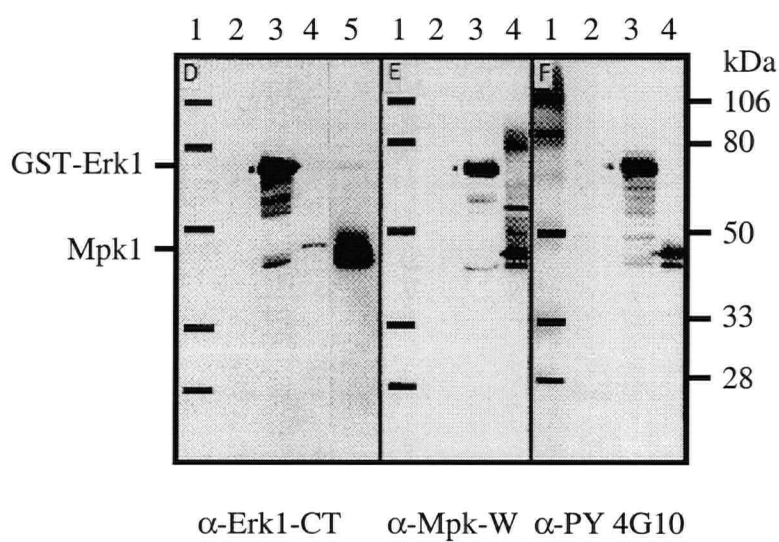
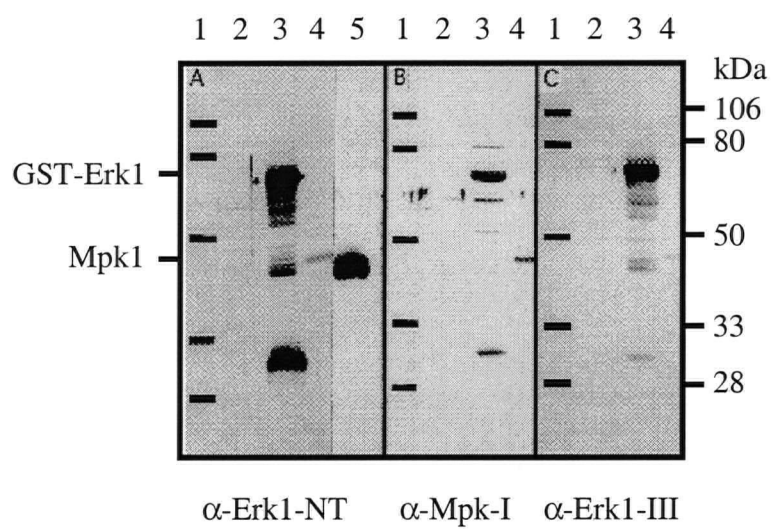


and transferred to nitrocellulose for Western immunoblotting. These anti-peptide antibodies recognize unique sequences (see Table 5 for details) near the amino terminal (anti-Erk1-NT [Figure 13A]) of human Erk1 (Charest *et al.*, 1993), the kinase subdomain III (anti-Erk1-III [Figure 13C]), and carboxy terminal (anti-Erk1-CT [Figure 13D]) sequences of rat Erk1 (Boulton *et al.*, 1990), as well as a peptide patterned after the ATP-binding site of subdomain I of Mpk1 (anti-Mpk-I [Figure 13B]). The purified Mpk1 (anti-Mpk-W [Figure 13E]) from sea star was also used to generate antibodies against the complete protein (Sanghera *et al.*, 1990; Sanghera *et al.*, 1992). A GST-fusion protein of approximately 70-kDa in size (molecular mass of Erk1, 44-kDa and of GST, 26-kDa) was recognized by all the antibodies from cell lysates of bacteria transformed with the pGEX-Erk1 expression vector (Figure 13, lane 3). The 44-kDa thrombin-cleaved Erk1 protein was also detected by the anti-Erk1-NT and -CT antibodies (Figure 13 lane 5). A ladder of smaller bands (Figure 13A, B and ) were also detected by anti-Erk1 peptide antibodies. These are probably the result of aborted translation or proteolytic cleavage since a similar pattern was not observed with thrombin cleaved Erk1 (Figure 13 compare lanes 3 and 5). No protein bands, however, were observed from control bacterial cell lysates expressing only GST (Figure 13, lane 2). Purified Mpk1 from sea star protein was more easily revealed by whole protein Mpk antibody than with any of the peptide antibodies (Figure 13, lane 4). These data indicate that human Erk1 can be expressed in *E. coli* and subsequently purified in reasonably large quantities for experimentation.

## 2.2 Characterization of autophosphorylated Erk1

In vivo MAP kinases are activated in response to growth hormones by becoming phosphorylated on threonyl and tyrosyl residues (Ray and Sturgill, 1988; Anderson *et al.*, 1990). The mechanism that regulated these phosphorylation events were not established at the time these enzymes were being cloned and sequenced. As a first step in

Figure 13: Expression and immunoreactivity of recombinant Erk1 protein from *E. coli* to MAP kinase antibodies. Isopropyl- $\beta$ -D-thiogalactopyranoside-induced bacterial lysates from pGEX control plasmid (lane 2), lysates from IPTG-induced pGEX-Erk1 plasmid (lane 3), 0.5 mg of purified sea star Mpk1 (lane 4) (Sanghara et al., 1990) and 1.0 mg of thrombin-cleaved glutathione affinity purified Erk1 (lane 5) were electrophoresed on a 10% SDS-polyacrylamide gel. The MAP kinase proteins were revealed by Western immunoblotting the nitrocellulose filters with anti-peptide antibodies Erk1-NT (A), Mpk-I (B), Erk1-III (C), Erk1-CT (D), whole protein antibodies Mpk1-W (E). The Roman numeral designation specifies the catalytic subdomain location used to synthesize the peptides (Hanks et al., 1988). Lane 1 shows the prestained standard proteins: bovine serum albumin, 87-kDa; ovalbumin, 50-kDa; carbonic anhydrase, 33-kDa soybean trypsin inhibitor, 29-kDa; and lysozyme, 21-kDa. The black marks indicate the positions of the markers.

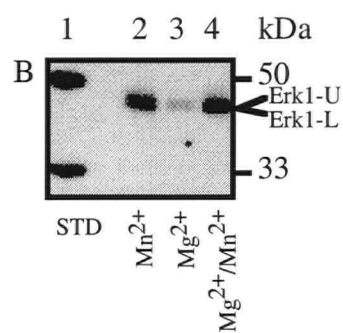
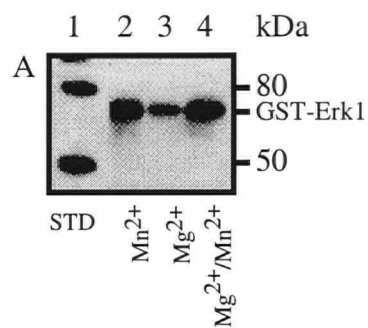


understanding the regulation of MAP kinases at a molecular level, the phosphorylation state of the human GST-Erk1 preparation was analyzed with the monoclonal antiphosphotyrosine antibody 4G10. While MAP kinase purified from sea star oocytes was shown to be regulated by tyrosyl phosphorylation (Figure 13, lane 4) (Sanghara *et al.*, 1994), we also detected the presence of phosphotyrosine in the non-activated form of Erk1 purified from bacteria (Figure 13, lane 3). The detection of Erk1 with tyrosyl-specific 4G10 antibodies seemed to be comparable to the levels observed with anti-Erk1-specific antibodies (compare panels A, C, D, and F of Figure 13). This seemed unusual since there have been no reports of tyrosyl phosphorylation in prokaryotes and no reactivity with 4G10 was detected in control bacterial lysates (Figure 13, lane 2) (Schieven *et al.*, 1986). Therefore, the most likely explanation for the presence of phosphotyrosine in GST-Erk1 was likely the result of an autophosphorylation reaction. As mentioned earlier, MAP kinases become fully activated by phosphorylation on threonyl and tyrosyl sites (Sturgill *et al.*, 1988; Anderson *et al.*, 1990). Because 4G10 is specific for phosphotyrosine, the data could not exclude the possibility of a threonine autophosphorylation reaction. A different approach to determining whether autophosphorylation leads to increased phosphothreonine is to perform an autocatalytic reaction in the presence of [ $\gamma$ - $^{32}$ P]ATP followed by phosphoamino acid analysis of the labelled protein.

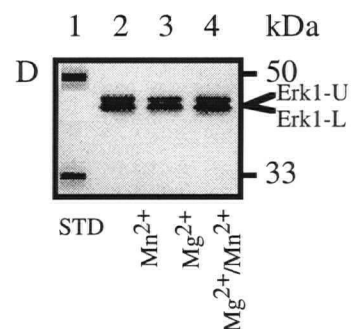
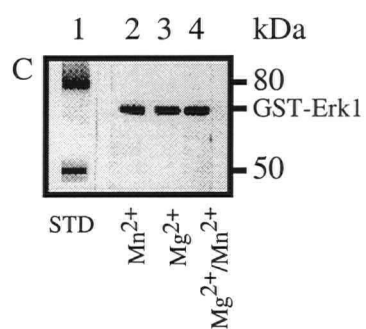
We examined the possibility that autophosphorylation of Erk1 might regulate its phosphotransferase activity. To do so, we linked GST-Erk1 to glutathione-agarose beads and incubated the fusion protein with [ $\gamma$ - $^{32}$ P]ATP under conditions that promoted autophosphorylation *in vitro*. A single radiolabelled protein band that was identical in size to the Erk1 immunoreactive bands was identified as Erk1 using MAP kinase-specific antibodies (Figure 14A and C). Similar results were obtained using identical assay conditions in which the thrombin-cleaved Erk1 was incubated with radiolabel (Figure

Chapter 14: Autophosphorylation and phosphoamino acid analysis of the Erk1 recombinant protein. GST-Erk1 fusion (A and C) and thrombin-cleaved Erk1 proteins, Erk1-U (U = upper band) and Erk1-L (L = lower band) (B and D) were incubated with  $\gamma^{32}\text{-P[ATP]}$  (9000 cpm/pmol) in the presence of either 10 mM  $\text{MnCl}_2$  (lanes 2) or  $\text{MgCl}_2$  (lanes 3) separately or combined (lanes 4). Autoradiograms are shown in panels A and B, and immunoblots in panels C and D. Phosphoamino acid analysis was performed on autophosphorylated bands of GST-Erk1 (E) and cleaved-Erk1 (F) proteins in the presence of  $\text{Mn}^{2+}$  or  $\text{Mg}^{2+}$ . The prestained protein standards (lanes 1) are displayed with radioactive ink (A and B) and with bars (C and D). The migration of the free-phosphate and phosphoamino acid standards are indicated in panels E and F.

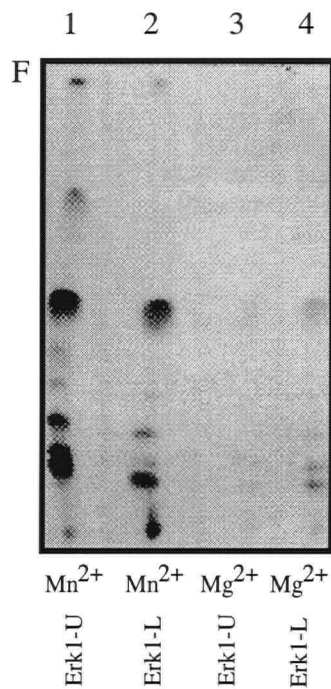
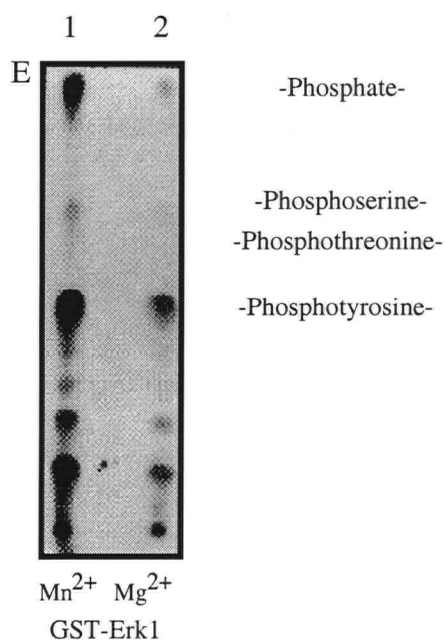
Autoradiograph



Western analysis



Phosphoamino acid analysis





14B and D). Thus, the presence or removal of the 26-kDa GST protein had no adverse effects on Erk1 enzyme activity. Interestingly, antiphosphotyrosine immunoblotting of GST-free Erk1 revealed two antibody-reactive proteins of approximately 43-kDa (Erk1-L) and 44-kDa (Erk1-U) while only a single band was observed with GST-Erk1 (Figure 14C and D). When the autoradiogram in Figure 14B was underexposed both forms were shown to be radiolabelled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Both species underwent autophosphorylation and were recognized by 4G10 antibodies. The most likely explanation for the doublet is the existence of a cryptic thrombin-cleavage site located at the carboxy-terminal end of the protein. Coincidentally, the kinase-inactive form of Erk1 (A71) also displayed a similar doublet after cleavage with thrombin. This result indicates that the observed doublet is probably due to improper thrombin-cleavage of the GST-Erk1 and not a band shift that has been associated with activation of the Erk1 protein kinase.

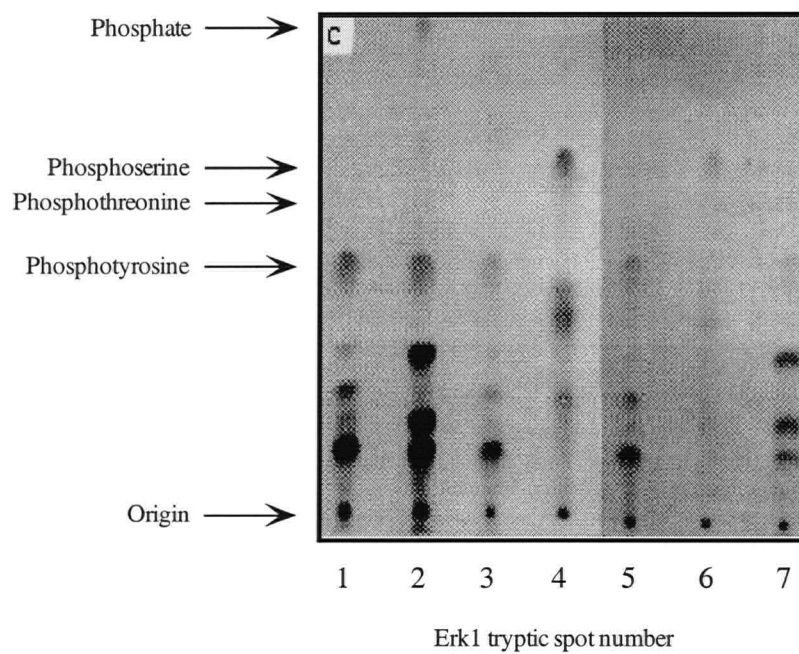
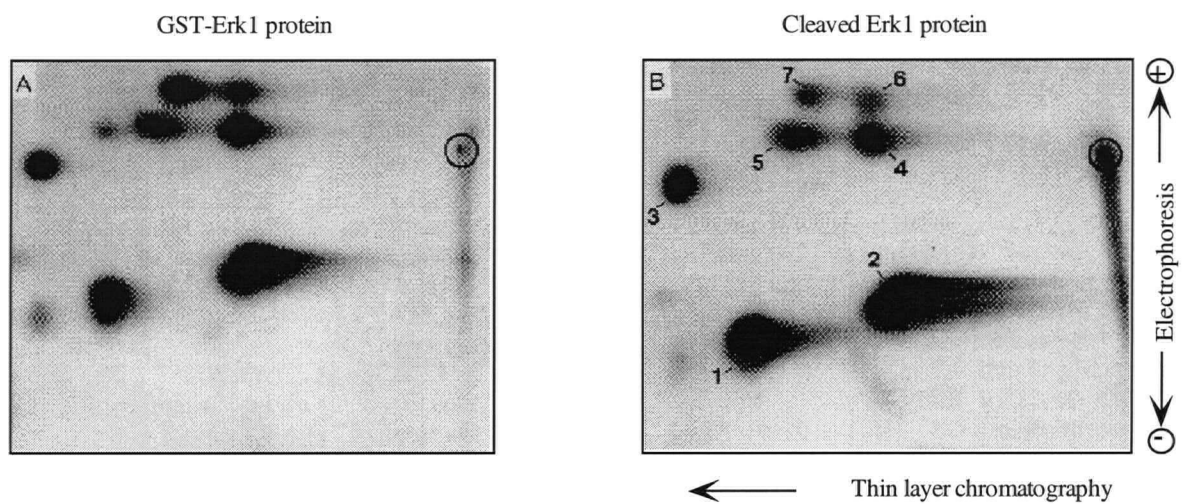
The divalent cation requirement for GST- and cleaved-Erk1 during autophosphorylation reactions was examined (Figure 14A and B).  $\text{MnCl}_2$  and  $\text{MgCl}_2$  were chosen since these two cations are required in most tyrosyl and seryl/threonyl phosphorylation reactions. The autophosphorylation activity was more efficiently stimulated in the presence of 10 mM  $\text{Mn}^{2+}$  than with 10 mM  $\text{Mg}^{2+}$  during the 30 min incubation period. A similar level of radiolabel incorporation was achieved with the combination of divalent cations as with  $\text{Mn}^{2+}$  alone.

The stoichiometry of GST-Erk1 or thrombin-cleaved Erk1 phosphorylation as assessed by  $^{32}\text{P}$  incorporation was approximately 0.01 mol of phosphate per mol of enzyme (data not shown). Since MAP kinases apparently require threonyl and tyrosyl phosphorylation to become activated, the low stoichiometry implied that Erk1 poorly autophosphorylated *in vitro*. Another possible explanation for these findings is that Erk1

was already heavily autophosphorylated in *E. coli* during the course of protein expression (Figure 14C and D). The amount of phosphate incorporated in a half hour reaction was quite low when compared to the phosphotyrosyl content that occurs *in situ* in bacteria. This is best illustrated when comparing the differences in  $^{32}\text{P}$  labelling of Erk1-U and Erk1-L proteins incubated in the presence of  $\text{Mn}^{2+}$  or  $\text{Mg}^{2+}$  divalent metal cations (Figure 14B, compare lanes 2 and 3). Although the autophosphorylation reaction was easily observed by radiolabelling with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , this increase in Erk1 phosphotyrosine did not result in greater immunoreactivity in Western blots with 4G10 antiphosphotyrosine antibody (Figure 14D, compare lanes 2 and 3). Perhaps the higher phosphotyrosine content of GST-Erk1 was attributable to the lengthy IPTG induction period (12 h) that was used to maximize GST-Erk1 protein synthesis. Since prokaryotes are unlikely to express endogenous tyrosyl phosphatases, Erk1 that autophosphorylated on tyrosyl residues could accumulate over a longer duration. Another possible explanation for the absence of any observable differences may be the lack of sensitivity in detecting subtle changes by the Western immunoblotting technique. MAP kinases have been reported to require threonyl in addition to tyrosyl phosphorylation to become activated *in vivo* (Anderson *et al.*, 1990; Payne *et al.*, 1991). Therefore, the *in vitro* autophosphorylated proteins in Figure 14A and B were further characterized by phosphoamino acid analysis. In the presence of either divalent cation, GST-Erk1 (Figure 14E lanes 1 and 2) and cleaved Erk1 (Figure 14F Erk1-U, lanes 1 and 3; Erk1-L, lanes 2 and 4) were phosphorylated on tyrosyl. Additionally, GST-Erk1 and Erk1-U were partially seryl phosphorylated in presence of  $\text{Mn}^{2+}$  (Figure 14E and F lane 1). However, almost no phosphothreonine was detected for any of the phosphorylated protein bands.

The GST-Erk1 and thrombin-cleaved Erk1 autophosphorylation sites were determined by two-dimensional tryptic phosphopeptide mapping. The phosphopeptide pattern of seven distinct spots was identical for both forms of Erk1 (Figure 15A and B).

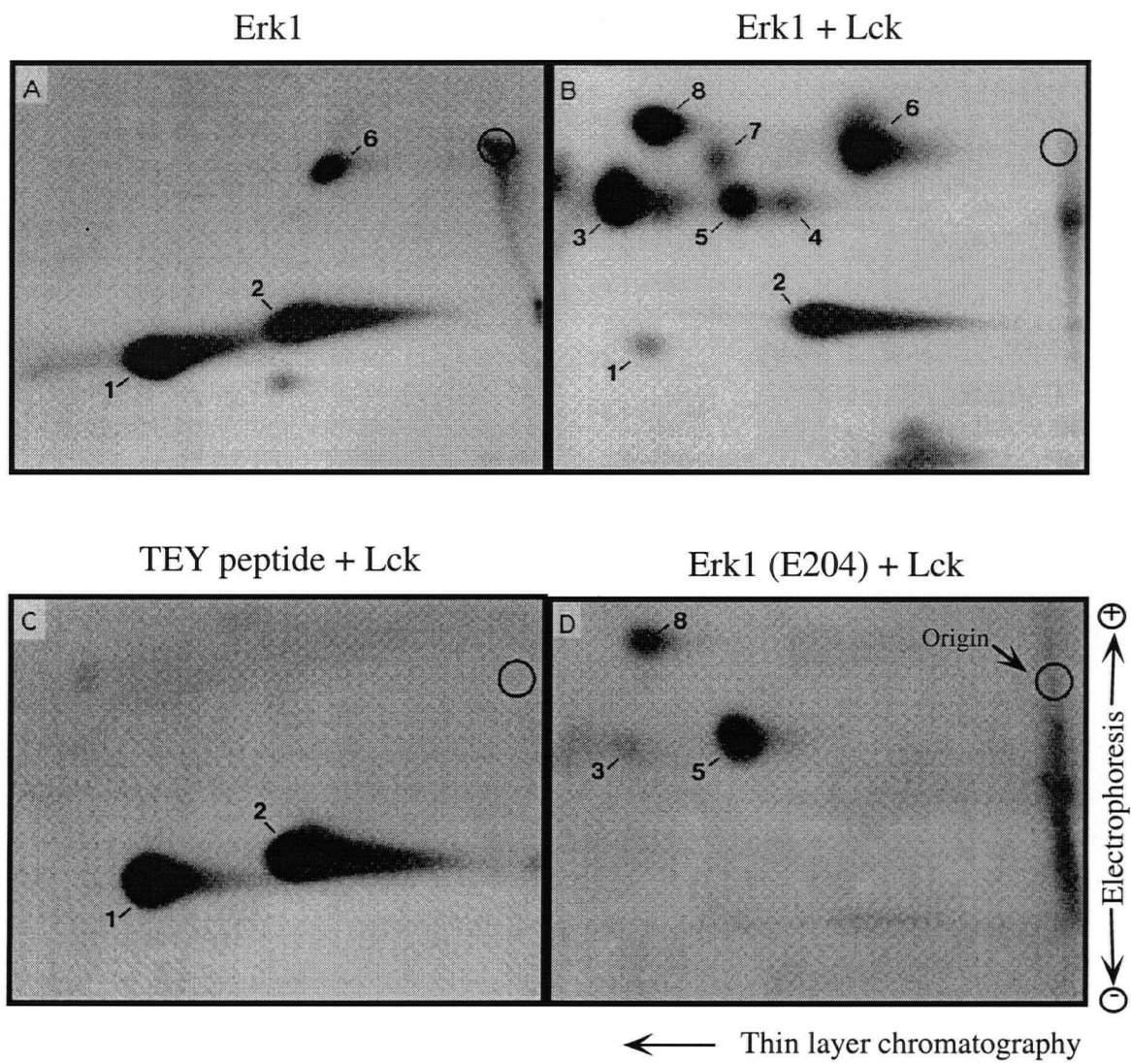
Figure 15: Two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis of autophosphorylated recombinant Erk1. GST-Erk1 fusion (A) and thrombin-cleaved Erk1 (B) proteins were incubated with [ $\gamma$ - $^{32}\text{P}$ ]ATP (10,000 cpm/pmol) and separated on 10% SDS-polyacrylamide gels before excising and digesting the bands with trypsin as described in Materials and Methods. The open circle marks the origin. The direction of the electrophoresis (positive and negative poles) and thin layer chromatography steps are indicated by arrows. The numbered spots in panel B were removed and further analyzed by phosphoamino acid analysis (C).



A number was assigned to each phosphopeptide for comparison purposes. Spots 1 and 2 appear to be the major phosphorylated peptides. The phosphopeptides were further subjected to phosphoamino acid analysis (Figure 15C). The majority of the phosphorylated spots (1, 2, 3, 5, and 7) were tyrosyl phosphorylated while the remaining two spots (4 and 6) were seryl phosphorylated. Interestingly, no threonyl phosphorylation was present. Therefore, these results indicate that Erk1 autophosphorylates at multiple tyrosyl and seryl residues *in vitro*. Although the protein was digested for close to 24 hours, it may be possible that an incomplete tryptic digest generated multiple phosphopeptides.

To determine which tryptic phosphopeptides in Figure 15 corresponded to potential *in vivo* regulatory sites (i.e., Tyr-204) murine Lck purified from baculovirus was used to phosphorylate recombinant thrombin-cleaved Erk1. A similar approach was used to tyrosyl phosphorylate and activate sea star Mpk1 (Ettehadieh *et al.*, 1992). Erk1 was initially preincubated with unlabelled ATP as a measure to reduce autophosphorylation prior to addition of [ $\gamma$ - $^{32}$ P]ATP and Lck. A similar tryptic phosphopeptide mapping pattern was achieved during the *in vitro* phosphorylation of Erk1 by Lck and some of this may be the result of autophosphorylation (Figure 16B). The major tyrosyl phosphopeptide sites 1 and 2 were differentially phosphorylated. The only novel Lck site was phosphopeptide 8. To confirm which tryptic phosphopeptides, if any, contained the Thr-202 and Tyr-204 (TEY peptide IADPEHDHTGFLTEYVATR) identified by Payne *et al.* (1991), a synthetic peptide patterned after this sequence was phosphorylated with Lck and subjected to the same trypsin treatment as performed for the Erk1 proteins (Figure 16C). Tryptic phosphopeptides sites 1 and 2 were generated with the TEY phosphorylation peptide and therefore Tyr-204 represents the major tyrosyl autophosphorylation sites in Erk1 (Figure 16C). The GST-Erk1 and TEY peptide both generated spots 1 and 2 after trypsin digest and electrophoresis. This result was

Figure 16: Two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis of p56<sup>lck</sup> phosphorylated recombinant Erk1. Panel A, Thrombin-cleaved Erk1 was incubated with [ $\gamma$ -<sup>32</sup>P]ATP under autophosphorylating conditions for 20 min; Panel B, the same thrombin-cleaved Erk1 protein was pretreated with unlabeled ATP as in panel A before incubation with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of Lck; Panel C, the Erk1 regulatory phosphorylation site TEY peptide (IADPEHDHTGFLTEYVATR) was phosphorylated by Lck and purified by high-pressure liquid chromatography before trypsin treatment; Panel D, thrombin-cleaved Erk1-E204 allele phosphorylated by Lck as in panel B. Variable migration was observed with spot 6.



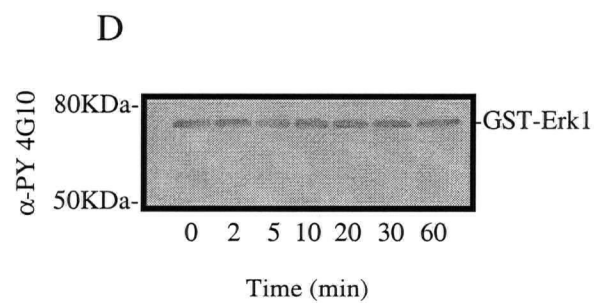
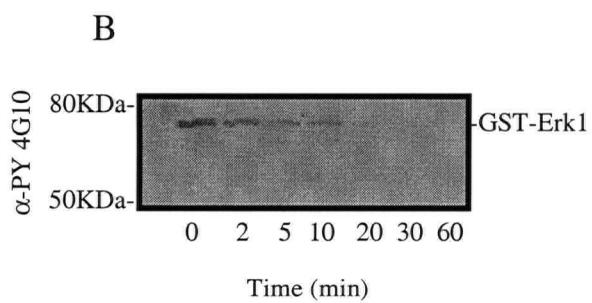
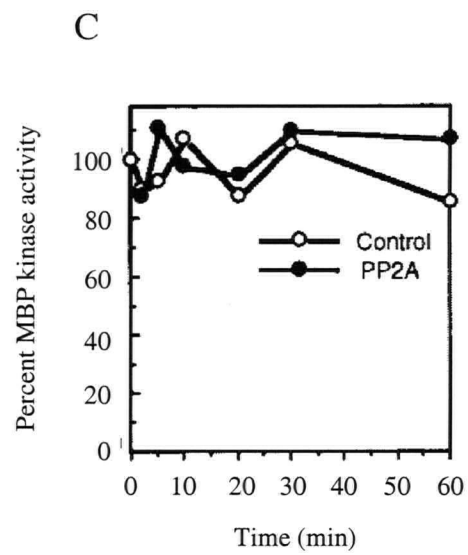
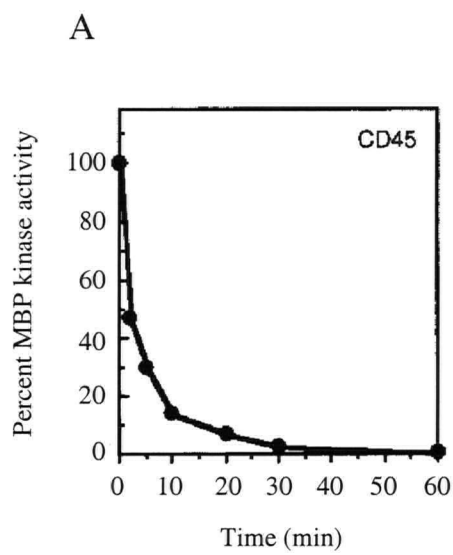
unexpected, since the sequence of the synthetic peptide contained just one tyrosyl residue. A possible explanation for this anomaly may be that the trypsin preparation was contaminated with another protease that cleaved the TEY phosphopeptide. A second experimental approach was used to confirm that Tyr-204 was the principal autophosphorylation and Lck phosphorylation site *in vitro*. A Tyr-204 phosphorylation site allele was created by converting it to a negatively charged glutamyl residue by polymerase chain reaction site-directed mutagenesis. In phosphorylation experiments with Lck, Glu-204 Erk1 substrate was phosphorylated specifically at phosphopeptide sites 5 and 8 (Figure 16D). No autophosphorylation was observed with the catalytically compromised Glu-204 Erk1. Furthermore, Lck was also demonstrated to phosphorylate the regulatory TEY peptide in a parallel experiment which is apparent from the appearance of spots 1 and 2 in Figure 16C. Hence, Lck is capable of phosphorylating Erk1 protein kinase on the Tyr-204 *in vitro*.

### 2.3 MBP phosphotransferase activity of GST-Erk1

A small amount of MBP phosphotransferase activity was detectable in recombinant GST-Erk1 and cleaved Erk1 purified from bacteria. MBP was phosphorylated at a rate of ~ 2 nmol/min/mg of enzyme, a rate that was substantially lower than the 324 nmol/min/mg obtained from the 42-kDa MAP kinase purified from *Xenopus* oocytes using the same substrate (Gotoh *et al.*, 1991). The importance of threonyl and tyrosyl phosphorylation in Erk1 activity was examined with phosphatases that selectively removed these residues *in vitro*. Tyrosine-specific protein phosphatase CD45 incubation with GST-Erk1 led to a 50% decrease in MBP phosphotransferase activity in 2 min and complete inactivation of the enzyme after 60 min of incubation (Figure 17A). This was associated with a comparable decrease in the amount of detectable tyrosyl phosphorylation in GST-Erk1 by Western analysis (Figure 17C).



Figure 17: Phosphatase treatment of recombinant Erk1. GST-Erk1 bound to glutathione-agarose beads was incubated with 0.5  $\mu$ g of purified protein tyrosine phosphatase CD45 (A and B) or  $\sim$ 4 U of human protein phosphatase 2A (C and D) for the specified times before quenching the reaction with 1 mM sodium orthovanadate or 1  $\mu$ M okadaic acid respectively. After washing the beads several times with Buffer G, the material was divided in half and subjected to MBP kinase assays (A and B) or Western immunoblotting analysis with the 4G10 antiphosphotyrosine antibody (C and D). The data are expressed relative to the non-phosphatase treated control GST-Erk1 beads. For protein phosphatase 2A, 1 U releases 1 nmol of phosphate per min from 15  $\mu$ M phosphorylase at 30°C.



However, treatment with the seryl/threonyl specific protein phosphatase 2A did not substantially reduce the MBP phosphotransferase activity of GST-Erk1 (Figure 17B). The phosphotyrosyl levels for GST-Erk1 as revealed by the antiphosphotyrosine antibodies remained constant throughout the incubation with PP2A (Figure 17D). Staining of the protein bands with Ponceau S solution revealed that the amount of Erk1 enzyme assayed was identical for each time point (data not shown). As a control, protein phosphatase 2A (PP2A) was shown to be active through its ability to dephosphorylate a synthetic phosphopeptide with the sequence Lys-Arg-Thr(P)-Ile-Arg-Arg (data not shown). Hence, autophosphorylation on Tyr-204 was necessary to minimally activate Erk1 phosphotransferase activity.

#### 2.4 Activation of GST-Erk1 by a MAP kinase kinase

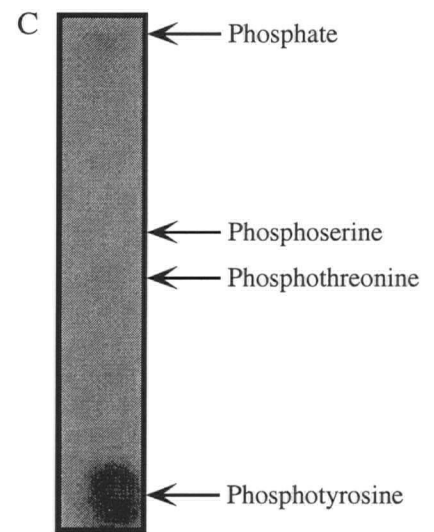
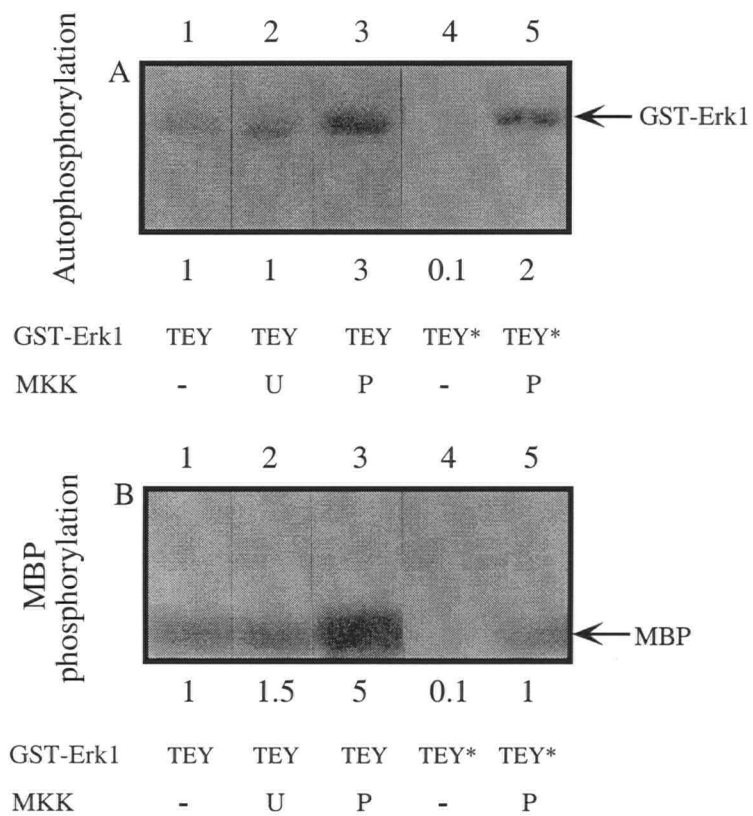
Lck phosphorylated Erk1 *in vitro* to a stoichiometry of 0.1 mol of P per mol on a number of different tyrosyl sites including the TEY containing sites 1 and 2 (Figure 17). However, no increase in Erk1 MBP phosphotransferase activity by Lck was noted during these kinase reactions even though it was reported that sea star Mpk was activated by Lck under similar circumstances (Ettehadieh *et al.*, 1992) (data not shown). Because recombinant expressed bacterial Erk1 is already substantially phosphorylated on tyrosyl, further phosphorylation by Lck may be insufficient to stimulate further its MBP kinase activity. It is also possible that Lck phosphorylates a tyrosyl site that inactivates the phosphotransferase activity of Erk1. The unique tryptic phosphopeptide site 8 which was only detected when Erk1 was incubated with Lck is a good candidate. One candidate tyrosyl site is located in the ATP-binding region (IGEGAYGMV) of subdomain I which is found in some MAP kinases. Phosphorylation of a homologous site in cyclin-dependent kinase 2 (CDK2) by the dual-specificity kinase Wee1 leads to the diminution

of its histone H1 phosphotransferase activity *in vivo* (Parker and Piwnicka-Worms, 1992; McGowan and Russell, 1993; Parker *et al.*, 1995).

MAP kinase activators that have been identified in different PMA-treated mammalian cells and apparently stimulate MBP phosphotransferase activity by phosphorylating the regulatory threonyl and tyrosyl phosphorylation sites of Erks (Wu *et al.*, 1991; Alessandrini *et al.*, 1992; Rossomando *et al.*, 1992). Since Lck was unable to activate Erk1 directly *in vitro*, a different approach using a GST-Erk1 two-step activation assay was used to identify possible regulatory kinases. This work was undertaken as part of a separate study to investigate the role MAP kinase pathway plays in the regulation of platelet aggregation (Samiei *et al.*, 1993).

The experimental treatment used to detect MAP and S6 protein kinases in sheep platelets was also used to investigate the presence of an upstream activator. Purified platelets were exposed to 200 nM PMA and homogenized after 5 min incubation period. The crude extract was applied to a DEAE-cellulose resin at neutral pH. The break through material was recovered and diluted in 10 volumes of MES buffer pH 6.5 before fractionation on a Mono S cation exchange column. Activation of the partially purified preparation of MAP kinase kinase (MAPKK) was determined by assaying for GST-Erk1 phosphotransferase activity in the presence of [ $\gamma$ - $^{32}$ P]ATP. A three-fold increase in phosphorylation was observed when compared with autophosphorylated Erk1 (Figure 18A, lanes 1 and 3). Analysis of the phosphoamino acid content of Erk1 phosphorylated with MAPKK showed an elevation in tyrosyl phosphorylation while the presence of seryl and threonyl was almost undetectable (Figure 18C). MAPKK purified from unstimulated platelets displayed almost no GST-Erk1 phosphorylation under the same experimental conditions (Figure 18A, lane 2). The increase in GST-Erk1 phosphorylation by PMA-activated sheep platelet MAPKK stimulated GST-Erk1 MBP phosphotransferase activity

Figure 18: MAP kinase kinase phosphorylation and activation of recombinant Erk1. Partially purified MAP kinase kinase from sheep platelets stimulated for 5 min with 200 mM PMA (P-MKK) and from unstimulated sheep platelets (U-MKK) was used to phosphorylate GST-Erk1 linked to glutathione agarose beads (A) and promote its phosphotransferase activity (B) by the technique outlined in Materials and Methods. Approximately 1  $\mu$ g each of Erk1 wild type (K71) and catalytically compromised (A71) in which the Lys that is essential for the phosphotransferase reaction is changed to an Ala (Kamps et al., 1994). SDS-PAGE loading buffer was added at the completion of the reaction. After electrophoresis, the proteins were transferred to nitrocellulose then autoradiographed for 15 min. The incorporated  $^{32}$ P was quantitated by liquid scintillation counting and is described as fold change above wild type with no extract. The top and bottom edges of each gel define the positions of the prestained standards: bovine serum albumin, 80-kDa; and ovalbumin, 50-kDa in panel A and soybean trypsin inhibitor, 28-kDa; and lysozyme, 19-kDa; in panel B. Lane 1, wild type Erk1 TEY with no extract; lane 2, wild type Erk1 TEY with untreated extract; lane 3, wild type Erk1 TEY with PMA-treated extract; lane 4, inactive Erk1 TEY\* without extract; lane 5, inactive Erk1 TEY\* with PMA-treated extract. The P-MKK phosphorylated GST-Erk1 (K71) in lane 3 of panel A was excised and subjected to phosphoamino acid analysis (C). Migrations of the free phosphate and phosphoamino acid standards are indicated.



by five-fold while the same kinase purified from control platelets had only marginal effects (Figure 18B lanes 1-3).

There was some speculation that the MAP kinase upstream regulator was a protein devoid of kinase catalytic activity that acted as an allosteric activator, since MAP kinases are capable of limited activation through autophosphorylation (Wu *et al.*, 1991; Seger *et al.*, 1991; Ahn *et al.*, 1991; Robbins and Cobb, 1992). To address this controversy, Lys-71 was mutated to an Ala in human Erk1 thereby compromising catalytic activity of GST-Erk1. A homologous Lys residue located in subdomain II of other protein kinases has been shown to be essential for the catalytic activity of protein kinases (Kamps *et al.*, 1984). The Ala71-GST-Erk1 allele displayed almost no autophosphorylating activity (6%) or MBP phosphotransferase activity (7%) in the absence of activated MAPKK from sheep platelets (Figure 18A and B, lane 4). However, Ala71-GST-Erk1 was phosphorylated to 80% of what was observed in the same experiment with wild type Lys-GST-Erk1 when treated with PMA-activated MAPKK and 38-fold greater than its autophosphorylation (Figure 18A compare lanes 3, 4 and 5). These results confirmed that the MAP kinase activator from PMA-treated sheep platelets was a *bona fide* protein kinase. Interestingly, MAPKK phosphorylation of catalytically compromised Ala71-GST-Erk1 unexpectedly stimulated its MBP phosphotransferase activity 16-fold above what was achieved with control autophosphorylation (Figure 18 lanes 4 and 5). This limited Ala-71-GST-Erk1 stimulation may be attributable to a second lysyl residue (Lys-72) that may compensate in a limited fashion for the mutation at Lys-71 (Figure 10).

### 3. PURIFICATION AND CHARACTERIZATION OF A SEA STAR MAP KINASE ACTIVATOR

#### 3.1 Detection of MAP kinase activator during oocyte maturation

Germinal-yesicle breakdown (GVBD) indicates meiotic maturation in sea star oocytes. This event, which occurs within ~80 min of oocytes being exposed to the hormone 1-methyladenine, is characterized by disintegration of the nuclear envelope and chromosomal condensation in a manner similar to meiotic and mitotic cell divisions in other model systems. This makes the sea star oocyte system suitable for studying cell cycle events, since large numbers of synchronized cells arrested in prophase I of meiosis I can be isolated for cell and biochemical studies. Unlike sheep platelets which yield an insufficient quantity of protein for purification, each sea star can yield millions of quiescent cells to provide gram amounts of protein for this same purpose.

Activation of several distinct protein kinases have been detected in maturing oocytes (Pelech *et al.*, 1988; 1991). One of these kinases, meiosis-activated MBP kinase (Mpk), was first identified because of its ability to phosphorylate the exogenous substrate MBP, was purified to homogeneity (Pelech *et al.*, 1988; Sanghera *et al.*, 1990). Peptide sequences derived from purified Mpk displayed a high degree of homology to rat and *Xenopus* MAP kinases (Posada *et al.*, 1990). Furthermore, a partial cDNA sequence obtained from RT-PCR using sea star mRNA yielded two contiguous fragments that displayed a large degree of similarity with human Erk1 in the regions of overlap (Figure 19). Of the seven conserved regions defined by Hanks *et al.* (1988), the amino acid sequence located between subdomains X and XI reveals the highest amino acid divergence (42% identical) between the partial sea star cDNA and the full-length human Erk1 clone. However, a high degree of identity was also observed in the central region of



Figure 19: Sequence comparison between Erk1 cDNA from human and an Erk-like cDNA from sea star oocytes. Full-length Erk1 cDNA obtained from the liver Hep G2 cell liver line was aligned with a partial sequence from several overlapping cDNA fragments identified by PCR amplification from sea star oocytes. The MAP kinase signature regulatory phosphorylation sites (TXY) are indicated by asterisks. Amino acid residues that are conserved in all kinases are highlighted in boldface type. The consensus sequence is indicated while non-matching residues are represented by a hyphen.

## V

Human Erk1	IQILLRFRHENVIGIRDILRASTLEAMRDVYTVQDLMETDLYKLLKSQQLSN
Sea star mpk	IKILTRFRHENIINIQDIHANTIDEMKDVYTVQSLMETALYKLLKTQKLSN
consensus	I-IL-RFRHEN-I-I-DI--A-T---M-DVYTVQ-LMET-LYKLLK-Q-LSN

## VI

## VII

Human Erk1	<u>D</u> HICYFLYQILRGLKYIHSANVLHRDLKPSNLLSNITCDLKIC <u>DFG</u> LARIAD
Sea star mpk	<u>D</u> HISYFLYQILRGLKYIHSANVLHRDLKPSNLLSNITCDLKIC <u>DFG</u> LARIAD
consensus	<u>D</u> HI-YFLYQILRGLKYIHSANVLHRKLKPSNLL-NTTCKLKIC <u>DFG</u> LARIAD

\* \*

## VIII

## IX

Human Erk1	PEHDHTGFLTEYVATRWYRAP <u>E</u> IMLNSKGYTKSI <u>D</u> IWSV <u>G</u> CILAEMLSNRPI
Sea star mpk	PVHDHTGFLTEYVATRWYRAP <u>E</u> IMLNSKGYNKSID <u>I</u> WSV <u>G</u> CILAEMLNKPI
consensus	P-EDHTGFLTEYWATRWYRAP <u>E</u> IMLNSKGY-KSID <u>I</u> WSV <u>G</u> CILAEML---PI

## X

Human Erk1	FPGKHYLDQLNHILGILGSPSQEDLNCTINMKARNYLQSLPSKTKVAWAKLF
Sea star mpk	FPGKHYLDQLNHILNILGSPSCEDLGCTHNDKAPGYMQSLPKKPTVPWKRLY
consensus	FPGKHYLDQLNHIL-ILGSPS-EDL-CI-N-KA--Y-QSLP-K--V-W--L-

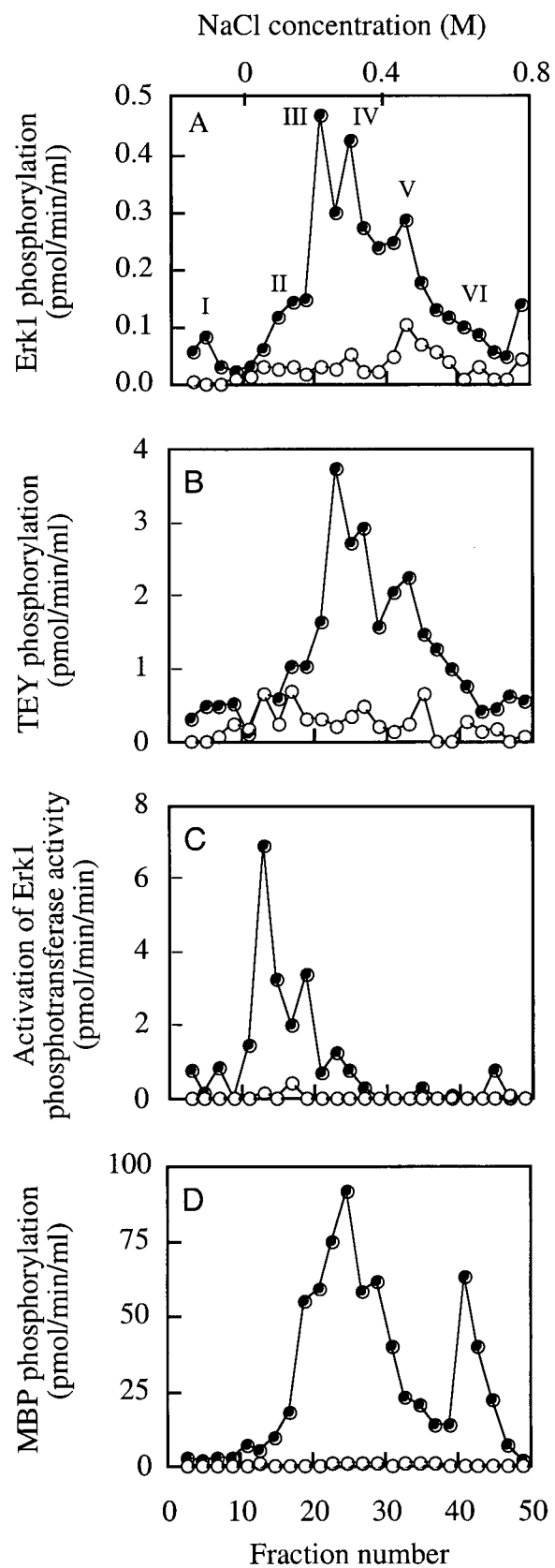
## XI

Human Erk1	----PKSDSKALDLLDRMLTFNPNK <u>R</u>
Sea star mpk	GAADPKS----LSLLDRILTFNPDK <u>R</u>
consensus	----PKS----L-LLDR-LTFNP-K <u>R</u>

the catalytic portion of partial Mpk protein (Figure 19). Therefore, there appeared to be sufficient conservation between human Erk1 and the partial Mpk clone to justify using the bacterial expressed recombinant human GST-Erk1 fusion protein to identify and purify to homogeneity a MAP kinase activator from sea star oocytes.

To determine the existence of a MAP kinase activator in sea star oocytes, 2 mg of cytosolic extract from immature oocytes and cells treated with 1-methyladenine were applied separately to a Mono Q anion exchange column. The protein was eluted with a 15 ml linear 0.8 M NaCl gradient and assayed for kinase activities toward a battery of substrates. As shown in Figure 20D, the MBP phosphorylating activity eluted as a broad peak at a concentration between 200-400 mM NaCl. The protein kinase displayed an identical chromatographic profile to the previously purified sea star MAP kinase, Mpk (Sanghera *et al.*, 1990). When these same fractions were assessed for MAP kinase kinase activity using the kinase-inactive GST-Erk1 fusion protein as a substrate, three major peaks of activity were observed in fractions 21, 25 and 33, respectively (Figure 20A). Additionally, three minor peaks of kinase activity were detected in fractions 7, 15 and 47 from the same Mono Q column. A similar kinase activity profile was observed using the TEY peptide as a substrate (compare Figure 20A and B). The 19 amino acid TEY peptide (IADPEHDHTGFLTEYVATR) was patterned after the regulatory phosphorylation sites Thr-202 and Tyr-204 and surrounding sequence in the activation loop of human Erk1 (Charest *et al.*, 1993). These phosphorylation sites were first uncovered at a similar position in the MAP kinase isoform Erk2 (Thr-183 and Tyr-185) by mass spectrometry (Payne *et al.*, 1991). Since phosphorylation of the full-length Erk1 and TEY peptide uncovered at least six distinct kinase activities that were capable of phosphorylating one or more of the threonyl or tyrosyl residues present in this peptide region, it was necessary to determine which one these phosphorylation events could regulate Erk1 phosphotransferase activity. A two-step Erk1 phosphorylation and

Figure 20: Detection of Erk1 activator activity in 1-methyladenine-treated sea star oocytes. Sea star extracts (2 mg) from immature (open circles) and mature (closed circles) were applied to a Mono Q anion exchange column and developed with a linear 0.8 M NaCl salt gradient and collected in 25  $\mu$ l fractions. A 10  $\mu$ l aliquot from every odd numbered fraction was tested for phosphotransferase activity toward (A) full-length recombinant GST-Erk1 protein; (B) a peptide termed 'TEY' which is patterned after the MAP kinase regulatory phosphorylation sites; (C) Erk1 phosphotransferase activity toward MBP following preincubation with column fractions (see Materials and Methods); and (D) myelin basic protein.



activation reaction was performed by first pre-incubating GST-Erk1 immobilized on glutathione beads with column fraction in the presence of 50 mM non-radiolabelled ATP. After 20 min, the beads were washed three times in buffer and the Erk1 assessed for MBP phosphotransferase activity. A maturation-stimulated MBP phosphorylating activity eluted in the 100 mM NaCl range just after the void volume from the Mono Q column. This indicated that the MAP kinase activator binds with low affinity to anion exchange resins equilibrated at pH 7.2. The MBP phosphotransferase activity for Erk1 was stimulated 7-fold by a MAP kinase activator that became stimulated when the oocytes were treated with hormone. However, the peak GST-Erk1 MBP phosphotransferase activity ( Figure 20C; fractions 11 through 15) was stimulated by the MAP kinase activator that eluted earlier from the Mono Q column. In fact this GST-Erk1 activation peak did not coincide with the GST-Erk1 nor the TEY phosphorylation peaks (Figure 20A and B; fractions 19 through 39) when these were used as *in vitro* substrates. In fact, Western blotting with anti-peptide antibodies revealed that peak IV was Casein kinase II. Therefore, the enzyme peaks that were capable of phosphorylating GST-Erk1 and TEY peptide did not overlap with the activation peak that stimulated Erk1 MBP phosphotransferase activity. This contradicted the result described earlier in sheep platelets (Figure 18 compare lanes 3 and 5). However, at that time, several research groups speculated that the upstream MAP kinase regulator may be a factor that could activate MAP kinase by an allosteric reaction (Wu et al., 1991; Seger et al., 1991; Ahn et al., 1991; Robbins and Cobb, 1992). Therefore, it was critical to determine if the sea star activating factor that regulated GST-Erk1 activity was distinct from the MAP kinase kinase detected in sheep platelets.

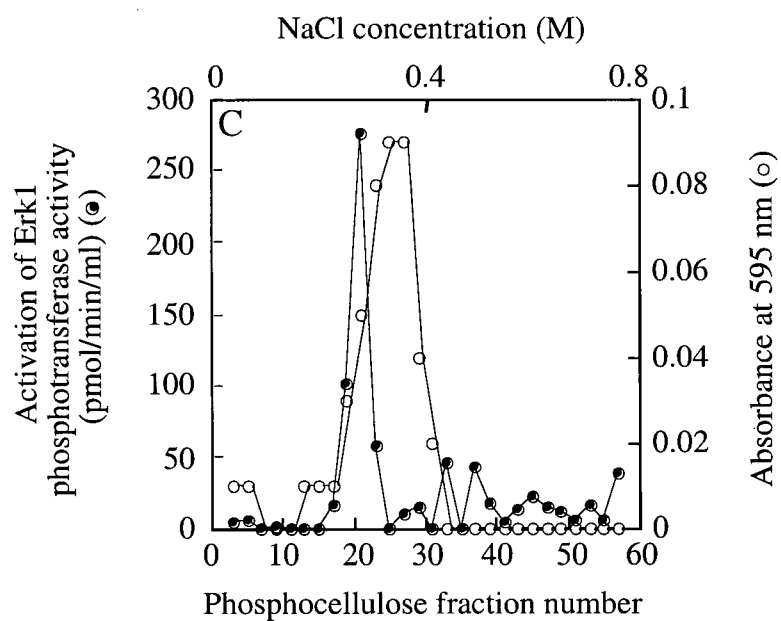
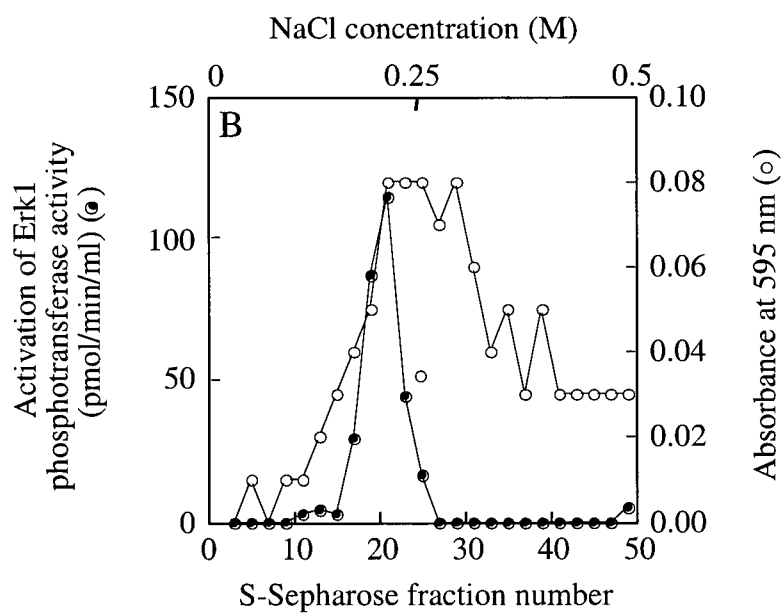
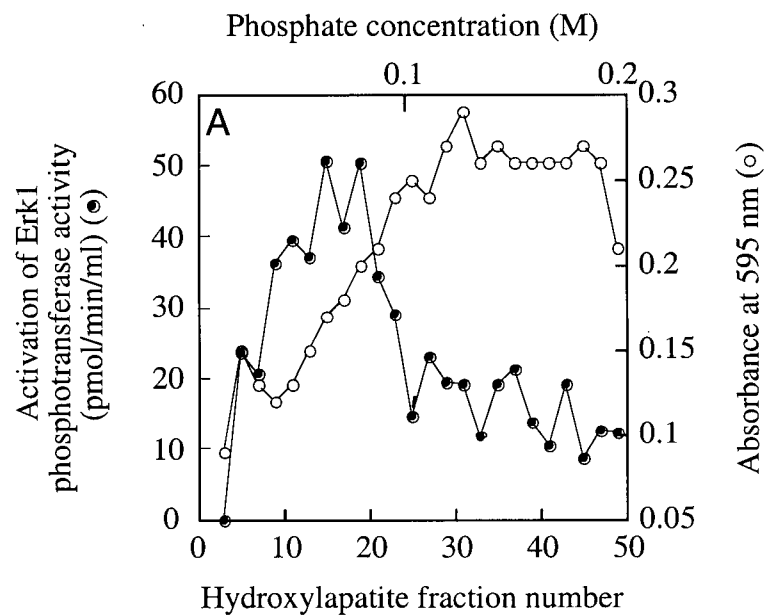
### 3.2 Purification of MAP kinase activator

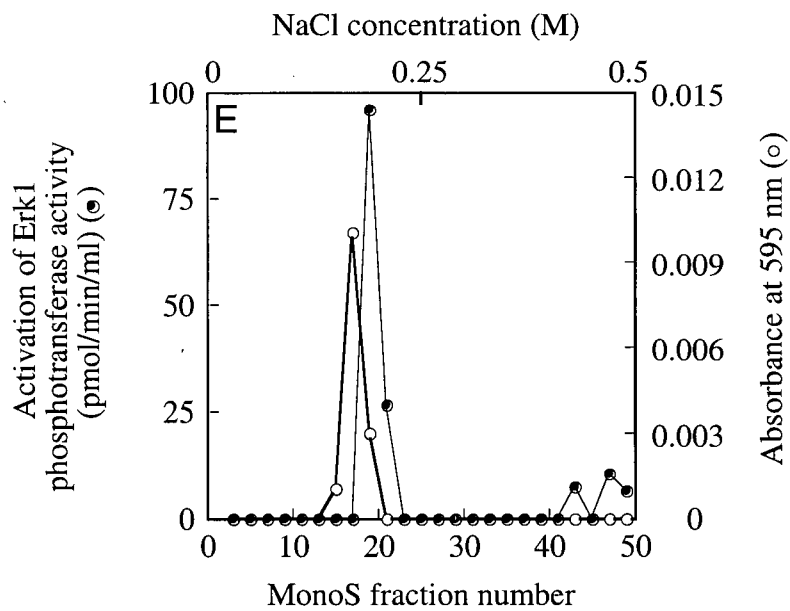
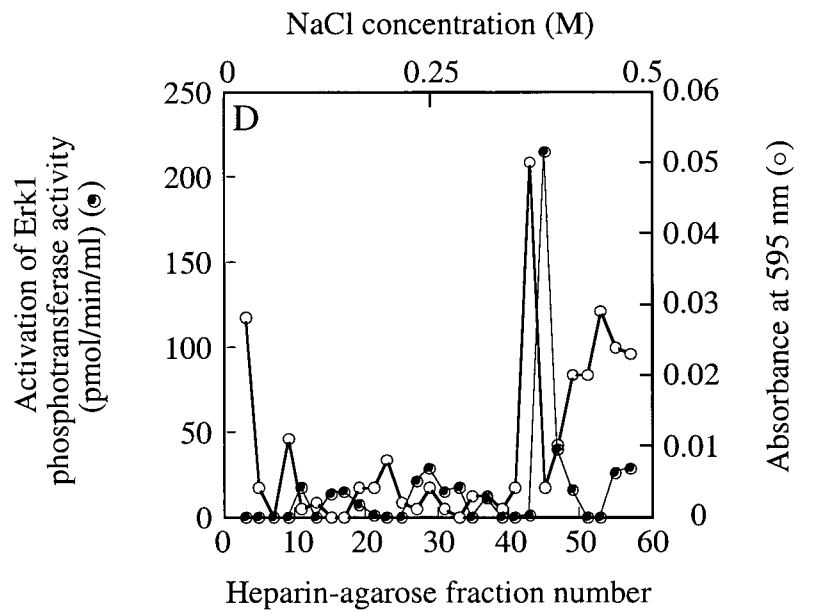
The major peak of MAP kinase activator activity was easily separated by anion exchange chromatography from the sea star MBP kinase Mpk (Figure 20 compare panels C and D). A purification strategy was devised for the isolation and characterization of this activating factor. Extracts prepared from the cytosolic portion of mature oocytes were applied to a DEAE-cellulose and the breakthrough material directly loaded onto a hydroxylapatite column linked in series. Assessment of the eluted DEAE-cellulose fractions for kinase activity revealed that none of the MAP kinase activator activity bound to this weaker anion exchange column (Figure 21A). However, the activator activity did adsorb to hydroxylapatite. It was released from the hydroxylapatite column as a broad peak between 20-100 mM phosphate, in a fractionation region separate from the bulk of the contaminating proteins (Figure 21A).

The fractions containing the highest activator activity were combined, and after dilution in buffer, adsorbed onto an S-Sepharose column. The highest Erk1 activator activity eluted as a narrow peak between 100-135 mM NaCl, with a large amount of contaminating proteins eluting in the later fractions (Figure 21B). The most active fractions were pooled and the relative salt concentration reduced by dilution in column buffer before application onto a phosphocellulose column. A sharp peak of activator activity was released from the phosphocellulose resin with ~270 mM-NaCl just prior to the elution of bulk of the contaminating proteins (Figure 21C). The combined fractions were diluted and loaded onto a heparin-agarose column. After development of the heparin-agarose with 200 mM linear NaCl gradient, the activator activity was eluted as a single peak with 150 mM salt (Figure 21D). Only minor amounts of contaminating protein was present in these fractions.

Figure 21: Purification of Erk1 activator from mature sea star oocytes. The sea star Erk1 activator was purified using six different chromatographic resins; DEAE-cellulose (reverse column); (A) hydroxylapatite; (B) S-Sepharose; (C) phosphocellulose; (D) heparin-agarose; and (E) Mono S. All columns were developed with a linear NaCl gradient except the hydroxylapatite which required potassium phosphate. Every odd numbered fraction was assayed for the presence activator indirectly by assaying for increased Erk1 MBP phosphotransferase activity (closed circles) as described in Material and Methods. The protein concentration (open circles) was determined at 595 nm for each fraction assayed.







For the remaining purification step, the most active fractions from the heparin-agarose column were combined and the pH reduced by dialyzing against MES buffer [pH 6.5]. The dialysate was applied to a Mono S column (Figure 21E). With this concentration step, the volume of the MAP kinase activator was reduced to one tenth the original volume. The most active fractions were detected at a concentration of 175 mM NaCl. A summary of the MAP kinase activator purification is outlined in Table 10. After six column chromatography steps, the MAP kinase activator was purified 536-fold for an overall recovery of 0.2%. Approximately 8  $\mu$ g of protein was obtained from 2 g of crude oocyte cytosolic extract. The hydroxylapatite column eliminated nearly 75% of the contaminating proteins, while both hydroxylapatite (8-fold) and heparin-agarose (7-fold) resins yielded the best overall purification of the activator.

This MAP kinase activator was a highly labile enzyme. Often the stimulatory activity of the activating factor was lost after two or three column purification steps for no apparent reason. Attempts to stabilize the protein by increasing the concentration of phosphatase inhibitors or by addition of protease inhibitors (soybean trypsin inhibitor, aprotinin, and protamine) had minimal effect on maintaining the enzyme activity during the purification. The purified enzyme was also inactivated following storage at 4°C for more than 3 h or after a freeze-thaw cycle. Therefore, it would appear that the sea star activator was highly susceptible to denaturation at lower protein concentrations. Consequently, the purification of the sea star MAP kinase activator was performed on a continuous basis over a 24 h period. Characterization of the enzyme was performed immediately following its purification.

	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification fold
Crude extract	1000	2000	12500	6.3	100	1
DEAE-cellulose *	1000	1500	9100	6.1	78	1
Hydroxylapatite	104	29	1400	48	11	8
S-sepharose	60	4	475	119	4	19
Phosphocellulose	21	1	348	348	3	55
Heparin-agarose	20	0.15	388	2590	3	411
Mono S	2	0.008	27	3380	0.02	536

Table 10: The Erk1 activator passed in the breakthrough material of the DEAE-cellulose. The protein concentration was determined from this material. One unit of MAP kinase activity was defined as the amount of enzyme that would activate the recombinant protein GST-Erk1, resulting in the phosphorylation of MBP at a rate of 1 pmol/min under assay conditions described in Materials and Methods.

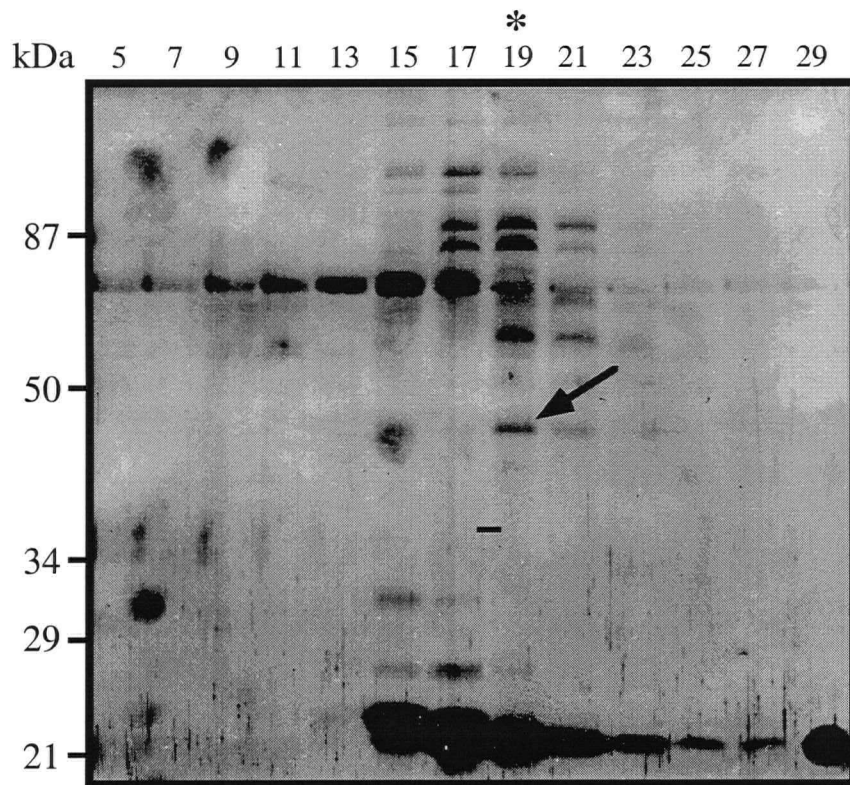
### 3.3 Identification of the purified MAP kinase activator

To determine the purity of the sea star MAP kinase activator after the final Mono S column, fractions were electrophoresed on SDS-PAGE and the proteins revealed by the silver staining technique (Figure 22A). Both high and low molecular mass contaminating proteins were observed. However, at least three silver-stained bands of 82-90-kDa, and 44-kDa displayed an enrichment that correlated with the Mono S fraction possessing the greatest ability to increase Erk1 MBP phosphotransferase activity in the two-step MAP kinase activator assay.

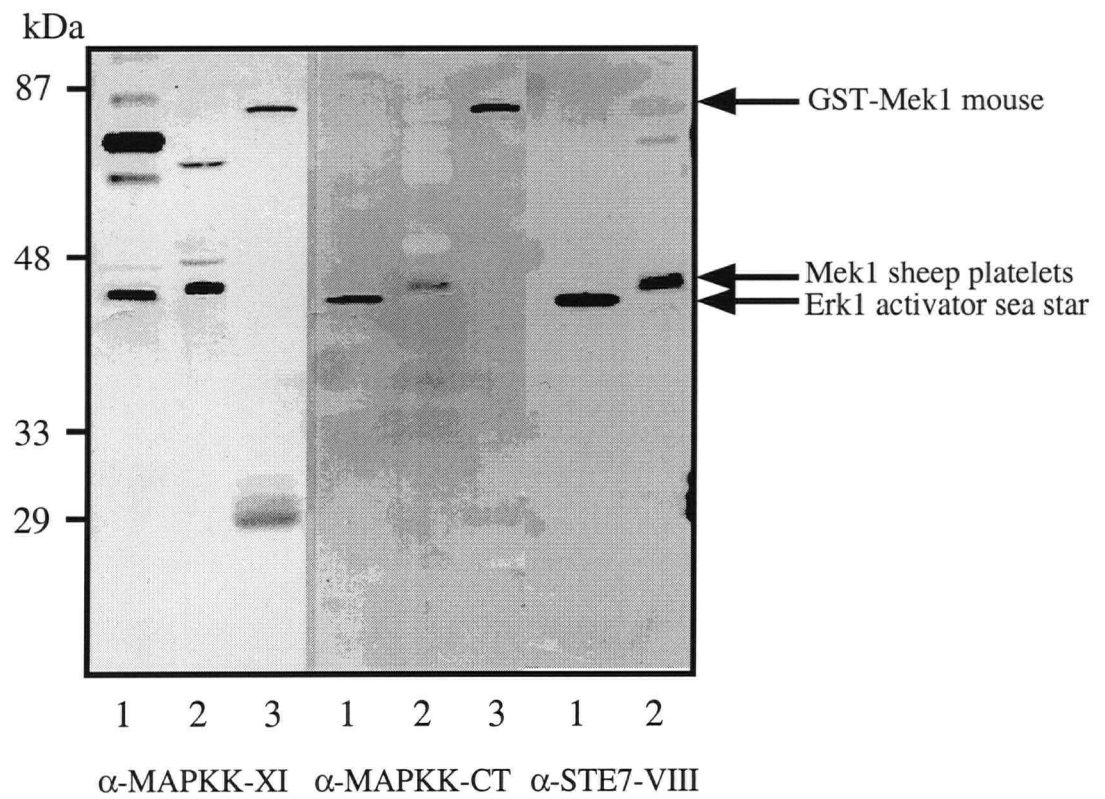
To clarify which band might be the active enzyme, the most purified preparation of MAP kinase activator was tested for its ability to undergo autophosphorylation in the presence of high specific activity  $\gamma^{32}\text{P}[\text{ATP}]$ . Although autophosphorylation *in vitro* is known to occur with many kinases, the purified MAP kinase activator failed to undergo any self-phosphorylation. Therefore, a different approach was necessary to confirm which of the silver-stained polypeptide bands represents the MAP kinase activator. Western blot analysis was performed on the most purified preparation. The Mono S fractions displaying the highest sea star Erk1 activator activity were combined before electrophoresis on SDS polyacrylamide gel. After protein transfer, the nitrocellulose was immunoblotted with 3 distinct MAP kinase kinase polyclonal antibodies. These antibodies were raised against peptides patterned after the carboxy-terminus and subdomain XI of mouse Mek1 and subdomain VIII of budding yeast *Saccharomyces cerevisiae* (Teague *et al.*, 1986; Crews *et al.*, 1992). All three anti-peptide antibodies detected a 44-kDa band (Figure 22B, lane 1). No other bands were consistently detected with all three antibodies. As a control, partially purified sheep platelet MAP kinase kinase and glutathione-agarose affinity purified mouse recombinant GST-Mek1 were also

Figure 22: Analysis of Erk1 activator purification (A) The purified Erk1 activator fractions from the final Mono S column step were electrophoresed on a 10% SDS-PAGE. The separated proteins were revealed by silver staining (see Material and Methods). The fraction containing the peak Erk1 activator activity is denoted by an asterisk. An arrow indicates the position of the putative sea star Erk1 activator. (B) The peak Erk1 activity fraction and the adjacent fractions were amalgamated for Western analysis. The combined material was separated on a 10% SDS-PAGE and transferred to nitrocellulose. Purified sea star Erk1 activator (lane 1); partially purified sheep platelet Mek1 (lane 2); and recombinant mouse GST-Mek1 (lane 3) were probed with polyclonal antibodies directed against peptides specific for mouse MAPKK-XI and MAPKK-CT as well as yeast *Saccharomyces cerevisiae* Ste7-VIII (where the Roman numerals refer to the kinase subdomains of these proteins). The molecular masses of prestained standards are indicated: bovine serum albumin, 87-kDa; ovalbumin, 50-kDa; carbonic anhydrase, 34-kDa; soybean trypsin inhibitor, 29-kDa; and lysozyme, 21-kDa.

A.



B.



separated on the same gel (Figure 22B, lanes 2 and 3). Both the mouse and sheep isoforms were recognized by these same antibodies. In summary, it appears that the sea star MAP kinase activator is homologous to mammalian Mek. This is supported by the fact that the echinoderm isoform was detectable with three antibodies that were derived from three different kinase subdomain regions in Mek isoforms from two disparate model species.

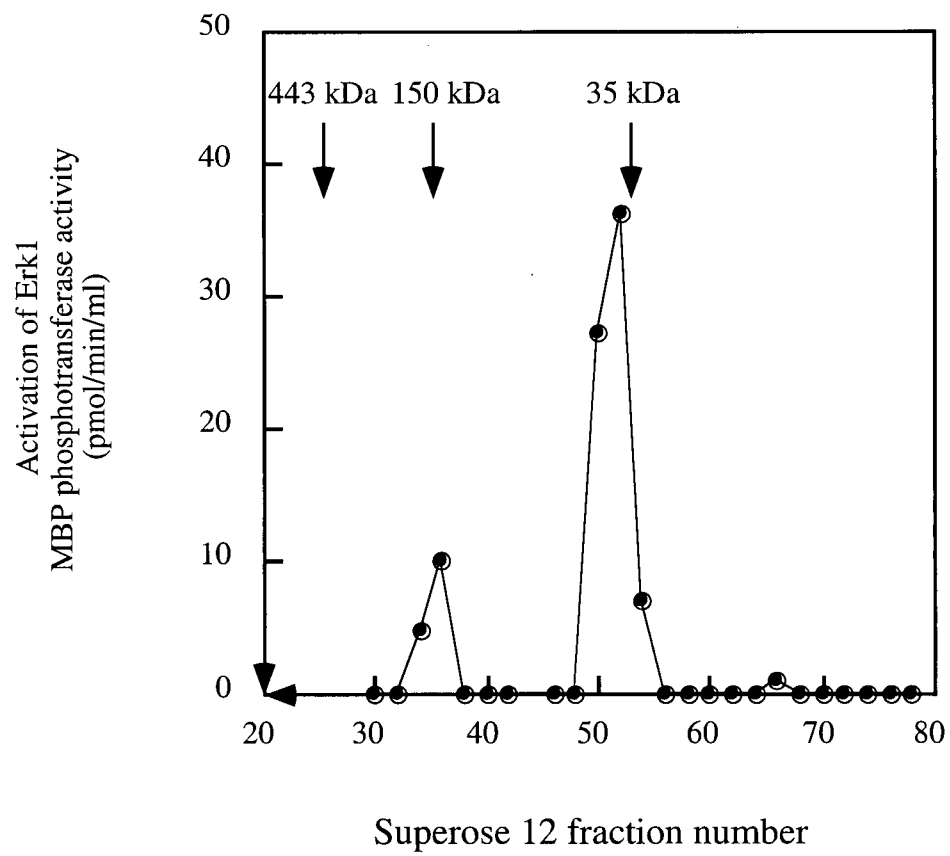
To verify the subunit composition of the MAP kinase activator, a small sample of the most purified material was subjected to gel filtration analysis on a Superose 12 column (Figure 23). The activator activity chromatographed as a double peak. The smaller peak eluted from the Superose column with an apparent molecular mass of 150 kDa. The larger peak was estimated to have a molecular mass of 40 to 44 kDa which was in the same size range as the enriched 44kDa band identified from the silver-stained gel (compare Figure 22A and B). These data cannot exclude the possibility that the smaller 44 kDa band was generated from the larger 150 kDa species observed from the Superose 12 column. It is feasible that the larger 150 kDa species from the Superose 12 column was a dimer composed of the smaller 44 kDa Mek-like protein and one of the enriched 80 to 90 kDa proteins that copurified. These results do not exclude the possibility that the larger species is a homotrimer of Erk1 activators

### 3.4 Mechanism of human Erk1 activation by the sea star MAP kinase activator *in vitro*

A number of research groups have reported that recombinant Erks expressed in bacteria were phosphorylated on tyrosyl *in vivo*. This phosphorylation was shown to be important for Erk kinase activity since treatment with the tyrosyl phosphatase CD45 caused its inactivation, while the seryl/threonyl protein phosphatase 2A had no effect on



Figure 23: Gel filtration chromatography of the Erk1 activator. A small volume (200  $\mu$ l) of activator protein purified from the final Mono S step was chromatographed on a Superose 12 gel filtration column in buffer B containing 150 mM NaCl. The fractions were analyzed for the presence of activator by assaying for recombinant Erk1 activation. Protein molecular mass standards used for calibrating the column are indicated: Blue Dextran, 443-kDa; alcohol dehydrogenase, 150-kDa; and  $\beta$ -lactoglobulin, 35-kDa.




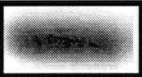

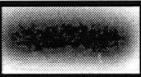
the enzyme (Seger *et al.*, 1991; Crews *et al.*, 1991; Wu *et al.*, 1991; Charest *et al.*, 1993). It was also shown that the Erks were capable of undergoing a slow intramolecular autophosphorylation on the regulatory Tyr-204 (Tyr-185 in Erk2) *in vitro* and that this was accompanied by a small increase in its MBP phosphotransferase (Rossomondo *et al.*, 1992; Robbins *et al.*, 1993; Charest *et al.*, 1993). These observations lead several researchers to speculate that MAP kinases may be regulated by an allosteric mechanism in which a non-enzymatic factor may be responsible for inducing conformational changes and thus increase its kinase activity (Seger *et al.*, 1991; Wu *et al.*, 1991).

To examine the mechanism of MAP kinase activation *in vitro*, affinity-purified recombinant Erk1 proteins immobilized on glutathione-agarose beads were incubated with the most purified preparation of sea star MAP kinase activator. It is evident that phosphorylation of Erk1 did increase in the presence of the sea star activator when compared to Erk1 alone (Figure 24A, compare lanes 2 and 4). Furthermore, the sea star activator was capable of augmenting phosphorylation on tyrosyl- threonyl- and seryl-residues on wild type Erk1 when assessed with its autophosphorylated counterpart (Figure 24C). It is noteworthy that the increased phosphorylation of wild type Erk1 resulted in a 3-fold increase in its phosphotransferase activity (Figure 24B, compare lanes 2 and 4). This is expected if a kinase is to be activated in a robust fashion after sea star oocyte exposure to the hormone 1-MeAde. However, the same sea star activator was unable to phosphorylate the catalytically-compromised Erk1 (TEY\*) in a similar assay to levels above what was obtained with Erk1 (TEY\*) alone (Figure 24A lanes 1 and 3). As expected, the kinase-inactive Erk1 (TEY\*) was unable to phosphorylate the MBP substrate (Figure 24B lanes 1 and 3). These data indicate that the purified sea star MAP kinase activator was capable of activating Erk1 by a phosphotransferase-independent mechanism perhaps by inducing a conformation change that opened the active site of the Erk1 kinase and promoted its activation by autophosphorylation. It is also possible that


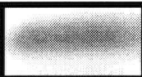
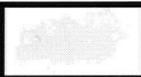
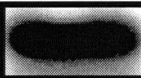
the sea star activator interacts weakly with human Erk1 and that a mutation in Lys-72 may lead to small conformational changes that could further destabilize interactions between the two proteins. In fact, comparison between human Erk1 and sea star Mpk1 MAP kinase isoforms that was cloned recently for our laboratory revealed that the two enzymes possess lower sequence homology in the amino- and carboxy-terminal regions of the enzymes (data not shown). Consequently, lower amino acid conservation combined with small conformational changes due to the Lys-72 substitution may be sufficient to prevent phosphorylation of the catalytically compromised Erk1 by the sea star MAP kinase activator.

Figure 24: Sea star Erk1 activator phosphorylation and activation of GST-Erk1. Recombinant kinase inactive (TEY\*) and wild type (TEY) GST-Erk1 were combined without (-) or with (+) purified sea star activator and  $\gamma$ - $^{32}\text{P}$ [ATP] (9000 cpm/pmol) and assayed for Erk1 phosphorylation (A) or activation of MBP phosphotransferase activity (B) during a 20 min time period. The reaction was quenched with 5X gel loading. After separating the proteins by 10% SDS-PAGE, they were transferred to nitrocellulose and the phosphate incorporation into Erk1 and MBP assessed by autoradiography. Quantitation was obtained by excising the phosphorylated bands for liquid scintillation counting. In a separate experiment, the wild type GST-Erk1 band in (A) was excised and hydrolyzed with constant-boiling HCl and the free amino acid assessed by phosphoamino acid analysis (C) as outlined in Materials and Methods.

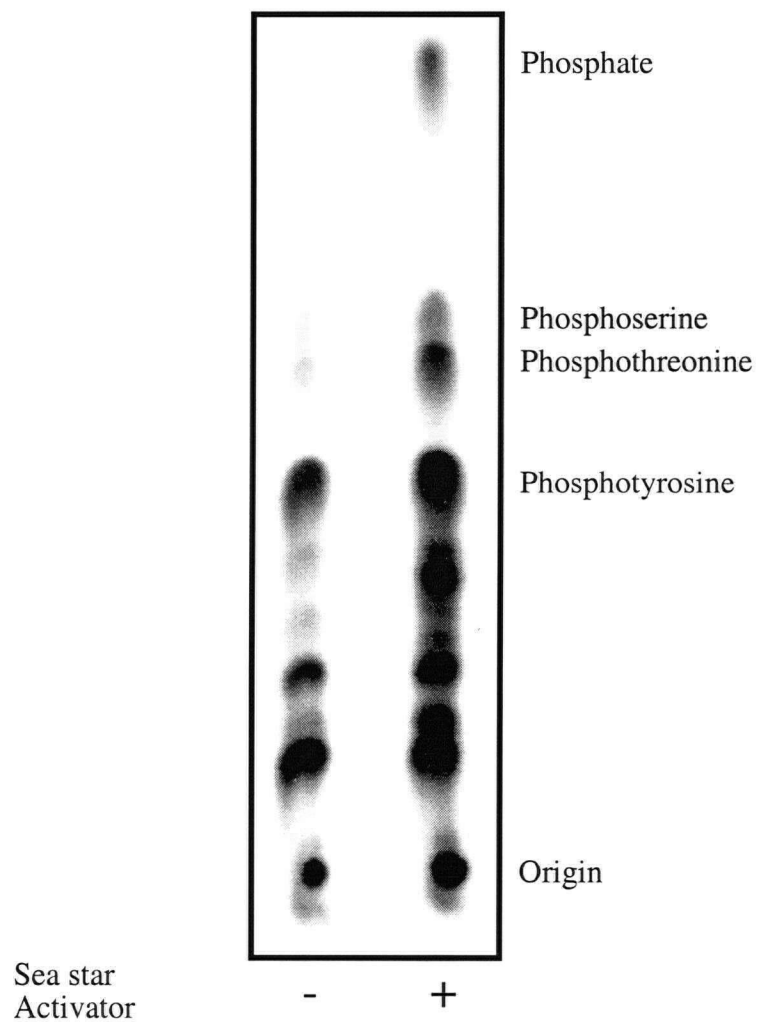
A

	TEY*	TEY	TEY*	TEY
GST-Erk1				
pmol/min/ml	0.01	1.5	0.05	2.4
Sea star Activator	-	-	+	+
Lane	1	2	3	4

B

	TEY*	TEY	TEY*	TEY
MBP				
pmol/min/ml	0.1	46	0.2	142
Sea star Activator	-	-	+	+
Lane	1	2	3	4

C



#### 4. MOLECULAR ANALYSIS OF THE REGULATORY PHOSPHORYLATION SITES (TEY) WITHIN THE L12 ACTIVATION LIP OF ERK1

##### 4.1 Phosphorylation and activation of Erk1 by activated Mek1 (EE)

To conduct Erk1 phosphorylation site studies, a constitutively active mouse Mek1 mutant was constructed that could be expressed and purified from bacterial lysates as a GST-fusion protein (see Appendix 20). Site-directed mutagenesis using specific oligonucleotides and PCR was employed to alter the Raf1 phosphorylation sites on Mek1 (Zheng and Guan, 1994). Both Ser-218 and Ser-222 amino acids were exchanged with glutamic acid mimetics of phosphoresidues. The recombinant double glutamic acid allele, Glu-218/Glu-222-GST-Mek1 (termed Mek1 (EE)), activated Erk1 MBP phosphotransferase activity 900-fold above what the dephosphorylated wild type Mek1 could activate this same enzyme under identical experimental conditions (Figure 42, Appendix 20).

Erk2 has been shown previously to be activated by phosphorylation on neighbouring Thr-183 and Tyr-185 residues *in vivo* (Thr-202 and Tyr-204 in Erk1) (Payne *et al.*, 1991). Subsequently, phosphorylation on these same sites in MAP kinase isoforms Erk1 and Erk2 *in vitro* was demonstrated by addition of partially purified preparations of Mek from mammalian tissue or with marginally activated forms of recombinant Mek from *E. coli* (Haystead *et al.*, 1992; Robbins *et al.*, 1993; Charest *et al.*, 1993; Butch and Guan, 1996). However, the results described in these publications were not conclusive since phosphoamino acid analysis revealed that Mek1 phosphorylated Erk protein principally on tyrosyl residues and to a lesser degree on threonyl residues. This contrasted *in vivo* labelling studies that showed that activation of Erk MBP

phosphotransferase activity required phosphorylation on neighbouring threonyl and tyrosyl residues (Payne et al., 1991).

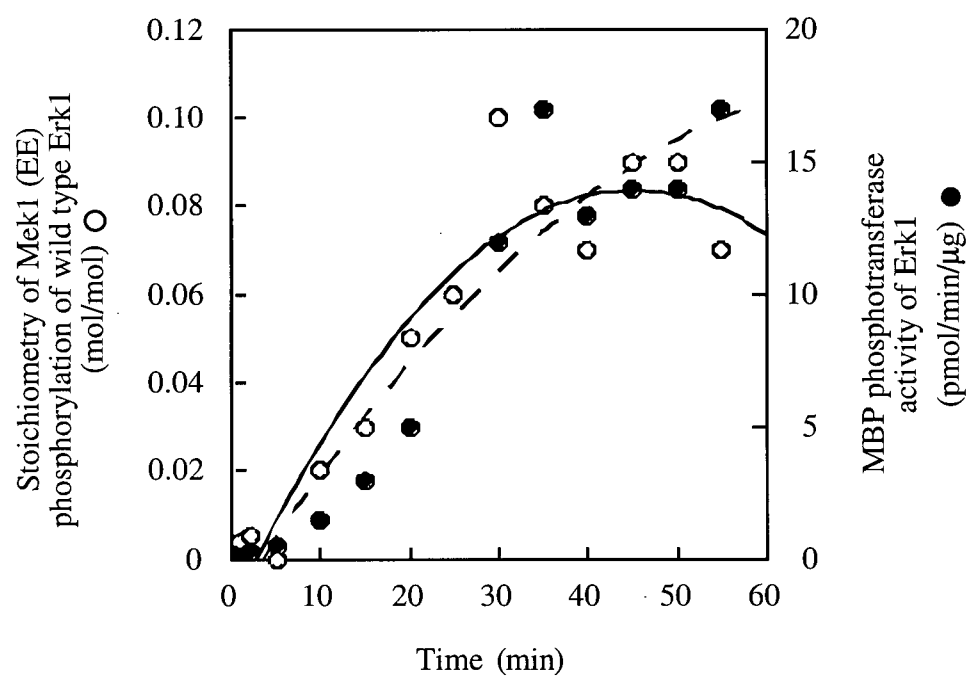
To confirm these previous reports, a time course of Erk1 activation by Mek1 (EE) was compared with the appearance of phosphate incorporation into the three hydroxyl containing amino acids, serine, threonine and tyrosine of this same enzyme. The experiment was initiated by addition of [ $\gamma^{32}\text{-P}$ ] ATP (10,000 cpm/pmol) to an Eppendorf tube containing the Erk1 and Mek1 (EE) enzymes as well as the required cofactors. Thirty seconds prior to the completion of each time point, an aliquot was removed from the reaction mixture and added to a second tube containing the Erk1 exogenous substrate MBP. After incubation for one half minute, the reaction was terminated by addition of gel loading buffer and the proteins separated by SDS-PAGE. The amount of radioactive  $^{32}\text{P}$  incorporated into Erk1 attained a maximum of  $\sim 0.08$  mol phosphate/ mol enzyme after the first thirty minutes of incubation with activated Mek1 (EE) (Figure 25A). As expected, Erk1 MBP phosphotransferase activity increased in parallel with Mek1 phosphorylation albeit at a slightly delayed rate (Figure 25B). The specific enzyme activity of Mek1 (EE) activated Erk1 was approximately 15  $\mu\text{mol}/\text{min}/\text{mg}$ . A steady state level of Erk1 phosphotransferase activity was reached within forty minutes post ATP addition. Mek1 (EE) phosphorylated Erk1 below stoichiometric levels (2 mol phosphate/mol enzyme). This resulted in a poor activation of Erk1 MBP phosphotransferase activity.

The Erk1 bands in Figure 25A were subjected to phosphoamino acid analysis. Separation of the hydrolyzed phosphoamino acids by thin layer chromatography revealed that incorporation of phosphate by Mek1 (EE) occurred in an ordered manner (Figure 25B). As will be discussed later, similar results were obtained with a more active Mek1

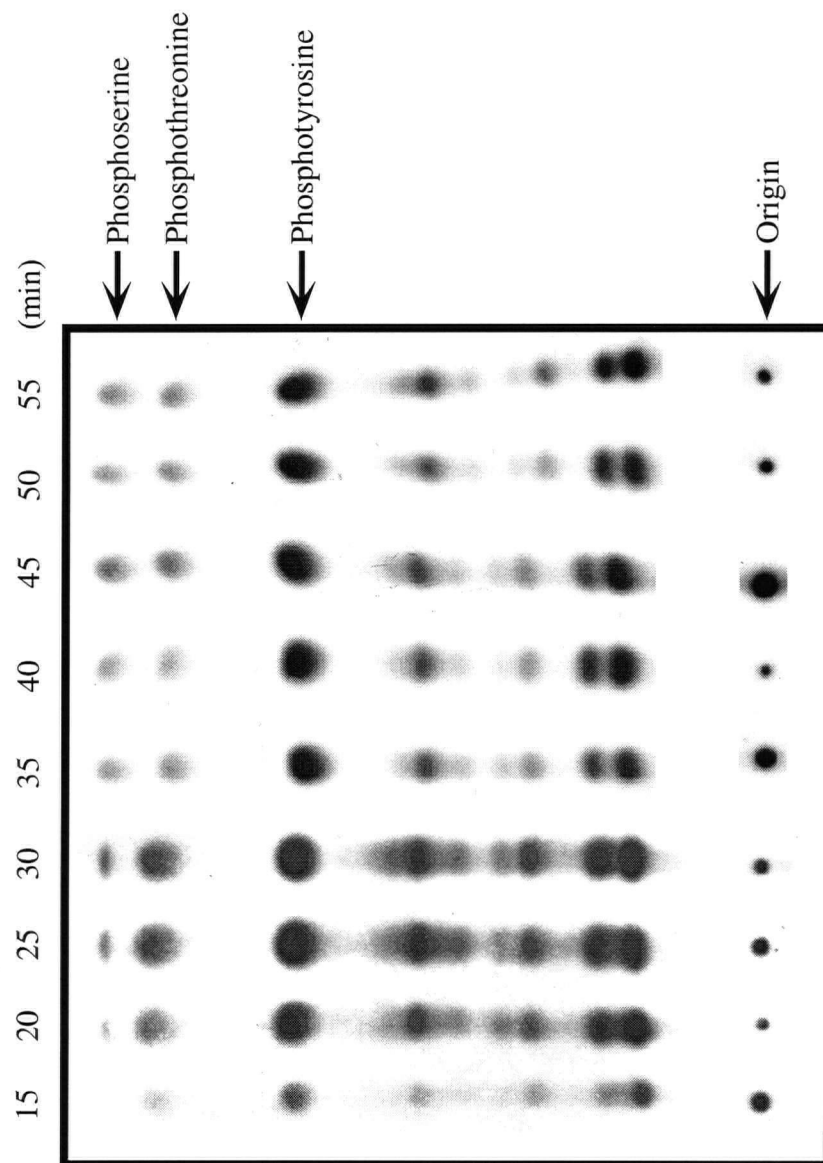


Figure 25: Time course of Mek1 (EE) phosphorylation, activation and phosphoamino acid analysis of wild type Erk1. Recombinant thrombin-cleaved Erk1 protein (~1  $\mu$ g) was incubated with eluted constitutively active GST-Mek1 (EE) (~53 ng) in the presence of [ $\gamma$ - $^{32}$ P] ATP (10,000 cpm/pmol) for the indicated times. At thirty seconds prior to completion of the reaction, a 5  $\mu$ l aliquot of MBP (5 mg/ml) was added to the mixture. The reaction was incubated for a further 30 sec before termination of the reaction by addition of 5  $\mu$ l of 4X gel loading buffer. The proteins were electrophoresed on a 10% Bio-Rad mini gel, transferred to PVDF and visualized by autoradiography. The radioactive bands were excised from the PVDF membrane. (A) The phosphorylated MBP bands were counted by liquid scintillation (dotted line, closed circles) while the phosphorylated Erk1 bands were counted by Cerenkov (solid line, open circles). (B) The recombinant Erk1 proteins were subjected to phosphoamino acid analysis. The hydrolyzed amino acids were separated by thin layer chromatography followed by autoradiography. The migration of the phosphoamino acid standards are indicated to the right of the panel

A.



B.



( $\Delta$ N3EE). In this experiment, the appearance of phosphotyrosine preceded that of phosphothreonine which, in turn, preceded phosphoserine (Figure 25B). However, all three phosphoresidues were present at their highest levels when Erk1 reached maximal activity. In fact, phosphoserine only became visible when Erk1 reached maximal MBP kinase activity. There have been no reports of Mek1 phosphorylating Erk1 on residues other than Thr-202 and Tyr-204 despite the fact that seryl autophosphorylation has been detected in MAP kinases phosphorylated *in vitro* (Robbins *et al.*, 1993; Charest *et al.*, 1993). In addition, Mek1 (EE) phosphorylation of Erk1 predominantly on tyrosine and to a lesser extent on threonine may explain the low specific enzyme activity achieved by Erk1 in these assays.

#### 4.2 Phosphorylation and activation of Erk1 by Mek1 ( $\Delta$ N3EE)

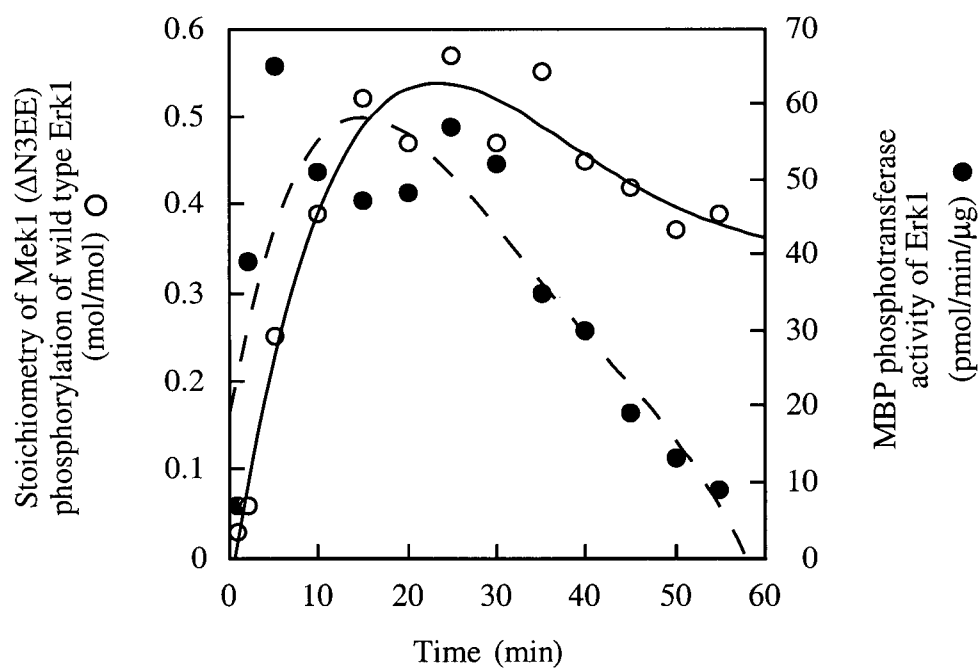
A second approach was used to create a more constitutively active Mek1 for stimulating Erk1 MBP phosphotransferase activity and mapping the sites of phosphorylation. To obtain a more active Mek1 protein kinase Mansour *et al.* (1994) identified an  $\alpha$ -helix structural motif (A-helix) in the amino-terminal region located outside the kinase catalytic domain that is implicated in the regulation of Mek1 protein kinase activity. Deletion of the hydrophilic region between residues 32 to 51 resulted in a mutant that was several fold more active than the basal activity of the recombinant dephosphorylated form of Mek1. Furthermore, the combination of deletion mutant and phosphorylation site substitutions with Glu-218 and Glu-222 caused an even greater Mek1 phosphotransferase activity toward Erk1 *in vitro* (Mansour *et al.*, 1994). This Mek1 hyperactive mutant induced a very dramatic increase in Erk1 MBP kinase activity when compared to the wild type protein.

To examine the pattern of phosphate incorporation into Erk1 by hyperactive Mek1 ( $\Delta$ N3EE), a deletion mutant using the mouse Mek1 (EE) cDNA clone was prepared as reported by Mansour *et al.* (1994). Identical reaction conditions and Mek1 protein concentration described in the previous Mek1 (EE) time course were used for the Erk1 phosphorylation and activation experiments with Mek1 ( $\Delta$ N3EE) hyperactive mutant (see Figure 25A). Mek1 ( $\Delta$ N3EE) phosphorylation of recombinant wild type Erk1 attained a maximum  $^{32}$ P incorporation of  $\sim 0.5$  mol phosphate/ mol of enzyme within ten minutes of initiating the reaction (Figure 26A). The amount of phosphate incorporated into Erk1 was 6-fold greater for the  $\Delta$ N3EE allele than what was observed with the EE allele. This steady state level was maintained for most of the experiment, except for a minor decline near the completion of the time course. Again the Erk1 enzyme was not phosphorylated to stoichiometric levels in this experiment. Mek1 ( $\Delta$ N3EE) phosphorylated Erk1 reached a maximum enzyme activity of  $\sim 50$   $\mu$ mol/min/mg within 10 min of initiating the reaction. In fact, Mek1 ( $\Delta$ N3EE) activated Erk1 three-fold higher in one third less reaction time it took Mek1 (EE) to phosphorylate Erk1 to its maximal level (compare Figure 25A with Figure 26A). However, in the later time points Erk1 MBP phosphotransferase activity declined to near basal levels.

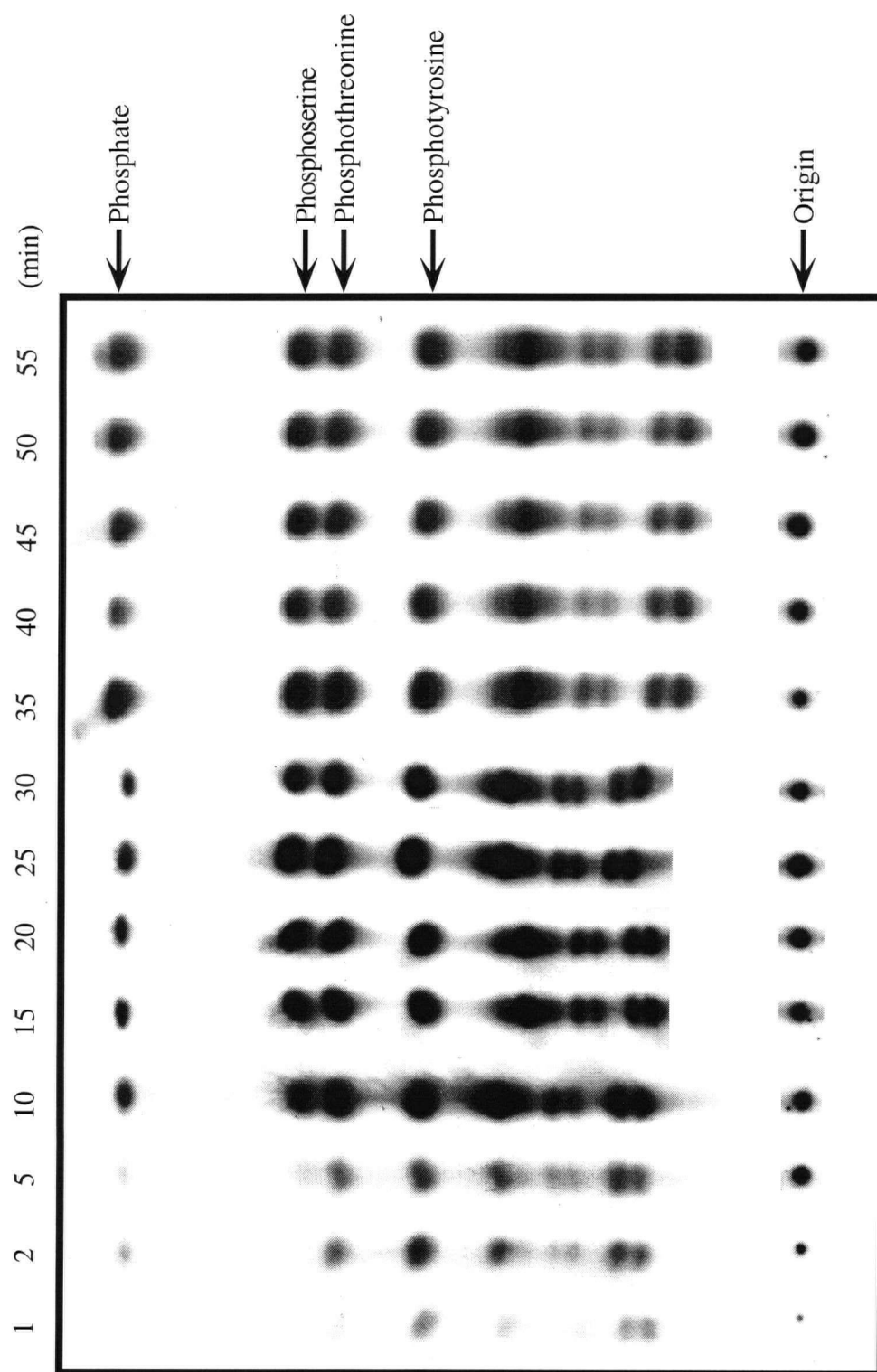
To determine the amino acids phosphorylated by Mek1 ( $\Delta$ N3EE), the Erk1 protein was hydrolyzed with acid and subsequently separated in one-dimension on a thin layer chromatography plate. After one minute, Erk1 was predominantly phosphorylated on tyrosyl (Figure 26B). As the reaction progressed phosphothreonyl became more apparent. Finally, all three phosphoamino acids became manifest when Erk1 reached full enzymatic activity at ten minutes. In fact, the appearance of phosphoserine with Erk1 peak activation was reminiscent of what occurred with Mek1 (EE) phosphorylation and activation of Erk1 in that the appearance of phosphoserine occurred when Erk1 reached maximal activity (Figure 26B). However, seryl phosphorylation in the presence of Mek1

Figure 26: Time course of Mek1 ( $\Delta$ N3EE) phosphorylation, activation and phosphoamino acid analysis of wild type Erk1. Recombinant thrombin-cleaved Erk1 protein ( $\sim 1 \mu\text{g}$ ) was incubated with eluted constitutively active GST-Mek1 ( $\Delta$ N3EE) ( $\sim 53 \text{ ng}$ ) in the presence of  $[\gamma\text{-}^{32}\text{P}] \text{ ATP}$  ( $10,000 \text{ cpm/pmol}$ ) for the indicated times. At thirty seconds prior to completion of the reaction, a  $5 \mu\text{l}$  aliquot of MBP ( $5 \text{ mg/ml}$ ) was added to the mixture. The reaction was incubated for a further 30 sec before termination of the reaction by addition of  $5 \mu\text{l}$  of 4X gel loading buffer. The proteins were electrophoresed on a 10% Bio-Rad mini gel, transferred to PVDF and visualized by autoradiography. The radioactive bands were excised from the PVDF membrane. (A) The phosphorylated MBP bands were counted by liquid scintillation (dotted line, closed circles) while the phosphorylated Erk1 bands were counted by Cerenkov (solid line, open circles). (B) The recombinant Erk1 proteins were subjected to phosphoamino acid analysis. The hydrolyzed amino acids were separated by thin layer chromatography followed by autoradiography. The migration of the free-phosphate and phosphoamino acid standards are indicated to the right of the panel.

A.



B.





( $\Delta$ N3EE) was more robust. These results revealed that Erk1 became phosphorylated on all three hydroxylamino acids in a Mek1-dependent manner. Although Mek1 has been demonstrated to phosphorylate catalytically-inactivated Erk1 on threonyl and tyrosyl residues both *in vivo* and *in vitro*, these data are unable to negate the possibility that Mek1 could phosphorylate sites distinct from the TEY regulatory motif. Alternatively, full activation of Erk1 by Mek1 ( $\Delta$ N3EE) may promote a more pronounced Erk1 autophosphorylation on serine.

#### 4.3 Mutational analysis of Erk1 regulatory phosphorylation sites

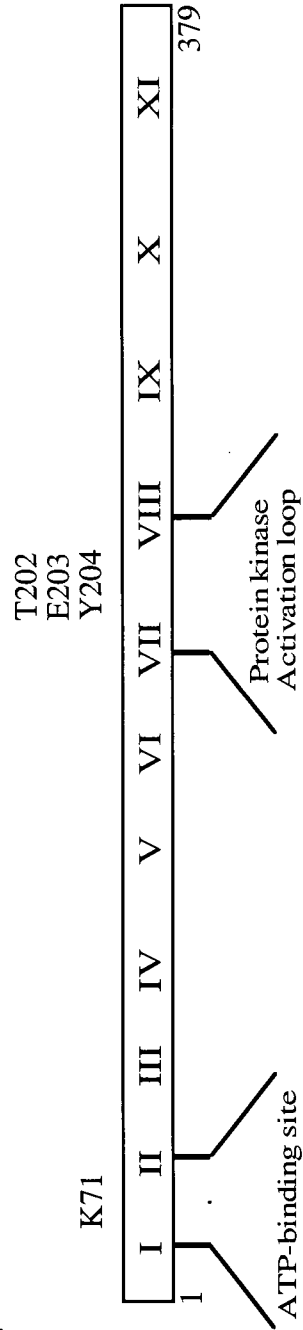
With the exception of Erk3 isoforms, all MAP kinases examined to date possess the canonical TXY phosphorylation sequence (Figure 27A). Erk3 appears to be regulated by phosphorylation on a seryl residue located at the same position as Thr-183 in Erk2 (Thr-202 in Erk1), while the homologous tyrosyl residue is substituted with a glycyl residue (Figure 27A). This implies that Erk3 may be regulated by a mechanism distinct from the other MAP kinase family members (Pelech and Charest, 1995). X-ray crystallographic studies revealed that Thr-183 and Tyr-185 phosphorylation residues in Erk2 protein are contained within a loop structure (L12) known as the activation loop that lies within the cleft of the active site (Figure 27B and see Zhang *et al.*, 1994). In the inactive form of Erk2, Tyr-185 (Tyr-204 in Erk1) is buried within the active site of the kinase, whereas Thr-183 (Thr-202 in Erk1) is exposed on the outer surface of the protein. It is predicted that phosphorylation of both these regulatory sites would cause a dramatic reorganization of enzyme structure and assist in stabilization of MAP kinase in the active conformation. Therefore, it is expected that the amino acid residues that comprise the TXY phosphorylation motif play a central role in recognition by the cognate upstream MAP kinase kinase activator.

Figure 27: Sequence alignments of MAP kinase isoforms. A. Sequence comparisons of the activation loop for several MAP kinase isoforms human (Charest et al., 1993); Erk2 (Gonzalez et al., 1992); Erk3 (Gonzalez et al., 1992); Jnk1 (Dérjard et al., 1994); Hog1 (Lee et al., 1994) and sea star Mpk1 (Posada et al., 1991). The two regulatory phosphorylation sites (TXY) are each designated with an asterisk and the intervening amino acid X is outlined with a box. B. Schematic representation for human Erk1 mutation sites (Lys-71, Thr-202, Glu-203 and Tyr-204). The site of ATP-binding, within the kinase catalytic domain is located in subdomains I and II; a region which is conserved in all kinases studied to date (Hanks et al., 1988). The regulatory phosphorylation sites for many kinase are located at within the enzyme activation loop between subdomains VII and VIII (Marshall, 1993).

A.

		<b>VII</b>		<b>VIII</b>
Erk1	human	DFGLARVADPDHDHTGFLT	* *	DFGLARVADPDHDHTGFLT
Erk2	human	DFGLARVADPDHDHTGFLT		DFGLARVADPDHDHTGFLT
Erk3	human	DFGLARIMDPHYSHKGHL		DFGLARIMDPHYSHKGHL
Jnk1	human	DFGLARTACTNFM	---	DFGLARTACTNFM
Hog1	Human	DFGLARHTDDE	---	DFGLARHTDDE
Mpk1	sea star	-----ADPV?D?TGFLT	!	-----ADPV?D?TGFLT

B.

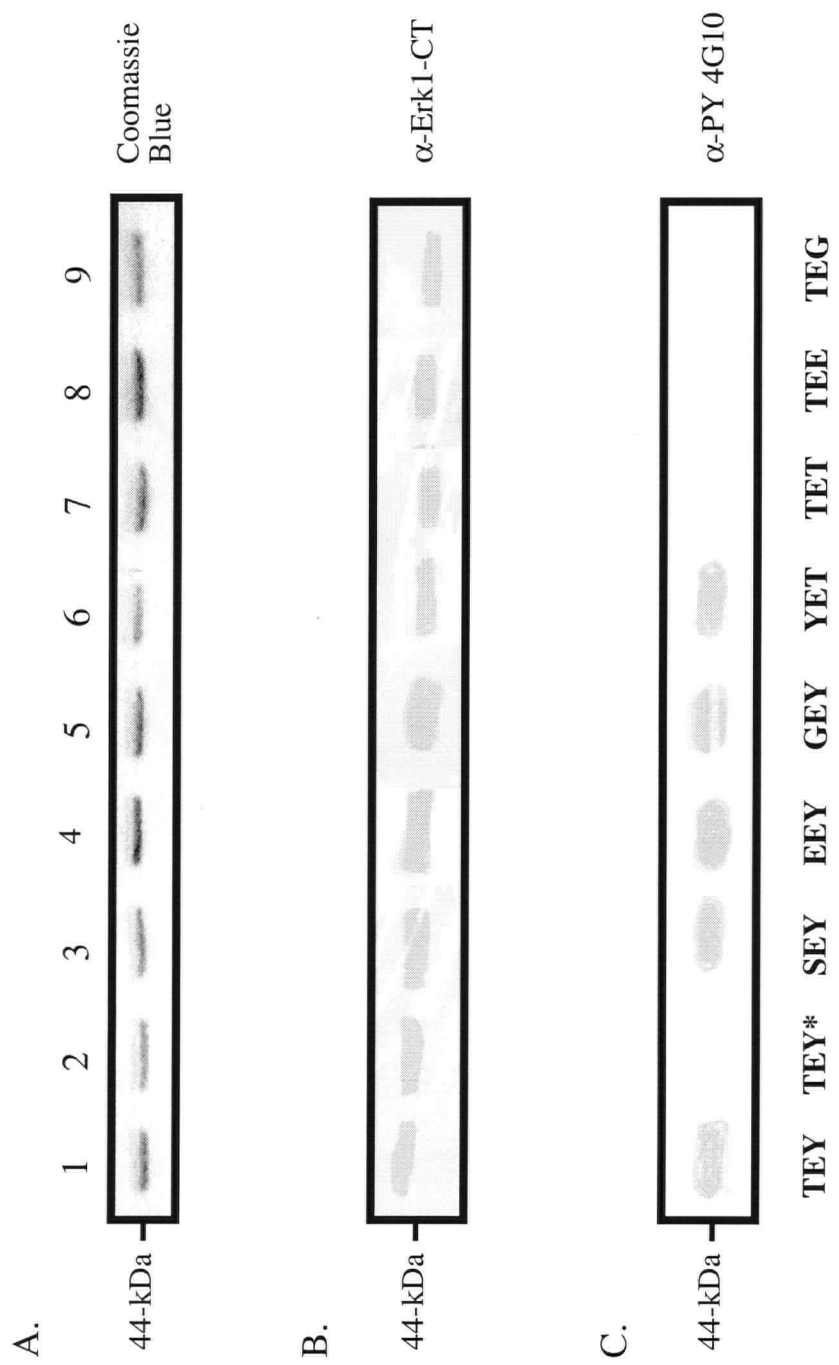


Mek1 displays a very narrow substrate specificity for Erk enzymes, since it is unable to phosphorylate non-native forms of the Erk1 or short peptides containing the TEY sequence (Seger *et al.*, 1992a). Furthermore, Mek1 also phosphorylates other exogenous substrates *in vitro* with reduced efficacy relative to Erk1 phosphorylation. To elucidate Mek1 substrate specificity, seven site-directed mutations were generated in Thr-202 and Tyr-204 regulatory phosphorylation sites of Erk1 (Table 9). Mek1 activation sites Thr-202 and Tyr-204 were exchanged with conserved phosphorylatable amino acids serine and threonine, respectively, to determine the effect these mutations have on Mek1 recognition. Also, a double mutant, in which the TEY phosphorylation site was inverted, was constructed to assess the effect orientation has on Mek1 phosphorylation. The non-polar glycyl amino acid that is present in Erk3 in place of tyrosyl found in other members of the MAP kinase family was used to substitute Thr-202 and Tyr-204 in Erk1, while the phosphorylation mimetic, glutamic acid, replaced these same regulatory sites.

#### 4.4 Analysis of autophosphorylation and basal MBP phosphotransferase activities of Erk1 regulatory phosphorylation site alleles

The different Erk1 alleles were expressed under identical growth conditions. Each allele was expressed as a GST-fusion protein and purified by adsorption onto glutathione-agarose resin. The Erk1 enzyme was released from the GST-beads by thrombin cleavage, resolved by SDS-PAGE and subsequently visualized by staining with Coomassie Blue dye or transferred to nitrocellulose and immunoblotted with Erk1-CT antipeptide antibody (Figure 28A and B). Erk1 migrated as a 42- and 44-kDa doublet (Figure 28A). The enzyme concentration was adjusted to 1 µg of protein for all kinase reactions (Figure 28A). MAP kinases Erk1 and Erk2 are known to autophosphorylate slowly on tyrosyl when expressed as recombinant proteins in bacteria (Seger *et al.*, 1991; Crews *et al.*, 1991; Wu *et al.*, 1991, Rossomondo *et al.*, 1992; Robbins *et al.*, 1993;

Figure 28: Expression, immunodetection and quantitation of recombinant Erk1 proteins from bacteria. The regulatory phosphorylation site mutant allele constructs were transformed into *E. coli* strain UT 5600 and subsequently grown in 2 x YT medium. The collected cells were disrupted in homogenization buffer P and the recombinant proteins purified by glutathione affinity chromatography. Thrombin digestion liberated the Erk1 protein from its GST fusion. Approximately 1  $\mu$ g of protein was subjected to 10% SDS-PAGE Bio-Rad mini-gels. The proteins were quantitated by Coomassie Blue staining (A) or transferred to nitrocellulose for Western blotting (separate gels) with Erk1-CT polyclonal antibody (B) or phosphotyrosine specific 4G10 monoclonal antibody (C).



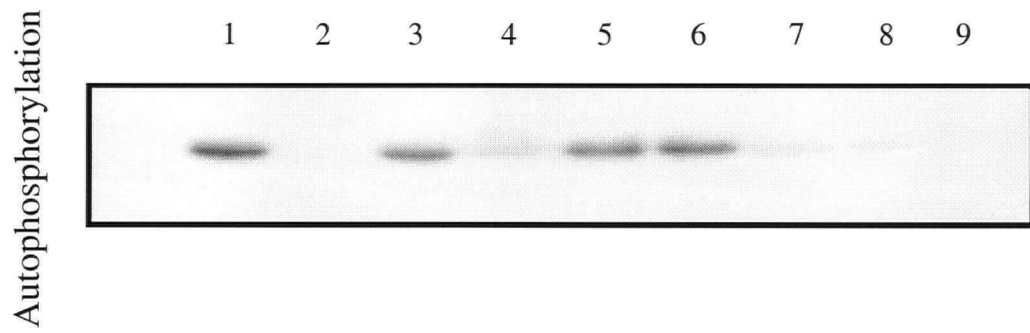
Charest *et al.*, 1993). As expected, Erk1 wild type TEY displayed tyrosyl phosphorylation after Western blotting with the antiphosphotyrosine 4G10 monoclonal antibody (Figure 4C). The catalytically-inactive Erk1 TEY\*, however, displayed no immunoreactivity with 4G10 antibody since mutation of Lys-71, an amino acid critical for phosphotransferase activity, compromised the capacity of the enzyme to autophosphorylate on tyrosyl residue (Figure 28C). Substitution of Thr-202 with seryl, glutamyl or glycyl residues seemed to have little effect on basal autophosphorylation, while similar mutations at Tyr-204 abolished the phosphotyrosyl signal (Figure 28C). These results indicate that Tyr-204 is the major site of tyrosyl autophosphorylation when Erk1 is expressed in bacteria. The dual phosphorylation site was reversed so that Thr-202 was replaced by tyrosine and Tyr-204 by threonine. Surprisingly, Erk1 YET displayed comparable immunoreactivity with the 4G10 antibody as seen with wild type TEY autophosphorylation. These preliminary data imply that there may be some flexibility with regards to the orientation and amino acid composition at these two phosphorylatable sites. To verify whether the narrow substrate specificity of Mek1 is determined by the class of amino acid present at positions 202 and 204, both constitutively active Mek1 mutants were used to phosphorylate and activate the Erk1 regulatory phosphorylation site mutants.

First it was important to test the ability of each Erk1 allele to further autophosphorylate *in vitro* was assessed in a ten and thirty minute assay reactions. These time points were selected based on the ability of Mek1 (EE) and ( $\Delta$ N3EE) to maximally activate wild type Erk1 MBP phosphotransferase activity. The pattern of autophosphorylation supports the observations made with the antiphosphotyrosine Western blot analysis in that those enzymes with an intact Tyr-204 present were able to undergo autophosphorylation (compare Figure 28C, 29A and 31A). The three Erk1 Thr-202 mutant alleles, SEY, GEY, EEY and the inverted YET allele autophosphorylated to

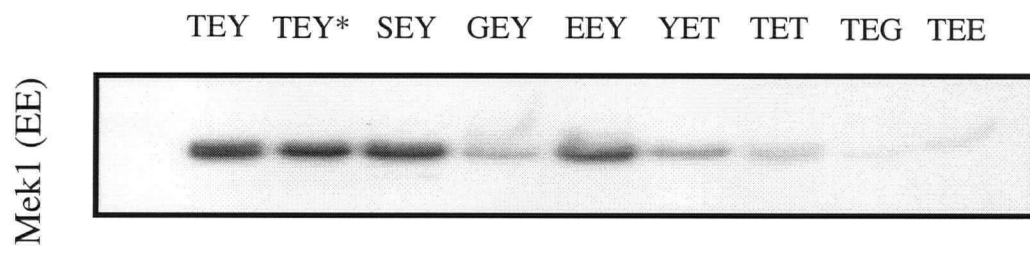




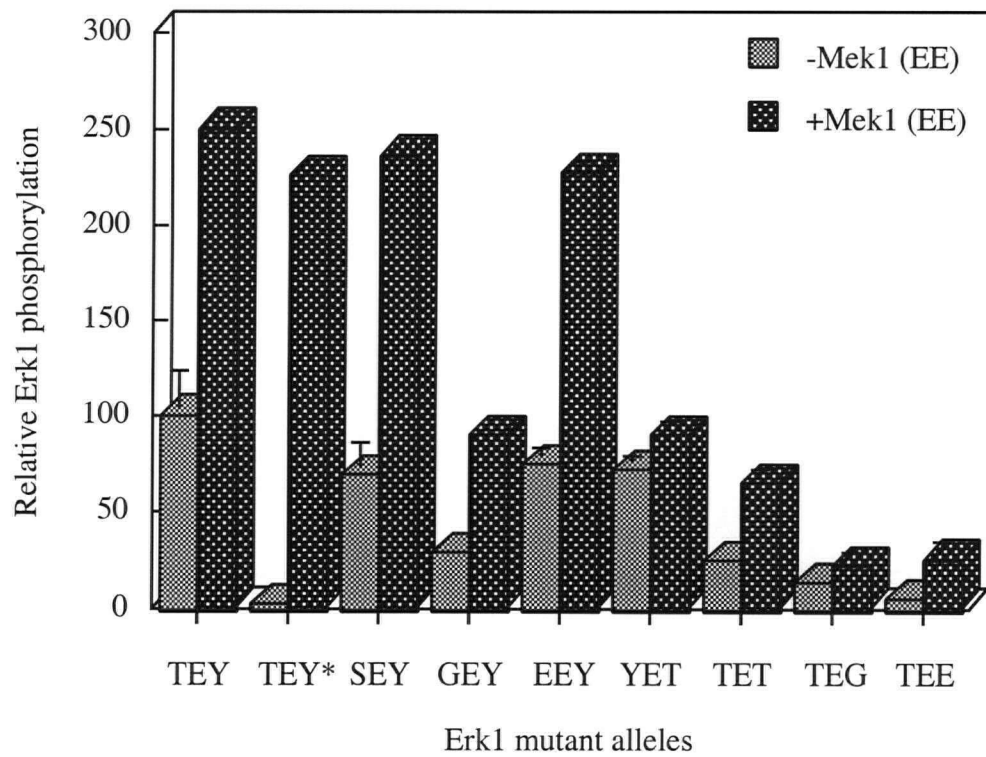
A.



B.



C.



40-75% of wild type TEY (Figure 29A and 31A). In contrast, the Tyr-204 phosphorylation site mutants TET, TEG, and TEE mustered on average less than one quarter of the self-phosphorylation of the Erk1 wild type (Figure 29A and 31A). No appreciable kinase activity was observed for any of the recombinant dephosphorylated forms of Erk1 in a 30 sec MBP phosphotransferase reaction (Figure 30 and 32). These data indicate alteration of either regulatory phosphorylation site causes a reduction of erk1 autophosphorylation, however, a more pronounced effect was observed at Thr-202 than with Tyr-204 mutants alleles. Overall, neither Erk1 wild type nor the mutant alleles displayed a strong MBP phosphotransferase activity in the absence of Mek1 phosphorylation.

To understand what effect the Erk1 phosphorylation site mutations may have on Mek1 substrate recognition and phosphorylation, the various Erk1 alleles were combined with constitutively active isoforms of Mek1 ( $\Delta$ N3EE) and (EE) for ten and thirty minute kinase reactions, respectively. Previously, Erk1 was demonstrated to be phosphorylated and activated maximally at these two time points (Figure 26 and 27).

#### 4.5 Mek1 (EE) phosphorylation and activation of Erk1 regulatory phosphorylation site alleles

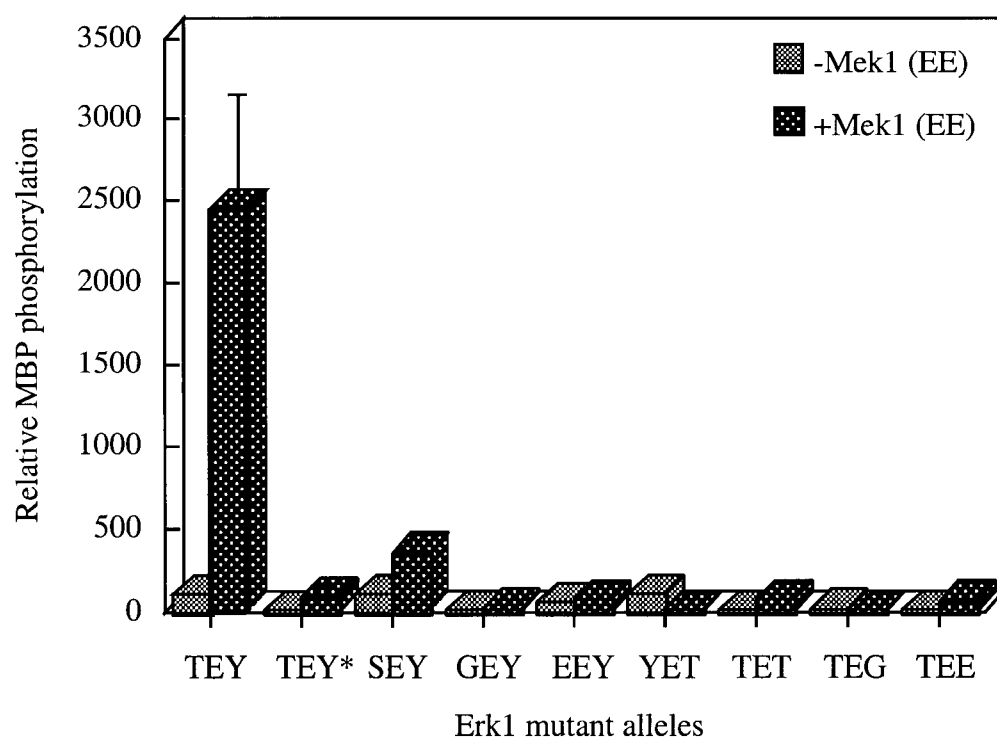
In a thirty minute assay, Mek1 (EE) phosphorylated Erk TEY, TEY\*, SEY, and EEY alleles 2.5- to 3.0-fold above the level of autophosphorylation of these same enzymes (Figure 29). Moreover, the analysis of variance test showed that the amount of radioactive  $^{32}\text{P}$  label incorporated into TEY\*, SEY and EEY was not significantly different from that of Erk1 wild type TEY; however only the wild type form of the Erk1 was effectively activated by Mek1 (EE). This constitutively active Mek1 (EE) caused a 25-fold stimulation of Erk1 wild type MBP phosphotransferase activity above that of

Figure 30: Mek1 (EE) activation of Erk1 regulatory phosphorylation site mutant alleles. The Erk1 activation was performed as a two-step assay in the presence of [ $\gamma$ - $^{32}$ P] ATP (1250 cpm/pmol). Erk1 protein (~1  $\mu$ g) was incubated without (light grey) and with (dark grey) constitutively active Mek1 (EE) (~53 ng) before performing the MBP kinase assay. At the completion of the 29 min preincubation described in Figure 25A, a 5  $\mu$ l aliquot of the exogenous substrate MBP (5 mg/ml) was added to the mixture. The reaction was incubated for a further 1 min before addition of 5  $\mu$ l of 4X gel loading buffer. The Mek1 activation activity is normalized to Erk1 wild type basal autoactivation activity which is given a value of 100; mean  $\pm$  s.d., n=3 (Erk1 wild type MBP phosphotransferase activity varied between 750-1550 cpm/1 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEE TEY\* YET YET TET GEY TEG GEY EEY TEE TET EEY TEY TEY\* SEY YET SEY TEY**

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This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutant alleles incubated in the absence (bold face) and presence of Mek1 (EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the kinase-inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.



Erk1 alone (Figure 30). In contrast, the Erk1 SEY allele, a mutant that possesses a conserved threonyl to seryl substitution, achieved only 20% of wild type activity even though both alleles were phosphorylated to identical levels with Mek1 (EE) (Figure 29). Analysis of variance revealed that the activation of SEY was significantly different from the remainder of the mutant alleles.

#### 4.6      Mek1 ( $\Delta$ N3EE) phosphorylation and activation of Erk1 regulatory phosphorylation site alleles

Under the identical reaction conditions described above for the double mutant (EE), the Mek1 ( $\Delta$ N3EE) was demonstrated to stimulate maximally Erk1 wild type enzymatic activity within 10 min (Figure 26). Erk1 wild type TEY and kinase-inactive TEY\* were robustly phosphorylated 12- to 15-fold above Erk1 autophosphorylation, respectively (Figure 31C). In addition, a pronounced band shift was detected for phosphorylated Erk1 which has been previously reported to be indicative of the stimulation of enzyme activity. Almost no change in the electrophoretic mobility was observed with the catalytically-inactive Erk1 (Figure 31B compare lanes 1 and 2). The threonyl phosphorylation site mutants SEY, GEY and EEY in the presence of activated Mek1 ( $\Delta$ N3EE) were phosphorylated 2- to 4-fold less than the non-phosphorylation site mutants TEY and TEY\* (Figure 31). Comparable mutations at the tyrosyl site limited the phosphotransferase activity of Mek1 ( $\Delta$ N3EE) toward Erk1 at 1- to 3-fold above autophosphorylated wild type Erk1. However, activation of the MBP phosphotransferase activity of these regulatory site mutant alleles was incongruent with amount each of these enzymes was phosphorylated by Mek1 ( $\Delta$ N3EE) during the same reaction. Analysis of variance revealed that only wild type Erk1 TEY and the conserved SEY mutant displayed significant activation by Mek1 ( $\Delta$ N3EE) (Figure 32). Erk1 TEY was activated 230-fold above the endogenous kinase activity and twice the level observed for Erk1 SEY allele.

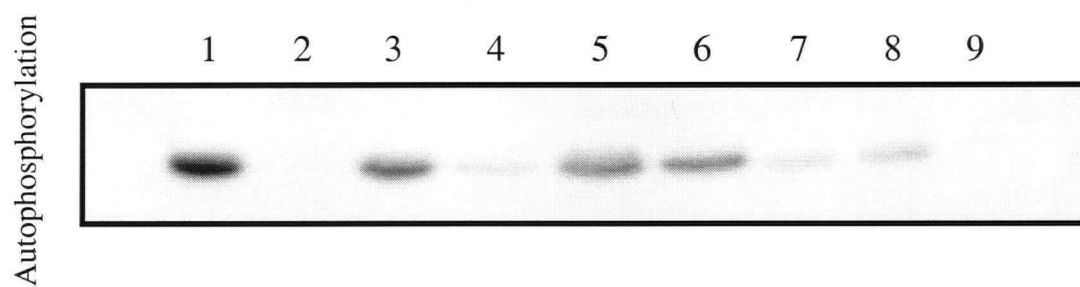
Figure 31: Mek1 ( $\Delta$ N3EE) phosphorylation of Erk1 regulatory phosphorylation site mutant alleles. Equal amounts of Erk1 recombinant proteins ( $\sim 1 \mu\text{g}$ ) were incubated without (A) or with (B) constitutively active recombinant Mek1 ( $\Delta$ N3EE) ( $\sim 53 \text{ ng}$ ) for 10 min in the presence of  $[\gamma\text{-}^{32}\text{P}] \text{ATP}$  ( $1250 \text{ cpm/pmol}$ ). The reaction was terminated by addition of 4X SDS-PAGE sample buffer. The proteins were separated on a 10% Bio-Rad mini gel, transferred to nitrocellulose and visualized by autoradiography. The radioactive bands were cut from Ponceau stained nitrocellulose and counted by liquid scintillation. (C), Erk1 phosphorylation without (light grey) or with Mek1 ( $\Delta$ N3EE) (dark grey) was normalized to Erk1 wild type autophosphorylation activity which was given a value of 100; mean  $\pm$  s.d.,  $n=3$  (Erk1 autophosphorylating activity varied between 5200-8300 cpm/10 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEY\* TEE GEY TEG TET SEY YET YET TEG EEY TEY TEE GEY TET EEY SEY TEY TEY\***

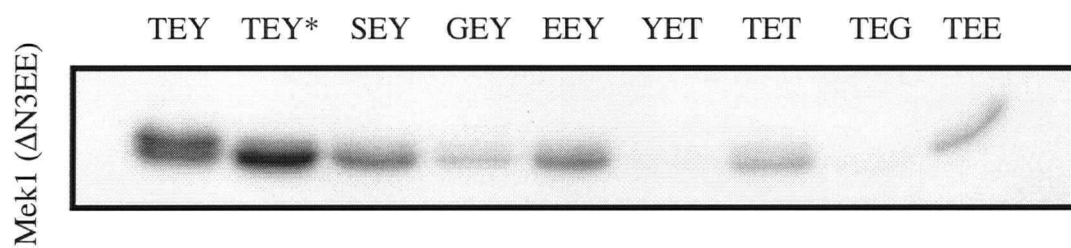


This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutants incubated in the absence (bold face) or presence of Mek1 ( $\Delta$ N3EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.

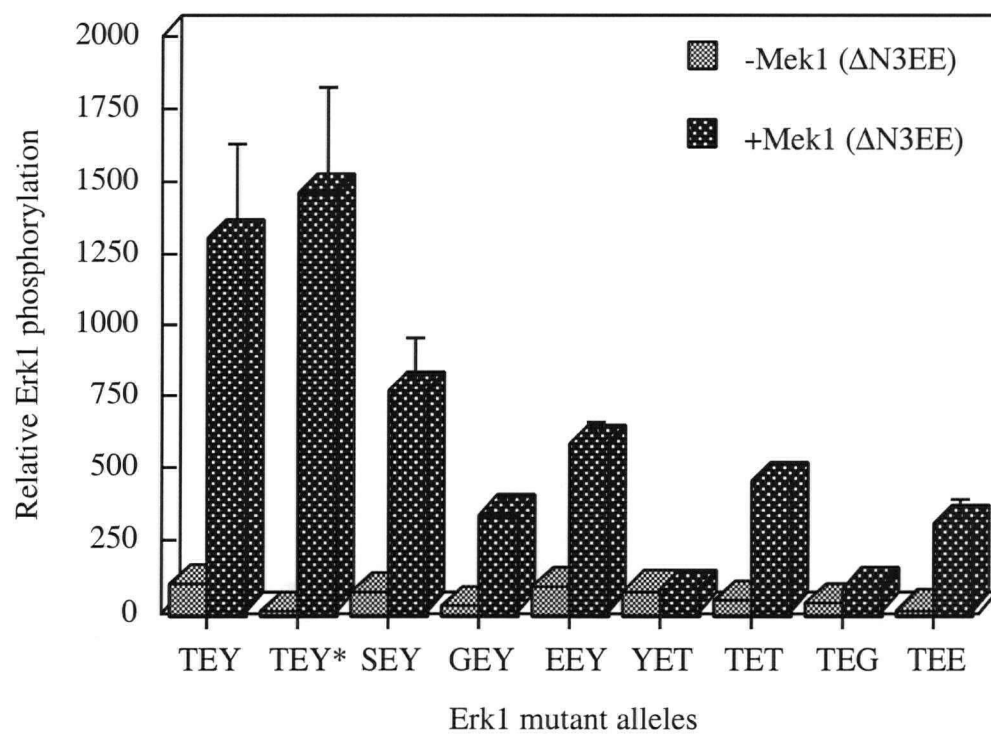
A.



B.



C.



No significant enzyme activation was observed for the remainder of the Erk1 alleles. Furthermore, comparisons between Mek1 (EE) and ( $\Delta$ N3EE) phosphorylation and activation of Erk1 proteins revealed that the triple mutant allele ( $\Delta$ N3EE) was able to stimulate both Erk1 TEY and SEY 10 to 20 times above the levels observed with the double mutant (EE) during a shorter incubation period (compare Figures 30 and 32).

Haystead *et al.* (1992) demonstrated that Erk protein kinases undergo phosphorylation in an ordered fashion. Initially, Mek1 phosphorylates Erk1 on tyrosyl followed by threonyl residues. Phosphoamino acid analysis of Erk1 phosphorylated by Mek1 (EE) revealed that the phosphotyrosyl content was 3- to 4-fold greater than phosphothreonine (Figure 25B). In comparison, the more active Mek1 ( $\Delta$ N3EE) phosphorylated wild type Erk1 equally on tyrosine and threonine (Figure 26B). The difference in the level of threonine phosphorylation by the two activated Mek1 alleles resulted in a ten-fold difference in the activation of Erk1 by each. Therefore, the phosphothreonine content of Erk1 correlates with the level of MBP phosphotransferase activity of this enzyme. With the exception of the Erk1 SEY allele and to a lesser degree the EEY allele, alteration of Thr-202 or Tyr-204 has a significant impact on the ability of Mek1 to phosphorylate Erk1. More importantly, only Erk1 SEY displays any significant MBP phosphorylating activity. The more robust Mek1 ( $\Delta$ N3EE) which has the deleted amino-terminal  $\alpha$ -helix appears to have less constraint in recognizing tyrosine when serine is present in position 202 of Erk1. The enzyme phosphorylated SEY 3-fold higher than Mek1 (EE) (compare Figure 29C and 31C) which resulted in 30-fold difference in Erk1 SEY kinase activity (compare figure 30 and 31). Further studies will be required to determine the degree to which Mek1 ( $\Delta$ N3EE) is able to phosphorylate Erk1 SEY.

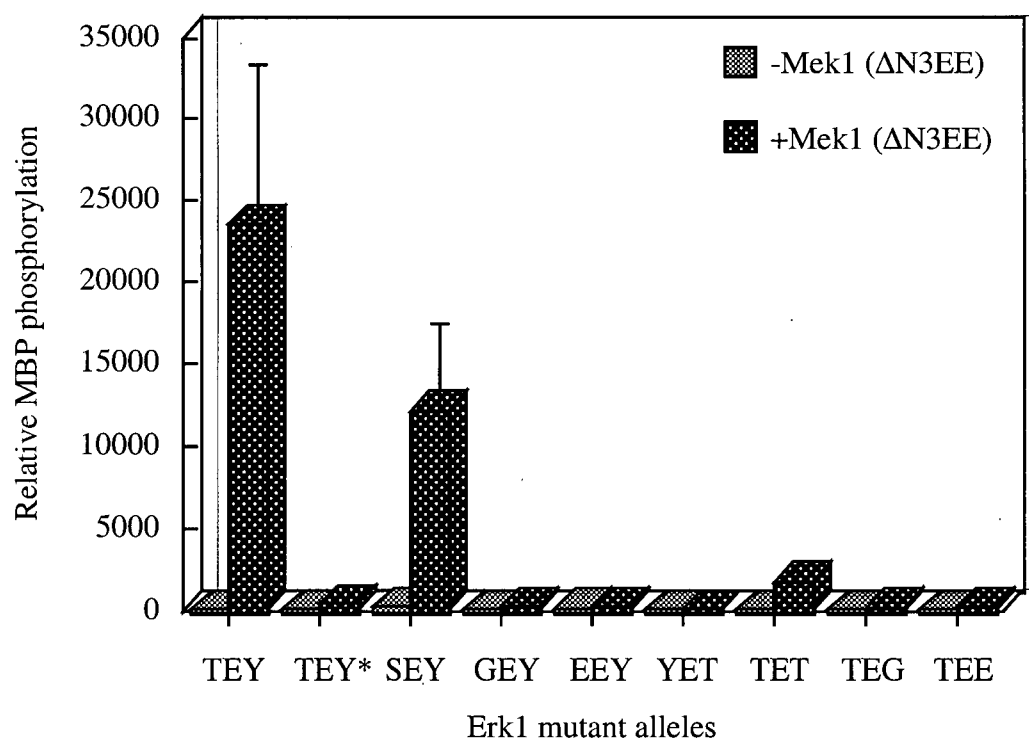


Figure 32: Mek1 ( $\Delta$ N3EE) activation of Erk1 regulatory phosphorylation site mutant alleles. The Erk1 activation was performed as a two-step assay in the presence of [ $\gamma$ - $^{32}$ P] ATP (1250 cpm/pmol). The Erk1 proteins ( $\sim$ 1  $\mu$ g) were incubated without (light grey) and with (dark grey) constitutively active Mek1 ( $\Delta$ N3EE) ( $\sim$ 53 ng) before performing the MBP kinase assay. At the completion of the 29 min preincubation described in Figure 26A, a 5  $\mu$ l aliquot of the exogenous substrate MBP (5 mg/ml) was added to the mixture. The reaction was incubated for a further 1 min before addition of 5  $\mu$ l of 4X gel loading buffer. The Mek1 activating activity is normalized to Erk1 wild type basal autoactivation activity which is given a value of 100; mean  $\pm$  s.d., n=3 (Erk1 wild type MBP phosphotransferase activity varied between 750-1550 cpm/1 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEE TEG TET YET TEY\* GEY YET TEY EEY TEG GEY TEE SEY EEY TEY\* TET SEY TEY**

---

This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutant alleles incubated in the absence (bold face) and presence of Mek1 ( $\Delta$ N3EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the kinase-inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.



#### 4.7 Mutational analysis of the intervening glutamic acid residue in TEY of Erk1

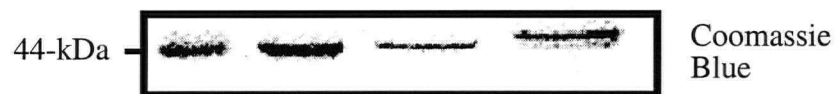
The MAP kinase family of protein kinases are defined by the dual phosphorylation on the threonyl and tyrosyl site motif (Ferrel, 1996). However, MAP kinases have been further classified into subfamilies; based on the length of the activation loop that extends between kinase catalytic subdomains VII and VIII and the intervening amino acid that separates the regulatory threonyl and tyrosyl residues (Figure 27A). The Erk subfamily possesses the longest activation loop sequence followed by the Jnk and Hog subfamilies, which individually are four and six amino acids shorter (Figure 27A). Furthermore, the threonyl and tyrosyl phosphorylation sites in the Erk1, Erk2, and Erk5 subfamilies are separated by the charged amino acid glutamic acid; in contrast, prolyl and glycyl residues divide these two same phosphorylatable residues in Jnk1 and Hog1, respectively. The combination of the activation loop length and the type of intervening amino acid is expected to have a dramatic effect on the regulation of these kinases by their specific upstream activators. To gain insight into the role the intervening amino acid has on the recognition and phosphorylation of MAP kinase by Mek1, the glutamyl residue in Erk1 was exchanged with the prolyl or glycyl residues normally present in the Jnk and Hog MAP kinase family members.

#### 4.8 Analysis of autophosphorylation and basal MBP phosphotransferase activities of Erk1 amino acid 203 mutant alleles

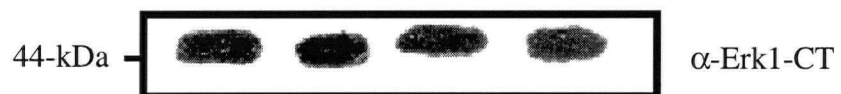
The MAP kinase alleles were affinity purified by binding to glutathione agarose beads and subsequently separated from the GST-fusion by thrombin cleavage. A small sample (~1 µg) was electrophoresed on an acrylamide gel and visualized by Coomassie Blue dye or transferred to nitrocellulose and probed with an antibody specific for the carboxy-terminal region of Erk1 (Figure 33A). As expected, the faster migrating 42-kDa

Figure 33: Expression, immunodetection and quantitation of recombinant Erk1 protein from bacteria. The constructs were transformed in *E. coli* strain UT 5600 and subsequently grown in 2 x YT medium. The collected cells were disrupted in homogenization buffer G. and the recombinant proteins purified by glutathione affinity chromatography. Thrombin digestion liberated the Erk1 protein from the GST-fusion. Approximately 1  $\mu$ g of protein was subjected to 10% SDS-PAGE Bio-Rad mini-gels. The proteins were quantitated by Coomassie Blue staining (A), or transferred to nitocellulose and Western blotted (seperate gels) with Erk1-CT polyclonal antibody. (B), or phosphotyrosine-specific 4G10 monoclonal antibody (C).

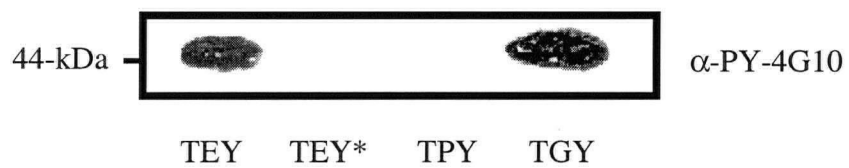
A.



B.



C.



band was present in higher concentrations than the slower 44-kDa protein band. Wild type Erk1 TEY displayed some autophosphorylation on tyrosyl following purification of the protein from *E. coli* (Figure 33C). The TGY mutant protein also cross-reacted with the anti-phosphotyrosine monoclonal antibody 4G10 in the same Western blots. However, no tyrosyl signal was observed with Erk1 TPY or the catalytically-inactivated TEY\* alleles (Figure 33C).

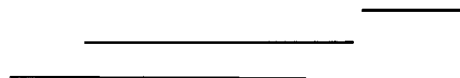
To evaluate how changes to the glutamyl residue may effect catalytic activity, Erk1 was assayed for basal autophosphorylation and MBP phosphotransferase activities. After a ten and thirty minute incubation period, only Erk1 TEY and TGY showed detectable levels of autophosphorylation activity (Figure 34A and 36A). The TPY allele, however, retained approximately 25% of wild type activity while TGY autophosphorylating activity remained the same as wild type after 30 minutes (Figure 34C and 36C). Since no phosphotyrosyl signal was detected with 4G10, Erk1 TPY may undergo seryl/threonyl autophosphorylation. Neither the prolyl nor the glycyl substitutions were able to relieve any conformational constraints imposed by Erk1 phosphorylation lip to activate the mutant alleles above wild type TEY basal activity in an autophosphorylation reaction (Figure 34 and 36).

#### 4.9 Mek1 (EE) phosphorylation and activation of Erk1 amino acid 203 mutant alleles

The Erk1 TEY and TEY\* recombinant proteins were phosphorylated by Mek1 (EE) to an average of 4.0- to 4.5-fold, above the threshold autophosphorylation levels (Figure 36A and C). In contrast, the prolyl and glycyl substitutions were phosphorylated only 50% above the level observed for autophosphorylated wild type Erk1 assayed under the same reaction conditions (Figure 36A and C). Analysis of variance demonstrated that Mek1 ( $\Delta$ N3EE) phosphorylation of Erk1 TPY and TGY alleles were not significantly

Figure 34: Mek1 (EE) phosphorylation of Erk1 intervening amino acid 203 mutant alleles. Equal amounts of Erk1 recombinant proteins (~1µg) were incubated without (A) or with (B) constitutively active recombinant Mek1 (EE) (~53 ng) for 30 min in the presence of [ $\gamma$ - $^{32}$ P] ATP (1250 cpm/pmol). The reaction was terminated by addition of 4X SDS-PAGE sample buffer. The proteins were separated on a 10% Bio-Rad mini gel, transferred to nitrocellulose and visualized by autoradiography. The radioactive bands were cut from Ponceau stained nitrocellulose and counted by liquid scintillation. (C), Erk1 phosphorylation without (light grey) or with Mek1 (EE) (dark grey) was normalized to Erk1 wild type autophosphorylation activity which was given a value of 100 mean  $\pm$  s.d., n=3 (Erk1 autophosphorylating activity varied between 350-1050 cpm/30 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

TEY\* TPY TGY TEY TPY TGY TEY TEY\*



This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutants incubated in the absence (bold face) or presence of Mek1 (EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.

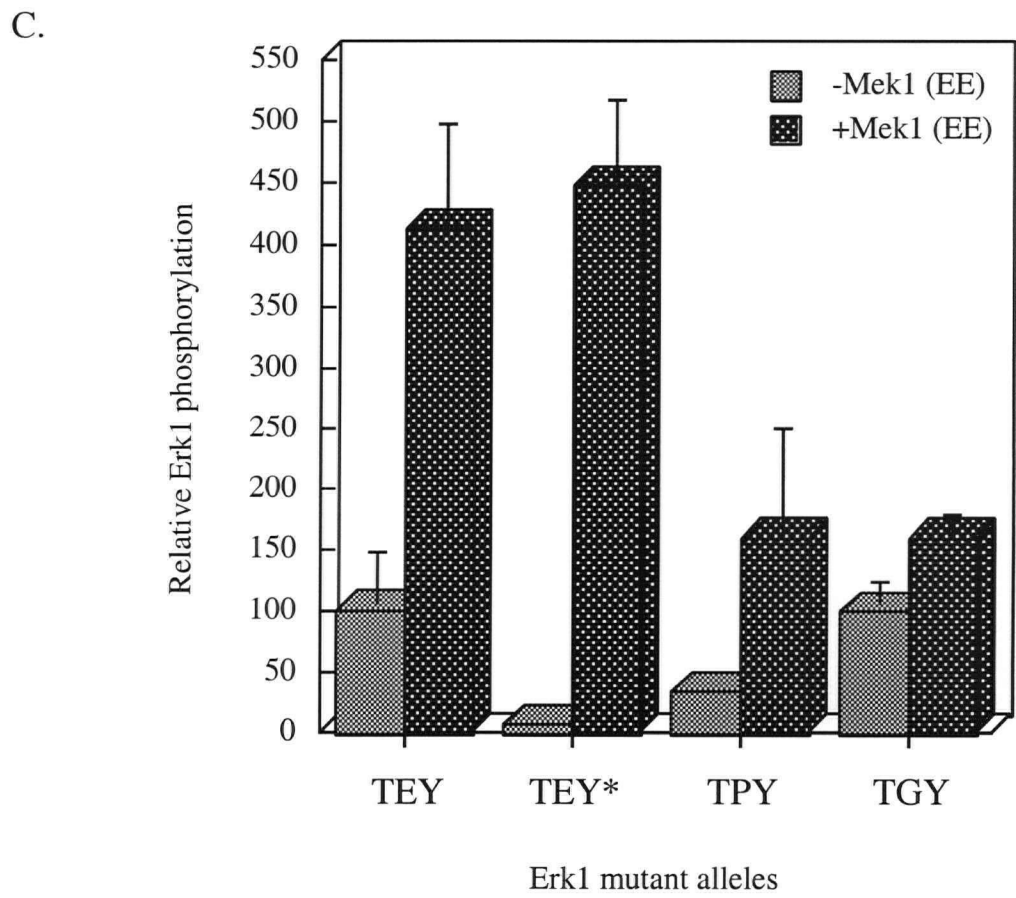
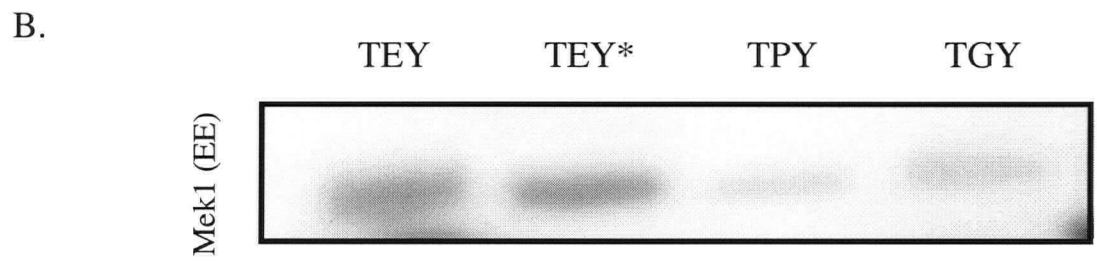
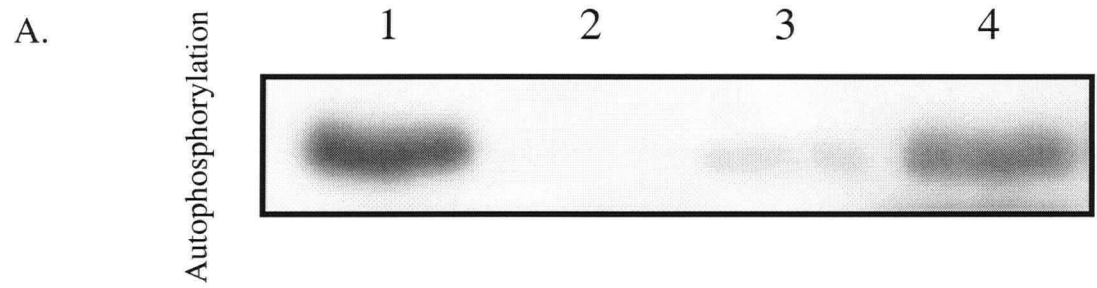


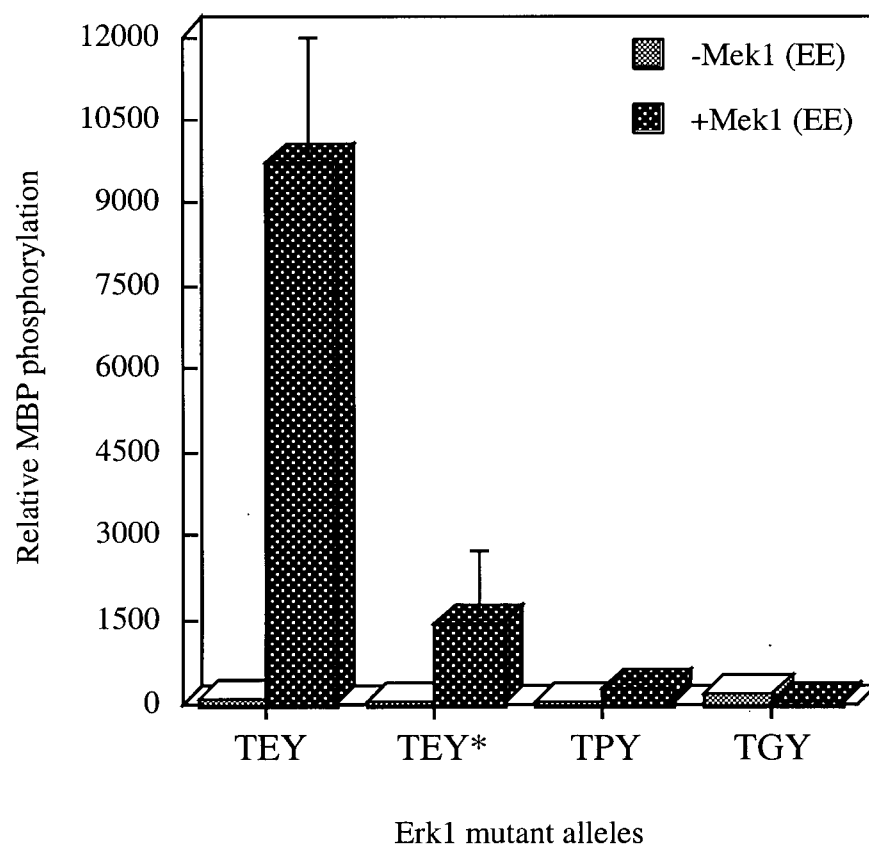


Figure 35: Mek1 (EE) activation of Erk1 intervening amino acid 203 mutant alleles. The Erk1 activation was performed as a two-step assay in the presence of [ $\gamma$ - $^{32}$ P] ATP (1250 cpm/pmol). The Erk1 proteins (~1  $\mu$ g) were incubated without (light grey) and with (dark grey) constitutively active Mek1 (EE) (~53 ng) before performing the MBP kinase assay. At the completion of the 29 min preincubation described in Figure 25A, a 5  $\mu$ l aliquot of the exogenous substrate MBP (5 mg/ml) was added to the mixture. The reaction was incubated for a further 1 min before addition of 5  $\mu$ l of 4X gel loading buffer. The Mek1 activating activity is normalized to Erk1 wild type basal autoactivation activity which is given a value of 100 mean  $\pm$  s.d., n=3 (Erk1 wild type MBP phosphotransferase activity varied between 100-200 cpm/30 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEY\* TPY TGY TEY TGY TPY TEY\* TEY**

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This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutant alleles incubated in the absence (bold face) and presence of Mek1 (EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the kinase-inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.



different than autophosphorylated wild type Erk1. Mutations at the Glu-203 caused an adverse affect on MBP kinase activity, since Mek1 (EE) was able to only activate Erk1 TEY (Figure 35). Therefore, Mek1 phosphorylation of Erk1 is acutely sensitive to the specific R-group present on the amino acid side chain at position 203.

#### 4.10 Mek1 ( $\Delta$ N3EE) phosphorylation and activation of Erk1 amino acid 203 mutant alleles

The more active Mek1 ( $\Delta$ N3EE) displayed the same specificity toward Erk1 TPY and TGY mutant alleles as the less active Mek1 (EE) (Figure 34C and 36C). Although Mek1 ( $\Delta$ N3EE) phosphorylated TPY and TGY proteins to one third the level of what was observed for the wild type enzyme and 10-fold higher than autophosphorylated wild type Erk1, no appreciable stimulation of the kinase activity was observed for these enzymes above the level observed for the kinase-inactive Erk1 TEY\* (Figure 34C and 35). In the presence of Mek1 ( $\Delta$ N3EE), wild type Erk1 TEY was activated 370-fold above Erk1 alone and 4-fold more than with Mek1 (EE) (compare Figure 35 and Figure 37). As noted above with Mek1 (EE), even the more active Mek1 ( $\Delta$ N3EE) recognition of Erk1 mutant alleles is effected by substitutions in Glu-203.

To date, Erk1 is the only known substrate for Mek1 (Seeger *et al.*, 1992a). Part of Mek1 substrate specificity for Erk1 lies in the three amino acid TEY motif. Substitution of Thr-202 and Tyr-204 in Erk1 with either hydroxyl or carboxyl group amino acids dramatically changed phosphorylation by Mek1. Even serine, which contains a hydroxyl group in place of a methyl group in its amino acid side chain, effects the ability of Mek1 to recognize Erk1 SEY. Another determinant in Mek1 recognition of Erk1 is the glutamic acid positioned between the regulatory threonyl and tyrosyl. Substitution of Glu-203 with glycyl or prolyl residues decreases the ability of Mek1 to properly

Figure 36: Mek1 ( $\Delta$ N3EE) phosphorylation of Erk1 intervening amino acid 203 mutant alleles. Equal amounts of Erk1 recombinant proteins ( $\sim 1\mu\text{g}$ ) were incubated without (A) or with (B) constitutively active recombinant Mek1 ( $\Delta$ N3EE) ( $\sim 53$  ng) for 10 min in the presence of  $[\gamma\text{-}^{32}\text{P}]$  ATP (1250 cpm/pmol). The reaction was terminated by addition of 4X SDS-PAGE sample buffer. The proteins were separated on a 10% Bio-Rad mini gel, transferred to nitrocellulose and visualized by autoradiography. The radioactive bands were cut from Ponceau stained nitrocellulose and counted by liquid scintillation. (C), Erk1 phosphorylation without (light grey) or with Mek1 ( $\Delta$ N3EE) (dark grey) was normalized to Erk1 wild type autophosphorylation activity which was given a value of 100 mean  $\pm$  s.d., n=3 (Erk1 autophosphorylating activity varied between 90-275 cpm/10 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEY\* TPY TEY TGY TGY TPY TEY TEY\***

This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutants incubated in the absence (bold face) or presence of Mek1 ( $\Delta$ N3EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.

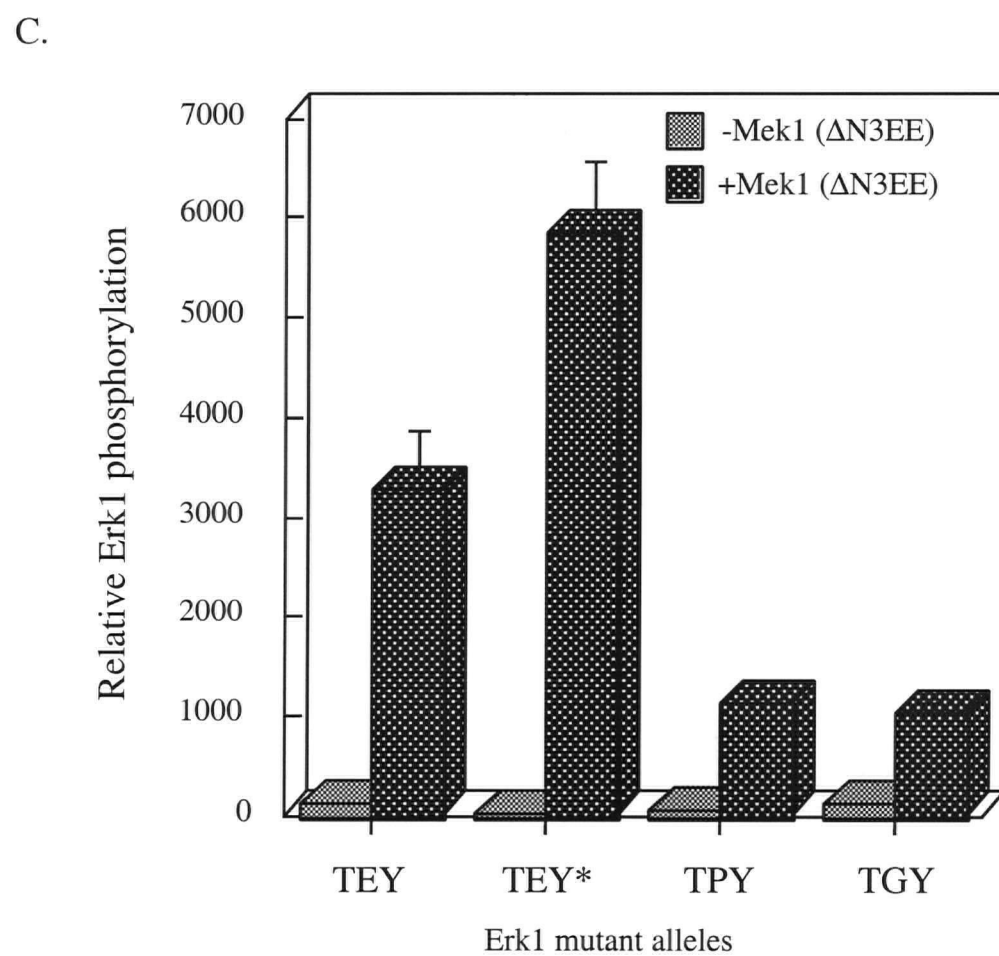
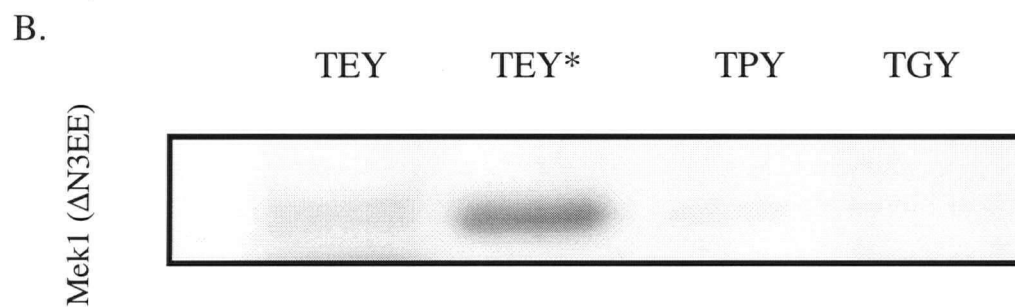
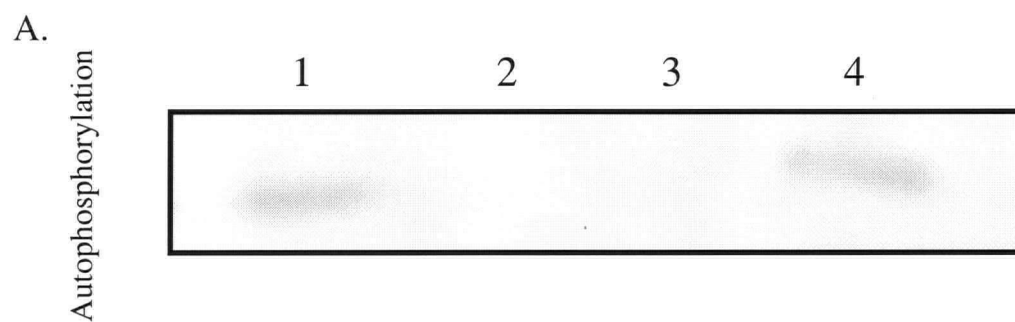
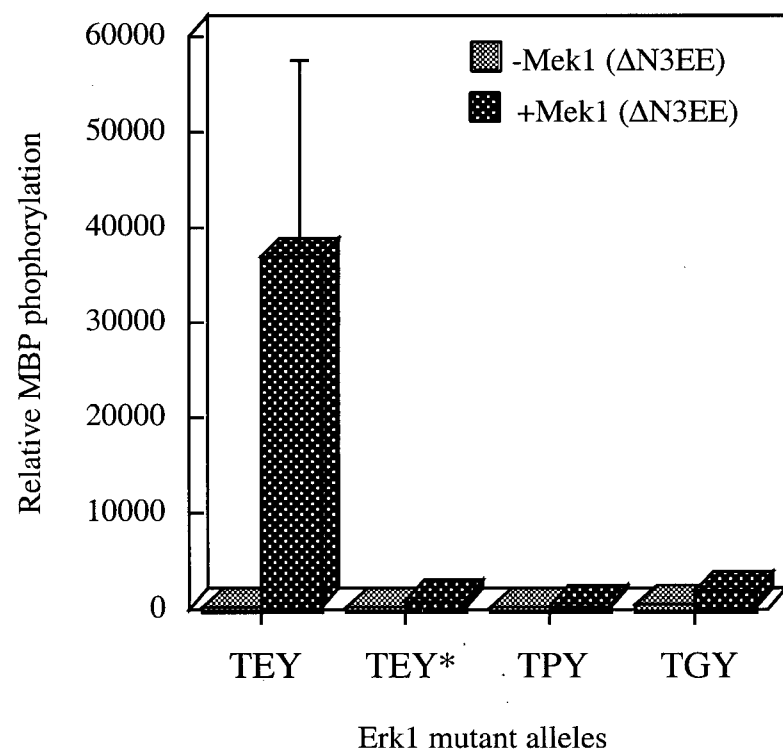


Figure 37: Mek1 ( $\Delta$ N3EE) activation of Erk1 intervening amino acid 203 mutant alleles. The Erk1 activation was performed as a two-step assay in the presence of [ $\gamma$ - $^{32}$ P] ATP (1250 cpm/pmol). The Erk1 proteins ( $\sim 1 \mu\text{g}$ ) were incubated without (light grey) and with (dark grey) constitutively active Mek1 ( $\Delta$ N3EE) ( $\sim 53 \text{ ng}$ ) before performing the MBP kinase assay. At the completion of the 9 min preincubation described in Figure 26A, a  $5 \mu\text{l}$  aliquot of the exogenous substrate MBP ( $5 \text{ mg/ml}$ ) was added to the mixture. The reaction was incubated for a further 1 min before addition of  $5 \mu\text{l}$  of 4X gel loading buffer. The Mek1 activating activity is normalized to Erk1 wild type basal autoactivation activity which is given a value of  $100 \text{ mean} \pm \text{s.d.}$ ,  $n=3$  (Erk1 wild type MBP phosphotransferase activity varied between 85-101 cpm/1 min reaction). The data were analyzed by the ANOVA (analysis of variance) test. Ranked sample means:

**TEY\* TPY TEY TPY TGY TEY\* TGY TEY**

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This is a graphical representation of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. The Erk1 mutant alleles incubated in the absence (bold face) and presence of Mek1 ( $\Delta$ N3EE) (normal) are indicated by their tripeptide sequence. The wild type enzyme is denoted by the three letter abbreviation TEY and the kinase-inactive Erk1 allele by TEY\*. All other alleles are indicated with their amino acid change.



phosphorylate Erk1. It appears that the charge, structure and size of the amino acid side chain of residue 203 plays a role in Mek1 recognition.



## DISCUSSION

MAP kinase was originally identified in a number of model systems following growth factor or hormone stimulation (Sturgill and Ray, 1986; Ray and Sturgill, 1987; Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Hoshi *et al.*, 1988). In the work presented here, a systematic approach was used to uncover the players involved in regulating the MAP kinase pathway in both sea stars and mammals. To isolate MAP kinase from human, a partial cDNA was obtained from A-431 epidermoid cell line by applying RT-PCR with paired forward and reverse oligonucleotide primers that were patterned after the partial rat Erk1 (Boulton *et al.*, 1990). Two partial overlapping cDNAs of the appropriate size were obtained using this approach. Sequence analysis revealed that these four and five hundred base pair fragments were ~98% identical within the same interval to the rat Erk1 (data not shown).

Before isolating the full-length human Erk1 from a Hep G2 cDNA library, the same hepatocellular carcinoma cell was verified for the presence of Erk1. Indeed, insulin stimulation of Hep G2 cells resulted in a two-fold activation of an MBP kinase activity relative to control cells. Immunoblot analysis of the fractionated extracts with an antipeptide antibody directed toward the carboxy-terminal region of Erk1 showed that the MBP kinase activity and the Erk1 enzyme co-eluted. Therefore, MAP kinase isoform Erk1 protein is expressed in the Hep G2 cell line.

The human Erk1 clone was obtained in a screen of a Hep G2 cDNA library. The 1850 base pair fragment excluding the poly(A)+ track corresponded in length with the published sequence from rat. However, an additional stretch of approximately one hundred base pairs were present in human Erk1 that were missing from the rat isoform (Charest *et al.*, 1993). Isolation of truncated forms of Erk1 from a number of model

systems may be due to the GC-rich content of this region that probably interfered with the synthesis of a full-length cDNA (Boulton *et al.*, 1990; Gonzalez *et al.*, 1992; Owaki *et al.*, 1992; Tanner and Mueckler, 1993). In contrast to previously reported rat Erk1 sequences, two potential translational initiation codons were identified at the 5' end of the cDNA. The two start codons located at nucleotides 73 and 160 conformed to the minimal consensus sequence defined by Kozak (1987). In fact, the DNA sequence surrounding the ATG start site was identical with respect to both methionines. To further complicate matters, no signal translational termination codon was located between the two initiation sites nor within the putative 72 base pair 5' untranslated region.

To define the 5' boundary of the Erk1 message, primer extension analysis of this region revealed that only 13 bases were missing from the message. The possible existence of a third AUG translation initiation site located so close to the 5' cap site was remote since ribosomes normally require longer stretches to initiate translation (Kozak, 1987). Therefore, this result supports the presence of a fixed site for translation initiation in Erk1 from Hep G2 cells. The assignment of cDNA nucleotide 73 as the translation initiation site for human Erk1 is supported by protein sequence analysis of different MAP kinase isoforms. Although there is a perfect alignment between the second in-frame methionine in human and those of MAP kinases Fus3 and Kss1 from *S.cerevisiae*, a valinyl residue is present at the exact position in rat Erk1 (Courchene *et al.*, 1989; Elion *et al.*, 1990; Boulton *et al.*, 1990; Charest *et al.*, 1993). In addition, the first initiating methionyl site followed by a string of alanyl residues (MAAAAA) is reminiscent of the predicted amino-terminal sequence of Erk2 from human. These results in combination with the full-length sequence from rat indicate that the first AUG is the most likely recognition site for translation from the human Erk1 mRNA (Marquardt and Stabel, 1992; Charest *et al.*, 1993).

Although Erk1 was more effectively autophosphorylated in the presence of the divalent metal cation  $Mn^{2+}$ . The reason for this requirement remains unclear since purified sea star Mpk1 MBP phosphotransferase activity was sensitive to elevated  $Mn^{2+}$  concentrations (Sanghera *et al.*, 1990a). Perhaps recombinant Erk1 expressed in bacteria has a slightly different conformation than the native form and consequently alters the enzymes divalent cation usage. Another possible explanation for this difference is that autophosphorylation on Tyr-204 in Erk1 requires the divalent cation  $Mn^{2+}$ .

Recombinant Erk1 purified from bacteria also displayed a minor amount of MBP phosphotransferase activity *in vitro*. This low level activity was likely due to the accumulation of phosphotyrosine within the bacteria, since treatment of the recombinant enzyme with the tyrosyl phosphatase CD45 inactivated the enzyme. The maximum level of Erk1 autophosphorylation following 20 min incubation was 0.01 mol of P per mol distributed at multiple sites. These data are similar to the results reported for the low activity structure of pTyr-185 in Erk2 (Seger *et al.*, 1991; Robbins *et al.*, 1993). Recombinant Erk1 autophosphorylation *in vitro* occurred at a very low stoichiometry considering the enzyme has two regulatory phosphorylation sites (Payne *et al.*, 1991). The explanation for the low level activation of Erk1 by autophosphorylation of Tyr-204 may be explained by examining the location of the residue within the context of the protein kinase topology. In Erk2, Thr-183 is exposed on the surface of the L12 activation loop, whereas Tyr-185 is buried in a hydrophobic pocket facing toward the active site (Zhang *et al.*, 1994). To become fully active, however, Erk protein kinases require dual phosphorylation on neighbouring threonyl and tyrosyl residues (Anderson *et al.*, 1990; Payne *et al.*, 1991; Canagarajah *et al.*, 1997).

This slow autophosphorylation on tyrosyl residues raised the possibility that a cellular 'enhancing factor' distinct from a protein kinase may stimulate this activity (Wu

*et al.* 1991; Seger *et al.*, 1991; Ahn *et al.*, 1991; Robbins and Cobb, 1992). However, the Src-related tyrosyl kinase Lck could directly phosphorylate the activating tyrosyl site in purified sea star Mpk1 and stimulate its MBP phosphotransferase activity (Ettehadiah *et al.*, 1992). In addition, Tyr-204 in GST-Erk1 was also shown to be the major site of phosphorylation by Lck. Unfortunately, this did not stimulate increased activation of human Erk1 MBP kinase activity. The reason for the discrepancy between Lck activation of purified sea star Mpk1 and recombinant human Erk1 is difficult to reconcile since both proteins display close to 80% identity at the amino acid level. It is quite possible that a contaminating protein in the Mpk1 preparation was activated in the presence of Lck. Since Lck was shown not to be an Erk1 activator, activated sheep platelets were used to assay for a MAP kinase activator. Indeed, a factor was detected in PMA-treated sheep platelets that stimulated GST-Erk1 phosphotransferase activity by nearly three-fold. To verify the mechanism of MAP kinase activation, a catalytically compromised Lys-71 mutant of Erk1 was used as a substrate. In a manner similar to wild type Erk1, the kinase-inactive version was phosphorylated to nearly identical levels, indicating that the activator possessed intrinsic phosphorylation activity. Moreover, the phosphorylation of GST-Erk1 by sheep platelet fractionated extract occurred predominantly on tyrosine and to a lesser extent on threonine. Evidence using catalytically compromised Erk2 microinjected into *Xenopus* oocytes revealed that a 'MAP kinase kinase' was responsible for phosphorylating the physiologically relevant sites (Posada and Cooper, 1992). Several groups reported identical observations in PMA- or growth factor- induced MAP kinase activation in mammalian cells (Alessandrini *et al.*, 1992; L'Allemain *et al.*, 1992; Shirakabe *et al.*, 1992). In an analogous approach, MAP kinase kinase partially purified from EGF-activated 3T3 cells displayed an identical amino acid specificity (L'Allemain *et al.*, 1992).

The high degree of amino acid sequence conservation between the partial sea star Mpk1 and the full-length human Erk1 cDNA implied that the mammalian isoform could be a useful reagent for purifying the sea star MAP kinase kinase. A MAP kinase activator, that was capable of increasing Erk1 MBP phosphotransferase activity above basal levels, was detected in sea star oocytes induced to mature with the hormone 1-MeAde. Like mammalian Mek1, the sea star isoform adsorbed very weakly on a Mono Q anion exchange resin at neutral pH (Gómez and Cohen, 1991; Ahn *et al.*, 1991; Shirakabe *et al.*, 1992; L'Allemain *et al.*, 1992). Major and minor peaks of Erk1 activator activity eluted at 100 and 200 mM NaCl, respectively. The detection of two activator peaks has been noted in NGF-, EGF- and phorbol ester-treated mammalian cells (Gómez and Cohen, 1991; Ahn *et al.*, 1991; Alessandrini *et al.*, 1992). However, the peak of Erk1 activation did not coincide with any of the 6 peaks of Erk1 protein phosphorylation. Previously, heat-denatured forms of MAP kinase as well as peptides harbouring the MAP kinase phosphorylation site were shown to be poorly recognized by the upstream activator (Ahn *et al.*, 1991). In control experiments, the TEY peptide phosphorylation profile superimposed that of the full-length Erk1 protein. These results indicate that the low level of Erk1 phosphorylation by the sea star activator coincides with a small activation of the Erk1 phosphotransferase activity. However, these results do not rule out the possibility of an allosteric activation of Erk1 by a factor unique to the sea star oocyte system.

The MAP kinase activator from sea star oocytes was purified using a protocol that incorporated six chromatographic resins including DEAE-cellulose (reverse column), hydroxylapatite, S-Sepharose, phosphocellulose, heparin-agarose and Mono S at acidic pH. Two or more of these column steps were also employed in the purification strategies of MAP kinase kinase from mammalian cells, rabbit skeletal muscle and *Xenopus* oocytes (Nakielnny *et al.*, 1992; Seger *et al.*, 1992b; Matsuda *et al.*, 1992; Wu *et al.*, 1992;

Crews and Erikson, 1992). The overall purification of MAP kinase activator from 1-MeAde-treated oocytes was about 500-fold. This recovery was much lower than the observed 5,000- to 40,000-fold purification of MAP kinase kinase obtained from other model systems.

Although the purified preparation of sea star MAP kinase activator was not homogeneous, an enriched polypeptide band of 42- to 44-kDa was identified on a SDS-PAGE silver stained gel. This band directly correlated with the peak activity of the MAP kinase activator in the final Mono S purification step. Furthermore, gel filtration chromatography indicated that the protein existed as a monomeric protein with an approximate molecular mass of 40- to 42-kDa. On occasion, a second peak of MAP kinase activator activity was eluted earlier from the Superose 12 column fractionation. This may be a complex of proteins which has as one of its components the sea star Erk1 activator. Purified fractions from the Mono S and Superose 12 column step were analyzed by Western blotting with amino- and carboxy-terminal antipeptide antibodies directed against a number of different mammalian, *Xenopus*, and yeast kinases including Ste11, Raf1, Mos and Src proteins. No immunoreactive bands were detected in these experiments (data not shown). However, these results cannot eliminate the possibility that the contaminating protein bands identified on the silver-stained gel are protein kinases that were unable to immunoreact with these antibodies due to species-specific sequence divergence at the amino acid level. Mek and Erk proteins are known to form complexes *in vitro* and *in vivo* and Erk2 has been shown to exist as protein dimers in crystal structures (Fukuda *et al.*, 1997; Canagarajah *et al.*, 1997). The size of the polypeptide band obtained after six distinct purification steps correlated to within 2-kDa of the molecular mass of the purified MAP kinase kinase from other sources (Nakielnny *et al.*, 1992; Seger *et al.*, 1992; Matsuda *et al.*, 1992; Wu *et al.*, 1992; Crews and Erikson, 1992). Several attempts were made to demonstrate that the MAP kinase activator was a

*bona fide* kinase by monitoring the activity of the kinase under reaction conditions that favored autophosphorylation. However, despite repeated attempts with different purified preparations of the protein, no autokinase activity was observed for the sea star MAP kinase activator. This contrasted the evidence presented from other MAP kinase kinase purifications. (Seger *et al.*, 1992; Nakielny *et al.*, 1992; Matsuda *et al.*, 1992).

Sequence analysis of tryptic peptides derived from a number of different purified MAP kinase kinase preparations revealed that the isolated Mek proteins were closely related to the yeast proteins Byr1 (bypass of Ras) and Ste7 (Nadim-Davis and Nasim, 1988; Teage *et al.*, 1986). Byr1 from the fission yeast *S. pombe* and its cognate, Ste7, from the budding yeast *S. cerevisiae*, have been implicated in cell cycle control during yeast mating. These two enzymes act as intermediary proteins that transduce signals emanating from receptors at the plasma membrane to specific MAP kinase effectors. The 45-kDa protein product of the Spk1 gene in *S. pombe* which has sequence similarity to *S. cerevisiae* Fus3 and Kss1 are highly related MAP kinases that regulate the conjugation (Teage *et al.*, 1986; Nadin-Davis and Nasim, 1990; Gartner *et al.*, 1992). In light of the difficulty in demonstrating that the single silver-stained ~42 to 44-kDa band observed by SDS-PAGE was the desired polypeptide, the combined peak Erk1 activator activity fractions were subjected to Western immunoblotting analysis using antibody probes specific for mouse Mek1 and budding yeast Ste7 MAP kinase kinases. Two antibodies directed against peptides patterned after amino acid sequences from highly conserved catalytic subdomain VIII in yeast Ste7 and subdomain XI in mouse Mek1 immunoreacted with a 42-kDa band from the most purified preparation of sea star Erk1 kinase activator. In contrast, the mouse Mek1 carboxy-terminal antibody displayed the least immunoreactivity with the purified protein. All three protein antibodies detected to varying degrees the bacterially expressed recombinant GST-Mek1 and partially purified Mek enzyme from TPA-treated sheep platelets. In terms of the size, chromatographic

behavior and immunoreactivity to Mek antibodies derived from two disparate species, the 42-kDa protein purified from maturing sea star oocytes appeared to be the sea star isoform of MAP kinase kinase. The most important difference, however, remains the relatively low degree of activation of Erk1 MBP phosphotransferase activity induced by the sea star Mek enzyme relative to purified isoforms from other model systems.

The mechanism of MAP kinase activation has been subject to much speculation given that Erk protein kinases were capable of being activated by a slow, linear autophosphorylation reaction that resulted in a detectable increase in the enzymes MBP phosphotransferase activity (Wu *et al.*, 1991; Seger *et al.*, 1991; Crews *et al.*, 1991; Rossomondo *et al.*, 1992b; Robbins *et al.*, 1993; Charest *et al.*, 1993). Many originally believed that Erk1 autophosphorylation may play a significant role in Erk1 regulation (Knighton *et al.*, 1991; Seger *et al.*, 1991; Crew *et al.*, 1991; Wu *et al.*, 1991; Robbins and Cobb, 1992). To determine the mechanism of Erk1 activation in the sea star oocytes, both the wild type and its catalytically compromised variant were analyzed in the presence of purified sea star Mek. As expected, addition of the sea star Mek promoted the Erk1 phosphorylation and a promoted a 4-fold activation of recombinant GST-Erk1 MBP phosphotransferase activity *in vitro* while at the same time failing to phosphorylate kinase-inactive Erk1. Analysis of the phosphoamino acid content of the sea star Mek 'phosphorylation' of Erk1 revealed that all three hydroxyl residues displayed a marked increase in phosphorylation. Similar results were observed with different sea star Mek preparations.

There are several possible scenarios that could explain the low level activation observed for human Erk1 by the sea star MAP kinase activator. It is plausible that purified sea star Mek may function as an allosteric activator of Erk1, perhaps via stimulation of MAP kinase autophosphorylation at the regulatory sites. Allosteric



activation has not yet been described between pairs of protein kinases. However, the transcription factor Elk1, which is a MAP kinase substrate, is able to increase the autophosphorylation of Erks and Mpk1 *in vitro* (Rao and Reddy, 1993; Rao and Reddy, 1994). Specific allosteric interactions between kinases may explain Mek's narrow specificity for its physiological substrates Erk1 and Erk2 (Ahn *et al.*, 1992; Charest *et al.*, unpublished data). In fact, Zhang *et al.* (1994) observed that because Tyr-185 in Erk2 is buried in a hydrophobic pocket in the low activity state, the activation loop must assume a completely different conformation when complexed with Mek. The mechanism of Erk1 activation by mammalian and sea star Mek is presently under investigation. We are in the process of analyzing how Mek-Erk association may stimulate Erk1 autophosphorylation and lead to the activation of MBP phosphotransferase activity. To do so we have constructed unique Mek1 mutant alleles that combine an Ala-97 mutation in the essential catalytic lysyl residue with Glu-218 and Glu-222 mutations at the regulatory seryl sites. In so doing, the catalytically-inactive but conformationally active Mek mutant can be tested as an allosteric activator of the Erk1 enzyme.

The low specific activity observed for the purified MAP kinase activator from sea star oocytes may result from the presence of a non-kinase 'activation factor' purified during the course of the purification procedure. This 'activation factor' may stimulate the *in vitro* autophosphorylation activity of Erk1 and consequently stimulate MBP phosphotransferase activity. Evidence of an 'autokinase-enhancing factor' which stimulates the intrinsic autokinase activity of Erk2 has been observed in Swiss 3T3 fibroblast cells (L'Allemain *et al.*, 1992). However, more detailed analysis of the 'enhancing factor' by Sturgill's research group revealed that this novel protein stimulated Mek phosphotransferase activity toward MAP kinase *in vitro* (L'Allemain *et al.*, 1992). Although the identity of the mammalian 'enhancing factor' remains unknown, it is similar to the 1-MeAde-stimulated sea star Mek activator in that the protein factor activity is

sometimes induced in mitogen-stimulated cells (L'Allemain *et al.*, 1992). A second Erk1 'enhancing factor' that activated recombinant Erk1 isolated from bacteria was detected in mammalian cells. The purified Erk1 activator was shown to be a 16 kDa thiol transferase enzyme. Apparently, Erk1 expressed in bacteria forms inactive kinase complexes through disulfide linkages between cystyl residues on the surface of the protein. Exposure to the thiol transferase causes the cleavage of the disulfide bonds and subsequent liberation of the active enzyme (Haystead, personal communication).

Many of the signalling components within each of the MAP kinase modules display a high degree of selectivity based on specific protein-protein interactions. In fact, the formation of large signalling complexes may be the *modus operandi* within the cell. In mammalian cells, individual kinases Raf1, Mek1/Mek2, Erk1/Erk2 and Rsk1/Mnk2 form protein contacts specifically with their immediate upstream activators and their downstream effectors. Discreet interactions between signalling kinases may be important for maintaining fidelity within the signal transduction pathway by limiting interaction with other MAP kinase modules or by regulating its activation/attenuation. The analogous situation exists in the *S. cerevisiae*, with the exception that the non-protein kinase, Ste5, provides specific docking sites for Ste11, Ste7 and Fus3/Kss1 kinases (Choi *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994). The specific interactions that occur between protein kinase pairs may explain the absence of a robust phosphorylation and activation of human Erk1 by sea star Mek activator. In support of this notion, Ste7 is unable to bind with other yeast (Mpk1 and Spk1) or mammalian (Erk2) MAP kinase homologues (Bardwell *et al.*, 1996). To further investigate the role of Mek/MAPK complexes in oocytes, we have isolated the full-length sea star Mpk1 cDNA by the RACE (rapid amplification of cDNA ends) method. Sequence comparison revealed that sea star Mpk1 and human Erk1 are identical in size (379-amino acid residues), in addition to both enzymes containing the signature TEY activation loop (data

not shown). However, both kinases display a great deal of variability within the amino- and carboxy-terminal regions (data not shown). Because purified sea star Mek poorly activates human Erk1, we are currently assessing whether constitutively active Mek1( $\Delta$ N3EE) is capable of stimulating the MBP phosphotransferase activity of sea star Mpk1. Furthermore, we are presently constructing protein chimerics of these enzymes by interchanging the amino- and carboxy-terminal regions between sea star Mpk1 and human Erk1 to determine whether these regions may regulate Mek binding to MAP kinase. In addition, we want to attempt to redirect mitogen signals in mammalian cells through expression of Erk1/Mpk1 chimeric proteins.

Since several high and low molecular mass proteins were present in the purified sea star Mek preparations, the data cannot exclude the possibility that a contaminating factor may regulate Erk1 activity. It is possible that one or more of the contaminating silver-stained bands observed on SDS-PAGE may promote the renaturation of misfolded Erk1 protein purified from bacteria. As a result, the relative amount of active enzyme that could undergo autophosphorylation would increase during the incubation period. This seems unlikely in light of the fact that the Erk1 activator appears to be stimulated during the course of oocyte maturation. Unexpectedly, however, we have observed a factor that is present in both 1-MeAde-stimulated and unstimulated sea star oocytes that is capable of inhibiting Erk1 MBP phosphotransferase activity in a concentration-dependent manner. Like sea star Mek, this inhibitory factor does not adsorb to anion exchange columns at pH 7.0. It may be possible that one of the contaminating protein bands in the purified Erk1 activator preparation may be a contaminating inhibitory factor. Investigations are presently underway to determine the identity of this heat-stable MAP kinase inhibitory factor.

MAP kinases are regulated by dual phosphorylation on neighbouring threonyl and tyrosyl residues in the L12 activation loop of catalytic subdomain VIII (Payne *et al.*, 1992; Canagarajah *et al.*, 1997). Although the presence of seryl phosphorylation has been observed by a number of investigators, it is assumed to play no role in the regulation of protein kinase activity (Robbins and Cobb, 1992). To understand the mechanism of Erk1 activation, a time course of phosphorylation and activation of Erk1 by constitutively activated forms of Mek1 was performed *in vitro* with recombinant proteins. The recombinant GST-Mek1 (EE) fusion protein which has the regulatory residues (Ser-218 and Ser-222) substituted with glutamic acid mimetics phosphorylated thrombin cleaved-Erk1 to 0.08 mol/mol. In fact, Erk1 was phosphorylated 60 to 80 percent more on tyrosyl than on the adjacent threonyl site during the first 30 min. No further changes were observed during the remainder of the reaction. The inability to achieve a stoichiometry of phosphorylation of 2 mol/mol is consistent with previous *in vitro* results (Robbin *et al.*, 1993; Scott *et al.*, 1995; Robinson *et al.*, 1996). Others have demonstrated that catalytic amounts of Mek resulted in the accumulation of the Tyr-185 form of Erk2 *in vitro* (Haystead *et al.*, 1992). In fact, a 40-fold excess of Mek was critical to achieve identical phosphotyrosyl and phosphothreonyl content in Erk (Scott *et al.*, 1995). As mentioned above, Haystead found that a substantial percentage of bacterial expressed Erk1 exists in a inactive complex and therefore may not accessible for activation by Mek.

However, proper signal transmission from Mek to Erk *in vivo* may require interactions with other proteins in a signalling complex. Ste5 in yeast has been proposed to act as a tether in the pheromone signalling pathway in budding yeast to assist in the sequential activation of the members in MAP kinase module. In mammalian cells, factors have been detected that stimulated the rate of Erk1 autophosphorylation and increase the rate at which this same enzyme is phosphorylated by Mek *in vitro* (L'Allemain *et al.*, 1992; Scott *et al.*, 1995). In support of this notion, removal of the

inhibitory amino-terminal NES sequence in Mek1 (EE) leads to a dramatic increase in the constitutive phosphorylation of Erk enzyme and transformation of mammalian cells (Mansour *et al.*, 1994). It will be interesting to determine whether the NES region in Mek1 becomes displaced by a non-kinase factor. This factor may induce a conformational change in the kinase and thereby increase Mek1 phosphorylation of Erk1. Mek1 ( $\Delta$ N3EE) phosphorylation of Erk1 reached a maximum between 5 and 10 min under identical conditions used for the Mek1 (EE) experiment. Furthermore, this 5-fold higher stoichiometry of phosphorylation ( $\sim 0.5$  mol/mol) translated into a similar increase in Erk1 MBP phosphotransferase activity. Although Erk1 phosphorylation was not stoichiometric, phosphoamino acid analysis revealed that Mek1 ( $\Delta$ N3EE) phosphorylated the enzyme equally on threonyl and tyrosyl residues.

In addition to phosphorylation on threonine and tyrosine, a very robust increase in serine phosphorylation of Erk1 was observed in the presence of Mek1( $\Delta$ N3EE). Following serine phosphorylation, Erk1 enzyme activity slowly declined to near basal levels during the remainder of the experiment. At the same time phosphoserine also coincided with the appearance a slower migrating Erk1 band on SDS-PAGE (data not shown). Several groups have demonstrated that overexpression of MAP kinase dual-specificity phosphatases can lead to the inactivation of Erk1 in mammalian cells (Campbell *et al.*, 1995). However, there are no reports of Erk down regulation by Mek phosphorylation or Erk autophosphorylation. Analysis of the primary sequence of Erk1 did not reveal any related SEY-like phosphorylation sites. In fact, the data indicate that serine phosphorylation may depend on the activity of Erk1, since phosphoserine appeared at the point where Erk1 attained full enzymatic activity. Interestingly, two canonical MAP kinase phosphorylation sites are present at Ser-74 and Ser-263 in Erk1. Ser-74 is positioned three amino acid carboxy-terminal to Lys-71 in the ATP-binding site. The Ser-263 site is located in the MAP kinase insert region. We are currently taking the

necessary steps to identify the site of serine phosphorylation in Erk1. The crystal structure has been solved for the active form of Erk2 (Canagarajah *et al.*, 1997). Conformational changes associated with phosphorylated Erk2 causes enzyme dimerization. Apparently, three residues at the carboxy-terminus (Tyr-356 to Ser-358) bind in the active site of the neighbouring molecule (Canagarajah *et al.*, 1997). Therefore, the association of activated Erk1 molecules in solution may lead to the slow inactivation of the kinase activity observed in these experiments. It will also be interesting to determine if phosphorylation on serine effects the dimeric interaction between individual Erk molecules

At present five distinct MAP kinase modules have been identified in mammalian cells. The individual pathways respond to specific stimuli or environmental perturbations by regulating a unique subset of effector molecules. In many instances, activation of one MAP kinase cascade occurs without stimulating parallel pathways. Some of the specificity appears to occur at the level of the Mek/MAP kinase nodal point. Primary sequence comparisons and crystal structure analysis of the members of the MAP kinase family revealed structural motifs that may be essential in regulating kinase function (Zhang *et al.*, 1994). Specifically, the dual phosphorylation motif, Thr-Xaa-Tyr, in the L12 linker of MAP kinase has been proposed to be an important determinant in the recognition and phosphorylation by upstream activators (Han *et al.*, 1994; Butch and Guan, 1996). Therefore, site-directed mutagenesis was used to examine the role of the intervening sequence Thr-Xaa-Tyr and the individual phosphorylation sites has on the mechanism of Erk1 activation.

To test the importance of Erk1 threonyl and the tyrosyl regulatory phosphorylation sites in Mek1 specificity, these residues were substituted with the small amino acid glycine, the hydroxy amino acid serine or the glutamic acid mimetic. The

SEY and EEY mutants were phosphorylated on tyrosine to the same extent as wild type Erk1. As discussed earlier, this limited phosphorylation on tyrosine leads to minimal activation of Erk1. However, in the presence of the more active Mek1( $\Delta$ N3EE) isoform, wild type Erk1 is phosphorylated 2-fold more than the SEY mutant indicating that serine is a poor substitute for threonine in this position. It has been shown previously that a serine substitution at position 183 in Erk2 and 202 in Erk1 was a poor phosphoacceptor site (Butch and Guan, 1996; Robinson *et al.*, 1996). It is possible that the methyl moiety present in the threonine R-group is critical for Mek1 recognition. To further analyze the role each phosphorylatable residue plays for Mek specificity, the orientation of the TEY motif was inverted in Erk1. Like the wild type enzyme, recombinant Erk1 YET contained a minor amount of phosphotyrosine after purification from bacteria. Further, YET displayed the same level of autophosphorylation *in vitro* as did the wild type enzyme. However, there was no observable increase YET phosphorylation in the presence of either activated forms of Erk1. These data demonstrate that at positions 202 and 204 in Erk1, Mek1 has a high degree of specificity for the amino acids threonine and tyrosine.

With the exception of the SEY allele, none of the mutants displayed any appreciable MBP phosphotransferase activity. This probably reflects the specific interactions Thr-202 and Tyr-204 must make with specific residues in the Erk1 molecule after phosphorylation by the Mek1. It has been hypothesized that the inactive form of MAP kinase would require both local and global conformational changes after phosphorylation that would be controlled by Erk1 phosphorylation (Zhang *et al.*, 1994). Indeed, phosphothreonine and phosphotyrosine interaction with conserved arginine residues induces structural changes that allow the enzyme to assume an active conformation (Canagarajah *et al.*, 1997).

The intervening residue between the regulatory threonine and tyrosyl phosphorylation sites differs among the MAP kinase family members. Each subfamily possesses a unique conserved residue (Ferrell, 1996). In the Erk isoforms, the intervening Xaa residue is occupied by glutamic acid whereas Jnk and Hog MAP kinase subfamilies encode proline and glycine, respectively, at this position. To examine what role the intervening amino acid plays in directing Mek substrate specificity, the glutamic acid residue in TEY was replaced with the cognate residue present in the two stress-activated MAP kinases. The TPY and TGY alleles were phosphorylated to approximately 30% of the original Erk1 wild type enzyme by Mek1. Phosphorylation of TPY and TGY did not stimulate any increase in MBP phosphotransferase activity of the mutant alleles. Previously, it has been suggested that the intervening sequence in Erk2 has no effect on Mek1 substrate specificity (Robinson *et al.*, 1996). Similarly, it has been reported that replacement of the glycine residue in Hog had no effect on the specificity of activation by Mkk3 or Mkk6 in transient transfection assays (Jiang *et al.*, 1997). The present work shows that replacement of an acidic amino acid with a residue containing a bulky or small side chain does indeed have a profound effect on Mek1 substrate recognition. The unusually long Mek/Erk2 preincubation period, transfection time (48 h) or absence of certain controls may account for why this was not apparent in the earlier studies (Robinson *et al.*, 1996; Jiang *et al.*, 1997).

These data demonstrate that Mek1 phosphorylates Erk1 sequentially on tyrosyl and threonyl residues that are positioned in the correct orientation. Further, the TEY motif plays a pivotal role in Mek substrate specificity. It seems likely that there are unique Mek determinants in the Erk protein kinase backbone that promote the specific association between these two enzymes. A MAP kinase-binding domain has already been mapped to the amino-terminal regions of Mek (Fukuda *et al.*, 1997). Using Hog-Erk chimera, Brunet and Pouyssegur (1996) have mapped a MAP kinase kinase



specificity determinant site to a region between catalytic subdomains III and V in MAP kinase. However, these same authors acknowledge that the chimeric molecules are substantially less active than the intact Hog and Erk proteins. We have recently identified a sea star isoform that contains the same number of amino acids as mammalian Erk1 (data not shown). Although the proteins share more than 80% identity at the amino acid level, mammalian Mek1 was unable to activate the sea star Erk1 enzyme to the same magnitude as the human isoform (data not shown). Since most of the sequence differences between the two enzymes map to regions outside the catalytic domain, it may be possible to create chimeric molecules that will uncover specific Mek1 determinant without compromising enzyme activity.

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## APPENDICES

### Appendix 1: Peptide conjugation to KLH

#### Peptide-coupling

1. Dissolve 5 mg of KLH (keyhole limpet hemocyanin) in a glass tube containing 1.0 ml of PBS (phosphate buffered saline) [pH 7.5]. Note: sometimes not all the KLH dissolves in PBS.
2. Prepare a fresh solution of 27 mg/ml SMCC (succinimidyl 4-(N-maleimidoethyl) cyclohexane-1-carboxylate) in dimethylformamide (DMF).
3. Add 30  $\mu$ l of SMCC to the KLH solution and let stir for 30 min at room temperature.
4. Prepare the peptide solution by dissolving 8 mg of peptide in 300  $\mu$ l of 4 M guanidine hydrochloride (Gu-HCl) in PBS, [pH 7.5]. Add 7-7.5 mg of solid dithiothreitol (DTT) and allow to stand for 10 min. This reduces the dimerization of the peptides through disulfide-linkage of cysteine residues.
5. Add the peptide solution to the top of the equilibrated Sephadex G10 column (see below) and centrifuge for 3 min at 100 x g. Wash the column with 250  $\mu$ l of 4M Gu-HCl in PBS. This removes the excess DTT.
6. Transfer the reaction eluate (~1550  $\mu$ l) to a fresh tube and allow the mixture to stir overnight.
7. The KLH cross-linked peptides are stored at -70°C until needed.

#### Sephadex G10 column preparation

1. Suspend 1 g of dried Sephadex G10 powder in distilled water. Decant the liquid and resuspend the resin a second time in distilled water. Allow swelling to continue overnight.
2. Decant the water and wash the resin once with 4 M Gu-HCl in PBS and then soak once overnight in the same solution.
3. Add Sephadex G10 previously equilibrated with 4 M Gu-HCl in PBS (see below) to a plastic, fritted column. Centrifuge the column at 1,000 rpm (125 g) in such a way that the excess Gu-HCl is collected into a receiving tube.

## Appendix 2: Antigen preparation

Complete Freund's adjuvant is used for the initial injection while incomplete adjuvant is used for subsequent injections. Several injections of decreasing KLH-peptide concentration is used for immunizations: first injection use 250 µg/rabbit, second and third use 200 µg/rabbit and subsequent boosts use 100 µg/rabbit.

1. Prepare 1 ml of Freund's adjuvant for each animal combined with 1 ml of PBS:

Number of rabbits	PBS (ml)	Adjuvant (ml)
1	1	1
2	1.5	1.5
3	2	2
4	2.5	2.5

2. Emulsify the PBS, KLH-peptide and Adjuvant by drawing and expelling the liquid using 18G1/2 gauge needle and syringe system. The emulsion is complete when a drop is unable to disperse in water.
3. Keep stored at 4°C until needed.

### Appendix 3: Antibody isolation, purification and quantitation

#### Isolation of rabbit sera

1. Trained animal technicians remove 30 ml of blood from the rabbit's ear. The blood is allowed to clot at room temperature. However, by the time the blood is received from the Animal Care Facility it is already partially clotted and can either be stored at 4°C overnight or processed immediately.
2. Centrifuge the 50 ml conical tubes at 1300 x g for 5 min to separate the serum from the haemopoietic cells. Discard clot in a biohazardous waste container.
3. Decant the serum to a fresh tube and recentrifuge to remove the remaining erythrocytes. It may be necessary to remove any remaining impurities by sequentially filtering the serum through a 0.45 µm filter followed by a 0.22 µm filter.
4. Decant the serum to a fresh tube and store at -20°C.

#### Affinity column antibody purification

1. Elute the storage buffer and wash the column with ~10 ml of PBS followed by one bed volume of 0.1 M glycine. Equilibrate the column with 10 ml of PBS and verify that the wash through pH is neutral.
2. Using a Pasteur pipette gently resuspend the affinity column affinity resin with 10 ml of serum. Transfer the beads to the 50 ml Falcon tube containing the remainder of the serum. Fasten the cap tightly and as a precaution secure the top of tube with Parafilm. Incubate the tube at 4°C on a vertical rotating table.
3. Allow the bead/serum slurry to equilibrate to room temperature. Prepare the column by pouring the slurry into the column while collecting the break-through material. Reapply the break-through material to the column a second time to maximize antibody binding.
4. Rinse the depleted serum from the column with one bed volume of PBS. Save an aliquot for ELISA analysis.
5. Remove unbound proteins and air bubbles from the gel by gently resuspending the beads in PBS with a Pasteur pipette. Allow the resin to settle before eluting the buffer.
6. Prepare to elute the antibody by running one bed volume of 0.1 M glycine, [pH 5.2] into the column.
7. Add more glycine buffer to the top of the column bed. Collect five-1 ml fractions. Verify that the second fraction is pH 2.5 (if not collect a sixth fraction). Immediately neutralize the eluted antibody fractions by adding 5 µl of Tris-base.
8. Remove and save 5 µl of the neutralized antibody fraction for ELISA testing.

9. The column is washed and reequilibrated with PBS then stored in PBS containing 0.5% sodium azide at 4°C.

#### Quantitation by enzyme-linked immunosorbent assay (ELISA)

1. Bind the peptide antigen to the microtitre plate by addition of 50 µl of antigen solution (10 µg/ml peptide diluted in 1X PBS containing 0.5% NaN<sub>3</sub>) to all the wells of a microtitre plate. Cover and incubate overnight at 4°C.
2. Remove the unbound peptide antigen by washing the plate in blocking solution (1X PBS containing 0.5% skim milk powder). The milk protein casein reduces the background by binding the unoccupied binding sites in the wells. Remove excess blocking solution by centrifuging in a lettuce drier. Repeat the washing step three times.
3. Dilute the primary antibody none applied and break through samples 1:1000 and the eluted fractions 1:100 with blocking solution. Add 100 µl of each dilution to column of wells. To the remainder of wells on the microtitre plate add 50 µl of blocking solution.
4. Titrate the primary antibody by performing sequential titrations in the blocking solution of the subsequent column. Continue the titration up to and including the eleventh column. Leave the twelfth column as a positive control. Incubate overnight at 4°C.
5. Wash the excess antibody solution three times in blocking solution.
6. Dilute the peroxidase labelled goat anti-rabbit secondary antibody 1:2000 in blocking solution. Add 50 µl of secondary solution to each well of the microtiter plate. Incubate for 2 h at ambient temperature.
7. Wash the plates three times with blocking solution then repeat three times with fresh distilled water.
8. Add 50 µl of substrate solution 1mg/ml of 2, 2'-azino-bis(3-ethylbenzthiazoline-sulfonic acid (ABTS) in citrate buffer and add 1:500 3% H<sub>2</sub>O<sub>2</sub> prior to using the solution) to each well of the microtiter plate. Allow the colour to develop for 1 h at room temperature and in the dark.
9. Read plates using an ELISA reader.

#### Appendix 4: Peptide affinity column preparation

1. Weigh 600 mg of thiol-activated Sepharose and combine it with distilled water in a 15 ml borosilicate tube. Gently suspend the resin by inverting the tube.
2. Centrifuge the tube at 1900 x g for 1 min and remove the supernatant by aspiration. Repeat the washing step twice with distilled water.
3. Equilibrate the thiol-activated Sepharose with 14 ml of PBS. Centrifuge and decant as above.
4. Prepare peptide by measuring 3-4 mg of lyophilized peptide per ml of gel resin.
5. The peptide solution is combined with the pre-swollen beads in a 50 ml conical tube and the reaction is allowed to incubate at room temperature for 3 days. The mixture should be swirled occasionally. The tube cap should be loosely fastened since gases are created as a by-product of the oxidation of disulphide groups.
6. At the completion of the reaction, centrifuge the beads and aspirate the supernatant. Wash the beads several times in PBS and store at 4°C in PBS containing 0.5% sodium azide.

Appendix 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Reagents:

Resolving gel buffer: 1.5 M Tris-HCl [pH 8.8], 0.4% SDS.

Dissolve 145.2 g Tris-base, 47.6 g Tris-HCl and 4 g SDS in 1,000 ml deionized H<sub>2</sub>O. There is no need to titrate with HCl.

Stacking gel buffer: 0.5 M Tris-base [pH 6.8], 0.4% SDS.

Dissolve 30.2 g Tris-base and 2 g SDS in 450 ml. H<sub>2</sub>O. Titrate pH to 6.8 with concentrated HCl. Increase the volume to 500 ml with deionized water.

Acrylamide buffer: 30% acrylamide, 0.8% bisacrylamide

Dissolve 300 g of acrylamide and 8 g of bisacrylamide in 1,000 ml deionized water.

Electrophoresis buffer: 10X concentration

Dissolve 30 g Tris-base, 144.2 g glycine and 10 g SDS in 1,000 ml deionized H<sub>2</sub>O.

Ammonium persulfate: 10% concentration

Dissolve 100 mg of ammonium persulfate in deionized H<sub>2</sub>O. Store for no more than a week at 4°C.

I. Polymerization of two regular size resolving slab gels:

Stock Solutions	9%	10%	11%	12%
Acrylamide buffer	24.0 ml	26.6 ml	29.4 ml	32.0 ml
Resolving buffer	26.4 ml	26.4 ml	26.4 ml	26.4 ml
Deionized H <sub>2</sub> O	29.6 ml	27.0 ml	24.2 ml	21.6 ml
Degas for 5 min				
TEMED	28.0 $\mu$ l	28.0 $\mu$ l	28.0 $\mu$ l	28.0 $\mu$ l
10% ammonium persulfate	160 $\mu$ l	160 $\mu$ l	160 $\mu$ l	160 $\mu$ l

Procedure:

1. Prepare the resolving gel solution as described above and degase for 5 min.
2. Add TEMED and ammonium persulfate to resolving solution, mix gently and pour carefully into the gel apparatus.

3. Overlay the polymerizing gel with water-saturated iso-butanol to create an even surface free of air bubbles and to prevent evaporation.
4. At the completion of polymerization (~ 1 h), rinse the gels with water and prepare to pour the stacking gel. Note: the gel(s) may be stored at 4°C if water is added to prevent dessication.

Polymerization of two regular size stacking gels:

Stock Solutions	4%
Acrylamide buffer	4.0 ml
Stacking buffer	7.5 ml
Deionized H <sub>2</sub> O	18.5 ml
Degas for 5 min	
TEMED	30.0 µl
10% ammonium persulfate	90.0 µl

5. Prepare the solution as described above and degas the stacking solution for 5 min.
6. Slowly pour the stacking solution over the polymerized resolving gel so as not to introduce air bubbles. Insert the gel comb and allow the gel to polymerize for 1 h. Note: gel combs are available with 10, 15 or 20 wells.

II. Polymerization of two mini-size resolving slab gels:

Stock Solutions	9%	10%	11%	12%
Acrylamide buffer	7.2 ml	8.0 ml	8.8 ml	9.6 ml
Resolving buffer	6.0 ml	6.0 ml	6.0 ml	6.0 ml
Deionized H <sub>2</sub> O	10.8 ml	10.0 ml	9.2 ml	8.4 ml
Degas for 5 minutes				
TEMED	10 µl	10 µl	10 µl	10 µl
10% ammonium persulfate	90 µl	90 µl	90 µl	90 µl

Procedure:

1. Prepare the resolving gel solution as described above and degase for 5 min.
2. Add TEMED and ammonium persulfate to resolving solution, mix gently and pour carefully into the gel apparatus.
3. Overlay the polymerizing gel with water-saturated iso-butanol to create an even surface free of air bubbles and to prevent evaporation.

4. At the completion of polymerization (~20 min), rinse the gels with water and prepare to pour the stacking gel. Note: the gel(s) may be stored at 4°C if water is added to prevent dessication.

Polymerization of two mini size stacking gels:

Stock Solutions	5%
Acrylamide buffer	1.3 ml
Stacking buffer	2.0 ml
Deionized H <sub>2</sub> O	4.7 ml

Degas for 5 min.

TEMED	20 µl
10% ammonium persulfate	60 µl

5. Prepare the solution as described above and degas the stacking solution for 5 min.
6. Slowly pour the stacking solution over the polymerized resolving gel so as not to introduce air bubbles. Insert the gel comb and allow the gel to polymerize for 20 min. Note: gel combs are available with 5 and 10 wells.



## Appendix 6: Silver staining

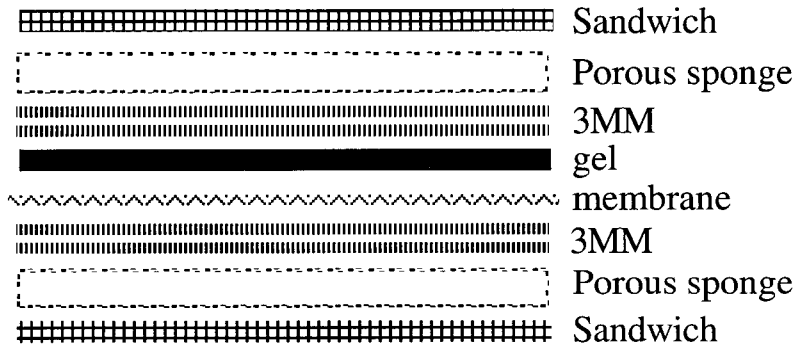
1. Fixative 1:	Combine methanol:acetic acid:water (40:10:50 by volume).	30 min
2. Fixative 2:	Combine ethanol:acetic acid:water (10:10:80 by volume).	2 x 15 min
3. Oxidizer:	Dissolve 200 mg $K_2Cr_2O_7$ and 40 $\mu$ l nitric acid in 200 ml deionized water.	5 min
4. Wash:	Remove excess oxidizer with deionized water.	3 x 5 min
5. Silver reagent:	Dissolve 408 mg $AgNO_3$ in deionized water.	20 min
6. Wash:	Remove excess silver reagent with deionized water.	1 min
7. Developer:	Combine the following in the fume hood 17.82 g $Na_2CO_3$ and 1 ml formaldehyde in 600 ml deionized water.	desired background
8. Stop:	Add a solution of 5% acetic acid.	5 min
9. Storage:	Place in deionized water.	overnight
10. Preservation:	Dry between two sheet of cellophane.	see Section 10, i

## Appendix 7: Gel transfer and Western immunoblotting

### Reagents

- 10X Transfer buffer: 250 mM Tris-base [pH 8.6], 1.92 M glycine.  
Dissolve 98.66 g of Tris-base and 360 g of glycine in 2 l of deionized water.
- 1X Transfer buffer: 25 mM Tris-base [pH 8.6], 192 mM glycine and 10 methanol.  
Dilute 500 ml of 10X transfer buffer and 1 l of methanol in 3.5 l deionized water.
- 10X TBS: 500 mM Tris-base [pH 7.5], 1.5 mM NaCl.  
Dissolve 60.55 g of Tris-base and 87.66 g NaCl in 1 ml deionized water.
- 1X TBS: Dilute 100 ml of 10X TBS in 900 ml deionized water.
- 1X TBST: Same as 1X TBS except add 500  $\mu$ l of Tween-20.
- Carbonate buffer: 100 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>-6H<sub>2</sub>O [pH 9.8].  
Dissolve 8.4 g of NaCO<sub>3</sub> and MgCl<sub>2</sub>-6H<sub>2</sub>O in 1 l of deionized water.
- 5% blocking solution: Dissolve 5 g skim milk powder in 100 ml 1X TBS.
- NBT solution: Dissolve 30 mg NBT in 1 ml 70% DMF.
- BCIP solution: Dissolve 15 mg BCIP in 1 ml DMF.
1. Cut the nitrocellulose membrane to the dimensions of the gel or to the size of the region of the gel where the protein migrates. Cut four pieces of Whatman 3MM chromatography paper to the exact size of the membrane.
  2. Soak the chromatography paper, transfer membrane and the porous sponge in the transfer buffer.
  3. Assemble the transfer sandwich as outlined below:

cathode (-ve)



anode (+ve)

4. Place the sandwich unit into the chamber containing 1X transfer buffer and transfer for 3 h at 300 A.
5. Disassemble the transfer sandwich and block the nitrocellulose membrane in 5% skim milk.
6. Wash the membrane for 5 min with three changes of TTBS.
7. Add the primary antibody (titre 1/256,000) diluted 1:500 in TTBS. Incubate at ambient temperature overnight.
8. Using three changes of TTBS, wash the membrane for 5 min followed by a final wash with TBS.
9. Develop the blot with 100 ml carbonate buffer containing NBT and BCIP solutions.
10. Allow the reaction to continue until the desired intensity is obtained.
11. Quench the reaction by placing the membrane in deionized water.

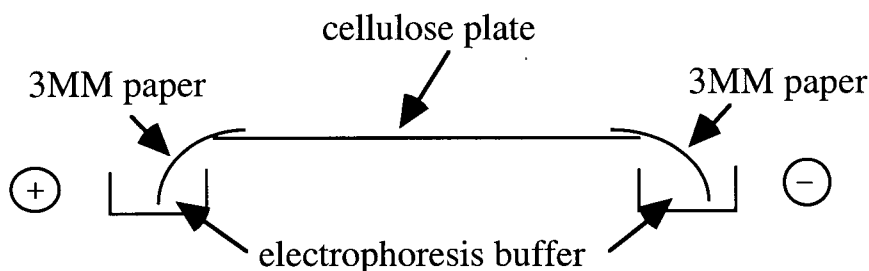
## Appendix 8: Phosphoamino acid analysis

### Reagents:

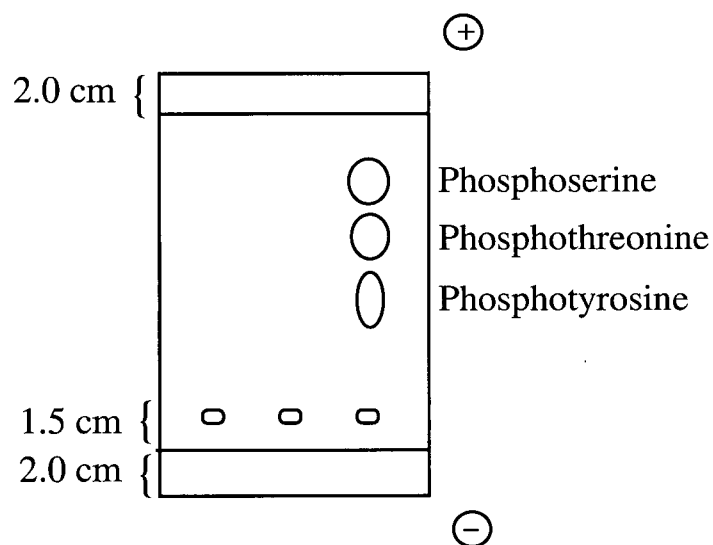
Electrophoresis buffer: pyridine:acetic acid:water (1:10:189 by volume).

Combine 5 ml pyridine and 50 ml glacial acetic acid with 945 ml deionized water.

1. At the completion of the SDS polyacrylamide gel electrophoresis (Section 9) the gel containing the radioactive band(s) is transferred to Millipore PVDF membrane (Appendix 7) and an autoradiogram is performed.
2. Excise the band of interest from the membrane. Mince the membrane using a scalpel into manageable pieces and place them into a Pierce reacti-vial.
3. Add 300  $\mu$ l of constant boiling 6N HCl (Pierce) and hydrolyse for 1 h at 105°C. Caution: heating for longer periods can destabilize the phosphate moiety of the phosphothreonine and phosphoserine residues.
4. Transfer the solution to an eppendorf tube and lyophilize the sample under vacuum. Wash the radioactive residue with several volumes of deionized water in alternation with vacuum drying. Reduce the amount of water used in each successive step i.e., 300  $\mu$ l, 150  $\mu$ l and 50  $\mu$ l.
5. Resuspend the sample in 5  $\mu$ l of electrophoresis buffer containing 1 mg/ml each of phosphoserine, phosphothreonine and phosphotyrosine. This will allow a direct comparison between the radioactive spot(s) detected by autoradiography and one or more of the phospho-standards.
6. Apply 1  $\mu$ l of the sample to a thin layer chromatography (TLC) cellulose plate (EM separations, NJ) and dry with a hair dryer to prevent diffusion. This is repeated until all the sample has been applied to the plate.
7. Install the plate. A sample diagram for installation of the plate on a LKB 2117 Multiphor II Electrophoresis Unit is shown below:



8. Premoisten the plate with a piece of 3MM chromatography paper saturated with electrophoresis buffer. For the area surrounding the applied sample, cut a hole in  
a 3MM paper twice the size of the spot on the plate and carefully place it over the area. The spot should be visible through the hole in the filter paper. This will enable the liquid to migrate uniformly toward the centre of the spotted samples. Absorb any excess liquid with a dry piece 3MM paper.
9. Perform the electrophoresis at 1,000 V for 50 min using a cooling apparatus.
10. Dry the plate using a hair dryer in the fume-hood. Reveal the positions of the phosphoamino acid standards by applying ninhydrin spray (0.2% in Ethanol) followed by heating in a 95°C oven for 5 min.
11. Autoradiograph. The figure shown below reveals the position of the three phosphoamino acid standards after electrophoresis:



## Appendix 9: Two-dimensional phosphopeptide mapping

### Reagents:

Digest buffer: 50 mM ammonium bicarbonate [pH 8.0].

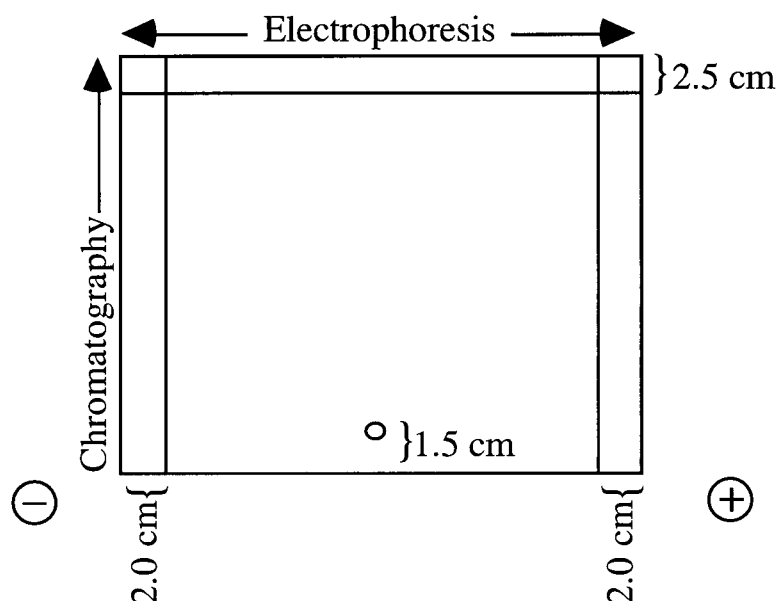
Dissolve 40 mg of ammonium bicarbonate in 10 ml deionized water. pH titration is not needed.

Electrophoresis buffer: 1% ammonium bicarbonate [pH 8.9].

Dissolve 10 g ammonium bicarbonate in 1 l of deionized water. Adjust pH with concentrated ammonia.

Chromatography buffer: butanol:acetic acid:water:pyridine (10:3:12:15, by volume).

1. After completing the SDS-PAGE (Methods, Section 9) fix the proteins and remove the SDS by soaking the gel in methanol:acetic acid:water (1:1:4 by volume) twice for 30 min.
2. Remove the acetic acid by soaking the gel twice for 20 min in absolute methanol.
3. Identify the radioactive band(s) by autoradiography.
4. Excise, swell and count by Cerenkov the bands of interest.
5. Mince the gel into small cubes and place it in a 2 ml screw-cap tube before drying them in a vacuum dessicator
6. Digest the radioactive protein in 1 ml of 50 mM ammonium bicarbonate containing 100 µg/ml TPCK-trypsin. Incubate in a rotating platform incubator at 37°C for 24 h. To ensure complete digestion add another millilitre of fresh trypsin and continue incubation for another 24 h.
7. Dry the sample in a vacuum concentrator. Wash the radioactive residue with water in alternation with vacuum drying. Reduce the amount of water used in each successive step i.e., 300 µl, 250 µl and 50 µl.
8. Determine the level of radioactivity by Cerenkov counting.
9. Resuspend the sample in electrophoresis buffer containing 0.5% phenol red and apply to a thin layer chromatography TLC cellulose plate as follows:



10. Premoisten the TLC plate using a Whatmann 3MM chromatography paper saturated with electrophoresis buffer. Do not touch the applied sample. For the area surrounding the applied sample, cut a hole in a piece of 3MM paper twice the size of the spot on the plate and carefully place the saturated 3MM over the plate. Allow the liquid to diffuse inward toward the centre of spot. Blot any excess liquid with a dry piece of 3MM paper.
11. Electrophorese at 700 V for 1 h. At the completion of the run (i.e. when the phenol red reaches to within 2 cm of the anode) remove the plate and dry.
12. Perform the second dimension chromatography by placing the plate in the chromatography buffer. Stop the ascent when the solvent front reaches within 2.5 cm from the top (~7 h).
13. Dry and autoradiograph.

## Appendix 10: RNA isolation

### Reagents:

RNA extraction buffer M: 4 M guanidinium thiocyanate, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol.

Dissolve 250 g guanidinium thiocyanate in 293 ml deionized water. Add 17.6 ml 750 mM sodium citrate [pH 7.0] and 26.4 ml 10% sarcosyl and heat to 65°C. Buffer I is prepared by the addition of 360 µl of 2-mercaptoethanol to the above stock solution.

DEPC-treated solutions: Treat all solution for RNA work with a 0.1% concentration of the ribonuclease chemical inactivator diethyl pyrocarbonate (DEPC). DEPC is converted to ethanol and CO<sub>2</sub> when thoroughly autoclaved. Non-autoclavable solutions can be heated at 60°C overnight. Caution! In solutions containing Tris, DEPC-treated water is used instead since DEPC is unstable in the presence of Tris buffers.

1. Grow cells in a monolayer to a confluency of 10<sup>8</sup>.
2. Decant the culture medium and wash the cell twice in 1X PBS (without magnesium and calcium).
3. Add 1 ml of PBS and dislodge the cells from the plate using a rubber policeman. Transfer the cells to Falcon 2059 polypropylene tube.
4. Collect the cells by centrifugation at 200 x g for 5 min at 4°C. Decant the supernatant.
5. Homogenize the cells in 1.8 ml of RNA extraction buffer I per 100-mm plate.
6. Sequentially add the following reagents in the order listed: 100 µl of 2 M sodium acetate; 1.0 ml water-saturated phenol (nucleic acid grade); 200 µl chloroform:isoamyl (49:1 by volume).
7. Mix the suspension thoroughly after adding each reagent. Once all the reagents are combined shake the tube vigorously for 10 sec.
8. Store on ice for 15 min.
9. Separate the aqueous from the organic phase by centrifugation at 10,000 x g for 20 min at 4°C.
10. Transfer the aqueous phase to a fresh 2059 tube, mix with 1 ml isopropanol and store at -20°C for a minimum of 1 h (better results are sometimes obtained with longer storage periods).
11. Sediment the RNA at 10,000 x g.



12. Aspirate the supernatant and resuspend the pellet in 300  $\mu$ l of buffer M. Transfer the solution to a 1.5 ml microfuge tube, reprecipitate the RNA with 300  $\mu$ l of isopropanol and store at -20°C for a minimum of 1 h.
13. Wash the pellet with 75% ethanol and vacuum dessicate.
14. Redissolve the RNA in 50  $\mu$ l of DEPC-treated water. To facilitate solubilization incubate the tube at 65°C for 10 min.

Appendix 11: Detection of DNA fragments by ethidium bromide staining of agarose gels.

Reagents:

50X Tris-acetate-EDTA (TAE): Combine 242 g Trizma with 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA [pH 8.0]. Adjust the volume to 1 l with deionized water.

5X gel loading buffer: Combine 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% glycerol (by volume) in 10 ml water.

I. Preparation of an agarose gel

Horizontal gel apparatuses for agarose gel are available in different sizes and designs depending on the manufacturer. Bio Rad gel molds and electrophoresis tanks were used throughout this study because their durability and simplicity of use. The percentage agarose gel used to analyse DNA ranged from 0.8-1.2% while the volumes used to caste the gel range from 100 ml for a small gel (7 cm x 10 cm) and 500 ml (10 cm x 15 cm) for a large gel.

1. Seal the edges of a clean and dry plastic gel casting tray with masking tape. Insure that the tray is set on a level horizontal section of the bench top.
2. Add an appropriate amount of powdered agarose to a specified volume of 1X TAE electrophoresis buffer in an Erlenmeyer flask so as to achieve the desired percent agarose gel concentration.
3. Heat the suspension in a microwave oven until the agarose is dissolved. Occasional swirling of the liquid in the flask prevents clumping of the agarose. Note, always wear protective gloves when manipulating the heated solution.
4. Allow the solution to cool to 60°C before mixing thoroughly the ethidium bromide (10 mg/ml) to a final concentration of 0.5 µg/ml. Caution! Ethidium bromide is a potent mutagen so protective clothing and gloves must always be worn.
5. Slowly pour the warm agarose solution into the gel tray to a thickness of between 3-5 mm. Remove any bubbles with a pipetteman. Install the comb to one end of the mold and verify that there are no bubbles
6. After casting the gel, allow 30-45 min for the agarose to solidify at room temperature.
7. Remove the comb and the masking tape. Place the mold in the electrophoresis tank and cover it with sufficient electrophoresis buffer (usually 2-3 mm above the gel line).
8. Carefully load the samples of DNA prepared in loading buffer in the wells of the submerged gel using a pipetteman.

9. Place the cover on the electrophoresis gel tank in such a way so as to insure that the negatively charged DNA migrates toward the anode (red lead). Attach the leads to the power supply and adjust the voltage to 1-5 V/cm (the measured distance between the electrodes).
10. When the bromophenol blue and xylene cyanol FF have migrated a sufficient distance (based on the size fragment to be observed) stop the power supply and detach the lid of the gel apparatus.
11. Examine the gel under ultraviolet light and take a photograph. Warning! Wear U.V. protective eyewear.

## Appendix 12: Digestion of DNA with restriction enzymes.

Throughout this study restriction enzymes were invaluable for generating physical maps based on restriction site frequency or reconfiguring DNA through subcloning or site-directed mutagenesis. All enzymes were purchased from New England BioLabs. The following protocol is used for routine analysis and manipulation of genetic information.

### Reagents:

Reaction Buffer: New England provides the 10X NEBuffer reaction buffer specific for each restriction endonuclease.

6X gel loading buffer: Combine 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% glycerol (by volume) in 10 ml water.

1. Pipette 1-2  $\mu$ g of DNA solution into a sterile Eppendorf microfuge tube. Note that the DNA should be free of any contaminants such as phenol, chloroform, EDTA, excessive salt or detergents which may inactivate the highly labile restriction enzyme.
2. Add 2  $\mu$ l of the appropriate 10X restriction endonuclease buffer. Adjust the volume with deionized water so that it will not exceed 20  $\mu$ l final once the enzyme is added to the reaction mix. Since most enzymes are supplied in a buffer containing 50% glycerol, it is important to reduce the glycerol concentration to less than 5% to assure maximal enzyme activity.
3. Add 2-3 units of restriction enzyme to the reaction and mix well by pipetting gently. Higher or lower concentration may be required depending on the stability and efficiency of the enzyme.
4. Incubate the reaction for the time and temperature recommended by the manufacturer.
5. If no other manipulations are to be performed, add 5  $\mu$ l of 6X gel loading buffer, otherwise most restriction endonucleases can be heat inactivated at 65°C for 20 min. In instances where a double digest is to be performed with incompatible buffer systems, it is important to perform the first restriction digest with the buffer containing the lower salt concentration.

## Appendix 13: DNA ligation into plasmid vectors

The ligation technique was most often used to clone cDNA fragments into appropriate vectors for sequencing or into prokaryotic expression plasmids for the production of recombinant GST-fusion proteins. The ligation of blunt-ended DNA and compatible cohesive termini was performed during the course of these studies.

### I: Ligation of compatible cohesive termini

1. Digest the supercoiled plasmid DNA to completion with the appropriate restriction enzyme(s) (Appendix 12). Verify that the digestion is complete by electrophoresing an aliquot of the reaction through an agarose gel (Appendix 11).
2. Purify the restricted plasmid with Sephaglas according to Pharmacia's specifications. After resuspending the DNA in the buffer provided, remove an aliquot of the DNA (~200 ng) and store at -20°C (see later).
3. To dephosphorylate the remainder of the DNA, add an appropriate volume of 10X Calf intestinal alkaline phosp hatase (CIP) buffer provided by the manufacturer and incubate at 37°C for 30 min.
4. At the completion of the incubation period, terminate the reaction by adding 0.5% SDS, 5 mM EDTA [pH 8.0] and 100 µg/ml proteinase K. Optional: incubate at 55°C for 30 min and purify with Sephaglas.
5. Digest the foreign DNA with a restriction enzyme that creates protruding termini compatible with the plasmid vector (Appendix 12). Purify the DNA fragment for ligation with Sephaglas.
6. For the ligation reaction, combine the fragment and the dephosphorylated vector in microcentrifuge in a molar ratio of 2:1. Add 2 µl of 10X T4 DNA ligase buffer, 0.1 unit of bacteriophage T4 DNA ligase and sufficient water to increase the reaction volume to 20 µl total. Prepare control ligations with non-dephosphorylated and dephosphorylated DNA vector.
7. Incubate overnight at 16°C.
8. Transform competent *E. coli* with the foreign and control ligations (Appendix 14).

### II. Ligation of blunt end termini

1. Prepare the vector and foreign DNA as described in steps 1 -4. except for the dephosphorylation reaction add the 1 unit CIP/2 pmoles of DNA and incubate at 37°C for 15 min. Continue the incubation by addition of another aliquot of CIP for a further 45 min at 55°C.
2. Perform the ligation reaction in a 20 µl final volume containing a 3:1 molar ratio of insert to vector, 2 µl 10X T4 DNA ligase buffer, 2 µl 5% polyethylene glycol 8000 (wt/vol) and 1 unit of bacteriophage T4 DNA ligase. Incubate at 16°C overnight.

3. Transform competent *E. coli* with the control and insert ligation as described (Appendix 14).

Appendix 14: Transformation of recombinant plasmid vectors  
into *E. coli*

I. Preparation of competent *E. coli* cells.

Competent bacterial cells with a transforming efficiency of  $5 \times 10^6$  to  $2 \times 10^7$  colonies per microgram of supercoiled plasmid DNA were prepared using a modified procedure first described by Cohen et al. (1972). *E. coli* strains XL.1 Blue, DH5 $\alpha$  and UT5600 were used during the course of this research.

1. Inoculate a 250 ml sterile Erlenmeyer flask containing 50 ml of 2 x YT (Appendix 15) with a bacterial colony selected from a freshly streaked 2 x YT agarose plate (grown for 12-16 h maximum). Incubate overnight at 37°C with vigorous shaking (220 cycles/min in a rotary shaker).
2. After 12-16 h of growth, add the 50 ml culture to a 1 l Erlenmeyer flask containing 250 ml of fresh 2 x YT. Incubate at 37°C in a rotary shaker. Monitor the number of viable cells by measuring their growth at OD<sub>600</sub> (optical density measurement at a wavelength of 600 nm) every 15-20 min. Since transformation efficacy decreases with increasing cell population density, it is essential that the spectrophotometer measurement fall within an OD range of 0.4-0.6.
3. Once the bacteria have reached the desired growth stage, aseptically transfer 50 ml of culture to 2070 Falcon conical tubes for centrifugation at 850 x g for 5 min. Carefully decant the medium and resuspend the bacterial pellet in 20 ml of sterile ice cold 50 mM CaCl<sub>2</sub> (Sterilize the CaCl<sub>2</sub> solution by filtering through 0.45  $\mu$ m filter). Incubate on ice for 20 min.
4. Recover the bacteria by centrifuging the suspension for 5 min at 800 x g. Decant the supernant and resuspend each pellet in 1.7 ml cold CaCl<sub>2</sub> solution. Add 300  $\mu$ l of glycerol to the suspension to a final concentration of 15 %.
- 5.. Transfer 200  $\mu$ l of the bacterial suspension to a sterile Eppendorf tube and immediately place the tube in a ethanol/dry ice bath to rapidly freeze the the cells. Store for later use at -70°C.

II. Transformation of *E. coli*

6. Thaw the frozen stock of competent bacteria on ice. Transfer the 200  $\mu$ l of bacterial suspension to a Falcon 2059 tube and add 500 ng of supercoiled plasmid DNA. Gently flick the tube to mix the contents before incubating on ice for 15 min. For ligation reactions add 1/4 to 1/2 of the mixture to the competent cells and 90  $\mu$ l of transformation buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>).
7. Place the tube in a preheated 42°C circulating water bath for 90 sec with gentle agitation.
8. Return the tube to the ice for another 5 min.

9. Add 3 ml of 2 x YT to the transformation and transfer the tube to a rotating shaker incubator (220 rpm) for 20 min at 37°C. This is recovery stage for the bacteria enabling them to grow and express the antibiotic resistance marker of the plasmid vector.
10. Recover the bacteria by centrifugation at 800 x g for 8 min. Decant the supernatant and resuspend the pellet in 200-1,000 µl of 2 x YT. Spread 50-200 µl evenly on a 2 x YT plate containing the appropriate antibiotic. Let stand at room temperature until the liquid is absorbed.
11. Invert the plates and incubate overnight at 37°C.



## Appendix 15: Preparation of 2 x YT and NZY bacterial medium

### I. Medium

2 x YT: In 900 ml of deionized water combine 16 g of bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl.

NZY: In 900 ml of deionized water combine 10 g NZ amine, 5 g NaCl, 5 g bacto-yeast extract, 1 g casamino acids and 2 g  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ .

Dissolve all the solutes before adjusting the pH to 7.0 with concentrated NaOH. Increase the volume to 1 l with deionized water and sterilize by autoclaving for 20 min at 15 lb/sq inch on liquid cycle.

### II. Top agarose and plates

2 x YT or NZY: Follow the recipe as outlined above except add 15 g agarose per litre of medium for plates and 7 g per litre for top agarose.

Thermolabile antibiotic compounds should be added when the temperature of the medium has been reduced to 50°C. Pour approximately 30-35 ml of warm medium per 90-mm plate. Remove bubbles using a flame from a bunsen burner. Allow the plates to cool to room temperature before inverting and placing then at 4°C until needed. For top agarose, allow the medium to harden in the bottle with the lid slightly loosened.

## Appendix 16: DNA purification.

- I. The boiling lysis protocol was adapted from Holmes and Quigley (1981) for the preparation of small-scale plasmids.

### Reagents:

STET buffer: 0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0] and 5% Triton X-100.

Dilute 10 ml of 1 M NaCl, 1 ml of 1 M Tris-HCl [pH 8.0], 200  $\mu$ l EDTA [pH 8.0] and 25 ml Triton X-100 in 64 ml of deionized/autoclaved water.

TE buffer: see Appendix 19.

1. Grow a 3 ml overnight culture in the appropriate antibiotic from a single colony on a bacterial plate.
2. Transfer 1.5 ml of bacterial culture to a sterile Eppendorf microcentrifuge tube and pellet the bacteria by centrifuging at 15,000 x g.
3. Decant the supernatant and resuspend the bacterial pellet in 350  $\mu$ l of STET.
4. Place the tube in a 100°C heating block for 40 sec.
5. Remove the bacterial debris by centrifuging the lysate at room temperature for 10 min at 15,000 x g.
6. Transfer the supernatant containing the DNA to a fresh sterile tube.
7. To the supernatant add 40  $\mu$ l of 2.5 M sodium acetate [pH 5.2] and 420  $\mu$ l of isopropanol. Mix thoroughly by vortex. Incubate the tube at room temperature for 5 min.
8. Pellet the nucleic acids by centrifugation at 15,000 x g for 5 min in 4°C in microfuge.
9. Carefully aspirate the supernatant using a 'pulled Pasteur pipette'. Allow the remaining liquid to drain away by standing the tube in an inverted position.
10. Wash the pellet with 70% ethanol and recentrifuge at 15,000 g for 2 min at 4°C in microfuge.
11. Again aspirate the supernatant as outlined in step 8. Leave the tube at room temperature to allow evaporation of the ethanol (5-10 min).
12. Redissolve the nucleic acid pellet in TE buffer [pH 8.0] containing 20  $\mu$ g/ml DNase-free RNase. Note: DNases may be heat-inactivated by boiling the RNase preparation at 100°C for 10 min.
13. Store at -20°C.

II. The TELT purification procedure was developed by He et al. (1989) as a one-step rapid purification of small quantities of plasmid DNA.

Reagents:

TELT buffer: 50 mM Tris-HCl [pH 8.0], 62.5 mM Na<sub>2</sub>EDTA, 2.5 M LiCl and 4% Triton X-100 (by volume)

Combine , 2.5 ml Tris-HCl [pH 8.0], and 6.25 ml 500 mM Na<sub>2</sub>EDTA 15.6 ml of 8.0 M LiCl, 10 ml 20% Triton X-100 in 15.65 ml of deionized/autoclaved water.

TE buffer: see Appendix 19

1. Select a single colony to grow a 3 ml overnight culture at 37°C in a temperature controlled shaker. Be careful to use the appropriate antibiotic.
2. Transfer 1.5 ml of bacterial culture to an autoclaved Eppendorf tube.
3. Centrifuge for 30 sec at 10,000 x g.
4. Resuspend the pellet in 400 µl TELT buffer.
5. Add an equal volume of phenol/chloroform and vortex for 15 sec.
6. Separate the phases by centrifuging at 15,000 x g for 60 sec at ambient temperature.
7. Transfer the upper phase to a fresh Eppendorf tube and precipitate the DNA by addition of 2 volumes of absolute ethanol.
8. Recover the DNA by centrifugation. Centrifuge at 15,000 x g for 60 sec at 4°C.
9. Wash the DNA pellet with 70% ethanol. Remove the supernatant and air dry the pellets for several minutes.
10. Dissolve the pellet in 25 µl TE containing 20 µg/ml DNase-free RNA.

III. The large-scale alkali lysis protocol was modified from the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

Reagents:

Solution I buffer: 50 mM glucose, 25 mM Tris-HCl [pH 8.0] and 10 mM [pH 8.0] EDTA [pH 8.0].

Mix 5 ml of 1 M glucose with 2.5 ml Tris-HCl [pH 8.0] and 1 ml EDTA [pH 8.0] in 91.5 ml deionized/autoclaved water. Filter, sterilize and store at 4°C.

Solution II buffer: 200 mM NaOH and 1% SDS.

Dilute 14 ml of concentrated 18.75 M NaOH and 2.5 ml of 20% SDS in 50 ml deionized/autoclaved water.

Solution III buffer: 3 M potassium acetate, 1.69 M glacial acetic acid.  
[pH 5.7]

Combine 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid in 28.5 ml deionized/autoclaved water. Ensure the pH is 5.7. Filter sterilize and store at 4°C.

TE buffer: Appendix 19.

1. Grow a 1 l overnight culture of plasmid transformed bacteria in 2 x YT (Appendix 15) containing the appropriate antibiotic.
2. Transfer the bacteria into two centrifuge bottles and sediment the cells at 12,000 x g for 10 min at 4°C.
3. Decant the supernatant and drain any remaining medium by inverting the bottles for several minutes.
4. Add 7.0 ml of chilled solution I to each bacterial pellet and mix thoroughly by vortexing. Transfer the slurry to a 50 ml conical tube.
5. All subsequent steps are to be performed on ice.
6. Add 14.0 ml of solution II to each tube and lyse the cells by gently inverting the bottles.
7. Place on ice for 10 min with occasional gentle inversion.
8. Add 7.0 ml of cold solution III to each tube and mix gently. Place the on ice for 15 min.
9. Transfer the coagulated mixture to a pre-chilled 50 ml centrifuge tube and spin at 18,000 x g for 10 min at 4°C.
10. Transfer the top aqueous layer to a 50 ml sterile conical tube and combine with an equal volume of isopropanol. Store on ice for a minimum of 15 min.
11. Transfer the 50 ml of precipitated DNA solution to two 50 ml pre-chilled centrifuge tubes (i.e. there should be four tubes containing 25 ml of solution) and centrifuge at 4°C for 15 min at 18,000 x g.
12. Discard the supernatants and air-dry the pellets briefly before resuspending each in 200 µl TE containing 20 µg/ml DNase-free RNase.
13. Combine the solution from two different centrifuge tubes into a single Eppendorf tube and incubate at 37°C for a minimum of 20 min.
14. Extract with equal volumes of phenol/chloroform (Appendix 19).

15. Transfer the top aqueous phase to a fresh Eppendorf tube and precipitate the plasmid DNA by filling the tube with 100% ethanol (-20°C). Combine phases by gently inverting the tube and storing at -20°C for a least 20 min.
16. Recover the plasmid DNA by centrifugation. Spin for 15 min at 4°C.
17. Decant the supernatant and dry the DNA pellet in a vacuum desicator.
18. Resuspend the purified plasmid in 500-1,000 µl of TE and store at -20°C.

## Appendix 17: DNA sequencing

### I. Denaturing polyacrylamide DNA gels

The protocol used for routine DNA sequencing analysis in this work is adapted from the gradient gel procedure described by Maxam and Gilbert (1980). This method is sufficiently sensitive that it can separate fragments that differ by a single nucleotide base.

#### Reagents:

Acrylamide solution: 38% acrylamide, 2% bisacrylamide.

Dissolve 380 g of acrylamide and N,N'-methylenebisacrylamide in 600 ml of deionized water by heating the solution to 37°C. Add water so that the final volume reaches 1 l. Pass the solution through a 0.45 µm filter and store in a aluminum foil covered bottle in a refrigerator.

10X TBE: 900 mM Tris-base, 900 mM boric acid and 20 mM EDTA.

Combine 105 g Tris-base, 55 g boric acid and 40 ml 500 mM EDTA [pH 8.0] with deionized water to 1 l.

Repel silane solution: 60% ethanol, 1.5% acetic acid 0.2% bind silane.

Combine 10 ml 70-100% ethanol, 2.5 ml 10% acetic acid, 37 µl bind silane with deionized water for a final volume of 17 ml.

Bind silane: Available commercially.

The majority of the sequence analysis conducted in this study was performed on an LKB/Pharmacia 2010 MacroPhor Electrophoresis Unit. There are two principles of the LKB/Pharmacia system which make it unique from other commercially available brands. First, the temperature gradient created during electrophoresis is minimized since the sequencing plate comes in direct contact with a thermostatic plate. This thermostatic plate is in essence a cooling jacket that is attached to a temperature controlled water circulator. The heat generated is easily dissipated while the temperature of the gel remains constant. Second, the acrylamide gel is dried directly onto the sequencing plate thereby eliminating the need to transfer the gel to 3MM paper.

1. Prepare the sequencing gel at least 2 h prior to electrophoresing the reaction samples. Soak the sequencing plate in water to soften the dried acrylamide. Gently scrape the gel from the glass using a plastic wedge. It may be necessary to do the same with the thermostatic plate.
2. Clean the sequencing and thermostatic plates with 70% ethanol and a lint-free tissue.
3. Complete all the following steps while wearing protective gloves.
4. Place the sequencing plate at a slight angle by raising one end. Apply 7-10 ml of bind silane to the plate and distribute the liquid evenly using a lint-free tissue. Allow time for the liquid to evaporate and the silane adhere to the glass.

5. Buff the plate with 70% ethanol.
6. Repeat steps 4 and 5 as outlined.
7. Place the thermostatic plate at a slight angle by raising one end. Apply 6 ml of repel silane to the plate and distribute evenly using a lint-free tissue. Allow enough time for the liquid to evaporate and the repel silane to adhere to the glass.
8. Buff the plate with 70% ethanol.
9. Repeat steps 7 and 8 as described.
10. Remove all traces of acrylamide from the spacers and combs.
11. Protect the working area with Bench-kote.
12. Install the thermostatic plate onto the Pharmacia/LKB Macromold. Clamp the spacers along each side of the thermostatic plate with several large bulldog binder clips. Using the supporting stand located at the bottom edge of the Macromold, place the sequencing plate in such a way that the top of the sequencing plate comes in contact only with the bottom 4 inches of the thermostatic plate.
13. Caution! Pour the gel without interruption.
14. For a 15 cm x 40 cm sequencing gel, combine 7 ml of 40% acrylamide solution with 5 ml 10X TBE and 20 ml deionized water. Dissolve 21 g of urea in the mixture. Increase the final volume of the acrylamide/urea solution to 50 ml with deionized water and filter with a 0.45  $\mu$ m filter.
15. Add 400  $\mu$ l of 10% ammonium persulfate to the solution and mix by swirling. Then add 40  $\mu$ l of TEMED and mix by swirling.
16. With the thermostatic plate and the sequencing plate in a slight inclined position (as described above), pour a small volume of acrylamide/urea solution in the grooved portion of the sequencing plate so as to create a puddle of liquid. Using a slow constant motion begin passing the sequence plate upward over the thermostatic plate. The acrylamide/urea solution will begin flowing downward between the two plates by gravitational forces and capillary action..
17. As the volume of the puddle decreases add more liquid. Continue displacing the sequencing plate in a sliding motion over the thermostatic plate until it both plates are directly aligned over one another.
18. Immediately install the flat side of the shark's tooth comb between the two plate at a depth of 0.5 cm into the gel solution.
19. Immediately clamp both plates tightly together using the bulldog binder clips and place the Macromold in a complete horizontal position.
20. Allow the gel to polymerize for a minimum of 1 h. The gel may be stored at room temperature for a maximum of 12-16 h without dehydration.

21. Install the gel mold and attach the thermostatic plate to the thermocycler as described by the manufacturer. Fill the top and bottom reservoirs with 1X TBE buffer.
22. Prime the sequencing gel by performing a mock electrophoresis at constant power (40 W and ~1,700 V) for 1-2 h. During this time slivers of acrylamide and undissolved urea will accumulate in the loading surface of the gel. These contaminants may interfere with the electrophoresis and therefore should be removed by washing area with 1X TBE buffer.
23. Clean the sharks tooth comb with 70% ethanol before intallation. Carefully place the comb into the loading surface of the gel. Slowly and gently push the teeth of the comb approximately 1-2 mm into the top surface of the gel.
24. Examine that there is no debris within the individual wells.
25. Load 1-2  $\mu$ l of each denatured sequencing reactions (see below) onto each of the gel slots while paying attention to the order they are being loaded. This first loading is considered the 'read long'.
26. Run the gel at constant power (40W and ~1700V).
27. Allow the bromophenol blue to reach the bottom of the gel before adding more reaction sample for a short read. For a 6% acrylamide gel the bromophenol blue dye comigrates with a single stranded DNA fragment with about a 26 nucleotide bases.
28. Stop the electrophoresis when the bromophenol blue dye from the second loading reaches the bottom the gel.
29. Drain the buffer from the reservoirs, disconnect the gel mold from the sequencing apparatus and thermocycler.
30. Using a very thin spatula, carefully separate the plates from the mold. The acrylamide gel should adhere to the sequencing plate.
31. Soak the gel plate into the dark box (provided by the manufacturer) containing 1 l 10% acetic acid for 15 min. Repeat once more with fresh acetic acid.
32. Finally fix the gel to the sequencing plate by drying with a hand-held hair dryer.
33. Autoradiograph by placing a sheet of film between the gel plate and a clean plate held together by bulldog binder clips.

## II. Sequencing Reactions.

1. Adjust the concentration of the template to 1.5-2  $\mu$ g per 8  $\mu$ l. Add 2  $\mu$ l of 2 M NaOH to denaturing the DNA. Vortex the mixture and collect the liquid by centrifugation. Incubate at room temperature for 10 min.
2. Add 3  $\mu$ l of 3 M sodium acetate [pH 4.5] and 7  $\mu$ l of deionized water



3. Add 60  $\mu$ l of 100% ethanol, vortex and store at -20°C for a minimum of 15 min to precipitate the DNA.
4. Collect the DNA by centrifugation at 15,000 x g. Aspirate the supernatant and wash the pellet with 70% ethanol. Recentrifuge, aspirate the supernatant and dry pellet briefly under vacuum.
5. Dissolve the DNA pellet in 10  $\mu$ l deionized water.
6. The sequencing reactions are performed according to the instructions outlined by Pharmacia or United States Biochemicals.

## Appendix 18: Synthesis of radiolabeled probes

### I. Random primer method

The method of Feinberg and Vogelstein (1983, 1984) was adapted for the preparation of high specific activity probes for screening a  $\lambda$  ZAP human Hep G2 cDNA library Erk1-like genes or determining the chromosome location for the genomic human Erk1-gene.

#### Reagents:

10X RP buffer: 900 mM HEPES [pH 7.6] and 100 mM  $\text{MgCl}_2$ .

Dissolve 214.5 g of HEPES and 20.3  $\text{MgCl}_2$  in 400 ml of deionized water. Adjust the pH with HCl. Increase the volume to 500 ml with deionized water. Autoclave.

1. Heat denature the 400 base pair Sephaglas purified partial Erk1 clone in a heating block at 100°C for 3 min.
2. Rapidly cool the tube on ice to prevent reannealing.
3. Transfer 50 ng of heat-denatured DNA to a microcentrifuge containing 2  $\mu\text{l}$  20 mM dithiothreitol, 3  $\mu\text{l}$  each (5 mM dATP, 5 mM dGTP, and 5 mM dTTP), 2  $\mu\text{l}$  10X RP buffer, 5  $\mu\text{l}$  [ $\alpha$ - $^{32}\text{P}$ ]dCTP (specific activity (10  $\mu\text{Ci}/\mu\text{l}$ ), 6  $\mu\text{l}$  deionized water and 1  $\mu\text{l}$  Klenow enzyme fragment (5 U).
4. Vortex gently, centrifuge briefly at 12,000 g for 10 sec and incubate at 37°C for 45 min.
5. Quench the reaction by adding 2  $\mu\text{l}$  of 250 mM EDTA. Purify the fragment from the unincorporated [ $\alpha$ - $^{32}\text{P}$ ]dCTP using a Stratagene Nucletrap<sup>TM</sup> push-column.
6. Disassociate the fragments by heating the probe at 100°C in a heating block and cool rapidly on ice.

### II. End-labelling method

The addition of  $\gamma$ - $^{32}\text{P}$  into the 5' phosphate site of the primer extension oligonucleotide was adapted from O'Farrell (1981) The radiolabelled probe was then used to measure the length of the 5'-untranslated region of the Erk1 mRNA by the primer extension method.

#### Reagents:

10X PK buffer: 700 mM Tris-HCl [pH 7.6], 100  $\text{MgCl}_2$  50 mM dithiothreitol.

The T4 polynucleotide kinase buffer was purchased by New England Biolabs.

1. Prepare the phosphorylation reaction by combining the following:
  - 2  $\mu\text{l}$  150  $\mu\text{g/ml}$  oligonucleotide primer
  - 5  $\mu\text{l}$  10X PK buffer
  - 20  $\mu\text{l}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (10  $\mu\text{Ci}/\mu\text{l}$ )
  - 12  $\mu\text{l}$  deionized water
  - 1  $\mu\text{l}$  T4 polynucleotide kinase (10 U/ $\mu\text{l}$ ).
2. Incubate the reaction at 37°C for hour. Heat inactivate the enzyme for 5 min at 70°C.
3. Dilute the reaction mixture to 100  $\mu\text{l}$  by addition of 50  $\mu\text{l}$  of deionized water and separate the labelled DNA from the unincorporated nucleotide by use of a Nucleotrap<sup>TM</sup> Push column according to Stratagene's specifications.
4. Recover the DNA by addition of 17  $\mu\text{l}$  deionized water and 133  $\mu\text{l}$  of 7.5 M ammonium acetate to the solution
5. Store at -20°C for 1 hour.
6. Resuspend the radiolabelled primer in 100  $\mu\text{l}$  of 0.3 M sodium acetate and count 1  $\mu\text{l}$  by Cerenkov counting.
7. A typical level of  $^{32}\text{P}$  incorporation is approximately  $2\text{--}4 \times 10^7$  cpm.

## Appendix 19: Buffer solutions

0.5 M EDTA:	Combine 186.1 g of disodium ethylenediaminetetraacetic [pH 8.0] acid • H <sub>2</sub> O with 900 ml of deionized water. Dissolve the EDTA by adjusting the pH to 8.0 with concentrated NaOH. Increase the volume to 1 l with deionized water. Autoclave.
1.0 M CaCl <sub>2</sub> :	Dissolve 54 g of CaCl <sub>2</sub> in 150 ml of deionized water. Increase the volume to 200 ml with deionized water. Sterilize the solution with a 0.22 µM filter and store in 1 ml aliquots at -20°C.
50X Denhardt's:	Dissolve 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V, Sigma) in deionized water to 500 ml. Aliquot in 50 ml volumes and store at -70°C.
1.0 M glucose:	Dissolve 90.1 g of glucose in 400 ml deionized water. Increase the volume to 500 ml with deionized water. Sterilize the solution with a 0.22 µM filter.
1.0 M IPTG:	Dissolve 2.4 g of IPTG in 8 ml of deionized water. Increase the volume with deionized water. Sterilize the solution with a 0.22 µM filter. Store at -20°C in 1 ml aliquots.
1.0 M LiCl:	Dissolve 42.39 g of lithium chloride in 900 ml of deionized water. Increase the volume to 1 l with deionized water. Autoclave.
1.0 M NaCl:	Dissolve 58.44 g of NaCl in 900 ml of deionized water. Increase the volume to 1 l with deionized water. Autoclave.
Phenol/ chloroform:	Combine equal volumes of phenol and chloroform. Equilibrate the mixture by several extractions with 0.1 M Tris-HCl [pH 7.6]. Store at 4°C with an equal volume of 0.01 M Tris-HCl in brown glass bottle.
5.0 KOAc:	Dissolve 491 g of KOAc in 900 ml of deionized water. Increase the volume to 1 l with deionized water. Autoclave.
1.0 M sodium citrate:	Dissolve 294.1 g in 900 ml of deionized water. Adjust the citrate pH with HCl. Increase the volume to 1 l of deionized water. Autoclave.
20% SDS:	Dissolve 200 g of SDS in 900 ml deionized water. Complete dissolution may require heat. Adjust the pH to 7.0 with 10 N NaOH. Increase the volume with deionized water to 1 l.
20X SSC:	3 M NaCl, 30 mM sodium citrate [pH 7.0]  Dissolve 175.3 g of NaCl and 88.2 g sodium citrate in 900 ml of deionized water. Using 10 M NaOH adjust the pH to 7.0. Increase the volume to 1 l with deionized water. Autoclave.

TE: Combine 1 ml of 1 M Tris-HCl [pH 7.4] with 200  $\mu$ l EDTA [pH 8.0] in 90 ml deionized water. Adjust the pH to 7.4 with HCl. Increase the volume to 100 ml with deionized water.

1.0 Tris-HCl: Dissolve 157.6 g of Tris-HCl in 900 ml of deionized water. Adjust [pH 7.4] the pH to 7.4 with NaOH. Increase the volume to 1 l with deionized water. Autoclave.

20% Triton:  
X-100 Dilute 50 ml of Triton X-100 in 200 ml deionized water.

## Appendix 20: Construction of a constitutively active Mek1.

The first MAP kinase kinase or Mek was initially cloned from a mouse library using a partial cDNA clone obtained by RT-PCR using degenerate oligonucleotides that were patterned after tryptic peptide sequences (Crews et al., 1992). To clone the coding region of the Mek1 enzyme, two non-degenerate oligonucleotides identical to the published 5' and 3' cDNA sequence were used in combination with total RNA isolated from mouse liver (Figure 38). *Bam* H1 restriction endonuclease sites, which are absent in the Mek1 cDNA, were introduced in the 5' end of the translational sequence of each oligonucleotide to facilitate the cloning of the amplified fragment. Because sequence specific primers were being used for the amplification, the PCR reaction was performed under very stringent conditions. The PCR product subsequently electrophoresed as a single band on a agarose gel with an approximate length of 1.2 kb which was within the expected size range (data not shown). The excised cDNA fragment was purified by Sephaglas<sup>®</sup> (Pharmacia) before being digested with *Bam* H1 and cloned into the identical site of the pGEX-2T prokaryotic expression vector (Figure 39). The proper orientation of Mek1 cDNA was verified by restriction analysis with *Eco* R1 (Figure 38). Those clones that yielded a fragment of 700 base pairs were retained for further characterization. Although the thermally stable VENT<sup>®</sup> DNA polymerase with its proof-reading capabilities was used for the PCR reaction, sequence analysis by the Sanger dideoxy method of a couple of clones revealed four point mutations when compared to the original sequence (Crews et al., 1992). A more detailed analysis of these single nucleotide-pair substitutions showed that they were the result of four separate nucleotide transitions (G205A, C227T, A391G and G907A) that probably occurred during the amplification procedure (Figure 38). Fortunately, these single base alterations resulted in silent mutations that altered the triplet codon but still coded for the original amino acid (Figure 38). The complete DNA nucleotide sequence and corresponding protein

Figure 38: Nucleotide and predicted amino acid sequence of mouse Mek1 cDNA from liver. The coding sequence for Mek1 was obtained from the full-length cDNA clone identified by Crews et al. (1992). The horizontal arrows situated above the protein sequence correspond to the location of the oligonucleotides used for the PCR amplification. The regulatory phosphorylation sites are indicated by asterisks. Vertical arrows indicate the nucleotide transitions that were most likely generated during the PCR reaction. The catalytic subdomains (Roman numerals) and their conserved sequences (boldface type and underlined) adhere to the nomenclature of Hanks et al. (1988).

→

CGCTCCCTGCTGAGTTGCAGGCTCTTTCCCGGCTGCAAGATGCCCAAGAAGAAGCCGACGCCCATCCAGCTGAACCCGGCCCCGATGGC  
 16 31 46 61 76 91

S A V N G T S S A E T N L E A L Q K K L E E L E L D E Q Q R  
 TCGGCGGTTAACGGGACCACTCGGCCGAGACCACTGGAGGCCTTGCAAGAAGCTGGAGGAGCTGGAGCTTGACGAGCAGCAGCGG  
 106 121 136 151 166 181

**I**

K R L E A F L T Q K Q K V G E L K D D D F E K I S E L G A G  
 AAGCGGCTCAGAGGCCTTTCTGAC@CAGAAGCAGAAGGTGGGGAACTGAAGGATGATGACTTTGAGAAGATCAGCGAACTGGGAGCTGGC  
 196 211 226 241 256 271

ⓐ ⓑ

**II**

N G G V V F K V S H K P S G L V M A R K L I H L E I K P A I  
 AACGGTGGAGTGGTCTTCAAGGTCTCCACAAGCCATCTGGCCTGGTTATGGCTAGAAAGCTGATCCACCTGGAGATCAAACCCGCAATC  
 286 301 316 331 346 361

**III** **IV**

R N Q I I R E L Q V L H E C N S P Y I V G F Y G A F Y S D G  
 CGGAACCAGATCATCCGGGAGCTGCAGGTACTGCACGAGTGCAACTCCCCGTACATCGTGGGCTTCTACGGGGCCTTCTACAGCGACGGC  
 376 390 406 421 436 451

ⓐ

**V**

E I S I C M E H M D G G S L D Q V L K K A G R I P E Q I L G  
 GAGATCAGCATCTGCATGGAGCACATGGATGGTGGTCCCTGGATCAAGTCTGAAGAAAGCTGGAAGAATTCTTGAGCAAATTTTAGGA  
 466 481 496 511 526 541

**VI**

K V S I A V I K G L T Y L R E K H K I M H R D V K P S N I L  
 AAAGTTAGCATGTGTGATAAAAGGCCTGACCTATCTTCGGGAGAAGCACAAGATTATGCACAGAGATGTCAAGCCATCCAACATTCTA  
 556 571 586 601 616 631

**VII** \* \*

V N S R G E I K L C D F G V S G Q L I D S M A N S F V G T R  
 GTGAACCTCAGCTGGGAGATCAAACCTCTGTGATTCTGGGGTCAGCGGGCAGCTAATTGACTCTATGGCCAACCTCCTTCGTGGGCACGAGA  
 646 661 676 691 706 721

**VIII** **IX**

S Y M S P E R L Q G T H Y S V Q S D I W S M G L S L V E M A  
 TCCTACATGTGCGCTGAGAGACTCCAGGGGACTCACTACTCTGTGCAGTCGACATCTGGAGCATGGGGCTCTCTCTGGTGGAGATGGCA  
 736 751 766 781 796 811

V G R Y P I P P P D A K E L E L L F G C H V E G D A A E T P  
 GTTGGGAGATACCCATTCTCTCTCTGATGCCAAGGAGCTGGAGCTACTGTTTGGATGCCATGTGAAGGAGACGACGCCGAAACACCA  
 826 841 856 871 886 901

**X**

P R P R T P G R P L S S Y G M D S R P P M A I F E L L D Y I  
 CCCAGGCCAAGGACCCCTGGGAGGCCTCTCAGCTCATATGGAATGGACGCCGACCTCCCATGGCAATTTTGTAGTTGTTGGATTACATT  
 916 931 946 961 976 991

ⓐ

V N E P P P K L P S G V F S L E F Q D F V N K C L I K N P A  
 GTCAATGAGCCTCCCAAACTGCCAGTGGAGTATTAGTCTGGAGTTTCAGGATTTTGTGAATAAATGCTTAATAAAGAACCCCTGCA  
 1006 1021 1036 1051 1066 1081

**XI**

E R A D L K Q L M V H A F I K R S D A E E V D F A G W L C S  
 GAGAGAGCAGATCTGAAGCAGCTCATGGTACATGCTTTTCATCAAAAGATCTGACGCCGAGGAGGTAGACTTCGAGGCTGGCTCTGCTCC  
 1096 1111 1126 1141 1156 1171

←

T I G L N Q P S T P T H A A S I Stop  
 ACCATTGGGCTTAACACAGCCAGCACACCAACCCACGCTGCCAGCATCTGAGCCTTTAGGAAGCAGCAAAGAGGAATTTCTGCCCCAGTG  
 1186 1201 1216 1231 1246 1261  
 GCATGCCATGTTGCTTTTCAGGCCTCTCCCATGCTTGTCTATGTTTCAGACGTGCATCTCATCTGTGACAAAGGATGAAGAACACAGCATGT  
 1276 1291 1306 1321 1336 1351  
 GCCAAATTGACTTGTGTCAATTTTAAATATCATTGTCTTTATCACTATGGTTACTCCCCTAAGTGGATTGGCTTTGTGCTTGGGGCTATT  
 1366 1381 1396 1411 1426 1441  
 TGTCTGTTTCATCAACACATGCCAGGCTGAACTACAGTGAACCCCTAGTGACCTGGGTGGTCTGTTCTTACTGATGTTTGCAGTCTGCTTTC  
 1456 1471 1486 1501 1516 1531  
 ATCGTGACTCACTAGCTGGCTGCCTGTATTTGTGAGGATCTCTGGACCTTGGTACTTCACTCTTGCTGGTGAACCTTCAGTCTGAGAGGGA  
 1546 1561 1576 1591 1606 1621  
 GCCTTGTGAGACCCCTTCACAGGCAGTGCATGCATGGAAGCATGCTTTGCTGCTACTGAAATGAGCATCAGAACGTGTACGTCATGGTAT  
 1636 1651 1666 1681 1696 1711  
 TTTTATTTTGTCTTTTGGTATAGAACTCAGCAATTTCCCATCAAAAAACCTAAGCAGAGCCCATCACTGCCATGATAGCTGGGCTTCAG  
 1726 1741 1756 1771 1786 1801  
 TCTGTCTACTGTGGTGATTTTGTAGACTTCTGGTGTATTTCTATATTTTAAATATACAGTGTGGGATACTTAGTGGTGTGTGTCT  
 1816 1831 1846 1861 1876 1891  
 CTAAGTTTGGATTAGTGTCTTAAATTTGGTGGTTATTTTGAATGTCACAAATGGATTAAAGCATCAATGTATCAAGAGTTCTATCTTTCT  
 1906 1921 1936 1951 1966 1981  
 TCCAGTCTAAGTACCAATGCTATTGTAAACAACGTGTATAGTGCCTACAAATTTGTATGAAACCCCTTTTAAACCACTTTAATCAAGATGTT  
 1996 2011 2026 2041 2056 2071  
 TATCAAACTAATCTTATTCTAATAAAATACTATCAAGTTAAAT  
 2086 2101 2116



sequence as well as the kinase catalytic subdomains are shown in figure 38. The Mek1 protein was subsequently expressed as a GST-fusion protein in *E. coli*. with an approximate molecular mass of 72-kDa (Figure 39). This was the expected size range for Mek1 (46-kDa) and GST (26-kDa) protein fusion. The GST-Mek1 fusion protein was purified in milligram amounts using 1 milliliter of glutathione-linked agarose beads. Recombinant Mek1 was detectable by immunoblotting with anti-peptide polyclonal antibodies directed against two distinct carboxy-terminal sequences (Figure 40). Furthermore, polyclonal antibodies that were generated against synthetic peptides obtained from yeast *S. cerevisiae* MAP kinase kinase Ste7 protein sequence displayed cross immunoreactivity with recombinant Mek1 from murine (Figure 40). As a control, the GST-specific antibody was used to detect the GST-Mek1 fusion protein (Figure 40).

To identify the regulatory phosphorylation sites in mouse Mek1, a protein sequence alignment was created between subdomains VII and VIII for all the known MAP kinase kinases identified in various model systems (Figure 41A). This region is known to harbour the activation sites for the seryl/threonyl family of protein kinases. Three conserved seryl/threonyl residues were identified from comparisons of mammalian, fruit fly, and yeast species including Ser-212, Ser-218 and Ser-222 in Mek1. As a further selection criteria to identify the most probable phosphorylation sites, a protein sequence alignment was created between several protein seryl/threonyl kinases that had been well characterized at the biochemical level and Mek1. The activating phosphorylation sites for cAMP-dependent protein kinase (PKA), cyclin-dependent kinase (Cdk2) and MAP kinase (Erk2) have been mapped to a region located between subdomains VII and VIII in the kinase catalytic domain (Shoji et al., 1981; Lee and Nurse, 1987; Boulton et al., 1991). In Mek1, Ser-218 and Ser-222 mapped closest to the regulatory phosphorylation sites of PKA, Cdk2 and Erk2 proteins (Figure 41B). Ser-212

Figure 39: Prokaryotic expression vector of the full-length mouse Mek1 protein cloned downstream of the glutathione-S-transferase (GST) gene. Construction of the vector is described in Materials and Methods. The 3' end of the GST protein is fused in frame with the 5' mouse Mek1 cDNA coding sequence. The thrombin cleavage site recognition sequence is indicated with a vertical arrow. The pBR322 origin of replication, ampicillin resistance gene, and the LacZ inhibitor protein gene, lacIq, are also presented.

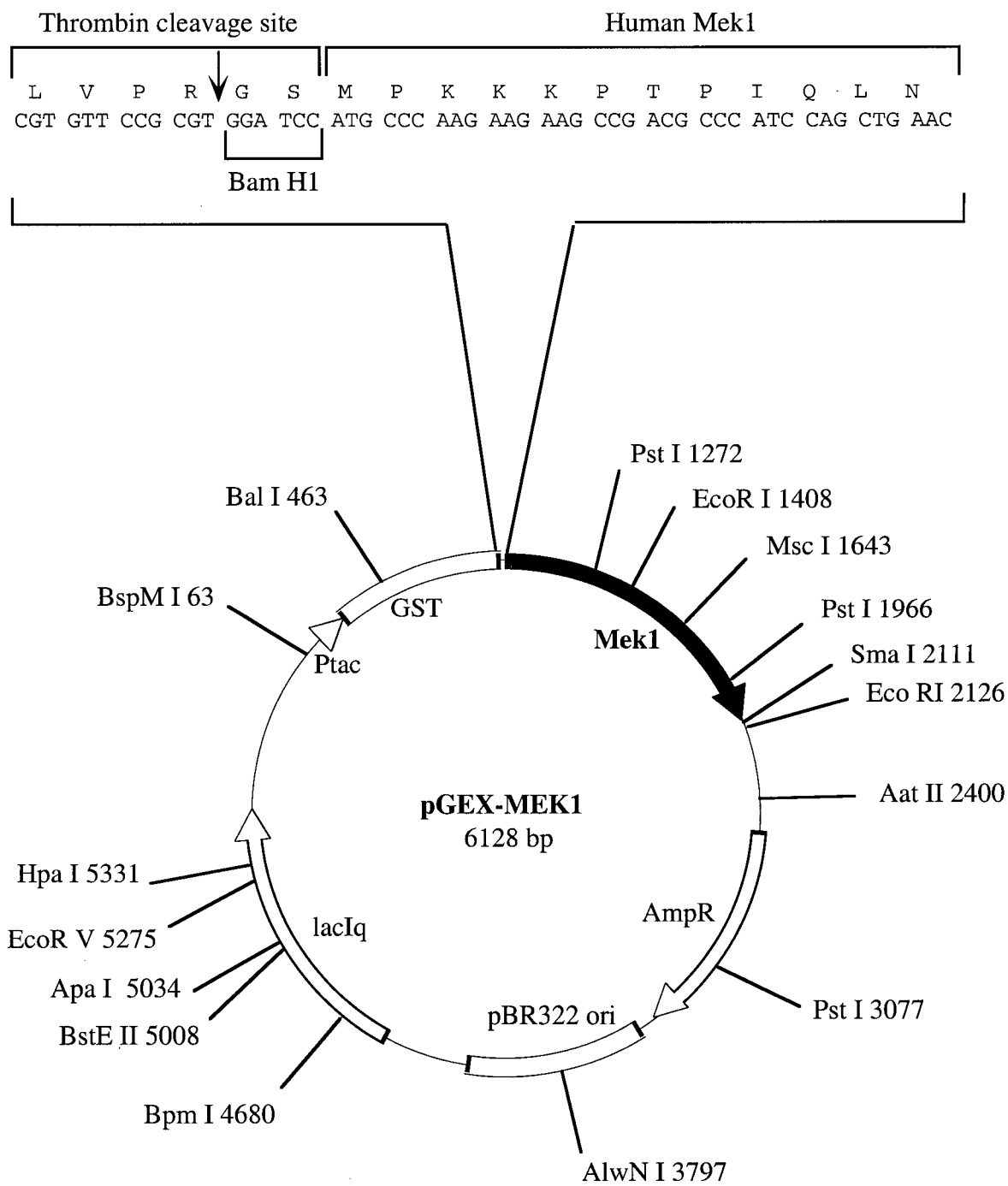
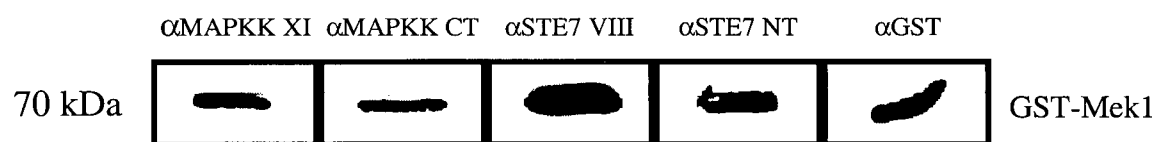


Figure 40: Expression and immunoreactivity of recombinant Erk1 protein from *E. coli* to MAP kinase antibodies. The Mek1 protein was expressed as a glutathione-S-transferase fusion protein under the control of the LacZ promoter. Approximately 1.0 mg of GST-Mek1 protein purified by glutathione-affinity chromatography from IPTG-induced bacterial homogenates was electrophoresed on an SDS-polyacrylamide gel and transferred to nitrocellulose for Western analysis. The blots were probed with a battery of MAP kinase kinase antibodies that recognize specific peptide sequences from mouse Mek1 and *Saccharomyces cerevisiae* Ste7 proteins (see Material and Methods). The Roman numeral designation specifies the catalytic subdomain location used to synthesize the peptides.



did not appear to be a logical candidate, since it was located several residues amino-terminal to the regulatory sites of these known kinases. To verify that these sites were essential for regulating Mek1 activity *in vitro*, these two seryl-residues were substituted with alanine by site-directed mutagenesis. This resulted in a catalytically compromised kinase that phosphorylated Erk1 at a level inferior to the wild type enzyme (data not shown). Exchange of these identical seryl-residues with mimetic glutamyl amino acids resulted in Mek (EE) becoming constitutively activated in the absence of upstream activator Raf1. Furthermore, the wild type Mek1 was 50-fold less active than hyperactive form of Mek1 (Figure 42).

Figure 41: Sequence alignments of several seryl/threonyl protein kinases. (A) The region extending between subdomains VII and VIII of Mek1 was aligned with corresponding regions that harbour the regulatory phosphorylation sites in cAMP-dependent protein kinase (PKA), cyclin-dependent protein kinase (Cdk1), and Erk2 (Shoji et al., 1981; Lee and Nurse, 1987; Boulton et al., 1991). (B) Alignments were also created between the mouse Mek1 sequence and several MAP kinase kinase isoforms from different model systems including: yeast, amphibian, fruit fly and mammals (Teague et al., 1986; Boguslawski and Polazzi, 1987; Nadin-Davis and Nasim, 1988; Warbrick and Fantes, 1991; Ashworth et al., 1992; Crews et al., 1992; Seger et al., 1992b?; Kosako et al., 1993; Irie et al., 1993; Otsu et al., 1993; Tsuda et al., 1993; Wu et al., 1993a, b; Zheng and Guan, 1993).

A.

	VII	VIII
PKA	DFGFAKRVKGRT-----W	TLCGTPEYLAPE
CDK2	DFGLARAFGVFVRTY---	THEVVTLWYRAPE
ERK2	DFGLARVADPDHDHTGFL	TEYVATR WYRAPE
MEK1	DFGVSGQLID-----	SMANSFVGTRSYMSPE

B.

		VII	VIII
mouse	<b>MEK1</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
<i>Xenopus</i>	<b>MKK1</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
rat	<b>MKK2</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
rabbit	<b>MKK1</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
human	<b>MEK1</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
human	<b>MEK2</b>	DFGVSGQLID	SMANSFVGTRSYMAPE
<i>Drosophila</i>	<b>Dsor1</b>	DFGVSGQLID	SMANSFVGTRSYMSPE
<i>S. pombe</i>	<b>byr1</b>	DFGVSEQLVNSVAQ	TFVGTSTYMSPE
<i>S. pombe</i>	<b>wis1</b>	DFGVSGNLVASISK	TNIGCQSYMAPE
<i>S. cerevisiae</i>	<b>STE7</b>	DFGVSKKLINSIAD	TFVGTSTYMSPE
<i>S. cerevisiae</i>	<b>PBS2</b>	DFGVSGNLVASLAK	TNIGCQSYMAPE
<i>S. cerevisiae</i>	<b>MKK1</b>	DFGVSGEAVNSLAT	TFTGTSTFYMAPE
<i>S. cerevisiae</i>	<b>MKK2</b>	DFGVSGEAVNSLAM	TFTGTSTFYMAPE



Figure 42: Expression and analysis of a constitutively active Mek1 allele. Wild type (SMANS) (lane 1), kinase-compromised (SMANS\*) (lane 2) and constitutively active (EMANE) (lane 3) Mek1 (~2 mg), which were expressed as a GST-fusion proteins, were combined with thrombin-cleaved Erk1 (~1  $\mu$ g), [ $\gamma$ - $^{32}$ P] ATP and kinase buffer G. The reaction was terminated with 5X SDS gel loading buffer. The phosphorylated proteins were electrophoresed, transferred to nitrocellulose and exposed to X-ray film. The positions of the GST-Mek1 and thrombin-cleaved Erk1 are located on the left of the panel while the position of the prestained standards are located on the right of the panel.

