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CLONING AND CHARACTERIZATION OF NOVEL KINASES FROM EMBRYONIC CELLS

Elizabeth Douville

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Department of Biochemistry
University of Ottawa,
Ottawa, Ontario
Canada

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ABSTRACT

Protein tyrosine kinases (PYKs) play key regulatory roles in the control of cell growth and differentiation. Attempts to identify novel PYKs through expression cloning strategies have led to the identification of a novel family of protein kinases, referred to as dual specificity kinases (DSKs). In addition to their immunoreactivity with antiphosphotyrosine antibodies, DSK family members have the ability to phosphorylate serine, threonine as well as tyrosine residues.

A novel protein kinase, Esk (Embryonal carcinoma Ser/thr/tyr Kinase), has been isolated from an embryonal carcinoma (EC) cell line using an expression cloning strategy. Sequence analysis of two independent cDNA clones (2.97 and 2.85 Kb) suggested the presence of two Esk isoforms in EC cells. The Esk-1 cDNA sequence predicted an 857 amino acid protein kinase with a putative membrane spanning domain, while the Esk-2 cDNA predicted an 831 amino acid kinase which lacked this domain. Genomic analysis revealed that the Esk transcripts could arise through alternative splicing of the same primary transcript to generate the cytoplasmic and transmembrane isoforms of the kinase. In adult mouse, Esk messenger RNA levels were highest in tissues possessing a high proliferation rate or a sizeable stem cell compartment suggesting that Esk may play some role in the control of cell proliferation or differentiation. As anticipated from the screening procedure, bacterial expression of the Esk kinase reacted with antiphosphotyrosine antibodies on immunoblots. Furthermore, in *in vitro* kinase assays Esk was shown to phosphorylate both itself and the exogenous substrate myelin basic protein on serine, threonine and tyrosine residues confirming that Esk is a novel member of the dual specificity family of protein kinases.

An antibody raised to the Esk kinase revealed that the protein was subject to developmental regulation; being highly expressed in rapidly proliferating cells, and absent in terminally differentiated cells and in adult mouse tissues. Finally, the Esk kinase was found to associate with the 85 kDa subunit of phosphatidylinositol 3-kinase (PI3K) in

proliferating stem cells. In vitro binding studies suggested that the interaction of the Esk kinase with the 85 kDa subunit of PI3K could be mediated via both the SH2 and SH3 domains of this protein.

The results presented in this thesis suggest that the Esk dual specificity kinase may play a role in the control of cell growth and differentiation and that the effects of the kinase could be mediated by the regulation of PI3K activity. The interaction of the Esk kinase with an SH2 domain containing protein, is the first indication for the physiological function of the tyrosine phosphorylating activity of this kinase in mammalian cells.

à mes parents, Jean et Lucille

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After six years and some, not to thank the people around me who made it all a bit easier would be ungrateful. At this point however, you are all probably more grateful to me for leaving than I to you!

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Pamela Icely, Pim-Pam, for BBQs and fun times,

My friend Ricardo Marius, for dart nights, camping trips, mountain biking and cross-country skiing sessions. No one else I know dances to Reggae with a pipetman,

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PREFACE

The work presented in this thesis has been or will be submitted for publication as described below:

CHAPTER 2

Douville, E.M.J., Afar, D.E.H., Howell, B.W., Letwin, K., Tannock,L., Ben-David, Y., Pawson, T. and J.C. Bell. 1992. Multiple cDNAs encoding the Esk kinase predict transmembrane and intracellular enzyme isoforms. Mol. Cel. Biol., 12: 2681-2689.

CHAPTER 3

Douville, E.M.J. and J.C. Bell. The Esk dual specificity kinase interacts with the 85 kDa subunit of phosphatidylinositol (PI) 3'-kinase. manuscript in preparation.

The experimental work presented in this thesis is entirely my own. Full length sequence of the Esk-2 cDNA was provided by Dr. Tony Pawson from the Mount Sinai Hospital Research Institute in Toronto. Chromosomal mapping of the murine Esk gene was done by Dr. Nancy Jenkins from the National Cancer Institute in Frederick, Maryland.

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LIST OF ABBREVIATIONS

ALL	Acute lymphocytic leukaemia
ATP	Adenosine triphosphate
CML	Chronic myelogenous leukaemia
DNA	Deoxyribonucleic acid
cDNA	complementary DNA
DSK	Dual specificity kinase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol Bis (β amino ehtyl ether) NNNN tetraacetic acid
EGFR	Epidermal growth factor receptor
GTP	Guanosine triphosphate
Kb	Kilobase
kDa	KiloDalton
LMAG	Large form of myelin associated glycoprotein
mRNA	Messenger ribonucleic acid
PDGFR	Platelet derived growth factor receptor
PGK	Phosphoglycerate kinase
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PIKkinase	Phosphatidylinositol 3-kinase kinase
PLC	Phospholipase C
PSK	Protein serine kinase
PYK	Protein tyrosine kinase
Ras-GAP	Ras-GTPase activating protein
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SH1	Src homology region 1 domain
SH2	Src homology region 2 domain

SH3	Src homology region 3 domain
ser	Serine
sscDNA	single stranded complementary DNA
thr	Threonine
tyr	Tyrosine
TCR	T cell receptor
TLC	Thin layer chromatography
TLE	Thin layer electrophoresis

GENERAL INTRODUCTION

1. PROTEIN KINASES

In order to respond to environmental cues, most organisms rely on complex pathways that involve rapid and reversible modifications such as protein phosphorylation. Protein kinases, the enzymes that carry out these reactions, have therefore evolved to fulfil very diverse functions depending on the host organism. In prokaryotes, phosphorylation is central to chemotaxis and allows bacteria to respond by adaptation or locomotion to environmental signals (Bourret *et al.*1991). Conversely, in higher eukaryotes, protein phosphorylation can regulate several cellular processes including hormone responsiveness, gene expression (Yamamoto *et al.*1988) and cell growth and division (Lorincz and Reed, 1984).

Although several amino acids can be modified by phosphorylation, most eukaryotic kinases phosphorylate the hydroxy amino acids serine, threonine or tyrosine. Historically, kinases have been classified into two distinct families depending on their amino acid specificity, protein serine/threonine kinases (PSKs) and protein tyrosine kinases (PYKs). It is now known that the control of cell growth and differentiation is regulated by the combinatorial actions of these enzymes. In general, PYKs are found at the plasma membrane in the form of growth factor receptors (Fantl *et al.*1993) or in close association with transmembrane glycoproteins (Veillette *et al.*1988). Alternatively, serine/threonine kinases are believed to be sequestered to the inner surface of the plasma membrane, the cytosol or in the nucleus.

Functionally, PYKs were thought to have evolved to mediate signals within and more importantly between eukaryotic cells, a hypothesis that was corroborated by the absence of these molecules in bacteria. In this regard, PSKs were thought to be the secondary effectors in the pathways concerned primarily with relaying signals within the cell. One simple model of signal transduction was the activation of tyrosine kinases by an extracellular signal, followed by phosphorylation and activation of intracellular protein serine/threonine kinases. The PSKs would then be responsible for transmitting the signal to the nucleus through phosphorylation of their own substrates.

While this model is correct, it has become apparent that signal transduction pathways are far more complex and that extensive cross-talk exists between the different families of protein kinases. Indeed, not only do PYKs use PSKs as substrates, but the reverse is also true. Furthermore, the subcellular and functional distinctions that existed between these families have been abolished with the identification of nuclear protein tyrosine kinases (Van Etten *et al.*1989) and transmembrane serine/threonine protein kinases (Walker and Zhang, 1990; Georgi *et al.*1990; Wrana *et al.*1992). Finally, while it has recently been recognized that *both* tyrosine and threonine (or serine) phosphorylations are required for the regulation of certain protein substrates, we and others have identified a novel family of protein kinases that can carry out these phosphorylations. These kinases, now referred to as dual specificity kinases (DSKs), can phosphorylate all three hydroxy amino acids and are therefore thought to play an integral role in the cross-talk that must exist between the PSKs and PYKs.

The objective of this work was to identify novel protein tyrosine kinases involved in the control of cell growth and differentiation. Our search for these kinases led to the characterization of members of the novel dual specificity kinase family. The following sections of the introduction will therefore describe protein tyrosine kinases as well as dual specificity kinases; their structure, and their mechanisms of activation and regulation. Finally, signalling pathways involving members of these two families will be illustrated in order to understand the functional importance of these kinases in controlling cell growth and differentiation.

1.1 PROTEIN TYROSINE KINASES

Protein tyrosine phosphorylation was first recognized with the identification of the transforming protein of Rous Sarcoma virus, $pp60^{v\text{-}src}$ (Collett and Erickson, 1978; Hunter and Sefton, 1980) and the cloning of its cellular homolog, $pp60^{c\text{-}src}$ (Takeya and Hanafusa, 1983). The oncogenic potential of this protein product together with the ancient evolutionary origin of this family of kinases (Hanks *et al.*1988) were indicative of a role for protein tyrosine kinases in the control of eukaryotic cell growth and division. Protein tyrosine kinase genes can be subgrouped into two major families: the cytoplasmic protein tyrosine kinases and the transmembrane growth factor receptor kinases represented by the $pp60^{v\text{-}src}$ oncogene and by the epidermal growth factor receptor (EGFr), respectively.

Unlike their oncogenic counterparts, cellular protein tyrosine kinases are strictly regulated in normal proliferating cells, and are only activated in response to proper regulatory signals. The following describes the mechanisms of activation, the regulation and possible functions of protein tyrosine kinases from the cytoplasmic and transmembrane receptor families.

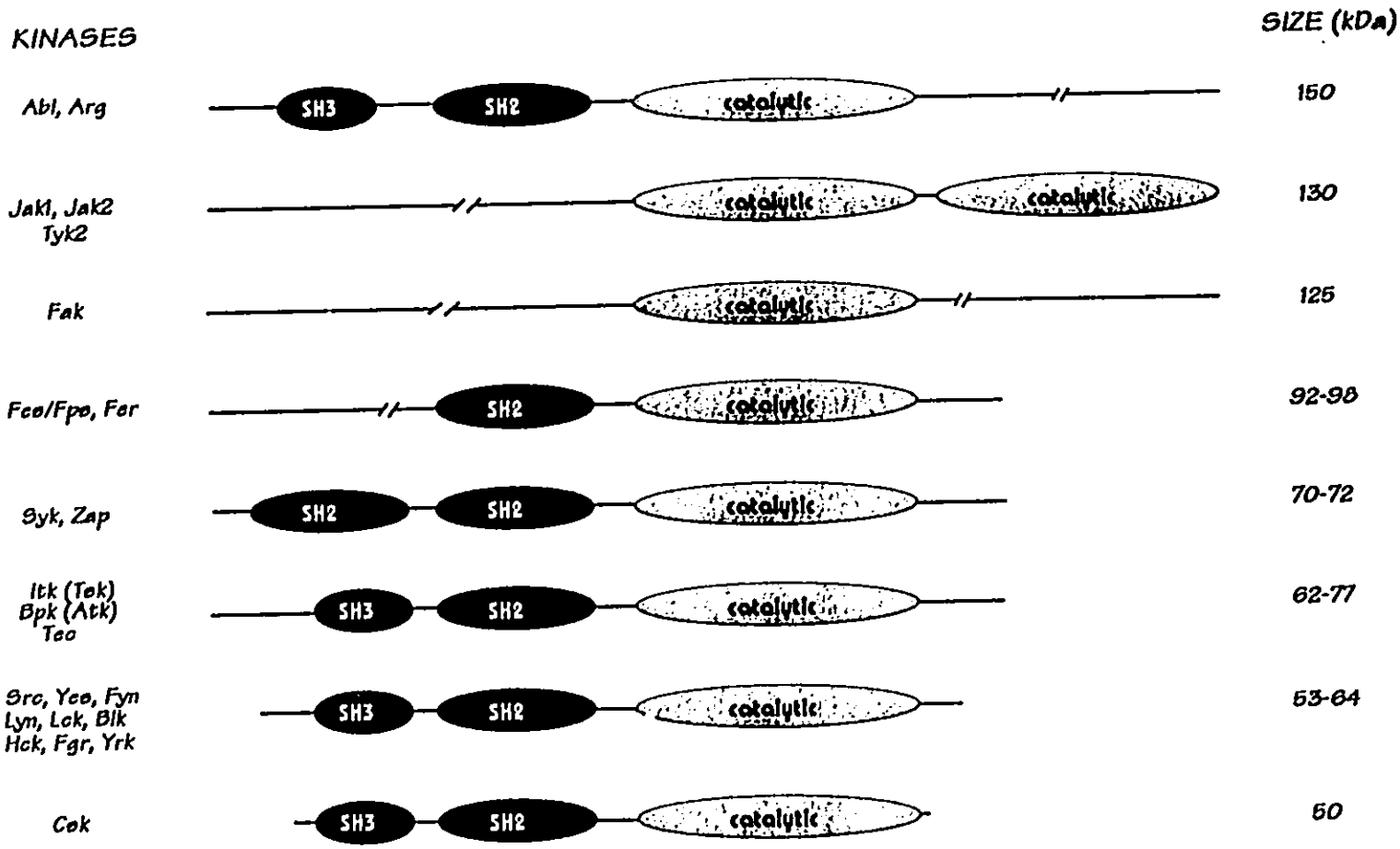
1.11 Cytoplasmic protein tyrosine kinases

The prototypical cytoplasmic PYK is the $pp60^{v\text{-}src}$ oncogene which was the first kinase identified with tyrosine phosphorylating activity (Hunter and Sefton, 1980). The involvement of this oncogene in the control of cell growth and division provoked a search for other src-related cytoplasmic kinases in the cellular genome. This search has led to the characterization of at least 24 different cytoplasmic tyrosine kinases (Bolen, 1993) that are shown in figure 1. The common feature of cytoplasmic protein tyrosine kinases is that, unlike their transmembrane receptor counterparts, they lack extracellular sequences that might be responsible for their activation. The cytoplasmic kinases are however, for the most part, membrane associated by virtue of a myristic acid moiety that anchors the kinases to the inner surface of the plasma membrane (Bolen, 1993). This post-translational modification, although not essential for protein tyrosine kinase activity, is indispensable for mediating the biological effects of cytoplasmic kinases (Kamps *et al.*1985; Cross *et al.*1984).

Figure 1

Structure of cytoplasmic protein tyrosine kinases.

Schematic representation of cytoplasmic PYKs, grouped into subfamilies. The names of the PYKs are listed in the left margin, while the average size is indicated in the right margin. The catalytic domain (src homology region 1, SH1) is represented by a shaded oval. The SH2 and SH3 domains involved in protein-protein interactions are depicted as black ovals.


KINASES
SIZE (kDa)
Abl, Arg
SH3
SH2
catalytic
150
Jak1, Jak2
Tyk2
catalytic
catalytic
130
Fak
catalytic
125
Fes/Fps, Fer
SH2
catalytic
92-98
Syk, Zap
SH2
SH2
catalytic
70-72
Itk (Tek)
Bpk (Atk)
Tec
SH3
SH2
catalytic
62-77
Src, Yes, Fyn
Lyn, Lck, Blk
Hck, Fgr, Yrk
SH3
SH2
catalytic
53-64
Csk
SH3
SH2
catalytic
50

The cytoplasmic PYKs shown in figure 1, however, are not all identical and can be further subgrouped based on structural similarities. With the exception of the Janus kinases (Jak1, Jak2 and Tyk2), most of the kinases shown in figure 1 comprise a single conserved kinase catalytic domain referred to as the src homology region 1 (SH1), and an amino regulatory region containing the conserved src homology regions 2 and 3 (SH2 and SH3 domains). The Janus family kinases, first identified by Andrew Wilks, are unusual in that they contain two conserved catalytic domains and no SH2 or SH3 domains (Wilks *et al.*1991). The functional significance of two catalytic domains in this family of kinases is unclear, however the recent observation that the Jak1 kinase may be a dual specificity kinase (Velazquez *et al.*1992) has led to the hypothesis that each catalytic domain could be independently involved in serine/threonine or tyrosine phosphorylating activities.

The SH2 and SH3 domains, which are absent in the transmembrane growth factor receptors, have been shown to mediate protein-protein interactions and are thought to be involved in the regulation of activity and substrate specificity of the cytoplasmic kinases. The functional significance of SH2 and SH3 domains will be discussed further in section 3.

1.111 Regulation of cytoplasmic protein tyrosine kinases

Although the viral oncogenic forms of the cytoplasmic kinases are deregulated and demonstrate no clear substrate specificity, their cellular homologs are strictly controlled

and phosphorylate only a subset of substrate molecules including themselves. Indeed, work on the src kinases ($pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$) has revealed two major sites of regulation, both mediated by tyrosine phosphorylation (Hunter, 1987). These sites, tyrosines 416 and 527 in the c-src kinase, are conserved in several cytoplasmic kinases which are thought to be subject to the same type of regulation (Okada *et al.*1991; Mustelin and Burn, 1993). Phosphorylation of the tyrosine residue at position 527 (outside the catalytic domain) of the c-src kinase is responsible for downregulation of its tyrosine kinase activity and is carried out by the C-src kinase (Csk) (Okada *et al.*1991). The C-src kinase is a misnomer since this enzyme can phosphorylate the equivalent site in other cytoplasmic tyrosine kinases including $p56^{lck}$ and $p59^{fyn}$ (Okada *et al.*1991; Amrein and Sefton, 1988). Phosphorylation at this site leads to a conformation that restricts the tyrosine kinase activity (Roussel *et al.*1991) (figure 8). Noteworthy, is the fact that the src oncogene ($pp60^{v\text{-}src}$) lacks this inhibitory phosphorylation site, resulting in constitutive activation of the viral enzyme. In leukocytes a transmembrane tyrosine specific protein phosphatase, CD45, is responsible for the dephosphorylation of this site and activation of the cytoplasmic kinases (Mustelin and Burn, 1993). Therefore, at least in blood cells, the regulation of cytoplasmic tyrosine kinase activity is achieved by a balance between the activities of CD45 and Csk. These results suggest that in other tissues, similar protein tyrosine phosphatases must be present to activate the cytoplasmic kinases.

In contrast to this site of negative regulation, phosphorylation of tyrosine 416 in the catalytic domains of both c- and v-src leads to activation of the kinase activity. The

tyrosine residue at position 416, and its equivalent in other kinases, is the major site of autophosphorylation in this family of protein kinases (Hunter and Cooper, 1985). Although the sequences surrounding tyrosine 416 are conserved in both $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, only the viral kinase has measurable phosphotyrosine at this position. The inability to detect the phosphorylated residue in the cellular homolog may reflect the restricted activity of this kinase.

Finally, while there are several serine/threonine phosphorylation sites present within the amino terminal portion of the src kinase, they are not conserved within the cytoplasmic kinase family members and therefore play a more restricted role in the overall regulation.

1.112 Activation of cytoplasmic protein tyrosine kinases

The src proto-oncogene is the most extensively studied of the cytoplasmic protein tyrosine kinases yet, until recently, little was know about the mechanisms of activation of this kinase and its physiological substrates. In contrast, the mechanism of activation of $p56^{lck}$ has been extensively studied and has led to a general scheme for the activation of cytoplasmic protein tyrosine kinases. $p56^{lck}$ is a lymphocyte specific protein tyrosine kinase found predominantly in developing thymocytes and in mature T cells (Molina *et al.*1992). The enzyme is localized to the inner surface of the plasma membrane and can associate with the CD4 and CD8 transmembrane glycoproteins (Veillette *et al.*1988). In mammalian cells, this interaction is mediated by cysteine containing motifs present in the

cytoplasmic carboxyl tail of the glycoproteins, and in the amino terminal regulatory region of the Lck kinase (Turner *et al.*1990). The proteins are brought together by the metal chelating properties of the cysteine residues. Work by Veillette *et al.* has shown that either MHC binding to, or antibody cross-linking of CD4 and CD8 molecules at the surface of T cells, can mediate the activation of the $p56^{lck}$ tyrosine kinase activity (Veillette *et al.*1989; Abraham *et al.*1991). While similar mechanisms of activation are employed by other cytoplasmic kinases, the nature of the interaction between the transmembrane glycoproteins and the kinases may not be identical to that described for Lck. Indeed, several cytoplasmic protein tyrosine kinases, including Src, Fyn, Lyn, Blk and Yes have been shown to associate with cell surface proteins in leukocytes, yet they lack the cysteine motif (Mustelin and Burn, 1993). These results suggest that other protein motifs are present in these kinases in order to mediate their association with transmembrane proteins.

1.113 Functions of cytoplasmic tyrosine kinases

Several cytoplasmic tyrosine kinases display restricted expression patterns in the developing embryo and in adult tissues (Adamson, 1987). For example, the c-src kinase is highly expressed both in the developing embryo, during organogenesis (Schartl and Barnekow, 1984), and in the adult mouse, in neurons (Cartwright *et al.*1988; Maness *et al.*1988) and platelets (Golden *et al.*1986). This expression pattern suggested a bifunctional role for the src kinase; one of proliferation in the early stages of

development, and one of differentiation in the mature neural cells (Adamson, 1987). The tissue specificity of the cytoplasmic kinases has therefore been helpful in answering questions regarding the possible functions of these kinases however, homologous recombination and the generation of kinase deficient mice have been an invaluable tool in this regard. It was generally believed that embryonic lethality would ensue from the disruption of certain kinase genes (including src), however, most of the kinase deficient mice generated thus far have survived to the adult stage. Indeed, mice deficient in Src, Lck or Fyn^T expression mature normally to the adult stage but develop osteopetrosis (Soriano *et al.*1991), display abnormal T cell development (Molina *et al.*1992) and display aberrant T cell receptor mediated signalling (Appleby *et al.*1992), respectively. The osteopetrotic phenotype of src deficient mice was puzzling given the expression pattern described above. However, it was later found that the osteoclasts, which are defective in the src deficient mice, also express high levels of the kinase (Tanaka *et al.*1992). Interestingly, mice lacking the Csk kinase display an embryonic lethal phenotype suggesting that the negative regulation of cytoplasmic tyrosine kinase family members is also important for normal embryonic development (Nada *et al.*1993).

Taken together these results suggest that redundancy must exist within the cytoplasmic protein kinases in order to compensate for the lack of a family member during the early stages of development. As the expression patterns of the different kinases are restricted during the later stages of development, the redundancy may be less apparent and give rise to the defects observed at the adult stage.

1.12 Receptor protein tyrosine kinases

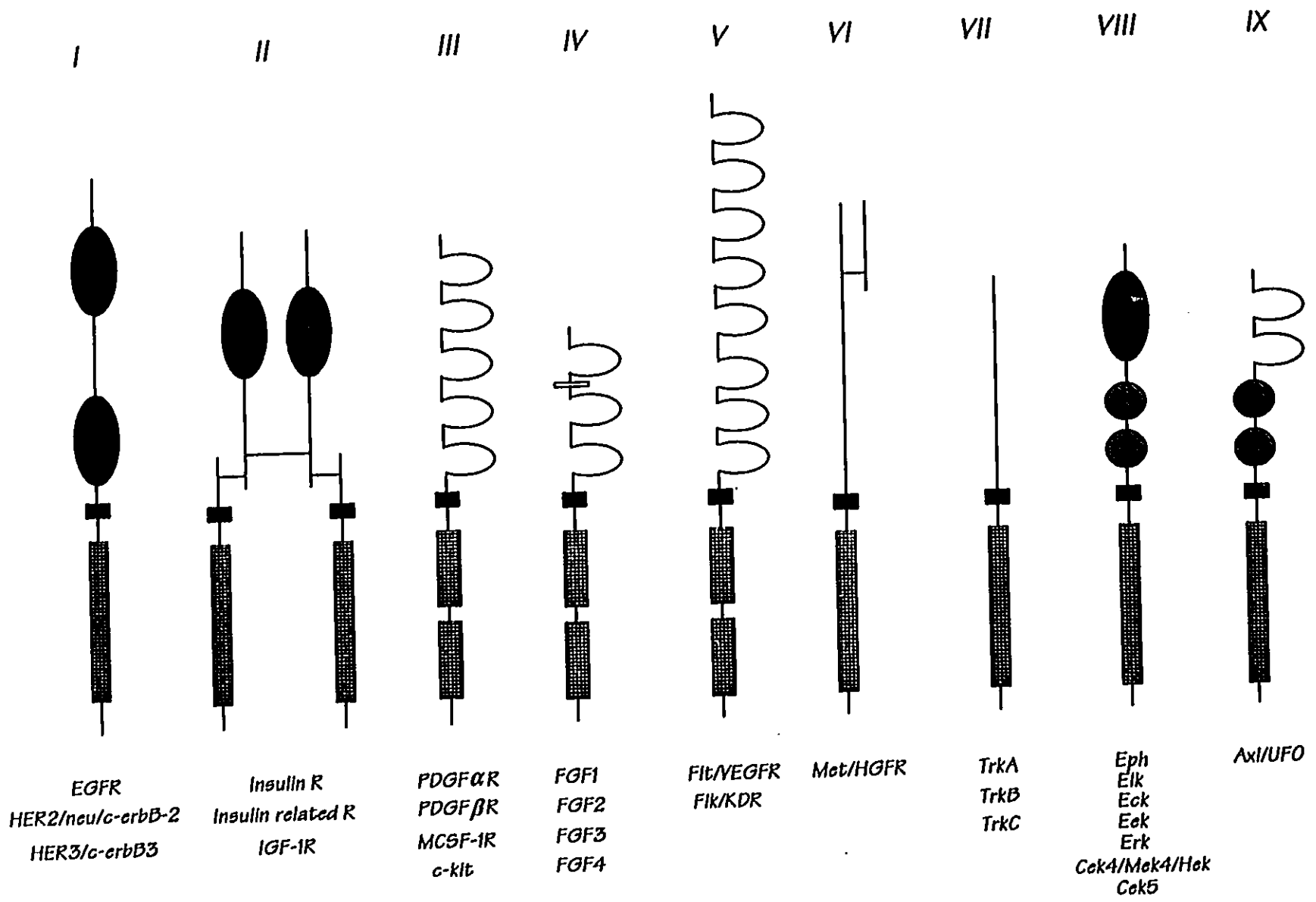
Members of the transmembrane growth factor receptor family are characterized by an extracellular ligand binding domain and an intracellular tyrosine kinase catalytic domain. Like their cytoplasmic counterparts, the receptor kinases can also be further subgrouped based on the structural features contained within their ligand binding and catalytic domains (Fantl *et al.*1993). As shown in figure 2 there are at least nine different receptor subfamilies.

The extracellular domain of PYK receptors, although having the common feature of ligand binding, do so through diverse functional domains. To date, there are three types of structural motifs that, alone or in combination, have been shown to be involved in ligand binding: cysteine rich motifs, immunoglobulin-like domains and fibronectin type III domains (figure 2). The intracellular catalytic domain is conserved throughout all protein kinases, however, in at least three receptor subgroups (PDGFr, Flt and FGFr), the catalytic domain is interrupted by a unique stretch of amino acids referred to as the kinase insert (KI) region. In the platelet derived growth factor receptor (PDGFr), the insert region contains several tyrosine autophosphorylation sites which are responsible for mediating binding of the receptor to secondary signal transducing molecules including phospholipase C-γ (PLCγ), phosphatidylinositol 3-kinase (PI3K), ras-GTPase activating protein (ras-GAP), raf-1 and src (Fantl *et al.*1993). Mutation of these sites does not affect the intrinsic tyrosine kinase activity of the receptors, but severely impedes their ability to mediate the proper growth regulatory signal (Fantl *et al.*1993).

Figure 2

Structure of transmembrane protein tyrosine kinase receptors.

Nine subfamilies of receptor kinases are shown here that differ in their extracellular ligand binding domains and their intracellular catalytic domain. Catalytic domains are represented as shaded rectangles, cysteine rich motifs in the extracellular domain are depicted as black ovals, while the immunoglobulin-like motifs and fibronectin type III motifs are depicted as semi-circles and hatched circles, respectively. The transmembrane domain is represented by the small black rectangle.


I
II
III
IV
V
VI
VII
VIII
IX
EGFR
HER2/neu/c-erbB-2
HER3/c-erbB3
Insulin R
Insulin related R
IGF-1R
PDGFαR
PDGFβR
MCSF-1R
c-kit
FGF1
FGF2
FGF3
FGF4
Flt/VEGFR
Flk/KDR
Met/HGFR
TrkA
TrkB
TrkC
Eph
Elk
Eck
Eek
Erk
Cek4/Mek4/Hek
Cek5
Axl/UFO

1.121 Activation and regulation of receptor protein tyrosine kinases

In the simplest model, receptor activation occurs only when ligand binds to the extracellular domain. The activation leads to dimerization of the receptor molecules followed by activation of the intracellular tyrosine kinase activity. The functional importance of dimerization in receptor activation is best demonstrated by tumor cells and retroviruses that have devised mechanisms to escape the tight controls that exist over receptor kinases. A truncated from of the epidermal growth factor receptor (v-erbB) is associated with the genome of the avian erythroblastosis retrovirus. The truncated receptor bipasses the requirement for ligand binding and dimerization, and is therefore constitutively activated in transformed cells (Hayman *et al.*1983; Downward *et al.*1984). Conversely, mutations within the transmembrane domain of certain growth factor receptors can lead to spontaneous dimerization and activation of the kinase, in the absence of ligand binding. Such a mutation has been described within the transmembrane domain of the HER2/Neu receptor kinase (from a valine to a glutamic acid) and is responsible for the activation and the transforming potential of this receptor (Schechter *et al.*1984; Bargmann *et al.*1986). Finally, dominant negative mutants of receptor molecules, defective in tyrosine kinase activity, can dimerize with wild type receptors and inhibit signal transduction. These types of dominant negative mutants are found in the c-Kit receptor kinase and are responsible for the White (W) spotting mutant mice (Tan *et al.*1990).

While there is controversy as to the role of tyrosine autophosphorylation in the activation of the receptor kinase activity and in the receptor-induced downregulation (Downward *et al.*1985; Wiley *et al.*1991), the importance of this autophosphorylation in receptor mediated signal transduction is unanimous. Indeed the interaction of secondary signal transducing molecules with the receptors is conditional upon tyrosine autophosphorylation and will be discussed further in section 3.

1.122 Function of receptor tyrosine kinases

Like their cytoplasmic counterparts, receptor protein tyrosine kinases may also play bifunctional roles in cell cycle regulation, with effects both on proliferation and differentiation. Indeed the HER2/neu receptor described above, although oncogenic when mutated or simply overexpressed, is actually a differentiation inducing receptor. The ligand for the neu kinase, neu differentiation factor (NDF), has recently been cloned and shown to promote the differentiation of certain mammary tumor cells (Peles *et al.*1992). Conversely, while the epidermal growth factor receptor (EGFr) promotes cell proliferation in most cell lines, it has also been shown to be developmentally expressed and may therefore play a role in inducing differentiation (Adamson, 1987). Therefore, the temporal and spatial expression patterns of receptor protein tyrosine kinases, combined with the availability of ligand and secondary effector molecules, are key determinants in dictating the proliferative or differentiation effects of a given receptor.

1.2 DUAL SPECIFICITY KINASES

1.21 Features of dual specificity kinases

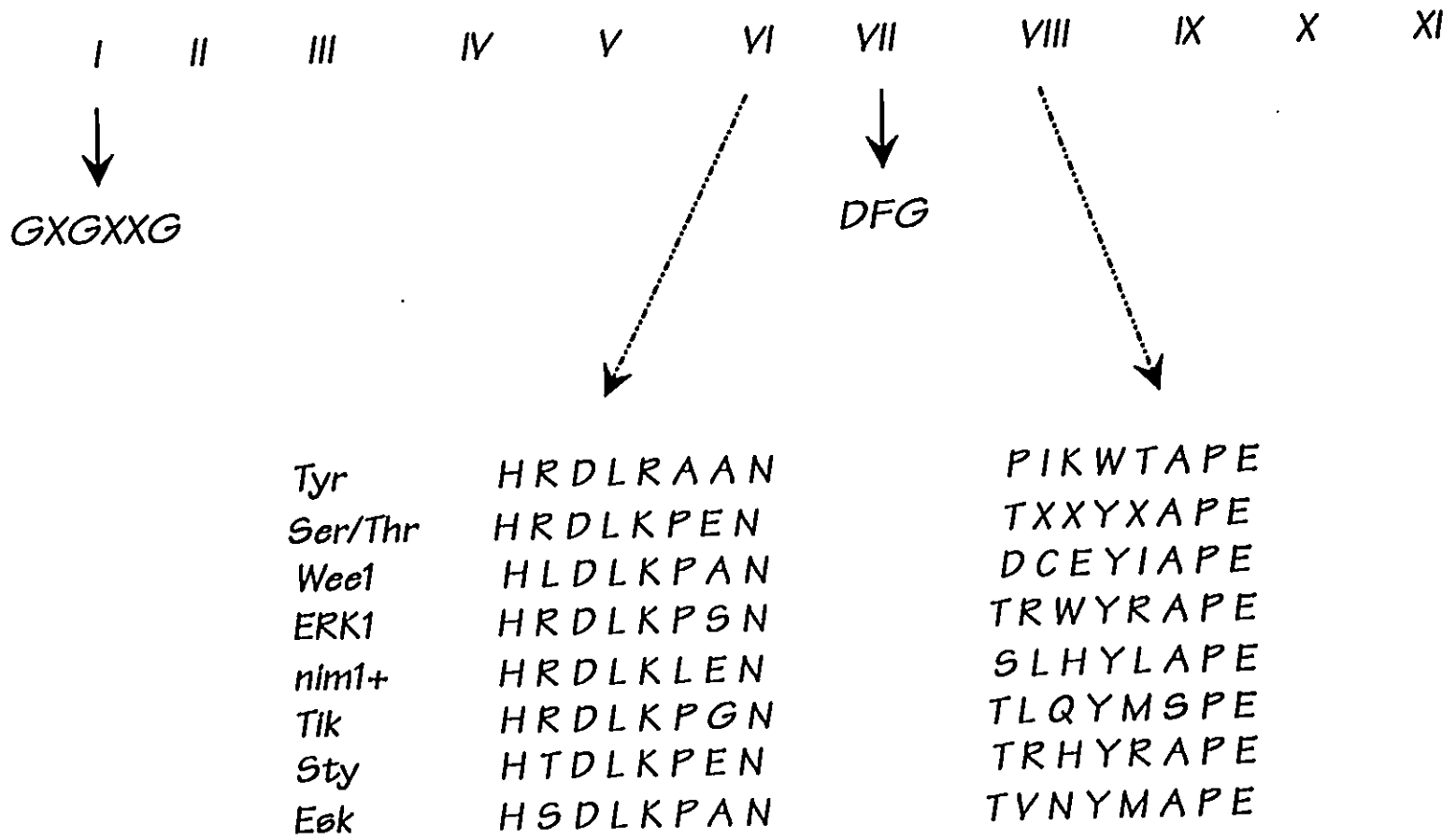
All protein kinases share common structural features including a conserved catalytic domain and an ATP binding site. Initially, members of the PSK and PYK families were thought to be sufficiently distinct, structurally and functionally, to be distinguishable. One could predict, based on the amino acid sequences contained within the catalytic domain, to which family a kinase belonged. Specifically, sequences contained within subdomains VI and VIII of the catalytic domain were indicative of whether a kinase belonged to the PSK or the PYK family (Hanks *et al.*1988; Lindberg *et al.*1992) (figure 3).

Over the last few years, the distinction that existed between the two families of protein kinases has become somewhat obsolete. Indeed, structurally and biochemically PSKs and PYKs are no longer as well defined. A novel family of protein kinases, now referred to as dual specificity kinases, has been identified and members have the potential to phosphorylate the three hydroxy amino acids; serine, threonine and tyrosine. These enzymes, originally characterized by our laboratory as well as several others, were identified through expression cloning strategies that made use of anti-phosphotyrosine antibodies (Letwin *et al.*1992; Ben-David *et al.*1991; Mills *et al.*1992; Lindberg *et al.*1993; Icely *et al.*1991; Stern *et al.*1991; Howell *et al.*1991; Douville *et al.*1991). While such a screening procedure should enrich for the cloning of protein kinases with structural features of PYKs, the kinases identified displayed features related to PSKs

Figure 3

Conserved subdomains within the catalytic domain of protein kinases.

The catalytic domain of all protein kinases can be subdivided into 11 conserved subdomains as defined by Hanks *et al.* (Hanks *et al.*1988). Specifically, the amino acid sequences contained within subdomains VI and VIII were thought to be indicative of whether a kinase belonged to the serine/threonine (Ser/Thr) or tyrosine (Tyr) family of protein kinases. Dual specificity kinases have the ability to phosphorylate tyrosine residues, yet the amino acid sequence of subdomains VI and VIII within these kinases reveals that they are related to members of the Ser/Thr family of protein kinases.


I II III IV V VI VII VIII IX X XI
GXGXXG
DFG
Tyr HRDLRAAN PIKWTAPE
Ser/Thr HRDLKPEN TXXYXAPE
Wee1 HLDLKPAN DCEYIAPE
ERK1 HRDLKPSN TRWYRAPE
nim1+ HRDLKLEN SLHYLAPE
Tlk HRDLKPGN TLQYMSPE
Sty HTDLKPEN TRHYRAPE
Esk HSDLKPAN TVNYMAPE

(figure 3). In fact, kinases initially thought to be serine/threonine specific were later found to be dual specificity kinases. These include the extracellular regulated kinases (ERKs) (Wu *et al.*1991; Crews *et al.*1991), Wee1 (Featherstone and Russel, 1991; Parker *et al.*1992) and the activin IIB receptor kinase (Nakamura *et al.*1992). To date there are over 20 members in the dual specificity kinase family (listed in table 1), and like their PSK and PYK counterparts, these enzymes are involved in all aspects of cell cycle regulation.

1.22 Regulation and function of dual specificity kinases

1.221 The cell cycle and DSKs

The distinguishing feature of dual specificity kinase regulation seems to be the occurrence of kinase cascades involving members of this new family. In eukaryotes the cell cycle is a tightly regulated process that involves several checkpoint genes responsible for coupling DNA replication to cell division. Key in directing the entry of cells into mitosis is the protein serine/threonine kinase $p34^{cdc2}$. The regulation of this enzyme has been extensively studied; in addition to the binding of $p34^{cdc2}$ to cyclin B, a series of phosphorylation and dephosphorylation reactions are required for full activation of this enzyme (figure 4) (Norbury and Nurse, 1992; Atherton-Fessler *et al.*1993; Draetta, 1993). In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), the Wee1 dual specificity kinase is responsible for phosphorylation of the cdc2-cyclin B complex on a tyrosine residue located in the ATP binding site of the kinase (Featherstone and Russel,

Table 1

List of dual specificity kinases.

The following table lists the dual specificity kinases that have been identified to date. The names of the kinases, the organism from which they are derived as well as the phosphoamino acids generated through autophosphorylation reactions are indicated. The last column lists the known substrates for these kinases.

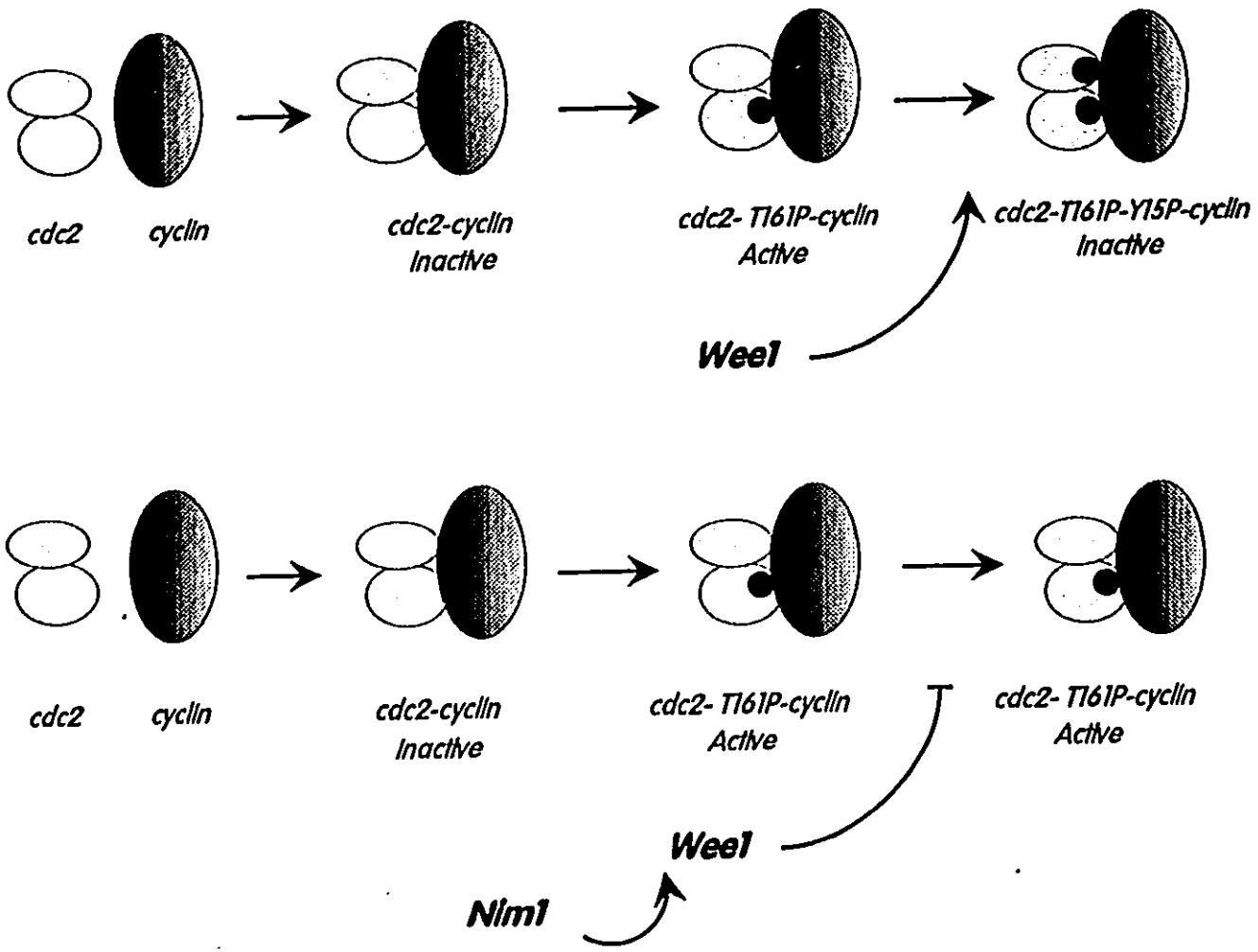
NAME	ORGANISM	PAAs/AUTOPHOSPHORYLATION	PHYSIOLOGICAL SUBSTRATES
Wee1	Schizosaccharomyces pombe	Ptyr, Pser	cdc2
Mik1	Sch. pombe	ND	cdc2
Nim1/Cdr1	Sch. pombe	Pser, Ptyr	wee1
byr1	Sch. Pombe	ND	spk1
Spk1	Saccharomyces cerevisiae	Pser, Pthr,Ptyr	
Ypk1	S. cerivisiae	Pser, Ptyr	
STE7	S. cerivisiae	Pser, Pthr, Ptyr	FUS3
Sty/Clk	mouse	Pser, Ptyr, Pthr	
Tik	mouse	Pser, Pthr	eIF2 alpha
Eek	mouse	Pthr, Pser, Ptyr	
Nek	mouse	Pser, Pthr, Ptyr	
Activin IIB receptor	mouse	Pser, Ptyr, Pthr	
JAK1	mouse	Ptyr, Pthr, Pser	
MAP kinase	mouse	Pthr, Ptyr	rsk
MAPK Kinase	Xenopus	Ptyr, Pthr	MAP kinase
Dsor1	Drosophila melanogaster	ND	
ERK1	Human	Ptyr, Pthr	
ERK2	Human	Ptyr, Pthr	
Tyk2/JAK3	Human	ND	ISGF3alpha?
TTK/PYT	Human	Pthr, Pser, Ptyr	

Figure 4

Dual specificity kinase cascade involved in the mitotic control of lower eukaryotes.

The cdc2 kinase in the monomeric state is inactive. The binding of cyclin allows for the phosphorylation of threonine 161 (Thr 161) within the cdc2 kinase, and its activation [Thr 161 phosphorylation is carried out by the cdc2-activating kinase (cak)]. The binding of cyclin also allows for the phosphorylation of Tyrosine 15 (Tyr 15) in the ATP binding site of cdc2, hence inactivating the enzyme. Tyr 15 phosphorylation is carried out by the Wee1 dual specificity kinase. When DNA replication has been completed, Tyr 15 is dephosphorylated by the tyrosine phosphatase cdc25, and cdc2 returns to the active state.

The regulatory role of the Wee1 DSK on the cell cycle is countered by the activity of the Nim1/Cdr1 DSK that can phosphorylate and inactivate the Wee1 kinase. Phosphorylations of Tyr 15 and Thr 161 are shown as black circles in the cdc2 kinase.


cdc2
cyclin
cdc2-cyclin
Inactive
cdc2- T161P-cyclin
Active
cdc2-T161P-Y15P-cyclin
Inactive
Wee1
cdc2
cyclin
cdc2-cyclin
Inactive
cdc2- T161P-cyclin
Active
cdc2- T161P-cyclin
Active
Wee1
Nim1

1991; Parker *et al.*1992). Hence, Wee1 temporarily blocks the entry of cells into mitosis by inhibiting the activity of the cyclin-kinase complex until the cellular contents have doubled and DNA replication has been completed. In turn, the Wee1 kinase is also subject to regulation via another dual specificity kinase, nim1/cdr1. Genetic and biochemical studies have demonstrated that nim1/cdr1 acts upstream of the Wee1 kinase and positively regulates the cell cycle by inhibiting the activity of Wee1 (Coleman *et al.*1993; Parker *et al.*1993; Wu and Russell, 1993). In lower eukaryotes, the cell cycle is therefore a tightly regulated process controlled by a cascade of phosphorylation events involving dual specificity kinases.

1.222 Growth regulation and DSKs

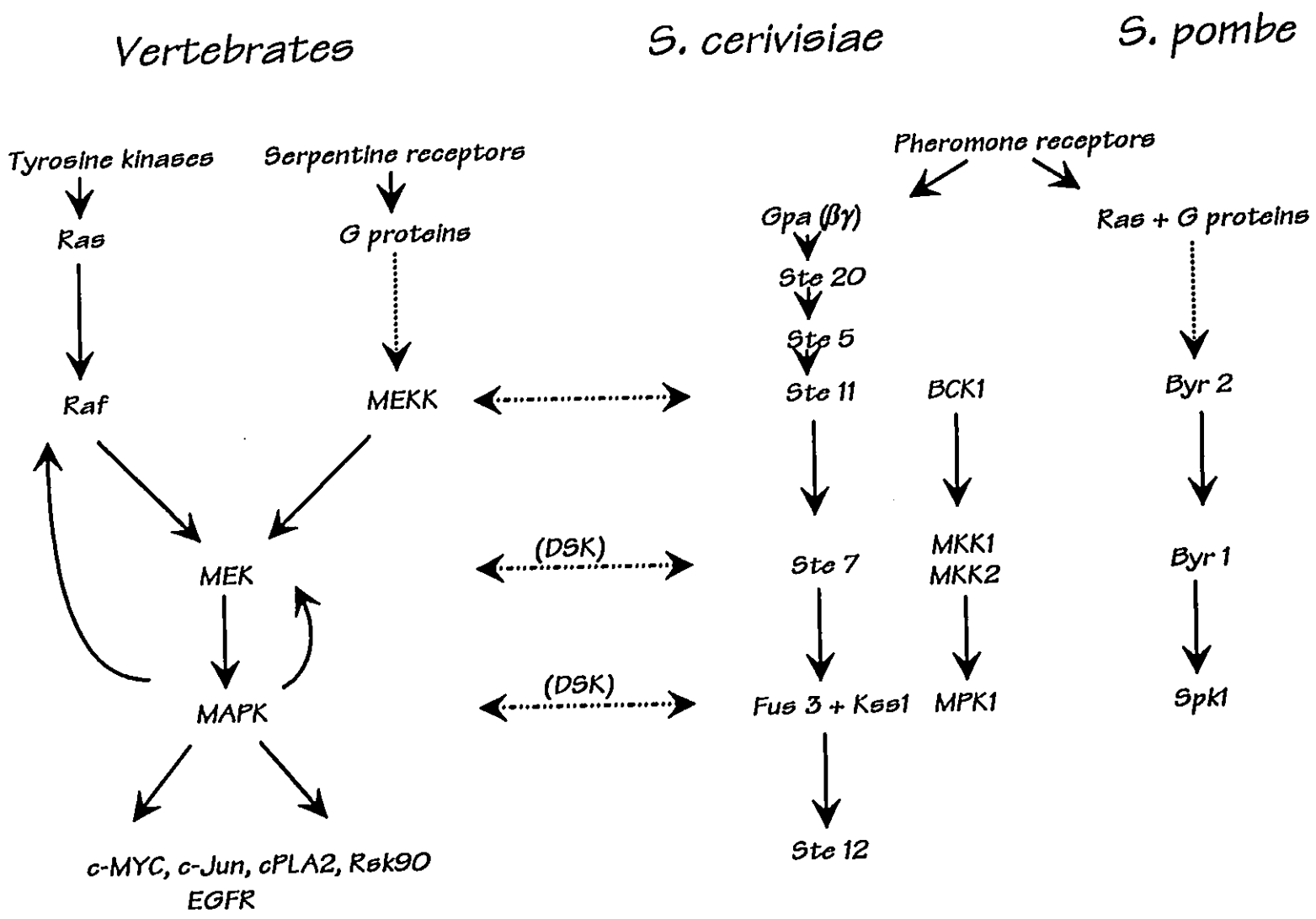
A cascade of phosphorylation events is also central to the response of cells to growth factor stimulation. Unlike the mitotic kinases, the enzymes involved in the growth factor induced kinase cascade are conserved from yeast to vertebrates and are thought to mediate diverse regulatory signals (figure 5).

In mammalian cells, the stimulation of growth factor receptors in response to extracellular stimuli leads to the rapid and reversible phosphorylation of the **MAP** Kinases (Mitogen Activated Protein Kinases) (L'Allemain *et al.*1992). The MAP kinases, also known as **ERK** kinases (Extracellular Regulated Kinases), were originally identified as serine/threonine kinases based on their amino acid sequence and on their activity towards exogenous substrates. The MAP kinases were later found to display a

Figure 5

Conserved kinase cascades in yeast and vertebrates.

Relationship of the vertebrate MAP kinases to those found in yeast (*S. cerivisiae* and *S. pombe*). The dashed arrows indicate related kinases acting in the vertebrates and yeast (e.g. MEKK-Ste11-BCK1-Byr2). The dotted arrows indicate links that have been hypothesized to occur. Note the regulatory feedback of MAPK on MEK and the Raf kinase.


Vertebrates
S. cerivisiae
S. pombe
Tyrosine kinases
Serpentine receptors
Ras
G proteins
Raf
MEKK
MEK
MAPK
c-MYC, c-Jun, cPLA2, Rsk90
EGFR
(DSK)
(DSK)
Pheromone receptors
Gpa (βγ)
Ste 20
Ste 5
Ste 11
Ste 7
Fus 3 + Kss1
Ste 12
BCK1
MKK1
MKK2
MPK1
Ras + G proteins
Byr 2
Byr 1
Spk1

slow intramolecular tyrosine kinase activity which was the basis for their reclassification into the dual specificity kinase family (Wu *et al.*1991; Crews *et al.*1991). The slow intramolecular autophosphorylation of MAP kinases was thought to potentiate the activation of these enzymes in response to growth factors (Wu *et al.*1991). Indeed, the activation of MAP kinases (MAPK) requires both threonine and tyrosine phosphorylation which has been shown to be carried out by the dual specificity kinase MAPK kinase (also known as MEK) (Kosako *et al.*1993). To date the MAPK kinase is the only enzyme which has been shown to display dual specificity in an *in vivo* context and actually mediate a biological effect through dual phosphorylation events.

The regulation of MEK has recently been shown to involve serine and threonine phosphorylation which can be carried out in mammalian cells by the MEK kinase (MEKK) or the raf-1 kinase, both members of the serine/threonine kinase family (Lange-Carter *et al.*1993). While the raf-1 kinase has been shown to function immediately downstream of $p21^{ras}$ in growth factor stimulation (Moodie *et al.*1993; Warne *et al.*1993; Zhang *et al.*1993; Vojtek *et al.*1993), it has been proposed that the MEK kinase may mediate G protein signals in response to activation of serpentine transmembrane receptors (such as the β-adrenergic receptor) (Lange-Carter *et al.*1993) (figure 5). Of interest, the regulation of MEK can also occur through MAP kinase which may be responsible for mediating a negative feedback loop (Nishida and Gotoh, 1993) (figure 5). In fact MEK (which displays a MAPK consensus phosphorylation site (Pro,X,Thr,Pro)) and raf-1, have both been shown to be substrates of MAPK (Matsuda

*et al.*1993; Lee *et al.*1992). This type of feedback loop might serve to tighten the control that exists over these kinases.

As discussed earlier, this protein kinase cascade has been evolutionarily conserved, and homologs for MAP kinase, MEK and MEK kinase have been cloned from yeast (*pombe* and *cerivisiae*) (figure 5), *Xenopus* (Kosako *et al.*1993), Drosophila and plants (*Arabidopsis thaliana*) (Nishida and Gotoh, 1993). In fact, these enzymes have been sufficiently conserved to allow for functional substitutions. In *S. pombe*, the MAP kinase homolog Spk1, has been shown to be essential for mating. While disruption of this kinase leads to defects in sporulation, preliminary results from Gotoh *et al.* have shown that the Xenopus MAPK can functionally substitute for Spk1 and restore sporulation (Nishida and Gotoh, 1993). Conversely, in *S.cerivisiae*, the *Xenopus* MAPK could substitute for the MPK1 kinase in the control of growth (Nishida and Gotoh, 1993). Finally, while it appears that yeast cells have devised several MAP kinase cascades to mediate different biological responses, a single MAP kinase cascade exists in mammalian cells that may serve to channel the different growth regulatory signals (figure 5).

1.23 The rationale for dual specificity in a kinase

It has recently been recognized that simultaneous serine/threonine and tyrosine phosphorylation events are required to mediate the full activation of certain enzymes. While members of the PSK and PYK family can independently converge onto a protein

to regulate its activity, an apparent alternative is for both functions to be intrinsic to a single protein kinase and mediate the same effect. The evolutionary conservation of dual specificity kinase members suggests that this mechanism of regulation is central to several organisms and may offer added functional features.

Key amongst these features, is the potential for dual specificity kinases to interact with SH2 domains. Pioneering work from Tony Pawson's group has shown that tyrosine phosphorylation serves as a molecular switch to create high affinity binding sites for proteins containing SH2 domains (discussed further in section 3). Even weak autophosphorylation on tyrosine residues could allow dual specificity kinases to bind to the SH2 domains of secondary signalling molecules such as PLCγ, ras-GAP and PI3K. In higher eukaryotes, dual specificity kinases may have therefore evolved to integrate into PYK signalling pathways by virtue of their tyrosine autophosphorylation and possible interactions with SH2 domains.

2. SECONDARY SIGNAL TRANSDUCERS

The stimulation of cells with growth factors, such as EGF and PDGF, leads to the activation of a number of secondary signal transducing molecules including PLCγ, PI3 kinase, Ras GTPase-activating protein and the Raf-1 serine/threonine kinase. The actions of these molecules are responsible, in part, for propagating the signals initiated at the surface of the cell. While the mechanisms of interaction of these molecules with growth factor receptors and cytoplasmic kinases will be discussed further in section 3,

the function of these secondary effectors in signal transduction will be described here. A particular emphasis will be put on the proteins involved in phosphoinositide metabolism: phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K).

There are two distinct pathways involving inositol phospholipid metabolism in mammalian cells. The canonical pathway involves the turnover of phosphatidylinositol-4,5-bisphosphate by PLC to generate the second messengers inositol-1,4,5-triphosphate and diacylglycerol. The second pathway, discovered more recently, involves the production by PI3K of 3-phosphorylated phosphoinositides, which are thought to function directly as second messengers. The enzymes involved in the synthesis and metabolism of these phospholipids are described below.

2.1 Phosphatidylinositol 4-phosphate pathway

Inositol-containing phospholipids are ubiquitous components of eukaryotic cells and constitute between 2 and 8% of total phospholipids. The three major inositol containing lipids are: phosphatidylinositol (PtdIns), phosphatidylinositol (4) phosphate (PI-4-P), and PtdIns (4,5) bis phosphate (PI-4,5-P_2). PtdIns accounts for over 80% of the phospholipids found in the membrane (Majerus, 1992).

Phosphatidylinositol biosynthesis was first demonstrated in the endoplasmic reticulum by the sequential phopshorylation of PtdIns by PtdIns 4-kinase and PtdIns-4-P 5-kinase to give rise to PI-4-P and PI-4,5-P_2 (Brockerhoff and Ballou, 1962) (Figure 6). PtdIns 4-kinase has been purified from several sources, including bovine uterus (Porter

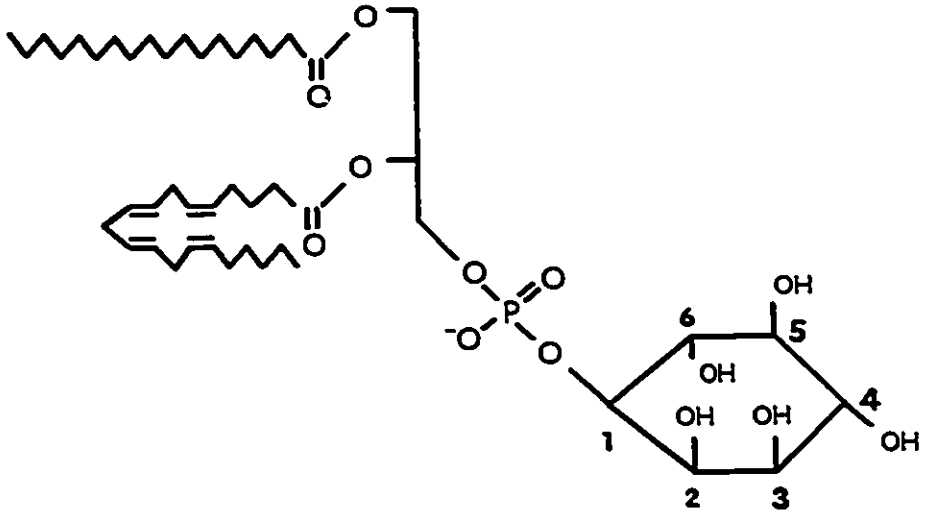
Figure 6

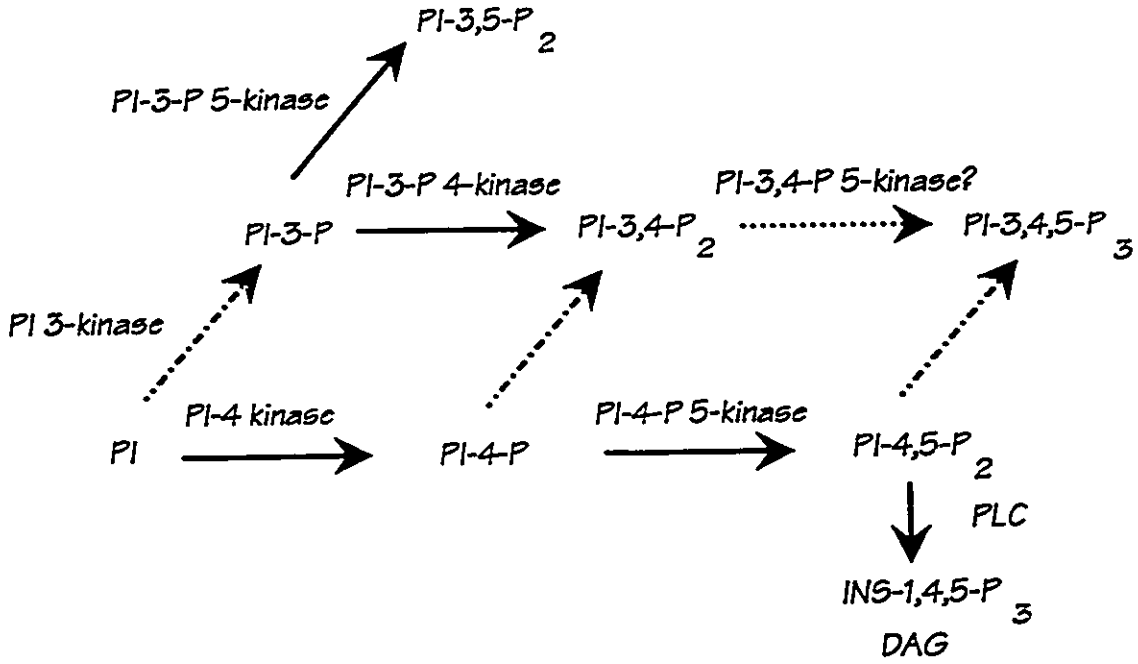
Structure and metabolic pathways of phosphatidylinositol (PI).

The structure of PI is shown in the top panel. The positions in the inositol ring that are relevant to the metabolic pathways (3-, 4-, 5- OH) are bolded. The sequential phosphorylation of PI by PI 4-kinase and PI-4-P 5-kinase generates PI-4,5-P (PIP_2), the substrate for the PLC enzymes. Hydrolysis of PIP_2 generates the second messengers Ins-1,4,5-P_3 (IP_3) and diacylglycerol (DAG).

Phosphatidylinositol 3-kinase (PI3-kinase) can use PI, PI-4-P and PI-4,5-P as substrates to generate to the 3-phosphorylated products which are thought to function directly as second messengers (pathways indicated by dashed arrows).

Phosphatidylinositol (PI)





*et al.*1988), rat brain (Yamakawa and Takenawa, 1988), porcine liver and A431 cells (Majerus, 1992) as a membrane bound protein. Two types of PtdIns 4-kinase (II and III) have been identified which differ in their sensitivity to adenosine and detergents but which are both regulated by cAMP, diacylglycerol, calcium ionophores and possibly GTPγS (Carpenter and Cantley, 1990). As with PtdIns 4-kinase, two types of PtdIns-4-P 5-kinase have been characterized from blood erythrocytes. The type II enzyme exists as both a soluble and particulate enzyme that displays product inhibition by PI-4,5-P_2, is activated by phosphatidylserine and will phosphorylate PI-3-P under certain conditions (see figure 6) (Carpenter and Cantley, 1990). The type I enzyme is only found in the membrane fraction of erythrocytes and appears to be the major enzyme responsible for the production of PI-4,5-P_2 (PIP_2). The mechanisms of regulation of the PtdIns-4-P 5-kinases is poorly understood but may involve G proteins and protein tyrosine kinases (Carpenter and Cantley, 1990).

2.11 Phospholipase C

The PtdIns-specific phospholipase C (PLC) enzymes catalyze the hydrolysis of phosphatidylinositols to produce messenger molecules. Indeed the hydrolysis of PtdIns, PtdIns-4-P and PtdIns-4,5-P (PIP_2) can yield six different *water-soluble* second messengers (including the well characterized Ins(1,4,5)P_3) as well as diacylglycerol (DAG), the lipid moiety (Majerus, 1992). These second messengers have been shown to directly activate secondary signal transduction pathways through calcium mobilization

and activation of protein kinase C (PKC) at the plasma membrane. The Ins(1,4,5)P_3 (IP_3) binding protein has recently been cloned and shares striking homology to the ryanodine receptor (Furuichi *et al.*1989). The IP_3 binding protein is a multispanning transmembrane receptor of 260 kDa that exists as a tetramer in membranes of the endoplasmic reticulum, the cis golgi apparatus and possibly the nuclear membrane (Ross *et al.*1989). The protein functions directly by displacing calcium ions across the lipid bilayers and displays high affinity for its ligand IP_3 (Ferris *et al.*1990).

There are nine forms of PLC identified to date that can be subdivided into four groups α, β, γ and δ (Majerus, 1992; Majerus *et al.*1990). Since the phospholipid substrates of these enzymes are localized to the plasma membrane, the PLC enzymes must bind to the lipid bilayer to reach their substrates. The phospholipase Cγ isoform, as will be discussed in the following section, is recruited to the plasma membrane by activated growth factor receptors. The tyrosine autophosphorylation of several growth factor receptors (including the PDGFR, the EGFR and the c-kit receptor), creates high affinity binding sites for the SH2 domain of PLCγ. The enzyme thus translocates to the plasma membrane where it is tyrosine phosphorylated and activated to hydrolyse PIP_2 to IP_3 and DAG (Kim *et al.*1991). In contrast, the PLCβ^1 isoform isolated from rat brain, appears to be activated 30-fold by a G protein (Gq) (Taylor *et al.*1990). These results suggest that in different tissues, the multiple isoforms of PLC may respond to different regulatory signals to mediate the hydrolysis of PIP_2.

2.2 Phosphatidylinositol 3-phosphate pathway

Phosphatidylinositol lipids phosphorylated in the D3 hydroxyl position are a recently discovered metabolite in the phosphatidylinositol metabolic pathway. These phosphorylated products include PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (figure 6). The PtdIns-3-P product is present in cells at all times and serves as a precursor for the polyphosphorylated 3-phosphate containing PtdIns. In contrast, the PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are formed only transiently in cells in response to agonists and growth factors. Indeed Whitman *et al.* first identified 3-phosphorylated PtdIns in transformed fibroblasts and proposed that this lipid directly played a role in controlling cell proliferation (Whitman *et al.*1988). In contrast to the 4-phosphorylated lipids (PI-4-P and PI-4,5-P_2) that are relatively abundant in resting cells and decline rapidly in response to hormones or growth factors, the 3-phosphorylated lipids (PI-3,4-P_2 and PI-3,4,5-P_3) are absent in quiescent cells and are rapidly produced upon stimulation. Moreover, the 3-phosphorylated lipids are not hydrolysed by the PLC enzymes. It is therefore unlikely that these lipids serve as precursors for water soluble second messengers, but rather may mediate their function as lipids per se.

2.21 Phosphatidylinositol 3-kinase (PI3K)

The enzyme that catalyzes the phosphorylation of PtdIns on the D3 position is a heterodimer composed of two subunits, 85 and 110 kDa. Phosphatidylinositol 3-kinase activity was initially purified from various sources, and subsequently cDNAs cloned for

each subunit (Hiles *et al.*1992; Skolnik *et al.*1991; Escobedo *et al.*1991; Otsu *et al.*1991). The 110 kDa subunit exits as two distinct isoforms and possesses PI3K activity, while the 85 kDa subunit, which also exists in two distinct isoforms, functions as an adaptor molecule to link the catalytic subunit to activated protein tyrosine kinases.

2.211 The p110 catalytic subunit

The cDNA for the catalytic subunit of PI3K was recently cloned from bovine brain by Waterfield's group (Hiles *et al.*1992). The cDNA displays greatest homology to Vps34p, a *S.cerivisiae* kinase involved in the sorting of proteins in the vacuole (Herman and Emr, 1990). The significance of this homology is unknown, however PI3K activity has been implicated in vesicle-mediated responses in higher eukaryotes. Indeed, ligand stimulation of platelets and neutrophils leads to the fusion of vesicular structures, and is associated with an increase in PI3 kinase activity (Kucera and Rittenhouse, 1990; Traynor-Kaplan *et al.*1988). Interestingly, both kinases lack the conventional glycine-rich ATP binding loop (GXGXXG) found in all protein kinases, but share sequences of other conserved subdomains (including the DFG motif involved in chelating magnesium in the active site) (see figure 3) (Hiles *et al.*1992). Whereas PI3-kinase activity has been observed in p85-p110 complexes purified from bovine brain (Hiles *et al.*1992; Fry *et al.*1992), it has also been observed in the p110 subunit isolated alone from bovine thymus (Shibasaki *et al.*1991), suggesting that the catalytic subunit need not be

complexed to p85 to be active. Further work will be needed to determine if these p110 proteins represent different isoforms of the catalytic subunit.

2.222 The p85 adaptor subunit

Two independent cDNAs encoding the p85 subunit have been isolated from bovine brain and are referred to as p85α and p85β (Lin *et al.*1991). The alpha and beta subunits are composed of a single SH3 and two SH2 domains (see figure 7 for structure) and are believed to function by linking the p110 subunit to activated protein tyrosine kinases (figure 9). In addition to the src homology regions, the cDNAs for the 85 kDa subunit of PI3K contain a domain related to members of the Rho/Rac family of GTPase-activating proteins including Rac-GAP, Rho-GAP, Bcr and recently inositol polyphosphate 5-phosphatase (Baldwin and Zhang, 1993). These GAP domains are unrelated to the Ras-GAP family of activators. Although GAP activity has not been demonstrated in p85, it suggests that this protein could function independently or in association with PI3 Kinase activity in regulating members of the G protein family. The cDNAs encoding the different p85 subunits display 62% overall identity at the amino acid level. The highest level of identity resides in the SH2 domains; 85% for the more amino terminal SH2 domain and 78% for the carboxyl SH2 domain (figure 7). Unexpectedly, the region located between the two SH2 domains of the alpha and beta subunits also displayed high homology (74%) (Otsu *et al.*1991). Recent evidence indicating that the binding of the 110 and 85kDa subunits could occur through this

intervening region would explain the conserved nature of these sequences (Klippel *et al.*1993). Finally, while the alpha and beta subunits are relatively conserved, they appear to be differentially regulated in their association with the catalytic subunit in tissues and cell lines. While PI3K activity has been found associated with the p85α subunit in bovine brain, investigators have only detected traces of lipid kinase activity associated with the p85β subunit (Fry *et al.*1992). This observation has been extended to several cell lines, including A431, cos-1 and NIH3T3, where again only traces of PI3K activity can be detected in p85β immunoprecipitates (Fry *et al.*1992; Gout *et al.*1992). These results suggest that p85β may represent an alternative regulatory subunit for the PI3 kinase complex or alternatively, the p85β subunit may be the regulatory subunit for another cellular activity.

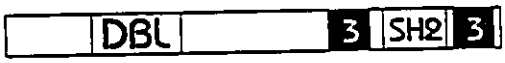
3. SRC HOMOLOGY REGIONS

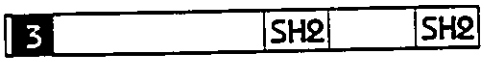
The src homology domains were initially characterized as conserved regions shared amongst cytoplasmic protein tyrosine kinases. The src homology 1 domain (SH1) corresponds to the catalytic domain of the src kinase and is conserved amongst all protein kinases. In contrast, the conserved src homology regions 2 and 3 (SH2 and SH3) are found in both kinase and non-kinase cytoplasmic proteins (figure 7). It has become apparent that both the SH2 and SH3 domains can mediate protein-protein interactions and can therefore regulate the activity, substrate specificity and subcellular localization of the molecules carrying these domains.

Figure 7

Structure of SH2 and SH3 containing proteins.

Proteins containing SH2 and SH3 domains have been grouped into two categories, those with a known cellular activity (PYKs, PLC, Ras-GAP) and those with no recognizable catalytic activity (p85, Crk, SHC and Nck). SH3 domains are indicated by the number 3 only.

	Activity	SH2/SH3 Enzymes
Src	Tyrosine kinase	
Abl	Tyrosine kinase	
Syk	Tyrosine kinase	
PTP1C	Phosphotyrosine phosphatase	
PLCγ	Phospholipase	
GAP	Ras GTPase Activation	
Vav	Guanine nucleotide exchange ?	

	Targets	SH2/SH3 Adaptors
p85 PI3K	PI3Kinase	
c-Crk		
SHC		
Nck		
Sem5/GRB2	Ras pathway	
ISGF3α	ISGF3γ	

3.1 SH2 domains

3.11 Structure and specificity of SH2 domains

The SH2 domain is a sequence motif of approximately 100 amino acids that was initially identified in the transforming oncogenes v-src and v-fps (Sadowski *et al.*1986). The non-catalytic SH2 domain was found to be important in mediating protein-protein interactions by recognizing and binding to sequences that contained tyrosine phosphorylated residues. The SH2 domains share at least five conserved subdomains (I to V) which dictate their phosphopeptide specificity (Koch *et al.*1991). Key amongst these domains, the FLVRES motif (subdomain II) is responsible for binding phosphotyrosine within the recognition motif. The crystal structure of the Src SH2 domain (with and without peptide) has revealed that this small modular domain forms a pocket into which the phosphotyrosine of the phosphopeptide can protrude. Within the pocket, the phosphotyrosine interacts with a lysine and two arginine residues including the invariant arginine in the FLVRES motif (Pawson and Gish, 1992). To date, only one phosphotyrosine independent SH2 interaction has been described and involves the gene product of the Philadelphia chromosomal translocation present in patients with chronic myelogenous leukaemia (CML) and acute lymphocytic leukaemia (ALL) (Hermans *et al.*1987). The translocation of chromosomes 9 and 22 involves the genes encoding the ABL protein tyrosine kinase and the BCR protein and results in the fusion product, BCR-ABL. Recently, the BCR portion of the fusion, which is highly phosphorylated on serine

residues, has been shown to bind to the SH2 domain of ABL providing the first example of serine phosphorylation promoting SH2 binding (Pendergast *et al.*1991).

The specificity of SH2 domains for phosphotyrosine containing peptides has been demonstrated extensively with the use of competing phosphopeptides and mutagenesis. Evidence that the binding of SH2 domains was dictated by the primary sequence surrounding the phosphotyrosine came from comparing the regions in the polyoma middle T antigen and the PDGFr which were responsible for binding phosphatidylinositol 3-kinase (PI3K) (Cantley *et al.*1991). The sequence motif PTyr-Met/Val-X-Met was found to be essential for binding and was also shared by other proteins which were predicted to bind PI3K. These observations led Cantley's group to generate a phosphopeptide library which would enable them to predict the binding sequence of SH2 domains (Songyang *et al.*1993). The important observation was that the amino acids in the position +1 and +3 (relative to the phosphotyrosine) were critical determinants of the sequence specificity of SH2 binding sites.

3.12 Functions of SH2 domains

3.121 SH2 domains associated with cytoplasmic protein tyrosine kinases

Most of the information gained on the functional significance of SH2 domains came from mutational studies on the viral cytoplasmic oncogenes. Indeed, mutations within the SH2 domains of the src and fps oncogenes gave rise to mutants that are temperature sensitive, defective or host-range dependent for their transformation

(Sadowski *et al.*1986; DeClue *et al.*1987). These observations suggested that the SH2 domains could play a role in regulating the kinase activity and substrate specificity of cytoplasmic PYKs.

The importance of the SH2 domain in controlling the kinase activity of cytoplasmic PYKs has been discussed in section 1.111 and involves the SH2 domain binding to the phosphorylated tyrosine residue at position 527 in the src kinase. The c-src kinase activity would therefore be repressed as a result of the kinase folding back on itself (figure 8). This model has been corroborated by the work of Liu *et al.* (Liu *et al.*1993) using bacterial expression of the src SH2 domain [as a glutathione-S-transferase (GST)- fusion product] and phosphopeptides corresponding to the sequences surrounding tyrosine 527. Dephosphorylation of this residue would release the SH2 domain, allow the kinase to unfold and resume activity. Consistent with these observations, discrete mutations within the SH2 domain of c-src which prevent self binding, also lead to kinase activation (Hirai and Varmus, 1990).

In the transforming oncogenes v-src and v-fps, the SH2 domain can also play a role in regulating substrate specificity as evidenced by mutations that impair the phosphorylation of the protein substrates p62 and GAP without altering the intrinsic kinase activity (Moran *et al.*1990; Bouton *et al.*1991; Koch *et al.*1989) (figure 8, bottom panel). The transforming ability of v-src is also compromised in these mutants which prevent the binding and phosphorylation of p62 and GAP suggesting therefore, that these interactions contribute to the transformed phenotype.

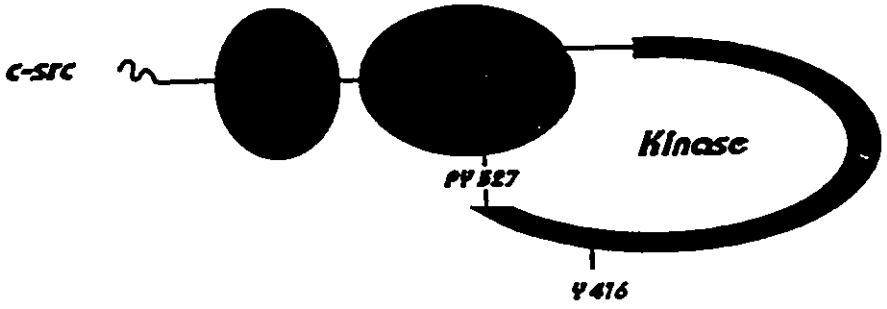
Figure 8

Regulation of Src kinase by its SH2 domain.

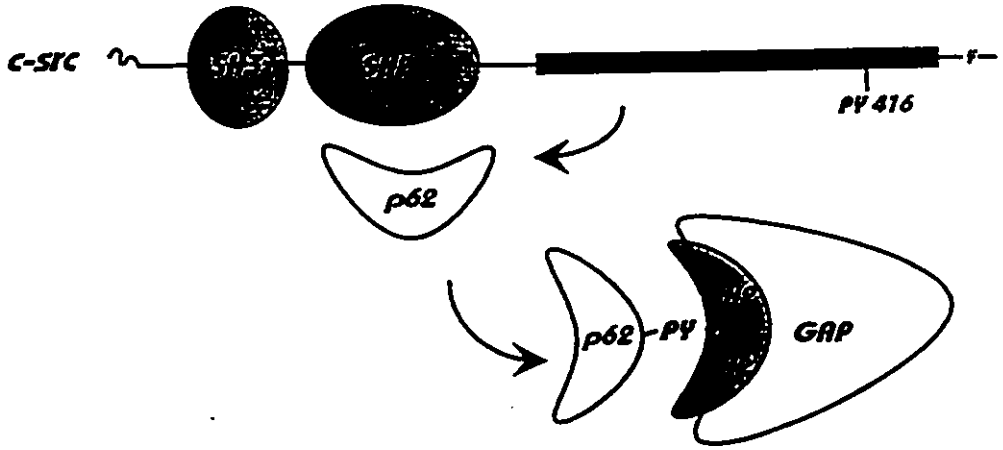
Tyrosine phosphorylation of the C-src kinase on residue 527 induces a conformational change that inhibits its protein kinase activity. The conformational change is created by the binding of the tyrosine-phosphorylated residue 527 to the SH2 domain within the N-terminal regulatory region. (Upper panel)

Dephosphorylation of Tyr 527 allows the c-src kinase to unfold and resume catalytic activity. The SH2 and SH3 domains are also thought to mediate substrate specificity. Mutations within the SH2 domain prevent the binding and phosphorylation of p62 which in turn binds to GAP, both shown here.

Kinase inactive



Kinase active



3.122 SH2 domains of linker/adaptor molecules

Although the SH2 domains were initially discovered associated with cytoplasmic PYKs, there now over 10 cytoplasmic proteins that contain SH2 and/or SH3 domains (figure 7). The SH2 domain containing proteins can be grouped into two categories; those which contain an associated catalytic activity such as src, and those that are found in proteins with no obvious catalytic activity such as the 85 kDa subunit of PI3K.

Indeed, the 85 kDa subunit of PI3K is the prototype adaptor molecule which has been shown to link activated growth factor receptors, such as the PDGF and CSF-1 receptors, to the 110 kDa catalytic subunit of PI3 kinase. Tyrosine phosphorylation of the activated growth factor receptor acts as a switch to allow binding of PI3K, and more importantly allowing its translocation to the plasma membrane where its substrates are located (Kashishian *et al.*1992; Escobedo *et al.*1991) (figure 9).

Recently, the Grb2 adaptor (sem5 in Caenorhabditis elegans, drk in *Drosophila*) was found to function similarly by linking the activated receptors for EGF and PDGF to the ras guanine nucleotide exchange factor, mSos1 (Son of sevenless in *Drosophila*) (Buday and Downward, 1993; Egan *et al.*1993; Olivier *et al.*1993). In this scheme, receptor activation leads to autophosphorylation and creates a binding site for Grb2. In turn Grb2 associates with mSos1 and recruits it to the plasma membrane where Ras activation is thought to occur (figure 9). The interaction of Grb2 and Sos1 has been characterized and involves the SH3 domain of Grb2 and a proline-rich region in Sos1. Interestingly, Sos1 activation of Ras can occur independently of the Grb2-Sos1 interaction

and moreover this interaction can occur in the absence of receptor activation (Buday and Downward, 1993). It is therefore believed that Ras activation is mediated by the recruitment of the complex to the plasma membrane which increases the local concentration of the Ras activator.

Taken together these results have brought forth a model whereby the adaptor-type SH2 domains that are found in SHC, Vav, Nck and Crk (figure 7) would play a similar role in linking activated growth factor receptors and possibly cytoplasmic kinases to downstream target proteins.

3.123 SH2 domains and growth factor receptors

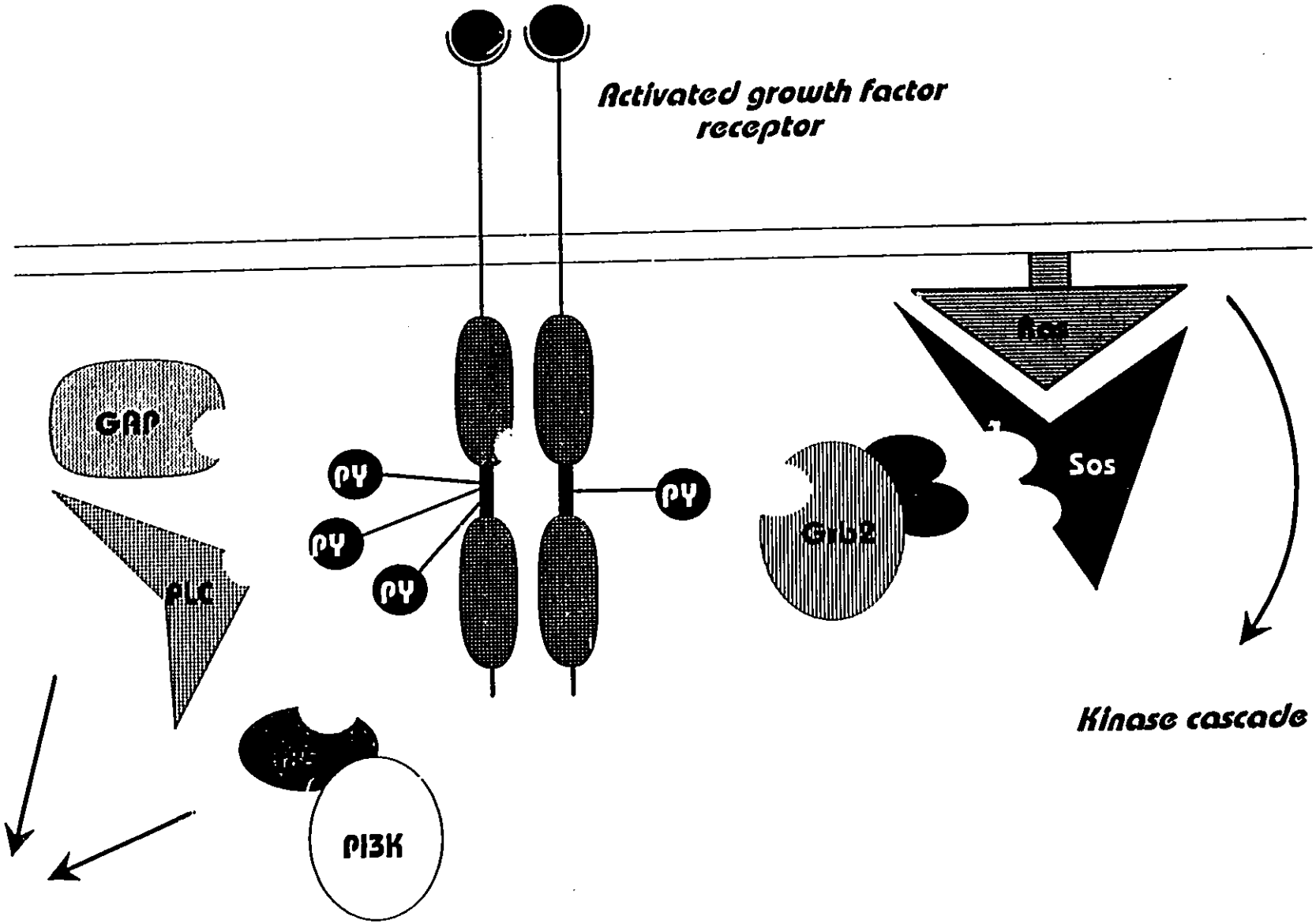
As discussed in earlier sections, growth factor activation of receptor kinases leads to autophosphorylation and the subsequent recruitment of secondary signal transducing molecules to the plasma membrane. These signalling molecules include PLCγ, PI3K and Ras-GAP and effect phosphatidylinositol metabolism and Ras activity respectively (figure 9). The interaction of effector molecules with activated growth factor receptors has been well characterized and involves the binding of effector SH2 domains to tyrosine phosphorylated sites present in the catalytic domain or kinase insert region of the activated receptors. Although the effector molecules are common to different signalling pathways, the SH2 binding specificity dictates to which receptor they will bind such that the PDGFr can bind all three, the EGFr, GAP and PLCγ and the CSF-1r, PI3K (Koch *et al.*1991).

Figure 9

Interaction of growth factor receptor with cytoplasmic SH2 adaptor molecules.

Activated growth factor receptors autophosphorylate on tyrosine residues which creates high affinity binding sites for secondary signal transducing molecules such as Ras-GAP, PLC-γ and PI3K.

The complete pathway from growth factor receptor to Ras has recently been elucidated. Receptor autophosphorylation creates a binding site for the SH2 domain of the Grb2 adaptor molecule. In turn, the SH3 domain of the Grb2 protein can bind a proline-rich sequence present in the Ras guanine nucleotide exchange factor, Sos. Since Ras can directly interact with the Raf-1 kinase (see figure 5), the entire pathway from receptor PYK to MAPK activation has now been described.


Activated growth factor receptor
GAP
PLC
PY
PY
PY
PY
PI3K
Grb2
Sos
Ras
Kinase cascade

The ability of activated growth factor receptors to recruit secondary signalling molecules to the plasma membrane may serve two purposes. The first, as discussed previously, may be to bring the effector molecules in close proximity to their substrates in the membrane, be it phospholipids for PI3K and PLCγ, or p21Ras for GAP. Alternatively, the translocation to the plasma membrane may allow the effector molecules to be regulated via the tyrosine phosphorylating activity of the growth factor receptors. Indeed the catalytic activity of PLCγ has been shown to be enhanced by tyrosine phosphorylation (Kim *et al.*1991). Alternatively, in v-src transformed cells, tyrosine phosphorylation of GAP induces the protein to associate with two other tyrosine phosphorylated proteins p62 and p190. The binding of GAP to p190 inhibits its Ras GTPase activity which ultimately results in activation of Ras (Moran *et al.*1991) and promotion of the transformed phenotype.

3.2 SH3 domains

The SH3 domains are short regions of approximately 50 amino acids that were first recognized to be present in the cytoplasmic protein tyrosine kinases. Like their SH2 counterparts, SH3 domains are evolutionarily conserved and have now been identified in several non-kinase cytoplasmic proteins (figure 7). However, the evolutionary conservation is distinct for both functional domains, since the SH3 domains are present in yeast which lack SH2 domains as well as cytoplasmic src-related protein tyrosine kinases (Pawson and Gish, 1992). These observations suggest that the SH3 domains

have evolved independently of tyrosine kinases and were acquired in metazoan organisms by SH2 containing proteins to couple tyrosine kinase signalling pathways to SH3-regulated pathways.

3.21 Recognition motif of SH3 domains

It is only recently that the recognition motifs of SH3 domains have been identified. The characterization of the Grb2-Sos1 interaction *in vitro* has enabled investigators to narrow down the binding of the Grb2 SH3 domain to a proline-rich sequence in the Sos1 Ras activator (Cicchetti *et al.*1992). Although a proline-rich region is necessary, the specific sequence required for binding has not been determined. Despite the lack of sequence specificity, the SH3 domains display differential binding affinities for the proline rich regions. An expression cloning strategy has enabled Ren *et al.* to identify proteins that bind to the SH3 domain of c-abl (Ren *et al.*1993). The proteins identified (3BP1 and 3BP2) were found to bind with higher affinity to the abl SH3 domain than to the SH3 domains of Src and Grb2, and were unable to bind to the SH3 domains of Nck and N-Src. The 3BP1 cDNA displayed high homology to GTPase-activating proteins of the Rho/Rac family, while the 3BP2 cDNA was homologous to the multispanning muscarinic acetylcholine transmembrane receptor.

3.22 Function of SH3 domains

The function of SH3 domains remains unclear, however, like their SH2 counterparts, they appear to be involved in protein-protein interactions. The presence of SH3 domains in proteins of the cytoskeleton suggested that these domains may play a role in regulating cytoskeletal architecture. Consistent with these observations, SH3 domains are also found in proteins involved in the regulation of small ras-like guanine nucleotide binding (G) proteins which in turn are involved in regulating the cytoskeleton. Indeed the small G protein family members Rho and Rac have been shown to be involved in membrane ruffling and the formation of actin stress fibers and focal contacts (Ridley *et al.*1992; Ridley and Hall, 1992).

In budding and fission yeast, SH3 domain containing proteins are associated with processes such as cell fusion (FUS1), budding (BEM1 and ABP1) and Ras guanine nucleotide exchange (CDC25) (Musacchio *et al.*1992). Consistent with a role in cytoskeletal organization, these cellular processes require cell and membrane polarization as well as bud formation. In budding yeast the ABP1 gene product is an actin-binding protein exclusively located to the bud structure and is required for the proper location of the bud site (Drubin *et al.*1988). In addition, genetic studies have shown that the BEM1 gene product interacts with the Bud5 gene product which is a guanine nucleotide exchange factor related to the CDC25 gene product (Chant *et al.*1991). These protein products are also involved in proper bud site assembly. Therefore in yeast, bud formation requires at least two SH3 containing proteins that affect membrane

organization either directly through binding to actin or alternatively, through binding to proteins involved in the control of ras-like G proteins.

In mammalian cells, the link between SH3 domains, cytoskeletal architecture and G protein regulation has also been shown. Cells transformed by the v-src oncogene usually display a fusiform morphology which arises as a result of a collapse in the cytoskeletal architecture. In normal cells, a p110 protein is only weakly associated with actin filaments whereas in src transformed cells, a substantial fraction of the p110 protein is translocated to the plasma membrane via the SH3 domain of v-src. Mutations within the SH3 domain of the v-src oncogene that prevent binding to p110, also fail to completely depolarize the actin filaments (Koch *et al.*1991). Recently, direct evidence linking SH3 domains and regulators of G proteins has been documented. Gout *et al.* have shown that the GTPase dynamin binds and is activated by a subset of SH3 domains (Gout *et al.*1993). The dynamin protein was originally characterized from bovine brain as a microtubule binding protein. Biochemical analysis has suggested that the protein functions as a motor protein for microtubules (Shpetner and Vallee, 1989). The work of Gout *et al.* therefore suggests that SH3 domains may have a direct effect on cytoskeletal architecture by binding to and activating the GTPase activity of dynamin (Gout *et al.*1993).

These results suggest that in higher eukaryotes, in the context of SH2 domains, the SH3 domains may link tyrosine kinase signalling pathways to those controlled by G proteins.

THESIS WORK

As mentioned previously, the main objective of this work was to identify novel protein tyrosine kinases involved in the control of growth and differentiation. This search led to the identification of a novel family of protein kinases, now referred to as dual specificity kinases.

The first section will describe the cloning and characterization of both protein tyrosine kinases and dual specificity kinases from embryonal carcinoma cells. The remaining sections will focus on the characterization of the novel Esk dual specificity kinase.

The second section will describe the cloning of the transmembrane (Esk-1) and cytoplasmic (Esk-2) isoforms of the Esk kinase. This section will also describe the initial characterization of the dual specificity of this kinase in a bacterial system.

The third section will deal with the expression of the Esk-2 kinase in mammalian cells as well as its interaction with the SH2 and SH3 domains of the 85 kDa subunit of the phosphatidylinositol 3-kinase.

Finally the characterization of the different Esk isoforms will be described in the last section. The mechanism by which both isoforms are generated as well as their expression pattern in mammalian cells will be illustrated.

CHAPTER 1

Cloning of novel kinases from embryonic stem cells

INTRODUCTION

The pivotal role that protein tyrosine kinases (PYKs) play in the control of cell growth and differentiation was only fully appreciated when it became apparent that several oncogenes transduced by retroviral insertion encoded protein tyrosine kinases. Indeed it was not till the late 1970's that the transforming protein of the Rous sarcoma virus was identified as being the cytoplasmic PYK, $pp60^{v\text{-}src}$ (Collett and Erickson, 1978; Hunter and Sefton, 1980). Since then, protein tyrosine kinases from both the transmembrane growth factor receptor and cytoplasmic subfamilies have been shown to be involved in all aspects of cell cycle regulation.

Several groups have elegantly shown that mutations, rearrangements and activation of members of the protein tyrosine kinase family can lead to abnormal growth and differentiation schemes, and often to tumorigenesis. The most striking example comes from the study of patients with chronic myelogenous leukaemia (CML) and acute lymphocytic leukaemia (ALL). It has been shown that rearrangements leading to the juxtaposition of chromosomes 22 and 9 in the human genome disrupts the normal coding sequences of the breakpoint cluster region (Bcr) and c-Abelson genes respectively (Hermans *et al.*1987). This rearrangement gives rise to a fusion product, bcr-abl with activated protein tyrosine kinase activity which is responsible for the malignant phenotype of this disease. More recently, mutations within the c-Kit receptor protein tyrosine kinase have been shown to be responsible for the White spotting mutant mouse (W). Mutations of the gene encoding this receptor not only lead to coat color abnormalities

(failure of melanocytes to migrate along the neural crest), but also give rise to severe blood anaemia (macrocytic) and sterility (Tan *et al.*1990). These results suggest that the c-Kit receptor PYK may be involved in controlling early events in embryogenesis and consequently affecting the fate of several differentiated cell types.

We have been interested in identifying novel protein tyrosine kinase genes involved in controlling cell growth and differentiation by making use of embryonal carcinoma (EC) cells as an *in vitro* model system. EC cells are pluripotent or multipotent stem cell lines derived from murine teratocarcinomas (Martin, 1981; Evans and Kaufman, 1981). When cells of a blastocyst are transferred to an extrauterine site, tumors form (teratocarcinomas) that comprise both stem cells and differentiated cells of multiple lineages. When reintegrated into a blastocyst, the stem cell population of these tumors can, in some cases, reconstitute all cell lineages and give rise to a normal mouse. Alternatively, the stem cells can be cultured *in vitro* and give rise to pluripotent (P19), multipotent or nullipotent (F9) embryonal carcinoma cell lines. P19 EC cells, first described by McBurney and Rogers (McBurney and Rogers, 1982), are a pluripotent stem cell line that can be induced to differentiate into cells of the mesodermal and neuroectodermal lineages (McBurney *et al.*1982). In the presence of retinoic acid (RA), P19 cells will differentiate into neurons, astroglia and even oligodendrocytes; alternatively, in the presence of dimethylsulfoxide (DMSO), the cells can differentiate into both skeletal and smooth cardiac muscle cells. Because EC cells are immunologically, biologically and genetically related to cells of the early mouse embryo,

they are a good model system for studying the events that regulate early embryonic development.

MATERIALS AND METHODS

Isolation of cDNA clones

A cDNA library was constructed in λgt11 from P19 embryonal carcinoma (EC) poly (A^+) RNA. First and second strand cDNAs were prepared from P19 EC cell poly (A^+) RNA with the cDNA Synthesis System Plus Kit (Amersham), and subsequently size selected for products larger than 1Kb by agarose gel electrophoresis (Howell *et al.* 1991).

The expression library was screened for active protein tyrosine kinases using an anti-phosphotyrosine antibody (PY 20 monoclonal antibody from ICN Biochemicals). Essentially, $5X10^5$ phage plaques were screened by infecting the bacterial strain Y1090 with recombinant bacteriophage, and incubating the cells at 42°C for 4 to 6 hours. The plates were then overlayed with filters presoaked in 10mM isopropyl-β-D thiogalactopyranoside (IPTG) and incubated for an additional 6 hours at 37°C. Positive clones were identified by probing the filters with the PY20 anti-phosphotyrosine monoclonal antibody (ICN Biochemicals), and an alkaline phosphatase conjugated secondary anti-mouse antibody (Gibco-BRL). The initial screen revealed 30 positive plaques which were cross-hybridized to identify unique clones.

Sib selection of cDNA clones isolated from P19 cells

Positive clones were cross-hybridized to identify distinct clones by the following method: 1 μl of phage solution from each clone was spotted onto a bacterial lawn of Y1090, allowed to adsorb for 20 min. at room temperature, and incubated for 8-12 hours at 37°C. Nitrocellulose filters were overlayed for 4 min., denatured in 1.5M NaCl, 0.5M NaOH for 2 min, neutralized in 1.5M NaCl, 0.5M Tris-HCl, pH8.0 for 5 min., and rinsed in 0.2M Tris-HCl, pH 7.5, 2X SSC for 30 sec. Filters were baked at 80°C under vacuum for 2 hours. The filters were probed with random-primed probes derived from the phage inserts. Prehybridizations and hybridizations were carried out with 6X SSC, 5X Denhart's solution (1% [wt/vol] Ficoll, 1% [wt/vol] polyvinylpyrrolidone, 1% [wt/vol] bovine serum albumin [BSA; Fraction V), 0.5% Sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA at 0.1mg/ml at 65°C for 6 hours to overnight. The filters were washed with 0.1X SSC and 0.1% SDS at room temp. for 20 min. The filters were then autoradiographed by exposure to Kodak XAR-5 x-ray film. The 30 clones were therefore reduced to 7 individual kinase clones.

Sequencing of cDNA clones

Six of the unique phage clones were digested with EcoR1 and subcloned into the EcoR1 site of PTZ19R (Pharmacia). To determine the identity of the clones, sequencing reactions were carried out using the universal and M13 reverse sequencing primers (Pharmacia). Single stranded DNA was generated from the plasmids, hybridized to the

primers and sequenced using the dideoxy chain termination method (Sanger *et al.*1977). The sequence obtained from each end of the clones was compared to the GenBank data base.

RNA isolation and Northern blot analysis

Total RNA was prepared from cells or tissues following the method of Auffray and Rougeon (Auffray and Rougeon, 1980). Poly A^+ RNA was selected by passage of total RNA through oligo(dT)-cellulose columns as described by Jacobson (Jacobson, 1987). Typically, aliquots of 5μg of poly A^+ RNA were electrophoresed in a 1% agarose gel containing 19% formaldehyde, 40mM MOPS, 10mM sodium acetate and 1mM EDTA, transferred to a Hybond N membrane (Amersham) and UV cross linked for 2 minutes. Filters were prehybridized at 42°C for 12-16 hrs in 50% formamide, 6X SSC, 5X Denhart's solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA (fraction V)], 5% dextran sulfate, 0.1% SDS and 0.5 mg/ml denatured salmon sperm DNA. Hybridizations were performed using random-primed ^{32}P-labelled cDNA inserts at $2X10^6$ cpm/ml for 12-16 hrs at 42°C. Filters were washed in 0.1% SDS, 0.1X SSC at room temperature and 42°C.

RESULTS AND DISCUSSION

P19 EC cells contain a number of novel dual specificity kinases.

In order to determine the functional role of protein tyrosine kinases in the control of cell growth and differentiation, an expression library derived from P19 EC cell poly (A^+) RNA was generated (Howell *et al.*1991). The library was screened for active protein tyrosine kinases using an anti-phosphotyrosine antibody (PY20). This strategy is feasible for two reasons, 1) protein tyrosine kinases are non-existent in bacteria and 2) these enzymes can autophosphorylate on tyrosine residues and are therefore immunoreactive with anti-phosphotyrosine antibodies. An initial screen of $5X10^5$ recombinants revealed 30 positives plaques which upon further characterization (sib selection) represented 7 different kinase clones (these clones are listed in table 1). Although four of the clones had been previously identified and were members of the tyrosine kinase family [Lyn, Fyn, Fer, Unk (Tyro3)], the three remaining clones (STY, Esk, Nek) were all novel kinases. Sequence analysis of the three novel clones revealed that they were members of a new family of kinases, now referred to as Dual Specificity Kinases (DSKs) (Lindberg *et al.*1992). This new family of enzymes, unlike their protein serine/threonine and protein tyrosine counterparts, could phosphorylate all three hydroxy amino acids.

The significance of numerous DSKs in P19 cells is unclear, however since our initial finding, several groups have identified a number of dual specificity kinases including homologs of the STY and Esk kinases from murine and human sources (Ben-David *et*

Table 1

Kinases identified through anti-phosphotyrosine screen of P19 cells.

The table lists the kinases isolated from P19 cells using the expression cloning strategy. The names of the kinases are listed along with the number of each clone isolated, and the family to which they belong [protein tyrosine kinase (PYK) versus dual specificity kinase (DSK)]. The reference for the initial cloning of each kinase is indicated in the last column.

NAME	CLONES	KINASE FAMILY	REFERENCE
LYN	3/11/26/27	PYK	Yamanashi et al. (1987) MCB 7:237-243
FYN	17/20	PYK	Kawakami et al. (1986) MCB 6:4195-4201
FER	16/18/24	PYK	Pawson et al. (1989) MCB 9:5722-5725
UNK/TYRO-3	4	PYK	Lai and Lemke (1991) Neuron 6:691-704
STY	2/9/13/22/23	DSK	Howell et al. (1991) MCB 11:568-472
ESK-1 ESK-2	14 7	DSK DSK	Douville et al. (1992) MCB 12:2681-2689
NEK	15/21	DSK	Letwin et al. (1992) EMBO 11:3521-3531

*al.*1991; Lindberg *et al.*1993; Mills *et al.*1992; Douville *et al.*1991). The characterization of the Esk DSK will be the subject of this thesis and will be described in chapters 2, 3 and 4. The four remaining clones (Lyn, Fyn, Nek and Unk) were further characterized at the level of expression by Northern blotting and are described here in more detail.

The Fyn^B cytoplasmic PYK is expressed in P19 cells.

The Fyn kinase was first described as a novel human src-like kinase with oncogenic potential. The cDNA, isolated from a normal human fibroblast cell line, encoded a protein of 537 amino acids with protein tyrosine kinase activity, and was termed p59^{fyn} (Kawakami *et al.*1986). Cloning of the murine homolog revealed that two isoforms of the Fyn kinase existed and arose through alternative splicing of the mutually exclusive exons, 7A and 7B (Cooke and Perlmutter, 1989). Transcripts containing exon 7B were unique to the T lymphocyte population (p59^{fyn_T}), while transcripts containing exon 7A were found in other tissues, particularly brain (p59^{fyn_B}). The Fyn^t isoform is fairly well characterized and is an integral part of the signal transduction pathway involving the T cell receptor (TCR) in thymocytes. In fact, the generation of Fyn^t null mice reveals that this kinase is not needed for normal T cell development but rather for proper signalling through the TCR (Appleby *et al.*1992). In contrast, the role of Fyn^b is less well characterized but has been implicated in long-term potentiation and spatial learning (Grant *et al.*1992). Recently, the Fyn^b isoform was found associated with the

large form of the cell adhesion molecule myelin associated glycoprotein (L-MAG) ((Umemori *et al.*1993) and M.L. Jaramillo and J.C. Bell, unpublished observations). The L-MAG cell adhesion molecule is important for the proper myelination of nerve cells in the developing brain (Owens and Bunge, 1991). The association of this cell adhesion molecule with Fyn^b suggests a role for this PYK in mediating MAG induced myelination signals.

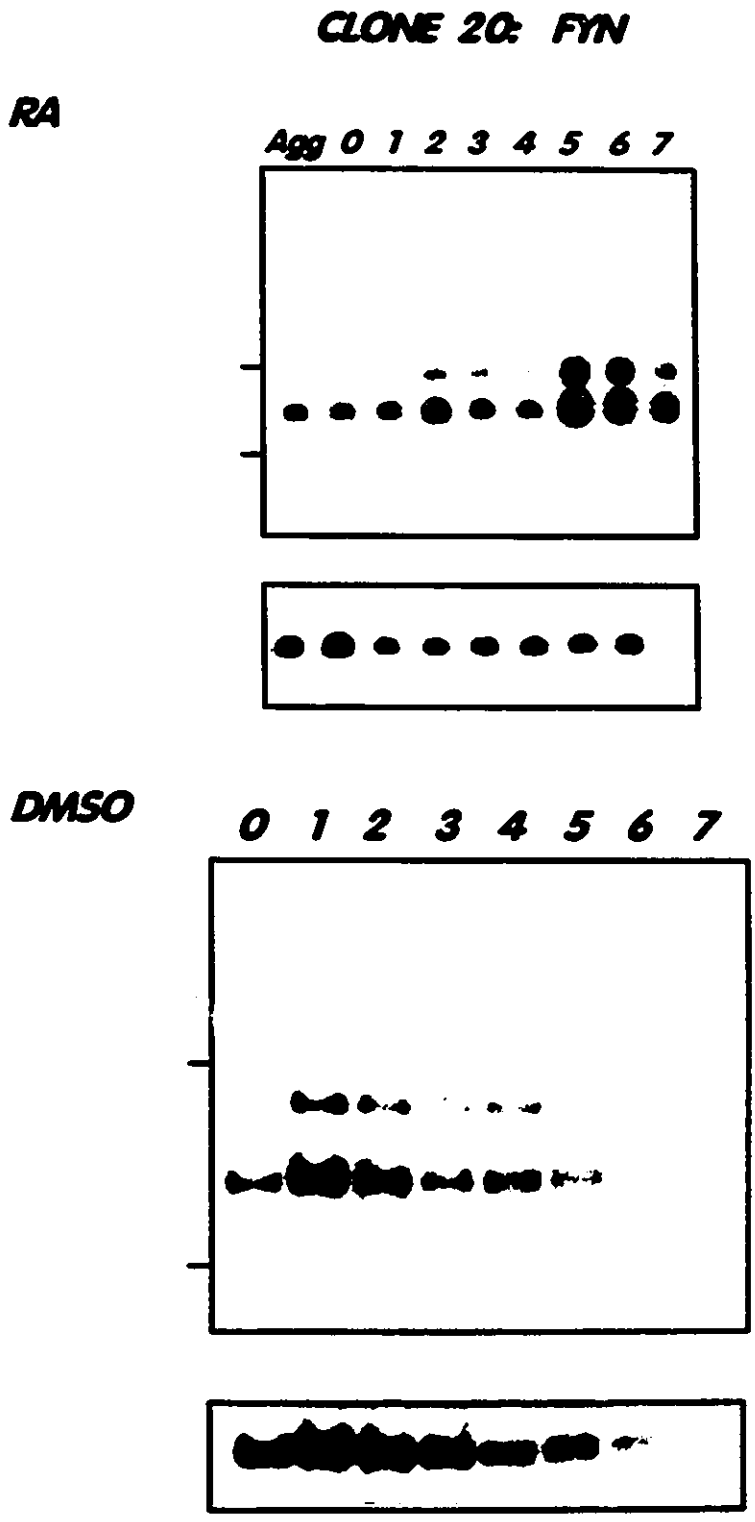
Two distinct Fyn encoded mRNAs can be detected on Northern blots (2.8 and 3.5 Kb) that differ in their 5' untranslated regions and may contain sequences from either exon 7A or 7B (Cooke and Perlmutter, 1989). Northern blots of P19 cells induced to differentiate into cells of the neural or muscle lineages (figure 1) were hybridized with our Fyn cDNA and revealed transcript sizes corresponding to those previously described (Appleby *et al.*1992; Cooke and Perlmutter, 1989). Interestingly, the levels of Fyn transcript were regulated during differentiation; increasing with RA induced differentiation and decreasing with DMSO treatment. Taken together these results suggest that the brain specific isoform of the Fyn kinase was cloned from P19 cells. Since the Fyn kinases are not critical to early embryonic development, as demonstrated by the generation of adult Fyn null mice (Appleby *et al.*1992), the role of Fyn^b in neural differentiation could be addressed in P19 cells. Defining the expression pattern of the Fyn kinase in neural differentiated derivatives could lead to the identification of membrane proteins other than MAG that may associate with Fyn^b.

Figure 1

Northern blot analysis of Fyn transcripts in differentiating P19 cells.

Five micrograms of poly(A+) RNA was isolated from P19 cells induced to differentiate with retinoic acid (RA) or dimethylsulfoxide (DMSO). The numbers above the lanes refer to the days of differentiation; lane "0" refers to stem cells, and lane "Agg" refers to P19 cells aggregated in the absence of an inducing drug. The blots were hybridized with a random-primed probe derived from a 2.3 Kb cDNA of the Fyn kinase.

The mRNA levels were standardized by probing the blots with a cDNA corresponding to the phosphoglycerate kinase-1 gene (PGK-1). Positions of 18S and 28S ribosomal RNAs were determined by UV visualization of an acridine orange-stained marker lane.


CLONE 20: FYN
RA
Agg 0 1 2 3 4 5 6 7
DMSO
0 1 2 3 4 5 6 7

The Lyn kinase is constitutively expressed during P19 differentiation.

Similar to the Fyn tyrosine kinase, the Lyn kinase was identified in an attempt to identify novel src-related kinases. Although initially cloned from a human placenta cDNA library, the Lyn transcript was found to be most abundant in human fetal liver (Yamanashi *et al.*1987). The Lyn kinase is associated with the membrane-bound immunoglobulin M (IgM) on the surface of B cells and may be involved in the regulation of the immune response (Yamanashi *et al.*1991). Recently, splicing variants have also been described for the Lyn kinase in mouse tissues and cell lines. Stanley *et al.* have shown that alternative splicing is probably responsible for the insertion of a 21 amino acid sequence within the amino terminal portion of the kinase (Stanley *et al.*1991). This group has also reported that the mRNAs encoding the different Lyn isoforms are thought to be present in all cells that express the Lyn gene. Although the function of this new isoform is still unknown, an insertion in the amino terminal portion of this kinase could regulate the association of the Lyn kinase with different types of membrane proteins.

The specific isoform of the Lyn kinase isolated from P19 cells has not been determined, however the transcript(s) were found to be constitutively expressed throughout differentiation (figure 2). In adult tissues the Lyn transcript was ubiquitously expressed with possibly higher levels in lung and kidney (figure 3). The functional significance of the Lyn kinase in P19 cells is unknown. Given that most cytoplasmic PYKs described to date are activated through their interaction with cell surface membrane proteins, the mechanism of activation of the Lyn kinase in P19 cells could lead to the

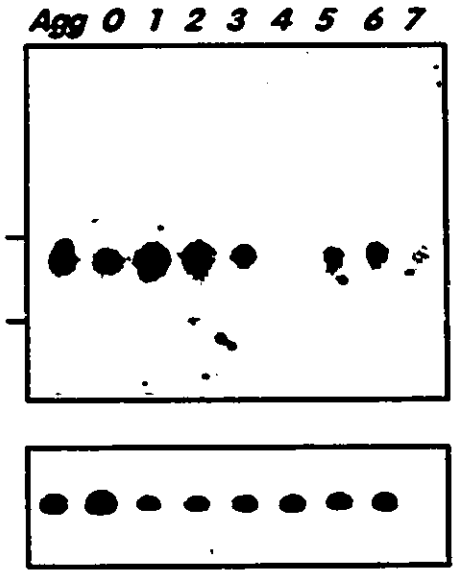
Figure 2

Northern blot analysis of Lyn transcripts in differentiating P19 cells.

As in figure 1, five micrograms of poly(A+) RNA was isolated from RA and DMSO treated P19 cells. The blots were hybridized with a random-primed probe derived from a 1.7 Kb cDNA of the Lyn kinase isolated from P19 cells.
The blots were standardized with the PGK-1 probe.

CLONE 26: LYN

RA



DMSO

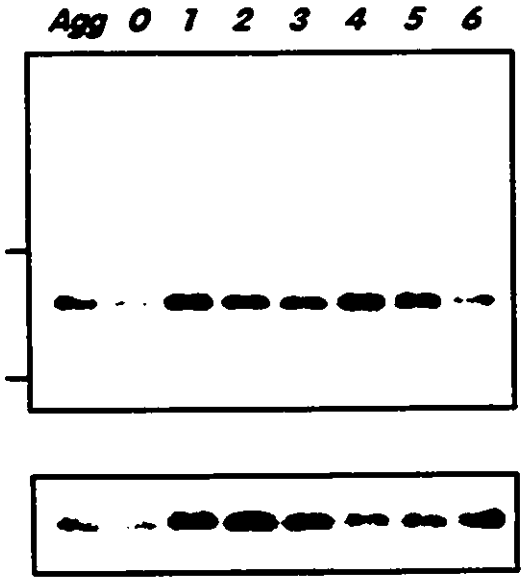
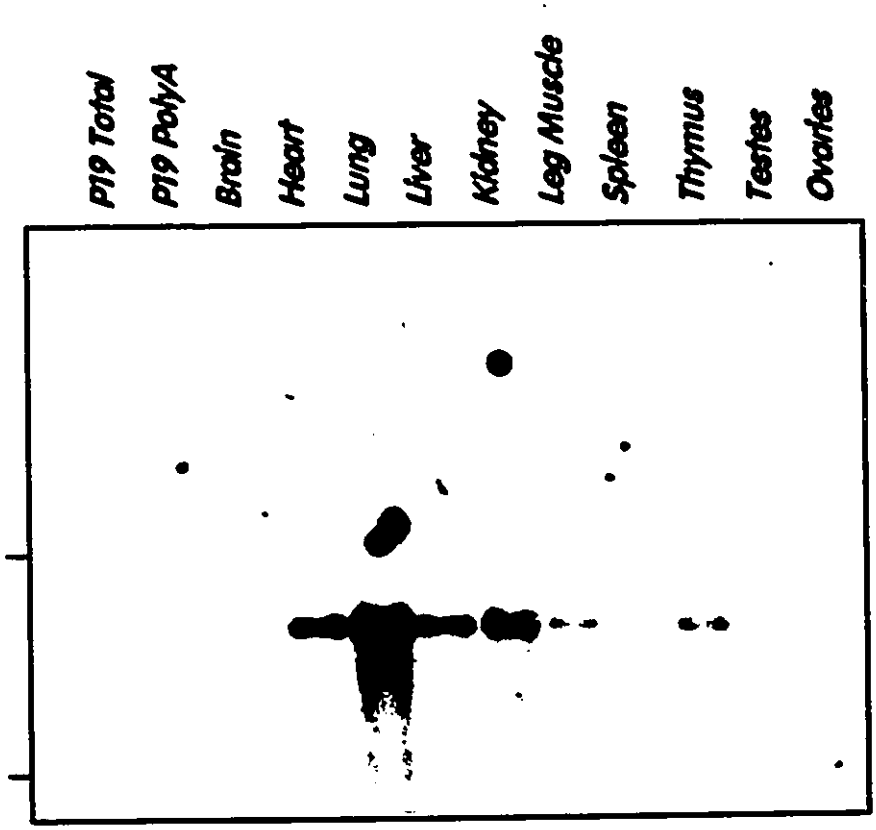


Figure 3

Northern blot analysis of Lyn transcripts in adult mouse tissues.

Five micrograms of poly(A+) RNA was isolated from the tissues indicated above the lanes. The blot was hybridized with a random-primed probe derived from the Lyn cDNA.
The blot was *not* standardized for even loading.

Clone 26: LYN


P19 Total
P19 PolyA
Brain
Heart
Lung
Liver
Kidney
Leg Muscle
Spleen
Thymus
Testes
Ovaries

identification new cell surface membrane proteins.

The dual specificity kinase Nek is expressed in P19 cells.

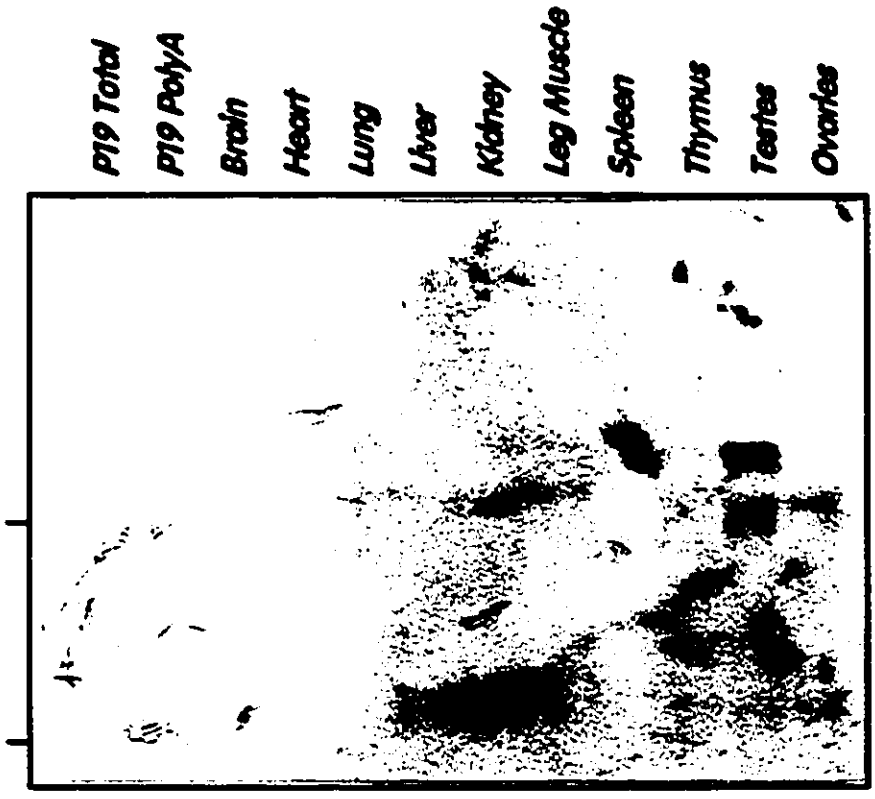
The Nek1 kinase was first identified by Tony Pawson's group in a murine erythroleukaemia cell line using the same type of cloning strategy as ours (Letwin *et al.*1992). Indeed this group also identified the STY and Esk kinases in the same screen (Ben-David *et al.*1991; Douville *et al.*1991). Despite the intrinsic tyrosine kinase activity of the Nek1 kinase, sequence analysis revealed that it was most closely related to members of the serine/threonine family of protein kinases, a hallmark of DSKs. Indeed the Nek1 kinase was found to be most homologous (42% identity in the catalytic domain) with the *nimA* kinase from the fungus *Aspergillus nidulans*. The *nimA* kinase is involved, independently of cdc2, in controlling the initiation of mitosis, spindle formation and nuclear envelope breakdown in the fungus (Osmani *et al.*1991). Letwin *et al.* have shown that the highest levels of expression by Northern blot analysis were found in testes and ovaries which displayed transcripts of 6.5 and 4.4 Kb (Letwin *et al.*1992). Consistent with these observations, the Nek1 cDNA isolated from our P19 library also hybridized to testes and ovary RNAs on Northern blots (figure 4). In contrast to the results of Letwin *et al.* where both transcripts were expressed at equal levels in testes, the 6.5 Kb transcript was more abundant in our poly (A^+) preparation of this tissue. Letwin *et al.* have also shown by *in situ* hybridization that the Nek1 transcript was most abundant in oocytes at the secondary follicular stage and persisted until ovulation. In

Figure 4

Northern blot analysis of the NEK dual specificity kinase transcripts in adult mouse tissues.

The same blot as in figure 3 was hybridized with a random-primed probe derived from the Nek1 cDNA (1.8 Kb) isolated from P19 cells.

Clone 15: NEK


P19 Total
P19 PolyA
Brain
Heart
Lung
Liver
Kidney
Leg Muscle
Spleen
Thymus
Testes
Ovaries

testes, the transcripts were highly expressed during spermatogenesis, in germ cells actively involved in meiosis, and decreased dramatically during the transition from round to elongated spermatids. Taken together these results suggested that the Nek1 kinase may play a role in the control of meiosis rather than mitosis (Letwin *et al.*1992). The expression pattern of the Nek1 transcript observed during RA and DMSO induced differentiation is intriguing and can not be explained in terms of mitotic index. In P19 cells, only one transcript can be detected (6.5Kb) which rapidly decreases during DMSO induced differentiation, but remains constant with RA treatment (figure 5). These results are consistent with the observations of Letwin et al. regarding the putative role of the Nek1 kinase in meiosis rather than mitosis, since the mitotic index of DMSO treated cells is higher than that of RA treated cells. Interestingly, the Nek1 transcript is virtually absent in adult brain (Letwin *et al.*1992), the constitutive expression of the Nek1 transcript during P19 induced neuronal differentiation suggests a novel role for this kinase in early brain development.

P19 cells express a novel receptor kinase related to Tyro-3.

The Tyro-3 partial cDNA (PCR product) was initially identified by degenerate oligonucleotide PCR amplification of cDNAs derived from the rat sciatic nerve and brain (Lai and Lemke, 1991). The oligonucleotides were engineered to specifically amplify receptor-type protein tyrosine kinases by virtue of the conserved sequences found in subdomain VI (HRDLAAR vs HRDLRAA for cytoplasmic kinases). PCR products

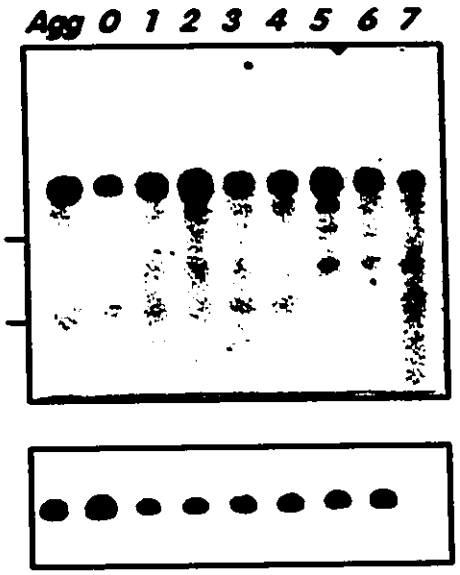
Figure 5

Northern blot analysis of Nek1 transcripts in differentiating P19 cells.

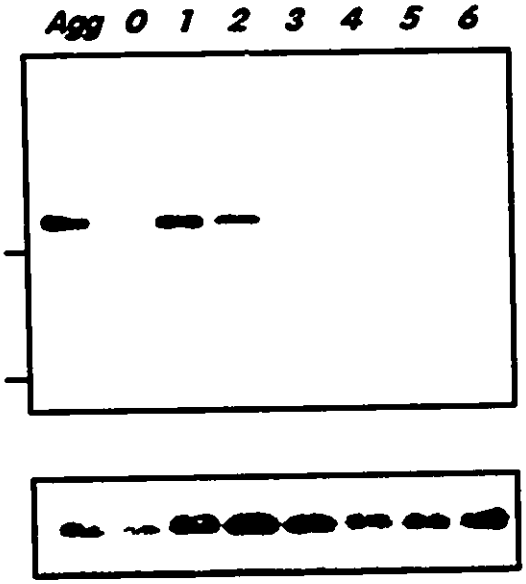
Five micrograms of poly(A+) RNA was isolated from RA and DMSO treated P19 cells. The same blots as in figure 2 were hybridized with a randon-primed Nek1 cDNA probe and standardized with the PGK-1 cDNA probe.

CLONE 15: NEK

RA



DMSO



corresponding to regions amplified between subdomains VI and VIII were subgrouped according to their homology with known kinases. The Tyro-3 product was most closely related to other Tyro PCR products than to known protein tyrosine kinases despite a weak structural homology to the insulin growth factor receptor related kinases (the sequence $Y(N)_3YY$ near subdomain VII) (Lai and Lemke, 1991).

Preliminary sequence data from the Unk cDNA (Unk stands for **Unk**nown for lack of a better name) revealed that it had 100% identity with the Tyro-3 PCR product. The Unk cDNA sequence was found to contain a conserved kinase catalytic domain and sequences reminiscent of a transmembrane domain consistent with Unk being a receptor protein tyrosine kinase (Jennifer Ingram, Maria Jaramillo and John Bell, unpublished observations). The expression pattern of the Unk transcripts during RA induced P19 differentiation (figure 6) was consistent with the results reported by Lai and Lemke (Lai and Lemke, 1991). Unk was found to increase during P19 induced neuronal differentiation as did the Tyro-3 transcript in rat brain from embryonic days 12 to 19. Also consistent was the expression of Unk in adult tissues, being primarily expressed in brain and in testes (figure 7). The catalytic domain of the Unk cDNA was also found to share homology with the Axl/UFO receptor PYK (see figure 2 of general introduction for structure). The Axl receptor kinase has been suggested to function as a cell adhesion molecule in certain systems by virtue of its immunoglobulin-like and fibronectin type III motifs (Faust *et al.*1992). If the Unk kinase is indeed related to this subfamily of receptor molecules, our results would indicate that we have identified a PYK from P19

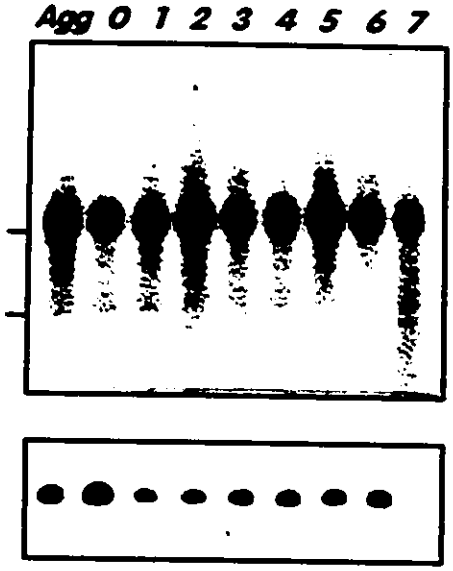
Figure 6

Northern blot analysis of Unk transcripts in differentiating P19 cells.

The same Northern blots as in figures 2 and 5 were hybridized with a random-primed probe derived from a 2.4 Kb Unk cDNA isolated from P19 cells.
The standardization was with the PGK-1 cDNA probe.

CLONE 4: UNK

RA



DMSO

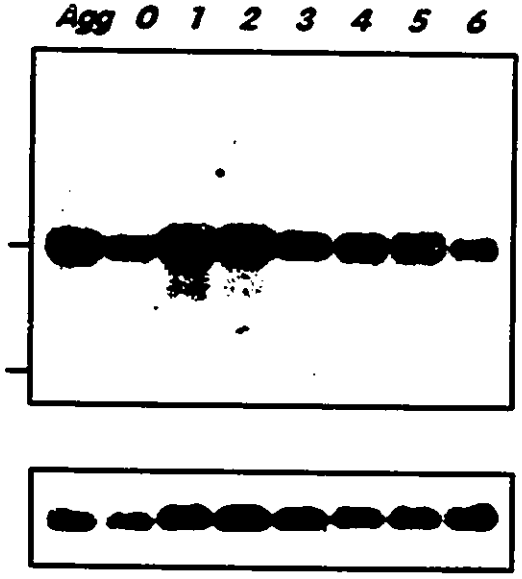
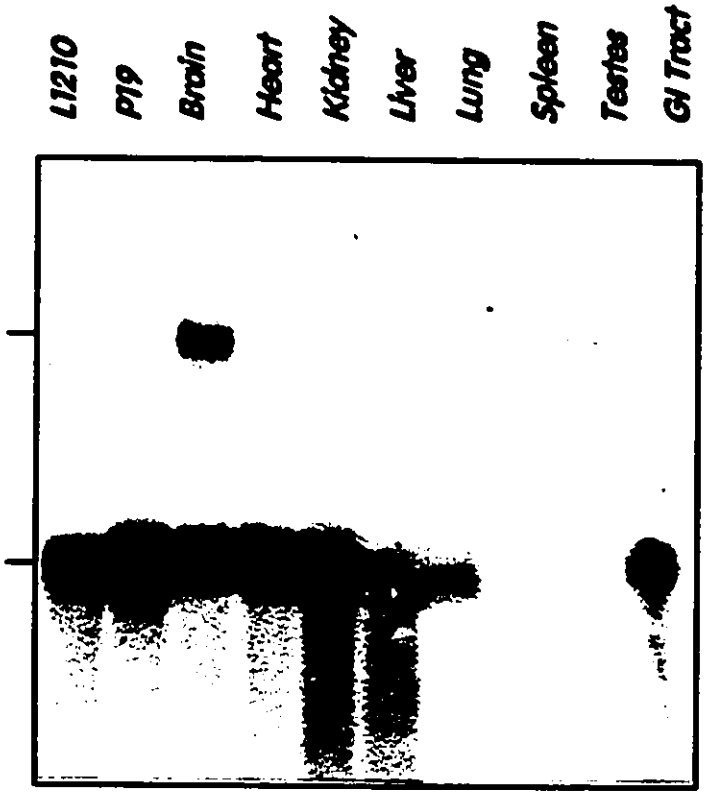


Figure 7

Northern blot analysis of Unk transcripts in mouse adult tissues.

Five micrograms of poly(A+) RNA was isolated from the tissues and cell lines indicated above the lanes. GI tract refers to the gastro-intestinal tract. The blot was hybridized with a random-primed probe derived from the Unk cDNA.
The blot was also probed simultaneously with the PGK-1 probe to standardize for mRNA levels.

Clone 4: UNK


L1210
P19
Brain
Heart
Kidney
Liver
Lung
Spleen
Testes
GI Tract

cells with potential cell adhesive properties. Full length sequencing of the Unk kinase will have to be carried out to determine the features of the extracellular domain which may hint towards identifying a ligand.

While EC cells express several cytoplasmic kinases of both the PYK and PSK families, interestingly they express very few PYK receptors. Vu *et al.* have shown that EC cells (F9 and PSA-1) express a truncated PDGF receptor transcript (4.2 Kb instead of 5.3) which is generated through the alternative usage of a cryptic promoter upstream of exon 6 in the gene (Vu *et al.*1989). The 4.2 Kb transcript is predicted to encode a truncated receptor lacking most of the extracellular domain. Expression of the transcripts is developmentally regulated in differentiating EC cells, with the truncated transcript decreasing with RA treatment while that of the full length receptor increases (Vu *et al.*1989). The functional significance of these isoforms of the PDGFr in differentiating EC cells remains unclear. Similarly, the EGF receptor is not expressed in P19 stem cells but can also be induced following differentiation (Adamson, 1987). Further work will be needed to determine if the Unk receptor kinase is expressed at the protein level in P19 cells and its role in signal transduction in this cell line.

Finally, we have shown that our cloning strategy was invaluable in identifying protein tyrosine kinases in P19 cells. While several previously identified cytoplasmic protein tyrosine kinases have been recloned using this strategy, at least one novel receptor PYK has been identified that may play a role in brain development and possibly in P19 cell physiology. More importantly, this cloning strategy has allowed for the

identification of a novel class of protein kinases, dual specificity kinases, that are related in sequence to PSKs but that display both serine/threonine and tyrosine kinase activities. The functional significance of these kinases in growth and differentiation will be addressed in terms of the Esk kinase in the following chapters.

CHAPTER 2

Cloning and characterization of the Esk dual specificity kinase

INTRODUCTION

The combinatorial actions of serine, threonine and tyrosine kinases are involved in the regulation of information transfer within and between cells (Boulton *et al.*1991; Hunter, 1987). In general, a hierarchy of functions has evolved for these different kinases such that growth and differentiation factor receptors receive extracellular signals and transmit these across the plasma membrane in the form of a tyrosine phosphorylation event. Downstream of the growth factor/receptor interaction is the activation by tyrosine phosphorylation of a number of cytoplasmic and nuclear serine/threonine kinases which are thought to be secondary effectors of the primary signal. There are however clear examples of variations to this theme. Nuclear isoforms of tyrosine kinases are known to exist (Dhut *et al.*1991; Van Etten *et al.*1989) and tyrosine phosphorylation of nuclear proteins has been demonstrated (Bell *et al.*1987). The transforming growth factor β receptor appears to be the first example of a mammalian transmembrane kinase which phosphorylates serine/threonine rather than tyrosine residues (Wrana *et al.*1992).

We have been involved in the cloning of novel tyrosine kinases from embryonic and transformed cells by screening λgt11 expression libraries with antibodies to phosphotyrosine. While a number of kinases which exclusively phosphorylate tyrosine residues (Kornbluth *et al.*1988; Letwin *et al.*1988; Lhoták *et al.*1991) have been identified in this way we have also isolated cDNAs encoding serine/threonine/tyrosine kinases (Ben-David *et al.*1991; Howell *et al.*1991).

Here we report the characterization of one of these kinases, Esk, and show that it exists in two isoforms, one of which appears to be a transmembrane molecule.

MATERIALS AND METHODS

Isolation of cDNA clones

Expression libraries were constructed from P19 embryonal carcinoma cells (Howell *et al.*1991) as well as from CB7 and DP28-9 erythroleukemia cell lines (Ben-David *et al.*1991) using poly (A^+) RNA, and screened for active protein tyrosine kinases using an antiphosphotyrosine antibody. Essentially, 5 x 10^5 plaques were screened by infecting the bacterial strain Y1090 with recombinant bacteriophage and incubating at 42°C for 4-6 hrs. The plates were then overlayed with filters presoaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 6 hrs at 37°C. Positive clones were identified by probing the filters with the PY20 antiphosphotyrosine monoclonal antibody (ICN biomedicals) and an alkaline phosphatase conjugated anti-mouse secondary antibody (Gibco-BRL).

Sequencing of cDNA clones

The Esk cDNA inserts were subcloned into the EcoR1 site of the PTZ19R plasmid (Pharmacia). Nested deletions were generated using an Exo-Mung deletion kit (Stratagene). Double stranded DNA sequencing was carried out on the deletion mutants using the dideoxy chain termination method (Sanger *et al.*1977).

P19 EC cell maintenance and differentiation

P19 EC cells were cultured and differentiated as described previously (Jones-Villeneuve *et al.*1982; McBurney *et al.*1982). Essentially, P19 cells were induced to differentiate into cells of the neural or muscle lineages by aggregating the cells in bacterial dishes in the presence of 0.5 μM retinoic acid or 1% dimethylsulfoxide for 3 days, and subsequently plating the cells onto coated tissue culture dishes to form monolayers in the absence of inducing drug.

RNA isolation and Northern blot analysis

Total RNA was prepared from cells or tissues following the method of Auffray and Rougeon (Auffray and Rougeon, 1980). Poly A^+ RNA was selected by passage of total RNA through oligo(dT)-cellulose columns as described by Jacobson (Jacobson, 1987). Typically, aliquots of 5μg of poly A^+ RNA were electrophoresed in a 1% agarose gel containing 19% formaldehyde, 40mM MOPS, 10mM sodium acetate and 1mM EDTA, transferred to a Hybond N membrane (Amersham) and UV cross linked for 2 minutes. Filters were prehybridized at 42°C for 12-16 hrs in 50% formamide, 6X SSC, 5X Denhart's solution [1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA (fraction V)], 5% dextran sulfate, 0.1% SDS and 0.5 mg/ml denatured salmon sperm DNA. Hybridizations were performed using a random-primed ^{32}P-labelled 1.5 Kb fragment of the Esk-2 cDNA at $2X10^6$ cpm/ml for 12-16 hrs at 42°C. Filters were washed in 0.1% SDS, 0.1X SSC at room temperature and 42°C.

Bacterial expression of Esk cDNA

A 1.5 Kb fragment of the Esk-2 cDNA from the PvuII site (position 901) to the EcoR1 site (position 2417) was blunt ended with T4 DNA polymerase and subcloned in frame into the unique BamH1 site of the pET11b bacterial expression vector (Novagen). This places the cDNA insert under the control of the T7 promoter; expression is induced by supplying T7 RNA polymerase to the host cell. In *Escherichia coli* pLysS bacteria (Novagen), the polymerase gene is under *lac*UV5 control and induction is achieved by adding IPTG to the culture.

Typically, pLysS bacteria expressing either the sense or antisense Esk expression vectors were grown to an $O.D._{600}$ of 0.4 in M9CA minimal medium containing 100 μg/ml ampicillin. The bacteria were induced for Esk protein production by adding IPTG to a final concentration of 0.4mM and incubating for an additional 2 hrs at 37°C.

Protein and phosphoamino acid analysis

Immunoblotting

Whole cell extracts from bacteria (pLysS) induced to express Esk were resolved on SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose (Schleicher and Schuell). Membranes were blocked with 20% serum in Tris-saline (140mM NaCl, 10mM Tris pH 7.5) for 1 hr at room temperature and incubated with a 1:1000 dilution of IgG2bk antiphosphotyrosine antibody (UBI) in blocking buffer for 1 hr at room temp. Blots were washed three times in Tris-saline plus 0.05% Tween 20 and then incubated

with an alkaline phosphatase conjugated anti-mouse secondary antibody. Blots were washed and developed with Nitro Blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

In vitro kinase assay

pLysS bacteria induced to express Esk were lysed in 10mM Tris (pH 7.5), 150mM NaCl, 5mM EDTA, 1% Triton X-100, 2mM NaF, 2mM sodium pyrophosphate, 500μM ammonium vanadate, 200μg/ml phenylmethylsulfonyl fluoride (PMSF), 2μg/ml aprotinin and 5μg/ml leupeptin, and sonicated on ice three times for 20 seconds. Lysates were cleared by centrifugation and supernatants were immunoprecipitated with either PY20 (ICN biomedicals) or IgG2bk (UBI) antiphosphotyrosine antibodies. The immunoprecipitates were assayed for kinase activity in lysis buffer supplemented with 20mM $MgCl_2$ and γ^{32}P-ATP or in buffers containing either 30mM Tris pH 7.5, 25mM N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) pH 7.1 or 20mM 2[N-Morpholino] ethanesulfonic acid (Mes) pH 6.5 all supplemented with 10mM $MgCl_2$, 2mM $MnCl_2$ and 0.2mCi/ml of γ^{32}P-ATP for 30 min at 22°C. Myelin basic protein and acid denatured enolase were added as exogenous substrates in some kinase assays. The reaction products were resolved on SDS-PAGE (10% polyacrylamide) and the gels transferred to nitrocellulose membranes at 0.8 Amps for 2 hours, or 0.3 Amps overnight. For phosphoamino acid (PAA) analysis, phosphoproteins were excised from the membrane, incubated in 300μl of 0.5% PVP (polyvinylpyrrolidone) in 100mM acetic

acid at 37°C for 30 min, rinsed with water and 50 mM ammonium carbonate (pH 8.0-8.5), and trypsinized overnight at 37°C in 90μl of 50mM ammonium bicarbonate and 10μl of trypsin (10mg/ml stock in 50 mM ammonium bicarbonate). Following trypsin treatment, the samples were diluted with 500μl of water and dried in vacuo. Phosphoproteins were hydrolyzed at 110°C for 75 min. in 6N HCl, diluted in 100μl of water and dried. After two washes in water, the samples were resuspended in phosphoamino acid standards and resolved by one or two-dimensional thin layer electrophoresis.

Renaturation kinase assay

The procedure was carried out essentially as described by Ferrell and Martin (Ferrell and Martin, 1991). Whole cell extracts from induced pLysS bacteria were resolved on SDS-PAGE (10% polyacrylamide) and transferred to Immobilon P membranes (Millipore) at 50V for 90 min. Blotted proteins were denatured in 6M Guanidinium HCl, 50mM Tris, 50mM dithiothreitol (DTT), 2mM EDTA (pH 8.3) for 1 hr at room temperature. Proteins were allowed to renature at 4°C for 12-16 hrs in 100mM NaCl, 50mM Tris, 2mM DTT, 2mM EDTA, 1% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) NP-40 (pH 7.5). Blots were blocked with 5% (w/v) BSA in 30mM Tris pH 7.5 for 1 hr at room temperature. Kinase assays were performed by incubating the blots in 30mM Tris pH 7.5, 10mM $MgCl_2$, 2mM $MnCl_2$ and 50μCi/ml γ^{32}P-ATP for 30 min at room temp. Blots were washed twice in 30mM Tris pH 7.5,

once in 30mM Tris pH 7.5, 0.05% NP-40 and once in 30mM Tris pH 7.5 (each wash was for 10 minutes). For phosphoamino acid analysis, regions of the membrane were excised and acid hydrolysed in 6N HCl for 75 minutes at 110°C as described by Kamps and Sefton (Kamps *et al.*1985).

Phosphatase treatment of Esk

Whole cell extracts of induced pLysS bacteria were resolved on SDS-PAGE (10% polyacrylamide) and proteins transferred to Immobilon P or nitrocellulose membranes as described above. Blots were blocked for 1 hr with 5% (w/v) BSA in 30mM Tris pH 7.5 prior to treatment with potato acid phosphatase (PAP) (Sigma) or with the human phosphotyrosine phosphatase 1B (a glutathione S-transferase PTP1B fusion protein, generous gift of Ben Neel).

PAP treatment was an adaptation of the procedure described by Morrison et al. (Morrison *et al.*1989). Membrane strips prepared as described above were incubated with 80 μg/ml of PAP in a reaction buffer containing 20mM Tris pH 7.4, 5% glycerol, 0.05% Triton X-100, 2.5mM $MgCl_2$, aprotinin (2μg/ml) and leupeptin (5μg/ml) for 1 hr at 37°C with gentle shaking. An additional amount of PAP enzyme (80μg/ml) was added and incubated for another hour at 37°C. In some experiments 1mM ammonium vanadate was added to the buffer to inhibit phosphatase activity. The reaction was stopped by blocking the blots with 20% serum in Tris-saline for 1 hour at room temperature. The

blots were probed with an antiphosphotyrosine antibody as described above, and detected using an ^{125}I labelled anti-mouse secondary antibody.

Isolation of the GST-PTP1B fusion protein. An overnight culture of *E. coli* DH5α (pGEX-PTP1B) was diluted 1:10 (with 100ug/ml ampicillin), grown for 1 hour at 37°C and induced for another hour by adding IPTG to 1mM. Bacteria were sonicated three times for 20 seconds in lysis buffer containing 10mM Imidazole-HCl pH 7.2, 1mM EDTA, 100mM NaCl, 1mM DTT and 1% Triton X-100. Lysates were cleared by centrifugation and the supernatants were incubated with 500ul of 50% glutathione coupled beads (Pharmacia) for 30 minutes at 4°C. The GST-PTP1B fusion was eluted twice with 1 ml of 5mM reduced glutathione (Boehringer Manheim) in phosphatase buffer. Glycerol was added to a final concentration of 33% (v/v) and aliquots of the enzyme (150ul PTP1B/ml reaction buffer) were used in phosphatase experiments as described above.

Sty kinase assay and phosphatase (Gst-PTP1B) treatment. TrpE-Sty fusion protein was prepared as described by Howell et al. (Howell *et al.*1991). The Sty fusion protein was immunoprecipitated using an anti-TrpE antibody (Oncogene Science) and allowed to autophosphorylate in the presence of γ^{32}P-ATP for 30 minutes at room temperature. The reaction products were resolved on SDS-PAGE (10% polyacrylamide), transferred to immobilon P membrane and individual strips were treated with phosphatase 1B as described above.

Nucleotide sequence accession number

GenBank accession number for the murine Esk-1 cDNA sequence is M86377.

RESULTS

Identification and expression of the Esk gene product

Messenger RNA isolated from P19 murine embryonal carcinoma cells was used to prepare a λgt11 cDNA library which was then screened with antibodies to phosphotyrosine as described previously (Ben-David *et al.*1991; Howell *et al.*1991; Lhoták *et al.*1991). A 2.97 kb cDNA isolated in this way was shown by sequence analysis to encode a novel protein kinase which we have named Esk (Embryonal carcinoma Serine/threonine/tyrosine Kinase).

To investigate the expression pattern of Esk, Northern blot analysis was performed on a number of adult mouse tissues and cell lines. Esk appears as a 3.0 kb mRNA (size estimated from ribosomal RNA markers) in tissues which contain a significant subpopulation of proliferating cells (figure 1). Organs such as brain, heart, lung and kidney had low amounts of Esk while testes, bone marrow, spleen and thymus all contained higher levels of Esk mRNA. The myeloma cell line SP10 and the B cell leukaemia lines L1210 and 70Z/3 also expressed a 3.0 kb Esk mRNA. It appears therefore that Esk transcripts are expressed in a limited spectrum of cell types with some capacity for proliferation. Following differentiation of P19 EC cells (Jones-Villeneuve *et al.*1982; McBurney *et al.*1982) into the neuronal (using retinoic acid) or the muscle

Figure 1

Northern blot analysis of Esk expression in adult mouse tissues.

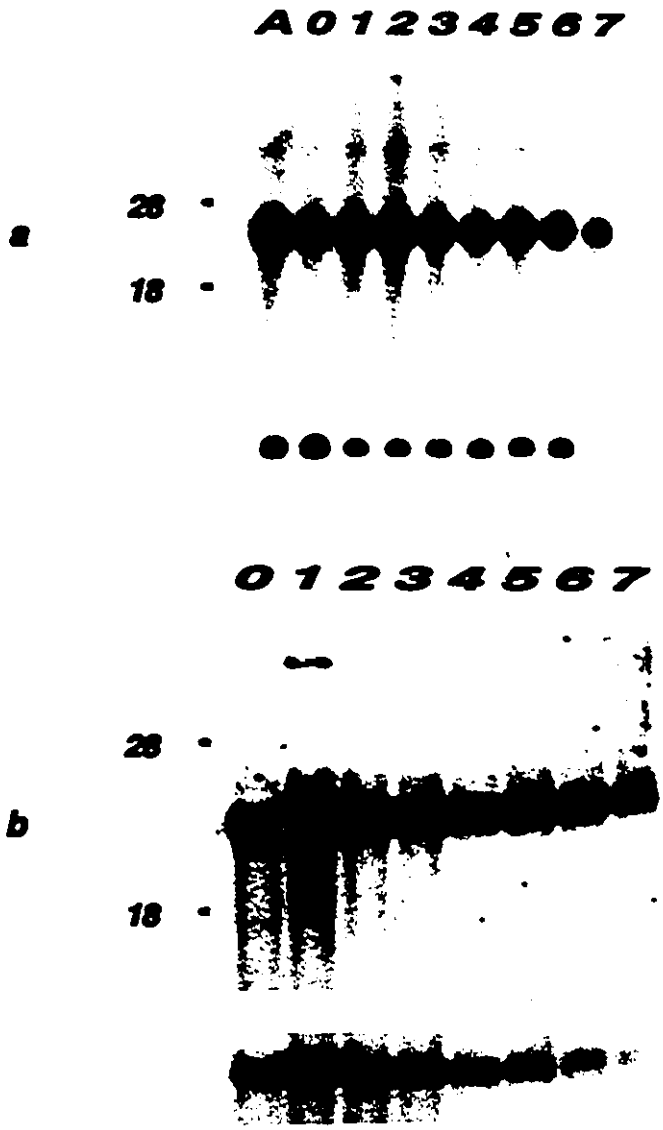
5μg of poly (A^+) RNA was isolated from the different adult mouse tissues and leukaemic cell lines indicated above the lanes (BM: bone marrow). The blot was hybridized to a random primed 1.5 Kb cDNA probe derived from the Esk-2 cDNA. Positions of 18 and 28S ribosomal RNAs were determined by ultraviolet visualization of an acrydine orange stained marker lane.
The lower panel represents the same Northern blot standardized with a β-2 microglobulin cDNA probe.

L1210
BM
thymus
spleen
testes
kidney
brain
lung
heart
7OZ/3
SP10
P388
28
18

Figure 2

Northern blot analysis of Esk expression during P19 differentiation.

5μg of poly (A^+) RNA was isolated from P19 cells induced to differentiate with retinoic acid (RA) in panel a or with dimethylsulfoxide (DMSO) in panel b. The blots were hybridized to the same 1.5 Kb cDNA probe as in figure 1. The numbers above the lanes refer to days of differentiation: "0" refers to the stem cells and "A" (in panel a) refers to P19 cells aggregated in the absence of an inducing drug. Messenger RNA levels were standardized by probing the blot with a cDNA to the phosphoglycerate kinase-1 gene (PGK-1).


A01234567
a
28
18
01234567
b
28
18

lineages (using dimethyl sulfoxide) Esk mRNA levels remained constant (figure 2). During the time course of differentiation, there remains a significant proportion of proliferating cells (Jones-Villeneuve *et al.* 1982) (M. McBurney personal communication), and we believe that it is these cells that express the Esk transcripts.

Two distinct Esk isoforms are expressed in P19 cells

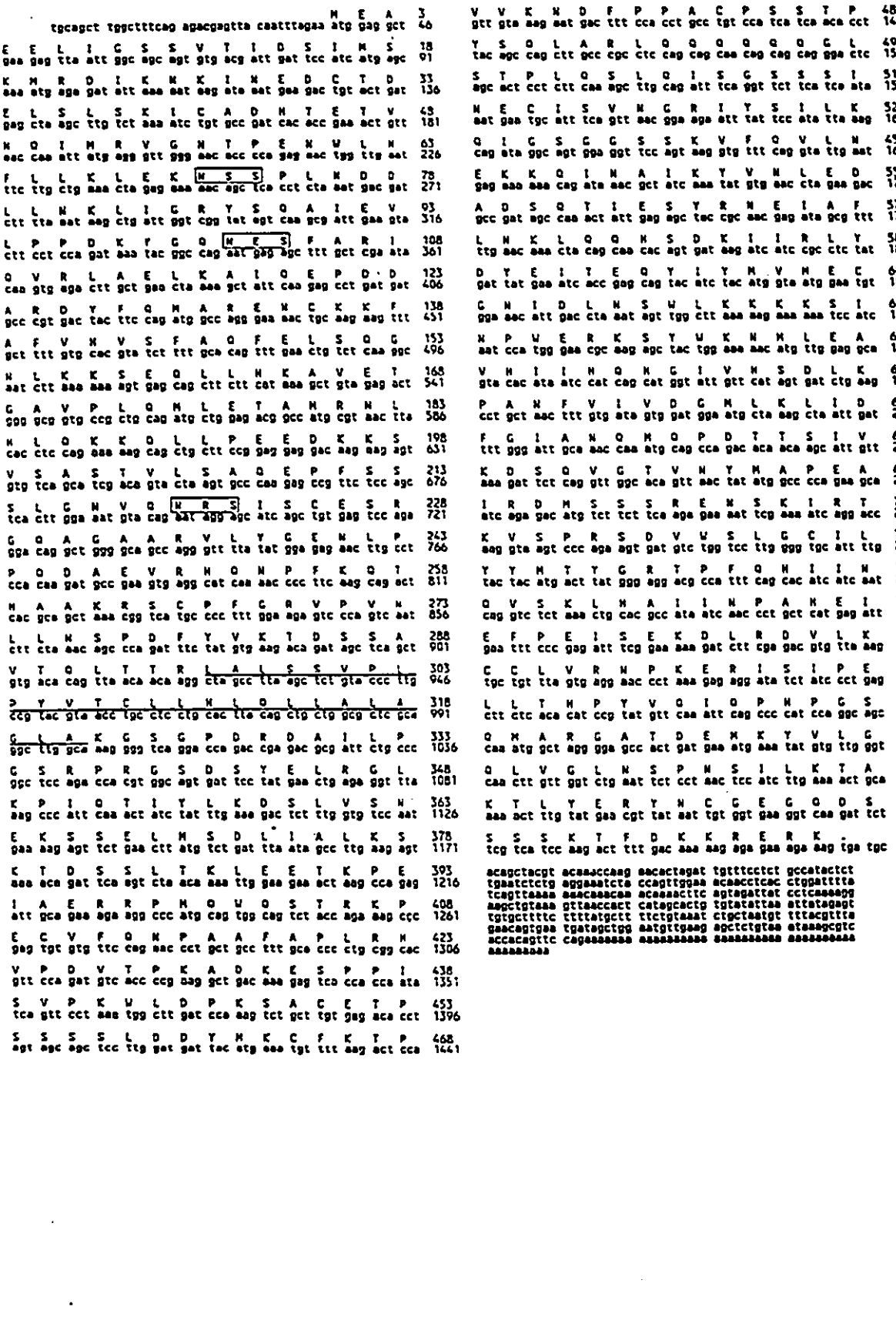
The nucleotide and deduced amino acid sequence of the 2.97 kb Esk cDNA is presented in figure 3. The first methionine codon in the longest open reading frame is located at position 38, with a purine in the -3 position and a guanine residue in the +4 position, consistent with the consensus sequence of Kozak (Kozak, 1987). No in frame stop codons are found upstream of this ATG, preventing a formal assignment of this methionine as the site of translation initiation. Following this first methionine is an open reading frame encoding 857 amino acids ending in a TGA termination codon at nucleotide 2606. It is followed by a 362 nucleotide 3'untranslated region containing a potential polyadenylation signal (AATAAA at position 2901) which ends in 46 consecutive adenosine residues.

Analysis of the predicted translation product of the Esk open reading frame revealed several interesting features. As expected the Esk protein contains the eleven subdomains which are thought to collectively make up the catalytic region of protein kinases (Hanks *et al.* 1988) (see figure 3 of General Introduction). Surprisingly, although Esk was cloned by virtue of its immunoreactivity with an antiphosphotyrosine antibody

Figure 3

The complete nucleotide and predicted amino acid sequence of the Esk-1 cDNA.

The ATG codon in position 38 fits the proper Kozak consensus sequence and marks the initiation of the predicted protein. Potential glycosylation sites at amino acid positions 71, 102 and 219 are boxed and the putative transmembrane domain between residues 296 and 321 has been underlined. The Esk-2 predicted protein is identical to that shown here, except it lacks amino acid residues 296 to 321, the putative membrane spanning domain.



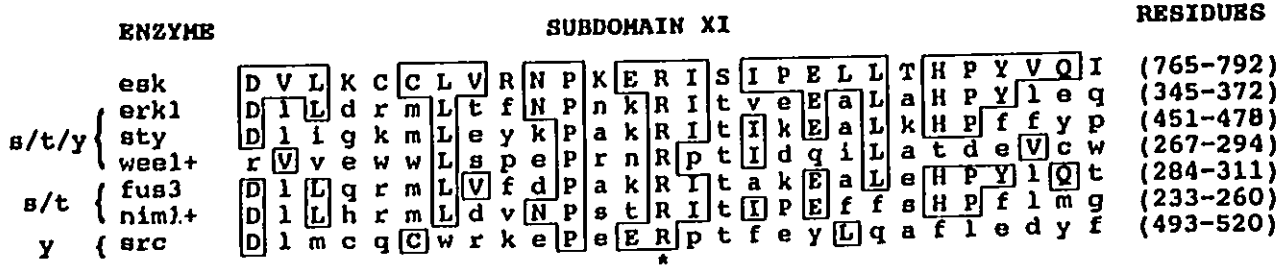
its catalytic domain shows greatest similarity to serine/threonine kinases when compared against the NBRF-PIR(r) data base using the Lipman-Pearson algorithm (Lipman and Pearson, 1985), including the yeast Snf1 ser/thr kinase (Celenza and Carlson, 1991) (27% identity over a 402 amino acid overlap), calcium/calmodulin dependent protein kinase (Tobimatsu *et al.*1988) (28% identity over 543 amino acids) and the human pim protein kinase (Zakut-Houri *et al.*1987) (25% over 329 amino acids). When individual subdomains were searched against the data base an interesting homology in subdomain XI was observed between Esk and other members of the dual specificity kinase family (Ben-David *et al.*1991; Featherstone and Russel, 1991; Howell *et al.*1991; Seger *et al.*1991) (see figure 4). It has been suggested by Seger et al. (Seger *et al.*1991) that the homology in this subdomain may be related to the novel specificity that these enzymes share. In this subdomain, Esk resembles several dual specificity kinases including STY (Howell *et al.*1991) and Erk-1, a member of the ERK kinase family (Seger *et al.*1991). The fus3 and nim1$^+$ kinases, although initially cloned as yeast ser/thr kinases (Toda *et al.*1991; Russell and Nurse, 1987), were recently shown to be dual specificity kinases (Errede *et al.*1993; Coleman *et al.*1993; Parker *et al.*1993). These protein kinases also display extensive homology to Esk and members of the DSK family in this subdomain (figure 4). While subdomain XI lies outside the catalytic domain, and may not be directly involved in specificity, these observations indicate that the sequence homology in subdomain XI may constitute a novel criteria to assign ser/thr/tyr specificity to a kinase.

Figure 4

Amino acid sequence comparison of subdomain XI.

Sequences from subdomain XI of Esk, erk-1, sty, wee1$^+$, fus3, nim1$^+$ and src were aligned without gaps or insertions. Amino acids identical to Esk have been capitalized and boxed. The arginine marked by an asterisk is the conserved residue of this subdomain. The kinases have been grouped according to their presumed amino acid specificity; s/t/y: serine/threonine/tyrosine kinase, s/t: serine/threonine kinase*, and y: tyrosine kinase.

* The fus3 and nim1$^+$ kinases were thought to be ser/thr specific kinases at the time of this publication, they have since been shown to be dual specificity kinases (please refer to text).


ENZYME
SUBDOMAIN XI
RESIDUES
esk D V L K C C L V R N P K E R I S I P E L L T H P Y V Q I (765-792)
erk1 D l 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 (345-372)
s/t/y sty D l i g k m L e y k p a k R I t I k E a L k H P f f y p (451-478)
wee1+ r V v e w w L s p e p r n R p t I d q i L a t d e V c w (267-294)
fus3 D l L q r m L V f d p a k R I t a k E a L e H P Y l Q t (284-311)
s/t nim1+ D l L h r m L d v N P s t R I t I p E f f s H P f l m g (233-260)
y src D l m c q C w r k e P e E R p t f e y L q a f l e d y f (493-520)
*

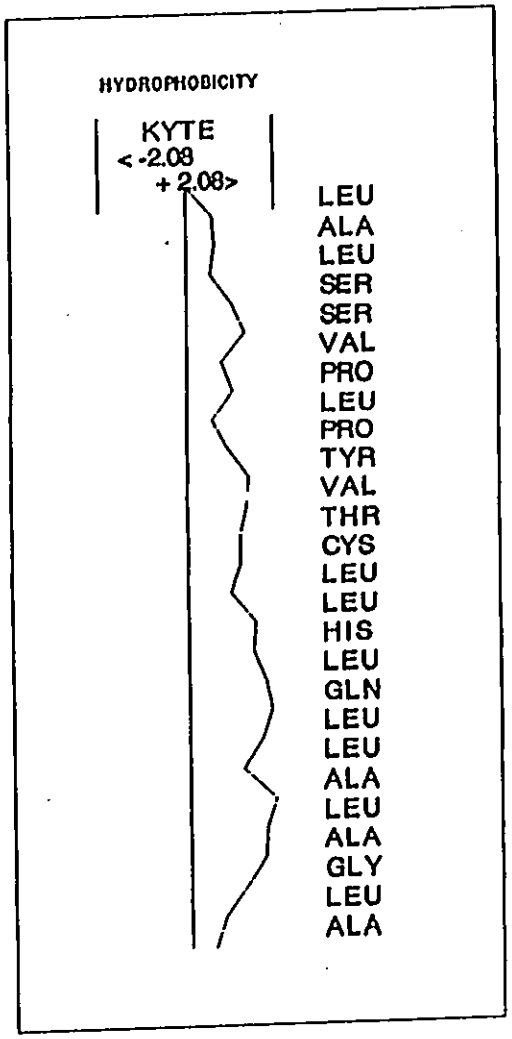
The non-catalytic domain of Esk contains a very hydrophobic stretch of 26 amino acids followed by a lysine residue between residues 296 and 321 (see figure 3, underlined amino acids and figure 5 for hydropathy plot). This configuration of amino acids is reminiscent of the transmembrane domains of receptor molecules (von Heijne, 1988) and would partition Esk into a 295 amino acid extracellular domain and a 536 amino acid cytoplasmic region. The putative extracellular portion of Esk contains three potential sites of N-linked glycosylation (NXS or NXT) at positions 71, 102 and 219. Taken together these observations suggest that this isoform of Esk, termed Esk-1, is a glycosylated transmembrane kinase. Inspection of the predicted amino terminus of Esk-1 did not reveal a convincing cleavable signal sequence peptide. There are several examples of integral membrane proteins lacking amino terminal signal peptides perhaps most relevantly the transmembrane protein tyrosine kinase Itk (Bernards and De la Monte, 1990).

Two additional Esk clones, a 2.85 kb cDNA isolated from a mouse erythroleukemia cell line library (Ben-David *et al.*1991; Letwin *et al.*1988) and a 2.0 kb cDNA isolated from the P19 library were sequenced and compared to the original Esk-1 cDNA. While the catalytic domains of all three cDNAs were identical, there was an interesting and discrete difference in the predicted non-catalytic regions of the cDNAs. Both Esk-2 cDNAs lack the amino acids which we believe form the membrane spanning domain of Esk-1. The poly A tract at the 3' end of the larger Esk-2 cDNA is 39 nucleotides shorter than the poly A tail of Esk-1 however the 5' ends of the two cDNAs

Figure 5

Hydropathy plot of the putative membrane spanning domain of Esk-1.

A Kyte-Dolittle plot of residues 296 to 321 of the Esk-1 kinase was produced with the DNASTAR program using a 9 amino acid window. There are no other extended hydrophobic regions within the Esk-1 protein. There is also a positively charged lysine residue immediately following this stretch which could act as a stop-transfer sequence.


HYDROPHOBICITY
KYTE
< -2.08
+ 2.08>
LEU
ALA
LEU
SER
SER
VAL
PRO
LEU
PRO
TYR
VAL
THR
CYS
LEU
LEU
HIS
LEU
GLN
LEU
LEU
ALA
LEU
ALA
GLY
LEU
ALA

are identical. Since these clones were isolated from two separate libraries it seems likely that either this represents the authentic 5' end of the Esk mRNA or there exists a strong stop for reverse transcriptase at this position preventing the isolation of full length cDNA clones. In either case, it appears that two distinct Esk isoforms are expressed in P19 embryonal carcinoma cells, one of which is likely a transmembrane molecule.

Esk has kinase activity in vitro

The Esk cDNAs were isolated by virtue of the fact that their gene products were recognized by an antiphosphotyrosine antibody. Paradoxically the predicted amino acid sequence of the Esk catalytic domain shows greatest similarity to serine/threonine kinases. Similar observations have been made for several members of the dual specificity family of protein kinases (Ben-David *et al.*1991; Howell *et al.*1991; Seger *et al.*1991; Stern *et al.*1991). To determine the specificity of the Esk kinase, the catalytic domain was subcloned into a pET bacterial expression vector under the control of the T7 promoter as described in materials and methods. Western blot analysis of bacterial extracts containing the Esk kinase with antiphosphotyrosine antibodies revealed an immunoreactive band of approximately 75 kd (figure 6 panel a). Cells transfected with the expression vector containing Esk in the antisense orientation did not contain any immunoreactive proteins. This immunoreactivity was specific for phosphotyrosine, since phosphoserine and phosphothreonine did not compete for antibody binding whereas phosphotyrosine completely abolished Esk immunoreactivity with the antiphosphotyrosine

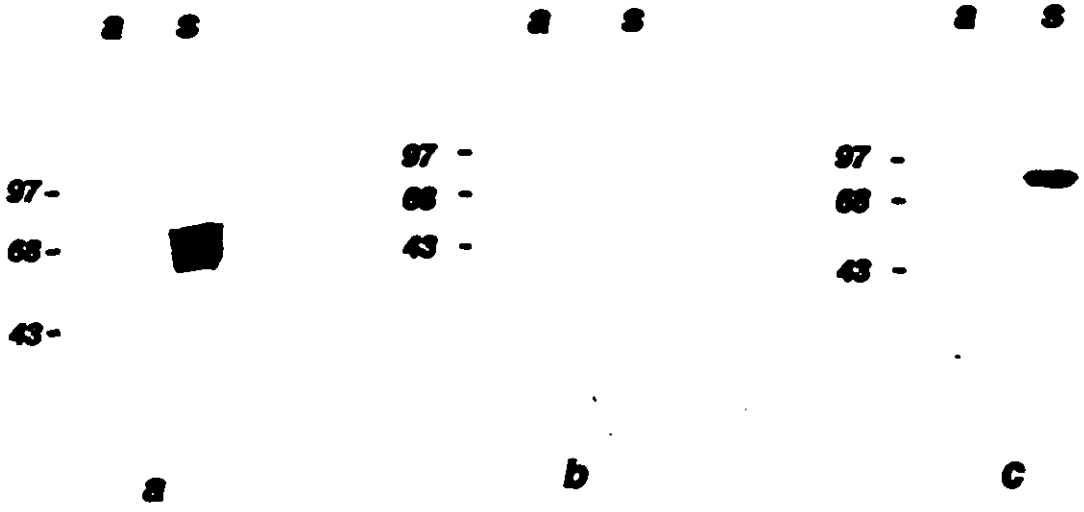
Figure 6

Esk displays kinase activity in vitro.

Panel a, Western analysis of Esk. Lysates of *E.coli* pLysS expressing Esk in the antisense (a) or sense (s) orientation were run on SDS-PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The membrane was probed with the IgG2bK antiphosphotyrosine antibody (UBI) and detected with an alkaline phosphatase conjugated anti-mouse secondary antibody.

Panel b, *In vitro* kinase assay. The Esk kinase was immunoprecipitated from pLysS bacterial extracts (either sense or antisense) using an antiphosphotyrosine antibody and assayed for kinase activity by adding γ^{32}P-ATP to the immunoprecipitates. The products were resolved on SDS-PAGE (10% polyacrylamide).

Panel c, Renaturation kinase assay. Lysates of *E.coli* pLysS expressing Esk in either orientations were run on SDS-PAGE (10% polyacrylamide) and transferred to immobilon P membranes. The proteins were denatured and renatured as described in materials and methods and assayed for kinase activity by adding γ^{32}P-ATP to the membrane.



a b c

antibody (data not shown). When the same antibody was used for immunoprecipitation followed by an *in vitro* kinase assay, a 75kd protein was again identified while control cells were negative (figure 6 panel b). To rule out the possibility that Esk phosphorylation in these immunoprecipitates resulted from a co-precipitating bacterial kinase, in situ kinase reactions were performed. Essentially, Esk bacterial extracts were resolved on SDS gels, transferred to Immobilon membranes, renatured and then incubated with γ^{32}P-ATP as described (Ferrell and Martin, 1991). As with the immunoblots and immunoprecipitates a 75 kd band was identified in Esk bacterial extracts but was absent in extracts prepared from bacteria expressing the antisense expression vector (see figure 6 panel c). Phosphoamino acid analysis was performed on Esk proteins labelled in immunoprecipitates or following renaturation and only phosphoserine and phosphothreonine residues were identified (figure 7 panels A and B). Although the Esk protein is immunoreactive with antiphosphotyrosine antibodies, only phosphoserine and phosphothreonine residues were labelled in these *in vitro* conditions.

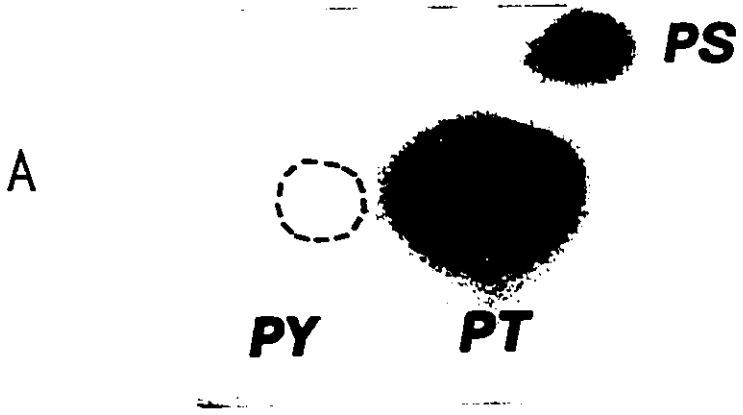
Phosphatase treatment of Esk

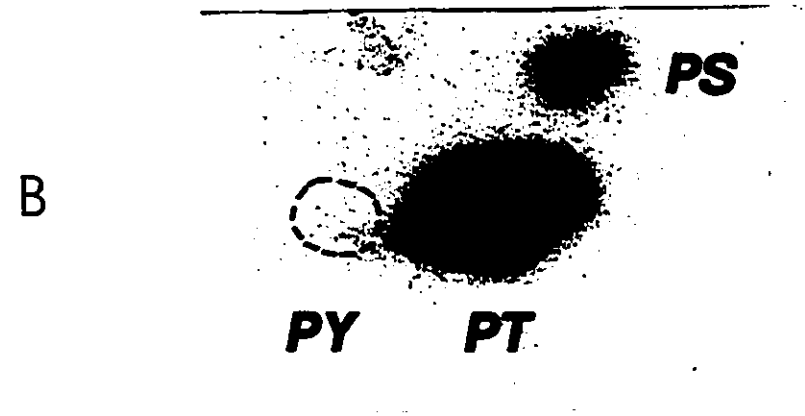
Since the Esk kinase was immunoreactive with antiphosphotyrosine antibodies, yet displayed no *in vitro* tyrosine kinase activity, we were concerned about the specificity of the anti-phosphotyrosine antibody. Previously, others had reported that certain monoclonal anti-phosphotyrosine antibodies cross react with 5'-mononucleotides, phosphohistidine, phosphoserine and phosphothreonine residues (Frackelton *et al.* 1983).

Figure 7

Phosphoamino acid analysis of labelled Esk.

Labelled Esk kinase derived from an *in vitro* kinase assay (panel A) or from a renaturation kinase assay (panel B) were acid hydrolysed and phosphoamino acids resolved by two dimensional thin layer electrophoresis. The positions of the phosphoamino acid standards were identified by ninhydrin staining. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.


A
PS
PY
PT


B
PS
PY
PT

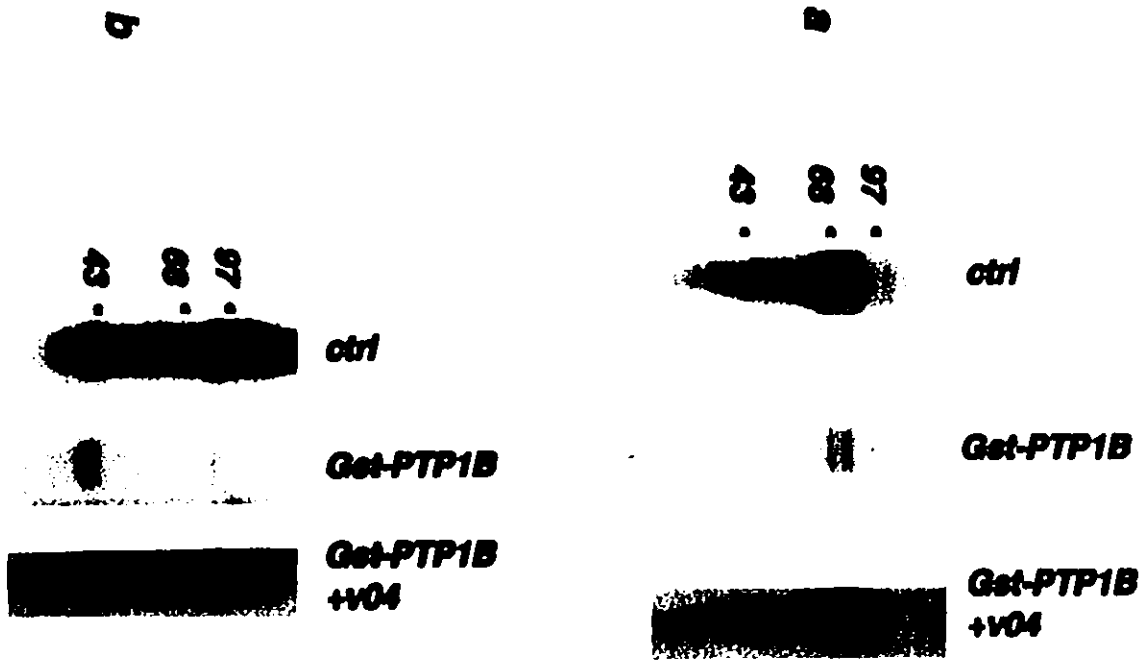
In view of this observation, phosphatase treatment of the Esk kinase was performed in an attempt to identify the residues responsible for its immunoreactivity. If the Esk kinase reacted with the antibody via a phosphotyrosine residue, then specific phosphotyrosine phosphatase treatment should abolish its immunoreactivity. Conversely, if Esk immunoreactivity was due to some other epitope, phosphatase treatment would not affect antibody binding. In the following experiments, Esk containing extracts were resolved on SDS gels, electroblotted onto nitrocellulose membranes and then treated with either potato acid phosphatase (PAP) or with the human phosphotyrosine specific phosphatase 1B (expressed as a GST fusion protein). Preparations of PAP are known to have reactivity towards phosphoserine, -threonine and -tyrosine, while phosphatase 1B preferentially dephosphorylates phosphotyrosine residues (Tonks *et al.*1988). The filters were then reacted with antibodies to phosphotyrosine and ^{125}I-labelled secondary antibodies. As shown in figure 8 (panel a) treatment of Esk with phosphatase 1B eliminated its immunoreactivity as did treatment with PAP (data not shown). Filters incubated with PAP or 1B in the presence of vanadate, a phosphatase inhibitor, retained the epitope recognized by the antiphosphotyrosine antibody confirming that the loss of immunoreactivity was due to phosphatase activity rather than a contaminating protease. To verify that our preparation of phosphatase 1B was specific for phosphotyrosine, it was tested on a substrate which is known to contain all three phosphorylated hydroxy amino acids. For these experiments we used Sty, a kinase which we have shown autophosphorylates on serine, threonine and tyrosine residues (Howell *et al.*1991). Like

Figure 8

Esk immunoreactivity is sensitive to a phosphotyrosine specific phosphatase.

Panel a, Esk bacterial extracts were resolved on SDS-PAGE and transferred to nitrocellulose as described in figure 8, except that the human phosphatase 1B (Gst-PTP1B fusion protein) was used to phosphatase treat the individual membrane strips. Ctrl lane is untreated, Gst-PTP1B lane is phosphatase treated and Gst-PTP1B+VO_4 is phosphatase treated in the presence of 1mM ammonium vanadate. Blots were probed with an antiphosphotyrosine antibody and ^{125}I-labelled secondary antibody followed by autoradiography.

Panel b, Bacterial extracts expressing a TrpE-Sty fusion protein (18) were treated exactly as in panel A as a control for Gst-PTP1B activity. The TrpE-sty fusion has a molecular weight of 85 Kd and a breakdown product runs at 43 Kd.


a
b
ctrl
Gst-PTP1B
Gst-PTP1B
+v04
ctrl
Gst-PTP1B
Gst-PTP1B
+v04

Esk, the Sty kinase lost its immunoreactivity with the anti-phosphotyrosine antibody when treated with phosphatase 1B (figure 8 panel b). To further define the specificity of the phosphatase, the TrpE-sty fusion protein was immunoprecipated and incubated with γ^{32}P-ATP in a kinase assay as previously described (Howell *et al.*1991). The products of the kinase reaction were run on SDS gels, transferred to Immobilon and treated with phosphatase 1B. The samples were acid hydrolysed and phosphoamino acids resolved by two dimensional thin layer electrophoresis. From figure 9, it is clear that the phosphatase 1B preparation used in these experiments removes phosphate groups from tyrosine but not serine or threonine residues. These results confirm that Esk contains a phosphotyrosine residue(s) which was not detected in our initial *in vitro* or renaturation kinase assays.

Esk has serine, threonine and tyrosine kinase activity

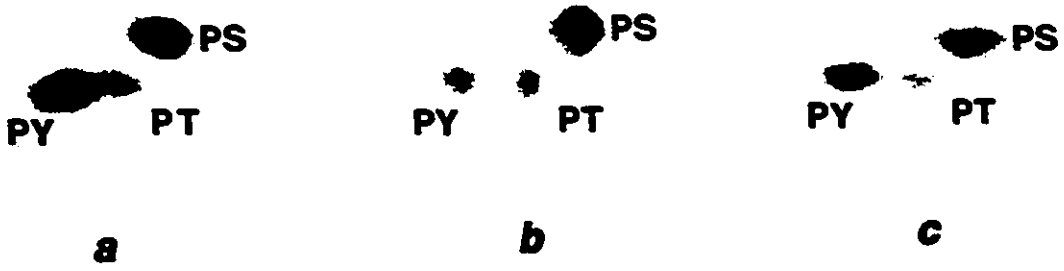
In the preceding experiments, kinase reactions were performed in a lysis buffer containing salt and detergents supplemented with magnesium (see materials and methods). We therefore attempted to define the buffer conditions that might reveal Esk tyrosine kinase activity *in vitro*. Kinase assays were carried out in a series of buffers (Hepes, Mes and Tris) at different pH conditions supplemented with magnesium and/or manganese. We found that the Esk kinase had optimum activity in buffers ranging between pH 6.5 and 7.0 containing 2mM manganese, and that a three to five fold decrease in Esk kinase activity was observed in buffers supplemented with magnesium

Figure 9

The Gst-PTP1B phosphatase is phosphotyrosine specific.

The Sty kinase was subjected to an *in vitro* kinase assay, resolved by SDS-PAGE and transferred to Immobilon. Membrane strips were treated with Gst-PTP1B or with Gst-PTP1B in the presence of 1mM ammonium vanadate. Phosphoamino acids (PAA) were generated from these different Sty samples and analyzed by two dimensional thin layer electrophoresis.

Panel a: PAA of untreated Sty,
Panel b: PAA of Sty treated with phosphatase and
Panel c: PAA of Sty treated with phosphatase in the presence of vanadate.



a *b* *c*

only (data not shown). Under these conditions, Esk was able to autophosphorylate and phosphorylate exogenous substrates on serine, threonine and tyrosine residues. The results of one such experiment are shown in figure 10. In panel A it is evident that not only can Esk autophosphorylate (lane 2), but it also has activity towards myelin basic protein (lane 4) and enolase (lane 6). Immunoprecipitates from extracts of bacteria expressing antisense Esk mRNA lacked kinase activity (lanes 1,3,5). Amino acid analysis of the phosphorylated products (panel B) revealed that threonine was the preferred target in all three substrates although phosphotyrosine and phosphoserine were also present. Taken together, the results obtained from Western blotting, the phosphatase experiments as well as from the *in vitro* kinase assays clearly demonstrate that the Esk kinase has serine, threonine and tyrosine kinases activity, and is therefore a novel member of the dual specificity family of protein kinases.

DISCUSSION

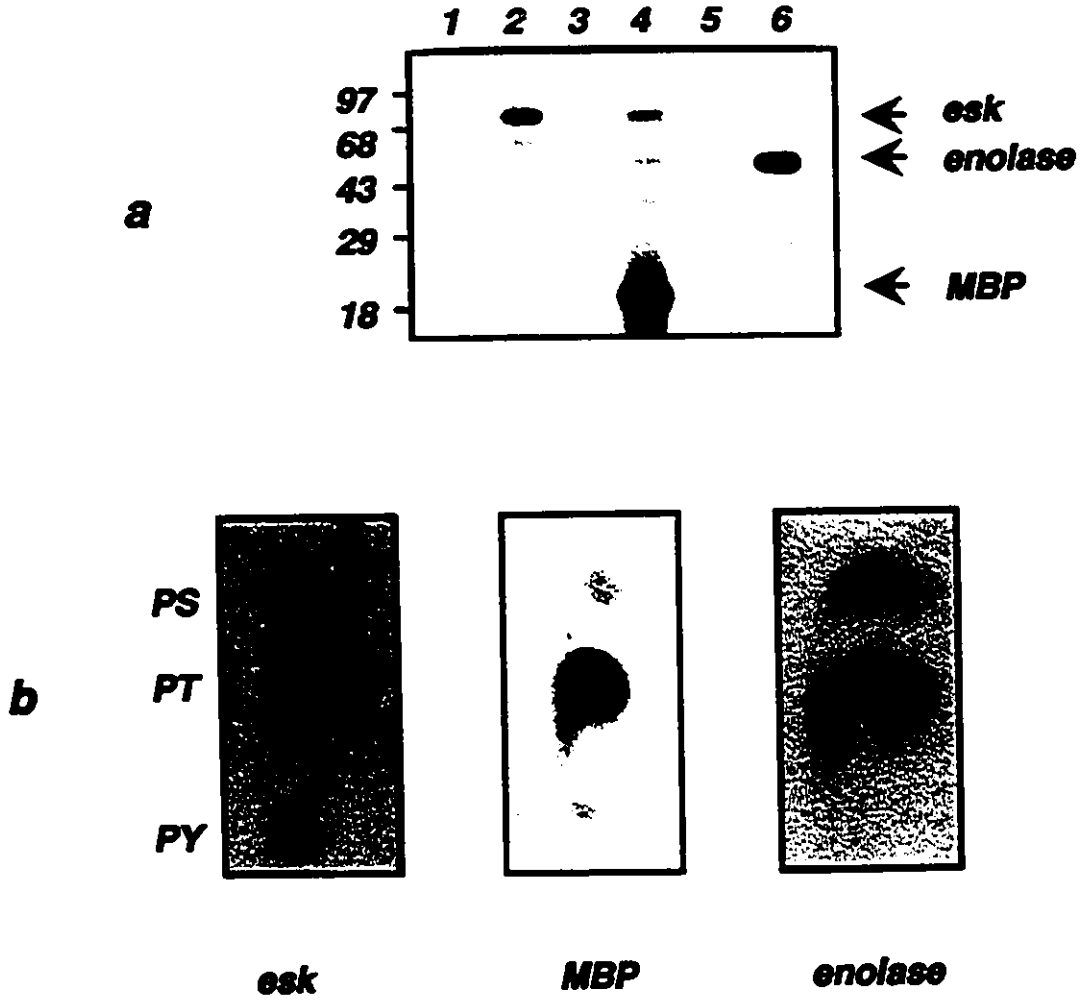
Several protein kinases appear to exist in multiple isoforms encoded by distinct mRNA species. In this regard Esk represents a novel departure from previously identified alternatively spliced kinases, in that Esk is the first example of a kinase which may exist as both a transmembrane molecule and as a soluble catalytically active enzyme. Indeed, most alternative splicing events in receptor protein kinases generate molecules with variant ligand binding affinities or specificities as for the FGF, ltk and activin receptors (Hou *et al.*1991; Haase *et al.*1991; Attisano *et al.*1992). Alternatively, these splicing

Figure 10

Esk has serine, threonine and tyrosine kinase activity.

Panel a, *In vitro* kinase assay. The Esk kinase was immunoprecipitated from pLysS bacterial extracts (either sense orientation lanes 2, 4, 6; or antisense orientation lanes 1, 3, 5) using an antiphosphotyrosine antibody and assayed for kinase activity by adding γ^{32}P-ATP to the immunoprecipitates. Myelin basic protein was added to the immunoprecipitates in lanes 3 and 4, while acid denatured enolase was added in the assays in lanes 5 and 6.

Panel b, Phosphoamino acids were generated from the labelled protein bands in lanes 2, 4 and 6 and resolved by one dimensional thin layer electrophoresis (as described in material and methods). The positions of the phosphoamino acid standards were identified by ninhydrin staining. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.


a
1 2 3 4 5 6
97
68
43
29
18
esk
enolase
MBP
b
PS
PT
PY
esk
MBP
enolase

events have also been shown to generate truncated and/or secreted receptors in the case of the neurogenic trkB kinase and in the epidermal growth factor receptor, respectively (Middlemas *et al.*1991; Petch *et al.*1990).

The cloned Esk cDNAs do not encode an amino terminal signal peptide and it remains possible that the putative transmembrane domain could itself be the signal peptide (von Heijne, 1988). Indeed the signals which determine the subcellular localization of proteins are incompletely understood and are likely encoded in the three dimensional structure of the protein (von Heijne, 1988). Furthermore, there appears to be a hierarchy of subcellular localization signals (Roberts *et al.*1987) and thus insertion of a transmembrane domain in the Esk-1 protein may override the localization signals shared between the Esk-1 and Esk-2 kinases, including a putative nuclear localization signal encoded by the last six amino acids of the Esk kinases (KKRERK).

To date, only one mammalian transmembrane kinase, the transforming growth factor β (TGFβ) receptor, is believed to have serine/threonine instead of tyrosine kinase activity (Wrana *et al.*1992). Despite the degree of homology of Esk and members of the serine/threonine kinase family, we have found very little sequence homology between Esk and the TGFβ receptor. Two other transmembrane serine/threonine kinases, daf-1 from C.elegans and Zmpk1 from maize also have very little homology to Esk (Georgi *et al.*1990; Walker and Zhang, 1990). Through the recent cloning of these novel serine/threonine receptor kinases (Georgi *et al.*1990; Mathews and Vale, 1991; Walker and Zhang, 1990) and the Esk-1 ser/thr/tyr receptor kinase, it has become clear that cell

proliferation and differentiation signals mediated by receptor kinases are not restricted to tyrosine phosphorylation events.

Amino acid sequence comparisons revealed that Esk shares greatest homology with members of the serine/threonine family of protein kinases, and in particular with the catalytic domains of the yeast kinase snf1 (Celenza and Carlson, 1991). Seger et al. (Seger *et al.*1991) have recently reported that conserved amino acid motifs exist in subdomain XI of a family of kinases with the capacity to phosphorylate serine, threonine and tyrosine residues. Since this region is generally not well conserved among kinases, it was suggested that it may play some role in determining substrate specificity. Both Esk and nim1$^+$ show considerable homology to the dual specificity kinases in this region, however, they have a more pronounced homology to each other. The similarity of this domain in the Esk and nim1$^+$ kinases may reflect conserved biochemical activities for these two enzymes.

Threonine and tyrosine phosphorylation events are important in regulating the activity of several signal transduction components. The activities of the epidermal growth factor receptor kinase, the cell cycle regulating kinase p34^{cdc2} as well as the MAP kinases have all been shown to be regulated through threonine and tyrosine phosphorylation events (Sibley *et al.*1987; Gautier *et al.*1991; Posada and Cooper, 1992). Since Esk appears to exist as both a transmembrane and soluble catalytically active enzyme, its threonine and tyrosine kinase activities could be involved in modulating different levels of signal transduction pathways.

The Esk kinase has a restricted pattern of expression in the adult mouse, found predominantly in tissues which have a significant stem cell component. We are currently determining the spatial and temporal expression patterns of the different Esk isoforms. Defining the specific distribution of the Esk-1 kinase will aid in the identification of its ligand.

CHAPTER 3

The Esk kinase interacts with the 85 kDa subunit of phosphatidylinositol 3'-kinase

INTRODUCTION

It is generally accepted that the control over cell growth and differentiation is achieved through the combinatorial actions of protein serine/threonine and protein tyrosine kinases. The recent identification of a novel class of kinases, referred to as dual specificity kinases (DSK) (Lindberg *et al.*1992), with serine/threonine and tyrosine phosphorylating activities, has broadened the spectrum of signal transduction pathways leading to growth regulation. Like their protein serine/threonine and protein tyrosine kinase counterparts it has become apparent that DSKs are involved in all aspects of cell cycle regulation as demonstrated by the presence of conserved kinase cascades across several species. Indeed in lower eukaryotes, the regulation of entry of cells into mitosis is accomplished by the sequential phosphorylation and inactivation of a number of kinases (Norbury and Nurse, 1992). The nim1 DSK has been shown to phosphorylate and inactivate the Wee1 DSK (Parker *et al.*1993; Wu and Russell, 1993) which in turn inactivates $p34^{cdc2}$, the mitotic kinase (Featherstone and Russel, 1991; Parker *et al.*1992). A cascade of phosphorylation events is also central in the response of cells to extracellular stimuli. As a result of growth factor stimulation, the MEKK or raf-1 kinases can activate the MEK dual specificity kinase (Lange-Carter *et al.*1993) which can in turn phosphorylate and activate the MAP kinase DSK (Kosako *et al.*1993).

Members of the dual specificity kinase family are uniquely positioned to integrate within both the PYK and PSK signalling pathways. The autophosphorylation of DSKs on tyrosine residues can lead to interactions with SH2 domain containing molecules

which in turn can be regulated via the serine/threonine phosphorylating activities of the DSK. While several protein tyrosine kinases have been shown to mediate signal transduction through the binding of SH2 domains (Koch *et al.*1991), no DSK has yet been shown to be involved in such an interaction. However, the recent identification of consensus SH2 binding sites for different downstream signalling molecules, has revealed the presence of cryptic binding sites within several proteins including the novel DSK, Esk (Songyang *et al.*1993). The Esk catalytic domain was found to harbour two overlapping SH2 binding sites for the 85 kDa subunit of phosphatidylinositol (PI) 3-kinase with the sequences YIYMVM (Y596 and Y598).

Phosphatidylinositol 3'-kinase (PI3K) was first described in cells transformed by the polyoma (Py) middle T antigen in a complex with the c-src proto-oncogene (Courtneidge and Heber, 1987). The enzyme was later found to be associated in a ligand dependent fashion with activated growth factor receptors (Koch *et al.*1991) as well as with several oncogenic cytoplasmic tyrosine kinases (Fukui and Hanafusa, 1989). The PI3 kinase enzyme exists as a heterodimer of an 85 and 110 kilodalton subunit. The catalytic activity of PI3K was found to reside within the 110 kDa subunit (Hiles *et al.*1992), while the 85 kDa protein was found to function as an adaptor molecule bridging activated tyrosine kinases to the catalytic subunit (Skolnik *et al.*1991; Escobedo *et al.*1991; Otsu *et al.*1991). Indeed the 85 kDa subunit was found to contain an amino terminal SH3 domain as well as two carboxyl SH2 domains which are thought to mediate the interaction of PI3K with activated protein kinases.

The Esk kinase, like several other dual specificity kinases was initially cloned by virtue of its immunoreactivity with anti-phosphotyrosine antibodies (Douville *et al.* 1991). When expressed in bacteria the kinase displayed serine, threonine and tyrosine kinase activities thus making it a member of the DSK family. The kinase was found to be expressed predominantly in tissues or cell lines with high proliferation rates and/or stem cell compartments, suggesting a possible role for this kinase in growth regulation.

This work describes the characterization of the Esk kinase from the murine P19 embryonal carcinoma cell line and its binding to the 85 kDa subunit of PI3K both *in vitro* using GST-p85 fusions and *in vivo*.

MATERIALS AND METHODS

Antibodies

A multiple antigen peptide (map, 8 branch lysine tree) corresponding to the sequence of the last twelve amino acids of the Esk kinase, (SSKTFDKKRERK, position 845 to 856), was synthesized and injected into rabbits. The crude serum was tested for immunoreactivity against the Esk kinase by immunoprecipitations and Western blotting. In both assays the antibody recognizes a protein of ~96 kDa consistent with the predicted molecular weight of the Esk-2 kinase (94 kDa). The antibody was affinity purified by passing the crude serum over a peptide column and used in all the experiments described herein. The peptide column was generated by linking the peptide to cyanogen-bromide activated sepharose beads as described by the manufacturer

(Pharmacia). The p85 PI3K antibody (generous gift of X. Liu) was raised to a GST-SH3 fusion in rabbits and has been described previously (Liu *et al.*1993). An antibody raised to the most amino SH2 domain of p85 was a generous gift of C. Carpenter (Carpenter *et al.*1993).

Synthetic peptides

The two Esk phosphopeptides used in these studies were consensus binding sites for the SH2 domains of the 85 kDa subunit of PI3K and corresponded to amino acids 591 to 603 in the kinase. The phosphopeptides had the following sequence EITEQYIYMVMEC with either Y596 or Y598 phosphorylated. The non-relevant phosphopeptide had the following sequence: DNDpYIIPLPDPK.

Cell culture and transfections

P19 EC cells were maintained in αMEM supplemented with 10% serum (50% fetal calf serum and 50% calf serum). Cos-1 cells were maintained in αMEM supplemented with 10% calf serum. The cos cells were transfected by electroporation using the Bio-Rad gene pulser set at 960 μFad and 220 Volts. Typically, 3 million cells were trypsinized and resuspended in 0.5 ml of serum-free αMEM containing 20μg of plasmid (pECE-Esk-2). The cells were kept on ice 10 minutes prior to electroporation and 30 minutes at room temperature post-electroporation. The cells were then plated back into tissue culture dishes and harvested for kinase assays 24 hours post-transfection.

The pECE-Esk-2 construct was generated by subcloning an Esk-2 cDNA (derived from an L1210 λgt10 library) into the unique Kpn1 site of the pECE expression vector (Ellis *et al.*1986).

Immunoprecipitations and kinase assays

Whole cells were lysed in buffer containing 10 mM Tris (pH7.5)- 150mM NaCl- 5mM EDTA- 1% Triton X-100- 2mM sodium pyrophosphate- 2mM sodium fluoride- 500μM ammonium vanadate- 200μg of phenyl-methylsulfonyl fluoride per ml- 2 μg of aprotinin per ml and 5μg of leupeptin per ml. The lysates were incubated on ice 30 minutes and cleared by centrifugation for 15 minutes. The supernatants were immunoprecipitated with anti-Esk (1:500) or anti-p85 (1:200) antibodies for 16 hours at 4°C. After a 30 minute incubation with protein-A sepharose (Pharmacia) (50μl of a 50:50 slurry), the immunoprecipitates were washed three times with lysis buffer and two times with kinase buffer [20mM 2[*N*-morpholino] ethanesulfonic acid (MES) pH 6.5 - 10mM $MgCl_2$- 2mM $MnCl_2$] and assayed for Esk kinase activity for 30 minutes at room temperature with the addition of 5μCi [γ^{32}P]-ATP. The kinase reaction was stopped by adding one volume of sample buffer. The products of the reaction were resolved by SDS-PAGE, and gels dried or transferred to nitrocellulose. For phosphoamino acid (PAA) analysis and tryptic peptide mapping, protein bands were cut out of nitrocellulose and trypsinized overnight (as described in chapter 2). For PAA analysis, the peptides were acid hydrolyzed at 95°C in 6N HCl for 75 minutes as described by Kamps *et al.*

(Kamps *et al.*1985) and in chapter 2. For tryptic peptide mapping, the proteins were oxidized in 50μl performic acid (made by incubating 900μl formic acid and 100μl hydrogen peroxide (H_2O_2) for 60 minutes at room temperature) for 60 min. on ice. The samples were diluted with 500μl of water, dried and resuspended in PAA standards and pH 1.9 buffer (see below). The resulting phosphopeptides were separated by ascending chromatography in tank buffer containing n-butanol (204 ml), glacial acetic acid (50 ml), pyridine (143 ml) and water (143 ml), followed by electrophoresis in pH 1.9 buffer [formic acid (50ml), acetic acid (156ml), water (1794ml)] at 1000 volts for 60 min. (anode to cathode).

Phosphatidylinositol 3'-kinase assays

PI3 kinase activity was measured following the procedure of Fukui and Hanafusa (Fukui and Hanafusa, 1989). Briefly, cells were lysed as described and immunoprecipitated with the Esk or p85α antibodies. The immunoprecipitates were incubated with Protein A-Sepharose (Pharmacia) for 30 minutes at 4° C, washed with lysis buffer three times followed by three washes in PI kinase (PIK) buffer (20mM Tris-HCl, PH 7.5, 100 mM NaCl, 0.5 mM EGTA). The protein A beads were finally resuspended in 50μl of PIK buffer and preincubated with 0.5μl of phosphatidylinositol (Sigma) (20 mg/ml in dimethylsulfoxide) for 10 minutes at room temperature. To start the reaction, [γ^{32}P]-ATP (10μCi) and $MgCl_2$ (20mM final concentration) were added. The reaction was allowed to proceed for 3 minutes (linear formation of

phosphatidylinositol-phosphate) and stopped by the addition of 150μl of chloroform-methanol- 11.6N HCl (100:200:2). The phases were separated by the addition of 100μl of chloroform. The organic (bottom) layer was washed with an equal volume of methanol-1N HCl (1:1). The lipid sample was concentrated in vacuo and the whole sample spotted onto a silica gel 60 coated glass plate (Merck & Co. Inc., Rahway, N.J.). The phospholipids were separated by ascending chromatography in tank buffer containing chloroform- methanol-28% ammonium hydroxide- water (43:38:5:7).

Bacterial expression of Esk and GST-fusions of p85 and in vitro binding assays.

(i) Bacterial inductions

Induction of bacterial Esk has been described before (Douville *et al.*1991). Briefly, *E.coli* pLysS containing an Esk expression vector were grown in M9CA medium to an $O.D._{600}$ of 0.4 and induced for 2 hours by the addition of IPTG (0.4 mM final). In the *in vitro* binding experiments described below, 1.5 ml of bacteria were pelleted, lysed by sonication (2 times 30 seconds on ice) and cleared by centrifugation 15 minutes.

GST-fusions of the SH2 domains (amino acids 314-723) and of the SH3 domain (amino acids 2-83) of the bovine p85α subunit of PI3K (generous gift of X. Liu) were induced as follows. The pGEX-3X vector (GST alone) was used as a negative control. Bacteria were grown at 37°C to an $O.D._{600}$ of 0.9 in LB containing ampicillin, and induced for an additional three hours with IPTG at 1mM final concentration. Typically, 10 ml of bacteria were pelleted, lysed in 1 ml of lysis buffer (described above), and

sonicated 2 times for 30 seconds on ice. The lysates were cleared and incubated with 40 μl of packed glutathione-sepharose beads (Pharmacia) for 60 minutes at 4°C. The beads were then washed three times with lysis buffer, and a fraction corresponding to 10μg of bound GST-fusion protein used in the binding studies.

(ii) in vitro binding assays

The induced GST-fusions (10μg of sepharose bound protein) were incubated with 1.5 ml of Esk bacterial lysates (both sense and antisense) for 60 minutes at 4°C. The beads were then washed three times with lysis buffer and the bound proteins eluted by boiling in sample buffer. The proteins were resolved by SDS-PAGE and the gels transferred to nitrocellulose. The GST-fusion bound Esk kinase was visualized by Western blotting with an anti-phosphotyrosine antibody (UBI) followed by an alkaline phosphatase conjugated secondary anti-mouse antibody (Gibco-BRL).

RESULTS

The Esk kinase can be immunoprecipitated from P19 cells.

The Esk mRNA transcripts have previously been characterized and shown to be abundant in tissues or cell lines with stem cell compartments and/or high proliferation rates (Douville *et al.*1991). In order to determine the functional role of this dual specificity kinase in growth control, antibodies were raised to a multiple antigen peptide (map) corresponding to the last twelve amino acids of the kinase. The antibody raised could immunoprecipitate the Esk-2 kinase expressed in cos cells as well as a protein with

kinase activity from P19 cells (figure 1 panel A). Both proteins corresponded to the predicted molecular weight of the Esk kinase, ~96 kDa. The polyclonal antibody was specific for the Esk kinase since the peptide inhibited its immunoprecipitation from P19 cells (data not shown). Analysis of the tryptic peptide maps of the proteins immunoprecipitated from cos transfected and P19 cells, revealed that they were similar and suggested that the Esk-2 kinase was the predominant isoform expressed in P19 EC cells (figure 2). When expressed in cos cells, the Esk-2 kinase is resolved by SDS-PAGE as a doublet which is visible upon western blotting of the immunoprecipitate (data not shown). We believe that the doublet corresponds to different phosphorylation forms of the kinase based on the subtle differences in the peptide maps (figure 2, upper panels peptides indicated by arrows). Of interest, the kinase immunoprecipitated from P19 cells appears to represent a composite of these peptide maps (figure 2, bottom map), suggesting that the distinct phosphorylated forms of Esk may exist in mammalian cells and represent different activation states of the kinase.

To determine the specificity of the Esk kinase isolated from mammalian cells, phosphoamino acid analysis was carried out. We have previously shown that when expressed in bacteria the Esk kinase, although clearly immunoreactive with anti-phosphotyrosine antibodies, displayed predominantly a manganese dependent serine/threonine kinase activity with traces of tyrosine kinase activity (Douville *et al.*1991). In mammalian cells, the Esk kinase also displayed predominantly threonine kinase activity, with no detectable tyrosine kinase activity in *in vitro* kinase assays (figure

Figure 1

The Esk polyclonal antibody immunoprecipitates a 96 kDa protein from mammalian cells.

Panel A, Esk-2 kinase was immunoprecipitated (ip) from cos-1 transfected cells and P19 EC cells using a rabbit polyclonal antibody raised to an Esk peptide (see materials and methods). The immunoprecipitates were subjected to a kinase assay and the products resolved on 7.5% SDS-PAGE. Lane 1: ip/kinase assay from cos transfected cells, lane 2: ip/kinase assay from P19 cells.

Panel B, Specificity of the Esk-2 kinase was determined by phosphoamino acid (PAA) analysis of the proteins in panel A. Left panel:PAA of the Esk-2 kinase expressed in cos cells, right panel: PAA of the endogenous Esk kinase in P19 cells. PSer: phosphoserine, PThr: phosphothreonine and PTyr: phosphotyrosine. The phosphoamino acids were resolved in one dimension at pH 3.5.

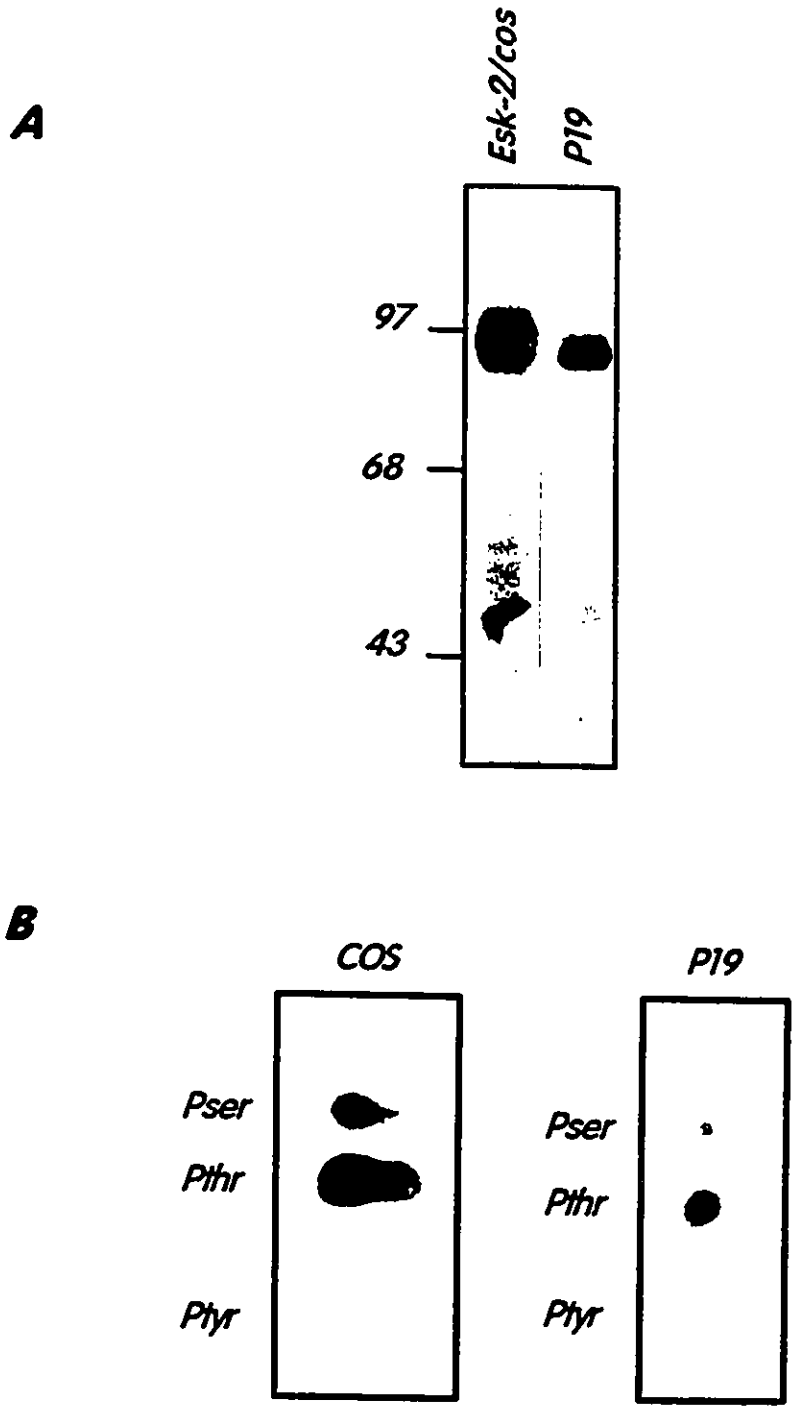

A
Esk-2/cos
P19
97
68
43
B
COS
P19
Pser
Pthr
Ptyr
Pser
Pthr
Ptyr

Figure 2

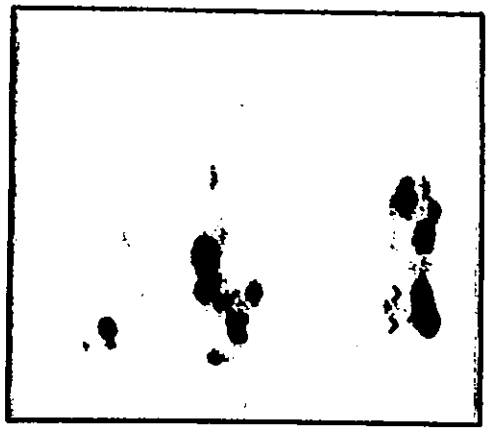
Tryptic peptide maps of the Esk-2 kinase.

Esk-2 kinase immunoprecipitated from P19 and cos transfected cells was subjected to an *in vitro* kinase assay and the labelled proteins trypsinized. Individual phosphopeptides were resolved by ascending chromatography followed by electrophoresis. The directions of electrophoresis (TLE) and chromatography (TLC) are indicated by arrows. Small arrowheads indicate unique phosphopeptides.

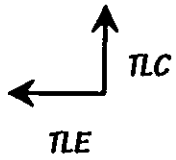
Upper panel: tryptic peptides maps of the upper and lower forms of the Esk-2 kinase transfected in cos-1 cells.

Lower panel: tryptic peptide map of the endogenous Esk-2 kinase immunoprecipitated from P19 cells.

COS
Esk-2 U



COS
Esk-2 L



P19

1 panel B) or from *in vivo* ^{32}P labelled cells (data not shown). Given the extent of the tyrosine phosphorylation seen with the bacterially expressed kinase, the tyrosine phosphorylation of the mammalian kinase may be a subtle regulatory event which may not be assayed by conventional techniques.

The Esk kinase has a consensus PI 3-K binding site.

The binding of phosphatidylinositol 3-kinase (PI3K) by several receptor tyrosine kinases has been implicated in the propagation of growth regulatory signals (Cantley *et al.*1991). Most of the proteins that bind the SH2 domains of the 85 kDa subunit of PI3K, with the exception of the hepatocyte growth factor/scatter factor receptor (Ponzetto *et al.*1993), have been shown to bind through the same tyrosine-phosphorylated sequence (figure 3). Cantley's group has corroborated these observations and has shown, using a random phosphopeptide library, that the preferred binding sequence of PI3K is pY-M/V/I-X-M (Songyang *et al.*1993). The predicted consensus sequence was not only found in proteins which are known to bind the 85 kDa subunit of PI3K, but it was also present in a subset of proteins which are thought to have the potential of binding PI3K. Included amongst the latter group of proteins, the Esk kinase was found to share this conserved sequence (figure 3). The sequence found in the Esk kinase corresponds to amino acids 591 to 603 (EITEQYIYMVMEC) and is found between subdomains IV and V in the catalytic domain. This amino acid stretch contained two tyrosine residues in a consensus sequence for binding to the SH2 domains of p85 , Y596 (YIXM) and Y598

Figure 3

Alignment of SH2 binding sites of the 85 kDa subunit of PI3K.

The p85α SH2 binding sites in the receptors for platelet derived growth factor β (PDGFRβ), colony stimulating growth factor-1 (CSF1R), hepatocyte growth factor/scatter factor (HGF/SCR) and in the polyoma middle T antigen (Py mT) have been aligned to emphasize the consensus sequence YMXM. The position of the tyrosine involved in the interaction is shown on the left and is highlighted in bold character. The sequences surrounding Y596 and Y598 of the Esk kinase are also shown and display high homology to the predicted consensus sequence.

p85 PI kinase binding site:

ESK	596	E	I	T	E	Q	**Y**	I	Y	M	V	M	E	C
ESK	598	T	E	Q	Y	I	**Y**	M	V	M	E	C	G	N
PDGF R β	740	E	S	D	G	G	**Y**	M	D	M	S	K	D	E
PDGF R β	751	D	E	S	V	D	**Y**	V	P	M	L	D	M	K
CSF1 R	721	Q	G	V	D	T	**Y**	V	E	M	R	P	V	S
mT	315	E	E	E	E	E	**Y**	M	P	M	E	D	L	Y
HGF/SF R	1356						**Y**	V	N	V				
HGF/SF R	1349						**Y**	V	H	V				
CONSENSUS							**Y**	X	X	M				

(YMXM). Since bacterially expressed Esk displayed serine/threonine and tyrosine kinase activities *in vitro*, we investigated the ability of the bacterial kinase to bind to PI3K.

The Esk kinase binds to phosphatidylinositol 3-kinase *in vitro*.

To assess the ability of the Esk dual specificity kinase to bind the 85 kDa subunit of PI3K, we made use of glutathione-S-transferase (GST) fusions of regions of p85α (generous gift of X. Liu). Essentially, GST fusions encompassing the SH2 or SH3 domains of p85α were used in *in vitro* binding studies to immunoprecipitate the Esk kinase from bacterial extracts. The GST-SH2 bound Esk kinase could then be visualized as a 75 kDa protein by Western blotting with anti-phosphotyrosine antibodies. As shown in figure 4, the Esk kinase could bind to the combined SH2 domains (amino and carboxyl) of p85α. This binding was inhibited by either phosphopeptide, pY596 (EITEQpYIYMVMEC) or pY598 (EITEQYIpYMVMEC), but not by an irrelevant phosphopeptide (DNDpYIIPLPDPK) suggesting that the binding of Esk to p85α was specifically mediated through this sequence. Unexpectedly, the Esk kinase was also found to bind to the SH3 domain of p85α (figure 4, right panel). In contrast to the SH2 interaction, the binding to the SH3 domain could not be competed with any of the phosphopeptides indicating that this interaction was mediated through another domain present in the catalytic domain of Esk. Since the Esk kinase did not bind to the GST portion of the fusions (figure 4, right panel), we conclude that the interactions with the p85 fusions were specific to the SH2 and SH3 domains. The bacterially expressed kinase

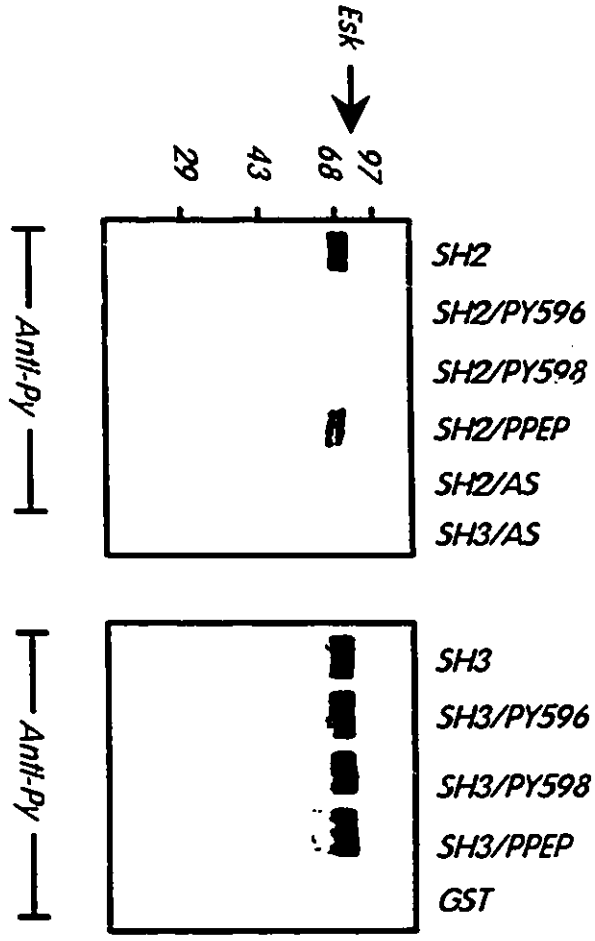
Figure 4

The Esk kinase binds to the SH2 and SH3 domains of the 85 kDa subunit of PI3K.

GST fusions of the SH2 and SH3 domains of the p85α subunit were expressed in bacteria and bound to glutathione-sepharose columns. The GST-fusion columns were used to immunoprecipitate the tyrosine phosphorylated Esk kinase from bacterial extracts. The bound proteins were eluted with sample buffer and resolved on 10% SDS-PAGE. The gels were transferred to nitrocellulose and probed with an anti-phosphotyrosine antibody followed by an alkaline-phosphatase conjugated secondary antibody.

Left panel: lane 1, Esk kinase bound to the GST-SH2 column in the absence of competing peptide,
lane 2, in the presence of 10μM of the Y596 phosphorylated peptide,
lane 3, in the presence of 10μM of the Y598 phosphopeptide,
lane 4, in the presence of 10μM of an irrelevant phosphopeptide,
lanes 5 and 6, the Esk kinase expressed in the antisense orientation (AS) bound to the SH2 and SH3 columns.

Right panel: lanes 1 to 4 same as in the left panel, except the Esk kinase was bound to the GST-SH3 column,
lane 5, the Esk kinase bound to GST alone.


Esk
29
43
68
97
Anti-Py
SH2
SH2/PY596
SH2/PY598
SH2/PPEP
SH2/AS
SH3/AS
Anti-Py
SH3
SH3/PY596
SH3/PY598
SH3/PPEP
GST

does not contain a domain with significant sequence homology to the prototypic 3BP-1 SH3 binding sequence (Cicchetti *et al.*1992). However, it does contain several stretches of proline rich sequences which could mediate binding to the SH3 domain. Taken together, these *in vitro* binding studies suggest that the Esk kinase interacts with the 85 kDa subunit of PI3K through both a phosphorylation dependent and independent mechanism that involves the SH2 and SH3 domains, respectively. The binding of Esk with the SH2 domains of p85α suggests a physiological function for the tyrosine phosphorylation of this dual specificity kinase.

The Esk kinase co-immunoprecipitates with the 85 kDa subunit of PI 3-K in P19 cells.

In order to determine if the Esk kinase interacted with p85 *in vivo*, a series of co-immunoprecipitations was carried out. Initial attempts at detecting the proteins through immunoprecipitation followed by Western blotting failed, however through the sensitivity of kinase assays, co-immunoprecipitation could be detected. Antibodies to p85α were used to immunoprecipitate PI3 kinase from P19 cells. Following immunoprecipitation, the proteins were subjected to a kinase assay under conditions where the Esk kinase is active (MES buffer pH 6.5 containing manganese). The products of the p85 immunoprecipitation were then resolved by SDS-PAGE. The results shown in figure 5 (left panel, lane 1) demonstrate that an antibody directed to the SH3 domain of p85α (gift of X. Liu) can co-immunoprecipitate a protein of 96 kDa which can be specifically re

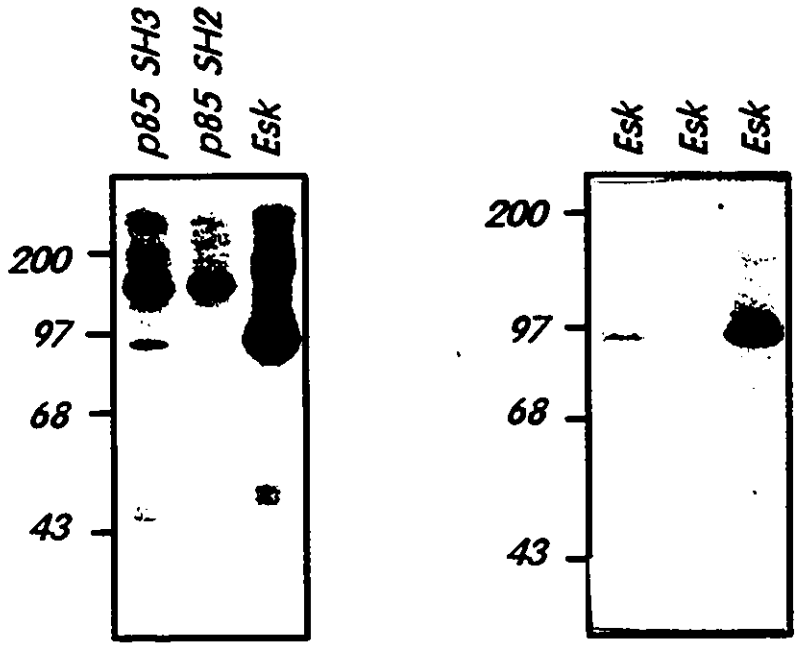
Figure 5

The Esk-2 kinase associates with the 85 kDa subunit of PI3K *in vivo*.

P19 cell extracts were immunoprecipitated with the p85α or Esk antibodies and subjected to an *in vitro* kinase assay. Half of the kinase reaction was run on 10% SDS-PAGE (left panel), while the other half was re-immunoprecipitated with the Esk antibody and subsequently run on SDS-PAGE (right panel)

Left panel: lane 1, the Esk kinase immunoprecipitated with a p85 antibody raised to the SH3 domain (p85SH3),
lane 2, the Esk kinase immunoprecipitated with a p85 antibody raised to the SH2 domain (p85SH2) and
lane 3, the Esk kinase immunoprecipitated with the Esk antibody.

Right panel: re-immunoprecipitations from the reactions in the left panel with the Esk antibody.


p85 SH3
p85 SH2
Esk
200
97
68
43
Esk
Esk
Esk
200
97
68
43

-immunoprecipitated with the Esk antibody following protein denaturation (right panel, lane 1). In contrast, a p85 antibody directed against the SH2 domain (gift of C. Carpenter) was unable to coprecipitate the Esk kinase suggesting that the SH2 domain may be directly involved in the interaction (figure 5, both panels lane 2). The tryptic peptide maps of the Esk kinase immunoprecipitated with the Esk antibody and those of the kinase immunoprecipitated with the p85 antibody are comparable and indicate that the Esk kinase indeed co-precipitates with p85 (figure 6). However, the tryptic peptide maps generated from the p85α immunoprecipitation display unique peptides not observed in the Esk immunoprecipitate (figure 6, peptides indicated by arrow heads). We have not determined if the novel peptides are responsible for the Esk kinase binding to p85α or arise as a result of the interaction with this protein. Although no phosphotyrosine could be detected on the Esk kinase associated with p85α on short exposures (figure 7, panel A), traces of phosphotyrosine could be detected after two weeks of exposure (figure 7, panel B). A small fraction of the total cellular Esk kinase was found associated with PI3K (figure 5, left panel, lanes 1 and 3) which may reflect the low levels of tyrosine phosphorylation of the kinase *in vivo*. Interestingly, the profile of phosphoamino acids of the p85α associated Esk kinase has changed, with serine being predominantly phosphorylated instead of threonine (figure 7). This difference may reflect a modification in the regulation of the Esk kinase upon binding p85α. These results therefore suggest that the Esk kinase interacts with the 85 kDa subunit of PI3K

Figure 6

Tryptic peptide maps of the Esk kinase immunoprecipitated with the Esk and p85 antibodies.

The Esk kinase was immunoprecipitated with the different antibodies and subjected to a kinase assay. The labelled proteins were trypsinized and phosphopeptides resolved by ascending chromatography (vertical direction) followed by electrophoresis in buffer at pH 1.9 (horizontal direction towards the left). The unique peptides in the Esk kinase immunoprecipitated with the p85(SH3) antibody are highlighted with arrowheads in the right panel.

Esk

p85

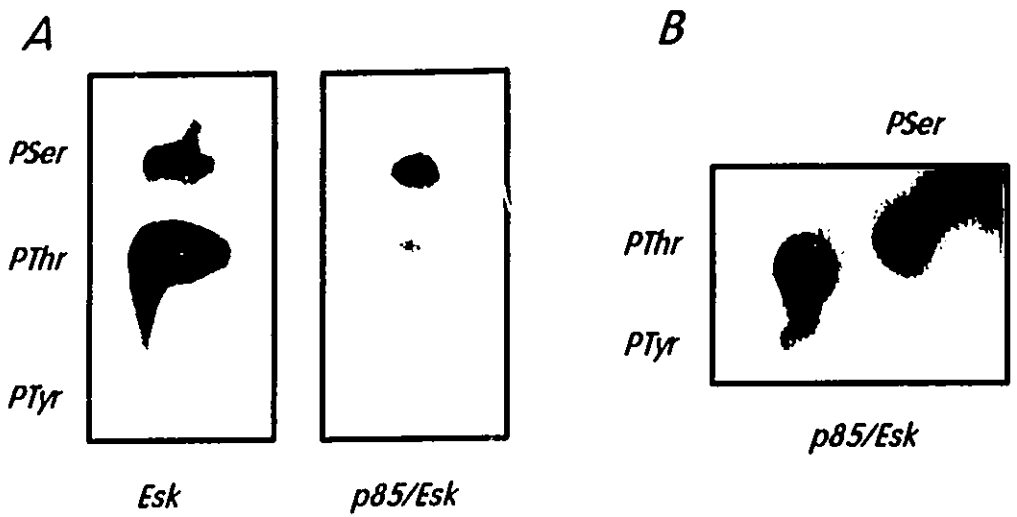
Figure 7

Phosphoamino acids analysis of the kinase immunoprecipitated with the Esk and p85 antibodies.

The Esk kinase immunoprecipitated as in figures 6 and 7 was subjected to a kinase assay. The proteins were acid hydrolysed and the phosphoamino acids resolved by one or two dimensional thin layer electrophoresis.

Panel A: phosphoamino acids of the Esk kinase immunoprecipitated with the Esk or p85 antibodies were resolved in one dimension in buffer pH 3.5. (exposed 16 hours -80°C with intensifying screen)

Panel B: the phosphoamino acids of the Esk kinase immunoprecipitated with the p85 antibody were resolved in two dimensions in buffers at pH 1.9 and 3.5 (exposed 14 days with screens at -80°C). PSer: phosphoserine, PThr: phosphothreonine and PTyr: phosphotyrosine.


A
PSer
PThr
PTyr
Esk
p85/Esk
B
PSer
PThr
PTyr
p85/Esk

in P19 cells and implies a physiological function for the tyrosine kinase activity of this dual specificity kinase *in vivo*.

The Esk-2 kinase does not coprecipitate PI3-kinase activity.

The antibody raised to the SH3 domain of the p85α subunit was found to coprecipitate the Esk kinase from P19 cells. To determine the levels of PI3 kinase activity associated with the p85α subunit and with the Esk kinase in P19 cells, PI kinase assays were carried out. P19 cell extracts were immunoprecipitated with antibodies to either p85α or Esk and the levels of associated PI3K activity measured by adding phosphatidylinositol (PI) in the kinase reaction. As shown in figure 8 (lane 1 and 4), both p85α antibodies (directed against the SH2 and SH3 domains) coprecipitated PI3 kinase activity from P19 cells as measured by the amount of phosphorylated PI (PIP). In contrast, the Esk antibody failed to coprecipitate any detectable PI3K activity (lane 2) suggesting that either the interaction is limited to the 85 kDa subunit of PI3K, or alternatively the Esk kinase is involved in the regulation of PI3 kinase activity in mammalian cells.

DISCUSSION

We and several other groups have identified a novel family of kinases that displays serine, threonine and tyrosine kinase activity, referred to as dual specificity kinases (DSKs) (Letwin *et al.*1992; Ben-David *et al.*1991; Mills *et al.*1992; Howell *et*

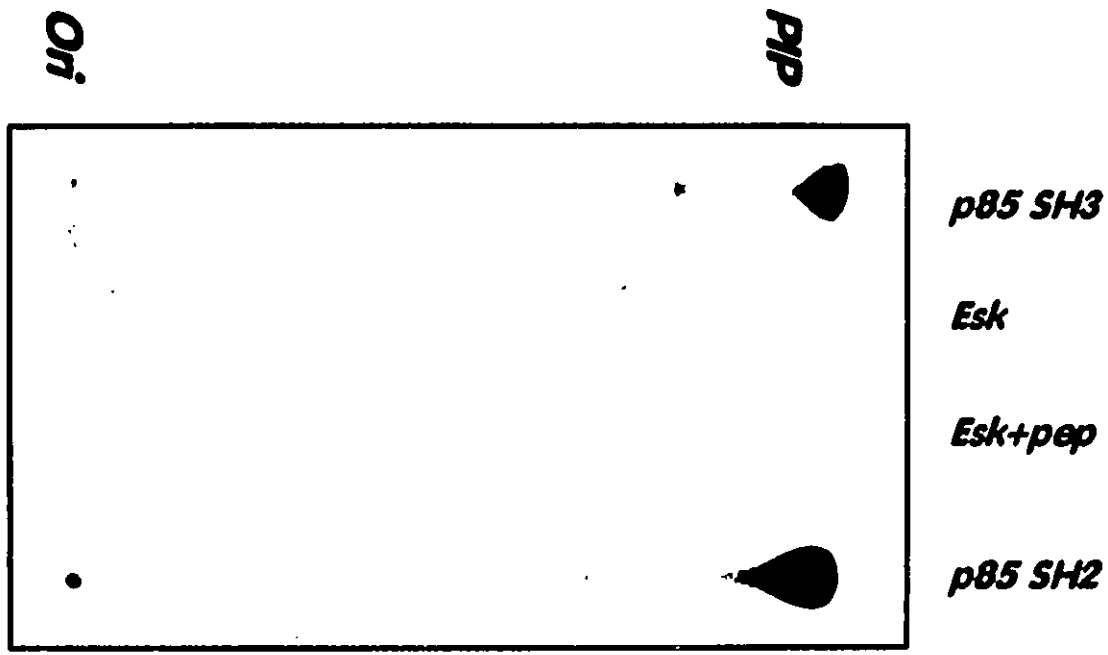
Figure 8

The Esk kinase does not coprecipitate PI3 kinase activity.

P19 cell extracts were immunoprecipitated with the Esk and p85 antibodies (SH2 and SH3). The activity of the associated PI3 kinase was measured by the addition of γ^{32}P-ATP and phosphatidylinositol (PI) to the immunoprecipitates. The phosphorylated phosphatidylinositol (PIP) was resolved by thin layer chromatography as described in materials and methods.

Lane 1, the PI3 kinase activity immunoprecipitated with the p85(SH3) antibody,
Lane 2, the PI3K activity associated with the Esk kinase in an Esk immunoprecipitate,
Lane 3, the PI3K activity associated with Esk in an Esk immunoprecipitate in the presence of competing antigenic peptide and
Lane 4, PI3K activity in a p85(SH2) antibody immunoprecipitate.

The spotting origin is marked by **ori** while the position of the phosphorylated phosphatidylinositol is marked by **PIP**.


Ori
PIP
p85 SH3
Esk
Esk+pep
p85 SH2

*al.*1991; Icely *et al.*1991; Stern *et al.*1991; Douville *et al.*1991). While most family members are immunoreactive with anti-phosphotyrosine antibodies, few have been shown to have serine/threonine and tyrosine kinase activity *in vivo*. To date, only the MAPK kinase (MEK), the activin IIB receptor and the STY kinase have been shown to autophosphorylate on all three hydroxy amino acids when isolated from mammalian cells ((Kosako *et al.*1993; Nakamura *et al.*1992) and P. Duncan and J.C. Bell, unpublished observations).

The recent identification of consensus SH2 binding sites present within several proteins (Songyang *et al.*1993) has lead to the identification of novel cryptic tyrosine phosphorylation sites. Indeed, the amino acid sequence EITEQYIYMVMEC, found in the Esk catalytic domain, displays two overlapping consensus binding sites for the 85 kDa subunit of PI3K. When expressed in bacteria, this site has been shown to be directly involved in the binding of Esk to the SH2 domains of p85α. Our results demonstrate that both the Y596 and Y598 phosphorylated peptides could efficiently prevent binding of Esk to the p85 GST-SH2 fusions (at concentrations of 10μM final). In mammalian cells, we have shown that the Esk kinase can co-immunoprecipitate with the p85α subunit of PI3K, however, not unlike the CSF-1 receptor, the *in vivo* tyrosine phosphorylation of this binding site is virtually undetectable. Indeed, Reedijk *et al.* have shown that the CSF-1 receptor Y721 residue, nested within a p85 SH2 consensus binding sequence, is directly involved in the interaction with PI3K *in vivo*. While this tyrosine residue is physiologically relevant, it represents only a minor phosphorylation site *in vivo*

and cannot be detected with conventional techniques (Reedijk *et al.*1992). The binding of the Esk kinase to the SH2 domains of p85 therefore suggests a physiological function for the low but detectable tyrosine kinase activity of this dual specificity kinase.

The Esk kinase expressed in bacteria can also bind the SH3 domain of p85α, thus we cannot rule out the possibility that the binding observed in P19 cells may be mediated through this domain. A similar model has been proposed to explain the binding of src to PI3K. While deletions within the SH2 domain of v-src decrease the *in vivo* binding to PI3K (Fukui and Hanafusa, 1989), Liu *et al.* have shown that the SH3 domain of the v-src oncogene can also mediate binding to p85 at the plasma membrane in the absence of tyrosine phosphorylation (Liu *et al.*1993). The SH3 binding sites of proteins have only recently been defined. While a proline-rich sequence is necessary for binding, the exact amino acid has not been determined. The Esk catalytic domain contains several proline-rich stretches which may mediate binding to the SH3 domain of p85, however, other SH3 domains will have to be tested in order to determine if the interaction is specific.

Until recently, most proteins found associated with phosphatidylinositol-3 kinase have been activated protein tyrosine kinases, their substrates or viral oncogenes (Songyang *et al.*1993). Indeed the levels of phosphorylated inositides in a cell can be directly correlated with cell growth and transformation (Courtneidge and Heber, 1987; Cantley *et al.*1991). To date, only one protein serine/threonine kinase has been found associated with PI3K. Carpenter *et al.* (Carpenter *et al.*1993) have shown that a manganese dependent serine/threonine kinase (PIK kinase) copurifies with PI3K and is

involved in the downregulation of the lipid kinase activity. Interestingly, the Esk DSK which coprecipitates with PI3K in P19 cells, displays predominantly a manganese dependent serine/threonine kinase activity and could similarly be involved in PI3K regulation. Although Carpenter *et al.* have not mapped the regions in PI3K responsible for binding the PIK kinase, we believe that the weak tyrosine autophosphorylation of the Esk DSK may be sufficient to trigger the binding of the 85 kDa subunit of PI3K. This interaction could then lead to the regulation of PI3 kinase activity through the serine and threonine phosphorylating activities of Esk.

Two different isoforms of the 85 kDa subunit of PI3K have been identified, p85α and p85β (Lin *et al.*1991). While the p85α subunit has been found to associate with PI3 kinase activity when purified form bovine brain (Otsu *et al.*1991), the β isoform has only been shown to coprecipitate low to undetectable levels of the lipid kinase activity (Fry *et al.*1992). To date, only one group has demonstrated PI3 kinase activity associated with the β isoform in mammalian cells (Reif *et al.*1993). While detectable, the levels of p85β and its associated PI3K activity are only ever between 3 and 14% of the levels of p85α and its associated PI3K activity (Reif *et al.*1993). Consequently, it has been proposed that the β isoform of p85 may serve as a bridging molecule between activated protein kinases and an as yet characterized protein. The Esk kinase may therefore be associated with the β isoform of p85, thus explaining our inability to detect any associated PI3 kinase activity. The apparent similarity of the p85 isoforms (62% identical) (Otsu *et al.*1991) could lead to antibody cross reactivity, and may explain the

low levels of Esk kinase immunoprecipitated with the p85α antibody. These results would suggest that a p85β antibody would immunoprecipitate higher levels of the Esk kinase from P19 cells. Alternatively, our inability to detect any PI3 kinase activity associated with the Esk DSK, may reflect the regulatory role of the Esk kinase on PI3K similar to that reported for PIK (Carpenter *et al.*1993).

The dual specificity of the Esk kinase positions it favorably to integrate into both PYK and PSK signalling pathways. The tyrosine kinase activity of the kinase may allow it to bind to downstream signalling molecules via their SH2 domains, while the serine/threonine kinase activity may serve a regulatory role. We are currently investigating the possible relationship between the Esk kinase and the different subunits (p85α and p85β) of PI3K.

CHAPTER 4

The Esk kinase isoforms arise through alternative splicing and are developmentally regulated

INTRODUCTION

Alternative splicing is the most common mechanisms employed by mammalian cells to generate diversity in the coding potential of messenger RNAs. The shuffling (addition, deletion, substitution) of exons within a given transcript increases the potential for the expression of protein products available to accomplish a certain task. The alternative usage of exons can confer both tissue specificity and specific subcellular localizations upon a given protein product. In addition, splicing can generate mRNAs that encode proteins with different affinities for ligand and/or substrate binding.

The superfamily of protein kinases takes advantage of alternative splicing to achieve the fine tuning of signal transduction observed in mammalian cells. Several growth factor receptors such as the activin IIB (Attisano *et al.*1992) and the basic fibroblast growth factor (bFGF) (Eisemann *et al.*1991) receptors, undergo alternative splicing in order to generate proteins with different ligand binding affinities. In the case of the activin IIB receptor, the different isoforms generated are thought to mediate the various effects of activin in early embryonic development and in adult tissues. During early embryonic development in *Xenopus*, activin has been shown to induce the formation of mesodermal structures (Thomsen *et al.*1990). In adult tissues, the effects of activin include the stimulation of hormone production from cells in the anterior pituitary, as well as from cells in the ovaries and placenta (Vale *et al.*1986; Petraglia *et al.*1989), neural cell survival (Schubert *et al.*1990) and inhibition of neural cell differentiation (Hashimoto *et al.*1990). Exon switching in the FGF receptor family is responsible for the generation

of autocrine FGF ligand and receptor loops in malignant epithelial tumor cells (Yan *et al.*1993).

Alternative splicing can also generate tissue specificity in protein kinases. For example, the Fyn cytoplasmic kinase and the Ltk transmembrane receptor both exhibit splicing variants in the brain and in cells of the lymphoid lineage (Cooke and Perlmutter, 1989; Bernards and De la Monte, 1990). Finally, several receptor protein kinases assume distinct subcellular localizations due to alternative splicing. A truncated, secreted form of the epidermal growth factor receptor (EGFr) has been observed in normal rat tissues (Petch *et al.*1990), whereas at least four different forms of the K-*sam* tyrosine kinase receptor can be generated through alternative splicing in different cell lines. Like the EGFr, K-*sam* can be secreted as a truncated form lacking a catalytic domain, however a full length secreted kinase is also predicted from cDNA sequencing, the function of which is unknown (Katoh *et al.*1992).

The Esk dual specificity kinase was initially cloned from an embryonal carcinoma cell line and found to be encoded by two distinct mRNAs. The cloned cDNAs, Esk-1 and Esk-2, differed by a unique stretch of 78 base pairs found only in the Esk-1 cDNA. This unique sequence predicted a protein segment with a high hydrophobic index which could correspond to a transmembrane domain (Douville *et al.*1991). Here we describe that the isoforms arise through alternative splicing of the same primary transcript to give rise to transmembrane (Esk-1) and cytoplasmic (Esk-2) proteins. As with other alternatively spliced protein kinases (Lynch *et al.*1986), we show that the Esk kinases are

subject to developmental regulation, expressed predominantly in cell lines with high proliferation rates, such as stem cells and absent in terminally differentiated cell lines and adult tissues.

MATERIALS AND METHODS

Screening and isolation of genomic clones.

A partial Esk-1 cDNA clone encompassing the transmembrane domain was used to screen a mouse embryonic stem cell line (D3) library (generous gift of D.A. Gray). Three positives clones were identified and plaque purified: EG101, EG81 and EG32. Partial restriction mapping using the Promega Lambda MAP™ system, and oligonucleotide probing were used to characterize the genomic clones and determine the exons that contained the transmembrane domain and translation initiation codon of the cDNA. Genomic clones EG101 and EG32 were identical and spanned from the 5'end of the cDNA to sequences downstream of the transmembrane domain. Genomic clone EG81 overlapped with the two others and also encompassed the transmembrane domain.

Rapid amplification of complementary ends (RACE).

To extend the 5' end of the Esk cDNA, a RACE procedure was carried out. First strand cDNA synthesis was carried out from 1μg of poly (A^+) mRNA derived from early mouse embryos using the procedure described by the manufacturer of Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL). Briefly, poly (A^+) RNA was

incubated with 100 picomoles of primer [Esk oligos XTL-1 (5'ATCGTCACACTGCTG CCAATT-3') or XTL-2 (5'-CCCAAGCTTCGGTGTGATCGGCACAG-3') directed to the 5' end of the cloned cDNA], denatured at 70°C for 10 min. and transferred to ice. Reaction buffer (5X), dithiothreitol (DTT) - 10mM final conc., dNTPs (0.5mM final conc. of each dATP, dCTP, dGTP and dTTP) and enzyme were added to a final volume of 20μl and incubated at 42°C for 60 min. The single stranded DNA (sscDNA) generated was ethanol precipitated, resuspended in 13.7μl water, and used for a oligo dG tailing reaction. Briefly, the sscDNA was incubated with terminal deoxynucleotidyl transferase (TdT) buffer (Gibco-BRL), dGTP (5μM final conc.) and TdT enzyme for 30 min. at 37°C in a final reaction volume of 25μl. The reaction was ethanol precipitated and resuspended in 10μl of water. For PCR amplification, 2.5μl of the tailed sscDNA was incubated with 75 picomoles of primers [oligo dC and Esk specific oligo, XTL3A (5'-TCTGAAAGCCAAGCTGCA-3'] and amplified with Taq polymerase (Gibco-BRL). After two rounds of PCR amplification using nested oligos (oligo dC and XTL3A), the reactions products were resolved on a 4% (3:1, Nusieve: Gico-BRL agarose) agarose gel. The cDNA product was stabbed with a pipette tip, and the contents sequenced using the New England Biolabs PCR sequencing kit and an Esk specific oligo (XTL3A).

Transfection of Cos-1 cells.

Electroporation of Cos-1 cells was as described in chapter 3 in materials and methods. The construction of the pECE-Esk-2 plasmid was also described in chapter 3.

The pECE-Esk-1 plasmid was generated from pECE-Esk-2 in a multi-step fashion. Briefly, a HindIII fragment encompassing the transmembrane domain of Esk-1 was exchanged for the equivalent fragment of the Esk-2 cDNA in pECE. The 5' end of the constructed pECE-Esk-1 plasmid, was reconstituted by swapping PstI fragments (PstI cuts once in Esk, and once in the ampicillin resistance gene of the pECE expression vector) between both pECE-Esk constructs. The chloramphenicol acetyl transferase (CAT) gene under the control of the same regulatory elements (SV40 promoter/enhancer) (Gorman *et al.*1982) was electroporated into Cos cells as a negative control.

Differentiation of P19 cells.

P19 cells were maintained in alpha MEM medium supplemented with 10% serum (50% fetal calf serum and 50% calf serum). For differentiation experiments, the cells were maintained in alpha MEM medium supplemented with 10% calf serum only. Typically, to induce P19 cells to differentiate into the neural lineage, $2X10^6$ cells were aggregated in bacterial dishes in the presence of 0.5μM retinoic acid (RA) for a total of four days (the aggregates were transferred to fresh medium containing RA after two days). At day four of differentiation, the aggregates were plated onto tissue culture dishes in the absence of drug for the remaining time course. Typically neurons were visible by day six of differentiation.

Immunoprecipitation and kinase assay from mouse adult tissues.

Adult mice were dissected and tissues collected for analysis of Esk protein kinase activity. Individual tissues were initially cut up (teased) in petri dishes prior to addition of lysis buffer. The lysates were homogenized on ice by sonication (three times 20 seconds) and the extracts cleared by centrifugation at 30 000 rpm (Beckman tabletop ultracentrifuge) for 20 min. at 4°C The cleared lysates were immunoprecipitated with 40 μl (1:200 dilution) of crude anti-Esk rabbit polyclonal antibody (described in chapter 3).

RESULTS

A unique exon encodes the putative transmembrane domain of the Esk-1 kinase.

The Esk cDNAs were originally cloned from an embryonic stem cell line and found to encode two distinct isoforms, putatively a transmembrane and a cytoplasmic isoform (refer to chapter two). While human homologs of the Esk-2 isoform have been cloned from both fibroblast (PYT) (Lindberg *et al.*1993) and NK cell lines (TTK) (Mills *et al.*1992), no homologs of the Esk-1 isoform have been identified to date. To verify the authenticity of the Esk-1 kinase isoform, a genomic library generated from an embryonic stem cell line (D3) (generous gift of D.A. Gray), was screened with a partial Esk-1 cDNA encompassing the transmembrane domain. Three overlapping clones spanning a total of 19.4 Kb of genomic DNA were identified: EG101, EG81 and EG32. The three clones were found to span from the 5'end of the Esk cDNA to sequences

beyond the transmembrane domain. A schematic representation of the clones EG101 and EG81 is shown in figure 1. Partial restriction mapping of the genomic clones was carried out (shown in figure 2) and sequences corresponding to the transmembrane domain of Esk-1 were found to reside on a unique 3.6 Kb HindIII fragment of clone EG81. Sequencing of this region with specific oligonucleotides revealed that the putative transmembrane domain resided on a single exon and that alternative splicing could generate mRNAs encoding both Esk isoforms (figure 3).

The Esk-1 cDNA sequence predicted a transmembrane domain however, no leader peptide necessary for the translocation of the protein to the plasma membrane, was encoded within the isolated cDNA. Partial sequencing of the genomic exons containing the 5' initiating methionine and the upstream untranslated region, failed to reveal the presence of a leader peptide. Interestingly, the 5' most exon sequenced contained nucleotide motifs reminiscent of promoter elements including CAAT boxes (Jones *et al.*1987; Dorn *et al.*1987), E boxes (Murre *et al.*1989) and GC boxes (Kageyama *et al.*1989) (figure 4). The presence of these putative promoter sequences, however, does not preclude the possibility of an upstream exon encoding a leader peptide. In such a scenario, the alternative usage of promoters could lead to the expression of different Esk isoforms as has been described for the PDGF receptor in EC cells (full length and truncated membrane-bound forms) (Vu *et al.*1989). Finally, in collaboration with Nancy Jenkins, the Esk gene was found to map on mouse chromosome 9 near the dilute locus (figure 5). This locus is found to contain a number of embryonic lethal genes and may

Figure 1

Schematic representation of Esk genomic clones EG101 and EG81.

Three genomic clones were isolated from an embryonic stem cell line library (D3). The clones were found to span a length of ~ 19.4 kilobases of DNA and encompass the 5'end and the transmembrane domain of the cDNA. The genomic clones EG101 and EG81 overlap over 7 kilobases and contain the 5' initiating methionine and transmembrane exons, respectively (highlighted by arrows).

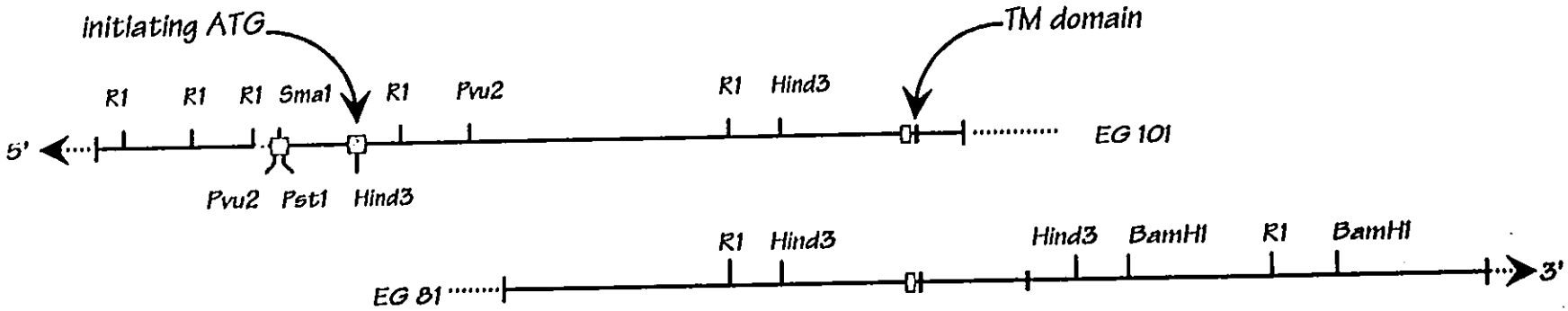
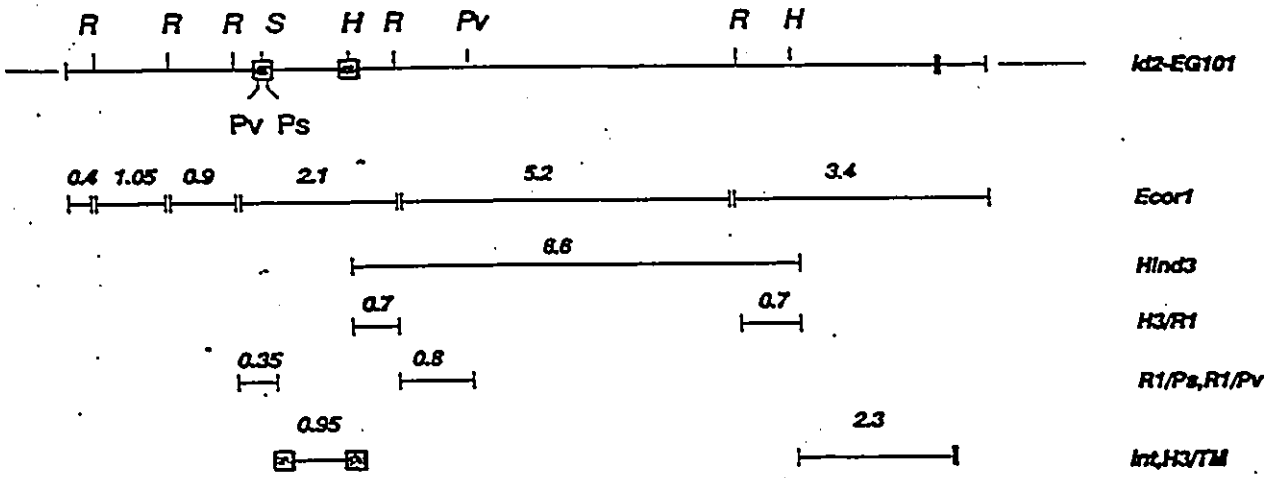

initiating ATG
TM domain
R1
R1
R1
Sma1
R1
Pvu2
R1
Hind3
5'
EG 101
Pvu2
Pst1
Hind3
R1
Hind3
Hind3
BamHI
R1
BamHI
EG 81
3'

Figure 2

Partial restriction mapping of genomic clones EG101 and EG81.

The genomic clones were mapped using the Promega LambdaMap™ system. The EcoR1 sites were mapped first, and all other restriction sites mapped relative to these. Exons are depicted as boxes; black boxes indicate exons for which the sizes and boundaries are known, grey boxes indicate exons for which one boundary has been set but not the other. The distances between the restrictions sites, exons and/or introns are indicated below each clone. The transmembrane domain exon was found to reside on a unique 3.6 Kb HindIII fragment in the genomic clone EG81. Abbreviations: ld2: lambda dash 2, R, R1: EcoRI, B, Bh1: BamHI, H: HindIII, S: SmaI, Pv: PvuII, Ps: PstI, Int: Intron. Note that the PvuII and PstI sites are not unique.


R R R S H R Pv R H
Pv Ps
kd2-EG101
0.4 1.05 0.9 2.1 5.2 3.4
Ecor1
6.6
Hind3
0.7 0.7
H3/R1
0.35 0.8
R1/Ps,R1/Pv
0.95 2.3
Int,H3/TM

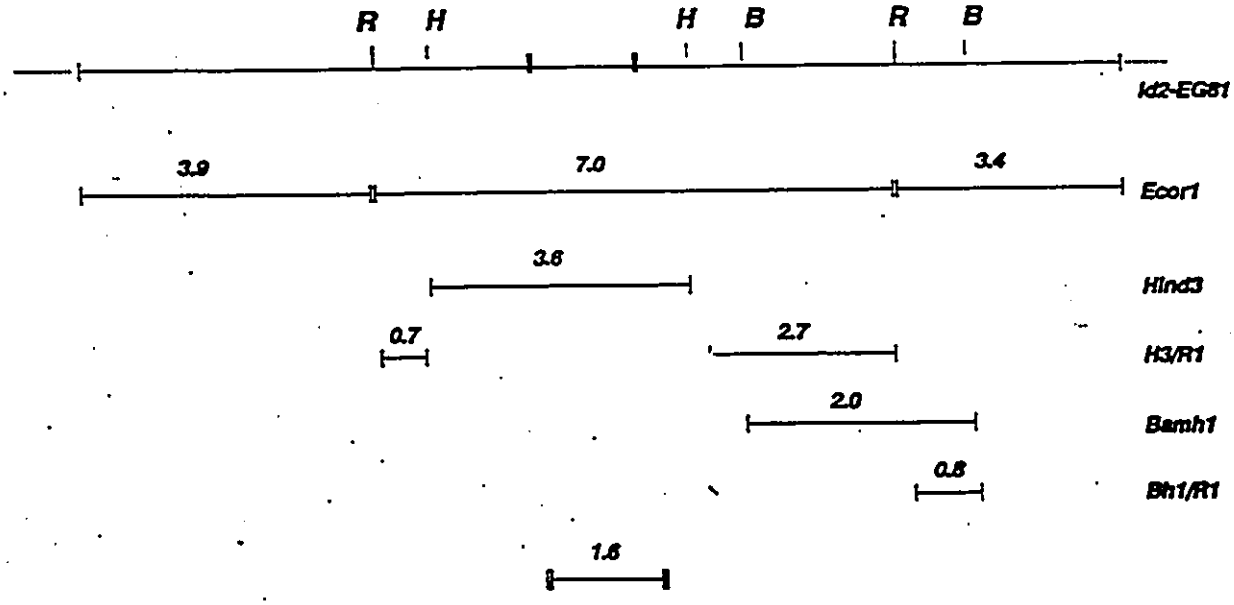

R H H B R B
kd2-EG81
3.9 7.0 3.4
Ecor1
3.6
Hind3
0.7 2.7
H3/R1
2.0
Bamh1
0.8
Bh1/R1
1.6

Figure 3

The transmembrane domain of the Esk-1 kinase is encoded by a unique exon.

The sequence of the intron/exon boundaries is presented, together with a scheme for the alternative splicing of the exons generating the Esk-1 and Esk-2 isoforms. The transmembrane domain exon is 78bp in length and can be spliced in or out of the transcript to generate in frame protein products.

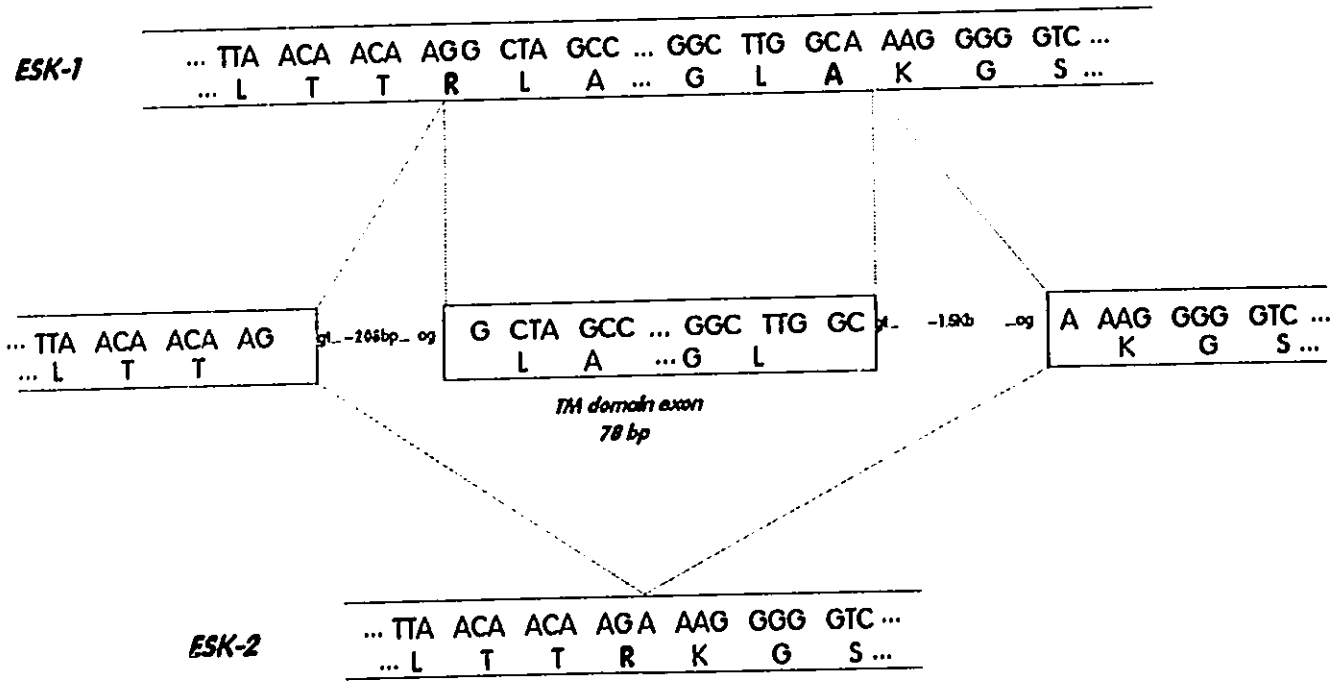

ESK-1
... TTA ACA ACA AGG CTA GCC ... GGC TTG GCA AAG GGG GTC ...
... L T T R L A ... G L A K G S ...
... TTA ACA ACA AG
... L T T
G CTA GCC ... GGC TTG GC
L A ... G L
TM domain exon
78 bp
A AAG GGG GTC ...
K G S ...
ESK-2
... TTA ACA ACA AGA AAG GGG GTC ...
... L T T R K G S ...

Figure 4

Sequence of the 5' exons of the Esk gene.

A combination of 5'RACE and genomic sequencing was used to extend the Esk cDNA sequence and ascertain the existence of a leader peptide. The initiating methionine of the Esk cDNAs has been outlined. 5'RACE (bold sequences) extended the Esk transcript a further 48 base pairs upstream of the cloned cDNA which is marked by an asterisk. Genomic sequencing revealed that the 5'untranslated region was interrupted by an intron (small letters in italics). Sequences upstream of the transcription initiation site were found to contain several transcription regulatory motifs: CAAT boxes (underlined), E boxes (italics) and GC boxes (double underlined). No conventional TATA boxes were found in these upstream sequences.

GC box

GAATTCTAAC AAAGAGAAGG GCGGGTCTTG GGttGAAGGC GAGGCCAGGG

CTCCcTGGaG GTaGTGCAAG GTCCAGCGGG AGAGCACCAA AGAAAAAGAC

E

CCTGACCCct GACcgTACAG GCGCAGGTCA GcACggcGAG CgcgTCGGcA

box

*gCTG*CGGGTA ACTCCCCGGG CCGCTTGAAG TGGGTGGGTC CGGCCTCAAC

GC box -73 E

GCAGGAGGGG CGGTGCGACG CAAGGAGCCA ATCACGGCCC ACGCTTCCAC

box -36

*GTG*GCGAGCC GTAACCAATG AGAGGTAGCG GGGAAGGCAG CGGGGAAATT

+1

CAAACGTGTT TCCGGAGAGG GGTTGGGGTG CCGCTGGTTT GGGCTGCAGC

TTGGCTTTCA GAG*gtgggtg catcagagcg ggcatggcag agaggctgcg*

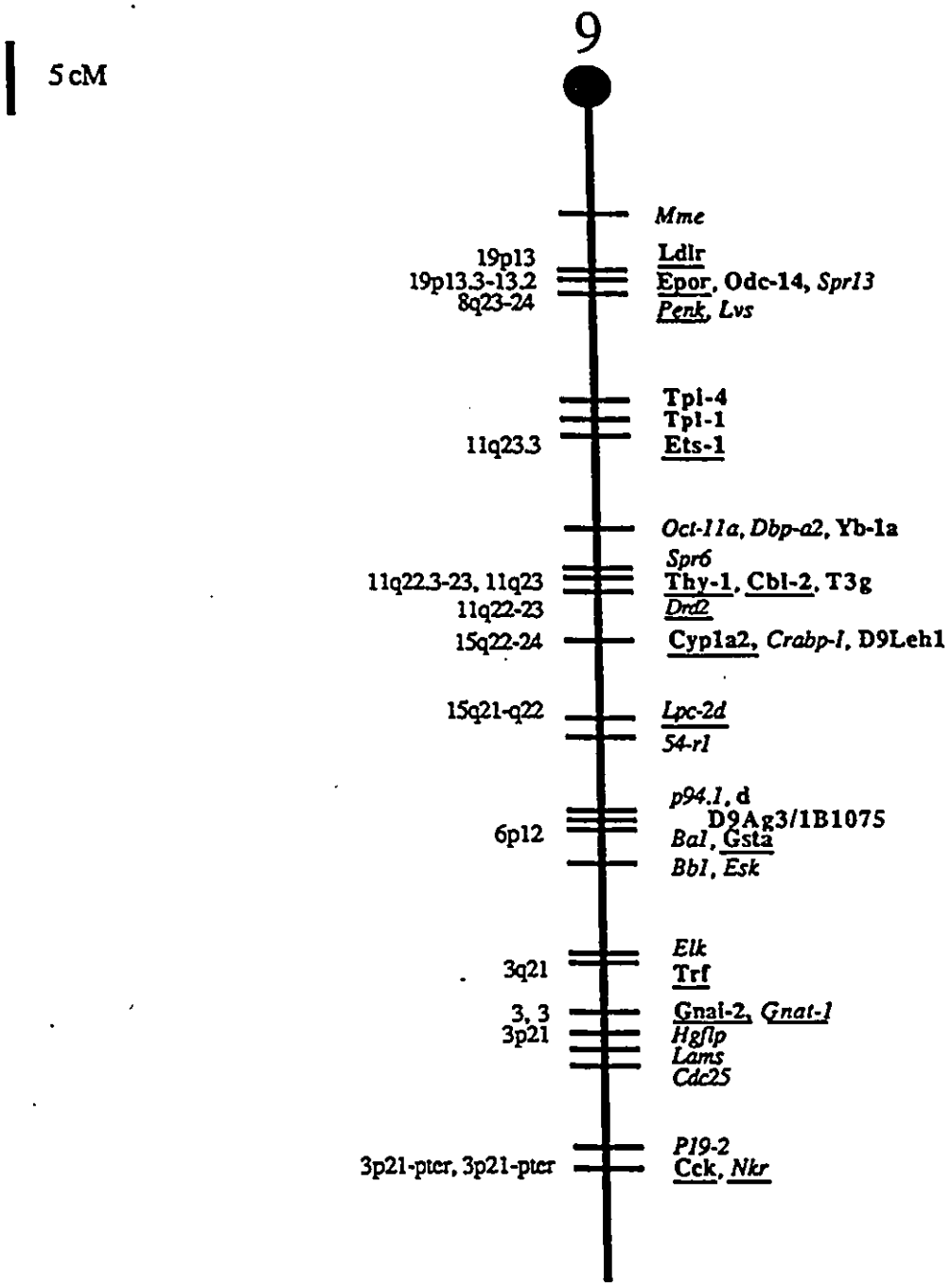
gccggcggga ggctggctgg gtgac .....intron.....

*ttccaaag*ACGAGTTACAATTTAGAAATG

Figure 5

The Esk gene is localized to mouse chromosome 9.

A non repetitive genomic probe of murine Esk was used to position the gene within a [(C57BL/6J X *Mus Spretus*)F_1 X C57BL/6J] interspecific backcross map. Loci that have been mapped in human chromosomes are underlined and the positions are indicated. Loci that are not italicized and are shown in bold print have been published. The map locations of loci shown in italics have not yet been published. The Esk gene was found to map near the dilute locus (**d**). Map provided by Nancy Jenkins.


5 cM
9
Mme
19p13
Ldlr
19p13.3-13.2
Epor, Odc-14, Spr13
8q23-24
Penk, Lvs
Tpi-4
Tpi-1
11q23.3
Ets-1
Oct-11a, Dbp-a2, Yb-1a
Spr6
11q22.3-23, 11q23
Thy-1, Cbl-2, T3g
11q22-23
Drd2
15q22-24
Cyp1a2, Crabp-1, D9Leh1
15q21-q22
Lpc-2d
54-rl
p94.1, d
D9Ag3/1B1075
6p12
Bal, Gsta
Bbl, Esk
Elk
3q21
Trf
3, 3
Gnai-2, Gnat-1
3p21
Hgflp
Lams
Cdc25
P19-2
3p21-pter, 3p21-pter
Cck, Nkr

indicate that the Esk kinases are involved in embryonic development.

The Esk kinase is downregulated during P19 induced neuronal differentiation.

To determine the expression pattern of the different Esk isoforms, immunoprecipitation/kinase assays were carried from P19 EC cells. While the peptide antibody generated against the terminal twelve amino acids can recognize both Esk isoforms when expressed in cos cells (figure 6), P19 cells were found to express predominantly the Esk-2, cytoplasmic isoform, as determined by tryptic peptide mapping analysis (refer to chapter 3, figure 1 and 2). However, given the migration pattern of the kinases in cos cells, we can not rule out the possibility that the observed kinase in P19 cells represents a mixed population of Esk-1 and Esk-2.

In P19 cells, a larger molecular weight protein of approximately 150 kDa was found to coprecipitate with the Esk kinase (figure 7, lane 1). This protein was specifically immunoprecipitated with the Esk antibody since addition of the immunogenic peptide to the cell lysate blocked its immunoprecipitation (figure 7, lane 2). Whereas the levels of the 96 kDa Esk kinase decreased with P19 neuronal differentiation (figure 7, lane 3), as revealed by kinase assay (figure 7) and Western blotting (data not shown), coincidently, the levels of the 150 Kda protein band increased (revealed by kinase assay only). In order to determine at which point the Esk kinase was downregulated, a differentiation time course of P19 cells was carried out. P19 EC cells were induced to differentiate with retinoic acid and harvested for kinase assays from day 0 to day 11.

Figure 6

The Esk antibody recognizes both Esk isoforms expressed in cos cells.

Cos cells were electroporated with the Esk-1 and Esk-2 cDNAs subcloned in the pECE expression vector, under the control of the SV40 promoter/enhancer elements (refer to materials and methods). Cos cells were tranfected with pSV_2CAT as a negative control. The Esk kinases were immunoprecipitated with Esk polyclonal antibody from the cos transfected cells and subjected to an *in vitro* kinase assay.

Lane 1: ip/kinase assay from cos cells transfected with the pSV_2CAT plasmid,
Lane 2: ip/kinase assay from cos cells transfected with pECE-Esk-1,
Lane 3: ip/kinase assay from cos cells transfected with pECE-Esk-2, and
Lane 4: kinase assay from untransfected cells immunoprecipitated with Protein-A sepharose beads only.

Positions of the Esk-1 and Esk-2 kinases are indicated by arrows.

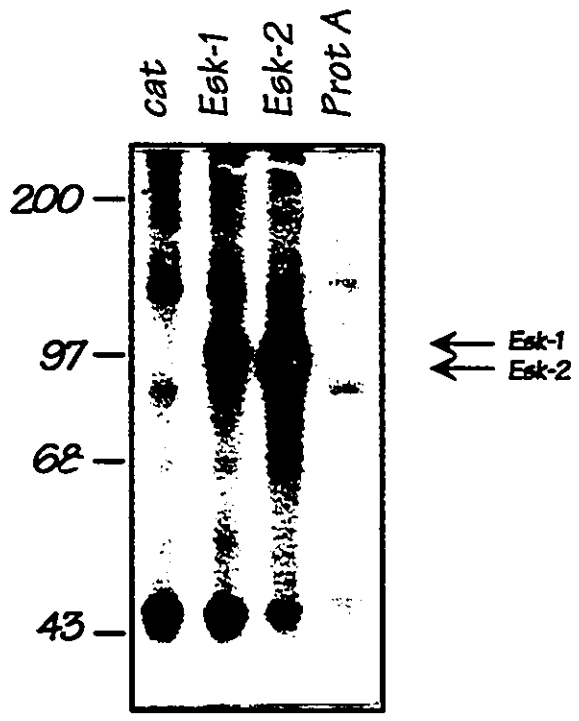

cat
Esk-1
Esk-2
Prot A
200
97
68
43
Esk-1
Esk-2

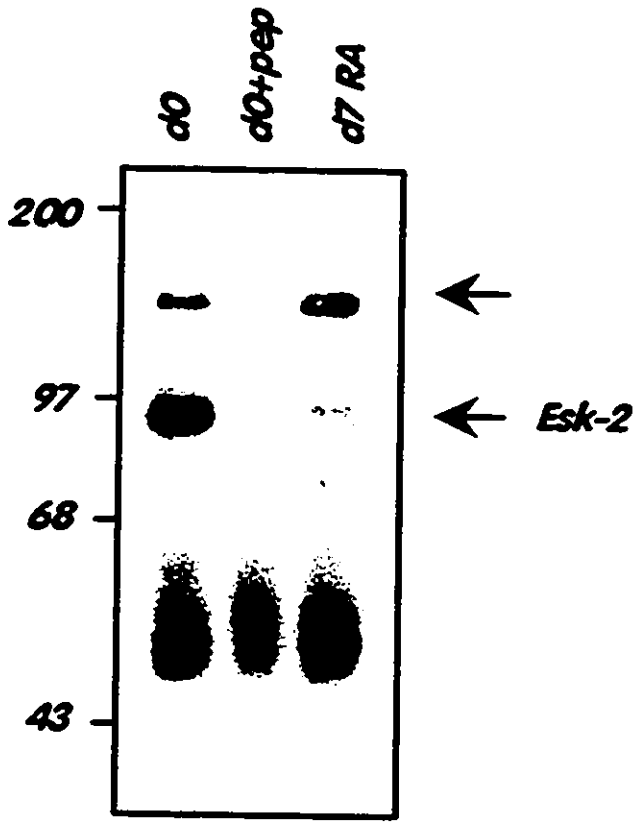
Figure 7

P19 EC cells express the 96 kDa Esk kinase and a 150 kDa protein.

Immunoprecipitation/kinase assays were performed from P19 cells and their differentiated derivatives using the rabbit polyclonal Esk antibody. P19 stem cells (day 0) and P19 cells induced to differentiate with retinoic acid (day 7) were lysed, and equal amounts of protein used for an immunoprecipitation (ip) followed by a kinase assay. The products of the kinase reaction were resolved on 10% SDS-PAGE.

Lane 1: ip/kinase assay from P19 stem cells,
Lane 2: same as lane 1 except in the presence of 25 μg immunogenic peptide,
Lane 3: ip/kinase assay from P19 cells induced to differentiate with RA and harvested at day 7.

The sizes of the molecular weight markers are indicated on the left. The Esk-2 kinase runs as a 96 kDa protein, and the position of the 150 kDa protein is indicated by an arrow.


d0
d0+pep
d7 RA
200
97
68
43
Esk-2

Cell lysates were immunoprecipitated with the Esk specific antibody and subjected to a kinase assay. The reaction products were then re-immunoprecipitated (rip) with the antibody in the presence or absence of competing peptide (figure 8). The results demonstrate that the Esk kinase activity disappears by day 6 of differentiation, while the 150 kDa protein appears by day 4 and peaks until day 8 (figure 8). Since the 150 kDa can be reprecipitated with the Esk antibody following protein denaturation, it is unlikely to be an associated protein. In order to determine if the 150 kDa protein was related to the Esk kinase, tryptic peptide mapping was carried out on the proteins immunoprecipitated from P19 cells. As shown in figure 9, the phosphopeptide maps of the 96 and 150 kDa proteins were distinct, however, in the absence of a mixing experiment, no conclusions can be drawn as to the relatedness of the proteins. Finally, since the proteins appear to share the same epitope, the results suggest that either the 150 kDa protein is an Esk related protein, or alternatively, the 150 kDa protein is an unrelated protein that cross-reacts with the Esk antibody.

The 150 kDa protein is expressed in murine adult tissues.

Northern blot analysis of the Esk messenger RNAs in adult tissues revealed that the transcripts were most highly expressed in cell lines with high proliferation rates including embryonic and leukaemic cell lines, or in tissues with stem cell compartments including spleen, thymus, bone marrow and testes (refer to chapter 2, figure 1). In order to determine the expression pattern of the Esk kinase in adult mice, tissues were

Figure 8

The Esk kinase is developmentally regulated during retinoic acid induced neuronal differentiation of P19 cells.

A time course experiment of RA treatment of P19 cells was carried out to determine the expression patterns of the Esk kinase and the 150 kDa protein. P19 cells were induced to differentiate with retinoic acid and harvested at time intervals over a period of 11 days. Equal amounts of protein were immunoprecipitated with the Esk antibody and subjected to a kinase assay. The final kinase assay reactions were split in two, and each half re-immunoprecipitated in the absence (-) or presence (+) of competing peptide (25μg). The products of the reaction were resolved on 10% SDS-PAGE. Each lane represents a re-immunoprecipitation at the day indicated above the lane. The positions of the 150 kDa protein and the Esk kinase are indicated by arrows.

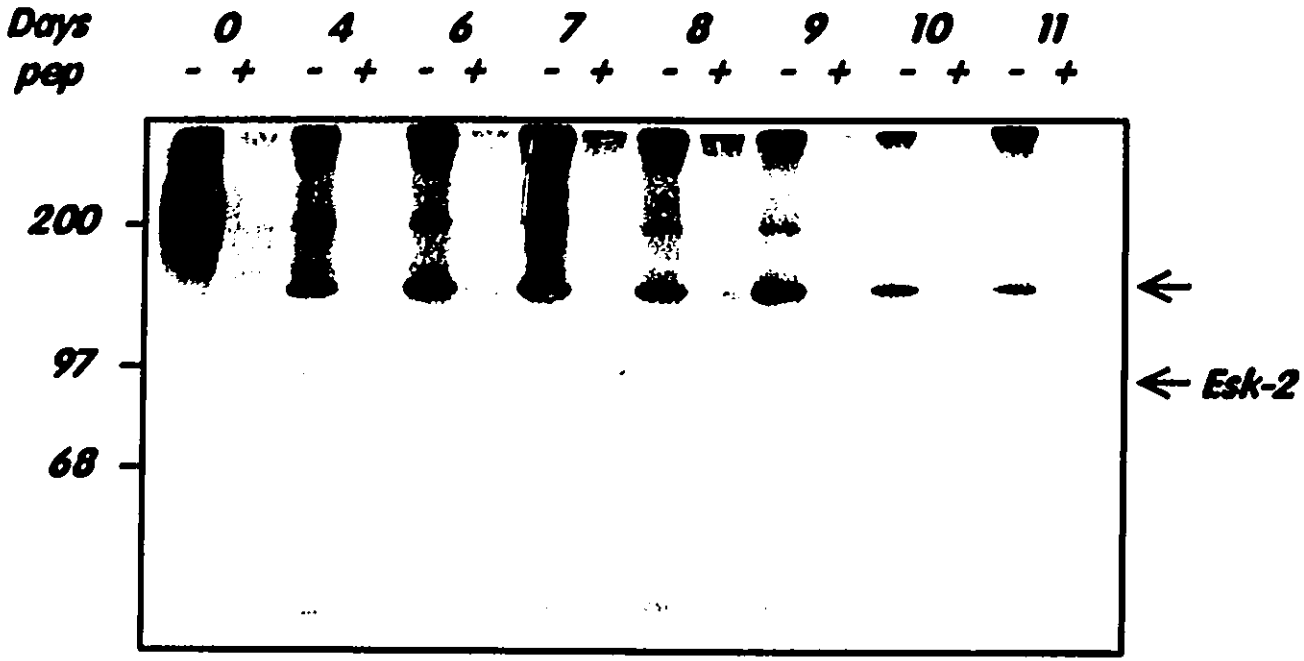

Days
0
4
6
7
8
9
10
11
pep
- + - + - + - + - + - + - + - +
200
97
68
Esk-2

Figure 9

Tryptic peptide maps of the 96 and 150 Kda proteins present in P19 cells.

Proteins corresponding to the 150 kDa protein and the Esk kinase were excised from a nitrocellulose filters and subject to trypsin digestion overnight. The individual phosphopeptides were resolved by ascending chromatography followed by electrophoresis in buffer at pH 1.9.

Left panel: tryptic peptide map of the 96 kDa Esk-2 kinase,
Right panel: tryptic peptide map of the 150 kDa protein.

The direction of the chromatography and electrophoresis is indicated by arrows.

p96
p150
IEF
2D

collected from mice at different ages, homogenized, immunoprecipitated with the Esk antibody and subjected to kinase assays. The samples were then re-immunoprecipitated as described in the previous section. As shown in figure 10 (third lane of each set), most tissues tested were found to express predominantly the 150 kDa protein, with the exception of testes which was also found to express the 96 kDa Esk kinase. This result, although unexpected, may be consistent with the notion that the Esk kinase is predominantly expressed in rapidly dividing cells, such as stem cells, while the 150 kDa protein is mainly associated with differentiated cell lineages. The presence of both proteins in testes may reflect the nature of this tissue which contains both stem cells (spermatids) and terminally differentiated cell types (Sertoli, Leydig). Although the identity of the 150 kDa protein is unknown, the results suggest that an inverse relationship exists in the expression pattern of the 96 kDa Esk kinase and the 150 kDa protein.

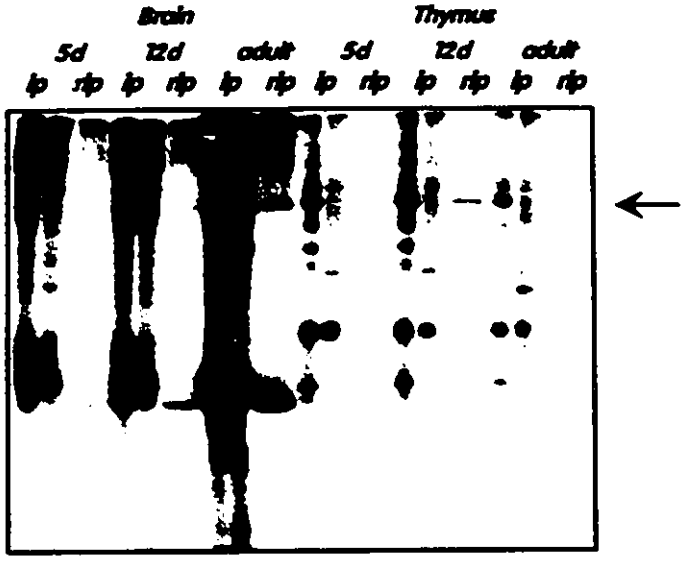
The 150 kDa protein associates with phosphatidylinositol 3'-kinase.

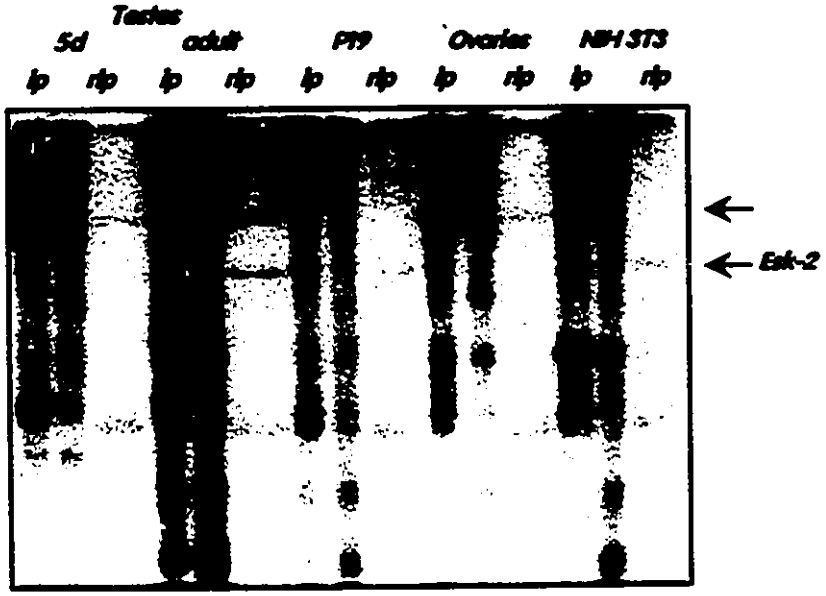
We have previously shown that the Esk kinase can associate with the 85 kDa subunit of PI3K *in vitro* using bacterial expression of the Esk catalytic domain and of the SH2 domains of the p85α protein (chapter 3). This association was also observed *in vivo* by co-immunoprecipitation of the Esk kinase with antibodies directed against p85α. In view of the interesting relationship between the 150 kDa protein and the Esk kinase, we wanted to determine if the p85 binding extended to the 150 kDa protein in mammalian

Figure 10

The 150 kDa protein is expressed in adult mouse tissues.

Mouse adult tissues were lysed, immunoprecipitated with the Esk antibody in the presence or absence of peptide and subjected to a kinase assay as described (first two lanes of each set of tissues indicated above the lanes). In the last lane of each set (rip), the initial kinase assay was subjected to a second round of immunoprecipitation in the absence of competing peptide. The products of the reaction were resolved on 10% SDS-PAGE. The initial immunoprecipitation was carried out from different amounts of protein. The positions of the 150 kDa and Esk proteins are indicated by arrows.


Brain
Thymus
5d
12d
adult
5d
12d
adult
lp
nlp
lp
nlp
lp
nlp
lp
nlp
lp
nlp
lp
nlp


Testes
5d
adult
P19
Ovaries
NIH 3T3
lp
nlp
lp
nlp
lp
nlp
lp
nlp
lp
nlp
Esk-2

cells. We chose to analyze this interaction in a more differentiated cell line, NIH 3T3 cells. The 96 kDa Esk kinase is predominantly expressed in proliferating NIH 3T3 cells, (figure 11, lane 1), however, preliminary data suggests that the expression of the 150 kDa protein is induced in quiescent NIH 3T3 cells (Marc Foot and John Bell, unpublished observations). In the experiment shown in figure 11, proliferating NIH 3T3 cells were lysed, immunoprecipitated with anti-Esk or anti-p85α antibodies and subjected to a kinase assay. While the 96 kDa Esk kinase was expressed at higher levels than the 150 kDa protein in NIH 3T3 cells, the 150 kDa protein co-immunoprecipitated with the p85 antibody with higher stoichiometry. These results suggest that similarly to the Esk kinase, the 150 kDa protein can also interact with the 85 kDa subunit of PI3K. In addition, the results suggest that the p150/p85 interaction occurs with higher affinity or alternatively is more stable than the Esk/p85 interaction.

DISCUSSION

The Esk kinase isolated from P19 embryonal carcinoma cells exists in two isoforms, putatively cytoplasmic and transmembrane. Isolation of the gene encoding the murine Esk kinase has revealed that a unique exon encodes the transmembrane domain, such that alternative splicing could account for both isoforms. Several protein kinases have been shown to be alternatively spliced in mammalian cells. Specifically, receptor protein kinases have been shown to undergo extensive alternative splicing to generate receptor molecules with different ligand binding affinities or alternatively, with distinct

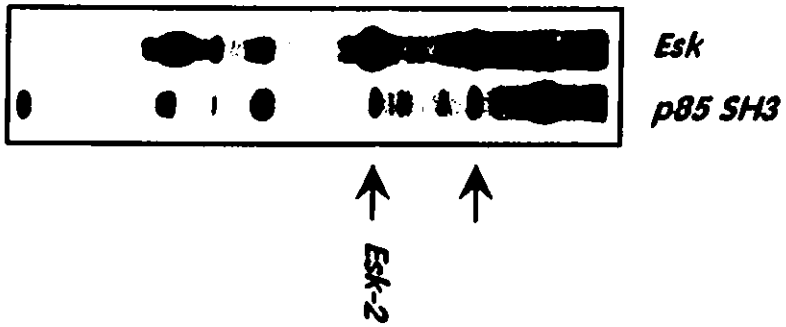
Figure 11

The 150 kDa protein and the Esk kinase interact with the 85 kDa subunit of PI3K in NIH 3T3 cells.

NIH 3T3 cells were lysed, immunoprecipitated with the Esk or p85α antibodies and subjected to a kinase assay.

Lane 1: anti-Esk immunoprecipitate,
Lane 2: anti-p85α immunoprecipitate.

Both lanes show the Esk kinase at 96 Kda and the 150 kDa protein. Proteins were resolved on 8.0% SDS-PAGE.


Esk
p85 SH3
Esk-2

subcellular localizations. Indeed the alternative splicing described for the EGF and FGF receptors as well as for the K-*sam* PYK predict membrane bound proteins as well as full length and/or truncated proteins that are secreted (Petch *et al.*1990; Katoh *et al.*1992; Eisemann *et al.*1991). In contrast, the alternative splicing described for the Esk kinase is unique in that it predicts a kinase that exists as both a soluble and membrane bound protein. The apparent lack of a leader peptide in the Esk-1 kinase might limit its localization to the endoplasmic reticulum or the nuclear membrane, in order to orient the catalytic domain of the kinase within the cell. However, proteins spanning the plasma membrane that lack leader peptides have been described, including the neu differentiation factor (Wen *et al.*1992). The functional significance of the Esk isoforms and their regulation is still unclear and may be best addressed by deleting the gene products through homologous recombination.

We have shown that P19 EC cells predominantly express the 96 kDa Esk kinase. This isoform of the kinase appears to be associated with stem cells, or cells with high proliferation rates, since it is highly expressed in the undifferentiated P19 cells, and is rapidly downregulated during retinoic acid induced neuronal differentiation. Consistent with a possible role in cell proliferation, the Esk kinase is virtually absent in all adult tissues examined with the exception of testes which are known to contain a stem cell compartment. Interestingly, we found that a larger molecular weight protein ($\sim$150 Kda) coprecipitated with the Esk kinase from P19 cells. In contrast to the Esk kinase which was found to be highly expressed in proliferating cells, the 150 Kda protein was

found associated with differentiated cells. The expression of this protein was induced in differentiating P19 cells and was also found in all adult tissues assayed.

The 150 kDa protein is unlikely to be an Esk-associated protein since it was specifically recognized by the Esk antibody. The immunoprecipitation of the 150 kDa protein was blocked by the addition of competing peptide, and it could be re-immunoprecipitated following protein denaturation. The 150 kDa protein could be an Esk related protein, since it appears to possess intrinsic kinase activity, and similarly to the 96 kDa Esk kinase, it can associate with the 85 kDa subunit of PI3K. In contrast, the 150 kDa protein is not recognized by the Esk antibody on Western blots, and when expressed in cos-1 cells, the Esk-1 kinase runs as a 98 kDa protein rather than 150 kDa (figure 6). While the migration of the Esk-1 kinase in cos cells suggests that the 150 kDa protein is not the Esk-1 isoform, we cannot rule out the possibility that the cloned cDNA represents an incomplete message. Further analysis of the Esk-1 transcript, and expression of epitope-tagged cDNAs may be informative as to the nature of the 150 kDa protein. However, while the identity of this protein remains unclear, its relationship to the Esk kinase may be relevant to the functional role of Esk in the control of cell growth and differentiation.

GENERAL DISCUSSION

The main objective of this thesis work was to identify novel protein tyrosine kinases involved in the control of cell growth and differentiation. The cloning strategy involved the isolation of active protein tyrosine kinases from an expression library derived from P19 embryonal carcinoma cells. This strategy was successful as protein tyrosine kinases were isolated and unexpectedly, a novel family of protein kinases was also identified. The kinase members of this novel family, now referred to as dual specificity kinases, have the ability to phosphorylate tyrosine as well as serine and threonine residues. While the intrinsic tyrosine kinase activity was the premise for their identification, DSK family members are more closely related to members of the serine/threonine family of protein kinases based on their primary amino acid sequence. The conventional cloning strategies utilized to identify protein tyrosine kinases originally made use of degenerate oligonucleotides corresponding to conserved subdomains (VI and VII). Since these subdomains are distinct between PSKs and PYKs, such a strategy would fail to identify the dual specificity kinases. Evidence that these enzymes are involved in all aspects of cell growth and differentiation continues to grow, as more members of the dual specificity kinase family are characterized.

The Esk kinase isoforms

The Esk kinase was cloned from P19 EC cells and found to be encoded by two distinct mRNAs that predicted transmembrane (Esk-1) and cytoplasmic (Esk-2) isoforms. The sequencing of genomic clones encoding the Esk kinase revealed that both isoforms

could be generated through the alternative splicing of the same primary transcript. While the Esk-1 cDNA sequence predicted a putative transmembrane domain, there are no sequences present within the cDNA that could encode a leader peptide. Preliminary sequence data from the genomic DNA also failed to reveal the presence of a leader peptide. Instead, the sequences immediately upstream of the transcription initiation site revealed an area rich in transcription factor consensus binding sites. Taken together, the results suggest one of two possibilities; the same transcription and translation initiation sites are used for both isoforms, and therefore the Esk-1 isoform putatively encodes a leaderless transmembrane protein, or alternatively, different initiation sites are used for the Esk kinases, such that the initiation sites (and leader peptide) for the Esk-1 transmembrane isoform lie further upstream in the gene.

Esk kinase displays dual specificity in bacteria

The initial characterization of the Esk kinase was carried out in bacteria where it was shown to display predominantly a manganese dependent threonine kinase activity. The dependence of the Esk kinase activity on manganese is unusual in that this property is an attribute of protein tyrosine kinases (Hunter, 1987). In addition, the immunoreactivity of the Esk kinase with anti-phosphotyrosine antibodies and the disappearance thereof by treatment with phosphotyrosine specific phosphatases, suggested that the Esk kinase should also have specificity for tyrosine residues. In fact, the tyrosine specific kinase activity of Esk was only revealed by lowering the pH and

omitting detergent from the kinase assay reactions. However, these strict assay conditions shed some doubt as to the physiological relevance of the Esk dual specificity in an *in vivo* context. Indeed, this discrepancy has been observed in other DSK members that fail to display dual specificity in a cellular context, including the Wee1 DSK (Parker *et al.*1992; McGowan and Russell, 1993).

The Esk kinase associates with the 85 kDa subunit of PI3K in mammalian cells

The identification of consensus SH2 binding sites has been an invaluable tool in identifying novel cryptic tyrosine phosphorylation sites. The tyrosine residue at position 721 in the CSF-1 receptor, has been shown to be directly involved in the binding of the receptor to PI3K in mammalian cells. While this tyrosine residue is nested within a perfect consensus sequence for the SH2 domain of p85, the *in vivo* phosphorylation of this residue has not been demonstrated (Reedijk *et al.*1992). Similarly, the work of Songyang *et al.* (Songyang *et al.*1993) revealed that a p85 SH2 consensus binding site existed within the catalytic domain of the Esk kinase. The results obtained from the bacterial binding studies suggested that the sequences surrounding this tyrosine residue were directly involved in the binding of the Esk catalytic domain to the 85 kDa subunit of PI3K. While the SH3 domain of p85 was also shown to bind to the bacterially expressed Esk kinase, the significance of this interaction is unclear. To determine the specificity of this interaction, other SH3 domains will have to be tested and deletion mutants of the Esk kinase employed to identify the region responsible for binding.

In mammalian cells, an antibody directed to p85α could specifically immunoprecipitate the Esk kinase. The p85 associated Esk kinase was phosphorylated on tyrosine, displayed higher specificity towards serine residues, and exhibited novel tryptic phosphopeptides. The results therefore suggest that two distinct pools of the Esk kinase exist in mammalian cells, one which is associated with the 85 kDa subunit of PI3K, and one which is not. More importantly, the p85/Esk interaction described in mammalian cells suggests a physiological importance for the tyrosine phosphorylation of the Esk dual specificity kinase.

Recently, Carpenter *et al.* have documented the presence of a manganese dependent serine/threonine kinase (PIK) tightly associated with PI3K in mammalian cells (Carpenter *et al.*1993). Further, they have demonstrated that the serine phosphorylation of PI3K by PIK leads to a three- to sevenfold decrease in the activity of PI3K. It is tempting to speculate that Esk is a PIK-related kinase for two reasons. First, our inability to detect any PI3K activity associated with Esk, may reflect the regulatory role of the Esk kinase on PI3K. Indeed, even if only a small fraction of both kinases are associated with each other, the downregulation of PI3-kinase activity by the Esk kinase could render it undetectable, or below the limits of detection. This question could be addressed by treating the co-immunoprecipitates with protein phosphatases in an attempt to rescue PI3K activity. Indeed Carpenter *et al.* have shown that the inhibitory effects of PIK on PI3K activity are reversible by such a treatment (Carpenter *et al.*1993). Second, while antibodies directed to the SH3 domain of p85α (from X. Liu)

immunoprecipitate detectable Esk kinase activity, antibodies directed to the SH2 domain (from C. Carpenter) fail to do so (chapter 3, figure 5). In contrast, p110 and p85 phosphorylated proteins are detected in such immunoprecipitates. Similar results have been obtained by Carpenter et al. in their immunoprecipitates of PI3K (Carpenter *et al.*1993) and have hindered their ability to purify and clone the PIK kinase. If indeed the Esk kinase can be co-immunoprecipitated with the p85SH2 antibody, our results obtained with the different p85 antibodies may suggest both SH2 and SH3 dependent interactions between Esk and p85, a hypothesis also supported by the bacterial studies.

The Esk kinase is developmentally regulated.

The characterization of the Esk isoforms at the molecular level indicated that both isoforms could exist. The cloned cDNAs predicted proteins of 94 and 96 kDa for the Esk-2 and Esk-1 kinases, respectively. In P19 cells, the antibody raised against the Esk kinase recognized a protein of approximately 96 kDa that comigrated with the Esk-2 kinase expressed in cos-1 cells. The proteins displayed similar tryptic peptides maps indicating they were related.

In P19 cells, a protein of ~150 kDa was found to coprecipitate with the Esk kinase at low levels. Peptide competition blocked its immunoprecipitation from P19 cells and the protein could be re-immunoprecipitated following denaturation suggesting that it was not an associated protein. To date, the evidence suggests that the 150 kDa protein is either an Esk related protein, or simply a cross-reacting protein. The observations that

the 150 kDa protein appears to have an intrinsic kinase activity, and that it associates with the 85 kDa subunit of PI3K argue in favor of it being an Esk related protein. The results would suggest that the 150 kDa protein is a different phosphorylation form of the 96 kDa Esk kinase, or alternatively it may be the Esk-1 kinase isoform. However, the observations that the 150 kDa protein is not detectable by Western blotting with the Esk antibody, that its tryptic peptide maps are distinct from those of Esk, and that the Esk-1 kinase migrates as an 98 kDa protein in cos cells, argue against the 150 kDa protein being the Esk-1 kinase or an Esk related protein. The latter argument may be invalid as the cloned Esk-1 cDNA may be incomplete. Indeed, if the Esk-1 kinase were to have a leader peptide its migration could be similar to that observed for the 150 kDa protein in P19 cells.

Interestingly, the 150 kDa protein was found to increase during P19 induced neuronal differentiation, while the levels of the Esk kinase decreased. The 150 kDa protein was the predominant protein isolated from adult mouse tissues, with the exception of testes which also expressed the 96 kDa Esk kinase. This evidence, although circumstantial, indicates that an inverse relationship exists between the Esk kinase and the 150 kDa protein that may relate to their roles in the control of cell growth and differentiation. If the Esk kinase is involved in the downregulation of PI3K activity, the following model could be proposed. In rapidly proliferating cells, such as stem cells, levels of 3-phosphorylated phosphatidylinositols (PtIns) are high and are therefore associated with higher PI3K activity. Under these circumstances only a small fraction

of the total cellular Esk kinase is associated with the enzyme. As the cells are induced to differentiate, a decrease in the amount of 3-phosphorylated PtIns would coincide with a decrease in the activity of PI3-kinase. Although the relationship between the Esk kinase and the 150 kDa protein is unclear, in this model, the downregulation of PI3K activity during differentiation could be mediated through its association with the 150 kDa protein which would be an Esk related protein. Coincidently, preliminary data suggests that the 150 kDa protein associates with a higher stoichiometry to the 85 kDa subunit of PI3K than does the Esk kinase (see chapter 4, figure 11).

Is the Esk-1 kinase a membrane protein?

The subcellular localization of the Esk-1 kinase still remains unclear. While the Esk-1 kinase has a potential transmembrane domain that predicts a corresponding localization, it also displays several unusual features that complicate the analysis. First and foremost, the Esk kinase displays a putative nuclear localization signal in the last six amino acids encoding the kinase (KKRERK). Additionally, a stretch of six glutamine residues in the juxtamembrane region, as well as a heptad repeat of leucine residues within the putative TM domain, both represent features reminiscent of transcription factors (Kassis *et al.*1986; Landschultz *et al.*1988). The heptad repeat of the Esk-1 kinase, albeit too hydrophobic to be a leucine zipper, may represent a protein binding motif that may be involved in the regulation of the kinase through its binding to other proteins. This model predicts that the Esk-1 kinase is a nuclear enzyme involved in the

regulation of transcription factors and other nuclear proteins. While attractive, this model does not account for the binding of the Esk kinase to the 85 kDa subunit of PI3K, which is a cytoplasmic protein.

Recently, a transmembrane serine/threonine protein kinase has been described in yeast that is involved in the transcriptional induction of genes encoding resident endoplasmic reticulum (ER) proteins (Mori *et al.*1993; Cox *et al.*1993). When unfolded proteins accumulate in the ER, an intracellular signal transduction pathway is activated that involves signalling from the ER to the nucleus. The signal translates into the transcriptional induction of resident ER genes including BiP (a molecular chaperon related to the HSP70 family) and PDI (protein disulfide isomerase, involved in the proper folding of proteins) (Mori *et al.*1993; Cox *et al.*1993). Genetic and biochemical evidence from Mori *et al.* and Cox *et al.* indicate that the ERN1 kinase (or IRE1) is the enzyme involved in the first step of this signalling pathway. Indeed, not only do ERN1 mutants fail to activate the induction of ER resident genes, but they become inositol auxotrophs. Interestingly, the ERN1 kinase shares several structural features related to the Esk kinase, including a large extracellular domain with no structural motifs, a transmembrane domain, a nuclear localization signal (in the juxtamembrane region in the case of ERN1), and a hydrophillic kinase insert between subdomains VIII and IX. In contrast to the Esk-1 kinase however, the ERN1 kinase possesses a leader peptide, has been shown to fractionate with the microsomal/cytosolic fraction and is thought to reside in the membrane of the ER (Mori *et al.*1993).

The investigators have proposed a model whereby an accumulation of unfolded proteins in the ER would trigger the ERN1 signalling pathway by activation of its intra-ER ligand binding domain. The signal could then be transmitted by two possible mechanisms. In the first scenario, the cytoplasmic catalytic domain of the ERN1 kinase could be cleaved and subsequently migrate to the nucleus. Alternatively, the entire kinase could migrate laterally along the plane of the membrane and ultimately negotiate its way through the nuclear pore (Mori *et al.*1993). In this respect, the Esk kinase isoforms would be uniquely positioned to integrate into this model if both membrane bound and soluble forms of the kinase were shown to exist. The predicted localization of the leaderless Esk-1 kinase to the ER or nuclear membranes would support such a model.

The inositol auxotrophy of ERN1 mutants is interesting and suggests that the synthesis of ER proteins is correlated with a synthesis of inositols lipids which are the major constituents of membranes in yeast cells. The Esk kinase has been shown to bind to the 85 kDa subunit of PI3K. While it is too early to determine to which isoform of the 85 kDa subunit the Esk kinase will bind, a similar correlation between the Esk kinase and inositol metabolism to that shown for ERN1 would be attractive.

Future prospects

The recent identification of dual specificity kinases as a novel family of protein kinases has changed the way investigators perceive signal transduction pathways. From

an evolutionary point of vue, protein tyrosine kinases are thought to have evolved from protein serine/threonine kinases; the possibility that dual specificity kinases might represent an intermediate step along this pathway is intriguing. At this point in time however, there does not appear to be any key structural features associated with DSKs. Nevertheless, correlations between structure and specificity may be drawn in the future as more thorough biochemical and structural analyses are carried out.

The evolutionary conservation of dual specificity kinase members suggests that these enzymes play key regulatory roles in signal transduction. Indeed, the work presented in this thesis on the Esk kinase suggest that this DSK family member may be involved in controlling certain aspects of cell growth and differentiation. In addition, the interaction of the Esk kinase with the SH2 domain containing protein, p85, suggests that dual specificity kinases may have evolved in the context of protein tyrosine kinases in order to integrate into SH2 domain signalling pathways.

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ELIZABETH MARIE-JOSEE DOUVILLE

ACADEMIC ADDRESS

Department of Medicine
University of Ottawa
451 Smyth Road
Ottawa, Ontario (K1H 8M5) (613) 787-6569

HOME ADDRESS

16 Cameron Avenue, Apartment 2
Ottawa, Ontario K1S 0W7

EDUCATION

1994 Ph.D. in Biochemistry, University of Ottawa, Ottawa

1987 B.Sc. in Biochemistry, McGill University, Montréal, Québec

1984 D.C.S.in Health Sciences, CEGEP Bois-de Boulogne, Montréal Québec

SCIENTIFIC BACKGROUND

1987 - present University of Ottawa, combined MSc/PhD in Biochemistry: Cloning and characterization of a novel dual specificity kinase, Esk. Laboratory of Dr. John C.Bell

1986, May - Aug Lady Davis Research Institute, Montréal, summer research project: Transactivation of *myc* genes by the *c-fos* protein. Laboratory of Dr. John Hiscott.

ACADEMIC AWARDS

1989-1992	Fonds pour la Formation de Chercheurs et l'Aide à la recherche (Fonds FCAR)
1989-1992	University of Ottawa Supplementary Research Scholarship
1988-1989	McGill Medical faculty award

DOCTORAL THESIS

Cloning and characterization of a novel kinases from embryonic cells.

PUBLICATIONS

1.- The Esk dual specificity kinase interacts with phosphatidylinositol (PI) 3'-kinase. E.M.J. Douville and J.C. Bell. manuscript in preparation.

2.- Identification of a novel receptor-type protein tyrosine kinase highly expressed in brain. M.L. Jaramillo, E.M.J. Douville, J. Ingram and J.C. Bell. manuscript in preparation.

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4.- ESK, Embryonal Carcinoma Serine/Threonine/Tyrosine kinase. E.M.J. Douville and John C. Bell. Chapter in The Protein Kinase FactsBook, Edited by Grahame Hardie and Steve Hanks. Published by Academic Press. in press.

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ABSTRACT

E.M.J. Douville and John C. Bell. Characterization of the Esk Dual Specificity kinases. (1993) Ninth Annual meeting on Oncogenes, June 22-26, Hood College, Frederick, Maryland.

E.M.J. Douville, Tony Pawson and John C. Bell. Multiple cDNAs encoding the Esk kinase predict transmembrane and intracellular enzyme isoforms. (1992) Eighth Annual meeting on Oncogenes, June 23-27, Hood College, Frederick, Maryland.

E.M.J. Douville and John C. Bell. Expression Cloning of a Unique Transmembrane Serine/Threonine Kinase. (1991) The 2nd Eastern Canadian Conference on Development and Cancer, September 23-25, Montréal, Québec. Speaker

E.M.J. Douville and John C. Bell. Expression Cloning of a Unique Transmembrane Serine/Threonine Kinase. (1991) Seventh Annual Meeting on Oncogenes, June 24-29, Hood College, Frederick, Maryland.

E.M.J. Douville and John C. Bell. Expression Cloning of Novel Protein kinases from Embryonal Carcinoma Cells. (1991) Eleventh Annual Great Lakes Mammalian Development meeting, March 22-24, Mount Sinai, Toronto.
Speaker

E.M.J. Douville and John C. Bell. Promoter activity in P19 Embryonal Carcinoma Cells. (1989) Mouse Molecular Genetics Meeting, August 26-30, Heidelberg, Germany.