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Characterization of Esk kinase Isoforms

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

The Esk dual specificity kinase has elevated mRNA levels in cell types that proliferate compared to terminally differentiated cells. Two isoforms of the kinase were cloned from embryonal cells suggesting that Esk may be important during murine development. *esk1* and *esk2* differ only by the splicing in of an additional exon present in the *esk1* mRNA that is absent from *esk2*. To address the importance of Esk in the normal development of the mouse, a targetting vector was made with the purpose of generating a null allele at the *esk* locus by homologous recombination. The vector contained 9.0kb of homology to the *esk* gene and homologous recombination was predicted to eliminate functional Esk1 and Esk2 protein products. No homologous recombination events were identified after transfection into J1 ES cells and screening 186 colonies. Myc epitope tagged Esk1 and Esk2 constructs enabled the properties of the two isoforms to be analyzed separately. M-Esk1 and M-Esk2 are catalytically active by *in vitro* autophosphorylation kinase assay and both isoforms are localized in the cytoplasm by immunostaining in COS-1 cells. Fractionation of endogenous Esk from P19 EC cells confirmed that Esk is predominantly a soluble cytoplasmic kinase. Site directed mutagenesis of Esk2 identified a kinase inhibitory phosphorylation site in the activation segment of the kinase. The mutant kinase, MT648A,T649A is activated 19 fold above M-Esk2 and 41 fold above the kinase impaired M-Esk2(LL`8'). Successful stable expression of MT648A,T649A but not WT Esk demonstrated that the mutant is regulated differently than the WT kinase. Mutation at three sites in the catalytic domain has been shown to affect the autophosphorylation kinase activity of Esk2 to different degrees. The T648A,T649A mutation leads to activated kinase activity by the

removal of a negative regulatory phosphorylation site. A L(758) to R mutation in subdomain XI retains kinase activity while a L(561) to R mutation in subdomain IV is kinase inactive.

To my parents, Bob and Lynda Foot
for all the support they have given me in all my endeavours

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PREFACE

The experimental work presented in this thesis is entirely my own. J1 ES cell genomic southern blots were performed by Simon Ginsberg of Dr. John Bell's lab. Some sequencing done using an ABI automated sequencing machine and performed by Doris Leopold and Suzana Drmanic at the Ontario Regional Cancer Center in Ottawa. Site directed mutagenesis of the *esk2* cDNA using the Promega, pAlter system performed by Ricardo Marius of Dr. John Bell's lab.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

List of Abbreviations

ATP	Adenosine triphosphate
BES	N-,N-bis(2-hydroxyehyl)-2-aminoehanol sulfonic acid
βGeo	β-galactosidase/neomycin fusion
BME	β-mercaptoethanol
bp	Base pair(s)
cAMP	Cyclic adenosine monophosphate
cAPK	cAMP dependent kinase
cdc	Cell division control
CDK	Cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CSK	C-terminal Src kinase
d	Day
dd	Double distilled
DSK	Dual specificity kinase
EC	Embryonal carcinoma
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMBL	European Molecular Biology Laboratory
Erk	Extracellular regulated kinase
ES	Embryonic stem
FAK	Focal adhesion kinase
FCS	Fetal Calf Serum
FIAU	1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-5- iodouridine
FITC	Fluorescein isothiocyanate
GANC	Gancyclovir
GSK3β	Glycogen synthase kinase 3β
GST	Glutathione-S-transferase
h	hour(s)
HPLC	High pressure liquid chromatography
hprt	Hypoxanthine phosphoribosyl transferase
HRP	Horseradish peroxidase
hsv-tk	Herpes simplex virus - thymidine kinase
IκB	Inhibitor κB
IRES	Internal Ribosome Entry Site
kb	Kilobase pair(s)
kDa	KiloDalton(s)
LIF	Leukaemia inhibitor factor
mAb	Monoclonal antibody
Mapk	Mitogen activated protein kinase

MEM	Minimal essential medium
MES	2[*N*- morpholino]ethanesulfonic acid
MPS	Monopolar spindle
mRNA	Messenger ribonucleic acid
neo^R	Neomycin resistance
NFκB	Nuclear factor κB
NK	Natural killer
NLS	Nuclear localization signal
nt	Nucleotide
ORF	Open reading frame
pA	Poly adenylation
PAA	Phosphoamino acid analysis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
pgk	Phosphoglycerate kinase
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMSF	Phenyl methyl sulfonyl fluoride
PS/TK	Protein serine/threonine kinase
puro	Puromycin
pY	Phosphotyrosine
PYK	Protein tyrosine kinase
pyt	*p*hosphotyrosine picked *t*hreonine kinase
Sapk	Stress activated protein kinase
SH2	Src homology 2
SH3	Src homology 3
SPB	Spindle pole body
SRE	Serum response element
SV40	Simian vacuolating virus 40
TBST	Tris-buffered saline - Tween
tet	Tetracyclin
TGFβR	Transforming growth factor β receptor
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
U	Units
Ub	Ubiquitin
UV	Ultraviolet light
VEGF	Vascular Endothelial Growth Factor
V	Volts
WT	Wild Type
X	Any amino acid

Chapter 1
General Introduction

1.1 Dual Specificity Kinases

The protein kinase superfamily is considered to be the largest protein superfamily derived primarily from eukaryotes. It has been estimated that protein kinase genes account for 1% of the genes in the mammalian genome (Hunter, 1987). Historically, this superfamily has been divided into sub-classes; the protein serine/ threonine kinases (PS/TKs) and the protein tyrosine kinases (PYKs). These groups were defined based both on activity and on differences in the catalytic domain at the amino acid level. These two groups can be further sub-divided into receptor and soluble kinases. Members of the protein serine/ threonine kinases family include cAPK, Mapk and TGFβR, where cAPK and Mapk are soluble kinases and TGFβR is a member of the serine/ threonine receptor family. Similarly, members of the protein tyrosine kinase family include Src and the EGFR where Src is a soluble kinase and the EGFR is a member of the receptor subfamily. More recently a new subclass has been identified that is thought to bridge signalling between the protein serine/ threonine and protein tyrosine kinases, this newer group of kinases are the so- called dual specificity kinases (DSK) or STY (Serine, Threonine, tYrosine) kinases. Members of this family of kinases have now been identified in plants (Ali et al., 1994), bacteria (Ostrovsky and Maloy, 1995), yeast (Zheng et al, 1993, Featherstone and Russell, 1991, Poch et al, 1994, Lauze et al, 1995), *xenopus* (Gotoh et al., 1994) and also in mammals (Howell et al, 1991, Letwin et al, 1992, Douville et al, 1992).

The importance of kinases in a wide variety of processes that lead to both cell growth and differentiation cannot be overstated. Phosphorylation events can signal the initiation

of a number of events within the cell. Transphosphorylation of the carboxy terminus of activated growth receptors signal the association of soluble SH2 domain containing proteins to the receptor and leads to the initiation of a variety of signalling cascades (Schlessinger and Ullrich, 1992). Both tyrosine and serine/ threonine kinase activity can be regulated by the kinases state of phosphorylation. For instance, Src Y527 phosphorylation causes a change in conformation of the kinase such that the catalytic domain becomes inaccessible to downstream substrates, therefore inactivating the kinase (Bagrodia et al., 1994). Similarly, activation of cdc2 kinase requires dephosphorylation of T14 and Y15, which removes steric hinderence at the active site of the kinase and permits substrates into the binding fold of the kinase (Gould and Nurse, 1989). Phosphorylation is also a critical signal leading to protein degradation mediated by the ubiquitin proteosome pathway. Phosphorylation of IκB signals rapid degradation of IκB and leads to NFkB translocation to the nucleus and activation of NFkB responsive genes (Siebenlist et al, 1994, Thanos and Maniatis, 1995). Finally, protein phosphorylation can lead to protein translocation. Activation of Mapk by phosphorylation causes a translocation from the cytoplasm to the nucleus (Chen et al, 1992). Similarly, phosphorylation of the HIV-1 matrix protein (MA) on tyrosine leads to translocation from the plasma membrane to the nucleus, an event that leads to infection of non- proliferating cell types (Gallay et al., 1995).

Looking on a broader scale, many of the functions described above for protein kinases are important parts of signal transduction pathways that take information from the external

environment, transmit this information across the plasma membrane and subsequently into the nucleus either directly by kinases or by phosphorylated regulation of transcription factors that shuttle between the nucleus and the cytoplasm. Dual specificity kinases are poised to play important roles in signalling cascades by mediating important crosstalk that must exist between the PYK family and the PS/TK family. While a number of DSKs have been identified in yeast and other lower eukaryotes (Douville et al., 1994) only a few have been identified in mammalian cells (Summarized in Table I). These DSKs have been identified in several species from rat to human and the kinases themselves have been cloned from embryonal carcinoma cells to lung fibroblasts, suggestive of important roles in early embryo development right through to the adult animal. Esk heads the list of DSKs with no known substrates, along with the LIM domain containing kinase Limk1implicated in neuronal differentiation and Nek1, a kinase related to the *Aspergillus nidulans* NIMA kinase that controls entry into mitosis indepently of Cdc2 activity. Several other DSKs have been shown to phosphorylate substrates *in vivo*, however no physiological role for the ability of these kinases to phosphorylate both S/T and Y residues has been identified. For instance, STY/ Clk kinase activity has recently been shown to affect mRNA splicing factors and specifically phosphorylate the SR protein ASF (Colwill et al., 1996). This phosphorylation was found to be only on S/ T residues, without tyrosine phosphorylation. Similarly, Tik/ PKR, the mouse homolog of human p68 PKR, the interferon inducible, double-stranded RNA dependent protein kinase phosphorylates eIF2α on serine in the absence of tyrosine phosphorylation to prevent translation initiation following virus infection (Tuazon et al., 1980). Phosphorylase kinase and

Table 1- Mammalian dual specificity kinase family members

Kinase	Mammal	Substrate	Reference
Esk	Mouse	--	(Douville et al., 1992)
Ttk/Pyt	Human (T cell/ Lung fibroblast)	--	(Mills et al., 1992; Lindberg et al., 1993)
Sty	Mouse	Asf/ SR proteins	(Colwill et al., 1996)
Clk	Human	Asf/ SR proteins	(Johnson and Smith, 1991)
Clk2/ Clk3	Human	????	(Hanes et al., 1994)
Nek1	Mouse	--	(Letwin et al., 1992)
Tik	Mouse	eIF2α	(Icely et al., 1991)
Gsk3β	Rabbit	glycogen synthase, eIF2B, Tau	(Wang et al., 1994)
Phosphorylase kinase	Rabbit	Phosphorylase b	(Yuan et al., 1993)
Limk1	Mouse	--	(Proschel et al., 1995)
Mek1	Human	Erk1/ Erk2	(Seger et al., 1992)
Mek2/Mkk2	Rat, Mouse	Erk1/ Erk2	(Wu et al., 1993: Brott et al., 1993)
Mkk4/ Sek/ Jnkk	Human	Jnk, p38/ HOG1	(Lin et al., 1995)
Mkk3	Human	p38/ HOG1	(Derijard et al., 1995)
Mek5	Rat	????	(English et al., 1995)

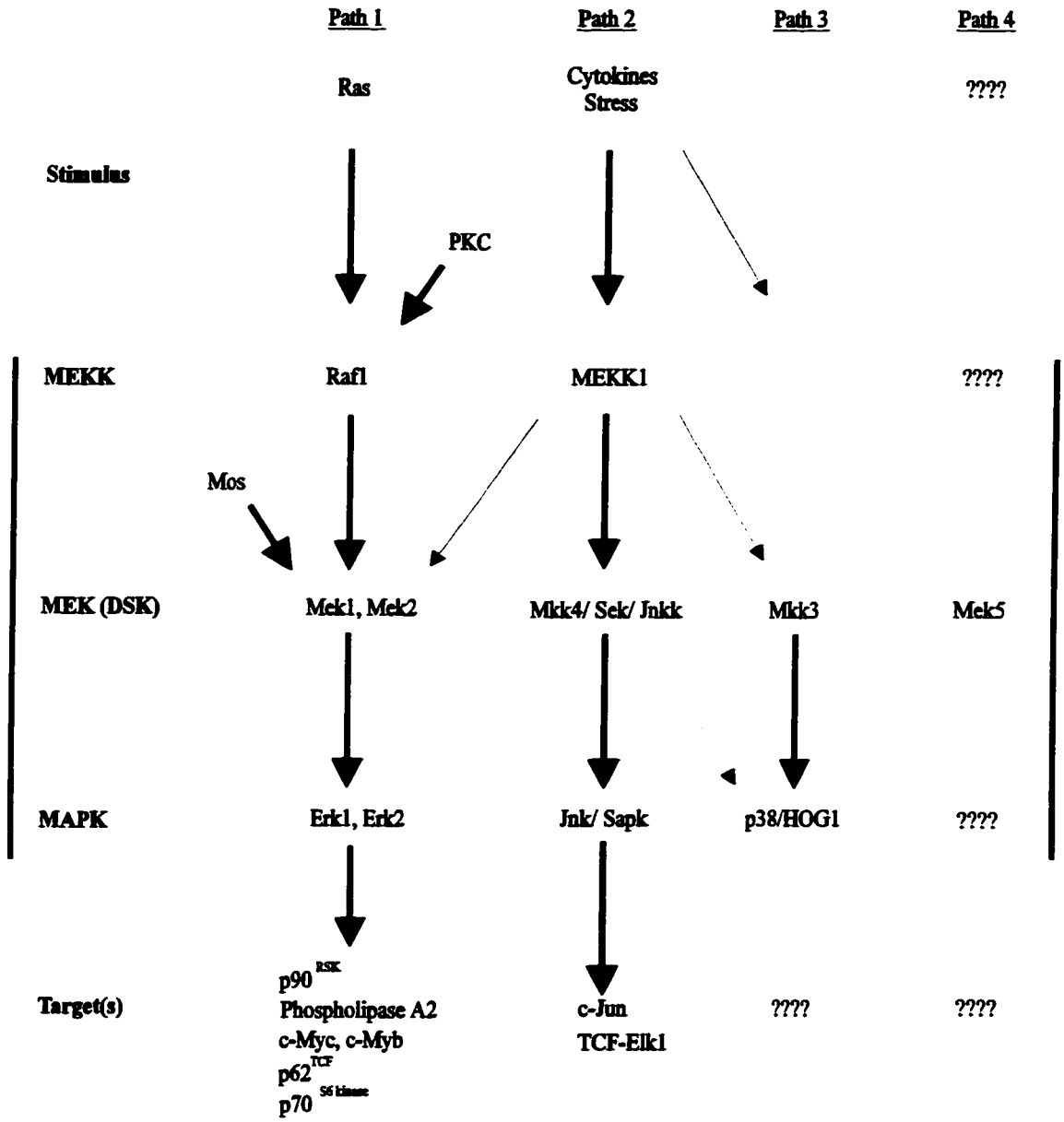
Dual specificity kinases are listed along with the species it was cloned from and known physiological substrates

GSK3β were initially identified as S/ T kinases exclusively but have recently been classified as DSKs based on new observations of their activity. Phosphorylase kinase is a calcium dependent protein kinase known to phosphorylate a single serine residue per subunit of phosphorylase b in its regulation of glycogen metabolism. Phosphorylase kinase contains a second metal ion binding site associated with its catalytic subunit. It has been shown that Mg^{2+} binding in this second binding site activates phosphorylase kinase phosphorylation of phosphorylase b while Mn^{2+} inhibits this activity (Kee and Graves, 1987). Further analysis of the regulation imparted by the divalent cations revealed that Mg^{2+} in fact activates seryl phosphorylation of an exogenous substrate while Mn^{2+} activates tyrosyl phosphorylation (Yuan et al, 1993). Phosphorylase kinase has dual specificity kinase activity based on phosphorylation of exogenous substrates and this activity is regulated by divalent cations, however like STY/ Clk and Tik no physiological relevance for the dual specificity activity has yet been shown. More interesting is the regulation of GSK3β, a kinase that was initially described for its regulation of glycogen synthase in glycogen metabolism but has also been shown to phosphorylate a variety of substrates including the microtubule associated tau proteins and the largest subunit of eIF-2B. GSK3β has been shown to phosphorylate substrates at a S-X-X-X-S(P) consensus however expression of GSK3β in bacteria demonstrated that it could autophosphorylate on S, T and Y residues. Dephosphorylation of S/ T residues activated the kinase while dephosphorylation on Y led to inactivation of the kinase. Thus it was shown for GSK3β, that tyrosine phosphorylation activates the kinase while autophosphorylation on S/ T leads to inactivation of the kinase *in vitro* (Wang et al, 1994). Therefore, GSK3β is an

example of a kinase apparently regulated in a contradictory fashion by tyrosine and serine/ threonine phosphorylation.

Classification of the kinases described above as dual specificity is based solely on their ability to be recognized specifically by antibodies to phosphotyrosine when expressed in bacteria and also by their ability to autophosphorylate on S/ T and Y when analyzed by PAA after expression in bacteria (all kinases in Table I) and in mammalian cells (Esk, Sty/ Clk, Tik). To date the only mammalian kinases whose dual specificity kinase activity has physiological relevance are the Mek/ Mkk/ Mapkk family of kinases. This family of kinases are part of the classic three kinase module that has been conserved from yeast to mammals (reviewed by Herskowitz, 1995). In mammals three such modules have been identified thus far but evidence suggests the existence of at least one more (Cobb and Goldsmith, 1995; English et al, 1995). Activation of the Mapk modules is best understood through receptor tyrosine kinases leading to activation of Ras, subsequent activation of Raf (a Mapkkk) which then specifically activates Mek1 or Mek2 but not Sek or Mkk3 (Cobb and Goldsmith, 1995)(Figure 1). Raf can be activated through Ras by certain hetero trimeric G proteins and independently of Ras, by PKC and also by Mos (Cobb and Goldsmith, 1995). These methods of activation converge on the best known Mapk module. Briefly, a Mekk (a Mek activator), a Mek (a Map kinase activator) and a Map kinase (any Erk homolog) constitute a distinct Mapk module. The best known consists of Raf-1, Mek1 (a DSK) , and Erk2 (Figure 1). Phosphorylation by Mek1 on Y-185 and T-183 is required (Haystead et al., 1992) for Mapk activation and thus constitutes

Fig. 1. **Mek DSKs are key regulatory proteins in Multiple Signal Transduction pathways**- The 3 kinase Mapk cascade has at least 4 distinct pathways in mammalian cells distinguished by unique Mek DSK isoforms (bordered). Raf1 can be activated by Ras dependent and independent mechanisms that can stimulate cell growth by activation of transcription factors such as c-Myc, c-Myb and $p62^{TCF}$ (Path 1). Mekk1, Mkk4 and Mkk3 are activated by cytokines and cell stress such as osmolarity changes and UV irradiation which eventually activates transcription factors such as TCF-Elk1 and c-Jun that will activate genes with a SRE (serum response element) (Path 2, Path 3). Mek5 is not activated by Raf or Mekk1 and does not activate Jnk, Erk1,2,3 or p38/HOG1 and therefore is suggested to be part of a distinct Mapk cascade (Path 4). Known signal transduction pathways are represented by the thick arrows, thin arrows indicate pathways that are less understood.


Path 1
Path 2
Path 3
Path 4
Ras
Cytokines
Stress
????
Stimulus
PKC
MEKK
Raf1
MEKK1
????
Mos
MEK (DSK)
Mek1, Mek2
Mkk4/ Sek/ Jnkk
Mkk3
Mek5
MAPK
Erk1, Erk2
Jnk/ Sapk
p38/HOG1
????
Target(s)
p90 RSK
Phospholipase A2
c-Myc, c-Myb
p62 TCF
p70 S6 kinase
c-Jun
TCF-Elk1
????
????

the only known physiological relevance for dual specificity kinase activity. Activation of a Mapk leads to its nuclear translocation (Chen et al, 1992) and subsequent activation of a variety of transcription factors including c-Myc, c-Myb and $p62^{TCF}$ by Erk2 (Wu et al, 1993) and c-Jun and ATF2 by JNK (Whitmarsh et al, 1995). Activation of this pathway can lead to proliferation and growth response, or differentiation, stress responses or growth arrest depending on the Mapk stimulated, the cell type (Marshall, 1995; Wu et al, 1993). It has been suggested that the varied responses depend both on the Mapk activated and on the transient versus sustained nature of the Mapk activation (Marshall, 1995). The second Mapk module that has recently been well described is the stress response pathway. This pathway is activated by stress and cytokines, such as UV radiation, anisomycin, IL-1 and TNF and consists of activation of MEKK1, not Raf-1, which activates SEK leading to JNK activation (Lin et al, 1995). As mentioned earlier, activation of SEK/ JNKK activates JNK and p38 specifically and does not activate Erks (Lin et al, 1995). The third pathway is somewhat less defined in terms of what activates the DSK. It has been shown that MEKK1 is capable of activating Mkk3, the dual specificity kinase which then specifically activates p38/ HOG1 on T and Y (Derijard et al., 1995). The last Mapk module is speculated to exist based on the cloning of Mek5, a homolog to dual specificity Mek isoforms. Mek5 is suggested to lie in an uncharacterized Mapk pathway because it does not phosphorylate any known Map kinases and is not phosphorylated by any of the known Mek kinases (English et al, 1995) (Figure 1). These signal transduction cascades demonstrate the existence of the dual specificity kinase family as well as the importance of these kinases in signalling cell growth, differentiation or cell

arrest in response to a variety of external stimuli.

1.2 Esk Background

The Esk kinase was cloned from an embryonal carcinoma (EC) cell line by virtue of its immunoreactivity to an antibody to phosphotyrosine. This type of expression strategy was used to clone a number of tyrosine kinases based on the ability of cDNAs to encode functional kinases that could autophosphorylate on tyrosine in bacteria(Letwin et al, 1988; Kornbluth et al, 1988; Lhotak et al, 1991). Sequence analysis of two independent clones identified from a P19 EC cell λGTll expression library revealed that the two clones were identical at the nucleotide level except that one clone contained an additional 78ntds absent from the shorter clone. Sequencing of the *esk* genomic locus later revealed that this 78ntd portion of the cDNA was contained within a single exon (Douville et al., 1994). Therefore, the 2.97kb *esk-1* cDNA and the 2.85kb *esk-2* cDNA were derived from the same gene via alternative splicing. Sequence analysis of *esk* also revealed that its catalytic domain shared greatest homology to S/ T kinases such as human *pim1* protein kinase (25% similar in catalytic domain) and less homology to Y kinases despite the fact that it was cloned based on its ability to autophosphorylate on tyrosine(Douville et al, 1992).

Phosphoamino acid analysis following expression of Esk in bacteria and later in mammalian cells revealed that Esk was capable of autophosphorylating on S,T and Y thus exhibiting dual specificity kinase activity (Douville et al, 1992).

esk mRNA expression is tissue restricted in the mouse. *esk* is highly expressed in the

bone marrow, spleen, thymus and testis and at much lower levels in kidney, brain, lung and heart (Douville et al, 1992). Therefore cell types that maintain a large proportion of proliferating cells have higher expression of *esk* mRNA. This observation was supported by the fact that esk mRNA was elevated in the myeloma cell line SP10 and the B-cell leukemia cell lines L1210, 7OZ/3 and P388 (Douville et al, 1992). A rat *esk* cDNA has been cloned from a rat embryo stomach library using an expression screening strategy identifying clones that could autophosphorylate on tyrosine in the same way that *esk* was originally cloned from mouse. Rat *esk* was found to be highly expressed in embryonal stomach and completely absent from adult forestomach or glandular stomach (Iwase et al, 1993). Elevated *esk* mRNA was identified in 2 of 3 human gastric cancer specimens but absent from the same specimens non-cancerous mucosa when probed with the rat *esk* cDNA (Iwase et al, 1993). Taken together this data indicates that *esk* mRNA expression is elevated in dividing cells and that *esk* appears to be important during development.

It was initially suggested that Esk-1 may represent a transmembrane form of the Esk-2 kinase. This suggestion was driven by the hydrophobic nature of the additional 26a.a. present in Esk-1 and by the fact that the hydrophobic stretch was followed by a lysine residue reminescent of a stop transfer signal that follows transmembrane domains and prevents their continued passage through the membrane (Douville et al, 1992). The absence of a cleavable signal sequence peptide and the presence of two prolines and a glycine which are known α-helix breakers within the 26a.a. stretch however, suggests that this stretch of amino acids may not be a transmembrane domain. At the outset of this

work the significance of the two isoforms on the regulation of the kinase, its activity or its substrates was unknown.

Apart from the catalytic domain, the two isoforms of the kinase share other protein epitopes that may play a role in the activity of the kinase. Using a phosphopeptide library approach to identify the specificity of SH2 domains for phosphorylated peptide epitopes it was shown that the SH2 domain of the p85 subunit of PI3 kinase recognized phosphorylated tyrosine epitopes having a consensus; pY-M/V/I-X-M (Songyang et al, 1993). This phosphopeptide sequence was then found to mediate the interaction between a number of receptor tyrosine kinases, Src family kinases and polyoma virus Middle T and murine p85. Esk was found to contain two of these consensus binding sites, in tandem nested within subdomain V of its catalytic domain. There is no evidence to suggest that these tyrosines are phosphorylated *in vivo* or that Esk regulates or is regulated by p85. Both Esk isoforms also contain a putative SV-40 large T antigen type nuclear localization signal (NLS) (Kalderone et al, 1984) which may be involved in the regulation of Esk activity by control of Esk subcellular localization.

Database searches immediately following the cloning of Esk indicated that it was a unique kinase with no homologs or family members. Since that time two kinases, Ttk and MPS1/ RPK1 have been cloned that are more similar to Esk than to any other kinase. Although there is some debate as to the relationship of these kinases to Esk, it is clear that some of the information that has been found for these kinases may also apply to Esk.

1.3 Esk Related Kinases

ttk was cloned from an Nk-like human cell line (T cell) in the same manner that *esk* was cloned from mouse. *ttk* is 71% homologous to *esk* across its entire length and seems to be expressed in a restricted pattern similar to that of *esk*. *ttk* mRNA is elevated in thymus and testis but not detectable in adrenal, kidney, lung or stomach. Furthermore, *ttk* mRNA is elevated in a squamous cell lung cancer leading to the suggestion that proliferating cells have elevated *ttk* mRNA(Mills et al, 1992). Additional experiments have suggested that Ttk is a cell cycle regulated kinase whose mRNA, protein and kinase activity are low in serum starved cells, but rise in late G1 following induction of cell cycle transit until they peak at the G2/ M border (Hogg et al, 1994; Schmandt et al, 1994). The *ttk* locus has been assigned to human chromosome 6 which is syntenic with the assignment of *esk* on mouse chromosome 9 (Mills et al., 1992; Douville et al., 1994).

Although Ttk is the most homologous kinase to Esk that has been cloned, has similar tissue specific expression and its location within the genome seems to have been conserved from mice to humans there are several observations that suggest that Esk and Ttk are only family members rather than homologs. i) Northern blots identify a 3.0kb mouse transcript while *ttk* is 3.5 to 4.0kb (Douville et al, 1992; Mills et al, 1992; Iwase et al, 1993). ii) No alternatively spliced *ttk* transcript has been found homologous to *esk-1* and genomic mapping and sequencing have failed to identify the existence of an alternatively splice exon. iii) Probing a human gastric cancer RNA blot with a human *ttk* cDNA probe identified a 3.5kb transcript elevated in 3 of 3 patients compared to non-

cancerous mucosa of the same specimens. Probing the same blot with a rat *esk* cDNA probe identified a 3.0kb transcript that was elevated in only 2 of 3 patients suggesting that there are in fact distinct but related mRNAs in the human stomach that code for Ttk and Esk (Iwase et al, 1993).

MPS1/RPK1 is a dual specificity kinase cloned independently from *Saccharomyces cerevisiae* by two groups. RPK1 was cloned initially based on the fact that its open reading frame(ORF) was very close in the genome to the ACT2 gene and sequencing of DNA adjacent to ACT2 revealed an ORF that turned out to be RPK1 (Poch et al., 1994). MPS1 was initially identified as part of a screen identifying temperature sensitive mutants for failure to duplicate their mitotic spindles (MonoPolar Spindle)(Winey et al., 1991). The *mps1-1* mutation had a number of interesting characteristics that separated it from other monopolar spindle mutants. The *mps1-1* mutation resulted in failure to duplicate the spindle pole body in late G1 and then while most mps mutants arrested in mitosis with a monopolar spindle, it was observed that the *mps1-1* mutation resulted in cells failing to arrest in mitosis and attempting to segregate their DNA and undergo cytokinesis leading to an accumulation of cells with polyploid DNA content causing death (Winey et al., 1991). *mps1-1* was subsequently cloned and found to be the same gene as *rpk1*. The MPS1 kinase exhibited dual specificity kinase activity and was found to be 45% identical to *esk* and 67% similar within the catalytic domain (Lauze et al, 1995). MPS1 was found to be an essential gene for yeast as replacement deletion of the gene resulted in a lethal mutation. This lethality could not be rescued by a kinase inactive *mps1*Δ allele suggesting

that kinase activity is required for the essential function of MPS1 (Lauze et al, 1995). Additional experiments suggest that MPS1 is required for two distinct activities, MPS1 is required to duplicate the SPB but if mps1-1 temperature sensitive yeast are arrested at the permissive temperature with hydroxyurea then the yeast will arrest with duplicated SPBs. If these yeast are then moved to the restrictive temperature, released from the hydroxyurea arrest but exposed to nocodazole to depolymerize micortubules then the yeast still fail to arrest at mitosis indicating that MPS1 is absolutely required during mitosis to arrest yeast at a G2/M checkpoint that monitors microtubule structure (Weiss and Winey, 1996).

1.4 Rationale

Dual specificity kinases are suggested to play critical roles in signal transduction pathways through their unique ability to regulate the activity of proteins through both S/ T and Y phosphorylation. Examples in yeast have suggested that DSKs do play critical roles in the regulation of key events in the cell. The Wee1 kinase is a serine, tyrosine kinases that phosphorylates Cdc2 inhibiting Cdc2 kinase activity and controlling initiation of the cell cycle in yeast (Featherstone and Russell, 1991). The mammalian MEK kinase family plays an equally important role in the regulation of cell growth and differentiation through its regulation of the Mapk pathway. Effects on transcription in response to external stimuli such as growth factors and environmental stresses are mediated by Mapk homologs such as Erk2 and Jnk. These kinases are activated only by phosphorylation on T and Y that is mediated by the MEK family (Haystead et al., 1992). Identifying the critical functions and the requirement for dual specificity activity of the Esk kinase

isoforms within the cell was the prime motive for the following experiments. This question was addressed in three distinct ways.

A vector was made for the purpose of generating a null allele of *esk* by homologous recombination. The generation of mice deleted of functional Esk is a potentially powerful way to identify specific developmental processes that normally require Esk activity. *esk* was cloned from a library of embryonal origin (Douville et al, 1992) and its mRNA is elevated in the embryonal stomach but absent in adult (Iwase et al, 1993). Furthermore, esk-1 cDNAs have only been isolated from embryonic cells suggesting that this isoform at least may play a critical role in development (Douville et al, 1994). In the absence of gross differences in mice that no longer express Esk protein, the identification of cooperative mechanisms for coping with the absence of Esk might identify proteins that are involved in common signalling pathways or cellular processes.

cDNAs encoding the *esk1* and *esk2* isoforms were epitope tagged at the amino terminus within a mammalian expression vector. Epitope tagging the Esk isoforms allows for analysis of the kinases in both their active and inactive states and experiments were conducted to gain information about the subcellular localization of both kinases, their activity and their regulation individually. Identification of a specific subcellular localization for the Esk kinase can shed light into its possible role within the cell. Furthermore, analysis of the Esk isoforms individually can suggest ways in which the two kinases differ in their activities or their function within the cell.

Characterization of mutants of Esk permits the investigation of methods that the kinase uses to regulate its activity and what events specifically are important to regulate Esk activity. Mutation may affect kinase activity, subcellular localization or interaction with signalling partners. Investigation into effects on kinase activity can specifically lead to an understanding of the critical activating phosphorylation sites or inactivating phosphorylation sites as well as potential protein-protein interactions. Changes in subcellular localization could potentially enable the characterization of protein motifs that direct the kinase to a specific site in the cell and that might also regulate specific protein-protein interactions.

Chapter 2
Gene Targeting at the *esk* allele

2.1 Introduction

Targeted homologous recombination in embryonic stem(ES) cells for the purpose of deriving a null allele of a gene is a powerful technique capable of providing information at the level of protein regulation, activation, and substrate/effector interactions. On a broader scale, this technique can suggest the necessity of a protein in development and morphogenesis. The phenotypes of several kinase deficient mice have led investigators to important insights into a variety of previously unappreciated functions. Mice deficient for *c-src* were osteopetrotic with no other abnormalities. The specific phenotypic observations were initially surprising, specifically because the highest protein expression of Src was thought to be in neurons and platelets (Soriano et al., 1991). Src has since been shown to be expressed at equally high levels in osteoclasts and has kinase activity similar to the activity seen in brain and platelets (Horne et al., 1992). Src is not required for osteoclast development since Src -/- mice have normal numbers of osteoclasts but Src -/- mature osteoclasts do not resorb bone (Boyce et al., 1992). Src has now been suggested to be critically involved in phosphorylation and activation of components involved in protease vesicle translocation and fusion at the apical membrane which is the active site of bone resorption (Hall et al., 1994). Targeted disruption of the *csk* allele demonstrated that the Csk kinase was a critical negative regulator of Src family PTKs. Csk -/- deficient mice were embryonic (d10.5) lethal and displayed elevated levels of phosphotyrosine(pY) in general including elevated $pp60^{src}$ and $pp59^{fyn}$ kinase activity.(Imamoto and Soriano, 1993; Nada et al., 1993). This coupled with *in vitro* evidence identified a direct relationship between the Csk kinase and the src family PTK's.

Esk was originally cloned from P19 embryonal carcinoma(EC) cells implying that Esk protein may play some role in the embryo during early development of the mouse. Esk mRNA expression is tissue restricted in the adult mouse. Of the tissues tested, the spleen, thymus, bone marrow and testes expressed significant levels of the Esk mRNA (Douville et al., 1992). Esk mRNA levels were elevated in the myeloma cell line SP10 and the B cell leukemia cell lines L1210, 70Z/3 and P388(Douville et al., 1992). This data suggests that high levels of *esk* mRNA expression are associated with cells that are proliferating.

Few kinases have been shown to phosphorylate on serine, threonine and tyrosine in mammalian cells (Douville et al., 1994; Lindberg et al., 1992). No causative role for Esk dual specificity activity has been identified in any developmental or differentiation path and no signal transduction paths have a known requirement for Esk. For this reason, generation of a null allele of Esk through homologous insertion of a selection cassette into the genome represented an attractive way to identify developmental processes, differentiation pathways or growth requirements for the Esk kinase. Identification of phenotypes associated with loss of function mutations can be suppressed by functional redundancy between family members. Functional redundancy was not anticipated to present a problem for the targeted disruption of Esk because no known paralogues or orthologues of Esk have been found in mouse and Esk is only distantly related to Ran1 and Pim1 protein kinases based on homology in the catalytic domain (Hardie and Hanks, 1995). Redundancy can occur because of the effective substitution of the missing protein with another, a selective increase in expression of another protein or a modification in

catalytic activity of other proteins to compensate for the absence of a targeted allele. Compensatory interactions have contributed to suppressed or absent phenotypes in a number of Src family PTK mutant mice such as Yes, Fyn and Src (Stein etal., 1994). The osteopetrotic phenotype observed for Src -/- mice is directly related to the fact that Fyn, Lyn and Yes cannot compensate for the loss of Src activity in osteoclasts as they are able to in other tissues because they are only expressed at 1-5% of the levels of Src in osteoclasts (Horne et al., 1992; Stein et al., 1994).

The absence of any paralogues or orthologues and the high expression of Esk in embryonic stem cells suggests that any critical functions of the Esk kinase could be identified by a loss of function mutation created by targeted disruption of the *esk* allele.

2.2 Methods

Maintenance of ES cells- J1 ES cells (Passage no. 12) derived from a male agouti 129/ terSv embryo (Li et al., 1992) were maintained on gamma irradiated (3000 rad) neo^R embryonic fibroblast feeder cells (Zijlstra et al., 1989) in ES cell media [DMEM(high glucose),15% FCS, 5mL 100x non-essential amino acids, 5mL 100x Glutamax, 5mL 100x Penstrep, 3.5uL beta- mecaptoethanol] supported with LIF(500u/mL).

Electroporation and Selection- $5x10^7$ J1 ES cells were resuspended in 5mL Electroporation Buffer (EB) (20mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM

Na_2HPO_4(dibasic), 6mM Dextrose, O.1 mM BME) followed by centrifugation and resuspension in 0.8mL EB containing 100μg of Xho1 linearized pKS-KO-9.0. ES cells and DNA were Electroporated using a BioRad GenePulser™ (400volts, 25μFa), grown without selection for 24h in ES cell media and LIF on $3x10^6$ feeder cells/8.4 x 10^6 J1 ES cells, then selected in G418(200μg/mL) and FIAU(0.2μM) for 10 days. Colonies were picked and placed individually into a 96u-well dish (Corning, Cat. 25850) containing 60μL 0.025% trypsin, 1mM EDTA in PBS. Colonies were pipetted 10 times, 30μL of ES cell suspension was transferred onto feeder cells (5x 10^4/well) in 96 flat-well dishes (Linbro, Cat. 76-003-05) and the remainder into 24 well (flat bottom) dishes (Baxter-Cat.25820). Following expansion, cells in the 96 well dishes were monodispersed with Trysin-EDTA and frozen in 2x freezing medium (20% DMSO, 20%FCS in DME) under 2 drops of ultra pure light mineral oil and stored at -80°C.

Isolation of Genomic DNA- DNA isolation from 24 well dishes has been previously described(Laird, P.W. et al, 1991). Briefly 0.5mL of Lysis buffer (100mM Tris-HCl, pH 8.5, 5mM EDTA, 0.2% SDS), 200mM NaCl, 100g Prot. K/mL) was added to each well, cells were initially lysed by micro pipette (3-5x), the lysate was then transferred to eppendorf tubes and rotated at 37°C, overnight. Equal volume of Isopropanol was added to the lysate and the samples were mixed for 30' on a rotator. The DNA precipitate was isolated and dissolved in 60uL TE with agitation to ensure homogenous DNA samples.

Construction of targeting vector- pPNT has been previous described (Tybulewicz et al.,

1991). pKS-NT vector was generated from pPNT to contain an expanded multiple cloning site (M.C.S.). The XhoI/HindIII fragment of pPNT was ligated, blunt end into the blunted EcoRI, BstX1 site of pBluescript KS+ to create pKS-NT where the *pgk-1* promoter drives the *neo* resistant gene and the herpes simplex virus *thymidine kinase* gene (Gift of Alexandra L. Joyner). Genomic clones from the *esk* locus were isolated from a mouse D3 ES cell genomic library. A 5.2kb EcoR1 fragment was cloned into the EcoR1 site of pKS-NT. Clones positive for the insert were sequenced for orientation such that the 5' end of the insert was adjacent to the M.C.S. to create pKS101-R1-5.2. A 3.75 kb Sal1/HindIII fragment extending from the 5' end of the *esk* gene to the first 100bp of exon 2 of the *esk* gene was ligated into the Sal1/HindIII sites of pKS101-R1-5.2 to create pKS101-KO-9.0, containing 9.0 kb total homology to the *esk* gene flanking a *neo* cassette driven by the *pgk1* promoter. pKS-KO-9.0(100μg) was linearized by digestion with 48units of Xho1 for 12h followed by phenol/ chloroform extraction (twice), ethanol precipitation and resuspension in 100μL of ultrapure ddH_2O.

DNA sequencing- pKS101-5.2 clones were sequenced using the dideoxy chain termination method for double stranded DNA (Sanger et al., 1977). The oligonucleotide primer, 3'KOOII [AGAGGACCCAGGTTCC-OH] is complimentary to genomic DNA sequence at the 5' end of the 5.25kb EcoR1 fragment cloned into pKS101-5.2. The XTL1-53 oligo [AATTGGCAGCAGTGTGACGAT-OH] is complimentary to nt 55-75 from the *esk1* cDNA (Douville et al., 1992) at the 5' end of the first translated exon.

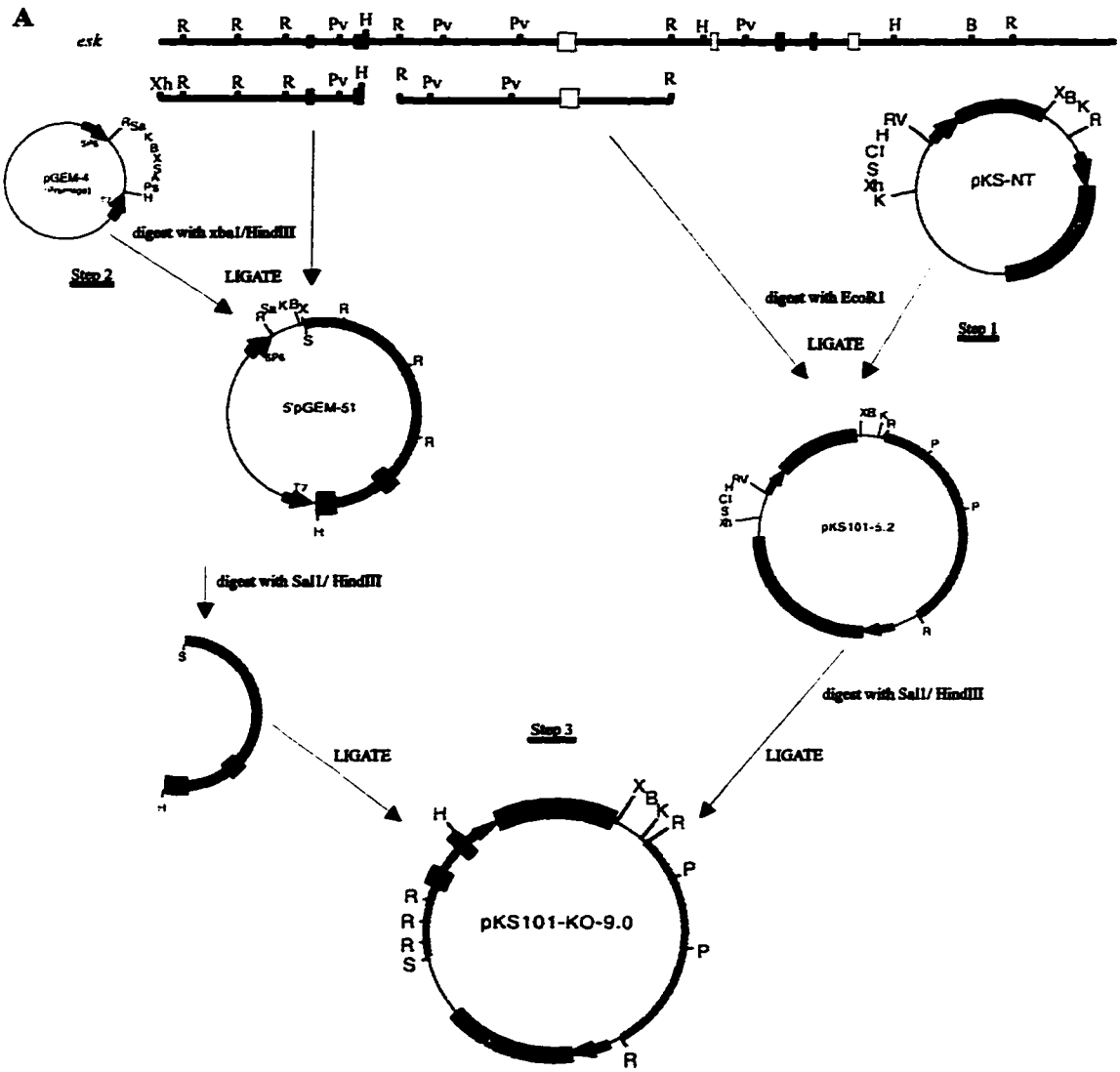
2.3 Results

Targeting the *esk* genomic locus

Genomic clones were isolated from a D3 ES cell genomic library using an *eskI* cDNA probe and preliminary mapping identified 3 overlapping clones containing 25kb of the esk locus corresponding to the 5' end of the cDNA extending past the alternatively spliced exon toward the 3' end of the gene. The isolated genomic clones contained predominantly the 5' end of the gene, therefore the targeting construct was designed such that the positive selection cassette would be inserted into the first coding exon (Figure 1a).

Experiments targeting the *hprt* locus had suggested that increasing the length of homology in the targeting vector between 3 and 12kb corresponded to a linear increase in absolute targeting frequency where maximum targeting frequency was achieved with total homology approaching 12kb (Capecchi, M.R., 1989). Typically targeting constructs are designed with 5-8kb of homology to permit easier construct manipulation while still maintaining significant total homology (Joyner, A.L., 1993). Construction of the *esk* targeting vector is outlined in Figure1a. To obtain targeted disruption of the *esk* allele a positive and negative selection strategy was employed using the targeting vector pKS-NT (A gift from A.L. Joyner). The vector consists of the *neo* expression cassette and *hsv-tk* both under the control of the *pgk-1* promoter and with the *pgk-1* poly (A) addition site (Tybulewicz et al., 1991). pKS-NT contains an expanded multiple cloning site taken from pBluescript KS and was derived from pPNT which has been used successfully in several

Fig. 1- **Esk Targeting vector construction-** (A) A 5.25kb EcoR1 fragment from *esk* genomic clone 101 was cloned into the EcoR1 site of pKS-NT (step 1). A 3.75kb 5' *esk* genomic clone was then subcloned into pGEM4 using an Xba1 site from the λdashII phage and a HindIII site in the first translated exon of *esk* (step 2). The 5' genomic fragment was then cloned into the multiple cloning site of pKS101-5.2 using a SalI site from the λdashII phage and the HindIII site from the first translated exon (step 3). The targeting vector, pKS101-KO-9.0 has 9kb of total homology to the *esk* locus and will insert the 1.74kb pgk-*neo* gene into the first translated exon replacing 0.7kb of the *esk* gene. The orientation of pKS101-5.2 (after step 1) was confirmed by sequencing back through the 5' end of the 5.25kb fragment and positive clones gave sequence corresponding to the pKS-NT polylinker (B).



B **Confirmation of pKS101-5.2 Orientation**

pKS-NT vector — XbaI

3'KOOII sequence 5' G CTT CTA ATT CCA TCA GAA GCT GAC TCT AGA

BamHI — KpnI — SstI — EcoRI

3'KOOII sequence GGA TCC CCG GGT ACC GAG CTC GAA TTC CCC T 3'

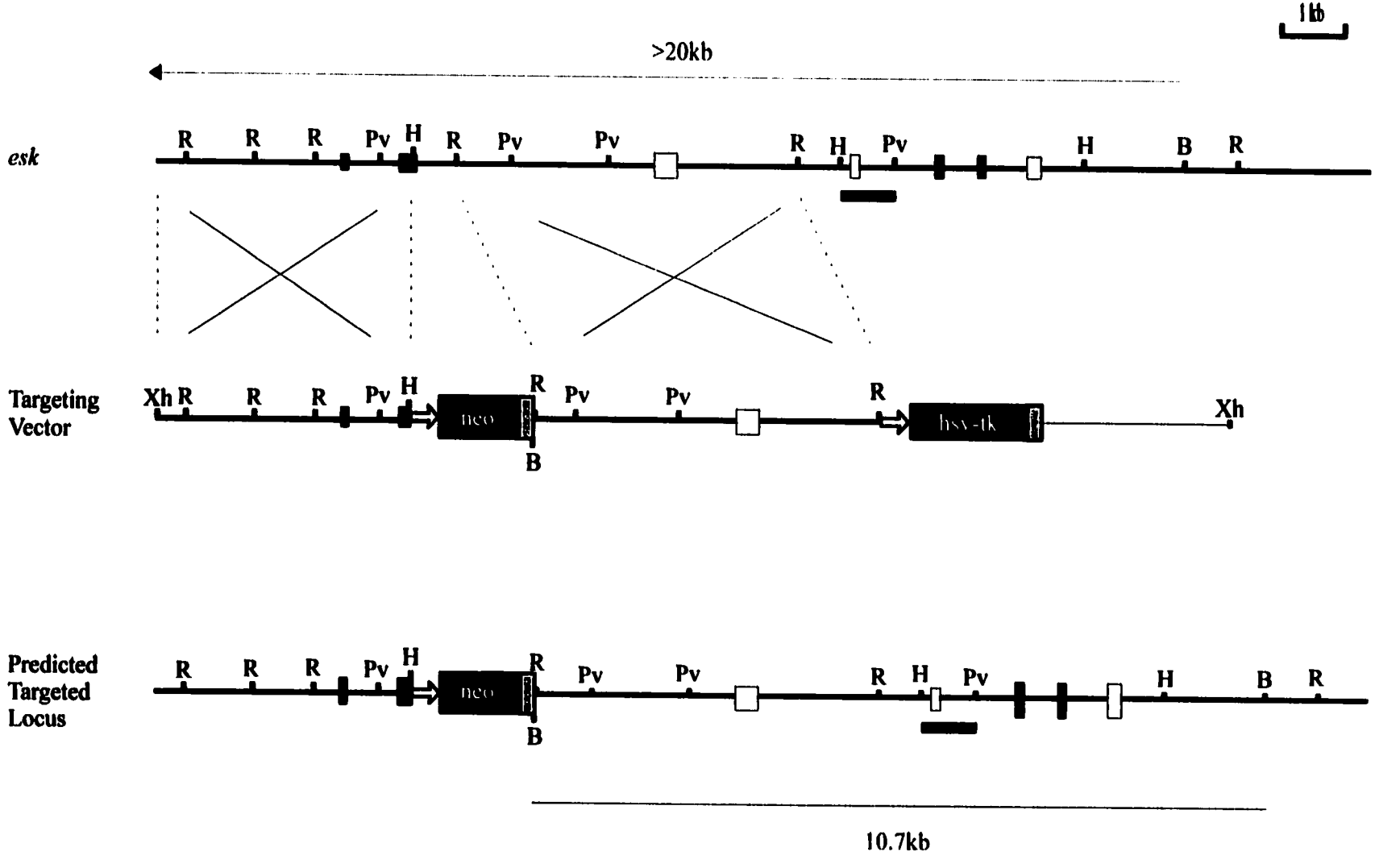
SmaI

targeting strategies (Tybulewicz, et al., 1991; Yamaguchi et al., 1994). In brief, the construction of the *esk* targeting vector involved cloning a 5.25kb EcoR1 fragment directly from an *esk* λdashII phage into pKS-NT. The positive clones were sequenced to confirm orientation (Figure 1b) prior to cloning in the second fragment containing 3.75kb of homology from the *esk* locus. The final targeting vector consists of 9.0kb of total homology and will result in the replacement of 0.8kb of the esk gene with the 1.74kb PGK-1 *neo*pA positive selection cassette into the first coding exon (figure 2). pKS-101-KO-9.0 was linearized at a unique Xho1 restriction site and transfected into J1 ES cells (Li et al., 1992) by electroporation. The electroporated ES cells were grown on γ-irradiated neo^R feeder cells (Zijlstra et al., 1989) and selected in G418 and FIAU for 10days. Colonies with good ES cell morphology were isolated and grown under continued selection to isolate DNA for southern blot screening.

Predicted mRNA species from Targeted Allele

Homologous recombination of pKS101-KO-9.0 is predicted to produce an *esk* mRNA interrupted by *pgk-neo* such that a translation stop codon will truncate the Esk polypeptide resulting in a 45aa protein that terminates prior to the alternatively spliced domain and the catalytic domain therefore abolishing the activity of both Esk1 and Esk2. The exon/ intron boundary at the 5' end of the first coding exon had been previously determined. To predict the outcome of the *esk* mRNA should the targeted exon be spliced out, the exon/ intron boundary at the 3' end of the first coding exon was determined (Figure 3a). The first coding exon was found to be 154nt in length and code for 45 1/3

Fig.2- **Homologous recombination predicted for the targeting vector pKS-101-9.0 at the *esk* locus.** Partial map of the *esk* gene is represented by the uppermost line. Closed boxes represent exons whose location within the gene has been identified. Open boxes represent exons whose location has not yet been mapped precisely. Restriction enzyme sites for EcoR1, Pvu11, Hind111, BamH1 and Xho1 are indicated by R, Pv, H and Xh respectively. Exon U is an untranslated exon, exon 1 contains the initiating methionine and exon A is alternatively spliced in for *esk1* mRNA and spliced out for *esk2* mRNA. The *neo*^{r} and *HSV-tk* genes are flanked by the PGK1 promoter (open arrow) and the PGK1 poly A signal (shaded box) and the plasmid backbone of pKS-101-9.0 is indicated by the thin line. Following homologous recombination a 700bp Hind111/ EcoR1 fragment of the *esk* gene will be deleted and replaced with the *neo*^{r} gene. The *esk* probe used for genomic southern blot analysis is a 600bp portion of the *esk* gene represented by the closed bar, the expected restriction fragments of the endogenous and targeted alleles are indicated.

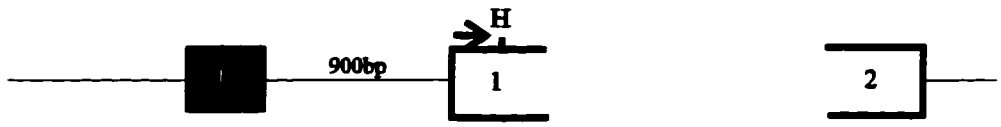

1kb
>20kb
esk
R R R Pv H R Pv Pv R H Pv H B R
Targeting Vector
Xh R R R Pv H neo R Pv Pv R hsv-tk Xh
B
Predicted Targeted Locus
R R R Pv H neo R Pv Pv R H Pv H B R
B
10.7kb

25a

Fig. 3. **Possible Esk mRNA species following homologous recombination-** (A) Schematic of the undefined exon/intron boundary between the 3' end of exon`1' and the 5' end of exon`2'. The 3' end of exon `1' was confirmed by sequencing using an oligo based on the*esk* cDNA sequence. Sequence derived from the XTL1-53 oligo is aligned with the published *esk* cDNA sequence and exonic sequence is shaded. Intron 2 begins with the consensus GT splice donor (underlined) after 154nt of esk cDNA sequence in exon `1' that codes for 45 complete amino acids plus 1nt. Intron 1 is 900bp in length, 104nt of intron 2 sequence have been confirmed but the splice acceptor site has not yet been found nor has exon `2' sequence corresponding to the *esk* cDNA. (B) Possible esk mRNA species (dark line) derived from the targeted allele and the corresponding size of the Esk polypeptides (Thick line) that would be produced are shown. Intron 1 is bordered by consensus splice donor and splice acceptor sites (underlined) between the untranslated exon and the first coding exon (shaded sequence). In the absence of exon skipping a truncated Esk polypeptide of 36aa would be translated, terminating upstream of the pgk promoter spliced into the esk mRNA (thin line). The initiating methionine is deleted in the event of exon skipping therefore polypeptides derived from the esk mRNA are dependent upon initiation at downstream ATG nucleotide sequences in the mRNA. Outcomes from the first two possible ATG sequences are shown. The first possible ATG is in frame with the Esk ORF and would produce a 804aa Esk protein, the second ATG is out of frame with Esk and a protein product of only 5aa would be produced.

A

Sequencing of the 3' Exon/ Intron Boundary of the targetted exon



HindIII

134

Esk cDNA (Published) GAT GAG CTA AGC TTG TCT AAA ATC TGT GCC GAT
XTL1-53 sequence CTA AGC TTG TCT AAA ATC TGT GCC GAT

167 173

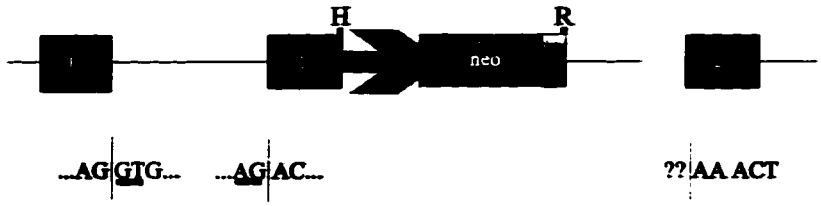
Esk cDNA (Published) CAC ACC GAA ACT GTT AAC CAA A
XTL1-53 sequence CAC ACC G GTTGA AGTTT TTTCT TTGCT TAAAA AA

T

XTL1-53 sequence
28 AAAAA GCCAA ACAAA AACCT AAAAA AAAAC GTT
61 TGAGG AGCCT CCGAG TTGAA GGTGA AGCAG TGT
94 TAACC ACCTT G — Intron —

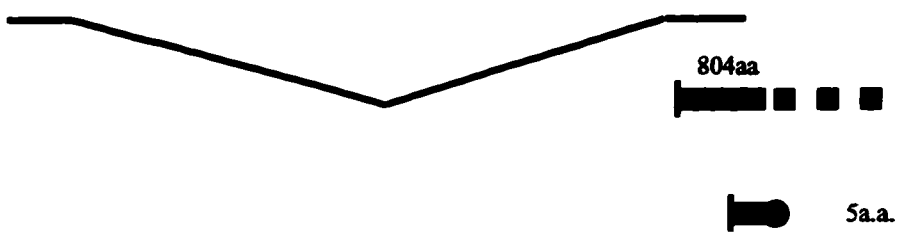
B

Alternative mRNA species from a Targetted allele



WT mRNA splicing

Exon Skipping



amino acids. The second intron is at least 104nt in length with the distance to the splice acceptor for the second coding exon still unknown. Splicing out the first coding exon will therefore produce an *esk* mRNA 2.75kb without the WT initiating methionine. Based on the *esk* cDNA (Douville et al., 1992) two alternative esk translation products initiating at downstream ATG sequences are outlined in Figure 3b. The significance of this alternative mRNA species and the ability of these cryptic ATG sequences to initiate translation is unknown.

Analysis of G418^R/ FIAU^R clones

Hybridization of HindIII and BamH1digested J1 ES cell DNA with a 600bp *esk* genomic probe was predicted to identify a >20kb BamH1 fragment and a 3.2kb HindIII fragment based on restriction mapping of the wild-type locus (Figure 4). The probe lies in a part of the *esk* gene not included in pKS101-KO-9.0, therefore non-homologous vector integration events will not be detected and hybridization of the probe will be specific for changes that result from integration events at the *esk* locus. The probe is predicted to identify a 10.7kb fragment in a correctly targeted locus (Refer to Figure 2). None of the 186 clones screened showed the predicted mutant fragment of 10.7kb. A representative series of clones screened shows only the endogenous > 20kb fragment from all informative individual clones (Figure 5). Table I summarizes the results of the electroporation experiment into J1 ES cells.

Fig.4- **Confirmation of screening strategy**. Based on the known restriction map of the *esk* locus, a 600bp genomic probe was identified that did not contain any repetitive elements and specifically identified the predicted *esk* genomic fragments on a genomic southern blot digested with HindIII (H) and BamH1 (B). (Refer to Fig.1)(Southern Blot courtesy of Simon Ginsberg)

H B

Fig.5- **Southern blot analysis of J1 ES cell clones transfected with pKS-101-9.0-** Shown are representative southern blots of DNA isolated from individual neo^r, FIAU^r J1 ES clones. DNA was digested with BamH1, blotted to nitrocellulose and hybridized with a 600bp genomic probe from the *esk* locus. As indicated in Fig.1, a >20kb genomic fragment is derived from the WT allele, the arrow indicates the approximate position of the predicted 10.7kb band that would indicate a targeted allele. (Southern blots courtesy of Simon Ginsberg)

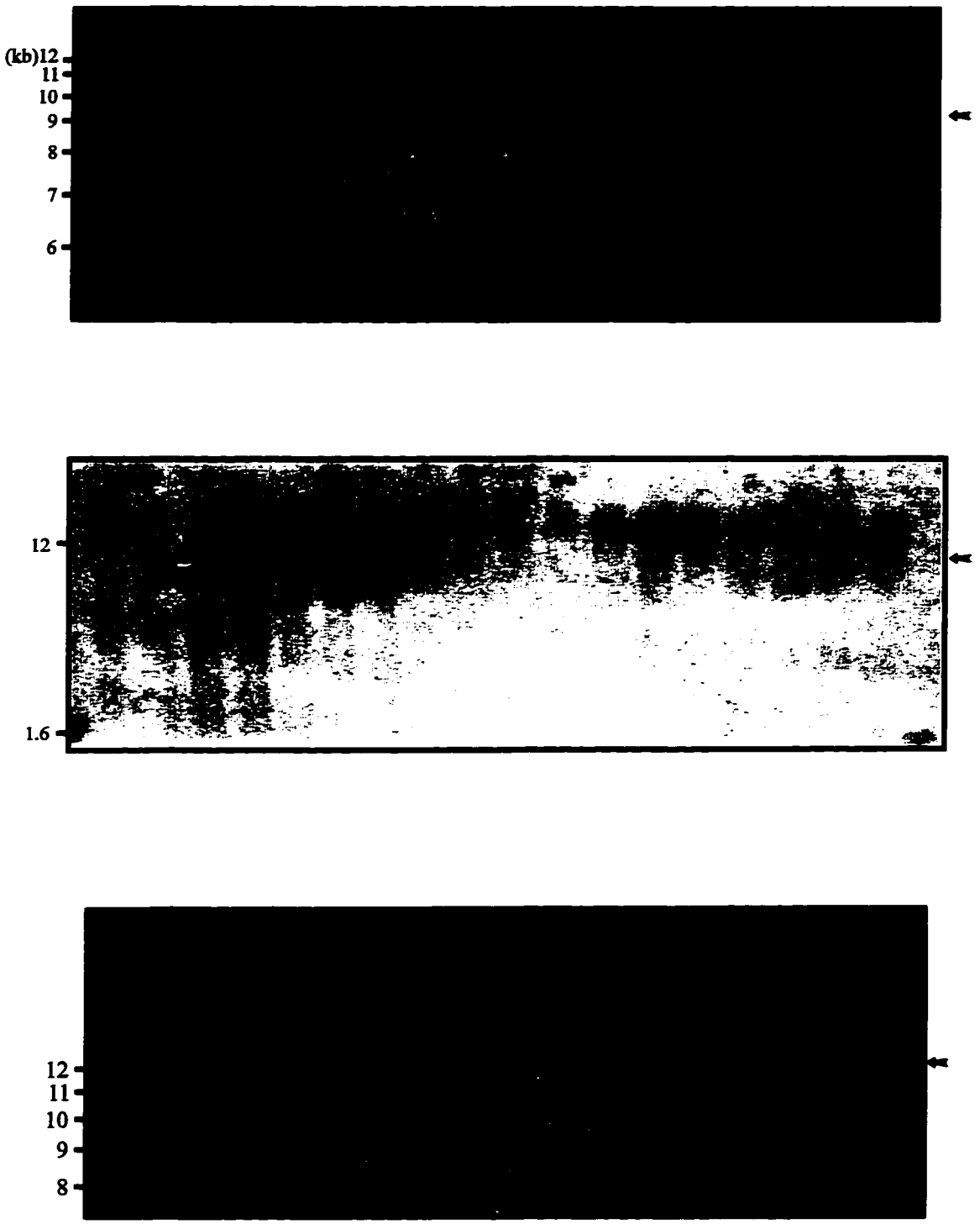

(kb)12
11
10
9
8
7
6
12
1.6
12
11
10
9
8

Table I- Summary of pKS101-KO-9.0 electroporation into J1 ES cells

J1 ES cells electroporated	5×10^7
G418 resistant, FIAU resistant colonies	238
G418 resistant, FIAU resistant colonies picked	214
G418 resistant, FIAU resistant colonies screened	186
Number of homologous recombination events	0

Electroporation and selection procedure is described in methods

2.4 Discussion

Gene targeting has been a useful approach to gain an understanding of the normal role of many genes. The observation of how a developing mouse copes in the absence of a gene can identify developmental pathways or suggest specific cell types where a genes function is normally important. The information gained from targeted disruption of a gene can arise from gross effects such as the *fgfr1* -/- mouse which die in utero as a result of embryonic growth defects and aberrant mesodermal patterning (Yamaguchi et al., 1994) or from more subtle observations such as those of the *c-src* -/- mouse which have reduced size compared to heterozygote littermates and whose incisors fail to erupt. Further investigation led to the conclusion that the *c-src* -/- mice had defective osteoclast function and were osteopetrotic (Soriano et al., 1991). Gene targeting was also able to expose subtle differences in the activities of the Flk-1 and Flt-1 receptor tyrosine kinases. Both receptors are activated by VEGF binding and both were believed to be important for the development of embryonic vasculature. Embryos homozygous for flk1(-/-) were embyonic lethal and indicated that the kinase was important for the development of haematopoietic cells and vasculogenesis (Shalaby et al., 1995). Embryos homozygous for flt-1 (-/-) underwent normal endothelial cell development but the endothelial cells were assembled in disorganized vascular structures which also led to embryonic lethality (Fong et al., 1995). It is clear that it would have been very difficult to identify these subtle differences without the benefit of gene targeting.

To effectively disrupt the normal function of a protein, the positive selection cassette is

usually targeted into an exon or in place of a series of exons that are important for the normal activity of the protein or into the exon containing the initiating methionine to effectively eliminate any functional mRNA and thus protein. Targeted disruption of IRF-1 and IRF-2 involved the insertion of the selection cassette into the exon that coded for DNA binding activity of the proteins, therefore it was presumed that elimination of this region of the protein would effectively eliminate protein function (Matsuyama et al, 1993). Targeted disruption of the Src protein is an example of effective elimination of the mRNA and protein by insertion of the positive selection cassette into the first coding exon therefore effectively eliminating any functional *c-src* mRNA or protein.(Soriano et al., 1991). Based on the information acquired by restriction digest of isolated *esk* genomic clones an approach that targeted the first coding exon was chosen over one that might target exons in the catalytic domain primarily because the genomic structural information available was from the 5' end of the *esk* gene and the exonic structure of the kinase domain was less well defined.

Fragments of the *esk* gene were cloned into the pKS-NT targeting construct (Figure 1) which contains both *neo* and *hsv-tk* expression cassettes under the control of the *pgk1* promoter. This vector was used successfully in the targeted disruption of many genes including the *c-abl* and *fgfr1* genes (Tybulewicz et al., 1991; Yamaguchi et al., 1994) and enables a positive-negative selection strategy to be used. Positive selection, selects for cells that grow in the presence of G418 and therefore contain the input targeting vector. Negative selection, selects for growth in the presence of FIAU/GANC and therefore

against random integrations of the input vector that would therefore incorporate the *hsv-tk* gene. Positive-negative selection has been suggested to enrich from 20-2000 fold for homologous versus non-homologous integrations (Mansour et al., 1988; Karaplis et al., 1994).

The final targeting construct contains 9kb of homology extending from the extreme 5' end of the gene to the first coding exon where the *neo*^{R} cassette is designed to insert into the HindIII site in the middle of exon 1. The 3' fragment extends towards the alternatively spliced exon and contains 3 or 4 exons that have not yet been mapped but contain 400-500 nt of the cDNA within the 5.25kb(Figure 2). Successful targeting constructs have been designed with as little as 3.5kb of total homology (Matsuyama et al., 1993) but typically contain 5-8kb of homology (Joyner, A.L., 1993). Homologous recombination of pKS101-KO-9.0 will result in the deletion of 0.8kb of the *esk* gene and insertion of the 1.74kb *neo* selection cassette. Generally, deletions of no larger than 3kb are preferred to avoid disruption of multiple genes located adjacent or internal to the targeted locus (Joyner, A.L., 1993). It has been reported that targeting constructs that deleted 19.2kb had the same targeting frequency as constructs that deleted as little as 3kb of the *hprt* locus (Zhang et al., 1994).

The length of an exon can affect the fidelity of the mRNA splicing machinery such that an unusually large exon with a positive selection cassette inserted may not be recognized as efficiently by the splicing machinery and therefore be skipped (Joyner,

A.L., 1993). The result is that a mRNA transcript initiated from an endogenous promoter may delete the mutated exon producing a transcript missing only the targeted exon. If the exon contains a unit number of codons then the result can be an in frame deletion and a protein that can retain partial or complete function. Sequencing the 3' exon/ intron boundary of the first coding exon confirmed that the exon codes for 45aa plus 1nt (Figure 3a) which in the event of exon skipping would normally produce a mRNA with a frame shift and prevent the translation of functional protein. However, because the targeted exon contains the initiating methionine, exon skipping will result in a mRNA deleted of 154nt but one which may be capable of initiating translation at any downstream ATG in frame or out of frame. In fact, consulting the published cDNA sequence suggested that the first such ATG would in fact produce an in frame protein product starting at aa 52 (Figure 3b). The significance of this situation is unknown at the present time. There are examples of gene targeting to the first coding exon, perhaps the most relevant is the *c-src* targeted disruption where the neo selectable marker was inserted into the second exon in the gene representing the first coding exon (Soriano et al., 1991). This situation mimics the *esk* targeting strategy and successfully produced a knockout of the *c-src* allele.

pKS101-KO-9.0 was electroporated into J1 ES cells following methods that have been used previously (Li et al., 1992). 234 colonies survived selection in G418 and FIAU from $5x10^7$ cells electroporated, 214 of these were isolated to be screened individually by southern blot. An ideal primary screening strategy uses an external

probe and a restriction digest that does not cut within the vector. Under these circumstances a single homologous integration event can be distinguished from concatamer integrations or integrations that include the *hsv-tk* gene by an increase in fragment size that can be distinguished from any other possibilities. An external probe only detects integration events that have occurred at the specific targeted locus and are preferred over probes that are internal to the targeting construct which will detect all integrations and can result in misdiagnosis if by chance, the informative band on southern blot is similar in size to the diagnostic band that indicates homologous integration (Joyner, A.L., 1993). Due to the large size of the pKS101-KO-9.0 targeting construct it was not possible to find a restriction enzyme that did not cut in the targeting construct and could still be resolved on a gel. Therefore the screening strategy that was chosen relied on the introduction of a BamH1 restriction site just 3' to the *neo* positive selection cassette between the two *esk* genomic fragments that were cloned into the vector and an external probe that will only differentiate homologous targeting events from the endogenous allele (Refer to figure 2). BamH1 digestion of the endogenous locus produces a fragment > 20kb (Figure 4) while the targeted allele is predicted to produce a fragment of 10.7kb. The drawback to this strategy is that it only analyses the 3' end of the integration site and therefore only distinguishes vector integration at the desired locus from random integration. To confirm that homologous recombination has occurred a second digest and southern blot using a probe to test the 5' integration site would also need to be done. The same primary screening strategy was used with success to confirm the targeted integration of a pPNT based targeting

vector that disrupted the parathyroid hormone-related peptide (PTHrP) gene (Karaplis et al., 1994).

The same positive-negative selection strategy and the same pPNT based vector have been used successfully in the disruption of genes such as *c-src*, *FGFR1* and *c-abl* , but screening 186 colonies failed to identify a homologous recombinant at the *esk* locus following electroporation of $5x10^7$ J1 ES cells with 100μg of linearized pKS101-KO-9.0 (Figure 5). It is not yet fair to say that using this approach will not successfully yield a disrupted *esk* allele. Prior to the positive-negative selection procedure, groups regularly reported screening 800-1000 colonies before identifying a successfully targeted allele. From 3 distinct electroporations using a positive-negative selection strategy, targeted disruption of the c-abl gene ranged from 1/10 to 1/83 (Tybulewicz et al., 1991). Others have documented efficiencies as low as 1 in $1x10^7$ electroporated cells and even 1 in $2x10^7$ electroporated cells (Matsuyama et al., 1993; Molina et al., 1992). However, using the same targeting construct and a vector with 8.7kb of homology (compared to 9.0kb for *esk*) the FGFR1 gene was disrupted in 1/11 G418 and FIAU resistant clones (Yamaguchi et al., 1994).

What is clear, is that the targeting efficiency can vary widely depending on the gene being disrupted and the targeting construct being used. There is every reason to believe that if the experiment was repeated and more colonies screened this strategy would produce a disrupted allele. What is also clear since the decision was made to disrupt

the *esk* locus is that improved targeting strategies have been developed to increase the efficiency of targeting a gene as well as improve the ability to detect such events. Our lab as well as others have seen the advantages of using a di-cistronic targeting construct that takes advantage of promoter trapping, cap-independent translation of a selectable marker and a β-galactosidase reporter gene that can indicate the success of the promoter trap/ homologous recombination event and enables sensitive detection of cellular sites of transcription from the endogenous promoter (Mountford et al., 1994). This vector increases sensitivity of a targeted event by forcing the positive selection to be driven from an endogenous promoter and takes advantage of an internal ribosome entry site (IRES) that links the homologous recombination event to the selectable marker. The only requirements are that the targeted gene must be a transcriptionally active gene in embryonic stem cells to drive the drug selection and the gene fragments integrated on either side of the IRES-βgeo must not contain any transcriptional control elements. We know that esk is expressed in embryonic cells because it was originally cloned from embryonal carcinoma cells. The current 5' fragment would be inappropriate for the IRES vector because it contains a large amount of the gene upstream of the transcriptional start site and likely contains promoter elements. An IRES based vector would be suitable to target the *esk* gene provided the 5' genomic fragment was reduced and did not contain any promoter elements.

Based on the suggested functions of the putative Esk family members, Ttk and Mps1 from human and yeast, the elimination of Esk from the mouse is expected to severely

affect the normal progression of the cell cycle. Mps1 is an essential gene in yeast where temperature sensitive mutations lead to 2 distinct effects, failure to duplicate the spindle pole body (Winey et al., 1991; Lauze et al., 1995) and failure to arrest at the G2/M mitotic check point in response to a monopolar spindle (Weiss and Winey, 1996). Mps1 kinase activity is required to rescue both functions implying that Esk kinase activity may be similarly important to the regulation of murine microtubule structures. There are many examples of signal transduction pathways that are conserved from yeast to mammals so it is not unreasonable to presume that Esk may be involved in microtubule maintenance. Study of the yeast Mapk pathway was one of the major reason for the eventual greater understanding of parallel pathways in mammalian cells (Herskowitz, I., 1995). Although Mps1 is only 67% similar in the catalytic domain to Esk and not homologous elsewhere it is entirely possible that conclusions drawn in yeast in the absence of Mps1 might be an indication of what to expect in the mouse nullizygous mutant of Esk. Ttk mRNA, protein and kinase activity are cell cycle regulated and peak at the G2/M transition (Hogg et al., 1994; Schmandt et al., 1994). Esk effect on cell cycle progression has yet to be demonstrated but Ttk function again points to a role for Esk in the cell cycle that may be demonstrated by targeting the esk allele. Whether as yet unidentified kinases act redundantly in the mouse and rescue Esk activity is unknown but how the mouse may develop in the absence of Esk remains an intriguing question and one that remains unanswered.

Chapter 3
Localization of the Esk kinase isoforms

3.1 Introduction

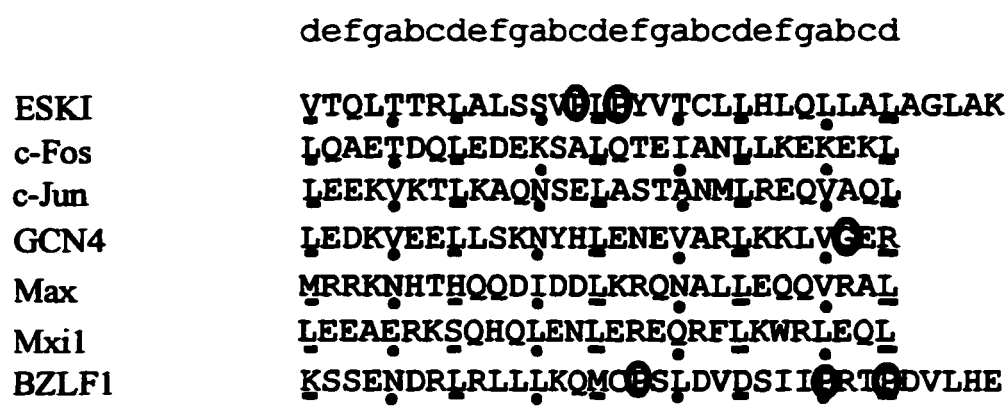
The primary sequence of a newly cloned kinase can suggest what function it may play within the cell. Several well established protein epitopes exist apart from the kinase domain that can imply a particular function. Hallmarks for receptor kinases include a signal sequence, a ligand binding domain such as EGF like repeats or fibronectin repeats, and a transmembrane domain followed by an intracellular kinase domain (Katoh et al., 1992). Cloning a kinase with these motifs immediately suggests that the kinase is a receptor kinase and that it is likely regulated by binding of a ligand from either the extracellular milieu or from within the soluble cytoplasm if the kinase is located in an internal membrane. Similarly, there are a number of motifs found in soluble kinases that can also suggest a function or at least a method of regulation. For instance, several kinases have been identified with SH3, SH2 or PH domains that are involved in specific protein-protein interactions or bind a subset of lipid structures (Koch et al., 1991; Songyang et al., 1993; Mussachio et al., 1993). A kinase with homology to these motifs suggests that specific protein-protein interactions are important in the regulation of this kinase. Identification of a protein motif within a cloned protein only suggests areas of study, it does not give direct evidence of a function for an unknown kinase. Identifying the subcellular localization of an unknown kinase that has no recognizable protein motifs has proved to be a useful way to gain information about the function of a kinase. The Focal adhesion kinase (FAK) is a 120kDa kinase lacking homology to any known protein motifs. Following the production of antiserum, the kinase was localized by immunostaining and found to be concentrated in focal adhesions implicating the kinase in

signal transduction pathways that respond to cell interactions with the extracellular matrix (Hanks et al., 1992). FAK has also been shown to be activated by cell adhesion (Hanks et al., 1992). Further study, concentrated on identifying the function of FAK at focal adhesions, has found that FAK is essential for focal adhesion turnover and mesodermal and endodermal motility during development (Ilic et al., 1995; Grant et al., 1995; Ilic et al., 1996). Similarly, the primary amino acid sequence of the STY/ Clk kinase suggested only that the kinase might be nuclear based on the homology of a basic stretch of amino acids to some nuclear localization signals (NLS) (Howell et al., 1991). The kinase was subsequently shown to be nuclear by immunostaining (Duncan et al., 1995) and further study of its role in the nucleus has suggested that the kinase is involved in regulating splicing factors in the nucleus (Colwill et al., 1996).

An *esk1* specific cDNA probe of a mouse D3 ES cell genomic library led to the cloning of 3 genomic portions of the esk allele. Sequencing of the genomic clones confirmed that the *esk1* and *esk2* mRNA transcripts were derived from the same gene by alternative splicing in of the 78nt exon to produce the *esk1* mRNA or splicing out to produce the *esk2* mRNA. This exon was initially described as an alternatively spliced transmembrane domain (Douville et al, 1992). An EMBL data library PROSITE database search of Esk to identify protein epitopes in Esk common to other proteins suggested that this alternatively spliced exon may also be a leucine zipper domain homologous to those found in transcription factors such as the mammalian c-fos and c-jun and yeast GCN4 (Figure 1a)

Fig. 1- **Conserved Protein Epitopes found in Esk primary Amino Acid sequence-** (A) An EMBL database PROSITE search suggested that the alternatively spliced exon in Esk1 is homologous to leucine zipper domains, defined as five heptad repeats of leucine residues. Nomenclature has defined these repeats as coiled coil structures where the amino acids at position 'd' (underlined, usually leucine) and 'a' (dotted, usually hydrophobic) of the repeat make up the dimerization face (Vinson et al., 1993). Leucine zipper domains have been defined for c-Jun and c-Fos (O'Shea et al., 1989b), GCN4 (O'Shea et al., 1989a), BZLF1 (Kouzarides et al., 1991) and Mad and Mxi1 (Zervos et al., 1993). Helix breakers such as glycine and proline are circled. (B) A stretch of 6 basic amino acids at the end of the Esk1 and Esk2 polypeptides is reminiscent of the SV40 Large T antigen type NLS defined by a basic stretch of amino acids downstream of a cluster of potential phosphorylation sites (Kalderone et al., 1984). The domain was more rigidly defined in proteins such as human c-Myc and *xenopus laevis* lamin L_1 as having a K-R/K-X-R/K consensus core (Chelsky et al., 1989). The nuclear STY kinase has a homologous stretch of basic amino acids that is important for nuclear localization (Howell et al., 1991; Duncan et al., 1995).

A



B

Consensus	$K^n/_kX^n/_k$	Location
SV-40 Lg T Ag	T- -S - -ST- PKKKRKVED--	N
Esk	SSSS -T- - KKRERK ·	??
STY	T- -SSSS - KRKKRSHSS	N
xenopus Laevis Lamin L_1	- TTKGKRKRI DV	N
Human c-Myc	- - KRVKLD	N

(O'Shea et al., 1989b; O'Shea et al., 1989a). Mutation in the leucine zipper domain of these transcription factors eliminates dimerization dependent DNA binding (Kouzarides et al., 1990; O'Shea et al., 1989a). Basic helix-loop-helix type transcription factors such as Myc with its heterodimerization partner Max use the leucine zipper to mediate protein-protein dimerization which is required for subsequent transcriptional activation (Blackwood and Eisenman, 1991). Mad and Mxi1 are examples of proteins that use leucine zipper to mediate dimerization in the absence of activating transcription (Zervos et al., 1993). A second motif found at the carboxy terminus of both Esk isoforms was a stretch of six basic amino acids downstream of a several potential S and T phosphorylation sites. This motif is reminiscent of an SV-40 large T antigen type nuclear localization signal (NLS) which follows a cluster of S and T phoshorylation sites (Figure 1b)(Kalderone et al, 1984). A similar stretch of basic amino acids has been shown to be required for the nuclear localization of human c-Myc, *xenopus Laevis* Lamin L_1 and important for directing murine STY kinase to the nucleus (Chelsky et al., 1989; Duncan et al., 1995).

Protein localization is thought to arise from a heirachy of epitopes within a protein. Signals involved in transmembrane protein kinase localization include a cleavable signal peptide and a hydrophobic stretch of at least 20aa followed by a basic residue which serves as a membrane anchor (von Heijne, G., 1988). Esk1 has no recognizable signal sequence suggesting that the internal hydrophobic stretch may direct the kinase on its own, or that perhaps this domain is important for protein-protein interactions rather

than membrane localization. Protein nuclear localization signals (NLSs) fall into one of 3 categories: (I) Rare motifs with a few basic residues that are required to drive the protein to the nucleus, (ii) bipartite NLSs which are present in 56% of nuclear proteins consist of 2 clusters of basic residues separated by 10-12aa and (iii) SV-40 large-T-Antigen type NLS consisting of a cluster of basic residues (Zacksenhaus et al., 1993). The relevance of these protein motifs in the function of the Esk1 and Esk2 kinases could help identify the importance of the two isoforms and suggest possible roles for the kinases within the cell.

The Esk kinase is a 97kDa protein identified as a doublet by *in vitro* autophosphorylation kinase assay from P19 EC cells. Esk1 and Esk2 cannot be distinguished in this assay and no antibody has been made that is specific to one isoform, therefore, questions addressing the functions of the two kinases have been difficult to answer.

To investigate the localization of the Esk isoforms separately the *esk* cDNAs were tagged with DNA coding for the six-repeat human myc epitope (Roth et al, 1991). Epitope tags such as glutathione-S transferase (GST) and the six repeat histidine tag have been exploited for protein purification and to demonstrate protein-protein interactions on affinity columns (Rindisbacher et al., 1995; Koland et al., 1991; Songyang et al., 1993). Haemaglutinin (HA), FLAG and the Myc epitope tags have high affinity antibodies that specific for the epitope (Howard et al., 1995; Knappit and

Pluckthun, 1994; Evan et al, 1985). These antibodies allow epitope tagged proteins to be detected with high specificity by immunofluorescence and immunoblot of cell lysates following transfection of mammalian expression vectors with an epitope tagged protein. Nuclear localization of the STY protein kinase was recently demonstrated by detection of a Myc epitope tagged Sty kinase following transient transfection in Cos-1 cells (Duncan et al, 1995). Using the six-repeat of the human Myc epitope in frame with Esk1 and Esk2 enables us to address questions about kinase oligomerization and test protein, protein interactions separately for the two isoforms of the kinase. The availability of a specific high affinity antibody for immunoblot will also enable the kinases to be examined when in a inactive state in addition to a kinase active state which is the only way to assay for Esk with the antibodies currently available. Most importantly, the epitope tagged proteins permit the analysis of the subcellular localization of the two isoforms of the Esk kinase which might identify a novel method of regulation between the two isoforms and identify differences in their functions within the cell.

3.2 Methods

Antibodies- The mouse monoclonal antibody (mAb) 9E10 (Evan et al, 1985) derived from a hybridoma supernatant detected the six-repeat of the human Myc epitope (Roth et al, 1991) and was followed by a goat anti- mouse fluorescein conjugated antibody (DAKO) for immunofluorescence or Goat anti- mouse HRP(Jackson Immunoresearch Lab., Inc.) for chemi-luminescence. Two rabbit polyclonal antibodies were used for

detection of Esk. For immunoprecipitation, an antibody directed against the carboxy terminal 12 amino acids (aa 845-856) generated in our lab was used. For immunoblots, an antibody raised against amino acids 838-856 of Esk (Santa Cruz Biotechnology, Inc) was used followed by a Goat anti-rabbit HRP conjugated antibody (ICN) for chemi-luminescence. mAb 104 is a mouse IgM which detects a phospho-epitope common to SR proteins (Roth et al., 1990). mAb 104 was detected using a biotin labelled anti mouse IgM secondary (Amersham) followed by streptavidin coupled HRP(Amersham). The p85 subunit of PI3 kinase was detected on immunoblot using a rabbit polyclonal antibody to full length p85 (Upstate Biotechnology, Inc., Cat. 06-195).

Cloning of the Myc epitope to the amino terminus of Esk1 and Esk2- The six repeat human Myc epitope tag at the amino terminus of the Sty kinase has been previously described (Duncan et al, 1995). Digestion with EcoR1 released the Sty cDNA, the EcoR1 sites were blunt ended with T4 DNA polymerase (Maniatas et al., 1982) and the blunt ended vector was then further digested with Pvu1, a unique site within the pECE ampicillin gene (Ellis et al., 1986). This 1.3kb Pvu1/blunt fragment containing a portion of the ampicillin gene and the six-repeat of the human Myc epitope tag was then cloned into a pECE construct containing the entire Esk2 cDNA by digestion of a pECE-Esk2 vector with Sal1, the restriction site was then filled in with T4 DNA polymerase and digested with Pvu1. The two fragments were then ligated to generate pECE-M Esk2. Sequencing of pECE-M Esk2 confirmed that the junction between the

epitope tag and Esk2 was in frame.

pECE-M Esk1 was then derived from pECE-M Esk2. A 1.9kb Pst1 fragment with the first 560ntds that code for Esk, the epitope tag and a portion of the pECE vector containing part of the ampicillin gene was cloned into a similarly digested pECE construct containing the entire Esk1 cDNA.

Both constructs were confirmed by restriction digest and by Cos-1 cell transient transfection which indicated that these constructs were immunoreactive with the monoclonal antibody 9E10 on western blot following an immunoprecipitation with a rabbit polyclonal antibody to the carboxy terminus of Esk.

DNA Sequencing- The nucleotide sequence of the junction between the six-repeat human *myc* epitope and the *esk2* cDNA (Douville et al., 1992) was sequenced using an oligo in the epitope itself (PDMP, [AGC TTG GGC GAC CTC ACC-OH]) and standard dideoxy chain termination methods for double stranded sequencing (Sanger et al., 1977).

Immunoblot and Kinase Assay- Cos-1 cells ($2x10^6$) were electroporated (220 volts, 960 μFa) using a BioRad Gene Pulser™ in serum free media with 20μg of DNA. The following day, the cells were lysed in immunoprecipitation buffer in the presence of protease inhibitors (20mM Tris (pH 7.5),150mM NaCl, 2mM EDTA, 1% Triton X-100, 2mM NaF, 2mM sodium pyrophosphate, 500μM ammonium vanadate, 200μg/ml phenylmethylsulfonyl flouride, 2μg/ml aprotinin, 5μg/ml leupeptin). The lysates were

cleared of the detergent insoluble fraction and immunoprecipitated with anti-Myc (mAb 9E10) and protein A-sepharose (CL4B) (Pharmacia) pre-coated with rabbit anti-mouse IgG(H+L)(Jackson ImmunoRes. Lab., Inc., Cat. 315-005-003). Autophosphorylation kinase assays were performed for 30min. in kinase buffer (20mM 2[*N*-morpholino]ethanesulfonic acid (MES; pH 6.5), 10mMMgCl_2, 2mM MnCl_2) with 0.5μCi/μl [γ-^{32}P]ATP (Amersham). The reactions were stopped with an equal volume of 4x Sample buffer and heating at 100°C for five minutes. Immunoprecipitations were resolved by 8% SDS-polyacrylamide gel electrophoresis. Protein transfer to nitrocellulose (Costar Sci. Corp., Bioblot-NC. Cat. 8812) was followed by autoradiography (kinase assay) or immunoblot with anti-Myc and chemiluminescence (Kirkegaard and Perry Laboratories).

Immunofluorescence Microscopy- Cos-1 cells were transiently transfected. After electroporation, 1x10^5-2x10^5 cells were plated onto gelatinized (0.15%/PBS) coverslips. 16-20h post transfection the cells were washed with PBS, fixed in 3% Paraformaldehyde in PBS for 10min and then permeabilized by a 0.2% Triton-X100 (BDH) extraction for 30min at room temperature. Sodium borohydride washes prepared the fixed cells for incubation with the 9E10 mouse monoclonal antibody (1/100 from supernatant). Coverslips were washed with PBS and incubated with an FITC conjugated anti-mouse antibody (1/20)(DAKO) and washed with PBS again. DNA was stained using Propidium iodide (20μg/mL in PBS) at room temperature for 10-30 minutes after RNaseA treating (100μg/mL) for 30 minutes at room temperature. Finally, the coverslips were washed

several times in PBS before mounting with Anti-Fade reagent (Molecular Probes, Inc; Cat. No. S-7461). Cells were viewed by confocal microscopy using an upright Leica Confocal Laser Scanning Microscope equipped with a 55mW kypton/argon air-cooled laser and a 63x Plan Apo oil immersion lens.

Cellular Fractionation- Isolation of cell fractions was previously described (Kampinga et al, 1995). Briefly, the nuclear fraction of P19 EC cells was isolated following a PBS wash, the cells were harvested in a second PBS wash by scraping the cells from the tissue culture dish. The cell suspension was pipetted several times to ensure a homogenous mix then centrifuged (600xg,3 min). The cell pellet was resuspended with TX-100 (1% Triton X-100, 0.08M NaCl, 0.01M EDTA, pH7.2), centrifuged (600xg,3 min) and again washed in TX-100. Following centrifugation , the cell pellet was subsequently washed in TNMP (10mM Tris-Base, 10mM NaCl, 5mM $MgCl_2$ and 0.1mM PMSF) and centrifuged leaving a pellet of clean nuclei. To isolate the cytoplasmic fraction, cells were washed with PBS, harvested in TNM (hypotonic) buffer (10mM Tris-HCL, pH7.5; 10mM NaCl, 1.5mM MgCl2, 1mM PMSF and 2μg/ml aprotonin and 5μg/ml leupeptin.) and swollen for 20 min. on ice. Dounce homogenization (8-10x) and centrifugation (10min,1000xg) pelleted the cell nuclei, leaving a supernatant of the cytoplasmic fraction. The supernatant was centrifuged a second time (60min, 100000xg) leaving a purified cytoplasmic fraction.

Esk was immunoprecipitated directly from the cytoplasmic fraction using a rabbit polyclonal antibody against Esk. Prior to immunoprecipitation of the nuclear fraction, the clean nuclear pellet was lysed in immunoprecipitation buffer, the insoluble portion was

cleared by centrifugation and the rabbit polyclonal antibody to Esk was then added to the purified nuclear lysate. Protein A-sepharose (CL4B) (Pharmacia) was added to the fractions which were then separated into Esk immunodepleted nuclear and cytoplasmic fractions and Esk immunoprecipitated nuclear and cytoplasmic fractions. Sample buffer was then added to the fractions and the proteins were resolved by 8% SDS-polyacrilamide gel electrophoresis. Protein transfer to nitrocellulose led to detection of Esk, SR proteins and p85 by immunoblot. Nitrocellulose was stripped by incubation for 30min at 50oC in 100mM β-Mercaptoethanol, 2% SDS and 62.5mM Tris-HCl(pH 6.7) and washed in TBST for 20min.

3.3 Results

Epitope Tagged Esk1 and Esk2

The human Myc epitope tag is a 12aa motif that is recognized with high affinity by the mAb9E10 (Roth et al., 1991). The Myc epitope that was cloned to the amino terminus of Esk2 and Esk1 codes for a six repeat of this 12aa sequence and has been described previously (Duncan et al., 1995). The construction of the Myc epitope tagged Esk2 and Esk1 mammalian expression vectors involved a series of pECE plasmid swaps (Figure 2). A schematic of the protein structure of the two Esk isoforms highlights the amino acids distinct between the kinases and also epitopes that are shared between Esk1 and Esk2 (Figure 3a). The Myc epitope provides an additional 102aa and sequencing demonstrated that the epitope fused inframe with the initiating methionine of Esk provided 16 novel amino acids (Figure 3b). The epitope was

Fig. 2- **Flow Chart depicting the Construction of Mesk2 and Mesk1**- pECE-M-Esk2(**4**) was made by swapping out a 1.0kb Pvu1, blunt ended Sal1 fragment consisting of a portion of the ampicillin resistance gene of pECE and the *SV-40* promoter from pECE-Esk2(**1**) and replacing it with a modified Pvu1 digested, EcoR1 digested and blunt ended 1.3kb fragment(**3**) consisting of the same portion of the ampicillin gene and *SV-40* promoter plus the six repeat human *myc* epitope from pECE-M-STY(**2**). This 1.3kb fragment was ligated with pECE-Esk2(**1**) digested with Sal1 and blunt ended at the 5' end of *esk2* and then digested with Pvu1 to contain the remainder of the Ampicillin resistance gene. The amino terminal portion of *esk2* up to the unique Pst1 restriction site (nt 560) was swapped out of pECE-M-Esk2(**4**) along with the *SV-40* promoter and part of the ampicillin resistance gene up to a Pst1 site. This 1.88kb fragment(**5**) was then cloned into pECE-Esk1(**6**) replacing a similar amino terminus that lacked the six repeat of the human *myc* epitope. Therefore pECE-M-Esk1(**7**) was made from the 1.88kb amino terminal fragment of pECE-M-Esk2(**5**) cloned into a 4.3kb Pst1 fragment with the remainder of the ampicillin gene and pECE-Esk1(**6**) lacking the amino terminal 560nt. Where Cl, H, K, N, P, PV, R, S, Sa, Sm, and X represent Cla1, HindIII, Kpn1, Nhe1,Pst1, Pvu1, EcoR1, Sal1, Sac1, Sma1 and Xba1 restriction sites.

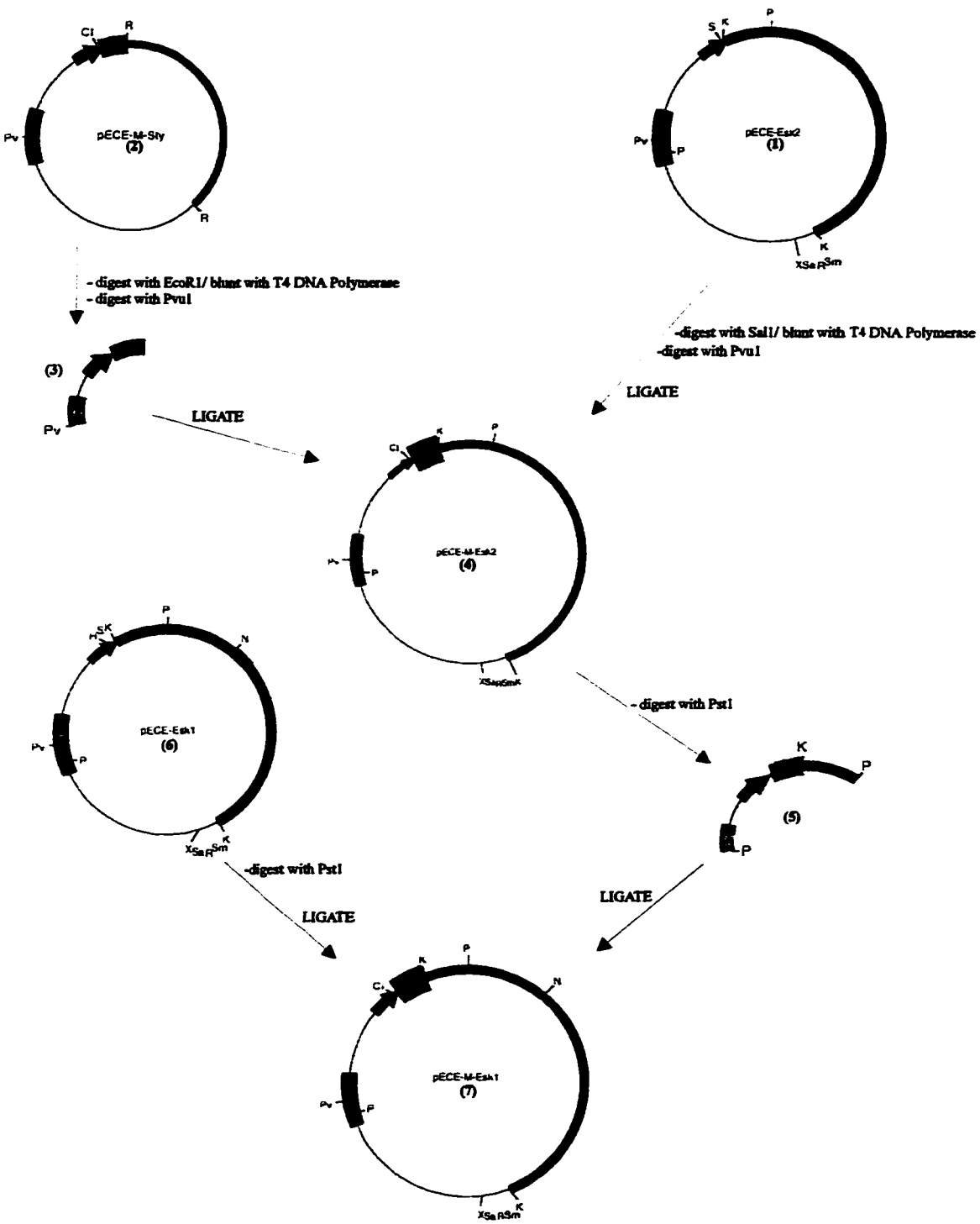
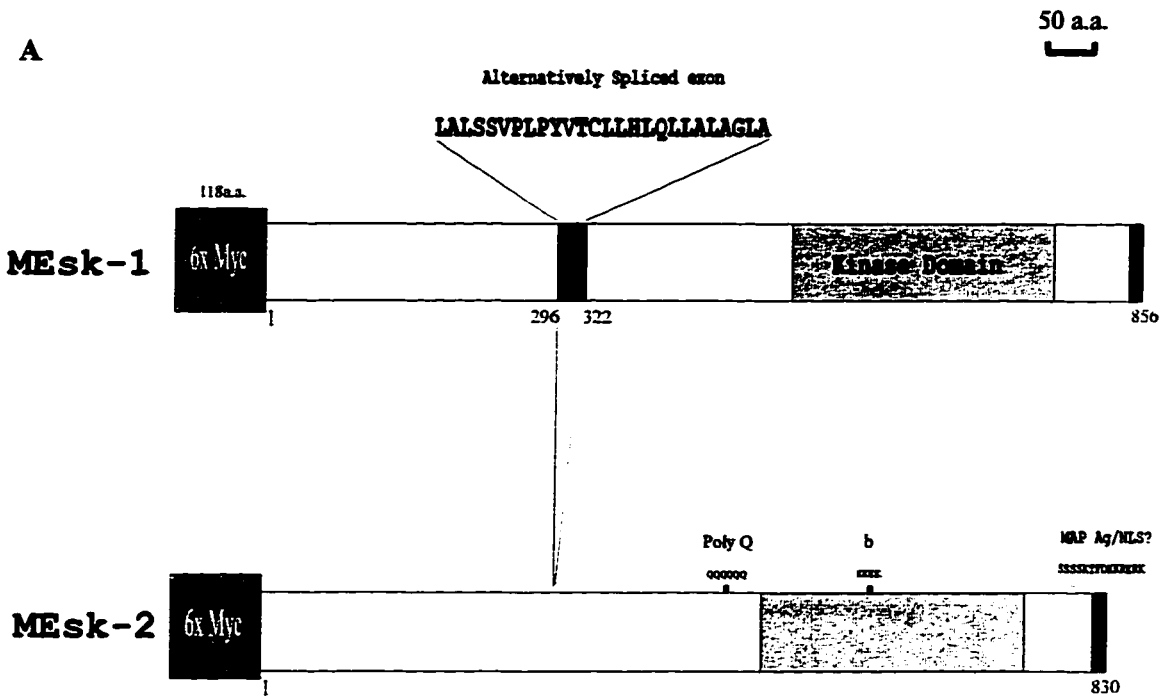

pECE-M-Sty
(2)
pECE-Esk2
(1)
- digest with EcoR1/ blunt with T4 DNA Polymerase
- digest with Pvu1
-digest with Sal1/ blunt with T4 DNA Polymerase
-digest with Pvu1
(3)
LIGATE
LIGATE
pECE-M-Esk2
(4)
- digest with Pst1
(5)
pECE-Esk1
(6)
-digest with Pst1
LIGATE
LIGATE
pECE-M-Esk1
(7)

Fig.3- **Schematic of the Esk isoforms in frame with the six-repeat of the human Myc epitope-** (A) MEsk1 can be distinguished by the presence of an additional 26 amino acids as a result of the splicing in of the alternate exon, spliced out of Mesk2.Common features include, the six-repeat of the human *myc* gene (Roth *et al.*, 1991) that codes for a protein epitope immunoreactive to the 9E10 mouse hybridoma (Evan *et al.*, 1985). The epitope recognized by the two Esk polyclonal antibodies is indicated (black box), this epitope contains a basic stretch of amino acids reminiscent of the SV40 virus large T antigen (Kalderone *et al.*, 1984). The catalytic domain is defined by all eleven sub-domains conserved between members of the protein kinase super-family (Hanks et al.,1988)(shaded box). A polyglutamine tract (poly Q: amino acids 491-496) and an additional basic stretch of amino acids (b: amino acids 613-616) are also found in both isoforms.(B) Sequencing with the PDMP oligo through the junction of the six repeat human *myc* epitope(bold) and the *esk* coding region(shaded) showed that the fusion(horizontal line) maintained the frame of Esk and encodes 16 novel amino acids including 5 phosphorylatable residues (2x Ser, 2x Thr and 1x Tyr).



B

Sequence of Junction between myc epitope and esk cDNA

sixth repeat of *myc* epitope

92

PDMP Sequence

ATT TCT GAA GAG GAC TTG AAT TTC GAC GGT ACC ATG GCT TGG

I S E E D L N F D G T M A W

1 2 3 4 5 6 7

Esk cDNA

PDMP Sequence

CTT TCA GAG ACG AGT TAC AAT TTA GAA ATG GAG GCT GAA ...

L S E T S Y N L E M E A E ...

8 9 10 11 12 13 14 15 16

therefore predicted to increase the size of the Esk polypeptides by 118 amino acids and an additional 13kDa.

MEsk1 and MEsk2 are Kinase active

To address whether the epitope tagged versions of the Esk kinase would mimic the activities of endogenous Esk kinase, the two kinases were overexpressed in COS-1 cells by transient transfection. Myc immunobot of the transfected COS-1 cell lysate indicated that the kinases were both expressed and that MEsk1 produced a protein product 120kDa while M-Esk2 was 110kDa (Figure 4a). Immunoprecipitation of the kinases with an Esk antibody directed against the final 12aa of the Esk protein followed by Myc immunoblot confirmed that the epitope tagged isoforms represented full length Esk protein in frame with the epitope tag (Figure 4b). The epitope tagged kinases were then tested for their ability to autophosphorylate in an *in vitro* kinase assay. Demonstrating the catalytic activity of the epitope tagged kinases further suggests that the epitope tagged kinases can be regulated in the same way that the endogenous Esk kinases would and that it may function when expressed in mammalian cells in the same way as the endogenous kinases. Expression in COS-1 cells, immunoprecipitation and the addition of [γ-^{32}P]ATP followed by SDS-PAGE and exposure to film indicated that both M-Esk1 and M-Esk2 were kinase active *in vitro* following expression in mammalian cells (Figure 5a,b).

Subcellular localization of the Esk kinase

Taking advantage of the specificity of the Myc antibody and the fact that M-Esk1 and

Fig.4- **Expression of the M-Esk proteins-** COS-1 cells were transfected with pECE-M-Esk1, pECE-M-Esk2 or the pECE-M expression vector alone. Cell lysates were resolved directly by 8% SDS-PAGE and transferred to nitrocellulose(A) or Myc tagged proteins were immunoprecipitated with an Esk polyclonal antibody, then resolved by 8% SDS-PAGE and transferred to nitrocellulose(B). M-Esk1 and M-Esk2 were detected by immunoblot with the anti-Myc mAb and enhanced chemiluminescence as ~120kDa and 110kDa proteins not detected from pECE-M transfected cells.

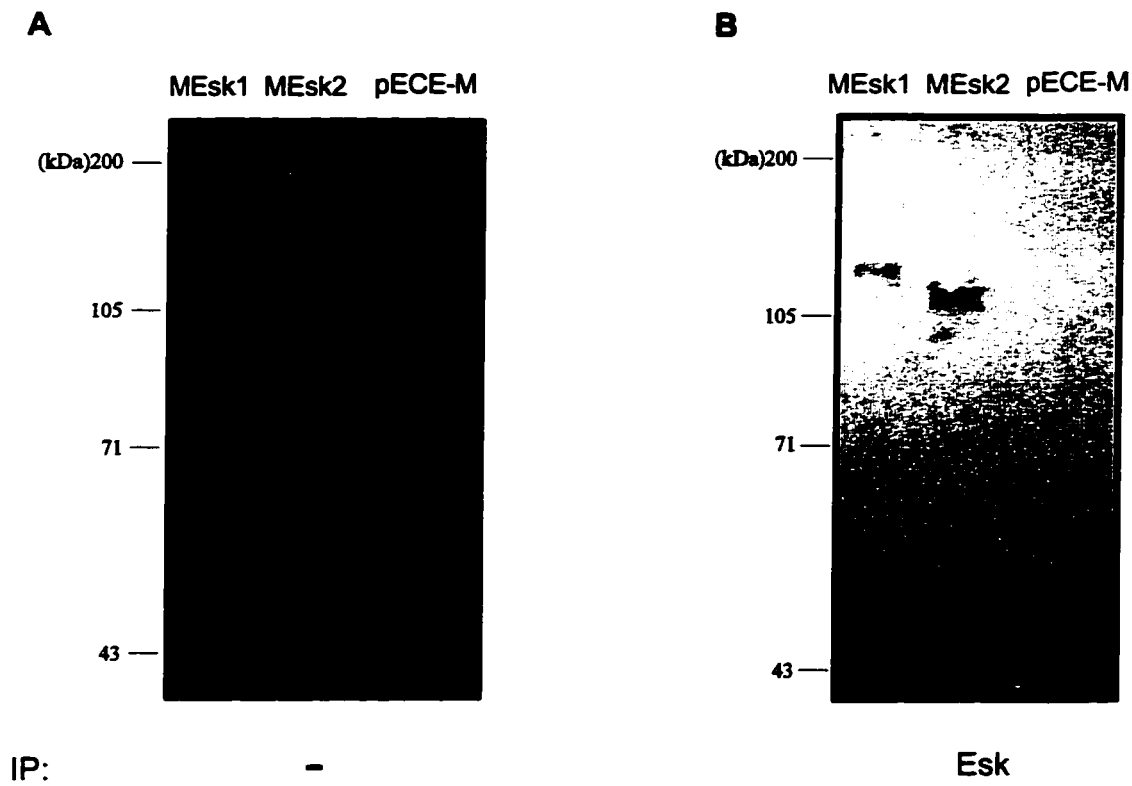
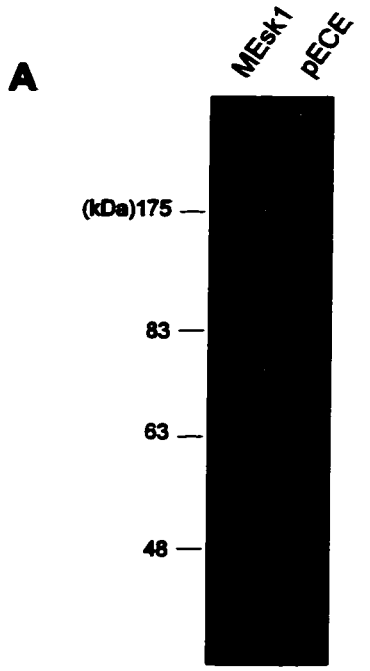
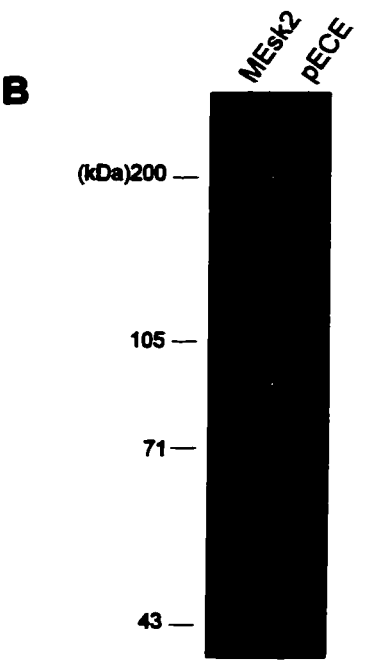

A
MEsk1 MEsk2 pECE-M
(kDa)200
105
71
43
IP: -
B
MEsk1 MEsk2 pECE-M
(kDa)200
105
71
43
Esk

Fig. 5. **Catalytic activity of M-Esk1 and M-Esk2-** Cos-1 cells were transiently transfected with M-Esk1 and pECE (A) or M-Esk2 and pECE (B). Myc tagged proteins were immunoprecipitated from cell lysates with the 9E10 anti-Myc mAb and subjected to *in vitro* autophosphorylation kinase assay. M-Esk1 is a ~130kDa kinase active protein not detected from pECE transfected cells (A). Similarly, M-Esk2 is a kinase active ~120kDa protein absent from pECE transfected cell lysates (B).

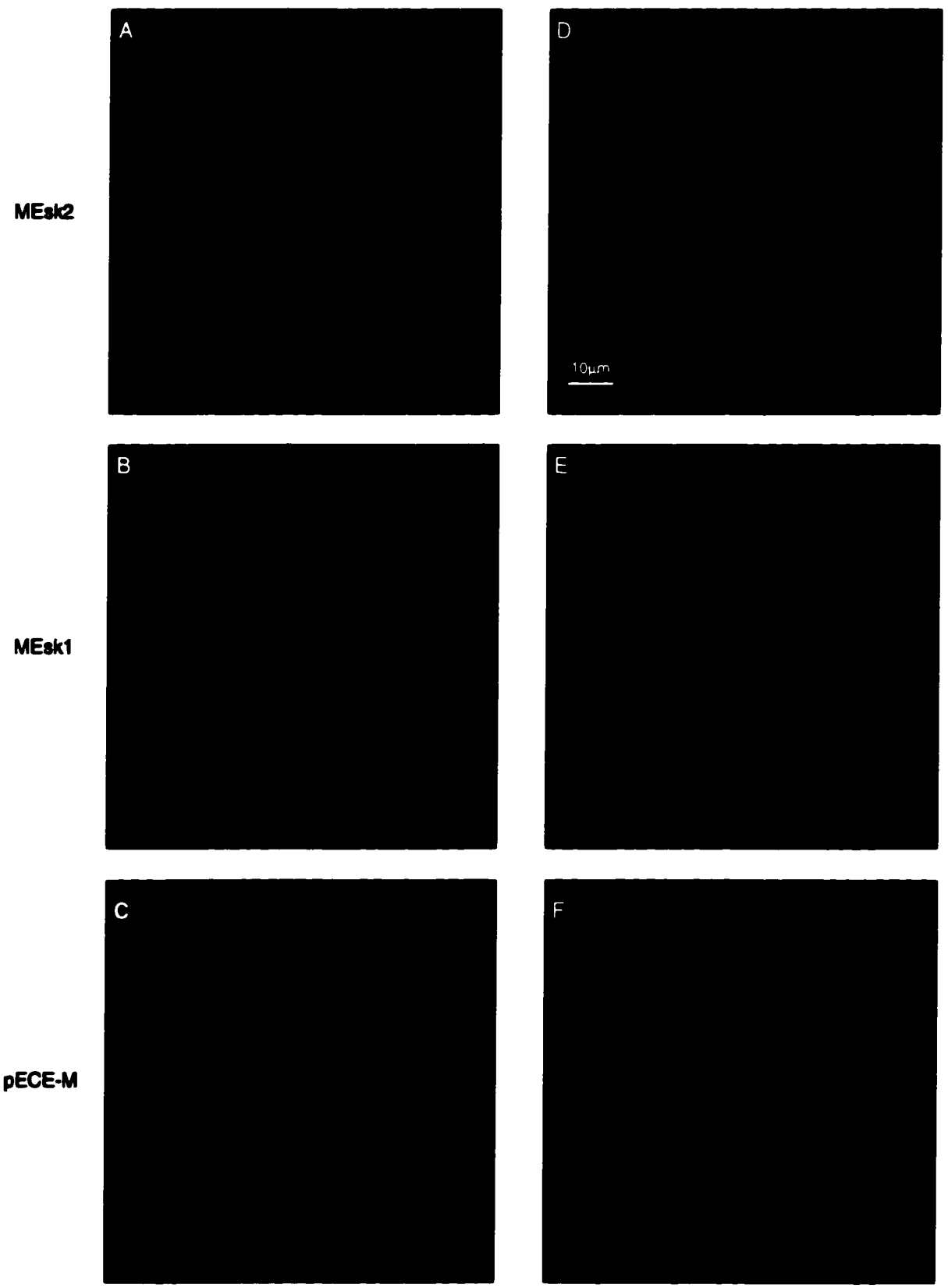

A
MEsk1
pECE
(kDa)175
83
63
48


B
MEsk2
pECE
(kDa)200
105
71
43

M-Esk2 seem to represent functionally active versions of the Esk kinase isoforms, the localization of Esk1 and Esk2 was assessed by transient transfection into COS-1 cells using M-Esk1 and M-Esk2. The anticipation was that the Esk1 kinase might be differentially regulated from the Esk2 kinase due to the stretch of hydrophobic amino acids that are present only in the Esk1 kinase. The possibility also existed that both kinases could be nuclear and at the present time few tyrosine kinases (ie. STY/Clk, c-Abl, Arg) have been found to be nuclear. Indirect immunoflourescence with the mAb 9E10 to the Myc epitope followed by a fluorescein conjugated secondary antibody was used to visualize the transfected epitope tagged constructs (Figure 6, a-f). Based on the immunoflourescence of Mesk1 and Mesk2 transfected into COS-1 cells it seems that in fact both kinases are cytoplasmic and do not appear to be associated with any particular subcellular compartment such as the endoplasmic reticulum, golgi apparatus, the plasma membrane or any internal membranes. Propidium iodide was used to highlight the DNA in the nucleus (Figure 6, a-c). The absence of any yellow fluorescence, from overlapping red and green fluorescence of DNA and Esk immunostaining clearly indicates the absence of either Esk isoform from the nucleus by this assay. It is possible that Esk may be endoplasmic reticulum or membrane associated and not detected with the sensitivity of immunofluorescence.

In an effort to address whether both Esk isoforms are truly in the soluble cytoplasm and that the epitope tagged Esk constructs are portraying the situation of the endogenous kinases, P19 EC cells were fractionated into nuclear and cytoplasmic

Fig.6- **Subcellular localization of MEsk1 and Mesk2 in COS-1 cells**- COS-1 cells were transiently transfected by electroporation with pECE-M-Esk2 (a,d), pECE-M-Esk1 (b,e) or pECE expression vector alone (c,f) and plated on gelatinized coverslips. Esk isoforms were detected by indirect immunofluorescence after fixation with the anti-Myc monoclonal antibody. DNA was stained using propidium iodide. Cells were viewed by confocal microscopy at 63x magnification and (a-c) are overlays of the anti-Myc and propidium iodide signals and represent a single plane 4μm thick through the cell. Extended focus images (d-f) represent the addition of 7 single plane images from the bottom of the cell to the top of the cell.

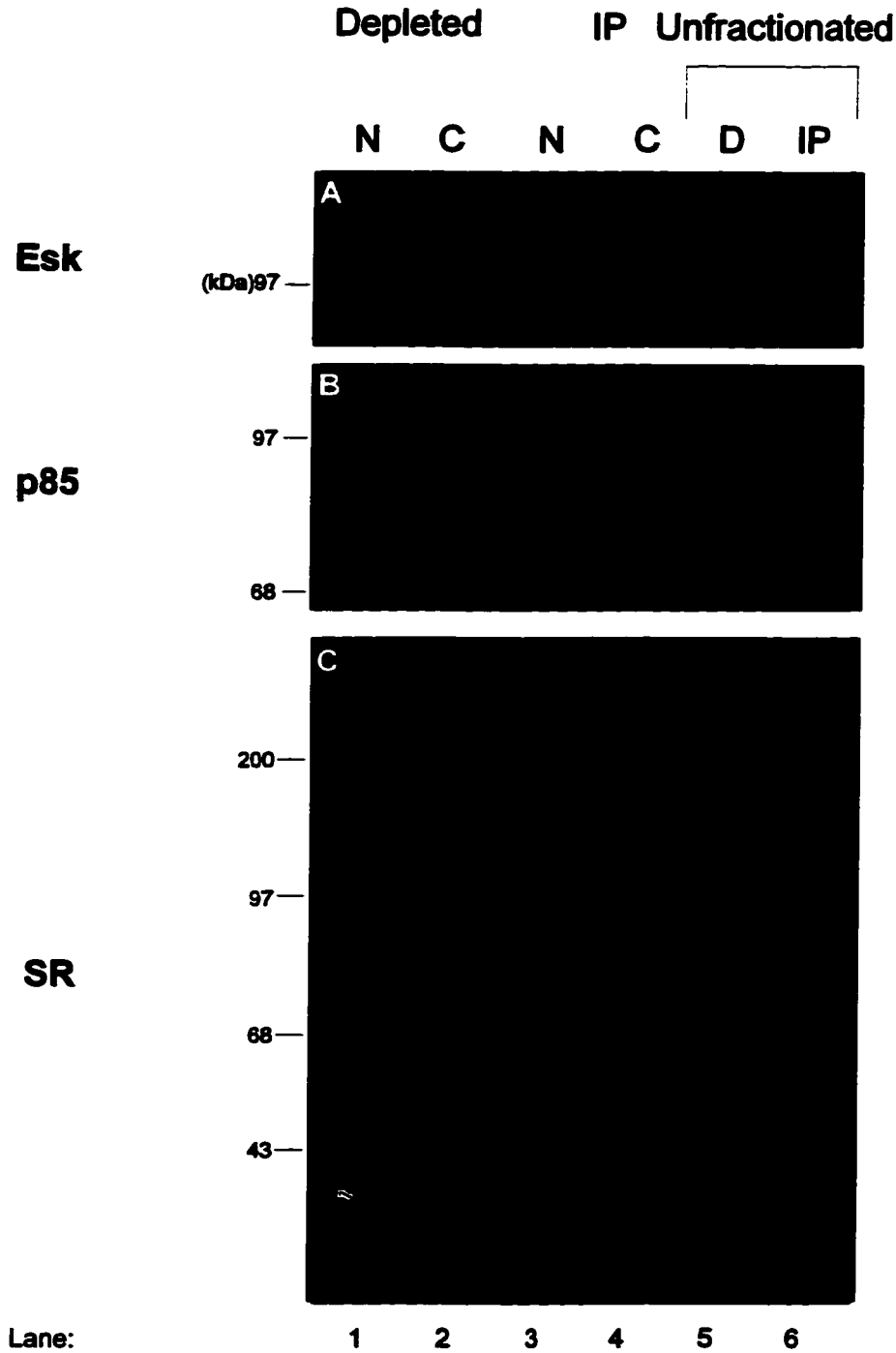

A
D
10μm
MEsk2
B
E
MEsk1
C
F
pECE-M

compartments for analysis by immunoblotting. Initially several distinct protocols were used to fractionate the cell lysates into nuclear and cytoplasmic compartments with varied success (Kampinga et al., 1995; Ruff and Leach, 1995; Prudovsky et al., 1995). Immunofluorescence of SR proteins using the mAb104 has shown that SR proteins are entirely nuclear in localization (Colwill et al., 1996). For this reason immunoblotting with the SR protein mAb104 was used to indicate that the nuclei were intact. To control against cytoplasmic capping of the nuclear fraction, an antibody to the p85 subunit of PI3K was used on immunoblot since it has been shown to be a predominantly cytoplasmic protein (Kapeller et al., 1993). Fractionation of P19 EC cells involved isolation of a detergent insoluble nuclear pellet and a cytoplasmic fraction by hypotonic lysis (Kampinga et al., 1995). p85 is found only in the cytoplasmic fraction and suggests no cytoplasmic contamination of the nuclear fraction (Figure 7b). SR proteins are predominantly nuclear indicating that the nuclei are largely intact and not contaminating the cytoplasmic fraction (Figure 7c). These controls indicate excellent fractionation of the nuclear and cytoplasmic compartments and indicates that the detectable endogenous Esk kinase in P19 EC cells is a soluble cytoplasmic protein (Figure 7a). Therefore, based on the fractionation of endogenous Esk in P19 EC cells and the immunofluorescence data using the epitope tagged MEsk1 and MEsk2 the conclusion is that both Esk1 and Esk2 are cytoplasmic and soluble in these assays.

3.4 Discussion

Alternative splicing of kinase mRNAs is important for regulation of ligand binding

Fig.7- **Endogenous Esk protein is predominantly Cytoplasmic**- Esk protein was immunoprecipitated from equivalent P19 EC cell fractions (lanes 3+4). Cell fractions depleted of Esk indicated the enrichment for nuclear and cytoplasmic proteins (lanes 1+2). Immunoprecipitated Esk from unfractionated P19 EC cells clearly identified Esk (p97) (lane 6) that is absent from the depleted fraction (lane 5). Endogenous Esk was immunoprecipitated using a rabbit polyclonal MAP antibody conjugated to the last 12 amino acids of Esk. Following immunoprecipitation, proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Esk was detected by an anti-Esk polyclonal antibody (Santa Cruz Biotechnology,Inc) followed by chemiluminescence only from the cytoplasmic fraction and was completely absent from the nucleus(A, lanes 3+4). p85 was detected with a polyclonal antibody (Upstate Biotechnology, Inc.)specifically in the cytoplasmic fraction (B, lanes 1+2) and SR proteins were detected using the mouse monoclonal antibody 104 and are highly enriched in the nuclear fraction(C, lanes 1+2).


Depleted
IP
Unfractionated
N
C
N
C
D
IP
A
Esk
(kDa)97
B
97
p85
68
C
200
97
SR
68
43
Lane:
1
2
3
4
5
6

through splicing in and out of specific extracellular domains involved in ligand binding of receptor tyrosine kinases such as the EGFR(Hardie and Hanks, 1995). Alternative splicing is also involved in the production of catalytic domain deficient receptors whose function is thought to be ligand sequestration and down regulation of receptor tyrosine kinase signal transduction (Katoh et al., 1992; Toyoshima et al., 1993). Cell type specific expression of the Fyn tyrosine kinase is regulated by mutually exclusive alternative splicing producing a T cell specific isoform and a brain specific isoform (Appleby et al., 1992). Examples of the developmental expression of proteins being regulated by alternative splicing exists for proteins such as the β-globin gene (Kazazian, H.H.Jr., 1987). Alternative splicing of the *K-Sam* mRNA is thought to produce a soluble, secreted kinase distinct from the receptor kinase form. This protein is predicted to be in the extracellular milieu because it has an intact signal sequence however the existence of this form of the protein has not been demonstrated (Katoh et al., 1992). The DM kinase mRNA has multiple splice variants including one that splices out a hydophobic region at its C-terminus that may produce soluble and membrane associated forms (Hardie and Hanks, 1995; Jansen et al., 1992). The Esk kinase has two isoforms that result from alternative splicing in of 78nt coding for 26aa in Esk1 and the splicing out of this exon producing Esk2 (Figure 3). The alternatively spliced 26aa initially suggested that Esk1 may encode a transmembrane form of the kinase (Douville et al., 1992). Demonstration that Esk1 was a receptor kinase and Esk2 a cytoplasmic soluble kinase would represent a method of kinase isoform regulation by differential sub-cellular localization that has not been shown for any other kinase. Homology searches have suggested that there are several motifs that may be

involved in regulating the localization of the two isoforms (Figure 1). Identification of the subcellular localization has led to investigation of specific potential functions for kinases such as FAK, STY/ Clk and Ltk (Hanks et al., 1992; Duncan et al., 1995; Bauskin et al., 1991). As a means of gaining information about the function of Esk1 and Esk2, experiments were conducted to determine their subcellular localization.

The kinase cDNAs were tagged at the amino terminal end with DNA coding for the six-repeat human Myc epitope (Figure 2)(Roth et al., 1991). The decision to tag the kinases at the amino terminus was based on concern over affecting the tertiary structure of the potential NLS at the extreme C-terminus of both isoforms. Epitope tags such as GST and 6xHis have proven useful for purification of proteins or peptides over glutathione-sepharose or Ni-sepharose columns and testing protein-protein interactions by passing lysates over tagged fusion proteins already immobilized on the sepharose columns (Songyang et al., 1993; Duncan et al., 1995). More importantly for identification of the subcellular localization of the Esk kinase isoforms is that many epitope tags have high affinity antibodies that detect the epitope specifically (Evan et al., 1985; Knappit and Pluckthun, 1994; Howard et al., 1995).

M-Esk1 and M-Esk2 were demonstrated to be full length in frame fusion proteins that could be immunoprecipitated with an antibody to the last 12aa of Esk and immunoblotted with a Mab to the human Myc epitope (Figure 4). The predicted size of the tagged proteins was 107kDa and 104kDa respectively, however based on western blot both

kinases appear to have the mobility of slightly larger proteins. This slower mobililty is likely due to *in vivo* phosphorylation of the proteins when expressed in mammalian cells. It is also important to note that the difference in mobility between the Esk1 and Esk2 kinases was clearly larger than the 3kDa predicted to separate the two isoforms based on the size of the alternatively spliced exon. There are two Ser, one Thr and one Tyr residue within the 26 amino acid addition to the Esk1 isoform. It is possible that at least one of these residues is modified by phosphorylation in the Esk1 kinase. It is also possible that this 26 amino acids directs the Esk1 kinase through an alternative post-translational modification pathway involving glycosylation in the ER. Esk1 has 3 putative glycosylation sites in its amino terminus and has been suggested to be a transmembrane kinase that might warrant such modification (Douville et al., 1992). The ER membrane kinase Ltk is modified by glycosylation leading to a large increase in size of the protein as seen by SDS-PAGE (Bauskin et al., 1991).

Given the homology of the Esk1 kinase to leucine zipper proteins, this isoform of the kinase may be a substrate in different signal transduction pathways or regulated by a different set of proteins based on regulation by the leucine zipper domain. These pathways could lead to increased phosphorylation specifically for the Esk1 protein kinase isoform. Based on *in vitro* autophosphorylation kinase assay, MEsk1 and MEsk2 were catalytically active and therefore were suggested to mimic endogenous Esk in terms of localization and regulation (Figure 5).

Expression of a Myc epitope tagged version of the STY/ Clk kinase into COS-1 cells indicated that STY was a nuclear tyrosine kinase (Duncan et al., 1995). The same approach was used in an effort to establish whether Esk1 and Esk2 were regulated differently by localization and potentially what functions the kinases were involved in. Expression of Esk1 and Esk2 in COS-1 cells followed by detection of the proteins by indirect immunofluorescence using an antibody specific for the epitope tag indicated that both Esk1 and Esk2 were cytoplasmic and soluble kinases (Fig.6). The immunofluorescence was specific to the expressed proteins because no immunofluorescence could be detected following transfection of pECE vector alone (Figure 6c,f). This indicated that both kinases were cytoplasmic, that Esk1 was not directed differently by the additional 26aa and that the putative NLS was not capable of directing either Esk isoform to the nucleus. It is however possible that factors such as epitope masking, the cell cycle, the cell type and the Myc epitope could influence Esk localization. Nuclear localization of the glucocorticoid receptor (GR) is regulated by the coordinated efforts of HSP90 and glucocorticoid binding to the receptor. The GR NLS is masked by HSP90 binding, however following ligand binding to GR, the receptor undergoes a conformational change that causes the release of HSP90, exposure of the NLS and translocation of GR to the nucleus (Schulman et al., 1994). It is possible that Esk is bound by a similar inhibitor of nuclear translocation and that stimulation of a specific signal transduction pathway might lead to nuclear translocation of transfected Esk protein. It is unlikely however, that such an inhibitor would be expressed at a high enough level to prevent targeting of Esk when overexpressed. Evidence from Esk kinase family

members in yeast and humans suggests that the Esk kinase might be cell cycle regulated. There are several examples of proteins whose subcellular localization is regulated by the cell cycle with PCNA being the most recognized example. During G1, PCNA is cytoplasmic but as the cell progresses through S phase PCNA is located in distinct regions in the nucleus (Celis and Celis, 1985; Vriz et al., 1992). M-Esk1 and M-Esk2 have never been observed in the nucleus following transfection into many different cell types. It is possible that at some point in the cell cycle, perhaps a small window at the G2/ M boundary, that Esk could be nuclear and that immunofluorescence has just not detected the translocation. FAK has been shown to have specific expression at focal adhesions (Hanks et al., 1992), however studies in neuronal cells found that FAK was distributed throughout the axonic cytoplasm, suggesting that proteins may have cell type specific localization (Grant et al., 1995). Transfection of M-Esk1 and M-Esk2 in human embryonal kidney cells, murine fibroblast and murine embryonal cells has shown only the diffuse cytoplasmic staining demonstrated in COS-1 cells. It is also possible that the addition of the 11.2kDa Myc epitope to the amino terminus of the Esk isoforms in some way affected the normal localization of the Esk isoforms. This question was addressed by fractionation of P19 EC cells to assess the distribution of endogenous Esk in the cell.

Esk1 and Esk2 were cloned from P19 EC cells and high levels of Esk kinase activity have been demonstrated by immunoprecipitation, autophosphorylation kinase assay indicating that the Esk kinase should be detectable in a fractionation, immunoblot assay. SR and p85 proteins were detected and suggested good fractionation of nuclei and

cytoplasm respectively in straight fractionation western blots. Esk was below the level of detection in both the nucleus and the cytoplasm by direct fractionation western blot. To overcome this problem, the number of cells used in each fraction was increased from $12x10^6$ to $30x10^6$ cells and detection of Esk was divided into two steps. The first step was to separate the nuclear pellet from the hypotonic soluble cytoplasm (described by Kampinga et al., 1995). The nuclear fraction was then solubilized in immunoprecipitation buffer (Duncan et al., 1995) and Esk was immunoprecipitated from both the nuclear and cytoplasmic fractions leaving immunoprecipitated and depleted nuclear and cytoplasmic fractions. The depleted nuclear and cytoplasmic fractions will indicate the success of the fractionation (Figure 7, lanes 1,2) while the immunoprecipitated fractions will indicate specifically if endogenous Esk is nuclear or entirely soluble as indicated by immunofluorescence of M-Esk1 and M-Esk2 (Figure 7, lanes 3 and 4). Unfractionated P19 EC cells serve as a control for the detection of Esk in the immunoprecipitated fraction (Figure 7, lane 6) with p85 and SR proteins being detected in the depleted fraction (Figure 7, lane 5). p85 was detected in the depleted cytoplasmic and unfractionated lanes, indicating that the nuclear fraction was free of any cytoplasmatic capping (Figure 7b). SR proteins were detected predominantly in the depleted nuclear and unfractionated lanes, suggesting that the nuclei were largely intact following fractionation (Figure 7c). While most of the SR proteins identified were entirely in the nuclear fraction, two apparent SR protein species at ~150kDa and 43kDa separated with the cytoplasmic fraction specifically, contrary to published immunofluorescence data with mAb 104 indicating that SR proteins are only nuclear (Colwill et al., 1996). This suggested that the fractionation

involved some degree of nuclear lysis but detection of only these two SR protein species. As anticipated based on the immunofluorescence data, endogenous Esk was identified only in the cytoplasmic fraction (Figure 7, lane 4) and in the unfractionated, immunoprecipitation control (Figure 7, lane 6). Based on these results it seems that endogenous Esk (Esk1 and/ or Esk2?) is predominantly cytoplasmic. It is possible that a portion of Esk exists in the nuclear/ ER fraction that is below level of detection by this assay. An experiment that would help confirm this result would be to follow immunoprecipitation of Esk from the nuclear and cytoplasmic fractions with a kinase assay for more sensitive detection of Esk in both fractions. It is possible that Esk in the cytoplasm represents an inactive pool of kinase and that following activation a portion of Esk undetectable by immunoblot is localized elsewhere. Mapk exists largely inactive in the cytoplasm prior to activation which leads to nuclear translocation (Chen et al., 1992; Marshall, C.J., 1995). It is also possible that a portion of Esk, endogenous Esk1 perhaps was localized to a membrane fraction (nuclear/ ER) and not solublized in immunoprecipitation buffer prior to immunoprecipitation. Certainly, SR proteins were not solublized as efficiently in Immunoprecipitation buffer compared to an SDS based buffer. A similar observation has been made with solubilization of the STY kinase from the nucleus (Duncan et al., 1995). However, the experiments to date suggest that apparently functional, recombinant Esk1 and Esk2 protein that are catalytically active expressed in COS-1 cells are entirely cytoplasmic. In addition endogenous Esk protein from P19 EC cells identified by immunoprecipitation, immunoblot is also only detected in the cytoplasmic fraction. Therefore both Esk isoforms appear to be soluble cytoplasmic

proteins.

It is still unclear at this point exactly why Esk1 and Esk2 are in the cytoplasm and what some of the differences in function might be between the two isoforms. It is interesting to note that two proteins that Esk has been speculated to be involved with are cytoplasmic proteins. p85 SH2 domains were shown, using a phosphotyrosine peptide library to bind a pY-M/V/I-X-M consensus sequence (Songyang et al., 1993). Esk was found to contain two of these consensus sequences in tandem in subdomain V of its catalytic domain. The region is not known to be an area of regulated phosphorylation among kinases and has not been shown to be a critical regulatory site in Esk but an understanding of the variety of ways that kinases are activated represents only a subset of kinases and the potential importance of tyrosine phosphorylation and the association of p85 in Esk function is still unknown. A role in tubulin regulation directly or indirectly has certainly been implied based on the important functions of Mps1 in yeast in G1 and at the G2/M boundary. Spindle pole body duplication is defective at the restrictive temperature of Mps1 temperature sensitive yeast and failure to arrest at the G2/M boundary in response to monopolar spindle formation has also been directly related to Mps1 function (Lauze et al., 1995; Weiss and Winey, 1996). Cytoplasmic Esk could definitely been involved in both of these pathways in mammalian cells. It should also be pointed out that Esk has several KKE(E/IV) motifs including one motif in the last 6aa that are homologous to repeats in MAP1b protein that have been shown to be critical for MAP1b binding to microtubules, although this motif is repeated 21 times in 200aa of MAP1b (Noble et al., 1989). The experiments in this chapter indicate that the 26aa in the Esk1 kinase are not capable of

directing Esk1 into membranes. Future experiments should perhaps be focused on this alternative exon as a potential Leucine zipper domain that may be involved in protein-protein interactions that are specific to Esk1 and not found in Esk2.

Chapter 4
Modulation of Esk kinase activity by mutation in the Catalytic Domain

4.1 Introduction

Regulated activity of protein kinases is critical to maintain cell homeostasis. The identification of many oncogenes as dominant acting deregulated versions of cellular kinases indicates how important it is for kinases to be tightly regulated for proper cell growth and differentiation (Cantley et al, 1991). Multiple sequence alignments between all protein kinases have identified 12 conserved sub-domains (Hanks et al, 1988). Many invariant amino acids have since been implicated in essential roles for kinase structure and function. Homology and crystal structure determinations have suggested that all protein kinases share a similar structure (Johnson et al, 1996), however what is clear is that despite a conserved general structure kinases have evolved to allow many different mechanisms of control.

Some of the control mechanisms that have been identified include: control by additional subunits or domains that may be regulated in response to second messengers (ie. cAPK); control by additional subunits whose level of expression is regulated by the state of the cell (ie. Cyclin regulation of CDKs); control by domains that target the kinase to different molecules or subcellular localizations (ie. SH2 and SH3 domains of Src family kinases); control by autoinhibitory domains (ie. Src pY-527 self associated to its own SH2 domain keeping the kinase in a closed activation state); and control by phosphorylation and dephosphorylation by kinases and phosphatases (Johnson et al,

1996). The latter mechanism of control can be quite variable and result from phosphorylation of serine, threonine or tyrosine at unique regulatory sites for different proteins. One common mechanism of activation for many kinases is centered around a specific phosphorylated residue within the activation segment between conserved subdomains VII and VIII in the catalytic domain (Johnson et al, 1996). This region between the conserved sequences DFG and APE, often contains an autophosphorylation site and phosphorylation at this site is a critical step in the conversion of many kinases from an inactive to active state. This conversion may result from conformation changes within the catalytic region, modification of substrate with some of the catalytic groups of the kinase or relief of steric blocking to allow the access of substrates. The importance of the site was first demonstrated for the activation of c-Src. Mutation of Tyr-416 to Phe resulted in a loss of growth in soft agar and a decrease in the formation of foci that were observed when the Src kinase was activated by an initial mutation of Tyr-517 to Phe (Kmiecik and Shalloway, 1987). The conclusion was that phosphorylation on Tyr-416 was critical for full activation of the Src kinase. Similar mutation at homologous sites of phosphorylation in a number of tyrosine kinases resulted in decreased autophosphorylation and decreased kinase activity (Hjermstad et al, 1993, Weinmaster et al, 1984). Phosphorylation on T160 of CDK2 when associated with Cyclin A is speculated to promote conformational changes and relieve steric hinderance for full activation of CDK2 (Jeffrey et al, 1995). Activation through phosphorylation within the activation segment can occur through autophosphorylation and phosphorylation by other kinases. Second messenger association of cAMP with the regulatory subunits of cAPK results in the

removal of the steric hinderance of the regulatory subunits of the protein kinase and subsequent autophosphorylation at T197 which is contained within a consensus phosphorylation site for cAPK (Hardie and Hanks, 1995; Johnson et al, 1996). Activation of Mapk by MEK1 requires the dual phosphorylation of T and Y residues within this region for full activation of Mapk (Chen et al, 1992; Johnson et al, 1996).

Not all kinases have a requirement of phosphorylation within this region for activation. It has been suggested that kinases that have the sequence HRDL and specifically have RD as part of the conserved subdomain VI are activated by phosphorylation between subdomains VII and VIII. The reason for this phosphorylation requirement is coordination between the positively charged R next to the catalytic base (D) and the negative charge that phosphorylation of S, T or Y between subdomains VII and VIII can provide (Johnson et al, 1996). Johnson et al., also suggests that non-RD type kinases do not have this requirement. Two examples of kinases that do no phosphorylate in the activation segment, twitchin kinase from *Caenorhabditis elegans* and phoshorylase kinase have either no requirement for coordination or have an alternate method for charge compensation. Twitchin kinase has a sequence HVDL and a L in the homologous site to Y416 of Src and it appears that proteolytic cleavage of the C-terminal tail is sufficient to activate the kinase. The phosphorylase kinase maintains the HRDL motif but uses a E carboxylate group for charge compensation (Johnson et al, 1996). Interestingly, the Esk and Clk dual specificity kinases have HSDL and HTDL motifs respectively and continue to have very negatively charged activation segments of DTTS(+8 to +11) and

YDDE(+4 to +7 away from subdomain VII) suggesting that these kinases may be regulated in some alternative fashion.

Given that the Esk kinase is a non-RD type kinase it is unclear from the literature whether phosphorylation between subdomains VII and VIII is important for Esk kinase activation. It has previously been shown that the major autophosphorylation site for the Esk family kinase Ttk, is in fact at a site homologous to Y416 of Src, T649 of Esk within the activation segment (Lindberg et al, 1993). As a means of investigating the regulation of the Esk kinase, site directed mutagenesis of T648 and T649 within the activation segment mutated these amino acids each to alanine. Given the results described in the literature it was expected that this kinase would have impaired autophosphorylation kinase activity. In fact, an activated Esk kinase with respect to its ability to autophosphorylate is described.

4.2 Methods

Tissue Culture- Human 293T cells (Graham and Smiley., 1977) and COS-1 cells (ATCC#CRL-1650) were maintained in minimal essential media with 10% calf serum (Gibco BRL) at 37°C in 5% CO_2 and passaged when necessary. P19 EC cells were maintained in minimum essential media supplemented with 10% fetal bovine serum (McBurney et al., 1982).

Site Directed Mutagenesis- pECE Esk2 was digested with KpnI and ligated into

pAlter(Promega) digested with KpnI. Restriction digest identified the correct orientation with the 5' end of Esk adjacent to the T7 promoter. pAlter-Esk2 was denatured in alkali then the Amp repair and EskTA mutagenesis oligo [5' tta aca atg ctt gct gcg tct ggc tgc a-OH] were annealed to the single-stranded DNA and the mutagenized strand was synthesized by T4 DNA polymerase and ligated with T4 DNA ligase. MutS bacteria were transformed with helper phage and pAlter-Esk2Mut vector. Virus was produced then introduced into JM109 by bacterial infection and selection on Ampicillin/ LB agar selected for mutated clones. Esk-Mut colonies were screened by restriction digest and sequencing of the mutagenized site using the dideoxy chain termination method (Sanger et al., 1977) with the EskYF oligo [C ACC GAG CAG TTC ATC TTC ATG GTA ATG G]. The mutant Esk cDNA was cloned from pAlter by digestion with AccI and PvuI back into pECE-MEsk2 to give pECE-MT648A,T649A.

Calcium Phosphate Transfection- For transient transfection into 293T cells, $3x10^6$ cells were transfected with 30μg DNA in 0.25M $CaCl_2$ and HBS, pH 7.05(50mM Hepes, 280mMNaCl, 1.5mM Na_2HPO_4, 10mM KCl, 12 mM Dextrose) for 12h, the cells were washed in PBS and incubated an additional 24h in fresh media.

For transient transfection into P19 EC cells, $2x10^5$ cells were plated onto coverslips in 35mm dishes and transfected with 10μg DNA in 0.5mM $CaCl_2$ and HBS for 8h with Chloroquine (0.1mM). Cells were washed in PBS and given fresh media for 24h before fixation.

For stable transfection into P19 EC cells, $0.5x10^6$ cells were plated into a 60mm tissue

culture dish. The following day 12μg of Mesk2, Mesk2(LL`8'), MT648A,T649A and pECE were co-transfected with 3μg pgk-puro (gift of Peter W. Laird) in BES, pH6.86(25mM BES[[N-,N-bis(2-hydroxyehyl)-2-aminoethanol sulfonic acid]], 140mM NaCl, 0.75mM Na_2HPO_4) and 0.5M $CaCl_2$ for 8h. The precipitate was cleared with 2x PBS washes and fresh media was added. After 48h, $2x10^6$ cells for each transfection were plated into a 150mm tissue culture dish with fresh media containing 2μg/ml puromycin. Colonies were well formed after 12d, trypsinized and grown in 24 well dishes to freeze in fetal bovine serum with 10% DMSO or 6 well dishes under continued drug selection for cell lysis and immunoblot.

Quantitative Immunoblot and Kinase Assay- 293T cells were lysed in immunoprecipitation buffer, immunoprecipitated with the 9E10 mAb and protein A sepharose pre-incubated with Rabbit anti-mouse IgG(H+L)(Jackson ImmunoRes. Lab., Inc.). Immunoprecipitated proteins are washed 4x with Immunoprecipitation buffer and 3x with kinase buffer before 30min incubation in kinase buffer with 0.5μCi [^{32}P γ-ATP]. Equal volume sample buffer and boiling at 100°C for 5min stops the reaction, the samples were then resolved by 8% SDS-PAGE and transferred to nitrocellulose.

For immunoblot, nitrocellulose was incubated in 5% Carnation powdered milk in TBST (1h, RT), washed in TBST and incubated with the primary antibody, 9E10 in 20% serum and TBST for 1h at room temperature and the nitrocellulose is washed again 3x for 5min. The blot is then incubated with goat anti-Mouse IgG(H+L) conjugated to Alkaline phosphatase (Gibco BRL, Cat. 97155A) in TBST for 1h and washed 3x 5min in TBST

prior to incubation with the Attophos™ (JBL Scientific, Inc.) detection reagent in 2.4M diethanolamine, 0.23mM $MgCl_2$ pH 10 which is a substrate for alkaline phosphatase which is converted enzymatically to a fluorescent product visualized using the Molecular dynamics Storm™ which excites the attophos product at 440nm for detection of fluorescence at 560nm and quantitation by measuring optical density.

^{32}P labelled proteins were detected by total counts detected using storage phosphor screens™ and the Molecular Dynamics Phosphorimager™.

Nomarsky Optics- P19 EC cells were fixed for 10min, room temperature in 3% paraformaldehyde, washed 3x 5min in PBS, excess praformaldehyde was cleared by 3x 4min washes in1mg/ml $NaBH_4$, followed by 3x 5min PBS wash before 9E10 mAb immunofluorescence and propidium iodide stain of the DNA. Coverslips were mounted using Anti-Fade™(Molecular probes, Inc). Slides were taken on Kodak elite 400asa colour film using a Zeiss Axiophot microscope at 40x magnification. Nomarsky optics (Differential interference contrast microscopy) fitted with a Zeiss Pan-Niofluor 40x/ 1.6 objective and a nomarsky prism. Pictures were taken using incident light.

Sequence Assemblages- Standard sequencing methods were used with the ABI sequencer and oligonucleotides located at various positions across the *esk* cDNA.

4.3 Results

Mutagenesis of Esk

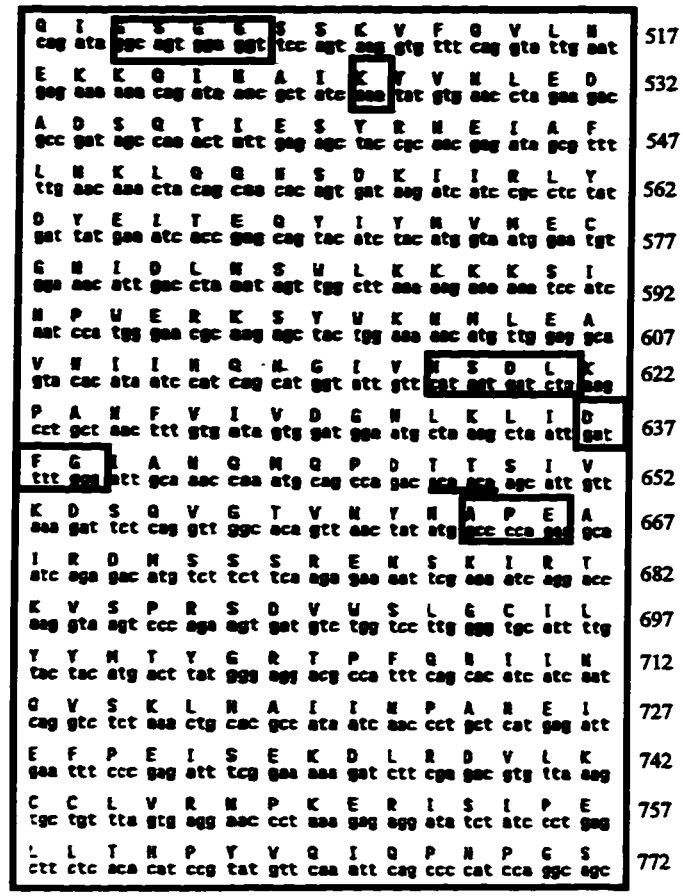
The kinase domain contains all 12 subdomains conserved between the protein kinase superfamily (Figure 1a). Subdomains I(GXGG), II (K), VI (HSDL), VII (DFG) and VIII are highlighted and play important roles in ATP binding and phosphate transfer. The Esk kinase is one of a small group of kinases that does not have an arginine (homologous to R165 of cAPK) in subdomain VI instead Esk has a serine residue (S645). The activation segment of several kinases are shown (Figure 1b). Known phosphorylation sites are indicated and all kinases shown have a number of phosphorylatable amino acids in this region. The major autophosphorylation site of Ttk is T676 (Lindberg et al., 1993). This amino acid is conserved in Esk (T649), but not in Mps1, although Esk, Ttk and Mps1 all have a phosphorylatable T (T648 in Esk) immediately preceding T649. To understand the importance of T649 phosphorylation in the regulation and activation of Esk, T648 and T649 were mutated to alanines by site directed mutagenesis with mismatched oligonucleotides. MT648A,T649A clones were confirmed by sequencing.

Characterization of MT648A,T649A

The activity of MT648A,T649A was compared to two versions of WT M-Esk2, and to the empty pECE vector alone. For each transfection, different fractions of the lysate were loaded so that the kinase activity could be compared to linear increases in protein. Myc immunoblot indicated that MT648A,T649A was ~120kDa with no differences in mobility when compared to M-Esk2 or MEsk2(LL'8') (Figure 2a). However, the MT648A,T649A mutant had elevated kinase activity when compared to both of the WT kinases (Figure 2b), in contradiction to similar mutations in Src, cAPK and MEK1 (Kmiecik and Shalloway, 1987; Adams et al., 1995; Cowley et al., 1994). Taking

Fig. 1- **Conserved features in the Catalytic domain of Esk2.** (A) The Esk2 catalytic domain contains all 11 of the subdomains conserved between protein kinases. Subdomains I, II, VI, VII and VIII (boxed) contain conserved residues that are involved in ATP binding and phosphotransfer activity. T648 and T649 in the activation segment are underlined. The amino acid sequence between subdomains VII and VIII are highlighted for a number of protein kinases (B). The invariant amino acids that make up subdomains VII and VIII are bolded, known autophosphorylated residues are marked by asterisks and the amino acids corresponding to the phosphorylated residue is in brackets. EskT648A,T649A is identical to Esk2 at the nucleotide level except for single nucleotide substitutions of guanine 1979 and guanine 1982 for adenosines that results in 2 single amino acid substitutions of T to A.

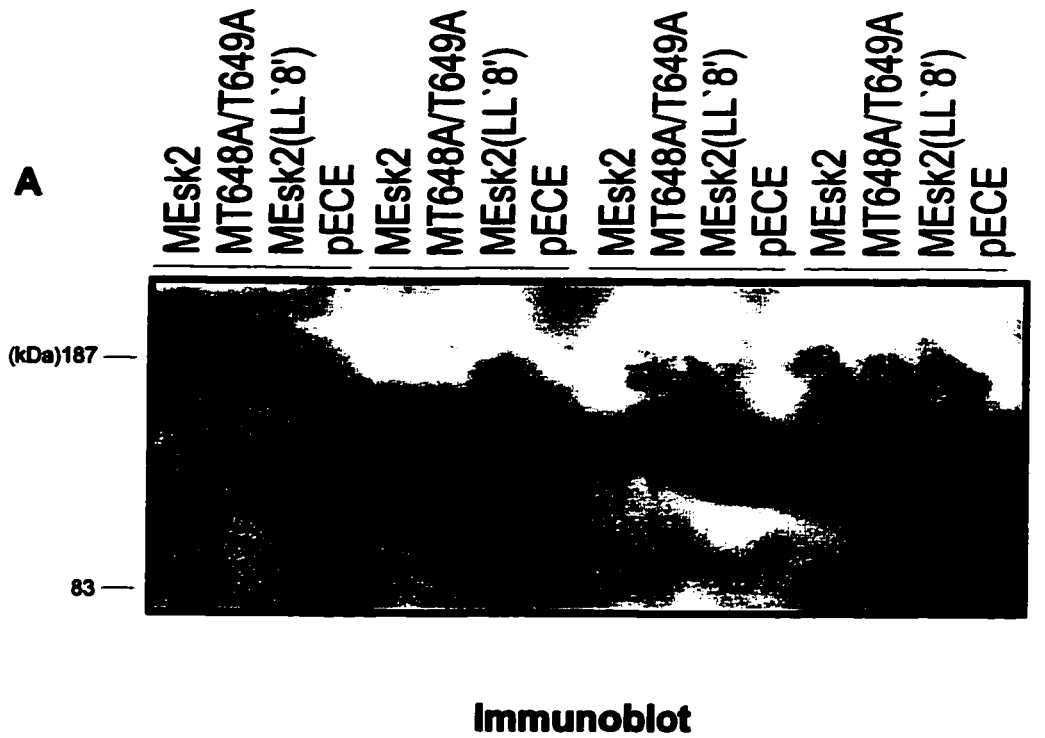
A

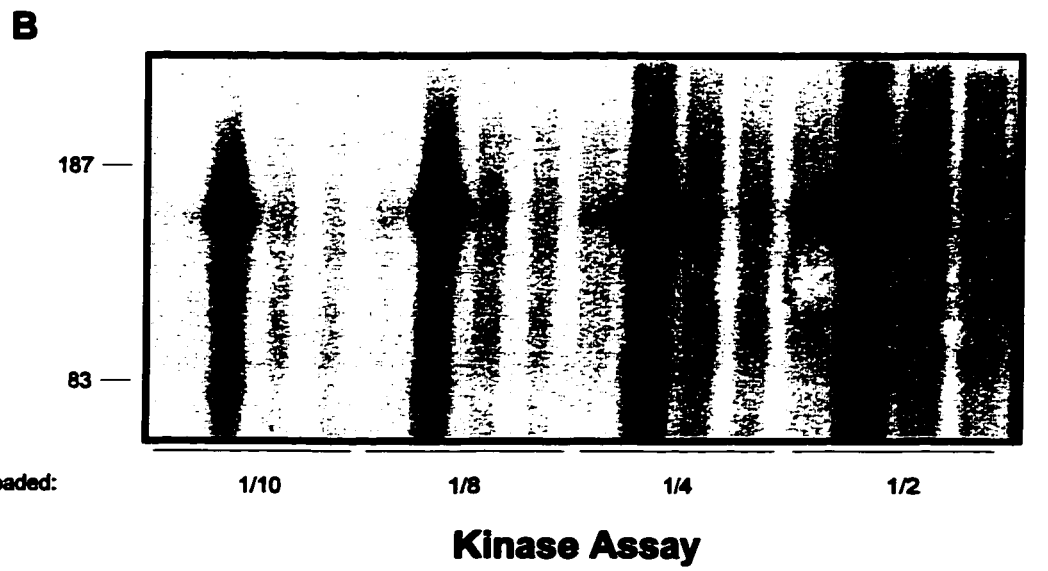


B

	VII	Activation Segment	VIII
Esk2 (???)	**DFG**	IANQMQPDTTSIVKDSQ--VGTVNYM	**APE**
T648A,T649A(648,649)		AA	
Ttk (676)	**DFG**	IANQMQPDTTSVVKDSQ--VGTVNYM	**PPE**
Mps1 (???)	**DFG**	IANAVPEHTVNIYRETQ--IGTPNYM	**APE**
Src (416)	**DFG**	LARLIEDNEYT-ARQGAK--FPIKWT	**APE**
Mek1 (217,221)	**DFG**	VSGQLIDSMANSF------VGTRSYM	**SPE**
Erk2 (183,185)	**DFG**	LARVADPDHDHTGFLTEY-VATRWYR	**APE**
Cdk2 (160)	**DFG**	LARAFGVPVRTYTHE----VVTLWYR	**APE**
PkcβII (500)	**DFG**	MCKENVFPGSTTRTF----CGTPDYI	**APE**
cAPK (197)	**DFG**	FAKRVKGRTWTL-------CGTPEYL	**APE**
HGFR (1234,1235)	**DFG**	LARDMYDKEYYSVHNKTGAKLPVKWM	**ALE**

Fig. 2- **MT648A,T649A Kinase Activity**. 293T cells were transfected by calcium phosphate with MEsk2, MT648A,T649A, Mesk2(LL'8') or pECE vector alone. Cell lysis and protein immunoprecipitation with the 9E10 mAb (Evan et al., 1985) were followed by *in vitro* autophosphorylation kinase assay. Lysate fractions of ½, 1/4, 1/8, 1/10 were separated by SDS-PAGE and transferred to nitrocellulose. (A) Esk protein was detected by anti-Myc immunoblot using an alkaline phosphatase conjugated secondary antibody and Molecular Dynamics attophos™ substrate. The ~120kDa epitope tagged Esk is detected from Esk transfected cells but absent from pECE transfected cells. (B) Phosphoproteins were detected with Molecular Dynamics phosphor screens™ and laser scanning image analysis.


A
MEsk2
MT648A/T649A
MEsk2(LL`8')
pECE
MEsk2
MT648A/T649A
MEsk2(LL`8')
pECE
MEsk2
MT648A/T649A
MEsk2(LL`8')
pECE
MEsk2
MT648A/T649A
MEsk2(LL`8')
pECE
(kDa)187
83
Immunoblot


B
187
83
Fraction Loaded:
1/10
1/8
1/4
1/2
Kinase Assay

advantage of the quantitation capabilities of the Molecular Dynamics Phosphorimager/ Flourimager™ the kinase activity and protein levels of the WT and mutant kinases was determined. Comparison of the mutant kinase, MT648A,T649A to the WT kinase, M-Esk2 indicates that the mutant has 19 fold greater autophosphorylation kinase activity (Table 1). M-Esk2(LL`8') has one half the kinase activity compared to the original WT kinase and 41 fold less activity than MT648A,T649A.

Localization of MT648A,T649A

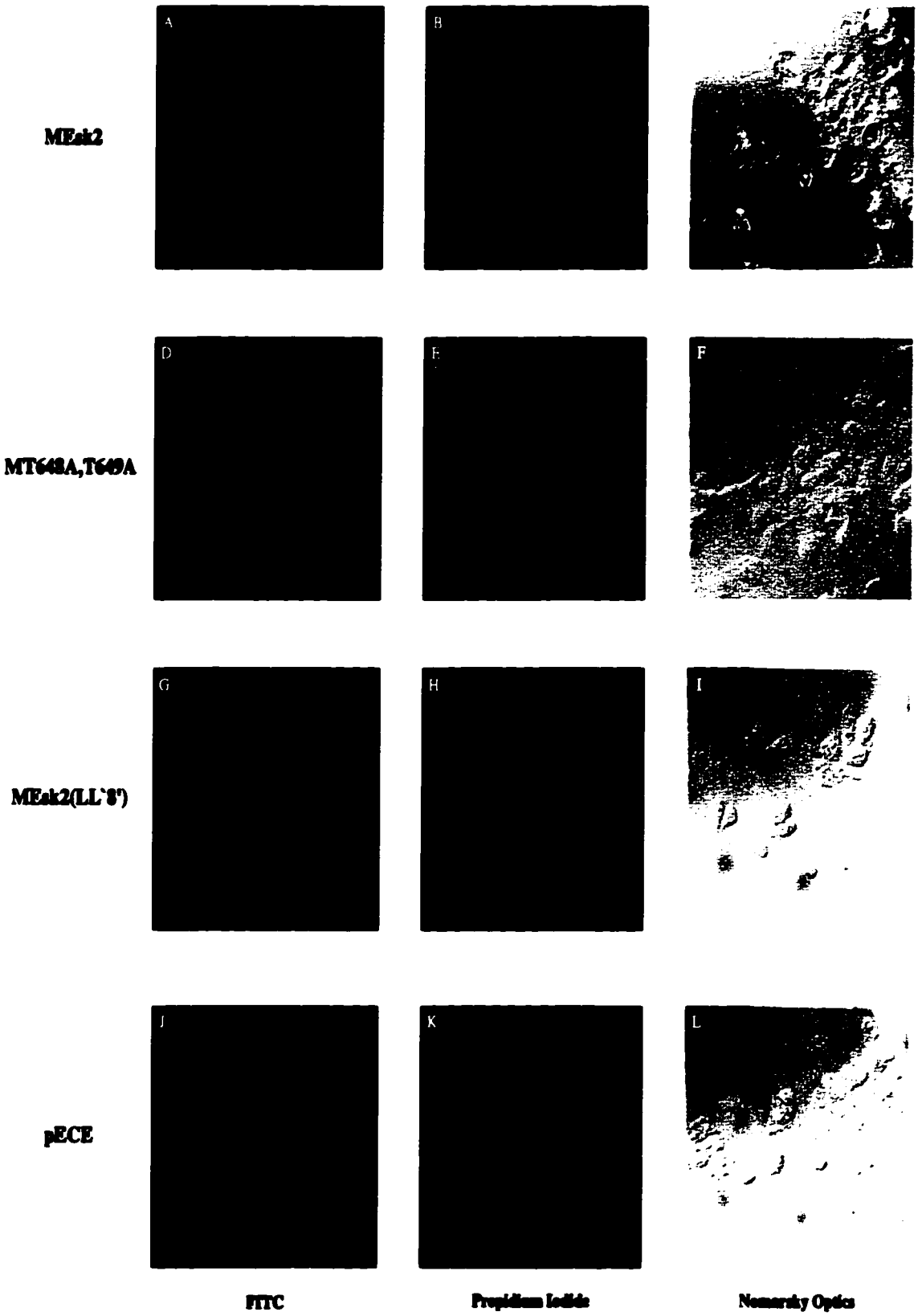
Kinases can be regulated by inhibitory proteins or autoinhibitory peptides, by absence of an activating ligand and by sequestration of the kinase from a particular subcellular localization or set of substrates. Mapk is sequestered in the cytoplasm prior to MEK activation which leads to accumulation of the activated kinase in the nucleus (Chen et al., 1992). The elevated level of kinase activity of MT648A,T649A suggests that some method of regulation for Esk has been overcome. Differences to the subcellular localization of MT648A,T649A were tested following expression of the mutant, WT kinases and pECE alone in P19 EC cells by calcium phosphate transfection. Indirect immunoflourescence using the mAb 9E10 to the human Myc epitope indicates that MT648A,T649A remains cytoplasmic despite its elevated kinase activity (Figure 3d). The subcellular localization is identical to M-Esk2 (Figure 3a) and M-Esk2(LL`8') (Figure 3g) and does not colocalize with the nucleus stained with propidium iodide (Figure 3 b,e,h,k). The cytoplasmic localization is specific to the transfected epitope tagged mammalian

Table 1 - Kinase Activity of M-Esk2, MT648A,T649A and MEsk2(LL`8')

	Kinase Activity (Total Counts)	Total Protein (Optical Density)	(Total Counts / Total Protein)	Average (Total Counts/Total Protein)	Fold Activation
M-Esk2	8036	77097	0.10	0.059± .029	—
	12224	393876	0.031		
	22635	446967	0.051		
	46578	832349	0.056		
MT648A,T649A	338297	302004	1.1	1.1± .21	19x
	507015	638698	0.79		
	1286366	994644	1.3		
	2725828	2393197	1.1		
M-Esk2(LL`8')	8166	179387	0.045	.027± .014	0.46x
	11333	591213	0.019		
	15536	1125074	0.014		
	50678	1693441	0.030		

Refer to Appendix II for explanation of quantitation of kinase activity and total protein levels

Fig. 3- **Immunoflourescence of MT648A,T649A compared to MEsk2, MEsk2(LL`8'), and pECE.** Calcium phosphate transfection of P19 EC cells was followed by 3% Paraformaldehyde fixation, indirect Immunofluorescence with mAb 9E10 and DNA staining using propidium iodide. MEsk2(a), MT648A,T649A(d) and M-Esk2(LL`8')(g) transfected cells show an identical diffuse cytoplasmic staining that is absent from pECE transfected cells(j) and clearly is not co-localized with the nucleus (b, e, h, k). Nomarsky optics shows a full field cells for MEsk2(c), MT648A,T649A(f), MEsk2(LL`8')(I) and pECE alone(l).


MEsk2
MT648A,T649A
MEsk2(LL`8')
pECE
A
B
D
E
F
G
H
I
J
K
L
FITC
Propidium Iodide
Nomarsky Optics

expression constructs because no FITC immunoflourescence is present in the pECE transfected cells (Figure 3j) which can be seen by propidium iodide and Nomarsky optics (Figure 3k,l).

Stable Expression of MT648A,T649A

Expression systems such as the *tet* repressor inducible promoter and the *lac* repressor inducible expression system (Miller and Rizzino, 1995) are often required to express high levels of proteins that would otherwise be lethal when expressed in mammalian cells. Esk has never been successfully over expressed stably in mammalian cells. The absence of Mps1 in yeast is lethal owing to loss of G2/ M checkpoint control. It is unclear specifically why overexpression of Esk is not tolerated in mammalian cells other than to speculate that cell cycle progression may be affected. MT648A,T649A is activated compared to WT Esk when assayed by autophosphorylation kinase assay. It is unclear whether the mutant is similarly activated in function towards endogenous Esk substrates. M-Esk2(LL`8') is virtually kinase inactive when assayed by autophosphorylation kinase assay, it is similarly unclear what effect this kinase will have on Esk substrates. M-Esk2(LL`8') could potentially act as a dominant negative kinase or it is possible that *in vitro* autophoshorylation kinase assay doesn't give a true indication of the kinase activity of Esk towards its endogenous substrates. The epitope tagged WT kinases and the activated mutant were stably transfected into P19 EC cells to test whether the activated mutant kinase is tolerated as poorly as WT Esk kinase when stably over expressed. Analysis of individual M-Esk2, MT648A,T649A, M-Esk2(LL`8') and pECE clones by

Myc immunoblot indicated that MT648A,T649A could be stably expressed in P19 EC cells while no expression of the WT kinases was found (Figure 4). From the clones tested, 40% of the MT648A,T649A clones were stably transfected compared to 0/18 tested for MEsk2(LL`8') (Table 2). Only 2 drug resistant colonies transfected with MEsk2 survived selection which is further indication of the detrimental effects of overexpression of the WT kinase. The pECE transfection control produced 10-200x more drug resistant colonies than cotransfection with an Esk mammalian expression vector. Only 50% of the isolated colonies have been tested for Esk expression, the absolute inablility to overexpress WT Esk cannot be confirmed until all the clones have been tested for Esk expression.

Identification of MEsk2 clones with increased kinase activity

M-Esk2 has been shown to be kinase active, however M-Esk2(LL`8') demonstrated very poor autophosphorylation kinase activity. In contrast to the literature MT648A,T649A has activated autophosphorylation kinase activity when compared to both WT kinases. Mutation at homologous autophosphorylation sites in Src, cAPK and MEK1 resulted in dominant negative, kinase inactive mutants not an activated mutant as seen with MT648A,T649A. The suggestion was that a secondary mutation had arisen in M-Esk2(LL`8') that led to impaired kinase activity relative to M-Esk2 and MT648A,T649A. Several additional M-Esk2 clones were screened to see whether all M-Esk2 clones exhibited low kinase activity or whether perhaps a secondary mutation was impairing the kinase activity of MEsk2(LL`8') specifically. Transient transfection into COS-1 cells of 7 clones, positive by restriction digest along with pECE and

Fig. 4- **Analysis of Individual P19 EC cell Stably transfected clones.** MT648A,T649A, MEsk2(LL`8') and pECE clones were picked after 13d in puromycin and grown under continued selection. Total protein lysates of a selection of drug resistant clones were resolved by 8% SDS-PAGE, transferred to nitrocellulose, immunoblotted with mAb 9E10 and detected on film using enhanced chemiluminescence. Myc epitope tagged Esk is identified as an ~120kDa protein (arrow) not detected in pECE transfected clones. β Tubulin (mAb E7) serves as the internal control for the amount of protein loaded into each lane.

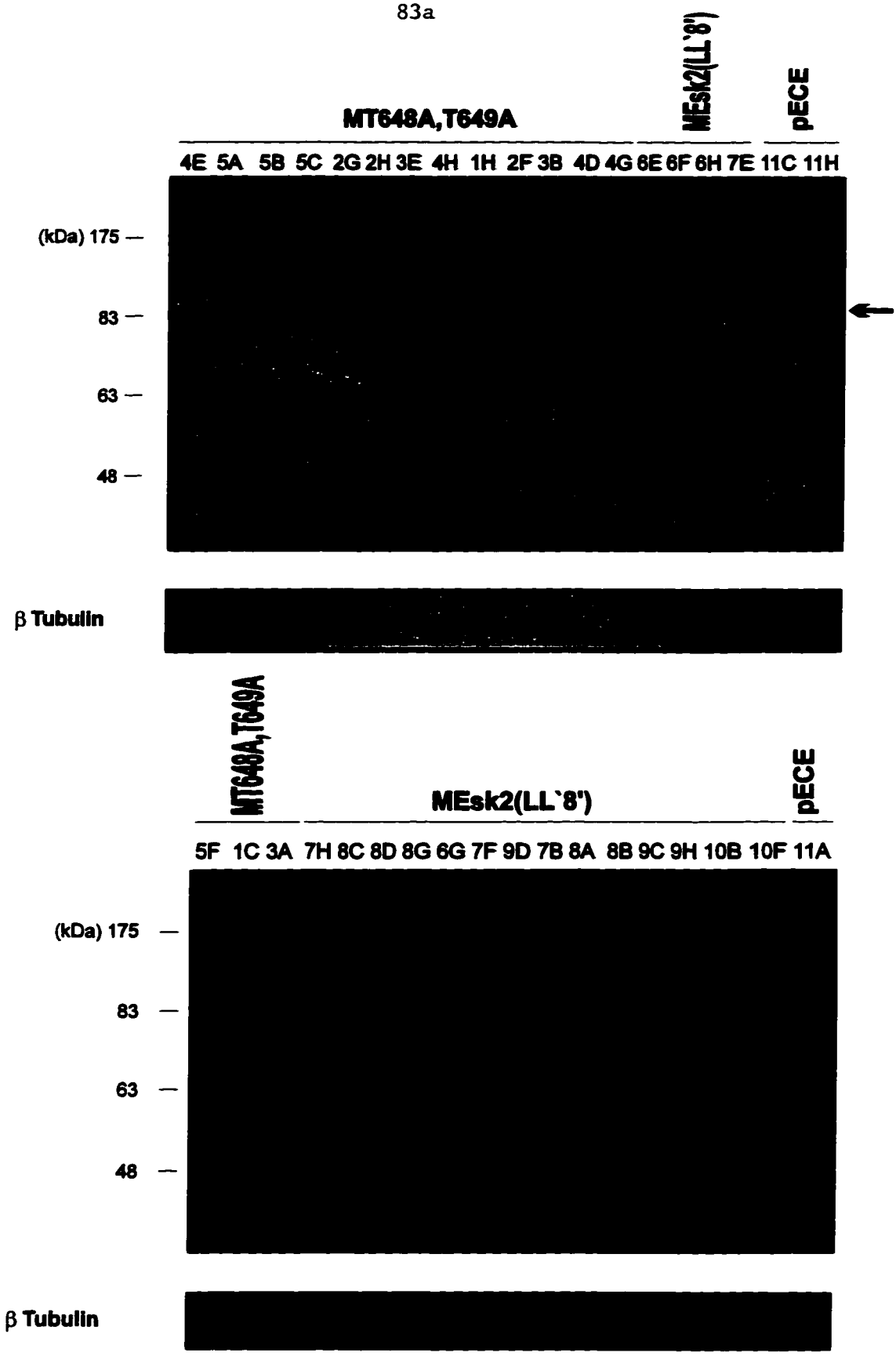

MT648A,T649A
MEsk2(LL`8')
pECE
4E 5A 5B 5C 2G 2H 3E 4H 1H 2F 3B 4D 4G 6E 6F 6H 7E 11C 11H
(kDa) 175
83
63
48
β Tubulin
MT648A,T649A
MEsk2(LL`8')
pECE
5F 1C 3A 7H 8C 8D 8G 6G 7F 9D 7B 8A 8B 9C 9H 10B 10F 11A
(kDa) 175
83
63
48
β Tubulin

Table 2- Summary of P19 EC cell stable transfection

	P19 EC cells transfected	Colonies after Selection	Colonies Picked	Expressing / Tested	%	Remaining
M-Esk2	$0.5x10^6$	5	5	0/0	na	2
MT648A,T649A	$0.5x10^6$	72	38	6/16	40	18
M-Esk2(LL`8')	$0.5x10^6$	120	40	0/18	0	22
pECE	$0.5x10^6$	>1000	8	na	na	5

MT648A,T649A led to the observation that 3 out of seven produced truncated protein products when analyzed by anti-Myc immunoblot (Figure 5a, lanes 4,5 and 6). The other 4 were apparently full length protein products when compared to MT648A,T649A (Figure 5a). *In vitro* autophosphorylation kinase assay (Figure 5b) identified that 1 of these 4 full length clones had impaired kinase activity similar to M-Esk2(LL`8') (Figure 5b, lane 2) however 3 new M-Esk2 clones had increased kinase activity suggesting that perphaps these clones were WT M-Esk2 clones without mutations (Figure 5b, lanes 1,3 and 7).

Mutation in the Catalytic domain of WT M-Esk2 constructs

To identify why M-Esk2(LL`8') is kinase inactive relative to M-Esk2 and to confirm that MT648A,T649A is identical to M-Esk2 at the amino acid level except at the mutagenized site, all the Esk kinases were sequenced. Amino acid alignment of the three WT Esk constructs and the mutant is shown (Figure 6). M-Esk2 contains a L(758) to R substitution in subdomain XI, at a position that is semi-conserved in the protein kinase superfamily adjacent to a conserved α-helical tertiary structure at the end of the catalytic domain. Only the βARK kinase has a basic amino acid at this position(Figure 7a). M-Esk2 is still kinase active and the relevance of this substitution is unclear. M-Esk2(LL`8') contains a L(587) to R substitution in subdomain IV, at a conserved hydrophobic residue that is at the start of a conserved β-strand. This residue is hydrophobic in 80% of the distinct kinases aligned by Hardie and Hanks, 1995. Only the human Clk kinase has an R at this position (Figure 7b). It is likely that this secondary mutation leads to impaired kinase activity, perhaps owing to global effects on tertiary structure. The only differences

Fig. 5- **Enhanced Kinase Activity associated with M-Esk2(D4-14).** M-Esk2 clones (lanes 1-7) were transfected into COS-1 cells by electroporation to identify full length clones with kinase activity compared to pECE vector alone (lane 8) and MT648A,T649A (lane 9). Immunoprecipitation from cell lysates with the anti-Myc mAb was followed by *in vitro* autophosphorylation kinase assay. Four full length M-Esk2 clones were identified (lanes 1-3,7) as ~120kDa proteins that had mobility similar to that of MT648A,T649A on immunoblot with the 9E10 mAb followed by enhanced chemiluminescence (A). Three of the full length clones (lanes 1,3,7) exhibited kinase activity approaching that of MT648A,T649A as detected using Molecular Dynamics Phosphor screens™ (B).

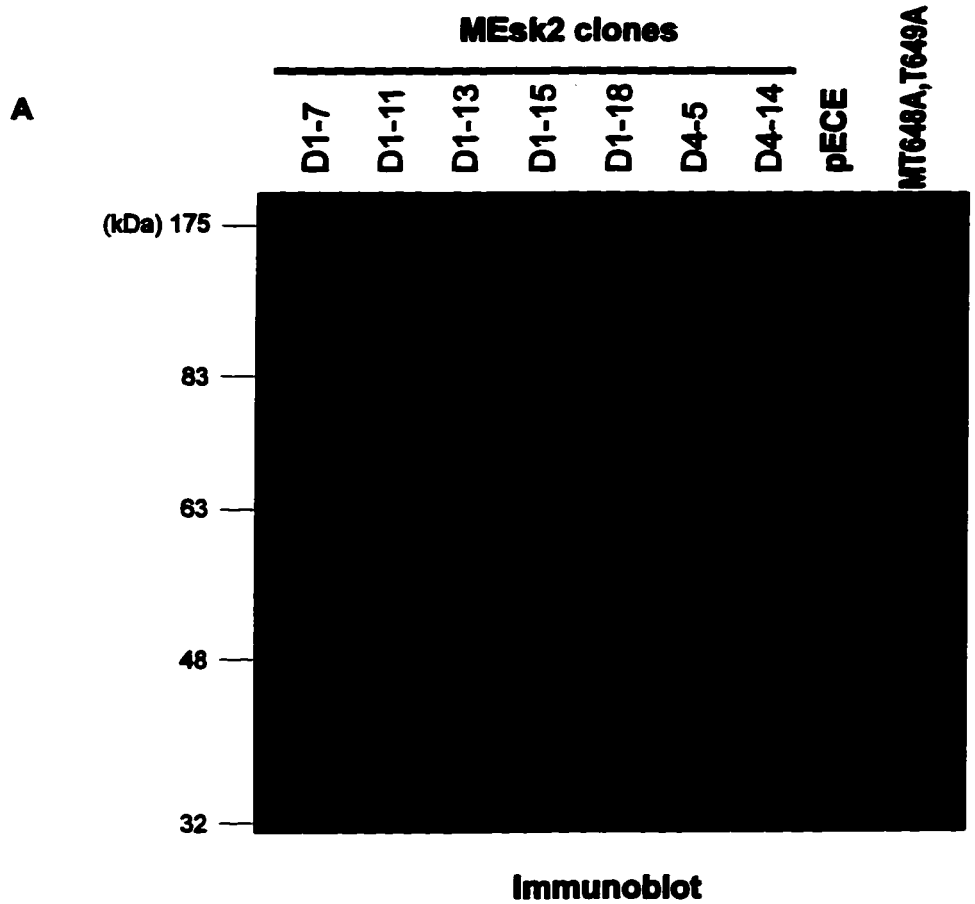

MEsk2 clones
A
D1-7
D1-11
D1-13
D1-15
D1-18
D4-5
D4-14
pECE
MT648A,T649A
(kDa) 175
83
63
48
32
Immunoblot
B
(kDa) 175
83
63
48
32
Lane:
1 2 3 4 5 6 7 8 9
Kinase Assay

Fig. 6- **Amino acid sequence alignment of the Catalytic domains of Esk WT and the mutant kinase.** M-Esk2, M-Esk2(LL`8'), M-Esk2(D4-14) and MT648A,T649A were sequenced in their entirety, the sequencing contigs are described in Appendix B. An alignment of the complete Esk ORF is shown for M-Esk2(Seq. 1), M-Esk2(LL`8')(Seq. 2), M-Esk2(D4-14)(Seq. 3) and MT648A,T649A(Seq. 4). No amino acid sequence differences are found outside the catalytic domain (aa 505-830). The ambiguity line (Seq. 5) identifies amino acids shared between all Esk sequences by a hyphen (-) and amino acids that are not conserved with an asterisk (*). Ambiguity (I) is at aa 758 where a L to R substitution is found in M-Esk2(Seq. 1) in subdomain XI. Ambiguity (II) is a L to R substitution at aa 561 of M-Esk2(LL`8')(Seq. 2) in subdomain IV. MT648A,T649A differs from M-Esk2(D4-14) at aa 648 and 649 only (Ambiguity III). These amino acids were mutagenized by site directed mutagenesis substituting threonine with alanine between subdomain VII and VIII in the activation segment.

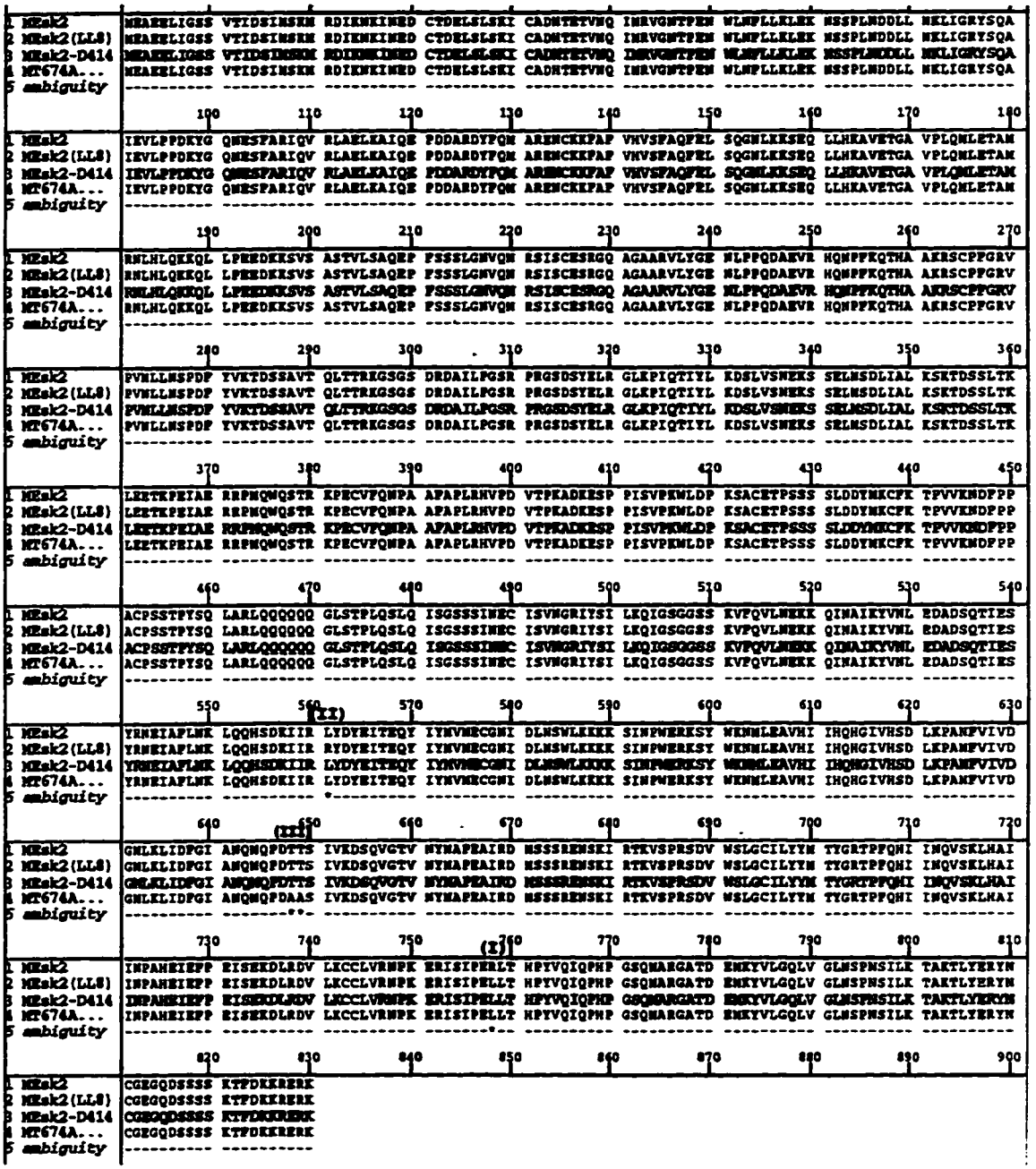


Fig. 7- **Mutation in the Catalytic domain of the Esk kinase.** (A) Mutation in subdomain XI of the catalytic domain is a L to R, +6 amino acids from the invariant R (100% conservation). 85% of kinases have a hydrophobic amino acid at position +6 (aa 758 in Esk). The βARK is the only kinase that also has a basic amino acid at this position (Hardie and Hanks, 1995). The mutated amino acid is adjacent to a conserved α-helical structure that marks the end of the of the catalytic domain. (B) Mutation in subdomain IV of the catalytic domain replaces a L(aa 561 in Esk) with an R, at position +5(+6 in Esk) from the conserved H (76% conservation) that is the focal point of this domain. The mutation is at a conserved hydrophobic aa between protein kinases, 80% of kinases have a hydrophobic aa at this position. The human Clk kinase is the only kinase with a basic amino acid at this position.

A

Subdomain XI

Consensus	E R -T/S- - - L/I- - -
MEsk2 mutation	E R I S I P E R L T H
	R(100%) h (85%)
βARK	R R L G C L G R G A Q

B

Subdomain IV

Consensus	H - -I/L/V/I R L - -
MEsk2(LL'8') mutation	H S D K I I R R Y D
	H(76%) h(80%)
	Y(16%)
hClk	D P N S T F R C V

between the MT648A,T649A amino acid sequence and the published Esk2 sequence are the amino acids that were changed by site directed mutagenesis. Therefore any differences in activity or regulation compared to the WT kinase can be directly related to effects that arise from the tandem T to A mutation. The M-Esk2(D4-14) amino acid sequence is identical to the published sequence and has significant kinase activity. Preliminary data suggests that the MT648A,T649A mutant is still 2-5 fold more active but this has not been quantified.

4.4 Discussion

Kinases have evolved to have several methods of reversible regulation. Autoinhibition by phosphorylated sites that mimics an SH2 binding site regulates the activity of the Src family kinases, association of regulatory subunits that inhibit substrate binding is an important part of the regulated kinase activity of cAPK and regulated phosphorylation can lead to conformational changes to activate or inactivate kinases. Regulatory phosphorylation sites are not conserved between kinases, however evidence suggests that for a number of kinases phosphorylation between the conserved subdomains VII and VIII is critical for activation of the kinase (Johnson et al., 1996). The method of regulation of the Esk kinase was unknown, however phosphorylation between subdomains VII and VIII was identified as being the major autophosphorylation site for an Esk related kinase cloned from human fibroblast (Lindberg et al., 1993). Alignment of the amino acid sequence for a number of kinases between subdomains VII and VIII indicated that critical activating autophosphorylation sites were contained in this region for Src, CDK2 and the HGFR

(Fig. 1b). Raf1 phosphorylation in this region has been shown to be critical for activation of MEK1 and MEK1 phosphorylation on T and Y of ERK2 is absolutely required for full activation of ERK2 (Cobb and Goldsmith, 1995). Phosphorylation in this region is critical for activation of a number of kinases and seems to represent a conserved method of activation (Johnson et al., 1996). To establish whether phosphorylation in the activation segment was important for the kinase activity of Esk2, the homologous T to the major autophosphorylation site of PYT was mutated by site directed mutagenesis to A. Similar mutagenesis of Src prevented activation of the kinase (Kmiecik and Shalloway, 1987) and mutagenesis of S217 and S221 to alanine prevented activation of MEK1 and acted dominant negatively to revert v-src and ras transformed cells and prevent PC12 differentiation (Cowley et al., 1994). Mutation of the homologous Y1234 in the HGFR showed only residual activity but the receptor was still activated due to redundant phosphorylation of Y1235. For full inactivation of HGFR both Y1234 and Y1235 had to be mutated to alanines (Longati et al., 1994). Adjacent to T649 which has been suggested to be the major autophosphorylation site of Esk is another phosphorylatable residue, T648 which is conserved between Ttk and Mps1 suggesting that it might be redundantly phosphorylated analogous to the HGFR situation (Fig1b). For this reason both T648 and T649 were mutated to alanine to evaluate the importance of phosphorylation in this region for Esk activation.

MT648A,T649A effect on Esk activity

Contrary to the literature which indicates that removal of phosphorylatable residues

from the region between subdomain VII and VIII will inactivate the kinase, MT648A,T649A led to increased autophosphorylation kinase activity (Figure 2a,b). Although the result was unexpected, other activating mutations have been described in this region. MEK1 can be activated by mutation of S217, S221 to glutamic acid, a negatively charged amino acid that is believed to mimic the co-ordination in the active site that would occur in the event of S phosphorylation to activate the kinase only this activation is unregulated since the negative charge is always present (Cowley et al., 1994). cAPK T197 mutation to D similarly led to increased activity , resulting from decreased complexation with the regulatory subunits of the kinase (Adams et al., 1995). Both of these examples however involve replacement of a phosphorylated residue with a constitutively negative amino acid not an uncharged amino acid like alanine. Several activating mutations have been described for the yeast GCN2 kinase within the catalytic domain based on deregulated expression of GCN4 whose translation is inhibited in the absence of GCN2 activity (Ramirez et al., 1992). The mechanism for hyper activation is unclear for all the mutants. One mutant, H792Y is within the activation segment and would add a phosphorylatable amino acid to the region, therefore the mechanism of activation may be phosphorylation that leads to novel charge coordination and increased activity. Another mutation, E734V would result in loss of a conserved negatively charged amino acid which may affect coordination of amino acids near the active site and perhaps leads to an activated conformation. Similarly, in subdomain VI a conservative amino acid E752K mutation at a non-conserved amino acid switches the charge from negative to positive which may affect coordination of amino acids and lead to an activated

conformation at the active site.

The fold activation of MT648A,T649A was quantified using laser scanning image analysis provided by the Molecular Dynamics™ phosphorimager and flourimager. MT648A,T649A was 41 fold more active than M-Esk2(LL'8') and 19 fold more active than M-Esk2 (Table 1). Activation could arise through mutagenesis of threonine to alanine if Thr phosphorylation was normally involved in autoinhibition by self association similar to Tyr527 of Src, or if phosphorylated Thr was important to bind a regulatory subunit that normally inhibited kinase activity similar to cAPK. In these cases, the removal of a phosphorylatable residue would remove the inhibitory mechanism. Phosphorylation can be involved in signalling protein degradation through Ub mediated proteosolysis. IκB phosphorylation is the critical first step leading to degradation of IκB (Thanos and Maniatas, 1995), therefore removal of a phosphorylation site that is involved in downregulation of the kinase by degradation would affect the total amount of activity observed but would also lead to increased protein levels and not necessarily increased kinase activity. Removal of the phosphorylatable T may also remove steric hinderance from the substrate binding site and the active site, similar to the required removal of phosphate from T14 and Y15 in CDK2 to allow activation, however typically this type of inhibition is found close to the ATP binding fold, not usually toward the active site and substrate binding site. Removal of T phosphorylation could also affect the subcellular localization of Esk by perhaps leading to removal of an inhibitor that traps Esk in the cytoplasm or exposure of a targeting signal normally hindered by T phosphorylation in the

activation segment.

Immunoflourescence of MT648A,T649A is cytoplasmic and diffuse, identical to that of M-Esk2 and M-Esk2(LL`8') (Figure 3). Therefore, activation of Esk does not appear to affect its subcellular localization suggesting that Esk remains cytoplasmic when activated and T phosphorylation does not regulate Esk activity by localization.

Attempts to stably express WT Esk mammalian expression constructs have been unsuccessful. Speculation is that Esk might affect check points involved in microtubule maintenance based on evidence from Mps1 in yeast. Why WT Esk cannot be stably overexpressed in mammalian cells is unclear, but cell cycle arrest leading to growth disadvantage is one possibility. To test whether activated Esk activity based on autophosphorylation kinase assay would translate into similar activated Esk function *in vivo* MT648A,T649A was stably transfected into P19 EC cells and the ability to express MT648A,T649A was compared to the ability to express M-Esk2 and M-Esk2(LL`8'). Of the individual clones tested 6/16 (40%) express a 120kDa protein that is immunoreactive to anti-Myc antibody by immunoblot after transfection with MT648A,T649A compared to 0/18 clones tested after transfection of M-Esk2(LL`8') (Figure 4). Very few drug resistant colonies grew up following transfection of M-Esk2 reiterating the inability of WT Esk constructs that are kinase active to be overexpressed stably in mammalian cells. M-Esk2(LL`8') has been shown to have impaired kinase activity and initially this was suggested to account for the high number of drug resistant colonies following transfection

and selection (Table 2). Although only 50% of the M-Esk2(LL`8') drug resistant colonies picked have been tested, it appears that it is still not possible to overexpress even a WT M-Esk2 with impaired kinase activity. The ability to overexpress the activated MT648A,T649A suggests that the mutant may not be involved in the same processes that WT Esk is involved in. This could allow the cell to tolerate its overexpression while overexpression of the WT may lead to growth inhibition. It may also indicate that *in vitro* autophosphorylation kinase assay is not a true measure of a kinases' activity *in vivo*. It is also possible that MT648A,T649A is in fact activated and Esk function normally involves phosphorylation of a substrate that allows cell growth, therefore an activated Esk kinase would permit growth and identification of stably transfected clones. In contrast, WT kinases are normally inactive when expressed and therefore act in a dominant negative fashion to interfere with cell growth and such that transfected cells are selected against because they are growth inhibited.

Kinase Activity and Secondary mutations of WT M-Esk2 clones

The lack of kinase activity of M-Esk2(LL`8') and the low activity of M-Esk2 compared to MT648A,T649A led to concern about whether the secondary mutations existed in the so called WT mammalian expression constructs that impaired their kinase activity or whether low kinase activity was normally associated with M-Esk2 expressed in mammalian cells. To address this question several more WT clones were tested for kinase activity by expression in COS-1 cells, immunoblot and *in vitro* autophosphorylation kinase assay (Figure 5). Of seven clones, four appear to encode full length protein products and

three of these have significant kinase activity. M-Esk2(D4-14) kinase activity has not been compared in a quantitative manner to MT648A,T649A but based on quantitation of the kinase activity of the two and their relative expression it appears that MT648A,T649A still has 2-5 fold more activity than M-Esk2(D4-14) compared to the 19 fold and 41 fold higher activity over M-Esk2 and M-Esk2(LL`8') respectively. This suggested that M-Esk2 and M-Esk2(LL`8') have secondary mutations that impair their kinase activity relative to true WT clones such as M-Esk2(D4-14).

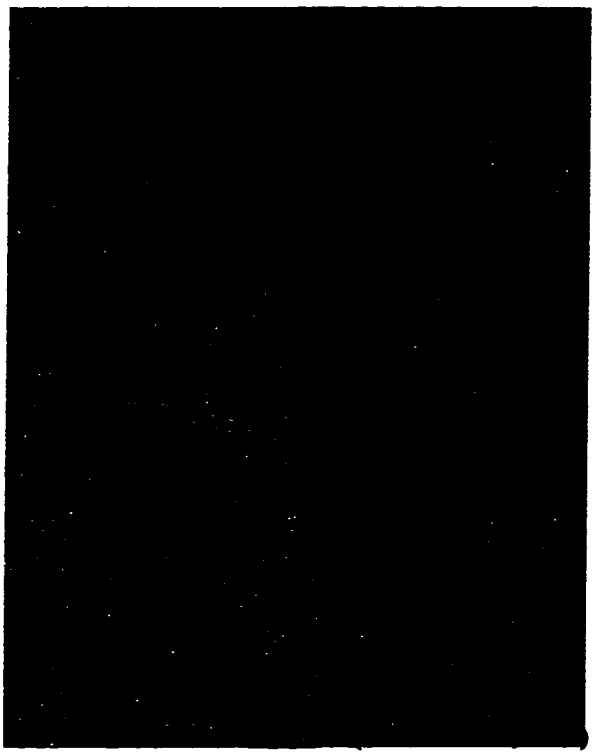
The entire ORF was sequenced for the kinases from the fusion between the six repeat *myc* epitope and the DNA coding for the *esk* initiating methionine to the translation stop codon at the end of the *esk* cDNA. The only ambiguities between the sequencing projects existed in the catalytic domain of the kinases (Figure 6). M-Esk2 has a single amino acid substitution in subdomain XI (Seq. 1, site I). A L to R substitution at a semi-conserved amino acid, +6 residues from the invariant amino acid that is the focal point of the domain (Figure 7a). The mutated amino acid is predominantly hydrophobic among the 51 distinct kinase domains looked at, 85% have a hydrophobic amino acid and only one has a basic amino acid (Hardie and Hanks, 1995). The mutation is adjacent to an α-helix that marks the end of the kinase domain and forms part of the tertiary structure of the large lobe of protein kinase domains (Johnson et al., 1996). The addition of charge at this position might affect the global structure of the kinase domain by changing the nature of a hydrophobic pocket that the leucine was in or by causing novel amino acid coordination to account for the new positive charge which put the kinase into a less active conformation.

M-Esk2 is still kinase active and it is unclear exactly what the relevance of this mutation is at this time. M-Esk2(LL`8') also had a single amino acid substitution of L to R, only this time in subdomain IV and the mutation a conserved hydrophobic amino acid (Figure 7b). Substitution of a hydrophobic amino acid with a positively charged amino acid could result in global effects on the tertiary structure of the kinase. The mutation would affect the fourth β-sheet that is part of the first lobe of the kinase domain. The sheet is far from the ATP binding fold and the active site, suggesting that the addition of charge to the β-sheet may have an affect on the global tertiary structure of the kinase domain or specifically alter the ATP binding fold, to impair the kinase activity of M-Esk2(LL`8'). MT648A,T649A differs from the WT kinases only at the amino acids mutated by site directed mutagenesis, T648 and T649 that are now alanines. Further experiments will need to be done to confirm that MT648A,T649A has elevated kinase activity.

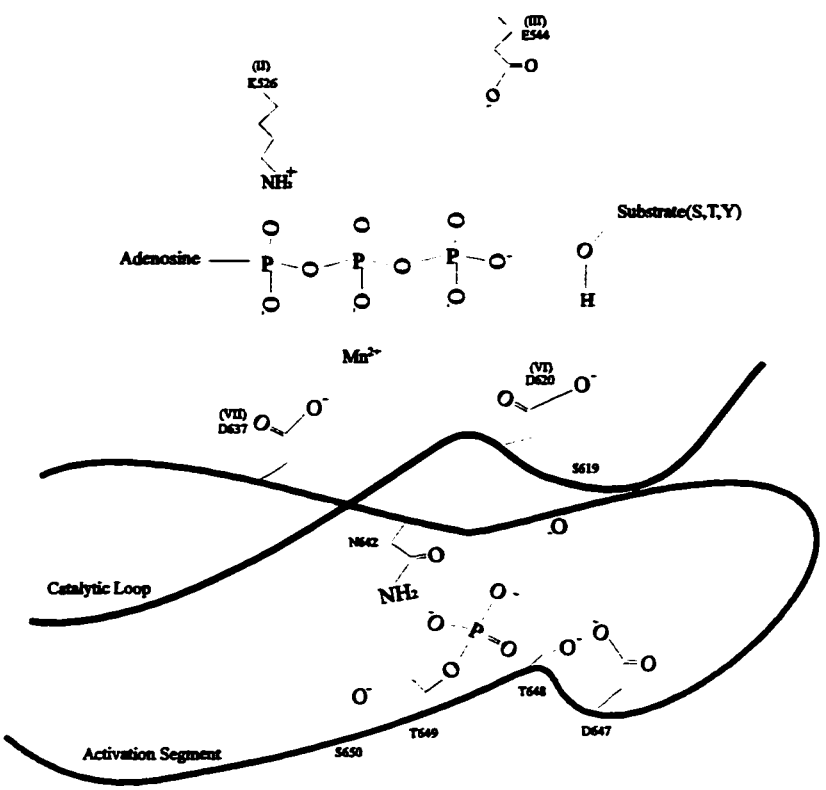
Experiments suggest that the mutation increases Esk kinase activity and that Esk is regulated by a novel mechanism distinct from other kinases phosphorylated in the activation segment. Based on crystal structure data and mutagenesis of a number of kinases in this region, phosphorylation between subdomains VII and VIII is coordinated specifically by an conserved Arg (165 of cAPK) that is part of subdomain VI (Refer to Figure 1a)(Figure 8a) and this specific coordination affects the conformation of the active site and activates kinases such as CDK2, cAPK, Src and MEK1 (Johnson et al., 1996). Amino acids in the active site of these kinases have been documented to move as much as 21Å from phosphorylation in the activation segment such that the kinase shifts from an

Fig. 8- **Charge co-ordination in the active site of the Esk kinase.** (A) Crystal structure analysis and experiments with site directed mutants have indicated that RD type kinases such as cAPK, CDK2 and MEK1 have similar tertiary structure at the active site. It has been suggested that phosphorylation in the activation segment (ie. Thr 197 of cAPK) forces conformational changes of Lys 169 and Arg 165 to co-ordinate the negatively charged phosphate. These conformational changes bring the catalytic base (Asp 166) closer to the γ-phosphate group of ATP and the substrate and enhances activity. (B) Arg 165 of cAPK corresponds to Ser 619 of Esk and Lys 169 is Asn 642. Phosphorylation at Thr 649 will not induce the same conformational changes as cAPK simply because these amino acids will not co-ordinate the phosphate as effectively. Phosphorylation at Thr 649 might induce repulsive effects on the conformation of the activation segment that prevents a tight association of the γ-phosphate of ATP, the substrate and the catalytic base (Asp 620) and therefore downregulates the activity of the kinase.

A



B


(III) E544
(II) E526
NH3+
Substrate(S,T,Y)
Adenosine
Mn2+
(VI) D620
(VII) D637
S619
N642
Catalytic Loop
T648
T649
D647
S650
Activation Segment

inactive state to an active one (Jeffrey et al., 1995). It has been suggested that phosphorylation in the activation segment is a critical regulatory event only for so-called RD kinases that have the conserved Arg as part of subdomain VI (Johnson et al., 1996). We have demonstrated that phosphorylation between subdomains VII and VIII is an important part of Esk regulation and that mutagenesis that prevents phosphorylation at this site leads to activation of Esk. Therefore phosphorylation between subdomains VII and VIII is an important regulatory event for non-RD type kinases and suggests that novel charge coordination may play a role in a novel regulatory mechanism for Esk where phosphorylation is inhibitory to kinase activation. Dephosphorylation of T649 may be required to remove repulsive forces that would exist due to the concentration of negative charge between the activation segment and the catalytic loop (Figure 8b). The dephosphorylation of T649 would then permit N642, and K622 to coordinate the remainder of the high negative charge in the region, opening the active site and permitting greater phosphate transfer.

Chapter 5
General Conclusion

5.1 Conclusion

A knockout vector was made to target the *esk* allele with 9.0kb of homology to the *esk* gene. A screening strategy was identified that could differentiate between a successfully targeted *esk* allele and the endogenous allele. Screening 186 individual clones from one transfection of J1 ES cells did not identify any targeted homologous recombination events. Therefore, no information was gained about the requirements of the Esk kinase in development or in specific cell processes by attempting to generate a mouse deficient of the Esk kinase.

The two isoforms of the Esk kinase, Esk1 and Esk2 were shown to be cytoplasmic, soluble kinases. Human Myc epitope tagged versions of Esk1 and Esk2 enable the isoforms to be analyzed separately. Transfection into mammalian cells followed by Immunofluorescence suggested that both isoforms of the kinase were not nuclear or membrane bound but cytoplasmic or perhaps in the endoplasmic reticulum. Fractionation of P19 EC cells which express both isoforms of the kinase confirmed that endogenous Esk was cytoplasmic.

Site directed mutagenesis of Esk2 has identified that threonine phosphorylation of the Esk kinase in the activation segment between the kinase subdomains VII and VIII is important for regulating Esk autophosphorylation kinase activity. It is unclear whether this regulatory phosphorylation site is important for regulating Esk kinase activity toward substrates in mammalian cells. Only the mutant kinase can be overexpressed in P19 EC

cell stable clones suggesting that MT648A,T649A is somehow regulated differently in the cell than the WT kinase allowing its overexpression to be tolerated. Two additional Esk mutations have been identified that affect Esk autophosphorylation kinase activity. The effect of kinase impaired Esk, WT Esk and activated Esk on a particular signal transduction pathway has not been demonstrated but further characterization of Esk using these Esk kinase isoforms should identify regulated Esk functions in the cell.

5.2 Future Work

The motive behind these experiments was to understand the importance of the Esk dual specificity kinase for specific signal transduction pathways and cell processes and to understand the differences in the two isoforms. While the experiments described begin to address these issues, there is still a lot to be done before there is a detailed understanding of the Esk kinase.

Targeted disruption of *esk* is a critical experiment in the understanding of this kinase. The current targeting vector and screening strategy is capable of generating and identifying a targeted allele following a second transfection of pKS101-KO-9.0 into J1 ES cells. However, improvements to knockout vectors have increased the targeting frequency and simplified the identification of targeted alleles. It might therefore be more advantageous to use an IRES-βgeo targeting vector that can provide information about the cell type specificity of *esk* promoter activity, in addition to increased frequency of targeted disruption. Generation of mice deficient for Esk may be important specifically for

analysis of the Esk1 isoform which has only be identified from embryonic cells. The current targeting vector will cause a disruption of both Esk1 and Esk2 but the additional exon spliced into the *esk1* mRNA could also be targeted to identify effects specific to a deficiency of this isoform.

MEsk1 and M-ESK2 will be useful tools for investigating protein-protein interactions and effects on signal transduction pathways by their overexpression. p85 has been shown to interact constitutively with α and β tubulin and in an insulin dependent manner with γ tubulin at the microtubule organization complex (Kapeller et al., 1995). There are two tandem repeats of the p85 SH2 domain consensus docking site in Esk (Songyang et al., 1993) and an Esk related kinase regulates spindle pole body duplication in Gl and a G2/M checkpoint of spindle integrity in yeast (Lauze et al., 1995; Weiss and Winey, 1996). Esk1 and Esk2 have now been shown to be cytoplasmic, soluble proteins and are capable of perhaps directly interacting with p85 and tubulin as part of Esk regulation of microtubule maintenance in mammalian cells. The sensitivity of the experiments that established Esk as a cytoplasmic kinase cannot eliminate the possibility that a subset of Esk, perhaps Esk1 specifically is in the ER. An additional biochemical experiment that would confirm that Esk is cytoplasmic and not in the endoplasmic reticulum involves immunoprecipitation of recombinant Esk from COS-1 cells or endogenous Esk from P19 EC cells, treatment with EndoH and EndoF followed by a kinase assay. Susceptibility to these endonucleases indicated by a decrease in size as seen by a kinase assay suggests that Esk is modified by glycosylation that is endoplasmic reticulum specific (Bauskin et al.,

1991). If Esk is entirely cytoplasmic then EndoH and EndoF will have no effect when compared to the Ltk kinase which is in the ER membrane.

Regulated Esk kinase activity is likely involved in cell cycle progression. Given that the absence of Mps1 leads to loss of a cell cycle checkpoint and that Ttk mRNA, protein and kinase activity peak at the G2/ M transition, activation of Esk may be important as a cell cycle inhibitor monitoring malformed spindles. It is also conceivable that Esk activity may be required for the formation of mitotic spindles prior to mitosis. Therefore understanding how Esk kinase activity is regulated may eventually lead to ways of regulating the cell cycle with either activated or inactivated Esk kinase isoforms. A greater understanding of the nature of the increased kinase activity of MT648A,T649A is the first step in understanding the regulation of Esk kinase activity. The first experiment to address this issue is phosphorylation of an exogenous substrate such as enolase to confirm that the increased autophosphorylation kinase activity of MT648A,T649A represents an increased abililty to phosphorylate substrates as well. It will also be important to confirm that MT648A,T649A has activated kinase activity compared to M-Esk2(D414) the third generation WT version of the kinase. Repeat of the linear assay experiment into 293T cells will confirm whether the mutant indeed has greater activity when compared to the true WT without secondary mutations. *In vivo* labelling, followed by isolation of Esk protein from nitrocellulose, tryptic digestion, reverse phase HPLC and peptide sequencing was used to identify the major autophosphorylation site in the Esk related kinase Ttk (Lindberg et al., 1993). Identification of T648 or T649

phosphorylation *in vivo* in a similar way would help establish that phosphorylation in the activation segment is an important part of Esk regulation. Based on the fact that it is possible to stably transfect MT648A,T649 but not the WT kinase, this suggests that there are some functions that the mutant does not participate in that lead to its tolerance within the cell. However, the stably transfected MT648A,T649A P19 EC cell clones should also be a valuable tool in experiments designed to address specific protein-protein interactions that might be involved in Esk regulation of microtubules or other signal transduction pathways within the cell.

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ABSTRACTS

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