Biochemical and Genetic Approach to the Characterisation of Tec function in the mouse

A thesis submitted to the University of Adelaide

for the degree of Doctor of Philosophy

by

Ines Irene Caterina Atmosukarto, B.Sc. (Hons)

Department of Molecular Biosciences
Discipline of Biochemistry
University of Adelaide
Adelaide, South Australia

JUNE 2001

TABLE OF CONTENTS

CHAPTER 2 MATERIALS AND METHODS

SUMMARY

The Tec family of intracellular protein tyrosine kinases (PTKs) currently lists five members: Btk, Itk, Txk, Bmx, and Tec. All members of this family are characterised by the presence of an N-terminal Pleckstrin Homology domain (except for Txk), that is followed by a Tec Homology (TH) motif characteristic of the Tec family, a Src Homology (SH) 3, a SH2 and a kinase domain. The expression of Tec family PTKs has been reported to be, at least to some degree, specific to lineages of the haematopoietic compartment. In addition, one member of this family, Btk, has so far been associated with the human immunodeficiency disorder X-Linked Agammaglobulinemia.

Numerous reports over the last five years have focussed on the characterisation of the signal transduction pathways that are dependent on Tec family kinase activity. These have uncovered a critical role for several members of this family of PTKs in antigen receptor signalling. It is currently believed that upon activation of the B and T cell receptors (BCR and TCR respectively), Btk and Itk form multiprotein complexes that are critical for the propagation of BCR- and TCR-initiated signals respectively. These complexes are nucleated by adaptor proteins of the BLNK family (either BLNK/Slp-65 or SLP-76), and include a member of the Tec kinase family (Btk or Itk), Syk or Zap-70 and PLCγ. The formation of such complexes enables the phosphorylation and activation of PLCγ, and is a pre-requisite for sustained calcium signalling upon receptor activation in these signalling systems.

In contrast to this role in antigen receptor signalling, functional studies of Tec have concentrated mostly on its role in cytokine signalling including SCF, IL-3, Epo, IL-6, thrombopoietin and GM-CSF signal transduction pathways; although more recently, Tec has also been implicated in BCR, and TCR/CD3 and CD28 signalling. This thesis describes a potential novel role for Tec in Fcγ receptor (FcγR) signal transduction, during the

internalisation of IgG opsonised exogenous particles, through a process more commonly referred to as phagocytosis.

FcγR signalling has been the subject of numerous studies and the following sequence of events have been uncovered to take place during particle internalisation. Internalisation of the target particle is dependent on the formation of a membrane bound structure known as the phagosome. This structure eventually fuses with components of the endocytic pathway to facilitate the degradation of the internalised particle. Fcγ receptors recognise IgG molecules on the surface of target particles. Particle binding induces the clustering of FcγRs which is followed by the phosphorylation of Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) of the γ chain, probably by a Src family kinase. The intracellular protein tyrosine kinase Syk is also recruited and activated at the activated receptor complex. Other downstream effectors of FcγRs have also been identified that are necessary for the actin-based engulfment of the target including PLCγ and a number of its effector proteins including PKC, MARCKS and MacMARKS all of which accumulate at the forming phagosome. Additionally, PI3-K has also been identified to be critical mainly for the closure of the forming phagosome.

Evidence presented in Chapter 4 demonstrates the involvement of Tec in phagocytosis. Such involvement is easily visualised by the distinct change in the subcellular localisation of Tec observed at the sites of particle binding during the formation of the phagocytic cups. Using immunofluorescence, Tec was shown to be rapidly recruited to phagocytic cups during the internalisation of IgG opsonised Zymosan A, where it colocalised with F-actin. Using Cytochalasin D, an inhibitor of actin polymerisation, actin polymerisation was shown not to be necessary for the redistribution of Tec to the phagosome. Recruitment of Tec is predicted to be mediated by an interaction between the lipid binding PH domain of Tec and $PI(3,4,5)P_3$ molecules at the plasma membrane. This is supported by the observation that Tec remains

diffuse in the cytoplasm of macrophage cells when phagocytosis was carried out in the presence of the PI3-K inhibitor LY294002.

In addition to a role in phagocytosis, preliminary studies described in Chapter 5 also suggest a possible role for Tec in the generation of macrophage cells, at least *in vitro*. In these studies, Tec-/- ES cells were unable to demonstrate the levels macrophage cell differentiation seen in wild type and Tec+/- ES cells. Although it remains to be fully characterised, this defect in macrophage differentiation was predicted to reflect either a defect in cytokine signalling, especially IL-3, or a defect in macrophage migration and/or adhesion that might prevent macrophage cells to migrate out of the differentiating embryoid body.

Finally, as shown in Chapter 3, two major isoforms of the Tec transcript, referred to as *TecIII* and *TecIV*, have been detected in various mouse embryonic and adult tissues, as well as in a number of different hematopoietic cell lines: Tec IV is the full length Tec protein with functional PH, TH, SH3, SH2 and Kinase domains, while *TecIII* is generated by the splicing out of exon 8 sequences to yield a shorter peptide with a non-functional SH3 domain. Using GFP-TecIII fusion proteins, this shorter isoform of Tec was shown to have biological characteristics that differed from TecIV. Briefly, GFP-TecIII appeared to be hyperphosphorylated and showed increased redistribution to plasma membrane ruffle structures when expressed in COS-1 cells. A potential role for TecIII in serum deprivation-induced apoptosis was also highlighted using this system. The distribution of GFP-TecIV on the other hand appeared to be mostly diffuse in the cytoplasm and nucleus of transfected cells. In addition, punctate fluorescence was also observed for both GFP-TecIII and GFP-TecIV suggesting the possible association of Tec with vesicle structures.

In summary, this thesis identified the involvement of Tec in signalling pathways other than that of cytokine signal transduction and uncovered a possible role for Tec in the phagocytosis of IgG-opsonised exogenous particles as a downstream effector of the Fcγ

receptor. In addition, a model system for the study of Tec function during macrophage differentiation was also generated using gene targeting in ES cells. This system should allow more detailed characterisation of Tec function both in macrophage differentiation and macrophage function as it allows studies to be carried out in a Tec null genetic background.

STATEMENT

This thesis contains no material that has been accepted for the award of any degree or diploma by any other University. To the best of my knowledge it contains no material that has been previously published by any other person, except where due reference has been made in the text.

I consent to this thesis, when deposited in the University library, being available for photocopying and loan.

Date: 18-6-01

Ines Atmosukarto

ACKNOWLEDGMENTS

I would like to thank the Department of Biochemistry at the University of Adelaide for giving me the opportunity to pursue these studies, and Prof. P.D. Rathjen as the Head of this Department and co-supervisor of my thesis. I am grateful for the Overseas Postgraduate Research Scholarship (OPRS) and University of Adelaide Scholarship that have supported me financially throughout the duration of my studies. Dr. G.W Booker has been most supportive and patient as my principal supervisor and his guidance and critical input during the preparation of this thesis has been most appreciated. I must also thank J. Wrin for all the time and effort (and frustration) he has invested during the microinjection procedures.

Anita Merkel, my faithful thesis editor has played major roles both in the early stages of these studies and during the preparation of this thesis. She has been a wonderful friend throughout many stressful times. Sharon and Gavin Chapman have definitely made the last few years a lot more fun and their attention in times of need will never be forgotten. It might be a while until Sharon and I will be able to share a cup of coffee, a strawberry tart and lots of gossip but her friendship will never be forgotten.

I have been blessed with the most supportive family. My mum Iona, my dad Soedjono and my sisters Ira and Indri have always been there especially when everything seemed too hard. My husband, Teddy, has been most patient throughout the years and our little girl (although unexpected) is by far the most perfect result of this Ph.D. She was forced to live a very unconventional life for the first two years of her life but has nonetheless blossomed into the most beautiful little girl. The help that Threes (Oma), my mother in law has lent me while she came to Adelaide and cared for Adeline was priceless. I must also mention my "surrogate" family in Adelaide, especially Mary who has been there for me since 1994 and who has allowed me to disrupt her household during the last stretch of this Ph.D.

Finally, I have throughout the years made wonderful friends here in Adelaide, Renate, Angelo, Viv and Jackie who were always there to make sure I remained sane. Last but not least I want to thank lab members past and present for their friendship Bec especially, who joined the lab at the most stressful stage of this Ph.D. (thanks for the laughs and the great big bear hugs), Terry, Carlie, Eric, Fil, Kathy and Leah.

CHAPTER 1:

INTRODUCTION

AND

LITERATURE REVIEW

1.1 Introduction

The work described in this thesis has concentrated mainly on the characterisation of the molecular mechanism of action of the Tec protein tyrosine kinase using biochemical and genetic approaches. This introductory chapter addresses the issues of signal transduction, the Tec family of protein tyrosine kinases, phagocytosis and gene targeting, all of which were fundamental to the formulation and design of the experimental procedures described in chapters 3-5.

1.2 Signal transduction

Signal transduction is the systematic passage, modification and amplification of a signal, from one carrier to another, in response to external physical and chemical stimuli. It is generally initiated by the interaction of an extracellular molecule with a ligand-specific receptor on a target cell. Receptor activation then stimulates intracellular biochemical pathways that are, depending on the nature of the stimuli, able to induce changes in gene expression, cytoskeletal structure, protein trafficking, cell adhesion and migration, or affect progression through the cell cycle. Signal transduction is essential for the existence of multicellular organisms, and a vast body of work carried out over the last few decades has not only uncovered the highly conserved nature of signal transduction pathways, but also recognised that defects in signalling are responsible for many human disorders (Uribe and Weinberg, 1998).

It is now well established that enzymes involved in signal transduction have highly specific catalytic domains, separated from other protein-protein or protein-lipid interaction modules, and that it is the later modules that determine the signal transduction pathway(s) that a particular enzyme will contribute to. Protein modules are compact, independently folding

units. Many have their N and C termini in close apposition such that they can be plugged into the surface of proteins and act as molecular adhesives that nucleate the formation of multiprotein complexes (Panayotou and Waterfield, 1993). These modules are generally 40-150 amino acids long and fold to generate one or more ligand binding pockets where a core motif of 3-6 amino acids determines the type of ligand recognised by a specific module, and the flanking amino acids dictate its specificity (reviewed in Pawson, 1995; Cohen et al., 1995; Sudol, 1998). Protein modules such as Src Homology (SH) 2, SH3 and Pleckstrin Homology (PH) domains are not restricted to any particular type of signal transduction proteins. They have in fact been identified in protein kinases, lipid kinases, protein phosphatases, cytoskeletal proteins, phospholipases and transcription factors, as well as non-catalytic adaptor proteins, but are rarely found in plasma membrane receptors.

1.2.1 Protein-protein interaction modules

1.2.1.1 Src Homology 2 (SH2) domains

SH2 domains are highly specific phosphotyrosine (pTyr) binding modules (Sadowski et al., 1986) which have essentially no affinity for unphosphorylated peptide sequences (Piccione et al., 1993; Felder et al., 1993; Panayotou et al., 1993; reviewed in Pawson, 1995). Because the interaction between SH2 domains and pTyr residues on tyrosine phosphoproteins - including activated receptors- is highly specific, tyrosine phosphorylation can function as an "on/off" switch for SH2 binding. Binding of SH2 domains to the pTyr residues of phosphoproteins can stimulate enzymatic activity, induce subcellular re-localisation and facilitate enhanced tyrosine phosphorylation of target proteins (reviewed in Pawson, 1995).

Structurally, SH2 domains have a highly conserved peptide fold that consists of a large central anti-parallel β sheet flanked by two α helices, which follow a general β–α–β–β–β–β–β–α–β pattern (Eck et al., 1993; reviewed in Cohen et al., 1995). The

binding pocket of the SH2 domain is bipartite. The first binding interface consists of an invariant arginine residue that forms hydrogen bonds with two pTyr phosphate oxygens, while the second interface recognises the three amino acids immediately C-terminal to the pTyr residue and largely determines binding specificity (Eck et al., 1993; Songyang et al., 1993). Two major groups of SH2 domains have been identified based on their binding specificity. The first group of SH2 domains selects pTyr-hydrophilic-hydrophilic-hydrophobic sequences, while the second interacts with pTyr-hydrophobic-X-hydrophobic sequences where X represents any amino acid residue (reviewed in Cohen et al., 1995; Cantley and Songyang, 1994).

1.2.1.2 Src Homology 3 (SH3) domains

SH3 domains have been identified as structural components of signalling and cytoskeletal proteins that act as highly specific molecular adhesives by interacting with specific left-handed poly-proline type II helices. SH3 domains typically consist of five anti-parallel β strands packed to form two perpendicular β sheets, with a binding pocket characterised by a hydrophobic patch of clustered aromatic amino acids that are surrounded by two charged and variable loops (reviewed in Cohen et al., 1995). The minimal consensus binding sequence of the SH3 domain has been identified as P-X-X-P-X-X-R/K or R/K-X-X-P-X-X-P (reviewed in Morton and Campbell, 1994), where X tends to be an aliphatic residue, R represents arginine, K is lysine and P is a scaffolding residue that is generally a proline. The conserved proline residues are crucial for binding affinity, and binding specificity is conferred by the interaction between non-proline residues in the ligand and the two variable SH3 loops that flank the main hydrophobic binding surface of the SH3 domain (reviewed in Pawson, 1995).

1.2.1.3 Pleckstrin Homology (PH) domains

The pleckstrin homology fold was originally identified as a 100 amino acid internal repeat in the protein Pleckstrin (Musacchio et al., 1993), and has since been recognised in proteins with a variety of functions such as phospholipases (PLC), GTPase regulating proteins (VAV, Dbl, SOS), protein kinases (Akt and Tec kinases), as well as cytoskeletal proteins such as spectrin (reviewed in Shaw, 1996; Blomberg et al., 1999). Structural studies have also revealed that despite the limited extent of sequence identity shared between the various PH domains, these generally share a common basic fold, itself similar to that of the Protein Tyrosine Binding (PTB) domains (Orengo et al., 1994).

Structurally, PH domains form a β barrel structure that consists of two orthogonal β sheets of three and four strands that create a β sandwich closed off at the C terminal end by an amphipathic α helix. While different PH domains have similar core structures, the loop regions between the β strands vary and are therefore predicted to be involved in ligand binding interactions. PH domains are able to incorporate a series of different binding sites on the same structural scaffold. This enables them to interact with a wide range of molecules, including βγ complexes of heterotrimeric G proteins (Touhara et al., 1994; Abrams et al., 1996), protein kinase C (PKC) (Yao et al., 1994; Kawakami et al., 1995), phosphorylated ligands such as inositol phosphates or phosphatidylinositol lipids (Hyvonen et al., 1995), and cytoskeletal components such as filamentous actin (Yao et al., 1999). PH domains are therefore able to interact not only with other signalling proteins but also with critical structural components of the cell such as the plasma membrane and actin cytoskeleton.

PH domains have an electrostatically positive polarised surface with high affinity for the negatively charged phospholipids of the plasma membrane which facilitates the recruitment of these domains to receptor signalling complexes at the plasma membrane. This strong positive potential surrounds the phosphoinositide recognition site of the domain and dictates

the relative affinity of PH domains for various inositol derivatives. Based on their affinity for phospholipids, PH domains are classified into four groups: group 1 interacts with $PI(4,5)P_2$ and $I(1,4,5)P_3$; group 2 binds $PI(3,4,5)P_3$ and $I(1,3,4,5)P_4$; group 3 binds $PI(3,4,5)P_3$ and $PI(3,4)P_2$, and group 4 shows no binding for phosphoinosides (Kavran et al., 1998; reviewed in Blomberg et al., 1999).

Phosphoinositide metabolism is a critical component of the signal transduction pathways necessary to control diverse cellular processes including cell survival, proliferation, differentiation and migration (Divecha and Irvine, 1995; Carpenter and Cantley, 1996). A wide range of receptor stimuli induce the production of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ by phosphoinositide 3-kinases (PI3-K). This family of phosphoinositide kinases catalyse the transfer of the γ-phosphate group of ATP to the D3 position of phosphoinositides resulting in a transient rise of phosphorylated lipids including $PI(3,4,5)P_3$. Binding between group 2 and group 3 PH domains and $PI(3,4,5)P_3$ *in vivo* is dependent on specific interactions between the negatively charged phosphates in the polar head groups of $PI(3,4,5)P_3$ and several conserved basic residues in the β1-β2 strand and β1-β2 loop region of the PH domain. It is the combinatorial strength of these interactions that dictates the relative affinity of $PI(3,4,5)P_3$ for PH domains as demonstrated by the detrimental effect of the mutagenesis of amino acid sequences that alter the overall positive charge of the domain or disrupt the binding pocket of PH on phosphoinositide binding (reviewed in Blomberg et al., 1999).

1.2.2 Modular domains as regulators of enzyme activity

In addition to their function as molecular adhesives, modular domains can also act as regulators of enzymatic activity. The mechanism by which these modular domains are able to control enzyme activity is currently best understood for Src (reviewed in Schwartzberg, 1998). Biochemical studies first identified critical roles for modular domains in regulating Src kinase

activity. An interaction between the SH2 domain of Src and the phosphotyrosine residue located just C-terminal to the Src kinase domain ($pTyr^{527}$) down-regulates Src kinase activity (Roussel et al., 1991; Liu et al., 1993). A critical role for the SH3 domain was also identified (Parsons and Weber, 1989) but the mechanism by which it affected Src kinase activity remained unclear until the three dimensional structure of downregulated Src and Hck was elucidated (Xu et al., 1997 and Sicheri et al., 1997; reviewed in Sicheri and Kuriyan, 1997). In combination, these studies and numerous others, suggest that although the SH2 domain of Src is able to interact with $pTyr^{527}$, this interaction occurs away from the active site of the kinase domain and as such can not completely inhibit Src kinase activity. A second unexpected intramolecular interaction involving the SH3 domain and the linker region that joins the SH2 and kinase domains stabilises the closed/inactive conformation of Src. The combination of these two intramolecular interactions is therefore responsible for regulating Src enzymatic activity. In the absence of a stimulatory signal, the two lobes of the kinase domain appear to be pushed close together thus enforcing a conformation in the small lobe that disables the active site. This in turn restricts the relative motion between the N and C-terminal lobe of the kinase and prevents the binding of ATP. Activation of the enzyme requires competitive interactions with SH3 or SH2 ligands to displace these intra-molecular interactions and destabilise the inactive conformation (Xu et al., 1997 and Sicheri et al., 1997 and Sicheri and Kuriyan, 1997).

1.2.3 Principles of signal transduction

The study of signal transduction pathways has revealed that the propagation of a signal from a plasma membrane activated receptor to appropriate effector pathways is dictated by several principles (Hunter, 2000). The first of these principles states that amino acid residues can be biochemically modified and that these modifications can affect the activation state of

enzymes and increase the diversity of interactions the modified peptides are able to participate in. Tyrosine phosphorylation is one such modification. It involves the sequence specific addition of the γ phosphate residue of an Adenosine triphosphate (ATP) molecule on to a specific tyrosine residue of a target protein, by a protein tyrosine kinase (PTK) enzyme. At any time, only 0.05% of total cellular proteins in a normal vertebrate cell show detectable levels of tyrosine phosphorylation (Hunter and Sefton, 1980). This extremely low basal level of tyrosine phosphorylation, coupled with the tight regulation of PTK activity, and the existence of high affinity pTyr binding domains such as the SH2 and PTB domains are the basis of a highly efficient inducible signalling system based on highly specific phosphotyrosine-mediated protein-protein interactions. Ligand induced protein proximity has recently emerged as a second principle of signal transduction. Induced proximity is dependent on the sequence specific recruitment of proteins to signalling foci, resulting in a local increase in protein concentration (Pawson et al., 1993; Pawson, 1993). A typical example is the recruitment of SH2- and PTB-containing proteins to phosphorylated receptors at the plasma membrane, where these proteins are able to interact with membrane targeted modifying enzymes and/or membrane targeted effector proteins. A third principle of signal transduction suggests that signal transduction occurs in a semi-solid-state manner with minimal free diffusion. Such compartmentalisation is facilitated by the association of signalling proteins with cytoskeletal structures nucleated by anchoring and scaffolding proteins, to form subcellular signalling-competent microenvironments. Compartmentalisation increases the efficiency and specificity of substrate modifications to allow the coordinated action of enzymes and their substrates in a particular position in the cell (Pawson and Scott, 1997). A final principle of signal transduction suggests that mechanisms exist in the cell that prevent spontaneous signal activation. These generally depend on the regulation of the subcellular localisation of the various signalling molecules to separate signalling complexes from effector systems. Under

such circumstances, long range movements of activated signalling components are necessary for the initial activator complex to activate the effector system. A typically example of such movement is the translocation of phosphorylated transcription factors from the cytoplasm to the nucleus.

Although a vast body of work is beginning to unravel the biomolecular details of various signal transduction pathways, the issues of redundancy and cross talk remain to be resolved. The mechanism by which different external stimuli can trigger overlapping signalling pathways and yet result in distinctive cellular responses is still unclear. Part of the answer for both phenomena probably lies in the fact that the repertoire of signal transduction proteins expressed by a given cell will influence the range and strength of the signal transduction pathway(s) being activated.

The area of signal transduction has had a dramatic impact on medical research. To date at least 18 protein tyrosine kinase genes have been identified as oncogenes either in acutely transforming retroviruses or in human tumors. Increasing numbers of human diseases have been shown to involve mutations in genes encoding protein kinases and/or phosphatases, or in genes encoding their regulators and/or effectors. Of particular interest has been the contribution that this area has made to the understanding of the molecular basis of inherited immunodeficiencies such as SCID, X-linked agammaglobulinemia, Wiskott-Aldrich syndrome and a number of other autoimmune diseases (Korpi et al., 2000). It is therefore becoming increasingly evident that the study of signal transduction pathways will enable further understanding of the molecular defects that underlie many such disorders and will facilitate the rational design of therapeutic compounds.

1.3 The Tec family of protein tyrosine kinases

The Tec family of protein tyrosine kinases belongs to the family of non-receptor/intracellular protein tyrosine kinases (Figure 1.1). It includes Tec (Mano et al., 1990), Btk (Thomas et al., 1993; Rawlings et al., 1993), Bmx (Tamagnone et al., 1994), Itk (Siliciano et al., 1992) and Txk (Sommers et al., 1995), making it the second largest family of intracellular PTKs. Like Src, Tec kinases are characterised by the presence of SH3, SH2 and kinase domains with approximately 48-59% sequence identity shared between the various members of the Tec family of PTK (Figure 1.2A and B), and 33-35% sequence identity to Src (Figure 1.2A and C). The N-terminal region of Tec kinases typically consists of a PH domain which is followed by a Tec Homology (TH) region (Vihinen et al., 1994) and one or two proline rich regions (PRR), except in Txk which lacks the characteristic PH region and has a shorter cysteine rich sequence instead (Sommers et al., 1995). The TH region is characterised by the presence of a conserved His-Cys_3 pattern, recognised as the Btk motif, which is necessary for Zn^{2+} binding (Hyvonen and Saraste, 1997; Vihinen et al., 1997). Together with the PH domain, the TH region forms a structural unit that is highly characteristic of the Tec family and is referred to as the PHTH domain with 38-50% sequence identity shared between Tec family members (Figure 1.2D) (excluding Txk).

A relatively extensive profile of Tec family kinase expression has been compiled and is diagrammatically represented in Figure 1.3. Briefly, Tec family kinases are generally restricted to cells of the hematopoietic lineage with some relatively high degree of lineage specificity, though various members of the family have been detected in a subset of non-hematopoietic tissues (Figure 1.3) (Mano et al., 1990, Siyanova et al., 1994; Heyeck and Berg, 1993; Smith et al., 1994; Tamagnone et al., 1994; Sommers et al., 1995).

Figure 1.1 Nonreceptor protein tyrosine kinases

(adapted from Hunter, 2000)

Intracellular protein tyrosine kinase families are arranged according to their secondary structure. Common domains are depicted as shaded boxes. The various domains represented in this diagram include the SH3, SH2 and Kinase domains as well as the PH and TH domains characteristic of the Tec family of protein tyrosine kinases. (SH: Src Homology, PH: pleckstrin Homology, TH: Tec Homology)

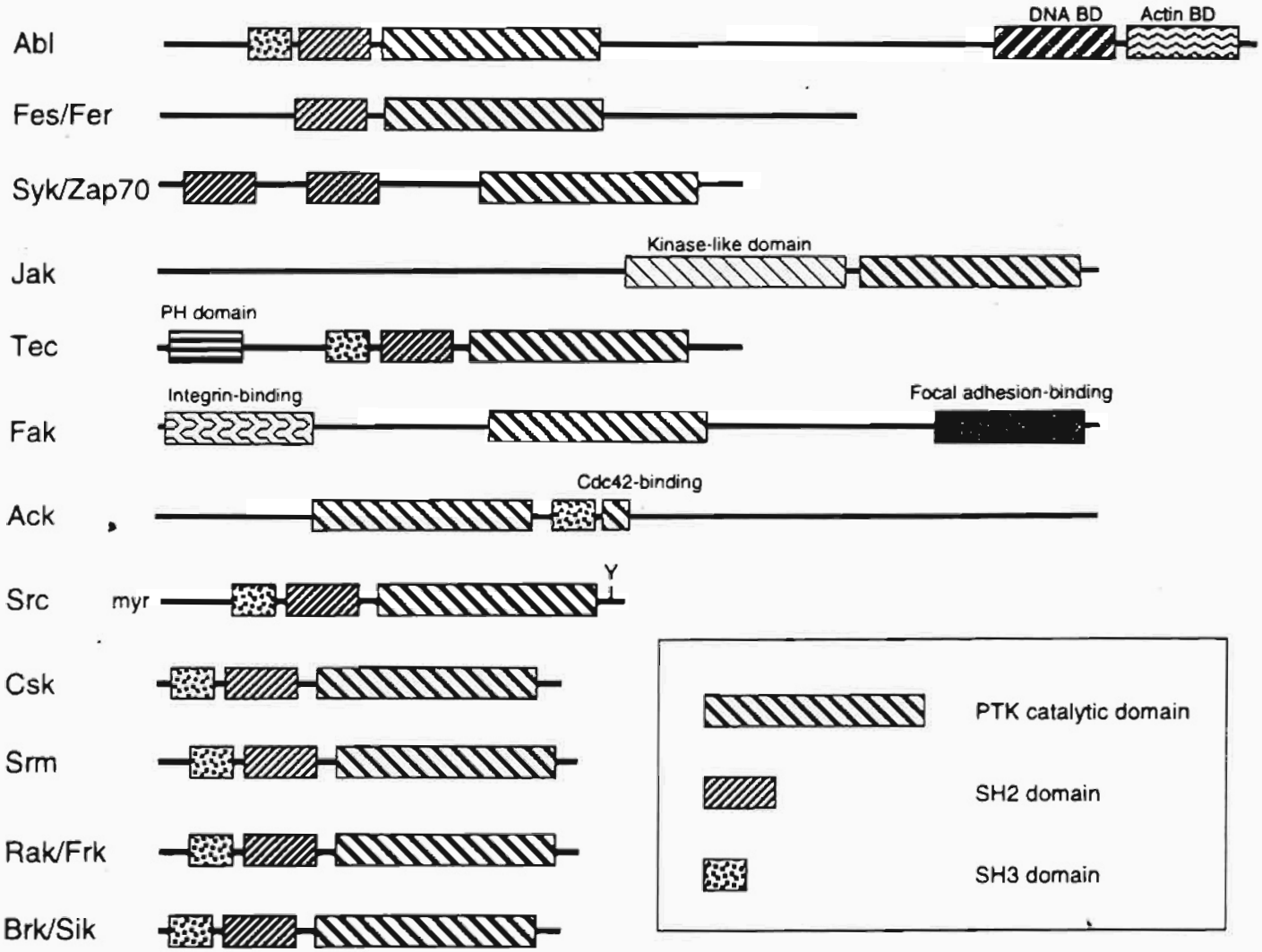

Abl
DNA BD
Actin BD
Fes/Fer
Syk/Zap70
Jak
Kinase-like domain
PH domain
Tec
Integrin-binding
Focal adhesion-binding
Fak
Cdc42-binding
Ack
Src
myr
Y
Csk
Srm
Rak/Frk
Brk/Sik
PTK catalytic domain
SH2 domain
SH3 domain

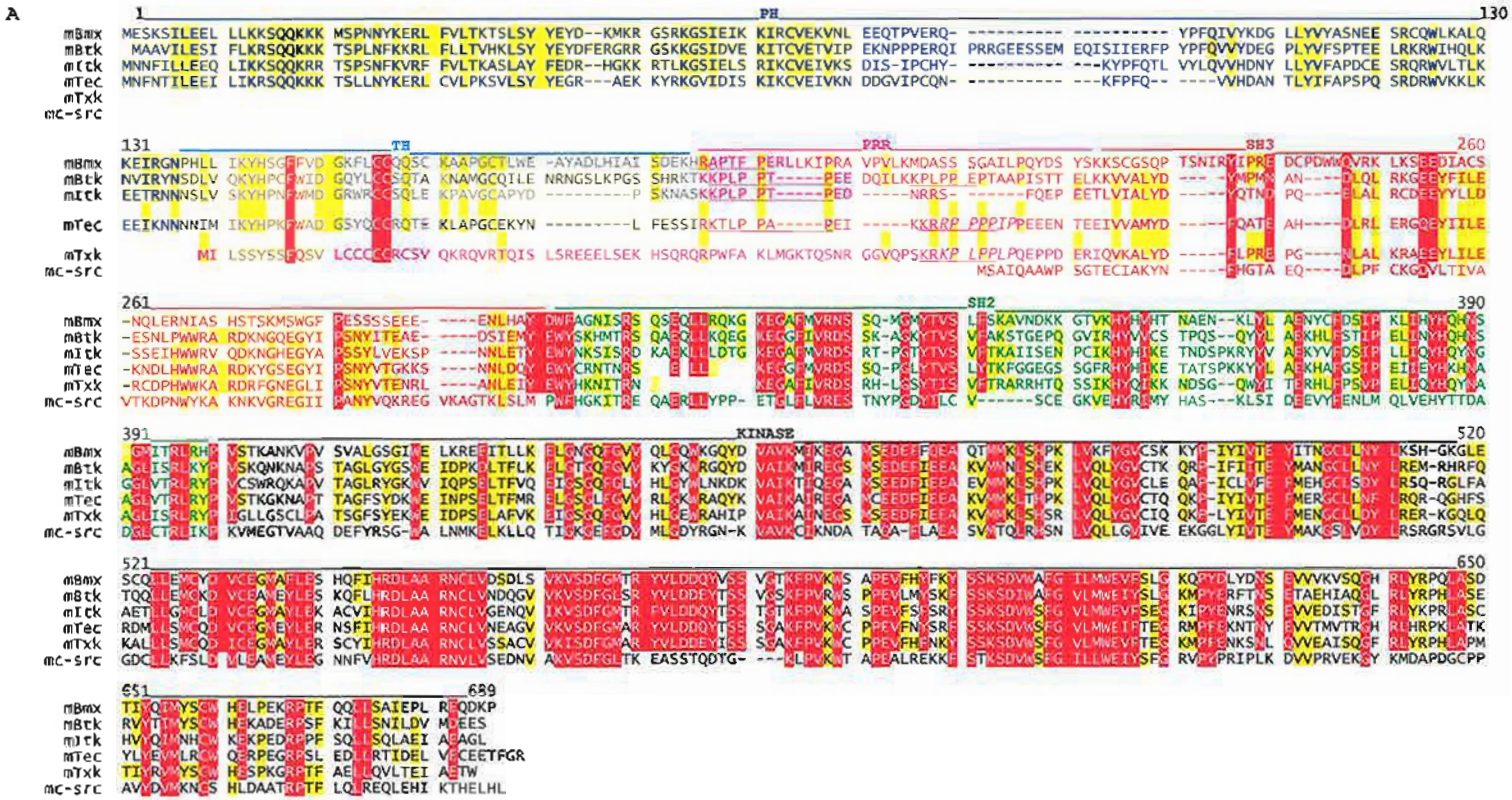
Figure 1.2 Alignment of the amino acid sequence of the members of the Tec family of non-receptor protein tyrosine kinases

The alignment of mouse Tec family kinase used sequences obtained from GenBank. The following GenBank accession number were used: Bmx: NP_033889, Btk: I49553, Itk: NP_034713, Tec: T01380, Txk: I49133 and c-src: NP_031809.

A. Alignment of mouse Tec family kinase sequences. The mouse Tec family kinase sequences were compared with that of c-Src to determine SH3 (pink), SH2 (green) and Kinase (black) domain boundaries. Poly-proline sequences characteristic of SH3 recognition sites are underlined. Where more than one site was identified, overlapping sites are marked in italics. The Tec kinase specific PHTH domain is shown in blue and amino acid sequences shared between the members of the Tec family are highlighted in yellow and those common to Tec family kinases and Src are in red.
B. Tabulation of the levels of amino acid identity identified between the full length amino acid sequences described above.
C. Tabulation of amino acid identity levels between the C-terminal SH3-Kinase protein sequences described above.
D. Tabulation of the levels of amino acid identity identified between the N-terminal sequence described above.

(SH: Src Homology, PH: Pleckstrin Homology, TH: Tec Homology)

A



B

	mBmx	mBtk	mItk	mTec	mTxk
mBmx					
mBtk	49%				
mItk	47%	52%			
mTec	46%	55%	56%		
mTxk	44%	48%	51%	53%	
mSrc	32%	33%	34%	35%	33%

C

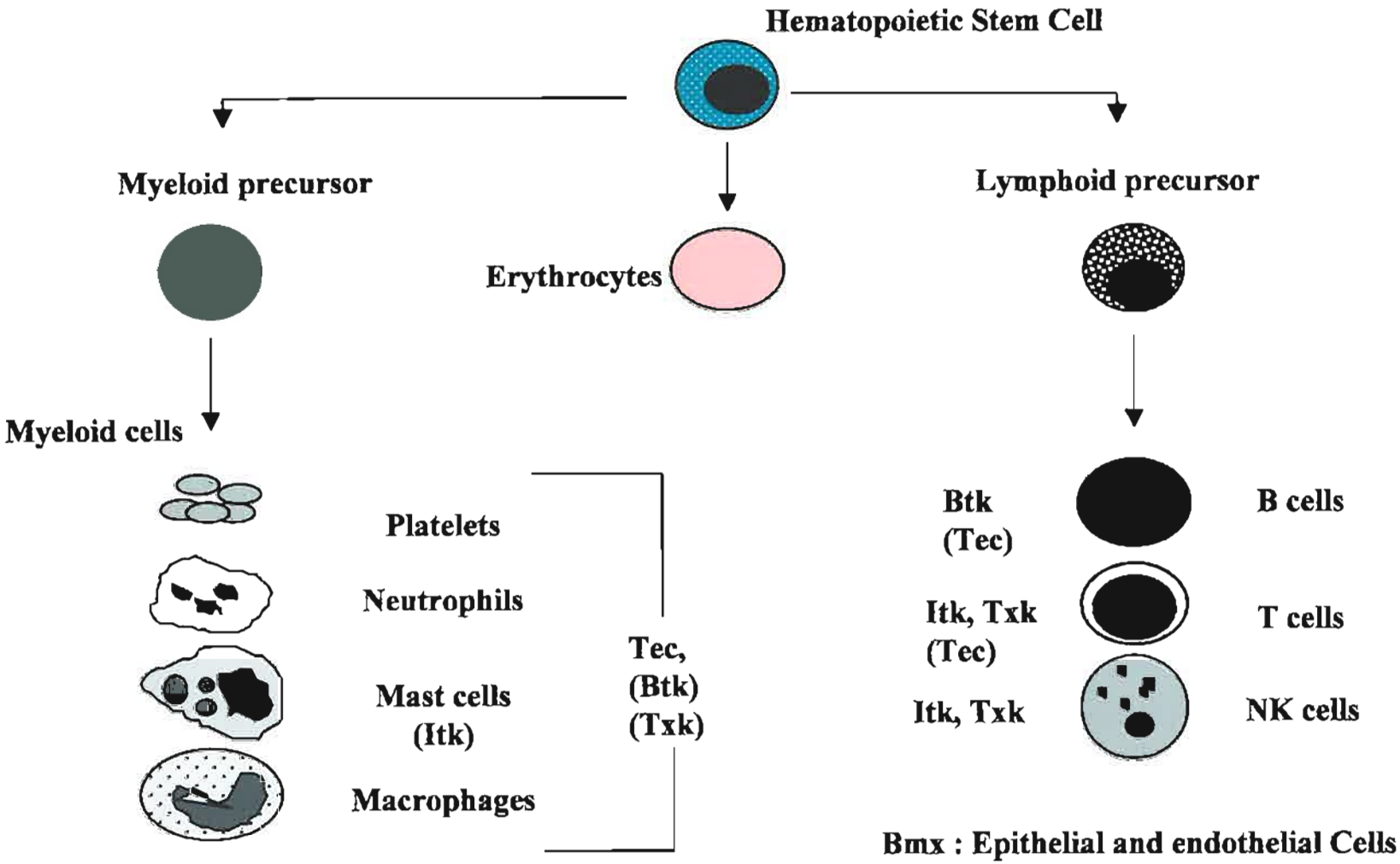
	mBmx	mBtk	mItk	mTec	mTxk
mBmx					
mBtk	52%				
mItk	50%	54%			
mTec	48%	59%	58%		
mTxk	48%	56%	59%	61%	
mSrc	33%	34%	34%	35%	33%

D

	mBmx	mBtk	mItk	mTec	mTxk
mBmx					
mBtk	40%				
mItk	38%	45%			
mTec	38%	43%	50%		
mTxk	7%	17%	22%	14%	
mSrc	9%	18%	18%	18%	18%

Figure 1.3 Diagrammatic representation of the expression pattern of Tec family kinases in hematopoietic lineages.

The major hematopoietic lineages are represented in this diagram. The expression of Tec family kinases in these lineages is listed adjacent to the corresponding lineage(s).


Hematopoietic Stem Cell
Myeloid precursor
Erythrocytes
Lymphoid precursor
Myeloid cells
Platelets
Neutrophils
Mast cells
(Itk)
Macrophages
Tec,
(Btk)
(Txk)
Btk
(Tec)
B cells
Itk, Txk
(Tec)
T cells
Itk, Txk
NK cells
Bmx : Epithelial and endothelial Cells

In general, three main characteristics differentiate Tec kinases from Src family kinases, the latter being the prototype of nonreceptor PTKs. These differences include:

(i) the lack of Src-like N-terminal modifications, including the post-translational addition of fatty acid chains such as N-myristoylation or palmitoylation (Mano et al., 1990);

(ii) the absence of a C-terminal regulatory tyrosine residue. This tyrosine residue (Y^{527}) in Src acts as a negative regulator of Src kinase activity (Matsuda et al., 1990 and Roussel et al., 1991). Its absence in Tec family kinases suggests that an alternative regulatory mechanism is likely to regulate Tec family kinase activity;

(iii) the presence of three functional regions in the N- terminal unique sequence of Tec family kinases consisting of the PH and TH regions-which form a novel fold (the PHTH domain) that is stabilised by cysteine mediated zinc binding (Vihinen et al., 1994; Hyvonen and Saraste, 1997), and the proline rich region (PRR) which has been proposed to act as an intramolecular SH3 domain ligand (Andreotti et al., 1997).

1.3.1 Bruton's Tyrosine Kinase (Btk) and B Cell Receptor (BCR) signalling

The vast body of research that has contributed to the characterisation of Btk was initially instigated by genetic linkage studies that positioned the Btk gene at the X-Linked agammaglobulinemia (XLA) locus (Vetrie et al., 1993). XLA is an X-linked immunoglobulin deficiency disorder that manifests itself as recurrent bacterial infections in young male patients (reviewed in Satterthwaite et al., 1998). This condition affects mainly cells of the B cell lineage, and is diagnosed by a lack of germinal cells, a severe deficit in mature B and plasma cells, and low IgM and IgG3 levels (Conley et al., 1994). Although mutations of the Btk gene in Xid mice result in a less severe phenotype, the same population of cells as those affected in XLA patients are developmentally abnormal in these mice (Rawlings et al., 1993). Xid B cells are functionally immature, they have a reduced half life *in vivo* (Oka et al., 1996) and show

increased spontaneous apoptosis *in vitro* (Woodland et al., 1996). At the molecular level, many signal transduction pathways are found to be defective in Xid B cells, including those downstream of BCR cross-linking (Wicker et al., 1986) and those involved in IL-5 (Hitoshi et al., 1993; Koike et al., 1995; Corcoran and Metcalf,1999), CD-38 (Kikuchi et al., 1995) and RP105 (Corcoran and Metcalf, 1995) signalling.

Btk is reportedly involved in the signal transduction pathways activated by a number of receptors (reviewed in Rawlings, 1999), including the B cell receptor (Khan et al., 1997), the B cell co-stimulatory receptor CD38 (Santos-Argumedo et al., 1995), the IL-5 receptor (Kikuchi et al., 1995) and the high affinity IgE receptor in mast cells (Kawakami et al., 1994). It is also required for G protein coupled receptor (Tsukada et al., 1994; Jiang et al., 1998) and collagen receptor (glycoprotein VI associated Fc receptor γ chain) signalling (Quek et al., 1998) as well as for alphaIIb/beta 3 integrin associated thrombin receptor-mediated signal transduction in platelets (Laffargue et al., 1999).

1.3.1.1 Tyrosine phosphorylation events at the BCR

Of these signalling systems, the B cell receptor (BCR) system has been characterised in most detail and will be reviewed below. The BCR, together with the T cell receptor (TCR), the receptor for the Fc portion of immunoglobulin-γ (IgG) (FcγR) and the receptor for Fc portion of immunoglobulin-ε (IgE) (FcεR), are grouped in the antigen receptor superfamily and share common structural features as well as signalling targets (Figure 1.4). These are multichain receptors that form heteromeric structures, with distinct extracellular ligand-binding subunits and intracellular cytoplasmic signal transduction compartments. The ligand binding subunit of the BCR is derived from membrane immunoglobulin (mIg), while signalling is mediated by immunoreceptor tyrosine-based activation motifs (ITAM) present in the cytoplasmic region of the Igα and the Igβ signalling subunits (Wienands et al., 2000). ITAM motifs consist of 6 conserved amino acids (D/E-X_7-D/E-X_2-Y-X_2-L/I-X_7-Y-X_2-L/I) (Cambier

Figure 1.4 Antigen receptor superfamily

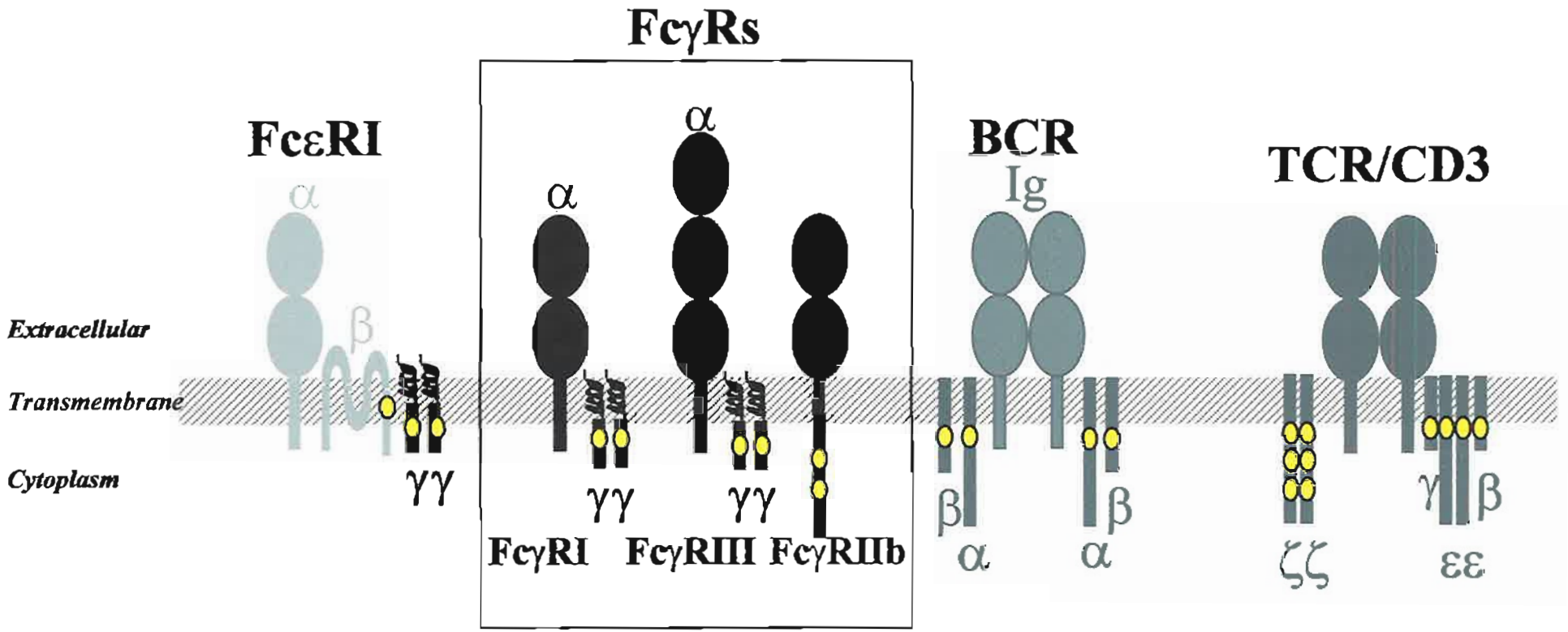
Diagrammatic representation of the structural components of the antigen receptor superfamily.

FcεRI : Receptor for Fc portion of IgE

FcγR : Receptor for Fc portion of IgG

BCR : B Cell Receptor

TCR : T Cell Receptor


FcγRs
FcεRI
BCR
Ig
TCR/CD3
Extracellular
Transmembrane
Cytoplasm
α
β
γγ
FcγRI
FcγRIII
FcγRIIb
ζζ
εε
: Immunoglobulin domains
: ITAM motif

et al., 1995) which act as phospho-acceptor sites for the pTyr/SH2-mediated recruitment of intracellular signalling components. Gene targeting studies indicate that the Igα and the Igβ signalling subunits differ in their signalling capacities. Igβ deficient mice are unable to assemble the remaining components of the BCR and exhibit a complete block in B cell development (Gong and Nussenzweig, 1996), while Igα-/- mice can assemble an incomplete BCR that is nevertheless able to support very inefficient progression through the pre-B cell stage (Torres et al., 1996).

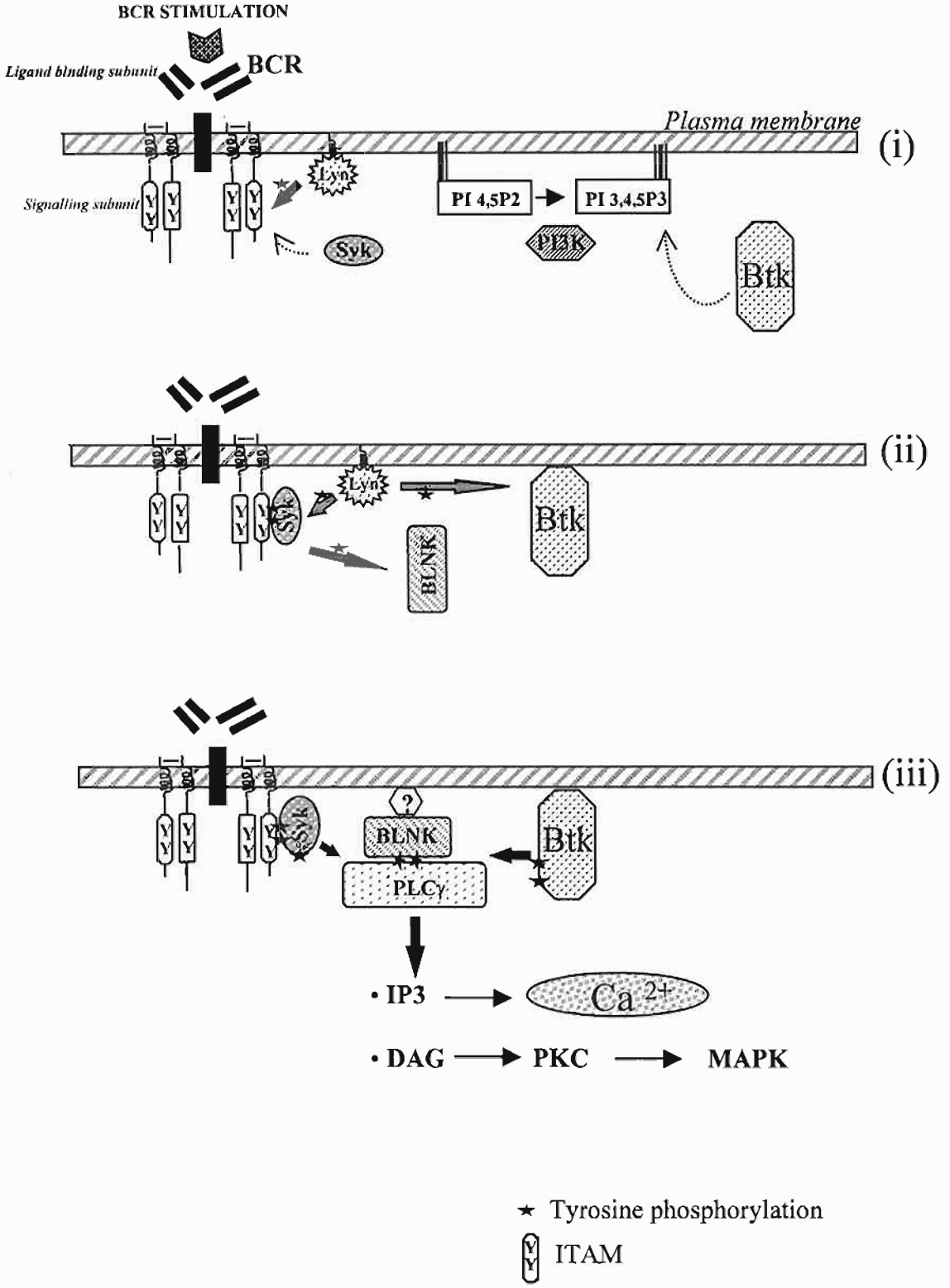
The sequential events that take place following BCR engagement are relatively well established. Igα and Igβ, the signalling components of the B cell receptor, form a heterodimer that associates with the ligand binding subunit (mIg) to form a functional receptor. As represented in Figure 1.5, upon stimulation, cross-linking of the BCR results in the sequential recruitment and activation of a number of signal transduction molecules, first Src family kinases, most probably Lyn and/or Fyn, and subsequently intracellular PTKs such as Syk and Btk (Saouaf et al., 1994). Lyn and Fyn but not Src are also able to interact with the resting BCR (Pleiman et al., 1994). Under these circumstances, receptor cross-linking alone is sufficient to induce auto/trans-phosphorylation and activation of the Lyn and Fyn kinase domains through induced proximity of the kinase domains (Cooper and Howell, 1993). Activation of Src family kinases at the BCR is also determined by the balance between the activities of the CD45 phosphatase (Yanagi et al., 1996) and the tyrosine kinase Csk (Hata et al., 1994). This balance determines the phosphorylation state of the C-terminal regulatory Y^{527} residue, which in turn dictates the level of Src family kinase activation and influences the strength of BCR signalling. The local increase in Src family kinase activity at the BCR complex is then responsible for ITAM phosphorylation, and the subsequent recruitment and activation of Syk at the activated complex (Kurosaki et al., 1994). An additional mode of activation for Syk is possible given that low levels of Syk have also been detected in

Figure 1.5 Membrane proximal events during B cell receptor signal transduction

(i) BCR engagement activates Lyn, leading to the phosphorylation of ITAM tyrosine residues. Tyrosine phosphorylated residues recruit Syk to the activated receptor and activation of PI3-K results in the phosphorylation of $PI(4,5)P_2$ to yield $PI(3,4,5)P_3$. This phospholipid mediates PH-dependent recruitment of Btk to the plasma membrane.

(ii) Activated Lyn phosphorylates and activates Syk and Btk and activated Syk phosphorylates the adapter protein BLNK.

(iii) Phospho-BLNK nucleates a multiprotein complex that brings Syk and Tec in proximity to their substrate PLCγ , and this results in the generation of IP_3 and DAG, an increase in intracellular calcium and the activation of PKC.


BCR STIMULATION
Ligand binding subunit
BCR
Plasma membrane
(i)
Signalling subunit
Lyn
Syk
PI 4,5P2
PI 3,4,5P3
PI3K
Btk
(ii)
Lyn
Syk
BLNK
Btk
(iii)
Syk
?
BLNK
PLCγ
Btk
• IP3
Ca 2+
• DAG
PKC
MAPK
★ Tyrosine phosphorylation
ITAM

association with the BCR complex prior to receptor activation. Aggregation of the receptor alone might be able to induce the auto/trans-phosphorylation and activation of a limited amount of Syk, independently of Src family kinase activity (Hutchcroft et al., 1992). In brief, it is currently believed that engagement of the BCR results in the activation of three families of PTKs. The first two, the Src family and Syk, are both critical for BCR activation as reflected by the total absence of tyrosine phosphorylation observed in Lyn-/-Syk-/- DT40 B cells despite BCR crosslinking (Takata and Kurosaki, 1996). The third PTK to be activated following B cell receptor crosslinking is the Btk tyrosine kinase. At the molecular level, Btk activation results from the phosphorylation of a highly conserved tyrosine residue located in the activation loop of the Btk kinase domain, Y^{551}, by a member of the Src family of tyrosine kinases, (Saouaf et al., 1994; Mahajan et al., 1995). Btk phosphorylation is initially dependent on Src kinase activity, but Syk is necessary for sustained phosphorylation (Kurosaki and Kurosaki,1997)

1.3.1.2 Phospholipid metabolism and BCR signalling

Changes in intracellular calcium levels follow receptor activation. These are initiated by the phosphorylation and activation of PLCγ (Padeh et al., 1991), an enzyme responsible for converting the plasma membrane phospholipid $PI(4,5)P_2$ into the secondary messenger IP_3. The initial activation of PLCγ is mediated by Syk (Takata and Kurosaki, 1996) and sustained PLCγ phosphorylation is dependent on phosphorylation by Btk (Fluckiger et al., 1998; Takata and Kurosaki, 1996). IP_3 molecules generated by PLCγ then trigger the release of calcium from endoplasmic reticulum (ER) stores, the majority of which are sensitive to low levels of IP_3. A distinct subset of ER calcium stores which are sensitive to higher levels of IP_3 release also trigger extracellular calcium influx through store-operated calcium channels (SOC) (Parekh and Penner 1997). It is the release of these stores of calcium that requires the phosphorylation of PLCγ by Btk (Fluckiger et al., 1998). The two levels of calcium signalling

control indicate that the amplitude and duration of calcium signalling is important in regulating the intensity of BCR signalling and is expected to determine the transcriptional program initiated following BCR engagement (Healy et al., 1997).

1.3.1.3 Adaptor protein and BCR signalling

A critical role for an emerging class of immune cell-restricted adaptor proteins in BCR signalling has recently begun to emerge. Adaptor proteins lack enzymatic and transcriptional activity. They encode multiple motifs and domains that nucleate the formation of multiprotein complexes and act as molecular scaffolds that co-localise enzymes and their appropriate substrates. The adaptor protein BLNK/Slp-65, plays a central role in PLCγ activation (Kurosaki and Tsukada, 2000). Mice deficient for BLNK exhibit a block in B cell development at the pro-B to pre-B cell transition stage, that mimics the defect seen in Xid mice (Pappu et al., 1999 and Xu et al., 2000). At the molecular level, the phosphorylation of BLNK by Syk facilitates the formation of a multiprotein complex, nucleated by BLNK, which includes Syk, Btk, and PLCγ (Hashimoto et al., 1999) and brings PLCγ in proximity to Syk and Btk thus facilitating PLCγ phosphorylation and activation.

1.3.1.4 Regulation of Btk kinase activity

Btk kinase activity is determined by a number of posttranslational modifications, and intra- and inter-molecular interactions that include :

(i) The phosphorylation of Y^{551} (Afar et al., 1996 and Rawlings et al., 1996). Phosphorylation of this tyrosine residue changes the conformation of the activation loop in the kinase domain and increases intrinsic Btk kinase activity.

(ii) The phosphorylation of Y^{223} which is located near the poly-proline binding pocket of the SH3 domain (Park et al., 1996). Addition of the phosphate residue sterically inhibits SH3-mediated interaction(s) and stabilises the active form of Btk through

yet to be characterised mechanisms. These could involve either the direct association of the SH3 domain with inhibitory proteins or intramolecular association with proline rich sequence(s) of Btk (Andreotti et al., 1997 and Patel et al., 1997). Using phospho-peptide specific antibodies raised against the two distinct phosphotyrosine residues, phosphorylation of Y^{223} was confirmed to temporally follow that of Y^{551} (Nisitani et al., 1999).

(iii) The interaction between the PHTH domain of Btk and $PI(3,4,5)P_3$ molecules (Salim et al., 1996) that accumulate at the plasma membrane following BCR receptor activation (reviewed in Marshall et al., 2000). This interaction ensures that Src family kinase-mediated phosphorylation and activation of Btk can only occur upon the translocation of Btk to the BCR signalling complex at the plasma membrane. The PHTH-$PI(3,4,5)P_3$ interaction positively affects membrane localisation and phosphorylation of Btk (Varnai et al., 1999, Nore et al., 2000; Laffargue et al., 1999; Li et al., 1997a and b). Structural studies of the PHTH region have demonstrated that XLA mutations in the PH region are generally clustered around the predicted lipid binding site, in the positively charged end of the domain (Hyvonen and Saraste, 1997). In addition, the E41K mutation which positively affects the affinity of the PH domain for membrane phospholipids generates an activated form of Btk that is constitutively targeted to the membrane (Nore et al., 2000).

The successful propagation of the BCR signal thus incorporates a combination of ITAM and PI3-K mediated signals that provide several levels of regulation to prevent spontaneous activation of Btk. Recently, basic residues in the first 10 amino acids of the PH domain of Btk were also shown to interact with actin thus positioning Btk as a possible link between calcium signalling events and actin cytoskeleton remodelling (Yao et al., 1999).

1.3.2 Tyrosine kinase Expressed in hepatocellular Carcinoma cells (Tec)

Tec is the founding member of the Tec/Btk family of protein tyrosine kinases. It exists as a single copy gene on mouse chromosome 5, and is closely linked to the Kit gene (Mano et al., 1993). *Tec* mRNA is expressed mainly in the liver (Mano et al., 1990) and is detected faintly in the heart, ovary and kidney (Mano et al., 1990) as well as in the spleen, small intestine, lungs, testis and brain (Siyanova et al., 1994). Expression of *Tec* is temporally and spatially regulated during mouse development (Kluppel et al. 1997). It is detectable first at day 7 of embryogenesis in the blood islands and maternal decidua, and in the placenta at day 9. At later somite stages, strong punctate expression of Tec is visible in the liver and AGM region, both of which are sites of embryonic hematopoiesis. Later, Tec expression broadens to include embryonic skin, blood vessels in the central nervous system, heart, kidneys, lungs and intestine (Kluppel et al., 1999). In the adult, like other members of this family, Tec is expressed in hematopoietic cells, although with a lower degree of lineage specificity. Its expression overlaps with that of Btk in B cells (Kitanaka et al., 1998) and platelets (Hamazaki et al., 1998), Itk and Txk in T cells (Mano et al., 1993; Yang et al. 1999). It is mostly abundant in megakayocytes (Dorsch et al., 1999), myeloid and monocytic cells (Mano et al., 1993; Yang et al., 1999; Yang and Olive, 1999).

Based on its homology to Src, Tec was initially reported to consist of 3 modular domains with significant homology to SH3, SH2 and Kinase domains (Mano et al., 1990). In total 5 transcripts have been reported for Tec in the literature and these will be described in detail in Chapter 3. The first transcript, *Tec* I (Mano et al., 1993) has a short N-terminus with no significant homology to any other known modules and no characteristic membrane targeting motifs (Mano et al., 1990) while all other transcripts *TecIIA*, *TecIIB*, *TecIII* and *TecIV* encode proteins with a PHTH N terminal domain (Mano et al., 1993). Other major differences between the Tec isoforms included differences in the SH3 and Kinase regions (Mano et al.,

1993). Of these, *TecIII* and *TecIV* encode peptides that differ by 22 amino acids at the C-terminus of the SH3 domain, with TecIII being the truncated isoform. How this deletion affects TecIII kinase activity has yet to be characterised. A constitutively active form of Tec (TecΔSH3) can however be generated by removing the majority of the Tec SH3 domain (Yamashita et al., 1996), suggesting a regulatory role for this domain similar to that of the Btk SH3 domain (Park et al., 1996). Removal of the 22 amino acids in TecIII and the 57 amino acids in TecΔSH3 are predicted to disrupt the three dimensional fold of the SH3 domain and effectively abolish SH3-dependent function(s)/interaction(s) involved in regulating kinase activity.

The regulatory mechanism(s) that control Tec activation are predicted to differ from that of Src family kinases since, like Btk, Tec lacks the C-terminal regulatory Tyr residue. Further insight into the regulation of Tec family kinase activity has come from multidimensional nuclear magnetic resonance (NMR) studies of the Tec family member Itk (Andreotti et al., 1997). These studies have revealed that the SH3 domain of Tec kinases is capable of interacting with poly-proline rich sequence(s) in the PRR region, of which Tec has two. Recently studies carried out in this laboratory have also identified an analogous interaction in Tec (Pursglove, 2001). This interaction is predicted to prevent the docking of other SH3 binding proteins thus effectively sequestering the SH3 from other signalling molecules.

In an effort to understand Tec-dependent signal transduction pathways, several Tec binding proteins have been identified. These include Kit, the receptor for SCF (Tang et al., 1994), Lyn (Mano et al., 1994), gp130 (Matsuda et al., 1995), Shc (Mano et al., 1995), Vav (Machide et al., 1995), PI3K, Jak1/Jak2 (Takahashi-Tezuka et al., 1997), and SOCS-1/JAB/SSI-1 (Ohya et al., 1997). Tec has also been implicated in a number of signal transduction pathways including those involving SCF (Tang et al., 1994), IL-3 (Mano et al.,

1995; Machide et al., 1995), Epo (Machide et al., 1995), IL-6 (Matsuda et al., 1995), integrin (Laffargue et al., 1997), thrombopoietin (Yamashita et al., 1997 and Dorsch et al., 1999), PI3-K (Takahashi-Tezuka et al., 1997), the BCR (Kitanaka et al., 1998), G protein coupled receptor and integrin mediated signalling (Hamazaki et al., 1998), heterotrimeric G proteins (Mao et al., 1998), GM-CSF (Yamashita et al., 1998), TCR/CD3 and CD28 (Yang et al., 1999; Yang and Olive,1999) as well as IL-2 and IL-4 (Yang et al., 1999). Tec is also reported to influence a range of effector molecules such as Serum Response Factor through Rho (Mao et al., 1998), and Stat5 (Isaksen et al., 1999). Despite these numerous reports the molecular details of Tec action remain poorly understood compared to that of other family members such as Btk and Itk.

1.3.3 Other Tec family members: Interleukin-2 (IL-2)-inducible T cell Kinase (Itk), T cell eXpressed Kinase (Txk) and Bone Marrow kinase in chromosome X (Bmx)

The Tec family kinase member Itk was originally isolated by a degenerate PCR screen which aimed to identify T cell specific tyrosine kinases (Siliciano et al., 1992). Its expression is apparently exclusive to the thymus, more specifically to T and Natural Killer cells (Siliciano et al., 1992; Gibson et al., 1993; Tanaka et al., 1993). Functional studies demonstrated Itk kinase activity to be important for T cell development. Mice deficient for Itk expression show reduced numbers of mature T cells, a reduced ratio of CD4 to CD8 cells, and compromised T cell proliferation in response to TCR stimulation (Liao and Littman, 1995). At the molecular level, stimulation of the TCR in these mice fails to fully activate PLCγ1 thus resulting in reduced levels of IP_3 which are sufficient to release calcium from intracellular stores, but inadequate to open plasma membrane calcium channels (Liu et al., 1998).

Overall, activation of the TCR parallels to some extent signalling from the BCR. Like the BCR, the signal transduction unit of the TCR belongs to the ITAM family. The

CD3γ, –δ, –ε subunits each contain one ITAM sequence and TCR ζ has three (Figure 1.4). Tyrosine phosphorylation of the ITAM by the Src family kinases (Lck and/or FynT) initiates a signalling cascade that culminates in calcium mobilisation, activation of the transcription factor NFAT and IL-2 production (Weiss and Littman, 1994). The extent of phosphorylation of the 6 ITAM tyrosine residues on the TCR ζ chain determines the strength of TCR activation (Neumeister et al., 1995), and is, at least in part, regulated by the tyrosine kinase Csk (Schmedt et al., 1998). Tyrosine phosphorylated ITAM motifs recruit the T cell specific homolog of Syk, Zap-70, to the signalling complex where Src family kinase-dependent phosphorylation of Zap-70 the Y^{315} and Y^{319} residues leads to increased ZAP-70 kinase activity (Di Bartolo et al., 1999). Two Zap-70-specific substrates include the adaptor proteins LAT and the BLNK homolog SLP-76 (reviewed Pivniouk and Geha, 2000). In activated T cells, the transmembrane protein LAT associates either directly or indirectly with many critical signalling molecules including Grb2, the p85 subunit of PI3-K, PLCγ, SLP-76, SOS and Cbl (Zhang et al., 1998b), while SLP-76 is rapidly phosphorylated following TCR engagement (Wardenburg et al., 1996). Deletion of SLP-76 result in a marked reduction in PLCγ1 tyrosine phosphorylation, calcium flux and ERK activation (Yablonski et al., 1998).

Functionally, the adaptor protein SLP-76 is able to couple Syk activation following TCR engagement to downstream effector pathways, including that of phospholipid metabolism, through its ability to nucleate multiprotein signalling complexes (Finco et al., 1998). These complexes are anchored at the plasma membrane by LAT and include Syk and Itk (Bunell et al., 2000). They ensure sustained PLCγ1 phosphorylation and calcium signalling (Finco et al.,1998; Yablonski et al., 1998). SLP-76 has also been implicated in cytoskeleton reorganisation through Rho-family dependent activation of Pak and WASP (Bubeck-Wardenburg et al., 1998) suggesting a role for this adaptor protein in coupling lipid metabolism and cytoskeleton rearrangement.

In addition to Tec and Itk, Txk is also expressed in cells of the T cell lineage (Sommers et al., 1995) The *Txk* locus is also closely linked to *Tec* on human chromosome 4 though the significance of such linkage has not been identified (Sommers et al., 1995). *Txk* mRNA is detectable from day 13.5 of gestation, the earliest stage at which foetal thymus can be identified and in the adult, expression is detected in T and mast cell lineages (Sommers et al., 1995). Structurally, Txk differs from other members of this family. It lacks a PH domain and possesses instead a palmitoylated cysteine ring motif that is proposed to facilitate membrane localisation (Debnath et al., 1999). An alternatively spliced isoform of *Txk* is also produced that is shorter and is targeted to the nucleus (Debnath et al., 1999). The functional significance of this isoform is yet to be determined. Functional characterisation of Txk is still limited. It is known not to be involved in the early signalling events associated with CD3ζ, CTLA4 or CD 28 signalling (Ellis et al., 1998). TCR stimulation induces the SLP-76-dependent association of Txk with PLCγ1 (Schneider et al., 2000) which mediates PLCγ1 phosphorylation, activation of ERKs and the synergistic up regulation of TCR driven IL-2 NFAT transcription (Schneider et al., 2000). Txk transgenic mice are hyper-responsive to TCR stimulation, exhibit enhanced PLCγ1 phosphorylation and increased calcium flux. Nevertheless, overexpression of Txk can only partially rescue the signalling defects of Itk deficient mice (Sommers et al., 1999). This suggests a complementary role for Txk and Itk kinase activity in regulating TCR signal transduction. The response to TCR stimulation although moderately impaired in Itk -/- mice, and even less in Txk -/- (Schaeffer et al., 1999), has major defects in numerous effector systems such as calcium mobilisation and IP_3 and IL-2 production in the absence of both kinases (Schaeffer et al., 1999).

Bmx is the most recent member of this family of protein tyrosine kinases. It was identified and cloned during a screen for novel tyrosine kinases expressed in human bone marrow cells and was shown to be localised to the X chromosome (Tamagnone et al., 1994).

Expression of Bmx is detectable in the spleen of late gestation embryos (Weil et al., 1997). In the adult it is expressed primarily in the bone marrow and at lower levels in the lung and heart (Weil et al., 1997). In contrast to other family members which show strong preference for hematopoietic cells, Bmx is preferentially expressed in epithelial and endothelial cells (Weil et al., 1997). Functionally Bmx induces the activation of Stat signalling pathways and is positively regulated by PI3-K(Saharinen et al., 1997).

In summary, Tec family members have proven to be critical for signal transduction from a wide range of receptors. Some details have begun to emerge mainly from the studies of antigen receptor signalling that have demonstrated the specific role that Btk, Itk and Txk play in PLCγ activation in B and T cell receptor signalling respectively. The relatively selective expression of these kinases in hematopoietic cells positions them as attractive therapeutic targets for the management of disorders of the immune system, and this in turn requires detailed understanding of their molecular mode of action.

1.4 Fcγ Receptor (FcγR)-mediated phagocytosis

Particle internalisation is a fundamental biological phenomenon that is responsible for the essential role of nutrient uptake in lower eukaryotes, and the more specialised function of pathogen clearance and destruction in higher eukaryotes. Based on the nature of the exogenous material being internalised. Particle internalisation is differentiated between the uptake of soluble material or pinocytosis and that of particulate matter. The uptake of particulate material is further subdivided based on the size of the exogenous particles. Material smaller than 0.5 μm in diameter is internalised through clathrin-dependent endocytosis, while larger material is taken up through phagocytosis (reviewed in Aderem and Underhill, 1999). Three types of phagocytic receptors have been recognised in higher eukaryotes. The first includes the mannose receptor (MR), DEC 205 and scavenger receptors (Stahl and Ezekowitz,

1998) which are responsible for the recognition of conserved motifs on pathogens including mannans in the yeast cell wall, formylated peptides in bacteria and lipopolysaccharides and lipoteichoic acids on the surface of Gram negative and Gram positive bacteria. The two remaining receptor systems recognise humoral components (Ig and C3bi) and include Fcγ receptors which are specific for immunoglobulins (Indik et al., 1995), and complement receptors which specifically recognise the C3bi fragment (Carroll, 1998). Given the conserved nature of the BCR, TCR and FcγR signalling systems, and the importance of Tec family kinases for signal transduction in the first two receptor systems, the work described in this thesis focussed mainly on the investigation of a possible role for Tec in FcγR -dependent phagocytosis.

During Fcγ receptor-mediated phagocytosis, exogenous particles greater than 0.5μm in diameter are internalised by professional phagocytic cells such as macrophages and neutrophils. This process is initiated by the specific interaction between the extracellular region of Fcγ receptors and the Fc portion of IgG opsonins present on the surface of exogenous particles. This interaction triggers the clustering of Fcγ receptors, induces the reorganisation of the underlying actin cytoskeleton to form the phagocytic cup and provides the driving force for particle internalisation. The phagosome is the membrane-derived structure that is formed following the pinching of the plasma membrane to enclose IgG-opsonised exogenous particles. Actin is shed after the particle has been engulfed as the phagosome matures through a complex series of fusion events with components of the endocytic pathway and eventually forms a mature phago-lysosome structure (reviewed in Aderem and Underhill, 1999).

1.4.1 Fc receptor biology

As previously mentioned, Fc receptors are part of the antigen receptor superfamily. In contrast to the B and T cell receptors, Fc receptors recognise the Fc portion of immunoglobulins and not the antigenic moiety on the surface of exogenous particles. As a

result, these receptors form a link between the binding of immunoglobulins to antigenic material and the inflammatory and cellular response(s) that are stimulated by this interaction (Raghavan and Bjorkman, 1996).

Five types of Fc receptors exist based on their immunoglobulin isotype specificity: FcγRs recognise IgG, FcεRs bind IgE, FcαRs interact with IgA, FcμRs are specific for IgM and FcδRs for IgD. Each antibody-receptor interaction triggers a distinct cellular response. Fc receptors for IgA and IgM facilitate the transport of antibodies through the epithelia without affecting cellular activation, while FcγRs and FcεRs mediate ITAM-dependent cellular responses, the intensity of which is dependent on the affinity of the receptor for its ligand (reviewed in Daeron, 1997):

The Fcγ receptor system is a relatively complex receptor system which consists of three major receptors:

(i) FcγRI/CD64 is a high affinity, multi-chain receptor complex that is involved in early immune responses when IgG levels are typically low (Shen et al., 1987). As shown in Figure 1.4, this receptor consists of a ligand-binding α chain with two Ig-like extracellular domains and a homodimer of the intracellular γ signal transduction unit (reviewed in Raghavan and Bjorkman, 1996). FcγRI is generally expressed on the surface of neutrophils, monocytes, granulocytes and macrophages (reviewed in Hulett et al., 1994) and *in vitro* studies have associated this receptor type with antibody-dependent cell-mediated cytotoxicity (ADCC), endocytosis and phagocytosis (Shen et al., 1987; Davis et al., 1995).

(ii) FcγRIII/CD16 is a multichain low affinity receptor that is expressed as a complex consisting of a single α chain with two extracellular Ig-like domains, and disulphide linked homodimers of γ subunits or γ/ζ heterodimeric subunits (Figure 1.4) (reviewed in Raghavan and Bjorkman, 1996). This type of receptor is expressed on macrophages, neutrophils and mast cells and is the only Fc receptor found on natural killer cells (Hulett et al., 1994).

Functionally, FcγRIII mediates similar responses to FcγRI (van de Winkel et al., 1991). A single FcγRIII isoform exists in the mouse while two isoforms of the receptor have been identified in human: FcγRIIIA which encodes a protein with a transmembrane region and a 25 amino acid cytoplasmic domain, and FcγRIIIB which is anchored at the plasma membrane by a glycophosphatidyl inositol linkage (Ravetch and Perussia, 1989).

(iii) The low affinity FcγRII/CD32 is a single chain receptor (Figure 1.4) (reviewed in Raghavan and Bjorkman, 1996) that is expressed mainly in myeloid and lymphoid cells (Hulett et al, 1994). The affinity of the extracellular domain of FcγRIIs for IgG is so low that under physiological conditions these receptors are predicted to interact with IgG only following immunoglobulin aggregation on multivalent antigens. Several isoforms of this receptor are encoded by three different genes FcγRIIA, FcγRIIB and FcγRIIC. They have identical extracellular ligand binding and transmembrane regions but differ in their cytoplasmic tail. FcγRIIC and the human specific FcγRIIA have a functional ITAM while FcγRIIB isoforms possess a single Y-X-X-L sequence and are predicted to function as putative negative regulators of immune complex-triggered activation (Takai et al., 1996).

1.4.2 Actin cytoskeleton remodelling and phagocytosis

FcγR-mediated ingestion of IgG opsonised particles occurs through zippering of the receptors on the plasma membrane with IgG molecules on the particle. Cryo-Electron Microscopy studies of phagocytosis have also shown that during particle ingestion, veils of the plasma membrane rise above the cell surface and tightly surround the particle before drawing it into the body of the macrophage (Allen and Aderem, 1996). Morphological remodelling of the cytoskeletal structures that underlie the plasma membrane thus appear to be essential for FcγR-mediated phagocytosis. Several line of studies have confirmed that the actin cytoskeleton plays a critical role in phagocytosis. Immunofluorescence studies have demonstrated that during phagocytosis, F-actin is present diffusely in the cortical cytoplasm directly beneath the phagocytic cup, and in pseudopod extensions that surround the exogenous particle where it co-localises with other known cytoskeletal proteins such as talin (Greenberg et al., 1990), vinculin, paxilin and α-actinin (Allen and Aderem, 1996). A critical role for F-actin assembly was further demonstrated using the actin assembly inhibitory drug Cytochalasin D. Pre-incubation of macrophage cells with Cytochalasin D abolishes FcγR-mediated phagocytosis without altering particle binding and the extent of inhibition is dependent on the size of the target particle (Koval et al., 1998). Unlike actin filaments, microtubule filaments do not appear to be necessary for the initial stages of particle ingestion as the microtubule disrupting compound Nocodazole has no effect on the formation of phagosomal structures (Newman et al., 1991).

Several signalling molecules are widely reported to affect actin assembly and cytoskeleton remodelling, including members of the Ras-related family of Rho GTPases (small guanosine triphosphatases). Rho is important for the assembly of contractile actomyosin filaments and for the stabilisation of the focal plaques that form at sites of cell attachment

(Ridley and Hall, 1992 and 1994). Other members of this family, Rac and Cdc42, control actin polymerisation during the formation of lamellipodial and filopodial structures (Nobes and Hall, 1995).

The evidence describing a role for Rho in particle ingestment is still controversial. Hackam et al. (1997) first reported complete inhibition of phagocytosis in macrophage cells using C3 (an exotoxin derived from *Clostridium botolinum* which is a documented inhibitor of Rho (Aktories, 1997)). This toxin impairs particle binding and results in a defect in FcγR clustering. In contrast, Toxin B (an inhibitor of all members of the Rho family), C3 transferase and a dominant negative Rho mutant were shown to inhibit phagocytosis while not affecting the binding of opsonised targets (Caron and Hall, 1998). Although the differences between these two reports remain to be addressed further, both are suggestive of a role for this family of signalling proteins in phagocytosis. Further studies are required to clarify whether this role is limited to the process of particle ingestion or includes that of particle binding.

The involvement of other families of GTPases during phagocytosis has also been suggested. The ARF family of GTPases has been implicated mostly in membrane trafficking events (reviewed in Moss and Vaughan, 1995) though recently, it has been associated with FcγR-dependent phagocytic events. Overexpression of ARF6 mutants in RAW cells blocks the accumulation of F-actin beneath attached particles and prevents the formation of phagocytic cups suggesting the involvement of this GTP-ase in the delivery of phagocytic effector membrane components to the plasma membrane (Zhang et al., 1998a). Dynamin 2 is another GTPase also involved in membrane trafficking at the trans-golgi network. Dominant negative forms of this GTPase block particle internalisation at the membrane extension stage, resembling the block observed upon PI3-K inhibition (Gold et al., 1999). Further characterisation of the role of these GTPases is necessary to elucidate the molecular details of their action during phagocytosis.

1.4.3 Protein tyrosine kinases involved in phagocytosis

A requirement for tyrosine kinases in FcγR-mediated phagocytosis was first demonstrated using the tyrosine kinase inhibitors Genistein and Herbimycin A (Greenberg et al., 1993). The addition of these compounds causes a dose-dependent and reversible decrease in the phagocytic index of macrophages, and reduce both the number of macrophages actively ingesting exogenous particles, as well as that of particles being ingested by single macrophage cells. An increase in the tyrosine phosphorylation of numerous intracellular proteins during particle ingestion is detectable by immunoblotting during particle ingestion (Strzelecka et al., 1997b). Furthermore, immunohistochemistry experiments have also identified the localisation of a substantial proportion of tyrosine phosphoproteins in the F-actin rich sub membranous regions beneath phagocytic cups (Greenberg et al., 1993).

To date, the activation of three classes of intracellular protein tyrosine kinases has been identified following crosslinking of FcγRs in monocytes: (i) Src family kinases such as Fgr, Lyn or Hck (Pan et al., 1999), (ii) Syk kinase (Pan et al., 1999, Greenberg et al., 1994) and (iii) FAK (Pan et al., 1999). Physical association between the Src family kinase members Hck/Lyn and FcγR is even detectable prior to FcγRI and FcγRII activation (Ghazizadeh et al., 1995). Receptor activation then induces an increase in Src tyrosine phosphorylation and Src kinase activity (Ghazizadeh et al., 1995) and the absence of three members of the Src family of tyrosine kinases (Fgr,Hck,Lyn) in macrophage cells abolishes FcγR-dependent particle internalisation (Crowley et al., 1997).

Syk is a 72 kD intracellular protein tyrosine kinase characterised by two N-terminal tandem Src Homology 2 (SH2) domains, a unique domain and a C-terminal kinase domain (Yagi et al., 1994). Although Syk expression has been detected in most lineages of the hematopoietic compartment, it is present at lower levels in thymocytes, mature T cells, mast cells, monocyte/macrophage and neutrophils and is highest in B cells (Chan et al., 1994). Syk

is phosphorylated following the activation of the γ subunit of Fc receptors (Greenberg et al, 1994). Stimulation of the FcγRIIIA and FcγRI receptors induces Syk tyrosine phosphorylation (Darby et al., 1994; Durden and Liu, 1994) and Syk kinase activity peaks within 5 minutes of receptor stimulation (Darby et al., 1994). Low levels of Syk are also detectable in association with the γ subunit of the Fc receptor prior to receptor stimulation though it increases significantly following receptor stimulation (Durden and Liu, 1994).

Clustering of the Syk kinase domain at the plasma membrane in response to a phagocytic stimuli initiates the redistribution of F-actin (Greenberg et al., 1996). COS cells can be rendered phagocytic by the transfection of exogenous phagocytic receptors (Indik et al., 1995; Park et al., 1995). Using this system, the introduction of exogenous Syk, but not Zap70 (Indik et al., 1995; Park et al., 1995) was shown to be required for phagocytosis. In contrast, the presence of Src family kinases, in the absence of Syk, appears insufficient to trigger particle internalisation by phagocytic COS cells (Greenberg et al., 1996; Taylor et al., 1997). The combination of these observations thus suggests that the requirement for Src family kinases in phagocytosis is limited to their role in phosphorylating ITAM residues to provide a docking platform for Syk. A critical role for Syk in phagocytosis was further confirmed *in vitro* using antisense oligonucleotides which significantly reduced FcγR-mediated phagocytosis in cultured human monocytes (Matsuda et al., 1996). *In vivo*, gene targeting of Syk in mice results in lethality early in the embryonic development of Syk deficient mice (Turner et al., 1995). Foetal liver macrophage cells derived from these mice can however be isolated *in vitro* and are able to initiate the formation of actin rich phagocytic cups but fail to complete the phagocytic process (Crowley et al., 1997). In these cells, exogenous particles remain bound to the receptors for several days in culture without further completion of phagocytosis (Crowley et al., 1997; Kiefer et al., 1998). At the molecular level, these cells exhibit a marked decrease in the phosphorylation of intracellular proteins including that of the receptor itself, thus

confirming a critical role for Syk for the successful propagation of the phagocytic signal (Kiefer et al., 1998).

1.4.4 Adaptor proteins and phagocytosis: SLP-76, BLNK/SLP-65 and LAT

A novel group of adaptor molecules have recently emerged as essential components of the signal transduction pathways initiated by antigen receptor stimulation. These proteins generally possess a number of modular domains that are able to mediate protein-protein and protein-lipid interactions to facilitate the formation of multiprotein signalling complexes such as those involved in BCR and TCR mediated PLCγ activation (Yablonski et al., 1998; Pappu et al., 1999).

SLP-76 is physically associated with the γ signalling subunit both in monocytic cells (Chu et al., 1998) and in platelets (Gross et al., 1999b) where it is required for PLCγ2 activation following collagen stimulation (Clements et al., 1999, Gross et al., 1999a). More recently, SLP-76 tyrosine phosphorylation was also reported in response to FcγRI and FcγRII/III crosslinking in bone marrow derived macrophage cells (Bonilla et al., 2000). These observations suggest a possible link between the stimulation of the γ signalling subunit and SLP-76-dependent activation of PLCγ. The molecular details that control these interactions remain to be fully characterised.

The 36/38 kD adaptor LAT (linker for activation of T cells) is a specific substrate for Zap70 in response to TCR stimulation (Zhang et al., 1998b) whose main function is to nucleate a large multi-protein complex that includes PLCγ1 (Finco et al., 1998), Grf40, a novel Grb2 family member (Asada et al., 1999) and GrpL (Law et al., 1999), as well as Vav (Wu et al., 1996), Cbl (Thien et al., 1999), PI3-kinase (Gibbins et al., 1998) and SLP-76 (Yablonski et al., 1998). In human monocytic cells, the 36 kD isoform of LAT constitutively associates with FcγRI and IIa, and clustering of these receptors induces the specific phosphorylation of the

p36 LAT isoform and its association with PLCγ1, Grb2 and the p85 subunit of PI3-kinase (Tridandapani et al., 2000). It is predicted that adaptor proteins are likely to be critical to the signal transduction pathways that are initiated at the FcγR during particle internalisation though their identity is yet unclear.

1.4.5 Phospholipase C γ (PLCγ) and phagocytosis

Phagocytic receptors have been reported to induce a rise in cytosolic calcium concentration that is greatest around the forming phagosome (Odin et al., 1991). In macrophage cells, calcium release from intracellular stores is mediated by the secondary messenger IP_3 which is produced by PLCγ in response to FcγR crosslinking (Kiener et al., 1993). The involvement of several effectors of PLCγ has also been identified during phagocytosis. A number of them are present at the forming phagosome including several substrates of PKC including pleckstrin (Brumell et al., 1999) and MARCKS (Allen and Aderem, 1995). PKC phosphorylates the actin-crosslinking protein MARCKS (Myristoylated alanine-rich C kinase substrate) and is involved in actin cytoskeleton remodelling (Hartwig et al., 1992). It is activated during particle internalisation and is rapidly recruited to the submembranous fraction of the nascent phagosome with kinetics similar to that of F-actin (Allen and Aderem, 1996).

1.4.6 Phosphoinositide 3-kinase (PI3-kinase) and phagocytosis

The inositol phospholipid $PI(3,4,5)P_3$ is generated by PI3-K-mediated phosphorylation of $PI(4,5)P_2$ and acts at the plasma membrane to recruit PH-containing proteins in response to a wide range of external stimuli (Toker and Cantley, 1997). One such stimulus is the crosslinking of FcγRs which induces a rapid rise in PI3-K activity (Ninomiya et al., 1994). Chimaeric receptors consisting of the extracellular ligand binding region of Fc receptors and

the cytoplasmic p85 coding region have PI3-K activity and are able to induce the polymerisation of actin necessary for the formation of phagocytic cups (Lowry et al., 1998).

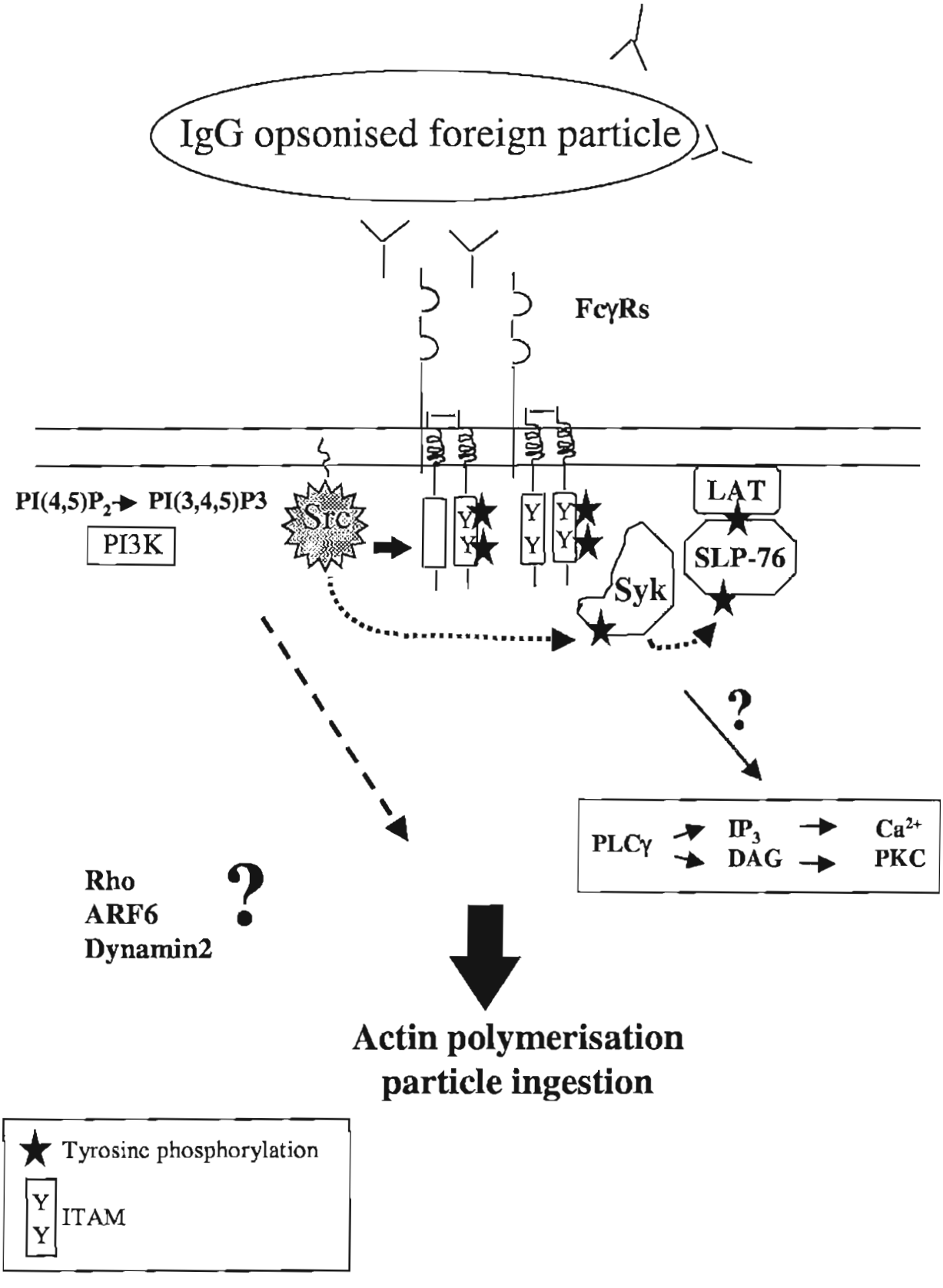
Wortmannin and LY294002, both of which inhibit PI3-K activity, prevent FcγR-mediated phagocytosis (Ninomiya et al., 1994, Cox et al., 1999; Araki et al., 1996). Detailed scanning electron microscopy studies of macrophage cells treated with either inhibitory compound show that this block in particle ingestion results from the failure of pseudopodia to successfully seal around the exogenous particle (Araki et al., 1996). This effect is most evident for phagocytic targets greater than 4.5 μM in diameter thus suggesting that PI 3-kinase inhibition limits the availability of plasma membrane components that are required for pseudopod extension (Cox et al., 1999).

1.4.7 Current model for FcγR-mediated signal transduction during phagocytosis

A current model describing the signal transduction pathways predicted to be involved in FcγR-dependent phagocytosis is diagrammatically represented in Figure 1.6. Briefly, IgG opsonised particles induce the clustering of FcγR on the surface of professional phagocytes. Receptor clustering activates pre-associated Src family kinases leading to the phosphorylation of ITAM motifs in the γ chain of the receptor. Phosphorylated ITAMs provide a platform for the recruitment of Syk kinase which in turn is responsible for the phosphorylation of the adaptor proteins. These adaptor molecules facilitate the formation of a multi-protein complex that recruits PLCγ to the receptor and permits phospholipid metabolism and the release of intracellular calcium. At the same time, receptor clustering also triggers the activation of PI3-K, the remodelling of the actin cytoskeleton and eventually phagosome closure. Other effectors of the phagocytic signalling pathway have also been identified that include Rho GTPases, PKC and its substrate, dynamin and ARF6 although the molecular details of how their action is coordinated have yet to be determined.

Figure 1.6 FcγR-mediated Phagocytosis

Exposure to opsonised exogenous particles results in the crosslinking of the FcγRs and stimulates Src family kinases to phosphorylate tyrosine residues of ITAMs. Syk is subsequently recruited to the receptor complex and is responsible for the phosphorylation of the adapter proteins such as SLP-76 and LAT. Syk activity is a prerequisite for the PLCγ-dependent increase in calcium levels. PI3-K activity is also required for the polymerisation of actin and the formation and closure of the phagosome.


IgG opsonised foreign particle
FcγRs
PI(4,5)P2 → PI(3,4,5)P3
PI3K
Src
Y
Y
Y
Y
Y
Y
Y
Y
LAT
SLP-76
Syk
?
PLCγ
IP3
DAG
Ca2+
PKC
Rho
ARF6
Dynamin2
?
Actin polymerisation
particle ingestion
Tyrosine phosphorylation
Y
Y
ITAM

1.5 Aims and approach

At least three members of the Tec family of protein tyrosine kinases (Btk, Itk and Txk) have recently emerged as critical elements of antigen receptor signalling in B and T cells respectively, while Tec has been reported mainly as a component of cytokine-mediated signal transduction pathways. The sequential events that initiate from the activation of B and T cell and Fcγ receptors have so far proved to be relatively well conserved. Given the role that the Tec family members Btk and Itk play in BCR and TCR signalling respectively, the work described in this thesis aimed to investigate a possible role for Tec in the signal transduction pathway that initiates at the FcγR in the myeloid lineage, especially macrophage cells.

Over the last decade, gene targeting has emerged as a powerful technology that enables the controlled modification of the genetic material of ES cells to generate mice with well defined mutations. This work was based mainly on the hypothesis that a novel role exist for Tec, that is distinct from its widely published involvement in cytokine signalling, by drawing analogy to the role of Btk and Itk in antigen receptor signal transduction. *In vitro* culture systems have also been developed for ES cells that can drive their differentiation into specific hematopoietic lineage(s). Together, these developments permit the characterisation of the molecular consequences of defined targeting events.

Three specific aims have driven the research described in this thesis. The first aim of this work was to characterise the relative abundance and biological characteristics of the multiple isoforms of the *Tec* transcript. In order to determine the biological relevance of each *Tec* isoform *in vivo*, the expression of each transcript was analysed in various mouse embryonic and adult tissues, and transcripts that were most abundant were identified. Molecular differences between the two most abundant isoforms of Tec were subsequently characterised *in vitro*, using green fluorescent protein (GFP) fusion proteins that were overexpressed in monkey kidney fibroblast COS-1 cells.

The second aim of this research was to establish a novel role for Tec in antigen receptor mediated signal transduction, specifically in the pathway initiated at the Fcγ receptor. These studies exploited a well established FcγR-dependent *in vitro* phagocytosis assay that uses the macrophage/monocytic cell line J774. This cell line supports antibody dependent particle internalisation and by-passes the need to isolate primary macrophage cells from mouse tissues. Commercially available antibodies were used to visualise changes in the subcellular localisation of Tec and other known effectors of FcγR-dependent phagocytosis and inhibitory drugs were used to dissect and position Tec in this signalling pathway.

Finally, gene targeting was used to generate genetically manipulated ES cells that can subsequently be reintroduced into recipient mouse blastocysts to generate mice carrying one or two copies of the modified locus. ES cells act as powerful tools that permit not only *in vivo* studies of Tec dysfunction in the mouse but also allow more rapid studies of lineage-specific defects *in vitro* using well established differentiation assays. *Tec* -/+ and *Tec* -/- ES cells were therefore used to investigate the consequence(s) of these mutation(s) on the developmental potential of ES cells to form macrophage cells *in vitro*.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Abbreviations

ATP	:	adenosine tri phosphate
DNA	:	deoxyribunucleic acid
Ig	:	immunoglobulin
l	:	litre
M	:	molar
µCi	:	microcurie
µg	:	microgram
mg	:	milligram
min	:	minutes
µl	:	microlitre
ml	:	millilitre
µM	:	micromolar
mM	:	millimolar
°C	:	degree centigrade
pfu	:	plaque forming unit
PI3-K	:	phosphoinositide 3 kinase
PLC	:	phospholipase C
PTK	:	protein tyrosine kinase
r.p.m.	:	rotation per minute
RNA	:	ribonucleic acid
sec	:	second
Tyr	:	tyrosine

2.2 Materials

The materials used in these studies and their main supplier are listed below.

2.2.1 Sheet Materials

3MM chromatography paper	Whatman Ltd
Biomax™ MR X-ray film	Kodak/Integrated Sciences
Colony/Plaque Screen™ nylon membrane	Du Pont
Hybond ™-C (nitrocellulose membrane)	Amersham
Hybond™-N$^+$ (nylon transfer membrane)	Amersham
Nitrocellulose	Schleicher and Schuell
X-ray film	Konica, AGFA

2.2.2 Chemicals and Reagents

All chemicals and reagents were of analytical grade, or of the highest purity available. The major source of chemicals are listed below.

Acrylamide (Acryl/bis 29:1)	Astral Scientific
Agarose type I	Sigma Chemical Co.
Amethopterin	Sigma Chemical Co.
Ampicillin	Sigma Chemical Co.
APS	BDH Chemicals Ltd.
ATP	Sigma Chemical Co.
Bacto-agar	Difco Labs Ltd.
Bacto-tryptone	Difco Labs Ltd.
BCIG (X-gal)	Progen

BCIP	Sigma Chemical Co.
β–mercaptoethanol	Sigma Chemical Co.
Bromophenol Blue	Sigma Chemical Co.
BSA	Sigma Chemical Co.
CsCl	Cabot
DMEM	Gibco BRL
DTE	Sigma Chemical Co.
DTT	Diagnostic Chemicals
EDTA	Sigma Chemical Co.
EGTA	Sigma Chemical Co.
Ethidium Bromide (Et Br)	Sigma Chemical Co.
Fetal bovine Serum	Commonwealth Serum Lab.
G418	Gibco BRL
Gelatin	Sigma Chemical Co.
Gentamycin	Schering Corporation
Human gamma globulin	Sigma Chemical Co
Hypoxanthine	Sigma Chemical Co.
IPTG	Progen
L-Glutamine	Sigma Chemical Co.
NBT	Sigma Chemical Co.
PEG	BDH Chemicals Ltd.
Phenol	BDH Chemicals Ltd.
Salmon sperm DNA	Sigma Chemical Co.
SDS	Sigma Chemical Co.
Sephadex G50	Pharmacia

Sepharose CL-6B	Pharmacia
Sequagel-6, Sequagel-XR	National Diagnostics
Temed	BIORAD
Thymidine	Sigma Chemical Co.
Triton X-100	Sigma Chemical Co
Tween 20 (polyoxyethylene- sorbitan monolaurate)	Sigma Chemical Co
Zymosan A	Sigma Chemical Co

2.2.3 Water Sterilisation

The Milli-Q^R Reagent Water System was used to filter the water used in the experiments described in this thesis. Tissue culture solutions were filtered *in-vacuo* using Corning disposable bottle top filters (0.22 μM pore size).

2.2.4 Bacterial Strains

The *Eschericia coli* DH5α strain was used for the propagation of all recombinant plasmids. The *E. coli* LE392 strain was used as host bacteria for genomic library screening. Stock cultures of these strains (and transformants) were stored as glycerol stocks at -80°C.

DH5α : *sup*E44, Δ*lac*U169 (phi80, *lac*Z,ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyrr*A96, *thi*1, *rel*A1.

LE392 : *supE,supF,hsdR* , permissive for vectors carrying amber mutation, will modify but not restrict DNA

2.2.5 Plasmids

pBluescript II SK$^+$	Stratagene
pBluescript II KS$^+$	Stratagene
pGEM-T	Promega
pBluescriptSK+TecIIB	Dr. J. Ihle (St Judes Hospital, Tennesse)
pGEX4T2-SH3	Sharon Pursglove (Laboratory of Dr Booker)
pEGFP-C2	Dr. S. McColl (Department of Molecular Biosciences, University of Adelaide).
pEGFP-C2-TecIII	Anita Merkel (Laboratory of Dr Booker)
pEGFP-C2-TecIV	Anita Merkel (Laboratory of Dr Booker)

2.2.6 Genomic library

The E14TG2a murine genomic library was supplied by Dr Martin Kennedy. The library consists of partially digested 18-23 kb DNA fragments of E14TG2a genomic DNA cloned into the *Bam HI* site of λ2001 (Karn et al., 1984).

2.2.7 Bacterial Growth Media

Growth media were prepared using double distilled water and were sterilised by autoclaving. When required, Ampicillin was added after the media solution had cooled to 50°C.

Luria broth (LB) : 1% (w/v) bacto-tryptone (Difco), 0.5 % (w/v) yeast extract (Difco), 1% (w/v) NaCl. The pH was adjusted to 7.0 with NaOH.

LMM broth : 1% (w/v) bacto-tryptone, 0.4 % (w/v) NaCl, 0.5% (w/v) maltose, 0.2% (w/v) $MgSO_4$.

NZCYM : 1% (w/v) NZ amine, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.1% (w/v) casamino acids, 0.2% (w/v) $MgSO_4$

L plates were prepared by supplementing the LB media with 1.5% (w/v) bacto-agar. LMM agarose plates were prepared by dissolving agarose (0.7% (w/v) in LMM broth.

2.2.8 Antibodies and staining reagents

Polyclonal goat anti-Tec (sc-1109) and polyclonal rabbit anti-Syk (sc-1077) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-phosphotyrosine antibody was a kind gift of Dr. S. McColl (Department of Molecular Biosciences, University of Adelaide). FITC-conjugated rabbit anti-goat IgG, FITC-conjugated goat anti-rabbit IgG, TRITC-conjugated rabbit anti-goat IgG, horseradish peroxidase-conjugated rabbit anti-goat IgG, TRITC-conjugated phalloidin, were obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase-conjugated sheep anti-mouse IgG was purchased from Silenius (AMRAD). All antibodies were diluted according to the manufacturer's recommendation.

2.2.9 Tissue Culture Material

1X PBS : 8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4, 0.115% (w/v) Na_2HPO_4. The solution was sterilised by autoclaving and stored at room temperature

β-mercaptoethanol/PBS : 1000X stock β-mercaptoethanol solution was generated by diluting 100µl β-mercaptoethanol in 14.1 ml PBS. β-mercaptoethanol/ PBS was stored at 4°C in a light-proof container for up to two weeks.

LIF conditioned medium : LIF conditioned medium was prepared following the method described by Smith (1991) with the exception that COS-1 cells were used and transfections were carried out by electroporation.

PBS/EGTA : 25 mM EGTA was prepared in PBS was stored at room temperature.

1000X stock FIAU (200μM) : A 100 mM solution was first made up in PBS. To dissolve FIAU, NaOH (2M) was added and the final volume was finally adjusted to 10 ml with PBS. This solution was filtered sterilised and stored at -20°C. When needed it was subsequently diluted 1:500 in PBS to yield the 1000X stock solution.

G418 stock solution : 50 mg/ml G418 in PBS (stored at 4°C)

100X HAT:

100X HT :1.375% (w/v) Hypoxanthine, 0.38% (w/v) Thymidine in MQ H_2O. The mixture was gently heated to 70°C to dissolve.

100X A : 17.6% (w/v) aminopterin was dissolved in MQ H_2O with 5mM NaOH.

1X Freezing Solution : 10%DMSO, 90% FCS stored at 4°C in a light proof container.

2.2.10 Tissue Culture Media

Incomplete ES cell medium : 1.348% (w/v) DMEM (high glucose, containing L-glutamine and no sodium bicarbonate nor sodium pyruvate), 0.37% (w/v) $NaHCO_3$, 0.125% (v/v) gentamycin. HCl was used to adjust the pH of the medium to 7.4. The medium was subsequently filter sterilised and stored at 4°C.

Complete ES cell medium : 85% (v/v) incomplete ES cell medium, 15% (v/v) FCS, 0.1%(v/v) LIF conditioned medium, L-glutamine1 mM, 0.1 mM β-mercaptoethanol

2.2.11 Polymerase Chain Reaction Primers

PHLexA5	GGC GAA TTC GAT GAA TTT CAA CAC
THLexA3	CCC GGT ACC TTA ACT ACT CTC AAA
TECSH3.5	TTT AGG ATC CGA CGT TGT AG
TECSH2.3	CTT GAA TTC TTA ACT GAC CGG GTA
5'EXON16	TTT GAG AAG ACC AAT TAC
3'EXON17	TCC AAA GAG GAT TAC TAC
5PRR	ATG GAT CCA GTA TAA GAA AGA CC
5'exon8	GGT ACT GCA GAA ATA CCA ACA GAA GCA AAG CAG
3-exon8	TGG TGT CGA CTG GAG GAG GCA CAC TCA
5-exon8	GAG CTC GAG GTA GAT GGG GAC TGG AAT AAG ATA
3'exon8	ATG GGT ACC GTT CTG AGG AGC TGT TCT GC
IA-K16-1	GTTAAAAGGCGGCCGCACCTGGTGTA

2.2.12 Cells Lines

The following cell lines have been cultured throughout the course of this study:

1) E14TG2a had originally been obtained from Dr. Austin Smith, CGR Edinburgh, UK,
2) W9.5 cells had originally been obtained from Dr. Richard Harvey, WEHI, Melbourne,
3) STO^R had originally been obtained from Dr. Richard Harvey, WEHI, Melbourne,
4) FDC-P1 cells had originally been obtained from Dr. Richard D'Andrea, IMVS, Adelaide,
5) J774 murine monocytic/macrophage cells had originally been obtained from Dr G.Mayerhofer (Department of Molecular Biosciences, University of Adelaide)

2.3 Molecular Biology Techniques

2.3.1 Small scale plasmid preparation

Approximately 5 µg of plasmid DNA was typically extracted from 2 ml bacterial cultures. Small scale cultures typically used LB supplemented with ampicillin (100 µg/ml) inoculated with a single transformant bacterial colony and incubated overnight at 37°C in an orbital shaker. Bacterial cells were collected by centrifugation at maximum speed for 2 min in 1.5 ml eppendorf tubes. Pellets were subsequently resuspended in approximately 100µl of supernatant and lysed by vortexing with 300µl of Megadeath solution (0.1M NaOH, 0.5 % (w/v) SDS, 10mM Tris pH 8.0, 1mM EDTA pH 8.0). Cellular proteins and chromosomal DNA were precipitated with 150µl of 5M NaAc pH5.2 and collected by 3 min centrifugation at maximum speed. To precipitate plasmid DNA, supernatant fractions were mixed with 1 ml ice cold 95 % ethanol and centrifuged for 5 minutes at maximum speed. Plasmid DNA pellets were finally washed with 70% ethanol, vacuum dried and resuspended in 20 µl of TE.

2.3.2 Large scale preparation of plasmid DNA

Large scale DNA preparations were obtained from 500 ml cultures of LB supplemented with ampicillin (100µg/ml) inoculated with a single bacterial colony and grown at 37°C with shaking overnight. Bacterial cultures were centrifuged at 5,000 r.p.m. for 10 min. Pellets were resuspended in 16 ml Solution 1 (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) and incubated at room temperature for 5 min. Cells were lysed in 40 ml Solution 2 (0.2 M NaOH, 1% (w/v) SDS) for 10 min on ice, and neutralised on ice with 30 ml Solution 3 (5M Potassium acetate, 11.5% (v/v) glacial acetic acid) for 10 min. Cellular proteins and

chromosomal DNA were removed by centrifugation at 13,000 r.p.m. for 15 min. To precipitate plasmid DNA, equal volumes of isopropanol were added and pellets were subsequently collected by 15 min centrifugation at 12,000 r.p.m. (4°C), resuspended in 400µl 1x TE and transferred to fresh 1.5 ml eppendorf tubes. Bacterial RNA was removed with 50 µl RNase A (10mg/ml) at 37°C for 30 min, and DNA preparations were further cleaned by phenol/chloroform and chloroform extractions. Plasmid DNA was finally precipitated with 2.5 volumes 95% ethanol at maximum speed. Pellets were then washed with 70% ethanol and resuspended in 1x TE.

2.3.3 CsCl purification of plasmid DNA

Plasmid DNA required for transfections into mammalian cells are required to be of high quality and purity. Typically, approximately 300 µg of plasmid DNA prepared following the protocol described in section 2.3.2 was resuspended in 720 µl 1x TE with 1.26 g CsCl. Following the addition of 120 µl of the DNA intercalating agent EtBr (10mg/ml), the DNA-containing solution was underlaid into a TL100 tube (Beckman) containing 1.4 ml CsCl (65% (w/v)). The tubes were sealed and centrifuged at 100,,000 r.p.m. for 3 hrs at 20°C. Plasmid DNA was drawn with a 1 ml syringe, transferred to 1.5 ml eppendorf tubes and extracted 3x with equal volumes of H_2O saturated butan-1ol, and precipitated with two volumes of 95% EtOH. DNA pellets were collected by centrifugation at maximum speed at room temperature for 10 min. Pellets were finally rinsed with 70% ethanol and the DNA was resuspended in 1x TE.

2.3.4 Colony cracking

Rapid screening for recombinant plasmids was carried out using the colony cracking method. Small scale bacterial cultures were grown overnight in LB supplemented with ampicillin

(100µg/ml), at 37°C in a rolling drum. Crude DNA preparations were obtained by mixing 25µl of overnight culture with an equal volume of 2X cracking solution (0.2M NaOH, 0.5% SDS, 20% sucrose). The mixture was vortexed, incubated at 70°C and centrifuged for 5 min at maximum speed. Plasmid DNA was visualised by TAE agarose electrophoresis (2.3.5) and recombinant plasmids were identified by differences in mobility compared to parental plasmids.

2.3.5 Agarose gel electrophoresis

Horizontal "minigels" were prepared by pouring 10-12 ml of molten 1-2% agarose gel solution prepared in 1X TAE (40 mM tris-acetate, 20mM sodium acetate, 1 mM Na_2EDTA) onto a 5.0 cm x 7.5 cm glass microscope slide, or 30 ml of gel solution poured into a 6.5 cm x 10 cm perspex minigel cast. Agarose gels were submerged in 1X TAE and samples were prepared with GLB*. These were electrophoresed at 70 V. The migration of DNA was generally visualised by staining with EtBr (5 µg/ml) followed with exposure to short wavelength UV light. To avoid uv-induced damage to DNA, preparative gels were visualised using long wave UV light and the desired DNA fragments were isolated using a fresh #10 scalpel blade and processed as described in 2.3.11.

* 10X GLB: 50% (v/v) glycerol, 0.1% (w/v) SDS, 0.05% (w/v) bromophenol blue, xylene cyanol 0.05% (w/v)

2.3.6 Restriction endonuclease digestion of DNA

Plasmid and phage DNA were digested with 2-4 units of restriction enzyme per 1μg of DNA, in a 20μl reaction for analytical purposes, or in a 40μl reaction when DNA fragments were to be isolated for cloning. Reactions were incubated at 37°C for 1-6 hours in 1X SD buffer*. The extent of digestion was assayed by agarose gel electrophoresis (2.3.5).

*10x SD buffer: 330 mM tris-Hac pH 7.8, 625 mM Kac, 100 mM MgAc, 40 mM spermidine, 5 mM DTE.

2.3.7 Endfill reactions

When 5′ overhangs generated by restriction enzyme digestion had to be removed prior to the use of the DNA fragments in blunt end ligations, up to 5μg of DNA with 5′ overhangs were endfilled at 37°C, for 1 hour in 1X Endfill Buffer*, with 5mM dNTP and 10mM DTT using 10 units DNA Polymerase Klenow Fragment.

*10x Endfill Buffer: 10mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 100mM NaCl.

2.3.8 Endchew reaction

3′ overhangs were removed from fragments required for blunt end ligation reactions by exonuclease digestion. Typically, up to 5μg of DNA with 3′ overhangs were treated with 5 units of T4 DNA Polymerase and 5 mM dNTPs in 1X T4 DNA Polymerase Buffer* at 37°C for 30 minutes.

*10x T4 DNA Polymerase Buffer: 700 mM Tris-HCl (pH 7.4), 100 mM $MgCl_2$, 50 mM DTT.

2.3.9 Dephosphorylation of 5′ protruding termini

To prevent recircularisation of the parental vector, the 5′ protruding termini of vector DNA fragments to be used in non-directional cloning reactions were dephosphorylated with 1 unit of Calf Intestinal Phosphatase in 1X CIP buffer (Boehringer Mannheim Biochemicals), at 37°C for 30 minutes.

2.3.10 Dephosphorylation of blunt and 3′ recessive termini

Dephosphorylation of blunt and 3′ recessive termini of linearised vector fragments used in non-directional cloning was carried out in 1x CIP buffer (Boehringer Mannheim, Biochemicals), with 2 units of enzyme. The reaction was initiated at 37°C for 15 minutes and was followed by a 30 min incubation at 55°C.

2.3.11 Purification of linear DNA fragments from agarose gels

To purify DNA fragments used as probes or in ligation reactions, restriction fragments were separated by agarose gel as described in 2.3.5. Linear fragments were extracted from the gel slices using the BRESACLEAN kit (GeneWorks) following the protocol suggested by the manufacturer.

2.3.12 Ligation reactions

Ligation reactions typically contained a 3:1 molar ratio of insert to vector DNA fragments and were carried out in 1X Ligation Buffer* with 1 mM ATP and 1 unit of T4 DNA ligase in a 20µl volume. Reactions were allowed to proceed at room temperature for 2 hours or overnight at 16°C when blunt-end ligations were performed.

*10X Ligation Buffer: 500 mM Tris-HCl pH7.6, 100 mM $MgCl_2$, 100mM DTT, (500 mg/ml BSA (optional)).

2.3.13 Preparation of calcium competent bacterial cells

Calcium competent cells used to transform ligation reactions were prepared as follows. A small scale bacterial culture was set up in LB from a single *E. coli* DH5α colony and was allowed to grow overnight at 37°C (shaking). The following morning, a 1% subculture was made in 500 ml of LB and the culture was expanded until an OD_{600} reading of 0.4 - 0.5 was reached. The culture was chilled on ice for 10 minutes and bacterial cells were collected by centrifugation at 5,000 r.p.m. (4°C) for 15 minutes. Pellets were then resuspended in 100 ml ice cold $CaCl_2$ solution*, centrifuged at 5,000 r.p.m. (4°C) for 15 minutes and concentrated to 20 ml ice cold $CaCl_2$ solution. Aliquots of approximately 200 µl were transferred into eppendorf tubes, snap frozen in a dry ice/ ethanol bath and stored at -80°C for up to six months.

*$CaCl_2$ solution: 0.06 M $CaCl_2$, 15% v/v glycerol.

2.3.14 Transformation of competent cells

Approximately 10 ng of circular plasmid or one half volume of ligation reaction was incubated with 100 µl of competent cells on ice for 20 minutes. Cells were heat shocked for 2 minutes at 42°C before returning to ice for 10 minutes and plated onto LB supplemented with ampicillin (100 µg/ml) plates. Bacterial plates were incubated overnight at 37°C. When blue/white colour selection was necessary, plates were spread with 40 µl of 100 mM IPTG and 40 µl of 20 mg/mL X-gal 30 minutes before transformant cells were plated.

2.2.15 Genomic library screening

i) Preparation of bacterial host

A 20 ml LMM broth culture was inoculated from a single *E. coli* LE392 colony. The bacterial culture was expanded overnight at 37°C in an orbital shaker. Bacterial cells were collected by centrifugation at 3000 r.p.m. for 5 minutes and were resuspended in 10ml of prechilled 10mM $MgSO_4$. Cells were stored on ice prior to infection with bacteriophage.

ii) Plating of genomic library

Aliquots of 0.7% (w/v) LMM agarose were warmed to 42°C prior to infection. The infection step was typically carried out using 200µL of. *E. coli* LE392 prepared in 2.2.15i and 7 x 10^4 λ2001 bacteriophage, in a 37°C water bath for 15 min. Following this, 10 ml of 0.7% (w/v) LMM agarose was added to the infected cells and the mixture was quickly overlayed onto prewarmed 15cm LB agar plates. Plates were left to set at room temperature for 10 minutes and were shifted to 37°C for 6-8 hours or until the plaques had grown to a sufficient size. They were subsequently stored at 4°C for at least 1 hour prior to plaque lifts. First round plates were generally plated at 40,000-50,000 pfu/plate whereas comparatively low plaque density (80-100 pfu/plate) was used in subsequent rounds.

iii) Plaque lifts

Nylon membrane circles were overlayed onto infected plates for 1 minute (first lift), 3 minutes (second lift) and 7 minutes (optional 3rd lift). Membranes were then air dried on 3MM Whatman paper for 10 minutes and autoclaved at 101°C for 2 minutes. Phage DNA was immobilised by uv crosslinking after membranes had been moistened with 6X SSC. Membranes were incubated with 10-25 ml prewarmed prehybridisation solution* at 42°C for at least 2 hours in plastic bacterial petri dishes.

*Prehybridisation Solution: 5.85% (w/v) NaCl, 40% (w/v) deionised formamide, 17% (w/v) SDS, 11% w/v PEG, 50 mM Tris-HCl pH 7.4, 5x Denhardt's reagent, 0.05 mg/ml salmon sperm DNA.

iv) Radiolabelling of linear DNA probes

Single stranded DNA probes were labelled with 30-50 μCi α-^{32}P-dATP using the Gigaprime DNA labelling kit as suggested by the supplier. The volume of the labelling reaction was increased to 100 μl using MQ water and unincorporated label was removed by centrifugation on a 0.5 ml Sepharose CL-6B column by centrifugation at 1800 r.p.m. for 3 minutes. Prior to its addition to the prehybridisation solution, the probe was denatured at 100°C for 2 minutes and snap cooled on ice

v) Hybridisation of labelled probes and detection of positive clones

Hybridisation was carried out overnight in petri dishes at 42°C. Genomic library screen lifts were washed twice in 1L of 2X SSC/0.1% (w/v) SDS at 42°C for 20 minutes. An optional higher stringency wash using 1L of 2X SSC/0.1% (w/v) SDS at 65°C for 15 minutes was performed when the radioactivity was greater than 5-10 counts per second. Genomic library screen lifts were exposed to X-ray film at -80°C for 72 hours.

vi) Isolation of single genomic clones

Positive plaques identified on duplicate filters were isolated by stabbing with a sterile pasteur pipette. Phage were eluted by submerging the agar stab in 1 ml PSB for 4 hours at 4°C. The eluate was transferred to a fresh tube and E.coli LE392 cells were lysed by the addition of a drop of chloroform. Phage eluates were stored at 4°C. The titre of the eluted phage was subsequently determined.

2.3.16 Large scale preparation of phage DNA (λprep)

i) Preparation and infection of cells

Small scale bacterial cultures were inoculated from a single *E. coli* LE392 colony and grown in LMM broth overnight at 37°C. One millilitre of bacterial host (approximately 2 x 10^9 bacterial cells) was subsequently incubated with 1 x 10^7 phage at 37°C, with intermittent shaking for 30min. One half litre of NZCYM was then inoculated with 1ml of bacteria/phage mixture, adjusted to 10mM $MgSO_4$ and expanded at 37°C overnight in an orbital shaker.

ii) Lysing cells and extraction of bacteriophage DNA

To lyse bacterial cells and release phage particles, 10ml of chloroform was added to the culture for 10 minutes on a tilt table. Bacterial RNA and genomic DNA were digested with RNaseA (1μg/ml) and DNase I (1μg/ml) respectively for 30 minutes on a tilt table. Solid NaCl was dissolved into the mixture to yield a 1M solution and cooled on ice for 1 hour. Cell debris were collected by centrifugation at 5,000 r.p.m. (4°C) for 20 minutes. Solid PEG (10% w/v) was slowly dissolved into the supernatant and the mixture was kept on ice for 1 hour. Bacteriophage particles were pelleted by centrifugation (3,000 g, 4°C) for 10 minutes and resuspended in PSB. PEG and any remaining bacterial debris were extracted with an equal volume of chloroform. Clean bacteriophage particles were collected from the supernatant fraction following centrifugation (5,000 r.p.m.; 4°C) for 15 minutes. These were lysed with 20 mM EDTA pH 8, 0.5 % w/v SDS, 50 μg/mL Proteinase K for 30 minutes at 37°C. Proteinase K was heat inactivated at 65°C for 30 minutes. Bacteriophage DNA was then subjected to phenol/chloroform extraction (centrifugation was carried out at 3,000 r.p.m. for 5 minutes using a benchtop centrifuge). Phage DNA was precipitated with 2 volumes of ice cold ethanol at 3,000 r.p.m for 10 minutes in a benchtop centrifuge. DNA pellets were washed with 70% ethanol, dried under vacuum and resuspended in 1X TE.

2.3.17 Southern blot analysis of plasmid and phage DNA

Following restriction enzyme digestion (2.3.6), DNA fragments were separated by 1% (w/v) TAE agarose gel electrophoresis (2.3.5) and visualised by EtBr staining under longwave length UV light. To facilitate the transfer of large molecular weight fragments, agarose gels were submerged in 0.25M HCl for 20 minutes to depurinate DNA fragments. Agarose gels were then rinsed in water and blotted onto a nylon membrane (Hybond™-N^+) in 0.4M NaOH for 90 minutes under vacuum according to the manufacturer's suggestion. Membranes were then rinsed in 5X SSC and DNA fragments were further immobilised onto the membrane by UV crosslinking or by air drying the membrane at room temperature overnight. Probes were labelled as in 2.3.15 (iv). For hybridisation, probes were denatured for 5 minutes and snapcooled on ice prior hybridisation in cylinders. Filters were hybridised at 42°C in a Hybaid midi oven in 10 ml/filter of hybridisation solution for a minimum of 2 hours or overnight. Filters were finally washed successively for 20 minutes in 2x SSC/0.1% (v/v) SDS, 0.5x SSC/0.1%(v/v) SDS and 0.1x SSC/0.1%(v/v)SDS at 65°C. Radioactivity was detected as described in section 2.3.18

2.3.18 Autoradiograph scanning and phosphorimager analysis

X-ray films were developed using a CURIX 60 X-Ray developer. Storage Phosphor Screens exposed to membranes were processed using a Molecular Dynamics PhosphorImager running the ImageQuant software.

2.3.19 Double stranded sequencing of plasmid DNA

(i) ***Manual sequencing using the dideoxy-mediated chain termination method***

Up to 2µg of plasmid DNA were denatured at 37°C for 15 minutes (166.6mM NaOH/166.6µM EDTA) and snap cooled on ice. Denatured DNA was purified on a

Sepharose CL-6B spin column by centrifugation (2,000g) for 5 minutes and annealed to sequencing primers (5ng/µl). Dideoxy sequencing reactions were carried out using the Pharmacia or Bresatec T7 polymerase sequencing kits according to the manufacturer's instructions, using α^{32}P-dATP or α^{33}P-dATP and optional $MnCl_2$, when sequencing was required in close proximity to the primer. Stop solution* was added to terminate the reactions and samples were denatured at 75°C. Fragments were resolved by electrophoresis on a denaturing 6% w/v polyacrylamide at 50mA. Gels were blotted onto 3MM Whatman paper, vacuum dried at 70°C for 1 hour and exposed to X-ray film at room temperature to detect radioactivity.

*Stop solution: 97.5 % (v/v) formamide, 10 mM EDTA pH 7.5, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol).

(ii) *Cycle Sequencing*

Automated DNA sequencing was carried out using the protocol of the supplier (Applied Biosystems). Briefly, approximately 500ng DNA template was added to the reaction mix (ABI Prism Dye Terminator Sequencing Ready Reaction Mix) with the appropriate sequencing primer (5ng/µl). Sequencing was carried out at 96°C (30sec), 50 °C (15sec), 60°C (4min) for 25 cycles. Sequencing products were subsequently precipitated by the addition of 0.1X volume 3M sodium acetate pH4.6 and 2.5X volume 95% ethanol at -80°C, followed by centrifugation at 14,000 r.p.m.

2.3.20 Small scale preparation of high molecular weight DNA from mammalian cells

High molecular weight genomic DNA was prepared from ES cells grown in 24-well plates. To each well, 300µl of lysis solution* was added and plates were incubated overnight at 37°C.

DNA was precipitated with an equal volume of isopropanol at room temperature on a rotating table until a precipitate was formed. DNA precipitates were subsequently picked with the tip of a 200µl pipetteman and transferred to 500µl of 70% (v/v) ethanol. DNA pellets were collected by centrifugation (14,000 r.p.m., 10 minutes) and air dried at room temperature. Pellets were finally resuspended in 100µl of 1X TE (37°C, overnight).

*Lysis Solution: 100 mM HCl pH 8.5, 50 mM EDTA, 0.2% (v/v) SDS, 200 mM NaCl, 0.1mg/ml Proteinase K

2.3.21 Southern Blot Analysis of Mammalian Genomic DNA

Approximately 20 µg of high molecular weight genomic DNA were digested at 37°C using 10-20 units of restriction enzyme in a total volume of 100µl. The digested DNA was precipitated with 95% ethanol, washed with 70% (v/v) ethanol and air dried. The pellet was finally resuspended in 10µl 1X TE. GLB was added and the mixture was run on a 0.7% (w/v) TAE agarose at 30V overnight. Gels were photographed the following morning under longwave UV light. They were depurinated (0.25 M HCl for 20 minutes), rinsed in MQ water and transferred to Hybond-N^+ membranes (Amersham) in 0.4M NaOH under vacuum. Membranes were then rinsed in 6X SSC and DNA was immobilised by UV crosslinking (120 J, Stratagene Inc., UV Stratalinker 1800). Radioactive probes were labelled using the Megaprime labelling Kit (Amersham) with 50µCi α-^{32}P-dATP following the protocol of the supplier. Hybridisation reactions were carried out in a Hybaid midi hybridisation oven at 65°C. Filters were prehybridised for a minimum of 2 hours at 65°C in 10 ml/filter of hybridisation solution. Probes were denatured for 5 minutes and snapcooled on ice prior hybridisation in cylinders. Filters were hybridised at 65°C overnight. Filters were then washed successively for 20

minutes in 2X SSC/0.1% (v/v) SDS, 0.5X SSC/0.1%(v/v) SDS and 0.1X SSC/0.1%(v/v)SDS successively at 65°C. Radioactivity was detected as described in section 2.3.18

*Prehybridisation Solution: 250mM $NaPO_4$ (pH. 7.2), 7%(v/v) SDS, 10%(w/v) PEG 6000, 1mM EDTA, 0.1mg/ml sonicated salmon sperm DNA.

2.3.22 Polymerase Chain Reaction

(i) Standard PCR

Standard PCR reactions used lincarised plasmid DNA templates and were carried out following the manufacturer's recommendation. Briefly, 20ng plasmid DNA were generally used as template. Fragments were amplified in a total volume of 25µl using 5ng/µl of each primer, 800µM dNTPs, 1 unit Taq polymerase, in 1X Taq reaction buffer (67mM Tris-HCl, pH8.8, 16.6mM $(NH4)_2SO_4$, 0.2mg/ml gelatin and 0.45% Triton X-100). $MgCl_2$ concentrations were optimised for each primer combination and typically ranged between 1mM and 2.5mM $MgCl_2$.

(ii) Long Distance PCR

The LD-PCR reactions were performed in a volume of 50µl containing 1.5mM Mg^{2+}, 60mM Tris-SO_4 (pH 9.1, at 25°C), 18mM $(NH_4)_2SO_4$, 200µM of each dNTP, 200nM each primer, 200ng of genomic DNA and 1µl Elongase Enzyme Mix (Life Technologies). Initial denaturation at 94°C (30 sec) was followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 60°C (30 sec) and extension at 68°C (4 min).

2.3.23 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Mouse adult and embryonic RNA samples were subjected to reverse transcription PCR (RT-PCR) analysis using the SUPERSCRIPT ONE STEP RT-PCR System (Life Technologies). Reactions were carried out in a total volume of 50µl containing 1X Reaction Mix (0.4mM each dNTP, 2.4mM $MgSO_4$), 40 Units of RNaseOut Ribonuclease Inhibitor (Life Technologies), 200nM each primer, 1µl RT/*Taq* Mix and 2 µg of RNA. The PCR profile consisted of a reverse transcription step at 50°C (40 min), denaturation at 94°C (2 min), 1 cycle of denaturation at 94°C (30 sec), annealing at 46°C (1 min) and extension at 70°C (1 min) and 40 cycles of denaturation at 94°C, annealing at 50°C and extension at 70°C. Twenty percent of each reaction was analysed by TAE agarose gel electrophoresis.

2.3.24 RNase Protection Assays

RNase protection assays were performed essentially as described in Merkel et al. (1999) Transcription reactions were generally labelled with 240 µCi α-[^{32}P] rUTP (mGAP and rGAP probes were labelled using 40 µCi of α-[^{32}P]-rUTP) using the appropriate RNA Polymerase enzyme. One millilitre Sephadex G50 spin columns (Pharmacia) were used to remove excess radiolabel by centrifugation at 3,000 r.p.m. (5 minutes). 150,000 counts/minute of Tec ribonucleotide probes and 3,700 counts/minute of antisense mGAP and rGAP ribonucleotide probes were added to 10 µg of RNA. After hybridisation, samples were digested, with a mixture of RNase T1 and RNase A at 42°C for 30 min. Samples were subsequently phenol/chloroform extracted and ethanol precipitated. The pellet was then resuspended in formamide loading buffer, loaded onto a Sequagel-6 (National Diagnostics) polyacrylamide gels and visualised by Phosphorimager analysis (Molecular Dynamics).

2.3.25 Preparation of cell lysates for immunoblotting

Cells were washed in PBS and collected in cold TEN buffer (40mM Tris, 1mM EDTA, 150mM NaCl). Following centrifugation cells were resuspended in cold lysis buffer (0.5% v/v Triton X-100, 60mM KCl, 5mM $MgCl_2$ 2mM EDTA, 2mM EGTA, 30mM HEPES pH 7.4, supplemented with 1mM $NaVO_4$, 50mM NaF, 2mM phenylmethylsulfonyl fluoride, 10μg/ml aprotinin) and lysed at 4°C for 20 minutes. Cytoskeleton fractions were separated from cellular fractions by centrifugation at 10,000 r.p.m. (4°C) for 15 minutes.

2.3.26 Immunoblotting analysis of cell extracts

Pellet fractions were resuspended in SDS-loading buffer (50mM Tris HCl pH6.8, 1% v/v 2 mercaptoethanol, 2% w/v SDS, 0.1% w/v Bromophenol Blue, 10% v/v glycerol). Cellular fractions were also prepared using SDS-loading buffer. All samples were denatured for 5 minutes at 100°C, separated by SDS-PAGE on 8% Tris-tricine acrylamide gels and electro-transferred to a nitrocellulose membrane (Hybond-C, Amersham). Nitrocellulose membranes were blocked overnight at 4°C in 5% w/v skim milk powder, 0.1% v/v Tween 20 in PBS. Immunodetection was carried out in 5% skim milk/PBS for 1 hour at room temperature and specific binding of antibody was visualised by ECL detection (Amersham).

2.3.27 Immunoblotting analysis of immunoprecipitates

Cells were washed in PBS, collected in cold TEN buffer and lysed by three rounds of sonication (30 sec each) in cold sonication buffer (0.02% v/v TritonX-100, 1mM EGTA, 50mM Tris pH 8, 1 mM $NaVO_4$, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin as described in Izaguirre et al., 1999). The resulting supernatant fractions were pre-cleared with Protein A-Sepharose (Sigma Chemical Co. St. Louis, MO) for a minimum of 4

hours at 4°C. Immunoprecipitations were carried out at 4°C for 4 hours with the appropriate antibodies. Immune complexes were collected on Protein A-Sepharose and were washed three times with cold lysis buffer. Prior to loading, pre-heated SDS-loading buffer was added to the immunoprecipitates which were further denatured for 5 minutes at 100°C. Proteins were visualised after SDS-PAGE electrophoresis and electro-transfer to nitrocellulose as described in 2.3.26.

2.3.28 Fluorescence microscopy

Cells were washed in PBS, fixed for 2 minutes in pre-chilled methanol and rehydrated in PBS for 15 minutes. To facilitate antibody penetration, cells were permeablised with PBS/0.1%(v/v) Triton-X 100 (Sigma Chemical Co. St. Louis, MO) for 10 minutes. Permeabilised and fixed cells were exposed to antibodies for 1 hour at room temperature and washed with PBS/0.1% Tween 20. Cells were mounted in PBS/50% glycerol and viewed using a Zeiss Axioplan microscope equipped for 3 channel fluorescence (Zeiss filter sets II, IX and XV) and photographed with a Zeiss MC 100 camera attachment using 35 mm ektachrome 160T film.

2.4 Tissue culture techniques

2.4.1 Preparation of STO^R feeder layers

STO^R cells were expanded in complete ES cell medium until they reached 70-80% confluency. Cells were washed twice with PBS and dislodged using 2ml of pre-warmed trypsin for less than 3 min. Trypsin activity was neutralised with 10 ml of complete ES cell medium and cells were collected by centrifugation at 1,200 r.p.m. for 5 minutes. Single cell suspensions of 10^7 cells/ml were subsequently γ irradiated (30 Grays). Irradiated cells were resuspended in 1x freezing medium (10% (v/v) DMSO, 90% FCS) at 2.5 x 10^7 cells/ml and frozen in $2x10^7$ cell aliquots at - 80°C for short term storage or in liquid nitrogen for long term storage. When required, aliquots were thawed at 37°C and plated onto pre-gelatinised 100 mm plates with 10 ml complete ES cell medium.

2.4.2 Routine maintenance of ES cells

(i) Culture conditions

To facilitate ES cell adherence, polystyrene cell culture dishes were pre-gelatinised with PBS/0.1% gelatine for 20 minutes. Cells were grown in complete ES cell medium in 10% CO_2 at 37°C. E14TG2a ES cells are feeder layer independent and were therefore grown directly onto pre-gelatinised plates. W9.5 ES cells require a feeder layer and were grown onto mitotically inactivated STO^R fibroblast cells.

(ii) Routine passage of ES cells

ES cells were routinely passaged to prevent differentiation of ES cell colonies. This is important for optimal contribution of ES cells to the germline of chimaeric mice. Briefly, cells

were washed twice with PBS and dislodged, by pipetting up and down with 1 ml pre-warmed trypsin per 100 mm diameter plates, for approximately 3 minutes at room temperature. Trypsin activity was neutralised by gentle mixing using an equal volume of complete ES cell medium and cells were collected by centrifugation at 1,200 rpm. for 5 minutes. Cells were then resuspended in 10 ml of complete ES cell medium and the appropriate volume of cells was finally added to pre-gelatinised plates in the case of E14TG2a cells or to feeder-coated plates in the case of W9.5 cells.

2.4.3 Freezing of ES cells

ES cells were grown to sub-confluence and harvested by trypsinisation. Cells were counted, centrifuged for 5 minutes at 1,200 r.p.m., and resuspended in 1x freezing medium at 6.25 x 10^6 cells per ml. ES cells were stored in cryotubes ($5x10^6$ cells/aliquots) at -80°C for short term storage or in liquid nitrogen for long term storage.

2.4.4 Electroporation of ES cells

Targeting vector DNA was prepared by the CsCl banding technique (2.3.3) and 200µg of the appropriate DNA was linearised by restriction enzyme digestion. The reaction was subsequently extracted with phenol/chloroform and chloroform and resuspended in sterile 1X TE.

(i) Electroporation of W9.5 ES cells

Typically, six 100 mm plates of ES cells were required per electroporation. Plates were fed with complete ES cell medium 4 hours prior to procedure. Plates were washed first with PBS and a second time with PBS/EGTA. Cells were trypsinised with 1ml pre-warmed trypsin per 100mm plate for a maximum of 3 minutes at room temperature. An equal volume of complete

ES cell medium was added to neutralise trypsin activity and cell suspension from all plates were pooled. Cells were further centrifuged at 1,200 r.p.m. for 5 minutes and the pellet was resuspended in 20ml PBS. A cell count was performed using a haemocytometer, cells were pelleted by centrifugation and resuspended in PBS at a concentration of 2.8×10^7 cell per ml. In an electroporation cuvette (Bio-Rad, 4 mm disposable electroporation cuvette), 25µg of linearised vector was mixed with 700µl of pre-chilled cell suspension. Electroporation was carried out using the Bio-Rad Gene Pulser at 230mV, 500µF. The entire contents of the cuvette were then transferred to 9ml of complete ES cell medium and divided over ten 100mm plates with feeder cells. Cells were grown in complete ES cell medium for 24 hours at 10% CO_2 after which selection was applied for up to 10 days. Cells were re-fed daily for the first six days to eliminate dead cells and every 48-72 hours from thereon.

(ii) Electroporation of E14TG2a cells

Electroporation was carried out as described above with the exception that ES cells were expanded in six $175cm^2$ flasks for the experiment. In this experiment, cells were resuspended in PBS to a concentration of 1.43×10^8 cells per ml. A mixture of 150µg of linearised targeting vector and 700µl cell suspension was immediately electroporated using the Bio-Rad Gene Pulser at 800mV, 3 µF.

2.4.5 Picking and expansion of resistant colonies

After 10 days of selection, resistant ES cell colonies were visible to the naked eye and could be picked. The plate was washed twice with PBS and PBS was added to cover the plate. Each colony was picked using a 20µl pipetteman (Gilson) and transferred to a 96 well plate containing 100µl of trypsin. After 8 colonies were picked, 100µl complete ES cell medium was added to each well to neutralise trypsin activity and clumps of cells were broken up by

moving the cell suspension up and down using a multichannel pipettor. In the case of W9.5 ES cells, when 24 colonies had been picked and treated as described, half of the content of each well were transferred to a pre-gelatinised 24 well plate and the other half to a pre-gelatinised 24 well plate with feeder layers. When E14TG2a cells were used the content of each well was divided over two pre-gelatinised 24 well plates. In both cases, the first plate was used for characterisation of the clones by Southern blot analysis (2.3.21) while the second were grown to sub-confluency and frozen for storage (2.4.3).

2.4.6 Freezing and thawing of ES cells in 24 well plates.

To avoid the time consuming task of freezing individual clones in freezing vials, cells could also be frozen directly into 24 well plates. Wells were washed twice with PBS, 100µl trypsin was added to each well and the plate was incubated at 37°C for 3 minutes. An equal volume of 2X freezing medium was then added and cell clumps were disrupted by pipetting using a multichannel pipettor. Plates were then sealed and immediately transferred to a -80°C freezer. To thaw cells, plates were placed in a 37°C incubator for 10 minutes and 1 ml of pre-warmed ES cell medium was added. The content of the well was then transferred to a fresh pre-gelatinised 10 mm well with or without feeder cells depending of the type of ES cells used.

2.4.7 Karyotyping ES cell lines

ES cells were grown in 60 mm plates at high density (10^6 cells per plate) and fresh complete ES cell medium was added to the plates 3 hours prior to the procedure. Cells were arrested in mitosis by the addition of colcemid (0.1µg/ml) (Sigma Chemical Co.) after which they were harvested by trypsinisation and the cell suspension was centrifuged at 1,000 r.p.m. for 10 minutes at 4°C. The pellet was then gently resuspended in 9 ml 0.075M KCl and remained at room temperature for 30 minutes. One millilitre of fresh pre-chilled methanol/acetic acid

solution (3:1) was added and cells were centrifuged for 10 minutes at 1,000 r.p.m. (4°C). The pellet was resuspended in 6ml of cold fixative, and washed twice in 6ml of methanol/acetic acid fixative solution. Following the final wash, 0.5ml supernatant was kept and a single cell suspension was generated. Microscope slides were soaked in methanol for 5 min and 2-3 drops of cell suspension were added to the microscope slides. The slides were finally stained for 5 minutes with Giemsa and spreads were viewed under phase contrast optics on the Zeiss Axioplan Universal microscope.

2.4.8 *In vitro* differentiation of ES cells

ES cells were differentiated to form macrophages by the methods of Wiles and Keller (1991) following modifications described in Lake et al (2000). Embryoid bodies were incubated in 1.25ml MC medium (0.9% methyl cellulose in IMDM, 15%FCS, 50mg/ml ascorbic acid, 0.45mM a-monothioglycerol) supplemented with 400 U/ml recombinant mouse IL-3 (a kind gift of Dr T. Gonda, Institute of Medical and Veterinary Science, Adelaide) and 10 ng/ml recombinant human M-CSF (R&D systems). Colonies were scored for the presence or absence of macrophages by microscopic examination after 8 days of culture. The ability of the resulting cells to phagocytose IgG-opsonised Zymosan A was tested in the same manner as for J774 cells.

2.4.9 Histochemical staining of cells for detection of β-Galactosidase

Two days after transfection, cells were fixed and stained to determine transfection efficiencies. Briefly, the medium was removed from the transfected cells and cells were washed twice with PBS. Cells were then fixed with 5ml of cold fixing solution and incubated at 4°C for 5 minutes. The fixing solution* was then removed and cells were washed three times in PBS. Five millilitres of staining solution** was then added to the plates and these were incubated

overnight at 30°C for the detection of β-galactosidase activity. Cells were then viewed under phase contrast and bright field optics using a Nikon Diaphot microscope.

*Fixing Solution: 2% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde)

**Staining Solution: 0.5mM potassium ferrocyanide, 0.5mM potassium ferricyanide, 1mM MgCl2, 40mg/ml BCIG

2.4.10 Fugene™ transfection of GFP construct in COS-1 Cells

Transient transfection required for immunohistochemical analysis of Tec isoforms were carried out in 6 well plates. Briefly, 10^5 cells were plated on glass coverslips 24 hours prior to transfection. Cells were transfected according to the manufacturer's instruction. Typically, 3µl transfection reagent were diluted in 97µl serum-free DMEM and incubated at room temperature for 5 min. The diluted transfection reagent was subsequently added to 1µg of purified plasmid and complex formation was carried out at room temperature for 15 min. This mixture was finally added to freshly changed medium on adherent cell layer. GFP and GFP fusion proteins were visualised after 24 hours in PBS using a Nikon inverted (ECLIPSE TE 300) microscope. To visualise nuclei, cells were fixed in pre-chilled methanol for 2 min, rehydrated in PBS for 15 minutes and permeabilised in PBS/0.1% TritonX-100 for 10 min. Nuclei were stained with Hoechst (1µg/ml) for 30 sec and thoroughly washed in PBS/0.1% Tween-20.

2.5 Phagocytosis-related protocols

2.5.1 Routine maintenance of J774 cells

The murine monocytic/macrophage cell line J774 was maintained at 37°C in DMEM (GIBCO, Life Technologies) supplemented with 10%(v/v) fetal bovine serum, under 10% CO_2. For immunofluorescence, cells were plated on glass coverslips in 6-well plates at 10^5 cells/well.

2.5.2 Preparation of phagocytic target

Opsonisation of Zymosan A (Sigma Chemical Co. St. Louis, MO) was carried out as previously described (Strzelecka et al., 1997b). Briefly, particles were incubated for 1 hour at 37°C with 20mg/ml human IgG (Sigma Chemical Co. St. Louis, MO) at a final concentration of 10mg/ml Zymosan A. Particles were then washed 3 times with PBS to remove unbound IgG and resuspended in PBS.

2.5.3 Phagocytosis with adherent cells

J774 cells prepared for immunohistochemistry were plated on glass coverslips and allowed to bind opsonised Zymosan A in PBS, for 20 minutes on ice. To initiate particle uptake, cells were transferred to pre-warmed DMEM/10% FBS and at the required time points, cells were processed for immunofluorescence analysis.

2.5.4 Phagocytosis with cells grown in suspension

J774 cells prepared for immunobloting analysis were trypsinised, resuspended in pre-warmed DMEM/10%FBS and kept in suspension at 37°C for approximately 30 minutes prior to the assay. Cells were then resuspended in pre-chilled PBS and particle binding was carried out on ice for 20 minutes. Phagocytosis was initiated by a temperature shift to 37°C in

DMEM/10%FBS for the required length of time. Particle uptake was stopped by plunging tubes into ice until further processing.

2.5.5 Inhibitor studies

J774 cells were exposed to inhibitory drugs for 30 minutes at 37°C prior to phagocytosis assays. Cytochalasin D (Sigma Chemical Co. (St. Louis, MO) was used at 50-500nM, LY294002 (BIOMOL Research Labs Inc.) at 30-80μM and Nocodazole (Sigma Chemical Co., St. Louis, MO) at 5μM. All inhibitors were diluted in DMSO (Sigma Chemical Co., St. Louis, MO) to final concentrations of 48 mM LY294002, 5 mM Nocodazole, 18 mM Brefeldin A and 2 mM Cytochalasin D. Cells were exposed to IgG opsonised Zymosan A for 30 minutes at 37°C, unbound particles were then washed washed gently and cells were process for either immunofluorescence or immunoblotting analysis.

2.6 Mouse manipulation and analysis

(i) Chimaera production

Chimaeric mice were produced by microinjection (J. Wrin, Department of Biochemistry, University of Adelaide). W9.5 targeted derivatives were introduced into C57Bl/6 blastocysts or B6D2F1 blastocysts (C57Bl/6 X DBA/2). Chimaera were identified by coat colour assessment based on the known coat colour genotypes: C57Bl/6 (a/a; B/B; D/D) results in a black coat colour phenotype and DBA/2 (a/a; b/b; d/d) manifested by a dilute brown phenotype. B6D2F1 progeny had the following possible genotype: a/a; B/?; D/? (black), a/a; b/b; D/? (brown), a/a; B/?; d/d (dilute black) and a/a; b/b; d/d (dilute brown). W9.5 ES cells have a dominant agouti allele.

(ii) Breeding

Chimaeric mice displaying 25% or more chimaerism were bred to C57Bl/6 mice and progenies were scored for the transmission of the dominant agouti allele.

CHAPTER 3:

BIOCHEMICAL ANALYSIS OF TEC ISOFORMS

3.1 Introduction

Tec is a single copy gene located on mouse chromosome 5 (Mano et al., 1993). As part of this thesis, partial cloning and characterisation of mouse Tec genomic fragments were carried out and are described in Chapter 5. In combination with studies by Merkel (1996), these studies have identified all exon-intron boundaries of the *Tec* gene. A complete description of the exon-intron boundaries of the *Tec* gene is presented in Merkel et al. (1999). Briefly, the *Tec* locus consists of 18 exons that are distributed over approximately 100kb with tightly linked 5' exons and more sparsely arranged 3' exons. Since Mano et al. first reported the isolation of the *Tec* transcript in 1990, a total of five *Tec* mRNA isoforms have been reported in the literature that differ in the PHTH, SH3 and kinase regions (Mano et al., 1990; Mano et al., 1993). As seen in Figure 3.1A, the exon/intron structure of the *Tec* locus suggests that all 5 *Tec* isoforms can theoretically arise from alternative splicing of exons 4, 8 and 18a. Such transcripts would give rise to proteins that vary in their PHTH, SH3 and kinase domains respectively. A diagrammatic representation of the molecular mechanisms responsible for the generation of these alternatively spliced transcripts is represented in Figure 3.1B.

Translation of *TecII*, *III* and *IV* occurs in reading frame 1 and initiates at the first ATG codon located in exon 2. The full length *TecIV* transcript encodes functional PHTH, SH3, SH2 and kinase domains, but no Src-like negative regulatory Tyr residue is present in the C-terminal tail of the kinase domain. Compared to TecIV, TecIII has a truncation of 22 amino acids that is predicted to remove critical structural components of the SH3 fold (Figure 3.2A and B). *TecIIA* and *TecIIB* are characterised by a 99 bp insertion in the 3' end of the kinase region of *TecIII* and *IV* respectively which exchanges the last 27 amino acids of the kinase region of *TecIIA* and *IIB* with an unrelated 21 amino acids sequence. This potentially influences the fold and/or stability of type II Tec kinases and might in turn affect kinase activity

Figure 3.1 Five alternative isoforms of *Tec* can arise by alternative splicing of *Tec* transcripts

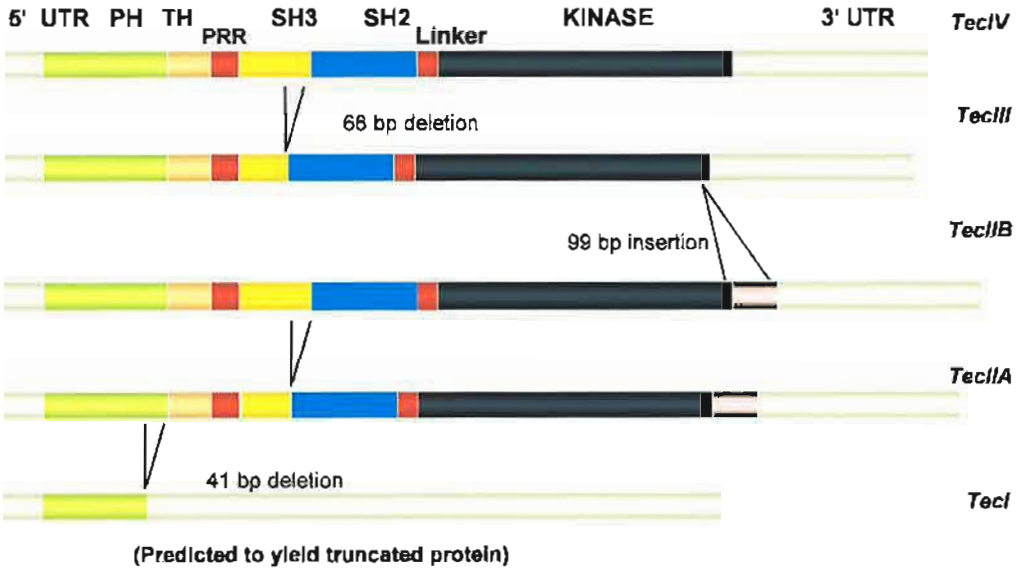
A. Diagrammatic representation of *Tec* isoforms as reported in the literature.

5'UTR: 5' untranslated region, PH: pleckstrin homology domain; TH: Tec homology motif, PRR: proline rich region; SH3: Src Homology 3 domain; SH2: Src Homology 2 domain; 3' UTR: 3' untranslated region.

The various domains represented in this diagram are based on translation initiated at the first ATG in exon 2.

B. Diagrammatic representation of splicing events proposed to occur at the *Tec* locus.

A)


5' UTR
PH
TH
PRR
SH3
SH2
Linker
KINASE
3' UTR
TecIV
68 bp deletion
TecIII
99 bp insertion
TecIIB
TecIIA
41 bp deletion
TecI
(Predicted to yield truncated protein)

B)

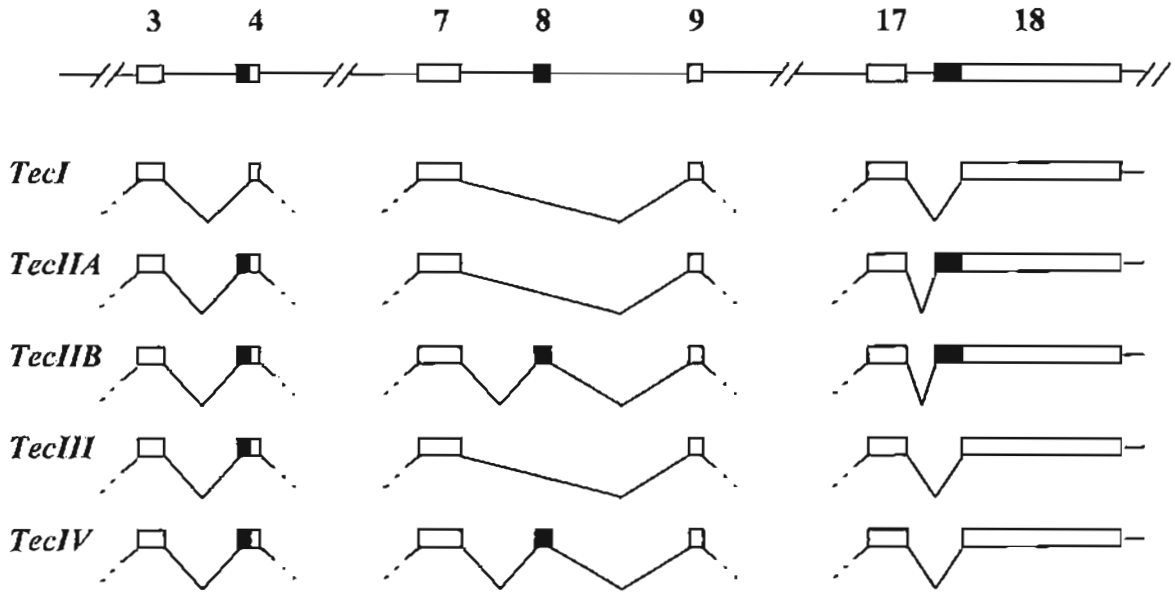

3
4
7
8
9
17
18
TecI
TecIIA
TecIIB
TecIII
TecIV

Figure 3.2 Representation of the structure of the TecIV SH3 domain

Diagrammatic representations of the TecIV SH3 domain are based on the solution structure (Pursglove et al., 2001).

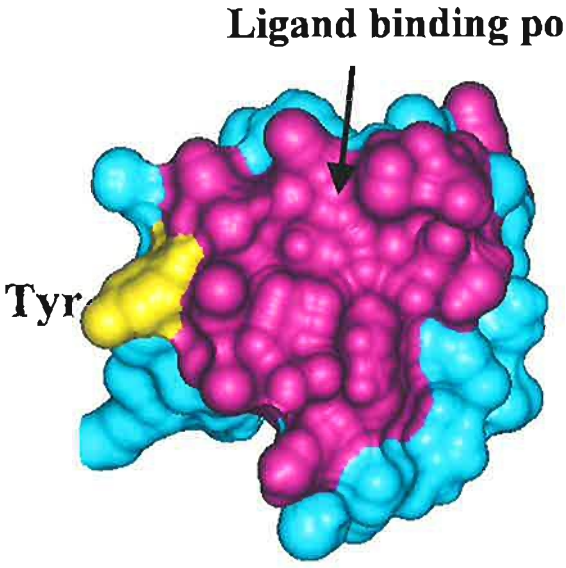
A. The molecular surface of TecIV SH3 domain.

   This orientation allows visualisation of the SH3 binding pocket (purple) and of the regulatory tyrosine residue (yellow).

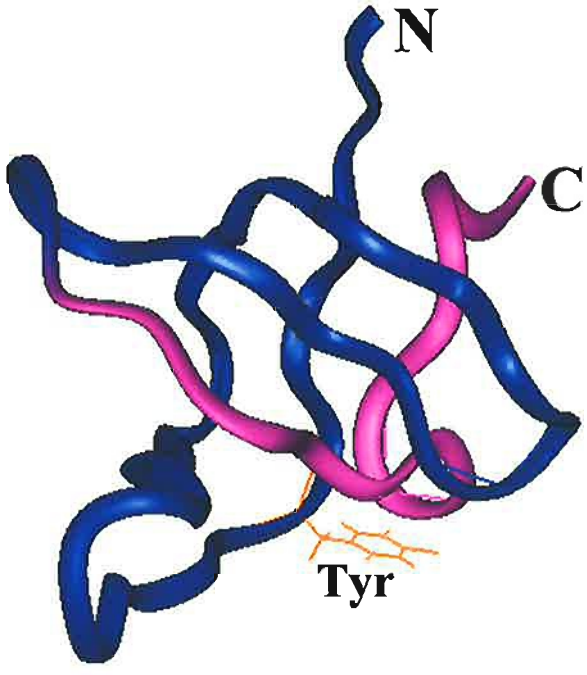
B. Ribbon diagram of the TecIV SH3 domain.

   This orientation displays the characteristic beta barrel conformation of the SH3 module and highlights the 22 amino acid stretch absent in TecIII (red). The regulatory tyrosine residue is highlighted in orange.

A)



B)



and/or specificity. Compared to *TecIV*, *TecI* has a 41 bp deletion in the PHTH region. Based on the sequence of the *TecI* transcript, it is predicted that its translation can initiate at two possible ATG sites. The first ATG is located in frame 1 of exon 2 (base pair 49 of exon 2). The same ATG codon facilitates the initiation of translation of *TecII, III* and *IV* transcripts. The use of this ATG to initiate the translation of *TecI* results in the synthesis of a short 100 amino acids long peptide that shares only 81 amino acids with the N-terminus of *TecIII* and *IV*. Alternatively, as initially suggested in Mano et al., 1990, translation of *TecI* could initiate in frame 3, from the first ATG in exon 3 (base pair 65 of exon 3) resulting in the expression of a unique N-terminal region, and functional SH3, SH2 and kinase domains. The N terminal unique region of this second peptide lacks traditional membrane targeting sequences and is reported to have no homology to known modular domains (Mano et al., 1990). In the cell, the TecI protein is predicted not to be targeted to the plasma membrane because of the lack of PH sequences otherwise present in all other Tec proteins. Consequently, distinct stimuli are expected to affect TecI subcellular localisation and/or kinase activity.

It is expected that the five isotypes of Tec potentially differ in their biological characteristics, including in the range of protein-protein interactions in which they can participate. All studies of Tec function have however to date mostly ignored this issue. Studies of Tec expression have also not differentiated between the different isoforms of the *Tec* transcript (Mano et al., 1990, Siyanova et al., 1994 and Kluppel et al., 1997).

Two distinct approaches were thus chosen to investigate the biological characteristics of the five isoforms of *Tec in vivo*. First, the *in vivo* expression pattern of each Tec transcript was characterised using RNase protection analysis (RPA) and/or reverse transcriptase PCR (RT-PCR) to provide an insight into the relative abundance of each isoform in mouse embryonic and adult tissues. To complement these studies, a second approach which utilised the power of the green fluorescent protein (GFP) was adopted to visualise the subcellular

localisation of the major Tec isotypes in living cells. This *in vitro* cell culture system also enabled the identification of some the biomolecular signals that affect the subcellular distribution of Tec in the absence of isoform-specific antibodies.

3.2 Results

3.2.1 *In vivo* characterisation of Tec isoforms by RNase Protection Analysis (RPA)

RNase protection analysis relies on the ability of RNA molecules to form RNA-RNA duplexes that are resistant to RNase digestion. Antisense radiolabelled RNA probes complementary to a transcript of interest are generated and allowed to hybridise to RNA samples. Hybridisation is followed by RNase digestion, and protected radiolabelled fragments are identified by gel electrophoresis. The expression of the transcript of interest is determined based on the presence and size of the specific protected fragment.

To characterise the expression of each *Tec* isoform, the studies described in this section utilised radiolabelled RNA probes complementary to *Tec* sequences, each encompassing one of the three regions characteristic of *TecI, TecII* and *TecIV*. These probes encode sequences complementary to the 41, 99 and 66 bp insertion regions respectively. Each region is typically flanked by regions of homology that range between 85 and 301 bp depending on the design of the probe to facilitate the formation of RNA-RNA duplexes. *TecI* and *TecIII* isoforms lack the 41 and 66bp regions of insertion respectively. The radiolabelled probes are expected to form single stranded loops in the insertion regions that are sensitive to RNase digestion resulting in the production of two protected fragments of radiolabelled probe. Their full length counterpart should be able to protect the entire length of the probe. Expression of the different isoforms of *Tec* can thus be monitored based on the sizes of the protected radiolabelled fragments recovered following RNase digestion.

3.2.1.1 Investigation of TecI Expression in Mouse Adult Tissues

A probe encoding the full length PHTH region was generated by PCR using the PHLexA5 and THLexA3 primer combination (2.2.11; 2.3.22i). This primer combination amplifies a 474bp PCR product that was subsequently cloned in pGEM-T (Stratagene). The orientation of the cloned PHTH fragment was determined by restriction enzyme digestion with *Acc* I (Figure 3.3A). SP6 RNA polymerase was used to generate the PH/TH probe described in Figure 3.3B. The predicted size of probe fragments that are protected by *TecI* versus *TecII, III* and *IV* mRNA sequences are depicted in Figure 3.3B. Briefly, two radiolabelled fragments of 244bp and 172bp are expected to be protected by the shorter *TecI* transcript, as the 41bp insertion in PH/TH-*TecI* duplexes should form a single stranded loop that is susceptible to RNase digestion. These two shorter fragments can easily be distinguished from the 457bp PH/TH fragment protected by *TecII, III* and *IV* transcripts using gel electrophoresis.

As shown in figure 3.3C, RNA samples from adult mouse liver, kidney and placenta tissues protected only the full length PH/TH fragment, and no *Tec* expression was detectable in the heart. In order to account for difference in the amounts of RNA recovered in each reaction, an antisense radiolabelled rGAP probe that monitors the expression of the house keeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

Given that *TecI* had originally been isolated from a mouse liver cDNA library (Mano et al., 1990), it is surprising that no *TecI* expression was detected in two independent liver RNA samples. The absence of *TecI* in the liver samples tested could however be explained as follows: First, *TecI* could represent an incompletely spliced form of *Tec* transcripts that had been captured during the synthesis of the cDNA library described in Mano et al. (1990). Alternatively, the *TecI* transcript could represent an extremely rare form of the *Tec* transcript that was not expressed in the liver tissues tested in these studies. It was thus decided that

Figure 3.3 Characterisation of the tissue distribution of *TecI* in mouse adult tissues

A. Diagrammatic representation of the pGEM-T-PHTH plasmid used to generate the radiolabelled antisense PH/TH probe.

T7: T7 RNA polymerase promoter; SP6: SP6 RNA polymerase promoter.

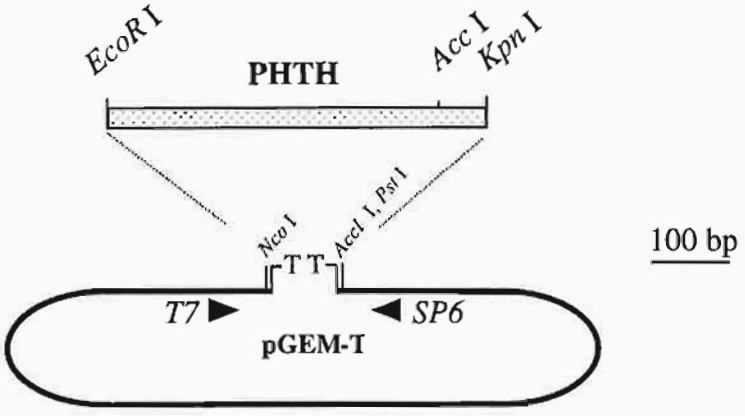
B. Diagrammatic representation of the ribonucleotide probe used in the RNase Protection Assay, together with the potential products generated following RNase digestion .

C. RPA analysis of mouse adult tissues.

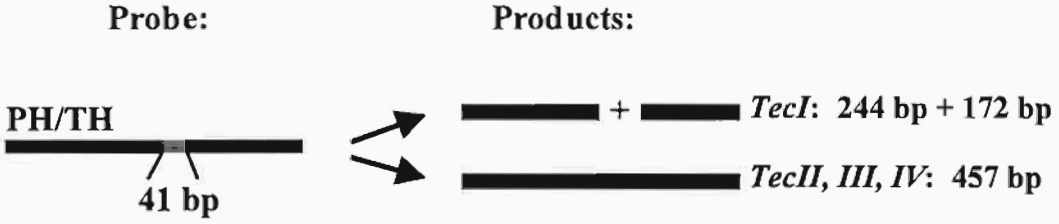
Ten micrograms of total RNA prepared from mouse adult liver, kidney, heart and placenta tissues were subjected to RPA using the PH/TH probe. The rGAP antisense probe was used as a loading control.

rGAP: rat glyceraldehyde-3-phosphate.

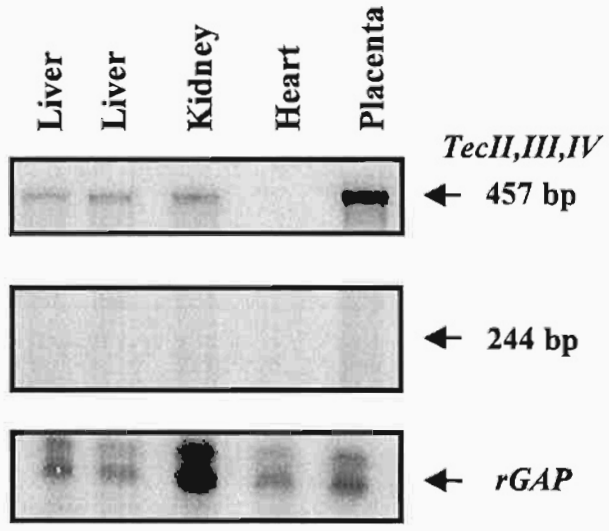
A)


EcoR I
PHTH
Acc I
Kpn I
Nco I
Acc I, Pst I
T T
T7
SP6
pGEM-T
100 bp

B)


Probe:
Products:
PH/TH
41 bp
+
TecI: 244 bp + 172 bp
TecII, III, IV: 457 bp

C)


Liver
Liver
Kidney
Heart
Placenta
TecII,III,IV
457 bp
244 bp
rGAP

TecI is only a minor isoform of the *Tec* transcript that might or might not be physiologically relevant, and was therefore not investigated further during the course of this Ph.D.

3.2.1.2 Investigation of the Expression of Type II Tec Transcripts

The abundance of the kinase-variant isoforms of *Tec* was investigated using the KINASE probe described in Figure 3.4B. The 99 bp insertion characteristic of *TecII* transcripts is located in the *Nco* I/*Bam*H I fragment at the 3' end of the *TecIIB* transcript. *Nco* I linearised pBluescript SK^+.TecIIB (Figure 3.4A) was used as a template to transcribe the KINASE antisense riboprobe using T7 RNA polymerase (Figure 3.4A). A 275 bp radiolabelled probe fragment is predicted to be protected by sequences in the 3' region of *TecII* transcripts. On the other hand the 99bp insertion sequence of the probe which has no complementary counterpart in type *III* or *IV* transcripts is susceptible to RNase digestion and should yield two fragments of 85 and 91bp (Figure 3.4B). As shown in Figure 3.4C, based on the size of the recovered protected fragments, no *TecII* transcripts were observed in adult mouse spleen, liver or placenta tissues, nor were *TecII* transcripts detectable in total RNA samples from day 16 embryos. ES cell RNA samples displayed no *Tec* expression at all despite efficient recovery of the rGAP control sequence.

The absence of type *II Tec* transcripts was further confirmed by RT-PCR analysis using the 5'EXON16 and 3'EXON17 primer combination (section 2.2.11). RT-PCR was carried out on liver, spleen and d16 total RNA samples and resulted in the amplification of only a 635bp fragment characteristic of *TecIII* and *IV* while no 734bp PCR product characteristic of *TecII* sequences was identified (Figure 3.4D). The identity of the amplified fragment was also confirmed by DNA sequencing and restriction enzyme digestion using *Nco* I/ *Sac* I, two

Figure 3.4 Characterisation of the tissue distribution of *TecII* transcripts in mouse adult tissues

A. Diagrammatic representation of the pBluescriptSK$^+$.TecIIB plasmid used to generate the radiolabelled antisense KINASE probe.

T7: T7 RNA polymerase promoter; T3: T3 RNA polymerase promoter.

B. Diagrammatic representation of the ribonucleotide probe used in the RNase Protection Assay (RPA), together with the potential products generated following RNase digestion.

C. RPA analysis of mouse adult tissues.

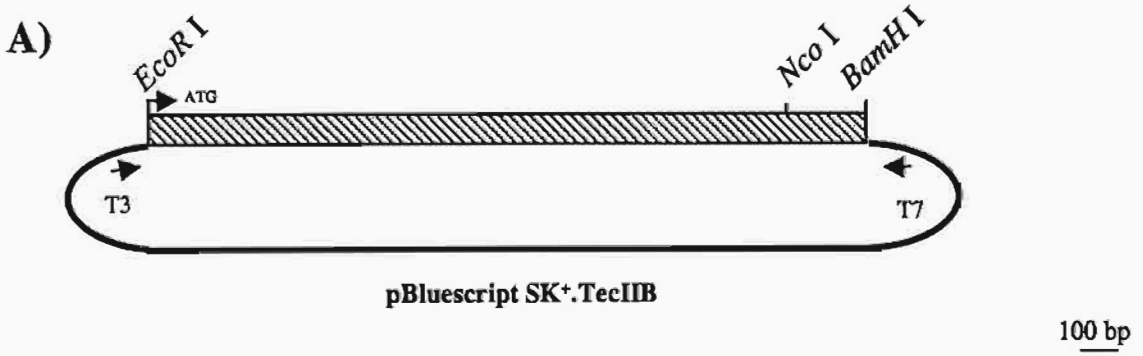
Ten micrograms of total RNA prepared from adult mouse liver, spleen, and placenta tissues as well as embryonic day 16 and ES cell total RNA were subjected to RPA using the KINASE probe. The rGAP antisense probe was used as a loading control.

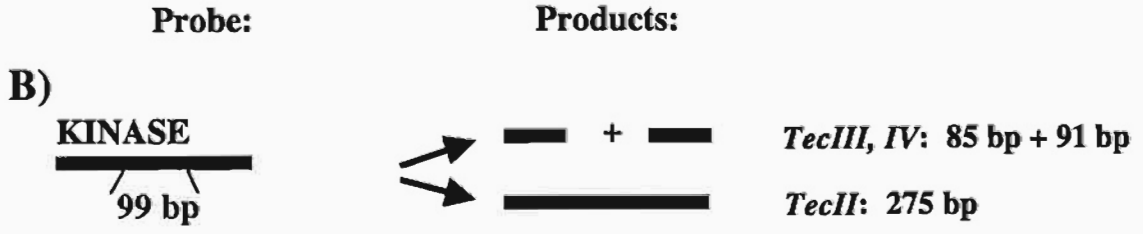
ES: Embryonic Stem cell; rGAP: rat glyceraldehyde-3-phosphate.

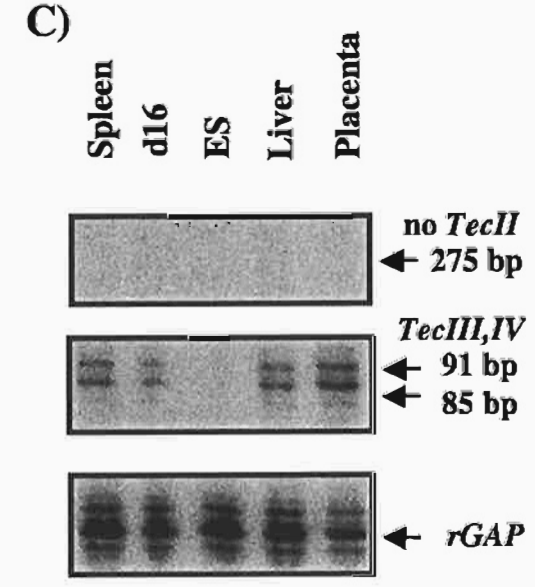
D. RT-PCR analysis of adult mouse liver and spleen tissues, and day 16 embryonic and ES cell total RNA.

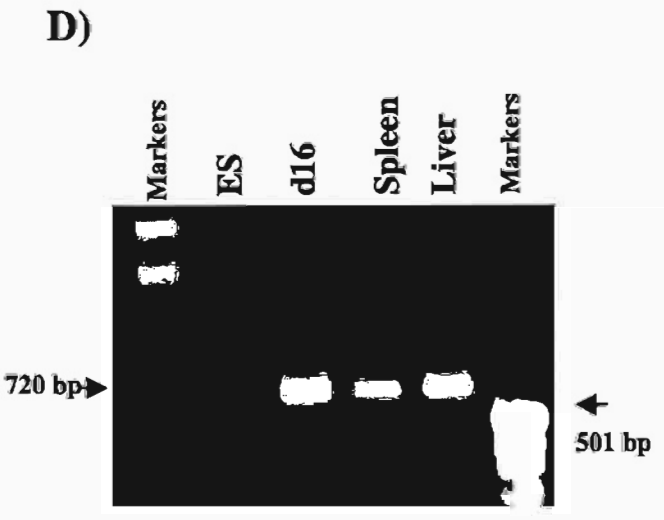
Five hundred nanograms of total RNA was tested in reactions that utilised the 5'exon16 and 3'exon17 primer combination and the SUPERSCRIPT ™ ONE-STEP™ RT-PCR System.

The products of the reaction were separated by 1% (w/v) TAE gel electrophoresis and visualiased by EtBr staining.


A)
EcoR I
ATG
Nco I
BamH I
T3
T7
pBluescript SK+.TecIIB
100 bp


Probe:
Products:
B)
KINASE
99 bp
+
TecIII, IV: 85 bp + 91 bp
TecII: 275 bp


C)
Spleen
d16
ES
Liver
Placenta
no TecII
275 bp
TecIII,IV
91 bp
85 bp
rGAP


D)
Markers
ES
d16
Spleen
Liver
Markers
720 bp
501 bp

restriction enzyme recognition sites known to be present in *TecIII* and *IV* kinase sequences (J. Doumanis, personal communication).

3.2.1.3 Investigation of TecIII and TecIV expression in mouse embryonic and adult tissues

Given that the expression of *TecI* and *II* in mouse tissues was found to be negligible, further studies concentrated on the distribution of *TecIII* and *IV* using the SH3/SH2 probe described in Figure 3.5B. This fragment had been amplified from pBluescriptSK $^{+}$TecIIB by PCR using the TecSH3.5 and TecSH2.3 primer combination (2.2.11; 2.3.22i). The amplified fragment had been subsequently subcloned in pBluescript SK^{+} using *Bam*H I and *Eco*R I restriction enzyme sites present in the primer sequences (Figure 3.5A). The antisense SH3/SH2 radiolabelled probe described in Figure 3.5B was transcribed from the T7 promoter of *BamH* I linearised pBluescriptSK^{+}SH3/SH2. This radiolabeled SH3/SH2 fragment is fully protected by *TecIV* sequences and yields a 495bp fragment after RNase digestion, while *TecIII* expression can be identified by the presence of a 301bp protected radiolabelled fragment (Figure 3.5B).

As seen in Figure 3.5C, analysis of mouse embryonic and adult tissues indicates that *TecIV* is the major *Tec* transcript in all tissue samples tested with the exception of adult liver and kidney, and embryonic day 16 limb tissues. In contrast to all other tissue tested, total RNA preparations from these latter tissues were found to protect only the *TecIII* 301bp fragment suggesting that *TecIV* expression in these samples was too low to be detected by this assay. In summary, the series of RPA experiments described in this section suggest that *TecI* and *TecII* transcripts are generally not expressed in embryonic and adult mouse tissues, while *TecIII* and *TecIV* are co-expressed at detectable levels except in adult liver kidney and embryonic d16 limb tissues where only *TecIII* can be detected by RPA.

Figure 3.5 Characterisation of the tissue distribution of *TecIII* and *TecIV* in mouse adult and embryonic tissues

A. Diagrammatic representation of the pBluescriptSK$^+$.SH3SH2 plasmid used to generate the radiolabelled antisense SH3/SH2 probe.

T7: T7 RNA polymerase promoter; T3: T3 RNA polymerase promoter.

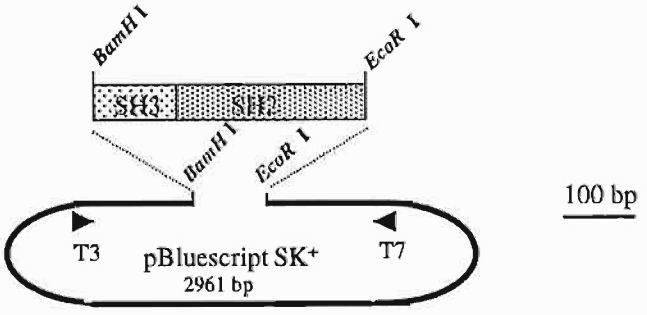
B. Diagrammatic representation of the ribonucleotide probe used in the RNase Protection Assay (RPA), together with the potential products generated in this type of experiment.

C. RPA analysis of mouse adult and embryonic tissues.

Ten micrograms of total RNA prepared from mouse adult liver, kidney, intestine, spleen, and placenta tissues as well as embryonic day 10.5 and 16 total RNA, embryonic day 16 brain, intestine, limb, lung and skin tissues were subjected to RPA using the SH3/SH2 probe. The mGAP antisense probe was used as a loading control.

mGAP: mouse glyceraldehyde-3-phosphate.

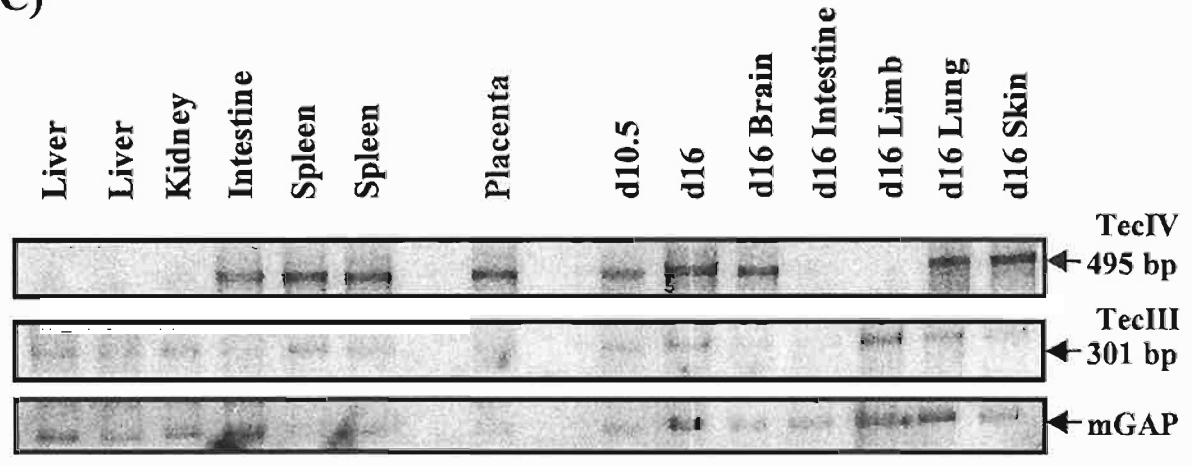
A)


BamH I
EcoR I
SH3
SH2
BamH I
EcoR I
100 bp
T3
pBluescript SK+
2961 bp
T7

B)


Probe:
Products:
SH3/SH2
66 bp
+
TecIII: 128 bp + 301 bp
TecIV: 495 bp

C)


Liver
Liver
Kidney
Intestine
Spleen
Spleen
Placenta
d10.5
d16
d16 Brain
d16 Intestine
d16 Limb
d16 Lung
d16 Skin
TecIV
495 bp
TecIII
301 bp
mGAP

3.2.2 RT-PCR analysis of TecIII and TecIV expression in adult and embryonic mouse tissues and hematopoietic cell lines

To complement the results described in section *3.2.1.3*, RT-PCR analysis was carried out on total RNA preparations from adult and embryonic mouse tissues, using the primer combination 5PRR and 3'exon8 (3.2.11; 2.3.23). This primer combination amplifies the PRR to SH3 region and identifies the presence of *TecIV* transcripts as a 353bp PCR product, and that of *TecIII* as a 287bp product (Figure 3.6A). To confirm the identity of the amplified fragments, Southern blot analysis (2.3.17) was carried out using an internal SH3 probe generated by *Bam*H I/*Eco*R I restriction enzyme digest of pGEX4T2-SH3 (Pursglove *et al.*, 1998). As shown in Figure 3.6B, expression of both *Tec* isoforms was detected in a range of adult and embryonic mouse tissues. Based on the intensity of the PCR products as detected by Southern blot analysis (Figure 3.6B), *TecIV* expression was greatest in the adult small intestine, spleen and placenta as well as embryonic day 16 brain, kidney, lung and skin. Surprisingly, although *TecIV* expression had been undetectable in adult liver and kidney tissues by RPA, RT-PCR assays carried out on the same RNA preparations amplified the *TecIV* specific 353bp product as well as the *TecIII* 287bp fragment.

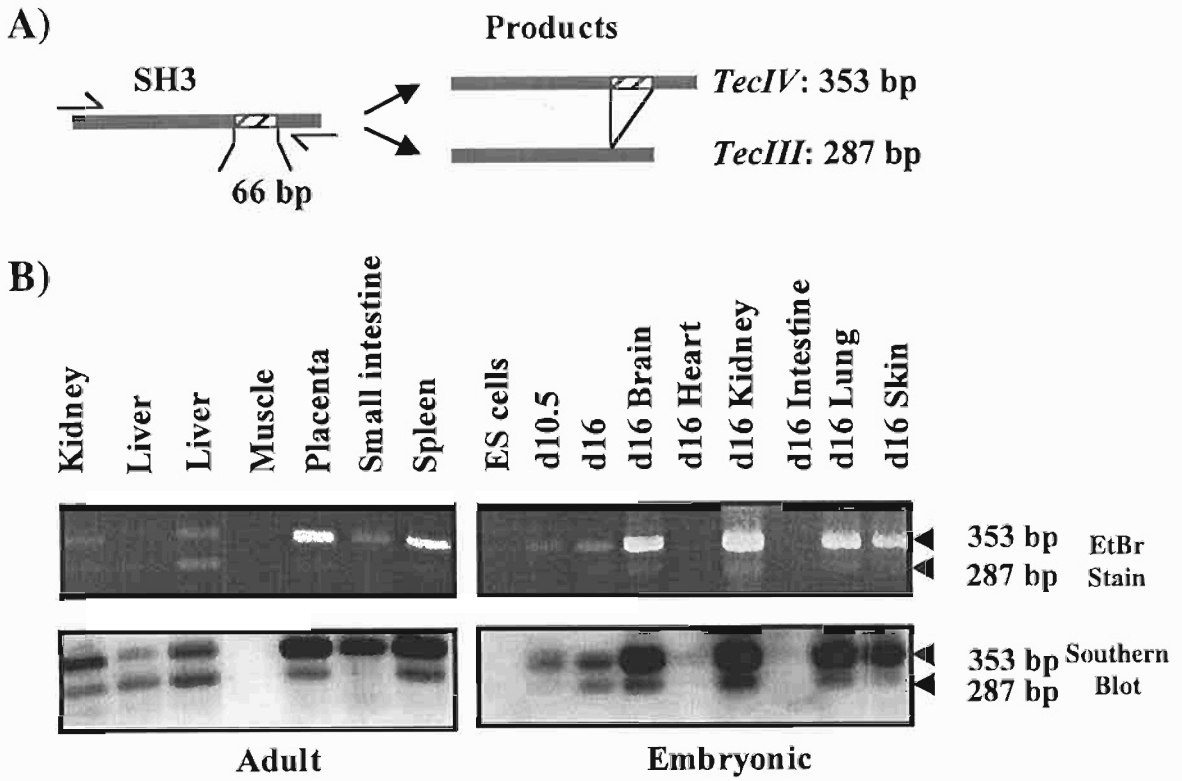
Given the discrepancy between the observations made in section *3.2.1.3* and those described in Figure 3.6, the possibility that the *TecIV* RT-PCR products observed in adult liver and kidney RT-PCR reactions had resulted from the amplification of contaminating genomic or plasmid DNA sequences needed to be excluded. Briefly, based on the structure of the *Tec* gene, amplification of genomic DNA using the 5PRR/3'exon8 primer pair (2.2.11) should result in a 4.5kb PCR product. The 353bp product observed in Figure 3.6 could therefore not have arisen from amplification from genomic DNA. Additionally, ES cell total RNA preparations which had been shown to lack *Tec* expression by RPA (Figure 3.3C and 4C) were used as a negative control. As shown in Figure 3.6B RT-PCR reactions with ES cell RNA as a

Figure 3.6 RT-PCR analysis of *TecIII* and *TecIV* transcripts in mouse adult and embryonic tissues

A. Diagrammatic representation of the RT-PCR products generated using the 5PRR and exon8 3' primer combination.

B. Five hundred nanograms of total RNA prepared from mouse adult liver, kidney, spleen, muscle, small intestine and placenta tissues as well as ES cell, embryonic day 10.5 and d16 total RNA and embryonic d16 brain, heart, kidney, intestine, lung and skin total RNA were tested using the 5'exon16 and 3'exon17 primer combination and the SUPERSCRIPT ™ ONE-STEP™ RT-PCR System. The products of the reactions were separated by 2% (w/v) TAE gel electrophoresis, visualised by Ethidium Bromide (EtBr) staining and blotted onto a nylon membrane and subjected to southern blot hybridisation using a radiolabelled Tec-SH3 fragment as a probe.

C. Five hundred nanograms of total RNA prepared from J774, CTLL, BaF3, FDCP1 and J2E tissue culture cells were tested in each reaction using the 5'exon16 and 3'exon17 primer combination using SUPERSCRIPT ™ ONE-STEP™ RT-PCR System. The products of the reactions were analysed as in (B).


A)
Products
SH3
TecIV: 353 bp
TecIII: 287 bp
66 bp
B)
Kidney
Liver
Liver
Muscle
Placenta
Small intestine
Spleen
ES cells
d10.5
d16
d16 Brain
d16 Heart
d16 Kidney
d16 Intestine
d16 Lung
d16 Skin
353 bp EtBr
287 bp Stain
353 bp Southern
287 bp Blot
Adult
Embryonic
C)
J774
CTLL
BAF3
FDCP1
J2E
WEHI
-VE
353 bp EtBr
287 bp Stain
353 bp Southern
287 bp Blot

template yielded no *Tec* product, thus confirming that no contamination of PCR reagents by plasmid DNA sequences (such as pBluescript SK^+.TecIIB or pBluescript SK^+.SH3/SH2) had occurred.

As *Tec* expression had previously been reported in various cells of the hematopoietic lineage, especially myeloid cells (Mano et al., 1993), the relative distribution of *TecIII* versus *TecIV* was investigated in various hematopoietic cell lines. Total RNA prepared from J774 (monocyte macrophage), CTLL (T lymphocytes), BaF3 (mouse pro-B cells), FDCP-1 (myeloblast/early promyelocyte) and J2E (erythroblast) cell lines were subjected to RT-PCR amplification of the PRR-SH3 region using the 5PRR/3'exon8 primer combination (2.2.11; 2.3.23). *TecIV* was identified in the three hematopoietic lineages tested, namely the myeloid lineage (J774, FDCP-1), lymphocyte lineage (CTLL and BaF3) and erythrocyte lineage (J2E). *TecIII* on the other hand was observed only in T lymphocyte (CTLL) cells, as well as in one of the two myeloid cell lines, the J774 cell line suggesting that *TecIII* expression showed some degree of specificity that might or might not be related to the origin of the lineage (Figure 3.6C).

It is interesting that the two myeloid derivatives showing different *Tec* expression differ in their morphological characteristics and represent different stages of myeloid differentiation. J774 cells are terminally differentiated macrophage cells that grow as flat, elongated adherent cells in culture. FDCP-1 cells in contrast are early promyelocyte cells that grow in suspension as small round cells, with relatively small cytoplasms. It is possible that these morphological differences, especially their distinct differences in substratum adherence, could provide some clues as to the biological differences between these two isoforms. More extensive studies on a larger panel of adherent and non-adherent cell lines would be necessary to investigate such a link.

3.2.3 Investigation of functional differences between TecIII and TecIV in mammalian COS-1 cells using GFP fusion proteins

3.2.3.1 Characterisation of the subcellular localisation of GFP-TecIII and GFP-TecIV

Biological differences between *TecIII* and *TecIV* were further investigated using fusion genes consisting of *TecIII* or *IV* cDNA sequences fused in frame to the C-terminus of the green fluorescent protein (GFP). These were routinely used in the laboratory of Dr. Booker (Merkel, personal communication). The fluorescent GFP moiety enables direct visualisation of the fusion protein in living cells by fluorescence microscopy without the need for fixation and immunostaining. This permits comparative studies of the subcellular distribution of the two isoforms of Tec to be carried out in living cells, in real time, and facilitates the characterisation of anticipated translocation(s) in the presence of specific stimuli. All transfections were transient to avoid the selection of non-representative clones that might arise from the procedure of establishing stable transfectants. Plasmids carrying GFP-Tec fusion sequences were transfected into COS-1 cells. COS-1 cells are routinely used as a model system for transient transfection experiments. They grow adherently as flattened cells with large cytoplasmic volumes which facilitates the visualisation of the subcellular distribution of fusion proteins in transfected cells. In addition, endogenous Tec expression had also been detected in COS-1 cells by western blot analysis (Merkel, personal communication). The subcellular localisation of Tec fusion proteins is thus expected to reflect that of its endogenous counterpart, although it is possible that the exogenous overexpression system used in these transfections might affect the fluorescence pattern observed.

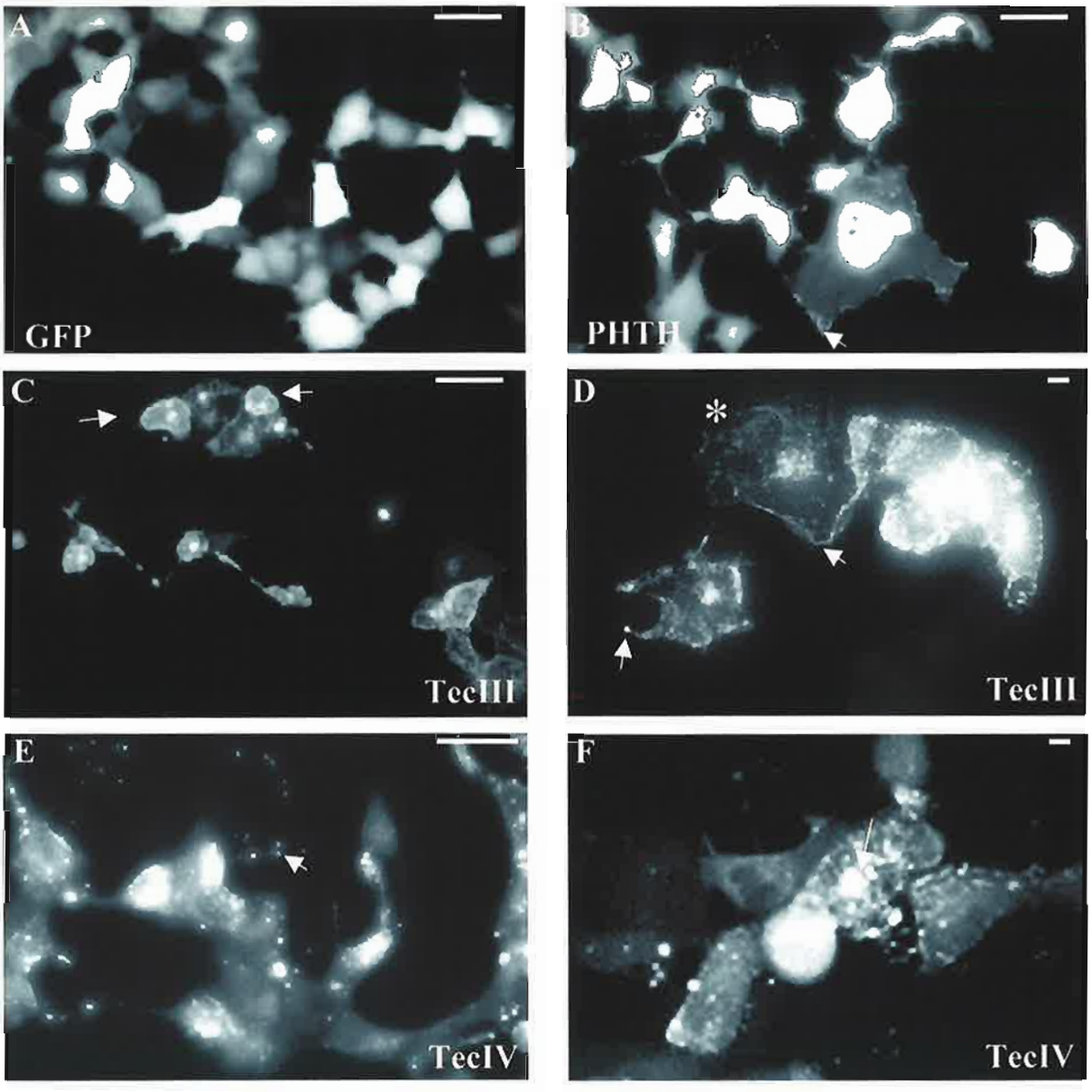
The sizes of the fusion protein products were confirmed by Western blotting analysis of whole cell lysates (Figure 3.7G). The GFP fusion partner alone yielded the predicted 29 kD GFP product, while GFP-TecIV and GFP-TecIII migrated at the 100kD mark. Surprisingly

Figure 3.7 Charaterisation of the subcellular localisation of GFP fusion proteins in COS-1 cells

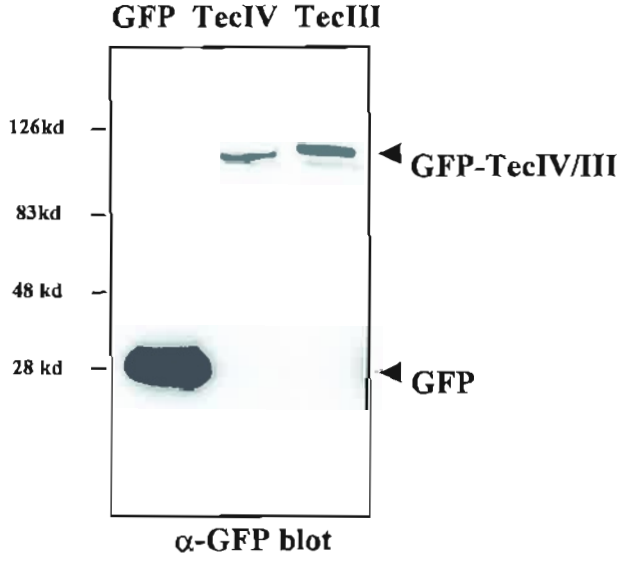
Fluorescence analysis of COS-1 cells expressing (A) GFP, (B) GFP-PHTH, (C-D) GFP-TecIII and (E-F) GFP-TecIV .

$5x10^6$ COS-1 cells were transfected with 2µg plasmid DNA (A) pEGFP-C2, (B) pEGFP-C2-PHTH, (C-D) pEGFP-C2-Tec3 and (E-F) pEGFP-C2-Tec4 for 18 hours at 37°C using the Fugene™ transfection system. Cells were grown at 37°C in complete medium for at least 24 hours prior to visualisation. The arrows in (B) mark membrane ruffle-like region characterised by plasma membrane fluorescence. The arrow in C marks cells with rounded morphology. The * in (D) highlights cells with asymmetrical distribution of plasma membrane GFP-TecIII and the arrows highlight fluorescent filopodia extensions. The arrow in (E) points to vesicular-like, punctate fluorescence of GFP-TecIV and the arrow in (F) shows juxtanuclear GFP-TecIV.
Bar = 100µM (A-C; E) and 10µM (D & F)

(G) Western immunoblot analysis of total cell lysates prepared from COS-1 cells expressing GFP, GFP-TecIV and GFP-TecIII. Protein samples were separated by SDS-PAGE electrophoresis on an 8% Tris-tricine polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond-C), probed with GFP-specific antibody and HRP-conjugated anti rabbit antibody, and detected by enhanced chemiluminescence.


A
GFP
B
PHTH
C
TecIII
D
TecIII
E
TecIV
F
TecIV

G


GFP TecIV TecIII
126kd
83kd
48 kd
28 kd
GFP-TecIV/III
GFP
α-GFP blot

GFP-TecIII appeared to migrate slower than GFP-TecIV despite its 2kD deletion. This is proposed to reflect differences in post-translational modification of the two peptides, most likely phosphorylation.

Visualisation of GFP transfected cells was carried out on a Nikon inverted (ECLIPSE TE300) microscope. GFP was present diffusely in the cytoplasm and nucleus of transfected cells and showed no specific subcellular localisation (Figure 3.7 A). The GFP-PHTH fusion protein was also present diffusely in the cytoplasm and nucleus of transfected cells (Figure 3.7B). Rarely, in less than 5% of transfected cells, GFP-PHTH expressing cells were identified that were phenotypically distinct from the rest of the population. These cells were mostly larger than other transfectants and displayed extensive cytoplasmic extensions and membrane ruffle-like structures that were enriched with GFP-PHTH (Figure 3.7B arrow).

When TecIV was fused to the C-terminus of GFP, a distinct change in the subcellular distribution of the fusion protein compared to that of GFP or PHTH alone was observed (Figure 3.7E-F). In general, within a single population of transfected cells, three distinct fluorescence patterns were observed that included: (i) diffuse staining in the cytoplasm and nucleus of transfected cells, undistinguishable from that of GFP alone. (ii) intense punctate staining generally in combination with diffuse fluorescence in highly fluorescent cells (Figure 3.7E arrow). This was suggestive of vesicular distribution and appeared to be associated with fine structures extending from the cytoplasm. An intense juxta-nuclear pool of fluorescence, suggestive of the endoplasmic reticulum/golgi network, was also observed in the majority of these cells (Figure 3.7F arrow). (iii) plasma membrane staining that was in general accompanied by punctate fluorescence.

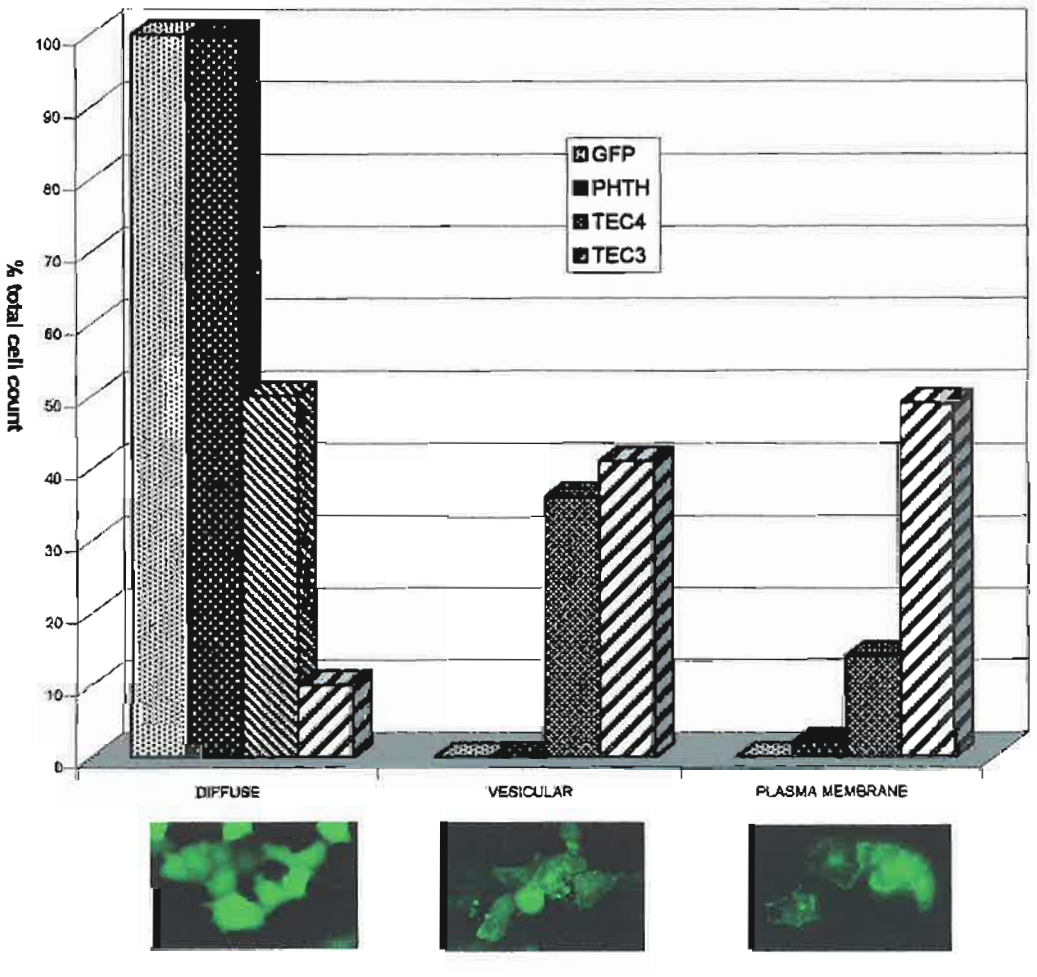
The pattern of fluorescence observed for GFP-TecIII fusion proteins was distinct from that described above. Although, overall the same three patterns were identified, a larger proportion of transfected cells showed intense plasma membrane localisation of the fusion

protein in ruffle-like membrane projections, as well as in fine filopodia-like extensions (Figure 3.7C-D). Interestingly, two distinct populations of GFP-TecIII cells appeared to exhibit plasma membrane localisation of the fusion protein. The first population of GFP-TecIII cells had large cytoplasmic regions, generally accompanied by an intense juxta nuclear pool of GFP-TecIII fusion proteins and plasma membrane localised GFP-TecIII in plasma membrane ruffle-like regions (Figure 3.7D asterisk and arrow; Figure 3.10 arrow) suggesting a potential role for TecIII in the formation of such structures. The second group of GFP-TecIII cells were smaller and had typically round cytoplasms, with distinctive intense plasma membrane fluorescence (Figure 3.7C arrow and 3.10A). Because they often possessed smaller, condensed nuclei as detected by Hoechst staining (Figure 3.10B) it was expected that these represent a subpopulation of poorly growing cells.

As previously described, the population of transfected GFP-TecIII and TecIV cells were heterogenous in their fluorescence pattern. The proportion of each characteristic pattern was thus quantified to provide an insight into the major characteristics that differentiate the two major isoforms of Tec. As described in Figure 3.8, 100% of GFP and approximately 98% of GFP-PHTH transfected cells showed diffuse cytoplasmic/nuclear localisation, while only 50% GFP-TecIV and less than 10% GFP-TecIII transfected cells showed the same diffuse staining pattern. Plasma membrane targeting was only observed in approximately 2% of GFP-PHTH cells, 15% of GFP-TecIV cells and 45% of GFP-TecIII cells. Plasma membrane targeting was thus most prominent in GFP-TecIII cells compared to GFP-TecIV cells. Punctate fluorescence was scored based on the presence of distinctive vesicular-like fluorescence, with or without the presence of an intense juxta-nuclear pool of fusion protein. When punctate fluorescence was observed in cells with an otherwise diffuse fluorescent pattern, those cells were counted as punctate. As seen in Figure 3.8, similar proportions of

Figure 3.8 Quantitation of subcellular pattern of GFP fluorescence in the population of COS-1 transfected cells

$5x10^6$ COS-1 cells were transfected with 2μg of the following plasmid DNA pEGFP-C2, pEGFP-PHTH, pEGFP-TecIII and pEGFP-TecIV as described in Figure 3.7. Staining patterns were quantitated from images captured using a Nikon (ECLIPSE TE300) inverted microscope. Briefly, cells were visualised using Adobe Photoshop 5.5 and approximately 200 cells were counted in each case. Cells expressing diffuse as well as punctate fluorescence were scored as punctate. An example of each subcellular localisation pattern is shown beneath the graph.


% total cell count
100
90
80
70
60
50
40
30
20
10
0
GFP
PHTH
TEC4
TEC3
DIFFUSE
VESICULAR
PLASMA MEMBRANE

Location

TecIII and GFP-TecIV expressing cells displayed vesicular punctate staining although the appearance of the punctate distribution was generally different (Figure 3.7C-F).

Biochemical differences between TecIII and TecIV fusion proteins were further investigated by immunoblotting. Total cell lysates and fusion protein immunoprecipitates prepared 24 hours following transfection, were probed for tyrosine phosphorylation (Figure 3.9). Phosphorylation of GFP-TecIII in total cell lysates as well as in GFP-immunoprecipitates was consistently higher than that observed for GFP-TecIV (Mano et al., 1995). This higher level of phosphorylation is also likely to be responsible for the slightly slower motility rate of GFP-TecIII identified by polyacrylamide gel electrophoresis.

Phalloidin-TRITC co-staining of GFP-TecIII and TecIV transfected cells identified the presence of regions, enriched in filamentous F-actin that strongly colocalised with GFP-Tec fusion proteins (Figure 3.10). GFP-TecIII transfected cells displayed regions enriched in F-actin that could clearly be identified by fluorescent cytoplasmic extension structures as they were labelled with both phalloidin-TRITC and GFP-TecIII (Figure 3.10A,C-D). In addition, GFP-TecIII cells with rounded morphology also displayed distinctive colocalisation of F-actin and GFP-TecIII at the periphery of the cells. This increased level of plasma membrane targeting of hyperphosphorylated GFP-TecIII coincides with high levels of filamentous actin at the plasma membrane.

The subcellular localisation of GFP fusion proteins was further investigated in serum starved quiescent cells to identify specific changes in the subcellular distribution of GFP-TecIII and TecIV in response to serum stimulation. Eighteen hours after transfection, cells were starved of serum for a further 18 hours after which GFP fluorescence was visualised. A subpopulation of cells from each transfection had also been set apart that were not subjected to serum starvation. These were monitored concurrently with experimental specimens at each

Figure 3.9 Western blot analysis of GFP-Tec fusion COS-1 transfected cells

$5x10^6$ COS-1 cells were transfected with 2μg plasmid DNA for 18 hours at 37°C using the Fugene™ transfection system. Total cell lysates were prepared and subjected to immunoprecipitation analysis using the GFP specific antibody for 18 hours at 4°C. Whole cell lysates and immunoprecipitates were subjected to SDS-PAGE analysis on 8% Tris-tricine polyacrylamide gels and blotted onto nitrocellulose membranes (Hybond-C). Membranes were immunoblotted using the GFP-specific and HRP-conjugated anti rabbit antibodies to confirm loading, stripped, and blotted with Tec specific and HRP-conjugated anti-goat antibodies to confirm the identity of the GFP-Tec fusion proteins. Phosphorylation levels were monitored using mouse monoclonal anti-pTyr and HRP-conjugated anti mouse antibodies. Detection was carried out using enhanced chemiluminescence.

(WCL: whole cell lysate; IP: immunnoprecipitation; IgHc: immunoglobulin heavy chain; GFP: green fluorescent protein)

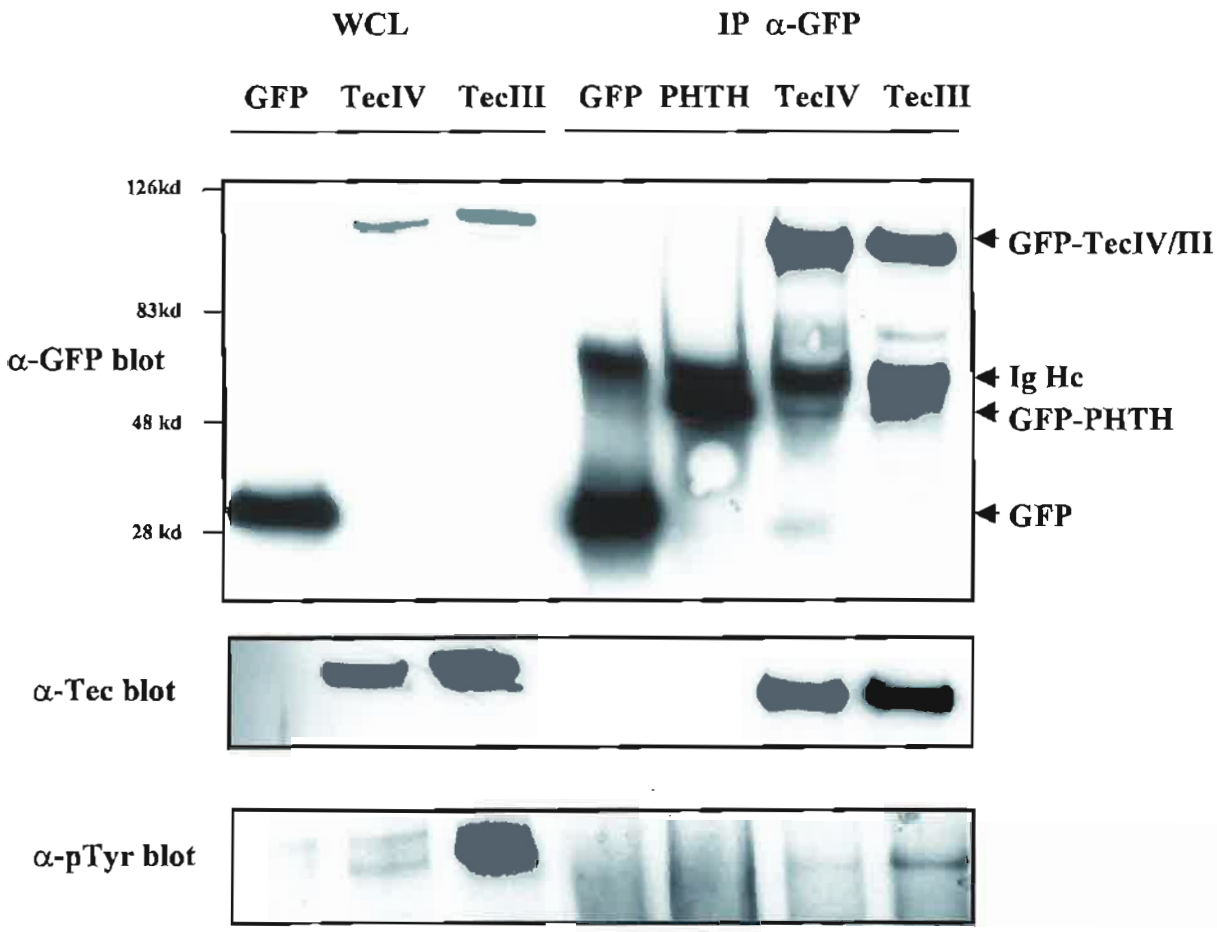

WCL
IP α-GFP
GFP
TecIV
TecIII
GFP
PHTH
TecIV
TecIII
126kd
83kd
48 kd
28 kd
α-GFP blot
GFP-TecIV/III
Ig Hc
GFP-PHTH
GFP
α-Tec blot
α-pTyr blot

Figure 3.10 Colocalisation of GFP-Tec fluorescence with F-actin in COS-1 cells

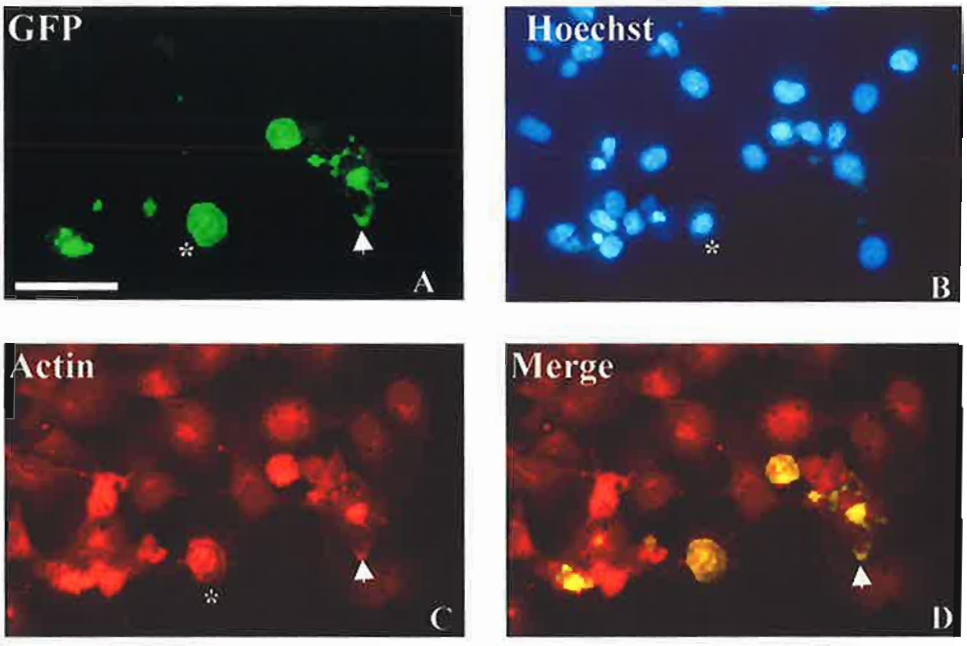
Transfections were carried out for 18 hours at 37°C using 2μg plasmid DNA per $5x10^6$ COS-1 cells, in presence of serum using the Fugene™ transfection system. Cells were fixed in methanol and F-actin was detected using TRITC-conjugated phallodin. Nuclei were visualised using Hoechst staining.

(A-D) pEGFP-TecIII and (E-H) pEGFP-TecIV transfected COS-1 cells. Images were visualised using a Nikon (ECLIPSE TE300) inverted microscope. D and H: merge of GFP fluorescence and F-actin staining showing colocalisation in yellow. A and E: GFP fluorescence; C and G staining of F-actin and B and F hoechst staining of nuclei.

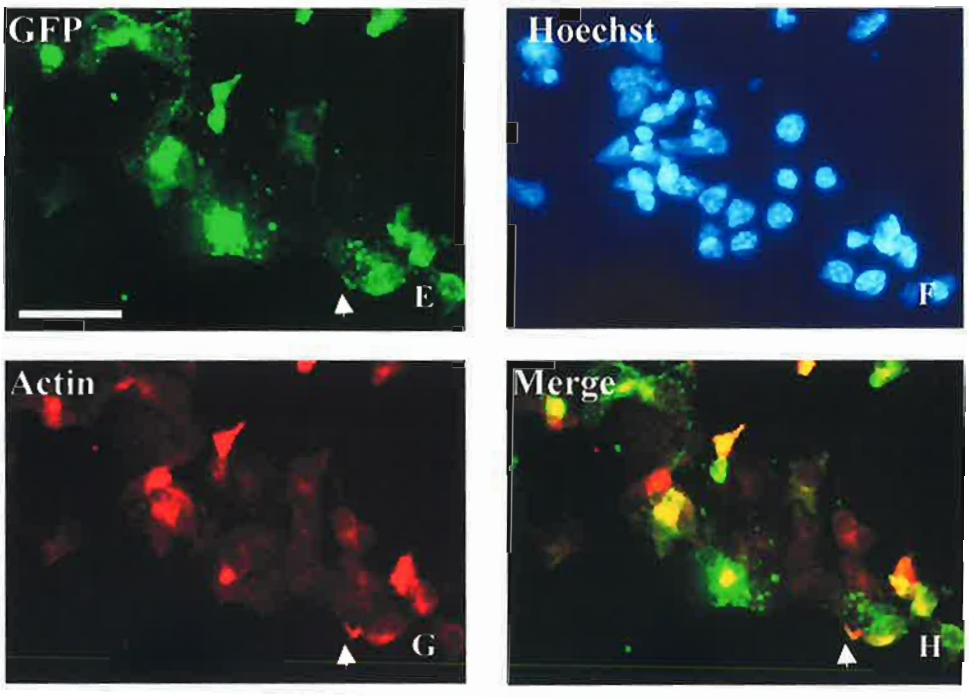
Bar = 100μM

Arrows mark colocalisation of GFP fluorescence with filamentous actin. * highlights cells with a characteristically round morphology, intense fluorescence and condensed nucleus.

GFP-TecIII transfected cells



GFP-TecIV transfected cells



time point and in all cases the fluorescence pattern of these controls was representative of those shown in Figure 3.7 (data not shown).

As represented in Figure 3.11D, the subcellular localisation of GFP-PHTH transfected cells was generally not affected by serum starvation. It remained largely diffuse in the cytoplasm although overall cells appeared to have smaller cytoplasms compared to cells cultured in the presence of serum (Figure 3.11A and D). Changes in the subcellular distribution of GFP-TecIV transfected cells were very distinctive (Figure 3.11B and E). In the absence of serum, GFP-TecIV cells were much smaller with less extended cytoplasms and showed diffuse distribution of GFP-TecIV (Figure 3.11E). Punctate fluorescence was rarely observed and no plasma membrane targeting of GFP-TecIV was observed, suggesting that serum stimulation was necessary for plasma membrane targeting, most probably as a result of phospholipid synthesis by PI3-Kinases. Re-exposure to serum for 3 hours restored punctate structures but plasma membrane distribution was rarely observed (Figure 3.11H). This suggests that under these culture conditions, additional external stimuli, are required to induce plasma membrane targeting of GFP-TecIV. Such stimuli could potentially accumulate with extensive culture periods and are probably responsible for the plasma membrane localisation of GFP-TecIV.

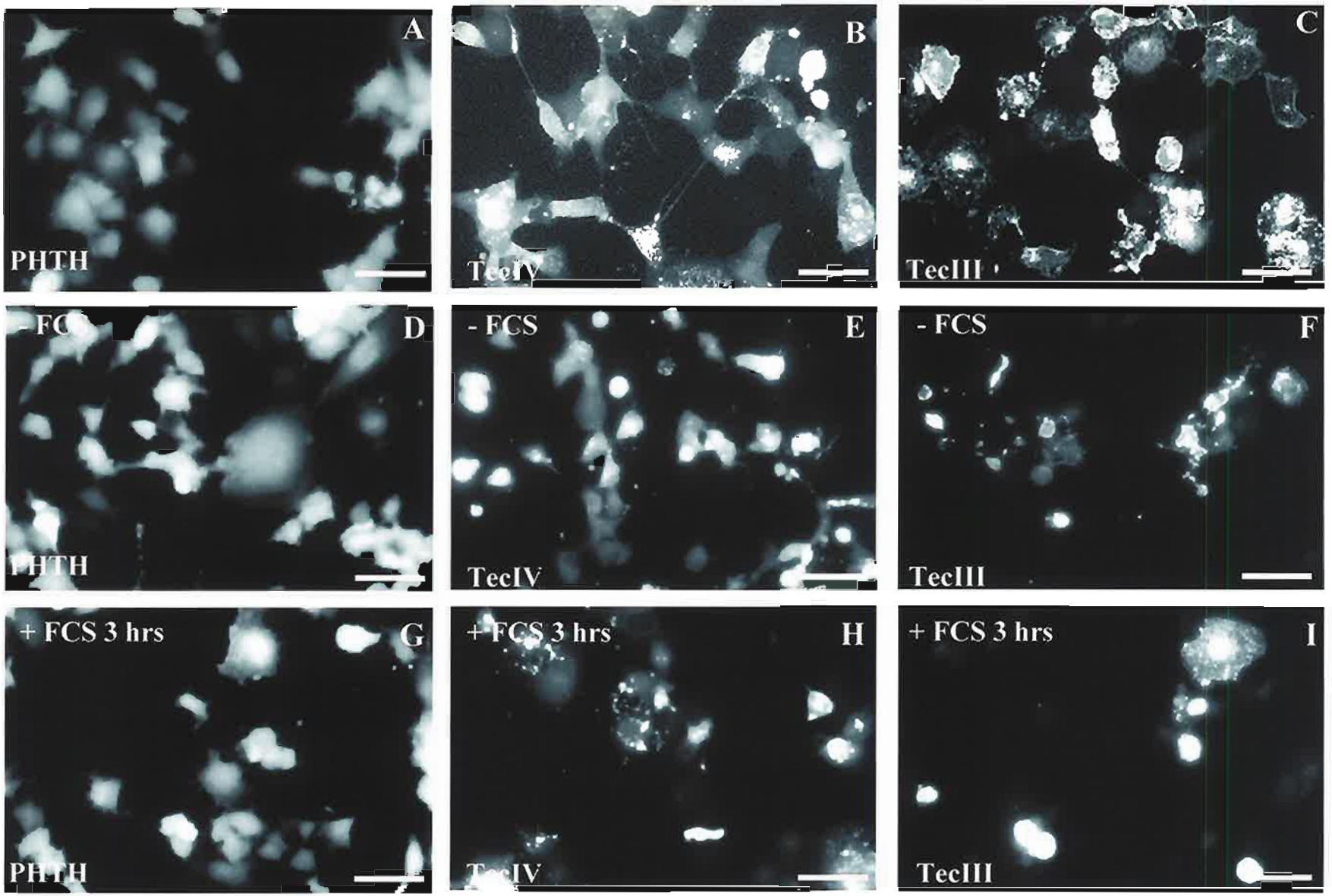
In serum free culture conditions, GFP-TecIII cells displayed a significant decrease in the size of GFP-TecIII expressing cells. Punctate fluorescence was less frequent plasma membrane targeted GFP-TecIII was still visible (compare Figure 3.11C and F). This suggested that even in the absence of growth factor stimulation, the absence of an intact SH3 domain facilitates plasma membrane targeting of TecIII. In general however, GFP-TecIII cells appeared smaller, round and shrivelled up, with almost no cytoplasmic extensions, suggesting that these cells were growing poorly (Figure 3.11F). The addition of serum did also not appear to fully restore the characteristic staining pattern of GFP-TecIII (Figure 3.11F and I).

Figure 3.11 Visualisation of changes in the subcellular localisation of GFP fusion proteins induced by serum deprivation of COS-1 transfected cells

Fluorescence patterns of (A) pEGFP-PHTH, (B) pEGFP-TecIV and (C) pEGFP-TecIII following 18 hours transfection with 2μg plasmid DNA per $5x10^6$ COS-1 cells using the Fugene™ transfection system; (D) pEGFP-PHTH, (E) pEGFP-TecIV and (F) pEGFP-TecIII transfected cells following 24 hours of serum deprivation at 37°C; and (G) pEGFP-PHTH, (H) pEGFP-TecIV and (I) pEGFP-TecIII transfected cells following the addition of FCS to 24 hours-serum starved cells for 3 hours.

Bar = 100μM

(FCS: fetal calf serum)


A
PHTH
B
TecIV
C
TecIII
D
PHTH
- FCS
E
TecIV
- FCS
F
TecIII
- FCS
G
PHTH
+ FCS 3 hrs
H
+ FCS 3 hrs
I
TecIII
+ FCS 3 hrs

In fact, a large proportion of transfected cells remained rounded up, appeared to have lost adherence to the tissue culture dish and could clearly be identified because of their intense fluorescence. It is therefore predicted that, in a proportion of GFP-TecIII expressing cells, serum deprivation induces changes that are irreversible and culminate in the loss of cell viability.

3.2.3.2 Characterisation of TecIII and TecIV subcellular localisation in the presence of LY294002 and Cytochalasin D

Membrane recruitment of PH containing proteins is dependent on the interaction of a positively charged phosphoinositide-binding pocket in the PH region of these proteins with phosphoinositides in the plasma membrane (Blomberg et al., 1999). The PHTH domain of the Tec kinases Tec and Btk have been reported to be highly specific for $PI(3,4,5)P_3$, a product of the lipid kinase PI3-K (Rameh et al., 1997). The role of PI3-K products in the plasma membrane targeting of TecIII and TecIV was thus investigated using the PI3-K inhibitor LY294002.

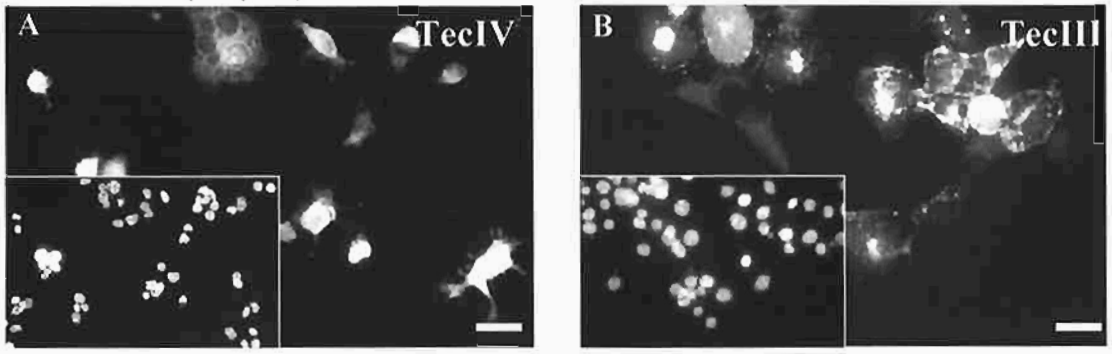
The PI3-K inhibitor was added to (i) cells 24 hours following transfection, (ii) transfected cells subjected to serum starvation for 18 hours. In all experiments, a subpopulation of cells had been set apart that were not exposed to inhibitory compounds. These were monitored concurrently with experimental specimens and in all cases the fluorescence pattern of these controls was representative of those shown in Figure 3.7 (data not shown).

The addition of LY294002 24 hours after transfection dramatically influenced the fluorescence pattern of GFP-TecIV (Figure 3.12A). The dramatic loss of punctate immunofluoresence in intensely fluorescent cells was most evident. The presence of the intense juxta nuclear pool of fusion protein was also less prominent in the presence of the

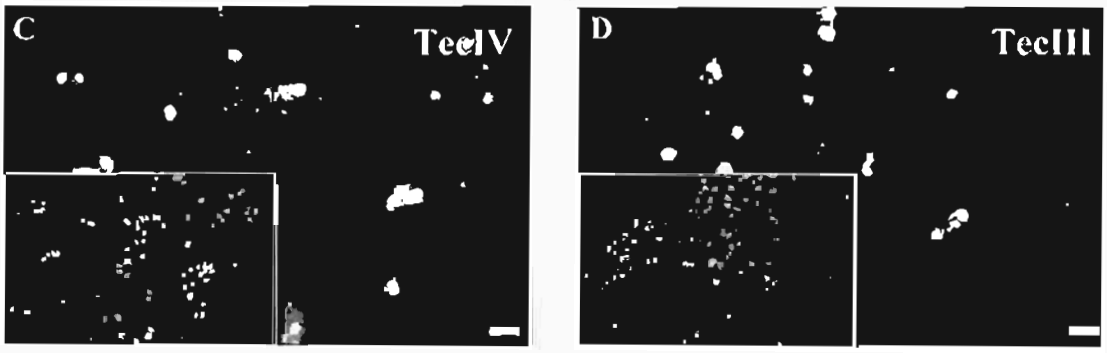
Figure 3.12 Characterisation of the effect of the PI3-K inhibitor (LY294002) on the subcellular localisation of GFP-TecIII and GFP-TecIV fusion proteins in COS-1 cells

Cells were transfected for 18 hours using Fugene™ transfection system. LY294002 (30 μM) was added to (A) pEGFP-TecIV and (B) pEGFP-TecIII transfected cells for 3 hours in the presence of serum at 37°C. Cells were serum starved overnight and LY294002 (30μM) was added to (C) pEGFP-TecIV and (D) pEGFP-TecIII transfected cells for 3 hours in the presence of serum at 37°C. Insets show Hoechst staining of respective fluorescence pictures. Bar = 100μM

LY294002 (30 μM)



Serum starved + LY294002 (30 μM)



inhibitor (Figure 3.12A). Together these suggest a possible role for PI3-K activity in the formation of GFP-TecIV-enriched vesicular-like structures.

In contrast, as depicted in Figure 3.12B, GFP-TecIII redistribution to the plasma membrane remained visible in the presence of LY294002, although it appeared less prominent. Vesicule-like structures remained present, and so was the juxta nuclear pool of GFP-TecIII. The persistence of plasma membrane targeting of GFP-TecIII suggests that the absence of an intact SH3 domain is able to potentiate plasma membrane targeting, even in the absence of $PI(3,4,5)P_3$. Alternatively, it is also possible that in the absence of an intact SH3 domain, SH3 binding sites present in the PRR become accessible to other SH3 domains and that this interaction might in turn be responsible for plasma membrane targeting of GFP-TecIII even in the absence of PI3-K activity.

To ensure that basal levels of $PI(3,4,5)P_3$ were minimal prior to the addition of the PI3-K inhibitor LY294002, cells were starved of serum prior to exposure to LY294002 (Figure 3.12C and D). This was carried out mainly because serum starvation is known to induce a quiescent state which was expected to result in the depletion of $PI(3,4,5)P_3$ at the plasma membrane. Under these conditions, the addition of serum (in the presence of LY294002) was expected to restore all cellular processes whilst preventing the activation of PI3-K. As seen in Figure 3.12C, faint punctate fluorescence was observed in a subset of GFP-TecIV transfected cells. These generally had a flattened appearance and lower levels of GFP-TecIV expression. Highly expressing cells in contrast were rounder and appeared to have lost adherence to the substratum due to a decrease in cell viability.

The combinatorial effect of serum starvation and PI3-K inhibition (Figure 3.12D) appeared to have a greater effect on the appearance of GFP-TecIII. Under these conditions, the majority of GFP-TecIII transfected cells appeared to have rounded up and lost adherence to the tissue culture plastic. As previously described however serum starvation alone can

induce such change in GFP-TecIII subcellular distribution and this appeared to be aggravated by the addition of LY294002. Thus, although the absence of $PI(3,4,5)P_3$ alone in GFP-TecIII cells is not sufficient to promote loss of cell viability (Figure 3.12B), addition of the inhibitor to serum starved GFP-TecIII cells appears to promote increased cell death.

A further requirement for an intact cytoskeleton in the distribution of fusion proteins was investigated using Cytochalasin D, an actin filaments-specific toxin (Figure 3.13). This inhibitory drug was added for three hours, 24 hours following transfection. In these experiments, a subpopulation of cells from each transfection had also been set apart that were not exposed to Cytochalasin D. These were monitored concurrently with experimental specimens and in all cases the fluorescence pattern of these controls was representative of those shown in Figure 3.7 (data not shown), thus confirming that any changes in fluorescence did not result from culture conditions but were directly affected by the addition of the inhibitory drug. When Cytochalasin D was added to adherent cultures of GFP-TecIII and TecIV, fluorescence was mostly diffuse, although, low levels of vesicular fluorescence could be identified in fine cytoplasmic structures (Figure 3.13A and B). These structures persisted even in the presence of the inhibitor, and were predicted to represent structures that had been established prior to the addition of the inhibitor.

3.2.3.3 Characterisation of vesicular and juxta-nuclear pool of GFP-TecIV using BrefeldinA

Brefeldin A is a fungal toxin that rapidly blocks anterograde exocytic transport through the golgi and results in the formation of uncoated membrane tubules through which golgi components redistribute to the endoplasmic reticulum and results in the failure to transport molecules out of this mixed golgi/ER system (Lippincott-Schwartz et al., 1991).

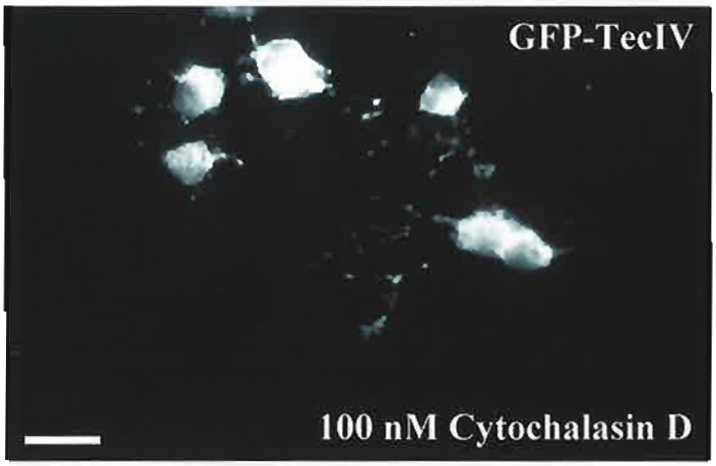
Brefeldin A was added to the culture medium of transfected cells in order to investigate the identity of the juxta-nuclear and punctate cytoplasmic pool of fluorescent GFP-TecIV.

Figure 3.13 Characterisation of the effect of Cytochalasin D on the subcellular localisation of GFP-TecIII and GFP-TecIV fusion proteins.

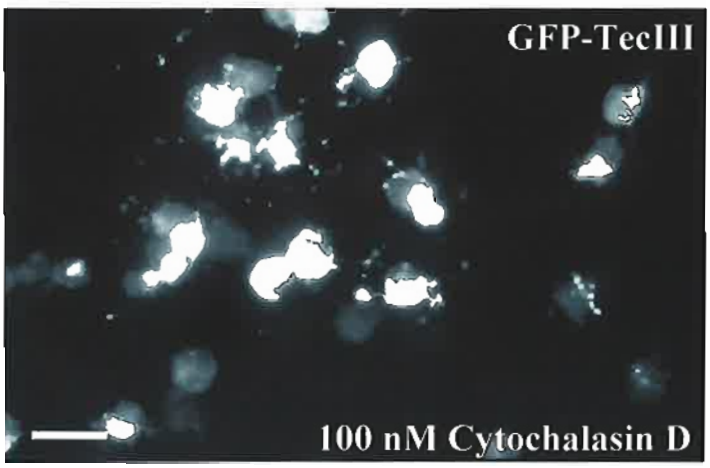
Cos-1 cells were transfected with (A) pEGFP-TecIV and (B) pEGFP-TecIII for 18 hours using the Fugene™ transfection system, trypsinised and replated in the presence of Cytochalasin D (100nM) for 3 hours.

Bar = 100μM

A


GFP-TecIV
100 nM Cytochalasin D

B


GFP-TecIII
100 nM Cytochalasin D

Addition of Brefeldin A abolished the presence of punctate cytoplasmic fluorescence suggesting that the formation of these vesicular-like structures was dependent on a functional golgi apparatus (Figure 3.14). Although the intense juxta-nuclear pool of fluorescence did not disappear, it was clearly disrupted and fragmented, resulting in a more diffuse fluorescence pattern. The effects of Brefeldin A were not permanent, and punctate fluorescence could be restored following removal of BFA. Punctate fluorescence of GFP-TecIV is therefore expected to represent vesicles that originate in the golgi.

3.3 Discussion.

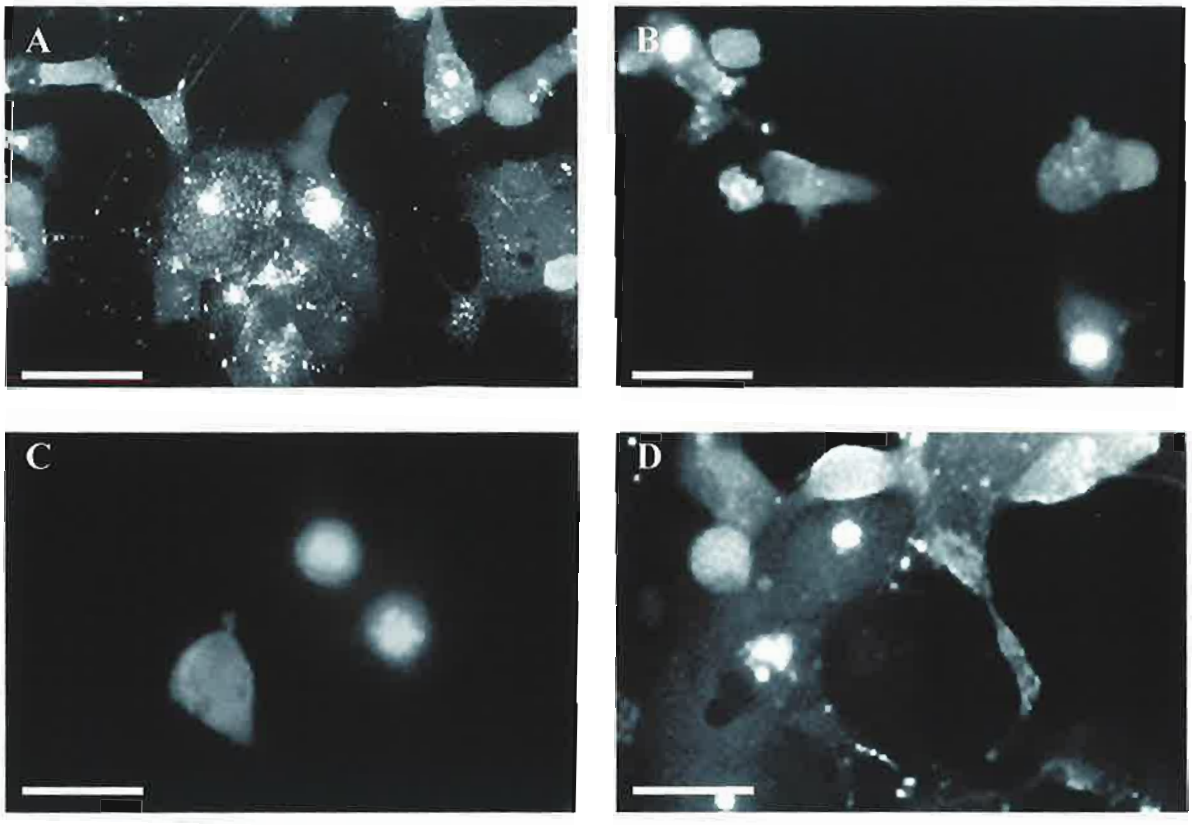
Protein-protein interaction domains are now recognised to have multiple functions. Their main role in signal transduction is to mediate inter-molecular protein-protein interactions and dictate the signalling pathway(s) to which a given protein will contribute. More recently, these domains have increasingly been found to function as critical regulators of enzymatic activity, typically through intra-molecular binding to internal ligand sites.

The Tec family of protein tyrosine kinases have three such modular domains and at least two of these, the PHTH and SH3 domains, have been shown to influence protein tyrosine kinase activity (Nore et al., 2000; Park et al., 1996 and Yamashita et al., 1996). Five isoforms of Tec have been reported in the literature that vary in the PHTH, SH3 and Kinase domains. Because these differences can potentially influence not only their level of kinase activity, but also the signal transduction pathway(s) each isoform is able to participate in, their expression effectively increases the complexity of Tec-dependent signal transduction pathway(s). This level of complexity had generally been ignored prior to the beginning of this Ph.D. The biochemical studies described in this chapter are therefore the first to address biological differences between the *TecI, IIA, IIB, III* and *IV* transcripts *in vivo*.

Figure 3.14 Characterisation of the effect of Brefeldin A on the punctate fluorescent pattern of GFP-TecIV transfected cells

Fluorescent pattern of (A) pEGFP-TecIV transfected cells 18 hours after transfection using the Fugene™ transfection system; (B and C) pEGFP-TecIV transfected cells exposed to Brefeldin A (5μM) for 1 hour and (D) cells grown at 37°C for 1 hour following the removal of BrefeldinA.

Bar = 100μM


A
B
C
D

3.3.1 Expression of Tec isoforms *in vivo*

Briefly, only two major isoforms of the Tec mRNA are present at detectable levels in mouse adult and embryonic tissues: *TecIII* and *TecIV*. Throughout these studies, the *TecI* and *TecII* transcripts described in Mano et al., 1993 remained undetectable despite the testing of numerous mouse embryonic and adult tissues. It is therefore predicted that *TecI* and *TecII* represent minor transcripts that could have resulted from either incomplete or incorrect splicing of the *Tec* mRNA. It is likely that these had been inadvertently captured during the synthesis of the cDNA library used in the studies of Mano et al. (1990 and 1993).

The expression of *Tec* in mouse tissues appears to be spatially and temporally regulated. Kluppel et al., (1997) were first to report detailed *in situ* hybridisation studies of the mouse embryo which identified Tec expression first at day 7 of embryogenesis in the blood islands and maternal decidua, and later in the placenta at day 9. In those studies *Tec* expression was also detected in the liver and AGM region at later somite stages as well as in embryonic skin, blood vessels in the central nervous system, heart, kidneys, lungs and intestine of older embryos. It is interesting that Tec expression is first detected at sites of embryonic hematopoiesis, suggesting a possible role for Tec in hematopoietic-specific functions. Unfortunately, the studies of Kluppel et al. (1997) could not differentiate between the two major isoforms of *Tec*. It remained unclear whether both isoforms are produced during early hematopoiesis.

The studies described in section 3.2 attempted to investigate the expression pattern of each major isoform *in vivo* and complement the report of Kluppel et al., 1997. In general, the expression of *TecIII* and *TecIV* was observed in both embryonic and adult tissues tested. Although the sensitivity of RT-PCR and RPA experiments appeared to differ, both types of experiments indicate that TecIV is generally the most prominent Tec isoform except in adult mouse liver, kidney and embryonic day 16 limb tissues were *TecIII* appeared to be more

prominent. The significance of such differential pattern of expression in a specific subset of tissues is yet to be determined and will require more detailed functional analysis using genetically modified animals.

3.3.2 Biological characteristic of major Tec isoforms

The two major isoforms of *Tec* differ by 22 amino acids in the SH3 region. This truncation removes the 3/10 helix and 6th β strand of the SH3 domain which are critical structural elements of the SH3 domain. The absence of such critical elements is predicted to affect the structure and function of the TecIII SH3 domain (Figure 3.2).

The importance of the SH3 region in modulating the activity of Tec family kinases has been best characterised for the Tec family member Btk. Btk activation is characterised by the phosphorylation of two tyrosine residues. First, Y^{551} in the activation loop of the kinase domain is phosphorylated by a Src family kinase (Rawlings et al., 1996, Mahajan et al., 1995 and Afar et al., 1996; Nisitani et al., 1999). A second phosphorylation event at Y^{223} residue near the ligand binding pocket of the SH3 domain follows and alters the ligand binding properties of the SH3 domain (Nisitani et al., 1999) (Figure 3.2). Both phosphorylation events are necessary for maximal Btk kinase activity. Deletion of the Btk SH3 domain and mutagenesis of Y^{223} (Y223F) result in increased Btk phosphorylation (Park et al, 1996). Recently, an SH3 binding protein (Sab) was isolated that inhibits the auto- and trans-phosphorylation activity of Btk (Matsushita et al., 1998). This suggests that SH3 dependent regulation of Btk kinase activity could at least in part be mediated by inter-molecular interactions with inhibitory proteins. Further complexity is added by reports that the SH3 domain of Tec family kinases is also able to mediate intra-molecular binding to an internal ligand site located in the PRR (Patel et al., 1997; Andreotti et al., 1997; Pursglove, 2001). The full implication of this association is yet to be fully understood although it is expected that

it is able to prevent binding to other signalling molecules and stabilise the down-regulated conformation of the enzyme through a mechanism that is yet to be identified. A role for the SH3 domain of Tec family kinases is therefore beginning to emerge as a negative regulator of Tec kinase activity through a combination of intra-molecular interactions with the PRR and intermolecular binding to negative regulatory proteins.

Based on this model, the biological characteristics of TecIII and TecIV are predicted to differ significantly. The 22 amino acid truncation in TecIII removes critical structural elements at the C terminus of the SH3 domain. In Btk, truncation of the C terminal tail prevents correct folding of the shorter peptide and gives rise to a random coiled coil structure (Patel et al., 1997). The absence of the last 22 amino acids in TecIII is likely to also disrupt the fold of the truncated SH3 domain such that surrounding electrostatic forces will determine the three dimensional fold of the truncated peptide. Under these conditions, SH3 dependent interactions are predicted to be dramatically weakened if not abolished (Patel et al., 1997). Previous reports have shown increased tyrosine phosphorylation and activation of a 48 amino acids deletion mutant TecΔSH3 (Yamashita et al., 1996). Although, the truncation in the TecIII SH3 domain removes a smaller region of the SH3 domain than that deleted in TecΔSH3, it is predicted to have a similar effect on Tec kinase activity. To support this, studies described in this chapter have found TecIII to be hyperphosphorylated. Given that phosphorylation of Tec family kinases is believed to reflect the activity of their kinase domain, TecIII is predicted to have intrinsically higher kinase activity than TecIV. The higher incidence of TecIII localisation to the plasma membrane, an event which in itself is recognised to result in activation of Tec (Li et al., 1997) provides further support to the idea that Tec III is intrinsically more active that TecIV.Further characterisation of the kinase activity of (partially) purified TecIII and TecIV will provide more insight into the biological differences between these isoforms.

3.3.3 Proposed mechanism for the regulation of TecIV and TecIII kinase activity

In the absence of external stimuli, full length GFP-TecIV is diffuse in the cytoplasm of transfected cells and shows lower levels of tyrosine phosphorylation than GFP-TecIII which shows higher incidence of plasma membrane targeting. This suggest a role for a functional SH3 domain in preventing plasma membrane targeting and activation of Tec.

Under normal conditions, it is expected that a functional SH3 domain, such as that of TecIV, interacts with negative regulators and/or internal SH3 binding sites to prevent the redistribution of TecIV to the plasma membrane. Phosphorylation of the activation loop tyrosine and subsequent phosphorylation of the SH3 domain tyrosine residue is predicted to displace such interaction(s) thus permitting plasma membrane targeting and activation of Tec.

The truncated SH3 domain of TecIII is unable to mediate these negative regulatory interactions. It is possible that plasma membrane targeting motifs of the PH domain of TecIII are largely exposed, and/or that SH3 binding sites in the PRR are also available to potential SH3 binding proteins. Either one, or the combination of these interactions, might then facilitate the targeting of the truncated protein to the plasma membrane. Redistribution of TecIII to the plasma membrane in turn spatially positions TecIII in proximity to plasma membrane localised Src family kinases that

are then able to phosphorylate and activate TecIII. Further characterisation of the subcellular localisation and phosphorylation of GFP fusion proteins encoding a specific kinase dead mutants (K397E) as well as mutants of the polyproline rich region of TecIV (Pursglove, 2001) will be necessary to further elucidate the molecular mechanism that regulate Tec activation.

3.3.4 Subcellular localisation of TecIII and TecIV

In transfected cells, GFP-TecIV fluorescence in highly expressing/fluorescent cells has a definite punctate appearance. Using Brefeldin A to disrupt golgi function, this pattern of distribution was shown to represent vesicle-like structures originating from the golgi. The trans golgi network is believed to produce both constitutive and regulated vesicles, and the molecular mechanisms that regulate these trafficking reactions is extremely complex. Recently, PI3-K activity was shown to be essential for constitutive vesicle formation (Jones and Howell, 1997) due to the requirement for PI(3)P phospholipid in regulating the multitude of steps necessary to sort molecules and form vesicles at the trans-golgi network (Jones et al., 1998). The sensitivity of TecIV-vesicle formation to LY294002 treatment suggests that TecIV-containing vesicles might arise from a PI3-K dependent pathway possibly that of constitutive vesicle formation. However, fluorescent vesicles appear more prominent in higher expressing cells, it thus remains possible that the formation of TecIV-containing vesicles might be biologically irrelevant and is an artefact of the overexpression system utilised in this study. This issue could easily be resolved using a weaker promoter to drive the expression of the GFP-TecIV fusion protein.

The difference in GFP-TecIII vesicle distribution, together with their seemingly different susceptibility to LY294002 treatment suggest a biological difference between TecIII and TecIV vesicles. A recycling compartment is known to exist that is responsible for the targeted delivery of plasma membrane components (Bajno et al., 2000) to replenish plasma membrane components necessary for processes dependent on the active remodelling of the plasma membrane necessary for lamellipodia formation, phagocytosis, macropinocytosis, cell spreading and chemotaxis. These vesicles are necessary to rapidly deliver signalling components to activated regions of the plasma membrane (Greenberg et al., 1999). It is then possible, given the plasma membrane-rich distribution of GFP-TecIII that recycling vesicles

could target GFP-TecIII, probably in association with other plasma membrane signalling components, to activated membrane ruffle-like regions hence continually replenishing activated TecIII at the plasma membrane. The absence of a functional SH3 domain might facilitate this process by exposing protein-protein interaction sites that facilitate such transport.

The absence of a functional SH3 domain thus appears to have a two fold affect on plasma membrane targeting of TecIII. Firstly by exposing the phospholipid binding pocket of the PH domain and SH3 binding sites in the PRR and secondly by facilitating its packaging into plasma membrane targeted vesicles.

3.3.5 A proposed role for Tec in apoptosis

Apoptosis is a critical component of immune system homeostasis. It is a common mode of eukaryotic cell death and is triggered by an inducible cascade of events that culminates in the activation of endonucleases responsible for the cleavage of nuclear DNA into oligo-nucleosome-length fragments. A pro-apoptotic role for Btk has recently been reported in B cells exposed to ionising radiation (Uckun et al., 1996), as well as in mast cells cultured in the absence of serum (Kawakami et al., 1997). Studies described in this chapter have also uncovered a possible role for Tec in serum deprivation-induced cell death that is most pronounced in TecIII expressing cells. Following the removal of serum, a larger proportion of TecIII expressing cells, compared to cells expressing TecIV, displayed signs of cell death. These include the characteristic round appearance of the cells, the loss of cytoplasmic extensions, and the appearance of brightly staining nuclei that reflect the increased permeability of the nuclear membrane to DNA-specific stains. In Btk, growth factor deprivation induced cell death appears to be dependent on the targeting of Btk to the plasma membrane and the activation of its kinase domain (Tomlinson et al., 1999). The decrease in cell viability observed in GFP-TecIII expressing cells is thus proposed to result from its increased redistribution to

the plasma membrane. No direct investigation of the role of Tec in apoptosis was presented in these experiments as it had been beyond the scope of this thesis. These preliminary observations will need to be followed up in more detail using apoptosis specific cell staining assays and DNA fragmentation analysis.

Preliminary evidence for Tec-dependent apoptosis has previously been reported in the literature. DT40 B cells are programmed to apoptose following BCR engagement. This response is impaired in the absence of Btk and can, although poorly, be partially restored by Tec (Tomlinson et al., 1999), suggesting that Tec can to some extent convey pro-apoptotic signals although not as efficiently as Btk. Interestingly, several reports have also suggested a pro-survival role for Tec family kinases. B cells derived from Xid mice have a reduced half life *in vivo* (Oka et al., 1996) and show increased apoptosis *in vitro* (Woodland et al., 1996). The number of peripheral B cells in Xid mice can be restored to normal by the expression of the anti apoptotic bcl-2 transgene (Woodland et al., 1996) suggesting the importance of Btk in activating pro-survival factors. This pro-survival signal is likely to involve bcl-xl which is up regulated in a Btk dependent manner (Anderson et al., 1996). Such apparently contradictory roles of Btk in apoptosis are likely to arise from differences in the type and/or stage of development of cells used in each assays. More interestingly, they highlight a possible function for Btk as a BCR threshold modulator that will determine the fate of a cell in response to BCR engagement based on the intensity of the BCR signal.

In conclusion, two major isoforms of the Tec transcript have been identified in the mouse with *TecIV* being the most prevalent. Although *TecIII* transcripts have been identified in various mouse tissues, the distribution pattern of the TecIII protein could not be characterised because TecIII and IV specific antibodies are yet to be generated. An *in vitro* system has also been identified that will facilitate further studies of the Tec function in plasma membrane remodelling.

CHAPTER 4:

PRELIMINARY INVESTIGATION OF TEC FUNCTION IN FCγ RECEPTOR-MEDIATED PHAGOCYTOSIS

4.1 Introduction

Macrophage cells are terminally differentiated myeloid cells with highly specialised roles in the innate and acquired immune systems. They are present ubiquitously in the stromal compartment of tissues under normal physiological conditions, and increase markedly in number with the onset and progression of many pathological states. The role of macrophages in innate immunity is mainly centred around their ability to internalise and degrade exogenous particles, a function more commonly referred to as phagocytosis, and their involvement in acquired immunity includes the production and secretion of regulatory molecules such as cytokines, chemokines and nitrogen oxide (Gordon, 1995).

Macrophage cell morphology can vary depending on surrounding environmental conditions as external stimuli can induce the formation of plasma membrane ruffles, fillopodia extensions and/or cytoplasmic vesicles, all of which modify the morphology of macrophage cells. *In vivo,* macrophages cells are highly motile, trafficking into and through tissues in response to various chemokines. *In vitro,* macrophage cells can be cultured as an adherent monolayer of cells. Cells generally have a flattened appearance due to the formation of cytoplasmic extensions and strong adherence to the tissue culture substratum.

Macrophage adherence and migration requires the rearrangement of cytoskeletal structures (Gordon, 1995). These changes are induced by the activation of plasma membrane receptor proteins some of which are also able to facilitate the recognition of exogenous particles for phagocytosis, as well as trigger the release of secretory molecules such as mediators of inflammation, growth factors, cytokines and cytocidal intermediates. Receptors with a specialised adhesion function include sialoadhesin, CD32, integrins, V-CAM and various receptors for extracellular matrix components, while plasma membrane molecules such as the Type 3 complement receptor (CR3), Fcγ receptor and MSR (macrophage scavenger

receptor) are able to participate in both cell adhesion and particle internalisation (Gordon, 1995). A complex relationship thus appears to exist between cell adhesion and particle internalisation, and components of the cytoskeleton are shared between the two processes. Adherent cells often change in shape and round up during particle internalisation, as known participants of cell adhesion including cytoskeletal components such as paxillin, talin, vinculin, α-actinin, protein kinase Cα, MARCKS (Myristiloylated Alanine Rich C Kinase Substrate) and MacMARCKS (Macrophage-enriched Myristiloylated Alanine Rich C Kinase Substrate) are recruited to the phagocytic cup (Allen and Aderem, 1995; 1996).

Professional phagocytes such as macrophage cells are highly specialised myeloid cells that are able to internalise exogenous particles with high efficiency. They act as professional scavengers that remove non-self material such as invading microorganisms, and clear altered-self material such as apoptotic cells, senescent erythrocytes, immune complexes and inflammatory products. Phagocytosis is consequently a complex process that can be initiated by a range of plasma membrane receptors. To simplify subsequent analysis, the work described in this chapter has concentrated on the study of the signal transduction pathway(s) involved in Fc gamma (Fcγ) receptor-dependent phagocytosis.

During Fcγ receptor-mediated phagocytosis, exogenous particles are recognised by immunoglobulin (Ig) molecules. The coating of invading particles by Ig molecules such as IgG marks them for degradation. IgG molecules are the major antibody class produced in secondary immune responses. Cross-linking of Fcγ receptors on the surface of macrophage cells by IgG molecules initiates the process of actin polymerisation that is necessary to form the phagosome, and culminates in actin-based internalisation of the particle (reviewed in Aderem and Underhill, 1999; Kwiatkowska and Sobota, 1999).

Some of the molecular events that take place during FcγR-mediated phagocytosis have also been identified. Immunoreceptor tyrosine-based activation motifs (ITAMs) located in the

gamma chain of the Fcγ receptor are first phosphorylated (Greenberg, 1994; Park et al., 1995) by Src family kinases found in association with clustered Fcγ receptors. The 72 kD Syk kinase is then recruited to phosphorylated ITAMs in the signalling compartment of the γ chain of the receptor via an SH2-pTyr interaction (Agarwal et al., 1993; Kiener, 1993). The association of Syk with activated receptors facilitates the phosphorylation and activation of Syk by Src family kinases (Crowley et al., 1997). This event is critical for cytoskeletal rearrangement in macrophage cells (Cox et al., 1996; Crowley et al., 1997). Other membrane proximal events that take place subsequent to Syk activation during FcγR-mediated phagocytosis are yet to be fully characterised. Recent reports however suggest that the phosphorylation of the 36 kD isoform of the anchoring protein LAT (Tridandapani et al., 2000) and that of the adaptor molecule SLP-76 (Bonilla et al., 2000) might be involved in FcγR-mediated signal transduction. The chronological details of the signal transduction pathway that induces actin rearrangement and phagosome formation are yet to be fully characterised. A number of signalling molecules have however been identified. These include members of the phosphatidylinositol 3-Kinase (PI3K) family (Ninomiya et al., 1994; Araki et al., 1996), the Rho family of small guanosine triphosphatases (Rho GTPases) (Hackam et. al, 1997; Caron and Hall, 1998), protein kinase C (PKC) (Zheleznyak et al., 1992) and myristoylated alanine rich C kinase substrate (MARKCS) (Allen et al., 1995) and MARCKS-related protein (Mac-MARKCS) (Zhu et al., 1995).

The aim of the work described in this chapter was to demonstrate a potential role for the Tec protein tyrosine kinase in macrophage cell function, especially in the process of FcγR-dependent phagocytosis. The rationale for this approach was derived from observations that the immediate events that follow the activation of the B and T cell receptor systems have so far proven to be conserved in the FcγR system. In fact, all antigen receptor systems appear to include the phosphorylation of ITAMs in the intracellular subunit of the receptors, the

activation of Src and Syk kinases and the subsequent increase in the levels of intracellular calcium and PKC function. In the B and T cell receptor system, the Tec family kinases Btk and Itk have been shown to act downstream of Src and Syk kinases to propagate the signal initiated by activated receptors (Schaeffer and Schwartzberg, 2000). Given the highly conserved nature of these receptor systems, Tec is proposed to perform an analogous role to that of Btk and Itk, in the FcγR system of macrophage cells.

The J774 macrophage cell line is a well established cell culture system that is widelyused in studies of FcγR-mediated phagocytosis (Hackam et al., 1997, Caron and Hall, 1998). The use of an established cell line to study phagocytosis provides a replenishable source of macrophage cells and eliminates the need to isolate primary macrophage cells from an animal source. J774 cells also have large cytoplasms that facilitate the visualisation of particle internalisation. This property is ideal for the use of immunofluorescence to follow the localisation of signalling proteins during phagocytosis. Zymosan A has been used as a phagocytic target in these studies. This phagocytic target consists of a preparation of yeast cell wall protein-carbohydrate complexes that can readily be opsonised with purified IgG molecules.

Two approaches were taken to identify a role for Tec in phagocytosis. Firstly, indirect immuno-fluorescence was used to follow the re-distribution of Tec, during particle internalisation. Similar methods have been used elsewhere to detect phagosomal enrichment of various signalling molecules including that of the protein tyrosine kinase Syk (Strzelecka et al., 1997b), substrates of the serine/threonine kinase PKC such as pleckstrin (Brumell et al., 1999) and MacMARCKS (Underhill et al., 1998) and various cytoskeletal proteins such as F-actin, talin (Greenberg et al., 1990) and cofilin (Nagaishi et al., 1999). Secondly, to further characterise the composition of the Tec signalling complex formed upon particle internalisation using immunoprecipitation and western blot analysis were carried out. Such studies are largely

dependent on the use of highly specific antibodies. Numerous attempts had previously been undertaken in this laboratory to generate antibodies that could differentiate between TecIII and TecIV all of which had unfortunately been unsuccessful. All studies presented in this chapter therefore utilised a commercially available polyclonal goat anti-Tec antibody (sc-1109, St Cruz) that recognises an epitope at the carboxy terminus of the mouse Tec protein and will therefore not differentiate between TecIII and TecIV-mediated processes. Time constraints did not permit further investigation of the role of each Tec isoform in FcγR-dependent signal transduction.

4.2 Results

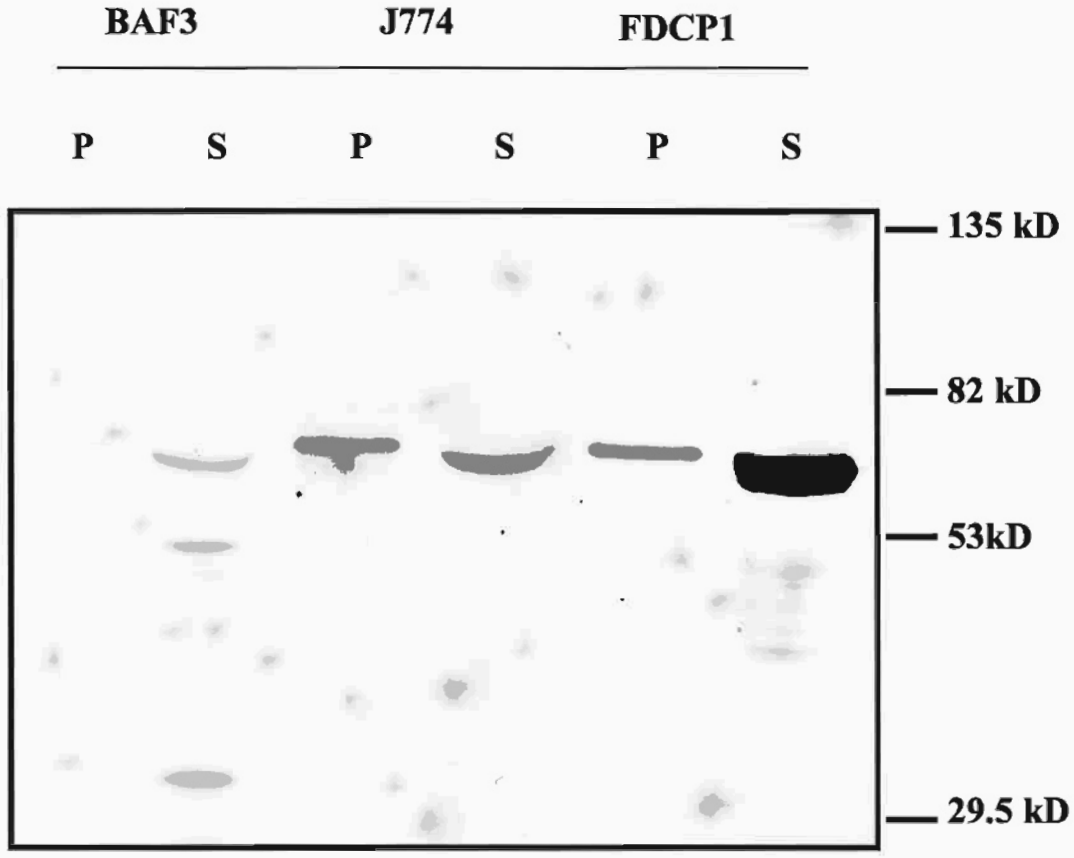
4.2.1 Characterisation of Tec protein expression in hematopoietic cells

As described in section 3.2.2 *TecIV* is the only *Tec* transcript identified in BaF3 pro-B cells and FDCP-1 promyelocytic cells, while both *TecIII* and *TecIV* transcripts are expressed by the J774 macrophage cell line. Given the differential expression observed for *TecIII* and *IV* mRNA in J774 cells, the distribution of the Tec protein in BaF3, FDCP-1 and J774 cells was also investigated by western blot analysis.

Triton-X 100 insoluble cytoskeletal rich pellets and Triton-X 100 soluble cytoplasmic fractions were prepared from J774, FDC-P1 and BaF3 cells as described in section 2.3.25. This method had previously been used by Strzelecka et al., 1997b to demonstrate the cytoskeletal recruitment of Syk during phagocytosis. Samples were resolved by polyacrylamide gel electrophoresis and probed for Tec expression using the sc-1109 antibody as described in section 2.3.26. As seen in Figure 4.1, a single Tec specific band, migrating at approximately 66 kD, was observed in each lane, thus confirming that the sc-1109 antibody was specific for mouse Tec. Interestingly, the staining pattern observed for Tec in J774 cells

Figure 4.1 Characterisation of Tec protein expression in hemapoietic cells

Western blot analysis of Tec in hemapoietic cell extracts using the polyclonal anti-Tec sc-1109 antibody. Whole cell lysates were prepared from $1X10^7$ J774, FDC-P1 and BaF3 cells respectively. Lysates were separated into Triton X-100 insoluble pellets and Triton X-100 soluble fractions by centrifugation. Western blot detection of Tec was carried out using the sc-1109 goat anti-Tec antibody and rabbit-anti goat-alkaline phosphatase conjugated secondary antibody and visualisation used NBT and BCIP substrates.



P = TritonX insoluble fraction
S = TritonX soluble fraction

was identical to that seen in BaF3 and FDC-P1 cells. These later cells had previously been shown to express only *TecIV* mRNA. It is therefore possible that only the TecIV isoform is expressed in J774 cells, despite the presence of both *TecIII* and *TecIV* transcripts in J774 cells. On the other hand it is possible that the 2 kD difference between the two Tec isotypes had not been resolved under the electrophoresis conditions used. It appears that despite the presence of both *TecIII* and *TecIV* transcripts in J774 cells, only the full length TecIV protein is present at detectable levels in J774 cells thus reflecting potential posttranscriptional regulation of *TecIII* or high turn over rate of the TecIII protein in J774 cells.

Two pools of Tec were identified in all cell lysates: a soluble cytoplasmic pool and non-soluble pool that precipitates with components of the cytoskeleton. Interestingly, the insoluble cytoskeletal-associated Tec specific band consistently migrated at a larger molecular weight than its soluble counterpart. This difference in molecular weight is not expected to reflect the presence of the TecIII and TecIV isoforms, as it had been observed in all cell lines tested, including those where *TecIII* transcripts were not present. It is likely that the apparently larger Triton-X 100 insoluble Tec protein results from posttranslational modifications of cytoskeletal-Tec. For the purpose of these studies, TecIV was thus considered to be the main Tec isoform expressed in the hematopoietic cells.

4.2.2 Visualisation of Tec subcellular localisation in hematopoietic cells

Immunohistochemical studies are dependent on the availability of highly specific antibodies that permit the detection of a specific epitope in the context of an intact, fixed cell. As seen in Figure 4.1, western blot analysis had shown that the sc-1109 antibody was highly specific for Tec in BaF3 and J774 cell extracts. This antibody was therefore used for subsequent immunofluorescent staining studies of Tec.

BaF3 cells are IL-3 dependent pro-B cells that grow in suspension and can be induced to adhere to fibronectin coated tissue culture plastic surfaces (Shibayama et al., 1998). J774 macrophage/monocyte cells in contrast exist as a mixed population of cells, the majority of which grow adherently. Cell adhesion is an important macrophage cell function. It induces changes in the morphological appearance of the cell that are primarily driven by the rearrangement of the underlying actin cytoskeleton. A range of morphological cell shapes have been observed during the culturing of J774 cells, and these generally correlate with the adherence of cells to the culture substratum. Unattached cells obtained by the displacement of monolayer cell cultures were typically round with little cytoplasm. Within 2-3 hours of seeding, adherent cells were typically flattened with visible plasma membrane ruffle structures. Upon prolonged culturing as an adherent monolayer, macrophage cells were found to generally form fine cytoplasmic extensions that could, in some cases, reach several times the length of the body of the cells.

Using the sc-1109 antibody, immunofluorescent staining studies were carried out in BaF3 and macrophage cells to investigate differences in the distribution of Tec and correlate these differences with specific cell morphology (Figure 4.2). As seen in Figure 4.2Ai, BaF3 cells were generally round with little cytoplasm and no cytoplasmic extensions and Tec was generally diffuse in the cytoplasm of BaF3 cells, except when clumps of cells were observed, in which case Tec was enriched at regions of contact between the cells (Figure 4.2Aiii). Rarely, BaF3 cells were identified that had adhered to the finbronectin coated glass coverslips that were morphologically distinctive from BaF3 cells grown in suspension due to the presence of plasma membrane ruffle structures enriched in Tec (Figure 4.2Aii). This suggests that in the absence of plasma membrane rearrangement, Tec is diffuse in the cytoplasm of BaF3 cells, and that upon contact either with other cells or with the culture substratum, changes in the plasma

Figure 4.2 Characterisation of Tec subcellular distribution in hemapoietic cells

Immunofluorescence detection of Tec in hemapoietic cells. Cells were fixed in methanol and permeabilised in 0.1% Triton X-100. Tec was detected using goat anti-Tec (sc-1109) and visualised using FITC-conjugated rabbit anti-goat antibody.

A) Immunofluorescent staining of Tec in BaF3 pro-B cells.

BaF3 cells were plated on fibronectin coated glass coverslips to facilitate adherence and processing of samples. (i-iii) show three different staining patterns typically observed for Tec in BaF3 cells. Arrows indicate regions of plasma membrane Tec enrichment.

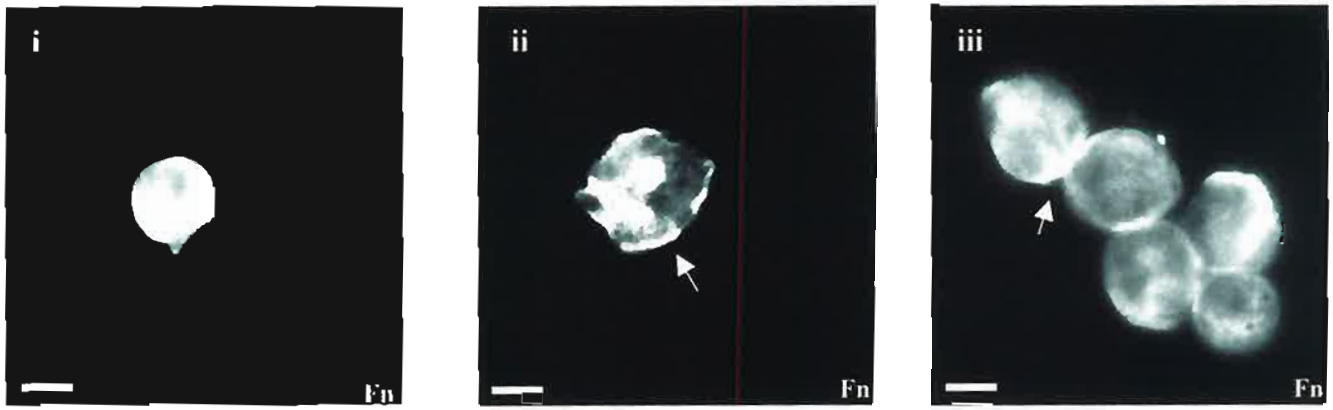
Bar = 10μM

B) Immunofluorescent staining Tec in J774 cells.

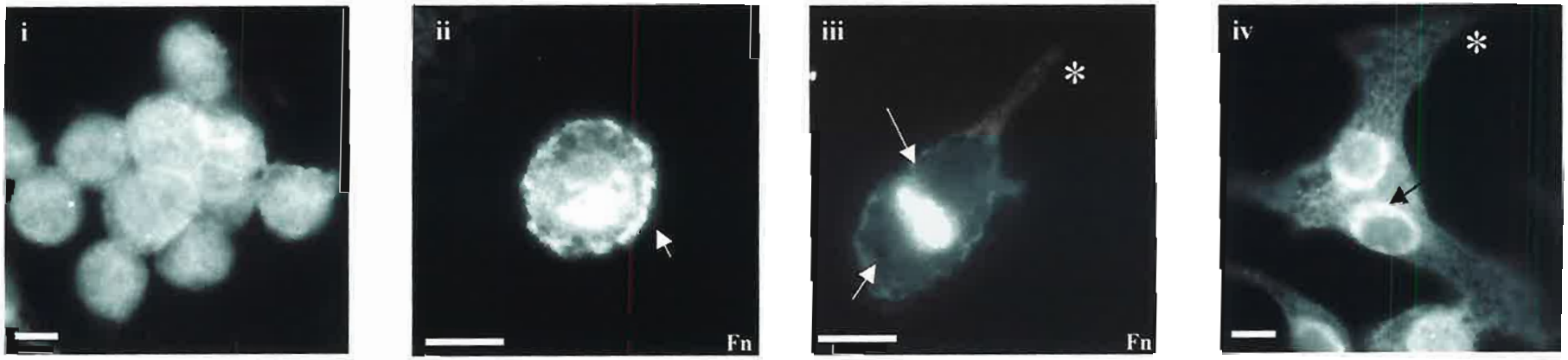
Tec staining of J774 cells (i) kept in suspension prior to fixing, or (ii) allowed to adhere on fibronectin coated glass coverslips for approximately 3 hours, or (iii) 6 hours. (iv) Tec staining in J774 plated on glass coverslips for at least 24 hours.

Bar = 10μM

A) Subcellular localisation of Tec in BaF3 cells



B) Subcellular localisation of Tec in J774 cells



membrane and/or underlying cytoskeleton are associated with the recruitment of Tec to the plasma membrane at sites of contact.

J774 cells kept in suspension were also typically round, with little cytoplasm and no cytoplasmic extensions, and Tec staining in these cells was predominantly diffuse in the cytoplasm (Figure 4.2Bi). When cells were allowed to adhere to glass coverslips for up to three hours, Tec staining remained detectable in the cytoplasm. A narrow strip of intense staining was generally visible at the periphery of the cell, indicating redistribution to either the plasma membrane or the underlying membrane cytoskeleton (Figure 4.2Bii). J774 cells cultured for 6-12 hours as adherent cells, readily produce flattened and elongated cells with membrane ruffle-like structures enriched in Tec (Figure 4.2Biii). Tec was also frequently observed to follow a filamentous, web-like staining pattern within the cytoplasm of flattened cells (Figure 4.2Biv). Overall, it appears that the distribution of Tec in J774 cells is highly heterogenous and dependent on the morphology of the cell.

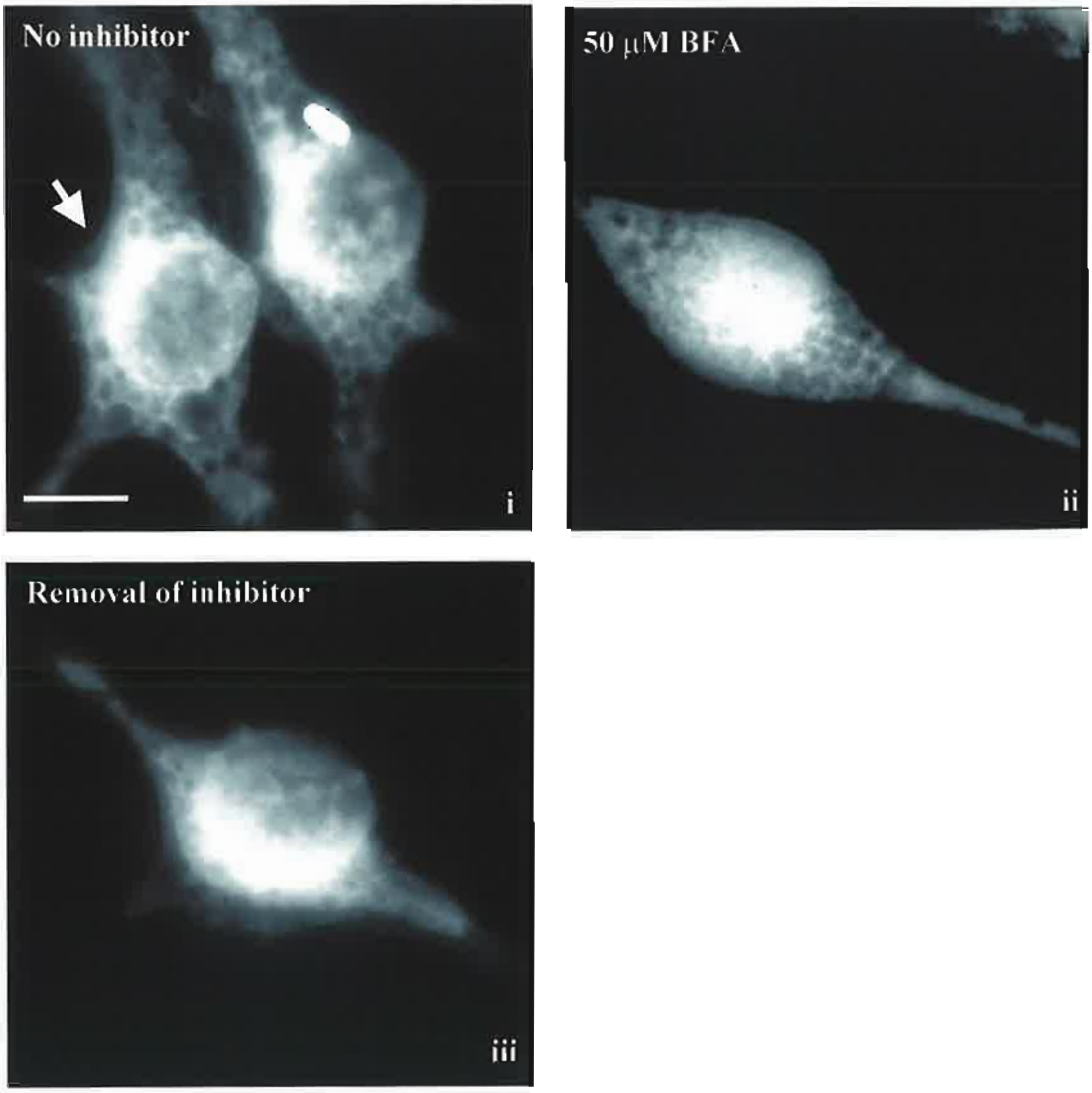
Throughout these studies, intense peri-nuclear Tec staining was often observed that was suggestive of the golgi (Figure 4.3i). This accumulation of Tec near the nucleus was further investigated using the golgi-disrupting compound Brefeldin A. The inhibitory compound was added to J774 cultures for 30 minutes after which cells were fixed and stained for Tec. As shown in Figure 4.3ii, addition of Brefeldin A induced a change in Tec staining. Tec was more diffuse in the cytoplasm as the perinuclear pool of Tec appeared to have been dispersed. When the inhibitor was removed and cells were allowed to recover in normal medium prior to fixing and staining, accumulation of Tec at the peri-nuclear region reappeared (Figure 4.3iii).

The presence of Tec in the Triton-X 100 insoluble cytoskeletal fractions of hematopoietic cells (Figure 4.1), together with the presence of Tec rich filamentous structures in adherent macrophage cells (Figure 4.2Biv) suggested its association with components of the

Figure 4.3 Characterisation of peri-nuclear pool of Tec in J774 cells

Immunofluorescence staining of Tec J774 cells (i) plated on glasscoverslips, (ii) exposed to 5 μM Brefeldin A and (iii) following removal of Brefeldin A. Cells were exposed to inhibitor for 30 minutes, fixed, permeabilised and stained for Tec using goat anti-Tec (sc-1109) and visualised using FITC-conjugated rabbit anti-goat antibody. The strong perinuclear accumulation of Tec in untreated cells is highlighted by arrows in (i).

Bar = 10μM


No inhibitor
i
50 μM BFA
ii
Removal of inhibitor
iii

cytoskeleton. Fibronectin is an extracellular matrix protein that promotes macrophage spreading and migration (Meng and Lowell, 1998), and induces actin polymerisation in neutrophils, monocytes and macrophages (Yang et al., 1994). To facilitate the visualisation of actin rich cytoskeletal structures, J774 cells were seeded on fibronectin coated coverslips and stained for Tec using sc-1109 and for filamentous actin using TRITC-labelled phalloidin. As seen in Figure 4.4, both staining patterns showed extensive web-like networks within cytoplasmic extensions (compare Figures 4.4A and D to 4.4B and E). Overlaying of the two images identified colocalisation of Tec and F-actin especially in the fine filamentous extensions found to protrude from the cytoplasm (Figure 4.4 C and F). These observations suggested that cell spreading induces remodelling of the actin cytoskeleton and promotes the redistribution of Tec from a diffuse cytoplasmic pool to the submembranous actin cytoskeleton.

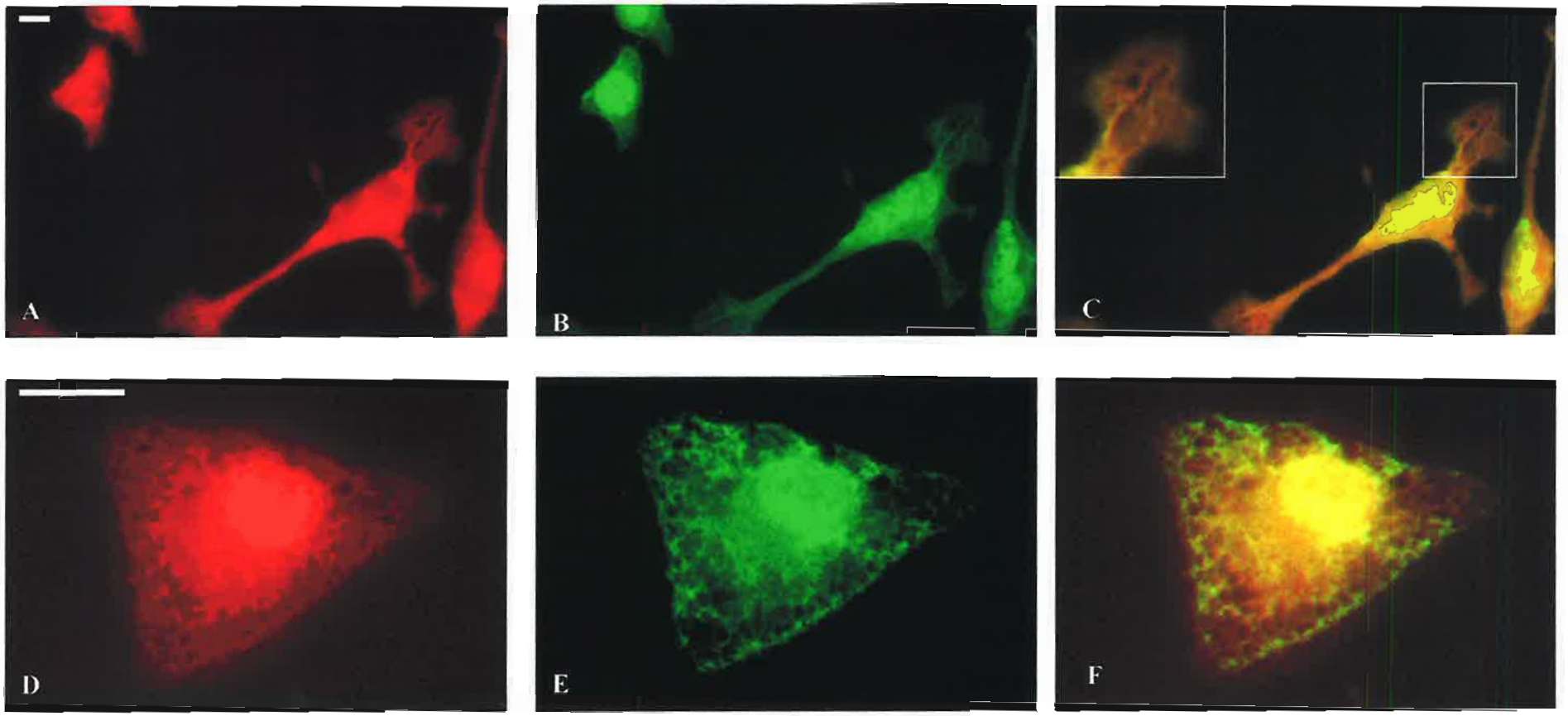
4.2.3 Immunofluorescence analysis of Tec during FcγR-mediated phagocytosis

Phagocytosis is a macrophage-specific function that requires extensive cytoskeletal rearrangement. Because Tec had been identified to colocalise with the actin cytoskeleton of adherent J774 cells, immunofluorescence was used to follow the subcellular distribution of Tec during the formation of FcγR-dependent phagocytic cups. J774 cells were exposed to Zymosan A opsonised with human IgG molecules at 4°C and phagocytosis was allowed to proceed at 37°C for increasing periods of time. Phagocytosis proceeded in a time dependent manner, with internalisation of the particle being essentially complete within 10 minutes. Screening of the secondary FITC-conjugated antibody had been carried out to confirm that it did not cross react with the opsonised Zymosan A particles or the J774 cells in the absence of an appropriate primary antibody (data not shown).

Figure 4.4 Colocalisation studies of Tec and F-actin in resting J774 cells

Co-localisation of (A and D) Tec detected with sc-1109, and (B and E) F-actin visualised with phalloidin-TRITC in cells plated on fibronectin-coated glass coverslips. (C and F) represent merges of A-B, and D-E respectively. Inset in C shows a higher magnification of the boxed area highlighting pseudopod extensions.

Bar = 10μM


A
B
C
D
E
F

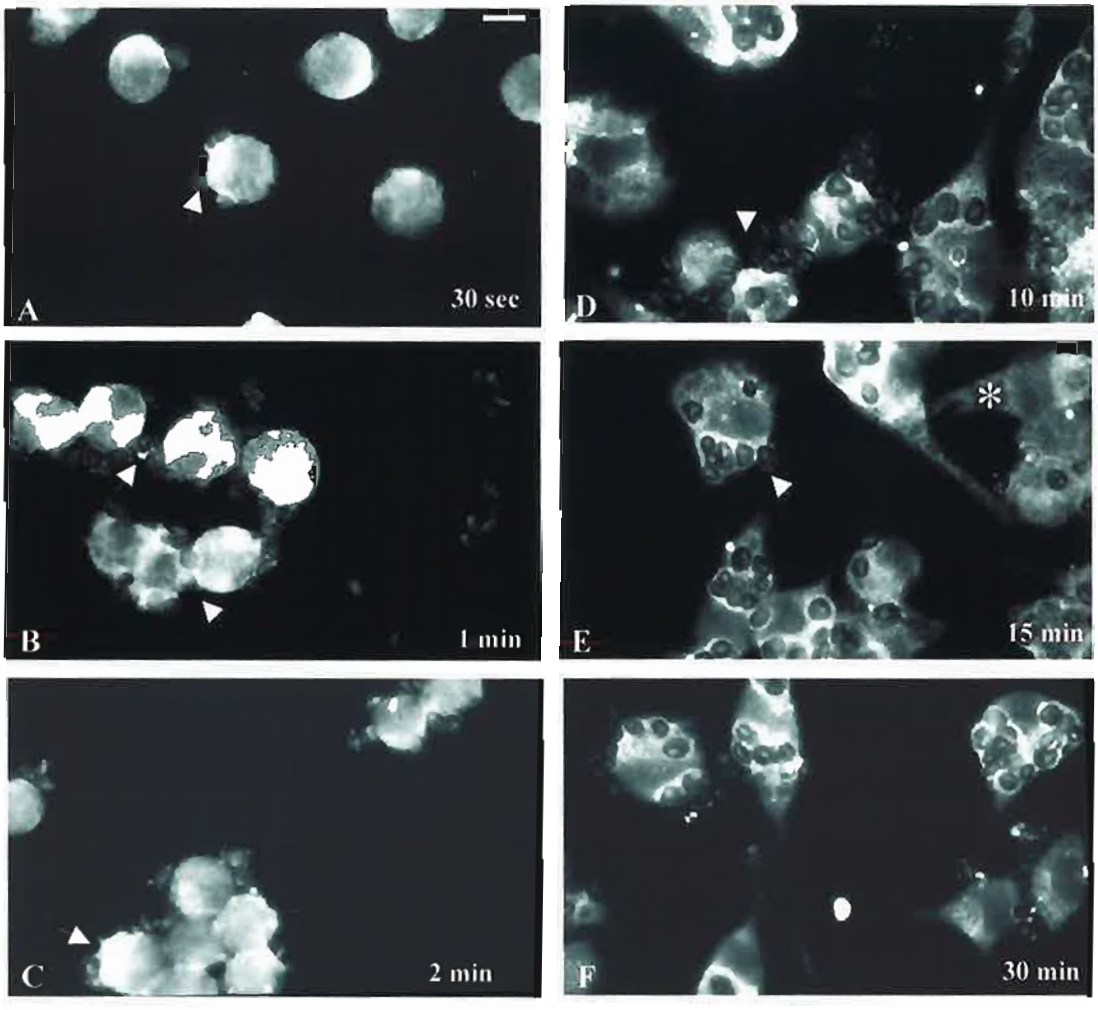
Comparison of the fluorescence pattern seen in Figure 4.5A with the fluorescence pattern of Tec in non-stimulated cells (Figure 4.2A) shows strong recruitment of Tec to the periphery of the macrophage cells following exposure to opsonised Zymosan A particles. In these cells, Tec was visible in peripheral regions of the cells that interacted with the exogenous particle. These structures are more commonly referred to as phagocytic cups. Tec was visible at the phagocytic cups within 30 seconds phagocytosis, persisted as membrane extended to engulf the particle and appeared to be shed following internalisation (Figure 4.5A i-vi). At any given time point, the population of phagocytosing cells was relatively heterogenous. This heterogeneity appeared to result from differences in the numbers of opsonised Zymosan A particles associated with a given phagocytic cup. Generally, where large numbers of particles were being internalised, particle internalisation appeared to be delayed compared to the internalisation of smaller numbers of exogenous particles. It is likely that this reflected larger requirements for cytoskeletal rearrangement and plasma membrane components necessary for the formation of a sealed phagosome. Consequently, even after 30 minutes, cells could be identified where Tec staining was observed at the phagosome, while the remaining cells which appeared to have completed particle internalisation had shed Tec (identified by the more diffuse staining of Tec in these cells) (Figure 4.5Av asterisk). In those later cells, the phagosome is expected to be undergoing maturation steps independent of Tec function.

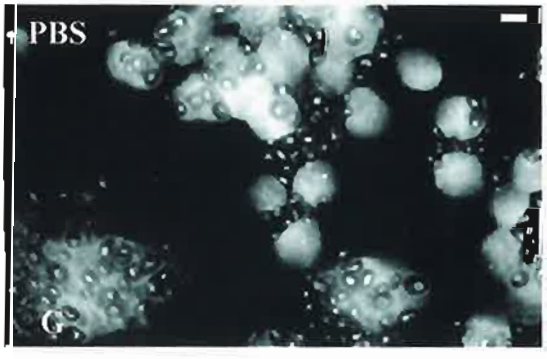
Because non-opsonised Zymosan A particles can also be internalised independently of the Fcγ receptor, it was necessary to confirm that the redistribution of Tec to the phagosome was dependent on opsonisation of target particles with IgG molecules. This control also confirmed that the apparent redistribution of Tec did not reflect the exclusion of cytoplasmic material from the region of particle ingestion. Phagocytosis assays were carried out using non-opsonised Zymosan A and cells were fixed and stained for Tec. As seen in Figure 4.5B non-opsonised particles were efficiently internalised by J774 cells, but Tec staining in these cells

Figure 4.5 **Characterisation of the subcellular distribution of Tec in J774 cells engaged in the phagocytosis of IgG opsonised Zymosan A**

J774 cells were exposed to *h*IgG-opsonised Zymosan A for 20 minutes at 4°C, and phagocytosis was allowed to proceed at 37°C for the appropriate period of time. Cells were fixed, permeabilised and stained at (A) 30 sec, (B)1 min, (C) 2 min, (D) 10 min, (E) 15 min and (F) 30 min of phagocytosis. (G) control experiment showing Tec staining pattern during the phagocytosis of PBS-opsonised Zymosan A. Tec staining and visualisation was carried out using goat polyclonal anti-Tec antibody (sc-1109) and FITC conjugated secondary rabbit-anti goat antibody. Arrows highlight the enrichment of Tec at the phagosome in (A-E). Asterisk in E indicates internalised particles that were devoid of Tec.

Bar = 10μM


A
30 sec
B
1 min
C
2 min
D
10 min
E
15 min
F
30 min


PBS
G

remained largely diffuse in the cytoplasm, independent of the extent of particle internalisation. The recruitment of Tec to the phagocytic cup therefore appears to be dependent on opsonisation of target particles with IgG molecules thus suggesting a role for Tec in FcγR dependent phagocytosis.

Syk is a well established component of the FcγR signalling system (Cox et al., 1996 and Crowley et al., 1997) and is also targeted to the phagosomal membrane (Strzelecka et al., 1997b). Co-staining of macrophage cells for Syk and Tec were originally planned to confirm the colocalisation of these signalling molecules at the phagosomal membrane. Unfortunately, the secondary antibodies available had been found to cross react. Immunofluorescence analysis of Syk during phagocytosis was thus carried out. (Figure 4.6). Syk was generally found to be enriched at the phagocytic cup prior to phagosome closure (Figure 4.6i). When particles had been drawn into the body of the cell, intense Syk staining was not observed to surround internalised particles and Syk was diffuse in the cytoplasm of these cells (Figure 4.6ii asterisks). The internalisation of IgG opsonised Zymosan A triggers the redistribution of two families of PTKs, Syk and Tec, to the phagosomal membrane. The redistribution of Syk to the phagosome appeared to be more transient than that of Tec. The signalling mechanisms that dictate the dissociation of Tec and Syk from the phagosomal membrane must thus differ.

Because targeting of Tec to the phagosome had been detected upon particle internalisation and given that F-actin had been shown to also accumulate at the phagosomal membrane (Greenberg et al., 1990), co-localisation of Tec and F-actin at the phagosomal membrane was also tested. As seen in Figure 4.7, strong colocalisation of Tec and F-actin was apparent at the forming phagosomes and was strongest prior to the closure of the phagosome.

Figure 4.6 Characterisation of the subcellular distribution of Syk during the internalisation of IgG opsonised Zymosan A

J774 cells were exposed to *h*IgG-opsonised Zymosan A for 20 minutes at 4°C, and phagocytosis was allowed to proceed at 37°C for 5 minutes. Cells were fixed, permeabilised and stained for Syk using SC-1077 and detected using a goat-anti-rabbit-TRITC conjugated secondary antibody. i and ii show two different populations of cells stained for Syk. Arrows highlight cells with intense accumulation of Syk at the phagosome. * highlights cells that appear to have undergone internalisation of particles.

Bar = 10μM

Subcellular localisation of Syk during FcγR-mediated phagocytosis in J774 cells

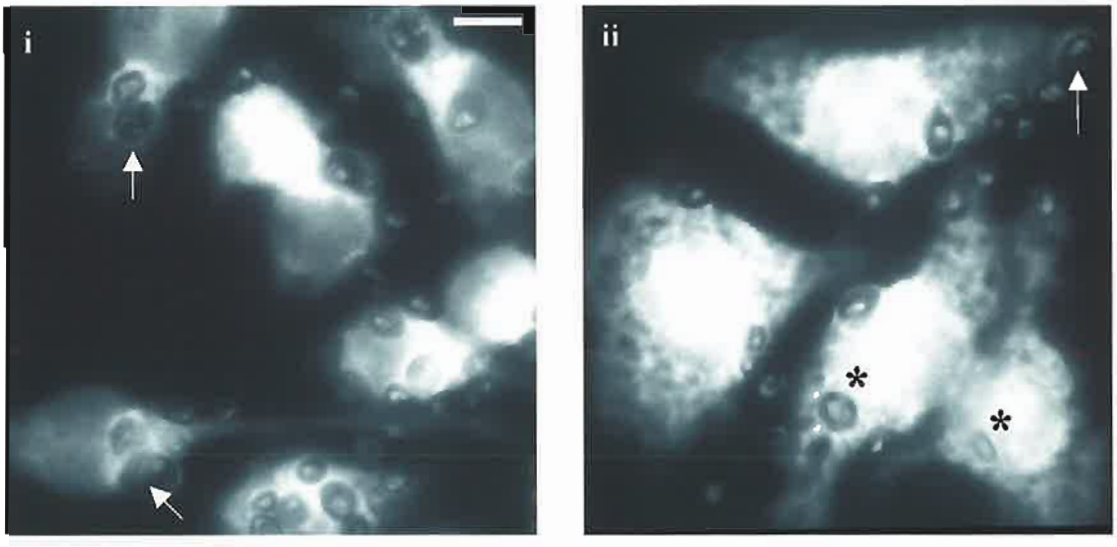
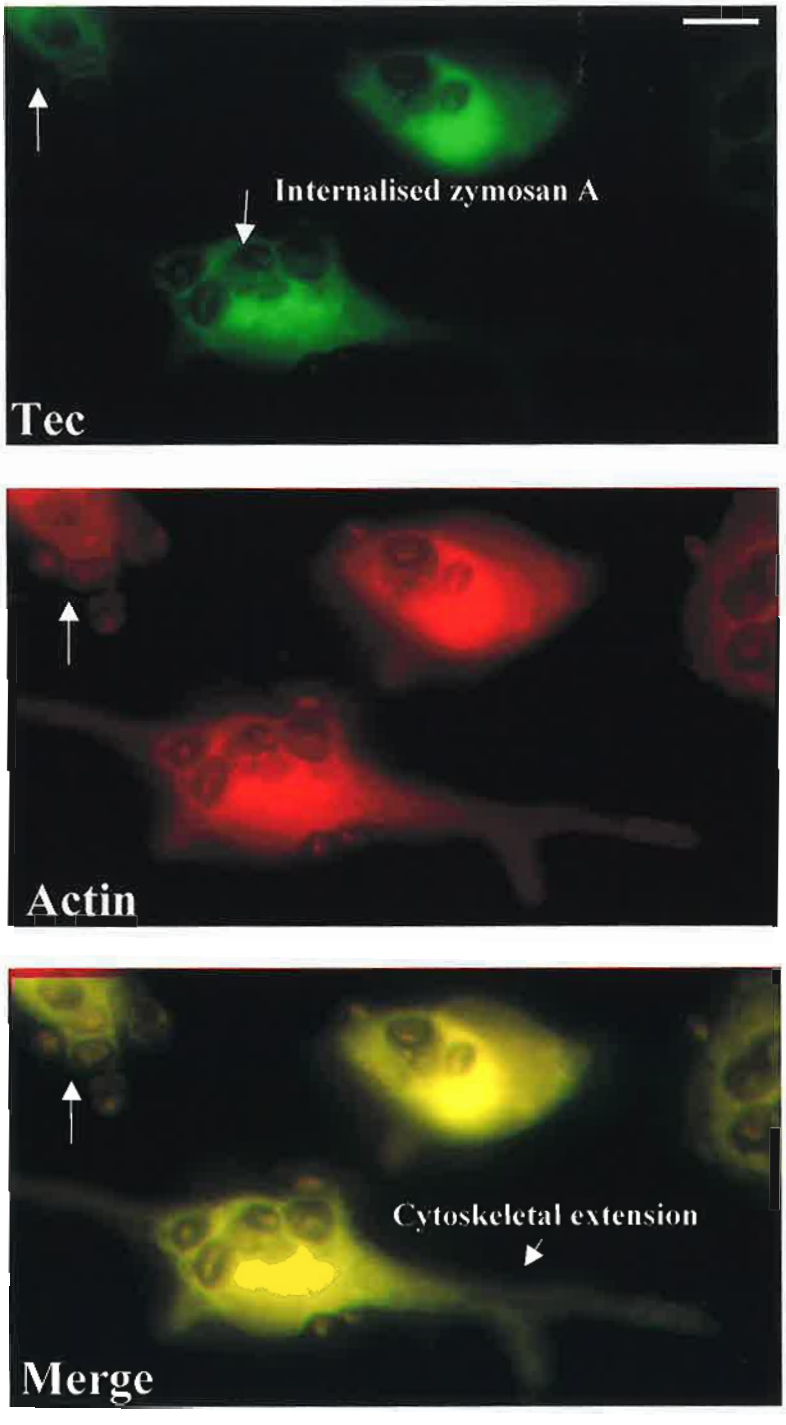


Figure 4.7 Characterisation of Tec and F-actin in J774 cells engaged in the internalisation of IgG-opsonised Zymosan A

J774 cells were exposed to *h*IgG-opsonised Zymosan A for 20 minutes at 4°C, and phagocytosis was allowed to proceed at 37°C for 5 minutes. Cells were fixed and stained for Tec using polyclonal goat anti Tec sc-1109 and FITC-conjugated rabbit anti-goat secondary antibody. Filamentous actin was detected using TRITC-cojugated phalloidin.

Tec staining is represented in green, F-actin staining is represented in red and a merge picture of the two, depicting colocalisation of the two in yellow.

Bar = 10μM


Internalised zymosan A
Tec
Actin
Cytoskeletal extension
Merge

4.2.4 Investigation of the localisation of Tec to the phagocytic cup in presence of the PI3K inhibitor LY294002

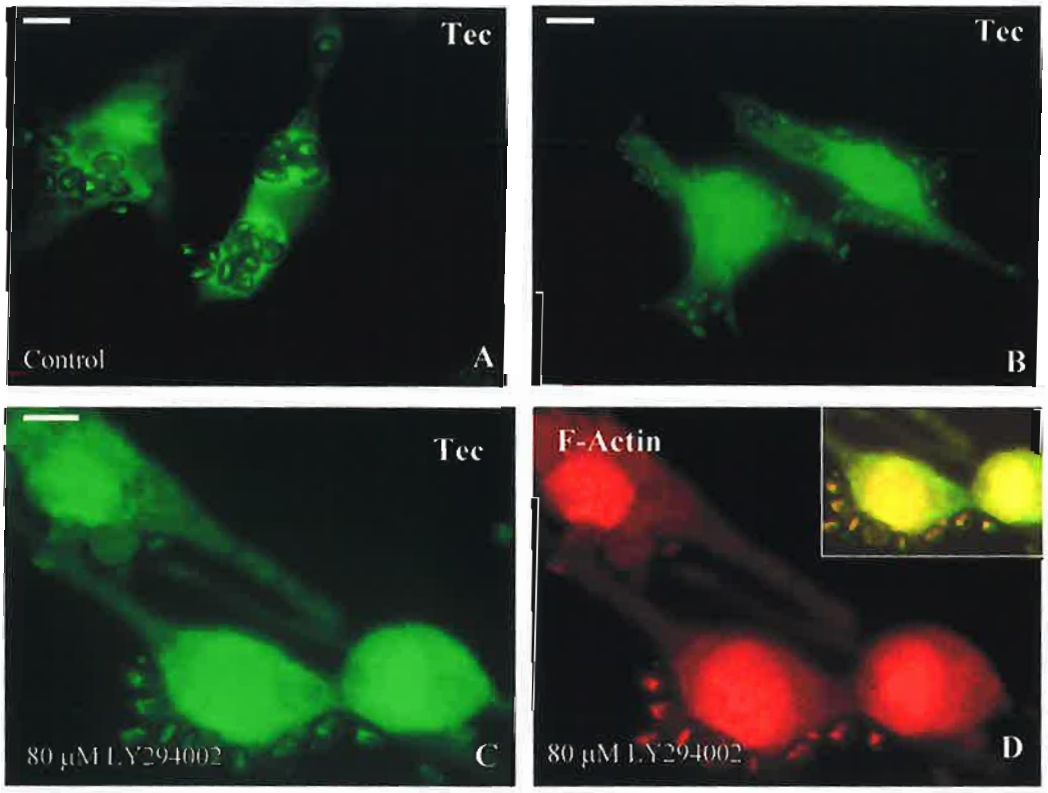
$PI(3,4,5)P_3$, is a product of the lipid kinase PI3-K which acts as a critical secondary messenger during phagocytosis. $PI(3,4,5)P_3$ binds the PH domain of Tec/Btk kinase (Hyvonen and Saraste, 1997) and targets Tec/Btk protein tyrosine kinases to the plasma membrane (Varnai et al., 1999). LY294002 is an inhibitor of PI3-K which has been reported to inhibit FcγR-mediated signal transduction and prevent particle internalisation (Ninomiya et al., 1994). As described in chapter 3, LY294002 has a visible effect on the subcellular distribution of GFP-tagged Tec expressed in COS-1 cells suggesting a possible role for Tec as a downstream effector of PI3-K. The effect of LY294002 on the subcellular distribution of endogenous Tec in J774 macrophage cells during phagocytosis was therefore investigated using immunohistochemistry.

During these studies, LY294002 concentrations had been tested that ranged between 5μM and 80μM. Figure 4.8 shows macrophage cells exposed to 40 μM (Figure 4.8B) and 80 μM (Figure 4.8C and D). As shown in Figure 4.8B-D, the addition of LY294002 affected particle internalisation in a dose dependent manner. Control samples showed the expected high levels of internalised particle after 30 minutes (Figure 4.8A). Figure 4.8B and C show that LY294002 blocked the internalisation of Zymosan A particles but did not abolish particle binding. At 40 μM, some particles could be identified that had been internalised after 30 minutes although the majority of Zymosan A particles remained bound at the periphery of the cells. Tec staining in these cells was generally diffuse and no redistribution to sites of particle binding could be identified (Figure 4.8B). Particle binding was significantly impaired at 80 μM of LY294002 as reflected by the small numbers of Zymosan A particles seen at the periphery of the cell after 30 minutes (Figure 4.8C). In these cells, the phagosomal membrane failed to

Figure 4.8 Investigation of the requirement for PI3-K activity in the recruitment of Tec to the phagosome using the inhibitory compound LY294002

Immunohistochemical analysis of Tec in (A) control cells, and (B-C) J774 cells undergoing phagocytosis in the presence of (B) 40 μM, and (C) 80 μM LY294002. Cells were exposed to LY294002 for 30 minutes prior to phagocytosis. Phagocytosis was carried out at 37°C for 30 minutes. (D) F-actin costaining of cells in (C); inset shows the mege of F-actin and Tec staining.

Bar = 10μM


Tec
Control
A
Tec
B
Tec
80 μM LY294002
C
F-Actin
80 μM LY294002
D

close despite the fact that phagocytosis had been allowed to proceed for up to 30 minutes and Tec generally showed diffuse fluorescence. Co-staining with phalloidin-TRITC showed that the absence of Tec at sites of particle binding generally corresponded to a lack of actin polymerisation in these regions (Figure 4.8D inset).

4.2.5 Investigation of the Localisation of Tec to the Phagosomal Membrane in Response to Cytochalasin D or Nocodazole Exposure.

As previously mentioned, the formation of the phagosome requires extensive remodelling of the actin cytoskeleton. Cytochalasin D is a potent inhibitor of actin polymerisation and has been reported to affect phagocytosis (Newman et al., 1991; Koval et al., 1998). It was thus used to determine whether the recruitment of Tec to the phagosome was dependent on a functional actin cytoskeleton. Cells were treated with Cytochalasin D for 30 minutes prior to exposure to IgG osponised Zymosan A and phagocytosis was carried out in the presence of the inhibitor. As seen in Figure 4.9A, Cytochalasin D inhibited FcγR-mediated phagocytosis in a dose dependent manner. At 50 nM, Cytochalasin D had no evident effect on particle internalisation and Tec remained targeted to the phagosome (Figure 4.9Aii). At 500nM, the morphology of J774 cells was dramatically affected and cells appeared to round up and loose their cytoplasmic extensions (Figure 4.9Aiii). It appears that this concentration of Cytochalasin D is toxic to macrophage cells. Particle binding in these cells was abolished and Tec staining was generally diffuse in the cytoplasm. At 100nM Cytochalasin D however, phagocytosis was partially impaired (Figure 4.9B). Phagocytic cups were formed but generally failed to extend and enclose Zymosan A particles. As F-actin staining could be detected at some phagosomes, suggesting that F-actin polymerisation had only been partially impaired. When F-actin was detected at the phagosomal membrane, F-actin always co-localised with Tec accumulation at the phagocytic cup. In contrast, phagocytic cups that lacked F-actin staining

Figure 4.9 Investigation of the dependence of Tec redistribution to the phagosomal membrane on actin and tubulin polymerisation

Cells were plated on glass coverslips and exposed to inhibitors for 30 minutes prior to assay. Phagocytosis was carried out for 30 minutes at 37°C in the presence of the inhibitory drugs.

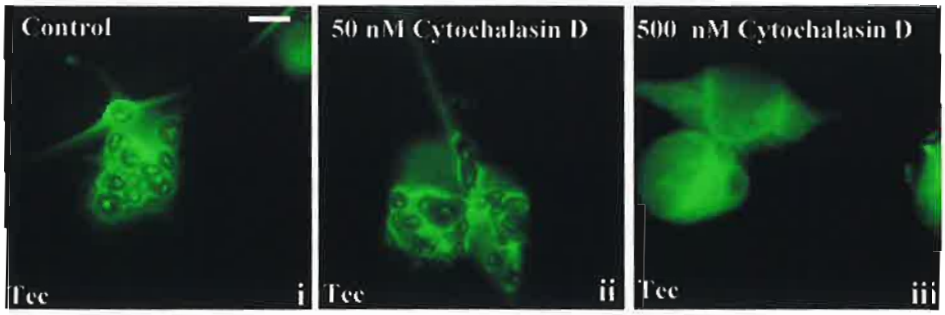
A. Tec staining in (i) control cells, and cells treated with (ii) 50 nM, (iii) 500 nM Cytochalasin D.

B. Tec and F-actin staining in phagocytosing cells in the presence of 100 nM Cytochalasin D. Tec staining is represented in green, F-actin in red and a merge of the two pictures shows colocalisation of Tec and F-actin in yellow.

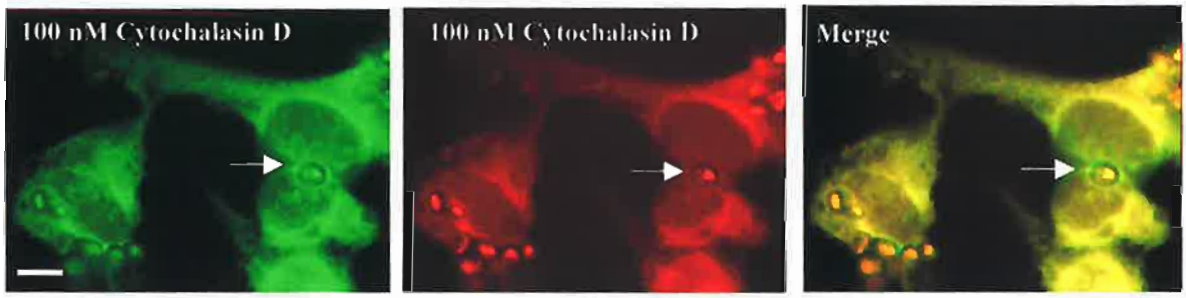
C. Tec and F-actin staining in phagocytosing cells in the presence of 5 μM Nocodazole. Tec staining is represented in green, F-actin in red and a merge of the two pictures shows colocalisation of Tec and F-actin in yellow.

Bar = 10μM

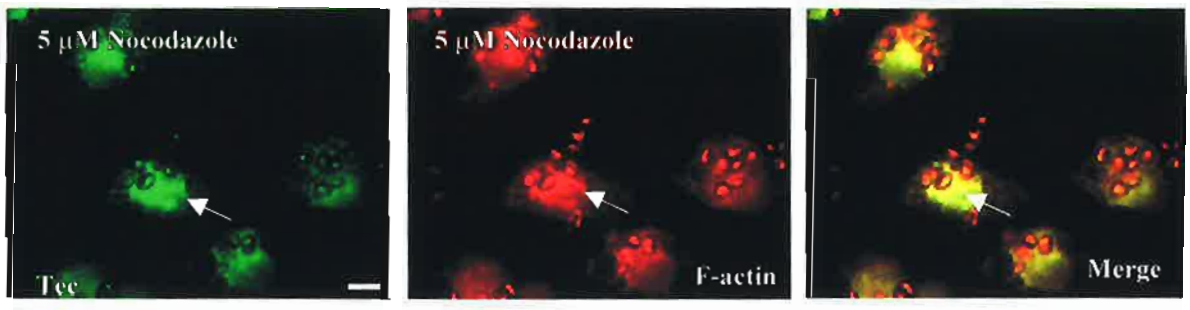
A. Tec staining in presence of Cytochalasin D



B. Tec/F-actin colocalisation in presence of Cytochalasin D



C. Tec/F-actin colocalisation in presence of Nocodazole



still displayed strong localisation of Tec (Figure 4.9B arrow). This suggests that although the rearrangement of cytoskeletal structures is closely associated with the recruitment of Tec to the phagosome during phagocytosis, it is not an essential pre-requisite for the redistribution of Tec to the phagocytic cup. It is therefore predicted that the recruitment of Tec to the phagosome precedes actin polymerisation during FcγR-dependent phagocytosis.

Since actin cytoskeletal structures surrounding the phagosome eventually exchange for microtubules, a possible role for microtubule assembly for the redistribution of Tec was investigated (Figure 4.9C). The microtubule inhibitory drug Nocodazole was added to J774 culture for 30 minutes prior to phagocytosis and particle internalisation was carried out in the presence of the inhibitor at concentrations previously reported to affect microtubule assembly (Newman et al., 1991). At 5μM Nocodazole did not affect particle engulfment and Tec remained localised to the phagocytic cup as the plasma membrane extended to surround bound particles and was shed following phagosomal closure as particles became internalised. The microtubule cytoskeleton does therefore not appear to be critical for the dynamics of Tec movement during phagocytosis.

4.3 DISCUSSION

The protein tyrosine kinase Tec is a non-receptor protein tyrosine kinase predicted to be primarily cytosolic in the absence of external stimuli. The PHTH, SH3 and SH2 domains of Tec can mediate protein-lipid and protein-protein interactions that will influence the subcellular localisation of Tec. The activation state of a cell, together with the presence of specific extracellular stimuli, are expected to dictate the subcellular localisation of Tec. This chapter describes the effect of FcγR stimulation during phagocytosis on the subcellular localisation of Tec in the J774 macrophage cell line.

4.3.1 Subcellular localisation of Tec in macrophage cells

Immunohistochemical studies presented in this chapter have confirmed Tec to be highly dynamic in macrophage cells. In these cells, the subcellular distribution of Tec appears to be dependent on the morphological shape of the cells, and at least four pools of Tec have been identified: (i) diffuse cytoplasmic Tec, (ii) plasma membrane targeted Tec, (iii) juxta-nuclear Tec, and (iv) Tec in association with the cytoskeleton.

Based on the current understanding of the Tec family kinase signal transduction pathways, the level of Tec phosphorylation and Tec kinase activity of each subcellular pool are expected to differ. Diffuse cytoplasmic Tec is predicted to represent a non-phosphorylated and thus inactive pool of Tec. In the presence of external stimuli (such as phagocytosis), Tec would be translocated to the plasma membrane, generally in ruffle-like regions and pseudopodial structures. Plasma membrane targeted Tec is expected then to be phosphorylated, most likely by a member of the Src family of tyrosine kinases, thus enabling it to participate in the signalling events required for plasma membrane ruffling and pseudpod extension. The significance of the juxta-nuclear pool of Tec is currently unknown although it appears to be dependent on golgi function. It is however interesting that this intensely staining region is most obvious in cells with extended cytoplasms, and is generally found in conjunction with plasma membrane or filamentous staining of Tec. This might reflect a golgi-dependent mechanism involved in targeting Tec to those locations in the cell. The association of Tec with actin filament web-like structures that extend from the cytoplasm of macrophage cells suggests the involvement of Tec in the molecular events necessary for the spreading of these cells. The phosphorylation and/or activation status of actin-associated Tec remains to be determined.

Tec-actin association is predicted to involve the PHTH domain. A yeast two-hybrid assay has been carried out in this laboratory that utilised the Tec PHTH domain as a bait (Merkel, personal communication). This work has identified the cytoskeletal protein α-actinin

as a Tec PHTH binding protein and the interaction between the two peptides is currently being characterised. In addition, the N-terminal region of the Btk PH domain, a region that is also conserved in Tec, has also been reported to interact with filamentous actin although it remains to be clarified whether this interaction is also mediated by an actin-binding protein or not (Yao et al., 1999). These reports suggests that a possible role for Tec in the rearrangement of the actin cytoskeleton. The phosphorylation of actin binding proteins, such as α-actinin, by Tec, would enable the rearrangement of filamentous actin structures, and facilitate the formation of plasma membrane ruffles and filopodia extensions, both of which are recognised to be associated with cell spreading/adhesion.

4.3.2 A possible role for Tec in cell adhesion

It is well documented that macrophage cells show extremely high adherence to tissue culture plastic and that the culturing of these cells on fibronectin coated surfaces induces the formation of filopodia structures characteristic of cell spreading. Several protein tyrosine kinase families are involved in cell spreading, including Src family kinases and Syk. Macrophage cells deficient in both Hck/Fgr, as well as Hck-/- Fgr-/- Lyn-/- triple mutants are unable to spread on fibronectin-coated surfaces and show impaired migration *in vivo* (Suen et al., 1999; Meng and Lowell, 1998). In the absence of functional Src kinases, PI3-K also fails to redistribute to the plasma membrane upon stimulation with fibronectin, suggesting that this plasma membrane targeted pool of PI3-K is also involved in the rearrangement of actin cytoskeletal structures necessary for cell spreading (Meng and Lowell, 1998).

Several observations suggest the involvement of Syk in the signalling events that are required for the formation of the cytoplasmic extensions that are characteristic of macrophage spreading. Firstly, fibronectin stimulates integrin receptors and leads to the phosphorylation of numerous proteins including that of Syk (Gotoh et al., 1997). Secondly, even in the absence of

fibronectin, it appears that Syk is phosphorylated upon the adherence of macrophage cells to tissue culture plastic alone (Hirano and Kanno, 1999). A critical role for Syk in the adherence of macrophage cells was further demonstrated using the Syk-specific inhibitor Erbstatin (Hirano and Kanno, 1999). Further involvement of Syk in the cell adhesion-dependent signal transduction pathways was also confirmed upon the characterisation of Hck-/-Fgr-/- double mutant macrophages. These cells exhibit significantly decreased phosphorylation of numerous proteins including that of Syk and several actin-associated proteins such as cortactin, vitronectin and tensin (Suen et al., 1999). In these cells, F-actin, paxillin and talin fail to accumulate at the leading edge of the cells thus explaining the absence of filopodial formation. This suggests a link between Syk phosphorylation and the rearrangement of the actin cytoskeleton.

It is thus apparent that several protein tyrosine kinases are involved the process of plasma membrane remodelling during cell adhesion and spreading. In macrophage cells, at least two families of protein tyrosine kinases have so far been implicated in this signalling pathway, both of which have been reported to be closely associated with Tec kinases in the antigen receptor signalling systems (BCR and TCR) (Schaeffer and Schwartzberg, 2000). Based on the subcellular distribution of Tec during macrophage spreading, this thesis proposes that Tec might be involved in the signal transduction pathways involved in cell adhesion and spreading, possibly through signalling from the γ chain of the integrin receptor.

4.3.3 A role of Tec in FcγR-dependent phagocytosis

The effect of the extracellular matrix environment on macrophage cell function is not restricted to its effect on cell spreading and migration as it affects other macrophage functions including phagocytosis (Yang et al., 1994). Macrophage cells plated on fibronectin-coated surfaces for example have an increased ability to phagocytose apoptotic cells (McCutcheon et

al., 1998). At the molecular level, this suggests that the signal transduction pathway(s) activated by cell adhesion must influence and/or overlap at least to some extent, with that involved in phagocytosis.

Mechanistically, phagocytosis requires the coordinated action of numerous molecules including that of cell membrane receptors, signalling proteins and cytoskeletal components. This study is the first to propose a role for the non-receptor tyrosine kinase Tec as a component of the early stages of signalling during FcγR-mediated phagocytosis.

The signal transduction events that follow antigen receptor stimulation are relatively well conserved between members of the antigen receptor family (Kwiatkowska and Sobota, 1999). During BCR, TCR and FcγR signalling, binding of exogenous ligands to membrane bound receptors induces the aggregation of these receptors and is rapidly followed by Src-dependent phosphorylation of ITAMs and phosphorylation and activation of Syk. Btk and Itk are well established effectors of the B cell receptor and T cell receptor respectively, and are critical for receptor function. This chapter proposes an analogous function for the myeloid cell-expressed protein tyrosine kinase Tec in FcγR signalling.

Immunostaining of Tec in macrophage cells exposed to IgG-opsonised Zymosan A indicates that Tec is redistributed to the phagocytic cup during particle ingestion. Similar redistribution has previously been reported for cytoskeletal components such as F-actin, talin, vinculin and actinin (Greenberg et al., 1990), as well as for the protein tyrosine kinase Syk (Strzelecka et al., 1997b). This report is however the first to position Tec, both spatially and temporally, at the site of particle ingestion during phagocytosis. It is expected that at the site of particle ingestion, Tec might come into proximity with other tyrosine kinases such as Src and Syk. Temporal and spatial colocalisation of the Tec family kinase Btk and Syk is also known to take place during BCR signalling through the formation of a multiprotein complex consisting of Syk, Slp-65, Btk and PLCγ. In this system, the adaptor protein Slp-65 plays a

critical role as a molecular adhesive that brings the two kinases in close proximity to their substrate PLCγ (reviewed in Rawlings, 1999). SLP-76 is a homologue of Slp-65 that is expressed in myeloid cells and is tyrosine phosphorylated upon the selective ligation of FcγRI and III (Bonilla et al., 2000). In T cells this SLP-76-dependent complex is anchored at the plasma membrane through the LAT anchoring protein, a protein that has also been implicated in FcγR signalling (Tridandapani et al., 2000). Although SLP-76 appears to be the logical candidate for the formation of Tec/Syk multiprotein complex during phagocytosis, it is apparently not essential during phagocytosis, as FcγR-mediated phagocytosis is not impaired in SLP-76 deficient cells (Clements et al., 1999). It is possible that another member of this family of adaptor proteins is responsible for the formation of a multiprotein signalling complex that includes Tec upon the stimulation of FcγRs.

FcγR stimulation causes a rapid rise in cellular $PI(3,4,5)P_3$ (Ninomiya et al., 1994). This phosphoinositol phospholipid acts as a secondary messenger molecule that recruits regulatory proteins to the plasma membrane via an interaction with phosphoinositide binding modules such as PH domains (reviewed in Martin, 1998). The phagocytic staining of Tec observed during FcγR-mediated phagocytosis suggests a possible role for Tec at the plasma membrane region that might include the remodelling of the plasma membrane during the formation of the pseudopod structures that engulf target particles. Plasma membrane targeting of Tec family kinases is predicted to involve the PHTH domain, probably through binding to $PI(3,4,5)P_3$ molecules in the plasma membrane. The PHTH region is also responsible for the redistribution of Btk to plasma membrane ruffles where it co-localises with F-actin upon cellular activation (Varnai et al., 1999, Nore et al., 2000). The LY294002 compound is a known inhibitor of the PI3-K family that blocks Fcγ-R mediated phagocytosis by interfering with the actin-driven extension of the phagosome surrounding the phagocytic target and preventing the closure of the newly formed phagosome (Araki et al., 1996). As shown in this

chapter, LY294002 also prevents the typically strong polarisation of Tec to sites of particle binding. This suggests that $PI(3,4,5)P_3$ synthesis is necessary for plasma membrane targeting. Tec thus appears to be involved in the PI3-K-dependent process of actin-driven membrane extension that closes the phagosomal membrane around bound particles.

The mechanism of F-actin polymerisation at the forming phagosome during particle internalisation is unclear. Tec family kinases, especially Btk have been shown to target to the membrane ruffle structures where it colocalises with F-actin upon growth factor stimulation (Varnai et al., 1999; Nore et al., 2000). Although the region of involved in this interaction is yet to be fully characterised, the first 10 amino acids of the Btk PH domain appear to be involved (Yak et al., 1999). Since this region is conserved in Tec it is possible that Tec also physically interacts with F-actin. This would be consistent with the redistribution of Tec to cytoskeletal structures seen during cell spreading or phagocytosis.

Preliminary evidence presented in this chapter suggests a novel role for Tec in FcγR-dependent phagocytosis. Tec was shown to relocate to the phagosome where it colocalises with previously reported effectors of the FcγR system including filamentous actin and Syk during phagocytosis. Based on studies of other antigen receptor systems, Tec is predicted to act as a central component of the early signalling events that take place at the plasma membrane. It is possible that Tec is important for sustaining PLCγ phosphorylation, an event required for extracellular calcium influx, in a manner reminiscent of the function of Btk in BCR signalling (Perrez-Villar and Kanner et al., 1999; Fluckiger et al., 1998). The work described in this thesis and independent observations made in this laboratory also suggest that Tec has a role in the remodelling of the actin cytoskeleton required for macrophage spreading and phagocytosis and that it acts as a link between PI3-K activation and actin cytoskeleton rearrangement.

CHAPTER 5:

GENE TARGETING AT THE TEC LOCUS

5.1 Introduction

5.1.1 Gene targeting

5.1.1.1 General overview

It is now over a decade since the first homologous recombination event was reported in embryonic stem cells (Thomas and Capecchi, 1987). Homologous recombination is a phenomenon that is initiated by the sequence specific recognition of endogenous sequences by an exogenous linear fragment of DNA, that is subsequently integrated through cross over events within the region of homology.

Gene targeting by homologous recombination is a powerful technique that exploits the phenomenon described above, and allows the controlled modification, generally the disruption, of a specific target gene (reviewed in Galli-Taliadoros et al., 1995). Over the past decade this procedure, although currently restricted in higher eukaryotes to the characterisation of mouse genes, has proven instrumental for the study of many physiological and developmental processes, especially in the areas of cell differentiation and gene function, as well as for the production of mouse models for many human genetic disorders.

Between 1981-1985, two significant technical developments occurred in the areas of cellular and molecular biology that were critical for the development of gene targeting. First, mouse pluripotent embryonic stem (ES) cells were isolated from the inner cell mass of mouse blastocysts and could be cultured *in vitro* without the loss of pluripotency (Evans and Kaufman, 1981 and Martin, 1981). Following their reintroduction into recipient blastocysts, ES cells were found to be able to contribute to all tissues of the developing embryo to produce phenotypically normal chimaeric animals (Evans and Kaufman, 1981 and Martin, 1981). Because of their pluripotent characteristic, ES cells are able to colonise the germline of these

chimaeric animals (Bradley et al., 1984) and are thus able to act as a bridge that allows direct access to the germline compartment. The development of protocols that enable the manipulation of the DNA of ES cells in a controlled and defined manner through homologous recombination (Thomas and Capecchi, 1987; and Doetschman et al., 1987) was the second major development to have an impact on gene targeting. Because ES cells are able to support homologous recombination events without affecting germline competence (Thompson et al., 1989), specific mutations can be introduced into the genetic material of these cells and can be passed on to subsequent generations thus allowing the production of animals with defined mutations.

Although gene targeting is theoretically straightforward, in practice, experiments are sensitive, time consuming and elaborate. ES cells are extremely sensitive to *in vitro* manipulation and culture conditions are crucial to the success of this procedure. Extensive pre-screening of reagents is generally required to avoid contamination by differentiating factors as these will decrease the efficiency of germline chimaerism. In spite of this, gene targeting has evolved into a relatively accessible technology that has been widely adopted for the study of gene function and dysfunction in the mouse.

5.1.1.2 Experimental design

Gene targeting experiments are initiated with the identification of a gene of interest and the isolation of appropriate target genomic sequences. A targeting vector is then constructed that consists of a selectable marker, DNA sequences homologous to the target gene and vector sequences necessary for the manipulation and propagation of the resulting plasmid. As described in Figure 5.1, two types of targeting vectors exist: *replacement* vectors and *insertion* vectors (reviewed in Bronson and Smithies, 1994). Replacement vectors are designed to effectively eliminate critical exonic sequences of the target gene, by replacing them with an appropriate selective marker. Structurally, replacement vectors consist of marker gene

Figure 5.1 Diagrammatic representation of gene targeting vectors

Wild type target sequences are diagrammatically shown at the top, with exonic regions represented as black boxes.

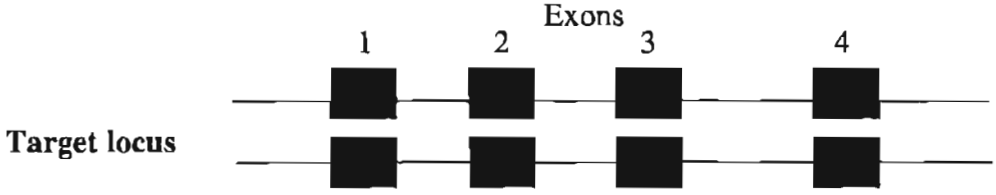
A. Typical replacement targeting events

A generic replacement vector is shown underneath the target locus. Marker sequences are cloned to disrupt exonic sequences. The targeting vector is linearised outside the region of homology. Integration of vector sequences requires two crossover events that can occur anywhere in the region of homology (represented as black crosses) that lead to the excision of endogenous sequences and its replacement with vector sequences.

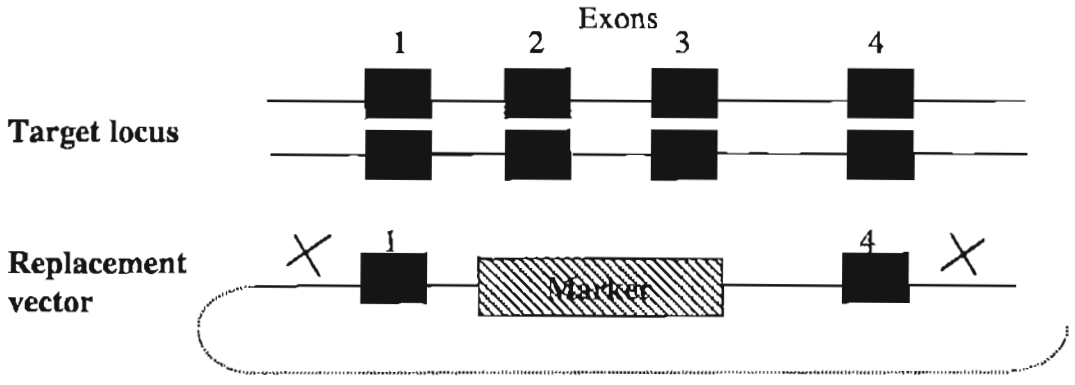
B. Typical insertion targeting events

A generic insertion vector is shown underneath the target locus. Marker sequences are cloned outside exonic sequences and the targeting vector is linearised inside the region of homology. Successful targeting events require a single crossover event (represented as a black cross) for the insertion of targeting vector sequences including that of vector sequences.

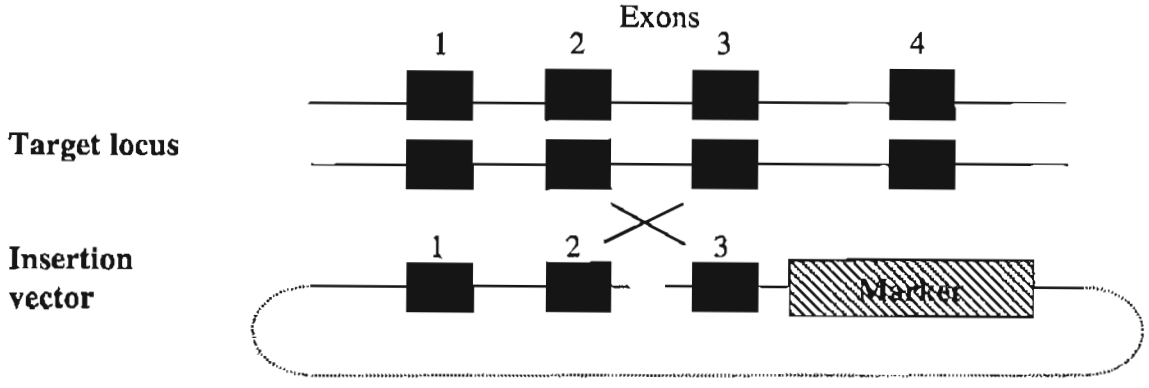
Genomic DNA sequences homologous to the target region are represented as full black lines, the selectable marker as a hatched box and plasmid vector sequences as a dotted line.


Exons
1
2
3
4
Target locus

A)


Exons
1
2
3
4
Target locus
Replacement vector
1
4
Marker

B)


Exons
1
2
3
4
Target locus
Insertion vector
1
2
3
Marker

sequences, flanked by DNA sequences homologous to the target gene. They are typically linearised outside the region of homology and two crossover events are required for the insertion of vector sequences into the target locus (Figure 5.1A). Insertion vectors on the other hand are designed to disrupt the target locus by duplicating target gene sequences and, as such, they are structurally different to insertion type vectors. In these vectors, the chosen selectable marker is inserted at the 3′ end of DNA sequences homologous to the target gene. Insertion vectors are typically linearised within the region of homology and a single crossover event leads to the duplication of the region of homology and insertion of the selectable marker (Figure 5.1B).

Targeting vectors are introduced into an embryonic stem cell line by standard transfection protocols, generally electroporation, under conditions that have been optimised for the chosen ES cell line. ES cells that carry integrated plasmid sequences are identified by antibiotic selection and homologous recombination events are differentiated from random integration of vector sequences using Southern blot or PCR analysis. As the parameters that influence the efficiency of homologous recombination events have become better defined, additional manipulations originally developed to eliminate ES cell clones with randomly integrated vector sequences have in many cases proven to be unnecessary.

To generate genetically manipulated mice, targeted ES cell lines need to be injected into mouse blastocysts. The genetic background of the recipient blastocysts is chosen such that the ES cell-derived coat colour differs from that of the recipient embryos thus allowing the visualisation of ES cell contribution. The efficiency of ES cell colonisation is judged by coat-colour chimaerism and highly chimaeric male pups are bred to identify successful germline transmitters. The breeding of chimaeric male pups allows for the screening of a large number of progenies as a single animal can be bred to numerous females. Additional breeding steps are

subsequently required to generate animals homozygous at the targeted allele, and these are identified by genotyping using either PCR or Southern blot analysis.

5.1.1.3 Parameters affecting the efficiency of gene targeting experiments

Over the last decade, numerous parameters have been shown to affect the efficiency of homologous recombination events including:

i) The use of isogenic DNA segments to construct targeting vectors.

Vectors constructed with isogenic DNA sequences generally display a significant increase in gene targeting efficiency (van Deursen and Wieringa, 1992). The magnitude of this positive effect appears to be dependent on the target gene (Deng and Capecchi, 1992).

ii) The length of uninterrupted perfectly matching sequences.

This appears to be more influential than the existence of a low percentage of mismatched bases (te Riele et al. 1992). However, given that non-isogenic DNA has successfully been used for the generation of a number of gene knock out mice, this parameter, though important, is obviously not an absolute pre-requisite for successful gene targeting (van Deursen and Wieringa, 1992; Deng and Capecchi, 1992).

iii) The length of homologous sequences shared between the targeting vector and the target gene.

A minimum of 2 kb has been identified as a prerequisite for successful homologous recombination events in ES cells (Hasty et al., 1991), and recombination frequency increases exponentially with increased length of homology (Deng and Capecchi, 1992).

iv) ES cell cycle rate.

Homologous recombination occurs during S-phase and almost exclusively within the first hour that follows transfection. Consequently, faster cycling cells reportedly support higher rates of gene targeting (Udy et al., 1997). This suggests that ES cell culture conditions should be monitored carefully during the targeting procedure.

The length of non-homologous DNA introduced into the target locus does not appear to affect the success of homologous recombination as various length fragments can be deleted successfully from the target gene (Mansour et al., 1990). Furthermore, because genes not expressed in ES cells have successfully been targeted (Johnson et al., 1989), target site transcription and thus chromatin structure is also not critical for the success of this approach.

5.1.1.4 Parameters affecting the efficiency of germline transmission

Germline transmission is the most time consuming and expensive step involved in the generation of genetically modified mice. Several parameters have been identified that affect the efficiency of germline transmission. Of these, the quality of ES cells used in the targeting experiments is most important. Decreased efficiency of germline transmission has been associated with chromosomal abnormalities found to be common in ES cells (Suzuki et al., 1997b), particularly trisomy 8 (Liu et al. 1997), as well as with increased passage numbers of ES cells (Nagy et al., 1990; Fedorov et al., 1997).

The choice of ES cell line used for homologous recombination and of the donor mouse strain used as a source of host blastocysts also appear to affect the success of germline colonisation by targeted ES cells (Lemckert et al., 1997). Ideally, preliminary studies are required to identify the optimal genetic background of recipient blastocysts to be used with a given ES cell line. To further increase the efficiency of this process, the genetic background of mice used to produce recipient blastocysts needs also to be sensitive to hormone treatment for

superovulation, produce high numbers of embryos and show high rate of germline colonisation by the chosen ES cell line.

5.1.2 *In Vitro* differentiation of ES cells

In vitro, ES cells are able to generate a range of specific cell types. This approach is often necessary to circumvent experimental difficulties encountered with the lethal phenotypes associated with genes necessary for early developmental processes. Elaborate protocols have been designed that enable a continuously growing ES cell population to be differentiated *in vitro* in a relatively well controlled manner. The hematopoietic system is ideal for *in vitro* differentiation studies. Many hematopoietic lineages are already well characterised, and a large number of recombinant growth factors that can act at different stages of hematopoietic differentiation are readily available.

In vitro differentiation protocols rely on the formation of embryoid bodies (EB). These are three-dimensional structures that form in response to the removal of the differentiation inhibiting factor LIF from suspension cultures of ES cells (Wiles and Keller, 1991). In the absence of exogenous factors, these structures are able to differentiate spontaneously to form many cell types including those of the hematopoietic system, though the efficiency of this process is generally unpredictable and the kinetics of differentiation are poorly defined (Evans and Kaufman, 1981; Martin, 1981 and Doetschman et al., 1985). *In vitro* differentiation conditions have been significantly improved with the use of a methylcellulose semi-solid culture system which acts as a three-dimensional matrix that supports the efficient, reproducible and synchronised differentiation of ES cells into numerous hematopoietic lineages, including erythroid, macrophage, neutrophil and mast cells (Wiles and Keller, 1991; Keller et al., 1993). The order of differentiation observed in those experiments also suggests

that hematopoietic differentiation of ES cells *in vitro* mimics to some extent the *in vivo* situation.

A modified *in vitro* differentiation system has been set up in the laboratory of Prof. P.D. Rathjen that has enabled the differentiation of ES cells into numerous cell types. This system includes the additional manipulation of ES cells to form a pluripotent cell population termed early primitive ectoderm-like (EPL) cells and results in the formation of terminally differentiated mesodermal cell types at higher levels than that obtained using ES cell embryoid bodies (Lake et al., 2000).

The ability to differentiate ES cells into various hematopoietic lineages has numerous advantages. Using this approach, the molecular and cellular events involved in establishing the primary hematopoietic system *in vivo* can be duplicated *in vitro* where cells are accessible to manipulation at all stages of differentiation. A broad range of genes can be studied through their inactivation and problems associated the generation of genetically modified animals as well as those encountered with embryonic lethality can be circumvented.

5.1.3 Approach

The work described in this thesis was initiated early in 1996, at a time when limited information was available on the physiological and molecular function of Tec and other members of the Tec family of protein tyrosine kinases. Since then, this family of protein tyrosine kinases has been the subject of increasing amount of interest, as reflected by the increasing number of publications in this area, all of which have along the way influenced the experimental design of this project.

Gene targeting has had a major impact on the field of immunology and has been instrumental in early studies of Btk, Itk and Txk function. This approach was thus chosen to study and identify a physiological function for Tec *in vivo*. Tec is an ideal candidate for gene

targeting. It is not expressed in ES cells and the disruption of the *Tec* gene is not expected to affect ES cell viability and function. In addition, the expression of Tec is first detected at day 7.5 of gestation and is therefore unlikely to be essential for early embryonic development.

As described in Chapter 3, *Tec* transcripts are expressed in various embryonic and adult tissues including cells of various hematopoietic lineages. Mice deficient for Tec (generated by homologous recombination) would thus be used to identify developmental processe(s) that are dependent on Tec function. This approach would also identify cell lineage(s), especially those of the hematopoietic compartment most affected by the lack of functional Tec.

ES cells targeted at the Tec locus can also be utilised for *in vitro* differentiation studies to investigate the role of Tec, if any, in hematopoietic differentiation. In light of the many problems encountered during attempts to generate Tec-/- mice, this second approach was expected to have several advantages. *In vitro* differentiation protocols for the generation of various lineages had been optimised in the laboratory of Prof. P.D. Rathjen (Lake et al., 2000) that showed high rates of macrophage differentiation. Given that studies described in Chapter 4 had suggested a role for Tec in macrophage function, this approach seemed appropriate to investigate further the role of Tec in macrophage-specific functions, including phagocytosis. Because genetically altered hematopoietic cells generated using this approach can easily be isolated, detailed analysis of Tec function in macrophage differentiation and function was also planned.

5.2 Results

The work described in this section focuses on the generation of targeted mutations at the *Tec* locus using mouse ES cells. These genetically altered ES cells were generated to facilitate the analysis of Tec function (i) *in vivo*, using genetically modified mice, or (ii) *in*

vitro, using a macrophage differentiation system (Keller et al., 1993; modified in Lake et al., 2000).

Although quite powerful, gene targeting is time consuming and expensive and the success of this approach is dependent on the strategy adopted to disrupt the locus of interest. Gene targeting vector design ideally requires knowledge of target protein structure and function to complement that of gene structure. Target gene sequences have traditionally been obtained through elaborate screens of genomic libraries, though recent developments in automated gene sequencing techniques and large scale genome sequencing projects are likely to eliminate the need for such elaborate and time consuming manual screening procedures.

The genomic library used in these studies was obtained from Dr. M. A. Kennedy and numerous gene sequences isolated from this library have been successfully used for targeting experiments in this department (Whyatt, 1996; Remiszewski, 2000). The library encodes 17-23 kb genomic DNA sequences prepared by partial restriction enzyme digestion of E14TG2a ES cell genomic DNA using *Sau*3A that had subsequently been cloned into the *BamH* I site of the vector λ2001 (Karn et al., 1984).

5.2.1 Partial characterisation of the mouse Tec locus

Prior to the beginning of this project, a genomic library screen had initially been carried out using PHTH cDNA sequences as the probe. It had successfully isolated two genomic library clones, λKS1.1 and λKS4.1 shown in Figure 5.2A (Stevens K., 1995). To facilitate the manipulation of genomic sequences, subclones of λKS1.1 and λKS4.1 had been generated using the restriction enzymes *Sac* I and *Xba* I (Stevens, 1995). They had been cloned into pBluescript II KS$^+$ (Stratagene) and had subsequently been subjected to restriction enzyme mapping. Using Southern blot analysis, DNA fragments encoding exon sequences had been

Figure 5.2 Tec genomic sequences

A. Diagrammatic representation of genomic clones λKS1.1 and λKS4.1

Hatched boxes representing the lambda genomic clones isolated during the first screen of the E14TG2a lambda genomic library (Stevens, 1995) are at the top. A *Sac* I restriction enzyme map that encompasses this region of the *Tec* locus is depicted underneath. Exons are numbered and represented as black boxes. The diagram also shows the genomic fragment probe used in Southern blot analysis to determine the overlapping region between the two genomic clones.

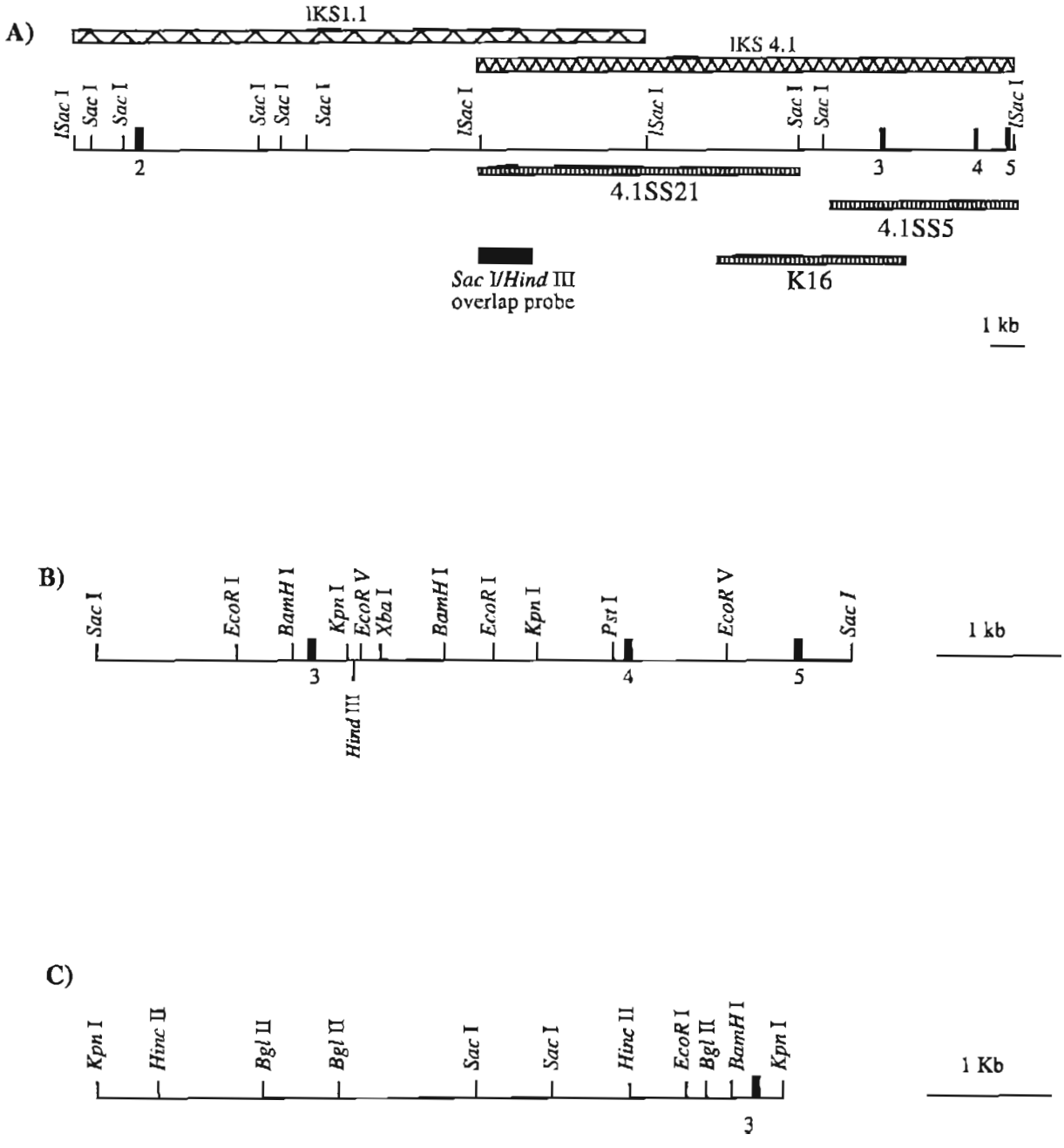
B. Restriction enzyme map of 4.1SS5

Restriction enzyme map of the 6 kb 4.1SS5 genomic DNA fragment encoding exons 3-5. Exon regions are numbered and represented as black boxes. The following MCS restriction enzyme sites were not identified in 4.1SS5: *Cla* I and *Not* I. 4.1SS5 was cloned into the *Sac* I site of pBluescript II KS^+ to generate p4.1SS5.

C. Restriction enzyme map of K16

Restriction enzyme map of 6 kb K16 genomic DNA fragment encoding exon 3 sequences (depicted as a black box). The following MCS restriction enzyme sites were not detected in K16: *Cla* I, *EcoR* V, *Not* I, *Sac* II, *Sal* I, *Sma* I. This fragment was cloned into pBluescript II KS^+ to generate pK16.

MCS: Multiple Cloning Site; l: lambda


A)
lKS1.1
lKS 4.1
lSac I
Sac I
Sac I
Sac I
Sac I
Sac I
lSac I
lSac I
Sac I
Sac I
lSac I
2
3
4
5
4.1SS21
4.1SS5
Sac I/Hind III
overlap probe
K16
1 kb
B)
Sac I
EcoR I
BamH I
Kpn I
EcoR V
Xba I
BamH I
EcoR I
Kpn I
Pst I
EcoR V
Sac I
3
4
5
Hind III
1 kb
C)
Kpn I
Hinc II
Bgl II
Bgl II
Sac I
Sac I
Hinc II
EcoR I
Bgl II
BamH I
Kpn I
3
1 Kb

identified and had been sequenced to confirm exon/intron boundaries for exons 2 and 3 (Stevens, 1995).

Further characterisation of λKS1.1 and λKS4.1 genomic DNA fragments was carried out during the course of this Ph.D to generate a detailed restriction enzyme map of the genomic DNA region surrounding exons 2 and 3 of the *Tec* locus. *Sac* I/*Sac* I and *Xba* I/ *Xba* I subclones of the two isolated genomic fragments were subjected to restriction enzyme analysis using a range of restriction enzymes. λ2001 vector multiple cloning site sequences were identified by manual sequencing and enabled the orientation of these genomic clones. The most 5' region of λKS4.1 was isolated by *Sac* I/*Hind* III restriction enzyme digestion. It was purified and used as a probe in Southern blot analysis to identify DNA fragments shared between λKS1.1 and λKS4.1 clones. Approximately 4 kb of DNA sequences were found to be shared between the two clones. Several *Sac* I sites, marked as λSac I, were identified during restriction enzyme mapping that were absent from the endogenous *Tec* gene suggesting that these had been derived from the multiple cloning site of λ2001 (Figure 5.2A). The K16 and 4.1SS5 fragments outlined in Figure 5.2A were characterised in detail, and their restriction enzyme maps are represented in Figure 5.2B and 5.2C. These genomic DNA fragments encode exon 2, and exons 3, 4 and 5 sequences respectively and as such were identified as potential targets for homologous recombination.

Although in theory removal of the ATG start codon located in exon 2 should disrupt the transcription of the *Tec* gene, several in frame ATG codons exist downstream of exon 2. One of these is located in exon 3 and is predicted to initiate the translation of the original *TecI* transcript (Mano et al., 1990). Exons located downstream of exon 2 were therefore identified as preferable targets for homologous recombination. Disruption of these exons is expected to interfere with the expression of all *Tec* transcripts and prevent the generation of truncated Tec sequences. The generation of the first gene targeting vector was limited to the genomic DNA

region encoded by λKS4.1 and λKS1.1. This initial targeting strategy therefore aimed to delete exons 3 and 4. These two exons, unfortunately, revealed to be clustered at the 3′ end of λKS4.1 thus limiting the length of DNA sequences available to use as the 3′ homology arm of the vector.

As described later in this chapter, this first targeting vector was unsuccessful in generating targeted ES cell clones for reasons that are unclear. Because shared homologous sequence length had been reported to influence the efficiency of homologous recombination (Hasty et al., 1991 and Deng and Capecchi, 1992), further attempts were made to isolate genomic DNA sequences by repeated screening of the E14TG2a genomic DNA library using the SH3/SH2 probe represented in Figure 5.3A. This probe which targets genomic sequences 3′ to λKS4.1 successfully identified a 15 kb clone labelled as λi81.1 (depicted in Figure 5.3B). Restriction enzyme analysis and DNA sequencing showed that λi81.1 encodes exons 3-7 suggesting extensive overlap with λKS4.1. In fact, only approximately 5 kb of genomic sequences were found to be unique to λi81.1. Restriction enzyme mapping was also used to characterise λi81.1. A 6.1 kb *Pst* I/*Sac* I fragment was identified to carry exonic sequences by Southern blot analysis using a radiolabelled SH3 fragment as a probe. As depicted in Figure 5.4A, this fragment encodes exons 6 and 7, as well as exons 4 and 5, thus extending genomic DNA sequences further 3′ by approximately 5 kb.

Independent experiments were subsequently carried out in the laboratory that aimed to isolate and characterise the entire mouse *Tec* locus (Merkel, 1996; Merkel et al., 1999). Several clones were identified using probes consisting of 3′ untranslated cDNA sequences (UTR) and exon 7/8 cDNA sequences and these are described in Merkel et al, 1999. One of these clones, λex78, was found to overlap extensively at the 5′ end with λi81.1 with approximately 6 kb of additional unique sequences including the 66 bp exon 8. This exon encodes the C-terminal region of the SH3 domain and is differentially spliced in *TecIII*

Figure 5.3 Isolation of lambda genomic clone using the SH3/SH2 probe

A. Diagrammatic representation of cDNA fragment used in genomic library screen.

Tec cDNA sequences are diagrammatically represented as hatched boxes that include the 5' untranslated region (5' UTR, Pleckstrin Homology (PH) domain, Tec Homology (TH) region, Proline Rich Region (PRR), Src Homology 3 (SH3) domain, Src Homology 2 (SH2) domain, kinase and 3′ untranslated region respectively.

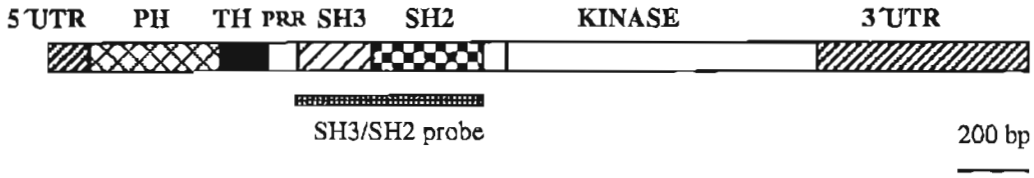
The SH3/SH2 probe used in the genomic screens was generated by PCR using the TecSH3.5 and TecSH2.3 primer sequences. The PCR product generated is diagrammatically represented underneath its corresponding cDNA region.

B. *Sac* I restriction enzyme map of λi81.1

Restriction enzyme map showing *Sac* I sites, sites unique to vector sequences are marked in italics. Sites derived from the lambda cloning vector are indicated with l. Exons are represented as black boxes. The SH3 cDNA fragment region is represented as a hatched box underneath exon 7. The PS6.1 cDNA fragment is also represented as hatched box underneath its corresponding genomic region.

l: lambda

A)


5'UTR
PH
TH
PRR
SH3
SH2
KINASE
3'UTR
SH3/SH2 probe
200 bp

B)

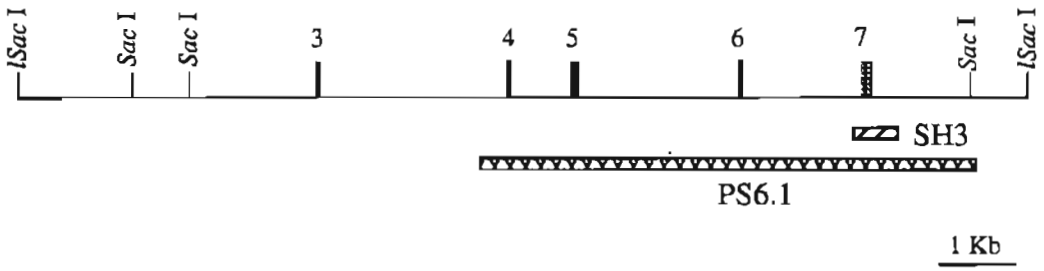

lSac I
Sac I
Sac I
3
4
5
6
7
Sac I
lSac I
SH3
PS6.1
1 Kb

Figure 5.4 Characterisation of Tec genomic sequences

A. Restriction enzyme map of PS6.1

A restriction enzyme map of PS6.1 cDNA fragment is depicted that includes exons 4 to 7 depicted as black boxes. The following MCS sequences were not identified in PS6.1: *BamII* I, *Cla* I, *Kpn* I, *Not* I, *Sal* I, *Xba* I. The BgS1.8 fragment isolated by *Bgl* II/*Sac* I restriction enzyme digestion is represented underneath.

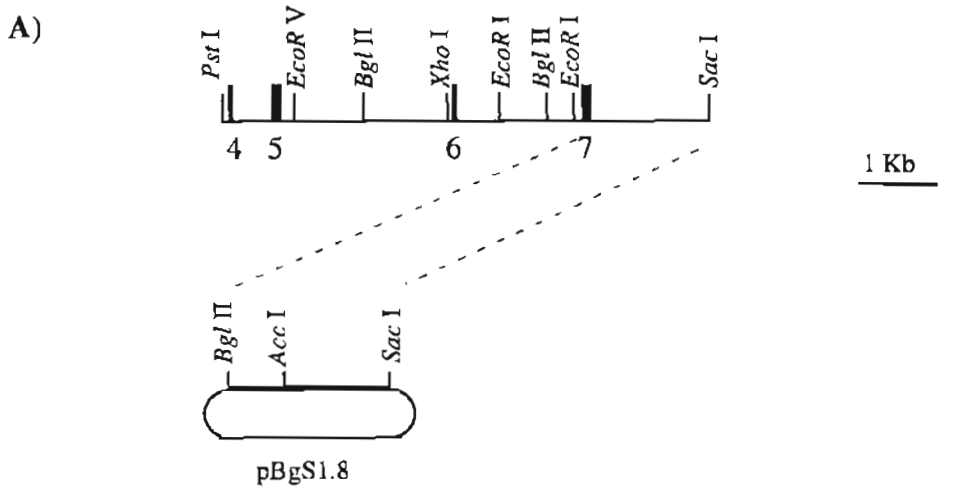
B. Diagrammatic representation of genomic fragment encoded by λex78 and λi81.1

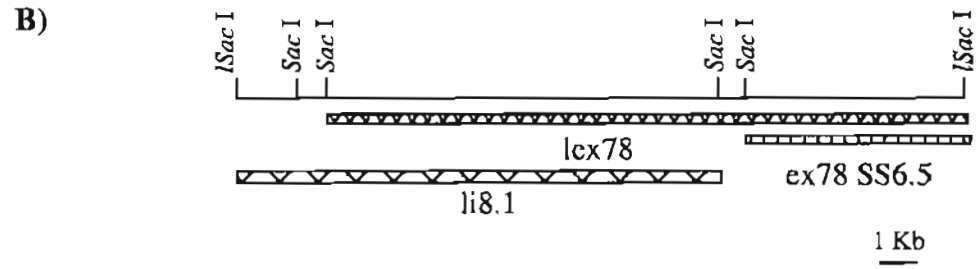
A *Sac* I restriction enzyme map is shown at the top that encompasses a region of the *Tec* locus encoded by the genomic clones λex78 and λi81.1. Hatched boxes represent DNA fragments λex78 and λi81.1 and ex78SS6.5.

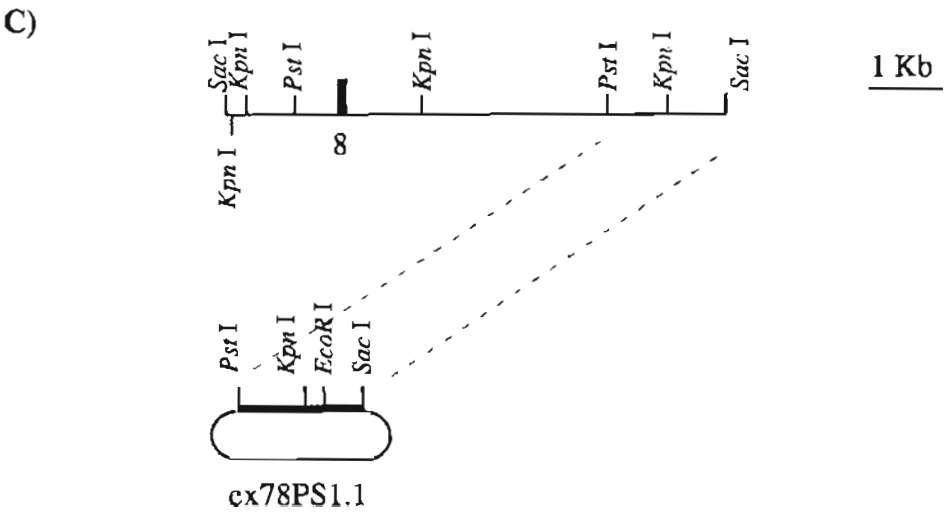
C. Restriction enzyme map of ex78SS6.5

Restriction enzyme map of ex78SS6.5 and of the PS1.1 fragment isolated by *Pst* I/*Sac* I restriction enzyme digestion of ex78SS6.5.

l: lambda


A)
Pst I
EcoR V
Bgl II
Xho I
EcoR I
Bgl II
EcoR I
Sac I
4
5
6
7
1 Kb
Bgl II
Acc I
Sac I
pBgS1.8


B)
lSac I
Sac I
Sac I
Sac I
Sac I
lSac I
lcx78
ex78 SS6.5
li8.1
1 Kb


C)
Sac I
Kpn I
Pst I
Kpn I
Pst I
Kpn I
Sac I
1 Kb
8
Kpn I
Pst I
Kpn I
EcoR I
Sac I
cx78PS1.1

compared with *TecIV*. A contiguous map of λex78 and λi81.1 is shown in Figure 5.4B. The ex78SS6.5 fragment shown in Figure 5.4B encodes a 6.5 kb DNA fragment located at the end of λex78. This DNA fragment was subcloned in pBluescript II KS$^+$ and mapped by restriction enzyme analysis (Figure 5.4C) before being used to generate the 3′ homology arm of targeting vectors described in section 5.2.2.2.

Numerous attempts to further screen the E14TG2a genomic library failed to isolate a genomic clone that encoded exon 9 and overlapped with λex78 and λAMC1.1.1 (Merkel, personal communication). In order to complete the mapping of the *Tec* locus and identify the exon/intron boundary of exon 9, LR-PCR was carried out using the 5-exon8/3′exon8 and 5′exon8/3-exon8 primer combinations. As shown in Figure 5.5A, two fragments were predicted to be amplified using these primer combinations: Fragment A and B. Gel electrophoresis analysis of the PCR products is shown in Figure 5.5B and C. Fragment A was 0.5 kb long and included an *EcoR* I site previously shown to be located at the 3′ end of λex78 and a *Pst* I site identified in exon 9. The 5′exon8/3-exon8 primer pair amplified two DNA fragments of 2 kb and 1.6 kb respectively. Upon the optimisation of Mg^{2+} concentrations, the larger of the two fragments appeared more prominent. This fragment was subsequently purified, subcloned in pBluescript II KS$^+$ and sequenced to confirm the presence of *Tec* exon 9 sequences.

In summary, several screening experiments using various regions of the Tec cDNA as probes have been necessary to isolate and characterise all exon/intron boundaries of the *Tec* gene. However, as highlighted in Figure 5.6, these screens failed to fully isolate intron 2 region thus leaving a gap of uncloned sequences. It is expected that this gap will be readily identified through the effort of the mouse sequencing project.

Figure 5.5 Isolation of exon 9 genomic sequences

A. Diagrammatic representation of the Long Range PCR (LR-PCR) strategy adopted to isolate genomic DNA sequences that include exon 9 and surrounding intronic sequences. A *Sac* I restriction enzyme map is depicted at the top that encompasses regions encoded by clones λi81.1, λcx78 and λAMC1.1.1, and highlights the gap in genomic sequences that includes exon 9 and surrounding intronic sequences. To bridge this gap, two rounds of PCR were carried out using ES cell genomic DNA template. Overlapping sequences of this region were isolated. Fragment A encodes intron 8 and exon 9 sequences while fragment B includes exon 9 and intron 9 sequences.

B. Gel electrophoresis of Fragment A PCR product

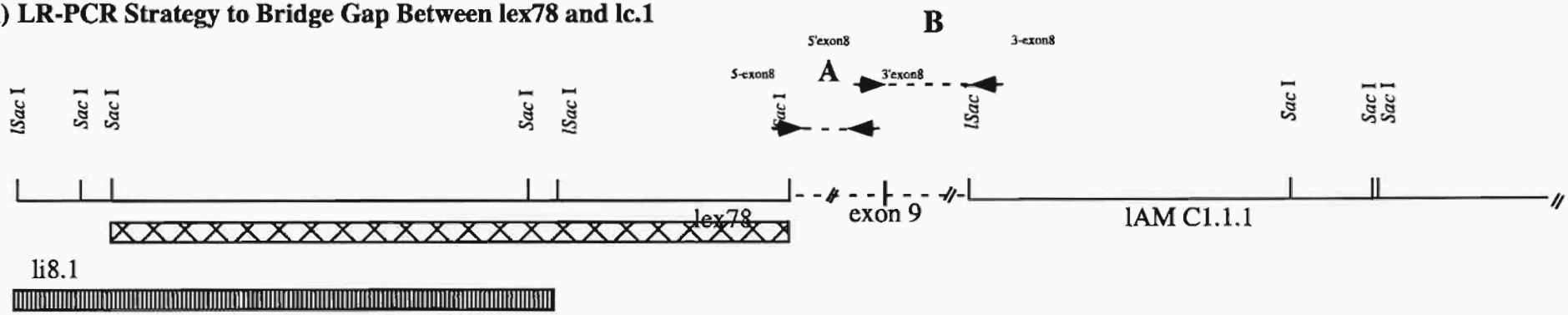
Fragment A was amplified using the 5-exon8/3'exon8 primer pair. Undigested PCR products, as well as *EcoR* I, *Kpn* I and *Pst* I restriction enzyme digests of the PCR product were analysed by 2% (w/v) TAE gel electrophoresis and compared with SPP1 and pUC19 DNA (Geneworks) markers for sizing.

C. Gel electrophoresis (2% (w/v) TAE) of Fragment B PCR product

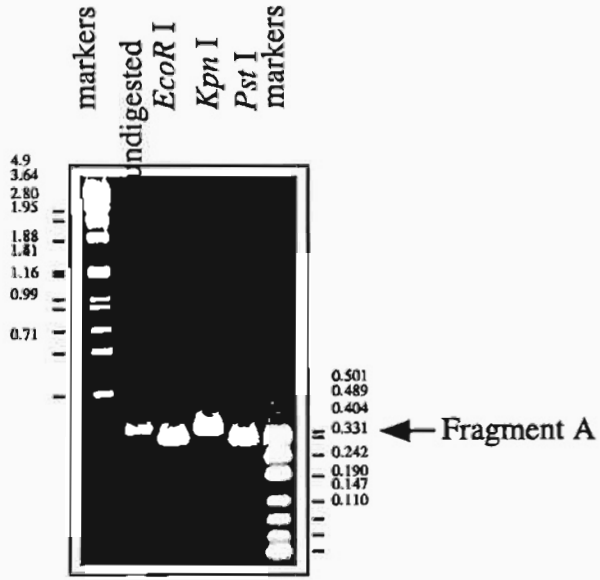
PCR was carried out using 5'exon8 and 3-exon8 primers. Two parameters of the reaction were tested including enzyme concentration and $MgCl_2$ concentration. A 2 kb fragment appeared most prominent at the higher $MgCl_2$ concentrations that was subsequently subcloned into pBluescript II KS^+ and subjected to automated DNA sequencing. SPP1 markers (Geneworks) were loaded onto the gel for size comparison.

The following program was used in the PCR reactions: 1 minute at 94°C, 30 seconds at 94°C, 30 seconds at 60°C, 4 minutes at 68°C, repeated steps 2-4 32 times.

A) LR-PCR Strategy to Bridge Gap Between lex78 and lc.1



B) Fragment A



C) Fragment B

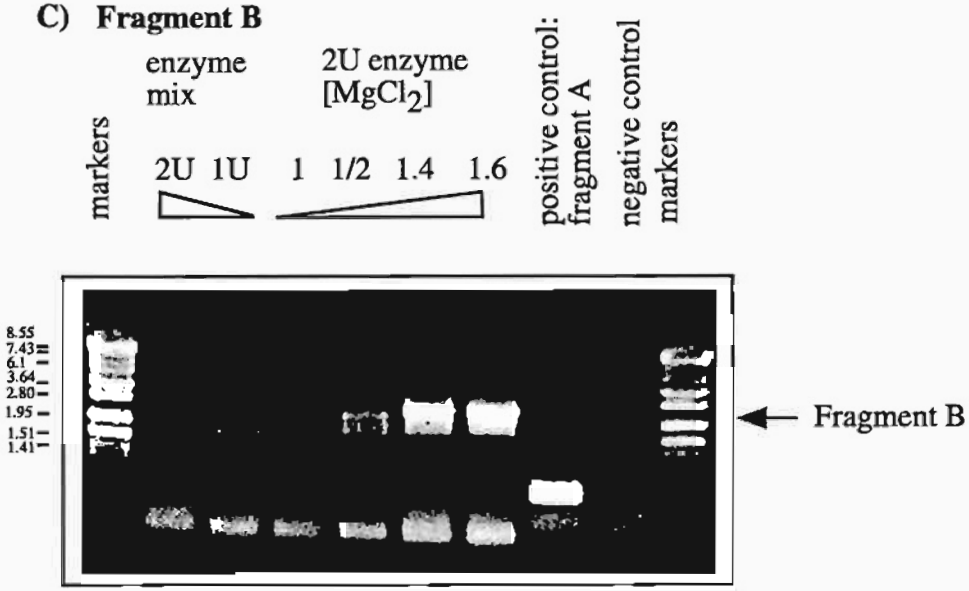
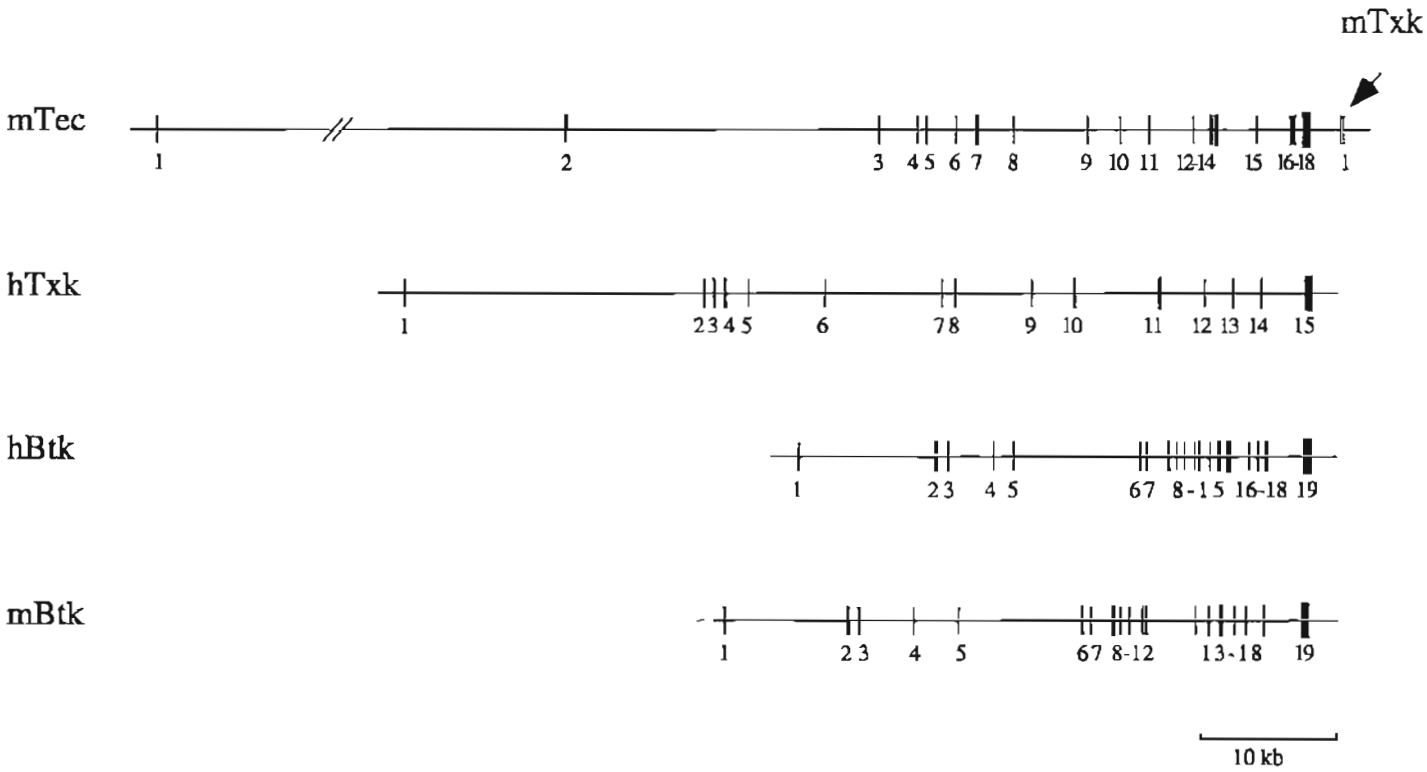


Figure 5.6 Diagrammatic representation of the Tec family kinases gene structure

Schematic representation of the mouse *Tec* (mTec), human *TXK* (hTxk), human *BTK* (hBtk) and mouse *Btk* (mBtk) loci showing the positions of the exons within the genes and the approximate sizes of the intervening sequences (*Txk* and *Btk* gene structures adapted from Ohta *et al.*, 1996 and Sideras *et al.*, 1994, respectively). Exon 1 of mouse *Txk* (mTxk) is also shown approximately 2.6 kb 3' of mTec exon 18.


mTxk
mTec
1
2
3 4 5 6 7 8 9 10 11 12-14 15 16-18 1
hTxk
1
2 3 4 5 6 7 8 9 10 11 12 13 14 15
hBtk
1
2 3 4 5 6 7 8-15 16-18 19
mBtk
1
2 3 4 5 6 7 8-12 13-18 19
10 kb

5.2.2 Gene targeting at the Tec locus

Several gene targeting vectors have been designed and constructed during the course of this project. Initial targeting experiments were carried out in E14TG2a ES cells, using the HPRT minigene as a selection marker, while subsequent experiments utilised W9.5 ES cells and the Neomycin resistance gene. These marker genes were chosen as they have been widely used in gene targeting experiments to confer resistance to HAT and Geneticin (G418 sulfate) respectively without affecting ES cell pluripotency.

5.2.2.1 Gene targeting in E14TG2a ES cells

The first round of gene targeting experiments was carried out in the ES cell line E14TG2a. This ES line is a feeder independent, HPRT deficient ES cell line derived from 129/SV/Ola mice that requires the Leukemia Inhibitor Factor (LIF) to prevent ES cell differentiation *in vitro*. It had, prior to the beginning of this project, been used successfully in this department for the generation of a number of targeted ES clones (Whyatt, 1996; Thonglairoam, 1994). Following injection into mouse blastocysts, targeted derivatives of this cell line had also successfully generated chimaeric animals that transmitted ES cell derived genetic material through their germline (Whyatt, 1996; Thonglairoam, 1994).

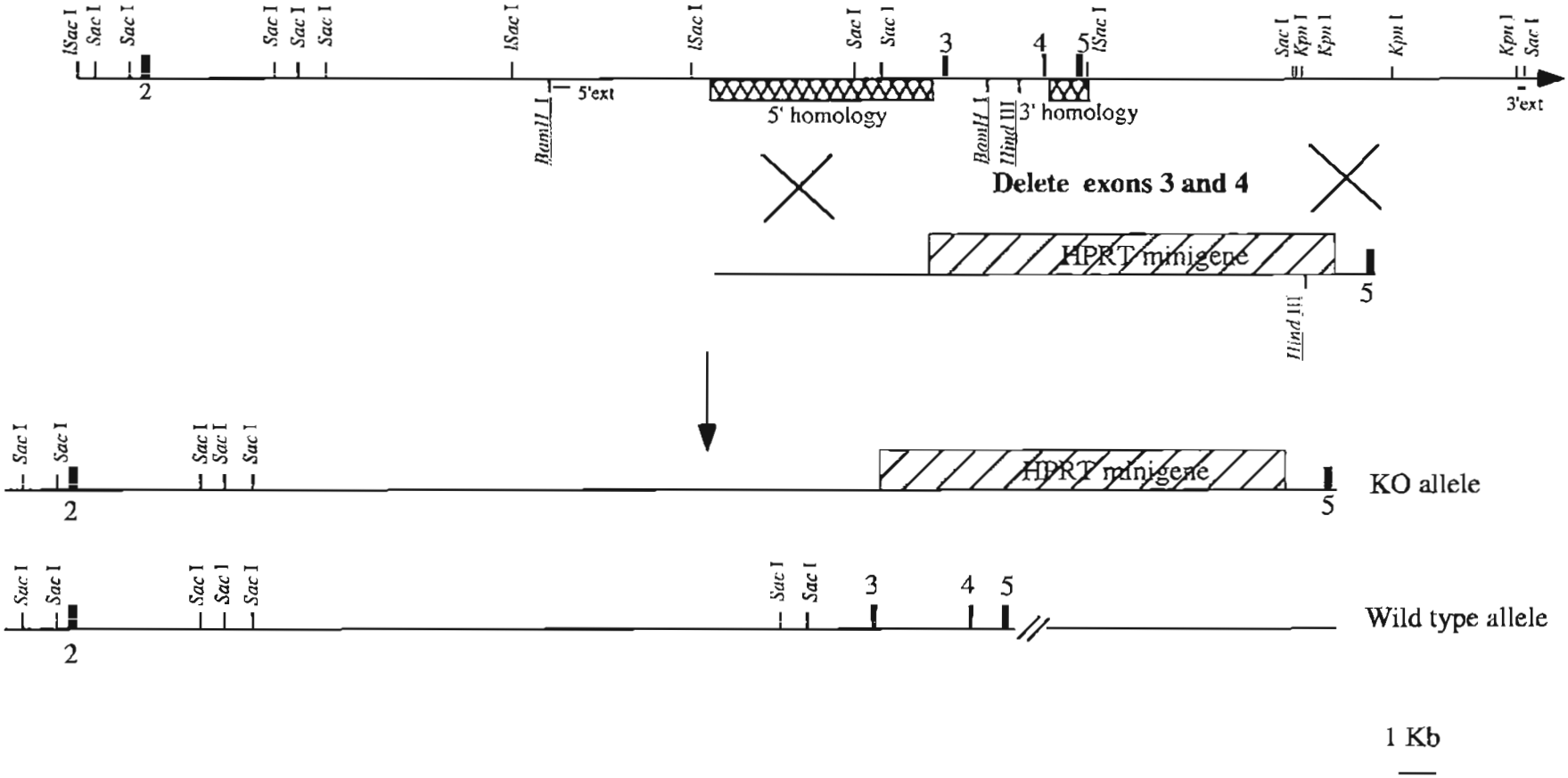
The gene targeting strategy adopted is diagrammatically represented in Figure 5.7. Removal of exons 3 and 4 is predicted to abolish the production of functional Tec sequences, although a 46 amino acid peptide can theoretically be generated from the translation of exon 2 sequences. This peptide encodes N-terminal amino acids of the PHTH domains which are predicted to be unstructured and have no biological activity. This strategy should therefore be effective in disrupting Tec function.

Figure 5.7 Diagrammatic representation of gene targeting strategy used to target the Tec locus in E14TG2a ES cells

A partial restriction enzyme map of genomic sequences encoded by lKS1.1 and lKS4.1 is represented at the top. Black boxes represent exon sequences and hatched boxes indicate 5′ and 3′ genomic sequences used to construct the targeting vector. Expected targeted and wild type alleles are shown below.

l: lambda

l KS1.1 and l KS4.1



5.2.2.1.2 Generation of the Gene Targeting Construct p5H.HPRT.3NS4

Exons 3, 4 and 5 are found clustered at the 3' end of λKS4.1. The design of this targeting vector therefore required that the length of DNA sequences used in the 3' homology arm of the targeting vector be maximised, whilst removing exons 3 and 4 sequences. A *Not* I restriction enzyme site was thus introduced by PCR in the 3′ exon4/intron4 boundary using the primer pair IA-K16-1 (2.2.11) and USP (universal sequencing primer, Stratagene). As described in Figure 5.8A, p4.1SS5PS had been generated by subcloning the 2 kb *Pst* I/*Sac* I fragment of p4.1SS5 into pBluescript II KS$^+$ and was used as the template for the PCR reaction. The PCR product amplified using IA-K16-1/USP was gel purified, digested with *Not* I/*Sac* I and subcloned into pBluescript II KS$^+$ to generate p3NS4 (Figure 5.8B and C). The identity of this plasmid was confirmed by restriction enzyme analysis using *EcoR* V. Manual sequencing using the Universal Sequencing Primer (Stratagene) was also used to confirm the presence of the exogenous *Not* I site.

The 5′ homology arm of the targeting vector was generated by *Hinc* II restriction enzyme digestion of pK16. The 4.8kb *Hinc* II fragment was isolated by gel electrophoresis, purified and ligated with *Hinc* II linearised p3NS4 to create p5H.NS4 (Figure 5.9A). Restriction enzyme analysis and gel electrophoresis was used to confirm the identity of the resulting plasmid. The HPRT marker gene was isolated from pnI2(I1S) (Reid et al., 1990) as a *BamH* I/ *BamH* I minigene fragment of approximately 6 kb and was subsequently cloned into *BamH* I linearised p5H.NS4 (Figure 5.9B). Restriction enzyme analysis of the resulting p5H.HPRT.3NS4 plasmid (Figure 5.10B) was carried out to generate a restriction enzyme map of the plasmid (Figure 5.10A). Prior to transfection into ES cell, p5H.HPRT.3NS4 was purified on a CsCl gradient, linearised with *Sal* I and extracted with phenol and chloroform. *Sal* I digested p5H.HPRT.3NS4 includes 2.9 kb of pBluescript II KS+ DNA sequences at the 3' end of targeting vector sequences. Non homologous regions at the extremities of targeting

Figure 5.8 Diagrammatic representation of cloning strategy used to generate the 3' homology arm of p5H.HPRT.3NS4

A. Restriction enzyme map of 4.1SS5 highlighting the *Pst* I/*Sac* I fragment used to generate the 3' homology arm of targeting vector as a hatched box.

B. Restriction enzyme map of the p4.1SS5PS plasmid and PCR strategy used to introduce a *Not* I restriction enzyme site at the exon4/intron4 boundary using the IA-K16-1/USP primer pair. Primers are diagrammatically represented as black arrows and exon sequences are represented as black boxes.

C. Diagrammatic representation of the subcloning step used to insert the NS4 fragment into pBluescript II KS^+.

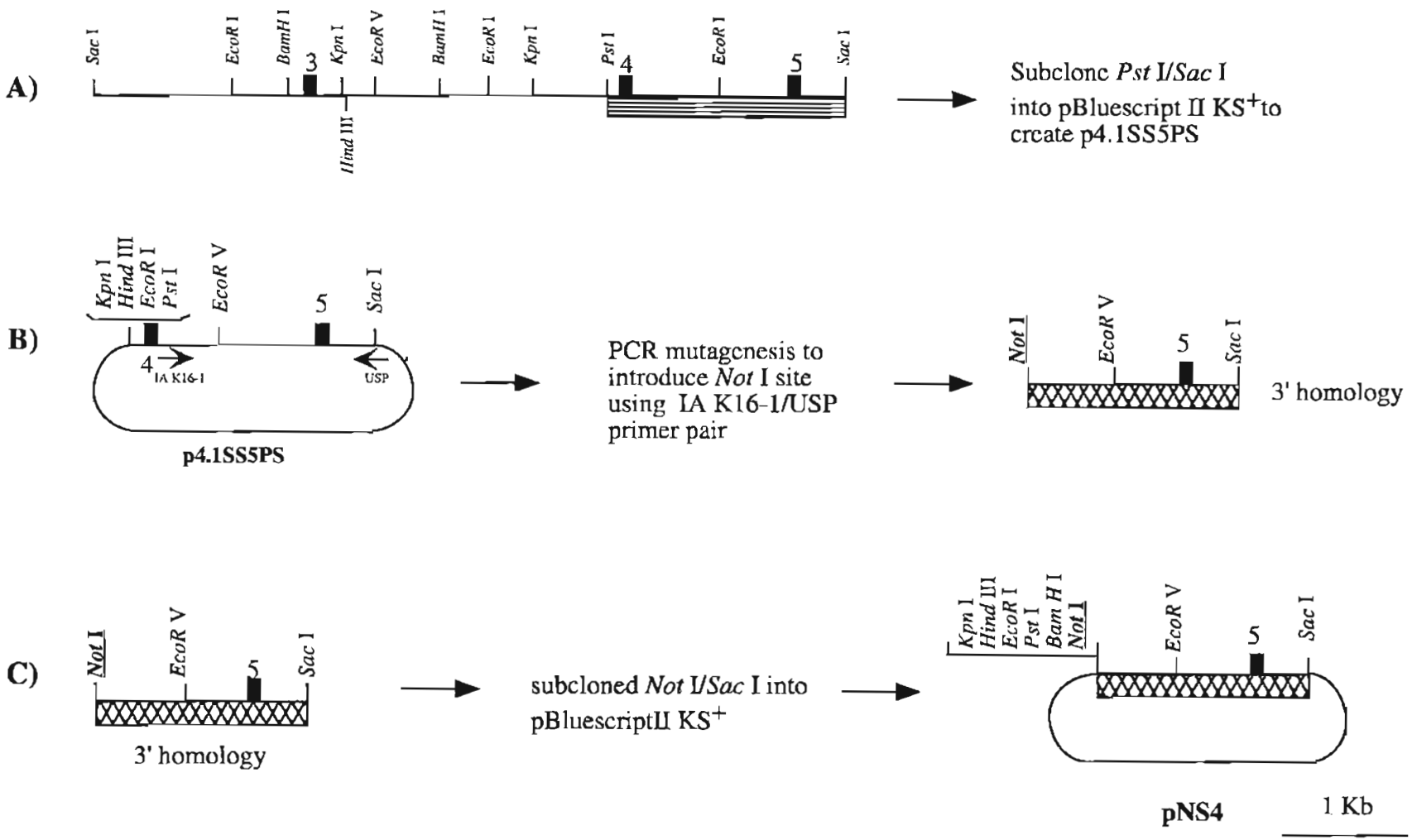

A)
Sac I
EcoR I
BamH I
3
Kpn I
EcoR V
BamH I
EcoR I
Kpn I
Pst I
4
EcoR I
5
Sac I
Hind III
Subclone Pst I/Sac I
into pBluescript II KS+ to
create p4.1SS5PS
B)
Kpn I
Hind III
EcoR I
Pst I
EcoR V
5
Sac I
4
IA K16-1
USP
p4.1SS5PS
PCR mutagenesis to
introduce Not I site
using IA K16-1/USP
primer pair
Not I
EcoR V
5
Sac I
3' homology
C)
Not I
EcoR V
5
Sac I
3' homology
subcloned Not I/Sac I into
pBluescriptII KS+
Kpn I
Hind III
EcoR I
Pst I
Bam H I
Not I
EcoR V
5
Sac I
pNS4
1 Kb

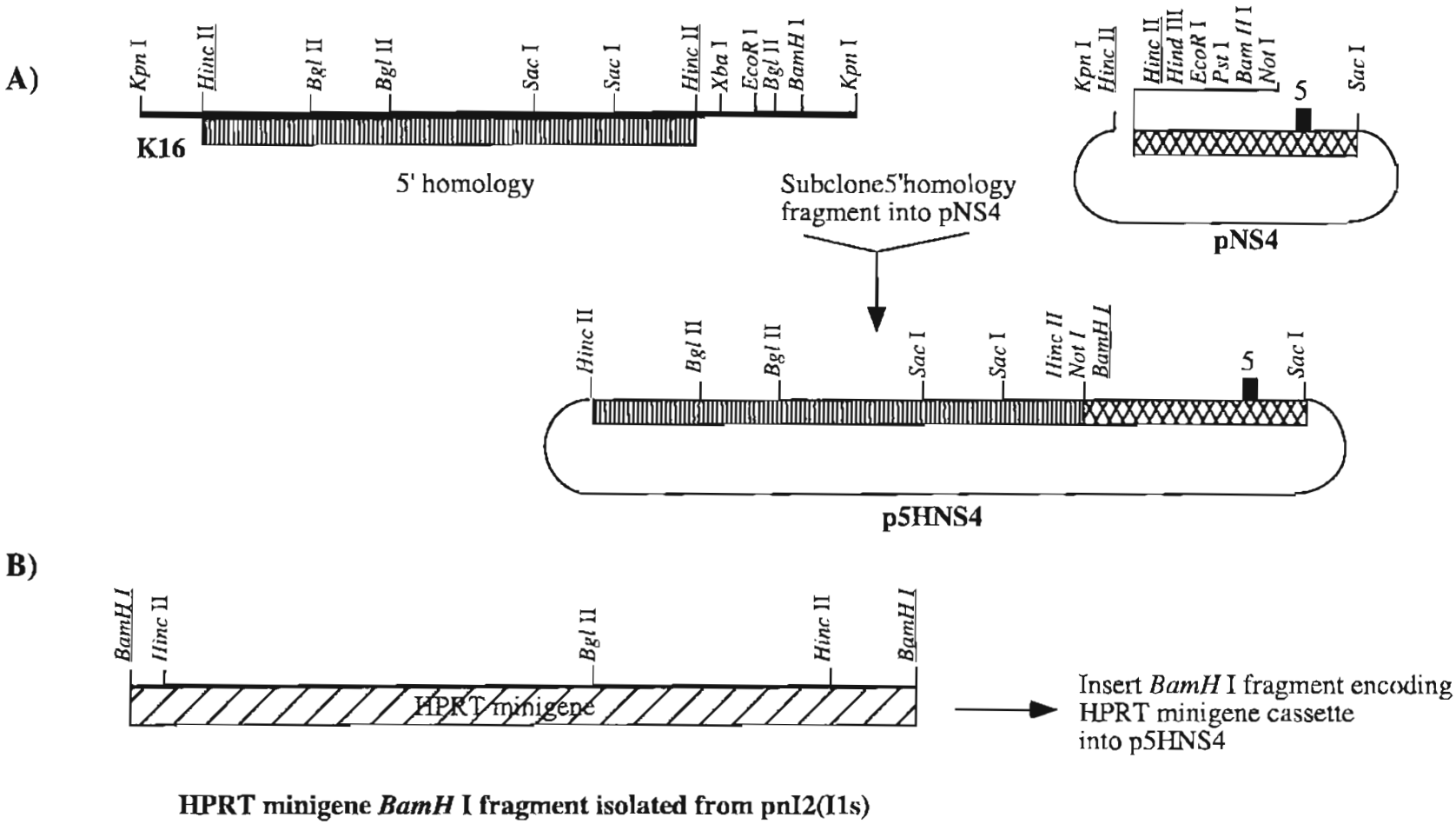
Figure 5.9 Diagrammatic representation of cloning strategy used generate targeting vector

A. Diagrammatic representation of cloning strategy used to generate the 5′ homology arm of targeting vector.

Restriction enzyme map of the K16 fragment is represented that highlights the *Hinc* II/*Hinc* II fragment used to generate the 5′ homology arm of the targeting vector as a hatched box. The subcloning strategy used to subclone this fragment into *Hinc* II linearised pNS4 is also represented. A map of the resulting p5HNS4 plasmid is also shown.

B. Diagrammatic representation of the HPRT minigene used as selection marker.

The HPRT minigene was isolated by *BamH* I restriction enzyme digestion of the pnI2(I1s) plasmid and inserted into *BamH* I linearised p5HNS4 to generate p5H.HPRT.3NS4.



HPRT minigene *BamH* I fragment isolated from pnI2(I1s)

Figure 5.10 Characterisation of p5H.HPRT.3NS4 targeting vector

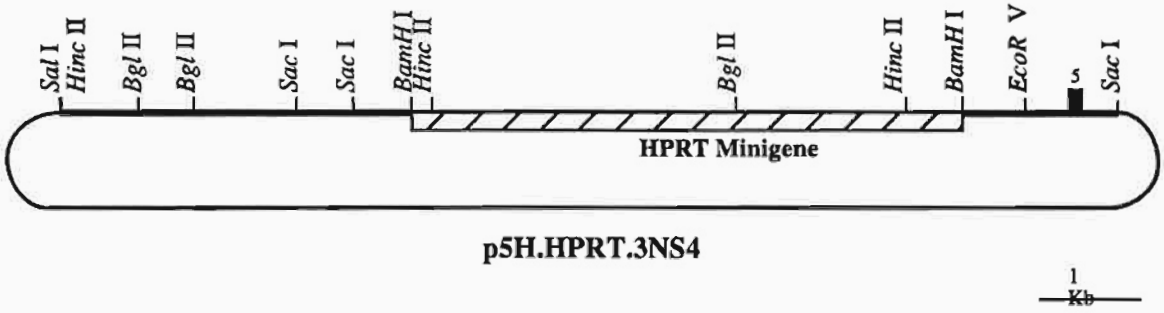
A. Restriction enzyme map of the p5H.HPRT.3NS4 targeting vector.

5' and 3' homology sequences are represented as full black lines, the HPRT minigene as a hatched box and exon 5 sequence as a black box.

B. Agarose gel electrophoresis of p5H.HPRT.3NS4 restriction enzyme digests

Restriction enzyme digests were carried out on CsCl purified p5H.HPRT.3NS4 DNA and products were separated by gel electrophoresis on a 1% (w/v) TAE agarose gel. SPP1 molecular weight markers were used for size comparison.

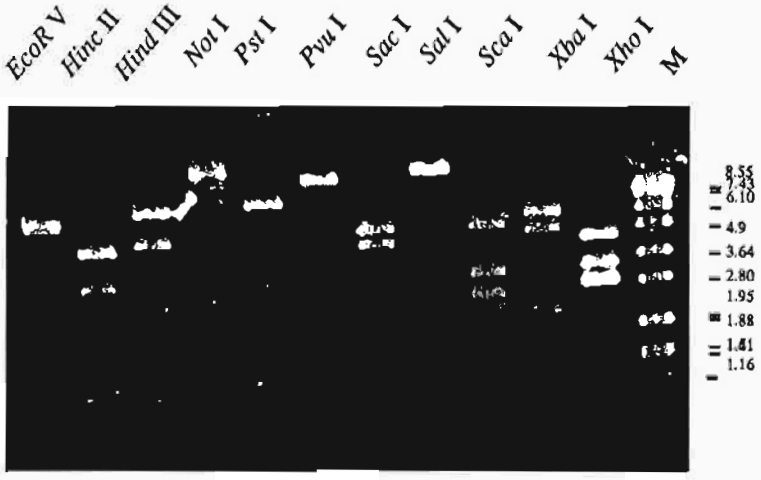
A)



p5H.HPRT.3NS4

1 Kb

B)



vectors were not removed prior to the transfection of the linearised vector into ES cells as they have previously been reported not to be incorporated into the target gene (Mansour et al., 1990) or affect targeting efficiency (Deng and Capecchi, 1992).

5.2.2.1.3 Transfection and characterisation of ES cell colonies

E14TG2a ES cells are deficient for the HPRT (hypoxanthine phosphoribosyl transferase) gene (Hooper et al., 1987) and as such are sensitive to selection using HAT (Hypoxanthine, Aminopterin, Thymidine). Because the activity of various batches of selective agents have been found to vary, selection conditions were determined for each batch of HAT using a standard toxicity assay. The optimal concentration of HAT required to effectively select against untransfected E14TG2a ES cells was determined as described in Figure 5.11A. During the course of this experiment, a final concentration of 100 μM hypoxanthine, 16 μM thymidine and 0.4 μM aminopterin was found to be sufficient to completely kill E14TG2a ES cells.

Transfection of E14TG2a ES cells was carried out by electroporation using parameters optimised to minimise cell death and prevent multiple integration of the targeting vector (Whyatt, 1996). The statistics of this experiment are shown in Figure 5.11B. Two hundred HAT resistant colonies were picked, expanded and subjected to genomic DNA extraction and Southern blot analysis. Briefly, genomic DNA preparations were subjected to restriction enzyme digestion using *Hind* III and the resulting products were separated by 0.8% agarose gel electrophoresis. Southern blot analysis was subsequently carried out using the 3' external probe which was prepared by *Acc I/EcoR* I restriction enzyme digestion of pBgS1.8 (Figure 5.4A). Unfortunately, no targeted clones were identified using this strategy. A blot representative of these experiments is shown in Figure 5.12A. To confirm the absence of successful homologous recombination events in these clones, genomic DNA preparations were also subjected to *Bam*H I restriction enzyme digestion, 0.8% agarose gel electrophoresis and

Table 5.11 Targeting of E14TG2a ES cells

A. Toxicity assay.

Determination of the optimal concentration of HAT needed to select against untransfected ES cells. Increasing concentrations of HAT were added to a sub-confluent layer of E14TG2a cells and cell death was measured 7 days after the start of the selection process. The selective medium was changed daily.

B. Statistics of electroporation of p5H.HPRT.2NS4 targeting vector into E14TG2a ES cells.

Selection was carried out at 100 μM hypoxanthine, 16 μM thymidine and 0.4 μM aminopterin for 14 days after which 200 HAT resistant colonies were picked manually and characterised by Southern blot analysis to identify successful targeting events. Mock transfection controls were carried out with no DNA. Transfected ES cells were also plated with no selection to characterise cell survival efficiency.

A)

Plate No.	Hypoxanthine,Thymidine	Aminopterin	% cell death
1	0 μM, 0 μM	0 μM	0%
2	100 μM, 16 μM	0.2 μM	85%
3	100 μM, 16 μM	0.267 μM	90%
4	100 μM, 16 μM	0.4 μM	100%
5	100 μM, 16 μM	0.534 μM	100%
6	100 μM, 16 μM	0.8 μM	100%

HT (Hypoxanthine, Thymidine) solution was prepared as a 100X stock solution
(10 mM Hypoxanthine, 1.6 mM Thymidine).
A (Aminopterin) solution was prepared as 100X stock solution (0.04 mM Aminopterin).

B)

	No Selection	HAT Selection
Cells Electroporated	1×10^8	1×10^8
Cells Plated	1×10^7	1×10^7
Cells Surviving	$2x\ 10^6$	3.6×10^4
Colonies Picked		200
Targeted Lines		0
Efficiency		0%

Figure 5.12 Characterisation of HAT resistant ES cells

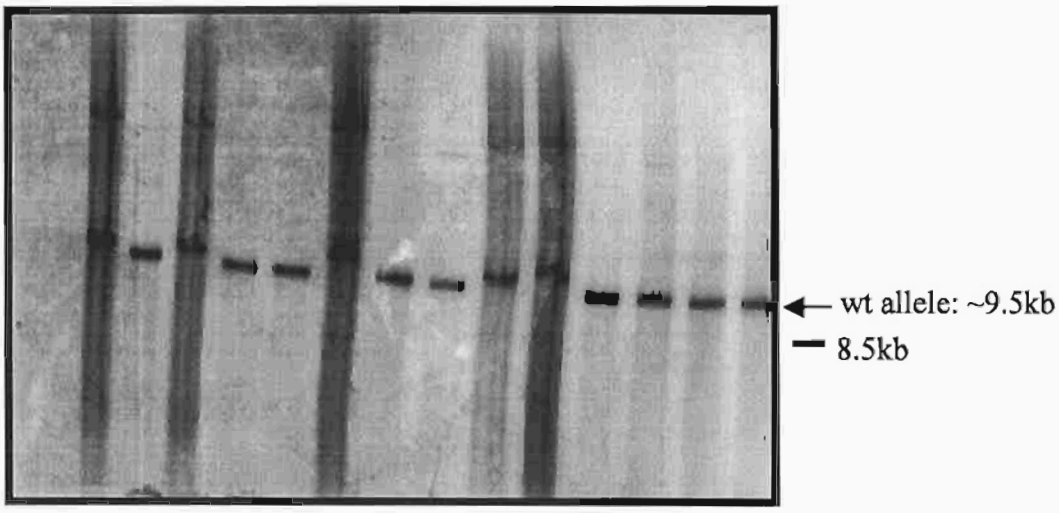
A. Southern blot analysis of HAT resistant E14TG2a ES cell DNA using 3' external probe

Approximately 20 µg of ES cell DNA was digested with *Hind* III. Restriction enzyme digests were separated by 0.8% (w/v) TAE agarose gel electrophoresis, transferred to a nylon membrane and probed with the 3' external probe.

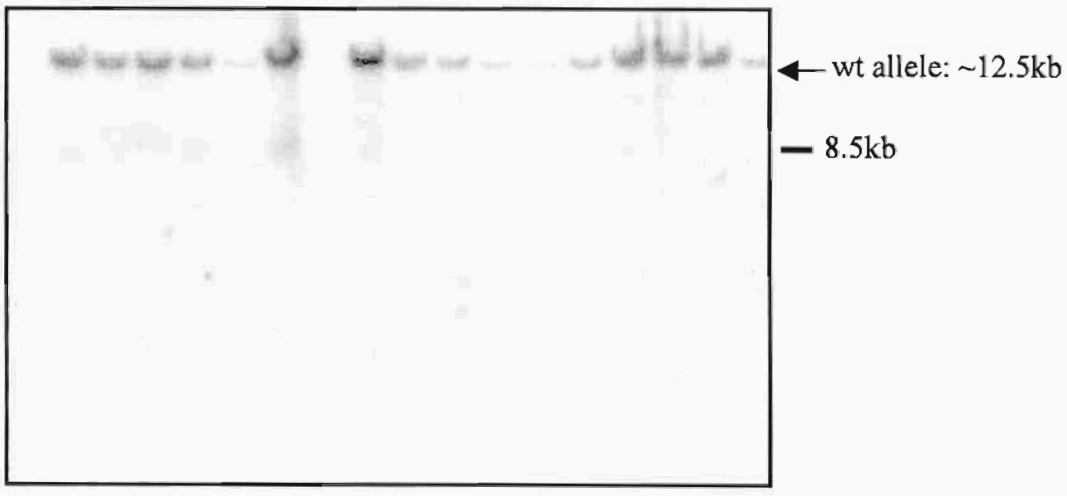
B. Southern blot analysis of HAT resistant E14TG2a ES cell DNA using the 5' external probe.

Approximately 20 µg of ES cell DNA was digested with *Bam*H I. Restriction enzyme digests were separated by 0.8% (w/v) TAE agarose gel electrophoresis, transferred to a nylon membrane and probed with the 5' external probe.

A) 3'ext probe



A) 5'ext probe



Southern blot analysis using the using the 5' external probe prepared by *BamH* I/ *Hind* III restriction enzyme digestion of p4.1S21 (Figure 5.2A). Figure 5.12B shows a typical Southern blot generated using the 5´ external probe.

5.2.2.2 Gene Targeting in W9.5 ES cells

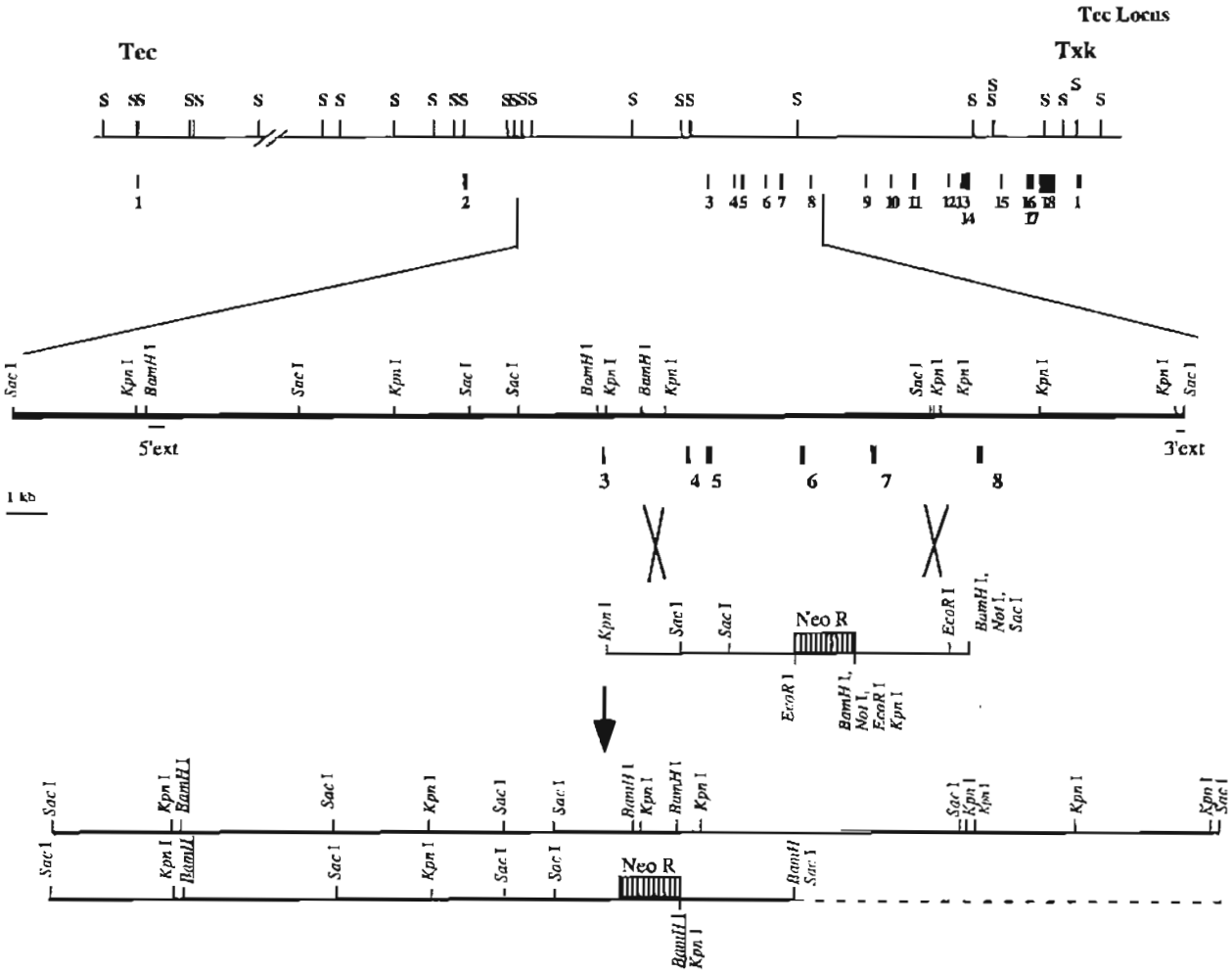
Because the approach described above failed to isolate E14TG2a-derived ES cell clones that had successfully been targeted at the *Tec* locus, an alternative targeting strategy was designed. Two critical changes were made to the original experimental approach that involved both the design of a new gene targeting vector and a change in the type of ES cell line used for homologous recombination.

This second replacement vector was designed to effectively remove exons 3-8 (Figure 5.13). These exons are clustered within a 10 kb region in the middle of the *Tec* locus and encode PHTH and SH3 sequences that are critical for Tec function. Removal of these exons is predicted to disrupt the *Tec* locus although a short 46 amino acid peptide that encodes N-terminal PHTH sequences can potentially be expressed from the ATG codon in exon 2. This peptide is predicted to be unstructured and have no biological activity. The absence of open reading frames from downstream exons suggests that this strategy should result in the complete abolition of Tec expression.

The use of a new targeting vector that encodes longer regions of homology compared to p5H.HPRT.3NS4 was expected to increase the efficiency of homologous recombination events at the *Tec* locus. As previously mentioned, the choice of ES cell line used for gene targeting experiments affects the success of these experiments. A consistent decrease in the germline transmission potential of E14TG2a ES derivatives had been observed in the gene targeting facility of this department while this work was in progress (Remizsewski, 2000; J. Wrin, personal communication). At the same time, W9.5 ES cells which have a similar genetic

Figure 5.13 Diagrammatic representation of the second gene targeting strategy used to generate Tec -/+ ES cells

A restriction enzyme map of the *Tec* locus is represented at the top that highlights *Sac* I sites while exons are represented as black boxes. A more detailed restriction enzyme map of the region targeted for homologous recombination is shown underneath. Homologous recombination events are represented as two black crosses and the resulting modified allele is represented under the arrow.


Tec Locus
Tec
Txk
5'ext
3'ext
1 kb
Neo R
Neo R

background to E14TG2a ES cells were made available to the department. These were reported to show a high rate of coat colour chimaerism and germline colonisation compared to the chimaeric animals obtained with E14TG2a ES cells (J. Wrin, personal communication).

W9.5 ES cells are feeder dependent adherent ES cells that are cultured in the presence of LIF. During the course of these studies, W9.5 ES cells were maintained on a confluent layer of gamma irradiated STO^R fibroblast cells. Because W9.5 ES cells are HPRT positive, HAT selection could not be used for selection. The choice of resistance markers was mainly determined by the feeder cell line(s) available. Two fibroblast cell lines were available that expressed antibiotic resistance: STO^R cells which carry a Neomycin resistance cassette and are therefore resistant to G418, and H200 cells which carry a Hygromycin resistance cassette that confers resistance to Hygromycin B. Given the extensive use of both selective agents for gene targeting, culturing of ES cells in the presence of these antibiotics was expected to have no affect on the pluripotent potential of ES cells.

5.2.2.2.1 Generation of the Gene Targeting Construct pKE.Neo.KP2.8

This targeting vector encodes the bacterial aminoglycoside phosphotransferase *neo* gene which confers resistance to the antibiotic G418. The *neo* selective marker was isolated as an *EcoR* I/*BamH* I fragment from the pMGD20Neo plasmid which includes pgk promoter and polyA tail sequences (Gassmann et al., 1992). To facilitate further manipulation, this fragment was subcloned in pBluescript II KS^+ to generate the pKS^+Neo plasmid shown in Figure 5.14B. The 5′ homology arm of the targeting vector was isolated as a 4.6 kb *Kpn* I/*EcoR* I DNA fragment from pK16, purified and subcloned into *Kpn* I/*EcoR* I linearised pBluescript KS^+ to generate the plasmid pKE (Figure 5.14A). The Neo cassette was isolated from pKS^+Neo and inserted into *EcoR* I/*BamH* I linearised pKE to produce the pKE.Neo plasmid. This plasmid encodes 5′ homology and marker gene sequences (Figure 5.14B). To derive the 3′ homology arm of the targeting vector, ex78SS6.5 (Figure 5.14C) was digested with *Kpn* I/*Pst* I and the

Figure 5.14 Diagrammatic representation of the cloning strategy to generate the 5′ homology arm and marker sequences of targeting vector

A. Restriction enzyme map of genomic fragment K16 that highlights the *Kpn* I/*EcoR* I fragment used as the 5′ homology arm of the targeting vector

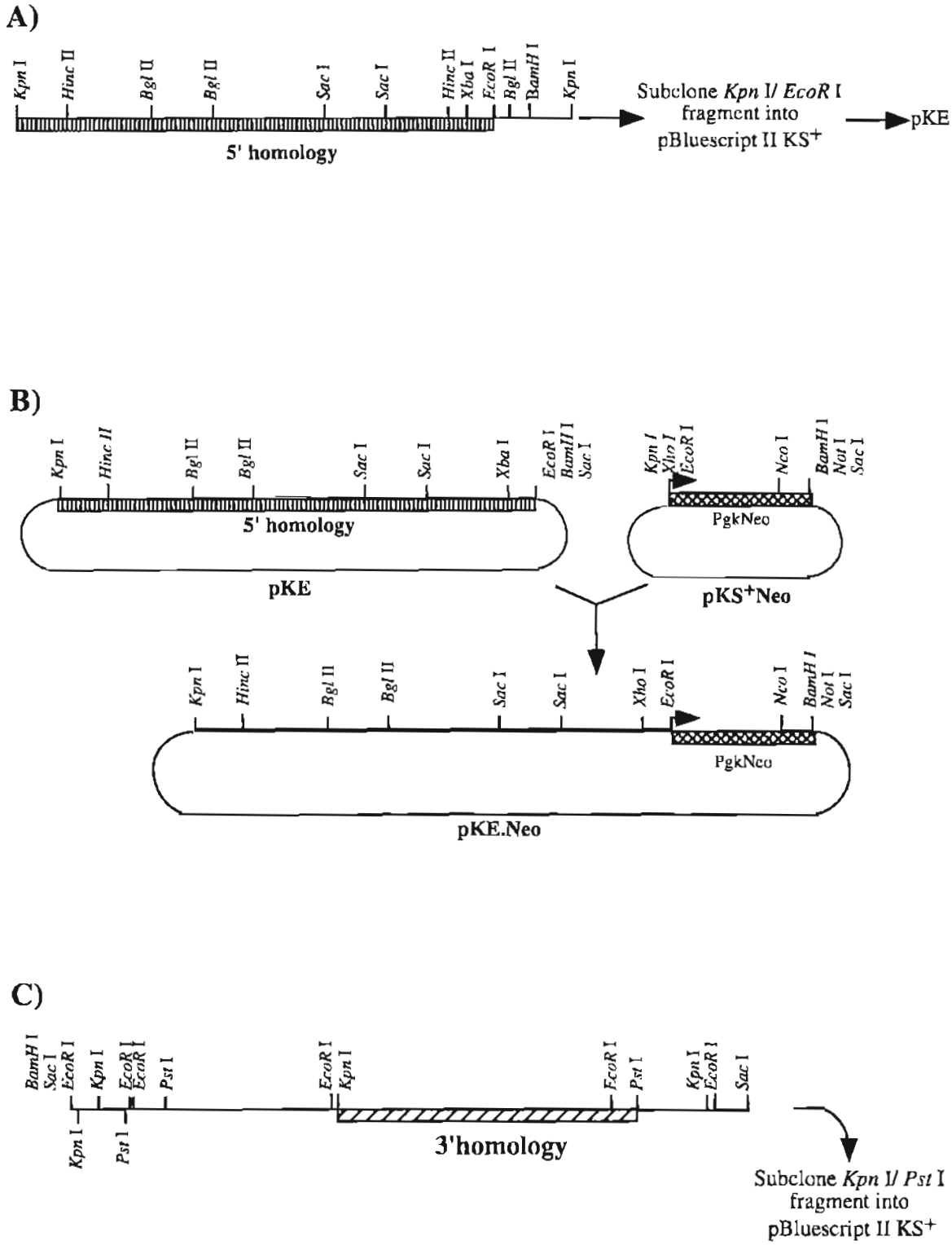
The 5′ homology arm of the vector was isolated using *Kpn* I/*EcoR* I restriction enzyme digestion of pK16 and was subsequently subcloned into pBluescript II KS+ to generate pKE.

B. Diagrammatic representing of the cloning step used to insert the Neo^R marker gene

The Neo^R resistance cassette was isolated by *BamH* I/*EcoR* I restriction enzyme digestion of pKS+Neo and was inserted into *EcoR* I/*BamH* I linearised pKE to construct the pKE.Neo plasmid.

C. Restriction enzyme map of the genomic fragment ex78S6.5 showing the *Kpn* I/*Pst* I fragment used as the 3′ homology arm of targeting construct

A restriction enzyme map of the ex78S6.5 fragment is shown that highlights the *Kpn* I/*Pst* I fragment used as the 3′ homology arm of the targeting vector. This fragment was subcloned into *Kpn* I/*Pst* I linearised pBluescript II KS+ to generate pex78KP2.8


A)
Kpn I
Hinc II
Bgl II
Bgl II
Sac I
Sac I
Hinc II
Xba I
EcoR I
Bgl II
BamH I
Kpn I
5' homology
Subclone Kpn I/ EcoR I fragment into pBluescript II KS+
pKE
B)
5' homology
pKE
PgkNeo
pKS+Neo
pKE.Neo
C)
3'homology
Subclone Kpn I/ Pst I fragment into pBluescript II KS+
1 Kb

2.8 kb genomic DNA fragment obtained was purified and subcloned into *Kpn* I/*Pst* I linearised pBluescript II KS$^+$ to generate pex78KP2.8 (Figure 5.14C). To facilitate the subcloning of 3' homology sequences into pKE.Neo, a *Not* I site needed to be introduced at the 5' end of the *Kpn* I site in the polylinker sequence of pex78KP2.8. Briefly, pex78KP2.8 and pBluescript II SK $^+$ were digested with *Sca* I/*Kpn* I. DNA fragments were purified by gel electrophoresis and the 1.87 kb *Sca* I/*Kpn* I fragment of pBluescript II SK$^+$ carrying multiple cloning site sequences was ligated to the 4 kb fragment of pex78KP2.8 which encodes 3' homology sequences to generate the plasmid pSK-KP2.8 (Figure 5.15A). The 3' homology arm of the targeting vector was isolated from pSK-KP2.8 by *Not* I restriction enzyme digestion. The 2.8 kb fragment was purified by gel electrophoresis and inserted into *Not* I linearised pKE.Neo (Figure 5.15B). This gene targeting vector, pKE.Neo.Kp2.8, was further characterised by restriction enzyme digestion and gel electrophoresis (Figure 5.16A and B). Prior to transfection into ES cells, large scale plasmid DNA preparations of pKE.Neo.Kp2.8 were finally purified on a CsCl gradient, linearised with *Sac* II, and extracted with phenol chloroform.

5.2.2.2.2 Generation and characterisation of Tec +/- ES cells

Toxicity assays were first carried out to optimise the concentration of G418 necessary to select against untransfected W9.5 ES cells. Unlike E14TG2a ES cells, W9.5 ES cells are ideally maintained on a layer of feeder cells. To identify the level of G418 resistance of the STO^R fibroblast cells used as feeder cells, STO^R cells were subjected to toxicity assays, the result of which are described in Figure 5.17A. Briefly, concentrations of G418 ranging between 200 and 2000 µg/ml were added to a subconfluent layer of STO^R cells. At 200 µg/ml G418, 100% STO^R cells appeared to survive selection after 7 days. Survival was significantly decreased at 600 µg/ml suggesting that higher concentration of G418 were not suitable for the culturing of STO^R cells. Unlike STO^R cells, W9.5 ES cells do not carry the *neo* cassette and

Figure 5.15 Subcloning of 3′ homology sequences

A. Generation of pSK-KP2.8 plasmid

Diagrammatic representation of cloning step required to generate pSK-KP2.8.

B. Final subcloning step required to generate the pKE.Neo.KP2.8 targeting vector

Diagrammatic representation of pKE.Neo and pSK-KP2.8 plasmids that highlights the cloning step required to generate pKE.Neo.KP2.8.

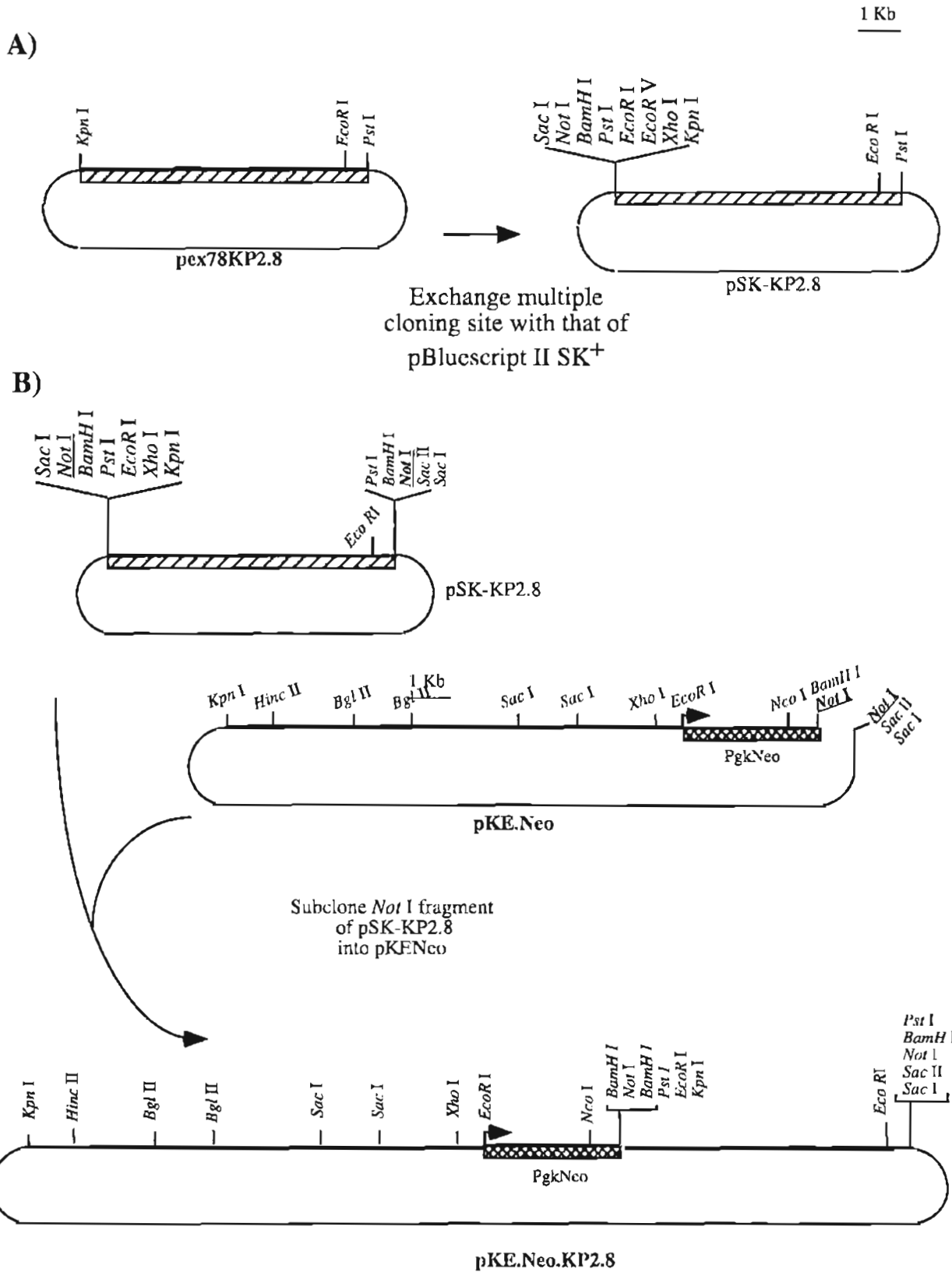

A)
1 Kb
Kpn I
EcoR I
Pst I
pex78KP2.8
Sac I
Not I
BamH I
Pst I
EcoR I
EcoR V
Xho I
Kpn I
Eco R I
Pst I
pSK-KP2.8
Exchange multiple
cloning site with that of
pBluescript II SK+
B)
Sac I
Not I
BamH I
Pst I
EcoR I
Xho I
Kpn I
Pst I
BamH I
Not I
Sac II
Sac I
Eco RI
pSK-KP2.8
Kpn I
Hinc II
Bgl II
Bgl II
1 Kb
Sac I
Sac I
Xho I
EcoR I
Nco I
BamH I
Not I
Not I
Sac II
Sac I
PgkNeo
pKE.Neo
Subclone Not I fragment
of pSK-KP2.8
into pKENeo
Kpn I
Hinc II
Bgl II
Bgl II
Sac I
Sac I
Xho I
EcoR I
Nco I
BamH I
Not I
BamH I
Pst I
EcoR I
Kpn I
Pst I
BamH I
Not I
Sac II
Sac I
Eco RI
PgkNeo
pKE.Neo.KP2.8

Figure 5.16 Characterisation of pKE.Neo.KP2.8

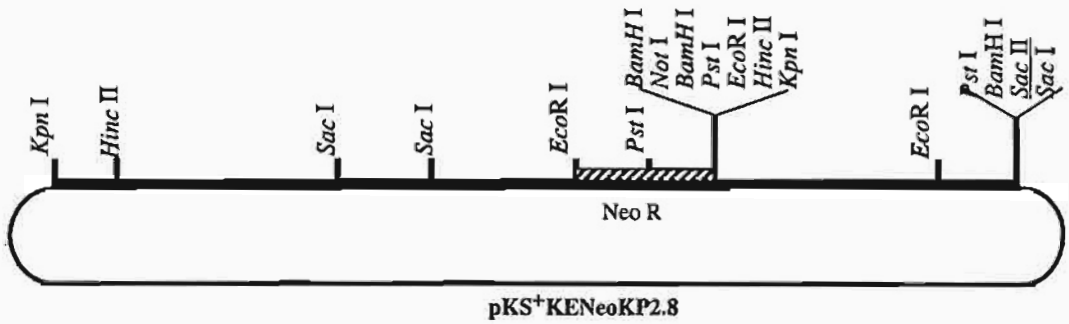
A. Restriction enzyme map of pKE.Neo.KP2.8

5′ and 3′ homology arms of the vector are represented as thick black lines and the Neo resistance cassette is represented as a hatched box. The *Sac* II restriction enzyme site used to linearise the vector is underlined.

B. Gel electrophoresis of restriction enzyme digests of pKE.Neo.KP2.8

Restriction enzyme digests of pKE.Neo.KP2.8 were separated on a 1% (w/v) agarose gel. SPP1 DNA molecular weight markers were used for size comparison.

A)


Kpn I
Hinc II
Sac I
Sac I
EcoR I
Pst I
BamH I
Not I
BamH I
Pst I
EcoR I
Hinc II
Kpn I
EcoR I
Pst I
BamH I
Sac II
Sac I
Neo R
pKS+KENeoKP2.8
1 Kb

B)

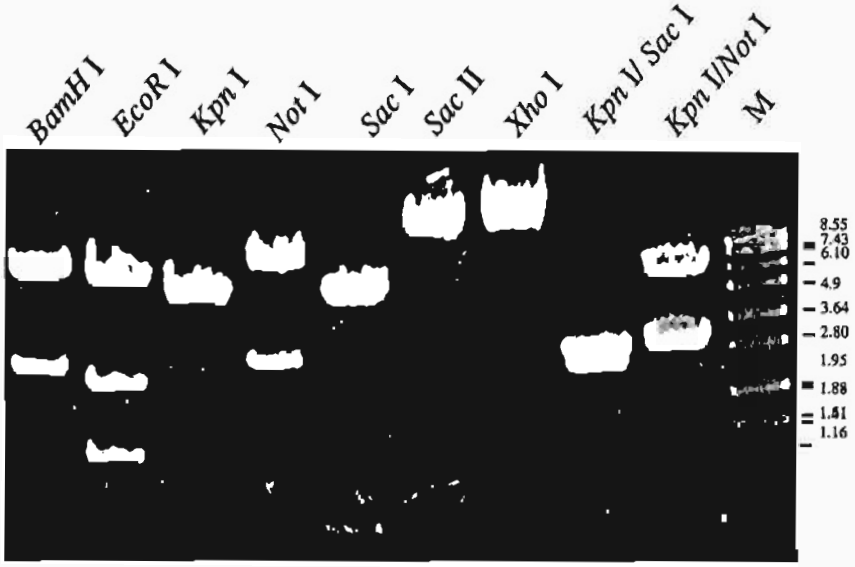

BamH I
EcoR I
Kpn I
Not I
Sac I
Sac II
Xho I
Kpn I/ Sac I
Kpn I/Not I
M
8.55
7.43
6.10
4.9
3.64
2.80
1.95
1.88
1.51
1.16

Figure 5.17 G418 toxicity assays

A. Determination of G418 toxicity on STO^R cells.

$1x10^6$ STO^R cells were plated on 100 mm plates. G418 was added 24 hours after plating and selection was carried out for 7 days. The percentage survival was determined based on the survival of cells plated in the absence of G418.

B. Determination of G418 toxicity on W9.5 cells.

$5x10^5$ W9.5 cells were plated on a confluent layer of STO^R cells in 100 mm plates. G418 was added after 24 hours and selection was carried out for 7 days. The percentage survival was determined based on the survival of ES cell colonies observed in the absence of G418.

A)

Plate number	G418 (μg/ml)	% cell survival *
1	0	100
2	200	100
3	600	80
4	1000	50
5	1500	30
6	2000	20

* 1×10^6 cells were plated.
Selection was started after 24 hours.
G418 resistant cells were counted after 7 days of selection.

B)

Plate number	G418 (μg/ml)	% cell survival *
1	0	100
2	150	< 5
3	175	< 5
4	200	< 1
5	225	< 1
6	250	< 1

* 5×10^5 cells were plated. Selection was started after 24 hours.
Remaining cells were stained and counted after 7 days of selection.

are therefore sensitive to G418 selection. As shown in Figure 5.17B, a minimum of 200 µg/ml G418 was shown to be necessary to achieve 99% W9.5 cell death after 7 days. In light of these observations,G418 selection was carried out at 250 µg/ml G418.

Sac II linearised pKe.Neo.Kp2.8 was transfected into W9.5 ES cells using the parameters described in Kwee et al. (1995). Transfected cells were cultured on STO^R cells and selection was started 24 hours following transfection and maintained for 8 days. ES cell colonies were picked using disposable yellow tips and expanded in duplicate for storage and Southern blot analysis. To identify homologous recombination events, genomic DNA preparations from G418 resistant ES cells were digested with *BamH* I, separated by gel electrophoresis on 0.8% TAE agarose gels and subjected to Southern blot analysis. The 3' external probe was derived by *Acc* I/*Sac* I restriction enzyme digestion of ex78PS1.1 (Figure 5.4C). This probe was used in the first round of screening. The Southern blot pattern expected for targeted clones using this probe is easily distinguished from that of its non-targeted counterpart due to the 10 kb difference between the two alleles. The 5' external probe was purified by *BamH* I/*Hind* III restriction enzyme digestion of the plasmid 4.1SS21 (Figure 5.2A) and was subsequently used to confirm homologous recombination events identified during the first screen. Multiple integration events of the vector were ruled out using the 300 bp internal Neo probe derived from *BamH* I/*Nco* I restriction enzyme digestion of pKS^+Neo . In practice, the 3' external probe identifies *BamH* I fragments of 20 kb and 10 kb for the wild type and targeted alleles respectively. The 5' external probe recognises a 12 kb *BamH* I fragment of the wild type allele and a 10.5 kb *BamH* I fragment of the targeted allele. The internal probe hybridises to a 10 kb *BamH* I fragment. Figure 5.18 shows typical southern blot membranes probed with the 3', 5' and internal probes respectively. To ensure that low frequency homologous recombination events would not be missed, plans were made to test a relatively larger pool of G418 resistant ES cell colonies. In total, 800 colonies that survived 7

Figure 5.18 Southern blot analysis of targeted W9.5 clones

A. Typical Southern blot analysis using the 3' external probe

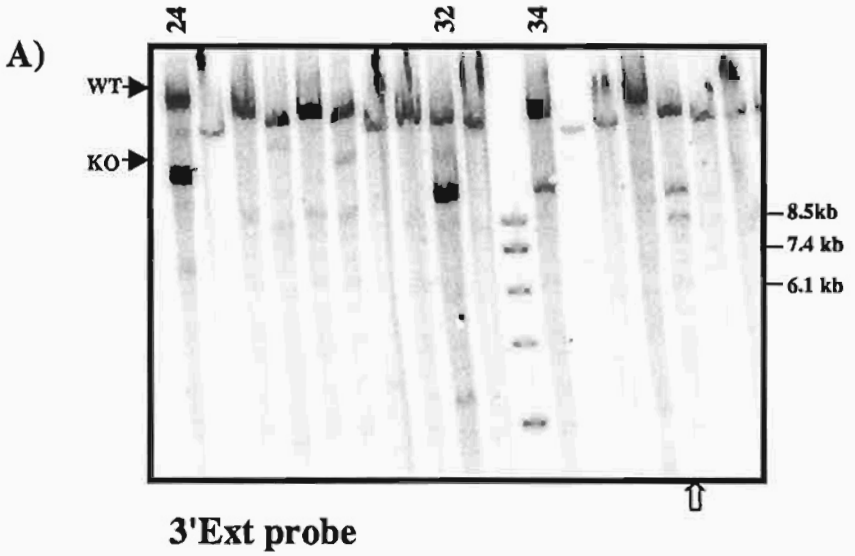
20 μg genomic DNA were digested with *BamH* I, transferred to a nylon membrane and subjected to southern blot analysis using the *Acc* I/*EcoR* I 3' external probe.

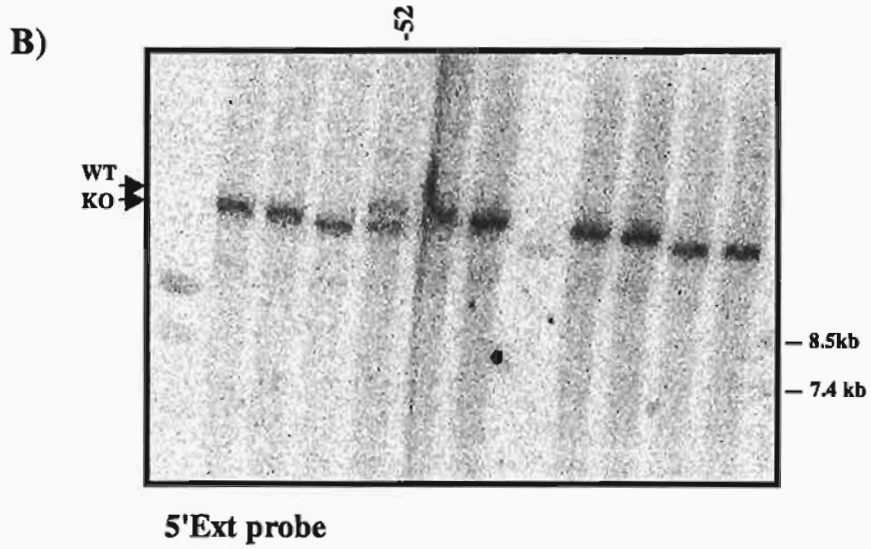
B. Typical Southern blot analysis using the 5' external probe.

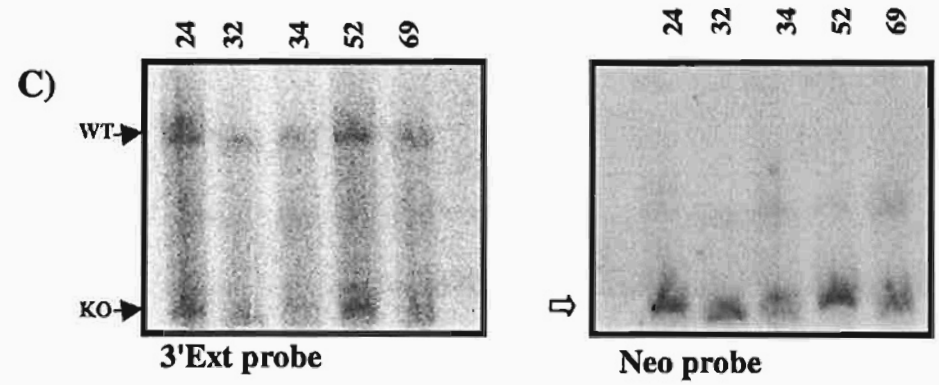
20 μg genomic DNA were digested with *BamH* I, transferred to a nylon membrane and subjected to southern blot analysis using the *Bam*H I/*Hind* III 5' external probe.

C. Southern blot analysis of targeted W9.5 derivatives using internal probe

20 μg genomic DNA were digested with *BamH* I, transferred to a nylon membrane and subjected to southern blot analysis using the *Nco* I/*Bam*H I 3′ external probe.


A)
24
32
34
WT
KO
8.5kb
7.4 kb
6.1 kb
3'Ext probe


B)
-52
WT
KO
8.5kb
7.4 kb
5'Ext probe


C)
24
32
34
52
69
WT
KO
3'Ext probe
24
32
34
52
69
Neo probe

days of selection at 250 µg/ml G418 were picked, of which 400 were immediately expanded in duplicate for storage and analysis, while the remaining 400 colonies were immediately frozen at -80°C as a backup pool of clones to be analysed only if necessary. Genomic DNA was prepared from each clone and were characterised by Southern blot analysis. Six of the first 130 ES cell clones tested displayed the Southern blot pattern expected for a targeted alleles.

These targeted ES cell clones were retrieved from storage, passaged and expanded. Upon expansion, two of the six targeted ES cell clones showed high levels of differentiation as determined by their phenotypic appearance and were thus discarded from subsequent manipulation and analysis. Numerous aliquots of the remaining 4 cell lines, subsequently labelled KENKP 24, 32, 52 and 69 respectively were prepared for long term storage in liquid nitrogen.

5.2.2.2.3 Generation and characterisation of Tec +/- chimaeras

It is critical to check the karyotype of targeted ES cell derivatives prior to their injection into recipient blastocysts as the presence of significant chromosomal abnormalities is known to affect ES cell pluripotence and inhibit colonisation of the germline of chimaeric mice (Suzuki et al., 1997). Four targeted ES cell lines were karyotyped. As summarised in Figure 5.19, all cell lines displayed a normal 2N karyotype of 40. They were thus retrieved from liquid nitrogen storage, cultured and prepared for blastocyst injection.

During the course of this Ph.D, numerous attempts were made to generate germline chimaeras using the KENKP-24, 32, 52 and 69 targeted ES cell lines. Blastocyst injection experiments were initiated at the end of 1997 using C57Bl6-derived blastocysts. All attempts made during the two years that followed however failed to generate germline transmitters. As summarised in Figure 5.20, a major limiting factor to these experiments was the relatively low number of recipients blastocysts obtained from C57Bl6 mice (Wrin, personal communication). Generally, when low numbers of blastocysts were reimplanted into recipient females no pups

Figure 5.19 Generation and characterisation of W9.5 ES cell derivatives targeted at the Tec locus.

A. Statistics of electroporation experiment of W9.5 ES cells

Selection was carried out at 250 µg/ml G418 for 8 days after which 800 G418 resistant colonies were picked manually and characterised by Southern blot analysis to identify successful targeting events. Mock transfection controls were carried out with no DNA. Transfected ES cells were also plated with no selection to characterise cell survival efficiency.

B. Karyotypic analysis of targeted ES cell derivatives.

Graphs showing the number of 2N chromosomes counted in the 4 Tec -/+ ES cell lines. Chromosome spreads were prepared and stained as described in 2.3.7 and viewed under bright field optics on the Zeiss Axioplan Universal microscope.

A.

	No Selection	G418 Selection
Cells Electroporated	3.5×10^7	3.5×10^7
Cells Plated	3.5×10^7	3.5×10^7
Cells Surviving	5.25×10^6	5.25×10^6
Cells Transfected		4.2×10^3
Colonies Picked		800
Lines Analysed		130
Targeted Lines		6
Efficiency		4.6%

B.

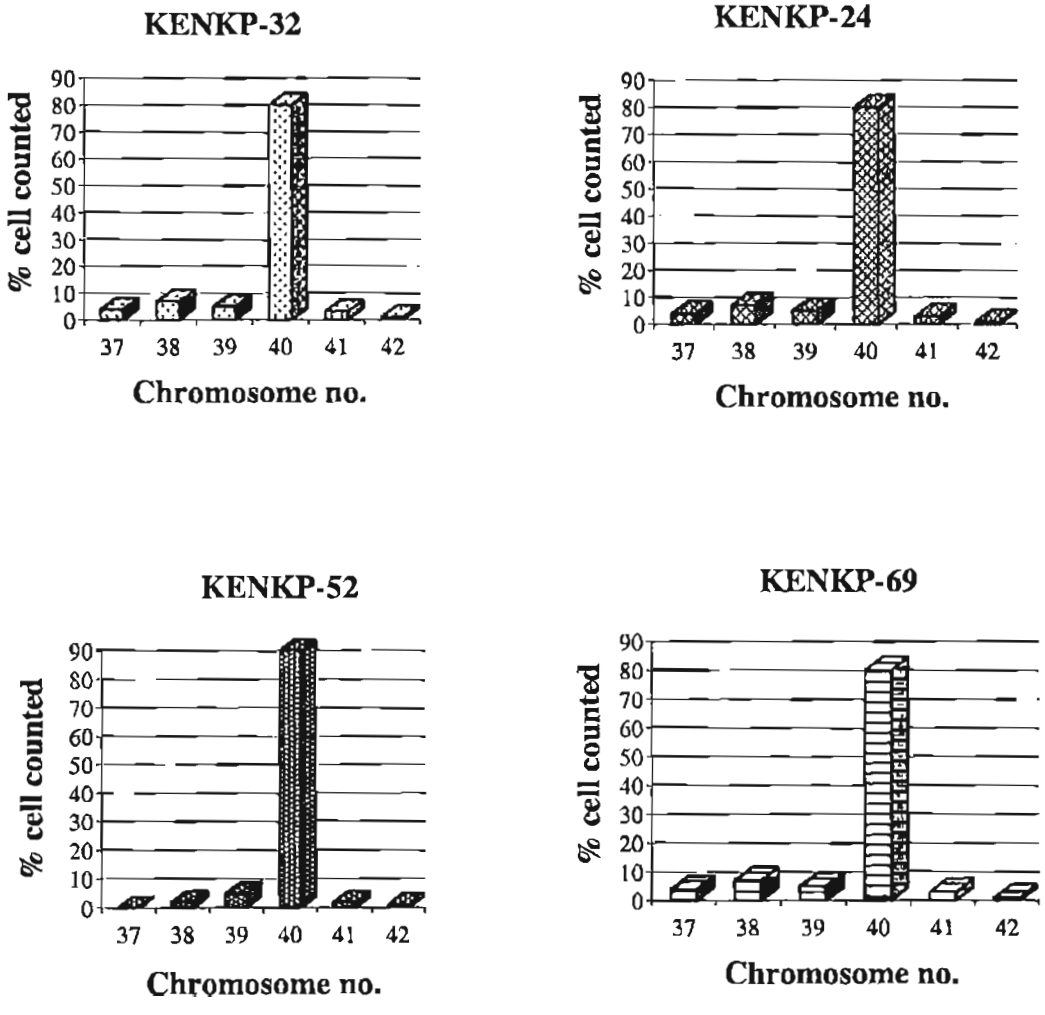


Figure 5.20 Summary of blastocyst injection experiments carried out using Tec-/+ ES cells

Table shows chronological summary of blastocyst injection attempts carried out between 1997 and 2000 according to the two mouse strains used to donate the blastocysts.

(A) Injections carried out using C57Bl6 blasotocysts.

(a) total number of blastocysts recovered from donor listed in brackets

(b) percentage chimaerism indicated in brackets

(A) Injections carried out using B6D2F1 blastocysts.

A)

Cells injected	Year	Blastocyst	No Blastocysts Injected [a]	No Pups born	Chimaeras[b]
W9.5	1997	C57Bl6	11 (11)	3	2
KENKP 52	1997	C57Bl6	13 (13)	0	
KENKP 32	1997	C57Bl6	10 (11)	4	1 (5%)
KENKP 52	1997	C57Bl6	24 (24)	1	0
KENKP 52	1998	C57Bl6	7 (7)	0	
KENKP 52	1998	C57Bl6	10 (10)	0	
KENKP 52	1998	C57Bl6	15 (18)	4	2 (>50%)
KENKP 32	1998	C57Bl6	15 (15)	3	2 (>50%)
KENKP 52	1998	C57Bl6	11 (11)	1	0
KENKP 52.2	1998	C57Bl6	4 (4)	0	
KENKP 52.2	1998	C57Bl6	7 (7)	0	
KENKP 52.2	1999	C57Bl6	13 (27)	0	
KENKP 52.2	1999	C57Bl6	NO BLASTOCYCTS	~	
KENKP 52.2	1999	C57Bl6	NO BLASTOCYCTS	~	
KENKP 52.2	1999	C57Bl6	NO BLASTOCYCTS	~	
KENKP 52.2	1999	C57Bl6	0 (5)	~	

B)

Cells injected	Year	Blastocyst	No Blastocysts Injected	No Pups born	Chimaeras
KENKP 52.2	2000	B6D2F1	26 (30)	~	~
KENKP 24.4	2000	B6D2F1	34 (100)	17	6
KENKP 52.2	2000	B6D2F1	14 (18)	3	2
KENKP 32	2000	B6D2F1	20 (48)	13	11
W9.5	2000	B6D2F1	12 (48)	~	~
KENKP 69	2000	B6D2F1	23 (25)	8	8

were born, thus reflecting the need for a critical number of embryos to ensure normal development of the transferred litters. In summary, only 5 chimaeric pups had been obtained using C57Bl6 blastocysts, three of which showed over 50% coat colour chimaerism. None of these showed germline transmission of the targeted *Tec* allele despite an extensive breeding program.

To address this problem, an alternative B6D2F1 blastocyst system which is compatible with a large number of ES cell lines was chosen. Larger numbers of blastocysts were expected from these mice as they appear to be responsive to hormone treatment (J. Wrin, personnal communication) . Injection of blastocysts was carried on five separate occasions and a significantly higher number of blastocysts were consistently obtained on each occasion. In general, approximately 50% of reimplanted blastcosysts developed to term (Figure 5.20). As mentioned in section 5.1, coat colour chimaerism is generally considered as an indicator of germline chimaerism. Percentage chimaerism is generally used to select chimaeric animals to be used for breeding. The percentage of coat colour chimaerism observed for each targeted ES cell line is summarised in Figure 5.21. In total, 27 chimaeras were obtained and coat colour chimaerism appeared to vary depending on the targeted ES cell used. Chimaeric mice generated with KENKP-69 showed the highest levels of coat colour chimaerism. Animals with a minimum of 25% agouti coat colour were bred to C57Bl6 mice to identify potential germline transmitters. As summarised in Figure 5.22, when possible, six litters were generated for each chimaeric animal including female chimaeras, unless chimaeric animals had died during the breeding program. Of these, three animals which include those with the highest level of coat colour chimaerism, appeared to be sterile. In total 436 pups were obtained, none of which showed germline transmission.

Figure 5.21 Analysis of coat colour chimaerism observed in mice produced with Tec-/+ ES cells

A. Picture of chimaeric animals obtained from injection using KENKP-32 cell line

B. The graph shows the percentage contribution of agouti coat colour observed on an otherwise black background. Percentages were determined based on surface area covered by agouti coat colour. Each column represent individual mice.

A.

B.

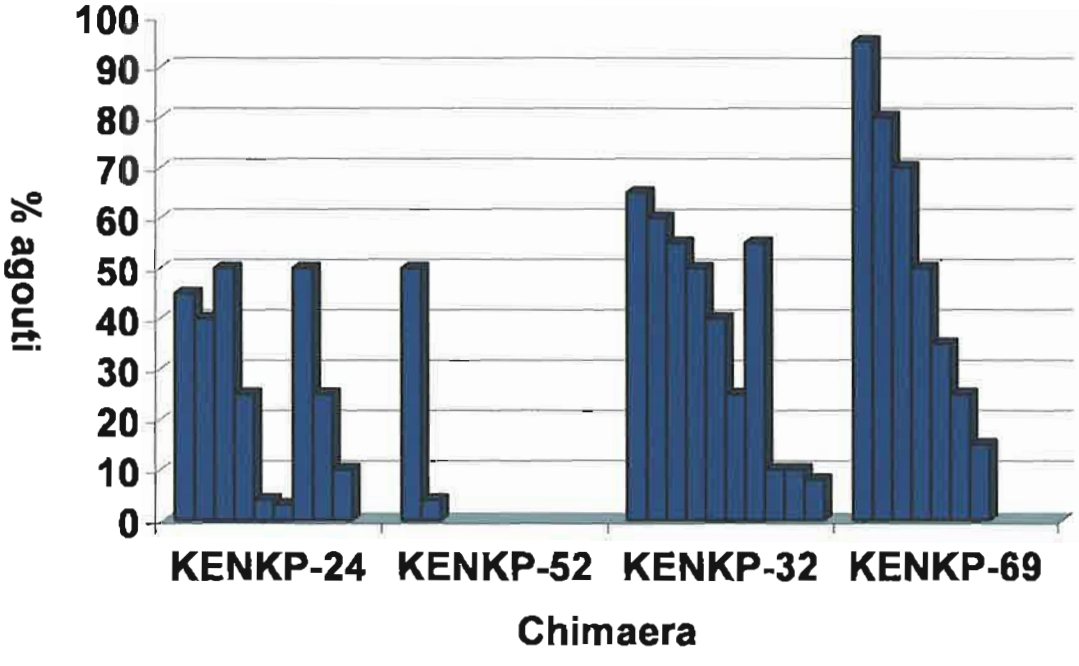

100
90
80
70
60
50
40
30
20
10
0
% agouti
KENKP-24
KENKP-52
KENKP-32
KENKP-69
Chimaera

Figure 5.22 Summary of breeding program carried out to identify germline transmitters

Both male and female chimaera that showed a minimum of 25% coat colour chimaerism were bred to C57Bl6 mice. Number of offsprings from each matings are listed. Germline transmission is identified by the transmission of the agouti coat colour. Mice that do not carry the W9.5-derived alleles are characterised by the black coat colour.

Chimaeras	M/F	#1	#2	#3	#4	#5	#6	Germline Transmitter
KENKP-24 45%	M	0	0	0	0	dead		0
KENKP-24 40%	M	*8*	*7*	10	11	9	14	0
KENKP-24 50%	M	*4*	*9*	7	7	6	8	0
KENKP-24 25%	M	*5*	*2*					0
KENKP-24 50%	F	*4*	*7*	9	2	8	14	0
KENKP-52.2 50%	M	*6*	*11*	4	4			0
KENKP-32 65%	M	*7*	*11*	8	8	9	4	0
KENKP-32 60%	M	*8*	*10*	11	11	5	11	0
KENKP-32 55%	M	*9*	9	7	9	7	10	0
KENKP-32 50%	M	*3 (1 runt)*	*10*	6	5	4	4	0
KENKP-32 40%	M	*9 (2 died)*	*7*	5	4	10	9	0
KENKP-69 95%	M	0	0	0	0	0	0	0
KENKP-69 80%	M	0	0	0	0	0	0	0
KENKP-69 *70%*	*M*	*5*	*9*	*10*	*10*	*12*	dead	0
KENKP-69 *50%*	*M*	*culled* [a]						
KENKP-69 50%	F	*10*	*11*	13	10			0
Total		*78*	*86*	47	81	70	74	0

5.2.2.3 Generation of Tec -/- ES cells

As previously mentioned, the power of ES cells lies not only in their ability to contribute to the developing embryo following their re-introduction into recipient mouse blastocysts, but also in their ability to differentiate *in vitro* thus allowing for critical information to be gathered in a relatively short time. Studies carried out using such *in vitro* differentiation approach are obviously less complex than those carried out in genetically manipulated mice as they generally concentrate on a specific cell lineage. As described in Chapter 4, studies of the phagocytic function of macrophage cells suggested a role for Tec in phagocytosis. *In vitro* differentiation protocols for ES cells had been reported that efficiently produce macrophage cells (Wiles and Keller, 1991; Keller et al., 1993; Lake et al., 2000). The role of Tec in macrophage differentiation and function *in vitro* was thus investigated further.

The overall strategy of this approach is described in Figure 5.23. To disrupt the non-targeted allele of Tec -/+ ES cells, the genomic fragment encompassing exons 3-8 of the *Tec* gene was replaced with a selectable marker to confer resistance to the antibiotic Hygromycin B. To confirm that phenotypic differences between Tec deficient cells and those expressing Tec were not due to a clonal effect, analysis was planned in targeted derivatives from at least two parental cell lines. Targeting was thus carried out in KENKP-24 and KENKP-32 Tec-/+ ES cells.

5.2.2.3.1 Generation of gene targeting construct

Briefly, the Hygromycin marker was isolated as a *EcoR* I/*Sma* I fragment from the plasmid pgkHygro/ΔLT20 (Gassman et al., 1992) and was subsequently cloned into *EcoR* I/*Sma* I linearised pBluescript II KS$^+$ to generate the pKS+Hygro plasmid shown in Figure 5.24A. 5' homology sequences were isolated from pK16 (Figure 5.24A) by restriction enzyme digestion with *Kpn* I. The 6 kb *Kpn* I/*Kpn* I fragment of pK16 was separated from vector sequences by gel electrophoresis, purified and cloned into *Kpn* I linearised pKS$^+$Hygro

Figure 5.23 Targeting strategy for the generation of Tec -/- ES cells

A restriction enzyme map of the Tec locus showing *Sac* I sites is shown at the top. A restriction enzyme map of the region of the locus that is targeted in this second round of gene targeting is shown underneath. Homologous recombination events of the gene targeting vector are represented as black crosses and the resulting targeted allele is represented under the black arrow. A second targeting event, using a vector containing a Hygromycin Resistance (HygroR) gene is indicated at the bottom. Tec -/- ES cells are expected to contain both NeoR and HygroR replacement vectors. Exons are represented as black boxes and the neomycin and hygromycin resistance cassettes as hatched boxes.

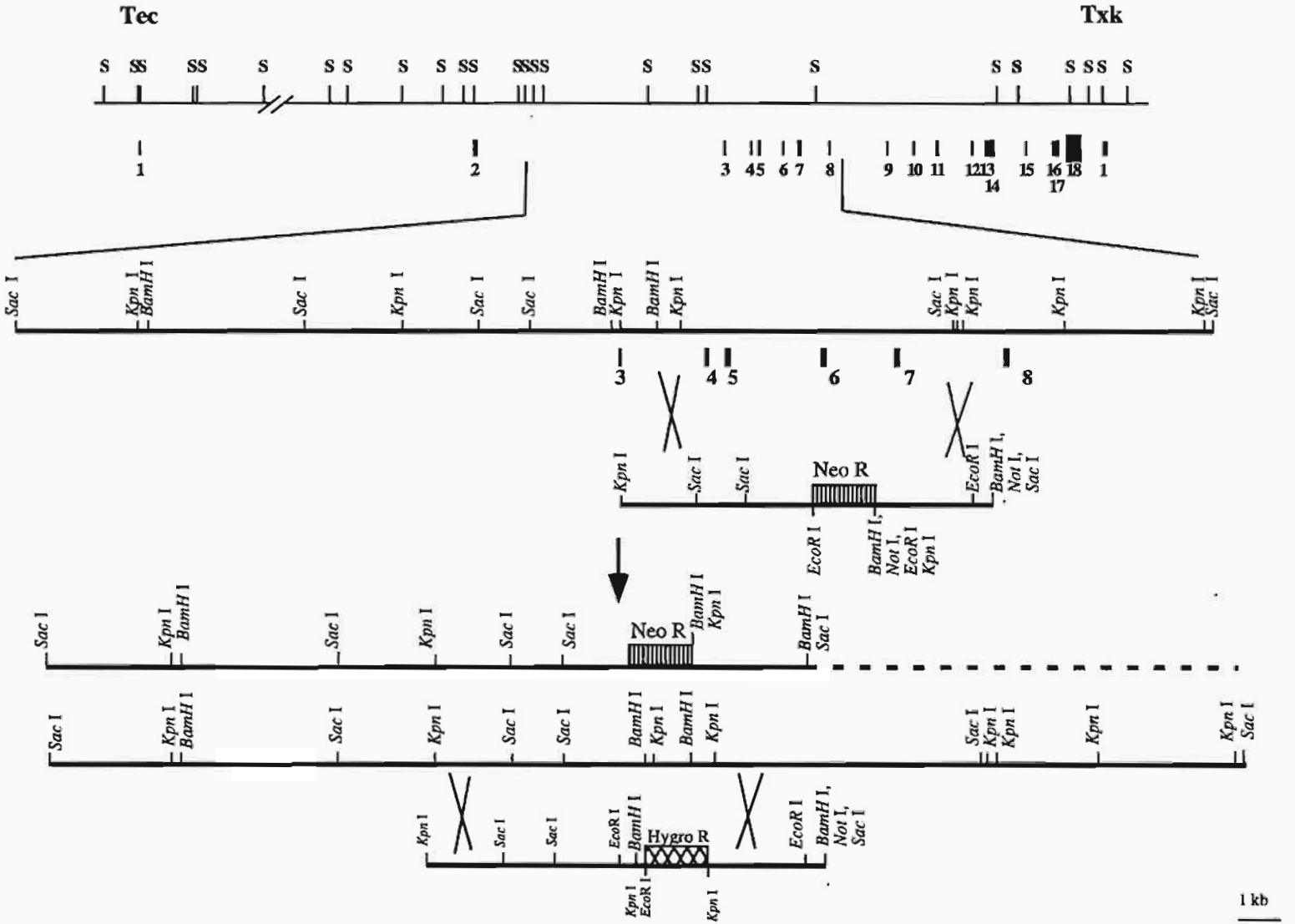
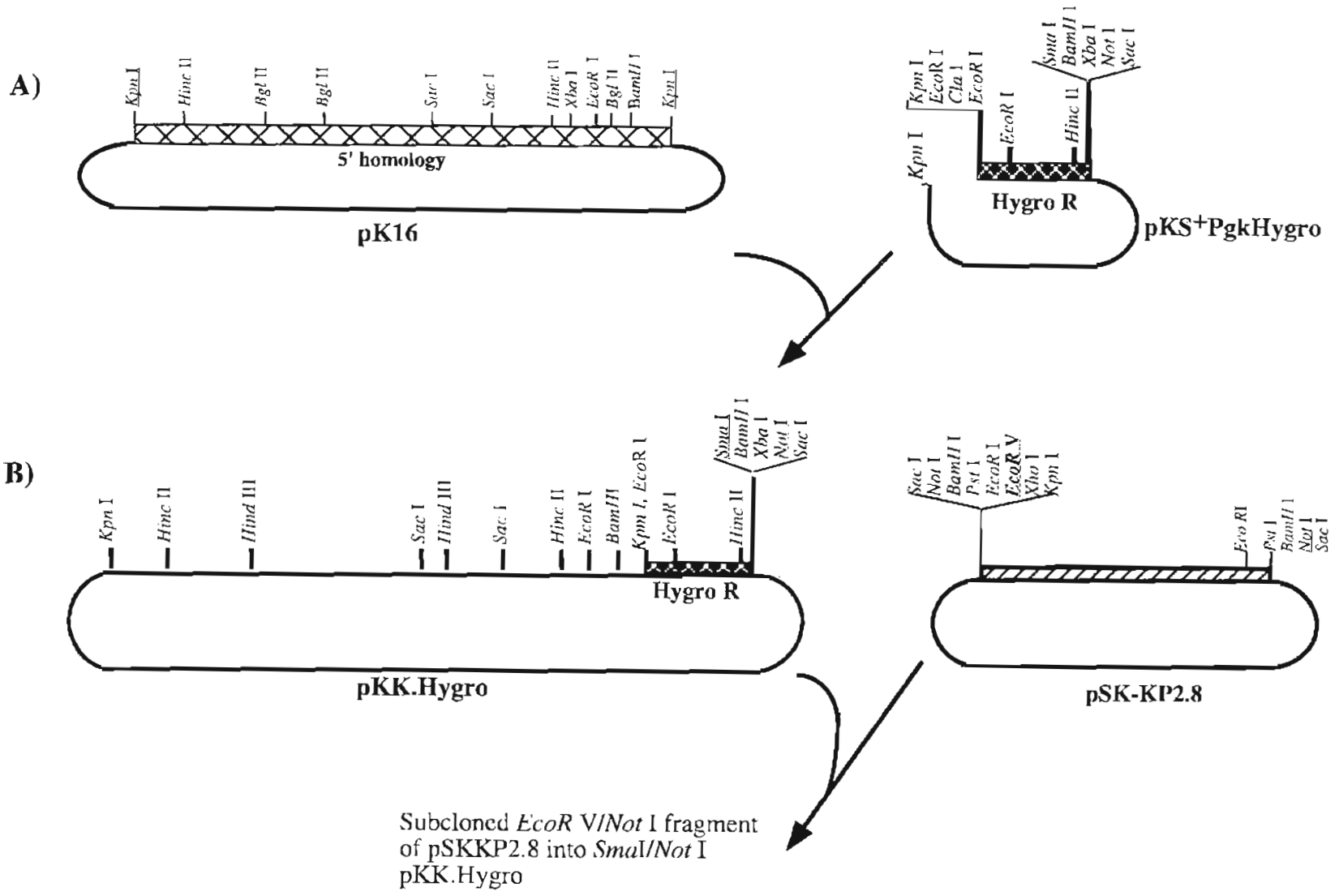

Tec
Txk
Neo R
Hygro R
1 kb

Figure 5.24 Diagrammatic representation of cloning steps involved in the generation of the pKK.Hygro.KP2.8 gene targeting vector

A. Restriction enzyme map of pK16 and pKS+Hygro. Restriction enzyme digestion of pK16 with *Kpn* I was used to isolate the 5' homology region of the gene targeting vector. This *Kpn* I/*Kpn* I fragment was inserted into *Kpn* I linearised pKS+Hygro.

B. Restriction enzyme map of pKK.Hygro and pSK-KP2.8 plasmids. The 3' homology arm of the targeting vector was inserted as *EcoR* V/*Not* I fragment into *Sma* I/*Not* I linearised pKK.Hygro.


A)
Kpn I
Hinc II
Bgl II
Bgl II
Sac I
Sac I
Hinc II
Xba I
EcoR I
Bgl II
BamH I
Kpn I
5' homology
pK16
Kpn I
EcoR I
Cla I
EcoR I
EcoR I
Hinc II
Sma I
BamH I
Xba I
Not I
Sac I
Hygro R
pKS+PgkHygro
B)
Kpn I
Hinc II
Hind III
Sac I
Hind III
Sac I
Hinc II
EcoR I
BamH I
Kpn I, EcoR I
EcoR I
Hinc II
Sma I
BamH I
Xba I
Not I
Sac I
Hygro R
pKK.Hygro
Sac I
Not I
BamH I
Pst I
EcoR I
EcoR V
Xba I
Kpn I
EcoR RI
Pst I
BamH I
Not I
Sac I
pSK-KP2.8
Subcloned EcoR V/Not I fragment
of pSKKP2.8 into SmaI/Not I
pKK.Hygro

to generate pKK.Hygro (Figure 5.24B). The 3' homology region of the targeting vector was derived from pSK.KP2.8 as an *EcoR* V/*Not* I fragment. The 2.8 kb fragment was subsequently purified and ligated into *Sma* I/*Not* I linearised pKK.Hygro (Figure 5.24B). A restriction enzyme map of the resulting plasmid, pKS$^+$KK.Hygro.KP2.8 is shown in Figure 5.25A and B. pKS$^+$KK.Hygro.KP2.8 was used as the gene targeting vector in second round targeting experiments. Prior to transfection into ES cells, plasmid preparation of pKS$^+$KK.Hygro.KP2.8 was purified on a CsCl gradient, digested with *Not* I and extracted with phenol chloroform.

5.2.2.3.2 Generation and characterisation of Tec -/- ES cells

To generate Tec -/- ES cells, two ES cell lines (KENKP-24 and 32) were electroporated with *Not* I linearised pKS+KK.Hygro.KP2.8 using the parameters described in Kwee et al., 1995. To prevent homologous recombination events at the pKE.Neo.Kp2.8 targeted Tec allele, selection was carried out in both Hygromycin B and G418 for 10 days. As no feeder cell line was available that was resistant to both antibiotics, and given time constraints that prevented the generation of such a feeder cell line, selection was carried out in the absence of a feeder layer on gelatinised plates. This was not expected to be detrimental to the experimental approach given that *in vitro* differentiation protocols require that ES cell lines be adapted to feeder-free culture conditions. As summarised in Figure 5.26A, 200 G418/Hygromycin B resistant colonies were picked after 10 days. Colonies were expanded in duplicate for liquid nitrogen storage and Southern blot analysis. The apparent efficiency of targeting events using this construct was much lower than expected based on first round targeting results. Although two Tec -/+ lines had been electroporated, only one Tec -/- clone was identified by Southern blot analysis (Figure 5.26B). This ES cell clone was subsequently expanded and frozen for long term storage. Karyotyping of the cell line confirmed the absence of gross chromosomal abnormalities (Figure 5.26C).

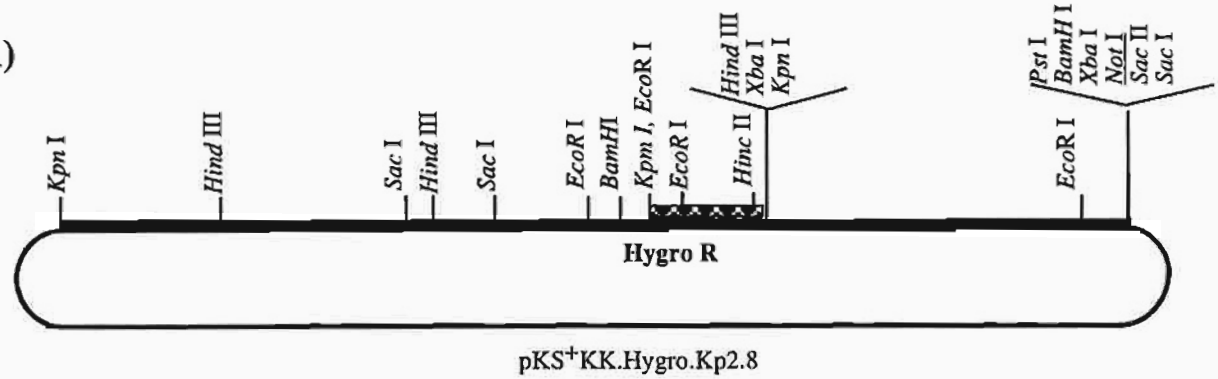
Figure 5.25 Characterisation of pKK.Hygro.KP2.8

A. Restriction enzyme map of pKK.Hygro.KP2.8 plasmid

Regions of homology are represented as thick black lines and the Hygromycin resistance cassette as a hatched box. Underlined is the Not I restriction enzyme site used to linearise the vector prior to transfection.

B. Gel electrophoresis of restriction enzyme digest products of pKK.Hygro.KP2.8

A)



pKS^{+}KK.Hygro.Kp2.8

1 Kb

B)

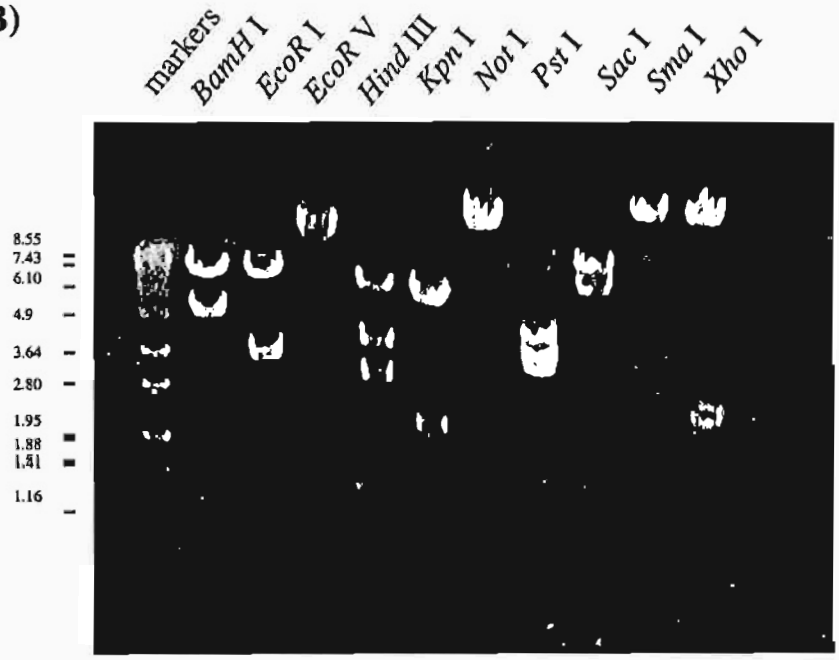


Figure 5.26 Characterisation of Tec-/- ES cell clones

A. Electroporation statistics for the generation of Tec-/- ES cell clones

Selection was carried out in Hygromycin (100μg/ml) and G418 (250 μg/ml) for 10 days after which a total of 200 colonies were picked and analysed by restriction enzyme digestion and Southern blot analysis. Transfected ES cells plated with no selection were used to determine the survival rate of transfected cells.

B. Southern blot analysis of G418/Hygromycin resistant ES cell using the 3' external probe. Note that lane 2 expected to represent a double knock out ES cell clone.

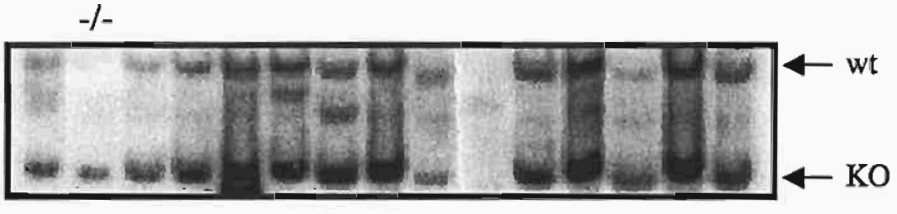
C. Karyotypic analysis of Tec -/- ES cell clone

Graphs showing the number of 2N chromosomes counted in the Tec -/- ES cell line. Chromosome spreads were prepared and stained as described in 2.3.7 and viewed under bright field optics on the Zeiss Axioplan Universal microscope.

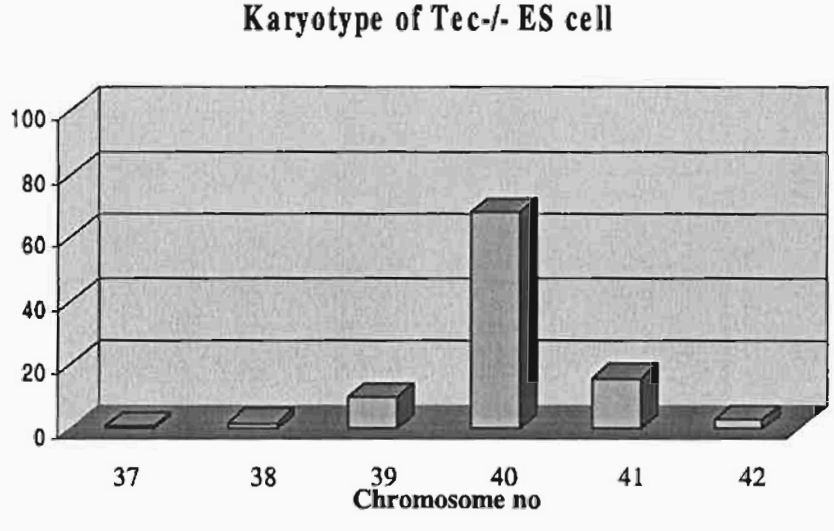
A)

	No Selection	Selection
Cells Electroporated	5.44×10^7	5.44×10^7
Cells Plated	5.44×10^7	5.44×10^7
Cells Surviving	1×10^6	1×10^6
Cells Transfected		1.8×10^3
Colonies Picked		200
Lines Analysed		200
Targeted Lines		1
Efficiency		0.50%

B)



C)



5.2.2.3.4 Analysis of phagocytic characteristics of ES cell in vitro differentiation product

The results described in this section represent observations made during three independent *in vitro* differentiation experiments. Each experiment utilised ES cells of the same passage number to ensure that differences were not due to extensive passaging of ES cell lines. Wild type W9.5 and Tec +/- KENKP-32 ES cells were adapted to feeder-free culture conditions for *in vitro* differentiation studies by multiple passages on gelatinised plates.

The *in vitro* differentiation system used followed the protocol optimised in the laboratory of Prof. P.D. Rathjen to ensure efficient differentiation of ES cells into macrophage cells (Remiszewsky,2000 and Lake et al., 2000). This *in vitro* differentiation system relies on the formation of EPL cells (Lake, 2000) prior to the formation of embryoid body structures and is reported to result in efficient hematopoietic differentiation from ES cells (Lake et al., 2000, Remischesky, 2000). In the presence of IL-3 and M-CSF, 80% of embryoid bodies reportedly release macrophage cells as confirmed by staining with the macrophage specific F4/80 antibody (Remiszewsky, 2000).

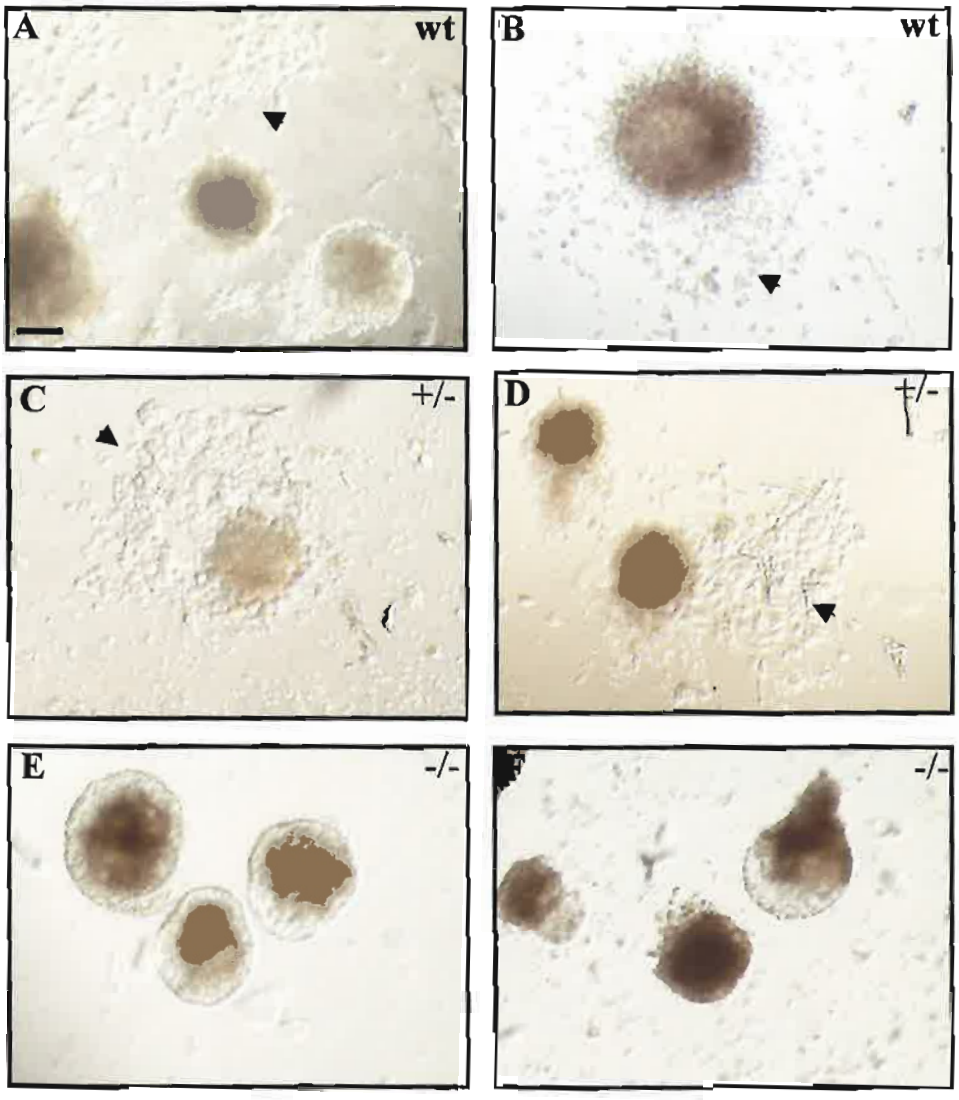
Differentiation in methylcellulose was carried out for 8 days. By this time embryoid bodies appeared to have ruptured and cells were identified that formed a halo surrounding the embryoid body structures which will be referred to as colony bursts. Colony bursts were visualised by phase contrast microscopy and images were captured using a Nikon inverted ECLIPSE TE 300 microscope. Photographs of typical embryoid body morphology obtained for W9.5 ES cells and its targeted derivatives are depicted in Figure 5.27. Wild type and Tec -/+ embryoid bodies were characteristically opaque in the centre and macrophage cells appeared to form an extending mass surrounding the embryoid bodies (Figure 5.27A and B). Tec -/- ES cells generally failed in all three attempts to generate similar structures (Figure 5.27C). Over the course of these experiments, embryoid bodies generally showed an extremely small colony

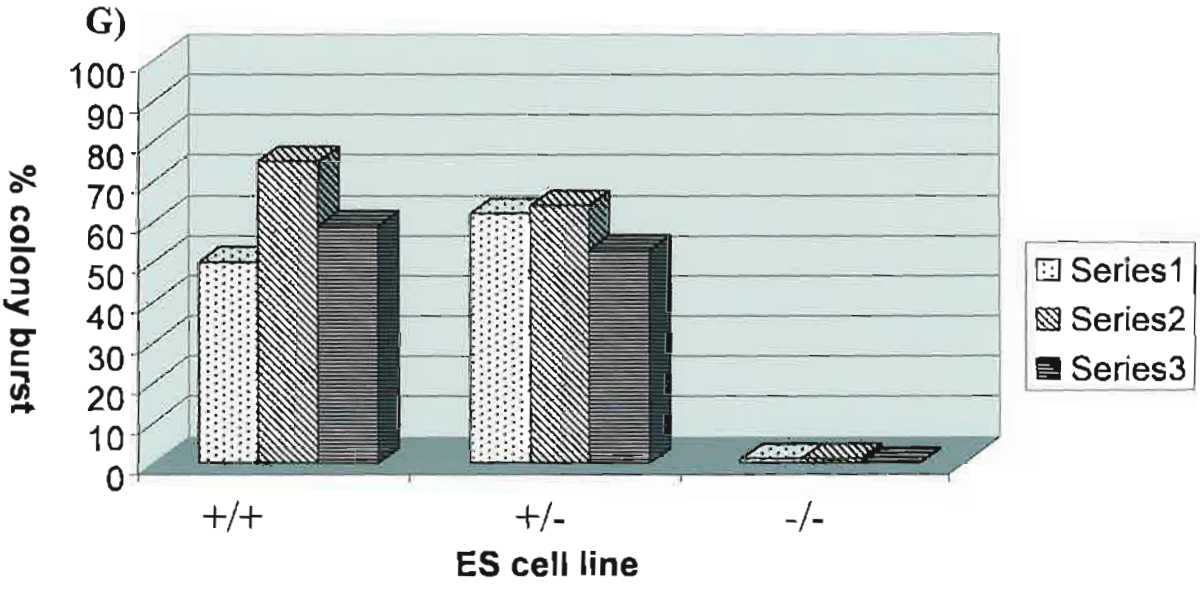
Figure 5. 27 In Vitro differentiation of ES cells into macrophage cells

Bright field images of embryoid bodies subjected to *in vitro* differentiation into macrophage cells in methylcellulose. (A-B) wild type embryoid bodies; (C-D) Tec -/+ embryoid bodies and (E-F) Tec -/- embryoid bodies.

G. Efficiency of differentiation quantitated based on the proportion of embryoid bodies forming the characteristic colony burst seen in A and B.

Bar = 100μM


A
wt
B
wt
C
+/-
D
+/-
E
-/-
F
-/-


G)
100
90
80
70
60
50
40
30
20
10
0
% colony burst
+/+
+/-
-/-
ES cell line
Series1
Series2
Series3

burst size-if any at all- compared to those obtained for Tec+/+ or Tec-/- ES cells (Figure 5.27C).

The phagocytic properties of macrophage cells generated from these embryoid bodies was subsequently confirmed by immunohistochemical analysis. In these experiments, *in vitro* differentiation products were first replated on glass coverslips. Tec+/+ and Tec+/- macrophage cells generally replated efficiently. Embryoid bodies could also occasionally be identified that had adhered to the glass coverslips. Phagocytic assays were generally set up 24 hours after replating using *h*IgG optimised Zymosan A as described in Chapter 4. As seen in Figure 5.28A although *in vitro* differentiation conditions had been optimised for macrophage differentiation, upon replating, cells with no phagocytic ability could also be identified (Left hand panel). The majority of cells obtained for Tec -/+ *in vitro* differentiation displayed macrophage-like characteristics that included adherence to the substratum and the presence of filopodial extensions. Most importantly these macrophage cells had the ability to bind and efficiently internalise *h*IgG opsonised exogenous material (Figure 5.28 inset). The internalisation of Zymosan A appeared to be highly efficient as numerous Zymosan A particles were characteristically internalised by single macrophage cells (Figure 5.28, inset). As previously mentioned, non phagocytic adherent cells were also identified in these preparations. These were generally morphologically distinctive from macrophage cells as they had a flattened appearance, relatively large cytoplasms and a prominent nucleus which were observed to extend from adherent embryoid bodies.

As expected Tec -/- ES cell differentiation experiments did not produce characteristic macrophage like cells after replating. Following overnight culture at 37°C fewer cells were observed on the glass coverslips, none of which resembled macrophage cells (Figure 5.28 right hand panel). This was not unexpected given that embryoid bodies had not ruptured in the methylcellulose matrix assay. Generally embryoid bodies could be identified that had adhered

Figure 5. 28 Characterisation of the phagocytic characteristic of embryoid-body derived macrophage cells

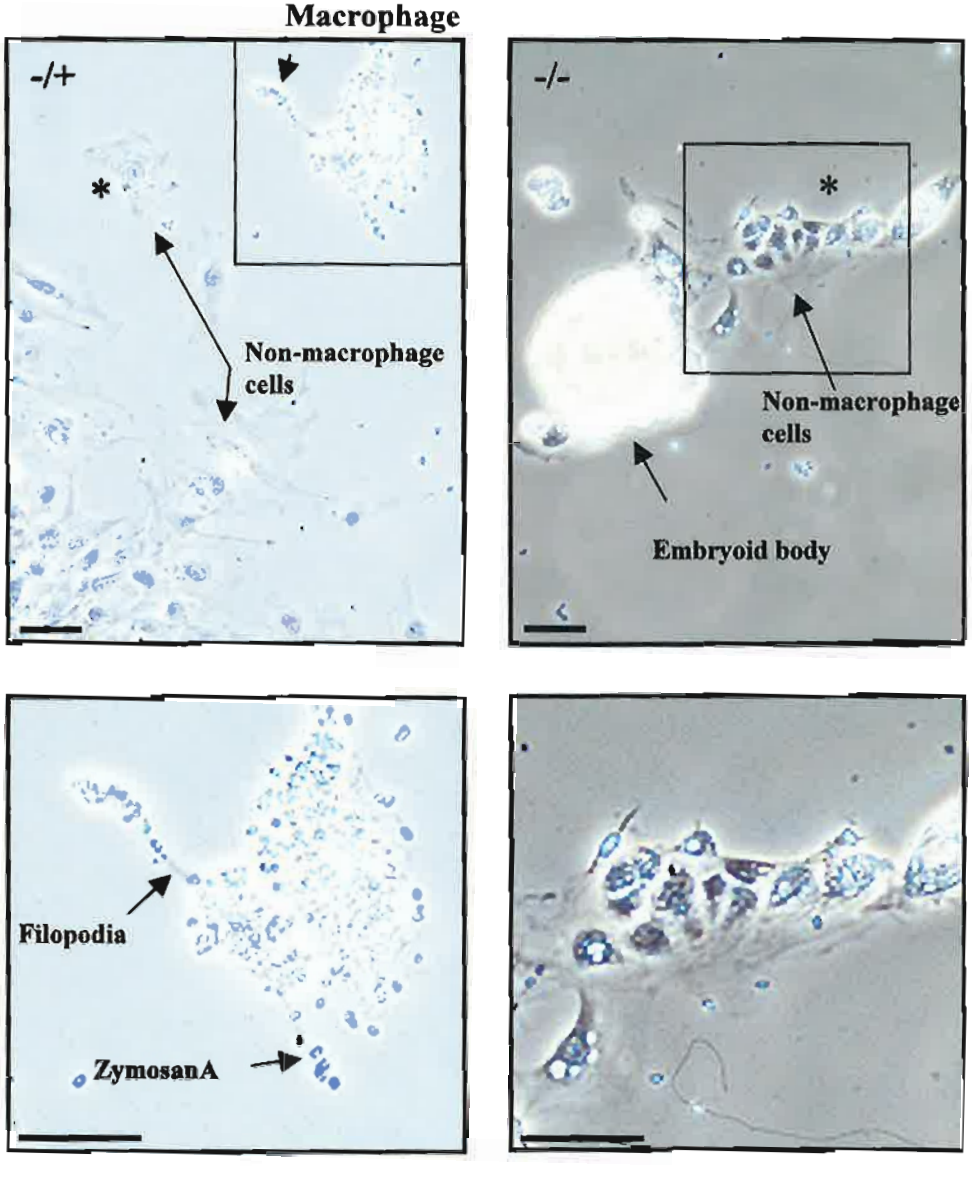
Embryoid bodies were subjected to *in vitro* differentiation into macrophage cells in methylcellulose. *In vitro* differentiation products were then washed in DMEM and re-plated onto glass coverslips. Phagocytic assays were carried out as described in Chapter 4.

Left hand panels depict *in vitro* differentiation product derived from Tec -/+ ES cells that had been replated on glass coverslips. The asterisk points to non-macrophage cells as determined by their inability to phagocytose IgG opsonised Zymosan A particles.

Right hand panels depict *in vitro* differentiation product derived from Tec -/- ES cells that had been replated on glass coverslips.

Bottom panels show a larger magnification of the boxed regions. The left hand panel depicts macrophage cells and highlights their extended filopodia. Large numbers of internalised Zymosan A particles are visible in this panel confirming their identity as macrophage cells. The right hand panel shows non macrophage cells recovered following the replating of Tec -/- *in vitro* differentiation products. Note the absence of internalised Zymosan A particles.

Bar = 100μM


Macrophage
-/+
*
Non-macrophage cells
-/-
*
Non-macrophage cells
Embryoid body
Filopodia
ZymosanA

to glass coverslips. Cells were observed around these that had a flattened appearance, relatively large cytoplasms and did not resemble macrophage cells morphologically. These cells were subjected to phagocytic assays but as expected where unable to ingest target particle (Figure 5.28 (inset)). This provided further indication that no macrophage cells were obtained from the Tec -/- ES cell line.

5.3 Discussion

The work described in this chapter aimed to characterise the function of Tec both *in vivo* using genetically manipulated mice and *in vitro* using a well established *in vitro* differentiation system for ES cells. Both approaches were highly dependent on partial characterisation of the structure of the *Tec* locus. The information gathered from these preliminary studies was subsequently used in the design of the gene targeting strategies described in this chapter.

Tec genomic sequences described in this thesis were isolated and characterised in collaboration with K. Stevens and A. Merkel. The structure of the Tec gene was shown to generally resemble that of other members of the *Tec* family of protein tyrosine kinases, including *Btk* and *Txk*. Exons located at the 5′ end of the locus are sparsely distributed, while 3′ introns are generally shorter. The 3′ end of the *Tec* locus is located within 1.5 kb of the *Txk* promoter regions suggesting that *Txk* could have arisen from the duplication of the Tec locus.

Using genomic DNA sequences isolated during gene mapping studies, gene targeting vectors were designed and constructed to disrupt the *Tec* locus in ES cells. Unfortunately, despite numerous attempts over a 3 year period, genetically altered heterozygous or homozygous mice could not be obtained from these ES cells. The first attempt which used E14TG2a ES cells failed to generate targeted ES cell clones, probably due to the relatively short 3′ homology arm of the construct. As reported in Deng and Capecchi, (1992) the rate of

homologous recombination events increases exponentially with increased length of homology, although targeting efficiency probably varies depending on the target loci.

The second targeting attempt used the feeder dependent W9.5 ES cell line which successfully generated targeted ES cell clones. This increase in efficiency can be attributed to at least two factors. First the increase in the length of homology shared between the target locus and second the identity of the ES cell line used. Studies by Udy et al., 1997 have demonstrated that targeting efficiencies can vary between ES cell lines, by 3 fold between isogenic cells line and as much as 12 fold between non isogenic cell lines. It remains possible that targeting efficiencies were also influenced by *in vitro* culture conditions of ES cells prior to transfection.

Following their return to recipient blastocysts, 4 of these lines, all of which displayed a normal karyotype, failed to show germline transmission despite high rates of coat colour chimaerism. The reason for this failure remains unclear. Wild type W9.5 ES cells had been tested prior to the beginning of this series of experiments and showed relatively high rates of germline transmission (J. Wrin, personal communication). However, three independent attempts made since this original test, using targeted derivatives of the W9.5 ES cell line (carrying mutation of different loci), have failed to generate targeted mice (J. Wrin, personal communication). It is possible that the difference in germline transmission efficiency observed between the parental W9.5 ES cell and its targeted derivatives has been caused by the extended culturing of ES cells necessary to generate targeted derivatives. The wild type W9.5 ES cell lines used to generate targeted derivatives had already been passaged at least 15 times prior to their use in these gene targeting experiments, and Tec -/+ ES cell derivatives required approximately 6 more passages prior to their reintroduction into blastocysts. Although Bradley et al. (1984) had shown that ES cells could still contribute to the germline after 15 passages, others had shown that this ability declines with the number of ES cell passages

(Nagy et al., 1993) probably because of the accumulation of chromosomal abnormalities associated with extended culture (Liu et al., 1997).

Because targeted ES cells failed to generate genetically altered mice, Tec function in these cells was subsequently studied using a well established *in vitro* differentiation system. Although Tec-/+ ES cells had failed to contribute to the germline of chimaeric mice, they appeared to retain their ability to differentiate *in vitro* and form macrophage-like cells with kinetics similar to that of wild type ES cells. To investigate the role of Tec in macrophage differentiation and function, Tec -/- ES cells were also generated through a second round of targeting using two independent Tec -/+ ES cell lines. The use of independent ES cell lines was proposed to avoid problems associated with clonal variation often observed with ES cells and would enable differences in macrophage cell differentiation and function to be attributed to the loss of Tec function. Unfortunately, only one targeted ES cell line could be generated from this experiment. It is therefore important to treat the observations made in these studies cautiously. An additional round of targeting using a different Tec -/+ parental cell line should thus ideally be performed. The experiment protocol for *in vitro* differentiation described in Lake et al. (2000) used feeder independent ES cell lines. Experiments described in this thesis however used W9.5 derivatives which needed to be adapted to feeder independent culture conditions thus requiring additional *in vitro* manipulation. All cell lines used in this series of *in vitro* differentiation assays (wild type, Tec -/+ and Tec -/-) were subjected to feeder independent culture conditions. It is thus possible that changes in culture conditions might affect the quality of ES cells and hence their ability to differentiate *in vitro*.

The *in vitro* differentiation system utilised in these studies is extensively used in this department and conditions have been optimised for macrophage cell differentiation originally detected by F4/80 staining (Hudson, personal communication). *In vitro* differentiation of Tec -/+ ES cells successfully generated macrophage cells which could clearly be identified by their

characteristic morphology. The kinetics of differentation observed for Tec -/+ ES cells are similar to that of wild type ES cells. Macrophage cells are detectable following 8-10 days in methylcellulose, and both wild type and Tec -/+ macrophage cells are able to internalise IgG opsonised Zymosan A following replating on glass coverslips. There appears not to be a dose dependent requirement for Tec during phagocytosis.

Interestingly no cells with macrophage morphology were isolated from three independent *in vitro* differentiation attempts of Tec -/- ES cells. Embryoid bodies in these experiments generally failed to display the characteristic burst seen with wild type and Tec-/+ ES cells suggesting a defect in macrophage differentiation *in vitro*. Because macrophage cells failed to be generated, the requirement for Tec in phagocytosis could not be characterised further. All cells isolated upon replating of the *in vitro* differentiation products were morphologically different from macrophage cells and resembled fibroblast cells. They appeared flattened in appearance with large cytoplasms and no pseudopodia extension. These typically resemble fibroblast cells. Phagocytic assays set up using these cells also confirmed their inability to internalise IgG opsonised Zymosan A.

At least three different reasons can be proposed to explain this defect in macrophage cell production. First, the absence of macrophage cells observed in these experiments might directly reflect a lesion in the macrophage differentiation pathway caused by the absence of a functional Tec gene. Secondly, the absence of macrophage cells in these assays could reflect the inability of differentiated macrophage cells to migrate out of the embryoid bodies, resulting in the absence of the characteristic colony burst structures seen in wild type and Tec-/+ differentiation experiments. This is not unlikely given the association of Tec with plasma membrane ruffles and cytoskeletal structures often seen during the spreading of macrophage cells (Chapter 4). These structures are also necessary for cell migration. To differentiate between these possibilities, embryoid bodies need to be tested for the presence of macrophage

specific markers both at the mRNA level, by RT-PCR or RNase Protection Analysis, and at the protein level by immunofluorescent staining for the macrophage specific markers such as F4/80. Alternatively, embryoid bodies could be disrupted mechanically to release a single cell suspension that can subsequently be stained for F4/80 and exposed to phagocytic targets to identify potential macrophage cells. Should macrophages not be detected by either methods, the role of Tec in macrophage differentiation can further be confirmed by complementation experiments. In these experiments, a Tec transgene could be introduced into Tec-/- ES cells by standard transfection protocols to generate transgenic ES cells. These could subsequently be subjected to *in vitro* differentiation protocols to test the ability of the Tec transgene to rescue the defective phenotype. More sophisticated mutations could also be introduced in this system to identify the role of each domain in macrophage cell differentiation. Finally, it remains possible that the lack of macrophage production by the Tec-/- cell line generated during these experiments reflects clonal variation or loss of pluripotency associated with the additional manipulation required to generate this cell line. Unfortunately no other targeted cell line had been identified to eliminate such possibility. Further characterisation of the differential potential of this cell line is therefore necessary. Preliminary conclusions from the studies described in this thesis suggest a potential role for Tec in macrophage cell differentiation *in vitro*. This report is the first to suggest such a role and more detailed studies will be required to confirm and characterise this possibility.

CHAPTER 6:

FINAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Final Discussion

Signal transduction is essential for cell metabolism, gene expression, cytoskeletal rearrangement, proliferation and apoptosis, all of which are cellular functions that are critical for the survival of multicellular organisms. Extra-cellular stimuli in the form of soluble ligands and/or proteins attached to extra-cellular matrix components are able to stimulate specific receptors at the plasma membrane and influence such functions. The molecular details of the events that follow receptor activation have, over the last two decades, been the subject of numerous studies, and many of the molecules responsible for the passage of the plasma membrane-initiated signals to specific intracellular effectors have been identified. These molecules are generally characterised by their ability to interact, in a specific and regulated manner, with other signalling molecules, and in some cases with structural components of the cell.

The Tec family of protein tyrosine kinases is characterised by the presence of three protein-protein interaction domains, namely the PHTH, SH3 and SH2 domains, in addition to the kinase domain, which acts as the critical enzymatic determinant of the protein. It has recently emerged as a major component of the signal transduction pathways activated by the family of antigen receptors (recently reviewed in Schaeffer and Schwartzberg, 2000).

To date, the role of Btk in B cells and, Itk and Txk in T cells has been characterised in detail. Recent reports suggest an important role for these tyrosine kinases for the maximal activation of phospholipase C-γ (PLCγ) (Fluckiger et al., 1998; Takata and Kurosaki, 1996; Liu et al., 1998; Schaeffer et al., 1999), through the formation of a multiprotein complex that is nucleated by LAT and SLP-76 in T cells, and BLNK/SLP-65 in B cells (Bunnell et al., 2000; Hashimoto et al., 1999; Shan and Wange et al., 1999; Su et al., 2000). The biological role of Tec is less well characterised . It is reportedly phosphorylated and activated in response to

numerous cytokines including stem cell factor (SCF) (Tang et al., 1994), IL-3 (Mano et al., 1995), IL-6 (Matsuda et al., 1995), granulocyte colony-stimulating factor (G-CSF) (Miyazato et al., 1996), erythropoietin (Epo) (Machide et al., 1995), thrombopoietin (Tpo) (Yamashita et al., 1997) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Yamashita et al., 1998).

6.1.1 Biological Significance of TecIII and IV isoforms

Several isoforms of the Tec transcripts have been reported in the literature of which two, *TecIII* and *TecIV* have been shown in this thesis to be most abundant in mouse embryonic and adult tissues (3.2.1). The structure of the *Tec* gene suggests that these two isoforms arise from alternative splicing of the *Tec* mRNA. The full length *Tec* transcript gives rise to *TecIV*, which is detectable in numerous tissues (3.2.1.3) while skipping of exon 8 generates the shorter *TecIII* transcript, which appears to be most predominant in the adult liver and kidney. While detailed characterisation of the distribution of *TecIII* and *TecIV* transcripts was carried out during the course of this Ph.D, the lack of suitable isoform specific antibodies prevented similar analysis to be carried out on appropriate protein extracts. It remains unclear whether TecIII and TecIV proteins are distributed in a manner similar to their respective transcripts. Theoretically isoform-specific antibodies can be generated using antigenic material consisting of recombinant peptides that encompass the full length and truncated SH3 region of TecIV and TecIII. Unfortunately, despite numerous attempts in this laboratory, this approach has failed to generate the desired antibodies (G. Booker, personal communication).

Studies carried out on hematopoietic cell extracts used the commercially available Tec antibody (sc 1109, St Cruz Biotechnology) (4.2.1), and have identified a single Tec specific band (based on electrophoretic mobility) in J774 and CTLL cells. This is in contrast to the identification of the two forms of the *Tec* transcript in these cells by RT-PCR (3.2.2). The

significance of this single band remains to be determined. It is possible that it reflects (i) post-transcriptional and/or post-translational control, (ii) instability of the TecIII protein given the relatively large section of unstructured peptide in the truncated SH3 region or (iii) tissue-specific slippage of the splicing machinery (although this is predicted to be least likely given the number of tissues that appear to express *TecIII*). TecIII-specific antibodies would facilitate further analysis of these protein extracts. It is possible that the two isoforms of Tec cannot be resolved under the electrophoresis condition used in these studies considering their small difference in molecular weight and given that the mobility of the two proteins is also affected by post-translational modifications of the peptides including tyrosine phosphorylation.

Yamashita et al. (1996) first reported that truncation of the C-terminal region of the SH3 domain of Tec results in hyperphosphorylation and activation of the truncated Tec protein. As shown in 3.2.3, exogenous GFP-TecIII, which includes the truncated SH3 domain, also appears to be hyperphosphorylated when overexpressed in COS-1 cells. Given that phosphorylation is generally considered to reflect the activation state of Tec family kinases (Aoki et al., 1994; Yamashita et al., 1996), it is predicted that TecIII will show higher levels of kinases activity even in the absence of a specific external stimulus. To further characterise differences between the two major isoforms of Tec, *in vitro* kinase assays need to be carried out to compare the kinase activity of the two Tec isoforms. These could be performed using various substrates including Tec to test for auto/trans-phosphorylation, and PLCγ (as a putative downstream effector and substrate for Tec family kinases).

The mechanism responsible for the hyperphosphorylation of TecIII is unclear. Fluorescence studies of GFP-TecIII transfected cells have however provided some clues. Visualisation of GFP-TecIII by fluorescence microscopy have identified high levels of plasma membrane-targeted GFP-TecIII in transfected cells, generally in regions associated with plasma membrane ruffles, suggesting a possible involvement for Tec in plasma membrane

remodelling. Constitutive plasma membrane targeting of other members of the Tec family, mainly Btk has been shown to result in their phosphorylation and activation. The E41K Btk mutant for example, generally referred to as Btk*, (Li et al., 1995) is phosphorylated on both tyrosine residues in the absence of specific stimuli (Li et al., 1995; Park et al., 1996). Targeting of Tec family kinases to the plasma membrane thus appears to be sufficient for kinase activation, probably as a result of their proximity to Src family kinases.

The mechanism by which the E41K mutation affects the phosphorylation of Btk is relatively well understood. This residue lies in the Btk PH domain and increases the affinity of the PH domain for plasma membrane phospholipids. The mechanism by which the absence of a functional SH3 domain in TecIII is able to facilitate the translocation of GFP-TecIII to the plasma membrane is not obvious. It is possible that in the absence of a functional SH3 region, proline rich sequences at the C terminus of the TH domain are exposed to SH3-containing proteins located at the plasma membrane and that such interactions facilitate the recruitment of TecIII to the plasma membrane. One such interaction has been reported between the TH region of Tec (including the PRR) and the plasma membrane anchored Src family kinase Lyn (Mano et al., 1996). Alternatively, the absence of the PRR-SH3 intramolecular interaction might influence the three dimensional orientation of the Tec PHTH domain rendering it more accessible to phospholipid binding. However, given the specificity of the PH domain of Tec for $PI(3,4,5)P_3$ molecules, (Shirai et al., 1998 and Okoh and Vihinen, 1999) it is difficult to explain the high incidence of plasma membrane targeting observed for GFP-TecIII in the absence of a specific extra cellular stimuli. In fact, the PI3-K inhibitor LY294002 which inhibits the synthesis of $PI(3,4,5)P_3$ molecules was shown not to affect the targeting of GFP-TecIII to the plasma membrane. It thus appears that signals other than phospholipid metabolism must affect the targeting of GFP-TecIII. It is also possible that the exposed PRR region of Tec might be able to facilitate the targeting Tec to the plasma membrane region via

some uncharacterised protein-protein interactions. The role of actin polymerisation in the plasma membrane targeting of GFP-TecIII also requires further characterisation.

The study of the mechanism(s) responsible for targeting GFP-Tec to the plasma membrane requires the generation of site-specific mutants. Mutation of essential proline residues in each of the PRR consensus sequences (Figure 1.2) results in loss of Tec-SH3-binding capability Pursglove (2001). These mutants could be used to investigate the role of the PRR in plasma membrane targeting. Using fluorescence microscopy, the subcellular localisation of GFP-TecIV and GFP-TecIII PRR mutants could be investigated and compared. It is predicted that plasma membrane targeting of GFP-TecIV PRR site1 mutants (Figure 1.2) would suggest a role for the PRR-SH3 interaction in affecting the orientation of the PH domain and its affinity for the plasma membrane. On the other hand, should the PRR site 1 itself be required for plasma membrane targeting then its mutation in GFP-TecIII would inhibit the localisation of the GFP-TecIII mutant to the plasma membrane resulting in diffuse fluorescence. Furthermore, mutation of the SH3 binding pocket of GFP-TecIV should prevent SH3-PRR binding and is predicted to result in a fluorescence localisation pattern that resembles that of GFP-TecIII, suggesting a role for the SH3 domain in preventing plasma membrane targeting. To investigate the role of the Tec-Lyn interaction in plasma membrane targeting, mutants of the PRR and TH region of GPF-TecIII should be generated to identify the region involved, if any at all, in such an interaction. Should this interaction be important in plasma membrane targeting of GFP-TecIII then mutants of this region are predicted to show diffuse fluorescence when expressed in COS-1 cells.

The biological role of this activated form of Tec is unclear. Plasma membrane regions enriched in GFP-TecIII appear to be enriched in filamentous actin and associated with membrane ruffling, suggesting active rearrangement of the underlying actin cytoskeleton and

remodelling of the plasma membrane. Activated Tec at the plasma membrane might thus be involved in active remodelling of the actin cytoskeleton.

Interestingly, ongoing studies in this laboratory have uncovered a physical association between the PHTH domain of Tec and the human actin binding protein Actinin-4 (Merkel, personal communication). Interestingly Actinin-4 has been associated with cell motility and tumour invasion in breast cancer (Honda et al., 1998). Phosphorylation of actinin is known to destabilise filamentous actin bundle structures and facilitate the rearrangement of the actin cytoskeleton. This allows the speculation that in the presence of specific stimuli, such as cell adhesion or the presence of migratory stimuli, activated Tec at the plasma membrane might be able to interact with and/or phosphorylate components of the underlying cytoskeleton including actinin, thus facilitating the remodelling of the cytoskeleton. Such possibility highlights a possible role for Tec in cell migration and metastasis.

6.1.2 Subcellular Localisation of Tec in macrophage cells

Immunofluorescence studies described in chapter 4 have identified at least four subcellular pools of Tec in macrophage cells. When cells do not adhere to the substratum and are processed in suspension, Tec appears to be diffuse in the cytoplasm of macrophage cells. When cells adhere to the tissue culture glass coverslips and cytoplasmic extensions become visible, Tec is present in ruffle-like regions at the plasma membrane, as well as in association with the actin cytoskeleton of these filopodia structures. A distinctive Brefeldin A-sensitive pool of juxta-nuclear Tec is also prominent in adherent cells. The significance of this pool of Tec remains to be determined. Throughout these studies nuclear localised Tec was also identified although the signals that affect this pool of Tec were not characterised. These observations suggest that Tec is a highly dynamic protein that cycles between various subcellular locations in the cell. It is expected that the subcellular localisation of Tec is

determined by the nature of extra-cellular stimuli. To complement observations made during this Ph.D, further quantitative analysis of the subcellular localisation of Tec in macrophage cells would require confocal microscopy analysis of Tec.

6.1.3 Tec-mediated signal transduction pathways

6.1.3.1 The role of Tec in antigen receptor signaling

The antigen receptor family includes amongst others the B cell receptor (BCR), T cell receptor (TCR) and Fcγ receptors (FcγRs). The roles of Btk and Itk as downstream effectors of the BCR and TCR have been studied in great detail (Schaeffer and Schwartzberg, 2000 for recent review). This thesis suggests a parallel role for Tec in antigen receptor signalling, as a downstream effector of the Fcγ receptor during the phagocytosis of IgG opsonised particles. In the BCR and TCR systems, activation of Btk and Itk occurs through a two-step sequence that involves the association of Btk or Itk with products of PI3-K, mainly $PI(3,4,5)P_3$, at the plasma membrane, the Src-family-dependent phosphorylation of the Tec family kinase domain activation loop tyrosine residue and the subsequent auto-phosphorylation of the SH3 domain tyrosine residue. The recruitment of Tec family kinases to the plasma membrane is critical for their activation and the important role that the PH domain plays in this interaction is reinforced by several observations. Firstly, PH domain mutations (such as the R28C mutation in Btk) that affect the affinity of the Btk PH domain for $PI(3,4,5)P_3$ molecules result in the Xid phenotype (Fukuda et al., 1996). Additionally, a decrease in $PI(3,4,5)P_3$ levels such as that seen in response to the activation of the phosphatase SHIP or in p85α -/- mice also result in the suppression of Btk activity (Bolland et al., 1998; Suzuki et al., 1999 respectively) thus reflecting the requirement for $PI(3,4,5)P_3$ molecules in Tec family kinase activation. During antigen receptor signalling, Tec family kinases phosphorylate and activate PLCγ, to facilitate the mobilisation of calcium from extracellular sources (Fluckiger et al., 1998; Takata and

Kurosaki, 1996; Schaeffer et al., 1999). They form a protein complex that includes Syk and PLCγ and is nucleated by the adaptor proteins LAT and SLP-76 in T cells, and SLP-65/BLNK in B cells (Bunnell et al., 2000; Hashimoto et al., 1999; Shan and Wange et al., 1999; Su et al., 2000). Such structure is critical for the phosphorylation of PLCγ by the two independent tyrosine kinase families.

The studies described in chapter 4 have uncovered the potential involvement of Tec in the signal transduction pathway activated by Fcγ receptors during the phagocytosis of IgG opsonised exogenous particles. Many aspects of phagocytosis have been characterised in detail (Greenberg, 1999; Aderem and Underhill, 1999). , The interaction between IgG-opsonised target particles and cell surface Fcγ receptors on macrophage cells is rapidly followed by receptor clustering. Although this had originally been assumed to result from simple diffusion of the receptors, recent reports suggest the involvement of signalling molecules such as GTPases including RhoA in this process (Hackam et al., 1997). Following receptor clustering, Src family kinases instigate a cascade of phosphorylation events (Wang et al., 1994) that initiates at ITAM tyrosine residues, in the γ subunit of the receptors (Greenberg et al., 1993, 1994; Duchemin et al., 1994). This sequence of events is believed to be the major activating pathway for phagocytosis. However, phagocytosis can be initiated, albeit with significantly lower efficiency, in Lyn-/-Fgr-/-Hck-/- macrophage cells (Crowley et al., 1997) thus suggesting an alternative mechanis might exist that is independent of these Src kinases. The phosphorylation of ITAM tyrosine residues then creates docking sites for the tyrosine kinase Syk (Crowley et al., 1997). Syk phosphorylation is proposed to result from a number of unrelated events. Firstly, low levels of Syk are associated with Fcγ receptors (Ghazizadeh et al., 1994) such that receptor clustering alone might be able to induce Syk auto-phosphorylation (Kurosaki et al., 1995) thus explaining the residual phagocytic activity seen in the absence of functional Src kinases (Crowley et al., 1997). In most cases however, receptor

aggregation is proposed to result in the activation of Src family kinases which can in turn phosphorylate and activate FcγR receptor-associated Syk (Sidorenko et al., 1995). Many of the subsequent downstream events that take place during phagocytosis have also been identified. These include rearrangement of the cytoskeleton to form the characteristic phagocytic cup (Allen and Aderem, 1996), calcium mobilisation (Odin et al., 1991), activation of PLCγ (Kiener et al., 1993), protein kinase C (PKC) (Zheleznyak et al., 1992) and MARCKS (Allen and Aderem, 1995). Other critical effectors of phagocytosis include PI3-K which is necessary for phagosome closure (Araki et al., 1996) and Rho family GTPases which are also required to control actin dynamics (reviewed in Chimini and Chavrier, 2000).

Work presented in this thesis suggests that Tec is also involved in phagocytosis. Tec is rapidly recruited to the phagocytic cup following particle binding, and is shed following particle internalisation as particles are drawn into the body of macrophage cells. The recruitment of Tec to the phagosome is dependent on PI3-K activity thus suggesting a critical role for the PH domain in the redistribution of Tec to the phagosomal membrane. Although Tec appears to co-localise with F-actin at the phagocytic cup, the redistribution of Tec is not dependent on the polymerisation of the underlying actin cytoskeleton as Tec can be detected at the phagocytic cup even in the presence of the inhibitor of actin polymerisation Cytochalasin D. Based on the molecular details of B and T cell receptor signal transduction, Tec is predicted to be part of a multiprotein complex nucleated by an adaptor protein such as that of the BLNK/SLP-76 family. At least two members of this family have been identified in macrophage cells: SLP-76 and BLNK (Bonilla et al., 2000). The SH2 domain of several Tec family kinases has also been reported to interact with SLP-76 (Su et al., 2000) and both SLP-76 and BLNK have been shown to cooperate with Tec and Syk to enhance PLCγ1 phosphorylation (Yamamoto et al., 2001). Surprisingly, SLP-76-/- macrophage cells retain their ability to phagocytose IgG opsonised exogenous particles (Myung et al., 2000),

suggesting that SLP-76 is not a critical component of the phagocytic signal transduction pathways in macrophage cells. Similarly, no defects in phagocytosis have yet been reported in BLNK-/- mice (Pappu et al., 1999; Xu et al., 2000). It is therefore possible that other as yet undefined family members, are important for macrophage function and/or that the absence of significant defects in these SLP-76 and BLNK deficient mice reflects complementation between members of this family.

Recent studies of SCF signalling in erythroid and megakaryocytic cell lines have also identified the formation of a PI3-K-dependent multiprotein complex that consists of Lyn, Tec and Dok-1 in response to SCF (van Dijk et al., 2000). Dok-1 is an adaptor molecule that has recently been shown to participate in BCR signal transduction where it is reported to associate with Tec in a phosphorylation-dependent manner (Yoshida et al 2000). In the B cell line Ramos, cross-linking of the B cell antigen receptor (BCR) results in tyrosine phosphorylation of Dok-1, and this effect is markedly inhibited by expression of dominant negative mutants of Tec. Overall these results indicate that Tec can mediate signalling from the BCR to Dok-1. Dok-1 tyrosine phosphorylation has also been associated with FcγRIIB signal transduction (Tamir et al., 2000). Physiologically, Dok-1 has been implicated in mitogenic signalling (van Dijk et al., 2000) as well as in the rearrangement of the cytoskeleton (through its interaction with the Wiskott-Aldrich protein) during cellular migration responses (Noguchi et al.,1999).

It is interesting that Tec has been shown to associate with at least two distinct families of adaptor protein, both of which have been associated with distinct signalling pathways in the cell. The role of these adaptors in FcγR-mediated signal transduction is yet to be clarified. Nevertheless the ability of Tec to participate in various multiprotein complexes would explain the apparent involvement of Tec in signal transduction mechanisms encompassing that range from phospholipid metabolism to cytoskeletal rearrangement and mitogenic signal transduction.

Additional experiments are required to further characterise FcγR-mediated signalling pathway(s). First the effect of phagocytosis on the phosphorylation of Tec needs to be characterised in detail. Preliminary experiments indicate that Tec might be phosphorylated in a time-dependent manner during phagocytosis and that such phosphorylation is inhibited by the addition of the PI3-K inhibitor LY294002 (data not shown). Putative interactions between BLNK and Tec, and between Tec and Syk need to be tested using a co-immunoprecipitation approach that should be carried out using commercially available BLNK antibodies (such as sc-8382, St Cruz Biotechnology). A search for potential novel family members of the BLNK/SLP-76 families in macrophage cells could also be carried out using a degenerate PCR approach. The predicted association between Tec and PLCγ needs to be confirmed using a co-immunoprecipitation approach. Furthermore, given the proposed role for Tec as an activator of PLCγ, *in vitro* kinase assays using immunoprecipitated material could also be performed to test the ability of Tec to phosphorylate PLCγ *in vitro*. Finally, it would be interesting to characterise the phosphorylation status of Tec and PLCγ in Lyn-/-Fgr-/-Hck-/- macrophage cells (Crowley et al., 1997; Fitzer-Attas et al., 2000) given the reported low level of phagocytic activity of these cells. It is predicted that in the absence of these Src family kinases, Tec would be hypo-phosphorylated despite the presence of phagocytic stimuli and that the low levels of particle internalisation observed would reflect low levels of PLCγ activation.

6.1.3.2 A possible role for Tec in IL-3 signaling

Interleukin 3 (IL-3), GM-CSF and IL-5 are cytokines principally released by activated T cells. Their activities include stimulation of proliferation, survival and differentiation of myeloid hematopoietic cells and enhancement of functions of terminally differentiated myeloid cells (Arai et al., 1990 and Guthridge et al., 1998). IL-3 receptors are present on early hematopoietic progenitor cells, on a range of committed myeloid cells, eosinophils and basophils (Arai et al., 1990). Numerous *in vitro* studies suggest that IL-3 can stimulate myelo-

monocytic cells and induce the differentiation of the granulocyte and macrophage populations, as well as stimulate effector functions of these cells, thus contributing to the body's defence against microbial pathogens (Nicola et al., 1996 and Arai et al., 1990).

In myeloid cells, IL-3 stimulation induces the association of Tec with the Src family kinase Lyn that is predicted to result in an increase in Tec phosphorylation and activation, thus positioning Tec as a downstream effector of the IL-3 receptor (Mano et al., 1994 and 1996). Interestingly, the Tec-/- ES cell line generated by gene targeting (chapter 5) appears to be unable to produce macrophage cells when subjected to *in vitro* differentiation protocols. The same protocol had previously been optimised for macrophage differentiation (Lake et al., 2000) and consistently produced the characteristic burst of macrophage cells for both wild type and Tec -/+ ES cells. These bursts are easily identified as a halo of cells that appear to have migrated out of the differentiating embryoid body. Upon replating, the cells adhere strongly to glass coverslips and form elongated cytoplasmic extensions such as those described for J774 macrophage cells (Chapter 4). Most importantly, *in vitro* generated macrophage cells successfully internalise IgG opsonised target particles. In contrast, no phagocytic cells could be isolated following the replating of Tec-/- embryoid bodies, even when attempts were made to disrupt embryoid body structure. It is possible that such attempts had not successfully released trapped macrophage cells. Additional manipulation of the embryoid bodies needs to be carried out using collagenase treatment and/or repeated passage through syringe needles to achieve a better single cell suspension and consequently better release of any potentially trapped macrophage cells.

It had originally been feared that the defects seen with Tec-/- ES cells reflected clonal variation and loss of pluripotency in these cells. While this thesis was in preparation, additional *in vitro* differentiation of Tec-/- ES cells was carried out in the laboratory of Dr P.D. Rathjen. These experiments show that Tec-/- ES cells are able to give rise to various cell lineages

including nerve cells, beating muscle and blood islets thus indicating some level of pluripotency (J. Rathjen, personnal communication).

Functionally, the absence of Tec-/- macrophage cells is thus proposed to reflect either (i) a defect in the macrophage differentiation pathway or (ii) a lesion in the signalling pathway required for the migration and/or adhesion of macrophage cells. The addition of IL-3 appears to significantly increase the efficiency of macrophage differentiation in this system (Wiles and Keller, 1991 and Keller et al., 1993). It is thus possible that the absence of Tec-/- macrophage cells results from a lesion in IL-3 and/or M-CSF signalling thus suggesting a possible role for Tec in IL-3 signalling. The role of Tec in the differentiation of macrophage cells *in vitro* remains to be clarified. Tec-/- embryoid bodies should be analysed for the expression of macrophage specific genes such as F4/80 or Mac-1 using RT-PCR or RNase protection analysis. The absence of these markers would confirm the inability of these cells to differentiate into macrophage cells. Further confirmation of macrophage differentiation defects should be carried out using rescue-type experiments where exogenous Tec, under the control of a macrophage specific promoter. Under macrophage differentiating conditions the expression of Tec is predicted to be able to restore the ability of Tec-/- ES cells to generate macrophage cells. Further characterisation of the phagocytic potential of such cells would be required to confirm the restoration of macrophage specific functions.

While this thesis was in preparation, Ellmeier et al. (2000) reported the generation and characterisation of mice deficient for Tec. These studies suggest that Tec deficient mice are viable and fertile. Surprisingly, hematopoiesis is also not obviously impaired in these mice. Tec-deficient B and T lymphocytes behave like wild type cells in both *in vitro* proliferation assays and *in vivo* immunization experiments. The myeloid lineage was characterised by FACS analysis and did not identify any developmental defects. However, it remains unclear whether Tec deficient myeloid cells, including macrophage cells behave normally. In light of the

preliminary results presented in this thesis, it would be interesting to test the phagocytic ability of Tec deficient macrophage cells. Further characterisation of the innate immunity response of Tec-deficient macrophage cells to a range of pathogens might identify possible Tec-dependent physiological processes. Btk/Tec double KO mice were also generated in those studies. Compared with Btk single KO mice, these mice suffered severe changes in splenic architecture and showed further disruption of B cell development. These results suggest that Tec family kinases are able to partially rescue each other although it appears that developing murine B cells have differential requirements for functional Tec family kinases. Btk (or an other Tec family member) thus appears to be able to fully compensate for the loss of Tec in Tec deficient mice while Tec can not compensate for the loss of Btk. This might reflect differences between the various members of this family in substrate specificity, binding affinities and or expression levels. These results contrast the *in vitro* differentiation results presented in this thesis, which identified the inability of Tec deficient ES cells to generate macrophage cells *in vitro*. It is possible that the absence of Tec-/- macrophage *in vitro* reflect limitations of the *in vitro* assay system used in the studies especially limitation in the variety of exogenous differentiating factors added to the differentiating medium. In summary, it appears that Tec is not required for the myelogenesis *in vivo* and that its requirement *in vitro* will require further characterisation.

6.1.3.3 A possible role for Tec in apoptosis

Conflicting evidence has been reported in the literature regarding the role for Tec kinases in apoptosis, and most studies have concentrated on the role of Btk in B cell apoptosis. On the one hand, Btk is required to induce *Bcl-xL* transcription in response to IgM stimulation and as such has a protective role (Anderson et al., 1996). On the other hand, it also appears necessary for radiation-mediated apoptosis (Uckun et al., 1996) as well as cytokine deprivation induced apoptosis (Kawakami et al., 1999) in B cells.

Preliminary evidence presented in chapter 3 suggests that hyperphosphorylated TecIII protein is associated with a higher incidence of cell death in the absence of serum when transfected into COS cells than TecIV. It is possible that this reflects a mechanism to eliminate cells that show hyperactivation of Tec kinase activity in the absence of an appropriate external stimulus. This would act as a selection mechanism to protect against overstimulation of Tec kinase activity. As apoptosis was not the focus of the research presented in this thesis, the nature of these high levels of cell death was not tested.

6.1.3.4 A possible role for Tec in macrophage adhesion and migration

The signal transduction pathways that are involved in macrophage cell adhesion and migration appear to overlap extensively with those necessary for phagocytosis (reviewed in Lowell and Berton, 1999). A number of signalling molecules identified as critical effectors of phagocytosis are also involved in myeloid cell adhesion/spreading and migration. These include tyrosine kinases such as Src family kinases and Syk (Yan et al., 1997; Suen et al., 1999; Felsenfeld et al., 1999, Lin et al., 1995 and Fernandez and Suchard, 1998), Rho family GTPases (Allen et al., 1997; Allen et al., 1998) and actin cytoskeleton-associated proteins such as α-actinin (Pavalko et al., 1993). Myeloid cell adhesion and/or migration are generally mediated by integrin receptors (Lowell and Berton, 1999). Effectors of this pathway include Src family kinases (Suen et al., 1999, Meng and Lowell, 1998), Syk (Lin et al., 1995; Yan et al., 1997, Hirano and Kanno, 1999), PLCγ, PKC and calcium signalling, PI3-K (Meng and Lowell, 1998 and 1999), Rho GTPases (Allen et al., 1997), and numerous cytoskeletal proteins (Hirano and Kanno, 1999). Some limited evidence also suggests the involvement of Tec in integrin signalling (Hamazaki et al., 1998).

As previously mentioned, it is proposed that Tec might be involved in macrophage migration and/or adhesion. The absence of macrophage cells observed in Tec-/- ES cell *in vitro* might thus reflect the inability of Tec-/- macrophage cells to migrate out of the embryoid

bodies and form the characteristic burst of macrophage cells seen in wild type and Tec-/+ cells. This is not unlikely considering the similarity between the membrane proximal events involved in cell spreading and/or adhesion and those identified in particle internalisation, and given the proposed involvement of Tec in both signalling pathways. The disruption of these embryoid bodies and replating of differentiation products were also unable to isolate phagocytic Tec-/- macrophage cells thus suggesting that any macrophage cells that might have been produced –if any, were unable to adhere to the culture substratum. Similar defects have been seen in macrophages lacking Src family kinases (Crowley et al., 1997; Fitzer-Attas et al., 2000) and Syk (Crowley et al., 1997).

6.2 Future Directions

As a result of the work carried out during the course of this Ph.D, several areas have been identified that will require further analysis. These include (i) *in vivo* analysis of Tec function in the mouse, (ii) the *in vitro* characterisation of the molecular details of Tec action in FcγR-mediated phagocytosis and (iii) the *in vitro* characterisation of other potential Tec-mediated signal transduction pathways identified during the course of this Ph.D.

It is obvious from the data presented in 5.2.2.2.3 that the Tec-/+ ES cell lines used in these studies are unable to colonise the germline of chimaeric animal. As previously mentioned, this difficulty is predicted to reflect a decrease of pluripotency which has since then been identified to result partly from the poor quality of the parental ES line used (J. Wrin, personal communication). Further characterisation of Tec function *in vivo* using a gene targeting approach would therefore require re-targeting of an appropriate ES cell line using the gene targeting vector described in 5.2.2.2.1. Targeting of Tec in the mouse has been reported to generate viable homozygous animals. Further characterisation of these animals is thus necessary to investigate the role of Tec in the myeloid compartment. FACS analysis of the

hematopoietic lineages should be carried out to determine the need for Tec in macrophage differentiation. Should viable macrophage cells be obtained, further analysis of Tec function needs then to concentrate on macrophage cell functions including FcγR-mediated phagocytosis as well as integrin-dependent cell adhesion and migration.

The work presented in this thesis has also identified a potential role for Tec in FcγR-mediated signal transduction during the internalisation of IgG-opsonised particles. For convenience, all the work presented in chapter 4 and 5 had been carried out using IgG-opsonised Zymosan A as target particles for phagocytosis assays. However, it appears that the use of IgG-opsonised fluorescently labelled latex beads might be more appropriate as it would enable better visualisation of the forming phagosome. Furthermore, F-actin accumulation at the phagocytic cup and the effect of PI3-K inhibitors have also been reported to be dependent on the size of target particle (Koval et al., 1998). Given that Tec is predicted to act as a downstream effector of PI3-K, the effect of target particle size on the redistribution of Tec to the phagocytic cup could also be characterised by immunofluorescence microscopy using various size latex beads. Most importantly, various protocols are now available that utilise latex beads, for the preparation of phagosomal membrane fractions. This provides a powerful tool that enables studies to focus on a single pool of Tec. Such an approach can be used (i) to purify and analyse the phosphorylation of phagosomal Tec, (ii) to purify and analyse the *in vitro* kinase activity of phagosomal Tec and (iii) characterise the kinetics of its redistribution to the phagosomal membrane.

Several parameters of Tec function during phagocytosis also need to be further characterised. For example, it remains unclear whether both isoforms of Tec are involved in FcγR-mediated phagocytosis and whether all types of FcγR are able to trigger the redistribution and activation of Tec. The first question could be addressed using the Tec-/- ES cell line described in chapter 5 while the second could be answered using specific monoclonal

antibodies to stimulate FcγRs. Briefly, cDNAs encoding each isoforms of Tec could be reintroduced in this cell line and the ability of each isoform to rescue the Tec-/- phenotype could be investigated. It is predicted that reintroduction of at least one isoform of Tec, if not both, should restore the ability of Tec -/- ES cells to differentiate into macrophage cells and the ability of such cells to carry out FcγR-mediated phagocytosis could then be investigated. This Tec null ES cell line is in fact a valuable tool that enables the characterisation of the roles of each domain of Tec in Tec function. For example, reintroduction of site specific mutants of Tec, such as the R28C PH domain mutant, would enable the characterisation of PH domain function in phagocytosis, and confirm the need for PH-domain dependent plasma membrane during particle internalisation. Reintroduction of a kinase dead mutant of Tec such as K397E would determine the need for Tec kinase activity during phagocytosis. This approach potentially enables one to dissect the role and function of each domain of Tec in FcγR-mediated phagocytosis.

Finally, several potential Tec-dependent signal transduction pathways have been identified in this thesis which suggest that Tec is able to participate in the signal transduction pathways activated by a range of different receptors including, cytokine, FcγR, integrin and apoptotic. While it remains unclear how specificity is achieved under such conditions, it is possible that the ability of Tec to participate in such a variety of signal transduction pathways enables the coordination of various cellular functions.

REFERENCES

References

Abrams, C. S., Zhao, W., and Brass, L. F. (1996). A site of interaction between pleckstrin's PH domains and G beta gamma. Biochim Biophys Acta 1314, 233-8.

Aderem, A., and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17, 593-623.

Afar, D. E., Park, H., Howell, B. W., Rawlings, D. J., Cooper, J., and Witte, O. N. (1996). Regulation of Btk by Src family tyrosine kinases. Mol Cell Biol 16, 3465-71.

Agarwal, A., Salem, P., and Robbins, K. C. (1993). Involvement of p72syk, a protein-tyrosine kinase, in Fc gamma receptor signaling. J Biol Chem 268, 15900-5.

Aktories, K. (1997). Rho proteins: targets for bacterial toxins. Trends Microbiol 5, 282-8.

Allen W.E., Jones G.E., Pollard J.W., Ridley A.J. (1997). Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. J Cell Sci. 110, 707-20.

Allen W.E., Zicha D., Ridley A.J., Jones G.E.. (1998). A role for Cdc42 in macrophage chemotaxis. J Cell Biol. 141, 1147-57.

Allen, L. A., and Aderem, A. (1996). Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J Exp Med 184, 627-37.

Allen, L. H., and Aderem, A. (1995). A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in Zymosan phagocytosis by macrophages. J Exp Med 182, 829-40.

Anderson, J. S., Teutsch, M., Dong, Z., and Wortis, H. H. (1996). An essential role for Bruton's [corrected] tyrosine kinase in the regulation of B-cell apoptosis [published erratum appears in proc Natl Acad Sci U S A 1996 Dec 24;93(26):15522]. Proc Natl Acad Sci U S A 93, 10966-71.

Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997). Regulatory intramolecular association in a tyrosine kinase of the Tec family. Nature 385, 93-7.

Aoki, Y., Isselbacher, K. J., and Pillai, S. (1994). Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. Proc Natl Acad Sci U S A 91, 10606-9.

Arai N., Tsuboi A., Iwai Y., Miyatake S., Yokota K., de Waal Malefyt R., Muramatsu M., Matsuda I., Nishida J., Baldwin D. (1990). Regulation of IL-3, IL-4 and GM-CSF genes and signal transduction by their receptors. Lymphokine Res. 9, 551-3.

Araki, N., Johnson, M. T., and Swanson, J. A. (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J Cell Biol 135, 1249-60.

Asada, H., Ishii, N., Sasaki, Y., Endo, K., Kasai, H., Tanaka, N., Takeshita, T., Tsuchiya, S., Konno, T., and Sugamura, K. (1999). Grf40, A novel Grb2 family member, is involved in T cell signaling through interaction with SLP-76 and LAT. J Exp Med 189, 1383-90.

Bajno, L., Peng, X. R., Schreiber, A. D., Moore, H. P., Trimble, W. S., and Grinstein, S. (2000). Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation [see comments]. J Cell Biol 149, 697-706.

Blomberg, N., Baraldi, E., Nilges, M., and Saraste, M. (1999). The PH superfold: a structural scaffold for multiple functions. Trends Biochem Sci 24, 441-5.

Bolland, S., Pearse, R. N., Kurosaki, T., and Ravetch, J. V. (1998). SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity 8, 509-16.

Bonilla, F. A., Fujita, R. M., Pivniouk, V. I., Chan, A. C., and Geha, R. S. (2000). Adapter proteins SLP-76 and BLNK both are expressed by murine macrophages and are linked to signaling via Fcgamma receptors I and II/III. Proc Natl Acad Sci U S A 97, 1725-30.

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309, 255-6.

Bronson, S. K., and Smithies, O. (1994). Altering mice by homologous recombination using embryonic stem cells. J Biol Chem 269, 27155-8.

Brumell, J. H., Howard, J. C., Craig, K., Grinstein, S., Schreiber, A. D., and Tyers, M. (1999). Expression of the protein kinase C substrate pleckstrin in macrophages: association with phagosomal membranes. J Immunol 163, 3388-95.

Bubeck Wardenburg, J., Pappu, R., Bu, J. Y., Mayer, B., Chernoff, J., Straus, D., and Chan, A. C. (1998). Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. Immunity 9, 607-16.

Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C., and Berg, L. J. (2000). Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. J Biol Chem 275, 2219-30.

Cambier, J. C. (1995). Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). J Immunol 155, 3281-5.

Cantley, L. C., and Songyang, Z. (1994). Specificity in recognition of phosphopeptides by src-homology 2 domains. J Cell Sci Suppl 18, 121-6.

Caron, E., and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 282, 1717-21.

Carpenter, C. L., and Cantley, L. C. (1996). Phosphoinositide kinases. Curr Opin Cell Biol 8, 153-8.

Carroll, M. C. (1998). The role of complement and complement receptors in induction and regulation of immunity. Annu Rev Immunol 16, 545-68.

Chan, A. C., van Oers, N. S., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994). Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. J Immunol 152, 4758-66.

Chimini G., Chavrier P.. (2000) Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. Nat Cell Biol.2,191-6.

Chu, J., Liu, Y., Koretzky, G. A., and Durden, D. L. (1998). SLP-76-Cbl-Grb2-Shc interactions in FcgammaRI signaling. Blood 92, 1697-706.

Clements, J. L., Lee, J. R., Gross, B., Yang, B., Olson, J. D., Sandra, A., Watson, S. P., Lentz, S. R., and Koretzky, G. A. (1999). Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. J Clin Invest 103, 19-25.

Cohen, G. B., Ren, R., and Baltimore, D. (1995). Modular binding domains in signal transduction proteins. Cell 80, 237-48.

Conley, M. E., Parolini, O., Rohrer, J., and Campana, D. (1994). X-linked agammaglobulinemia: new approaches to old questions based on the identification of the defective gene. Immunol Rev 138, 5-21.

Cooper, J. A., and Howell, B. (1993). The when and how of Src regulation. Cell 73, 1051-4.

Corcoran, L. M., and Metcalf, D. (1999). IL-5 and Rp105 signaling defects in B cells from commonly used 129 mouse substrains. J Immunol 163, 5836-42.

Cox D., Chang P., Kurosaki T., Greenberg S. (1996). Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. J Biol Chem. 271, 16597-602.

Cox, D., Tseng, C. C., Bjekic, G., and Greenberg, S. (1999). A requirement for phosphatidylinositol 3-kinase in pseudopod extension. J Biol Chem 274, 1240-7.

Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., and DeFranco, A. L. (1997). A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. J Exp Med 186, 1027-39.

Daeron, M. (1997). Fc receptor biology. Annu Rev Immunol 15, 203-34.

Darby, C., Geahlen, R. L., and Schreiber, A. D. (1994). Stimulation of macrophage Fc gamma RIIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. J Immunol 152, 5429-37.

Davis, K., Boros, P., Keltz, M., Unkeless, J. C., and Fleischmajer, R. (1995). Circulating Fc gamma receptor-specific autoantibodies in localized and systemic scleroderma [see comments]. J Am Acad Dermatol 33, 612-6.

Debnath, J., Chamorro, M., Czar, M. J., Schaeffer, E. M., Lenardo, M. J., Varmus, H. E., and Schwartzberg, P. L. (1999). rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol 19, 1498-507.

Deng, C., and Capecchi, M. R. (1992). Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol Cell Biol 12, 3365-71.

Di Bartolo, V., Mege, D., Germain, V., Pelosi, M., Dufour, E., Michel, F., Magistrelli, G., Isacchi, A., and Acuto, O. (1999). Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling. J Biol Chem 274, 6285-94.

Divecha, N., and Irvine, R. F. (1995). Phospholipid signaling. Cell 80, 269-78.

Doetschman T. C., Eistetter H., Katz M., Schmidt W., Kemler R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines:formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 87, 27-45.

Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., and Smithies, O. (1987). Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature 330, 576-8.

Dorsch, M., Danial, N. N., Rothman, P. B., and Goff, S. P. (1999). A thrombopoietin receptor mutant deficient in Jak-STAT activation mediates proliferation but not differentiation in UT-7 cells. Blood 94, 2676-85.

Duchemin A. M., Ernst L. K., Anderson C. L. (1994). Clustering of the high affinity Fc receptor for immunoglobulin G (Fc gamma RI) results in phosphorylation of its associated gamma-chain. J Biol Chem. 269, 12111-7.

Durden, D. L., and Liu, Y. B. (1994). Protein-tyrosine kinase p72syk in Fc gamma RI receptor signaling. Blood 84, 2102-8.

Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993). Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56lck. Nature 362, 87-91.

Ellis J. H., Sutmuller R. P., Sims M. J., Cooksley S. (1998). Functional analysis of the T-cell-restricted protein tyrosine kinase Txk. Biochem J. 335, 277-84.

Ellmeier W., Jung S., Sunshine M. J., Hatam F., Xu Y., Baltimore D., Mano H., Littman D. R. (2000). Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med. 192, 1611-24.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154-6.

Fedorov, L. M., Hacgel-Kronenberger, H., and Hirchenhain, J. (1997). A comparison of the germline potential of differently aged ES cell lines and their transfected descendants. Transgenic Res 6, 223-31.

Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, S. E., and Schlessinger, J. (1993). SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. Mol Cell Biol 13, 1449-55.

Felsenfeld D. P., Schwartzberg P. L., Venegas A., Tse R., Sheetz M.P. (1999). Selective regulation of integrin cytoskeleton interactions by the tyrosine kinase Src. Nat Cell Biol. 1, 200-6.

Fernandez R., Suchard S. J. (1998). Syk activation is required for spreading and H2O2 release in adherent human neutrophils. J Immunol. 160, 5154-62.

Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998). LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity 9, 617-26.

Fitzer-Attas C.J., Lowry M., Crowley M. T., Finn A. J., Meng F., DeFranco A. L., Lowell C. A. Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J Exp Med. 191, 669-82.

Fluckiger, A. C., Li, Z., Kato, R. M., Wahl, M. I., Ochs, H. D., Longnecker, R., Kinet, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998). Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation. Embo J 17, 1973-85.

Fukuda, M., Kojima, T., Kabayama, H., and Mikoshiba, K. (1996). Mutation of the pleckstrin homology domain of Bruton's tyrosine kinase in immunodeficiency impaired inositol 1,3,4,5-tetrakisphosphate binding capacity. J Biol Chem 271, 30303-6.

Galli-Taliadoros, L. A., Sedgwick, J. D., Wood, S. A., and Korner, H. (1995). Gene knock-out technology: a methodological overview for the interested novice. J Immunol Methods 181, 1-15.

Gassmann M., Donoho G. and Berg P. (1995). Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. Proc. Natl. Acad. Sci. U S A 92:1292-1296.

Ghazizadeh S., Bolen J. B., Fleit H. B. (1994). Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. J Biol Chem. 269, 8878-84.

Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1995). Tyrosine phosphorylation and association of Syk with Fc gamma RII in monocytic THP-1 cells. Biochem J 305, 669-74.

Gibbins, J. M., Briddon, S., Shutes, A., van Vugt, M. J., van de Winkel, J. G., Saito, T., and Watson, S. P. (1998). The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. J Biol Chem 273, 34437-43.

Gibson, S., Leung, B., Squire, J. A., Hill, M., Arima, N., Goss, P., Hogg, D., and Mills, G. B. (1993). Identification, cloning, and characterization of a novel human T-cell-specific tyrosine kinase located at the hematopoietin complex on chromosome 5q. Blood 82, 1561-72.

Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A., and Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. J Exp Med 190, 1849-56.

Gong, S., and Nussenzweig, M. C. (1996). Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. Science 272, 411-4.

Gordon, S. (1995). The macrophage. Bioessays 17, 977-86.

Gotoh, A., Takahira, H., Geahlen, R. L., and Broxmeyer, H. E. (1997). Cross-linking of integrins induces tyrosine phosphorylation of the proto-oncogene product Vav and the protein tyrosine kinase Syk in human factor-dependent myeloid cells. Cell Growth Differ 8, 721-9.

Greenberg S. (1999). Modular components of phagocytosis.J Leukoc Biol. 66, 712-7

Greenberg, S., Burridge, K., and Silverstein, S. C. (1990). Colocalization of F-actin and talin during Fc receptor-mediated phagocytosis in mouse macrophages. J Exp Med 172, 1853-6.

Greenberg, S., Chang, P., and Silverstein, S. C. (1993). Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. J Exp Med 177, 529-34.

Greenberg, S., Chang, P., and Silverstein, S. C. (1994). Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. J Biol Chem 269, 3897-902.

Greenberg, S., Chang, P., Wang, D. C., Xavier, R., and Seed, B. (1996). Clustered syk tyrosine kinase domains trigger phagocytosis. Proc Natl Acad Sci U S A 93, 1103-7.

Gross, B. S., Lee, J. R., Clements, J. L., Turner, M., Tybulewicz, V. L., Findell, P. R., Koretzky, G. A., and Watson, S. P. (1999a). Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. J Biol Chem 274, 5963-71.

Gross, B. S., Melford, S. K., and Watson, S. P. (1999b). Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. Eur J Biochem 263, 612-23.

Guthridge M. A., Stomski F. C., Thomas D., Woodcock J. M., Bagley C. J., Berndt M. C., Lopez A. F. (1998). Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors. Stem Cells. 16, 301-13.

Hackam, D. J., Rotstein, O. D., Schreiber, A., Zhang, W., and Grinstein, S. (1997). Rho is required for the initiation of calcium signaling and phagocytosis by Fcgamma receptors in macrophages. J Exp Med 186, 955-66.

Hamazaki, Y., Kojima, H., Mano, H., Nagata, Y., Todokoro, K., Abe, T., and Nagasawa, T. (1998). Tec is involved in G protein-coupled receptor- and integrin-mediated signalings in human blood platelets. Oncogene 16, 2773-9.

Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C., and Aderem, A. (1992). MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature 356, 618-22.

Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T., and Tsukada, S. (1999). Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK--functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. Blood 94, 2357-64.

Hasty, P., Rivera-Perez, J., and Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. Mol Cell Biol 11, 5586-91.

Hata, A., Sabe, H., Kurosaki, T., Takata, M., and Hanafusa, H. (1994). Functional analysis of Csk in signal transduction through the B-cell antigen receptor. Mol Cell Biol 14, 7306-13.

Healy, J. I., Dolmetsch, R. E., Timmerman, L. A., Cyster, J. G., Thomas, M. L., Crabtree, G. R., Lewis, R. S., and Goodnow, C. C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity 6, 419-28.

Heyeck, S. D., and Berg, L. J. (1993). Developmental regulation of a murine T-cell-specific tyrosine kinase gene, Tsk. Proc Natl Acad Sci U S A 90, 669-73.

Hirano, S., and Kanno, S. (1999). Syk and paxillin are differentially phosphorylated following adhesion to the plastic substrate in rat alveolar macrophages. Immunology 97, 414-9.

Hitoshi, Y., Sonoda, E., Kikuchi, Y., Yonehara, S., Nakauchi, H., and Takatsu, K. (1993). IL-5 receptor positive B cells, but not eosinophils, are functionally and numerically influenced in mice carrying the X-linked immune defect. Int Immunol 5, 1183-90.

Honda K., Yamada T., Endo R., Ino Y., Gotoh M., Tsuda H., Yamada Y., Chiba H., Hirohashi S. (1998). Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J Cell Biol. 140, 1383-93.

Hooper M., Hardy K., Handyside A., Hunter S., Monk M. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature. 326, 292-5.

Hulett, M. D., Witort, E., Brinkworth, R. I., McKenzie, I. F., and Hogarth, P. M. (1994). Identification of the IgG binding site of the human low affinity receptor for IgG Fc gamma RII. Enhancement and ablation of binding by site-directed mutagenesis. J Biol Chem 269, 15287-93.

Hunter, T. (2000) Signalling-2000 and beyond. Cell 100, 113-127.

Hunter, T., and Sefton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci U S A 77, 1311-5.

Hutchcroft, J. E., Harrison, M. L., and Geahlen, R. L. (1992). Association of the 72-kD protein-tyrosine kinase PTK72 with the B cell antigen receptor. J Biol Chem 267, 8613-9.

Hyvonen, M., and Saraste, M. (1997). Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinaemia. Embo J 16, 3396-404.

Hyvonen, M., Macias, M. J., Nilges, M., Oschkinat, H., Saraste, M., and Wilmanns, M. (1995). Structure of the binding site for inositol phosphates in a PH domain. Embo J 14, 4676-85.

Indik, Z. K., Park, J. G., Hunter, S., and Schreiber, A. D. (1995). The molecular dissection of Fc gamma receptor mediated phagocytosis. Blood 86, 4389-99.

Isaksen, D. E., Baumann, H., Trobridge, P. A., Farr, A. G., Levin, S. D., and Ziegler, S. F. (1999). Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction. J Immunol 163, 5971-7.

Izaguirre G., Aguirre L., Ji P., Aneskievich B., Haimovich B. (1999). Tyrosine phosphorylation of alpha-actinin in activated platelets. J Biol Chem 274, 37012-20.

Jiang, Y., Ma, W., Wan, Y., Kozasa, T., Hattori, S., and Huang, X. Y. (1998). The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. Nature 395, 808-13.

Johnson, R. S., Sheng, M., Greenberg, M. E., Kolodner, R. D., Papaioannou, V. E., and Spiegelman, B. M. (1989). Targeting of nonexpressed genes in embryonic stem cells via homologous recombination. Science 245, 1234-6.

Jones, S. M., Alb, J. G., Jr., Phillips, S. E., Bankaitis, V. A., and Howell, K. E. (1998). A phosphatidylinositol 3-kinase and phosphatidylinositol transfer protein act synergistically in formation of constitutive transport vesicles from the trans-Golgi network. J Biol Chem 273, 10349-54.

Jones, S. M., and Howell, K. E. (1997). Phosphatidylinositol 3-kinase is required for the formation of constitutive transport vesicles from the TGN. J Cell Biol 139, 339-49.

Kam, J., Matthes, H. W., Gait, M. J., and Brenner, S. (1984). A new selective phage cloning vector, lambda 2001, with sites for XbaI, BamHI, HindIII, EcoRI, SstI and XhoI. Gene 32, 217-24.

Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998). Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. J Biol Chem 273, 30497-508.

Kawakami, Y., Kitaura, J., Hata, D., Yao, L., and Kawakami, T. (1999). Functions of Bruton's tyrosine kinase in mast and B cells. J Leukoc Biol 65, 286-90.

Kawakami, Y., Miura, T., Bissonnette, R., Hata, D., Khan, W. N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S. E., Yao, L., Alt, F. W., and Kawakami, T. (1997). Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proc Natl Acad Sci U S A 94, 3938-42.

Kawakami, Y., Yao, L., Miura, T., Tsukada, S., Witte, O. N., and Kawakami, T. (1994). Tyrosine phosphorylation and activation of Bruton tyrosine kinase upon Fc epsilon RI cross-linking. Mol Cell Biol 14, 5108-13.

Kawakami, Y., Yao, L., Tashiro, M., Gibson, S., Mills, G. B., and Kawakami, T. (1995). Activation and interaction with protein kinase C of a cytoplasmic tyrosine kinase, Itk/Tsk/Emt, on Fc epsilon RI cross-linking on mast cells. J Immunol 155, 3556-62.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol 13, 473-86.

Khan, W. N., Nilsson, A., Mizoguchi, E., Castigli, E., Forsell, J., Bhan, A. K., Geha, R., Sideras, P., and Alt, F. W. (1997). Impaired B cell maturation in mice lacking Bruton's tyrosine kinase (Btk) and CD40. Int Immunol 9, 395-405.

Kiefer, F., Brumell, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase is essential for Fcgamma receptor signaling in macrophages and neutrophils. Mol Cell Biol 18, 4209-20.

Kiener, P. A., Rankin, B. M., Burkhardt, A. L., Schieven, G. L., Gilliland, L. K., Rowley, R. B., Bolen, J. B., and Ledbetter, J. A. (1993). Cross-linking of Fc gamma receptor I (Fc gamma RI) and receptor II (Fc gamma RII) on monocytic cells activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. J Biol Chem 268, 24442-8.

Kikuchi, Y., Yasue, T., Miyake, K., Kimoto, M., and Takatsu, K. (1995). CD38 ligation induces tyrosine phosphorylation of Bruton tyrosine kinase and enhanced expression of interleukin 5-receptor alpha chain: synergistic effects with interleukin 5. Proc Natl Acad Sci U S A 92, 11814-8.

Kitanaka, A., Mano, H., Conley, M. E., and Campana, D. (1998). Expression and activation of the nonreceptor tyrosine kinase Tec in human B cells. Blood 91, 940-8.

Kluppel, M., Donoviel, D. B., Brunkow, M. E., Motro, B., and Bernstein, A. (1997). Embryonic and adult expression patterns of the Tec tyrosine kinase gene suggest a role in megakaryocytopoiesis, blood vessel development, and melanogenesis. Cell Growth Differ 8, 1249-56.

Koike, M., Kikuchi, Y., Tominaga, A., Takaki, S., Akagi, K., Miyazaki, J., Yamamura, K., and Takatsu, K. (1995). Defective IL-5-receptor-mediated signaling in B cells of X-linked immunodeficient mice. Int Immunol 7, 21-30.

Korpi, M., Valiaho, J., and Vihinen, M. (2000). Structure-function effects in primary immunodeficiencies. Scand J Immunol 52, 226-32.

Koval, M., Preiter, K., Adles, C., Stahl, P. D., and Steinberg, T. H. (1998). Size of IgG-opsonized particles determines macrophage response during internalization. Exp Cell Res 242, 265-73.

Kurosaki T., Johnson S. A., Pao L., Sada K., Yamamura H., Cambier J. C. (1995). Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J Exp Med. 182, 1815-23.

Kurosaki, T., and Kurosaki, M. (1997). Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. J Biol Chem 272, 15595-8.

Kurosaki, T., and Tsukada, S. (2000). BLNK: connecting Syk and Btk to calcium signals. Immunity 12, 1-5.

Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, T., and Yamamura, H. (1994). Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. J Exp Med 179, 1725-9.

Kwee, L., Baldwin, H. S., Shen, H. M., Stewart, C. L., Buck, C., Buck, C. A., and Labow, M. A. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. Development 121, 489-503.

Kwiatkowska, K., and Sobota, A. (1999). Signaling pathways in phagocytosis. Bioessays 21, 422-31.

Laffargue, M., Monnereau, L., Tuech, J., Ragab, A., Ragab-Thomas, J., Payrastre, B., Raynal, P., and Chap, H. (1997). Integrin-dependent tyrosine phoshorylation and cytoskeletal translocation of Tec in thrombin-activated platelets. Biochem Biophys Res Commun 238, 247-51.

Laffargue, M., Ragab-Thomas, J. M., Ragab, A., Tuech, J., Missy, K., Monnereau, L., Blank, U., Plantavid, M., Payrastre, B., Raynal, P., and Chap, H. (1999). Phosphoinositide 3-kinase and integrin signalling are involved in activation of Bruton tyrosine kinase in thrombin-stimulated platelets. FEBS Lett 443, 66-70.

Lake J., Rathjen J., Remiszewski J., Rathjen P.D. (2000). Reversible programming of pluripotent cell differentiation. J Cell Sci 113, 555-66.

Law, C. L., Ewings, M. K., Chaudhary, P. M., Solow, S. A., Yun, T. J., Marshall, A. J., Hood, L., and Clark, E. A. (1999). GrpL, a Grb2-related adaptor protein, interacts with SLP-76 to regulate nuclear factor of activated T cell activation. J Exp Med 189, 1243-53.

Lemckert, F. A., Sedgwick, J. D., and Korner, H. (1997). Gene targeting in C57BL/6 ES cells. Successful germ line transmission using recipient BALB/c blastocysts developmentally matured in vitro. Nucleic Acids Res 25, 917-8.

Li T., Tsukada S., Satterthwaite A., Havlik M. H., Park H., Takatsu K., Witte O.N. (1995) Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. Immunity. 2, 451-60.

Li, T., Rawlings, D. J., Park, H., Kato, R. M., Witte, O. N., and Satterthwaite, A. B. (1997a). Constitutive membrane association potentiates activation of Bruton tyrosine kinase. Oncogene 15, 1375-83.

Li, Z., Wahl, M. I., Eguinoa, A., Stephens, L. R., Hawkins, P. T., and Witte, O. N. (1997b). Phosphatidylinositol 3-kinase-gamma activates Bruton's tyrosine kinase in concert with Src family kinases. Proc Natl Acad Sci U S A 94, 13820-5.

Liao, X. C., and Littman, D. R. (1995). Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity 3, 757-69.

Lin J., Burkhardt A. L., Bolen J. B., Justement L. B. (1995). Potentiation of B-cell antigen receptor-mediated signal transduction by the heterologous src family protein tyrosine kinase, src. Ann N Y Acad Sci. 766, 214-5.

Lippincott-Schwartz J., Yuan L., Tipper C., Amherdt M., Orci L., Klausner R. D. (1991). Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell. 67, 601-16.

Liu, K. Q., Bunnell, S. C., Gurniak, C. B., and Berg, L. J. (1998). T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J Exp Med 187, 1721-7.

Liu, X., Brodeur, S. R., Gish, G., Songyang, Z., Cantley, L. C., Laudano, A. P., and Pawson, T. (1993). Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene 8, 1119-26.

Liu, X., Wu, H., Loring, J., Hormuzdi, S., Disteche, C. M., Bornstein, P., and Jaenisch, R. (1997). Trisomy eight in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. Dev Dyn 209, 85-91.

Lowell C. A., Berton G. (1999). Integrin signal transduction in myeloid leukocytes. J Leukoc Biol. 65, 313-20.

Lowry, M. B., Duchemin, A. M., Coggeshall, K. M., Robinson, J. M., and Anderson, C. L. (1998). Chimeric receptors composed of phosphoinositide 3-kinase domains and FCgamma receptor ligand-binding domains mediate phagocytosis in COS fibroblasts. J Biol Chem 273, 24513-20.

Machide, M., Mano, H., and Todokoro, K. (1995). Interleukin 3 and erythropoietin induce association of Vav with Tec kinase through Tec homology domain. Oncogene 11, 619-25.

Mahajan, S., Fargnoli, J., Burkhardt, A. L., Kut, S. A., Saouaf, S. J., and Bolen, J. B. (1995). Src family protein tyrosine kinases induce autoactivation of Bruton's tyrosine kinase. Mol Cell Biol 15, 5304-11.

Mano H., Yamashita Y., Miyazato A., Miura Y., Ozawa K. (1996). Tec protein-tyrosine kinase is an effector molecule of Lyn protein-tyrosine kinase.FASEB J. 10, 637-42.

Mano, H., Ishikawa, F., Nishida, J., Hirai, H., and Takaku, F. (1990). A novel protein-tyrosine kinase, tec, is preferentially expressed in liver. Oncogene 5, 1781-6.

Mano, H., Mano, K., Tang, B., Koehler, M., Yi, T., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. (1993). Expression of a novel form of Tec kinase in hematopoietic cells and mapping of the gene to chromosome 5 near Kit. Oncogene 8, 417-24.

Mano, H., Sato, K., Yazaki, Y., and Hirai, H. (1994). Tec protein-tyrosine kinase directly associates with Lyn protein-tyrosine kinase through its N-terminal unique domain. Oncogene 9, 3205-11.

Mano, H., Yamashita, Y., Sato, K., Yazaki, Y., and Hirai, H. (1995). Tec protein-tyrosine kinase is involved in interleukin-3 signaling pathway. Blood 85, 343-50.

Mansour, S. L., Thomas, K. R., Deng, C. X., and Capecchi, M. R. (1990). Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination. Proc Natl Acad Sci U S A 87, 7688-92.

Mao, J., Xie, W., Yuan, H., Simon, M. I., Mano, H., and Wu, D. (1998). Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13. Embo J 17, 5638-46.

Marshall, A. J., Niiro, H., Yun, T. J., and Clark, E. A. (2000). Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway [In Process Citation]. Immunol Rev 176, 30-46.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78, 7634-8.

Martin, T. F. (1998). Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. Annu Rev Cell Dev Biol 14, 231-64.

Matsuda, M., Mayer, B. J., Fukui, Y., and Hanafusa, H. (1990). Binding of transforming protein, P47gag-crk, to a broad range of phosphotyrosine-containing proteins. Science 248, 1537-9.

Matsuda, M., Park, J. G., Wang, D. C., Hunter, S., Chien, P., and Schreiber, A. D. (1996). Abrogation of the Fc gamma receptor IIA-mediated phagocytic signal by stem-loop Syk antisense oligonucleotides. Mol Biol Cell 7, 1095-106.

Matsuda, T., Takahashi-Tezuka, M., Fukada, T., Okuyama, Y., Fujitani, Y., Tsukada, S., Mano, H., Hirai, H., Witte, O. N., and Hirano, T. (1995). Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. Blood 85, 627-33.

Matsushita, M., Yamadori, T., Kato, S., Takemoto, Y., Inazawa, J., Baba, Y., Hashimoto, S., Sekine, S., Arai, S., Kunikata, T., Kurimoto, M., Kishimoto, T., and Tsukada, S. (1998). Identification and characterization of a novel SH3-domain binding protein, Sab, which preferentially associates with Bruton's tyrosine kinase (BtK). Biochem Biophys Res Commun 245, 337-43.

McCutcheon, J. C., Hart, S. P., Canning, M., Ross, K., Humphries, M. J., and Dransfield, I. (1998). Regulation of macrophage phagocytosis of apoptotic neutrophils by adhesion to fibronectin. J Leukoc Biol 64, 600-7.

Meng, F., and Lowell, C. A. (1998). A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. Embo J 17, 4391-403.

Merkel, A. L. (1996). Identification and characterisation of murine Tec gene. B.Sc. Honours Thesis. University of Adelaide. Adelaide.

Merkel, A. L., Atmosukarto, II, Stevens, K., Rathjen, P. D., and Booker, G. W. (1999). Splice variants of the mouse Tec gene are differentially expressed in vivo. Cytogenet Cell Genet 84, 132-9.

Miyazato, A., Yamashita, Y., Hatake, K., Miura, Y., Ozawa, K., and Mano, H. (1996). Tec protein tyrosine kinase is involved in the signaling mechanism of granulocyte colony-stimulating factor receptor. Cell Growth Differ 7, 1135-9.

Morton, C. J., and Campbell, I. D. (1994). SH3 domains. Molecular 'Velcro'. Curr Biol 4, 615-7.

Moss, J., and Vaughan, M. (1995). Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. J Biol Chem 270, 12327-30.

Musacchio A., Gibson T., Rice P., Thompson J., Saraste M. (1993). The PH domain: a common piece in the structural patchwork of signaling proteins.Trends Biochem Sci. 18, 343-87.

Myung P. S., Clements J. L., White D. W., Malik Z. A., Cowdery J. S., Allen L. H., Harty J. T., Kusner D. J., Koretzky G. A. (2000). In vitro and in vivo macrophage function can occur independently of SLP-76. Int Immunol 12, 887-97

Nagaishi, K., Adachi, R., Matsui, S., Yamaguchi, T., Kasahara, T., and Suzuki, K. (1999). Herbimycin A inhibits both dephosphorylation and translocation of cofilin induced by opsonized Zymosan in macrophagelike U937 cells. J Cell Physiol 180, 345-54.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. Development 110, 815-21.

Neumeister, E. N., Zhu, Y., Richard, S., Terhorst, C., Chan, A. C., and Shaw, A. S. (1995). Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. Mol Cell Biol 15, 3171-8.

Newman, S. L., Mikus, L. K., and Tucci, M. A. (1991). Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. J Immunol 146, 967-74.

Nicola N. A., Robb L., Metcalf D., Cary D., Drinkwater C. C., Begley C. G. (1996). Functional inactivation in mice of the gene for the interleukin-3 (IL-3)-specific receptor beta-chain: implications for IL-3 function and the mechanism of receptor transmodulation in hematopoietic cells. Blood. 87, 2665-74.

Ninomiya, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O., and Ui, M. (1994). Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. J Biol Chem 269, 22732-7.

Nisitani, S., Kato, R. M., Rawlings, D. J., Witte, O. N., and Wahl, M. I. (1999). In situ detection of activated Bruton's tyrosine kinase in the Ig signaling complex by phosphopeptide-specific monoclonal antibodies. Proc Natl Acad Sci U S A 96, 2221-6.

Nobes, C. D., and Hall, A. (1995). Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. Biochem Soc Trans 23, 456-9.

Noguchi T., Matozaki T., Inagaki K., Tsuda M., Fukunaga K., Kitamura Y., Kitamura T., Shii K., Yamanashi Y., Kasuga M. (1999). Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. EMBO J. 18, 1748-60.

Nore, B. F., Vargas, L., Mohamed, A. J., Branden, L. J., Backesjo, C. M., Islam, T. C., Mattsson, P. T., Hultenby, K., Christensson, B., and Smith, C. I. (2000). Redistribution of Bruton's tyrosine kinase by activation of phosphatidylinositol 3-kinase and Rho-family GTPases. Eur J Immunol 30, 145-54.

Odin, J. A., Edberg, J. C., Painter, C. J., Kimberly, R. P., and Unkeless, J. C. (1991). Regulation of phagocytosis and [Ca2+]i flux by distinct regions of an Fc receptor. Science 254, 1785-8.

Ohya, K., Kajigaya, S., Yamashita, Y., Miyazato, A., Hatake, K., Miura, Y., Ikeda, U., Shimada, K., Ozawa, K., and Mano, H. (1997). SOCS-1/JAB/SSI-1 can bind to and suppress Tec protein-tyrosine kinase. J Biol Chem 272, 27178-82.

Oka, Y., Rolink, A. G., Andersson, J., Kamanaka, M., Uchida, J., Yasui, T., Kishimoto, T., Kikutani, H., and Melchers, F. (1996). Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the XID and CD40 deficiency genes. Int Immunol 8, 1675-85.

Okoh M. P, Vihinen M. (1999). Pleckstrin homology domains of tec family protein kinases. Biochem Biophys Res Commun. 265, 1-7.

Orengo, C. A., Jones, D. T., and Thornton, J. M. (1994). Protein superfamilies and domain superfolds. Nature 372, 631-4.

Padeh, S., Levitzki, A., Gazit, A., Mills, G. B., and Roifman, C. M. (1991). Activation of phospholipase C in human B cells is dependent on tyrosine phosphorylation. J Clin Invest 87, 1114-8.

Pan, X. Q., Darby, C., Indik, Z. K., and Schreiber, A. D. (1999). Activation of three classes of nonreceptor tyrosine kinases following Fc gamma receptor crosslinking in human monocytes. Clin Immunol 90, 55-64.

Panayotou, G., and Waterfield, M. D. (1993). The assembly of signalling complexes by receptor tyrosine kinases. Bioessays 15, 171-7.

Panayotou, G., Gish, G., End, P., Truong, O., Gout, I., Dhand, R., Fry, M. J., Hiles, I., Pawson, T., and Waterfield, M. D. (1993). Interactions between SH2 domains and tyrosine-phosphorylated platelet-derived growth factor beta-receptor sequences: analysis of kinetic parameters by a novel biosensor-based approach. Mol Cell Biol 13, 3567-76.

Pappu, R., Cheng, A. M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B. P., and Chan, A. C. (1999). Requirement for B cell linker protein (BLNK) in B cell development. Science 286, 1949-54.

Parekh, A. B., and Penner, R. (1997). Store depletion and calcium influx. Physiol Rev 77, 901-30.

Park, H., Wahl, M. I., Afar, D. E., Turck, C. W., Rawlings, D. J., Tam, C., Scharenberg, A. M., Kinet, J. P., and Witte, O. N. (1996). Regulation of Btk function by a major autophosphorylation site within the SH3 domain. Immunity 4, 515-25.

Park, J. G., and Schreiber, A. D. (1995). Determinants of the phagocytic signal mediated by the type IIIA Fc gamma receptor, Fc gamma RIIIA: sequence requirements and interaction with protein-tyrosine kinases. Proc Natl Acad Sci U S A 92, 7381-5.

Parsons J., Weber M. J. (1989). Genetics of src: structure and functional organization of a protein tyrosine kinase.Curr Top Microbiol Immunol. 147, 79-127.

Patel, H. V., Tzeng, S. R., Liao, C. Y., Chen, S. H., and Cheng, J. W. (1997). SH3 domain of Bruton's tyrosine kinase can bind to proline-rich peptides of TH domain of the kinase and p120cbl. Proteins 29, 545-52.

Pavalko F. M., LaRoche S.M. (1993). Activation of human neutrophils induces an interaction between the integrin beta 2-subunit (CD18) and the actin binding protein alpha-actinin. J Immunol. 151, 3795-807.

Pawson, T. (1993). Signal transduction--a conserved pathway from the membrane to the nucleus. Dev Genet 14, 333-8.

Pawson, T. (1995). Protein modules and signalling networks. Nature 373, 573-80.

Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science 278, 2075-80.

Pawson, T., Olivier, P., Rozakis-Adcock, M., McGlade, J., and Henkemeyer, M. (1993). Proteins with SH2 and SH3 domains couple receptor tyrosine kinases to intracellular signalling pathways. Philos Trans R Soc Lond B Biol Sci 340, 279-85.

Perez-Villar, J. J., and Kanner, S. B. (1999). Regulated association between the tyrosine kinase Emt/Itk/Tsk and phospholipase-C gamma 1 in human T lymphocytes. J Immunol 163, 6435-41.

Piccione, E., Case, R. D., Domchek, S. M., Hu, P., Chaudhuri, M., Backer, J. M., Schlessinger, J., and Shoelson, S. E. (1993). Phosphatidylinositol 3-kinase p85 SH2 domain specificity defined by direct phosphopeptide/SH2 domain binding. Biochemistry 32, 3197-202.

Pivniouk, V. I., and Geha, R. S. (2000). The role of SLP-76 and LAT in lymphocyte development. Curr Opin Immunol 12, 173-8.

Pleiman, C. M., D'Ambrosio, D., and Cambier, J. C. (1994). The B-cell antigen receptor complex: structure and signal transduction. Immunol Today 15, 393-9.

Pursglove S. E. (2001). Biophysical analysis of Tec kinase regulatory regions: implications for the control of kinase activity. PhD Thesis. University of Adelaide, Adelaide.

Pursglove, S. E., Mulhern, T. D., Hinds, M. G., Norton, R. S., and Booker, G. W. (1998). Assignment of 1H and 15N resonances of murine Tec SH3 domain. J Biomol NMR 12, 461-2.

Quek, L. S., Bolen, J., and Watson, S. P. (1998). A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. Curr Biol 8, 1137-40.

Raghavan, M., and Bjorkman, P. J. (1996). Fc receptors and their interactions with immunoglobulins. Annu Rev Cell Dev Biol 12, 181-220.

Rameh L. E., Arvidsson A., Carraway K. L., Couvillon A. D., Rathbun G., Crompton A., VanRenterghem B., Czech M. P., Ravichandran K. S., Burakoff S. J., Wang D. S., Chen C. S., Cantley L. C. (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J Biol Chem. 272, 22059-66.

Ravetch, J. V., and Perussia, B. (1989). Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J Exp Med 170, 481-97.

Rawlings, D. J. (1999). Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. Clin Immunol 91, 243-53.

Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., and et al. (1993). Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science 261, 358-61.

Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinet, J. P. (1996). Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science 271, 822-5.

Reid L.H., Gregg R.G., Smithies O., Koller B.H. (1990). Regulatory elements in the introns of the human HPRT gene are necessary for its expression in embryonic stem cells. Proc Natl Acad Sci U S A 874, 299-303.

Remiszewski J. L. (1999). The role of Gbx 2 in murine development. PhD Thesis. University of Adelaide, Adelaide.

Ridley A. J., Hall A. (1994). Signal transduction pathways regulating Rho-mediated stress fibre formation:requirement for a tyrosine kinase.EMBO J. 13, 2600-10.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70, 389-99.

Roussel, R. R., Brodeur, S. R., Shalloway, D., and Laudano, A. P. (1991). Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. Proc Natl Acad Sci U S A 88, 10696-700.

Sadowski, I., Stone, J. C., and Pawson, T. (1986). A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. Mol Cell Biol 6, 4396-408.

Saharinen, P., Ekman, N., Sarvas, K., Parker, P., Alitalo, K., and Silvennoinen, O. (1997). The Bmx tyrosine kinase induces activation of the Stat signaling pathway, which is specifically inhibited by protein kinase Cdelta. Blood 90, 4341-53.

Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. Embo J 15, 6241-50.

Santos-Argumedo, L., Lund, F. E., Heath, A. W., Solvason, N., Wu, W. W., Grimaldi, J. C., Parkhouse, R. M., and Howard, M. (1995). CD38 unresponsiveness of xid B cells implicates Bruton's tyrosine kinase (btk) as a regular of CD38 induced signal transduction. Int Immunol 7, 163-70.

Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S. A., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. (1994). Temporal differences in the activation of

three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. Proc Natl Acad Sci U S A 91, 9524-8.

Satterthwaite, A. B., Li, Z., and Witte, O. N. (1998). Btk function in B cell development and response. Semin Immunol 10, 309-16.

Schaeffer E. M., Schwartzberg P. L. (2000). Tec family kinases in lymphocyte signaling and function. Curr Opin Immunol. 12, 282-8

Schaeffer, E. M., Debnath, J., Yap, G., McVicar, D., Liao, X. C., Littman, D. R., Sher, A., Varmus, H. E., Lenardo, M. J., and Schwartzberg, P. L. (1999). Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science 284, 638-41.

Schmedt, C., Saijo, K., Niidome, T., Kuhn, R., Aizawa, S., and Tarakhovsky, A. (1998). Csk controls antigen receptor-mediated development and selection of T-lineage cells. Nature 394, 901-4.

Schneider, H., Guerette, B., Guntermann, C., and Rudd, C. E. (2000). Resting lymphocyte kinase (Rlk/Txk) targets lymphoid adaptor SLP-76 in the cooperative activation of interleukin-2 transcription in T-cells. J Biol Chem 275, 3835-40.

Scholz G., Cartledge K., Dunn A. R. (2000). Hck enhances the adherence of lipopolysaccharide-stimulated macrophages via Cbl and phosphatidylinositol 3-kinase. J Biol Chem. 275, 14615-23.

Schwartzberg, P. L. (1998). The many faces of Src: multiple functions of a prototypical tyrosine kinase. Oncogene 17, 1463-8.

Shan, X., and Wange, R. L. (1999). Itk/Emt/Tsk activation in response to CD3 cross-linking in Jurkat T cells requires ZAP-70 and Lat and is independent of membrane recruitment. J Biol Chem 274, 29323-30.

Shaw, G. (1996). The pleckstrin homology domain: an intriguing multifunctional protein module. Bioessays 18, 35-46.

Shen, L., Guyre, P. M., and Fanger, M. W. (1987). Polymorphonuclear leukocyte function triggered through the high affinity Fc receptor for monomeric IgG. J Immunol 139, 534-8.

Shirai T., Tanaka K., Terada Y., Sawada T., Shirai R., Hashimoto Y., Nagata S., Iwamatsu A., Okawa K., Li S., Hattori S., Mano H., Fukui Y. (1998). Specific detection of phosphatidylinositol 3,4,5-trisphosphate binding proteins by the PIP3 analogue beads: an application for rapid purification of the PIP3 binding proteins. Biochim Biophys Acta. 1402, 292-302.

Sicheri, F., and Kuriyan, J. (1997). Structures of Src-family tyrosine kinases. Curr Opin Struct Biol 7, 777-85.

Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck [see comments]. Nature 385, 602-9.

Sidorenko, S.P, Law C.L., Chandran K.A., Clark E.A. (1995). Human spleen tyrosine kinase p72Syk associates with the Src-family kinase p53/56Lyn and a 120-kD phosphoprotein. Proc Natl Acad Sci U S A 17, 359-63

Siliciano, J. D., Morrow, T. A., and Desiderio, S. V. (1992). itk, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. Proc Natl Acad Sci U S A 89, 11194-8.

Siyanova, E. Y., Serfas, M. S., Mazo, I. A., and Tyner, A. L. (1994). Tyrosine kinase gene expression in the mouse small intestine. Oncogene 9, 2053-7.

Smith A.G. (1991). Culture and differentiation of embryonic stem cells. J.Tiss.Cult. Meth. 13, 89-94

Smith, C. I., Baskin, B., Humire-Greiff, P., Zhou, J. N., Olsson, P. G., Maniar, H. S., Kjellen, P., Lambris, J. D., Christensson, B., Hammarstrom, L., and et al. (1994). Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. J Immunol 152, 557-65.

Sommers, C. L., Huang, K., Shores, E. W., Grinberg, A., Charlick, D. A., Kozak, C. A., and Love, P. E. (1995). Murine txk: a protein tyrosine kinase gene regulated by T cell activation. Oncogene 11, 245-51.

Sommers, C. L., Rabin, R. L., Grinberg, A., Tsay, H. C., Farber, J., and Love, P. E. (1999). A role for the Tec family tyrosine kinase Txk in T cell activation and thymocyte selection. J Exp Med 190, 1427-38.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell 72, 767-78.

Stahl, P. D., and Ezekowitz, R. A. (1998). The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol 10, 50-5.

Stevens K. (1995). Cloning and characterisation of the mouse Tec gene. B.Sc. Honours Thesis.University of Adelaide, Adelaide.

Strzelecka, A., Kwiatkowska, K., and Sobota, A. (1997a). Tyrosine phosphorylation and Fcgamma receptor-mediated phagocytosis. FEBS Lett 400, 11-4.

Strzelecka, A., Pyrzynska, B., Kwiatkowska, K., and Sobota, A. (1997b). Syk kinase, tyrosine-phosphorylated proteins and actin filaments accumulate at forming phagosomes during Fcgamma receptor-mediated phagocytosis. Cell Motil Cytoskeleton 38, 287-96.

Su Y. W., Zhang Y., Schweikert J., Koretzky G.A., Reth M., Wienands J. (1999). Interaction of SLP adaptors with the SH2 domain of Tec family kinases. Eur J Immunol. 29, 3702-11.

Sudol, M. (1998). From Src Homology domains to other signaling modules: proposal of the 'protein recognition code'. Oncogene 17, 1469-74.

Suen, P. W., Ilic, D., Caveggion, E., Berton, G., Damsky, C. H., and Lowell, C. A. (1999). Impaired integrin-mediated signal transduction, altered cytoskeletal structure and reduced motility in Hck/Fgr deficient macrophages. J Cell Sci 112, 4067-78.

Suzuki, H., Kamada, N., Ueda, O., Jishage, K., Kurihara, Y., Kurihara, H., Terauchi, Y., Azuma, S., Kadowaki, T., Kodama, T., Yazaki, Y., and Toyoda, Y. (1997). Germ-line

contribution of embryonic stem cells in chimeric mice: influence of karyotype and in vitro differentiation ability. Exp Anim 46, 17-23.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., and Koyasu, S. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science 283, 390-2.

Takahashi-Tezuka, M., Hibi, M., Fujitani, Y., Fukada, T., Yamaguchi, T., and Hirano, T. (1997). Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. Oncogene 14, 2273-82.

Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J. V. (1996). Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. Nature 379, 346-9.

Takata, M., and Kurosaki, T. (1996). A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. J Exp Med 184, 31-40.

Tamagnone, L., Lahtinen, I., Mustonen, T., Virtaneva, K., Francis, F., Muscatelli, F., Alitalo, R., Smith, C. I., Larsson, C., and Alitalo, K. (1994). BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TXK family located in chromosome Xp22.2. Oncogene 9, 3683-8.

Tamir I., Stolpa J. C., Helgason C. D., Nakamura K., Bruhns P., Daeron M., Cambier J. C. (2000) The RasGAP-binding protein p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. Immunity. 12, 347-58.

Tanaka, N., Asao, H., Ohtani, K., Nakamura, M., and Sugamura, K. (1993). A novel human tyrosine kinase gene inducible in T cells by interleukin 2. FEBS Lett 324, 1-5.

Tang, B., Mano, H., Yi, T., and Ihle, J. N. (1994). Tec kinase associates with c-kit and is tyrosine phosphorylated and activated following stem cell factor binding. Mol Cell Biol 14, 8432-7.

Taylor, N., Jahn, T., Smith, S., Lamkin, T., Uribe, L., Liu, Y., Durden, D. L., and Weinberg, K. (1997). Differential activation of the tyrosine kinases ZAP-70 and Syk after Fc gamma RI stimulation. Blood 89, 388-96.

te Riele, H., Maandag, E. R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc Natl Acad Sci U S A 89, 5128-32.

Thien, C. B., Bowtell, D. D., and Langdon, W. Y. (1999). Perturbed regulation of ZAP-70 and sustained tyrosine phosphorylation of LAT and SLP-76 in c-Cbl-deficient thymocytes. J Immunol 162, 7133-9.

Thomas, J. D., Sideras, P., Smith, C. I., Vorechovsky, I., Chapman, V., and Paul, W. E. (1993). Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 261, 355-8.

Thomas, K. R., and Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51, 503-12.

Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L., and Melton, D. W. (1989). Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell 56, 313-21.

Thonglairoam V. (1994). Histone gene "knock out" in mouse embryonic stem cells. PhD Thesis. University of Adelaide, Adelaide.

Toker, A., and Cantley, L. C. (1997). Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 387, 673-6.

Tomlinson, M. G., Kurosaki, T., Berson, A. E., Fujii, G. H., Johnston, J. A., and Bolen, J. B. (1999). Reconstitution of Btk signaling by the atypical tec family tyrosine kinases Bmx and Txk. J Biol Chem 274, 13577-85.

Torres, R. M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996). Aberrant B cell development and immune response in mice with a compromised BCR complex [see comments]. Science 272, 1804-8.

Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994). Binding of G protein beta gamma-subunits to pleckstrin homology domains. J Biol Chem 269, 10217-20.

Tridandapani, S., Lyden, T. W., Smith, J. L., Carter, J. E., Coggeshall, K. M., and Anderson, C. L. (2000). The adaptor protein LAT enhances fcgamma receptor-mediated signal transduction in myeloid cells. J Biol Chem 275, 20480-7.

Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994). Binding of beta gamma subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. Proc Natl Acad Sci U S A 91, 11256-60.

Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature 378, 298-302.

Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. (1996). BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. Science 273, 1096-100.

Udy, G. B., Parkes, B. D., and Wells, D. N. (1997). ES cell cycle rates affect gene targeting frequencies. Exp Cell Res 231, 296-301.

Underhill, D. M., Chen, J., Allen, L. A., and Aderem, A. (1998). MacMARCKS is not essential for phagocytosis in macrophages. J Biol Chem 273, 33619-23.

Uribe, L., and Weinberg, K. I. (1998). X-linked SCID and other defects of cytokine pathways. Semin Hematol 35, 299-309.

van de Winkel, J. G., Ernst, L. K., Anderson, C. L., and Chiu, I. M. (1991). Gene organization of the human high affinity receptor for IgG, Fc gamma RI (CD64). Characterization and evidence for a second gene. J Biol Chem 266, 13449-55.

van Deursen, J., and Wieringa, B. (1992). Targeting of the creatine kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors. Nucleic Acids Res 20, 3815-20.

van Dijk T. B., van Den Akker E., Amelsvoort M. P., Mano H., Lowenberg B., von Lindern M. (2000). Stem cell factor induces phosphatidylinositol 3'-kinase-dependent Lyn/Tec/Dok-1 complex formation in hematopoietic cells. Blood. 96, 3406-13.

Varnai, P., Rother, K. I., and Balla, T. (1999). Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. J Biol Chem 274, 10983-9.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Bobrow, M., and et al. (1993). The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases [published erratum appears in Nature 1993 Jul 22;364(6435):362] [see comments]. Nature 361, 226-33.

Vihinen, M., Nilsson, L., and Smith, C. I. (1994). Tec homology (TH) adjacent to the PH domain. FEBS Lett 350, 263-5.

Vihinen, M., Nore, B. F., Mattsson, P. T., Backesjo, C. M., Nars, M., Koutaniemi, S., Watanabe, C., Lester, T., Jones, A., Ochs, H. D., and Smith, C. I. (1997). Missense mutations affecting a conserved cysteine pair in the TH domain of Btk. FEBS Lett 413, 205-10.

Wang A. V., Scholl P. R., Geha R. S. (1994). Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. J Exp Med. 180, 1165-70.

Wardenburg, J. B., Fu, C., Jackman, J. K., Flotow, H., Wilkinson, S. E., Williams, D. H., Johnson, R., Kong, G., Chan, A. C., and Findell, P. R. (1996). Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J Biol Chem 271, 19641-4.

Weil, D., Power, M. A., Smith, S. I., and Li, C. L. (1997). Predominant expression of murine Bmx tyrosine kinase in the granulo-monocytic lineage. Blood 90, 4332-40.

Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. Cell 76, 263-74.

Whyatt L.M. (1996). Systems for the analysis of developmental control genes of the mouse. PhD Thesis. University of Adelaide, Adelaide.

Wicker, L. S., and Scher, I. (1986). X-linked immune deficiency (xid) of CBA/N mice. Curr Top Microbiol Immunol 124, 87-101.

Wienands, J. (2000). The B-cell antigen receptor: formation of signaling complexes and the function of adaptor proteins. Curr Top Microbiol Immunol 245, 53-76.

Wiles, M. V., and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111, 259-67.

Woodland, R. T., Schmidt, M. R., Korsmeyer, S. J., and Gravel, K. A. (1996). Regulation of B cell survival in xid mice by the proto-oncogene bcl-2. J Immunol 156, 2143-54.

Wu, J., Motto, D. G., Koretzky, G. A., and Weiss, A. (1996). Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity 4, 593-602.

Xu, S., Tan, J. E., Wong, E. P., Manickam, A., Ponniah, S., and Lam, K. P. (2000). B cell development and activation defects resulting in xid-like immunodeficiency in BLNK/SLP-65-deficient mice. Int Immunol 12, 397-404.

Xu, W., Harrison, S. C., and Eck, M. J. (1997). Three-dimensional structure of the tyrosine kinase c-Src [see comments]. Nature 385, 595-602.

Yablonski, D., Kuhne, M. R., Kadlecek, T., and Weiss, A. (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. Science 281, 413-6.

Yagi, S., Suzuki, K., Hasegawa, A., Okumura, K., and Ra, C. (1994). Cloning of the cDNA for the deleted syk kinase homologous to ZAP-70 from human basophilic leukemia cell line (KU812). Biochem Biophys Res Commun 200, 28-34.

Yamamoto T., Matsuda T., Junicho A., Kishi H., Yoshimura A., Muraguchi A. (2001). Hematopoietic cell-specific adaptor proteins, SLP-76 and BLNK, effectively activate NF-AT as well as NF-kappaB by Syk and Tec PTKs in non-lymphoid cell lines. FEBS Lett. 491, 272-8.

Yamashita, Y., Miyazato, A., Ohya, K., Ikeda, U., Shimada, K., Miura, Y., Ozawa, K., and Mano, H. (1996). Deletion of Src homology 3 domain results in constitutive activation of Tec protein-tyrosine kinase. Jpn J Cancer Res 87, 1106-10.

Yamashita, Y., Miyazato, A., Shimizu, R., Komatsu, N., Miura, Y., Ozawa, K., and Mano, H. (1997). Tec protein-tyrosine kinase is involved in the thrombopoietin/c-Mpl signaling pathway. Exp Hematol 25, 211-6.

Yamashita, Y., Watanabe, S., Miyazato, A., Ohya, K., Ikeda, U., Shimada, K., Komatsu, N., Hatake, K., Miura, Y., Ozawa, K., and Mano, H. (1998). Tec and Jak2 kinases cooperate to mediate cytokine-driven activation of c-fos transcription. Blood 91, 1496-507.

Yan S. R., Huang M., Berton G. (1997). Signaling by adhesion in human neutrophils: activation of the p72syk tyrosine kinase and formation of protein complexes containing p72syk and Src family kinases in neutrophils spreading over fibrinogen. J Immunol. 158, 1902-10.

Yanagi, S., Sugawara, H., Kurosaki, M., Sabe, H., Yamamura, H., and Kurosaki, T. (1996). CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. J Biol Chem 271, 30487-92.

Yang K. D., Augustine N. H., Shaio M. F., Bohnsack J. F., Hill H. R. (1994). Effects of fibronectin on actin organization and respiratory burst activity in neutrophils, monocytes, and macrophages. J Cell Physiol. 158, 347-53.

Yang, W. C., and Olive, D. (1999). Tec kinase is involved in transcriptional regulation of IL-2 and IL-4 in the CD28 pathway. Eur J Immunol 29, 1842-9.

Yang, W. C., Ghiotto, M., Barbarat, B., and Olive, D. (1999). The role of Tec protein-tyrosine kinase in T cell signaling. J Biol Chem 274, 607-17.

Yao, L., Janmey, P., Frigeri, L. G., Han, W., Fujita, J., Kawakami, Y., Apgar, J. R., and Kawakami, T. (1999). Pleckstrin homology domains interact with filamentous actin. J Biol Chem 274, 19752-61.

Yao, L., Kawakami, Y., and Kawakami, T. (1994). The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. Proc Natl Acad Sci U S A 91, 9175-9.

Yoshida K., Yamashita Y., Miyazato A., Ohya K., Kitanaka A., Ikeda U., Shimada K., Yamanaka T., Ozawa K., Mano H. (2000). Mediation by the protein-tyrosine kinase Tec of signaling between the B cell antigen receptor and Dok-1. J Biol Chem. 275, 24945-52.

Zhang, Q., Cox, D., Tseng, C. C., Donaldson, J. G., and Greenberg, S. (1998a). A requirement for ARF6 in Fcgamma receptor-mediated phagocytosis in macrophages. J Biol Chem 273, 19977-81.

Zhang, W., Trible, R. P., and Samelson, L. E. (1998b). LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity 9, 239-46.

Zheleznyak A., Brown E. J. (1992). Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation. Evidence for protein kinase C translocation to phagosomes. J Biol Chem. 267, 12042-8.

Zhu, Z., Bao, Z., and Li, J. (1995). MacMARCKS mutation blocks macrophage phagocytosis of Zymosan. J Biol Chem 270, 17652-5.

APPENDIX

Merkel A.L., Atmosukarto I.I.C., Stevens K., Rathjen P.D. and Booker G.W. (1999)
Splice variants of the mouse Tec gene are differentially expressed in vivo.
Cytogenetics and Cell Genetics, v. 84 (1/2), pp. 132–139, 1999