Exploring the Role of the Small GTPase Rho in T Lymphocyte Biology

Stephen Charles Cleverley

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Lymphocyte Activation Laboratory
Imperial Cancer Research Fund
44 Lincoln's Inn Fields
London WC2A 3PX

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Rho, a member of the small GTPase superfamily, acts as a molecular switch cycling between an inactive GDP bound state and an active GTP bound form and has been reported to regulate many cellular processes. In the present thesis the role of Rho in thymocyte development has been examined. The bacterial enzyme, C3-transferase from *Clostridium botulinum* selectively ADP-ribosylates Rho within its effector-binding domain and thereby abolishes its biological function. Previous work has used the proximal p56lck promoter to drive thymocyte-specific expression of C3-transferase generating transgenic mice with the first thymocyte progenitors and subsequent subsets devoid of Rho function. Rho inactivation severely impaired the production of normal numbers of thymocytes and peripheral T cells in the lck-C3 transgenic mice. The main problem caused by lack of Rho function was that early thymocyte progenitors underwent massive apoptosis in the absence of Rho function. An initial aim of the present thesis was to extend analysis of lck-C3 transgenic mice to gain greater insight into the molecular basis for the regulation of cell survival by Rho.

In the lck-C3 transgenic mice the survival defect is caused by loss of Rho function in the earliest T-cell progenitors. To study Rho function in later stages of T cell development the CD2 antigen locus control region (CD2-LCR) was used to generate a series of transgenic mice in which C3 transferase is not expressed in the earliest progenitors but is targeted to a later stage of thymic development, the pre-T cell population. Elimination of Rho at this later stage results in a differentiation block of pre-T cells, but no apparent survival defect and has led to the elucidation of an additional role for Rho; as a controlling intracellular switch for the critical thymic checkpoint of TCR β selection.

Cell survival is controlled by a complex network of signals mediated by death-domain containing receptors, Bcl-2 family members and the tumour suppressor p53. Studies of lck-C3 transgenic mice described in the present thesis have established that Rho controls survival of thymocytes by both p53 dependent and independent signalling mechanisms. The link between Rho and the tumour suppressor p53 was intriguing because during routine maintenance of the lck-C3 transgenic mice, it was observed that

the mice were becoming sick and dying prematurely. Analysis revealed that loss of Rho function in the thymus was associated with the development of aggressive thymic lymphoma. These observations suggest that Rho itself may be involved in the suppression of malignancy in the thymus.

The results presented in the thesis highlight the diversity of the roles of Rho within T cell biology. One way in which such diversity could arise is by this GTPase interacting with a number of downstream effectors. In an attempt to identify Rho effector molecules within T lymphocytes, a protein affinity chromatography approach was taken to purify proteins that can interact with activated GTP bound recombinant RhoA. These experiments identified the serine kinase PRK-1 and the actin regulatory protein mDia-1 as putative Rho effector molecules within T lymphocytes.

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List of Publications

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Abbreviations

APC	Allophycocyanin
ATP	adenosine 5'-triphosphate
bp	base pair
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
CRIB	Cdc42/Rac interactive binding
CRIK	Citron Rho-interacting kinase
D. melanogaster	*Drosphilia melanogaster*
DH	Dbl Homology
DMEM	Dulbecco's modified Eagle's medium
DN	Double Negative ($CD4^-8^-$)
DNA	deoxyribonucleic acid
DP	Double Positive ($CD4^+8^+$)
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EGTA	[ethylene-bis(oxyethylenenitrilo)] tetra-acetic acid
ERK	extracellular signal-regulated kinase
ERM	Ezrin, radixin and moesin proteins
ES cell	Embryonic Stem Cell
FADD	Fas-associated death-domain containing protein
FCS	foetal calf serum
FITC	Fluroscein Isothiocyanate
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hr	hour
HPRT	Hypoxanthinephosphoribosyltransferase
HRP	Horse-radish peroxidase
IL	Interleukin
IL-2R	Interleukin 2-Receptor
IL-7R	Interleukin 7-Receptor
IPTG	isopropyl-2-D-thiogalactopyranoside
JNK	Jun N-terminal kinase
kd	Kilodalton
LAT	Linker for Activation of T cells

M	Molar
m	milli
μ	micro
MAP	mitogen-activated protein
mDia	Mammalian homolog of *Drosophila* diaphanous
MEK	MAP/ERK Kinase
MHC	major histocompatibility complex
MLC	Myosin Light Chain
MLK	Mixed Lineage Kinase
min	minute
Mr	relative molecular weight
n	nano
NFAT	nuclear factor of activated T cells
NFκB	Nuclear Factor kappa-B
NK	Natural Killer Cell
NLC	Non-transgenic littermate control
NP-40	Nonidet P-40, r-*tert*-octylphenyl 9.6 ethoxylate
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PdBu	phorbol 12,13-dibutyrate
PDK	3-phosphoinositide-dependent kinase
PE	Phycoerythrin
PH	pleckstrin homology
PHA	Phytohemagglutinin A
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	Phospholipase C
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3'-hydroxyl kinase
PIP_2	Phosphatidylinositol (3,4)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PIP5-K	Phosphatidylinositol 4-phosphate 5-kinase
PIPES	Piperazine-N,N'-bis(ethanesulphonic acid)
POR	Partner of Rac
pre-TCR	pre-T cell antigen receptor
PRK	Protein Kinase C-related kinase
PTK	Protein tyrosine kinase
PVDF	polyvinylidene difluoride
RAG	Recombinase Activating Gene

ROCK	Rho-associated coiled-coil kinase
ROK	RhoA-binding kinase
RNA	ribonucleic acid
RT	Room Temperature (25°C)
RTK	Receptor tyrosine kinase
S. cerevisiae	*Saccharomyces cerevisiae*
SAPK	Stress-Activated protein kinase
SCID	Severe combined immunodeficency disease
SDS	sodium dodecyl sulphate
SH	src homology
SLP76	SH2-domain containing leukocyte protein of 76 kd
SOS	Son of Sevenless
SP	Single positive (either $CD4^+8^-$ or $CD4^-8^+$)
SRE	serum response element
SRF	serum response factor
TCF	ternary complex factor
TCR	T cell antigen receptor
TNF	Tumour Necrosis Factor
Tris	tris(hydroxymethyl)aminomethane
v/v	volume/volume
v/w	weight/volume
WASP	Wiscott-Aldrich syndrome protein
WT	Wild Type

CHAPTER 1
Introduction

1.1 - Rho Family GTPases and their Regulation

1.1.1 - Rho Family GTPases and their role in the Immune System

Small GTPases are a family of guanine nucleotide binding proteins with intrinsic GTPase activity. Almost a decade ago the small GTPase Ras was shown to be activated in response to antigen receptor triggering in T-cells. Since then Ras has been further characterised as a central molecule for the regulation of signal transduction pathways in lymphocytes. Work on Ras signalling prompted considerable interest into the possible role of other GTPases in lymphocyte biology, particularly relatives of the Rho family. Today it is well established that Rho GTPases act as unique molecular switches at several critical checkpoints in lymphocyte development and function. Additionally, a new and critical concept in GTPase signalling has taken shape over the last couple of years in that small GTPases are able to regulate quite diverse cellular processes in the immune response by linking to multiple biochemical effector pathways.

The following chapter will discuss the initial identification of the archetypal small GTPase Ras, then the role played by the Rho family of small GTPases and the way in which they are regulated before moving on the roles they play in biology. Finally, their importance in the immune system is explored.

1.1.2 - Discovery of the Ras Family of Small GTPases

The viral oncogenes of certain retroviruses with potent carcinogenic properties were the first ras genes (v-ras) to be identified (Andersen *et al.*, 1981; DeFeo *et al.*, 1981; Ellis *et al.*, 1981). The recognition that retroviral oncogenes, including v-ras, are derived from normal cellular genes was followed by the demonstration that many of the transforming genes identified through the NIH 3T3 cell transformation assay represented cellular c-ras genes that had been activated by point mutation (Eva *et al.*, 1983; Barbacid, 1987). The role of ras gene products in transformation was further strengthened by the

discovery that many human tumours contained mutated Ras genes, conferring a constitutively active state (Bos, 1989). The highest incidence of ras gene mutations were found in adenocarcinomas of the pancreas (90%), the colon (50%) and the lung (30%).

Ras biological activity is controlled by a regulated GDP/GTP cycle (Figure 1.1A). Binding of GTP mediates a transition of the protein from the inactive, GDP-bound state into the activated, GTP-bound form. The GDP/GTP binding cycle of small GTPases is regulated by two main classes of proteins, first identified from studies in *Saccharomyces cerevisiae*. In yeast, activation of Ras is catalysed by the product of the CDC25 gene, a protein that promotes removal of GDP bound to Ras and its replacement with free GTP (Robinson *et al.*, 1987), thereby shifting the equilibrium towards the activated, GTP-bound state. Analyses of Ras control in other systems has identified similar upstream activators, or Guanine Nucleotide exchange factors (GEFs) such as Sos in *Drosophila Melanogaster* (Simon *et al.*, 1991), and its mammalian homologs Sos1 and Sos2 (Bowtell *et al.*, 1992). Upon binding to GTP, a large conformational change occurs within the GTPase, allowing interaction with effector molecules and thus mediated downstream signalling (Figure 1.1B). Ras proteins are turned off by hydrolysis of GTP to GDP, a reaction that can be mediated by their own GTPase activity. Although this intrinsic GTPase activity is too low to be effective, the rate of hydrolysis can be enhanced by GTPase activating proteins (GAPs). The *S.cerevisiae* proteins IRA1 and IRA2 were originally identified as negative regulators of the Ras mediated cAMP pathway (Tanaka *et al.*, 1990); however, they were only found to be GAPs for Ras following comparisons with the mammalian GAP, p120GAP, identified by Trahey and McCormick (Trahey and McCormick, 1987).

In 1993, it emerged from comparisons of experimental observations from genetic analyses of *D.melanogaster, S.cerevisiae* and *C.elegans* as well as biochemical and biological studies that Ras was playing a central role in a range of diverse organisms. *Drosophila* eye development was shown to be controlled by a signalling pathway that begins with ligand stimulation of the Sevenless Receptor Tyrosine Kinase (RTK), leading to activation of a Ras homolog, which in turn activates a mitogen activated protein kinase (MAPK) cascade (Rubin, 1991). An almost identical scheme was seen for the pathway that regulates development of the vulva in *C.elegans*, involving a RTK-mediated

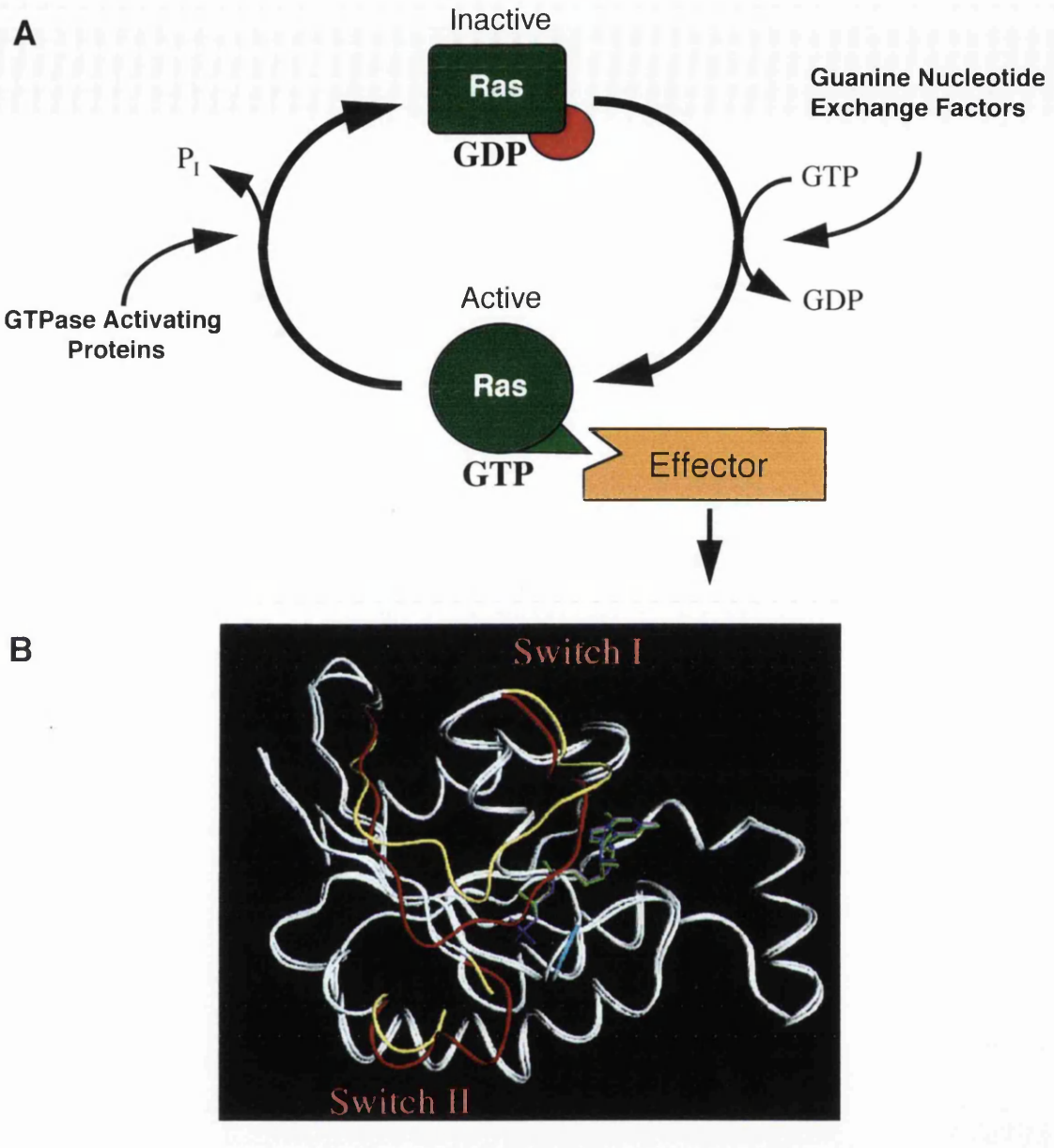


Figure 1.1 - Upon GTP binding small GTPases undergo large conformation changes allowing them to interact with downstream effectors

(A) Small GTPases act as Molecular Switches within the cell, cycling between active GTP-bound and inactive GDP-bound forms. Conversion from inactive to active is catalysed by Guanine Nucleotide Exchange Factors, while GTPase Activating Proteins enhance the intrinsic GTPase hydrolysis activity of the small GTPases, thus bringing about their inactivation. Conversion from inactive to active is accompanied by a large conformational change, allowing the activated GTPase to interact with effector molecules and thus mediate downstream signalling events.

(B) Superposition of Cα-carbon atom tracings of RhoA V14-GTPγS (magenta) and RhoA N25 bound to GDP (green) with segments displaying large displacements in red and yellow, respectively. These include switch I and the C-terminal flanking region (residues 28–44) and the N-terminal region of switch II (residues 62–69). Val 14 of RhoA V14 is highlighted in blue. (Figure from Ihara *et al.* 1998)

pathway that activates the Ras-homolog Let-60 (Han and Sternberg, 1990). *S.pombe* Ras1 also activated a MAPK cascade (Nielsen *et al.*, 1992). Studies of mammalian systems revealed a similar role; ligation of growth factor receptors caused Ras activation, which in turn activated the MAPK, Extracellular-signal regulated kinase (ERK) 2 (de Vries-Smits *et al.*, 1992; Leevers and Marshall, 1992). What emerged from all these studies was the linear pathway depicted in Figure 1.2. In the example shown, growth factor receptor ligation results in autophosphorylation of specific tyrosine residues in its cytoplasmic domain, creating phosphotyrosyl binding sites for association with adapter proteins such as Shc and Grb2 (Reviewed in McCormick, 1993). As Grb2 is stably associated with the RasGEF, SOS, association of Shc/Grb2 with the receptor localises SOS to the membrane where it can activate Ras (Rozakis-Adcock *et al.*, 1992; Egan *et al.*, 1993; Schlessinger, 1993). One of the key downstream effectors of Ras is the ser/thr kinase Raf-1. Upon activation, Ras forms a high-affinity complex with Raf, translocating Raf to the membrane (Warne *et al.*, 1993; Hallberg *et al.*, 1994; Marais *et al.*, 1995), where subsequent events lead to activation of its kinase function; these events are complex and not fully understood (Morrison and Cutler, 1997). Upon activation, Raf phosphorylates and activates both MAP/ERK Kinase (MEK) 1 and 2 (Crews and Erikson, 1993), which subsequently phosphorylate and activate two MAPKs, ERK-1 and ERK-2 (Crews *et al.*, 1992). Upon activation, the MAPKs translocate to the nucleus and activate a number of effectors, including the Elk-1 transcription factor thus regulating gene expression (Marais *et al.*, 1993). Today, it is recognised that this simple linear pathway represents only a minor component of Ras-mediated signalling. Ras is now known to regulate an array of signalling networks in which cross-talk, feedback loops and branch-points are recurring themes (Reviewed in Katz and McCormick, 1997).

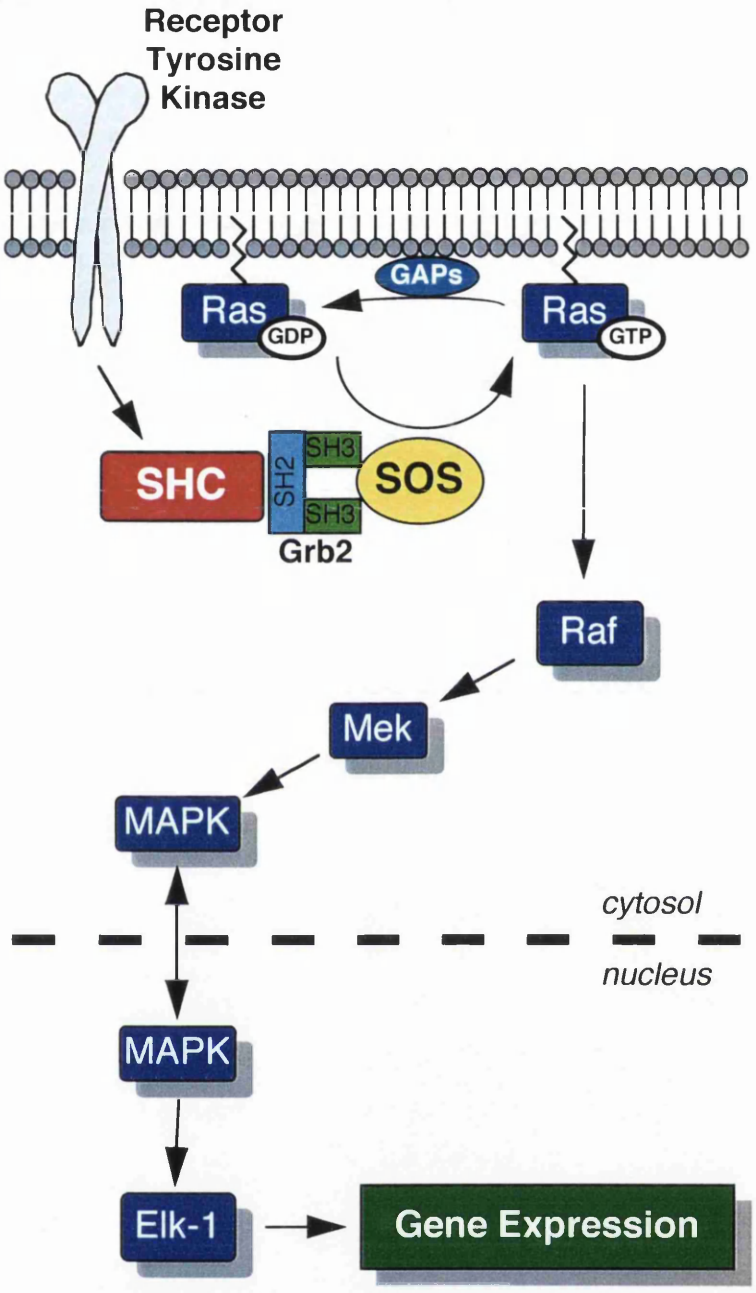


Figure 1.2 - The Ras/MAPK Cascade

Ras functions downstream of receptor tyrosine kinases and upstream of a cascade of serine/threonine kinases (Raf->MEK->MAPK) resulting in the regulation of gene transcription.

1.1.3 - Small GTPase Family Members

Since the initial identification of Ras as an oncogene, a large superfamily of Ras-related small GTPases (>80 mammalian members) have emerged and can be categorised into at least eight distinct branches. These include the Ras, Rab, Rho, Ran, Rheb, Rad/Gem, Rin/Rit, and Arf families. Rho family GTPases constitute one of the three major branches of the Ras superfamily and its members share approximately 30% amino acid identity with four Ras proteins (Chardin, 1993). The phylogenic relationship between the Ras and Rho superfamily of GTPases is shown in figure 1.3A. Presently, at least 14 mammalian Rho family proteins have been identified: RhoA, RhoB, RhoC, RhoD, RhoE/Rnd3, Rnd1/Rho6, Rnd2/Rho7, RhoG, Rac1, Rac2, Rac3, Cdc42, TC10, and TTF that share significant amino acid identity with each other. A sequence comparison of the mammalian Rho family members allows 5 distinct sub-families to be identified: 1) RhoA, B, and C, 2) Rnd1, Rnd2, RhoE, 3) Rac1, 2, 3, and RhoG, 4) TC10 and two mammalian Cdc42 isoforms, and 5) TTF and RhoD (Figure 1.3B). The following sections will review what is known about regulation of the guanine nucleotide binding cycle and hence activity state of Rho family GTPases.

1.1.4 - Switching the Rho GTPases On

Ras and Rho GTPases are activated upon exchange of GDP for GTP; a reaction mediated by guanine nucleotide exchange factors, or GEFs. The first GEF to be identified for the Rho family of small GTPases was Dbl, which was identified as a transforming oncogene in a diffuse B-cell lymphoma (Eva and Aaronson, 1985). The first clue to Dbl's function as a GEF came from the observation that it contains 29% sequence identity with the *S.cerevisiae* cell division protein Cdc24, which by genetic analysis was placed upstream of the yeast small GTPase Cdc42 (Ron *et al.*, 1991). Since this initial identification of Dbl as a GEF for Rho family GTPases, a growing number of oncogene products and growth regulatory molecules have been shown to contain a domain homologous to a 180 amino acid sequence in Dbl, and designated the Dbl homology (DH) domain. Deletion analysis revealed that the DH domain was essential and sufficient for exchange activity and was also necessary to induce oncogenicity (Ron *et al.*, 1991; Hart *et al.*, 1991; Hart *et al.*, 1994).

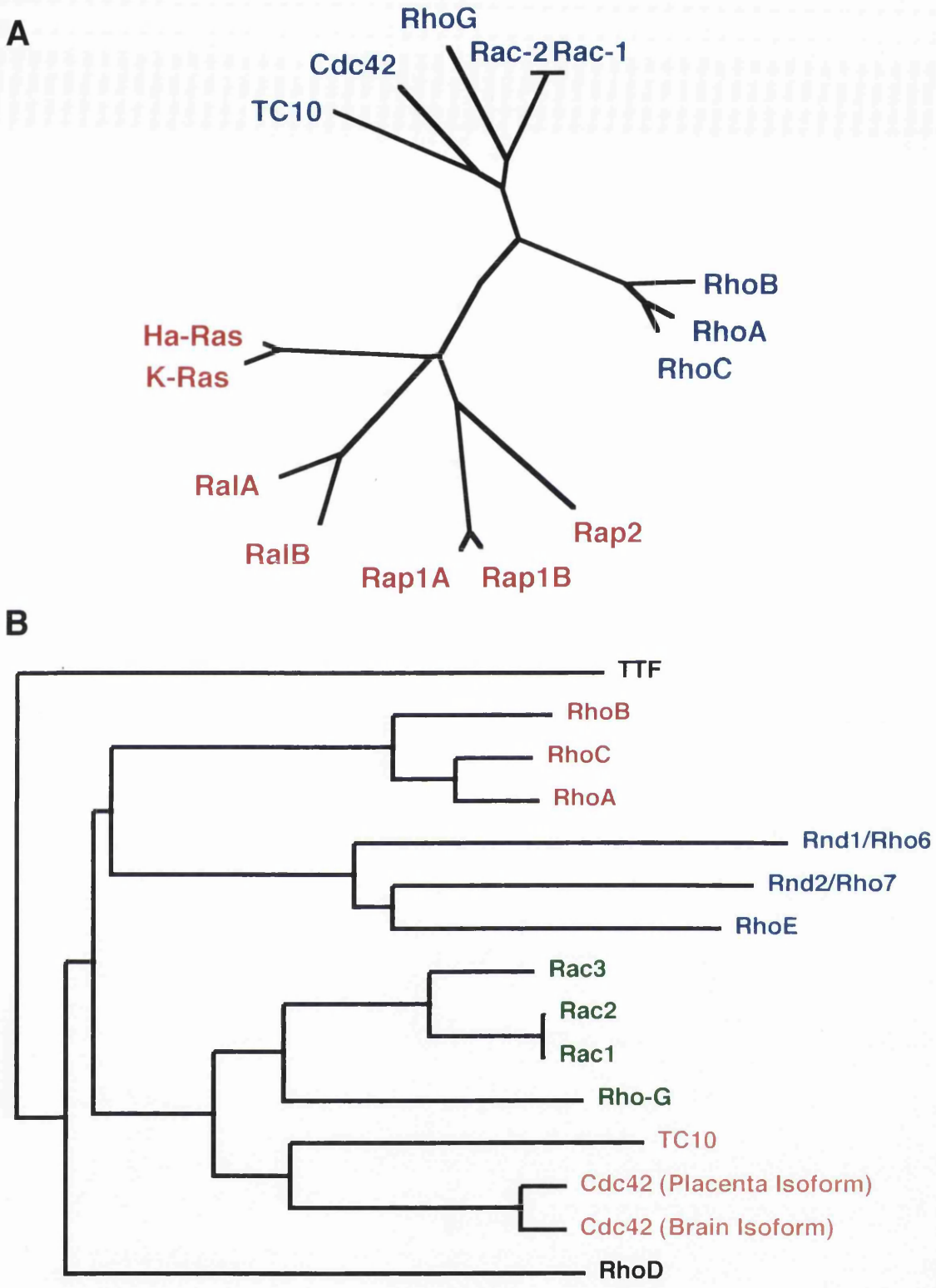


Figure 1.3 - Phylogenic relationship members of the small GTPase Superfamily

(A) Sequence comparision between members of the Rho (Blue) and Ras (Red) family of small GTPases reveals them to be two inter-related yet distinct families.

(B) Sequence comparisions of the Rho family of small GTPases reveal 5 distinct Rho-subfamilies: 1) RhoA, B, and C (Red), 2) Rnd1, Rnd2, RhoE (Blue), 3) Rac1, 2, 3, and RhoG (Green), 4) TC10 and two mammalian Cdc42 isoforms (Orange), and 5) TTF and RhoD (Black).

In addition to a DH domain, all family members have a second, 100 amino acid conserved domain called the pleckstrin homology (PH) domain. PH domains function as protein-protein or protein-lipid interaction motifs and it is generally believed that they serve to recruit proteins to the cell surface by lipid binding (Lemmon *et al.*, 1996). The PH domain is invariably found immediately Carboxy-terminal to the DH domain and this conserved relationship between the two suggests a functional interdependence. In addition to DH and PH domains, many of the exchange factors also have other domains that are commonly found in signalling molecules, such as src homology (SH3) domains and diacylglycerol binding motifs, providing further insight into the regulation of these molecules (Cerione and Zheng, 1996). Table 1.1 describes the source, activity and biochemical function of some of the dbl-family exchange factors.

Table 1.1 - The Dbl-family of Oncogenes and their Biological functions

	Source/Distribution	Biological Activity	Biochemical Function
Dbl	Diffuse B-cell lymphoma/brain, adrenal glands, gonads	Oncogenic	GEF for Rho and Cdc42Hs
Cdc24	*S.cerevisiae*	Yeast cell polarisation and gene activation	GEF for Cdc42
Ost	Osteosarcomas/brain, heart, liver, lung	Oncogenic	GEF for RhoA and Cdc42Hs, and binds to active Rac1
Tiam-1	T-lymphoma cells/brain, testis	Metastatic and oncogenic	GEF for Rac and Cdc42
Ect-2	Keratinocytes/testis, kidney, liver, spleen	Oncogenic	Binds to RhoA and RhoC, and Rac
Vav	Hematopoietic cells	Oncogenic	GEF for Rac and Ras?
Lbc	Myeloid leukemias/heart, lung, skeletal muscle	Oncogenic	GEF for Rho
Lfc	Myeloid cell, haemopoietic cells, kidney, lungs	Oncogenic	?
Brc	Brain	Implicated in chronic myelogenous leukemia	GAP for Rac; GEF for Rho-like proteins

Modified from (Cerione and Zheng, 1996)

1.1.5 - Switching Rho GTPases Off

Ras and Rho GTPases are turned off by the hydrolysis of bound GTP to GDP; a reaction regulated by GTPase activating proteins, or GAPs. Many potential GAPs have now been identified in mammalian cells including p50 RhoGAP, p190GAP and Bcr (Diekmann *et al.*, 1991; Settleman *et al.*, 1992; Lancaster *et al.*, 1994). Also, several Rho GAPs have been discovered in *S cerevisiae*, *D melanogaster*, and *C.elegans* (Agnel *et al.*, 1992; Chen *et al.*, 1994c; Zheng *et al.*, 1994; Stevenson *et al.*, 1995). These proteins all share a related GAP domain of 140 amino acids, but bears no resemblance to GAPs for the Ras GTPases. Although some Rho GAPs exhibit GTPase activity for several Rho GTPases in cell-free assays, their microinjection into fibroblasts reveals them to have a more restricted substrate specificity *in vivo*. For example, while RhoGAP shows activity towards both Rac and Rho *in vitro*, microinjection into fibroblasts can block the Rho-mediated formation of stress-fibres in response to serum, but not PDGF-induced Rac-mediated membrane ruffles, thus demonstrating that RhoGAP is specific for Rho *in vivo* (Ridley *et al.*, 1993).

1.1.6 - Spatial Control of Rho GTPases - GDIs

To date, guanine nucleotide dissociation inhibitors have only been described for the Rho and Rab subfamilies of small GTPases. GTP-bound Rho is generally associated with a membrane compartment whereas Rho-GDP is cytoplasmic. GDIs function by binding to the GTPases, burying their lipid modifications within a hydrophobic pocket. The resulting heterodimer exists within the cytoplasm and has been shown to interact with potential Rho effectors (Tolias *et al.*, 1998). Upon mitogen activated or cell cycle dependent association of Rho with membrane compartments, an interaction between the F-actin binding proteins ezrin, radixin, and moesin (ERM) and the GDI results in the displacement of the GTPase (Takahashi *et al.*, 1997). The action of GEFs then produces a Rho-GTP which is able to activate effector molecules. The subsequent GAP-enhanced GTP hydrolysis results in a membrane bound Rho-GDP which is extracted from the membrane by the GDI (Sasaki and Takai, 1998).

Little is known about the physiological function of the Rho GDIs *in vivo*. Microinjection studies have shown that Rho GDI inhibits several downstream functions of Rho (Nishiyama *et al.*, 1994; Coso *et al.*, 1995). Deletion of the D4-GDI gene in Embryonic stem (ES) cells results in a defect of superoxide production in D4-GDI-/- macrophages, a function reported to be mediated by Rac (Guillemot *et al.*, 1996). Moreover, the Rho GDI, D4-GDI has also been identified as a target of ICE/CED-3 cysteine proteases and is cleaved in response to Fas cross-linking in T cells (Na *et al.*, 1996). These data provide an intriguing link between the process of apoptosis and the regulation of Rho GTPases by GDIs.

1.1.7 - Regulation of Rho GTPases - Upstream Signalling

Clues as to the upstream regulators of Rho GTPases comes from observations that exchange factors for Rho family GTPases have PH domains. The PH domain is thought to serve two roles. Firstly, it may promote membrane translocation of the Dbl-proteins. Secondly, the PH domain may serve as an intramolecular regulator of the DH domain. Thus, upstream signalling pathways that cause activation of heterotrimeric G proteins, and the subsequent release of $\beta\gamma$ subunits, or upregulation of phosphatidyl-inositol lipids may regulate Dbl family protein function via interaction with the PH domain (Han *et al.*, 1998; Kiyono *et al.*, 1999). Further evidence for G-protein regulation of Rho family small GTPases comes from the observation that the external stimuli reported as Rho activating signals include LPA, thrombin, endothelin, and bombesin (Ridley and Hall, 1992; Jalink *et al.*, 1994; Rankin *et al.*, 1994). All of these substances act on a cell surface receptor coupled to a heterotrimeric G protein, and thus the heterotrimeric G protein-mediated pathway is implicated in Rho GTPase activation.

As well as GEF regulation by $G_{\beta\gamma}$ subunits, in fibroblasts, the $G_{\alpha}13$ subunit has been shown to be able to induce Rho-dependent stress-fibre formation and focal adhesions (Buhl *et al.*, 1995); an effect later reported to be mediated by $G_{\alpha}13$ stimulation of p115 RhoGEF activity (Hart *et al.*, 1998). Thus, it is possible that G_{α} subunits can also regulate Rho GTPases through the action of GEFs. However, it is not clear whether interaction with G_{α} subunits occurs via the PH domain of Rho family exchange factors.

In addition to binding to $G_{\beta\gamma}$ subunits, PH domains also mediate protein-lipid interactions, in particular with PIP_3, a product of Phosphatidylinositol 3'-hydroxyl kinase (PI3K) activity. The importance of PI3K-mediated signals in the regulation of Rac exchange factors was demonstrated by experiments in which expression of constitutively active PI3K mutants were able to induce Rac-mediated changes in the actin cytoskeleton (Reif *et al.*, 1996). It has been suggested that binding of the lipid products of PI3K to PH domains of Rho family GEFs either mediates an increase in exchange activity through a conformational change or induces relocalisation of the GEF to the plasma membrane where activation can occur; binding of PIP_3 to the PH domain of the exchange factor Vav1 has been reported to facilitate Vav1 tyrosine phosphorylation by the tyrosine kinase $p56^{lck}$, thereby promoting activation of Vav1 catalytic function (Han *et al.*, 1998). Thus, it is possible that the phospholipid products of PI3K play a critical role in localisation of Rho family GEFs to the membrane where they can be activated via receptor-mediated phosphorylation. Regulation of exchange activity by phosphorylation is not restricted to PIP_3 regulated GEFs. Tyrosine kinase inhibitors were recently shown to prevent $G_{\beta\gamma}$-mediated activation of a GEF with exchange activity for Rac, Ras-GRF1 (Kiyono *et al.*, 1999).

Upstream regulation of Rho family GTPases is mediated by modulation of GEF localisation and activity. GEF activation appears to be a two step process: first, the GEF is localised to the plasma membrane via interaction of its PH domain with either $G_{\beta\gamma}$, a product of heterotrimeric G protein activation, or PIP_3, a product of PI3K activity. Second, membrane proximal GEF is activated through phosphorylation by tyrosine kinase receptors, or membrane-proximal tyrosine kinases.

Stimulation of Rho activity by GEFs may only be one mechanism of regulation. Lang *et al.* have demonstrated that RhoA can be phosphorylated by cAMP dependent protein kinase A (PKA), increasing the affinity of GTP-bound RhoA for GDI, and thereby translocating RhoA from the membrane to the cytoplasm (Lang *et al.*, 1996). Clearly, the regulation of Rho-family GTPases is a complex process with multiple levels of regulation and the identification of additional upstream signalling molecules will be required to gain further insight into the activation of Rho GTPases in response to extracellular signals.

1.2 - Role of the Rho GTPases

1.2.1 - Cytoskeletal effects

The actin cytoskeleton plays a vital role in the control of eurkaryotic cell shape and its degree of attachment to the substratum. In fibroblasts, polymerised actin is assembled into a variety of distinct structures (Recently reviewed in Small *et al.*, 1999). Lamellipodia are curtain-like extensions that consist of thin protrusive actin sheets. Membrane ruffles represent lamellipodia that have lifted from the substratum at the leading edge of cells. Actin stress fibres consist of actin bundles that transverse the cell and promote cell attachment to the extracellular matrix via focal adhesions. Focal adhesions consist of integrins and cytoplasmic proteins such as vinculin and talin. Filipodia are thin, finger-like cytoplasmic extensions that contain tight actin bundles and may be involved in the recognition of the extracellular environment.

Rho GTPases were first implicated in controlling the actin cytoskeleton when it was observed that treatment of cultured Vero cells with the Rho-inhibitor, C3-transferase (See Section 1.5 for details) caused cell rounding and dissolution of actin fibres (Chardin *et al.*, 1989). Since this initial observation, Cdc42, Rac, and Rho have all been shown to play essential roles in the assembly and organisation of the actin cytoskeleton and have been placed in a hierarchical cascade in which Cdc42 activates Rac which in turn, activates Rho (Ridley *et al.*, 1992; Nobes and Hall, 1995) (Figure 1.4). In addition, Ras has been shown to activate Rac (Ridley *et al.*, 1992), although the molecular link between these small GTPases remains unclear.

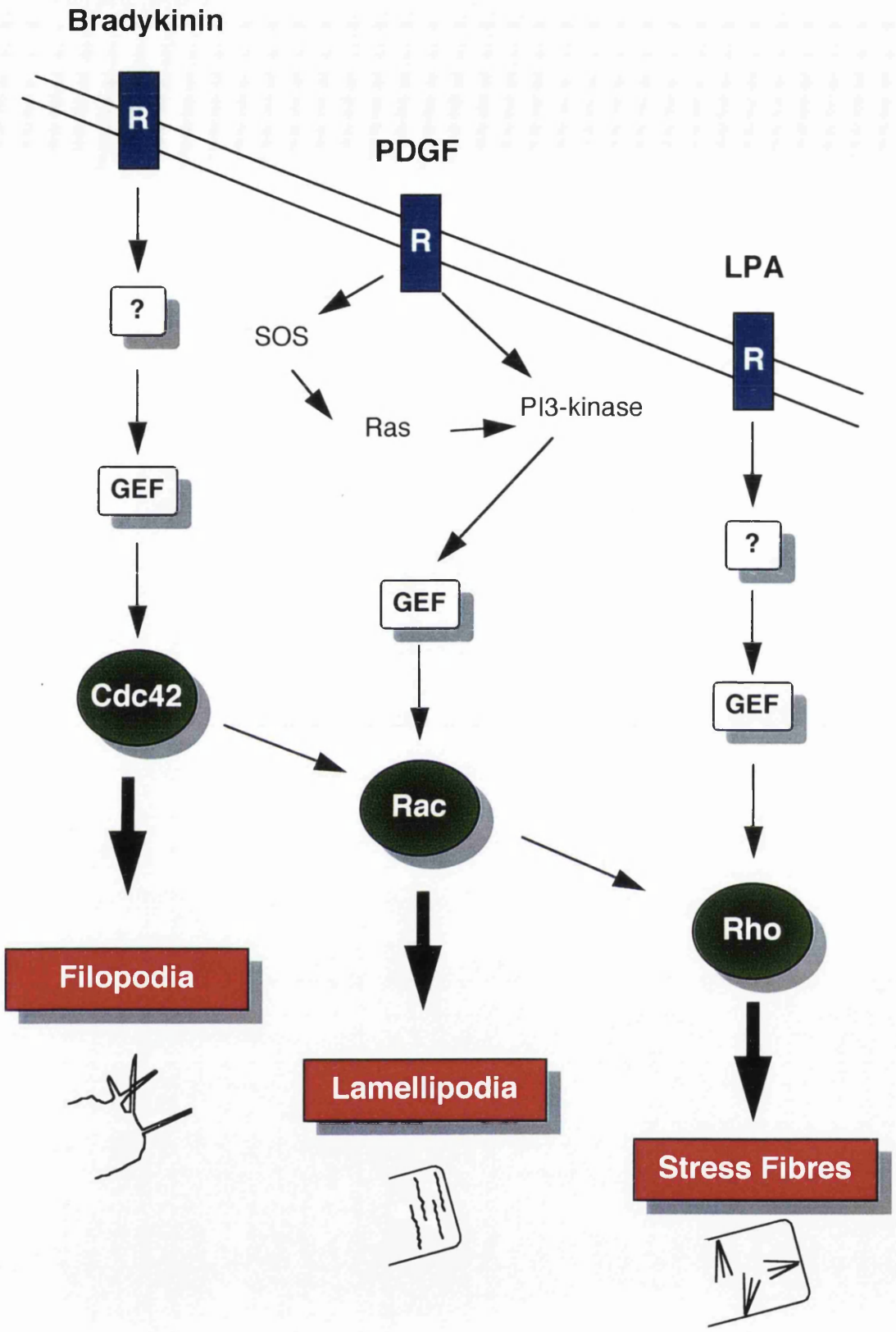


Figure 1.4 - The Regulatory inputs and cross-talk of Rho-family GTPases in Fibroblasts

Various extracellular stimuli trigger the activation of Cdc42, Rac, and Rho and elicit responses such as the formation of filapodia, lamellipodia and stress fibres. Cdc42 is able to activate Rac which, in turn is able to activate Rho, although the molecular mechanism of this cross-talk remains to be elucidated.

In fibroblasts, extracellular stimuli activate the Rho GTPase cascade at different points. For example, addition of LPA to quiescent fibroblasts is able to induce the formation of stress fibres; a response that is completely blocked by microinjection of the specific Rho-inhibitor, C3-transferase (Ridley and Hall, 1992). Moreover, stimulation of cells with growth factors such as PDGF, cause membrane ruffling and lamellipodia formation in addition to actin stress fibres (Ridley *et al.*, 1992; Nobes and Hall, 1995). Ruffling in response to growth factor stimulation can be inhibited by microinjection of dominant negative Rac mutants, thereby establishing a connection between growth factor receptors, membrane ruffling and actin polymerisation (Ridley *et al.*, 1992). Furthermore, activation of Cdc42 by bradykinin results in the formation of filopodia and the subsequent formation of lamellipodia (Kozma *et al.*, 1995). Filopodia formation can be inhibited by dominant negative Cdc42 mutants whereas dominant negative Rac inhibits only membrane ruffling (Kozma *et al.*, 1995; Nobes and Hall, 1995).

Insight into the molecular pathway connecting Rho and the actin cytoskeleton has come from the identification and characterisation of the Rho interacting protein, Rho-Kinase (ROKα). Leung *et al.* first observed that microinjection of full-length ROKα promoted formation of actin stress fibres and focal adhesions, consistent with it being an effector for RhoA (Leung *et al.*, 1996a). Kinase activity, but not the Rho-binding domain was required for this response. Furthermore, it has been demonstrated that although a kinase-negative Rho-binding defective mutant blocked Rho-induced formation of stress fibres and focal adhesions in HeLa cells, it was still capable of eliciting an enhancement of actin polymerisation. These results suggest that the pathways leading to actin polymerisation and formation of stress fibres and focal adhesions may be mediated by distinct effectors (Ishizaki *et al.*, 1997). Reports that the myosin binding subunit (MBS) of myosin light chain (MLC) was the substrate for Rho-kinase provided greater understanding of the Rho/actin cytoskeleton link (Kimura *et al.*, 1996; Matsui *et al.*, 1996). Phosphorylation of MBS leads to a decrease in MLC phosphatase activity, causing an accumulation of the phosphorylated form of MLC (Kimura *et al.*, 1996). Phosphorylated MLC can induce a conformational change in myosin, thereby increasing its binding to actin filaments and subsequently the formation of stress fibres (Chrzanowska-Wodnicka and Burridge, 1996). Furthermore, Rho-kinase has also been reported to phosphorylate MLC,

thus increasing the pool of phosphorylated MLC (Amano *et al.*, 1996a). The cascade shown in Figure 1.5 may be one way in which Rho can regulate changes in the cytoskeleton.

Through the action of Rho-kinase, Rho appears to be able to induce the formation of contractile stress fibres by the reorganisation of existing actin filaments. However, Rho is also involved in the process of actin assembly from free actin monomers; a process distinct from that described in figure 1.5 (Norman *et al.*, 1994; Hall, 1998). One possible mechanism connecting Rho and actin polymerisation is through the stimulation of phosphatidylinositol 4-phosphate 5-kinase (PIP5-K). Although reports of Rho binding to PIP5-K directly are conflicting (Chong *et al.*, 1994; Tolias *et al.*, 1995; Ren *et al.*, 1996), Rho (and Rac) have been shown to stimulate the synthesise of phosphatidylinositol bisphosphate (PIP_2) through the action of PIP5-K (Chong *et al.*, 1994; Hartwig *et al.*, 1995). The observation that PIP_2 binds to and potentially regulates the function of many actin-associated proteins, led to the proposal that Rho-mediated PIP_2 production may induce actin polymerisation. Further evidence for Rho-mediated PIP_2 regulation of the cytoskeleton was provided by Gilmore *et al.*, who showed that injection of anti-PIP_2 antibodies into fibroblasts inhibited LPA/Rho-induced stress fibre formation (Gilmore and Burridge, 1996). An additional molecule implicated in the control of actin assembly is mDia-1. mDia-1 is reported to bind to Rho in a GTP-dependent manner (Watanabe *et al.*, 1997; Chapter 7) and is thought to be involved in the recruitment of profilin to the plasma membrane. Here, profilin, possibly in co-operation with PIP_2, acts to catalyse the polymerisation of actin from the free actin pool (Watanabe *et al.*, 1997; Watanabe *et al.*, 1999).

The control of the cytoskeleton by the Rho GTPases is a complex and dynamic process involving many effectors. Rho itself is proposed to be involved in both the formation of actin stress fibres through the action of PIP5-K and mDia as well as their contraction via Rho-kinase. However, Rho is also involved in focal adhesion formation; a process that can be prevented by treatment with tyrosine kinase inhibitors (Chrzanowska-Wodnicka and Burridge, 1996), and shown to involve the phosphorylation of several focal adhesion proteins including p125FAK (focal adhesion kinase), p130 and paxillin (Flinn and Ridley, 1996). It is currently unclear whether stress fibre and focal adhesion formation are separate or interdependent events although there are several lines

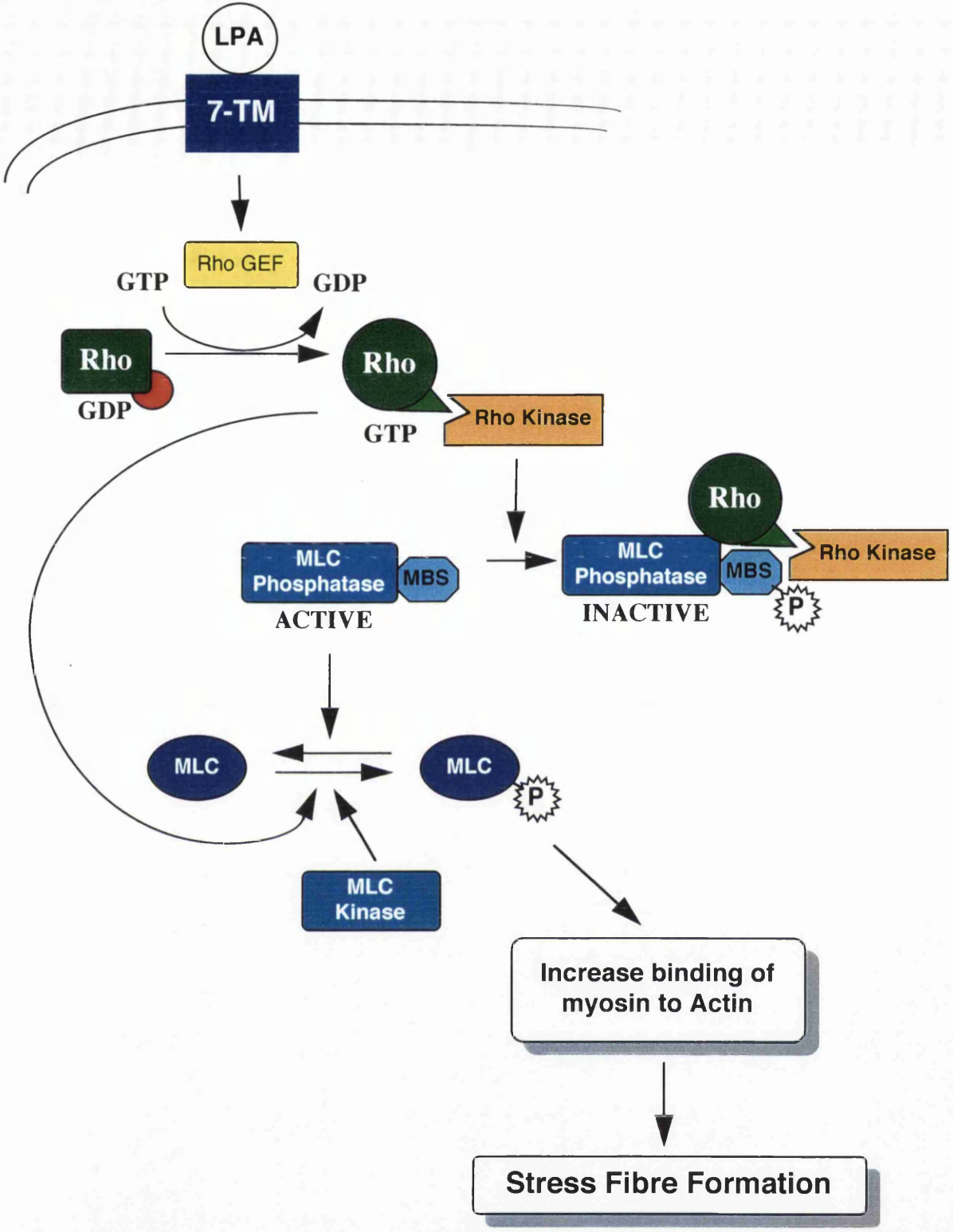


Figure 1.5 - A Putative Molecular Mechanism for Rho-induced Stress Fibre Formation.

Extracellular factors (LPA) bind to 7-transmembrane receptors (7-TM) and cause Rho activation via increased GEF activity. Active Rho binds to and activates Rho kinase, which in turn phosphorylates the myosin binding subunit (MBS) of myosin light chain phosphatase, inactivating it. Once active, Rho kinase also directly phosphorylates Myosin light chain (MLC) resulting in increased binding of myosin to actin and stress fibre formation.

of evidence to suggest that the latter is the case (Flinn and Ridley, 1996; Chihara *et al.*, 1997).

Although the molecular basis for Rho-mediated regulation of the cytoskeleton is beginning to be understood in some detail, until recently, little was known about the link between Cdc42, Rac and actin reorganisation. One Cdc42 interacting protein is WASP, the Wiskott-Aldrich syndrome protein and may provide a link between Cdc42 and the actin cytoskeleton. WASP expression is restricted to haemopoietic cells and binds to Cdc42 in a GTP dependent manner (Symons *et al.*, 1996). Moreover, expression of WASP in rat kidney epithelial cells or the Jurkat T cell line induces actin cluster formation; a response that cannot be prevented by co-injection of a dominant negative Rac or Rho, but is ablated by dominant negative Cdc42 (Aspenstrom *et al.*, 1996; Symons *et al.*, 1996). It is now known that WASP is one member of a family of WASP-related proteins including N-WASP, which shares 50% homology with WASP itself and is expressed in the brain (Fukuoka *et al.*, 1997). Other WASP-family members include Scar1/WAVE, WAVE2 and WAVE3 (Bear *et al.*, 1998; Miki *et al.*, 1998; Suetsugu *et al.*, 1999). Interestingly, WAVE appears to mediate Rac-induced changes in the actin cytoskeleton as a dominant negative WAVE mutant can suppress Rac induced membrane ruffling, but not Cdc42-induced microspike formation (Miki *et al.*, 1998). Moreover, N-WASP and the WAVE isoforms have been shown to link Rac and Cdc42 to the Arp2/3 complex (Rohatgi *et al.*, 1999; Suetsugu *et al.*, 1999). Insight into the function of the Arp2/3 complex has come from genetic studies with *S.cerevisiae* and also from biochemical studies using *in vitro* assays. In yeast, the Arp2/3 complex is required for assembly and/or organisation of actin patches and for endocytosis (Moreau *et al.*, 1997; Moreau *et al.*, 1996). *In vitro*, the Arp2/3 complex nucleates new actin filaments (Mullins *et al.*, 1998). Cdc42 and Rac have thus been suggested to stimulate actin polymerisation through regulation of the nucleation activity of the Arp2/3 complex (Ma *et al.*, 1998).

Another protein shown to be involved in Rac-mediated changes in the actin cytoskeleton is POR1 (Van Aelst *et al.*, 1996). Isolated from a yeast-two hybrid screen, POR1 interacts specifically with GTP-bound Rac. Deletion mutants of POR1 were able to inhibit active Rac induced membrane ruffling while wild-type POR1 synergistically co-operated with active Rac.

1.2.2 - Regulation of Gene transcription

As well as regulating the actin cytoskeleton, there is considerable data implicating Rho family GTPases in receptor-mediated control of gene transcription. Gene transcription involves the transmission of extracellular signals to the nucleus and over the past decade, has been shown to involve the sequential activation of several cytoplasmic protein kinases collectively known as mitogen-activated protein kinase (MAPK) cascade. One classical MAPK cascade leading to transcriptional regulation is that involving receptor mediated control of Extracellular-signal-regulated kinase (ERK). A well-documented role for the MAP kinase ERK-2 is in the phosphorylation and regulation of Elk-1 (Gille *et al.*, 1995), one of a family of proteins that can form a ternary complex with the transcriptional-activator serum response factor (SRF) and can thus play a key role in regulation of the serum response element (SRE) found in the promoter enhancer region of many growth factor regulated genes (Treisman, 1990).

Following the discovery that Ras was involved in activation of ERKs (de Vries-Smits *et al.*, 1992), many studies have explored the role of the Rho GTPases in MAPK activation. MAP kinase cascades with the potential to be regulated by Rho family GTPases are the JNK (Jun N-terminal Kinase) and the reactivating kinase p38 pathways. These MAPK pathways lead to the activation of JNK and p38 which then translocate to the nucleus and phosphorylate transcription factors (Seger and Krebs, 1995). Expression of constitutively active Rac and Cdc42 mutants have been shown to activate JNK and p38 in a number of cells (Coso *et al.*, 1995; Minden *et al.*, 1995). JNK and p38 are strongly activated by inflammatory cytokines, tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), and a diverse array of cellular stresses such as heat shock, UV and ionising radiation (Kyriakis and Avruch, 1996).

The identity of the molecules that link Rac and Cdc42 to JNK are not fully understood. One family of serine-threonine kinases originally proposed to play a role in JNK and p38 activation via Rac and Cdc42 are the PAKs. This family of kinases have been shown to become activated upon binding to either GTP-bound Rac or Cdc42 (Manser *et al.*, 1995). However, although certain constitutively active forms of PAK have been reported to stimulate the activity of JNK and p38 (Bagrodia *et al.*, 1995), others did not report an increase in JNK activity following PAK and active Rac or Cdc42 expression

(Teramoto *et al.*, 1996). There are also conflicting reports as to whether effector mutants of Rac, which fail to bind PAK, are able to activate JNK (Lamarche *et al.*, 1997; Westwick *et al.*, 1997). It has been suggested that other kinases, such as the Rac and Cdc42 interacting Mixed Lineage Kinase (MLK)-3 and MAP/ERK Kinase Kinase (MEKK)-4 selectively activate the JNK and p38 pathways (Gallo *et al.*, 1994; Teramoto *et al.*, 1996; Tibbles *et al.*, 1996).

Although Rac and Cdc42 are clearly established as regulators of the JNK pathway, interestingly, Rho itself has only been demonstrated to activate JNK in one cell type (Teramoto *et al.*, 1996). More recent evidence of a Rho involvement in the activation of JNK comes from the observation that the DH domain containing GEF mNET1 is able to activate the JNK pathway through a C3-transferase sensitive component (Alberts *et al.*, 1998a).

Rho and its family members have also been implicated in the regulation of the ERK pathway through cross-talk with the JNK pathway (Frost *et al.*, 1997). In a study which investigated cross-cascade activation of ERKs and ternary complex factors (TCFs) by Rho family proteins, it was shown that small GTPases such as Rac-1 and Cdc42hs, which can activate the JNK pathway, are able to co-operate with Raf-1 in the activation of ERK. Furthermore, examination of the ERK pathway kinases reveals that the MEKs are a focal point for cross-cascade regulation. Rho family proteins, signalling through either Rac or Cdc42hs, it was suggested, are most likely to contribute to MEK activation through the stimulation of the Rac effector protein kinase, PAK. However, since interfering forms of Rac and Cdc42hs each inhibit ERK activation by RhoA and Raf-1, while interfering mutants of RhoA does not block Rac or Cdc42hs-mediated co-operation with Raf-1, they suggest that RhoA uses endogenous Rac or Cdc42hs to stimulate ERK activity.

Rho family GTPases have also been implicated in transcriptional regulation through activation of the transcription factor, SRF (Hill *et al.*, 1995). SRF complexes with ternary complex factors such as Elk-1 to stimulate transcription of genes containing serum response elements in their promoter enhancer regions (Treisman, 1990; Treisman, 1994). Rho was shown to be required for LPA, serum and aluminium fluoride ion (AlF_4) induced transcriptional activation of SRF. The activation of SRF by Cdc42 and Rac however could not be prevented by C3-transferase expression and thus does not require Rho function,

suggesting that at least two signalling pathways controlled by Rho family GTPases lead to SRF activation. The identity of these signalling pathways remains unclear and neither JNK nor p38 are sufficient to mediate SRF activation alone (Hill *et al.*, 1995). Furthermore, as Ras has been shown to activate TCF via the Raf-MAPK pathway (Treisman, 1994) and activation of TCF and SRF act synergistically to induce the SRE-containing promoter region of c-fos (Hill *et al.*, 1995), the SRE may act as a convergence point for Ras and Rho dependent signals.

The role of Rho GTPases in the regulation of SRF has been further explored by Alberts *et al.* (Alberts *et al.*, 1998b). In their report they demonstrate that although active Rho is sufficient to activate extrachromosomal SRF reporter genes, active Rho mutants are insufficient to activate either chromosomal c-fos, or chromosomally-integrated SRF-reporter genes unless additional signalling pathways are triggered. These co-operating signals may be either JNK dependent or independent and can be substituted by treatment of cells with deacetylase inhibitors. Furthermore, they show that one of the targets for the co-operating signals is the hyperacetylation of histone H4, and thus demonstrate that chromatin remodelling is a prerequisite for SRF activation.

An additional mechanism through which Rho GTPases are reported to regulate transcription is via activation of the transcription factor NFκB (Nuclear Factor κB) (Perona *et al.*, 1997). NFκB plays an important role in immune function and inflammation and is activated by extracellular stimuli such as TNFα and UV irradiation (Baldwin, 1996). Active mutants of RhoA, Cdc42, and Rac1 all efficiently induce the transcriptional activity of NFκB by a mechanism that involves phosphorylation of the inhibitory subunit IκB. Interestingly, the activation of NFκB by TNFα depends upon Cdc42 and Rho, but not Rac; activation of NFκB by UV light is independent of Rac, Rho and Cdc42 (Perona *et al.*, 1997).

Rho family GTPases play an important role in the process of transcriptional regulation. However, what emerges from comparisons of cytoskeletal and transcriptional control by Rho GTPases is that a linear cascade of Cdc42→Rac→Rho is not conserved in all systems. Moreover, a recent study by Sahai *et al.* showed with the use of a panel of Rho effector mutants, each able to bind only a subset of downstream effectors, that the processes of cytoskeletal rearrangement and transcriptional activation are mediated by different effector pathways (Sahai *et al.*, 1998). Although the effector pathways leading

to changes in the actin cytoskeleton are beginning to be understood in some detail, much more work is required to establish the exact mechanism by which Rho family GTPases control gene transcription.

1.2.3 - Rho in Cytokinesis

Cytokinesis, the process of cytoplasmic division of cells during mitosis, is mediated by the contraction of an actin 'contractile ring' formed in the cleavage furrow in the middle of the cell body. The Rho family GTPases were first implicated in cytokinesis when it was noted that incubation of cultured cells with the Rho inhibitor, C3-transferase led to the appearance of multinucleated cells (Rubin *et al.*, 1988). The role of Rho GTPases in cytokinesis was further explored by microinjection of either C3 or Rho-GDI into fertilised Xenopus and sea urchin eggs (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993). After injection, cytokinesis following nuclear division did not occur and ongoing cytokinesis was aborted. Nuclear division however continued, resulting in multinucleated cells. These finding indicate that the small GTPase Rho plays a critical role in cytokinesis, receiving a signal of completion of nuclear division to induce and maintain cytokinesis; in the absence of Rho function, nuclear division is uncoupled from cell division. Two recent studies have shown that Rho and its effectors Rho-Kinase (Kosako *et al.*, 1999) and the related Citron-Kinase (Madaule *et al.*, 1998) accumulate in the cleavage furrow during cell division. Moreover, expression of a kinase-active mutant of Citron-kinase results in abnormal contraction of the contractile ring during cytokinesis. Thus, it is proposed that contraction of the actin contractile ring is mediated by Rho through the effector Citron-Kinase.

A role of another Rho family GTPase, Cdc42 in cytokinesis has been investigated by Drechsel *et al.*; by microinjection of mutant Cdc42 into Xenopus embryos, they suggest a requirement for Cdc42 (Drechsel *et al.*, 1997). Although the downstream effector by which Cdc42 mediates its effect on cytokinesis was not investigated in this report, analysis of cytokinesis in *S.cerevisiae* has implicated the PAK homolog Cla4p as playing an essential role downstream of Cdc42 in this process (Benton *et al.*, 1997). Whether mammalian PAKs play a role in cytokinesis remains to be investigated.

1.2.4 - Cell Growth and Proliferation

Rho family proteins were first implicated in the regulation of cell growth by the observation that expression of RhoB is regulated by growth factor stimulation (Jahner and Hunter, 1991). In addition to its expression being regulated by receptor tyrosine kinase activation, RhoB was also induced by non-receptor tyrosine kinases, UV irradiation, and by DNA damaging agents (Fritz *et al.*, 1995). In addition, RhoG expression was also found to be growth inducible (Vincent *et al.*, 1992), while Rac2 expression was induced in T cells following phytohemagglutinin A (PHA) growth stimulation (Reibel *et al.*, 1991).

Rho GTPases have also been shown to be involved in cell cycle progression. Inhibition of Rho function by C3-transferase causes serum-stimulated Swiss 3T3 cells to accumulate in the G1 phase of the cell cycle (Yamamoto *et al.*, 1993). This was found to be due to a decreased rate of G1/S progression in the absence of Rho function. Conversely, microinjection of active mutants of Rho, Rac, and Cdc42 are able to cause quiescent fibroblasts to enter into cell cycle in the absence of serum (Olson *et al.*, 1995).

Further evidence for a connection between the control of cell growth and division and Rho family GTPases is their involvement in cellular transformation. The transforming activities associated with many Dbl family proteins provides evidence that upregulation of Rho family GTPases can cause growth deregulation. *Dbl*, the first such oncogene to be identified was isolated from a human diffuse B-cell lymphoma by Eva *et al.* in 1985 (Eva and Aaronson, 1985) and later *proto-dbl* was shown to act as an exchange factor for Cdc42 (Hart *et al.*, 1991). Many other dbl-domain containing proteins have been found subsequently, including *ost.* First isolated from an osteosarcoma, *ost* has been shown to catalyse guanine nucleotide exchange for both RhoA and Cdc42 *in vitro* (Horii *et al.*, 1994). An exchange factor of particular interest is Ibc. Oncogenic *Ibc* was first identified from a screen to identify transforming genes involved in leukaemias (Toksoz and Williams, 1994) and has been reported to have guanine nucleotide exchange activity specific to RhoA *in vitro*. Furthermore, microinjection of oncogenic Ibc is able to induce stress fibres indistinguishable from those induced by active Rho (Zheng *et al.*, 1995b). Other dbl-containing oncogenes reported to have nucleotide exchange activity towards Rho family GTPases include Tiam-1, an exchange factor for Rac and Cdc42 (Van Leeuwen *et al.*, 1995), Vav (Adams *et al.*, 1992), and Ect-2 (Miki *et al.*, 1993). The existence of these

oncogenes, all with exchange activity for Rho family GTPases, implicates de-regulation of Rho, Rac and Cdc42 activity in the process of oncogenesis.

The role of Rho family GTPases is transformation is further strengthened by the early observations that Ras-transformed cells undergo changes to their cytoskeleton including loss of stress fibres, and decrease substratum adhesion and increased membrane ruffling and motility (Bar-Sagi and Feramisco, 1986). Subsequently, the Rho family GTPases RhoA, RhoB, Rac1, Cdc42, and RhoG have all been implicated in Ras-mediated transformation (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995b; Qiu *et al.*, 1995a; Qiu *et al.*, 1995b; Roux *et al.*, 1997; Qiu *et al.*, 1997). Expression of dominant negative Rho family GTPase mutants are all able to attenutate the focus-forming activity of oncogenic Ras in rodent fibroblasts to some extent. Although active mutants of the Ras-effector Raf-1 are able to induce transformation, Ras-mediated transformation is thought to involve downstream effectors other than Raf (Marshall, 1996). Support for this view is provided by the observation that although constitutively active versions of Rho GTPases could synergise with active Raf-1 mutants to transform NIH3T3 or Rat1 rodent fibroblasts, dominant negative Rac1 and RhoG failed to block Raf mediated transformation (Roux *et al.*, 1997). Thus, Rho family GTPases may act downstream of Ras, but independently from Raf-1 in mediating cellular transformation.

This function of Rho in cellular transformation has recently been reported to be mediated by the Rho effector ROCK-1, a serine/threonine kinase (Sahai *et al.*, 1999). ROCK-1 also participates in Ras mediated transformation *in vitro* although interestingly, it is clear that not all cell transformation is dependent on ROCK-1 function; inhibitors of this kinase block Ras mediated transformation but not transformation induced by expression of v-src (Sahai *et al.*, 1999), thus suggesting that v-src transformation is Rho-independent.

1.2.5 - Rho and Cell Growth: A Molecular Basis?

The molecular mechanism through which Rho family members regulate cell growth is uncertain. One recent report has suggested a role for Rho in Ras-regulated cell proliferation (Olson *et al.*, 1998). In this model, they propose that Ras activates the ser/thr kinase Raf, sending positive signals down a kinase cascade and promoting G1/S progression. Excessive signalling from Ras induces the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, blocking entry into cell cycle; Rho overcomes this cell cycle block by suppressing expression of $p21^{waf1/cip1}$ (Figure 1.6).

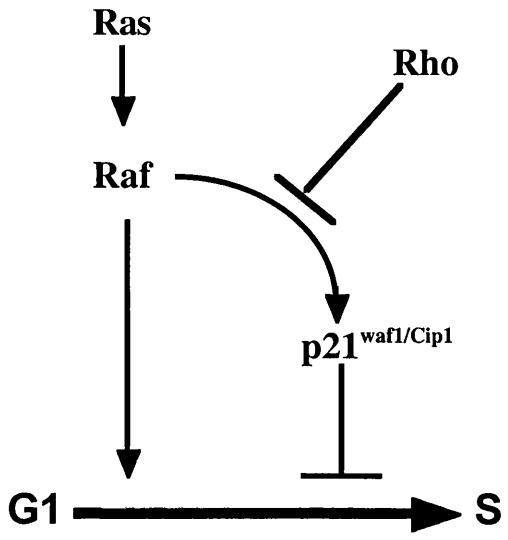


Figure 1.6 - Regulation of Ras-dependent DNA synthesis by Rho

The proposed model may offer a possible explanation for the synergistic action of activated Rho in Ras- and Raf- mediated transformation; activation of Rho may suppress the signals acting through $p21^{waf1/cip1}$ to suppress cell cycle progression, thus eliminating one level of growth control. Deregulation of Dbl-family exchange factors may have a similar effect, up-regulating Rho activity and removing $p21^{waf1/cip1}$ control of cell proliferation, contributing to transformation. Whether $p21^{waf1/cip1}$ expression is downregulated in Dbl-transformed cells remains to be determined.

In summary, Rho family GTPases have been shown to regulate a diverse range of cellular processes including rearrangement of the actin cytoskeleton, gene transcription, cytokinesis, and cell growth and transformation. These roles have been elucidated in a number of different cellular systems and it is not clear whether Rho family GTPases control all of these processes in all cell types. Once the molecular basis for the Rho family-mediated events are understood, analysis of the expression patterns of the Rho

family GTPases and their effector molecules will help to establish the cell-type specificity of these processes.

1.3 - The Immune System

1.3.1 - The Battle against the Environment

The life of every organism is constantly threatened by the other organisms it encounters every day. In response, each species has evolved protective systems ranging from camouflage to toxins. In response to their continual battle against micro-organisms, vertebrates have evolved an elaborate set of protective measures, collectively known as the immune system.

The process central to the immune system is that of recognition. The system must be able to distinguish between foreign organisms and the natural constitutes of the body; failure to recognise a potential threat would result in a chronic life-threatening condition, while inappropriate recognition would unleash the power of the immune system in the destruction of the body's own cells. Hence, the immune system must be carefully regulated. Central to the processes of both recognition and regulation are the lymphocytes.

1.3.2 - Role of the T Lymphocyte

The role of the T lymphocyte or T cell, is to recognise intracellular pathogens and to initiate an immune response. Instead of bearing several receptors recognising a conserved surface molecule of a pathogen, each naive lymphocyte bears receptors of only a single specificity. The specificity and diversity of these receptors occurs during T cell development in the thymus where through a process of germ-line gene rearrangements, hundreds of different variants of the genes encoding the receptor molecules are generated. The receptors present on the surface of the T cell are able to recognise short peptide sequences derived from pathogens, known as antigens; however, recognition can only occur when the antigen is 'presented' to the T cell by specialised antigen-presenting cells (APCs). These specialised cells are able to process

pathogens and present short peptide sequences on their surface in a complex called the Major Histocompatability Complex (MHC). The APCs also have several other surface receptors that provide costimulatory signals to the T cells.

Upon interaction of the T cell antigen receptor (TCR) with antigen in the context of MHC, along with the necessary costimulation, a sequence of intracellular events are initiated leading to the secretion of cytokines and G_o to G_1 transition; this is the process of T cell activation. During this process, over 60 different gene products become expressed in T cells (Ullman *et al.*, 1990; Kelly and Siebenlist, 1995). Among these molecules are the lymphokine interleukin-2 (IL-2), its receptor (IL-2R), and other cytokines such as Interferon-γ, transforming growth factor-β, tumour necrosis factor-β, IL-3, IL-6, IL-9, IL-11, IL-13 and IL-15 (Crabtree and Clipstone, 1994). In addition to the secretion of cytokines, activation involves clonal expansion of the activated T cell. However, ligation of the TCR complex and its coreceptors does not initiate G_o to G_1 transition; these signals are provided by the cytokine IL-2 (Cantrell and Smith, 1984). Upon activation, a range of cytokines are released from the T cell including IL-2. In addition, the activated T cell expresses an IL-2 receptor and it is ligation of this receptor by the cytokine IL-2 that initiates entry into cell cycle and the subsequent clonal expansion. The secreted cytokines also mediates an inflammatory response, recruiting of other cells of the immune system to the area of infection. Hence the role of the T cell is both recognition of pathogens via its antigen receptor co-ordination of the immune response through secretion of cytokines.

1.3.3 - Regulation of T Cell Development

All cellular components of the blood originate from hematopoietic stem cells in the bone marrow. These pluripotent cells divide to produce two more specialised types of stem cells, a common lymphoid progenitor which gives rise to T, B and NK cells, and a myeloid stem cell, which gives rise to leukocytes and erythrocytes (Reviewed in Shortman and Wu, 1996).

The intricate process of lymphocyte development involves the homing of progenitor cells to an appropriate microenvironment, commitment to a specific lineage, and the differentiation of progenitor cells along defined lineage pathways. Although T and B cells

originate from the same progenitor cell in the bone marrow, only the B cells remain and develop there: common lymphoid progenitor cells destined to become T cells migrate to the thymus where they undergo proliferative expansion, rearrangement of their T cell receptor chains and positive and negative selection. These thymic development events ensure sufficient cells with the appropriate immune repertoire exit to the periphery.

Different developmental stages of thymocytes can be distinguished by the differential expression of two surface receptors, CD4 and CD8, the coreceptors for MHC class II and class I respectively. Immature T-cell progenitors are found within the $CD4^-8^-$ double-negative (DN) compartment which comprises about 3% of the total thymocyte population. These progenitors can be further subdivided into four developmental stages on the basis of expression of CD44 and CD25. Earliest progenitors not yet fully committed to the T-cell lineage are $CD44^+25^-$ followed sequentially by the $CD44^+25^+$, $CD44^-25^+$, and $CD44^-25^-$ stages (Godfrey *et al.*, 1993; Godfrey and Zlotnik, 1993). Pre T-cells at the $CD44^-25^+$ stage that have successfully rearranged their TCR β-locus and express a functional receptor complex known as the pre-TCR proliferate rapidly, down-regulate CD25 expression and differentiate into $CD4^+8^+$ double positive (DP) cells (Fehling *et al.*, 1995). Allelic exclusion, the process which ensures that CD4/8 DP cells express a single productively rearranged β chain is also initiated by the pre-TCR. DP thymocytes then undergo TCR α-locus rearrangement and are subjected to positive and negative selection processes upon expression of a mature and functional αβTCR complex (von Boehmer, 1992).

The processes of TCR gene rearrangement, cell death and proliferation are all dependent upon signals initiated by antigen and cytokine receptors (DiSanto and Rodewald, 1998; von Boehmer *et al.*, 1999). Considerable insight into the control of thymocyte development by these receptors has been gained by the analysis of naturally occurring mouse mutants, in addition to new mutant mice generated by targeted gene disruption. Such analyses also define the branch-points and cytokine-dependency of other lymphoid cells within the thymus such as NK cells and T-cells bearing γδ T cell receptors. The following sections describe the progress that has been made in understanding the roles played by the growth factors and surface receptors, and the insight this has provided into the regulation of thymocyte development.

c-kit/SCF

The Stem Cell Factor (SCF) tyrosine kinase receptor, c-kit is expressed on the earliest thymocyte progenitors detectable in the thymus, the $CD44^{+}25^{-}$ cells. Expression is maintained on $CD44^{+}25^{+}$ cells, but is lost on subsequent $CD44^{-}25^{+}$ cells (Moore and Zlotnik, 1995). Thus c-kit appears to be expressed up until the point where cells start to rearrange their β chains. Mutations in c-kit or SCF genes affect the development of several cell lineages and consequently, c-kit knockout mice die 10 days after birth. Similarly homozygous loss of SCF is embryonic lethal (Fleischman, 1993; Galli *et al.*, 1994). Analysis of the thymic phenotype of c-kit null mice has revealed that in addition to the general hematopoietic effects resulting in early death, SCF/c-kit plays an important role in early T cell development *in vivo*. In c-kit null mice, the earliest thymocyte progenitor population in the thymus ($CD44^{+}25^{-}$) is reduced by approximately 40 fold (Rodewald *et al.*, 1995). Much work has done to establish the exact cause behind the depletion of the first progenitor population in the absence of c-kit mediated signalling. *In vitro* experiments from other cell systems has suggested that c-kit/SCF signals can act in an anti-apoptotic manner (Brandt *et al.*, 1994; Carson *et al.*, 1994; Iemura *et al.*, 1994), and thus it seems likely the c-kit mediates signals that ensure the continued survival of the early thymocyte progenitors on which it is expressed.

An additional receptor-ligand pair has also been suggested to regulate thymopoiesis at this early stage. flt-3, a member of the receptor tyrosine kinase family is structurally similar to c-kit; furthermore, its expression is restricted to uncommitted hematopoietic progenitor cells and early lymphoid precursor cells (Matthews *et al.*, 1991; Rosnet *et al.*, 1991). *In vitro* experiments have shown that the flt-3 ligand, FL can induce proliferation of the earliest thymic progenitors, the $CD44^{+}25^{-}$ cells, but has no effect on the subsequent $CD44^{+}25^{+}$ or $CD44^{-}25^{+}$ cells (Moore and Zlotnik, 1997). However, targeted disruption of the flt-3 gene alone does not cause any alteration in thymus phenotype or cell numbers, although mice lacking expression of both flt-3 and c-kit display defects in stem cell populations (Mackarehtschian *et al.*, 1995). These results suggest that although flt-3 may be important for the expansion and/or differentiation of the very early common lymphoid progenitors, some functional redundancy between flt-3 and c-kit may exist.

IL-7R and common γ chain containing receptors

Mice that fail to produce or secrete the cytokine IL-7 show a severe reduction in the numbers of both pro-B and pro-T cells and reduced differentiation of these cells to subsequent stages (Von Freeden-Jeffry *et al.*, 1995). The main intrathymic block in these mice is from the $CD44^+25^-$ stage to the $CD44^+25^+$ stage where T cell precursors are normally actively proliferating. The few αβ T cells that get beyond this stage are able to develop further in the absence of IL-7; however, γδ T cell development is completely prevented (Moore *et al.*, 1996). Similarly, mice lacking expression of the IL-7 Receptor (IL-7R) α chain also have reduced numbers of thymocytes and lack γδ T cells (He and Malek, 1996; Maki *et al.*, 1996). However, in contrast to IL-7 -/- mice, a large proportion of IL-7Rα -/- mice have a more complete block in T cell development with the thymus being composed solely of CD4/8 DN cells. Although the reason for this variability is unclear, the IL-7α knockout mice further strengthen the claim that IL-7 signals are important for the survival and/or proliferation of early thymocyte progenitors and essential for the development of γδ T cells.

All the features of the previous two mice described are seen in the common gamma chain (γ_c) deficient mice, a component not only of the IL-7R, but also of the cytokine receptors for IL-2, IL-4, IL-9 and IL-15 (Cao *et al.*, 1995; DiSanto *et al.*, 1995; Ohbo *et al.*, 1996). The similarity of the phenotypes between the γ_c -/- and the other IL-7 mutant mice suggests that other signals dependent on the γ_c (i.e. IL-2, IL-4, IL-9 and IL-15 (Sugamura *et al.*, 1996)) are not required at this stage of αβ T cell development. However, absence of the γ_c does result in the absence of NK and NK-T cells and leads to the hypothesis that one or more of IL-2, -4, -9 or -15 are essential for the development of these cells (DiSanto *et al.*, 1996; Lantz *et al.*, 1997). (Analysis of IL-2R β chain null mice has recently implicated the IL-2/IL-15 pathways in the development of NK and NK-T cells (Suzuki *et al.*, 1997)).

One of the signalling molecules known to function downstream of the γ_c is the tyrosine kinase JAK3. Strikingly, mice lacking expression of JAK3 show similar defects in thymocyte development to those deleted for the γ_c including severely reduced numbers of T and B cells and the absence of NK cells (Park *et al.*, 1995).

Analysis of IL-7 and γ_c mice has revealed that $CD44^+25^+$ cells have drastically reduced levels of the survival factor Bcl-2 in the absence of IL-7R mediated signals. Furthermore, culturing DN thymocytes from an IL-7-/- mice in the presence of IL-7 results in Bcl-2 upregulation (von Freeden-Jeffry *et al.*, 1997). These findings are further substantiated by experiments in which expressing the Bcl-2 transgene was found to partially restore αβ T cell development in IL-7Rα and γ_c -/- mice (Akashi *et al.*, 1997; Kondo *et al.*, 1997). It is therefore seems reasonable to assume that IL-7 signalling results in upregulation of Bcl-2, leading to continued survival at the $CD44^+25^+$ stage.

To address the issue of proliferation in mice defective in IL-7 signalling, a detailed study was performed analysing the cell-cycle status of each of the thymocyte progenitor populations in wild type and IL-7 -/- mice (von Freeden-Jeffry *et al.*, 1997). This revealed that the fraction of cycling $CD44^+25^-$ cells was comparable to wild type. However, in subsequent populations, the fraction of cycling cells decreased progressively in the absence of IL-7. These experiments highlight a role for IL-7 signalling in both thymocyte survival and proliferation.

Pre-T cell Receptor

Analysis of severe-combined immunodeficient (SCID) naturally occurring mouse mutant and those deficient for the recombinase-activating genes RAG-1 and RAG-2 reveal an essential requirement for gene rearrangement at the $CD44^-25^+$ stage of thymocyte development; an inability to mediate gene rearrangement results in a block in thymocyte differentiation at the $CD44^-25^+$ stage (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992; Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995). Defects in thymocyte development due to failure to rearrange could be overcome by expression of a functional TCRβ chain (Shinkai *et al.*, 1993). Expression of TCRα chains at this stage were not able to mediate this process and thus revealed that TCR β chain and not α chain rearrangement was rate limiting at the $CD44^-25^+$ stage of development. This raised the question of how a TCRβ chain in the absence of a functional α chain could promote T cell development. The answer came with the discovery of the pre-TCR. The pre-TCR consists of a rearranged β chain and a covalently associated 33kd type I transmembrane protein termed the pre-Tα chain (Groettrup *et al.*, 1993; Groettrup and von Boehmer, 1993; Saint-Ruf *et al.*, 1994). Belonging to the immunogobulin superfamily, the pre-Tα chain is encoded by a non-

rearranged gene, and hence its expression is not dependent upon RAG activity. Mice deficient for pre-Tα chain show reduced numbers of CD4/8 DP cells, similar to the phenotypes of RAG-deficient mice (Fehling *et al.*, 1995).

A role of the pre-TCR in signal transduction has been highlighted by comparisons of the pTα-deficient mice with those lacking expression of the src-tyrosine kinase $p56^{lck}$ (Molina *et al.*, 1992). The similarity in phenotypes suggests that the pre-TCR acts as a mediator of signalling through $p56^{lck}$. Furthermore, introduction of a constitutively active $p56^{lck}$ into pTα-deficient mice is able to rescue the wild-type phenotype in terms of CD4/8 DP numbers (Fehling *et al.*, 1997).

Thus, during early thymocyte development, in a process termed β selection, $CD44^{-}25^{+}$ cells rearrange their TCRβ chains which associate with the invariant pre-Tα chain and forms a pre-TCR complex on the surface, providing the necessary signals for pre-T cell differentiation and proliferation to the CD4/8 DP stage. Interestingly, it appears as though there is no extracellular ligand for the pre-TCR as thymocyte development is unaffected in mice lacking the extracellular immunoglobulin domains of the pre-Tα chain (Irving *et al.*, 1998). It is thus proposed that the primary purpose of the pre-Tα chain is to provide structural stability for assembly at the membrane with the TCRβ and other components of the pre-TCR such as CD3ζ chains (O'Shea *et al.*, 1997).

In addition to providing differentiation signals, the pre-TCR has also been shown to mediate allelic exclusion (Aifantis *et al.*, 1997), the process by which further β loci rearrangements are prevented once a functional TCR β chain has been generated, and thus insuring only one β chain is expressed on the surface of the lymphocyte.

The Mature αβ T cell Receptor

During the transition from CD4/8 DN to DP, thymocytes initiate rearrangement of their TCR α chain loci. This step is under the control of the thymic microenvironment, since isolated $CD44^{-}25^{+}$ cells are unable to differentiate into CD4/8 DP cells *in vitro* (Suda and Zlotnik, 1991). Once CD4/8 DP cells express an αβ T cell receptor, the process of positive and negative selection can occur (von Boehmer, 1992). At this stage, the majority of CD4/8 DP thymocytes are destined to die by neglect as their αβ T cell receptors cannot recognise the self-MHC molecules expressed on the surface of the thymic epithelial cells.

CD4/8 DP thymocytes expressing αβ TCRs capable of recognising self-MHC are protected from death and hence, are positively selected. MHC class II restricted TCRs undergo positive selection to the CD4 lineage, while the MHC class I restricted TCRs are positively selected to the CD8 lineage. Negative selection eliminates potentially harmful, self-reactive cells with too high affinities for self-MHC. The exact nature of the signalling pathways that control positive and negative selection are currently being elucidated (Reviewed in Mariathasan *et al.*, 1999). What is clear however is that TCR-induced signals alone do not appear to be sufficient to mediate T cell selection. In a study by Page and colleagues, ligation of the TCR in DP cells only induced down-regulation of CD4 and CD8, and accessory molecules were required to mediate negative selection (Page *et al.*, 1993). Furthermore, positive selection has been shown to require additional signals provided by thymic epithelial cells (Anderson *et al.*, 1994).

Thymocyte development is controlled by overlapping signals provided by flt-3, c-kit, IL-7, pre-TCR and mature T cell receptors. These signals mediate cell survival, proliferation and differentiation, allowing the development of mature single positive T cells able to recognise self-MHC with the correct affinity and express only one αβ T cell receptor on their surface. In addition to controlling αβ T cell development, cytokine driven signals are also required for the development of other thymocytes such as γδ and NK cells. Figure 1.7 summarises the requirements for receptor-mediated signals and illustrates the branch points of γδ and NK cells.

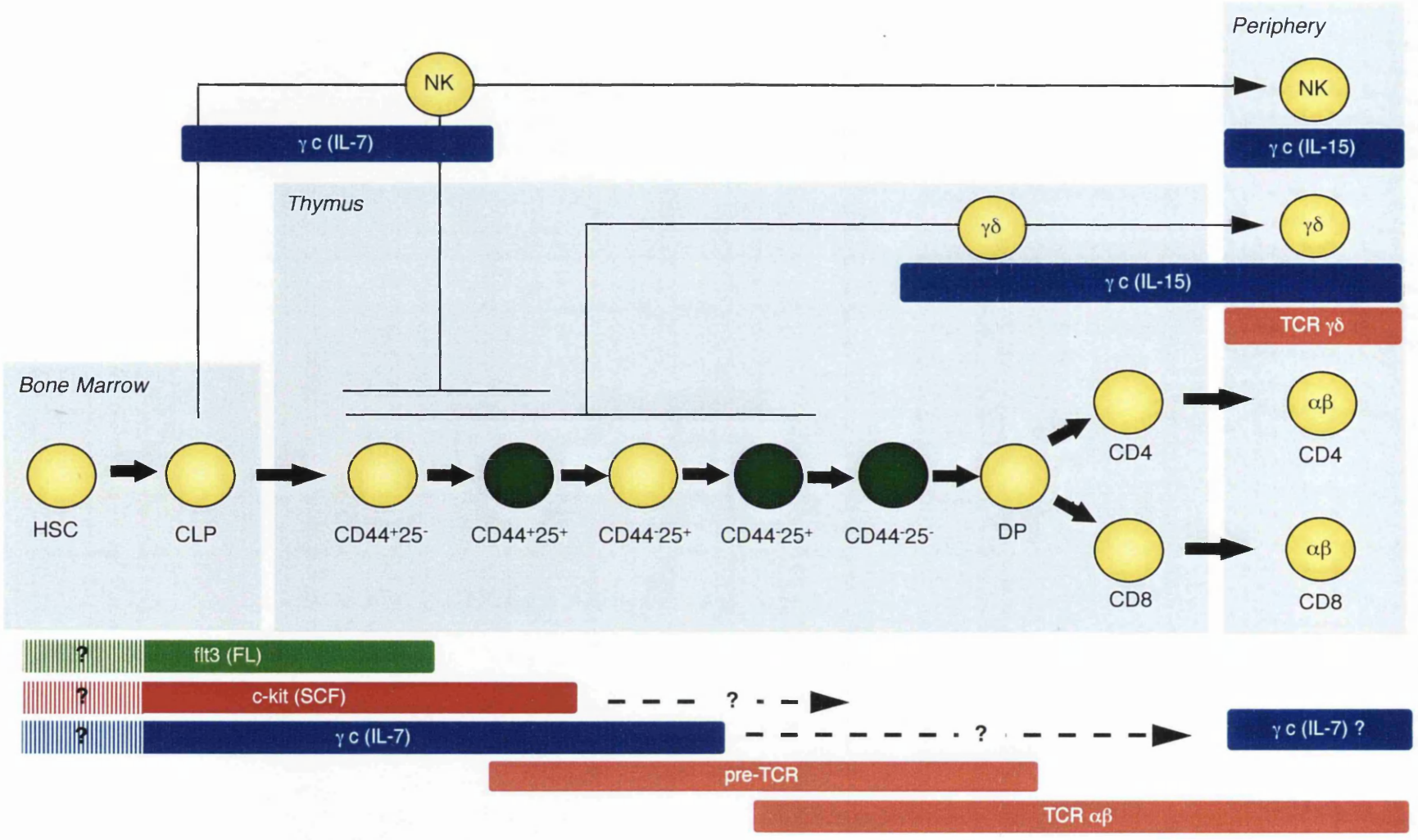


Figure 1.7 - Lymphocyte Development is controlled by overlapping cytokine and antigen receptor derived signals

Overlapping signals from fl3, c-kit and γc (via the IL-7R) are necessary for early thymocyte development. For αβ development, signals from the pre-TCR and αβ TCR complete maturation. Green cirles represent strongly proliferating populations *(Modified from DiSanto and Rodewald 1998).*

1.3.4 - T cell Receptor signalling

The T cell antigen receptor, in conjunction with co-stimulatory molecules such as CD28 regulate the activation and growth of T lymphocytes. The TCR/CD3 complex comprises of the TCR α and β subunits in a noncovalent association with the invariant TCR ζ and η chains and the γ, δ, and ε chains of the CD3 complex (Weiss, 1993). Signal transduction across the cell membrane is mediated by the cytosolic domains of the CD3 complex and the ζ and η chains of the TCR (Weiss and Littman, 1994) which all contain a common motif, termed the immunoglobulin receptor family tyrosine-based activation motif (ITAM).

Upon ligation of the TCR complex, the earliest biochemical response is the activation of the protein tyrosine kinases (PTKs), Zap-70, and two src-family kinases $p56^{lck}$ and $p59^{fyn}$ (Samelson and Klausner, 1992; Gauen *et al.*, 1994). The activation of the receptor proximal PTKs occurs in a highly ordered manner beginning with activation of the two src kinases, phosphorylation of the ITAMs followed by the recruitment of ZAP-70 through interaction of its SH2 domains with the phosphorylated ITAMs, mediating its subsequent tyrosine phosphorylation and activation (Iwashima *et al.*, 1994).

Activation of PTKs results in the recruitment and/or tyrosine phosphorylation of enzymes such as phospholipase Cγ1 (PLCγ1), PI3K and GTPase exchange factors (Reviewed in (Cantrell, 1996)). These membrane proximal events lead to initiation of signalling cascades resulting in cytokine gene transcription and cytoskeletal reorganisation. However, analysis has revealed that PTKs are unable to interact and activate PLCγ1, PI3K and exchange factors directly; rather, adapter molecules such as LAT (Linker for activation of T cells) and SLP76 (SH2-domain containing leukocyte protein of 76 kd) are involved (Clements *et al.*, 1998; Finco *et al.*, 1998). Both LAT and SLP76 are substrates for the PTK ZAP-70.

LAT is an integral membrane protein with a short extracellular region and a long cytosolic tail with nine tyrosine residues conserved between mouse and human (Zhang *et al.*, 1998). Upon tyrosine phosphorylation in response to antigen-receptor triggering, LAT interacts with the PLCγ1 SH2 domain thereby recruiting this enzyme to the plasma membrane where it is able to mediate the hydrolysis of inositol phospholipids. In addition to LAT, the adapter molecule SLP76 is also important for PLCγ1 activation although its

mechanism of action is not yet established. The products of inositol phospholipid hydrolysis, phosphoinositol 3-phosphate and diacylglycerol regulate increases in intracellular calcium and activate serine/threonine kinases of the protein kinase C family respectively. Sustained elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) is absolutely critical during the initial phases of T cell activation both for induction of cytokine gene expression and for controlling T cell cytolytic function (Rao *et al.*, 1997; Crabtree, 1999).

In addition to the recruitment of PLCγ1 to the membrane, adapter proteins such as LAT and TRIM (Bruyns *et al.*, 1998) are also involved in the activation of PI3K. There are multiple isoforms of PI3K and antigen receptors are thought to stimulate the activity of a PI3K complex that comprises a regulatory p85 and a catalytic p110 subunit. Upon activation, PI3K phosphorylates PIP_2 on the D-3 position of the inositol ring to produce PIP_3 (Ward *et al.*, 1996). The products of PI3K activity bind to the plextrin homology (PH) domains of proteins and either allosterically modify their activity or induce relocalization of the protein to defined regions of the cell. One of the proteins whose localisation has been suggested to be regulated by PI3K is the Rho family exchange factor, Vav1 (Discussed in section 1.1.7).

The third class of enzymes regulated by TCR ligation and mediated by adapter proteins are the Ras exchange factors. The Ras GEF Sos, the mammalian homologue of the Drosophila 'Son of Sevenless' protein associates constitutively with the SH3 domains of an adapter molecule Grb2 (McCormick, 1993). In TCR activated cells the Grb2 SH2 domain interacts with tyrosine-phosphorylated residues in the cytoplasmic tail of the adapter LAT thereby forming protein complexes that regulate the membrane localisation and catalytic activity of Sos (Clements *et al.*, 1999). Once Ras is activated it couples to multiple biochemical effector signalling pathways including the Raf-1/MEK/ERK1,2 kinases and signalling pathways controlled by the Rac/Rho GTPases (Genot *et al.*, 1996; Genot *et al.*, 1998). In the leukaemic Jurkat T cell line, Ras is important for pathways that activate transcription factors including AP-1 and nuclear factor of activated T cells (NFAT) leading to the transcription of cytokine genes (Genot *et al.*, 1996; Woodrow *et al.*, 1993). However, Ras, nor any one signalling pathway alone can activate transcription of cytokine genes; rather, multiple pathways are required that integrate at the level of these

transcription factors (Crabtree, 1989). In quiescent cells NFAT proteins are found in the cytosol but are imported into the nucleus upon receptor stimulation (Rao, 1995). Here, they form transcriptionally active complexes by combining with AP-1, a dimer consisting of proteins of the fos and jun family of transcription factors. The activation of NFAT requires the co-ordinated interaction of multiple receptor-induced Ras effector pathways and Ca/calcineurin signals (Rao *et al.*, 1997).

1.3.5 - *In Vivo* Analysis of T cell Signalling

Much of the knowledge of the TCR signalling pathway described in the previous section has come from both biochemical studies and transient transfection approaches. However, over the past few years, the roles of these signalling molecules in both T cell development and function have started to be analysed using transgenic technologies. Figure 1.8 shows a simplified version of TCR mediated signalling. Naturally occurring mutants, gene knockouts and transgene expressing mice have been used to explore almost every layer of this cascade and it is these that will now be reviewed.

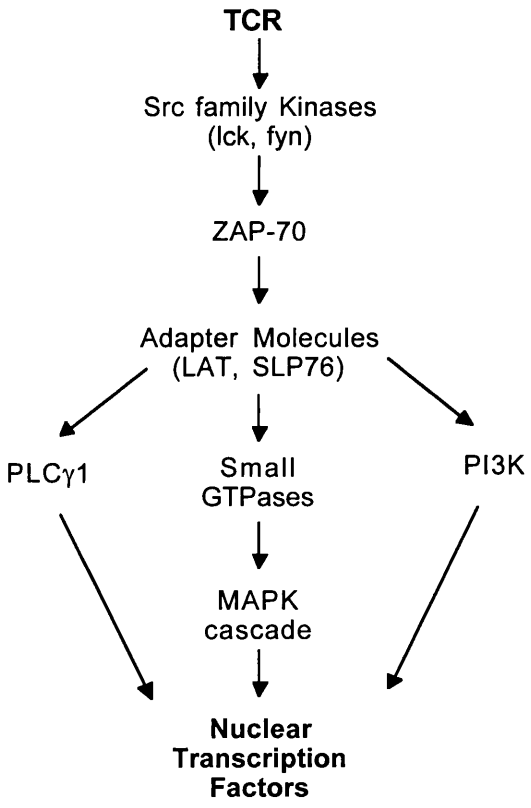


Figure 1.8 - Simplified Scheme of TCR signalling

Src Family Kinases

The essential role for src family kinases in T cell biology has been highlighted by the generation of mice deficient in either lck, fyn and both. Mice deficient in the PTK lck show reduced numbers of CD4/8 DP thymocytes but a few peripheral T cell manage to develop and are partially responsive to TCR-mediated stimulation (Molina *et al.*, 1992;

Penninger *et al.*, 1993). Moreover, mice lacking expression of fyn show normal numbers of CD4/8 DP and peripheral T cells (Appleby *et al.*, 1992; Stein *et al.*, 1992). Deletion of both fyn and lck, however, produces a more dramatic phenotype, with total ablation of all CD4/8 DP and peripheral T cells and a differentiation block at the $CD44^{-}25^{+}$ stage of development, similar to that seen in RAG -/- mice (van Oers *et al.*, 1996). These results highlight the potential for redundant functions mediated by src family PTKs and reveal crucial roles for lck and fyn in T cell development. Reports that expression of active lck mutants can overcome the block in T cell development caused by failure to rearrange β chains further supports a critical role for src-family kinases in pre-TCR mediated signalling (Mombaerts *et al.*, 1994).

ZAP-70

The importance of the protein tyrosine kinase ZAP-70 has been highlighted by analyses of patients lacking ZAP-70 due to gene mutations. Absence of ZAP-70 results in a severe combined immunodeficiency (SCID) disease (Chan *et al.*, 1994a). Although $CD4^{+}$ (and in some cases $CD8^{+}$) T cells develop in ZAP-70 deficient patients, the $CD4^{+}$ cells fail to proliferate following TCR crosslinking *in vitro*. However, pharmacological T cell stimulation with phorbel esters and calcium ionophores can induce proliferation, thus suggesting a TCR proximal signalling defect. Interestingly, mice lacking expression of ZAP-70 have a different phenotype to ZAP-70 deficient patients. Homozygous deletion of murine ZAP-70 results in a complete absence of CD4 and CD8 SP cells, and ZAP-70 -/- thymocytes are not deleted by peptide antigens (Negishi *et al.*, 1995). These data highlight a role for ZAP-70 in TCR-driven positive and negative selection in mice.

Mice deficient in the ZAP-70 related PTK, Syk, suffer severe haemorrhaging as embryos and die perinatally (Cheng *et al.*, 1995). Analysis of lymphoid cells reveals impaired differentiation of B-cells, and only a slight reduction (by 28%) in peripheral T cells. Unlike ZAP-70 deficiencies, CD4 and CD8 cells are present in the normal ratios in Syk -/- mice. These results suggest an absolute requirement for ZAP-70 in TCR mediated signalling that cannot be compensated for by the related PTK, Syk. Conversely, Syk appears to be required for the development of B cells and its absence only has a slight affect on T cell development. This differential requirement for Syk may be partly explained

by differences in the levels of expression between B and T cells with peripheral B cells expressing 12-15 fold more Syk than found in peripheral T cells (Chan *et al.*, 1994b).

Adapter Molecules

Mice lacking expression of the adapter molecule SLP76 exhibit subcutaneous and intraperitoneal haemorrhaging and impaired viability owing to a requirement for SLP76 in platelet formation (Pivniouk *et al.*, 1998). Analysis of lymphoid cells reveals a profound block in thymic development with absence of CD4/8 DP thymocytes and peripheral T cells. This block cannot be overcome with anti-CD3 treatment and V-D-J rearrangement appears unaffected (Clements *et al.*, 1998; Pivniouk *et al.*, 1998). Similarly, deletion of the linker protein LAT also results in the absence of mature peripheral T cells and a developmental block at the CD4/8 DN stage. However, LAT-deficient mice do not show any defects in viability (Zhang *et al.*, 1999). These results suggest that both LAT and SLP76 are required for pre-TCR mediated signals that drive CD4/8 DN differentiation and proliferation.

PLCγ 1

Although mice deficient in PLCγ1 have been produced, homozygous disruption results in embryonic lethality at approximately day 9 of embryogenesis (Ji *et al.*, 1997). To date, there have been no reports of analysis of its role in T cell biology *in vivo*.

PI3Kinase

The classical PI3K though to be involved in lymphocyte signal transduction comprises of two subunits: a p110 catalytic subunit and a smaller p85 positive regulatory domain. Furthermore, T cells have been reported to express two p85 isoforms, p85α and p85β (Reif *et al.*, 1993). Although deletion of the regulatory p85α subunit in mice results in death within the first week of life, attempts have been made to study the role of PI3K in lymphocyte development and function using the RAG-2 deficient blastocyst complementation system. In this system, ES cells lacking both alleles of the gene of interest are injected in RAG-deficient blastocyts and viable chimaeric mice obtained whose lymphocytes are ES-cell derived (as assessed by flow cytometry). Since RAG-deficient mice have no lymphocytes, but otherwise develop normally, the chimaeric mice have a 'lymphocyte-specific' gene knockout (Chen *et al.*, 1993).

Analysis of viable chimaeric mice reveals normal T cell development in the absence of the p85α subunit of PI3K, but a 90% reduction in splenic B cell numbers (Fruman *et al.*, 1999; Suzuki *et al.*, 1999). Although such experiments have highlighted a role for PI3K in B cell development, the failure to see a defect in T cell development in mice lacking expression a p85α may be due to functional redundancy between the p85 regulatory subunits of PI3K (Ward *et al.*, 1996).

Small GTPases

The role of Ras in T cell development has been assessed using transgenes to target expression of either constitutively active or dominant negative Ras mutants specifically into lymphoid cells. Transgenic expression of dominant negative Ras has been shown to partially block positive selection, highlighting the importance of Ras-mediated signalling at this stage of thymocyte development (Alberola-Ila *et al.*, 1995). However, Ras may also play a role at an earlier stage of thymocyte development; analysis of the ability of constitutively active Ras transgenes to cause thymocyte differentiation in the absence of a pre-TCR has highlighted a potential role for Ras in pre-TCR signalling (Swat *et al.*, 1996). By transfecting active Ras mutants into RAG-1 deficient embryonic stem cells, injecting them into RAG-2 deficient blastocysts, and assessing the ability of the resultant transgenic mice to generate mature T cells, the ability of Ras-mediated signals to promote DN thymocyte differentiation was investigated. It was found that in the absence of a pre-TCR, expression of active Ras allowed the transition of thymocytes from the CD4/8 DN to the DP stage and restored thymocyte cell numbers from $1x10^6$, as observed in RAG deficient mice to around $100x10^6$ when V12Ras was expressed. Such a rescue of differentiation and proliferation had been observed previously by expression of TCRβ and activated p56lck transgenes and thus suggests that activated Ras can mediate signalling events downstream of TCRβ and lck (Swat *et al.*, 1996). However, in a second report, they show that although Ras-mediated signals can compensate for the absence of a Pre-TCR with regard to DN thymocyte expansion and differentiation, Ras signals are unable mediate other Pre-TCR driven events such as the inhibition of TCR β chain rearrangements and thus insufficient to cause allelic exclusion (Gartner *et al.*, 1999). These studies highlight a potential role for Ras in pre-TCR mediated differentiation and proliferation, but suggest allelic exclusion is mediated by a Ras-independent pathway.

MAPK cascades

Ras activation is known to initiate a number of signalling cascades, only one of which leads to the activation of ERK2. However, further evidence for the role of the Ras/ERK2 pathway in the pre-TCR mediated signalling was provided by experiments in which an activated Raf transgene was used to stimulate the maturation of T cells in the absence of a pre-TCR. Iritani *et al.* have shown that a transgenic mouse expressing a constitutively active form of c-Raf-1 (Raf-CAAX) under the control of the lck promoter leads to a three-fold increase in basal MEK activity in isolated thymocytes and that this is a similar level of activation achievable by TCR-crosslinking (Iritani *et al.*, 1999). By breeding these transgenic mice onto a RAG 2 -/- background, both thymocyte differentiation and proliferation can be restored. They conclude that as the only well-defined substrate for Raf is MEK, the ser/thr kinase that phosphorylates and activates ERK2, that the activation of the Ras/Raf/MEK/ERK2 pathway controls maturation and expansion of lymphocyte progenitors. However, as observed with expression of active Ras on a RAG -/- background, allelic exclusion appears to be mediated by Pre-TCR generated signals not dependent on this pathway.

Raf has also been implicated in the process of positive selection. Transgenic expression of dominant negative Raf mutants decreases thymocyte numbers by approximately two-fold and results in a decrease in CD8 SP thymocytes in female mice doubly transgenic for the mutant Raf and a MHC class I restricted HY-TCR (O'Shea *et al.*, 1996). Similarly, expression of a dominant negative MEK, the downstream effector of Raf also decreases positive selection (Alberola-Ila *et al.*, 1996). Conversely, expression of activated MEK (MKK1) mutants are sufficient to provide positive selection signals. Moreover, expression of activated MKK6, the activator of the p38 signalling pathway is able to mediate negative selection of T cells, thus implicating different MAPK cascades in the processes of positive and negative selection (Sugawara *et al.*, 1998).

In addition to analysis of the roles of the ERK and p38 in T cell development, the MAPK JNK has also been implicated in playing a role in T cell development. Inhibition of JNK signals in the thymus results in reduced *in vivo* deletion of CD4/8 DP cells, thus implicating JNK-mediating signals in apoptosis in response to TCR-derived and other thymic microenvironment-mediated signals at the CD4/8 DP stage (Rincon *et al.*, 1998). However,

since several isoforms of JNK exist in mammalian cells, and mice lacking expression of JNK1 and JNK2 show normal T cell development (Dong *et al.*, 1998; Yang *et al.*, 1998), the JNK isoform that mediates apoptosis in the thymus remains to be elucidated.

Transcription factors

The roles of NFAT and its components c-jun and c-fos have been explored *in vivo*. Disruption of the NFATc1 gene results in an embryonic lethal phenotype before day 14.5, and so to circumvent this situation, a RAG blastocyst complementation system has been used. NFATc1 -/- chimaeric mice show a 50% reduction in thymus cellularity compared to wild-type mice, due to a significant decrease in the population of CD4/8 DP and CD4 SP thymocytes (Yoshida *et al.*, 1998). Absence of NFATc1 also results in defective proliferative expansion of immature $CD44^-25^-$ thymocytes. Strikingly, there is no reduction in peripheral T cells in these mice, although proliferation of peripheral T cells in response to TCR/CD28-mediated signals is inhibited by 70%. Thus, NFATc1 is implicated in proliferative expansion mediated by either the pre-TCR at the $CD44^-25^-$ stage or the mature TCR once cells are in the periphery.

One of the AP-1 components of NFAT, c-fos appears to play a less important role in T cells as mice deficient for c-fos are comparable to wild-type mice both in terms of T cell development and peripheral T cell function (Jain *et al.*, 1994). The role of the second component of AP-1 has also been explored *in vivo*. Although homozygous loss of c-jun results in embryonic lethality (Johnson *et al.*, 1993), the role of c-jun in T cells has been explored using the RAG-2 deficient blastocyst complementation system. T and B cells deficient in c-jun both develop and function normally (Chen *et al.*, 1994b). These result suggests that other Jun and Fos family members may be capable of substituting functionally for c-fos and c-jun in T cell development and function.

1.4 - Rho GTPases in T Lymphocyte Biology

1.4.1 - Rho GTPases in Lymphocyte Signalling

TCR ligation results in the activation of a number of receptor proximal PTKs which, through adapters such as LAT and SLP76 mediate changes in intracellular calcium,

phosphoinositol lipid metabolism and activation of the Ras/MAPK pathway. The result of all these events are cytokine gene expression and changes in the lymphocyte cytoskeleton. It was not until recently that a role for the Rho family GTPases was identified in T cell activation and the following section reviews what is known about the roles of Rac, Cdc42 and Rho in the biology of a T cell.

Rac

One of the first indications that Rac was important in TCR signalling came from analysis of the signals that regulate NFAT. One well established effector system for Ras in lymphocytes is the Raf-1/MEK/ERK2 pathway (Izquierdo *et al.*, 1993). However, initiation of this signalling pathway by Ras is not sufficient for activation of NFAT proteins. Experiments with activated and inhibitory mutants of Rac reveal a crucial role for this Rho family GTPase in mediating Ras signals to NFAT activation via AP-1 (Genot *et al.*, 1996). Additionally, the Rac effector kinase Pak1 has recently been shown to act downstream of Vav in the activation of NFAT by the TCR (Yablonski *et al.*, 1998). These data support a model which would describe Rac as a Ras effector molecule linking Ras to the regulation of NFAT and cytokine gene transcription (Figure 1.9 summarises this current model). However, the mechanism through which Ras and Rac networks are linked in lymphocytes is unclear.

The role of Rac in the immune system goes far beyond its role in mediating Ras signal transduction. In mast cells, for instance, it appears that activation of the Raf-1/MEK/ERK2 pathways is not required for the activation of NFAT, although the activation of Rac is essential (Turner and Cantrell, 1997). The import of NFAT into the nucleus is a critical stage in the activation of this transcription factor which is known to be controlled by Ca^{2+}/calcineurin signals (Rao *et al.*, 1997). It is now recognised that in mast cells, receptor-regulated NFAT-translocation is controlled by Rac, whereas Ras does not play a role in this process at all. Thus, Rac can be placed in at least two different effector pathways crucial for NFAT activation: antigen receptor controlled subcellular localisation of NFAT and Ras mediated control of AP-1 expression and function.

This example of two different roles for Rac in NFAT activation may serve to illustrate what today is regarded as an important common feature in signalling by small

GTPases. Functionally distinct pools of a GTPase may exist within the same cell at different subcellular localisations and regulate different effector targets and therefore cellular functions in response to the same upstream signal. Accordingly, Rac would be able to regulate NFAT subcellular localisation or NFAT transcriptional activity because of its interaction with distinct effector cascades. Indeed, Rac effector mutants that can induce different subsets of Rac responses in fibroblasts have been characterised and been used to link specific effector pathways with the initiation of specific cellular responses (Lamarche *et al.*, 1997; Westwick *et al.*, 1997).

In addition to being one of the most studied Rho family GTPases in terms of function in T cell signalling, the regulation of Rac activity is also understood in some detail. One way in which the activity of Rac is regulated is through the haemopoietic specific exchange factor, Vav1. Belonging to the Dbl family of exchange factors, Vav1 contains both a DH and PH domain and its activity has been shown to be regulated by antigen-receptor mediated tyrosine phosphorylation (Crespo *et al.*, 1997; Han *et al.*, 1997). In addition, interactions between the T cell costimulatory receptor CD28 and its physiological ligands B7-1 and B7-2 can induce a rapid and sustained tyrosine phosphorylation of Vav1 in the absence of simultaneous TCR ligation (Rudd, 1996). Vav1 activity is also regulated through its PH domain by $PI(3,4,5)P_3$, a product of PI3-Kinase activity (The dual regulation of Vav and Dbl-family exchange factors is discussed in Section 1.1.7).

Further evidence for the role of CD28 costimulation in the regulation of Rho family GTPases came from the characterisation of proteins which are substrates for CD28-activated tyrosine kinases but are not phosphorylated in response to TCR stimulation. Binding of B7 to CD28 induces tyrosine phosphorylation of the adapter protein $p62^{dok}$ (Nunes *et al.*, 1996; Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), which forms a protein complex with the two GAP proteins p120rasGAP and p190RhoGAP. Current models propose that CD28/B7 interaction triggers tyrosine phosphorylation of $p62^{dok}$ which modulates the binding to both GAP proteins. $p62^{dok}$ would thus sequester these negative regulators away from activated Ras and Rho GTPases and thereby promote the activation of Rac and Rho downstream effector pathways. The ability of CD28 to regulate Vav tyrosine phosphorylation combined with its link to $p62^{dok}$ indicates that this T-cell

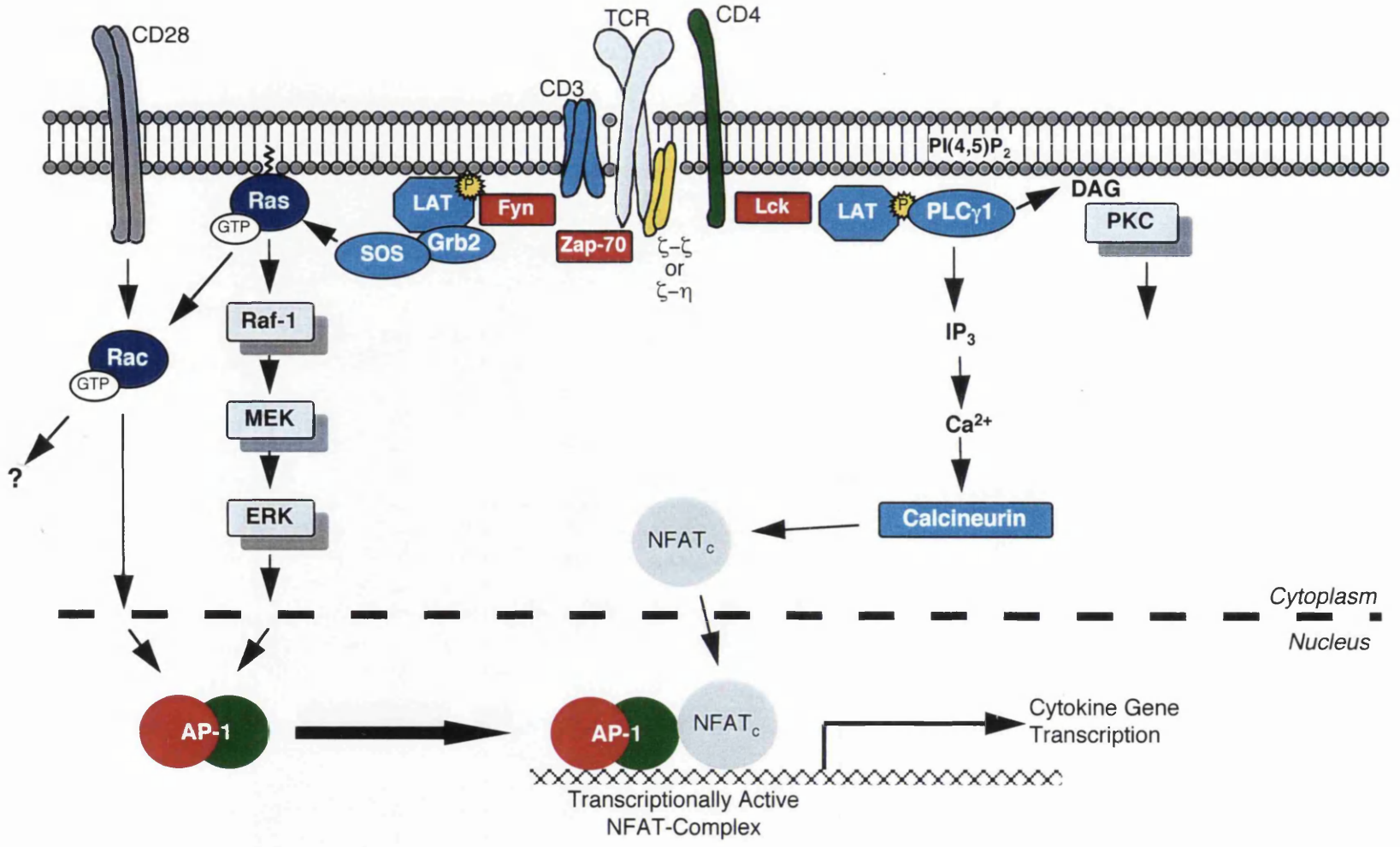


Figure 1.9 - Small GTPases play a central Role in the Process of T cell Activation

TCR-complex ligation initiates the activation of the membrane proximal tryosine kinases fyn, lck and Zap-70, leading to the tyrosine phosphorylation of the adapter protein LAT. The TCR is thus coupled to PLCγ1 and the Ras-regulated signalling pathways, resulting in the formation of a transcriptionally active NFAT complex, and the transcription of cytokine genes. Ras and the co-stimulatory molecule CD28 are known to activate the Rho family GTPase Rac and has been shown to be essential for NFAT activation.

coreceptor must be tightly coupled to the regulatory networks that control the activity of Rho family GTPases. Thus, in the current model shown in figure 1.10, upon initial contact between T cell and APCs, a series of events are initiated through ligation of the TCR and costimulatory molecule CD28, resulting in the activation of Rho family exchange factors such as Vav1 and the sequestering of the negative regulators of small GTPases, p190RhoGAP and p120Ras GAP (Henning and Cleverley, 1999).

Cdc42

Although little is known about the regulation of the Rho family GTPase Cdc42, studies using activated and inhibitory mutants of Cdc42 have provided evidence implicating it in the regulation of TCR-induced cytoskeletal changes in T cells. In particular, Cdc42 function is essential for regulating T cell polarisation towards APCs (Stowers *et al.*, 1995), a process that is critical for efficient T cell-APC contact and the directed release of cytokines. Furthermore, the importance of Cdc42 signalling in T cells is illustrated by the observation that Wiscott-Aldrich immunodeficiency syndrome is caused by a absence of the Cdc42 effector protein, WASP (Kirchhausen and Rosen, 1996). WASP-deficient cells have a paucity in microvilli on the cell surface and poor response to protein antigens, possibly due to defective T cell-APC contacts. Cdc42 has also been suggested to play a role in an apoptotic signalling pathway in T cells. In a report by Chuang *et al.*, active Cdc42 mutants were shown to induce cell death through the JNK pathway and a role for Cdc42 in co-ordinating morphological changes during apoptosis was proposed (Chuang *et al.*, 1997).

Rho

Recently, a role for the small GTPase Rho has been suggested in the potentiation of transcription through the transcription factor AP-1. Using a Jurkat cell line, constitutively active Rho was shown to potentiate AP-1 activity in the presence of the pharmacological PKC activator, phorbel myristate acetate (PMA) (Chang *et al.*, 1998). This observation, coupled with biochemical data showing an association between Rho and PKC has led to the suggestion that PKC and Rho may co-operate in the process of T cell activation through regulation of AP-1. Although this report does not address the role of Rho in AP-1 regulation by the TCR, the role of Rho in T cell activation has also been explored using

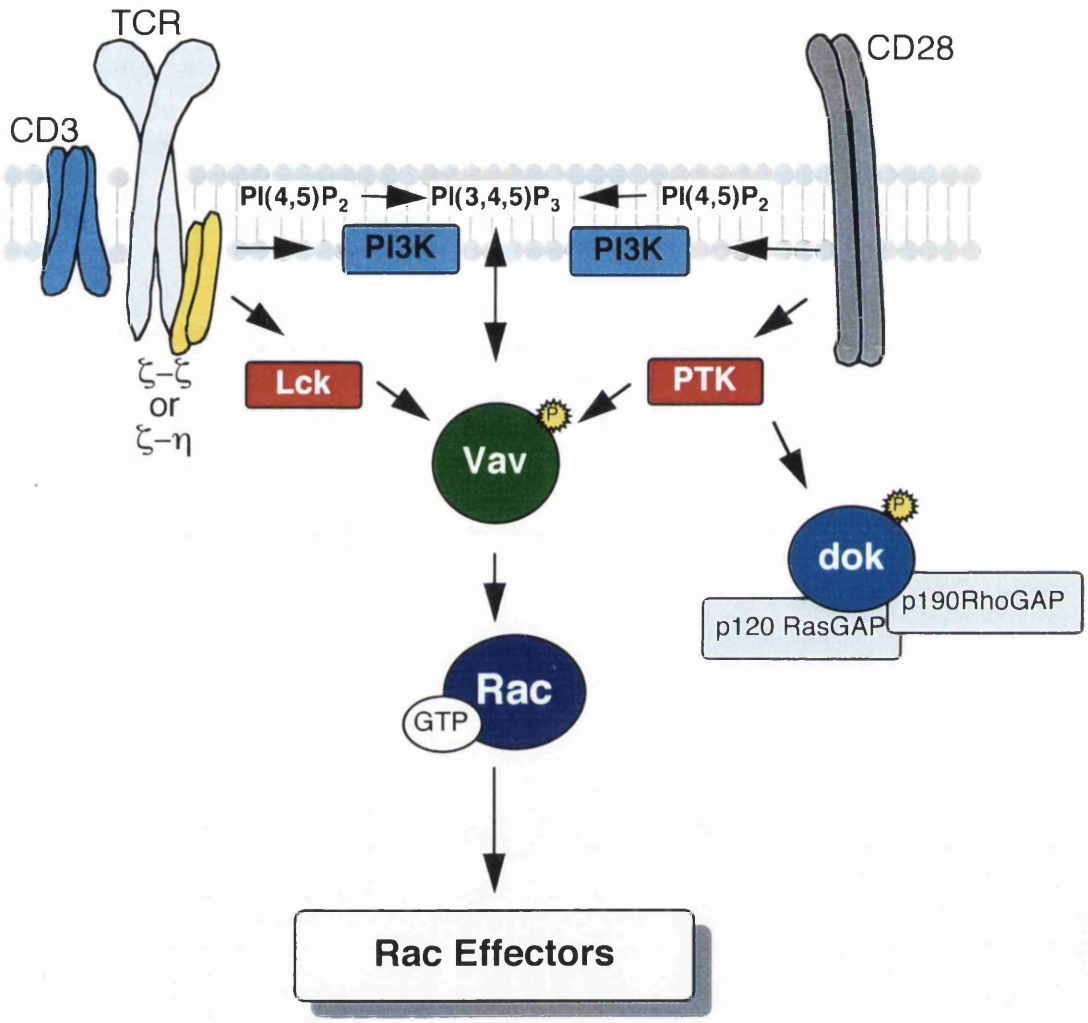


Figure 1.10 - Regulation of the Rac Exchange factor Vav by both PI3K and PTKs

TCR and CD28 are both able to activate PI3Kinase and PTKs, resulting in an increase in the exchange activity of Vav. In addition, ligation of CD28 results in the phosphorylation of dok, which in turn is able to sequester the negative regulators of the small GTPases, p120RasGAP and p190rhoGAP. Thus, TCR and CD28 mediated signals synergise in the regulation of the immune response.

peripheral blood human T cell scrape loaded with C3-transferase (Woodside *et al.*, 1998). Here, inhibition of Rho function inhibits IL-2 production following TCR and CD28 costimulation. Strikingly however, IL-2 production induced by PMA and ionomycin is unaffected by the presence of C3-transferase. Although neither of these reports provide a mechanism through which Rho is able to regulate T cell activation, they do nevertheless provide evidence that Rho function may play a role in TCR/CD28 mediated T cell activation.

Rho has also been implicated in integrin-mediated adhesion and cell-mediated cytotoxicity. In a *in vitro* model used to study the intracellular mechanisms that trigger adhesion through the heterotrimeric G-protein coupled chemoattractant receptors, inhibition of Rho function using C3-transferase blocks integrin-mediated adhesion to fibrinogen (Laudanna *et al.*, 1996). These data establish a requirement for Rho function in signalling between the heterotrimeric G-protein coupled chemoattractant receptors and integrin mediated adhesion. The requirement for Rho function in T cell cytotoxicity was investigated following the discovery that human cytotoxic natural killer cells expressed high levels of mRNA for RhoA relative to the amounts for Rac2, RhoB and RhoC (Lang *et al.*, 1992). Furthermore, inhibition of Rho function by C3-transferase transfection into both cytotoxic T cell lines and NK cell line drastically decreases cytotoxicity. Although the molecular basis for the requirement of Rho in cytotoxicity is unclear, the connection between Rho and the cytoskeleton has led to the suggestion that inhibition of Rho function may prevent the exocytosis of cytotoxic granules (Lang *et al.*, 1992).

1.4.2 - Rho family GTPases in Thymocyte development

Although little is known about the role of Cdc42 in thymocyte development, Rac has been implicated by studies of mice lacking expression of the Rac guanine nucleotide exchange factor Vav1. These animals display various defects in T and B cell development including defective positive selection (Turner *et al.*, 1997). Moreover, peripheral T cells in Vav1 -/- mice fail to proliferate in response to TCR engagement (Fischer *et al.*, 1995); an effect which is due to failure of Vav1 -/- T cells to produce the T cell growth factor IL-2. Thus Rac is suggested to play a role in both positive selection in the thymus and T cell proliferation.

In contrast to Rac, the small GTPase Rho has been shown to play a role at a much earlier point in T-cell development, namely in the control of critical survival and proliferative signals in early thymocyte progenitors. With use of the p56lck proximal promoter to drive thymocyte specific transgene expression, transgenic mice expressing the Rho inhibitor C3-transferase have been made in which the first thymic progenitors and subsequent subsets are devoid of Rho function. Rho inactivation severely impairs the generation of normal numbers of thymocytes and peripheral T cells in the lck-C3 transgenic mice (Gallandrini *et al.*, 1997; Henning *et al.*, 1997). The main failure caused by lack of Rho function results from survival defects of early thymocyte progenitors; $CD44^+25^+$ and $CD44^-25^+$ thymocytes undergo massive apoptosis in the absence of Rho function which leads to a severe depletion of these and subsequent thymic subpopulations. As discussed previously and depicted in figure 1.7, the survival of $CD44^+25^+$ and $CD44^-25^+$ thymocytes is normally controlled by IL-7. In this context, it is interesting that the phenotype of thymocytes lacking Rho function resembles in some way that of mice deficient for signalling components of the IL-7R complex. These observations suggest that Rho is a component of the signalling pathways used by the IL-7R to control cell survival (Peschon *et al.*, 1994; Cao *et al.*, 1995; Thomis *et al.*, 1995; Von Freeden-Jeffry *et al.*, 1995). Current models propose that the IL-7R regulates thymocyte survival by controlling cellular levels of the survival protein Bcl-2 (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1997; von Freeden-Jeffry *et al.*, 1997). Interestingly, the expression of a thymocyte-specific Bcl-2 transgene in the lck-C3 mice was able to rescue the survival defect seen in the $CD44^+25^+$ and $CD44^-25^+$ subsets, and partially restore thymic cellularity. This raises the possibility that Rho acts as an intracellular link between the IL-7R and events that control expression or function of Bcl-2 family proteins.

The phenotype of mice lacking Rho function during T cell development indicated that Rho might also be important for pre-TCR function. Although lck-C3 mice were able to generate DP and SP thymocytes, they were produced at very reduced levels. Further analysis revealed that loss of Rho function resulted in decreased cell proliferation in late pre-T cells ($CD44^-25^-$) due to a partial block in G1/S cell cycle progression. Cell cycle progression in $CD44^-25^-$ thymocytes is normally regulated by the pre-TCR complex and a

proliferative defect in these cells could be indicative of a problem in pre-TCR signal transduction (Hoffman *et al.*, 1996).

The signal transduction pathways used by the pre-TCR to control this stage of thymocyte development involve the activation of tyrosine kinases. In particular $p56^{lck}$ has been shown to play a critical role in the transmission of pre-TCR initiated signals (Molina *et al.*, 1992; Levin *et al.*, 1993; Mombaerts *et al.*, 1994; Fehling *et al.*, 1997). Active $p56^{lck}$ is able to initiate all the events that occur after this critical thymic checkpoint: onset of rapid cell cycle progression, allelic exclusion at the TCR β-locus and ultimately expression of CD4 and CD8. Experiments in which activated $p56^{lck}$ transgenes were expressed in the thymus of lck-C3 mice have provided evidence that the proliferative signals generated by $p56^{lck}$ are suppressed in mice lacking thymic Rho function. In contrast, the ability of active $p56^{lck}$ to mediate allelic exclusion and suppress the expression of αβTCR complexes is unimpaired in the absence of Rho function (Henning and Cantrell, 1998). Thus $p56^{lck}$ uses different signals to control different aspects of pre-T cell development; signalling pathways mediating proliferation are dependent on Rho function, whereas signals for regulation of antigen receptor expression are not.

Studies of the lck-C3 mice have thus revealed a requirement for Rho function in early thymocyte development at a stage where survival is dependent upon signals from the IL-7R. This is not the only report suggesting that Rho can play a role in the survival of T cells. Using the murine lymphoma-derived T cell line, EL4, C3-transferase treatment has been shown to induce formation of multinucleated cells, presumably by inhibiting Rho-mediated cytokinesis, and to cause apoptosis (Moorman *et al.*, 1996). Although it could be reasoned that these two observations are inter-related, with the apoptotic defect being a result of inhibited cytokinesis, inhibition of Rho function in cell cycle arrested EL4 cells also caused apoptosis and thus suggests that multinucleated cell formation and apoptosis are independent.

In the lck-C3 mice, the few cells that manage to escape the massive apoptosis at the $CD25^{+}$ stage go on to develop into CD4/ DP and SP cells, but display proliferative defects. As proliferation at this stage of development is suggested to driven by the pre-TCR, it has been suggested is that Rho function is also required for pre-TCR mediated cell cycle progression (Gallandrini *et al.*, 1997). An alternative possibility to account for the

reduced proliferative capacity of late pre-T cells is that the survival pressures exerted on the cells caused by loss of Rho function earlier in development has selected for cells with a reduced proliferative capacity. What is clear in the lck-C3 mice is that any late pre-T cell that develops has been selected to avoid apoptosis due to loss of Rho function at the $CD25^+$ stage of thymocyte development.

1.5 - C3-transferase as a Tool to study Rho GTPases

Small GTPases have been studied intensively since 1982, when the first evidence of the involvement of Ras in human oncogenesis was revealed (Lowy and Willumsen, 1993). Much work has involved the use of dominant negative mutants of Ras which have a higher affinity for GDP than for GTP and are reported to function by competing with normal Ras for binding to Ras exchange factors (Quilliam *et al.*, 1994). Dominant negative mutants cannot interact with downstream target proteins within cells, so when they are expressed they bind to GEFs and form 'dead-end' complexes. (Reviewed in Feig, 1999).

Although the use of dominant negative mutations in small GTPases has led to great advances in the field, there are a number of potential pitfalls with this type of approach. The major issue is that dominant negative GTPases inhibit the catalytic domain of exchange factors rather than the endogenous GTPase itself. Thus, a dominant negative mutant of Rac would bind to and inhibit all GEFs with exchange activity for Rac. However, from *in vitro* studies, Rac is reported to share a number of exchange factors with other small GTPases (Zheng *et al.*, 1995a), and hence expression of a mutant Rac may not only block Rac-mediated signalling but also pathways mediated by GTPases that share Rac exchange factors. An additional concern when using a dominant negative approach to study signal transduction is that a sufficient amount of the mutant GTPase is expressed to compete effectively with the endogenous protein. Nevertheless, the use of individual inhibitory Rho-family GTPases has produced remarkably specific phenotypes in cells.

An additional tool used in the functional analysis of Rho-family GTPases is the bacterial exoenzyme C3-transferase. First isolated from *Clostridium botulinum* in 1988, C3-transferase was shown, in the presence of [^{32}P] NAD to label a 21kd protein in various tissues (Aktories *et al.*, 1988). The observation that the ribosylation reaction was inhibited

by the presence of either free divalent cations or non-hydrolysable GTP homologs led to the conclusion that C3 transferase labelled GTP-binding proteins. The GTP-binding 21kd substrate was later shown to be distinct from p21ras (Rubin *et al.*, 1988). Furthermore, microinjection of C3-transferase caused morphological changes in a variety of cell lines. In NIH 3T3 cells, profound rounding was observed and binucleated cells accumulated upon C3 injection (Rubin *et al.*, 1988). The substrates of C3-transferase were identified as Rho family GTPases and ribosylation on Asn-41 (Sekine *et al.*, 1989) shown to block interaction with downstream effector proteins (See Figure 1.11). Although initial reports suggested that the substrates of C3 transferase were Rac and Rho (Aktories *et al.*, 1992), it was later shown in both biochemical and functional studies that C3-transferase only inactivated Rho A, B and C, and not Rac or Cdc42 (Menard *et al.*, 1992; Ridley and Hall, 1992). Although Rac and Cdc42 are ribosylated by C3-transferase *in vitro*, this is inefficient compared to Rho A, B, and C.

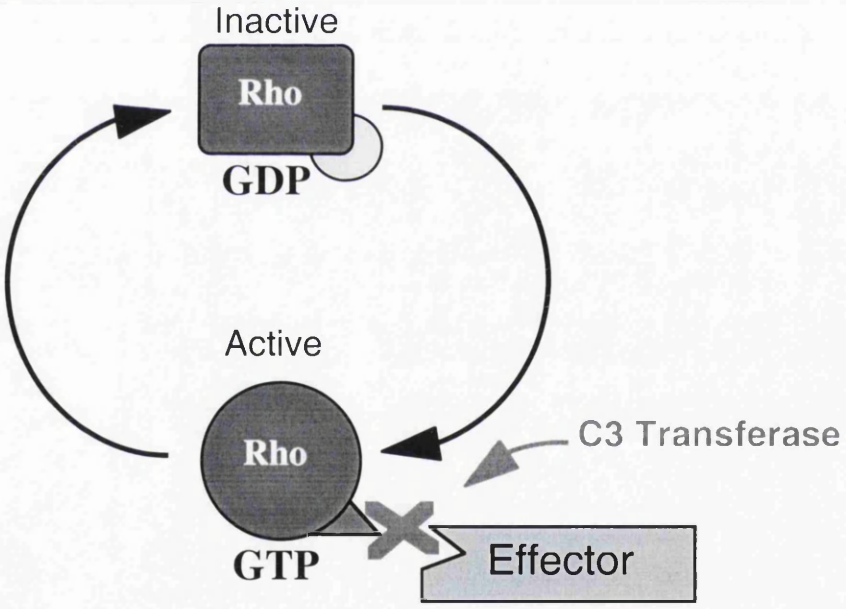


Figure 1.11 - C3-Transferase as a tool to study Rho function
C3-transferase ribosylated RhoA, B, C on Asn41 and blocks interaction with downstream effector molecules.

The use of C3-transferase as a tool to study Rho function within the cell has a number of advantages over a dominant negative approach. For example, as inhibition by C3-transferase is an enzymatic process, much less protein would need to be expressed than with a dominant negative approach in order to fully inactive all the endogenous Rho. In addition, the substrates of C3-transferase have been fairly well characterised *in vitro*,

and thus the effects seen through C3 expression can be correlated to the inactivation of specific GTPases.

The results obtained using C3-transferase to inactivate Rho have been verified using alternative approaches such as expression of RhoGDIs and dominant negative Rho mutants (Kishi *et al.*, 1993; Thomson *et al.*, 1997). Also, active Rho mutants have been used to complement the data obtained (Ridley and Hall, 1992; Olson *et al.*, 1995). However, in a recent report concerns over the use of C3-transferase as a tool to study Rho function were raised. In their report, Beltman and colleagues showed that in Rat-1 fibroblasts, C3-expression stimulated the stress-activated protein kinases JNK and p38, potentiating c-Jun expression and phosphorylation, and suggested that studies using C3-transferase must be interpreted with caution; C3 may not only be inhibiting Rho function, but also inducing activation of stress-induced kinases (Beltman *et al.*, 1999). Although the data presented is convincing, activation of JNK/SAPK by C3-transferase is not a universal phenomena and other studies have reported no such effect of C3-transferase expression (Sun *et al.*, 1999). Moreover, activation of the SAPK/JNK pathway by the Dbl-family exchange factor mNET1 can be inhibited using either C3-transferase or dominant negative Rho mutants (Alberts and Treisman, 1998). Furthermore, in the report by Beltman *et al.*, C3-expression did not affect the ability of LPA to activate either ERK-1 or ERK-2; a result that is notably different from previous studies which have shown C3 inhibits LPA stimulated ERK activation by 50% in Swiss 3T3 cells, and 80-90% in NIH3T3 cells (Kumagai *et al.*, 1993; Hill *et al.*, 1995). Moreover, unpublished data from the Lymphocyte Activation Lab supports a role for Rho in receptor-stimulated ERK activation in T cells as C3 expression attenuates TCR-mediated ERK activation.

The effects on JNK/SAPK activity and ERK activation seen in Rat-1 fibroblasts upon treatment with C3-transferase clearly differ from those reported in other cell systems. Although this may be due to inherent differences in the signalling pathways and their requirements for Rho between Rat-1 fibroblasts and other systems, it may also be caused by variation in experimental design; the Beltman group use electroporation of C3 protein rather than an mammalian expression vector encoding the bacterial exoenzyme. It is also unclear from their report whether their control transfection samples included an

irrelevant protein in similar amounts to the C3 protein used in order to account for any stress-kinase activating effects of protein transfection.

To date, C3-transferase remains a widely used tool in the analysis of Rho function. There is considerable and compelling evidence in support of the specificity of C3-transferase towards Rho itself and not other members of the Rho family of GTPases. In addition, it offers several advantages over the use of dominant negative approaches including the requirement for lower expression levels, and the direct inactivation of Rho, not its exchange factors. It is for these reasons that C3-transferase has been chosen to investigate Rho function throughout the study presented here.

1.6 - Aims of this Thesis

This introductory chapter has established that Rho family GTPases play an important role in the regulation of many biological processes including regulation of the actin cytoskeleton, cytokinesis, transcription and cell growth and differentiation. In the context of the immune system, the Rho GTPases have been suggested to be important for TCR-mediated responses, T cell:APC interactions, and thymocyte development. However, there are still a number of questions which remain with regard to the role of Rho and its family members in the immune system. Accordingly, this thesis aims to address three issues:-

Aim 1: Studies of lck-C3 mice has highlighted an essential role for Rho in thymocyte development. In lck-C3 transgenic mice, C3 transferase is expressed in early $CD44^{+}25^{-}$ thymocyte progenitors (Gallandrini *et al.*, 1997; Henning *et al.*, 1997). The main failure caused by loss of Rho function results from survival defects in $CD44^{+}25^{+}$ and $CD44^{-}25^{+}$ thymocytes with evidence for proliferative problems in late pre-T cells. One aim of the present thesis was to explore whether Rho has independent functions in other thymocyte subsets by generating a second series of transgenic mice in which C3 transferase is not expressed in early progenitors but is targeted to a later stage of thymic development. With use of the CD2-LCR to generate CD2-C3 transgenic mice, expression of C3-transferase directly into pre-T cells should bypass the major survival problem caused by loss of Rho function in the $CD44^{+}25^{+}$ thymocytes as observed in the lck-C3 mice, and allow the role of Rho to be explored independently at later stages of thymocyte development.

Aim 2: To extend to analysis of the lck-C3 transgenic mice to gain greater insight into the molecular basis for the regulation of cell survival by Rho and make a comparative study of the previously described lck-C3 transgenic mice and the CD2-C3 mice described here.

Aim 3: The comparisons of the lck-C3 and CD2-C3 mice highlights the diversity of the roles of Rho within T cell biology. One way in which such diversity could arise is by a small GTPase interacting with a number of downstream effectors. Accordingly, the expression of downstream effectors of Rho in lymphocytes is explored.

CHAPTER 2
Materials and Methods

2.1 - Materials

2.1.1 - Solutions

Phosphate Buffered Saline Dulbecco's A (PBSA) (137mM sodium chloride, 3.3mM potassium chloride, 1.7mM Potassium dihydrogen orthophosphate (anhydrous), 10mM disodium hydrogen orthophosphate (anhydrous), adjusted to pH7.4 with HCl), L-broth bacterial growth media (0.5% bacto-yeast extract, 1% bactro-tryptone, 1% NaCl, final pH7.6), Brain Heart Infusion (BHI) media (3.7% BHI powder, final pH7.6), and RPMI-1640 and DMEM cell culture media were produced by the ICRF Central Cell Services.

2.1.2 - Chemicals and Reagents

ECL reagents and [γ^{32}P]ATP (370 Mbq/ml) were from Amersham International (UK). Proteinase K was from Boehringer Mannheim. dNTPs were from Amersham Pharmacia Biotech. Ficoll-Hypaque was from Nycomed. Foetal Calf Serum, DNase and Reverse Transcriptase enzyme were from Gibco (UK). ConA was from Calbiochem, recombinant IL-2 from Proleukin and Phorbol 12,13-dibutyrate (PDBu) from Sigma Aldrich Company (UK). Pre-stained molecular weight markers were from Gibco (UK). Protogel acrylamide solution was from National Diagnostics. Taq polymerase was produced by ICRF Central Services. Keyhole Limpet Hemocyanin (in 50% glycerol) was from Calbiochem Novabiochem. Non-radioactive ATP, GTPγS and GDPβS was from Calbiochem. All other reagents were from standard suppliers or as indicated in the text.

2.1.2 - Antibodies - Flow Cytometry

Antibodies and secondary reagents used for surface marker expression analysis are listed below:-

Antibody	Clone	Isotype	Company
CD25	3C7	Rat IgG2b,k	Pharmingen
CD25	7D4	Rat IgM,k	Pharmingen
γδTCR	GL3	Hamster IgG	Pharmingen
αβTCR	H57-597	Hamster IgG	Pharmingen
CD4	RM4-5	Rat IgG2a,k	Pharmingen
CD8	53-5.8	Rat IgG1,k	Pharmingen
CD3ε	145-2C11	Hamster IgG	Pharmingen
B220	RA3-6B2	Rat IgG2a,k	Pharmingen
CD2	RM2-5	Rat IgG2b,k	Pharmingen
Mac-1	M1/70	Rat IgG2b,k	Pharmingen
Gr-1	RB6-8C5	Rat IgG2b,k	Pharmingen
CD44	IM7	Rat IgG2b,k	Pharmingen
CD69	H1.2F3	Hamster IgG	Pharmingen
NK (Dx5)	Dx-5	Rat IgM, k	Pharmingen
CD117 (c-Kit)	2B8	Rat IgG2b,k	Pharmingen
NK (2B4)	2B4	Mouse IgG2b,k	Pharmingen
NK-1.1	PK136	Mouse IgG2a	Pharmingen
RatIgG2a,k	R35-95	Mouse IgG2a,k	Pharmingen
RatIgG1,k	R3-34	Mouse IgG1,k	Pharmingen
MouseIgG2b,k	49.2	Mouse IgG2b, k	Pharmingen
CD16/CD32	2.4G2	Rat IgG2b,k	Pharmingen
Thy1.2	53-2.1	Mouse IgG2b	Pharmingen
Vβ8	F23.1	Mouse IgG2a	Pharmingen
Vβ11	RR3-15	Rat IgG2b, k	Pharmingen
HY-TCR	T3.70	Hamster IgG	Dr. H. Von-Boehmer Institut Necker, Paris
Streptavidin-Tricolor			Caltag
Streptavidin-Allophycocyanin			Pharmingen

2.1.3 - Antibodies - Western Blotting

For Western Blot analysis, the following antibodies were used:-

Antibody	Species	Source
mDia (N-term)	Rabbit	S.Narumiya, Japan
mDia (C-term)	Rabbit	S.Cleverley, ICRF
PRK-1	Mouse	Signal Transduction Laboratories
Myc (9E10)	Mouse	ICRF Hybridoma Unit
Murine CD3 (2C11)	Hamster	ICRF Hybridoma Unit
Human CD3 (UCHT-1)	Mouse	ICRF Hybridoma Unit
Anti-Rabbit HRP	Goat	Amersham
Anti-Mouse HRP	Donkey	Amersham

2.1.4 - Antibodies - Intracellular Staining

For intracellular staining and subsequent flow cytometry, the following antibodies were used:-

Antibody	Clone	Isotype	Company
TCR β Chain	H57-597	Hamster IgG	Pharmingen

2.1.5 - Antibodies - Immunohistochemistry

The anti-medullary epithelium antibody, MTS-10 was obtained from Pharmingen and used for visualisation of murine thymic medullar.

2.1.6 - Peptide Synthesis

The following peptide was synthesised by Nicola O'Reilly at the ICRF Oligosynthesis Service:-

PTILEEAKELVGRAS

2.1.7 - Oligonucleotide Synthesis

Oligonucleotides used for both DNA sequencing and PCR-based transgenic mouse screens were produced by the oligonucleotide synthesis lab within the ICRF.

2.1.8 - Glutathione S-Transferase Fusion Proteins

Plasmids encoding the following Glutathione S-Transferase (GST) fusion proteins were a gift of Alan Hall's Laboratory, UCL:-

Fusion	Molecule
GST	GST Alone
GST-Rho	Wild-type Human RhoA
GST-V14Rho	Constitutively Active Human RhoA

2.1.9 - Transgenic Mice

The transgenic mice used in the studies reported in this thesis are detailed below:-

Transgenic	Source	Reference
p56lck-C3	Dr. D. Cantrell Imperial Cancer Research Fund	(Henning *et al.*, 1997)
CD2-C3	Dr. D. Cantrell Imperial Cancer Research Fund	(Cleverley *et al.*, 1999)
v-Raf	Dr. M. Owen Imperial Cancer Research Fund	(O'Shea *et al.*, 1996)
v-Myb	Dr. K. Weston Institute of Cancer Research	(Badiani *et al.*, 1996)
FADD-DN	Dr. G. Evan Imperial Cancer Research Fund	(Zornig *et al.*, 1998)
p53 -/-	Dr. D. Cantrell Imperial Cancer Research Fund	(Clarke *et al.*, 1993)
HY-TCR	Dr. H. Von-Boehmer Institut Necker, Paris	(Swat *et al.*, 1991)

2.2 - Experimental Methods

2.2.1 - Cell Culture

Peripheral T cells were prepared from human peripheral blood by the discontinuous Ficoll-Hypaque gradient centrifugation method described by Cantrell and Smith (Cantrell and Smith, 1984). The Jurkat line JHM-1 (bearing the human muscarinic acetylcholine receptor) was maintained in RPMI containing 10% Heat-Inactivated Foetal Calf Serum. Passage stocks were kept in 1mg/ml G418 to ensure stability of the HM-1 clone. Murine T cell blasts were obtained by isolating lymph nodes from C57BL/6 mice, preparing a single-cell suspension and stimulating with ConA (5ug/ml), PdBu (50ng/ml), and IL-2 (20ng/ml) for 72h.

2.2.2 - Preparation of heat-shock competent DH5α *E. coli.*

A single DH5α colony was picked from a minimal plate and grown overnight in 25ml L-broth. The culture was subsequently diluted 1:200 in fresh L-broth and grown to logarithmic phase (OD_{550}=~0.4). The cells were chilled on ice, collected by centrifugation at 3500g for 10 min at 4°C and resuspended in 160ml of ice-cold transformation buffer I (30mM KAc, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol, final pH 5.8). After a 30 min incubation on ice the cells were pelleted by centrifugation at 3500g for 10 min at 4°C and resuspended in 30ml of transformation buffer II (10mM PIPES, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% glycerol, final pH 6.5) for 30 min on ice. Aliquots of 400µl were snap frozen on dry ice and stored at -70°C.

2.2.3 - Agarose Gel Electrophoresis

Resolution of DNA fragments for analysis or preparative purposes was by agarose gel electrophoresis in standard agarose in 1 x TAE buffer (0.04M Tris-acetate, 2mM EDTA pH8.0) . Agarose was melted in 1 x TAE buffer, allowed to cool, and ethidium bromide added to 0.3µg/ml. The agarose was poured into a gel casting tray with an appropriate gel comb to form loading wells.

DNA samples were loaded with one sixth volume of 6x loading buffer (30% glycerol (v/v), 0.25% bromophenol blue (w/v)). Gels were electrophoresed at 1-10 volt/cm. DNA was visualised by ultra violet illumination.

2.2.4 - DNA Subcloning

Plasmid DNA (1μg) was digested in a final volume of 10μl (in the appropriate buffer) at 37°C for 1hr, using 1 unit of restriction enzyme (final concentration) (New England Biolabs). DNA fragments were separated on agarose gels using 1xTAE as the running buffer. For subcloning purposes, desired fragments were isolated using a Qiagen Gel Purification Kit (according to the manufacturer's instructions). DNA fragments were ligated into the desired plasmid using T4 DNA ligase (Gibco). Typically, a 3x molar excess of DNA fragment was used relative to the plasmid, using <100 ng of DNA in total, in a final volume of 10 μl. Ligation reactions were performed at RT for 2 h.

Ligation mixtures was then used to transform DH5α *E.coli* (or in the case of GST fusion protein constructs, BL21 protease deficient *E.coli*) Circularised plasmid DNA was mixed with 100μl competent *E.coli* cells and incubated on ice for 30 min. The cells were then heat-shocked at 45°C for 45 sec and returned to ice for 5 min. 200μl L-Broth medium was added and the cells were left to recover at 37°C for 30 min before plating onto LB-agar plates, containing 50mg/ml ampicillin, and incubating at 37°C overnight.

Single colonies obtained from transformation reactions were grown overnight in 5ml L-Broth (containing 50μg/ml ampicillin). Plasmid DNA was isolated from 2ml aliquots using Qiagen Miniprep Kits, according to the manufacturer's instructions. Colonies containing the desired plasmid DNA were identified by restriction enzyme digestion.

2.2.5 - Preparation of DNA for Transgenic Mouse Generation

CsCl purification of plasmid DNA was carried out as follows. Briefly, 400ml culture of *E. coli* carrying the desired plasmid was grown overnight in BHI (brain heart infusion) medium. Bacteria were pelleted (3500g, 4°C, 20 min) and resuspended in 40ml solution I (50mM glucose, 25mM Tris pH 7.4, 10mM EDTA). Bacterial cells were lysed by adding

80ml of solution II (1% SDS, 0.2M NaOH). Denatured proteins, chromosomal DNA and cellular debris were precipitated by adding 40ml of solution III (60% v/v 5M potassium acetate, 11.5% v/v glacial acetic acid, in water). Precipitates were removed by centrifugation (3500g, 4°C, 20 min). Supernatant DNA was precipitated using 90ml isopropanol followed by centrifugation (3500g, 4°C, 20 min) The DNA pellet was resuspended in 5ml distilled water to which 5ml 7.5M ammonium acetate was added to precipitate RNA. Samples were centrifuged as above and supernatants ethanol precipitated for 1h at -20°C. After centrifugation (3500g, 4°C, 20 min), DNA pellets were resuspended in 8.5ml dH_2O and 0.5ml 10μg/ml ethidium bromide. 8.8g CsCl was dissolved in this solution and the mixture divided between two heat-sealable ultracentrifugation tubes and centrifuged in a vertical rotor (VTi 65.2, Beckman) at 385,000g for 4h at 20°C. The plasmid DNA was removed and the ethidium bromide extracted with water saturated *n*-butanol. The DNA was precipitated with 2 to 3 volumes of ethanol and resuspended in dH_2O.

2.2.6 - Generation of the cDNA for production of the CD2-C3 Transgenic Mice

The C3-Transferase cDNA was subcloned into the VAh-CD2-LCR bluescript vector. The 11.2kb DNA fragment encoding the CD2 promoter - C3-transferase - LCR was excised with a *KpnI/NotI* restriction digest. The products of the restriction digests were loaded and run on a 0.8% agarose/Tris-acetate-EDTA (TAE) gel. The bands were visualised with ethidium bromide, viewed under a long wavelength UV light and excised. The linear DNA fragment was purified using a Gene-Clean II kit (Stratech Scientific). Briefly, the gel slices were then weighed and three volumes of a saturated solution of sodium iodide added, followed by incubation for 10-15 minutes at 50°C to dissolve the agarose. The glass powder suspension was added and binding allowed to take place for 10 minutes on ice. Following brief centrifugation, the pellet of glass powder was washed first with 0.5ml saturated sodium iodide and then three times with 1ml of a Tris/NaCl solution in 50% ethanol (new wash). Finally, the DNA was eluted by resuspending the glass powder in TE buffer (10mM Tris pH 8.0, 1mM EDTA) and incubating for 10 min at 50°C. In order to provide a pure DNA fragment preparation for injection, the eluted DNA

was passed through an Elutip column (Schleicher and Schnell). The column was prepared according to the manufactures instructions and the DNA, made up in 0.5ml of 0.2M NaCl, passed slowly through the filter unit and column. Following washing of the column with 0.2M NaCl, the DNA was eluted by slow passage of 0.5ml of 1M NaCl in TE. The eluted DNA was then precipitated with ethanol, washed twice with 70% ethanol and redisolved at a final concentration of 5ng/μl in transgenic injection buffer (10mM Tris pH7.4, 0.1mM EDTA made up in Millipore Ultrapure water).

2.2.7 - Pronuclear Transgenic Fragment injection and generation of founder lines

The purified 11.2kb KpnI/NotI fragment (2ng/ml) containing the CD2-C3-LCR transgene was injected into the pronuclei of day 1 F1 mouse zygotes (C57BL/6/J x CBA/J). Embryos were transferred into pseudo-pregnant F1 foster mice which gave birth to litters of 5 to 10 mice 20 days later. Stable lines were generated by backcrossing transgene carrying founder mice with C57BL/6/J mice.

2.2.8 - Preparation of DNA from mouse ear discs

Ear discs were placed in an Eppendorf and 50μl of buffer (50mM Tris pH8.0, 25mM NaCl, 0.1% SDS) and 5μl of 10mg/ml Proteinase K added. Ears were digested overnight at 55°C in a water bath. Following digestion, tubes were removed and 250μl dH_2O added. The Proteinase K was inactivated by heating to 100°C for 8 min.

2.2.9 - Screening of Transgenic Mice by PCR

Transgene carrying mice were identified by PCR employing the specific primers shown below. PCR was performed in 20mM ammonium sulphate, 75mM Tris-HCl (pH9.0), 0.01% Tween, 1.2mM $MgCl_2$, 200mM dNTP, 0.5U TaqDNA polymerase, and 1mM of each primer. Genomic DNA was purified from mouse ear punches and used as template for 35 cycles of polymerase chain reaction (72°C elongation, 95°C denaturation, and the annealing temperature as shown in the table).

	Primers	Sequence	Annealing Temp.
CD2-C3	VAh2 + C3NT	gtcccaacccagctttccctg ctgatttgcttagtccatac	57°C
	VAh4 + C3CT	ggggacaatgagttttctgctg gggcacagctatcaatcc	57°C
lck-C3	p56SA + C3NT	gagggaacccagtcaggagc ctgatttgcttagtccatac	57°C
	C3CT +HgHas	gggcacagctatcaatcc gcgcttacctgtagccattgc	57°C
v-Raf	RafIntA + RafIntB	tacactccttatgaccct atgtcaattagctggaac	45°C
	RafInt1 +Mo142	catgcaggtgtttgtaga gcggttacctgtagccattgc	50°C
v-Myb	myb1 +myb2	ccatggaagccgtcattaagaaccgg cgcatggtggaattccagtggttcttg	57°C
FADD-DN	p56SA + FADD2	gagggaacccagtcaggagc acagaattctcaggacgcttcggaggtagatgcgtc	57°C
	sFADD1 +Mo142	accatggacttcgaggcggg gcggttacctgtagccattgc	57°C
p53 -/-	5NTL +3W2TL +F8NEO	gtgggagggacaaaagttcgaggcc atgggaggctgccagtcctaaccc tctcctgtcatctcaccttgc	57°C
Actin	A1 +A2	gtggccatctcctgctcgaagtc gtttgagaccttcaacacccc	57°C

2.2.10 - Cell Preparation

Thymi and spleens were obtained by dissection from 4-6 week old mice. Tissue was dissaggregated by mincing with fine forceps and forced through a fine mesh filter to obtain a single cell suspension. Splenocytes were additionally subjected to hypotonic shock to lyse red blood cells. Total cell numbers were determined by microscopic observation of trypan blue negative cells using a Neubauer hemocytometer.

2.2.11 - NK-T cell isolation

Livers were obtained by dissection from 10-12 week old mice. Tissue was dissaggregated by mincing with forceps and forced through a fine metal mesh filter to

obtain a single cell suspension. Lymphocytes were obtained by discontinuous density centrifugation over Percoll (Amersham Pharmacia Biotech). Percoll solutions were made by mixing 9 parts Percoll with 1 part 10x PBS, then preparing 40% and 80% solutions with Percoll/PBS by diluting in DMEM (No serum). The isolated cells were washed in PBS and resuspended in 15ml 40% Percoll in a 50ml tube, then underlayed with 15ml 80% Percoll. Following centrifugation at 1000g, RT, the layer of lymphocytes were recovered from the 40%-80% interface and washed in 50ml cold PBS with 5% serum.

2.2.12 - Flow Cytometric Analysis

Antibodies were obtained conjugated to either fluorescent isothiocyanate (FITC), phycoerythrin (PE), Allophycocyanin (APC) or biotin. Biotinylated antibodies were revealed using either streptavidin-TRICOLOR or streptavidin-APC. Prior to staining, Fc Receptors were blocked with an anti-FcγRII blocking monoclonal antibody. Cells were then stained with saturating concentrations of antibody at 4°C for 20 min at $2x10^6$ cells per sample in a 96 well V-bottom shaped microtitre well plate in 100µl phosphate buffered saline containing 1% bovine serum albumin. Cells were washed with this buffer between incubations and prior to analysis on a FACS Calibur (Becton Dickinson).

Events were collected and stored ungated in list-mode using CellQuest (Becton Dickinson) software. Live cells were gated according to their FSC and SSC profiles and data analysed using CellQuest software.

2.2.13 - Intracellular Staining

Thymocytes were isolated and surface receptors stained as described above. Cells were fixed in 1% Paraformalderhyde in PBSA for 10 min at RT, washed in PBSA and permeabilsed with 0.3% Saponin permeabilisation buffer (0.3% v/w Saponin, 5% Foetal calf serum, and 10mM HEPES pH7.4 in PBS) for 10 minutes at RT. Permeabilised cells were then washed, incubated with an anti-FcγRII blocking monoclonal antibody, and stained with an antibody to the intracellular protein of interest. All washes following permeabilisation

were performed in Saponin wash buffer (0.1% v/w Saponin, 5% Foetal calf serum, and 10mM HEPES pH7.4 in PBS).

2.2.14 - Apoptosis Assay

Thymocytes were stained with a FITC-labelled mAb reactive to CD25 in RPMI supplemented with 10% Foetal Calf Serum. After washing, cells were incubated with 7-amino actinomycin D (7AAD) (Calbiochem, San Diego, CA) for 20 minutes at 4°C in the dark. Cells were analysed by flow cytometry, with apoptotic cells being identified by 7AAD staining as described (Schmid *et al.*, 1994).

2.2.15 - Cell Cycle Analysis

Cellular DNA content was assayed by standard techniques using propidium iodide staining. Briefly, $2x10^6$ thymocytes were stained with FITC conjugated mAb against CD25 as described previously and fixed in cold 70% ethanol for 30 min at 4°C. Cells were washed free of ethanol, RNase (1mg/ml) treated for 15 min at RT and resuspended in propidium iodide (Sigma) (50mg/ml) to stain cellular DNA. Events were collected and analysed on a FACS Calibur (Becton Dickinson) using a doublet discrimination module. Propidium iodide fluorescence was measured above 600nm.

2.2.16 - Timed pregnancies and embryo studies

CD2-C3 and lck-C3 embryos were obtained by mating C57BL/6 females with transgenic adult males. Embryo thymi were obtained from time-mated pregnant mice on various days of gestation. The date of finding the vaginal plug was taken as day 0 of gestation.

2.2.17 - RT-PCR from Fetal Thymi or sorted cell populations

For embryonic RNA preparation, thymic lobes (days 13 - 15) and total embryos (days 10 - 12) were dissolved in 500µl RNazol (Cinna Scientific). For sorted cell DNA

isolation, FACS sorted cells were dissolved in 500µl RNazol. Total RNA was extracted according to the manufacture's protocol and subjected to DNase treatment. The reverse transcriptase (RT) reaction was performed using random hexamer primers, and the cDNA obtained used as template for PCR reactions employing primers specific for hypoxanthinephosphoribosyltransferase (HPRT) (5'-CACAGGACTAGAACACCTGC-3' and 5'-GCTGGTGAAAAGGACCTCT-3'), p56lck (5'-GCCCATCCGGAATGGCTCTG-3' and 5'-CGCCACGAAGTTGAAGGG-3') and C3 transferase (5'-GAGGGAACCCAGTCAGTCA GGAGC-3' and 5'-CTGATTTGCTTAGTCCATAC-3'). PCR reactions were performed in 20mM ammonium sulphate, 75mM Tris-HCl (pH9.0), 0.01% Tween 20, 1.2mM $MgCl_2$, 200mM dNTP, 0.5 U TaqDNA polymerase, and 1mM of each primer. DNA was amplified for 35 cycles at an annealing temperature of 57°C. PCR products were separated in 2% agarose gels and stained with ethidium bromide.

2.2.18 - Fusion Protein Preparation

Fusion proteins were prepared as follows. *E.coli* bearing fusion proteins encoded on the pGEX2T plasmid were inoculated into 400ml L-Broth containing 50mg/ml Ampicillin and grown to OD_{550} = 0.6-0.8 before induction of fusion protein expression for 4h using 0.5mM isopropyl β-D thio-galactopyranoside (IPTG) (Sigma). Cultures were then centrifuged and resuspended at 4°C in 10ml PBSA containing 1% Triton X-100, 2mM EDTA and 1mM PMSF. Lysis was by sonication and cell debris pelleted by centrifugation at 20000g for 10 min at 4°C. Initial purification of the fusion protein from lysate was by 2h rolling incubation with 1ml of a 50% (v/v) solution of Glutathione Agarose beads which had been washed 3 times in cold PBSA containing 2mM EDTA. The suspension was then centrifuged at 3000g for 30 seconds and washed twice in the above lysis buffer and twice in 2mM EDTA/PSBA.

The protein was eluted from the beads using 25mM glutathione in Tris buffer (pH 8.0) and its OD at 280nm measured. The protein fractions collected after elution were dialysed in Visking tubing (Mr cut-off 5000-8000 Daltons) against PBSA/2mM EDTA for 12h with 3 changes of buffer. Finally, fusion proteins were recoupled to glutathione agarose beads and stored in 50% glycerol, 50mM HEPES (pH 7.4) and 50mM NaCl at -20°C.

2.2.19 - Nucleotide Loading of GST-RhoA

GST-Rho coupled to glutathione beads were loaded with the non-hydrolysable analogues of either Guanine Nucleotide Di- or Tri-phosphate (GDPγS or GTPγS). Briefly, 30µl of coupled GST-RhoA (10mg/ml stock) was added to equivolume 2x loading buffer (40mM Tris pH7.4, 10mM EDTA, 2mM DTT) and 1µl of a 10mM stock of nucleotide added. After incubating for 10 minutes at 37°C, the loading reaction was terminated by adding 1µl 0.5M $MgCl_2$ before washing once in 1ml of lysis buffer (0.1% Triton X100, 20mM Tris pH 7.4, 100mM NaCl, 10mM $MgCl_2$, 10% glycerol, 1mM DTT, 1mM Benzamidine,1mM PMSF).

2.2.20 - GST-RhoA Protein Affinity Chromatography

Cells were lysed at between $20x10^6$/ml and $30x10^6$/ml of lysis buffer (0.1% Triton X100, 20mM Tris pH 7.4, 100mM NaCl, 10mM $MgCl_2$, 10% glycerol, 1mM DTT, 1mM Benzamidine,1mM PMSF). Cell lysis was performed on ice for 20 min, and cell debris removed by centrifugation at 20000g, 8min. Lysates were then precleared by rotating for 20 min at 4°C firstly with 20µl 10% Protein A insoluble solution (Sigma) and then with 20µl of a 50% Glutathione beads coupled to GST protein alone (10mg protein/1ml gluthione beads). Precleared lysate was then added to 20µl of a 50% Glutathione beads coupled to GST-Rho, preloaded with nucleotide, and rotated at 4°C for 90 min. Beads were washed 3 times in 1ml cold lysis buffer and dried with a Hamilton syringe. The samples were then either subjected to an *in vitro* kinase assay (Described below) or 30µl 2x Reducing sample buffer (1M Tris-HCl, pH 6.8, 0.1mM Na_3VO_4, 6% SDS, 0.5M EDTA, 4% 2-β mercapto-ethanol, 10% glycerol and 0.01% bromophenol blue) added and boiled for 8 minutes prior to SDS PAGE.

2.2.21 - *In vitro* Kinase Assay

Following protein affinity chromatography, GST-RhoA beads were washed three times in lysis buffer (0.1% Triton X100, 20mM Tris pH 7.4, 100mM NaCl, 10mM $MgCl_2$, 10% glycerol, 1mM DTT, 1mM Benzamidine, 1mM PMSF), and two times in kinase buffer (20mM HEPES pH7.5, 100mM NaCl, 10mM $MgCl_2$ and 1mM Na_3VO_4). After the final wash, the

beads were dried with a Hamilton syringe and the kinase assay initiated by adding 25µl kinase buffer containing 1.5µCi [^{32}P]-γ-ATP and 20µM cold ATP. After a 20min incubation at 30°C, *in vitro* phosphorylation was terminated by adding 25µl 2x reducing sample buffer and boiling for 8 min prior to SDS PAGE.

2.2.22 - Resolution by SDS-PAGE and Western blotting.

Proteins were resolved by SDS-PAGE using Protogel (National Diagnostics) 30% acrylamide, 0.8% bis-acrylamide stock solutions. The percentage of the acrylamide resolving gels (pH 8.8) are indicated in the figure legends. The stacking gels were 5% acrylamide (pH 6.8). The running buffer used contained 25mM Tris pH8.3, 190mM glycine, 3.5mM SDS.

Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting in a transfer buffer containing 10mM CAPS pH11. Presence of protein on the membrane was checked by exposure for 30 sec to 2% Ponceau S stain and immediate de-stain by rinsing in dH_2O.

Membranes were blocked by incubation in 5% non-fat milk solution (w/v in PBSA) for 1h at room temperature. Typically primary antibodies were using diluted in PBS/0.02% sodium azide/0.5% non-fat milk. Developing antibodies were horseradish peroxidase conjugated goat anti-rabbit or donkey anti-mouse IgG diluted in PBSA/0.05% Tween-20/0.5% non-fat milk. Washes between antibody incubations were standardised at four washes of 5 min in approximately 50ml PBSA/0.05% Tween-20.

Where required, PVDF membranes were stripped of antibodies by incubation in a rolling hybridisation oven for 30 min in 25ml strip buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris pH 6.7) at 55°C. The membrane was rinsed three times in 50ml PBSA/0.02% Tween-20 before blocking for 1h in 5% non-fat milk and re-probing.

2.2.23 - Mass Spec fingerprinting and sequencing

Mass Spectroscopy fingerprinting and sequencing was performed by Darryl Pappin, Protein Sequencing Laboratory at the ICRF. Briefly, the electroblotted proteins

were stained with sulforhodamine B (0.05% w/v in 30% v/v aqueous methanol/0.1% v/v acetic acid) using a rapid-staining protocol (Coull and Pappin, 1990). The dried, stained proteins were then digested *in situ* on the PVDF membrane with trypsin (Boehringer, modified) for 18 h at 30°C and the peptides extracted with 1:1 v/v formic acid:ethanol (Sutton *et al.*, 1995). Aliquots were sampled and directly analysed by matrix-assisted laser desorption ionisation (MALDI) time-of flight mass spectroscopy using a LaserMat 2000 mass spectrometer (Thermo Bioanalysis, UK) (Mock *et al.*, 1992). Additional aliquots were quantitatively esterified using 1% v/v thionyl chloride in methanol and also analysed by MALDI to provide acidic residue composition (Pappin *et al.*, 1996). Native and esterified peptide masses were then screened against the MOWSE peptide mass fingerprint database (Pappin *et al.*, 1993). The remaining digested peptides (>90% of total digest) were then reacted with N-succinimidyl-2(3-pyridyl) acetate in order to enhance b-ion abundance and facilitate sequence analysis by tandem mass spectroscopy (Sherman *et al.*, 1995). Dried peptide fractions were treated with 7µl of 1% w/v with N-succinimidyl-2(3-pyridyl) acetate in 0.5M HEPES (pH 7.8 with NaOH) containing 15% v/v actonitrile for 20 min of ice. The reaction was terminated by addition of 1µl heptafluorobutyric acid (HFBA) and diluted with an equal volume of water. The solution was then injected in 3 x 5µl aliquots onto the capillary reverse-phase column (300µm x 15cm) packed with POROS R2/H material (Perspective Biosystems, MA) equilibrated with 2% v/v acetonitrile/0.05% v/v TFA running at 3µl/min. The adsorbed peptides were washed isocratically with 10% v/v actonitrile/0.05% v/v TFA for 30 minutes at 3µl/min to elute excess reagent with HEPES buffer. Derivatised peptides were eluted with a single step gradient to 75% v/v acetonitrile/0.1% v/v formic acid and collected in a single 3-4 µl fraction. The derivatised peptides were then sequenced *de novo* by low-energy collision-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitter with a nanoelectrospray source (Hunt *et al.*, 1986; Wilm and Mann, 1996). CAD was performed using 2.5 mTorr argon with collisional offset voltages between -18V and -28V. The product-ion spectra were collected with Q3 scanned at 500amu/sec.

2.2.24 - Sequence Comparisons and Phylogenic Tree Construction

Sequences were aligned to calculate a distance matrix and a guide tree constructed from the distance matrix using the Clustal W algorithm (Thompson *et al.*, 1994; Higgins *et al.*, 1996).

2.2.25 - Generation of anti-mDia (C-terminal) antisera

A peptide corresponding to the C-terminal 15 amino-acids (residues 1241-1255) of mDia-1 was synthesised. The peptide was then coupled to Keyhole Limpet Hemocyanin, via a gluteraldehyde coupling method, to cross-link NH_2 groups. Briefly, 4mg of peptide was dissolved with 4mg of Keyhole Limpet Hemocyanin (KLH) in 0.1M $NaHCO_3$, in a final volume of 2ml. 0.05% ultra pure gluteraldehyde was added, the adjusted to pH 8 with NaOH and then mixed overnight in the dark at RT. The following day glycine ethyl ester (pH 8) was added to a final concentration of 0.1M and incubated at RT for 30 min. The coupled peptide-KLH proteins were then precipitated with 5 volumes of cold acetone at -70°C for 30 min and pelleted by centrifugation at 10,000g for 5 min. The pellet was then dried and resuspended in sterile PBS at a concentration of 1mg/ml. The KLH-coupled peptides were used to generate rabbit antisera using standard immunisation techniques and was carried out by the ICRF Biological Resources Unit.

CHAPTER 3
Analysis of CD2-C3 transgenic mice

3.1 - Introduction

In lck-C3 transgenic mice, C3 transferase is expressed in early $CD44^{+}25^{-}$ thymocyte progenitors (Gallandrini *et al.*, 1997). To determine whether Rho has independent functions at later stages of development, a second series of transgenic mice were generated in which C3 transferase is not expressed in early progenitors but is targeted to a later stage of thymic development; namely to the $CD44^{-}25^{+}$ early pre-T cell population. This was achieved by using the human CD2 locus control region (CD2-LCR) to control expression of C3-transferase. The CD2-LCR has been shown to direct position-independent tissue specific expression of linked genes in murine T cells (Greaves *et al.*, 1989; Zhumabekov *et al.*, 1995). Murine CD2 is not expressed in early thymocyte progenitors but is induced at the $CD25^{+}$ stage at the time these cells rearrange their β chain loci and express the pre-TCR complex (Rodewald *et al.*, 1993). The CD2-LCR transgene has been reported to have a similar onset of expression to mouse CD2 (Melton *et al.*, 1996) and the patterns of expression of the p56lck and CD2 promoter are shown in figure 3.1. Using the CD2-LCR to express C3 transferase directly in pre-T cells should bypass the major survival problem caused by loss of Rho function as observed in the lck-C3 mice, and allow the role of Rho to be explored independently at later stages of thymocyte development.

The following chapter summarises the initial characterisation of the CD2-C3 mice before going on to present evidence for the role of Rho in pre-TCR signalling. Finally, the peripheral phenotype of the CD2-C3 mice is investigated, and the requirement of Rho function in other cell lineages assessed.

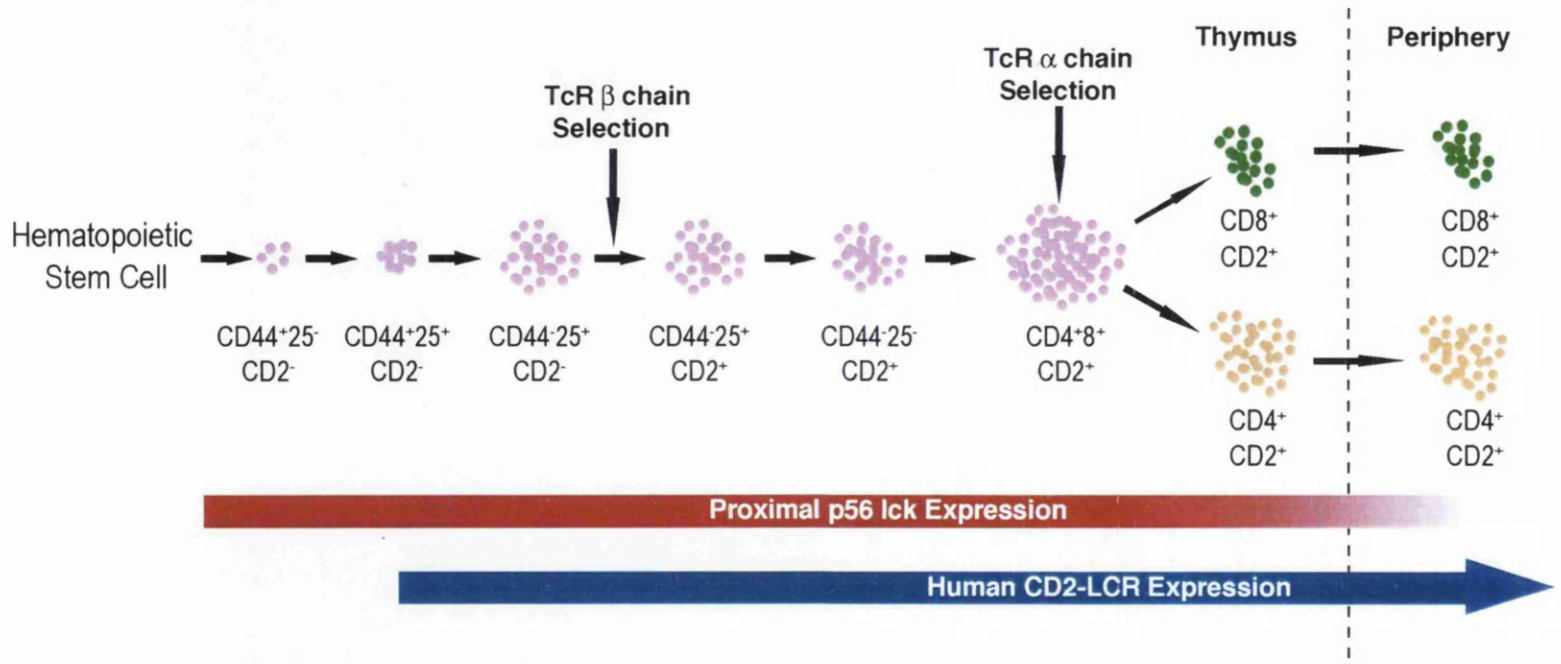


Figure 3.1 - Schematic diagram showing kinetics of p56lck and CD2 expression.

The p56lck proximal promoter is active in the early $CD44^{+}25^{-}$ thymocyte population until cells exit the thymus into the periphery, while CD2 expression begins in the $CD44^{+}25^{+}$ population as cells start to rearrange their β chain loci.

3.2 - Results

3.2.1 - Generation of the CD2-LCR C3-transferase transgenic mice

The bacterial enzyme C3-transferase is a powerful and specific inhibitor of Rho function. A 0.8kb cDNA encoding C3-transferase was ligated into the CD2-LCR-bluescript vector between the CD2-promoter and LCR. The 11.2kb *KpnI/NotI* fragment comprising the CD2-C3-LCR was excised and purified before injecting into F1 fertilised oocytes (C57BL/6J x CBA/J) (Figure 3.2). The CD2-LCR has been reported to confer position-independent copy-number dependent transcription of transgenes under its control (Zhumabekov *et al.*, 1995). A PCR-based screen was developed using primer pairs. The first pair produced a 300bp fragment with the forward primer, VAh2 priming from the human CD2 promoter and paired with the reverse primer C3-Nterm, priming from the N-terminal region of the C3-transferase cDNA. The second pair of primers produced a 250bp fragment with the forward primer, C3-Cterm priming from the C-terminal region of C3-transferase, and paired with the reverse primer VAh4, binding to a complementary sequence in the locus control region. Transgene carrying mice were identified with this screen and backcrossed onto C57BL/6 inbred mice to generate founder lines. Three mouse lines were initially obtained and the T cell phenotype analysed.

3.2.2 - Initial CD2-C3 founder line analysis

Thymi from the different lines of CD2-C3 transgenic mice were all reduced in size compared to those of normal littermate controls. Two of the founder lines, 1610A and 1611A showed between a 50 and 100-fold reduction in thymocyte numbers; line 1610B+C had a 10-fold reduction in thymocyte number compared to those of littermate controls (Table 3.1).

When spleens were removed, cell suspensions produced and splenocytes stained with antibodies against the αβ-TCR and the B cell marker, B220, flow cytometric analysis revealed that mice from all lines displayed severely reduced numbers of peripheral αβ T cells; only 10% of normal levels at 4 weeks of age. Splenic B cell numbers appeared unchanged in all but one of the lines (1611A), which displayed a 2 fold-reduction in B cell numbers compared to that of a wild-type mouse. It is clear from these data that expression

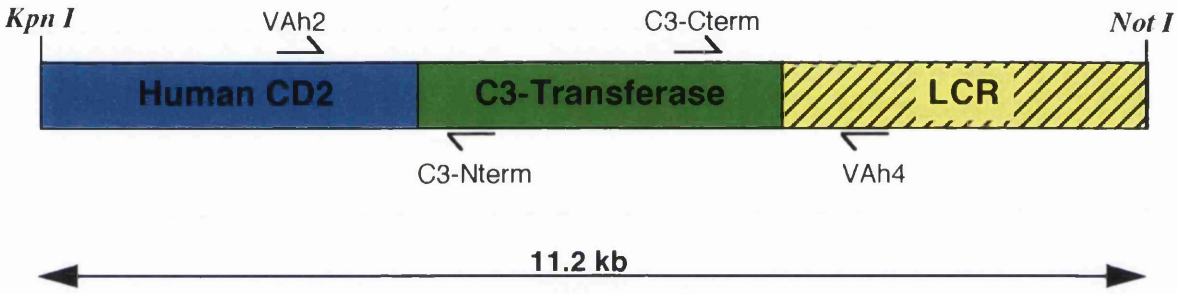


Figure 3.2 - Schematic of the CD2-C3 LCR construct used to generate the CD2-C3 Transgenic Mice.

The transgenic vector consists of the cDNA encoding C3-transferase fused between the human CD2 promoter and the locus control region. The 11.2kb KpnI/NotI fragment was excised and purified before injecting into F1 fertilised oocytes (C57BL/6J x CBA/J). Positions of the primers used for PCR-based screening are indicated.

$x10^6$	NLC	1610A	1611A	1610B+C
Thymocyte Nos	180	2	2.6	20
Splenocyte Nos	100	78	50	72
Splenic T cells	32	3.9	4	2.9
Splenic B cells	54	59.3	27.5	53.8

Table 3.1 - Thymic and Splenic Cellularity of the 3 Founder lines of CD2-C3 Mice.
The thymi and spleens were isolated from mice from founder lines 1610A, 1611A and 1610B+C, along with Non-transgenic Littermate Controls (NLC). The total thymic and splenic cell numbers were determined. The absolute numbers of T and B cells in the spleens were calculated by staining for αβ TcR and B220 and these data used to determine total T and B cell numbers in the spleen. Data shown is representative of 3 mice analysed for each line.

of C3 transferase under the control of the CD2 promoter severely reduces production of both thymic and peripheral αβ T cells.

3.2.3 - Maintenance of the CD2-C3 Transgenic Mice

For practical reasons, it was decided to reduce the number of initial founder lines. The mice all had very similar phenotypes; the founder lines 1611A had only one litter and it proved impossible to obtain further litters from this line. Breeding of 1610A was continued as a representative CD2-C3 transgenic line. The majority of the work presented is based on studies of this line.

3.2.4 - Analysis of Thymic Cellularity and Structure in the CD2-C3 Mice

Further analysis of the representative CD2-C3 transgenic line were consistent with initial observations that there was a problem in αβ T cell development. A study of thymocyte numbers post natally showed that loss of Rho function resulted in a severe reduction in thymocyte numbers at all ages of mice studied. Thymi from wild-type mice contained around $40x10^6$ thymocytes at 1 week of age, and this increased rapidly to around $200x10^6$ at 4 weeks, before gradually reducing to $100x10^6$ at 12 weeks (Figure 3.3). When cell numbers from CD2-C3 mice at various ages were investigated, they were in the region of $2x10^6$ at 1 week of age, increasing slightly at 4 weeks to 3-4 $x10^6$ before falling back to $2x10^6$ as the mice matured.

Figure 3.4A shows representative thymi from a 4 week old CD2-C3 mouse and that of a non-transgenic littermate control. Thymi isolated from CD2-C3 transgenic mice appeared 5 to 6 times smaller than those from nontransgenic littermate controls. Although the overall size of the thymus was reduced, it was of interest to determine whether this was accompanied with a loss of thymic structure. Therefore, cross-sections were prepared from both CD2-C3 and wild-type thymi, and stained with an antibody reactive to medullary epithelium. Stained wild-type thymic cross-sections showed clear medullar and cortex regions. CD2-C3 thymic cross-sections showed a severely disrupted thymic morphology with no clear medullary-cortical boundary (Figure 3.4B).

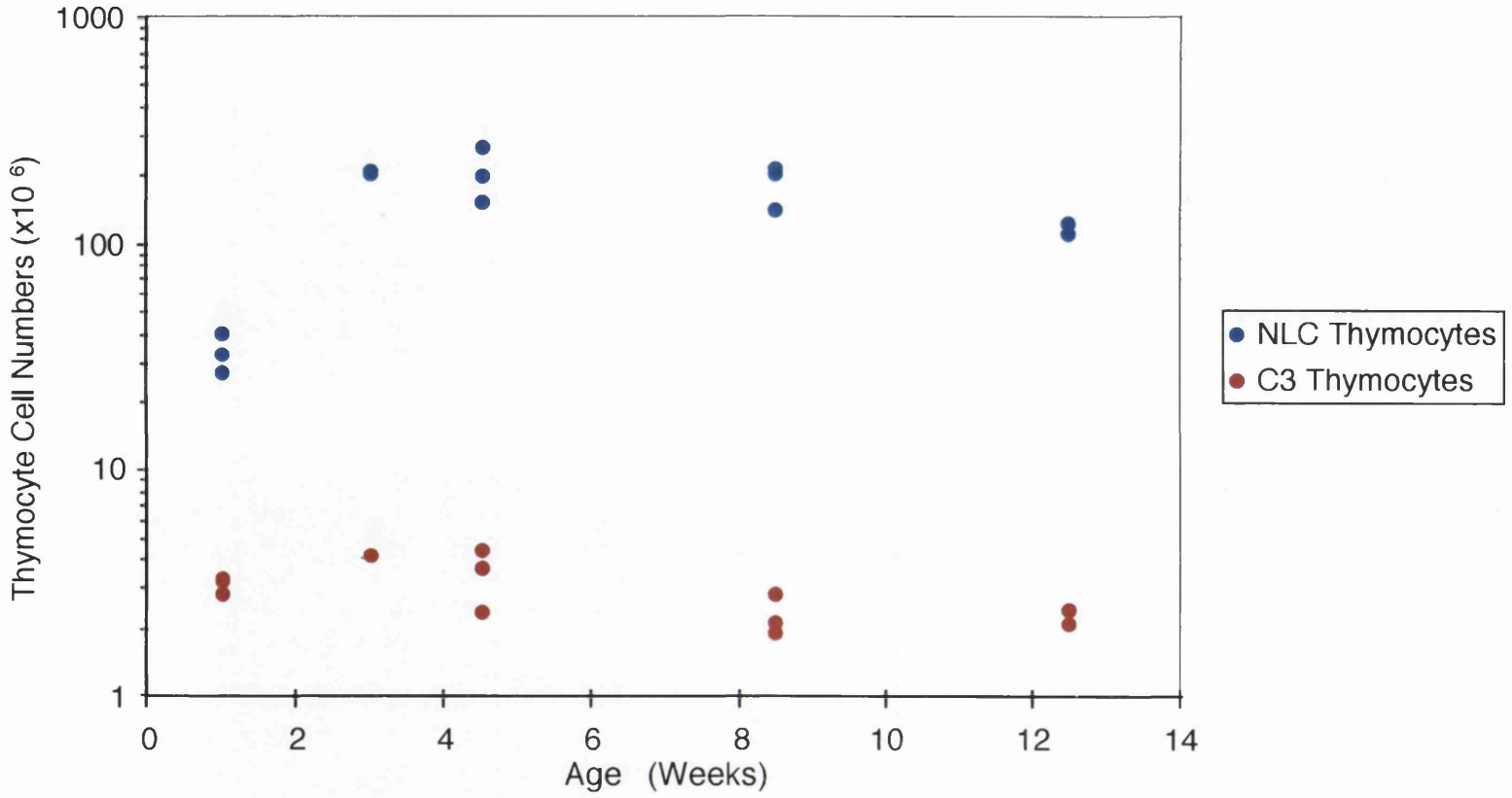
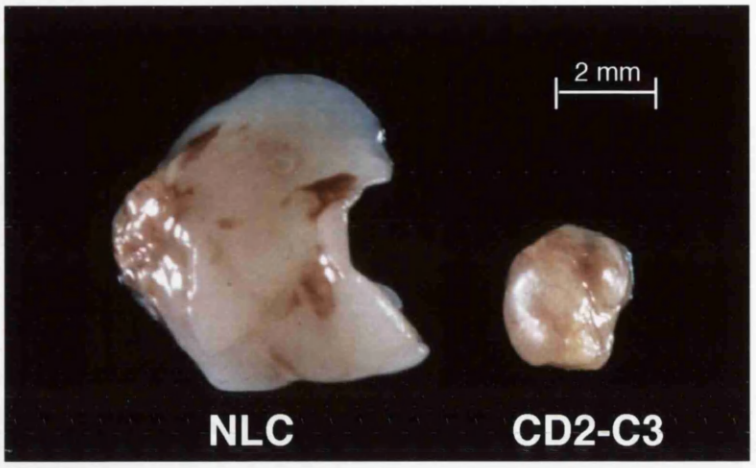


Figure 3.3 - Graph of thymocyte numbers from NLC and CD2-C3 Mice aged between 1 -13 weeks.

Thymocytes were isolated and counted from CD2-C3 transgenic and Non-transgenic Littermate Control mice aged between 1 and 13 weeks of age.

A



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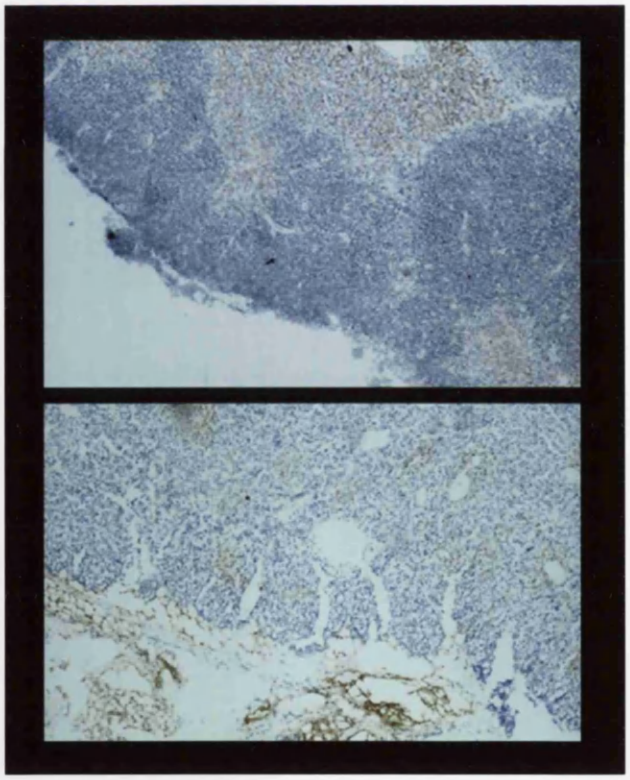

Figure 3.4 - Morphology of thymi isolated from CD2-C3 transgenic and non-transgenic littermate control mice.

(A) Thymi isolated from a 4 week old non-transgenic littermate control (NLC) and a CD2-C3 transgenic mouse.

(B) Thymus cross-sections stained with an antibody cross-reactive with thymic medullary epithelium (MTS-10) (Pharmingen) from a 4 week old non-transgenic littermate control (top panel) and a CD2-C3 transgenic (bottom panel).

3.2.5 - Thymocyte differentiation in CD2-C3 transgenic mice

Reduced thymic cellularity may result from a failure of thymocyte proliferative expansion or from a block in thymocyte differentiation prior to the main point of proliferative expansion which occurs as cells acquire CD4 and CD8 markers and enter the CD4/8 DP population. Figure 3.5 shows that thymi from CD2-C3 mice almost exclusively contain CD4/8 DN cells. Typically this subpopulation comprises 2-4% of a normal thymus whereas they represented 95% of the transgenic thymus. Calculations of absolute cell numbers show that the levels of CD4/8 DN cells were within the normal range in CD2-C3 mice, but all further stages of thymocyte development were effectively lacking (Table 3.2). Hence, loss of Rho function in CD2-C3 transgenic mice causes a block in thymocyte differentiation at the CD4/8 DN stage.

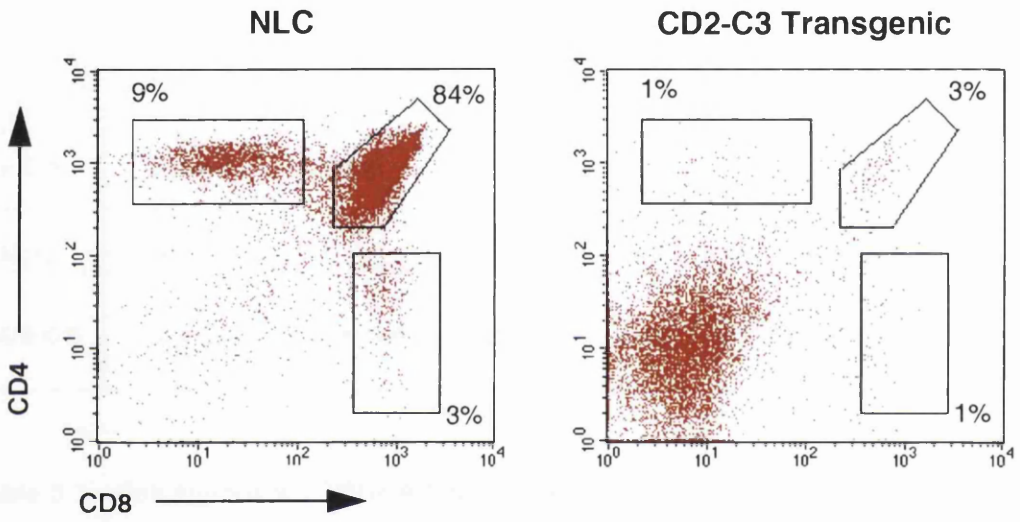

Figure 3.5 - Analysis of the CD4/8 thymic subsets in CD2-C3 transgenic mice.

Thymocytes from CD2-C3 mice and nontransgenic littermate controls were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 and analysed by flow cytometry.

x 10^6	Total	$CD4^-8^-$	$CD4^+8^+$	$CD4^+$	$CD8^+$
NLC	160 ± 9	5.1 ± 1	130 ± 12	19.3 ± 7	5.3 ± 2
CD2-C3	2.5 ± 0.5	2.3 ± 0.4	0.13 ± 0.1	0.10 ± 0.07	0.04 ± 0.01

Table 3.2 - Cell Numbers of CD4/8 Thymocyte Subsets in Normal and CD2-C3 Transgenic Mice.

Mean±SD cell numbers of CD4/8 DN, DP and SP cells from Normal (NLC) and Transgenic animals (n = 8).

3.2.6 - Characterisation of the CD4/8 DN thymocytes in CD2-C3 Mice

CD4/8 DN thymocytes can be further subdivided on the basis of the surface markers CD25 and CD44 (see Figure 3.1). Therefore to further characterise the nature of the CD4/8 DN differentiation block seen in the CD2-C3 mice, CD4/8 DN thymocytes were analysed for expression of CD25 and CD44. Staining and flow cytometry revealed that thymocytes isolated from CD2-C3 mice contained a marked increase in frequency of the $CD44^-25^+$ population compared to non-transgenic littermate controls (Figure 3.6A). When absolute cell numbers were calculated from the CD25 and CD44 FACS profiles, it became evident that $CD44^+25^-$, $CD44^+25^+$ and $CD44^-25^+$ cells were all present at normal levels, but the forth population of CD4/8 DN cells, the $CD44^-25^-$ population was effectively lacking (data not shown).

During the final stages of CD4/8 DN maturation, concomitant to CD25 down-regulation, CD2 expression is up-regulated, resulting in a final CD4/8 DN stage in which cells are $CD25^-2^+$ (Rodewald *et al.*, 1993). Figure 3.6B shows the CD25/CD2 profile of CD4/8 DN thymocytes from a normal control mouse as compared to that of a CD2-C3 mouse. These data clearly show that the mature $CD25^-2^+$ DN population is effectively absent in CD2-C3 transgenic mice, and thus confirm that lack of Rho function results in a differentiation block at the $CD44^-25^+$ stage of thymocyte development.

Further analysis also revealed that CD25 levels from CD4/8 DN thymocytes from CD2-C3 mice were elevated compared to CD25 levels on control cells (Figure 3.7). CD25 is normally down-regulated upon expression of a pre-TCR complex on the thymocyte surface membrane. Failure of the pre-TCR complex to either express or signal correctly results in increased levels of CD25 expression (Groves *et al.*, 1996; Aifantis *et al.*, 1997; Krotkova *et al.*, 1997). Elevated levels of CD25 in cells from CD2-C3 transgenic mice are therefore indicative with a block in pre-T cell expression/function and thus suggest that the differentiation block owing to loss of Rho function may be due to a defect in pre-T cell signalling.

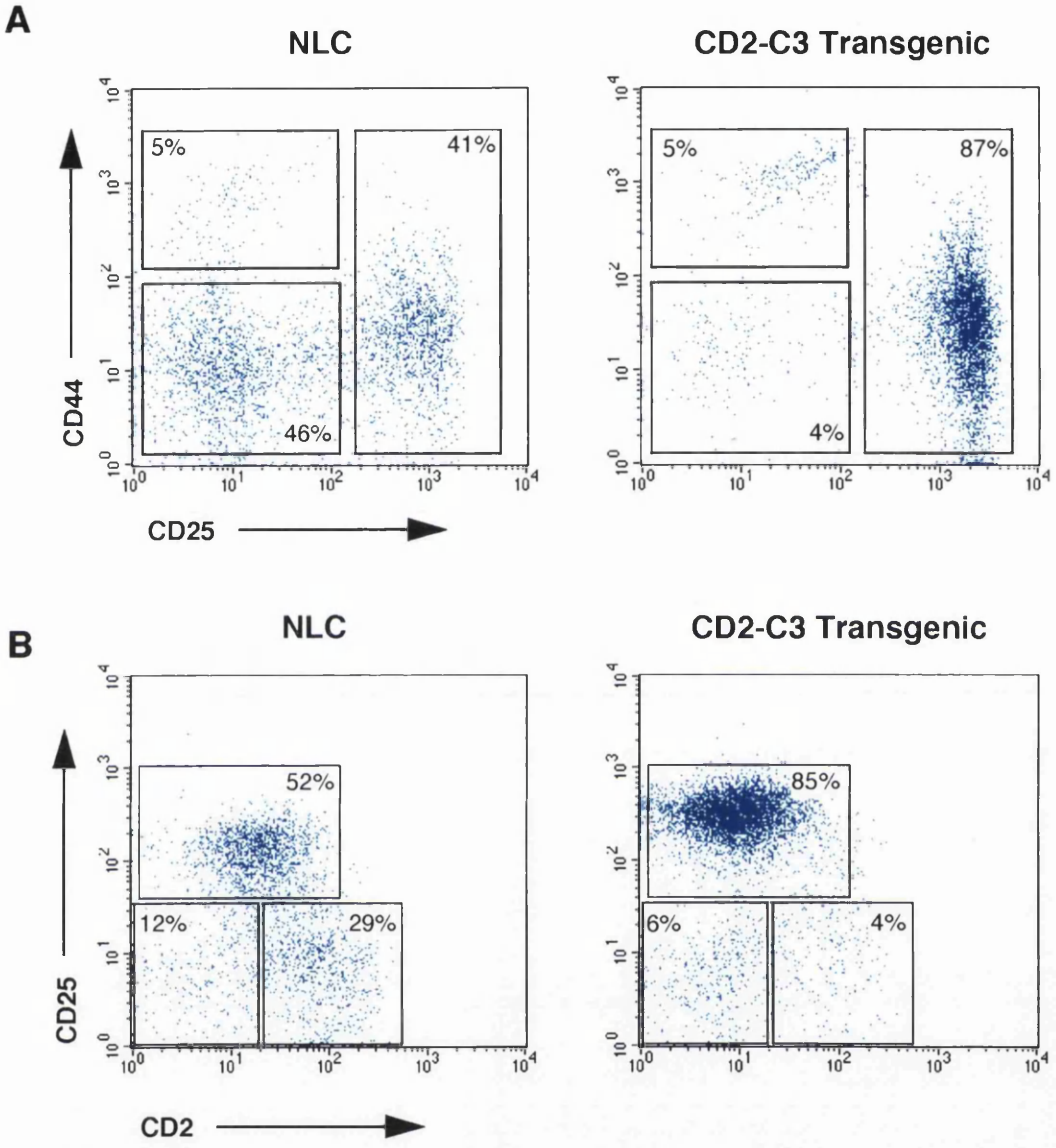

Figure 3.6 - Analysis of the CD4/8 DN population from CD2-C3 transgenic mice.

(A) Thymocytes were analysed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes as well as all cells of non-T cell lineage using a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr1bio) revealed with streptavidin tricolour, and co-stained with anti-CD44-PE and anti-CD25-FITC.

(B) Thymocytes were analysed for CD25 and CD2 expression by lineage exclusion of all CD4/8 DP and SP thymocytes are well as all cells of non-T cell lineage using a panel of biotinylated antibodoes revealed with streptavidin tricolour and co-stained with anti-CD2-FITC and anti-CD25-PE.

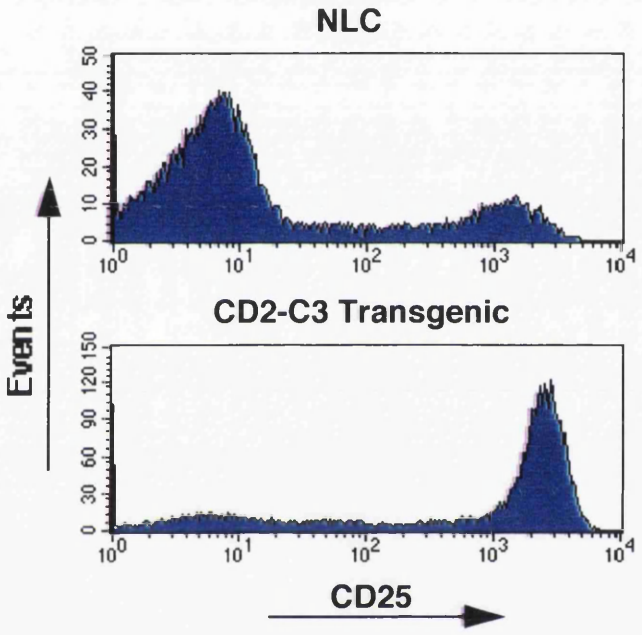

Figure 3.7 - Level of expression of CD25 on CD4/8 DN thymocytes from NLC and CD2-C3 transgenic mice.

CD25 expression levels on lineage negative thymocytes from NLC and CD2-C3 transgenic mice was determined by antibody staining with a panel of biotinylated antibodies and anti-CD25-PE and subsequent flow cytometry analysis.

3.2.7 - Analysis of β chain rearrangement in CD2-C3 Mice

Differentiation of $CD44^{-}25^{+}$ thymocytes is dependent upon cells successfully rearranging their β loci and expressing a functional pre-TCR on their surface; failure of β chain rearrangement results in a differentiation block as observed in RAG-1 or -2 deficient mice. Therefore, in order to investigate the cause of the differentiation defect seen in the absence of Rho function, the presence of rearranged β chains in $CD25^{+}$ cells from CD2-C3 mice was investigated by intracellular staining with an antibody reactive to a common β chain epitope (Buer *et al.*, 1997). β chain rearrangement is accompanied by a transition from small 'E' cells (β chain negative) to large blastoid 'L' cells (all of which express rearranged β subunits) (Hoffman *et al.*, 1996). $CD25^{+}$ E and L cells are present in CD2-C3 mice. $CD25^{+}$ L cells from CD2-C3 transgenic mice all expressed readily detectable levels of intracellular TCR β subunits, although at slightly lower than normal levels (Figure 3.8). These data suggest that loss of Rho function does not prevent β chain rearrangement and provides further evidence that Rho may play an essential role in pre-TCR signalling.

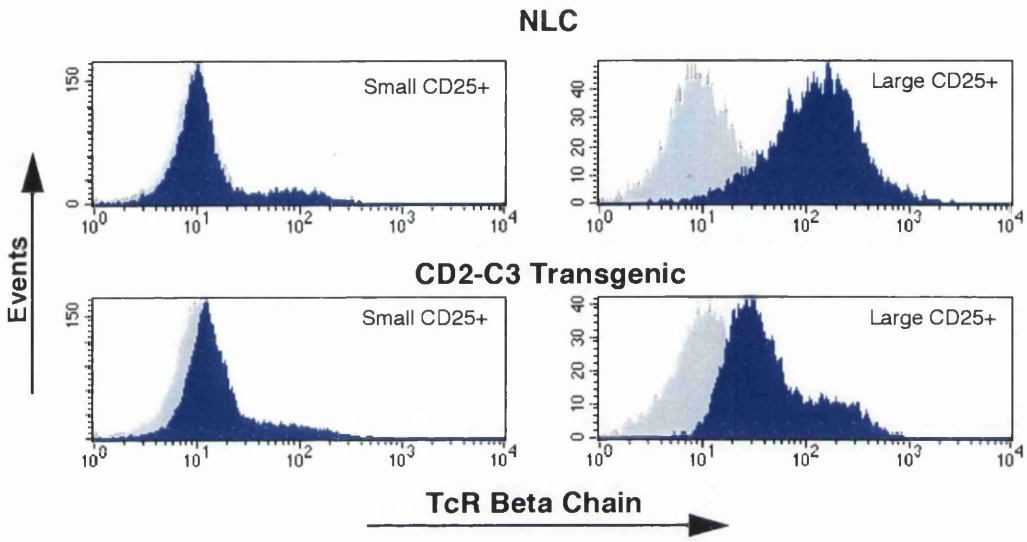

Figure 3.8 - Expression of rearranged β chain in CD25⁺ thymocytes

Thymocytes from NLC and CD2-C3 mice were stained with anti-CD25-PE before saponin permeabilisation and staining with an antibody to the common β chain epitope. Histograms show levels of intracellular β chain (Black region) and surface β-chain (Grey region) on both small 'E' and large 'L' CD25⁺ thymocytes.

3.2.8 - Expression of a functional TCR in CD2-C3 Mice

Although loss of Rho function does not prevent TCR β chain rearrangement, CD2-C3 mice displayed slightly reduced levels of intracellular β chain in $CD25^+$ L cells. These reduced levels may be either a cause or a consequence of a pre-TCR signalling defect in the absence of Rho function; like many antigen and cytokine surface receptors, the expression of the Pre-TCR is thought to undergo positive feedback in such a way as to generate signals to up-regulate its own expression .

One way to explore this issue further was to investigate whether expression of a functional TCR complex in CD4/8 DN cells can overcome the block in thymocyte development that occurs in the CD2-C3 mice; the thymocyte differentiation block in cells which fail to express a pre-TCR complex has been shown to be overcome by expression of transgenes encoding TCR αβ chains. For example, RAG-deficient mice lack expression of a functional pre-TCR as thymocytes fail to rearrange their β loci. T cell differentiation is blocked at the $CD44^-25^+$ stage resulting in a small hypocellular thymus similar to that seen in the CD2-C3 mice. Introduction of a functional TCRβ transgene into the RAG-1 or RAG-2 deficient background promotes thymocyte differentiation to the CD4/8 DP stage and leads to an increase in thymic cellularity to normal levels (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1993).

To determine whether TCR expression could induce the differentiation of thymocyte progenitors into CD4/8 DP cells in CD2-C3 transgenic mice, these mice were crossed with HY-TCR transgenic mice. The HY-TCR recognises a male antigen-derived peptide in the context of MHC class I molecule and thus male HY-TCR transgenic mice show massive deletion of CD4/8 DP thymocytes (Kisielow *et al.*, 1988; Swat *et al.*, 1991). Conversely, female HY-TCR transgenic animals show positive selection of CD8 single positive thymocytes (Figure 3.9). HY-TCR surface expression levels were similar on CD4/8 DN thymocytes isolated from both HY-TCR and HY-TCR/CD2-C3 transgenic mice (Figure 3.10A). Female HY-TCR/CD2-C3 double transgenic mice contain no CD4/8 DP cells (Figure 3.10B). Thus, expression of a stable, mature TCR complex cannot induce the differentiation of pre-T cells from the CD4/8 DN into the DP stage in CD2-C3 mice. These

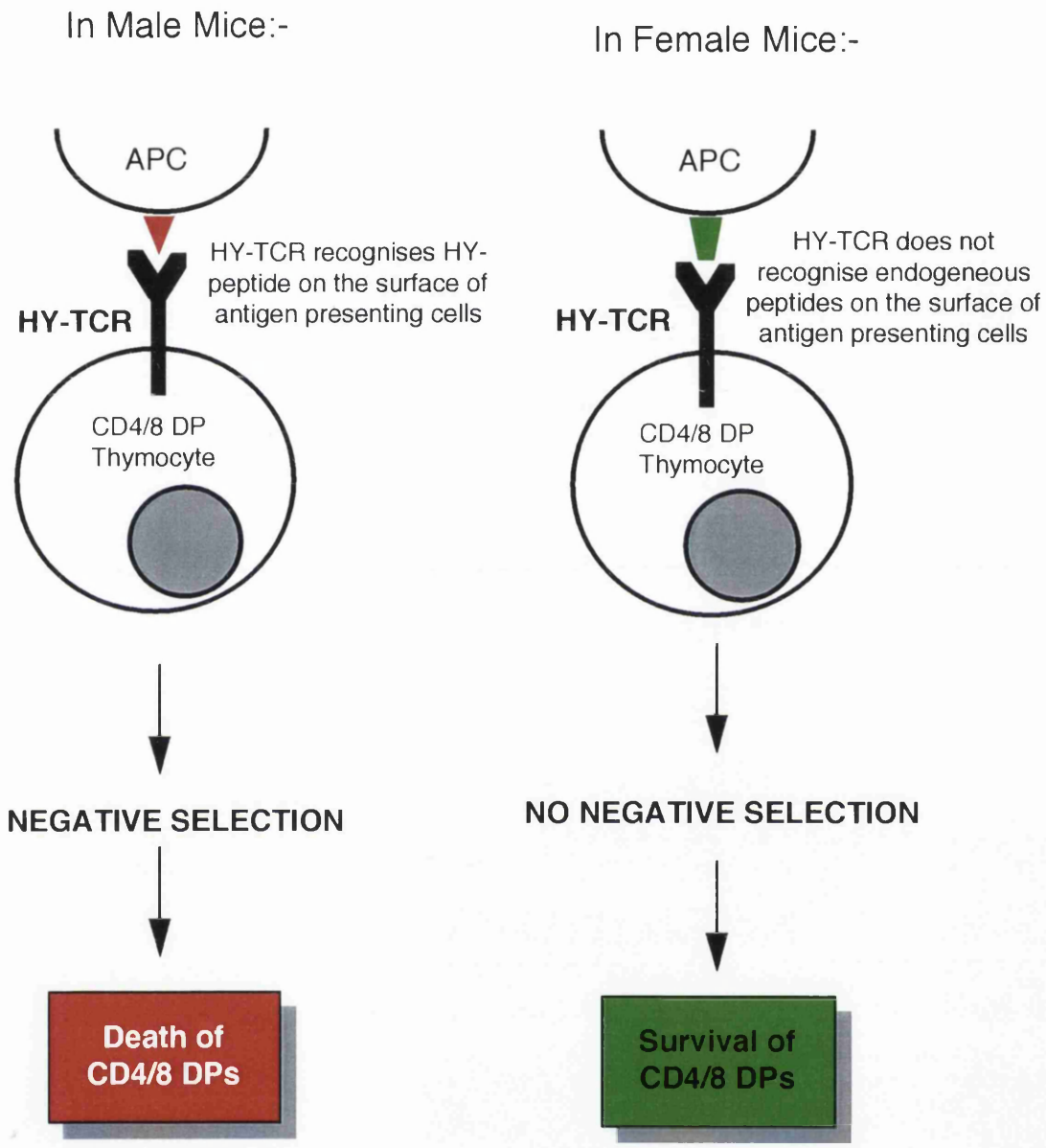

Figure 3.9 Model of HY-TCR recognition of Male surface peptides but not Female peptides.

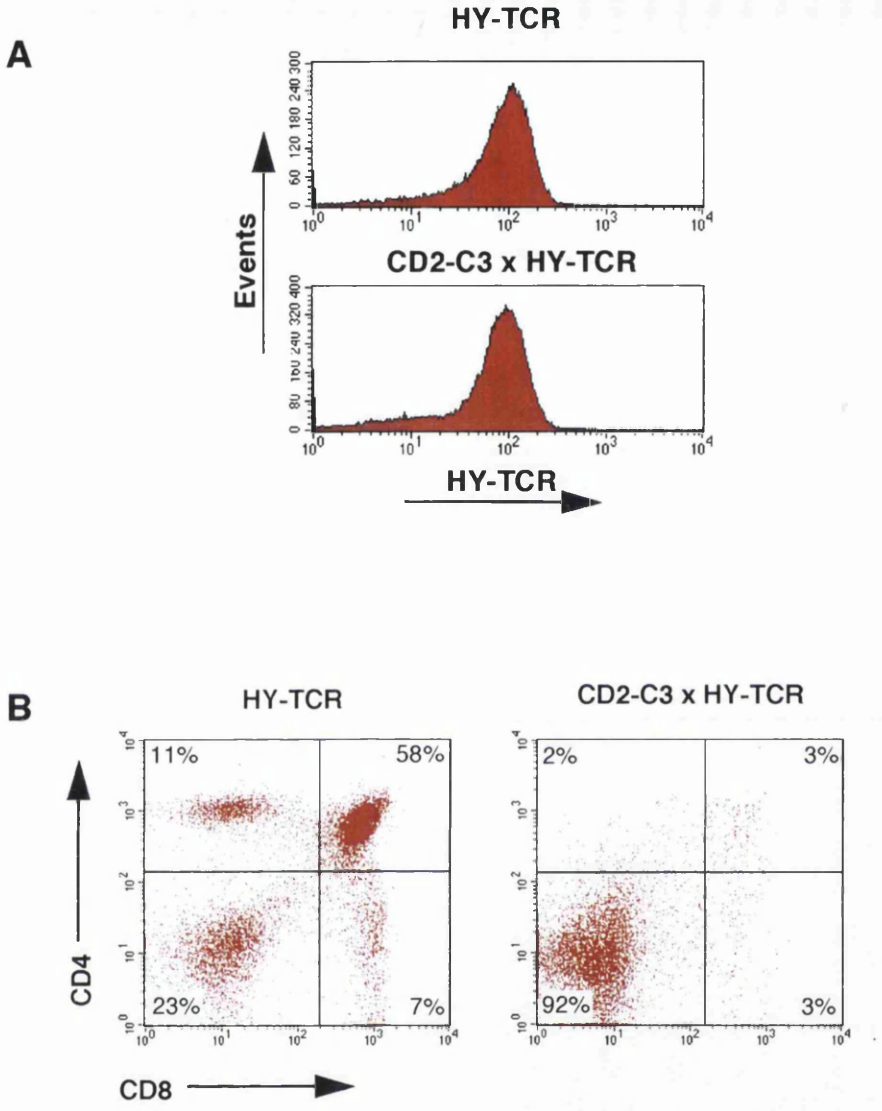

Figure 3.10 - Effect of functional TCR expression on CD2-C3 thymocytes

(A) Expression of the HY-TCR on CD4/8 DN thymocytes isolated from female HY-TCR transgenic littermate controls and female CD2-C3/HY-TCR double transgenic mice. Thymocytes were stained with anti-CD4-PE, anti-CD8-FITC, and anti-HY-Bio, revealed with streptavidin tricolour and the DN cells analysed for expression of HY-TCR.

(B) Analysis of the CD4/8 populations in female HY-TCR and CD2-C3/HY-TCR mice. Thymocytes were stained with anti-CD4-PE, anti-CD8-FITC, and anti-HY-Bio, revealed with streptavidin tricolour and analysed. CD4/8 profiles are shown for HY-TCR positive thymocytes.

results do not preclude that Rho might regulate surface expression/stability of a pre-TCR complex. Nevertheless, they show that when there is stable TCR expression, antigen receptor signalling requires endogenous Rho function to drive thymocyte differentiation at the pre-T cell stage. Hence, these studies show that Rho function is required for signalling by the pre-TCR complex and identify Rho as an intracellular switch for the critical thymic checkpoint of TCR β selection.

3.2.9 - Peripheral Phenotype of the CD2-C3 Mice

Initial analysis had indicated that CD2-C3 mice had very few peripheral T cells. To characterise this defect further and to assess whether the CD2-C3 transgenics acquired peripheral αβ T cells with increasing age, the total number of splenocytes and number of peripheral αβ T cells in the spleens of a series of CD2-C3 transgenic mice and their non-transgenic littermate controls were calculated at various ages from 1 week to 13 weeks after birth (Figure 3.11 and 3.12). This analysis revealed that the absolute numbers of αβ T cells were reduced at all ages studied. Surprisingly however, this reduction in the number of αβ T cells was not accompanied by a decrease in the total cellularity of the spleen. This would suggest that the absence of αβ T cells in the spleen is being compensated by an increase in another type of cell.

Clearly expression of C3 under the control of the human CD2 promoter severely reduced the numbers of peripheral αβ T cells. However, it was of interest to investigate whether inhibition of Rho function during thymocyte development resulted in a reduction in the numbers of other lymphoid lineages, for example, γδ T cells and NK cells. γδ T cells were identified by staining splenocytes with antibodies reactive to the γδ TCR and CD3, and subsequent flow cytometry, while NK cells were identified with use of the NK-specific antibody, NK1.1. The graph in figure 3.13 shows the absolute numbers of peripheral αβ, γδ, NK and B cells in 6 week old wild-type and CD2-C3 mice. Although αβ T

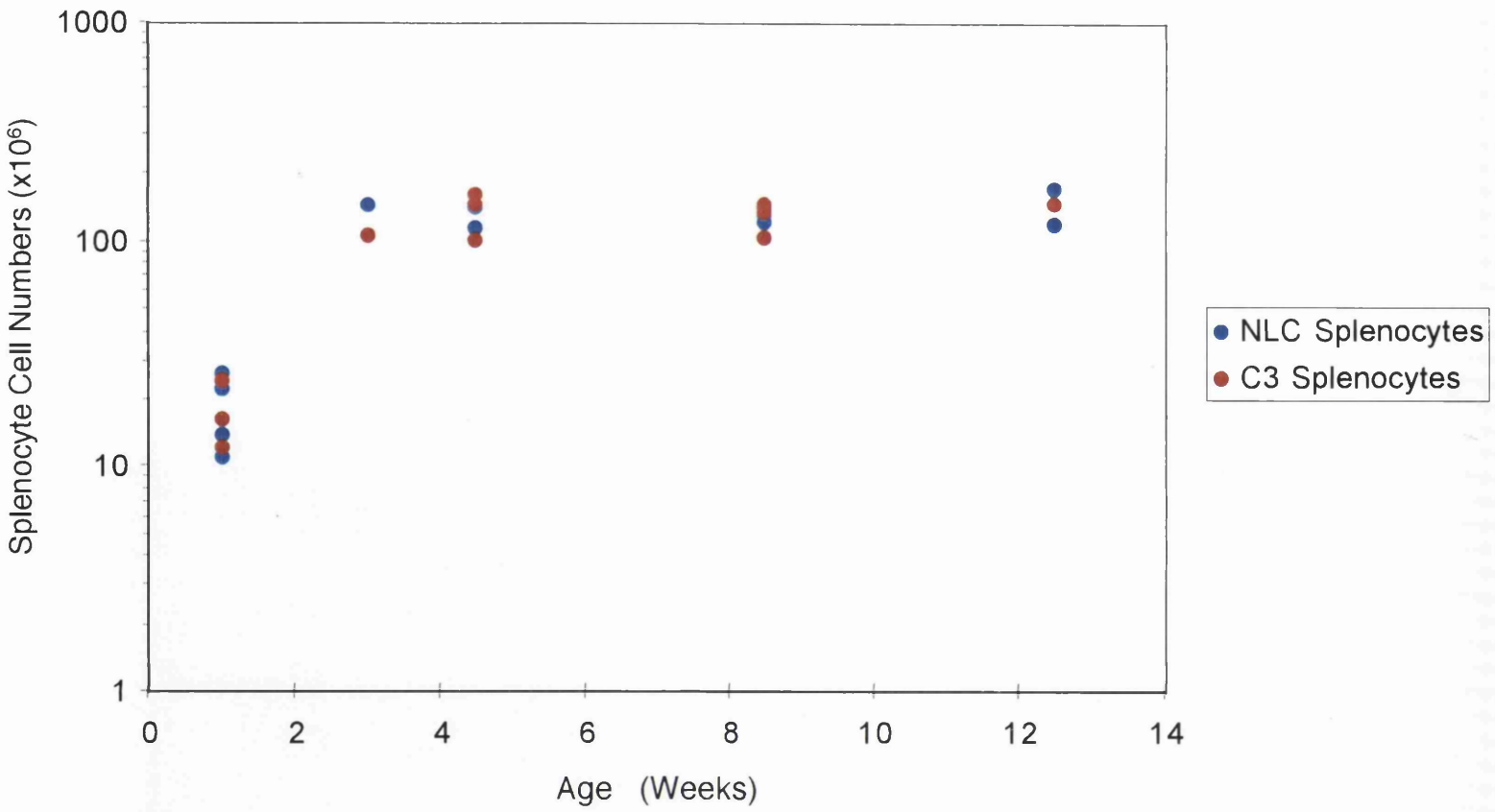

Figure 3.11 - Graph of splenocyte numbers of NLC and CD2-C3 Mice aged between 1 -13 weeks.

Splenocytes were isolated and counted from CD2-C3 transgenic and Non-transgenic Littermate control mice aged between 1 and 13 weeks of age

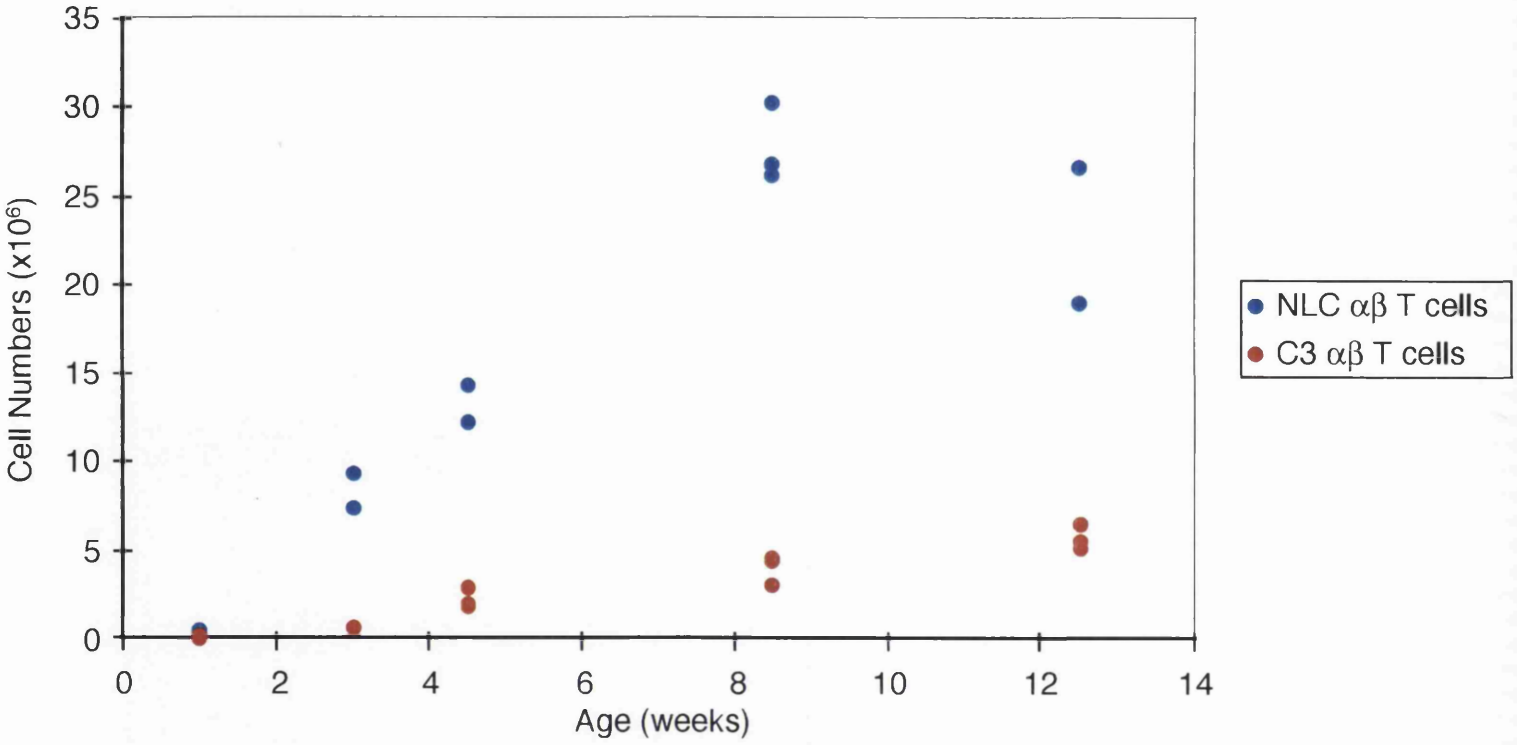

Figure 3.12 - Graph of peripheral αβ T cell numbers from NLC and CD2-C3 aged between 1 -13 weeks.

Splenocytes were isolated from CD2-C3 transgenic and Non-transgenic Littermate control mice aged between 1 and 13 weeks of age, stained with antibodies reactive to the αβ Tcell receptor and analysed by flow cytometry.

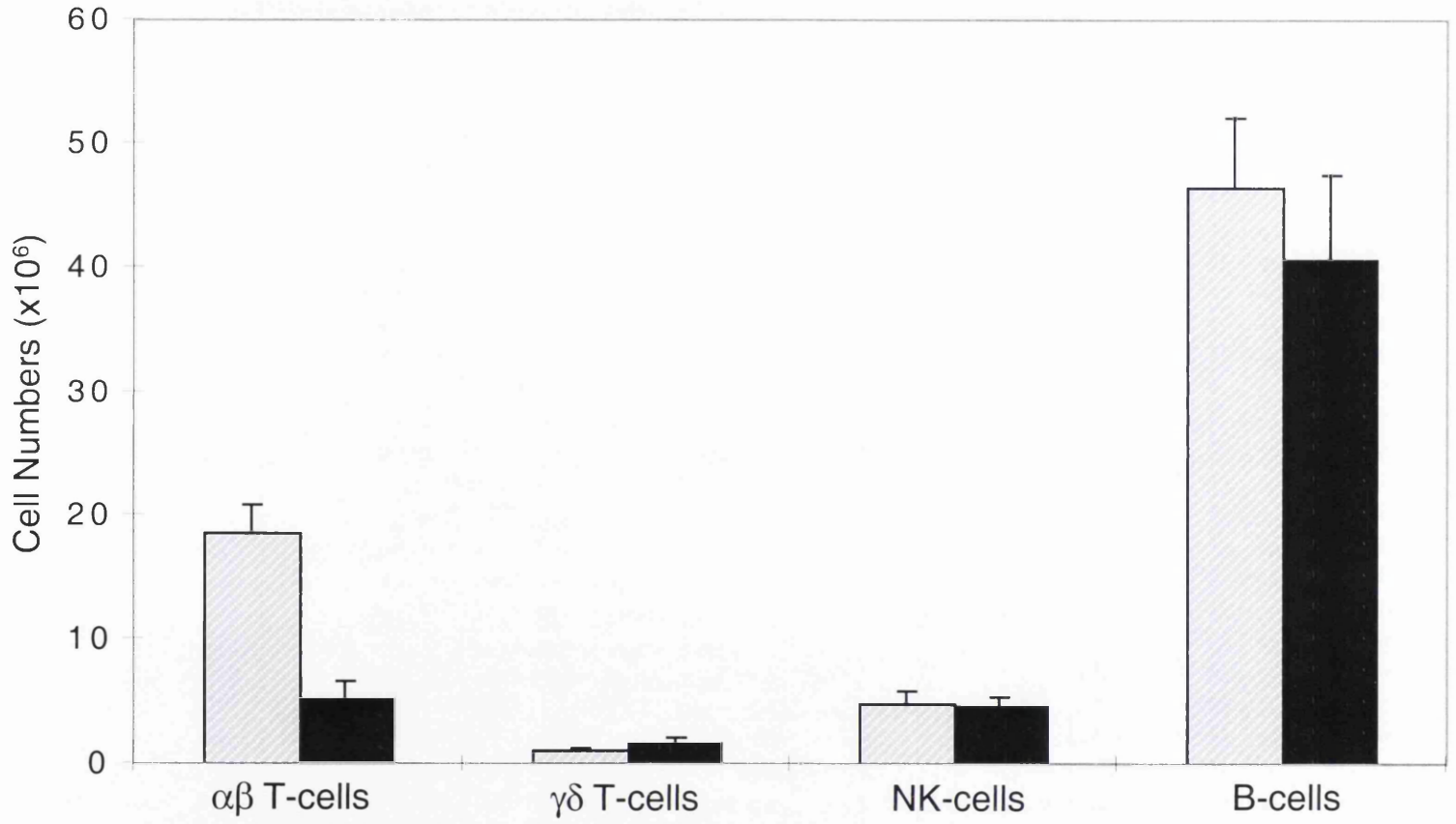

Figure 3.13 - Peripheral Cell numbers in CD2-C3 transgenic and Wild-type mice

Absolute numbers of peripheral cells from 4-6 week old non-transgenic (shaded bars) and CD2-C3 transgenic mice (solid bars). Splenocytes were stained with antibodies reactive to αβ, γδ, NK, and B220 surface markers, analysed by flow cytometry and absolute cell numbers calculated on the basis of percentages obtained (n=8).

cells are reduced in number, the absolute numbers of splenic γδ, NK, and B cells all appear to be within the normal range in CD2-C3 mice.

As murine CD2 is known to be expressed on both γδ and NK cells, it was of interest to investigate the expression of the human CD2-LCR driven C3-transgene in peripheral NK and γδ cells in these mice (Haas *et al.*, 1993; Fahlen *et al.*, 1997). Spleens were isolated from both normal and transgenic mice, stained with antibodies reactive to γδ and NK surface markers, and 100,000 cells sorted on a FACS Vantage cell sorter under sterile conditions. RNA was prepared from these samples, reverse transcribed to make single-stranded cDNA, and the presence of the C3-transferase determined by a PCR-based screen. Figure 3.14 shows that C3-transcript was not detectable in NK nor γδ cells although was clearly present in sorted $CD25^+$ cells from a CD2-C3 thymus. Thus, in the transgenic mice described here, C3 transferase is not expressed in either NK or γδ cells and these cells develop normally in the CD2-C3 mice.

A further subset of lymphoid cells reported to develop along a similar lineage to αβ T cells are the NK-T cells. These cells share receptor structures common to both NK cell and αβ T cells, namely the NK1.1 receptor, the αβ TCR, and CD3 (Reviewed in MacDonald, 1995). Whereas most mature αβ T cells express either CD4 or CD8 coreceptors, NK-T cells are almost exclusively either $CD4^+$ or $CD4^-8^-$. Although little is known about the expression of the human CD2 promoter in these cells, their similar developmental pattern to αβ-T cells suggests that the CD2 promoter may be expressed. As the highest abundance of NK-T cells is in the adult liver, livers were removed from both transgenic and wild-type 10 week old mice, single cell suspensions made before density-gradient centrifugation to isolate mononuclear cells. These were then stained with antibodies reactive to the NK1.1 surface receptor and the αβ TCR and analysed by flow cytometry. The data in figure 3.15 show that in the CD2-C3 mice, the NK-T cell population is effectively absent as are αβ T cells in the liver. However, as observed in the spleens of CD2-C3 transgenic mice, the number of classical NK cells is within the normal range. These data reveal that Rho function is essential for the development of NK-T cells.

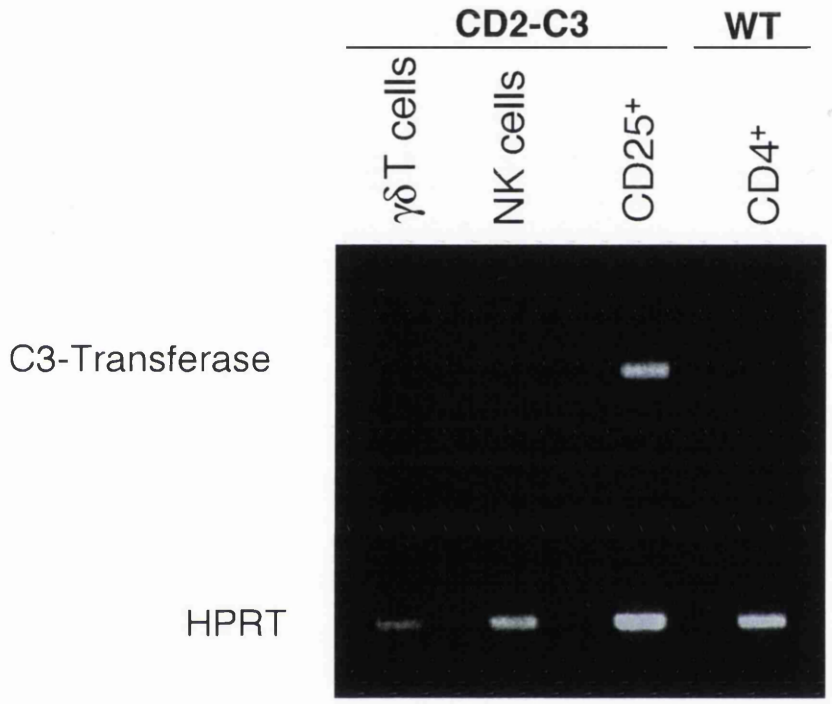

Figure 3.14 - Analysis of C3-transferase transcript in γδ and NK cells.

RT-PCR analysis of C3 Transferase expression in 100,000 sorted γδ, NK and $CD25^+$ cells from CD2-C3 transgenics and $CD4^+$ T cells from wild type mice. RNA was isolated by standard protocols and cDNA prepared. The presence of the C3 and HPRT transcripts was determined by PCR.

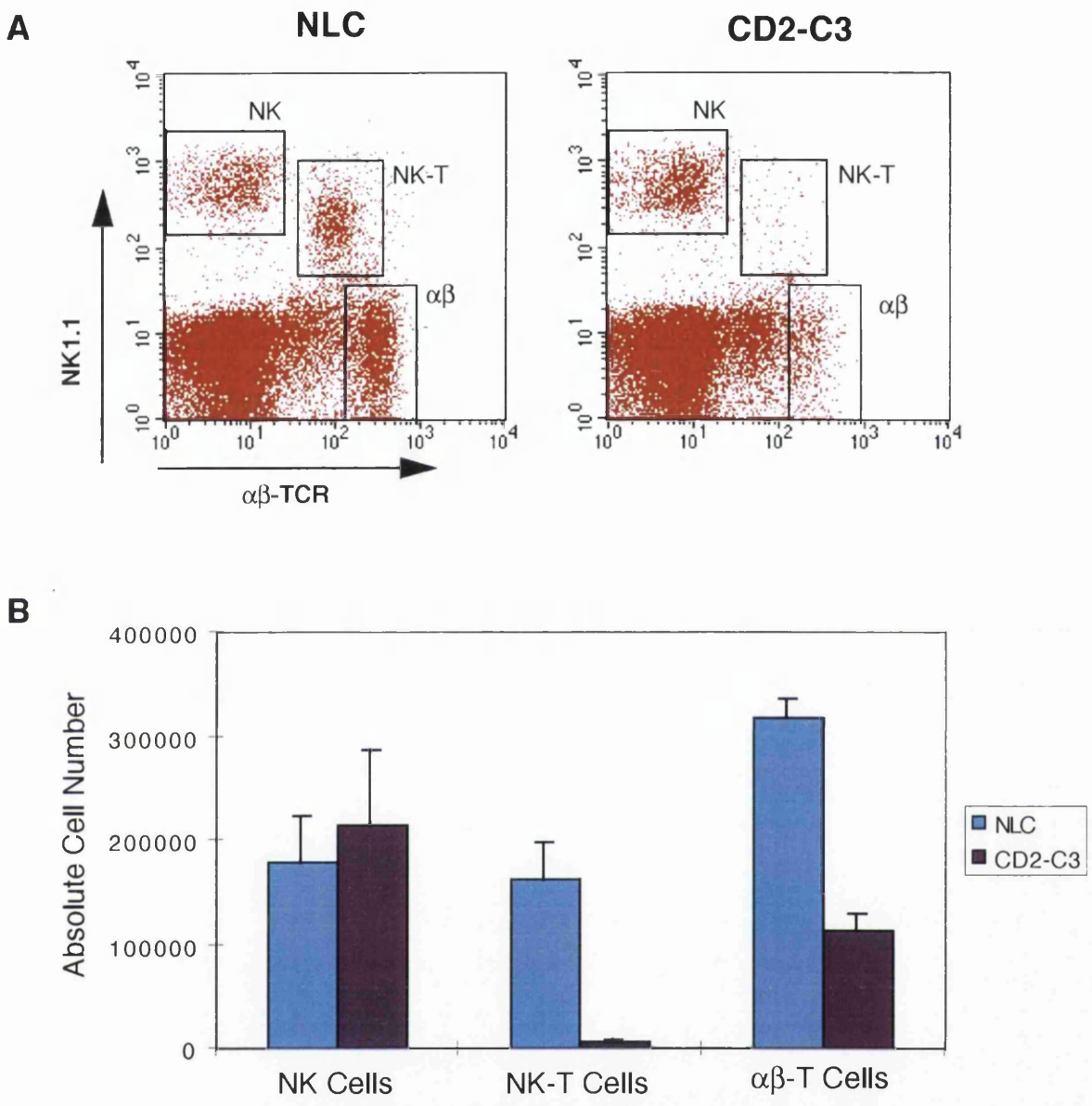

Figure 3.15 - Analysis of NK-T cells in the Liver of CD2-C3 Mice.

(A) The cell preparations from livers of CD2-C3 transgenic and Non-transgenic littermate control mice were stained with an antibody reactive to the NK cell marker, NK1.1 and an antibody reactive to the αβ-TCR prior to analysis by flow cytometry.

(B) Absolute cell numbers of NK, NK-T and αβ-T cells in the liver were calculated from CD2-C3 and non-transgenic littermate control mice (n=5).

3.3 - Discussion

The present study has used the human CD2-locus control region to generate transgenic mice that have selectively eliminated function of the GTPase Rho in $CD25^+$ early pre-T cells. Analyses of these mice show that inhibition of Rho function in pre-T cells causes a developmental block at the $CD44^-25^+$ stage of thymopoiesis. Thymocytes from CD2-C3 mice show elevated levels of CD25 expression at this stage; CD25 is normally down-regulated upon β-chain rearrangement and expression of a pre-TCR complex on the thymocyte surface membrane. Mice lacking expression of the pre-Tα chain or the src kinases p56lck and p59fyn show a differentiation block at the $CD44^-25^+$ stage and have elevated levels of CD25 (Groves *et al.*, 1996; Aifantis *et al.*, 1997; Krotkova *et al.*, 1997). Therefore, a development block at the $CD44^-25^+$ stage, accompanied with an increase in CD25 expression is indicative of failure of the pre-TCR complex to either be expressed or to signal correctly. In order to determine whether loss of Rho function at the pre-T cell stage resulted in an expression or signalling defect, intracellular β chain levels in $CD25^+$ cells were analysed. The presence of rearranged β chain in the CD2-C3 mice suggests that the block is due to a defect in pre-TCR signalling and not expression. Indeed, this differentiation block at the $CD44^-25^+$ DN stage cannot be rescued by expression of a heterologous antigen receptor complex, demonstrating that antigen receptor signalling requires endogenous Rho function to drive thymocyte differentiation at the pre-T cell stage. These results thus identify the GTPase Rho as a controlling intracellular switch for the critical thymic checkpoint of TCR β selection.

The reduction in the numbers of peripheral αβ T cells is a direct result of the differentiation block at the $CD25^+$ stage of thymocyte development. More striking however, is the effective absence of NK-T cells; cells of lymphoid origin that follow a similar developmental path to that of αβ T cells. The data presented here would suggest that Rho function is essential for the development of these cells as their numbers are severely reduced in the CD2-C3 mice. The reduction in peripheral cell numbers is not global, as CD2-C3 mice develop normal numbers of both 'classical' NK cells and γδ T cells, in neither of which can C3-transferase expression be detected.

Although γδ T cells and NK cells are both known to express murine CD2 on their surface, expression of murine CD2 and the human CD2-LCR are known to not always share the same pattern of expression; B cells isolated from mice are known to express murine CD2, but have been shown not to express the human CD2-LCR (Zhumabekov *et al.*, 1995). Despite these differences in expression, the CD2-LCR has previously been used to express transgenes in NK cells. In a study by Fahlen *et al.*, the inhibitory Ly49A receptor was expressed on thymocytes, peripheral T cells and NK cells (Fahlen *et al.*, 1997). It is therefore intriguing that C3-transferase was not expressed in the NK cells isolated from CD2-C3 mice. This lack of expression of C3 in the NK cells may be due to differences in the integration sites between the CD2-C3 mice and those described elsewhere. This idea however challenges the model in which the CD2-LCR gives position-independent transcription of transgenes under its control. Although the CD2-LCR confers position-independent transcription in T-cells, this may not be the case in all cell-lineages.

As discussed in Chapter 1, γδ cells and αβ T cells originate from the same progenitor population, with γδ cells branching off at the $CD25^+$ stage of development. As the human CD2-LCR is reported to express at this stage, it may be expected that γδ-cell progenitors express C3-transferase in the CD2-C3 transgenic mice. The failure to see expression in peripheral γδ cells of the CD2-C3 mice suggests that there is either heterogeneity in expression within the $CD25^+$ progenitor population with cells destined to become αβ T cells expressing C3-transferase and those committed to the γδ lineage not expressing the transgene, or that lineage commitment takes place prior to expression of C3-transferase. As no surface markers exist for γδ precursors, these issues would be difficult to resolve.

The introduction of transgenic technologies over the last few years has led to huge advances in understanding T cell development. Of particular interest and relevance to the study presented here, the use of transgenic models has allowed the molecular basis of pre-T cell signalling to be explored. Much of this work has employed RAG-deficient mice as their absence of a pre-TCR receptor enables the role of various signalling molecules downstream of the pre-TCR complex to be investigated (Chen *et al.*, 1994a). For example, the protein tyrosine kinase $p56^{lck}$ has been implicated in signalling downstream of the pre-TCR as expression of a constitutively active $p56^{lck}$ transgene on a

RAG null background allows differentiation and proliferation of pre-T cells to the CD4/8 DP stage (Mombaerts *et al.*, 1994). These results strongly suggest that $p56^{lck}$ may normally be involved in pre-TCR mediated signalling. Such genetic crosses provide a powerful method to explore the role of signalling molecules in receptor-mediated signalling *in vivo* and this type of approach can also be applied to analysis of the transgenic mice described here. The thymocytes from the CD2-C3 mice have a defect in pre-TCR mediated signalling caused by loss of Rho function. The use of these mice should provide a model in which to study the involvement of other molecules in Rho-mediated pre-TCR signalling. Transgenic mice exist that express constitutively active signalling molecules which may function downstream of Rho. By breeding these transgenics with the CD2-C3 mice, it may be possible to position Rho within a pre-TCR initiated signalling cascade. For example, if expression of an active kinase rescued thymocyte development in the CD2-C3 mice, then Rho would be implicated in having a role upstream of the rescuing kinase. (Such an analysis is presented in Chapter 6 where the role of the serine/threonine kinase Raf is explored in pre-T cells lacking Rho function.)

Further insight into the molecular basis of pre-TCR signalling may be gained by applying biochemical techniques. By isolating thymi from CD2-C3 mice, a relatively pure population of cells, all blocked at the pre-T cell stage can be obtained. These thymocytes, in which pre-TCR mediated signalling has been blocked by inhibition of Rho function could then be compared to wild type pre-T cells in which pre-TCR signalling is intact. By using techniques such as kinase assays and Western blots, it should be possible to ask what pre-TCR mediated pathways are inactive in the pre-T cells lacking Rho function, and hence gain greater insight into why Rho is required for pre-TCR mediated signalling.

In summary, previous work had shown that Rho regulates survival and proliferation of thymocyte progenitors but found no evidence that this GTPase controlled signalling events that regulate thymocyte differentiation. One of the initial aims of generating the CD2-C3 mice was to investigate whether the proliferative defect seen in the lck-C3 mice was a independent from the survival defect caused by loss of Rho function, or whether the cells selected for their ability to avoid apoptosis had simply reduced proliferative capacity. Analysis of the CD2-C3 mice reveals neither a survival defect nor a proliferative problem; rather a complete block in pre-T cell differentiation.

Therefore, although the phenotype of the CD2-C3 mice has made it impossible to resolve the survival/proliferation issue arising from the lck-C3 mice, it has revealed a novel role for Rho in the process of pre-T cell differentiation. Thymocytes expressing an inhibitor of Rho function directly in $CD25^+$ pre-T cells show a block in differentiation and is consistent with a model in which Rho function is required for signalling by the pre-TCR complex either directly or as part of parallel signalling pathways operating at this stage of thymocyte development. Further analysis of these mice provide the exciting possibility to understand the role of Rho in pre-TCR signalling on a molecular level.

CHAPTER 4
A Comparison of the CD2-C3 and lck-C3 Transgenic mice

4.1 - Introduction

Previously, the generation of transgenic mice expressing C3-transferase under the control of p56lck proximal promoter have been described (Henning *et al.*, 1997). lck-C3 mice have two defects; increased apoptosis of both $CD44^{+}25^{+}$ and $CD44^{-}25^{+}$ cells, and a partial proliferative inhibition of $CD44^{-}25^{-}$ cells (Henning *et al.*, 1997; Gallandrini *et al.*, 1997). The data presented in chapter 3 describes the generation and characterisation of a second series of C3-transgenic mice. With use of the CD2-LCR, Rho function has been inhibited at a later development stage and reveals a role for Rho in pre-T cell differentiation.

The difference between the CD2-C3 and the lck-C3 transgenic mice is in the timing of expression of the C3 transgene. The present chapter compares and contrasts the phenotype of the lck-C3 and the CD2-C3 transgenic mice and shows that cell type specific inhibition of Rho can reveal different functions of this small GTPase.

4.2 - Results

4.2.1 - Gross Morphology of Thymi from CD2-C3 and lck-C3 Mice.

The photograph in figure 4.1A shows a thymus from an age-matched lck-C3, CD2-C3 and wild type C57BL/6 mouse. The thymi from lck-C3 mice are small and hypocellular compared to nontransgenic littermate controls and in terms of gross external morphology are difficult to distinguish from CD2-C3 thymi. Thymocyte cell numbers are also drastically reduced in both C3-transgenics with lck-C3 thymi containing $8 \pm 3x10^6$ thymocytes and CD2-C3 thymi $2.5 \pm 0.5 \times 10^6$ cells as compared to $160 \pm 10x10^6$ in a nontransgenic thymi (Figure 4.1B).

4.2.2 - Patterns of Thymocyte Differentiation in CD2-C3 and lck-C3 Mice.

Although initial comparison in terms of gross morphology of the two C3-transgenics shows little difference, when thymocytes from lck-C3 and CD2-C3 mice are analysed on the basis of CD4 and CD8 expression, two completely different patterns of thymocyte differentiation are seen. lck-C3 thymocytes comprise CD4/8 DN, DP and SP cells whereas, as shown in the previous chapter, CD2-C3 thymi are lacking all DP and SP populations. Moreover, analysis of the CD4/8 DN cells from lck-C3 and CD2-C3 mice reveal striking differences. CD4/8 DN cells from lck-C3 thymi are depleted of $CD25^+$ cells and comprise largely of $CD44^+25^-$ progenitors; in CD2-C3 transgenic mice $CD44^-25^+$ cells are the predominant population (Figure 4.2).

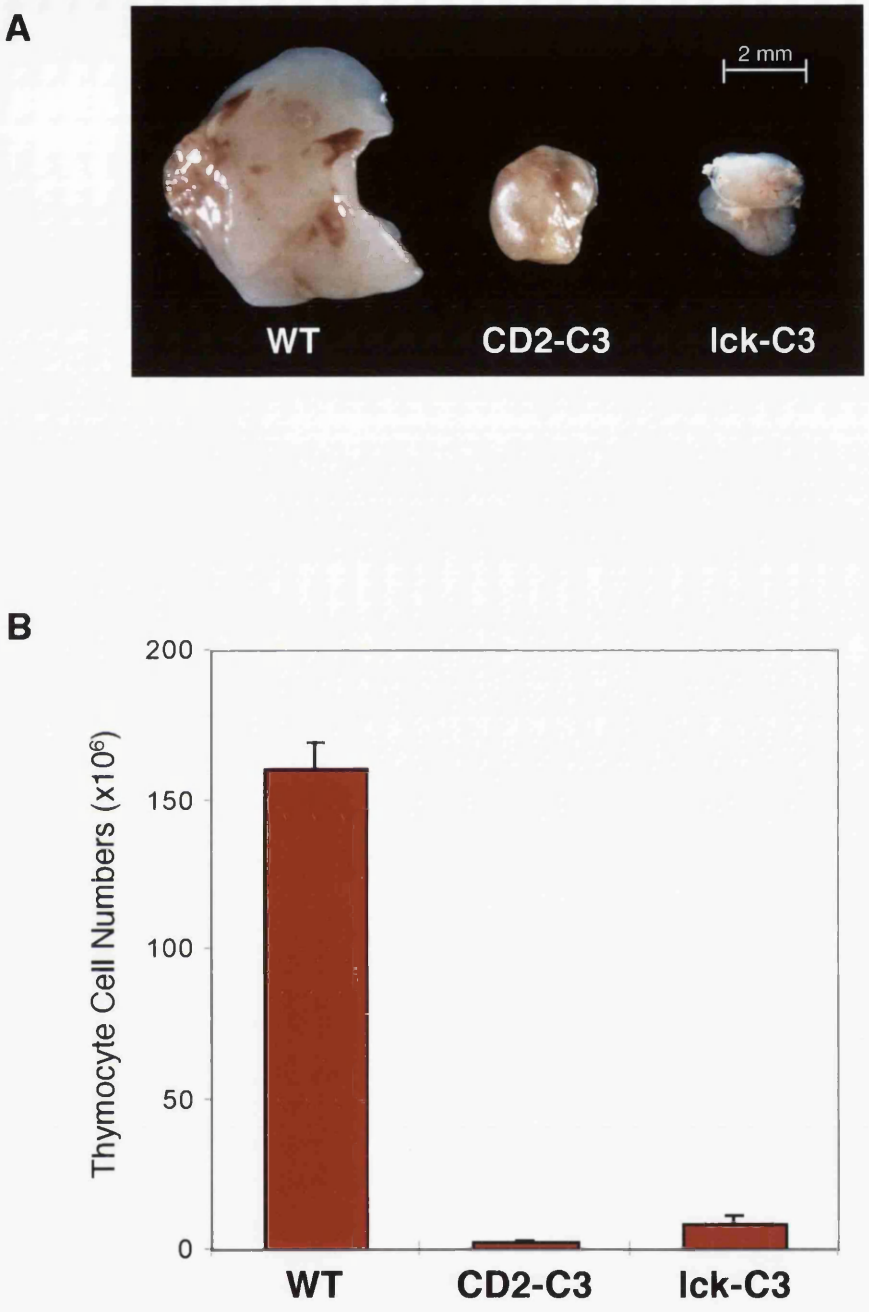

Figure 4.1- Comparision of the phenotype of CD2-C3 and lck-C3 transgenic mice

(A) Thymi isolated from a 4 week old wild type (WT), CD2-C3, and lck-C3 transgenic mice.

(B) Mean ± SE thymocyte cell numbers of 4 week old wild type (WT), CD2-C3, and lck-C3 transgenic mice (n=10).

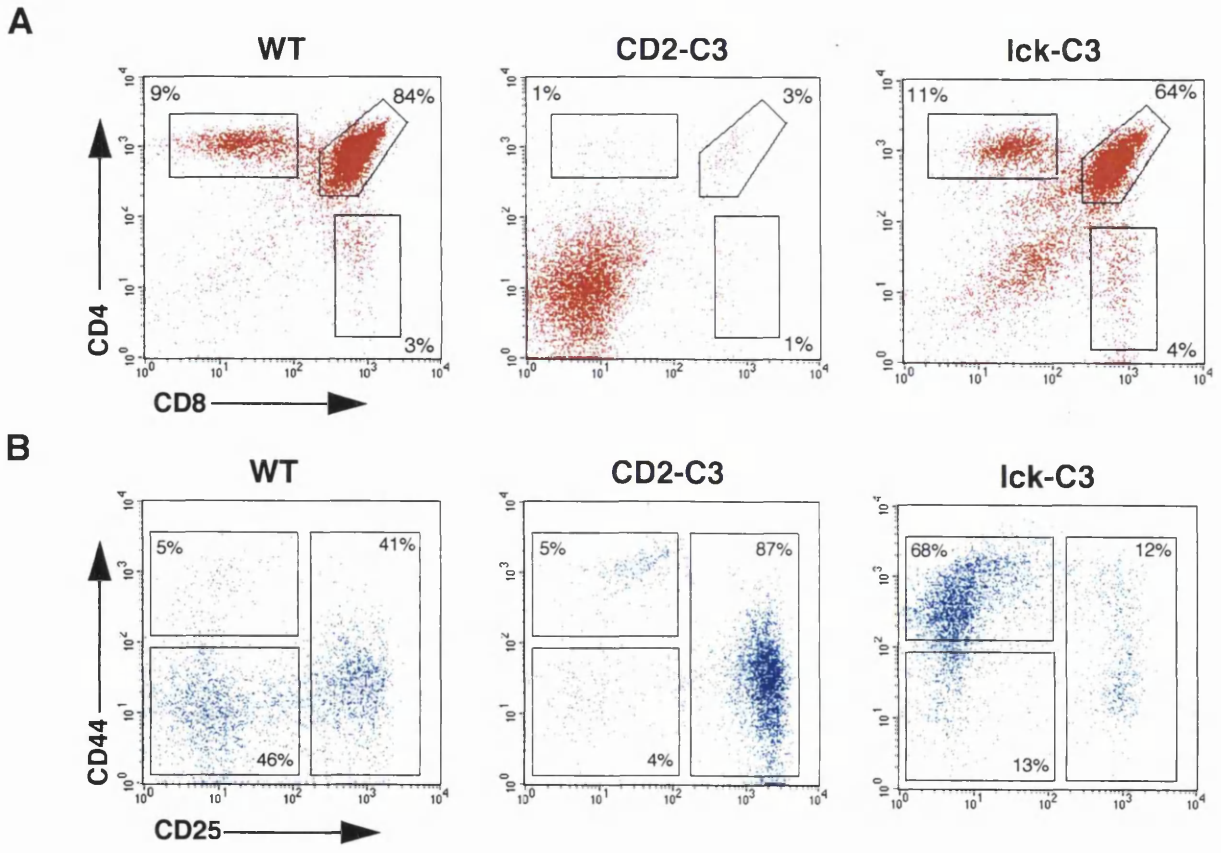

Figure 4.2 - Comparision of the thymic phenotype of CD2-C3 and Ick-C3 transgenic mice

(A) Analysis of the CD4/8 thymic subsets in CD2-C3 and Ick-C3 transgenic mice. Thymocytes from Ick-C3, CD2-C3 mice and wild type (WT) controls were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8.

(B) Analysis of the CD4/8 DN population from WT, CD2-C3 and Ick-C3 mice. Thymocytes were analysed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes plus all cells of non-T cell lineage using a panel of biotinylated antibodies revealed with streptavidin tricolour, and co-stained with anti-CD44-PE and anti-CD25-FITC.

4.2.3 - Analysis of Apoptosis in Early Thymocyte Progenitors.

The depletion of $CD25^+$ thymocytes in lck-C3 transgenic mice is explained by massive apoptosis of these cells (Gallandrini *et al.*, 1997). The increased levels of apoptosis can be visualised by staining the lck-C3 thymocytes with the fluorescent dye, 7-amino actinomycin D (7AAD) that detects apoptotic cells on the basis of increased cell membrane permeability (Schmid *et al.*, 1994). In contrast to lck-C3 mice, there was no significant difference in the numbers of apoptotic cells in the $CD25^+$ thymocytes isolated from normal or CD2-C3 transgenic mice (Figure 4.3). Hence, loss of Rho function by expression of C3-transferase under the control of the human CD2-LCR does not lead to increased apoptosis of $CD25^+$ cells.

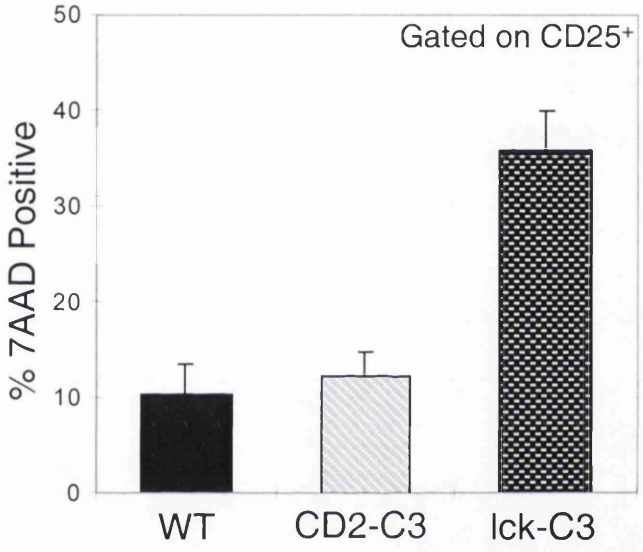

Figure 4.3 - Level of apoptosis of CD25+ cells from CD2-C3 and lck-C3 Mice.

Thymocytes isolated from 4 week old mice were stained with anti-CD25-FITC and the fluorescent dye 7AAD. Shown are the Mean ± SE percentages of 7AAD positive thymocytes present in the CD25+ compartments of WT, CD2-C3 and lck-C3 mice (n = 5).

4.2.4 - Kinetics of Expression of C3 under the control of the p56lck and CD2-LCR promoters

The reasons for these strikingly different phenotypes between the lck- and the CD2-C3 mice is either level or kinetics of expression of the transgene. Attempts to measure the level of C3-transferase protein expression in the transgenic mice by a number of different techniques including intracellular staining and Western blotting proved unsuccessful. However, it has previously been shown that all of the endogenous Rho from the lck-C3 mice is completely ribosylated, and hence inactivated (Henning *et al.*, 1997). The CD2-LCR is known to be a stronger promoter system than the proximal p56lck promoter (D.Cantrell, Personal Communication and Zhumabekov *et al.*, 1995) and it thus seems reasonable to assume that expression of C3 under the CD2-LCR would also cause complete inactivation of Rho within the cell. Although the levels of expression could not be measured and compared directly, it was possible to determine the kinetics of transgene expression by a RT-PCR based approach. RNA was prepared from total embryos (Days 10-12) or from embryonic thymi (Days 13-15) from both lck-C3 and CD2-C3 transgenic mice. Following reverse transcription, PCR was performed to detect the presence of the C3-transferase transcript. Figure 4.4A shows that the CD2-C3 transgene could be readily detected on day 12 of embryogenesis whereas the lck-C3 transgene was detectable on day 11. The kinetic differences in expression were also confirmed by RT-PCR from 50,000 $CD44^+25^-$, $CD44^+25^+$, and $CD44^-25^+$ FACS sorted cells from adult lck-C3 and CD2-C3 mice. These data, presented in figure 4.4B, confirmed expression of C3-transferase in $CD44^+25^-$ cells from lck-C3 mice; the CD2-driven C3 transgene was expressed later as $CD25^+$ cells appear.

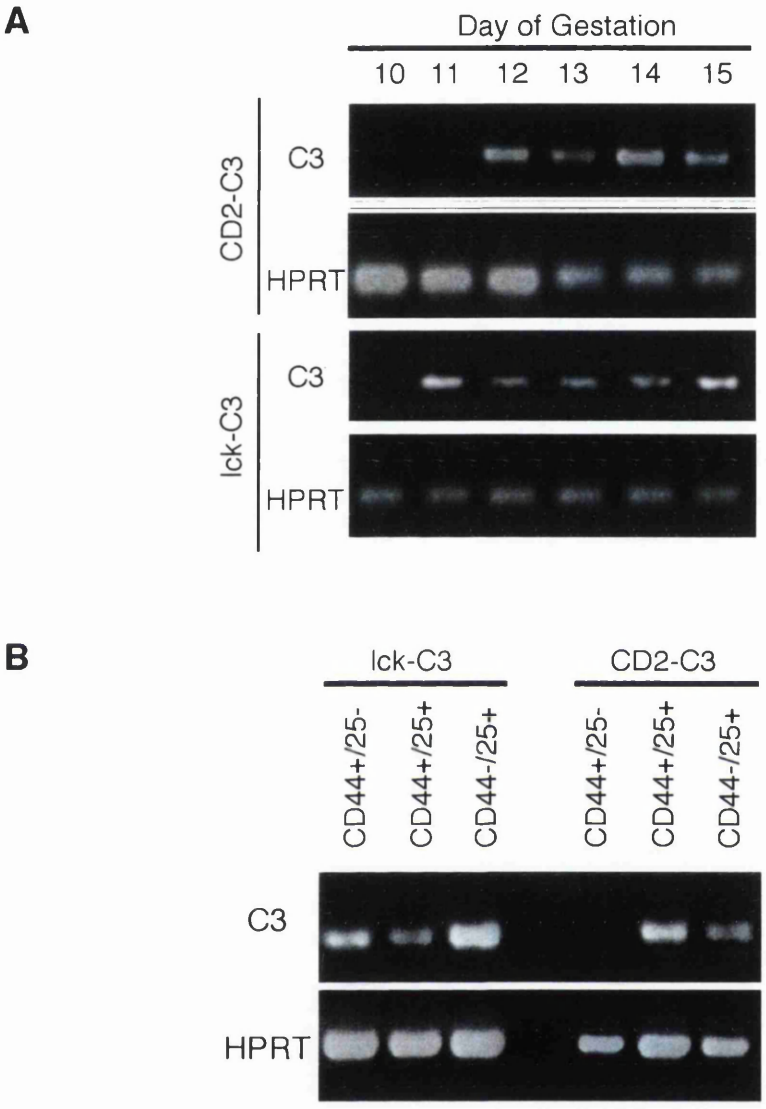

Figure 4.4 - Temporal Expression of C3-Transferase in CD2-C3 and lck-C3 mice.

(A) RT-PCR analysis of C3 Transferase expression in Fetal Thymocytes isolated from lck-C3 and CD2-C3 Transgenic Mice. RNA was prepared from total embryos (Day 10-12) and thymic lobes (Day 13-15), and cDNA prepared. The presence of the C3 and HPRT transcripts was determined by PCR.

(B) RT-PCR analysis of C3 Transferase expression in sorted $CD44^{+}25^{-}$, $CD44^{+}25^{+}$ and $CD25^{+}44^{-}$ thymocytes from lck-C3 and CD2-C3 Transgenic Mice. RNA was isolated by standard protocols and cDNA prepared. The presence of the C3 and HPRT transcripts was determined by PCR.

4.3 - Discussion

Henning *et al.* have previously analysed the role of Rho in thymocyte development in mice expressing C3-transferase targeted by the proximal p56lck promoter (Henning *et al.*, 1997; Gallandrini *et al.*, 1997). In the lck-C3 mice, Rho function is eliminated in the earliest thymocyte progenitors prior to expression of the CD25 marker as well as all subsequent populations. In CD2-C3 mice, Rho function is only eliminated in the $CD44^+25^+$ cells. Hence one difference between the lck-C3 and the CD2-C3 transgenic mice is the timing of expression of the C3 transferase. Comparisons of the phenotype of lck-C3 and CD2-C3 mice thus allow the effects of cell-type specific elimination of Rho function to be studied. These comparisons demonstrate that cell-type specific elimination of Rho can reveal different functions of this GTPase in thymocyte development. In the lck-C3 mice, Rho function is eliminated from the earliest detectable thymocyte population onwards. Loss of Rho function in the lck-C3 transferase mice causes massive apoptosis in $CD44^+25^+$ pro-thymocytes and in $CD44^-25^+$ early pre-T cells. Accordingly, Rho^- thymi from lck-C3 transgenic mice have very low levels of viable $CD25^+$ cells. Using the CD2-LCR promoter it has been possible to inhibit Rho function at a later stage of thymocyte development. The data presented here show that in the CD2-C3 transgenic mice, the CD2-LCR directs expression of C3-transferase directly into the $CD44^+25^+$ cells and has thus allowed the role of Rho in regulating survival of early pre-T cells to be investigated independently of its role in earlier thymocyte populations .

These results show that expression of C3 transferase directly in the $CD44^+25^+$ and $CD44^-25^+$ compartments may prevent thymocyte differentiation but does not apparently result in increased apoptosis (Summarised in Figure 4.5). Increased levels of cell death in $CD25^+$ cells from lck-C3 transgenic mice is thus not a cell autonomous consequence of loss of Rho signalling in early thymocyte progenitors. The death of $CD44^-25^+$ pre-T cells in lck-C3 transgenic mice must be explained by a model in which signals that regulate survival of this population are generated by Rho dependent mechanisms in earlier progenitor populations at the $CD44^+25^-$ stage. Once these signals are induced, there is no further requirement for Rho function for cell survival in either the $CD44^+25^+$ or $CD44^-25^+$ thymocyte compartments. Hence direct inactivation of Rho function within the $CD44^+25^+$ or $CD44^-25^+$ thymic subpopulations has no influence on apoptosis. This result affords the

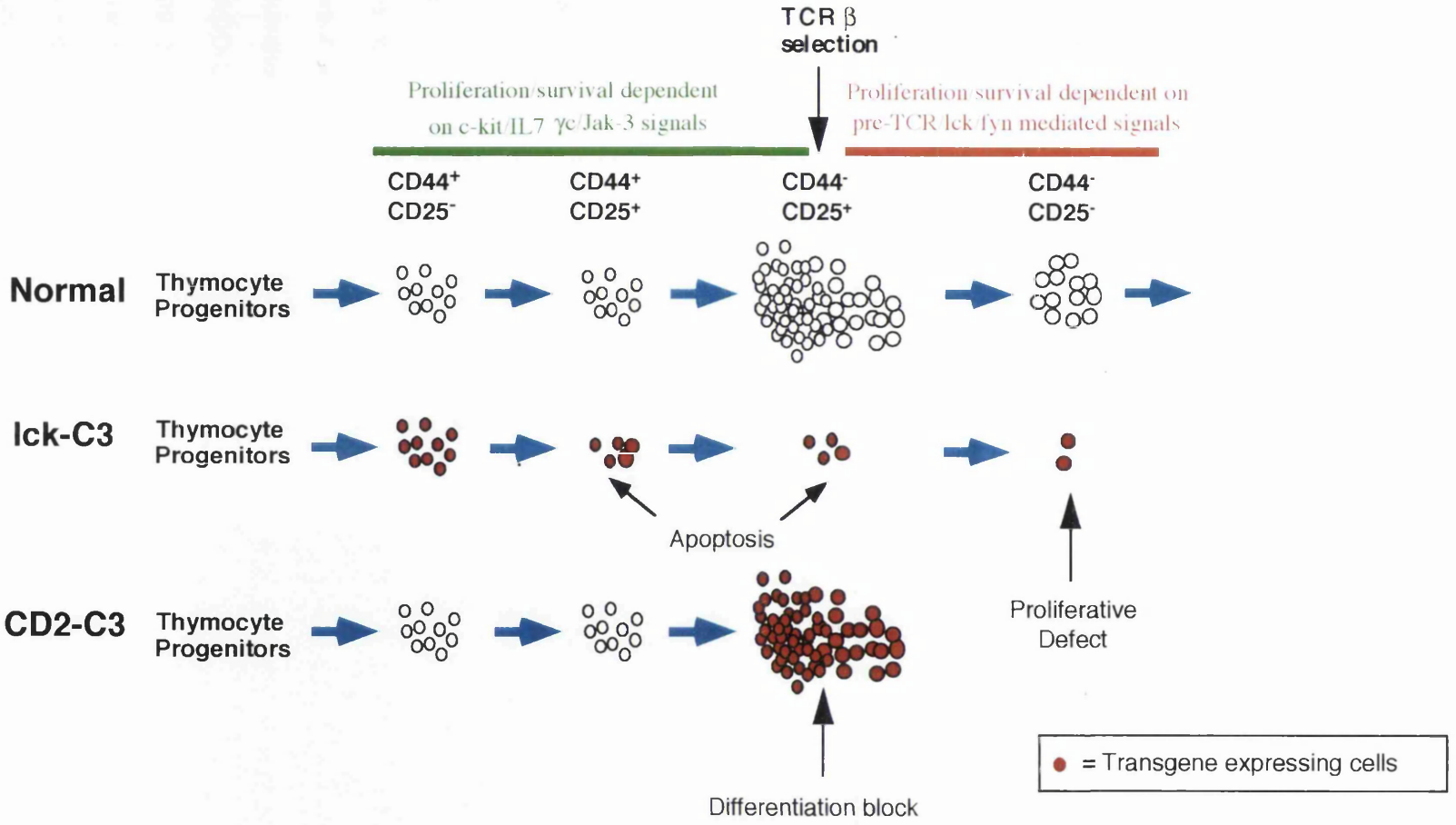

Figure 4.5 - Diagramatic comparision of the lck-C3 and CD2-C3 Mice.

Expression of C3-transferase under the control of the p56lck promoter reveals a role for Rho in survival of $CD44^{+}25^{+}$ and $CD44^{-}25^{+}$ cells; the few surviving cells (less than 1% normal levels) fail to undergo normal proliferation at the $CD44^{-}25^{-}$ stage. Analysis of the CD2-C3 mice reveals a differentiation block at the $CD44^{-}25^{+}$ stage and highlights a role for Rho in Pre-TCR mediated signalling.

insight that survival signals for early pre-T cells are generated by the extracellular signals that act on earlier thymocyte precursors.

$CD25^+$ pre-T cells in lck-C3 mice may show increased apoptosis but the few that survive (less than 1% of normal levels) are able to differentiate into CD4/8 DP and SP cells. $CD25^+$ pre-T cells in CD2-C3 mice fail to differentiate, hence CD4/8 DP and SP cells are absence. This striking difference raises a second issue that is not easily explained by differences in kinetics of transgene expression. One possibility is that in the lck-C3 transgenic mice, the huge survival pressure exerted on early thymocyte progenitors has selected for pre-T cells that are able to compensate for loss of Rho function. These compensating signals allow them to both escape apoptosis and go on to differentiate without Rho function but with reduced proliferative capacity. The direct inactivation of Rho in pre-T cells in CD2-C3 mice allows no opportunity to select cells with a compensating signalling pathway.

An alternative reason for the failure to see a differentiation block in the lck-C3 mice would be that there is heterogeneity of transgene expression. It is possible that in the lck-C3 mice, not all of the thymocytes express C3-transferase at the same time or to the same level. This would mean that not all cells have inactive Rho; the cells in which Rho inactivation is either incomplete or delayed are able to escape apoptosis and go on to differentiate into CD4/8 DP and SP cells.

Although both compensation and heterogeneity of expression can explain the absence of a differentiation block in the lck-C3 mice, there are a number of reasons why the compensation argument is favoured over that of heterogeneity. In Chapter 6, I will describe how mice expressing a FADD-DN transgene under the control of the p56lck promoter have been bred with the lck-C3 transgenics (As will be reviewed in chapter 6, FADD-DN acts as an inhibitor of Death-Domain containing receptor-mediated apoptosis and has been described to inhibit antigen receptor mediated proliferation in mature T cells (Newton *et al.*, 1998)). Transgenic mice expressing FADD-DN under the control of the lck promoter show no problems in early thymocyte development. However, when FADD-DN mice are bred onto lck-C3 mice, analysis of the thymi from double-transgenic mice reveals the presence of only a few $CD44^+25^-$ cells and a complete absence of all subsequent

stages. This phenotype is not leaky; in the lck-C3/FADD-DN double transgenic mice, if a sub-population of cells were not expressing C3 as suggested by the heterogeneity argument, then these cells would behave like FADD-DN cells and develop normally. However, when tested experimentally, this is not the case; mice transgenic for both C3 and FADD-DN have a complete block in thymocyte development. With no evidence for heterogeneity, the only way to explain this phenotype is if all cells express C3-transferase.

An additional observation that supports the idea that there is compensation rather than heterogeneity of expression in the lck-C3 transgenic mice is that the appearance of CD4/8 DP and SP in these animal is delayed relative to wild type mice (Gallandrini *et al.*, 1997); CD4/8 DP cells normally start to appear at around day 16 of embryogenesis but are not present in the lck-C3 mice until 1 week after birth. This delay in appearance would be compatible with the idea that time is required for the selection of cells with the ability to compensate for loss of Rho function. If heterogeneity in lck-C3 expression was occurring, then no delay in the appearance of CD4/8 DP cells would be expected as the CD4/8 DP cells developing from progenitors with lower or delayed C3 expression would arise at the same stage of embryogenesis as the CD4/8 DP cells in a normal mouse.

Taken together, it thus seems likely that cells that escape apoptosis in the lck-C3 mice have compensated for loss of Rho function. It is always a concern that cells may compensate for loss of a signal transduction molecule. It is also suspected that cells may react to the loss of function of molecules by developing complex secondary phenotypes. The present result shows that these are real concerns that can only be addressed by conditional or cell type specific elimination of gene function.

As discussed in chapter 1, thymocyte development is controlled by a complex array of receptor mediated signals; during early thymocyte development, survival is regulated through c-kit and IL-7R mediated signals (DiSanto and Rodewald, 1998). At the pre-T cell stage, thymocyte differentiation and proliferation is regulated by the pre-TCR (von Boehmer *et al.*, 1999). The data presented here suggests that Rho may be involved in both these receptor driven processes. During early thymocyte development, Rho may mediate signals from the c-kit or IL-7R receptors and mediate cell survival through

interaction with its effectors. Later, at the pre-T cell stage, Rho appears to be involved in pre-TCR signalling and the regulation of differentiation. One question this raises is how Rho can be involved in the regulation of two very different processes?

C3-transferase is known to ribosylate and inactive RhoA, B, and C (Aktories *et al.*, 1992) and hence studies involving the C3-transferase transgenic mice are unable to distinguish between inactivation of these Rho family members. It is thus possible that different Rho family members are involved at different stages of thymopoiesis. This theory is experimentally testable; by isolating different thymocyte subpopulations is should be possible to check for expression of the 3 Rho-family members known to be substrates for C3-transferase. Although it may prove difficult to isolate sufficient numbers of the earliest thymocyte sub-populations to study protein levels by Western blot, sufficient numbers of cells could be isolated to enable a PCR-based approach to be taken. However, such an approach would only be of use if the changes in Rho expression were at the level of transcription and even if this were the case, quantification would be complex. The theory is also testable functionally with a strategy involving ribosylation resistant mutants of Rho. By producing a second transgenic mouse expressing mutant of RhoA that cannot be inactivated by C3 (Asn 41→Ile 41) (Hill *et al.*, 1995) under the control of the lck proximal promoter and breeding this mouse both the lck-C3 and the CD2-C3 transgenics, it will be possible to assess the role of RhoA in the processes of survival and differentiation. For example, if the ribosylation resistant mutant of RhoA rescued the differentiation defect in the CD2-C3 mice, but not the survival defect in the lck-C3 transgenics, then RhoA would be implicated as being required for Pre-TCR mediated signalling, but not required for early thymocyte survival. The role of the other Rho family members inactivated by C3 could be investigated using a similar approach. Although the production of additional transgenic mice is expensive and time-consuming, this type of rescue experiment would provide unequivocal data as to the differential requirement for Rho-family GTPases during thymopoiesis.

The work presented in this chapter highlights the importance of condition or cell type specific elimination of gene function. Recently, advances have been made in this field by the use of conditional or inducible gene knockouts. With the use of the Cre-Lox system, researchers are beginning to be able to eliminate gene function in a tissue-specific manner

(Gu *et al.*, 1994; Jiang and Gridley, 1997). The technique involves flanking the gene to be deleted with LoxP target sequences, transfecting into ES cells, and selecting for homologous recombination of the gene of interest with the LoxP flanked gene. Mice are then generated from the selected ES cells, and made homozygous for the LoxP flanked gene. These mice are then bred with transgenic mice expressing the Cre-recombinase transgene under a tissue-specific promoter. Alternatively, an inducible promoter such as the Interferon sensitive promoter, Mx1 can be used to drive Cre-expression, allowing the LoxP flanked gene to be inducibly deleted upon addition of IFN (Kuhn *et al.*, 1995). The expression of Cre causes the deletion of the gene flanked by the LoxP sites, resulting in elimination of gene function only in the cells in which Cre is expressed. Although there have been some problems with 'leakiness' of the Cre-LoxP system (Kuhn *et al.*, 1995), these issues are currently being resolved and will result in a powerful tool through which to study cell type specific elimination of gene function.

In summary, analysis of the lck-C3 has previously defined a role for Rho in the survival of early thymocyte progenitors. By targeting expression of C3-transferase to the $CD25^+$ population with use of the human CD2-LCR, this survival defect can be bypassed, revealing an addition role for Rho in pre-TCR mediated signalling. These comparisons demonstrate that cell type specific elimination of Rho can reveal different functions for this GTPase in thymocyte development, and hence demonstrates that the only way to fully understand the function of a protein is to study the immediate consequences of its cell-type specific elimination.

CHAPTER 5
Thymic Lymphoma in the lck-C3 Mice

5.1 - Introduction

As described in the previous chapters the role of Rho in T cell biology has been studied in transgenic mice expressing C3-transferase under the control of the p56lck promoter and the CD2 promoter. The following chapter described a further defect in the lck-C3 mice; the development of thymic lymphoblastic lymphomas between four and eight months of age as a consequence of loss of Rho function. Previously, the specific role of Rho in tumour development has only been studied in cell lines where it has been identified as a mediator of cell transformation (Prendergast, *et al.* 1995; Qiu, *et al.* 1995). These studies reveal the GTPase Rho to have a previously unsuspected role in suppressing malignant transformation; i.e. loss of Rho function in the thymus results in rapid onset of aggressive thymic lymphoma and thus the *in vivo* analysis presented here provides important new insight into the biological role of the small GTPase Rho.

5.2 - Results

5.2.1 - lck-C3 transgenic mice develop thymic lymphomas

Maintenance of two independently derived lck-C3 transgenic lines revealed that although initially healthy, mice were dying prematurely in comparison with non-transgenic littermates. 80% of transgenic mice (n=70) died between four and eight months of age, with no transgenic mice from either line surviving beyond twelve months. The normal life span of wild type C57BL/6 mice is at least 24 months in a specific pathogen free environment. Post-mortem examination indicated that the deaths occurring in the lck-C3 mice were a result of compression of the heart and lungs by a grossly enlarged thymus (Figure 5.1, top panel). The mean cell number of the enlarged thymus was $9x10^8$ in comparison with $5x10^7$ observed in age matched littermate controls. Histological analysis showed that the characteristic structures of the thymic medulla and cortex were no longer evident (Figure 5.2, top panel).

Further post mortem examination of the lck-C3 transgenic mice revealed enlarged secondary lymphoid organs including the spleen (Figure 5.1 centre panel), lymph nodes (Figure 5.1, bottom panel) and in most cases the liver. In the spleens of the lck-C3 mice, infiltration of lymphoblasts almost entirely replaced the normal white and red pulp regions (Figure 5.2, centre panel). In the liver, infiltration of lymphoblasts was seen throughout the organ most notably around blood vessels (Figure 5.2, bottom panel). In no instance were these secondary manifestations observed in the absence of an enlarged thymus. This observation coupled with morphological and cell surface marker analysis strongly suggests that cells infiltrating peripheral organs originated from the thymic lymphoma.

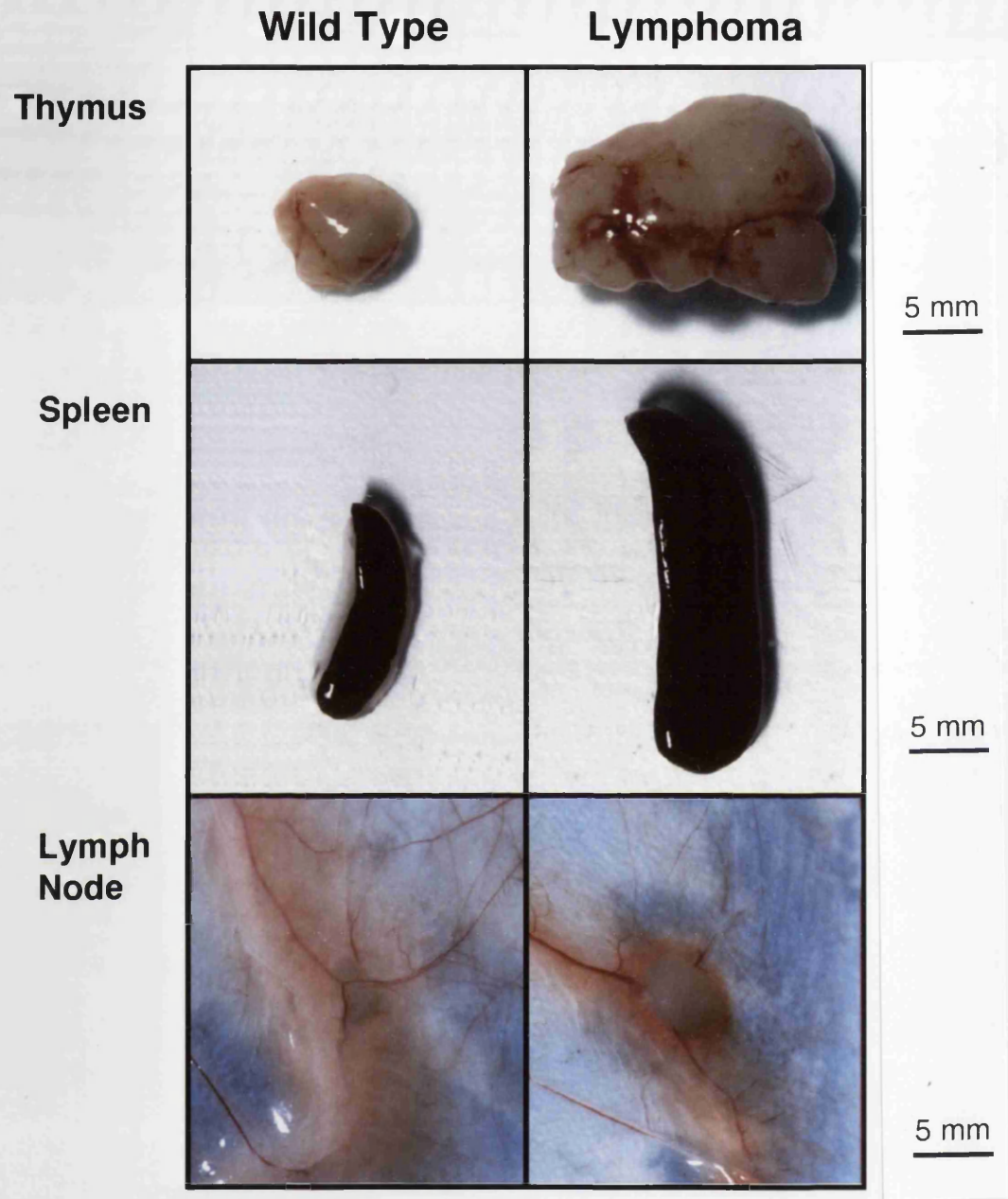

Figure 5.1 - Lymphoma Development in lck-C3 mice.

Thymus, spleen and lymph node of a 6 month old lck-C3 mouse exhibiting thymic lymphoma (right panel) compared to those of an age matched C57BL/6 wild type mouse (left panel).

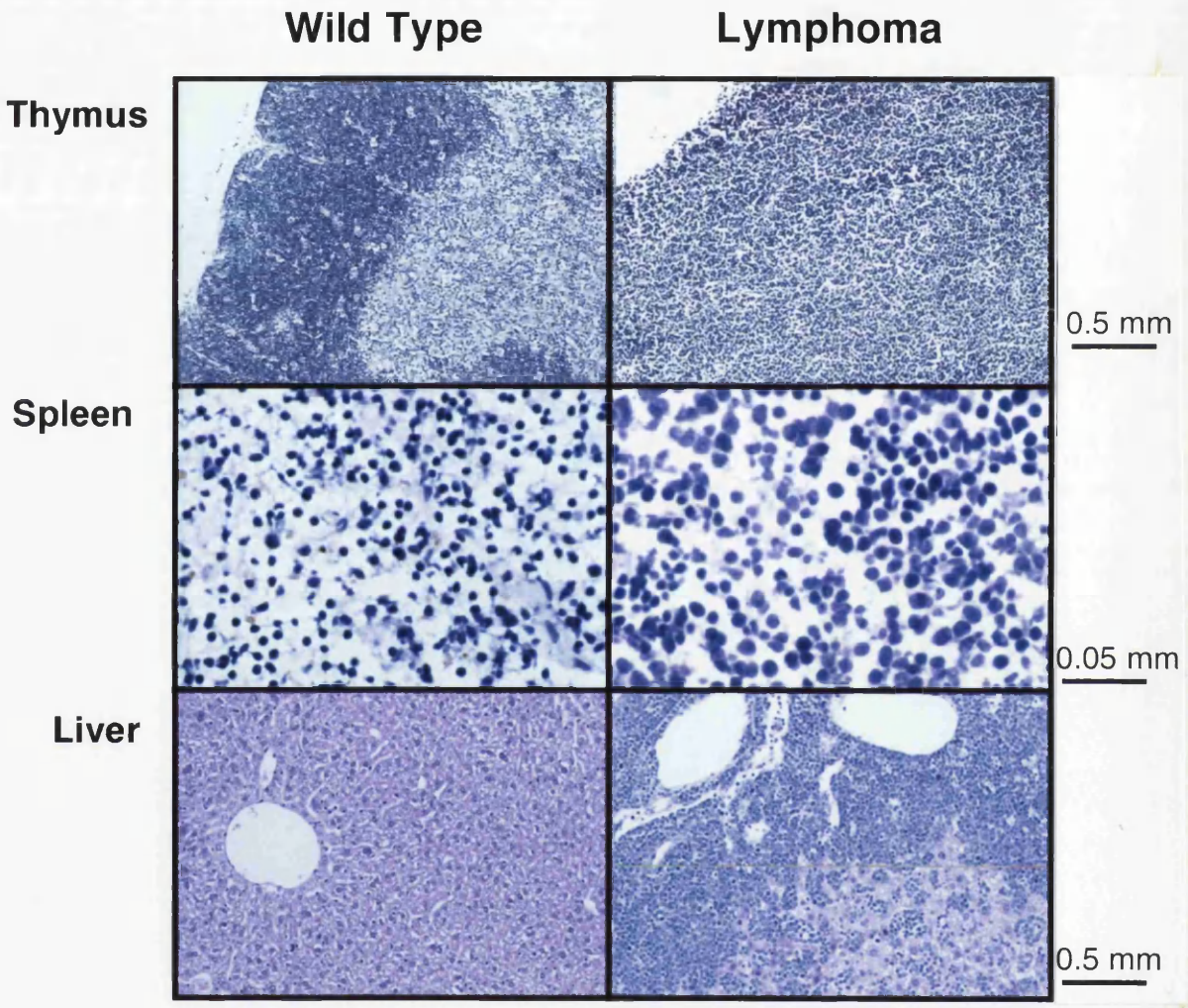

Figure 5.2 - Histology of lck-C3 thymomas.
Haematoxylin-stained sections of frozen thymus, spleen and liver of a 6 month lck-C3 mouse with thymic lymphoma (right panel) compared to those of an age matched C57BL/6 wild type mouse (left panel).

5.2.2 - Thymic lymphomas are of T cell origin

The pathology of the dead mice diagnosed thymic lymphoma. Confirmation of the T cell origin of the lymphomas came from flow cytometric analysis of thymic tumours from the two independently derived transgenic lines (1222D and 1222C) which revealed the cells to express Thy-1 and the T cell antigen receptor/CD3 complex (data not shown). The T cell origin of the tumours was also confirmed by the fact that CD4 and/or CD8 (T cell co-receptors for MHC molecules) were co-expressed on all tumours analysed. Representative CD4 and CD8 profiles of tumours observed in the two lck-C3 lines are illustrated in figure 5.3. Two subtly distinct CD4/8 profiles were seen: CD4 and CD8 double positive cells and a CD4/CD8 profile skewed towards the CD8 compartment. 7 out of 16 of tumours analysed by flow cytometry co-expressed CD4 and CD8 markers, characteristic of the $CD4^{+}8^{+}$ double positive (DP) thymocyte sub-population; curiously however, all of these cells expressed intermediate levels of CD3 more typical of mature thymocytes. The other CD4/8 profile (referred to as IDP on figure 5.7) seen in the tumours (9 out of 16) was intermediate levels of CD8, low levels of CD4 and again intermediate levels of CD3 analogous to those seen on mature thymocytes. FACS analysis of lymph nodes, spleen and bone marrow in tumour bearing mice showed that these organs were composed almost entirely of cells phenotypically identical to those observed in the thymic tumour; an indication of the aggressive recirculating nature of the tumour-derived cells described above (data not shown).

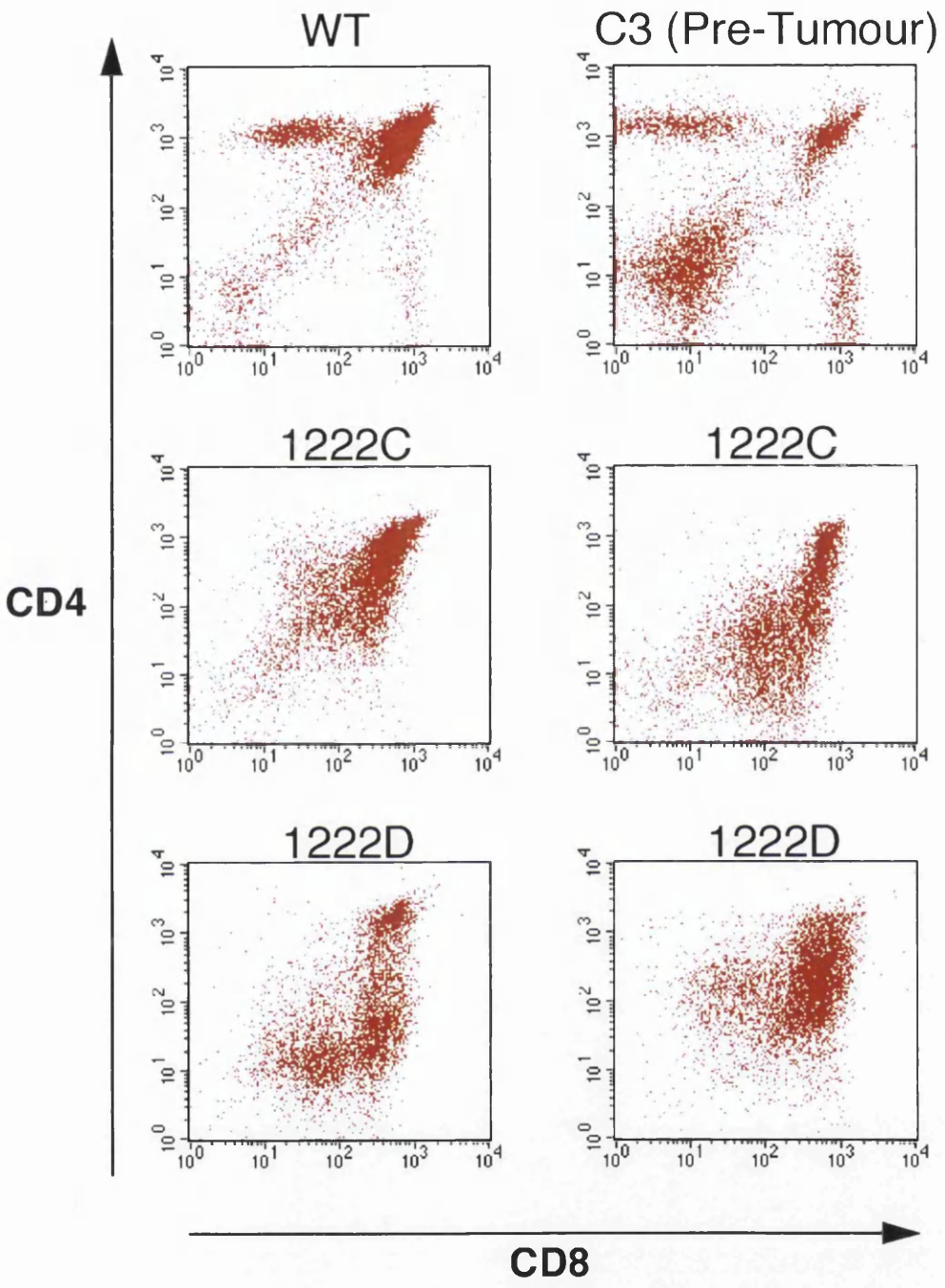

Figure 5.3 - CD4/8 Analysis of Thymic Lymphomas from lck-C3 mice

Flow cytometric analysis of thymocytes isolated from wild type, pre-tumour lck-C3 and lck-C3 mice exhibiting thymic lymphomas stained for surface expression of CD4 and CD8 co-receptors. 1222C and 1222D represent two independently-derived transgenic lines.

Cells isolated from lck-C3 derived thymic lymphomas were analysed for expression of various T cell activation markers including CD25, CD69, and CD62L. The thymic lymphomas did not express CD69 or CD62L but did express CD25 (the alpha subunit of the IL-2 receptor). Typically 5% of thymocytes express CD25 whereas this molecule was expressed on all lymphoma cells. Figure 5.4 shows the elevated levels of CD25 present of the surface of cells isolated from the lymphomas compared to those from an age-matched wild type thymus.

Further analysis revealed the lymphoma cells to be blastoid and much larger than quiescent thymocytes. These cells were not particularly abnormal in size, rather their size was comparable to the size of normal activated and proliferating splenic derived lymphoblasts (Figure 5.4B). The cells also showed a DNA staining profile typical of normal lymphoblasts in exponential growth, with 2n, intermediate or 4n DNA content. No lymphoma cells were polyploid (Figure 5.5).

Normal thymocytes from lck-C3 transgenic mice are devoid of functional Rho GTPase because of expression of *Clostridium Botulinum* C3-transferase which ribosylates Rho thereby inactivating its function (Henning *et al.*, 1997). Expression of the C3 transgene in tumour samples was analysed by RT-PCR. In four independent tumour samples analysed, endogenous p56lck was expressed and expression of the lck-C3 transgene was maintained (Figure 5.6).

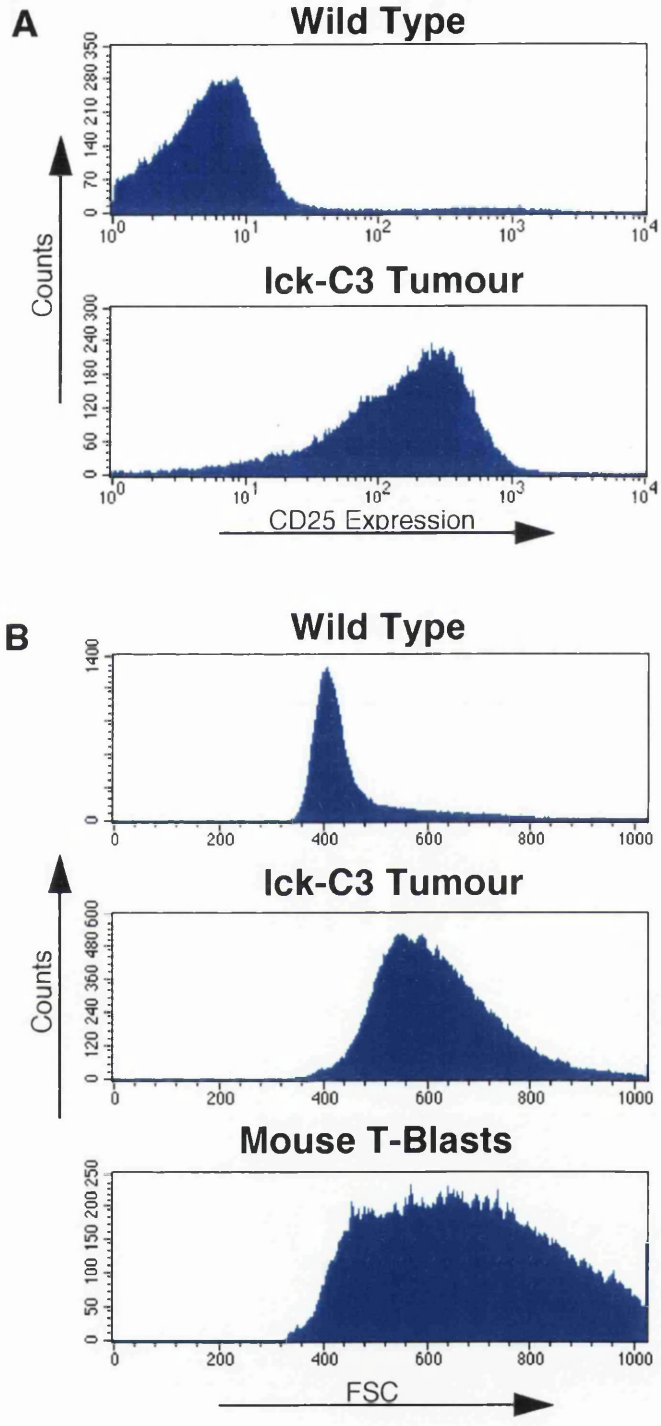

Figure 5.4 - Flow Cytometric Analysis of the lymphoma cell population.

(A) Analysis of surface CD25 expression on wild type thymocytes and lck-C3 lymphoma cells.

(B) Flow cytometric forward scatter analysis of thymocytes isolated from thymic lymphoma compared with those of an age-matched wild type mouse and wild type T-blasts.

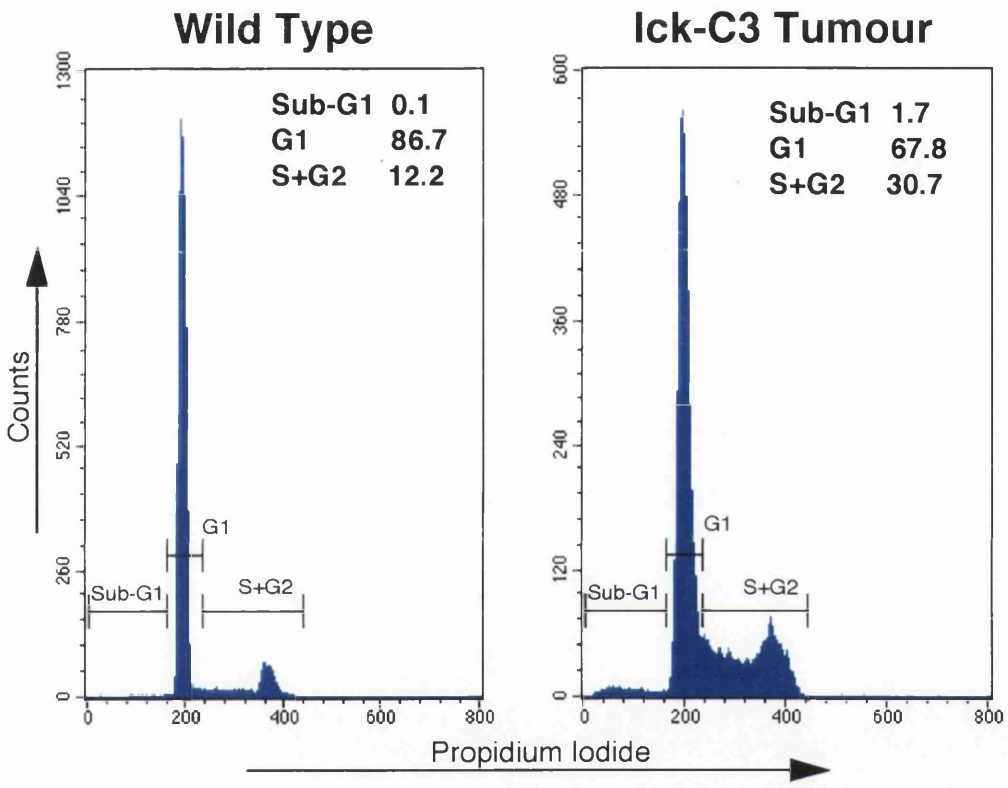

Figure 5.5 - Cell Cycle Analysis of cells isolated from age-matched wild type and lck-C3 mice exhibiting thymic lymphoma.

Thymocytes were fixed and permeabilised in 70% ethanol, stained with propidium iodide and analysed on a FACS Calibur.

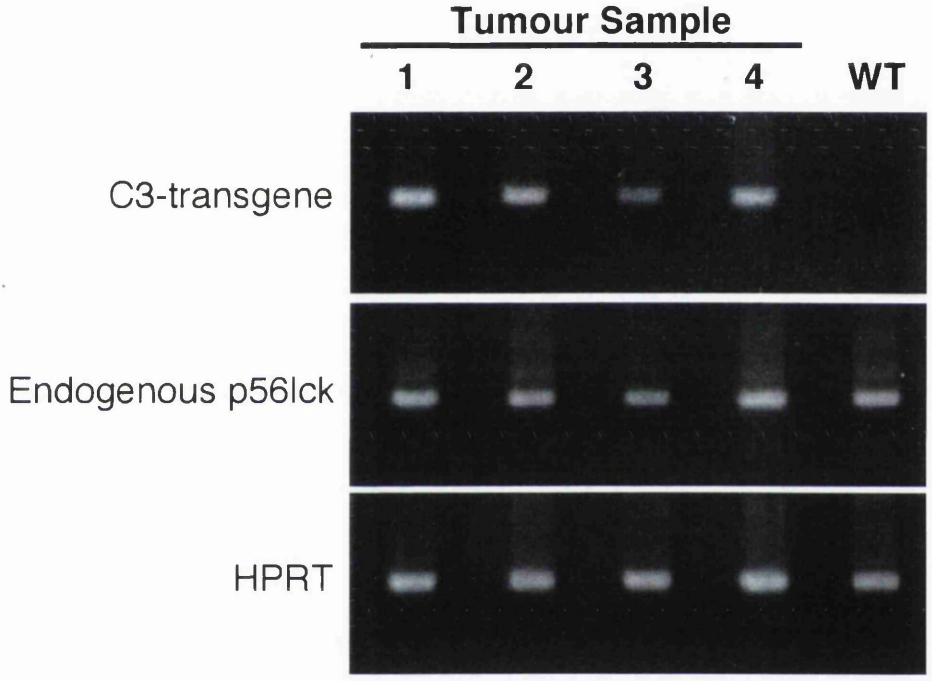

Figure 5.6 - Expression of C3-Transgene, p56lck, and HPRT mRNA in Tumour Tissue.

Cells were isolated from four typical lck-C3 thymic lymphomas and a wild type thymus, total RNA prepared and RT-PCR performed to detect expression of mRNA specific for the C3-transgene, endogenous p56lck, and HPRT.

5.2.3 - lck-C3 transgenic mice exhibit a median survival age of six months

The data in Figure 5.7 show a survival curve of lck-C3 transgenic mice. All the lck-C3 mice exhibited the development of thymic lymphomas over the period of study. The first thymic tumours were seen in mice at three months of age with the median age for tumour development being six months. The incidence of thymic lymphoma in the wild type C57BL/6 strain is 0-2% in the first 12 months of age (Bronson, 1990). The cell surface expression of CD8 and CD4 on tumour cells is indicated (where determined).

5.2.4 - Lymphomas developing in lck-C3 mice are Monoclonal in Nature

Having established that the tumours were of T cell origin, it was of interest to determine whether the tumour cells were polyclonal or monoclonal in nature. All tumour cells expressed the T-cell antigen receptor complex which normally comprises TCR α and β subunits coupled to the invariant CD3 antigen complex. A clonal T cell population would express a single TCRβ chain whereas polyclonal populations would show usage of multiple vβs (See Figure 5.8). In C57BL/6 mice, the most commonly used vβs are vβ8, typically expressed by 20% of the T cell population and vβ11 comprising approximately 5% of the population. vβ subgroup staining was routinely performed to investigate the TCR repertoire of normal and tumour derived T lymphocytes. Assuming transformation was a rare event, occurring within a single cell without bias, one would expect approximately 20% of the tumours to be vβ8 positive and 5% to be vβ11 positive. Interestingly, 3 out of the 16 tumours analysed (18%) exclusively expressed vβ8 while 1 out of 16 (6.25%) expressed vβ11. The remaining 12 out of 16 tumours were negative for both vβ8 and vβ11 demonstrating that the normal repertoire of vβ expression was absent and thus transformation was not occurring simultaneously throughout the whole $\alpha\beta$ T cell population. Representative profiles are illustrated in Figure 5.9. These phenotypes are not consistent with a polyclonal origin of the thymic lymphomas and strongly indicates that the tumours are monoclonal in nature, arising from a single rare transformation event.

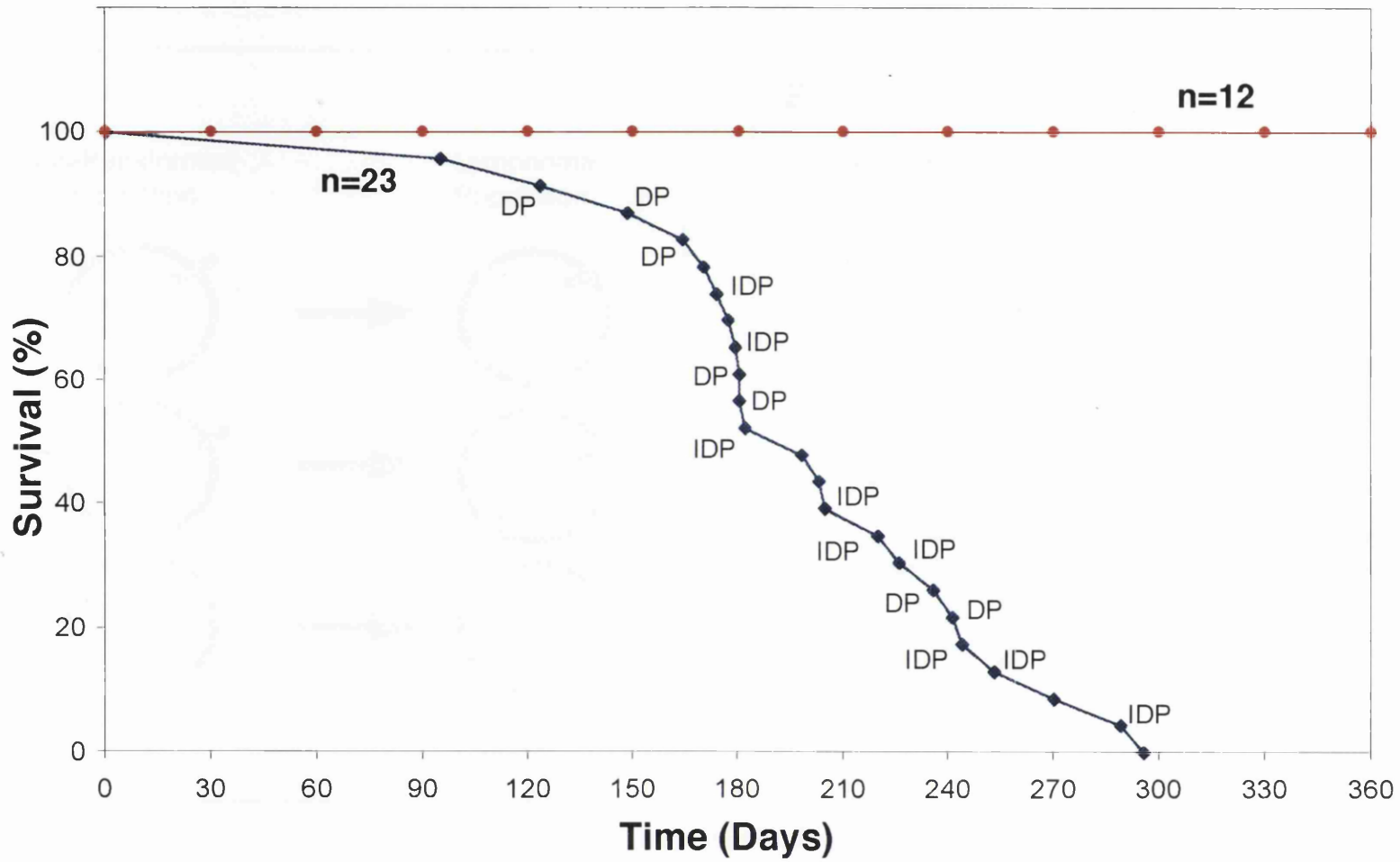

Figure 5.7 - Latency of thymic lymphoma development in lck-C3 transgenic mice maintained in a specific pathogen-free environment.
Percentage survival of wild type C57BL/6 (•) and transgenic mice (♦) is plotted versus age of detection (in days). Where determined, the CD4/CD8 phenotype analysed by flow cytometry is indicated by DP (CD4/CD8 Double Positive) or IDP (CD4/CD8 Intermediate Double Positive).

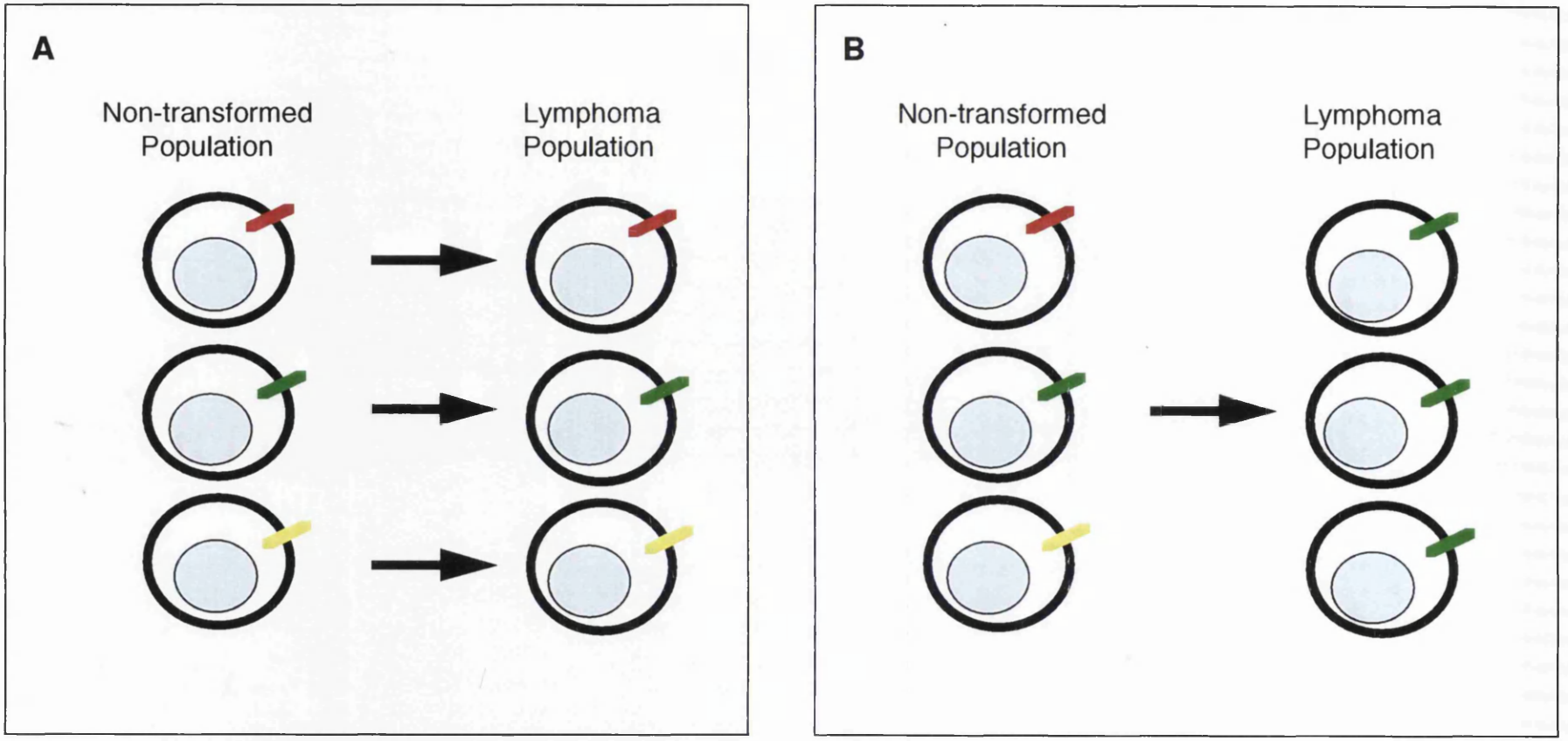

Figure 5.8 - Strategy used to determine clonality of Lymphoma cell population

(A) If all the cells lacking Rho function have become transformed, the lymphoma cell population will express the full repertoire of vβ chains.

(B) If the lymphoma cell origin is from only one cell, all cells of the lymphoma will express the same vβ chain.

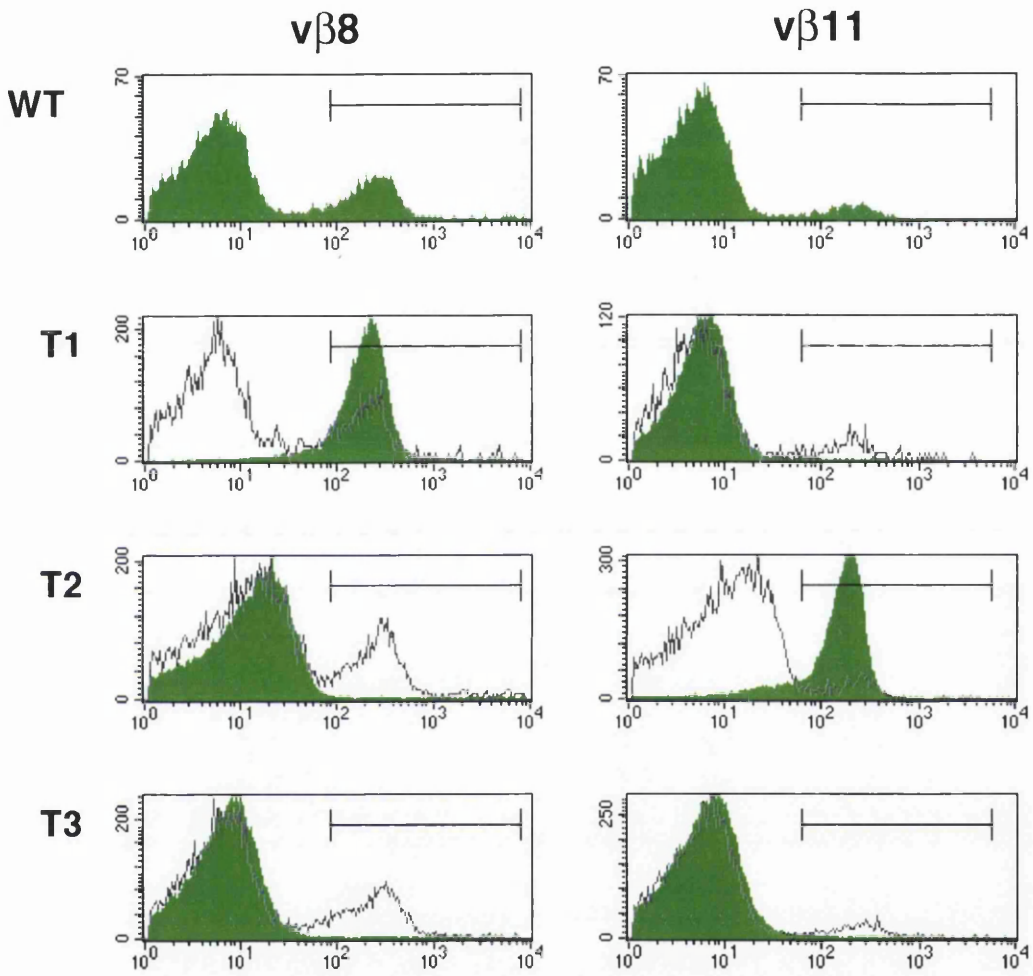

Figure 5.9 - Clonality of lck-C3 Lymphoma Cell Populations.

Flow cytometric analysis vβ subgroup expression on CD3 positive thymocytes (shaded region) isolated from an aged matched C57BL/6 wild type (WT) and three representative lck-C3 mice exhibiting thymic lymphomas (T1, T2, T3). Displayed are the vβ8 and vβ11 histograms of CD3 positive cells. Each histogram is overlayed with the same profile for that of a wild type mouse analysed on the same day (Grey Line).

5.2.5 - Incidence of Thymic Lymphoma in the CD2-C3 Transgenic Mice

Strikingly, mice expressing C3-transferase under the proximal p56lck promoter developed thymic lymphoma at around 6 months of age. Expression of C3-transferase under the control of the human CD2-LCR leads to an absolute block in T cells development at the pre-T cell stage, but does not result in survival defects as observed in the lck-C3 mice. It was therefore of interest to determine whether the CD2-C3 showed similar tumour development to the lck-C3 mice.

12 CD2-C3 mice were maintained in an SPF unit and their incidence of tumour development investigated. All CD2-C3 mice survived for at least 12 months. Post-mortem analysis revealed no incidence of lymphoma, although some mice died of infections, probably owing to their immuno-compromised status. This suggests that inactivation of Rho function at a later developmental stage does not give rise to thymic lymphoma.

5.3 - Discussion

5.3.1 - Loss of Rho function results in development of Thymic Lymphoma

Previously the essential role of the small GTPase Rho has been demonstrated in both thymocyte survival and pre-TCR mediated signalling. Here, it is shown that lck-C3 mice strikingly go on to develop aggressive thymic lymphoma and suggests a previously unsuspected role for Rho in the suppression of malignant transformation.

Perturbation of thymocyte development in itself does not *a priori* lead to tumour development. Blocks in thymocyte development in transgenic mice that show thymocyte survival defects comparable to those seen in young lck-C3 mice (for example, those expressing dominant negative Myb, or null for bcl-x expression (Badiani *et al.*, 1994; Ma *et al.*, 1995)) do not develop lymphoma. Furthermore, loss of function of other GTPases such as Ras or Rac does not result in predisposition to thymic lymphoma (Tarakhovsky *et al.*, 1995; Alberola-Ila *et al.*, 1996). It should also be noted that although the lck-C3 mice exhibit reduced numbers of peripheral T cells and are thus immuno-compromised, there are many examples of mice with immuno-deficiencies which do not go on to develop lymphomas (eg: Vav1-/-, CD45-/-, Dominant negative Raf) (Tarakhovsky *et al.*, 1995; Byth *et al.*, 1996; O'Shea *et al.*, 1996).

Typically, transgenic mice that develop thymic lymphomas are either those that express activated oncogenes such as lck, Myb, and PIM1 in the thymus (van Lohuizen *et al.*, 1989; Abraham *et al.*, 1991; Badiani *et al.*, 1996) or those lacking expression of tumour suppressor genes (eg: p53 or Brca-2) (Purdie *et al.*, 1994; Connor *et al.*, 1997). Figure 5.10 compares the latency of thymic lymphoma onset in a range of transgenic mice including the lck-C3 mice. Mice that develop thymic lymphomas tend to do so within a well-defined period of time (typically 2-4 months). lck-C3 mice have a relatively early onset of lymphomas, with 80% of mice dying between 4 and 8 months of age. Analysis suggests that there is little correlation between latency of onset, whether the lymphoma is caused by a gene deletion or transgene expression, and whether lymphoma development is preceded by disrupted thymic development.

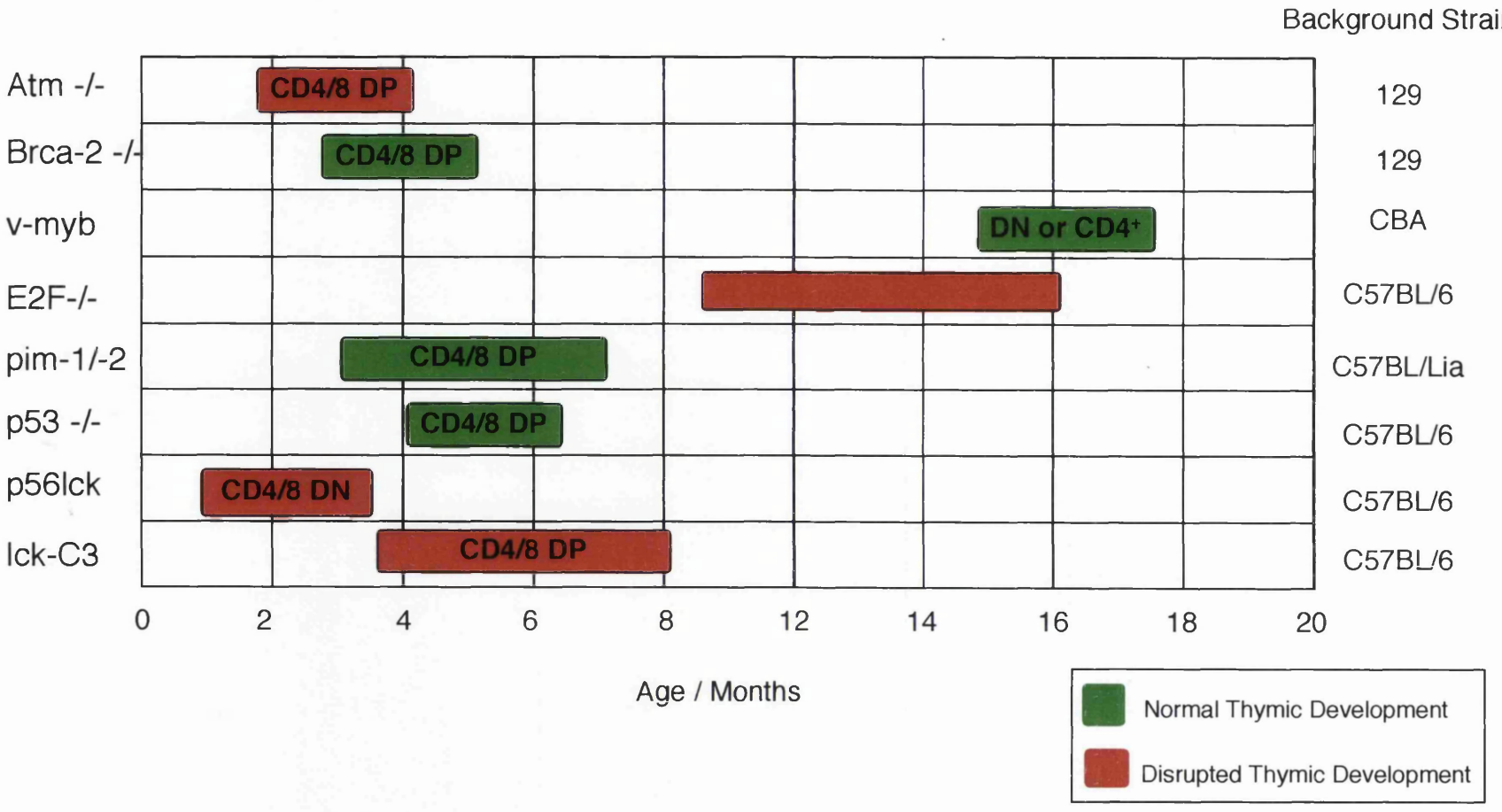

Figure 5.10 - Kinetics of Onset of Thymic Lymphoma in Transgenic Mice.

5.3.2 - Origin of the Thymic Lymphomas

All tumours in the lck-C3 mice were of T cell origin and expressed a mature TCR complex. The observation that the tumour-derived cell population lacked heterogeneity of vβ expression (i.e. expressed one vβ rather than the full repertoire) indicates that these tumours have arisen from a single cell and are thus monoclonal rather than polyclonal tumours. The lack of polyclonality of the tumours in the lck-C3 transgenic mice would suggest that loss of Rho function is not sufficient for direct transformation of T cells; rather lack of Rho function predisposes cells to further rare transforming events. This is not unusual; a single genetic insult never directly transforms cells and multiple genetic lesions are required for the development of malignancy (Hunter, 1991). The thymic lymphomas in the lck-C3 mice express some phenotypic markers typically associated with thymocytes; notably the co-expression of CD4 and CD8 molecules. Prior to the onset of the lymphomas most thymocytes in lck-C3 animals die as early progenitors so that CD4/8 DP thymocytes are generated at greatly reduced numbers (5-10% of normal). However, it is not easy to correlate the surface marker phenotype of thymic lymphomas to the population from which they arose; by eliminating either RAG-1 or RAG-2 on a p53 null background it has been shown that CD4/8 DP tumours can still arise even in the absence of V(D)J recombination (Nacht and Jacks, 1998). Therefore, in the lck-C3 mice, it is not clear whether it is the few CD4/8 DP thymocytes that manage to develop or an earlier thymocyte progenitor that give rise to the tumours.

It is interesting that expression of C3-transferase under the control of the CD2-LCR does not predispose cells to transformation; however, this observation provides insight into the origin of the thymic lymphomas observed in the lck-C3 mice. As discussed in Chapter 3, inactivation of Rho function within $CD44^{+}25^{+}$ and $CD44^{-}25^{+}$ cells leads to a block in pre-T cell differentiation owing to defective pre-TCR signalling with CD2-C3 mice failing to develop any CD4/8 DP or SP cells. The lack of development of lymphoma in these mice suggests that it is not inactivation of Rho *per se* that leads to malignancy; rather, certain thymic subsets are more susceptible to transformation initiated by loss of Rho function. Furthermore, a comparison of the CD2-C3 and lck-C3 mice provides insight into the population of cells from which the tumours arise. Three theories as to the origin of the transformed cells are presented below and summarised in figure 5.11:-

1. One possibility is that the thymocytes becoming transformed in the absence of Rho function are the earliest thymocyte progenitors, the $CD44^{+}25^{-}$ cells. Rho function within this population is only abolished in the lck-C3 mice and not in the CD2-C3 mice and would thus fit with the observations that lck-C3 but not CD2-C3 mice develop lymphoma. Interestingly, the thymic lymphomas consist predominantly of CD4/8 DP cells, and thus if the initial transforming events were occurring within the earliest thymic subset, transformed cells would have to follow a similar developmental pattern to non-malignant cells, acquiring expression of CD4/8 and expressing β chain on their surface.

2. In addition to the kinetics of C3 expression, the other major difference between the two C3-transgenics is the presence of CD4/8 DP cells in the lck-C3 mice. Thus, it is possible that the transforming events caused by loss of Rho function are occurring within these cells and may simply be a case of CD4/8 DPs being more susceptible to transformation by loss of Rho function; hence lymphomas do not arise in CD2-C3 mice as these mice lack CD4/8 DPs.

3. As discussed in Chapter 4, it is possible that the CD4/8 DP cells arising in the lck-C3 mice do so because early thymocyte progenitors have compensated for loss of Rho function and hence escape apoptosis. Inactivation of Rho at a later stage would offer no opportunity for cells to compensate for loss of Rho function and hence no CD4/8 DPs develop. Thus, if this is truly the case, the process of compensating for loss of Rho function may predispose cells to transformation.

To resolve the issue of which population of cells gives rise to the thymic lymphomas in the lck-C3 mice, more experiments would need to be performed. For example, breeding the lck-C3 mice onto a RAG-deficient background would help resolve whether the transforming cells originated from the early thymocyte progenitors, or the CD4/8 DPs. On a recombination-deficient background, it would be predicted that lck-C3 mice would not be able to develop any CD4/8 DP cells. Thus, if lymphoma development was prevented in these mice, it would be possible to eliminate the first hypothesis of transformation occurring within the $CD44^{+}25^{-}$ population. Hence, the CD4/8 DP cells would be implicated as giving rise to the lymphomas. To resolve whether the CD4/8 DP cells give

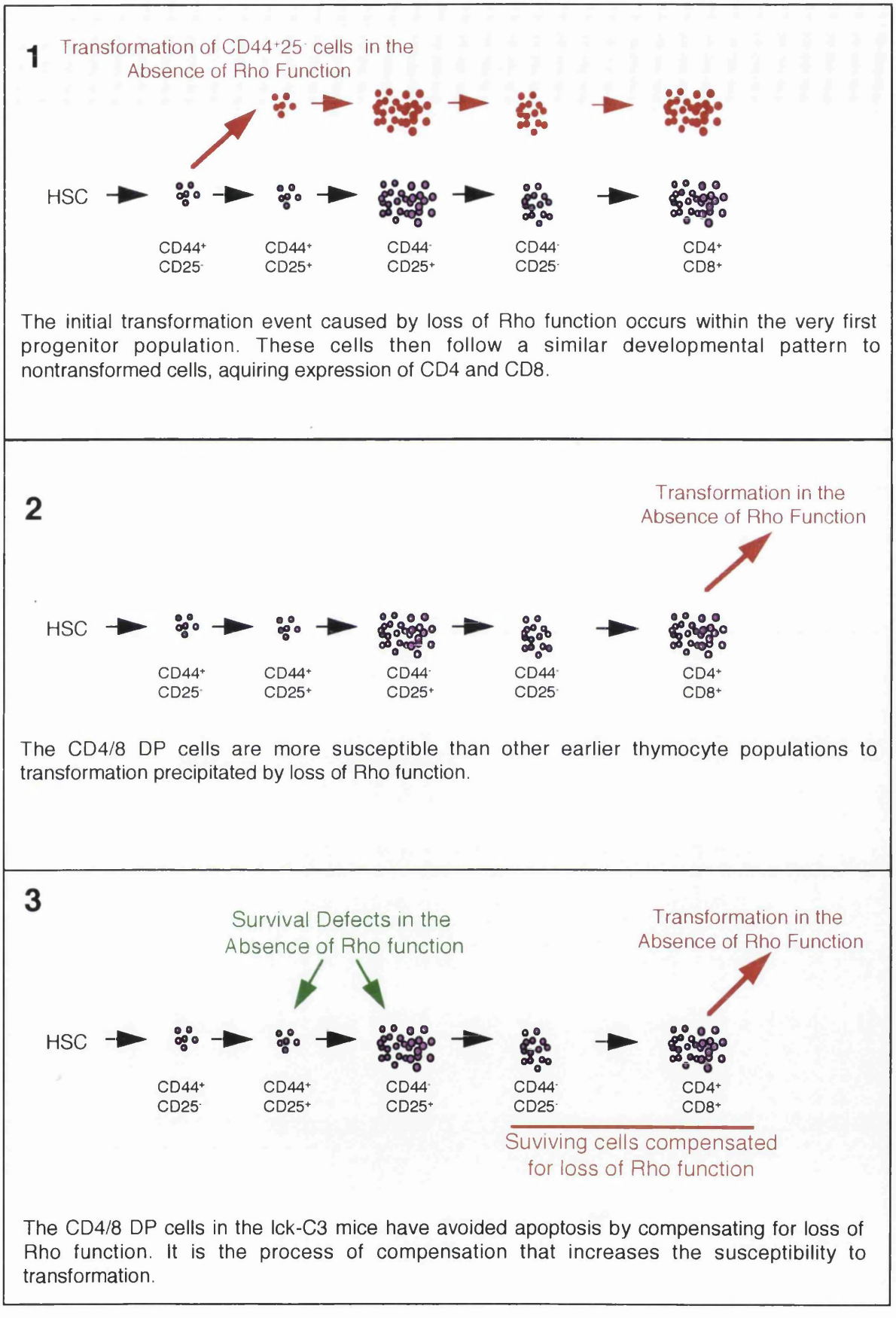

Figure 5.11 - The hypotheses for the origin of lymphomas in lck-C3 transgenic mice

rise to the lymphomas because of an increased susceptibility to transformation or a compensatory process for loss of Rho function would be more difficult.

5.3.3 - Inhibition of Rho Function as a Therapeutic Strategy?

Studies by a number of groups have highlighted a role for the small GTPase Rho in transformation (discussed in section 1.2.4) and has prompted much interest in the idea Rho GTPases may be useful targets for anti-tumour therapy (Olson *et al.*, 1998; Itoh *et al.*, 1999; Sahai *et al.*, 1999). Indeed recently, the small GTPase Rho and its effector ROCK have been implicated as having an essential role in metastasis and tumour cell invasion (Itoh *et al.*, 1999). The study made use of an *in vitro* model system in which cultured rat MM1 hepatoma cells migrate through a mesothelial cell monolayer in a serum-dependent, Rho-mediated manner. Expression of a dominant active mutant of ROCK in the hepatoma cells was able to confer invasive activity independent of serum and Rho. In contrast, treating cells with a specific ROCK inhibitor (Y-27632), or expression of a dominant negative kinase-dead ROCK were both able to block Rho-mediated activation of actomyosin and invasion of these cells. The study was extended by an *in vivo* approach in which the rat MM1 cells were transfected with constitutively active V14 Rho, implanted into the peritoneal cavities of rats and the effect of the ROCK inhibitor on the invasive capacity of the tumour cells assessed. Using implanted osmotic pumps to continuously deliver the ROCK inhibitor, it was possible to significantly reduce incidence of tumour cell dissemination and tumour nodule formation. The results of this study are interesting and suggest that inhibition of the Rho effector ROCK may be of therapeutic value in prevention of tumour invasion and metastasis. However, the study does not address whether Rho activation itself is a cause of malignancy, and there is no evidence to suggest that activating Rho mutations occur during transformation. The only way in which to truly address whether inhibition of ROCK activity is of potential therapeutic value would be to use a mouse model that develops invasive tumours spontaneously.

Genetic approaches to molecular function *in vivo* can lead to the discovery of roles for cellular proteins that were unsuspected from those elucidated by *in vitro* experiments. Previously, the specific role of Rho in tumour development has only been studied in cell

lines where it has been identified as a mediator of cell transformation (Prendergast *et al.*, 1995a; Qiu *et al.*, 1995b). The present study is the first to experimentally examine Rho function *in vivo* and in the context of oncogenesis. The observation that loss of Rho function in the thymus results in rapid onset of aggressive thymic lymphoma is striking. The present data reveals that it may be interesting to pursue whether loss of Rho function through mutagenesis/translocation of Rho regulatory proteins or their effectors is associated with human leukaemias or lymphoma. Furthermore, inhibiting Rho function has been suggested as a therapeutic strategy for treatment of Ras-transformed tumours (Olson *et al.*, 1998; Sahai *et al.*, 1999). This idea is based on the fact that strategies to inhibit Rho function in cell lines result in loss of *in vitro* transforming capacity. The generation of lck-C3 transgenic mice represents the first experimental approach to eliminate Rho function *in vivo* and here loss of Rho function is causally related to promotion of cell transformation capability. Any attempt to block Rho function *in vivo* would thus need to be used with caution; Rho may function as an oncogene in fibroblast cell lines but in lymphocytes loss of Rho function is associated with tumour development.

CHAPTER 6
Attempts to Genetically Characterise Signalling Pathways that can Rescue Thymocyte Development in the Absence of Rho Function

6.1 - Introduction

Transgenic mice provide an invaluable tool for probed gene function *in vivo*. The availability of mice expressing constitutively active or being null for expression of signalling molecules allows the inter-relationship of such molecules to be explored by simple genetic crosses. For example, by breeding transgenic mice expressing the constitutively active signalling molecules with those null for expression of essential components of antigen receptors, the role of these molecules can be explored in a physiological context.

As reviewed in section 1.3.3, early thymocyte survival is dependent upon overlapping signals provided by c-kit, IL-7R and the pre-TCR. Moreover, the IL-7R is implicated in regulation of intracellular Bcl-2 levels. Evidence for IL-7R in the regulation of survival comes from experiments in which mice expressing survival factors are bred into a receptor-deficient background. For example, breeding Bcl-2 transgenic mice onto a IL-7R null background rescues the survival defects seen in the absence of IL-7R, thus implicating signals mediated by the IL-7R in regulation of Bcl-2 (Maraskovsky *et al.*, 1997). This connection was confirmed by biochemical analysis studying the intracellular Bcl-2 levels in IL-7 null mice (von Freeden-Jeffry *et al.*, 1997). Although this type of approach establishes a genetic link between two proteins, further investigation is required to provide a mechanistic connection.

Similarly, genetic analysis has been used to assess the requirement of various signalling molecules in the process of pre-TCR mediated differentiation and proliferation. For example, the role of lck and Raf have been explored by expressing constitutively active mutants of these signalling molecules in RAG-deficient mice. Both active lck and Raf are able to rescue differentiation and proliferation in the absence of β chain

rearrangement, thus implicating these molecules in mediating signalling events downstream of the pre-TCR (Mombaerts *et al.*, 1994; Iritani *et al.*, 1999).

With use of the lck-C3 mice, the role of Rho in the thymus has previously been explored by breeding lck-C3 mice with those expressing either Bcl-2 or the src-kinase $p56^{lck}$ (Gallandrini *et al.*, 1997; Henning and Cantrell, 1998). In the following chapter, the role of Rho in T cell development is further probed using four similar genetic crosses. In the first two experiments, survival defects in the thymus of the lck-C3 mice are analysed and the requirement for the tumour suppressor protein p53 and Fas-mediated signalling investigated. In the second set of experiments, using the CD2-C3 mice, the ability of constitutively active Myb and Raf to compensate for loss of Rho function in pre-T cell differentiation is explored.

6.2 - Results - Analysis of the Survival Defects in lck-C3 Mice

6.2.1 - Rho mediated thymocyte survival is controlled by p53

Gallandrini *et al.* have previously shown that expression of Bcl-2 in lck-C3 thymocytes was able to overcome the survival defect in the $CD25^+$ cells, and give a partial rescue of thymic cellularity (Gallandrini *et al.*, 1997). As Bcl-2 has been reported to antagonise p53-dependent apoptosis (Chiou *et al.*, 1994), the role of p53 in survival of $CD25^+$ cells lacking Rho function was investigated. Indeed, although p53 null mice show no defects in thymocyte development the absence of p53 does appear to be able to compensate for cells with defective pre-TCR signalling. For example, the absence of p53 is able to promote the differentiation of thymocytes lacking a CD3γ chain (Haks *et al.*, 1999). Additionally, the absence of p53 on a RAG null background allows $CD25^+$ cells to differentiate into CD4/8 DP cells (Jiang *et al.*, 1996). These observations suggest that p53 is functional during early thymocyte development, but may have a negative regulatory role.

Considering the apparent importance of p53 during T cell development combined with its role in regulating apoptosis, it was of interest to investigate whether the survival defect in the lck-C3 mice caused by loss of Rho function was p53 dependent. p53 knockout mice were crossed with the lck-C3 transgenics and the p53 heterozygous offspring backcrossed to produce mice expressing the C3-transgene on a p53 null background.

Initial analysis of the gross morphology and cell numbers revealed no differences between lck-C3 and p53 null/lck-C3 mice (Table 6.1). However, upon analysis of the CD4/8 DN subset on the basis of CD25/44 surface expression, the absence of p53 appeared to rescue both the $CD44^+25^+$ and $CD44^-25^+$ cells (Figure 6.1). Careful numerical analysis confirmed the rescue of $CD25^+$ cells in the lck-C3/p53-/- double transgenics (Figure 6.2).

Although absence of p53 in the lck-C3 mice resulted in an apparent restoration of $CD44^-25^+$ cells, it did not restore subsequent populations nor rescue thymic cellularity. One possibility is that the apparent rescued population are not true $CD44^-25^+$ pre-T cells, thus explaining the absence of subsequent populations. At the $CD44^-25^+$ stage of development, cells undergo β chain rearrangement and express a pre-TCR on their surface. In order to

Mouse	Total Thymocytes ($x10^6$)
p53 +/-	196 ± 23
p53 -/-	202 ± 16
lck-C3/p53 +/-	9.92 ± 3.3
lck-C3/p53 -/-	11.9 ± 6.1

Table 6.1 - Thymic cellularity of lck-C3/p53-/- Mice.

Mean ± SD cell numbers of thymi isolated from p53+/-, p53-/-, lck-C3/p53+/- and lck-C3/p53-/- transgenic mice (n=8).

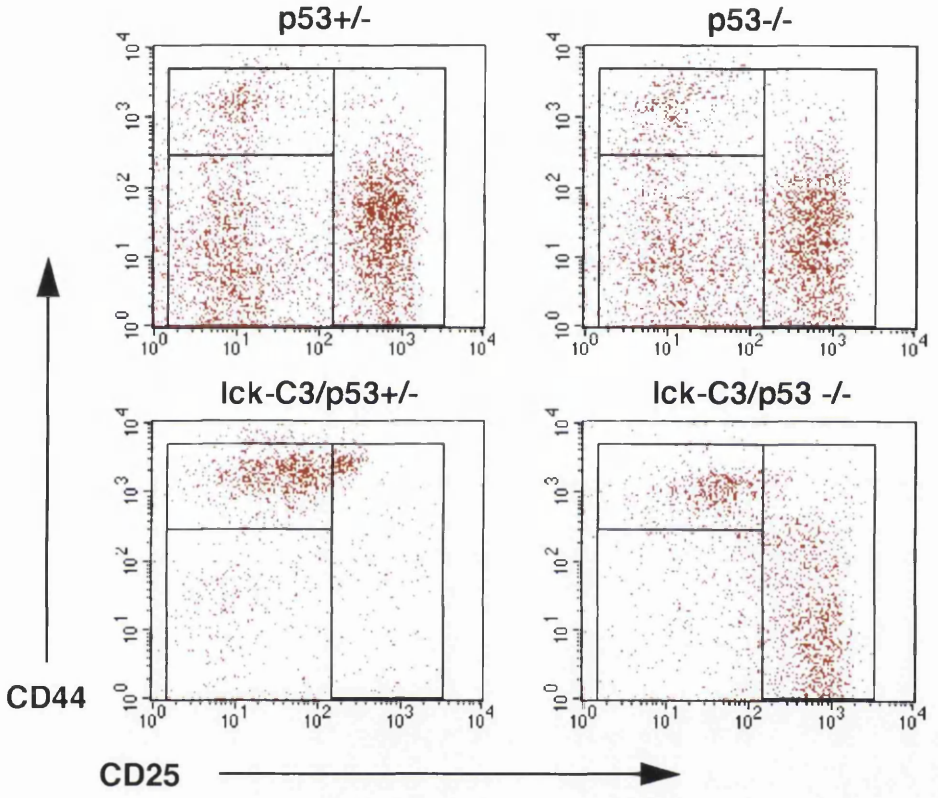

Figure 6.1 - Analysis of CD4/8 DN Thymocyte Populations in Ick-C3/p53-/- Mice.

Thymi were removed and thymocytes isolated from p53 +/-, p53-/-, Ick-C3/p53+/- and Ick-C3/p53-/- transgenic mice and analysed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes and all cells of non-T cell lineage using a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr1bio) revealed with streptavidin tricolour, and co-stained with anti-CD44-PE , anti-CD25-FITC and Thy1-APC.

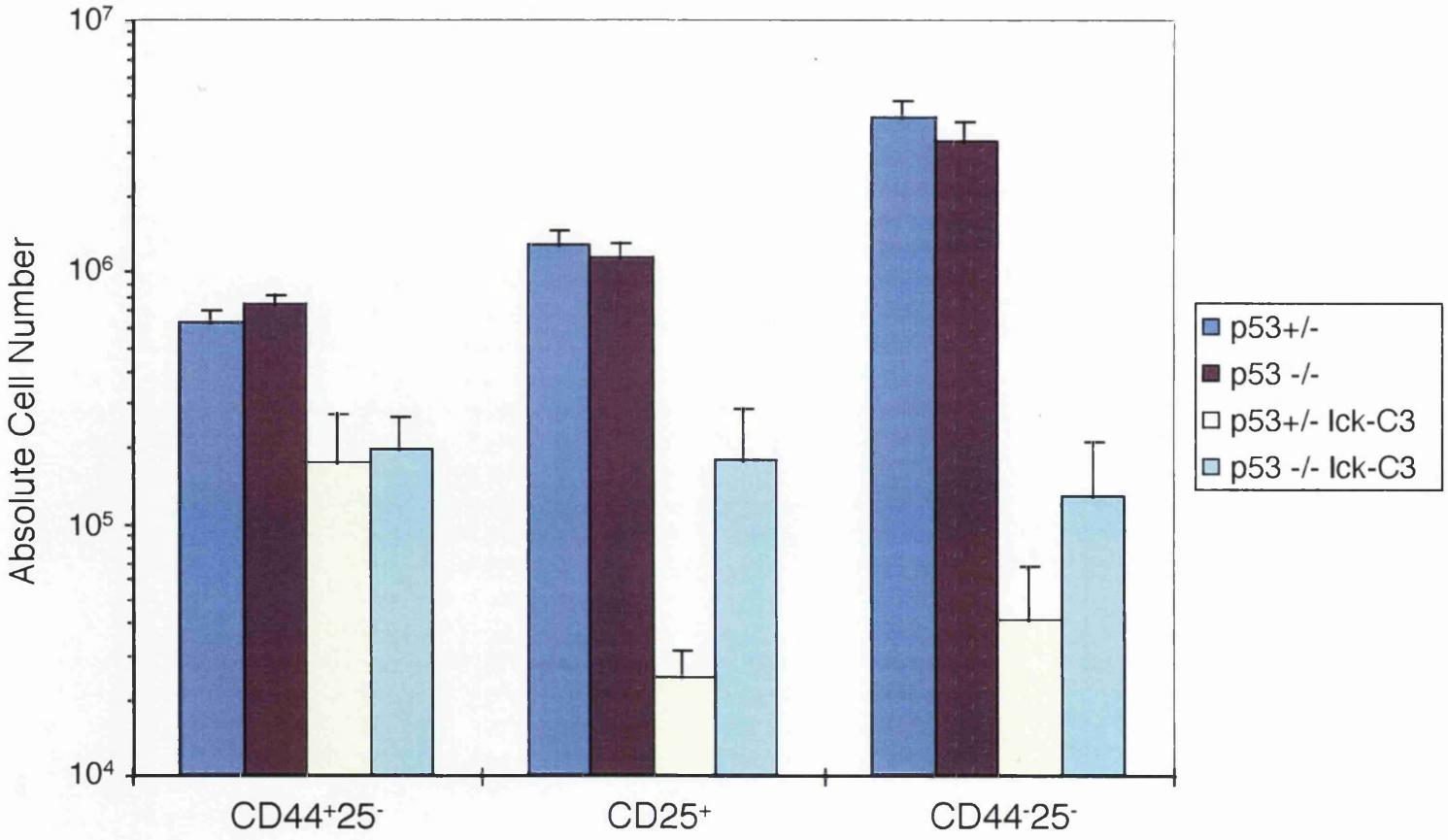

Figure 6.2 - Cell Number of CD25/44 thymic populations in lck-C3 / p53-/- Mice.

Cell numbers (Mean ± SD) of $CD44^{+}25^{-}$, $CD25^{+}$ and $CD44^{-}25^{-}$ thymic populations were calculated from p53 +/-, p53-/-, lck-C3/p53+/- and lck-C3/p53-/- transgenic mice.

confirm that the restored cells in the lck-C3/p53 null mice were pre-T cells, intracellular β chain staining was performed to determine whether β chain rearrangement and expression was occurring in these cells (Buer *et al.*, 1997). Although there were insufficient numbers of viable $CD25^+$ cells from lck-C3 mice, $CD25^+$ cells from p53+/-, p53 -/- and lck-C3/p53-/- mice all expressed readily detectable levels of intracellular TCR β subunits (Figure 6.3). The presence of intracellular β chain would thus suggest that the absence of p53 does indeed rescue survival in $CD25^+$ cells lacking Rho function. These results suggest that in the absence of Rho function, $CD25^+$ thymocytes die in a p53 dependent manner, thus implicating Rho in controlling p53-mediated survival.

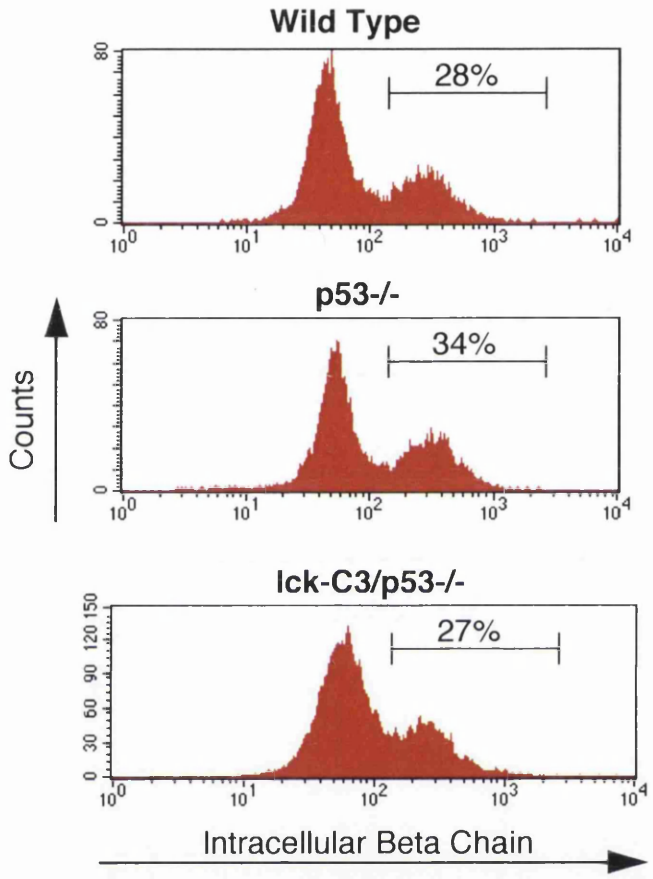

Figure 6.3 - Intracellular Beta Chain staining of CD25⁺ thymocytes from lck-C3/p53-/- Mice.

Thymocytes from p53 +/-, p53-/-, and lck-C3/p53-/- transgenic mice were stained with anti-CD25-PE and a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr1bio) revealed with streptavidin tricolour before saponin permeabilisation and staining with an antibody to the common β chain epitope.

6.2.2 - Analysis of FADD mediated signals in Thymocyte Survival

A recent report suggested that p53 is able to induce apoptosis by a transient increase in surface Fas (CD95) expression, inducing Fas-FADD binding and leading to caspase activation (Bennett *et al.*, 1998). One way to investigate the role of FADD death domain signalling in Rho regulated survival in the thymus would be to assess the consequences of blocking Fas and TNF-R family signalling on apoptosis induced by loss of Rho function. Several groups have generated transgenic mice expressing a dominant negative mutant of the cytoplasmic adapter molecule, FADD (FADD-DN). FADD-DN expression has been reported to inhibit all death-domain containing receptor induced apoptosis in both thymocytes and peripheral T cells (Newton *et al.*, 1998; Walsh *et al.*, 1998; Zornig *et al.*, 1998). Furthermore, in a report by Newton *et al.,* blockade of Fas and TNF-R family signalling with the dominant negative FADD mutant caused defective thymocyte development (Newton *et al.*, 1998). Thus, it is possible that in $CD25^+$ cells in the lck-C3 mice, loss of Rho function results in p53-mediated apoptosis via a Fas-dependent mechanism. Therefore, in order to investigate this possibility, the lck-C3 transgenic mice were crossed with mice expressing a dominant negative FADD mutant expressed under the control of the p56lck promoter. Expression of FADD-DN in the absence of Rho function in $CD25^+$ cells should allow the role of Fas-mediated apoptosis to be investigated at this stage of thymocyte development.

FADD-DN mice were crossed with lck-C3 mice and offspring analysed at 4-6 weeks of age. Examination of thymocytes isolated from FADD-DN/lck-C3 double transgenic mice on the basis of size (Forward Scatter vs. Side Scatter) and expression of the T cell marker, Thy-1 revealed the thymi to be almost devoid of thymocytes (Figure 6.4). Analysis of the few thymocytes present for surface expression of CD25 and CD44 showed that thymocytes from FADD-DN/lck-C3 double transgenic mice failed to develop past the $CD44^+25^-$ stage; all subsequent populations were completely absent (Figure 6.5). Thus, expression of a FADD-DN molecule in the lck-C3 mice appears to completely prevent the transition of early thymocytes onwards from the $CD44^+25^-$ stage of development, resulting in a complete absence of $CD44^+25^+$ thymocytes and all subsequent populations. These results suggest that inhibition of death-domain containing receptor signalling increases the severity of the lck-C3 phenotype.

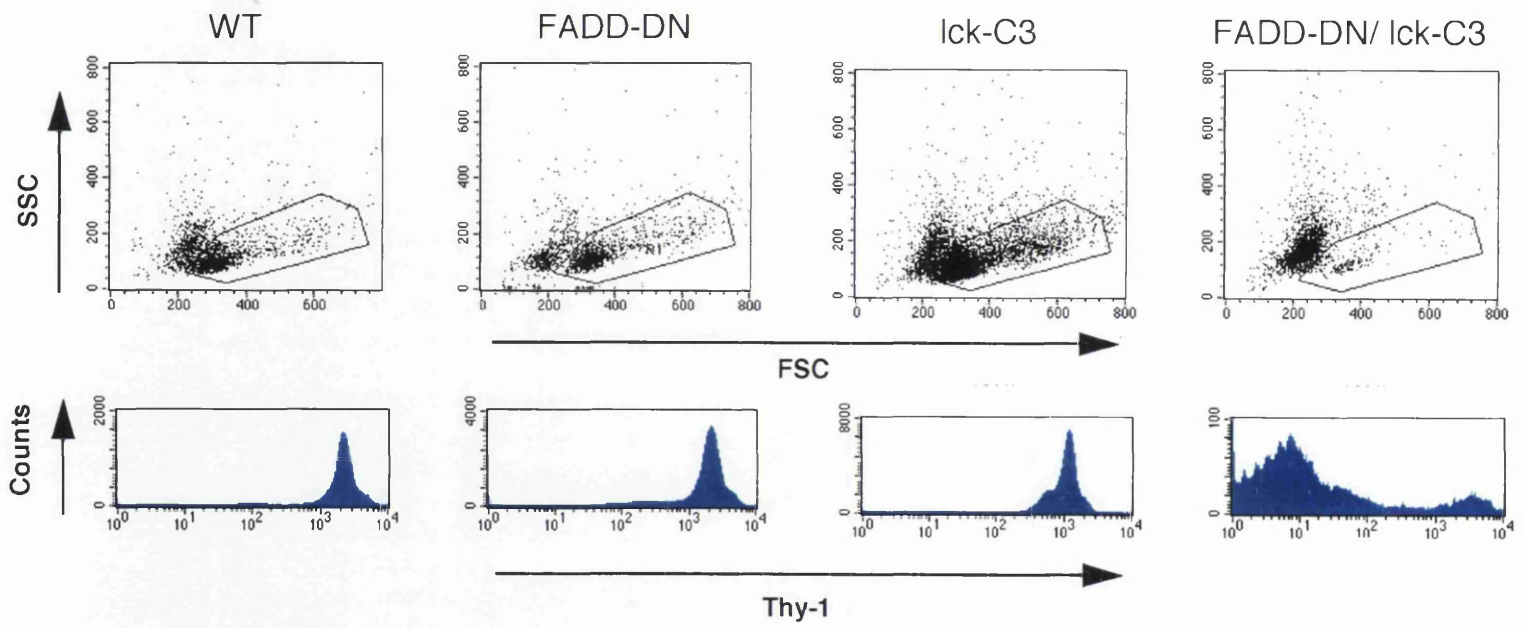

Figure 6.4 - Analysis of the FSC/SSC and Thy-1 expression levels on thymocytes isolated from FADD-DN/lck-C3 Transgenic Mice.

Thymocytes from WT, FADD-DN, lck-C3 and FADD-DN/lck-C3 double transgenic mice were isolated, stained with APC-conjugated anti-Thy-1 and analysed by flow cytometry. The top panels represents the Forward Scatter (FSC)/Side Scatter (SSC) of the transgenic mice and the botton panels the levels of Thy-1 expression.

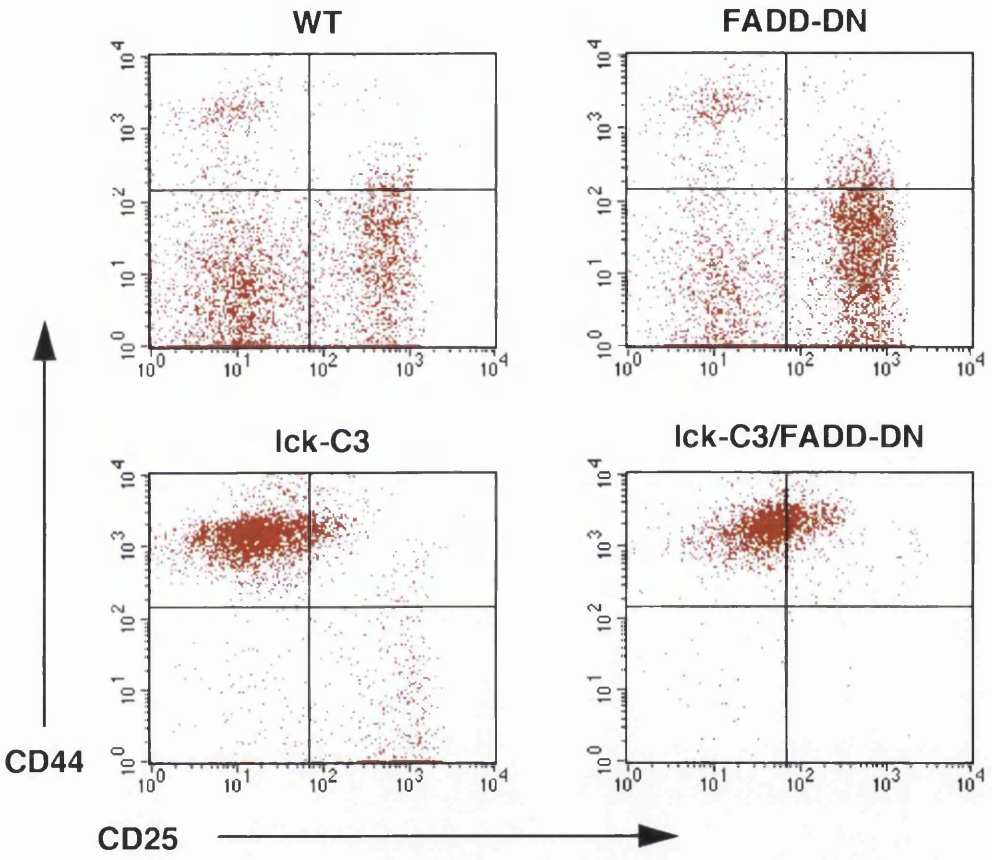

Figure 6.5 - Analysis of CD25/44 Thymocyte Populations in lck-C3/FADD-DN Mice.

Thymi were removed and thymocytes isolated from WT, FADD-DN, lck-C3 and lck-C3/FADD-DN transgenic mice and analysed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes and all cells of non-T cell lineage using a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr1bio) revealed with streptavidin tricolour, and co-stained with anti-CD44-PE , anti-CD25-FITC and Thy1-APC.

6.3 - Results - Analysis of the differentiation block in the CD2-C3 Mice.

6.3.1 - Analysis of the Role of Myb in Thymocyte differentiation

The Myb family of transcription activators play important roles in controlling the proliferation and differentiation of haemopoietic cells, as well as in a number of other cell types (Weston, 1998). During T cell development, c-Myb is expressed at high levels in immature thymocytes, but is downregulated as cells mature. Interestingly, c-Myb expression is upregulated again when resting T cells in the periphery are stimulated to proliferative by exposure to antigen (Stern and Smith, 1986; Churilla *et al.*, 1989). The ubiquitous nature of Myb family proteins has meant that little information can be obtained by conventional gene knockout approaches as mice null for expression of c-Myb, the predominant form expressed in haemopoietic cells, die during early embryogenesis (Mucenski *et al.*, 1991). To overcome this limitation, Badiani *et al.*, have taken a transgenic approach to eliminate c-Myb function. Mice expressing a dominant interfering Myb allele (DN-Myb) under the control of the CD2-LCR have severely impaired thymocyte development and a reduced proliferative capacity of peripheral T cells (Badiani *et al.*, 1994). Thymi isolated from mice homozygous for expression of the dominant interfering Myb allele are reported to contain approximately 8 x 10^6 thymocytes, comprising of 50% CD4/8 DN cells. Strikingly, the few cells that develop into CD4 and CD8 single positives are skewed towards the CD8 lineage in the absence of Myb function. Conversely, mice expressing the oncogenic form of c-Myb (v-Myb) under the control of the CD2-LCR do not have defects in thymocyte development (Badiani *et al.*, 1996). Rather, the v-Myb mice show increased numbers of $CD4^+$ cells and reduced thymic involution with age.

Analysis of CD2-C3 and lck-C3 transgenic mice reveals a number of interesting parallels between mice lacking Rho function and those expressing dominant interfering Myb alleles. Both C3 and DN-Myb mice show severely reduced thymocyte cell numbers and defective development of CD4/8 DN cells. This similarity raises the possibility that loss of Rho function during thymocyte development results in defective Myb expression and/or activation. The availability of transgenic mice expressing active Myb under the control of

the CD2-LCR allowed this hypothesis to be explored further by breeding the CD2-C3 mice onto those expressing active Myb.

In order to investigate the possibility that Rho is regulating Myb function during thymocyte development, CD2-C3 mice were crossed with mice expressing active Myb. Mice transgenic for both v-Myb and CD2-C3 were analysed at 6-8 weeks of age. Comparisons of thymocyte cell numbers of thymi isolated from wild type, CD2-C3, v-Myb and CD2-C3/v-Myb double transgenic mice revealed that expression of v-Myb was unable to rescue cellularity in the absence of Rho function (Figure 6.6A). Furthermore, analysis of CD4/8 thymic subsets showed that v-Myb expression was insufficient to promote differentiation of CD4/8 DN cells devoid of functional Rho (Figure 6.6B).

Therefore, although both loss of Rho function and c-Myb function result in very similar phenotypes in terms of both thymocyte cell number and CD4/8 DN differentiation, expression of a constitutively active Myb molecule cannot compensate for loss of Rho function in the CD2-C3 mice.

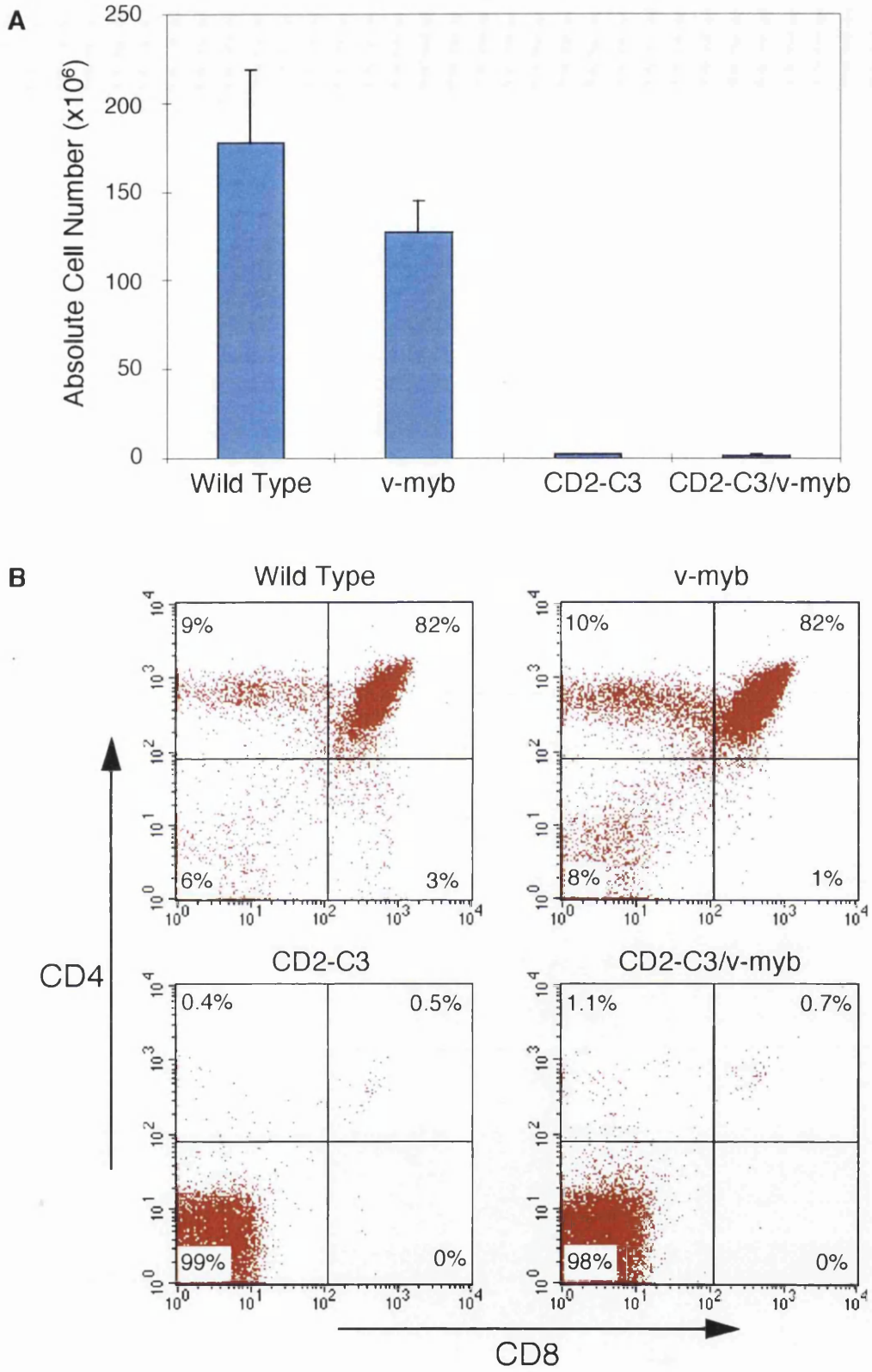

Figure 6.6 - Phenotypic Analysis on CD2-C3 /v-myb Double transgenic mice.

(A) Thymocyte cell numbers (Mean ±SD) of CD2-C3, v-myb, CD2-C3/v-myb double transgenics and Wild type mice at 4-6 weeks of age (n=5).

(B) Analysis of CD4/8 thymocytes isolated from CD2-C3, v-myb, CD2-C3/v-myb double transgenics and Wild type mice. Thymi were removed, cell prepared and stained with antibodies reactive to CD8 and CD4 surface molecules prior to analysis by flow cytometry.

6.3.2 - Analysis of the Role of Raf in Thymocyte differentiation.

As described in Chapter 3, the CD2-C3 mice show a differentiation block at the $CD25^+$ stage of thymocyte development. Although this is a similar phenotype to that observed in the RAG -/- mice, the defect in the CD2-C3 mice is not due to the absence of a pre-TCR; expression of a functional mature TCR can restore thymocyte development in RAG -/- mice (Shinkai *et al.*, 1993), but it unable to mediate differentiation in the CD2-C3 mice. Rho is thus implicated in playing an essential role in pre-TCR signalling.

One pathway established to play a central role in pre-TCR mediated differentiation and proliferation is the Ras induced Raf/MEK/MAPK pathway. In the study by Iritani *et al.*, constitutively active Raf mice were generated by attaching a farnesylation sequence (CAAX) to the carboxy-terminus of c-Raf, thus causing its localisation to the membrane, hence mimicking the Ras-mediated events that ordinarily activate Raf (Stokoe *et al.*, 1994). By breeding these mice onto a RAG-2 deficient background, both thymocyte differentiation and proliferation can be restored in the absence of a pre-TCR complex (Iritani *et al.*, 1999). Hence, Raf is able to substitute for the pre-TCR complex and drive pre-T cell differentiation and thus has been suggested to play an essential role in signal transduction downstream of the pre-TCR.

A role for Raf has also been highlighted at a later stage of development where it is required for positive selection in the thymus (O'Shea *et al.*, 1996). The mice used in this study expressed a p56lck promoter-driven transgene encoding oncogenic v-Raf, a gag-Raf fusion protein. v-Raf is constitutively active due to loss of the negative regulatory N-terminus which is replaced by gag (Rapp *et al.*, 1983) and has been shown to promote the differentiation of CD4/8 DP to SP in HY-TCR transgenic mice (O'Shea *et al.*, 1996).

As an active Raf transgene can promote both differentiation and proliferation in the absence of a pre-TCR (Iritani *et al.*, 1999), it was of interest to determine whether active Raf could rescue the thymocyte differentiation defect caused by loss of Rho function and thus gain insight into the possible link between Rho and Ras/MAPK signalling *in vivo*. Evidence for involvement of Rho in Ras/Raf/MAPK signalling comes from fibroblast studies in which active Rho mutants have been shown to co-operate with Raf in transformation assays (Roux *et al.*, 1997).

In order to investigate the ability of an active Raf-1 mutant to rescue the differentiation block caused by loss of Rho, the v-Raf transgenic mice described by O'Shea *et al.* were bred with the CD2-C3 transgenics and mice analysed at 4-6 weeks of age. The data in figure 6.7A show that there is no increase in thymocyte cell number in the CD2-C3/v-Raf double transgenics over that of the CD2-C3 mice. To investigate the possibility that v-Raf expression was promoting cell differentiation but not restoring proliferation in the absence of Rho function, the CD4/8 thymocyte populations were analysed. Figure 6.7B clearly shows that expression of v-Raf in the absence of Rho function does not promote transition of CD4/8 DN cells to later developmental stages. Analysis of thymocytes on the basis of CD25 and CD44 expression also confirmed that no rescue of thymocyte differentiation caused by loss of Rho function at the pre-T cell stage was occurring (Figure 6.8).

These data thus suggest that expression of an active Raf transgene in pre-T cells lacking Rho function is insufficient to promote their differentiation to subsequent stages of development and thus provides evidence that Rho mediates pre-TCR signals other than those that can be provided by an active Raf mutant.

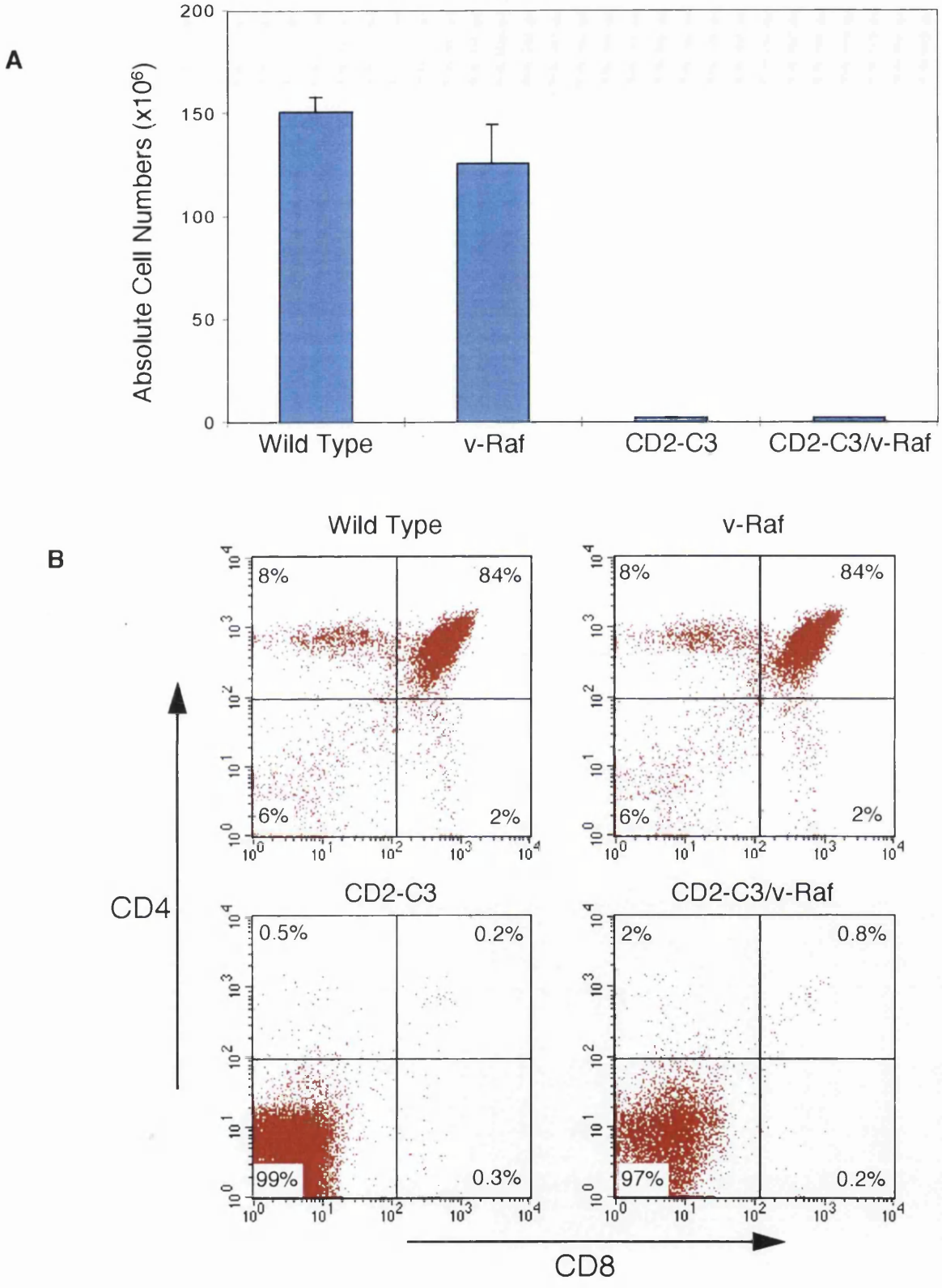

Figure 6.7- Phenotypic Analysis on CD2-C3 /v-Raf Double transgenic mice.

(A) Thymocyte cell numbers (Mean ±SD) of CD2-C3, v-Raf, CD2-C3/v-Raf double transgenics and Wild type mice at 4-6 weeks of age (n=5).

(B) Analysis of CD4/8 thymocytes isolated from CD2-C3, v-Raf, CD2-C3/v-Raf double transgenics and Wid type mice. Thymi were removed, cell prepared and stained with antibodies reactive to CD8 and CD4 surface molecules prior to analysis by flow cytometry.

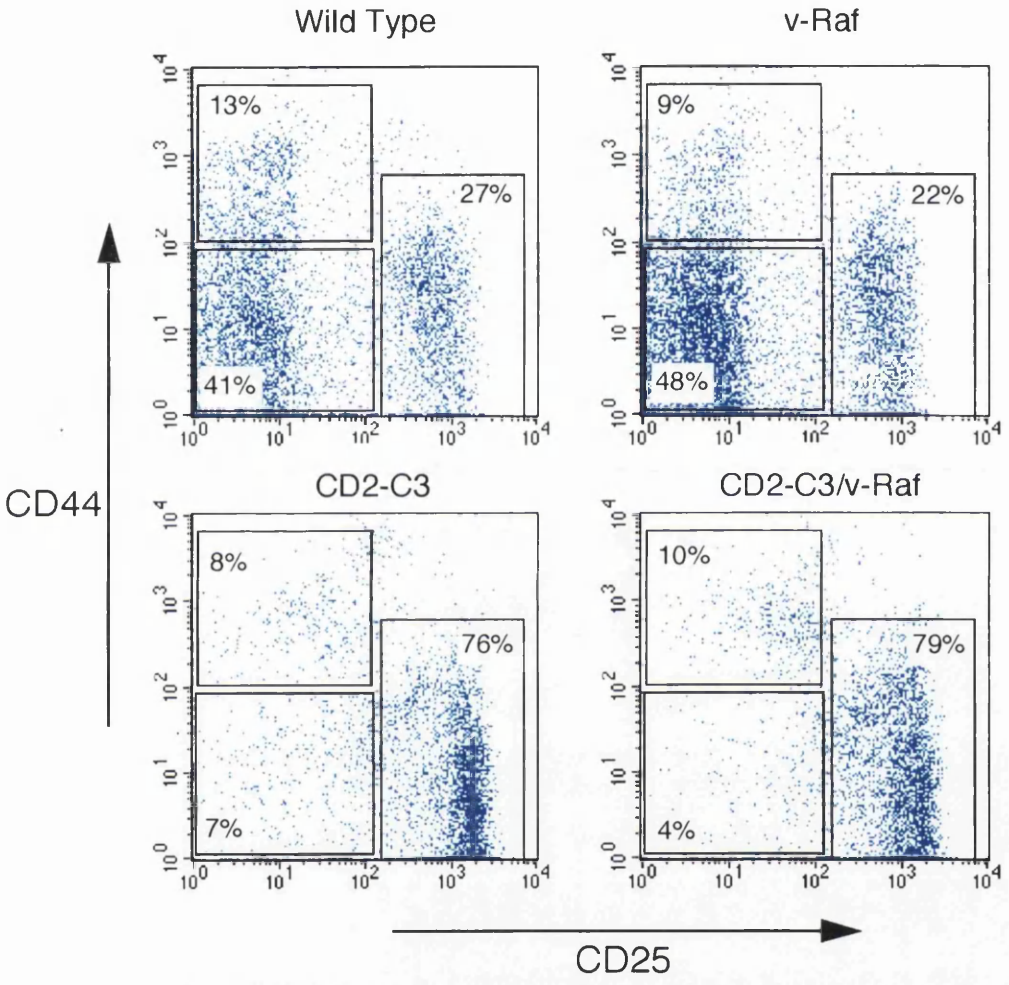

Figure 6.8 - Phenotypic Analysis of CD4/8 DN cells isolated from CD2-C3/v-Raf Double transgenic mice.

Thymocytes were analysed for expression of CD25 and CD44 by lineage exclusion of all CD4/8 DP and SP thymocytes and all cells of non-T cell lineage using a panel of biotinylated antibodies (CD4bio, CD8bio, CD3bio, B220bio, Mac-1bio, NKbio, γδ-bio, and Gr-1bio) revealed with streptavidin tricolour, and co-stained with anti-CD44-PE and anti-CD25-FITC.

6.4 - Discussion

6.4.1 - Analysis of the role of p53 in Rho-mediated Thymocyte Survival

Here, the characterisation of the previously reported survival defect in lck-C3 mice has been extended and the massive apoptosis previously observed in the CD25 population shown to be dependent upon p53. Apoptosis mediated by p53 may be antagonised by the expression of anti-apoptotic survival factors (Chiou *et al.*, 1994). Consistent with this, ectopic overexpression of the anti-apoptotic factor Bcl-2 is able to alleviate death of the $CD25^+$ population (Gallandrini *et al.*, 1997). Rho has also been suggested to regulate Bcl-2 expression (Gomez *et al.*, 1997); however, analysis of thymic subpopulations from lck-C3 mice reveals that Bcl-2 expression is not affected by the absence of Rho function (P. Costello, unpublished data). When Bcl-2 is ectopically overexpressed the balance is shifted in favour of survival and does not *a priori* mean that the original cellular defect was loss of Bcl-2. This observation would suggest that Rho has a role in maintaining the balance between anti- and pro- apoptotic factors though is not implicitly involved in the regulation of Bcl-2 expression.

Interestingly, absence of p53 allows $CD44^+25^+$ and $CD44^-25^+$ cells to survive in the absence of Rho function, it does not restore the $CD44^-25^-$ populations nor rescue thymic cellularity. This is in striking contrast to the effects of ectopic expression of Bcl-2 in the lck-C3 mice where not only does Bcl-2 expression rescue all stages of thymocyte development, but also partially restores thymic cellularity (Gallandrini *et al.*, 1997). Currently, data is emerging that loss of Rho function in the lck-C3 mice may give rise to other survival defects in addition to those seen in the $CD25^+$ cells. Furthermore, not all apoptosis in the thymus is p53 dependent (Clarke *et al.*, 1993). Thus, one explanation for the more complete rescue observed by Bcl-2 expression as compared to that obtainable by absence of p53 may be that expression of Bcl-2 is able to overcome other survival defects in the lck-C3 mice that are independent of p53. Further work will be required to resolve this issue.

6.4.2 - Analysis of the role of Fas-signalling in Rho-mediated Thymocyte Survival

A recent report suggesting that p53 is able to induce apoptosis through a transient increase in surface Fas (CD95) expression, inducing Fas-FADD binding and leading to caspase activation prompted the analysis of the role of Fas-signalling in the survival of $CD25^+$ thymocytes from lck-C3 mice (Bennett *et al.*, 1998). Surprisingly, the only population of cells present in mice transgenic for both FADD-DN and lck-C3 were the early $CD44^+25^-$ thymocytes, suggesting that blockade of death-domain receptor signalling in lck-C3 thymocytes completely prevented thymocyte development past the $CD44^+25^-$ stage. Although this meant it was not possible to assess the role of Fas-mediated signalling in $CD25^+$ cell survival, the data does reveal a role for death domain containing receptor signals in early thymocyte development only detectable when an additional survival pressure is exerted on the cells (i.e.; loss of Rho function).

The FADD-DN mice used in this study have not been reported to have defective thymocyte development (Zornig *et al.*, 1998). However, two other groups have produced similar FADD-DN mice with strikingly different phenotypes to those used here (Newton *et al.*, 1998; Walsh *et al.*, 1998). The FADD-DN mice reported in a study by Walsh *et al.* showed retarded thymocyte development, a feature most evident in homozygous FADD-DN mice (Walsh *et al.*, 1998). Thymi from homozygous FADD-DN mice were approximately one-third the size of littermate controls. Further analysis revealed a partial block in development at the $CD44^-25^+$ stage; as expression of transgenic TCRs do not rescue the block, it was suggested that this was due a defect in pre-TCR signalling. FADD-mediated signals were not found to be required for negative selection as male FADD-DN/HY-TCR mice were indistinguishable from male HY-TCR mice in terms of CD4/8 subsets. In a similar FADD-DN transgenic approach, Newton and colleagues reached a different conclusion; FADD-DN expression enhances negative selection (Newton *et al.*, 1998). Also, no reduction in thymic cellularity in their FADD-DN mice was reported. The FADD-DN mice used in this study were characterised in a study by Zornig *et al.* (Zornig *et al.*, 1998). Consistent with both studies, they observed inhibition of peripheral T cell proliferation. No defects in thymopoiesis were seen and the involvement of FADD-mediated signals in positive and negative selection was not assessed. However, analysis of these mice in

this study reveals a severe defect in thymocyte development in the absence of Rho function.

The differences between the three studies is intriguing. All used the proximal p56lck promoter to express a dominant negative truncated FADD molecule comprising solely of the death domain. One possible explanation for these differences maybe level of transgene expression and to what degree FADD-mediated signals were inhibited by these dominant negative approaches. This theory is supported by the observation that only mice homozygous for the FADD-DN transgene have thymocyte developmental defects (Walsh *et al.*, 1998). It is always a concern with dominant negative approaches that a sufficient amount of truncated molecule is expressed to inhibit signals normally transduced by the full-length endogenous version (Discussed in section 1.5); conversely, if expression is too high, the dominant negative molecule may attenuate other signalling pathways in which it is not normally involved.

Co-expression of C3 and FADD-DN in thymocytes results in a complete block in thymocyte development at the $CD44^{+}25^{-}$ stage with thymi from these mice being almost devoid of thymocytes. Although this has made it impossible to assess the role of death-domain containing receptor mediated signalling in $CD25^{+}$ lacking Rho function, these observations highlight a previously unrecognised role for death-domain mediated signalling in early thymocyte proliferation and differentiation.

6.4.3 - Analysis of the Role of Rho in Myb regulation

The similarity in phenotypes between mice devoid of Rho function and those expressing dominant interfering Myb alleles prompted the investigation of whether active Myb could rescue the block in differentiation seen in the CD2-C3 mice. Analysis revealed that active Myb expression was insufficient to either restore thymic cellularity or rescue CD4/8 DN differentiation in the absence of Rho function. These data suggest that Rho function is either a) required for processes in addition to Myb regulation during thymocyte development, or b) plays no role in the regulation Myb, and is thus essential for other processes regulating development.

The role played by Myb during thymocyte development has been suggested to be one of protection from apoptosis. Myb was first implicated as a regulator of cell survival by a study in which switching off an inducible Myb construct resulted in apoptosis, accompanied by down-regulation of Bcl-2 (Frampton *et al.*, 1996). The role of Myb in regulation of Bcl-2 was further strengthened by the observation that the Bcl-2 promoter contains a number of functional Myb-binding sites. In another study, induction of DN-Myb caused apoptosis in a murine T cell line and correlated with loss of Bcl-2 expression (Taylor *et al.*, 1996). Similarly, the effect of Myb on Bcl-2 expression was shown to be via conserved Myb-binding sites in the Bcl-2 promoter region. Moreover, it was suggested that the defect in thymopoiesis seen in mice expressing DN-Myb under the control of the CD2 promoter is due to increased apoptosis resulting from lack of Bcl-2 expression (Taylor *et al.*, 1996). However, the phenotype of mice lacking expression of Bcl-2 is less severe than those expressing DN-Myb (Veis *et al.*, 1993), suggesting that regulation of Bcl-2 is only one of the roles played by Myb .

The apparent requirement for Myb function in thymocyte survival through the regulation of Bcl-2 expression may explain why active Myb expression does not rescue thymocyte survival in the CD2-C3 mice; CD2-C3 mice have a defect in pre-TCR signalling and not thymocyte survival. Conversely, lck-C3 mice have defective survival which is rescuable by expression of Bcl-2 (Gallandrini *et al.*, 1997). One possible explanation is that loss of Rho function leads to defects in Myb activation, resulting in lack of Bcl-2 expression. Hence it may prove more beneficial to assess the role of Myb in the lck-C3 mice. However, this would require an alternative strategy to the one presented here as expression of the v-Myb transgene is driven by the later expressing CD2-LCR promoter.

6.4.4 - Analysis of the role of Rho in Raf/MAPK signalling

Raf has previously been suggested to play an important role in thymocyte development, both in pre-TCR mediated signalling and in positive selection (O'Shea *et al.*, 1991; Iritani *et al.*, 1999). As expression of a constitutively active Raf transgene is able to overcome the differentiation block at the $CD25^+$ stage in mice lacking a pre-TCR complex, it

was of interest to determine whether active Raf could also overcome the differentiation block caused by loss of Rho function in the CD2-C3 mice.

Analysis on mice transgenic for both v-Raf and CD2-C3 revealed that active Raf was not sufficient to restore thymocyte differentiation in the CD2-C3 mice. As active Raf has been shown to be sufficient to rescue thymocyte differentiation in the absence of a pre-TCR complex, this failure to rescue pre-T cells lacking Rho function may suggest that Rho is important for pre-TCR signal transduction via the Ras/Raf/MAPK pathway. However, there are a number of problems with this kind of interpretation. One important factor to be considered is whether or not the failure of an active Raf transgene to rescue thymocyte differentiation in the CD2-C3 mice is indeed due to an absolute requirement for Rho in Raf-mediated pre-TCR signalling or whether the failure is due to other defects caused by loss of Rho function, not rescuable by active Raf.

As Rho and its family members have previously been implicated in the regulation of MAPK cascades (discussed in section 1.2.2), it is tempting to speculate that Rho may be required in Raf-mediated pre-TCR signalling. However, analysis of the role of Rho GTPases in transformation suggests that Rho may not function downstream of Raf in all systems; although constitutively active versions of Rho GTPases can synergise with either active Ras or Raf to transform NIH3T3 or Rat-1 rodent fibroblasts, dominant negative Rho GTPases fail to block Raf mediated transformation (Roux *et al.*, 1997). These data suggest that Rho family GTPases may act downstream of Ras, but independently from Raf in mediating cellular transformation.

Although the failure of active Raf to rescue the differentiation defect in the CD2-C3 mice is intriguing, further work is clearly required to explore the genetic connection between Rho and Raf in pre-TCR signalling. One way in which to explore this link further would be to express a constitutively active signalling molecule that lies downstream of Raf in the Ras/MAPK cascade. Expression of a constitutively active MEK, for example, would further define the point at which Rho-mediated signals are required for pre-TCR initiated MAPK activation.

6.4.5 - Summary

In summary, using genetic crosses, the *in vivo* role of Rho-mediated signalling has been investigated. The death of $CD25^+$ cells in the absence of Rho function has been shown to be dependent upon p53. One way in which p53 mediates apoptosis is by inducing Fas-signalling. Although, an unsuccessful attempt to analysis the role of Fas-signalling in the apoptosis of $CD25^+$ cells was made, a previously unrecognised defect in thymocyte development was revealed in the FADD-DN mice used in this study. Expression of neither active Myb nor active Raf transgenes in CD2-C3 mice were able to overcome the block in thymocyte differentiation at the pre-T cell stage seen in the absence of Rho function. These data suggest that Rho in involved in pre-TCR signalling cascades other than those involving Raf or Myb.

CHAPTER 7
Identification of Rho Effector Molecules in T Lymphocytes

7.1 - Introduction

7.1.1 - Rho signalling diversity - A model

The work presented in the previous chapters has demonstrated that the GTPase Rho can have very different roles during a dynamic process such as thymocyte development. For example, the receptors mediating cell survival during early thymocyte survival and the pre-TCR both function via Rho although the final outcome of these signals is very different; continued cell survival in the first instance and cell differentiation in the latter. How can such signals both be mediated by Rho yet result in such different outcomes? One way in which this diversity of the processes Rho regulates could be generated is by differential expression of Rho effector molecules at different stages of thymocyte development. For example, during early thymocyte development, the receptors involved in cell survival activate Rho which in turn binds to and activates an effector specific to a survival pathway. As cells mature and start to express the pre-TCR, a different Rho effector involved in mediating cell differentiation and proliferation may be expressed (Figure 7.1). The effector involved in cell survival could then be inactivated by a secondary signal, degraded, or localised to a region of the cell where it can no longer interact with Rho. This is only one possible mechanism by which Rho signalling diversity could be generated; there are many others, for example the Rho effector molecule may be the same for both cell survival and differentiation, and the diversity occurs further downstream. Alternatively, Rho may regulate the recruitment of adapter molecules to the membrane that interact directly with the receptors. Nevertheless, given the large variety of Rho effector molecules already known, it was of interest to determine which, if any, interacted with Rho predominantly in lymphocytes.

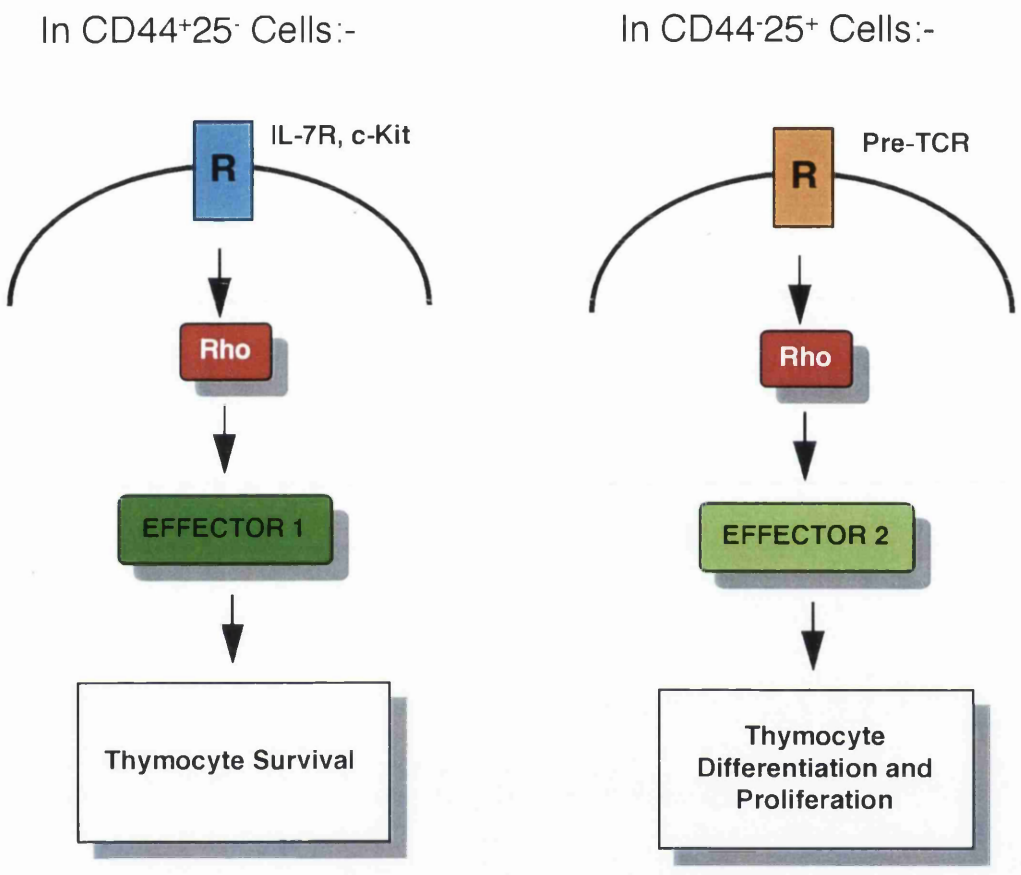

Figure 7.1 - Concept of differential effector expression within Thymocyte Progenitors

During thymocyte development, one way in which different receptors may signal through Rho yet mediate different responses is by the differential expression of Rho effectors at different stages of thymocyte development.

7.1.2 - Known Rho Effectors

At the time of beginning this study, it was becoming evident that Rho interacted with numerous effector molecules in a GTP-dependant manner. The majority of the proteins identified as interacting with Rho were implicated in the regulation of the actin cytoskeleton. Indeed, even today, the effector molecules linking Rho to processes such as transcriptional regulation are poorly understood. The molecules binding to Rho fall into two distinct categories; those with serine/threonine kinase activity and those with no catalytic domains and thus proposed to serve as adapter molecules.

One of the first Rho effectors to be identified was PRK-1 (also known as Protein Kinase N (PKN) owing its similarity to Protein Kinase C) (Amano *et al.*, 1996b). Although the substrates of PRK-1 are currently unknown, its autophosphorylation is stimulated by LPA *in vivo*; a response that is suggested to require Rho function as C3-transferase expression prevents LPA-induced PRK-1 activation (Amano *et al.*, 1996b). Another member of the PRK family is p140 PRK-2. First identified as a NCK-associated kinase, PRK-2 also binds Rho in a GTP-dependent manner but whether Rho binding activates PRK-2 is unclear (Quilliam *et al.*, 1996). Interestingly, PRK-2 has been reported to co-operate with activated Rho in transcriptional activation via the c-fos serum response factor (Quilliam *et al.*, 1996; Vincent and Settleman, 1997). Although no such effect has been reported for PRK-1, this may be due to the fundamental difference that PRK-2 can bind to the adapter molecule NCK while PRK-1 cannot and may therefore mediate very different signals.

Another serine/threonine kinase that interacts with Rho is ROCK-1, with its kinase domain within its N-terminus, a long amphipathic α helix capable of forming a coiled-coiled structure in the middle and the pleckstrin homology domain split by a cysteine-rich zinc finger in the C-terminal (Fujisawa *et al.*, 1996; Ishizaki *et al.*, 1997). This kinase shows significant homology in its N-terminus to myotonic dystrophy kinase, the product of the causative gene of myotonic dystrophy (Mahadevan *et al.*, 1993). A homolog of ROCK-1 has also been isolated and is called either ROCK-2, ROKα or Rho-kinase (Leung *et al.*, 1996b; Matsui *et al.*, 1996). The ROCKs has been shown to bind specifically to GTP-Rho *in vitro*, undergoing autophosphorylation upon activation by GTP-bound Rho and showing kinase activity towards exogenous substrates. The function of the ROCK family of Rho

effectors have been eluded to by two lines of experiments. Firstly, Kimura *et al.* have found that ROCK-2 is associated with a p130 binding subunit of myosin phosphatase and can phosphorylate this subunit thereby suppressing its phosphatase activity (Kimura *et al.*, 1996). Thus, ROCK has been implicated in Rho-mediated enhancement of myosin-based contractility (Reviewed in section 1.2.1). The second line of evidence as to the function of ROCK has come from expression of ROKα in cultured cells. Expression of full-length or truncation mutants have revealed that the kinase activity is required to induce both focal adhesion-like structures and stress-fibre bundles in the cells (Ishizaki *et al.*, 1997). ROCK has also been implicated as being essential for Rho mediated transformation in a study using the potent specific ROCK inhibitor Y-27632 (Itoh *et al.*, 1999).

Similar in structure to the ROCKs are the two known isoforms of Citron Rho-interacting kinase or CRIK. These are two differentially spliced kinases which unlike ROCK are expressed in a restricted number of tissues, suggesting that these kinases may serve a more specialised function in specific cell types (Di Cunto *et al.*, 1998). Little is known about the function of the shorter isoform of CRIK (called CRIK-SK) or indeed whether it is even able to bind to Rho as it consists almost solely of the N-terminal kinase domain. The larger splice variant has been reported to play a role in cytokinesis; overexpression of CRIK mutants lead to defective cell division resulting in multinucleated cells (Madaule *et al.*, 1998).

In addition to the Rho effectors mentioned above, all with kinase domains, there are several other effector molecules that lack such a catalytic domain and are thus thought to serve as adapter molecules. These include the PRK-related effectors, Rhotekin and Rhophilin (Reid *et al.*, 1996; Watanabe *et al.*, 1996). Although little is known about the exact function of these molecules, interestingly, sequence comparisons between PRK, Rhotekin and Rhophilin have enabled the identification of a consensus Rho-binding sequence, referred to a Rho effector motif class I (Reid *et al.*, 1996). This motif is distinct from that found within the ROCK and Citron-related effectors (Narumiya *et al.*, 1997).

7.1.3 - The Search for Rho effectors

Table 7.1 - Known Rho Effectors and their identification

Effector	Size (Kd)	Method of Identification	Reference
PRK-1 PKN-1	120	Yeast Two Hybrid Affinity Chromatography	Wanatabe et al.1996, Amano et al 1996
PRK-2	140	Yeast Two Hybrid Affinity Chromatography	Quilliam et al 1996 Vincent et al. 1997
ROCK-1	160	Affinity Chromatography	Fujisawa et al. 1996
Rho-Kinase	164	Affinity Chromatography	Matsui et al. 1996
CRIK Citron Kinase	240	Degenerate PCR Degenerate PCR	DiCunto et al 1998 Madaule et al 1998
CRIK-SK	54	Degenerate PCR	DiCunto et al 1998
Rhophilin	71	Yeast Two Hybrid	Watanabe et al. 1996
Rhotekin	61	Yeast Two Hybrid	Reid et al 1996
Citron	183	Yeast Two Hybrid	Madaule et al 1995
mDia-1	140	Yeast Two Hybrid	Watanabe et al 1997

It is evident from table 7.1 that a number of different methods have been employed in the search for Rho effector molecules. The most commonly used is that of yeast two hybrid screening (Fields and Song, 1989). This technique is based upon the requirement for both a DNA-binding domain and an activation domain to initiate transcription from an upstream activating sequence (UAS) in yeast. Typically, the UAS is upstream of a reporter gene such as β-GAL or HIS3. Briefly, a plasmid is constructed from the cDNA encoding for the protein of interest (referred to as 'Bait') fused to the DNA-binding domain. This is used to 'fish' for interacting proteins ('Prey') from a cDNA expression library consisting of a plasmids containing cDNAs fused to the activation domain sequence. When both the bait and library cDNAs are co-expressed within a single yeast, if the bait interacts with another protein fused to the activator domain, transcription is initiated from the UAS and the reporter gene transcribed. The reporter gene usually confers some selective

advantage on the yeast cell, and a colony is produced. Isolation of the viable colonies then enables the cDNA encoding the interacting protein to be isolated and sequenced.

Limitations of this technique are that a cDNA expression library needs to be constructed and the technique is prone to 'false positives'; these arise when non-physiologically relevant 'Bait-Pray' interactions take place and are able to confer the selection advantage on the yeast cell. The problem of false positives is widespread and can be time-consuming to eliminate (Hengen, 1997). Also, yeast two hybrid systems are unable to give any indication of the strength of protein-protein interaction or whether such an interaction is of physiological relevance within a mammalian cell. Despite these limitations, the yeast two hybrid system has been successfully used to identify an incredible number of protein-protein interactions.

The second method, and the one employed in this study is that of protein affinity chromatography. In this approach, the bait protein is immobilised on a matrix and mixed with a cell lysate. The matrix, bait, plus any bound proteins are recovered and resolved by SDS-PAGE. Following Western blotting, the membrane can be probed with an antibody raised to a known effector. This approach is of course limited to finding known proteins to which antibodies are commonly available. In order to use this method for identifying novel effectors, the process of mass spectroscopy fingerprinting and peptide sequencing must be used (Pappin *et al.*, 1996). This involves scaling up the protein affinity chromatography to such an extent that the effector protein(s) can be visualised when the membrane is stained with a dye such as Ponseau-S. The band of interest can then be excised, digested with trypsin and a mass spectroscopy fingerprint produced. The fingerprint is unique to each protein, and represents the mass of each peptide fragment produced following tryptic digestion. The fingerprint can be used to search a database in which all known proteins have been theoretically 'fingerprinted'; that is to say the masses of each fragment if the protein were to be digested with a specific protease have been calculated. However, this method is again limited to finding known proteins whose fingerprints have been calculated. To extend this approach to find novel effectors, the peptides must be sequenced. This can be done by mass spectroscopy peptide sequencing and involves processing of the tryptic digests and sequencing by low-energy collision-activated dissociation (CAD). The peptide sequences obtained can then be used to search a protein

database. Protein sequence databases now contain an ever-increasing number of ESTs, or expressed sequence tags. These are partial sequences obtained by rapid single-pass sequencing of cDNAs. For many, no function is known. If protein database searching is unproductive, the peptide sequences can be used to make degenerate oligonucleotides and these in turn used to screen a cDNA library. Figure 7.2 shows the steps involved in this approach.

One criticism of this technique is that it will bias detection to the most abundant effector molecules or those with the highest affinities for Rho, making it more difficult to pick up Rho effectors of low abundance. It is also possible that one might isolate proteins that do not bind directly to Rho but indirectly via complex formation with 'real' Rho effectors. In addition, the technique is susceptible to contaminants binding to the either the matrix itself or being transferred to the PVDF membrane during manipulations. Nevertheless, this method does offer the possibility to identify novel Rho binding proteins.

A third method of searching for interacting proteins is to use degenerate PCR techniques. In this approach, total RNA is prepared from the cells of interest and reverse transcribed to produce single-stranded cDNA (Holzman *et al.*, 1994). Degenerate oligonucleotides corresponding to a particular domain (i.e.: a serine/threonine kinase domain) or consensus sequence are then used in a PCR reaction. Any resultant DNA amplification products are sub-cloned in a vector, transformed into bacteria and DNA minipreps made. The cloned DNA is then sequenced and used to search a DNA sequence database.

This technique allows the rapid identification of expressed effector molecules either containing a particular domain of interest or in the cases of CRIK and CRIK-SK, related to a previously known effector molecule. The obvious limitation is that the sequence of the domain of interest must be known first.

In the following chapter, the approach that was taken leading to the identification of two Rho effectors in lymphocytes is described, and the possible roles for these molecules discussed.

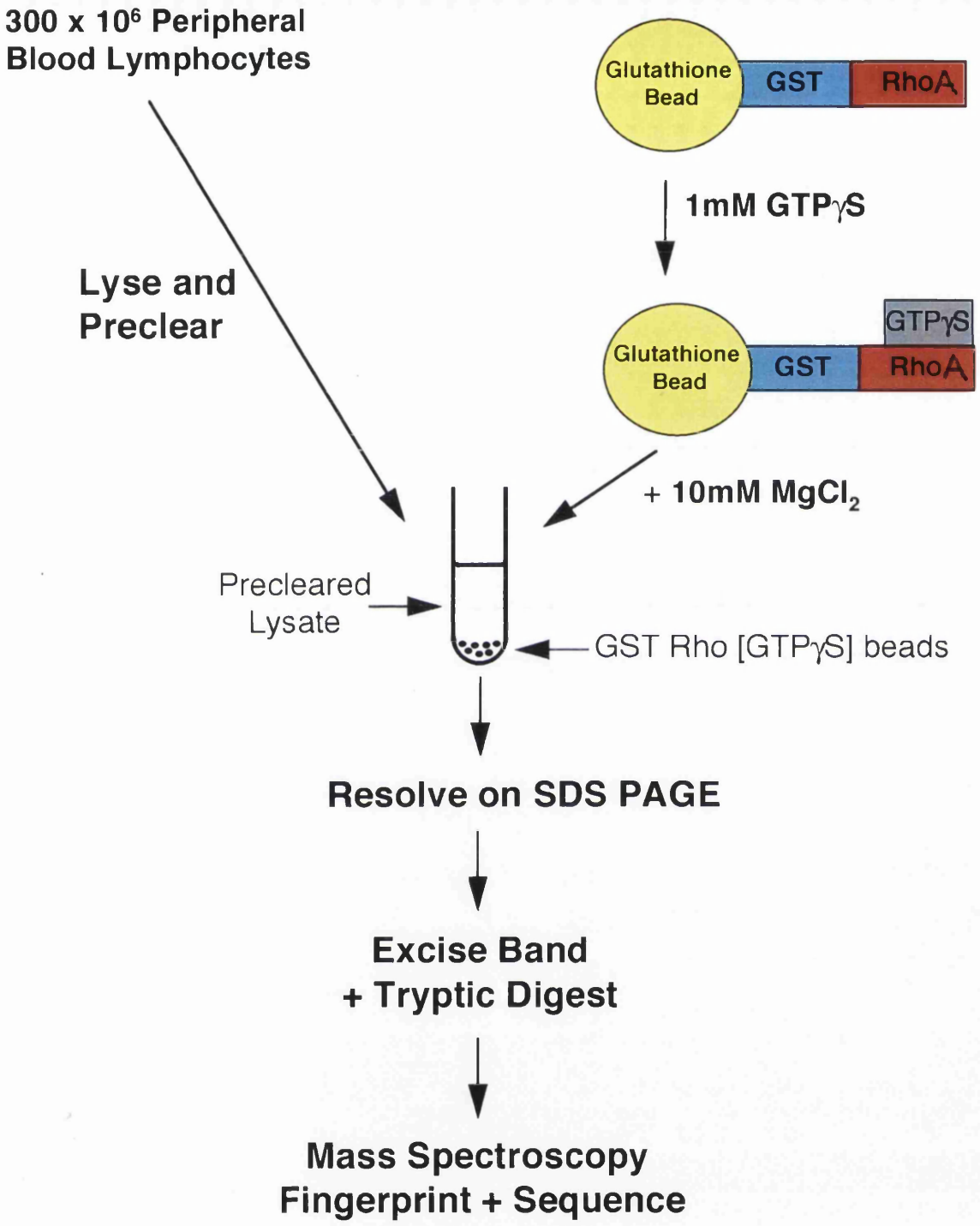

Figure 7.2- Strategy used to identify novel Rho Effectors from T cell Lysates.

Recombinant GST-RhoA was coupled to Glutathione beads, loaded with GTPγS, and incubated with a T cell lysate. Rho-binding proteins were then separated by SDS-PAGE, bands excised, digested and identified by Mass Spectroscopy fingerprinting and/or sequencing.

7.2 - Results

7.2.1 - Initial Analysis of Rho-binding proteins by *In vitro* Kinase assay

Using bacterially expressed RhoA, a protein affinity chromatography approach has been adopted to isolate Rho-interacting molecules from T cell lysates. Briefly, recombinant Rho fused to Glutathione-S-Transferase was expressed in bacteria and purified. The fusion protein was then bound to Glutathione-Agarose beads, and loaded with either non-hydrolysable GDPβS or GTPγS. A T cell lysate was prepared from 7 day old peripheral blood lymphocytes and pre-cleared with Glutathione-S Transferase bound to Glutathione-Agarose in order to remove any contaminants that may bind to either the agarose matrix or the GST protein itself. Guanine nucleotide loaded GST-RhoA was then mixed with a T cell lysate, before recovering the GST-RhoA and any interacting proteins that may be bound to it.

It has previously been reported that the Rho binding kinases PRK-1, -2 and ROCK-1 all autophosphorylate *in vitro* (Amano *et al.*, 1996b; Ishizaki *et al.*, 1997; Vincent and Settleman, 1997). As a first attempt to determine which Rho binding kinases were present in T-cells, the GST-Rho plus any interacting proteins were subjected to an *in vitro* kinase assay using [^{32}P]-γ-ATP, resolved on a SDS PAGE gel and exposed to film (Figure 7.3). The data show that there was no *in vitro* kinase activity detectable in the unloaded GST-Rho or GST-Rho[GDPβS] lanes. Two predominant bands associating selectively both with the GTPγS loaded Rho and constitutively active V14RhoA were observed.

The upper band runs at approximately 120kd; the lower band at around 80kd. The Rho binding kinase PRK-1 migrates at 120kd in SDS PAGE gels, whereas the Rho binding kinases, PRK-2 and ROCK-1 migrate at approximately 140kd and 160kd respectively (Quilliam *et al.*, 1996; Ishizaki *et al.*, 1997). It is not possible to be sure whether proteins detected in these *in vitro* kinase assays are autophosphorylated kinases or their substrates. There are no known Rho binding proteins of 80kd, but PRK-1 is a candidate for the 120kd protein labelled in the *in vitro* phosphorylation reaction.

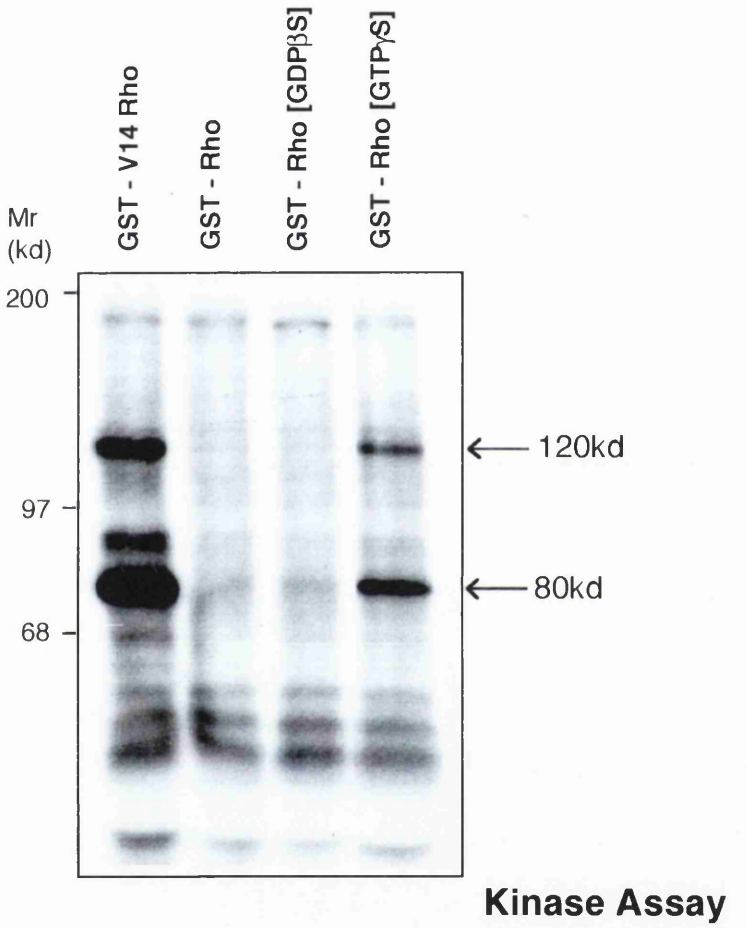

Figure 7.3 - Purification and *In vitro* Kinase assay of Rho Binding Proteins.

GST-V14 Rho and GST-WT Rho (unloaded, loaded with GDPβS and loaded with GTPγS) were used in a protein affinity chromatography approach to isolate Rho interacting proteins from peripheral blood lymphocytes, revealed with an *in vitro* kinase assay prior to running on a 7.5% polyacrylamide gel.

7.2.2 - PRK-1 Interacts with Rho in a GTP-dependent manner

The hypothesis that PRK-1 was interacting with Rho in T cells was tested further; proteins isolated from a T cell lysate with the different Rho fusion proteins were subjected to Western blot analysis with a monoclonal antibody to PRK-1. Figure 7.4 shows a 120 kd band recognised by the antibody, and therefore presumed to be PRK-1. PRK-1 could only be purified from T cell lysates by GTPγS loaded Rho and V14Rho, but not with GDPβS loaded Rho, thus demonstrating GTP-binding dependance for PRK-1/Rho interactions.

7.2.3 - Identification of a 'novel' Rho binding protein by Mass Spectroscopy fingerprinting

To further characterise Rho binding proteins in T cells and in particular to identify the 80kd protein phosphorylated in the *in vitro* kinase assay, the protein-affinity chromatography experiments were scaled up; Rho interacting proteins were resolved by SDS-PAGE and visualised using Ponceau-S staining. Mass spectroscopy fingerprinting and sequencing was then used to identify any proteins revealed.

Upon Ponseau-S staining, a number of bands in the region of 110-180kd were observed, although these bands were also present in the first lane of fusion protein alone, and thus represents bacterial protein contaminants binding to the fusion protein at the time of preparation. However, a protein migrating at 80kd was clearly visible in the lanes containing proteins that could bind to GST-Rho [GTPγS] and GST-V14Rho [GTPγS]. As this band represented a protein interacting with Rho in a GTP-dependant manner, the band was excised from the PVDF membrane, and subjected to digestion with trypsin (Trypsin cleaves C-terminal to Lys or Arg). Derived peptides were then directly analysed by matrix-assisted laser desorption ionisation (MALDI) time-of flight mass spectroscopy using a LaserMat 2000 mass spectrometer (performed by Darryl Pappin, Protein Sequencing Laboratory, ICRF, London). The protein-mass fingerprints obtained were analysed using the molecular weight search (MOWSE) peptide-mass database at the SERC Daresbury Laboratory, UK (Pappin *et al.*, 1993). The MOWSE database comprises calculated weights of all peptide fragments derived from a set of specific enzyme or cleavage rules, derived at the time from over 61,000 proteins. The fragment database is generally searched with

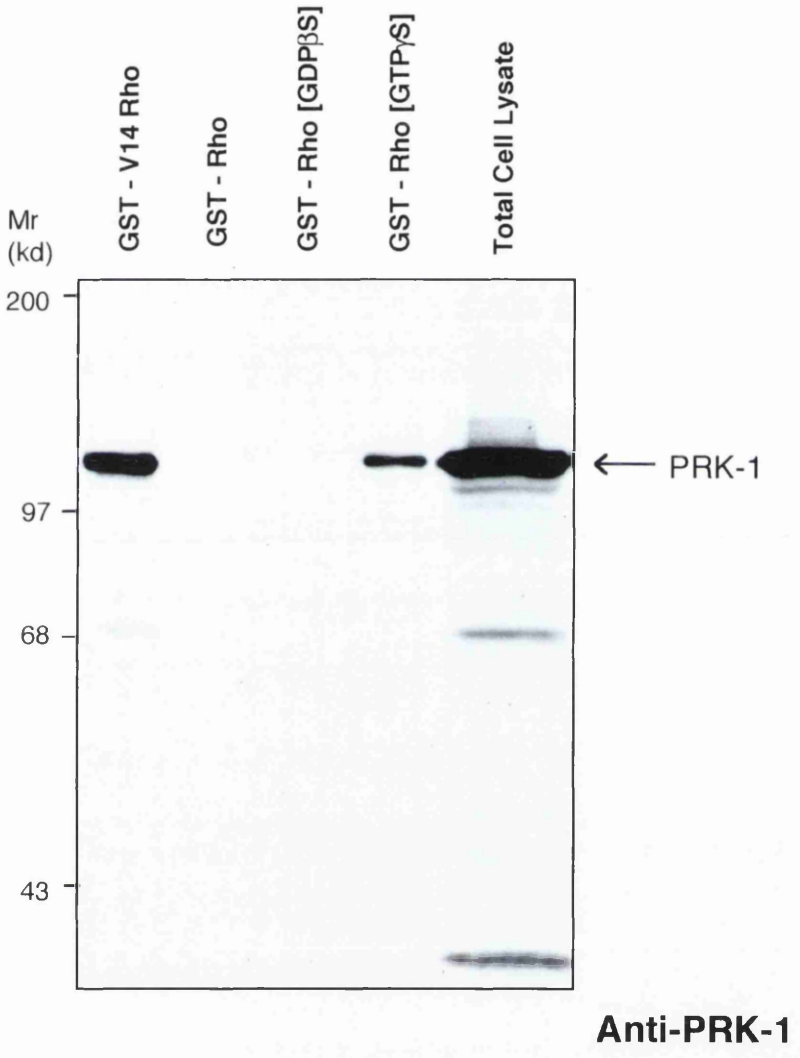

Figure 7.4 - Identification of PRK-1 as a Rho binding protein in lymphocytes
GST-V14 Rho and GST-WT Rho (unloaded, loaded with GDPβS and loaded with GTPγS) were used to isolate Rho-interacting proteins from peripheral blood lymphocytes, resolved on a 12.5% PAGE gel, transferred to a membrane and probed with a polyclonal antibody reactive to PRK-1.

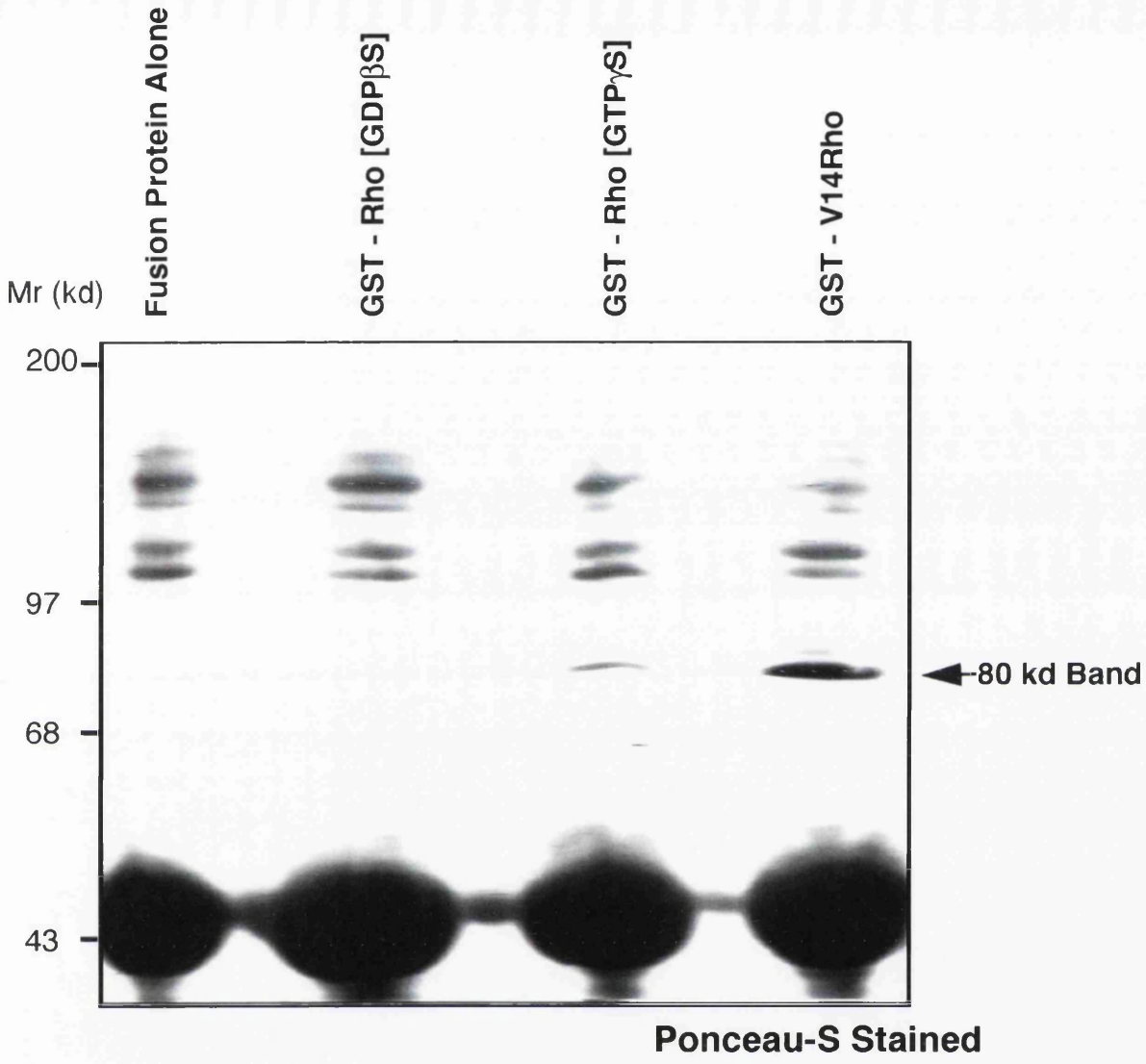

Figure 7.5 - Purification of Rho Binding Proteins by Protein Affinity Chromatography

GST- WT Rho (loaded with GDPβS or GTPγS) and GST-V14 Rho were used in protein affinity chromatography approach to isolate Rho-binding proteins from peripheral blood lymphocytes. The Rho-interacting proteins were then resolved on a 7.5% polyacrylamide gel, transferred to a membrane and revealed with Ponseau-S staining. The 80kd band was excised and sequenced by mass spectroscopy. 'Fusion Protein Alone' lane represents a sample a GST-Rho not exposed to a T cell lysate. The GST-Rho fusion protein migrates just above the 43Kd marker.

the following parameters: peptide mass-fingerprint, the cleavage reagent used, and the molecular weight of the intact protein (optional). Analysis of the 80kd band excised from the PVDF membrane did not yield any matches in the MOWSE database and hence was likely to be a novel protein. Moreover, it was interesting to note that no known Rho effector molecules have a molecular weight of 80kd.

7.2.4 - Mass Spectroscopy Peptide sequencing of the 80kd Rho-interacting Protein

In order to further characterise the 80kd Rho-interacting protein, mass spectroscopy peptide sequencing was performed. The remaining aliquots of the trypsin-digested 80kd band were processed and sequenced by low-energy collision-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitted with a nanoelectrospray source (performed by Darryl Pappin, Protein Sequencing Laboratory, ICRF, London). This approach identified the four peptides shown in table 7.2.

Table 7.2 - Peptide Sequences obtained by Mass Spectroscopy sequencing

	Peptide Sequence	Length
Peptide 1	SPDELPATGGDGGK	14
Peptide 2	EMVSQYLHTSK	11
Peptide 3	SAMMYIQELR	10
Peptide 4	TMLETEEGILLLVR	14

7.2.5 - Identification of mDia as a Rho-interacting protein in T cells

Using the four peptide sequences obtained, a search was performed of the most recent protein databases, and the sequences found to be homologous to those within a newly identified Rho effector molecule, p140mDia (for Mammalian homolog of Diaphanous) (Watanabe *et al.*, 1997). When a sequence alignment was performed using the software package MACAW, all of the peptides were present in the N-terminal region of p140mDia (Figure 7.6). Interestingly, the band from which these peptide sequences were obtained migrated at 80kd, and not 140kd, the mass of p140mDia.

```
mDia-1        mepsggglgpgrgtrdkkkgrSPDELPATGGDGGKhkkflerftsmrikkekekpnsahr    60
Peptide 1     ----------------------SPDELPATGGDGGK------------------------    14
Peptide 2     ------------------------------------------------------------     0
Peptide 3     ------------------------------------------------------------     0
Peptide 4     ------------------------------------------------------------     0

mDia-1        nssasygddptaqslqdisdeqvlvlfeqmlvdmnlneekqqplrekdivikrEMVSQYL   120
Peptide 1     ------------------------------------------------------------    14
Peptide 2     ----------------------------------------------------EMVSQYL     7
Peptide 3     ------------------------------------------------------------     0
Peptide 4     ------------------------------------------------------------     0

mDia-1        HTSKagmnqkessrSAMMYIQELRsglrdmhllscleslrvslnnnpvswvqtfgaegla   180
Peptide 1     ------------------------------------------------------------    14
Peptide 2     HTSK--------------------------------------------------------    11
Peptide 3     --------------SAMMYIQELR------------------------------------    10
Peptide 4     ------------------------------------------------------------     0

mDia-1        slldilkrlhdekeetsgnydsrnqheiirclkafmnnkfgikTMLETEEGILLLVRamd   240
Peptide 1     ------------------------------------------------------------    14
Peptide 2     ------------------------------------------------------------    11
Peptide 3     ------------------------------------------------------------    10
Peptide 4     ---------------------------------------------TMLETEEGILLLVR---    14

mDia-1        pavpnmmidaakllsalcilpqpedmnervleamteraemdeverfqplldglksgtsia   300
Peptide 1     ------------------------------------------------------------    14
Peptide 2     ------------------------------------------------------------    11
Peptide 3     ------------------------------------------------------------    10
Peptide 4     ------------------------------------------------------------    14
```

Figure 7.6 - Alignment of the four peptide sequences with N-terminal region of p140mDia-1.

BLAST Database searching revealed the four peptides to be homologous to the N-terminal region of p140mDia. The alignment shown above was perfomed using MACAW.

7.2.6 - p140mDia is an abundant Rho effector in T cells

p140mDia was identified only a few weeks prior to this study by Watanabe and colleagues in a yeast two hybrid screen for novel Rho-binding proteins using a mouse embryo cDNA library (The recent identification was the reason for its peptide fingerprint not being present in the MOWSE database) (Watanabe *et al.*, 1997). In their report, they showed mDia, the mammalian homolog of *Drosophila* diaphanous (Castrillon and Wasserman, 1994), to bind both to both the GTP-bound form of RhoA, and to profilin through different regions of the molecule. The structure was proposed to be 2 globular domains, joined by a poly-proline rich linker region with the Rho-binding domain being proximal to the N-terminus. It was from this N-terminal domain that the four peptide sequences were obtained (Diagrammatically depicted in Figure 7.7).

In order to confirm the findings that Rho interacted with mDia in T cells, an antibody specifically reactive with p140mDia was obtained from the Wanatabe *et al.* group, Japan. When the mDia antibody was used to probe a membrane similar to the one shown in Figure 7.5, the mDia antibody recognised the 80kd protein fragment that was excised and sequenced (Data not Shown). However, by freshly preparing T cell lysates from a variety of cells, it was noted that under analytical conditions the antibody recognised a 140 kd band in a range of different T cells (Figure 7.8).

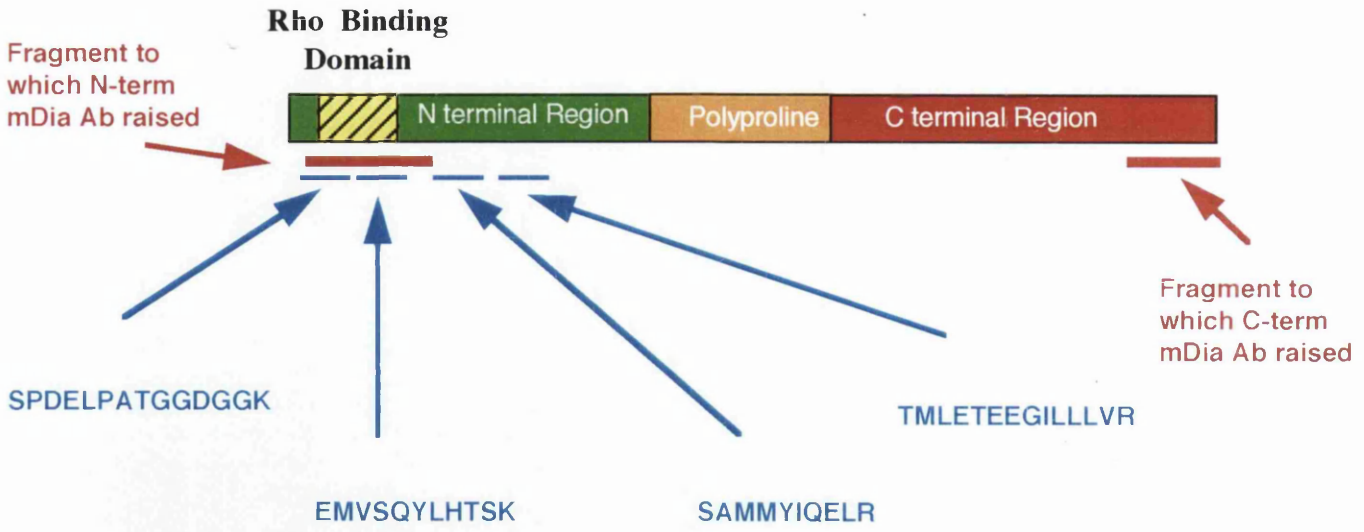

Figure 7.7 - Diagramatic representation of the positions of the peptides identified by Mass Spectoscopy sequencing.
All four peptides were homologous to sequences within the N-terminus of p140mDia; the terminus where the Rho-binding domain is located.The regions to which the N-terminal and C-terminal anti-mDia antibodies are raised are also shown.

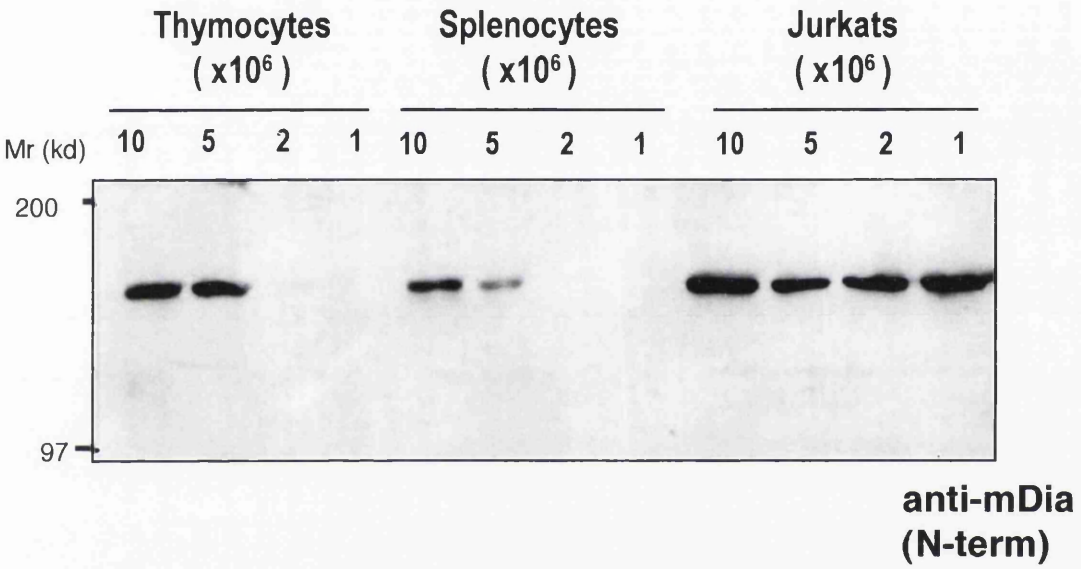

Figure 7.8 - Expression of p140mDia in Murine Thymocytes, Splenocytes and the Jurkat human T cell line.

A polyclonal antibody reactive to the N-terminal region of mDia was used to probe a panal of T cell lysates. The antibody recognises a 140kd protein, present in all cell types, albeit at variable levels.

7.2.7 - A novel 80kd mDia isoform or proteolysis of p140mDia?

Interestingly, the protein recognised by the N-terminal mDia antibody binding to GST-Rho [GTPγS] in these experiments migrates at 80kd, and not 140kd as reported for mDia. One possible explanation for this discrepancy in molecular weights is that there was proteolysis of mDia during the large scale protein purification. It is interesting to note that there is a long proline-rich sequence between the N- and C- terminal domains of mDia which may be particularly susceptible to proteolytic cleavage. This theory would predict that if proteolysis was to occur under 'normal' lysis conditions, the C-terminal specific antibody would recognise, in addition to native p140mDia, an additional smaller band when degradation occurs. An alternative explanation is that the smaller 80kd band represents a previously unrecognised truncated version of mDia. This is also testable with a C-terminal mDia antibody; if a shorter mDia isoform were present, the C-terminal antibody would not recognise an additional band.

Accordingly, a C-terminal mDia polyclonal antibody was raised to the last 15 amino acids of mDia. The anti-sera recovered was used to probe a blot of T cell lysates (Figure 7.9). In addition to recognising a band migrating at 140kd, the antibody also recognised a band migrating at around 80kd in one of the lanes. This band, present in the lysate of the larger number of cells would appear to be the C-terminal fragment of mDia. When the N-terminal mDia antibody was used to probe a similarly prepared blot, long exposure of the ECL-treated membrane to film revealed an 80kd band, representing the N-terminus of the degraded mDia protein. It thus seems unlikely that a novel, truncated version of mDia exists in T cells, but rather the full-length protein is very susceptible to proteolytic cleavage within its poly-proline rich region.

7.2.8 - *In vivo* or *in vitro* Cleavage of p140mDia?

The data presented so far suggested that mDia is subject to proteolytic cleavage. It was therefore of interest to determine whether this cleavage occurs *in vivo*, prior to cell lysis or *in vitro*, during the process of lysis and affinity chromatography. PBLs, Jurkats and the IL-2 dependent cell line, KIT225 were lysed by either NP40 lysis buffer, under the same conditions as used in the Rho pull-down experiments or by boiling

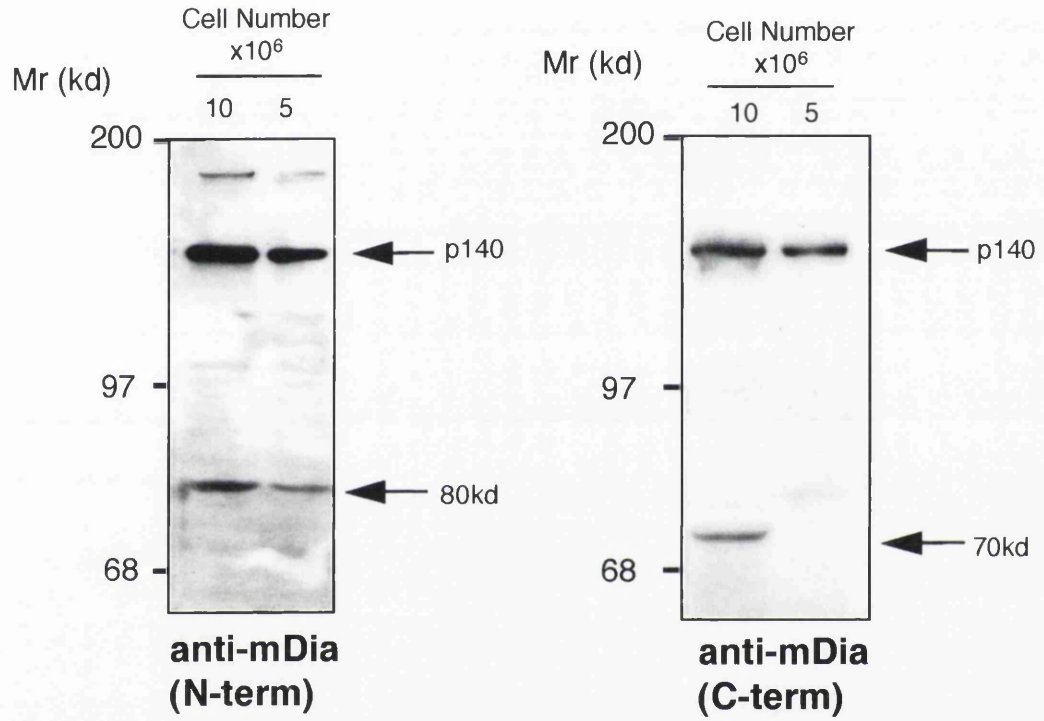

Figure 7.9 - The N-terminal mDia antibody recognises a 140kd and a 80kd protein while the C-terminal mDia antibody recognises a 140kd and 70kd protein

(A) The N-terminal antibody was used to probe of blot of total lysates prepared from 10 x10^6 and 5 x10^6 PBLs. Two bands are present; an intense band migrating at 140kd, and a weaker band at 80kd.

(B) The C-terminal mDia polyclonal antibody, raised to the C-terminal 15 amino acids was used to probe a similar blot. The antibody recognises a band migrating at 140kd and a fainter band, present only in the lane with the higher number of cells, migrating at 70kd.

the cells in SDS. By lysing in SDS, cells are immediately lysed and their proteins denatured so there is no opportunity for proteases to act. The data in figure 7.10 show that when PBLs, Jurkats and KIT225 cells were lysed with SDS, Western blotting with the C-terminal mDia antibody revealed a 140kd protein present in all samples. A similar 140kd protein was also detected in all samples lysed with NP40; however, NP40 lysis of PBLs resulted in an 70kd protein, detectable with the C-terminal mDia antibody. These data suggest that cleavage of mDia is an *in vitro* phenomena that occurs during lysis in NP40 lysis buffer. Proteolysis appears to be more of a problem in PBLs than the other types of cells used in this experiment and may suggest that proteases that degrade mDia are more abundant or active in primary cells.

7.2.9 - mDia appears to be phosphorylated *in vitro*

It was of interest to note that in the kinase assay shown in Fig 7.3, the lower phosphorylated band migrated with a similar molecular weight to that of the 80kd mDia fragment. To test whether this band indeed represented the phosphorylated degraded mDia, the kinase assay blot shown in figure 7.3 was probed with the N-terminal mDia antibody (Figure 7.11). This showed that the band revealed by this antibody exactly overlaid with the lower band in the kinase assay. Although it is not possible to exclude that it is a different 80kd migrating at exactly the same molecular weight as the mDia fragment that is being phosphorylated, the most probable explanation is that mDia is being phosphorylated by another kinase binding to activated Rho. Whether this is of physiological relevance remains to be determined.

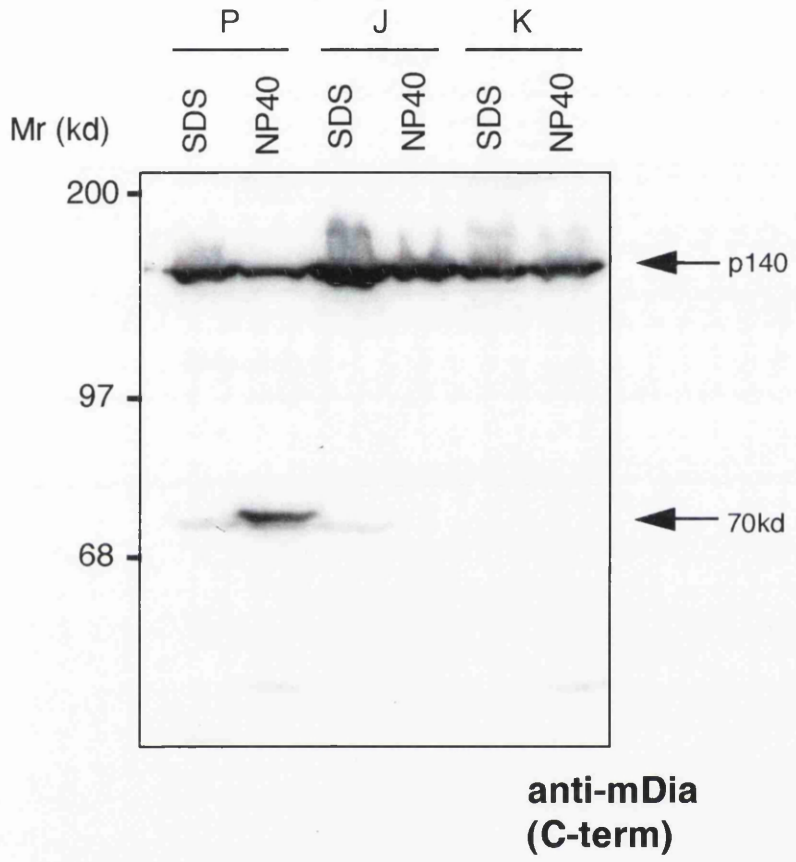

Figure 7.10 - T cell type and lysis conditions affects the proteolysis of p140mDia

The C-terminal mDia antibody was used to blot of total cell lysates from Peripheral Blood Lymphocytes (P), the jurkat T cell line (J), or the KIT225 T cell line (K). The different T cells were lysed by either boiling a cell pellet in SDS (SDS lanes) or adding 1% NP40 (NP40 lanes). (Data kindly provided by Isabel Corre, Lymphocyte Activation Lab)

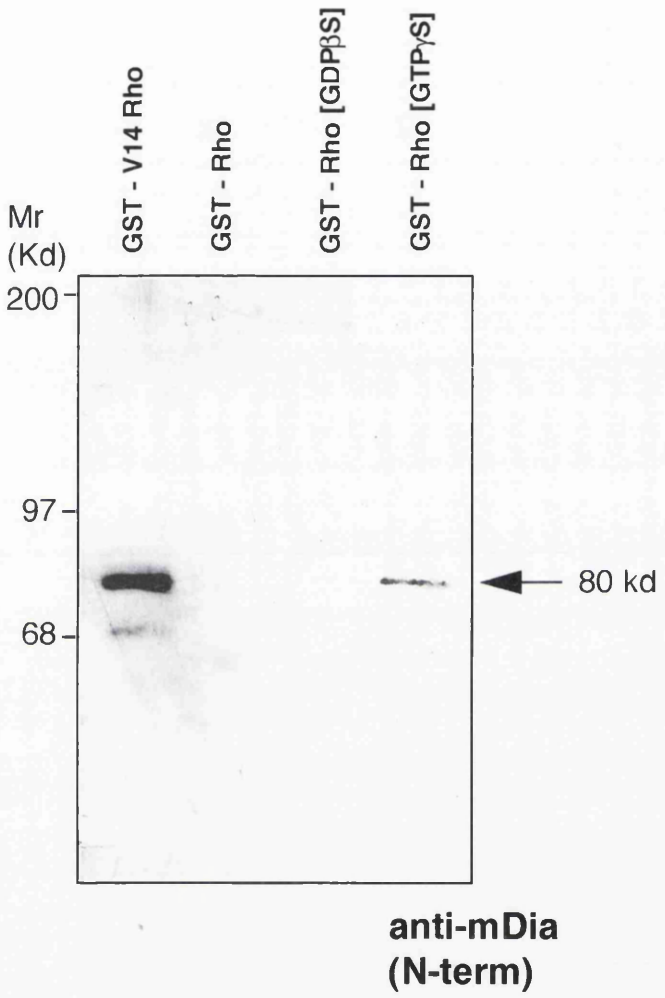

Figure 7.11 - The mDia N-terminal antibody recognises a band migrating at 80kd when the Kinase Assay blot from Figure 7.3 is re-probed.

GST-V14 Rho and GST-WT Rho (unloaded, loaded with GDPβS and loaded with GTPγS) were used in a protein affinity chromatography approach to isolate Rho interacting proteins. When the blot is probed with an antibody reactive to the N-terminal region of mDia, an 80kd band is recognised and runs in the same position as the 80kd band seen in Fig 7.3

7.3 - Discussion

Here, an affinity chromatography-based approach has been used to identify proteins interacting with Rho in a GTP-dependant manner. This study has shown that PRK-1 is a Rho effector in T cells and is autophosphorylated in an *in vitro* kinase assay. In addition, using a combination of mass spectroscopy peptide fingerprinting and sequencing, mDia has been identified as being an abundant Rho-effector in lymphocytes. mDia, the mammalian homolog of the *Drosophila* protein diaphanous is susceptible to proteolysis. Analysis has revealed that proteolysis occurs during cell lysis and subsequent processing, and is thus an *in vitro* phenomena. In addition, a new reagent has been generated; a polyclonal antibody reactive to the C-terminal 15 amino acids of mDia.

Interestingly, in a total of four independent GST-Rho pull-down experiments performed during the course of this study, full-length 140kd mDia was never observed binding to GST-Rho [GTPγS] nor GST-V14Rho when the Western blots were probed with the anti-N-terminal mDia antibody. Rather, the antibody recognised the shorter 80kd cleavage fragment of mDia. This data is in contradiction to the initial mDia identification report where Narumiya *et al.* shows an interaction between mDia and GST-Rho (Watanabe *et al.*, 1997). In their report, no mention of a 80kd mDia fragment is made. There are several possible explanations for these discrepancies. Firstly, purified peripheral T cells may contain more proteases than the Swiss 3T3 cell line. The data presented in figure 7.10 shows that this is indeed a possibility; mDia is proteolytically cleaved in primary T cells but not in the two T cell lines studied following NP40 lysis. Thus, the absence of full-length mDia associating with active Rho may simply be due to complete proteolysis of mDia during the lysis and affinity chromatography process. Alternatively, only some proteolysis may occur but the 80kd N-terminal fragment of mDia may bind with higher affinity to the GST-Rho [GTPγS], hence no full-length mDia is seen as binding to Rho is prevented due to competition. mDia is thought to adopt an inactive folded conformation in which the N- and C-termini domains interact in the absence of active Rho (Watanabe *et al.*, 1999). A difference in affinity may arise if a full-length mDia required a second *in vivo* signalling event in order to alter its conformation in such a way as to allow active Rho to bind. Further experiments are required to resolve these issues.

Function of PRK

The function of PRK has for a long time been elusive. Upon interaction of GTP-bound small GTPases with kinase-domain containing effectors, an increase in kinase activity is usually observed. Indeed, this seems to be the case with the interaction of PRK-1 with Rho-GTP where an increase in autophosphorylation has been shown on *in vitro* incubation with RhoA-GTP (Amano *et al.*, 1996b; Watanabe *et al.*, 1996). Although the extracellular signals that activate PRK are currently unknown, data is gradually emerging that suggests interaction between PRK-2 and the 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Balendran *et al.*, 1999). In a proposed model (Flynn, 1999), active Rho localises PRK to the membrane and mediates a conformational change resulting in the PRK phosphorylation site becoming exposed. PDK-1 can then bind to PIP_3 in the membrane and phosphorylate PRK, conferring an increase in PRK kinase activity. One of the substrates of PDK-1 has been reported to be the anti-apoptotic ser/thr kinase PKB (Alessi *et al.*, 1997), and hence in such a model, the Rho/PRK/PDK-1 complex may result in PDK-1 being able to cause activation of PKB. This remains to be proven, but does provide an intriguing link between Rho, the effector PRK and cell survival.

Function of mDia

mDia was originally identified by a yeast two hybrid screen for Rho-binding proteins from a embryonic mouse library (Watanabe *et al.*, 1997). Since its identification, a number of other mDia homologs have been found. Interestingly, a recent study has identified a human homolog of mDia1, called *hDia1*. Lynch *et al.* have shown that a truncation mutant of hDia1 is responsible for nonsyndromic deafness (Lynch *et al.*, 1997). Based on the role of mDia1, they hypothese that this mutation affects the actin cytoskeleton and that proper actin polymerisation is essential for amplification and detection of sound waves. An additional murine homolog, referred to as mDia-2 has also been reported by Alberts *et al.* although no function has been assigned (Alberts *et al.*, 1998a). mDia-1 and -2 are reported to interact with Rho through a binding domain that is distinct from either the PRK/Rhophilin/Rhotekin Rho binding domain (Class I) (Reid *et al.*, 1996), and the ROCK/Citron/Citron-kinase Rho binding domain (Class II) (Madaule *et al.*, 1998) and thus their discovery allows a third class of effectors to be defined. The three

classes of Rho effectors and their structural relationships to each other are shown in figure 7.12.

Recent biochemical studies performed by Watanabe *et al.* have provided evidence that mDia-1 and ROCK co-operate to induce actin reorganisation (Watanabe *et al.*, 1999). In their report, they suggest that Rho binds to the N-terminal region of mDia, thus disrupting its intramolecular interactions. They propose that in its resting inactive state, the N-terminus and C-terminus of mDia interact and thus prevent the formin-homology domains (FH1 and FH2) from inducing actin fibres. However, upon conversion from its inactive to active state, Rho binds to mDia within its N-terminal region and mediates its unfolding, thus exposing the FH1 and FH2 domains which in turn interacts with profilin and causes the polymerisation of actin monomers into F-actin structures. In their model, they propose mDia and ROCK work co-operatively with mDia being responsible for the assembly of thin actin fibres while at the same time ROCK mediates the condensing of these actin fibres and thus the balance between ROCK and mDia activity controls the thickness and densities of the stress fibres produced. This model raises a number of questions. Firstly, if a mDia and ROCK function co-operatively to control the thickness and densities of stress fibres, there must be an alternative mechanism, in addition to Rho activation, by which the activities of mDia and ROCK are modulated. Without such a mechanism, the activities of mDia and ROCK would correlate exactly to the level of GTP-bound Rho in the cell. This additional regulation may be either through transcriptional regulation of mDia and ROCK or through control of their cellular localisation. An alternative mechanism may be phosphorylation of either ROCK or mDia by a differentially regulated kinase.

Although lymphocytes do not make large actin bundles as found observed in fibroblasts, they appear to undergo much more subtle cytoskeletal changes. Lymphocytes migrate through blood and lymph vessels, transgress vessel walls, home into lymphoid organs, and adhere with target cells; all processes that require changes in cell morphology. These changes are dependent upon a functional tubulin and actomyosin cytoskeleton. In addition, since many signalling molecules are associated with cytoskeletal scaffolds, the cytoskeletal structure and scaffold geometries have been suggested to regulate the molecular dynamics of signalling and biochemical responses (Penninger and Crabtree, 1999). During T cell activation by APCs, the TCR stimulation leads

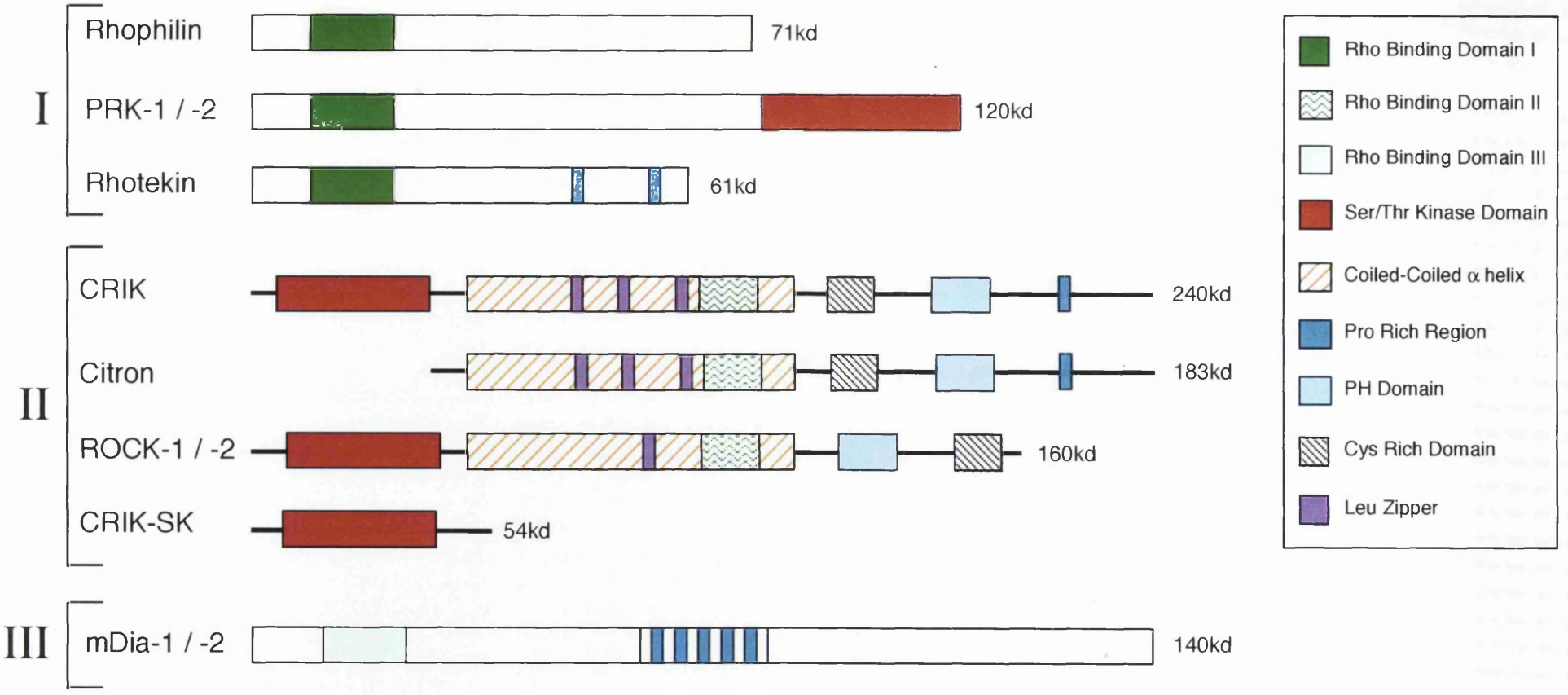

Figure 7.12 - Structural Relationship of the Rho effector molecules.

Rho effectors can be divided into three main groups on the basis of similarities in their Rho binding domains. Rhophilin, the PRKs and Rhotekin all have similar Rho binding domains (Rho binding domain I), while CRIK, Citron, and the ROCKs share certain domain similarities with each other and interact with Rho through a domain (Rho binding domain II) that has little similarity to Rho binding domain I. mDia-1 and -2 represent another class of Rho effectors and have a putative Rho binding domain (Rho binding domain III) distinct from either of the other two classes.

to the organisation of supramolecular clusters (SMACs) at the interfaces of physical contact with the APCs (Monks *et al.*, 1997). The SMACs consist of antigen receptors, co-receptors, and adhesion and signalling molecules; three dimensional immunofluorescence experiments have revealed that the SMAC is highly organised with the TCR, CD3, $p56^{lck}$ and $p59^{fyn}$ kinases, while the peripheral ring comprises of the adhesion molecule LFA-1 (Monks *et al.*, 1998). It has also been proposed that the co-receptors CD4 and CD8, as well as CD28 and CD2 all co-localise to the central region, while the phosphatase CD45 and adhesion molecule CD43 are excluded from the central region. Figure 7.13 shows the current model of SMAC formation, as described in a recent study by Grakoui *et al.* (Grakoui *et al.*, 1999). The forces driving the initial formation of the SMACs and their subsequent reorganisation require actin polymerisation; this would suggest that upon initial contact of between the T cell and APC, a signalling pathway is activated in order to mediate the reorganisation of the cytoskeleton.

One possibility is that Rho is involved in this initial actin polymerisation, and thus plays an integral role in SMAC formation. Indeed, the Rho family GTPase exchange factor Vav1 has been shown to be involved in antigen-receptor induced actin polymerisation; thymocytes and T cells from Vav-deficient mice show defects in receptor-mediated actin polymerisation (Fischer *et al.*, 1998). Although Vav is known to serve as an exchange factor for Rac and not Rho, this result nevertheless highlights a central role for Rho-family small GTPases in antigen-receptor driven actin reorganisation. Whether co-operation between ROCK and mDia has any significance in this receptor-driven process within lymphocytes has yet to be explored.

In summary, with the use of a protein affinity chromatography approach, it has been shown that both PRK-1 and mDia-1 are abundant in T cells and interact in GTP-dependent manner with the small GTPase RhoA. Although the function of the PRK family members remains to be established experimentally, an intriguing connection between PRK and PDK-1 and the regulation of the survival factor PKB has been suggested. mDia-1 has been reported to co-operate with another Rho effector, ROCK in regulation of actin polymerisation; a process central to T cell-APC interactions.

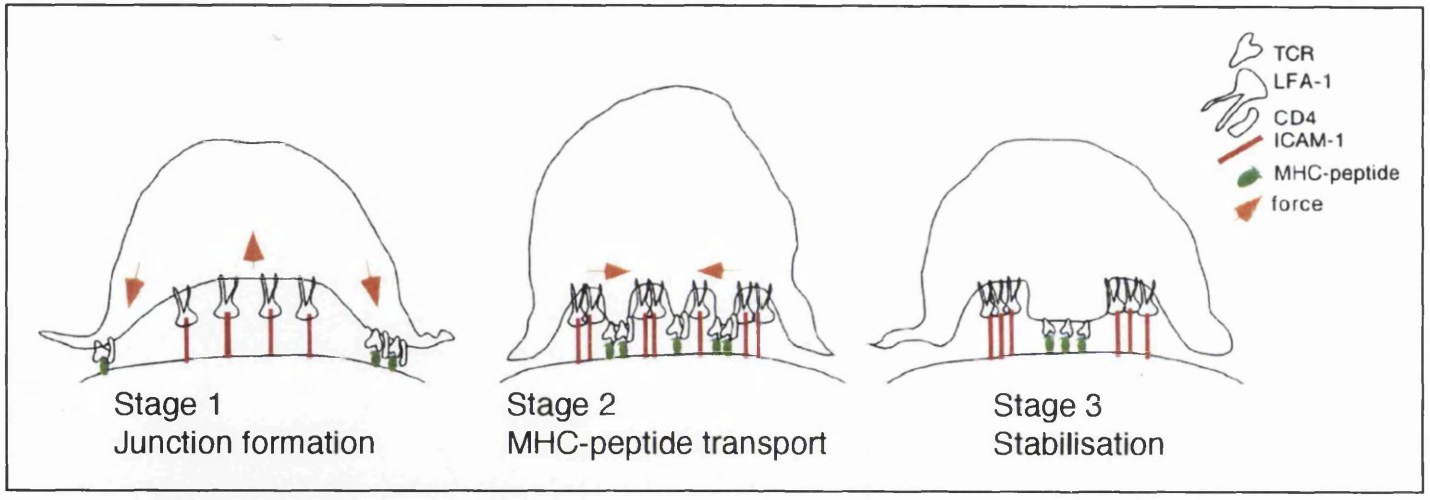

Figure 7.13- Model of the Immunological Synapse.

Stage 1: LFA-1 anchors the central region of the nascent immunological synapse, providing a falcrum for cytoskeletal protrusive mechanisms that force an outermost ring of T cell membrane into close apposition with the substrate. This allows TCR sampling of the MHC-peptide complex. Early signals from the TCR and CD4 stop migration.

Stage2: Transport of MHC-peptide to the centre of the synapse. This process is suggested to be mediated by the actin cytoskeleton.

Stage3: The clustered MHC-peptide complexes are 'locked-in'.

(Modified from Grakoui et al. 1999)

CHAPTER 8
General Discussion

8.1 - Conclusions

Over the last few years evidence has accumulated that Rho GTPases play an important role in the regulation of different cellular responses essential for immune function. The work presented here has provided greater understanding as to the role of the small GTPase Rho in T cell development and function. The following sections describe the findings of this work and discusses future possibilities for extending these studies.

8.1.1 - A Role in Pre-TCR Signalling

Previously, using the p56lck proximal promoter to drive expression of the Rho inhibitor C3-transferase, a role for Rho in the survival of $CD25^+$ thymocytes was discovered (Henning *et al.*, 1997; Gallandrini *et al.*, 1997). At this stage in development, survival is dependent upon the cytokine IL-7 and hence Rho may lie downstream of the IL-7R. Here, with use of the CD2-LCR, these survival defects are circumvented by inactivation of Rho function at a later developmental stage. This reveals a previously unrecognised role for Rho in pre-TCR signalling. Furthermore, comparisons between the lck-C3 and CD2-C3 mice reveal that thymocytes from lck-C3 mice are able to compensate for loss of Rho function during early development, and develop into mature T cells, avoiding the defect in pre-TCR signalling seen in the CD2-C3 mice. These comparisons show that cell type specific elimination of Rho can reveal different functions for this GTPase in thymocyte development, and hence demonstrates that the only way to fully understand the function of a protein is to study the immediate consequences of its cell-type specific elimination. The data presented here, combined with the previously reported analyses of the lck-C3 mice allows a model to be devised in which during early thymocyte development, Rho is required for IL-7R/c-kit signalling mediating thymocyte survival, while at the later stages, namely the $CD44^-25^+$ pre-T cell stage, Rho is required to mediate pre-TCR initiated differentiation and proliferation. This model is shown in figure 8.1.

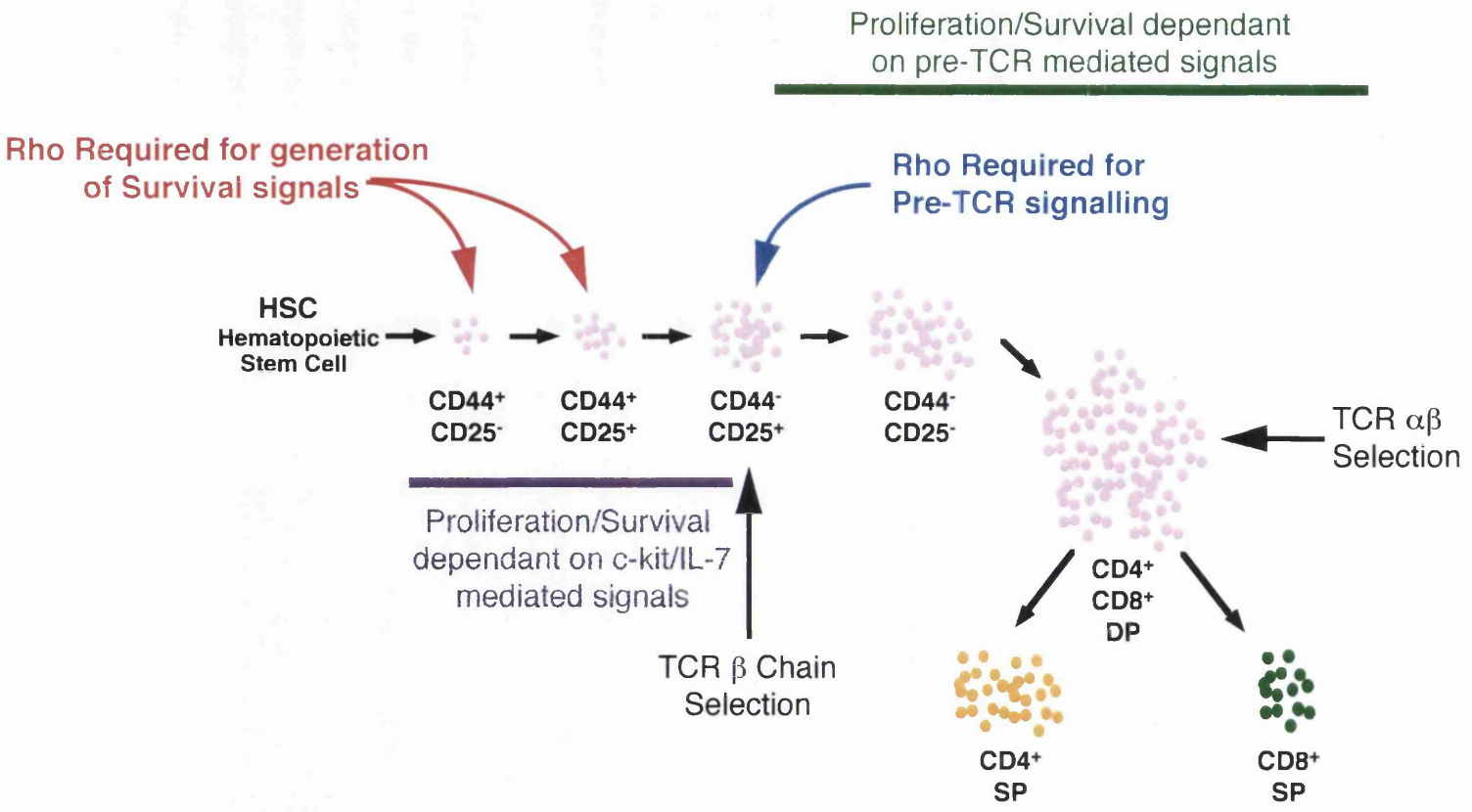

Figure 8.1 - Multiple Roles for the small GTPase Rho during thymocyte development.

Rho is required for the generation of survival signals at the $CD44^{+}25^{-}$ and $CD44^{+}25^{+}$ stage of development. However, there is no further requirement for Rho-mediated survival signals at the $CD44^{-}25^{+}$ stage; rather, Rho is necessary for Pre-TCR mediated signalling.

8.1.2 - A Role in Survival

Analysis of the lck-C3 mice has also been extended and the basis of the requirement for Rho function in early thymocyte survival explored. Previously, it was shown that in the absence of Rho function, $CD25^+$ thymocytes fail to receive the necessary survival signals and die by apoptosis; expression of Bcl-2 could alleviate death in the absence of Rho function (Gallandrini *et al.*, 1997). Here, the role of the tumour suppressor protein p53 in this process has been investigated. Elimination of p53 in lck-C3 mice allows $CD25^+$ cells to survive and hence implicates Rho in controlling thymocyte survival in a p53 dependent manner. Although levels of endogenous Bcl-2 appear to be normal in lck-C3 mice, Bcl-2 has been reported to block p53-dependent apoptosis (Chiou *et al.*, 1994) and hence may explain why ectopic expression of Bcl-2 in the lck-C3 mice rescues survival of $CD25^+$ cells. Interestingly, unlike expression of Bcl-2 transgenes, absence of p53 in the lck-C3 mice does not rescue all stages of thymocyte development nor restore cellularity. These results suggest that there are p53 dependent and independent forms of cell death occurring in the absence of Rho function. Work is currently underway to explore survival defects in thymocytes at subsequent developmental stages. The findings presented here are the first to link Rho and p53, and provide the basis of new and exciting future work to establish the molecular mechanism of such a link.

The studies involving the lck-C3 mice are not the only reports to implicate Rho and its effectors in the process of survival; there are a number of reports that may one day form the basis of a mechanism connecting Rho with the process of apoptosis. As discussed in section 7.3, the Rho effector PRK-1 has been suggested to regulate the serine/threonine kinase, PKB whose anti-apoptotic functions include suppression of Fas-induced caspase activation (Cardone *et al.*, 1998). Hence, Rho may mediate anti-apoptotic signals by interaction and activation of PRK-1 which in turn activates PKB (Flynn, 1999). However, the involvement of Rho and its effectors in the suppression of apoptosis may not be so straightforward. Fas-mediated caspase activation has been reported to result in the cleavage of PRK between the amino-terminal regulatory domain and the carboxyl-terminal catalytic domain, generating a constitutively active PRK-1 fragment (Takahashi *et al.*, 1998). Moreover, Fas-induced activation of caspases has also been shown to mediate

the destruction of a Rho regulatory protein, D4-GDI and thus, Fas-triggered apoptosis may regulate Rho localisation (Na *et al.*, 1996). Clearly the regulation of apoptosis and cell survival by Rho and its effectors is a complex process, possibly involving feedback control. Identification of the downstream targets of Rho effectors may speed the development of a model linking Rho-mediated signalling to cell survival.

8.1.3 - A Role in Transformation

Analysis of the lck-C3 mice reveals that loss of Rho function in the thymus predisposes mice to thymic lymphoma. lck-C3 mice have a mean survival age of 6 months and develop aggressive lymphoblastic lymphoma. Tumours are monoclonal in origin, suggesting that loss of Rho function is not sufficient for transformation; rather loss of Rho function predisposes the cells to accumulation of the other genetic mutations leading to malignancy.

Prior to analysis of the lck-C3 mice, there had been several reports that linking Rho GTPases in the process of transformation. Overexpression of RhoC gene has been reported to correlate with progression of ductal adenocarcinoma of the pancreas (Suwa *et al.*, 1998). A study employing a RT-PCR approach to examine levels of RhoA, B and C as well as K-ras found elevated levels of RhoC gene expression in patients with perineural invasion and lymph node metastasis compared to patients without these manifestations (Suwa *et al.*, 1998). No mutations were found in any of the Rho gene sequences, but 92% of cases studied had the K-ras gene mutation. Furthermore, the Rho GTPase TTF was first identified its association with the lymphoma associated LAZ3 gene (Dallery *et al.*, 1995). LAZ3/BCL6 is a gene encoding a potential transcription factor, disrupted in non-Hodgkin's lymphomas and the GTPase TTF gene has been shown to translocate and fuse to LAZ3. This observation raises the interesting possibility that Rho family GTPases may linked to transformation through chromosomal translocations, rather than activating mutations as observed with the Ras family. Further analysis of lymphoma tissue samples may reveal that chromosomal translocation or upregulation of expression of Rho family GTPases is a more widespread phenomenon than first thought.

Rho GTPases are closely linked to growth regulation and transformation. Although more work will be required to establish the molecular basis between loss of Rho function and the development of thymic lymphoma, there are a number of possibilities worth investigating. First, Rho function may be required to regulate a DNA repair mechanism. Hence, Rho inactivation would prevent DNA repair, and predispose the cell to accumulating transforming mutations. Such an effect is seen with loss of function of Brca2 or *atm*; loss of function of either of these genes results in defective DNA repair as well as the development of thymic lymphoma (Barlow *et al.*, 1996; Connor *et al.*, 1997). The connection between thymic lymphoma and ineffective DNA repair suggests that the process of T cell development requires DNA-repair mechanisms to be active, possibly because gene rearrangement is occurring. The development of lymphoma in lck-C3 mice is consistent with a role of Rho in DNA repair. Failure to observe transformation in fibroblasts transiently transfected with C3-transferase may suggest that DNA repair mechanisms are not active over the relatively short periods used for transformation assays. An alternative possibility is that loss of Rho function causes growth deregulation. Although this would be contrary to many reports showing it is Rho activation, rather that loss of function that results in transformation, it is always possible that Rho plays different roles in different cells. Maybe a more feasible explanation however, is that loss of Rho function during early thymocyte development selects for cells able to avoid apoptosis and thus exhibit growth deregulation to some extent. Again, such an effect would not be seen in fibroblast transformation assays as Rho does not appear to control survival in these cells.

Although the molecular basis for how loss of Rho can result in transformation is purely speculative at present, the observation that lck-C3 mice develop thymic lymphoma is striking and escapable. Recently, inhibition of Rho and its effectors has been suggested as a therapeutic strategy for the treatment of Ras-transformed tumours (Olson *et al.*, 1998; Itoh *et al.*, 1999; Sahai *et al.*, 1999). The data presented here suggests that such a strategy would need to be used with caution.

8.1.4 - A Role in Cytoskeletal Reorganisation

By taking a protein-affinity chromatography approach to identify Rho-interacting proteins in T cells, PRK-1 and mDia-1 have both been shown to interact with Rho in a GTP-dependent manner. Furthermore, from *in vitro* kinase assays, it appears as though mDia is phosphorylated; the physiological relevance of this event remains to be investigated. mDia has been suggested to be involved in the control of the actin cytoskeleton by, in co-operation with ROCK, regulating the formation and density of stress fibres (Watanabe *et al.*, 1999). Changes in the actin cytoskeleton of lymphocytes may regulate molecular dynamics of signalling and biochemical responses (Penninger and Crabtree, 1999). For example, during T cell activation by APCs, TCR stimulation leads to changes in the organisation of antigen receptors, co-receptors, adhesion and signalling molecules (Grakoui *et al.*, 1999; Monks *et al.*, 1998). Such cytoskeletal changes may be mediated by mDia in conjunction with other Rho effectors and hence it may prove interesting to investigate the intracellular localisation of mDia during the process of T cell activation by APCs.

8.2 - Studying Rho function *in vivo*

The use of C3-transferase as a tool for the inactivation of Rho *in vivo* overcomes the problems associated with dominant negative approaches and knockouts. When dominant negative small GTPase mutants are expressed, it is always a concern that a sufficient amount of protein is expressed to completely prevent the endogenous GTPase becoming activated. In transient transfection assays, this problem can be addressed by titrating the amount of cDNA encoding the dominant negative GTPase. To study the role of GTPases in transgenic models using a dominant negative approach, complete inactivation of the endogenous protein would be a greater concern as the only way to vary the amount of protein expressed would be to make a series of transgenic mice, each with a different copy number of the transgene.

Another alternative to studying gene function *in vivo* is targeted gene disruption. However, generation of a Rho knockout mouse was not considered a feasible option as a knockout in which all cells lineages lacked expression of Rho is likely to give an embryonic

lethal phenotype owing to the requirement for Rho in the essential process of cytokinesis (Drechsel *et al.*, 1997; Madaule *et al.*, 1998; Kosako *et al.*, 1999). If the Rho knockout mice were viable, then it may be due to functional redundancy between the Rho family members. Nevertheless, the advent of conditional and cell-type specific knockouts, and their continued improvement may make elimination of Rho family members *in vivo* a realistic option for the future. Although such an approach would allow the findings of the present study to be further explored, perhaps a better strategy for the analysis of Rho-mediated signalling pathways *in vivo* may be knockout effectors of Rho, rather that Rho itself. With the plethora of publications in the past few years characterising Rho effectors, the first reports of knockouts of these proteins must be imminent.

The use of C3-transferase has allowed the role of the small GTPase Rho to be studied *in vivo* and an essential role in thymocyte survival and pre-TCR defined. Given the merits, and potential pitfalls of such an approach, could the transgenic expression of a different bacterial exoenzyme be used to study other small GTPases? This is indeed a possibility as there exists a range of different toxins (See Table 8.1) that will act upon small GTPases.

For example, expression of the cytotoxin necrotizing factor-1 (CNF-1) is able to deamidate and active Rho, while expression of Toxins from *Clostridium difficile* mediate the glycosylation and inactivation of Rac, Rho and Cdc42. Expression of such toxins using a transgenic approach may provide greater insight into the role of the Rho family GTPases *in vivo*. However, what has been learnt from analysis of C3-transgenic mice is that timing and patterns of expression are of critical importance. Therefore, if a transgenic approach is to be taken in the future to analyse the role of small GTPases in thymocyte development, analysis would be much simplified by incorporation of a fluorescence tag, such as GFP in order to easily monitor expression of the transgene.

Table 8.1 - Covalent modification of Rho GTPases by Bacterial Endotoxins

Toxin and Source	Modification	Effect on cells	Reference
C3 from *Clostridium botulinum*	ADP ribosylates Asn41 and inactivates Rho	Rounding of Swiss 3T3 cells Decrease F-actin in HL60 cells Blocks migration of neutrophils	(Ehrengruber *et al.*, 1995; Koch *et al.*, 1994)
CNF1 from *E.coli*	Deamination and activates Rho	Increases total F-actin pool	(Fiorentini *et al.*, 1995; Schmidt *et al.*, 1997)
Toxin A and Toxin B from *Clostridium difficile*	Glucosylates and inactivates Rho, Rac, and Cdc42	Rounding of cells and cell death	(Dillon *et al.*, 1995; Just *et al.*, 1995)
C3-related Toxin from *Bacillus cereus*	ADP ribosylates Asn41, inactivates Rho	Same as C3	(Just *et al.*, 1995)
Exoenzyme from *Clostridium linosum*	ADP ribosylates Asn41, inactivates Rho	Same as C3	(Just *et al.*, 1992; Koch *et al.*, 1994)
DNT from *Bordetella bronchiseptica*	Rho mobility-shift on gels, activates Rho	Inhibits cytokinesis Stimulates stress fibres and focal adhesions stimulates protein and DNA synthesis	(Horiguchi *et al.*, 1995)

Modified from (Machesky and Hall, 1996)

8.3 - The Future of Rho (The RHOad goes on)

Here, the role of Rho in thymocyte development has been analysed. The future of Rho research in the context of the immune system will lie in understanding of the role of the Rho family GTPases in T cell function. Although such analysis is not possible with the current C3-transferase transgenics owing to the absence of peripheral cells, expression of C3-transferase using inducible promoter systems will move the analysis of Rho out of the thymus and into the periphery. This will be complemented by analysis of mice expressing constitutively active Rho mutants. In the past 6 months, transgenic mice expressing constitutively active RhoA have indeed been produced and are beginning to be characterised (I.Corre, personal communication). As expression of active Rho does not result in reduced thymocyte numbers, biochemical analysis may be a realistic option. It is

hoped that analysis of these mice may provide further insight into the role of Rho in T cell biology.

Studies of Rho function in thymocyte development have revealed that there are no universal outcomes of activating Rho family members but rather unique responses in different cell population; a notion that is becoming a common theme in many other cell types. There are several models to explain the global role in regulating a diverse range of cellular processes; here I present only two. First, the pleotrophic functions performed by the Rho GTPases can be explained by a unique expression pattern of effector molecules in different cells. This is testable experimentally and will be greatly assisted by the advent of Rho effector knockouts. Second, Rho in conjunction with adapter molecules and the cytoskeleton may act as an intracellular regulators of the spatial separation between molecules. In such a model, Rho could act by regulating the proximity between members of a signalling cascade and hence explain the diverse roles it plays. Insight into such a role may be gained by fluorescence tagging of Rho effectors and the tracking of their movements during processes such as T cell activation and adhesion. Resolution of these issues will no doubt involve some fascinating biological insights and will most likely represent an area of active and exciting research over the next few years.

CHAPTER 9
References

Abraham, K.M., Levin, S.D., Marth, J.D., Forbush, K.A. and Perlmutter, R.M. 1991. Thymic tumorigenesis induced by overexpression of $p56^{lck}$. *Proc. Natl. Acad. Sci. USA*, **88**, 3977-3981.

Adams, J.M., Houston, H., Allen, J., Lints, T. and Harvey, R. 1992. The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. *Oncogene*, **7**, 611-8.

Agnel, M., Roder, L., Vola, C. and Griffin-Shea, R. 1992. A Drosophila rotund transcript expressed during spermatogenesis and imaginal disc morphogenesis encodes a protein which is similar to human Rac GTPase-activating (racGAP) proteins. *Molecular & Cellular Biology*, **12**, 5111-22.

Aifantis, I., Buer, J., von Boehmer, H. and Azogui, O. 1997. Essential Role of Pre-T Cell Receptor in Allelic Exclusion of the T Cell Receptor Beta Locus. *Immunity*, **7**, 601-607.

Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R. and Weissman, I.L. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*, **89**, 1033-1041.

Aktories, K., Mohr, C. and Koch, G. 1992. Clostridium botulinum C3 ADP-ribosyltransferase. *Current Topics in Microbiology and Immunology*, **175**, 115-131.

Aktories, K., Roesner, U., Balschke, U. and Chatwal, S. 1988. Botulinum ADP-ribosyltransferase C3. *European Journal of Biochemistry*, **172**, 445.

Alberola-Ila, J., Forbush, K.A., Seger, R., Krebs, E.G. and Perlmutter, R.M. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature*, **373**, 620-623.

Alberola-Ila, J., Hogquist, K.A., Swan, K.A., Bevan, M.J. and Perlmutter, R.M. 1996. Positive and negative selection invoke distinct signaling pathways. *Journal of Expermental Medicine*, **184**, 9-18.

Alberts, A.S., Bouquin, N., Johnston, L.H. and Treisman, R. 1998a. Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. *Journal of Biological Chemistry*, **273**, 8616-22.

Alberts, A.S., Geneste, O. and Treisman, R. 1998b. Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. *Cell*, **92**, 475-87.

Alberts, A.S. and Treisman, R. 1998. Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1. *EMBO Journal*, **17**, 4075-85.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B. and Cohen, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology*, **7**, 261-9.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. 1996a. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *Journal of Biological Chemistry*, **271**, 20246-9.

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A. and Kaibuchi, K. 1996b. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. *Science*, **271**, 648-650.

Andersen, P.R., Devare, S.G., Tronick, S.R., Ellis, R.W., Aaronson, S.A. and Scolnick, E.M. 1981. Generation of BALB-MuSV and Ha-MuSC by type C virus transduction of homologous transforming genes from different species. *Cell*, **26**, 129-34.

Anderson, G., Owen, J.J., Moore, N.C. and Jenkinson, E.J. 1994. Thymic epithelial cells provide unique signals for positive selection of $CD4^{+}CD8^{+}$ thymocytes *in vitro*. *Journal of Experimental Medicine*, **179**, 2027-31.

Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X. and Perlmutter, R.M. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of $p59^{fyn}$. *Cell*, **70**, 751-763.

Aspenstrom, P., Lindberg, U. and Hall, A. 1996. Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldrich syndrome. *Current Biology*, **6**, 70-5.

Badiani, P., Corbella, P., Kioussis, D., Marvel, J. and Weston, K. 1994. Dominant interfering alleles define a role for c-myb in T-cell development. *Gen.Develop.*, **8**, 770-782.

Badiani, P.A., Kioussis, D., Swirsky, D.M., Lampert, I.A. and Weston, K. 1996. T-cell lymphomas in v-Myb transgenic mice. *Oncogene*, **13**, 2205-12.

Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. 1995. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *Journal of Biological Chemistry*, **270**, 27995-8.

Baldwin, A.S. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annual Review of Immunology*, **14**, 649-83.

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C.P. and Alessi, D.R. 1999. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Current Biology*, **9**, 393-404.

Bar-Sagi, D. and Feramisco, J.R. 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science*, **233**, 1061-8.

Barbacid, M. 1987. ras genes. *Annu Rev Biochem*, **56**, 779-827.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D. and Wynshaw-Boris, A. 1996. Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell*, **86**, 159-71.

Bear, J.E., Rawls, J.F. and Saxe, C.L.r. 1998. SCAR, a WASP-related protein, isolated as a suppressor of receptor defects in late Dictyostelium development. *Journal of Cell Biology*, **142**, 1325-35.

Beltman, J., Erickson, J.R., Martin, G.A., Lyons, J.F. and Cook, S.J. 1999. C3 Toxin Activates the Stress Signalling Pathways, JNK and p38, but antoagonizes the Activation of AP-1 in Rat-1 Cells. *Journal of Biological Chemistry*, **274**, 3772-3780.

Bennett, M., Macdonald, K., Chan, S.W., Luzio, J.P., Simari, R. and Weissberg, P. 1998. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science*, **282**, 290-3.

Benton, B.K., Tinkelenberg, A., Gonzalez, I. and Cross, F.R. 1997. Cla4p, a Saccharomyces cerevisiae Cdc42p-activated kinase involved in cytokinesis, is activated at mitosis. *Molecular & Cellular Biology*, **17**, 5067-76.

Blunt, T., Finnie, N.J., Taccioli, G.E., Smith, G.C., Demengeot, J., Gottlieb, T.M., Mizuta, R., Varghese, A.J., Alt, F.W., Jeggo, P.A. and Jackson, S.P. 1995. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell*, **80**, 813-823.

Bos, J.L. 1989. ras oncogenes in human cancer: a review. *Cancer Research*, **49**, 4682-4689.

Bowtell, D., Fu, P., Simon, M. and Senior, P. 1992. Identification of murine homologues of the Drosophila Son of Sevenless. *Proc. Natl. Acad. Sci. USA*, **89**, 6511-6518.

Brandt, J.E., Bhalla, K. and Hoffman, R. 1994. Effects of interleukin-3 and c-kit ligand on the survival of various classes of human hematopoietic progenitor cells. *Blood*, **83**, 1507-14.

Bronson, R.T. 1990. Rate of occurance of lesions in 20 inbred and hybrid genotypes of rats and mice sacrificed at six month intervals during the first year of their life. *In Genetic effects on aging II, D. E. Harrison ed.*, Caldwell, New Jersey: Telford Press Inc., 279-358.

Bruyns, E., Marie-Cardine, A., Kirchgessner, H., Sagolla, K., Shevchenko, A., Mann, M., Autschbach, F., Bensussan, A., Meuer, S. and Schraven, B. 1998. T cell receptor (TCR) interacting molecule (TRIM), a novel disulfide- linked dimer associated with the TCR-CD3-zeta complex, recruits intracellular signaling proteins to the plasma membrane. *Journal of Experimental Medicine*, **188**, 561-575.

Buer, J., Aifantis, I., DiSanto, J.P., Fehling, H.J. and von Boehmer, H. 1997. Role of different T cell receptors in the development of pre-T cells. *Journal of Experimental Medicine*, **185**, 1541-7.

Buhl, A.M., Johnson, N.L., Dhanasekaran, N. and Johnson, G.L. 1995. Gα12 and Gα13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *Journal of Biological Chemistry*, **270**, 24631-4.

Byth, K.F., Conroy, L.A., Howlett, S., Smith, A.J.H., May, J., Alexander, D.R. and Holmes, N. 1996. CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of $CD4^+CD8^+$ thymocytes, and in B-cell maturation. *Journal of Experimental Medicine*, **183**, 1707-1718.

Cantrell, D. 1996. T cell antigen receptor signal transduction pathways. *Annual Review of Immunology*, **14**, 259-274.

Cantrell, D.A. and Smith, K.A. 1984. The interleukin-2 T-cell system: a new cell growth model. *Science*, **224**, 1312-1316.

Cao, X., Shores, E.W., Hu-Li, J., Anver, M., Kelsall, B.L., Russell, S.M., Drago, J., Noguchi, M., Grinberg, A., Bloom, E.T., Paul, W.E., Katz, S.I., Love, P.E. and Leonard, W.J. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity*, **2**, 223-238.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282**, 1318-21.

Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B. and Clarkson, B. 1997. p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell*, **88**, 197-204.

Carson, W.E., Haldar, S., Baiocchi, R.A., Croce, C.M. and Caligiuri, M.A. 1994. The c-kit ligand suppresses apoptosis of human natural killer cells through the upregulation of bcl-2. *Proc. Natl. Acad. Sci. USA*, **91**, 7553-7.

Castrillon, D.H. and Wasserman, S.A. 1994. Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. *Development*, **120**, 3367-77.

Cerione, R. and Zheng, Y. 1996. The Dbl family of oncogenes. *Current Opinion in Cell Biology*, **8**, 216-222.

Chan, A.C., Kadlecek, T.A., Elder, M.E., Filipovich, A.H., Kuo, W.L., Iwashima, M., Parslow, T.G. and Weiss, A. 1994a. ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*, **264**, 1599-601.

Chan, A.C., van Oers, N.S., Tran, A., Turka, L., Law, C.L., Ryan, J.C., Clark, E.A. and Weiss, A. 1994b. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *Journal of Immunology*, **152**, 4758-66.

Chang, J.H., Pratt, J.C., Sawasdikosol, S., Kapeller, R. and Burakoff, S.J. 1998. The small GTP-binding protein Rho potentiates AP-1 transcription in T cells. *Molecular & Cellular Biology*, **18**, 4986-93.

Chardin, P. (1993) *GTPases in Biology I, Dickey, B.F. and Birnbaumer L. (eds)*. Springer-Verlag, Heidelberg, 159-176

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. 1989. The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO Journal*, **8**, 1087-1092.

Chen, J., Lansford, R., Stewart, V., Young, F. and Alt, F.W. 1993. RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proc. Natl. Acad. Sci. USA*, **90**, 4528-32.

Chen, J., Shinkai, Y., Young, F. and Alt, F.W. 1994a. Probing immune functions in RAG-deficient mice. *Current Opinion in Immunology*, **6**, 313-9.

Chen, J., Stewart, V., Spyrou, G., Hilberg, F., Wagner, E.F. and Alt, F.W. 1994b. Generation of normal T and B lymphocytes by c-jun deficient embryonic stem cells. *Immunity*, **1**, 65-72.

Chen, W., Blanc, J. and Lim, L. 1994c. Characterization of a promiscuous GTPase-activating protein that has a Bcr-related domain from Caenorhabditis elegans. *Journal of Biological Chemistry*, **269**, 820-3.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B. and Pawson, T. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*, **378**, 303-6.

Chihara, K., Amano, M., Nakamura, N., Yano, T., Shibata, M., Tokui, T., Ichikawa, H., Ikebe, R., Ikebe, M. and Kaibuchi, K. 1997. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *Journal of Biological Chemistry*, **272**, 25121-7.

Chiou, S.K., Rao, L. and White, E. 1994. Bcl-2 blocks p53-dependent apoptosis. *Molecular & Cellular Biology*, **14**, 2556-63.

Chong, L.D., Traynor, K.A., Bokoch, G.M. and Schwartz, M.A. 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell*, **79**, 507-13.

Chrzanowska-Wodnicka, M. and Burridge, K. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *Journal of Cell Biology*, **133**, 1403-15.

Chuang, T.H., Hahn, K.M., Lee, J.D., Danley, D.E. and Bokoch, G.M. 1997. The small GTPase Cdc42 initiates an apoptotic signaling pathway in Jurkat T lymphocytes. *Molecular Biology of the Cell*, **8**, 1687-98.

Churilla, A.M., Braciale, T.J. and Braciale, V.L. 1989. Regulation of T lymphocyte proliferation. Interleukin 2-mediated induction of c-myb gene expression is dependent on T lymphocyte activation state. *Journal of Experimental Medicine*, **170**, 105-21.

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L. and Wyllie, A.H. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849-852.

Clements, J.L., Boerth, N.J., Lee, J.R. and Koretzky, G.A. 1999. Integration of T cell receptor-dependent signaling pathways by adapter proteins. *Ann. Rev. Immunol.*, **17**, 89-108.

Clements, J.L., Yang, B., Ross-Barta, S.E., Eliason, S.L., Hrstka, R.F., Williamson, R.A. and Koretzky, G.A. 1998. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science*, **281**, 416-9.

Cleverley, S., Henning, S. and Cantrell, D. 1999. Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function. *Current Biology*, **9**, 657-660.

Connor, F., Bertwistle, D., Mee, P.J., Ross, G.M., Swift, S., Grigorieva, E., Tybulewicz, V.L. and Ashworth, A. 1997. Tumorigenesis and a DNA repair defect in mice with a truncating Brca2 mutation. *Nature Genetics*, **17**, 423-30.

Coso, O., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of JNK/SAPK signaling pathway. *Cell*, **81**, 1137-1146.

Coull, J.M. and Pappin, D.J.C. 1990. A Rapid fluoresent staining procedure for proteins electroblotted onto PVDF membranes. *Journal of Protein Chemistry*, **9**, 259-260.

Crabtree, G.R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science*, **243**, 355-361.

Crabtree, G.R. 1999. Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. *Cell*, **96**, 611-614.

Crabtree, G.R. and Clipstone, N.A. 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Ann. Rev. Biochem.*, **63**, 1045-1083.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S. and Bustelo, X.R. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the *vav* proto-oncogene product. *Nature*, **385**, 169-172.

Crews, C.M., Alessandrini, A. and Erikson, R.L. 1992. Erks: their fifteen minutes has arrived. *Cell Growth & Differentiation*, **3**, 135-42.

Crews, C.M. and Erikson, R.L. 1993. Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell*, **74**, 215-217.

Dallery, E., Galiegue-Zouitina, S., Collyn-d'Hooghe, M., Quief, S., Denis, C., Hildebrand, M.P., Lantoine, D., Deweindt, C., Tilly, H., Bastard, C. and et al. 1995. TTF, a gene encoding a novel small G protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene*, **10**, 2171-8.

de Vries-Smits, A., Burgering, B., Leevers, S., Marshall, C. and Bos, J. 1992. Involvement of p21ras in activation of extracellular regulated kinase 2. *Nature*, **357**, 601-604.

DeFeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowy, D.R., Scolnick, E.M. and Ellis, R.W. 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. *Proc. Natl. Acad. Sci. USA*, **78**, 3328-32.

Di Cunto, F., Calautti, E., Hsiao, J., Ong, L., Topley, G., Turco, E. and Dotto, G. 1998. Citron Rho-interacting Kinase, a Novel Tissue-specific Ser/Thr Kinase Encompassing the Rho-Rac-binding Protein Citron. *Journal of Biological Chemistry*, **273**, 29706-29711.

Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. 1991. Bcr encodes a GTPase-activating protein for p21rac. *Nature*, **351**, 400-2.

Dillon, S.T., Rubin, E.J., Yakubovich, M., Pothoulakis, C., LaMont, J.T., Feig, L.A. and Gilbert, R.J. 1995. Involvement of Ras-related Rho proteins in the mechanisms of action of Clostridium difficile toxin A and toxin B. *Infection & Immunity*, **63**, 1421-6.

DiSanto, J.P., Guy-Grand, D., Fisher, A. and Tarakhovsky, A. 1996. Critical role for the common cytokine receptor gamma chain in intrathymic and peripheral T cell selection. *Journal of Experimental Medicine*, **183**, 1111-8.

DiSanto, J.P., Mueller, W., Guy-Grand, D., Fischer, A. and Rajewsky, K. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA*, **92**, 377-381.

DiSanto, J.P. and Rodewald, H.R. 1998. *In vivo* roles of receptor tyrosine kinasesa and cytokine receptors in early thymocyte development. *Current Opinion in Immunology*, **10**, 196-207.

Dong, C., Yang, D.D., Wysk, M., Whitmarsh, A.J., Davis, R.J. and Flavell, R.A. 1998. Defective T cell differentiation in the absence of Jnk1. *Science*, **282**, 2092-5.

Drechsel, D.N., Hyman, A.A., Hall, A. and Glotzer, M. 1997. A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos. *Current Biology*, **7**, 12-23.

Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. and Weinberg, R.A. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, **363**, 45-51.

Ehrengruber, M.U., Boquet, P., Coates, T.D. and Deranleau, D.A. 1995. ADP-ribosylation of Rho enhances actin polymerization-coupled shape oscillations in human neutrophils. *FEBS Letters*, **372**, 161-4.

Ellis, R.W., Defeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R. and Scolnick, E.M. 1981. The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. *Nature*, **292**, 506-11.

Eva, A. and Aaronson, S.A. 1985. Isolation of a new human oncogene from a diffuse B-cell lymphoma. *Nature*, **316**, 273-275.

Eva, A., Tronick, S.R., Gol, R.A., Pierce, J.H. and Aaronson, S.A. 1983. Transforming genes of human hematopoietic tumors: frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. *Proc. Natl. Acad. Sci. USA*, **80**, 4926-30.

Fahlen, L., Khoo, N.K., Daws, M.R. and Sentman, C.L. 1997. Location-specific regulation of transgenic Ly49A receptors by major histocompatibility complex class I molecules. *European Journal of Immunology*, **27**, 2057-65.

Fehling, H.J., Iritani, B.M., Krotkova, A., Forbush, K.A., Laplace, C., Perlmutter, R.M. and von Boehmer, H. 1997. Restoration of thymopoiesis in pTα -/- mice by anti-CD3ε antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity*, **6**, 703-14.

Fehling, H.J., Krotkova, A., Saint-Ruf, C. and von Boehmer, H. 1995. Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. *Nature*, **375**, 795-798.

Feig, L. 1999. Tools of the Trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nature Cell Biology*, **1**, E25-27.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interactions. *Nature*, **340**, 245-6.

Finco, T.S., Kadlecek, T., Zhang, W., Samelson, L.E. and Weiss, A. 1998. LAT is required for TCR-mediated activation of PLCγ1 and the Ras pathway. *Immunity*, **9**, 617-26.

Fiorentini, C., Donelli, G., Matarrese, P., Fabbri, A., Paradisi, S. and Boquet, P. 1995. Escherichia coli cytotoxic necrotizing factor 1: evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infection & Immunity*, **63**, 3936-44.

Fischer, K.-D., Kong, Y.-Y., Nishina, H., Tedford, K., Marengere, L.E.M., Kozieradzki, I., Sasaki, T., Starr, M., Chan, G., Gardener, S., Nghiem, M.P., Bouchard, D., Barbacid, M., Bernstein, A. and Penninger, J.M. 1998. Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Current Biology*, **8**, 554-562.

Fischer, K.-D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A. and Guidos, C. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient $CD4^+CD8^+$ thymocytes. *Nature*, **374**, 474-476.

Fleischman, R.A. 1993. From white spots to stem cells: the role of the Kit receptor in mammalian development. *Trends in Genetics*, **9**, 285-90.

Flinn, H.M. and Ridley, A.J. 1996. Rho stimulates tyrosine phosphorylation of focal adhesion kinase, p130 and paxillin. *Journal of Cell Science*, **109**, 1133-41.

Flynn, P. 1999. Regulation of the Protein Kinase C-related Kinase Family. *Ph.D*, University College, London.

Frampton, J., Ramqvust, T. and Graf, T. 1996. v-Myb of E26 leukemia virus up-regulates bcl-2 and suppresses apoptosis in myeloid cells. *Genes and Development*, **10**, 2720-2731.

Fritz, G., Kaina, B. and Aktories, K. 1995. The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments. *Journal of Biological Chemistry*, **270**, 25172-7.

Frost, J.A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P.E. and Cobb, M.H. 1997. Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO Journal*, **16**, 6426-38.

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W. and Cantley, L.C. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85α. *Science*, **283**, 393-7.

Fujisawa, K., Fujita, A., Ishizaki, T., Saito, Y. and Narumiya, S. 1996. Identification of the Rho-binding domain of p160ROCK, a Rho-associated coiled-coil containing protein kinase. *Journal of Biological Chemistry*, **271**, 23022-8.

Fukuoka, M., Miki, H. and Takenawa, T. 1997. Identification of N-WASP homologs in human and rat brain. *Gene*, **196**, 43-8.

Gallandrini, R., Henning, S. and Cantrell, D.A. 1997. Different functions for the GTPase Rho in prothymocytes and late pre-T cells. *Immunity*, **7**, 163-174.

Galli, S.J., Zsebo, K.M. and Geissler, E.N. 1994. The kit ligand, stem cell factor. *Advances in Immunology*, **55**, 1-96.

Gallo, K.A., Mark, M.R., Scadden, D.T., Wang, Z., Gu, Q. and Godowski, P.J. 1994. Identification and characterization of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity. *Journal of Biological Chemistry*, **269**, 15092-100.

Gartner, F., Alt, F.W., Monroe, R., Chu, M., Sleckman, B.P., Davidson, L. and Swat, W. 1999. Immature thymocytes employ distinct signaling pathways for allelic exclusion versus differentiation and expansion. *Immunity*, **10**, 537-46.

Gauen, L.K.T., Zhu, Y.X., Letourneur, F., Hu, Q., Bolen, J.B., Matis, L.A., Klausner, R.D. and Shaw, A.S. 1994. Interactions of $p59^{fyn}$ and Zap-70 with T-cell receptor activation motifs - defining the nature of a signaling motif. *Molecular and Cellular Biology*, **14**, 3729-3741.

Genot, E., Cleverley, S., Henning, S. and Cantrell, D.A. 1996. Multiple p21ras effector pathways regulate nuclear factor of activated T cells. *EMBO Journal*, **15**, 3923-3933.

Genot, E., Reif, K., Beach, S., Kramer, I. and Cantrell, D. 1998. p21ras initiates Rac-1 but not phosphatidylinositol-3-kinase/PKB, mediated signaling pathways in T lymphocytes. *Oncogene*, **17**, 1731-1738.

Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M.H. and Shaw, P.E. 1995. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO Journal*, **14**, 951-62.

Gilmore, A.P. and Burridge, K. 1996. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. *Nature*, **381**, 531-5.

Godfrey, D.I., Kennedy, J., Suda, T. and Zlotnik, A. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of $CD3^-CD4^-CD8^-$ triple-

negative adult mouse thymocytes defined by CD44 and CD25 expression. *Journal of Immunology*, **150**, 4244-4252.

Godfrey, D.I. and Zlotnik, A. 1993. Control points in early T-cell development. *Imunnology Today*, **14**, 547-553.

Gomez, J., Martinez-A., C., Giry, M., Garcia, A. and Rebollo, A. 1997. Rho prevents apoptosis through Bcl-2 expression: implications for interleukin-2 receptor signal transduction. *European Journal of Immunology*, **27**, 2793-2799.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M. and Dustin, M.L. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science*, **285**, 221-7.

Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. 1989. Human CD2 3'-flanking sequences confer high-level, T cell specific, position-independent gene expression in transgenic mice. *Cell*, **56**, 979-986.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M.J., Hayday, A.C. and von Boehmer, H. 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. *Cell*, **75**, 283-294.

Groettrup, M. and von Boehmer, H. 1993. A role for A pre-T-cell receptor in T cell development. *Immunology Today*, **14**, 610-614.

Groves, T., Smiley, P., Cooke, M.P., Forbush, K., Perlmutter, R.M. and Guidos, C.J. 1996. Fyn can partially substitute for Lck in T-lymphocyte development. *Immunity*, **5**, 417-428.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science*, **265**, 103-6.

Guillemot, J.C., Kruskal, B.A., Adra, C.N., Zhu, S., Ko, J.L., Burch, P., Nocka, K., Seetoo, K., Simons, E. and Lim, B. 1996. Targeted disruption of guanosine diphosphate-dissociation inhibitor for Rho-related proteins, GDI-D4: normal hematopoietic differentiation but subtle defect in superoxide production by macrophages derived from *in vitro* embryonal stem cell differentiation. *Blood*, **88**, 2722-31.

Haas, W., Pereira, P. and Tonegawa, S. 1993. Gamma/delta cells. *Annual Review of Immunology*, **11**, 637-85.

Haks, M.C., Krimpenfort, P., van den Brakel, J.H. and Kruisbeek, A.M. 1999. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity*, **11**, 91-101.

Hall, A. 1998. Rho GTPases and the Actin Cytoskeleton. *Science*, **279**, 509-514.

Hallberg, B., Rayter, S.I. and Downward, J. 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *Journal of Biological Chemistry*, **269**, 3913-3916.

Han, J., Das, B., Wei, W., vanAelst, L., Mosteller, R.D., Khosravi-Far, R., Westwick, J.K., Channing, J.D. and Broek, D. 1997. Lck regulates Vav activation of members of the Rho family of GTPases. *Molecular and Cellular Biology*, **17**, 1346-1353.

Han, J., Luby-Phelps, K., Das, K., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A. and Broek, D. 1998. Role of substrates and products of PI3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science*, **279**, 558-560.

Han, M. and Sternberg, P.W. 1990. let-60, a gene that specifies cell fates during C. elegans vulval induction, encodes a ras protein. *Cell*, **63**, 921-931.

Hart, M.J., Eva, A., Evans, T., Aaronson, S.A. and Cerione, R.A. 1991. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. *Nature*, **354**, 311-4.

Hart, M.J., Eva, A., Zangrilli, D., Aaronson, S.A., Evans, T., Cerione, R.A. and Zheng, Y. 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the *dbl* oncogene product. *Journal of Biological Chemistry*, **269**, 62-65.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C. and Bollag, G. 1998. Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Gα13. *Science*, **280**, 2112-4.

Hartwig, J., Bokoch, G., Carpenter, C., Janmey, P., Taylor, L., Toker, A. and Stossel, T. 1995. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell*, **82**, 643-653.

He, Y.W. and Malek, T.R. 1996. Interleukin-7 receptor alpha is essential for the development of γδ T cells, but not natural killer cells. *Journal of Experimental Medicine* **184**, 289-293.

Hengen, P.N. 1997. False positives from the yeast two-hybrid system. *Trends in Biochemical Sciences*, **22**, 33-4.

Henning, S., Galandrini, R., Hall, A. and Cantrell, D.A. 1997. The GTPase Rho has a critical regulatory role in thymocyte development. *EMBO Journal*, **16**, 2397-2407.

Henning, S.W. and Cantrell, D.A. 1998. p56lck signals for regulating thymocyte development can be distinguished by their dependency on Rho-function. *Journal of Experimental Medicine,* **188**, 931 - 939.

Henning, S.W. and Cleverley, S.C. 1999. Small GTPases in Lymphocyte Biology: Rho Proteins take Center Stage. *Immunological Research*, **20**, 29-42.

Higgins, D.G., Thompson, J.D. and Gibson, T.J. 1996. Using CLUSTAL for multiple sequence alignments. *Methods in Enzymology*, **266**, 383-402.

Hill, C.S., Wynne, J. and Treisman, R. 1995. The Rho family GTPases RhoA, Rac1 and CDC42Hs regulate transcriptional activation by SRF. *Cell*, **81**, 1159-1170.

Hoffman, E.S., Passoni, L., Crompton, T., Leu, T.M., Schatz, D.G., Koff, A., Owen, M.J. and Hayday, A.C. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development *in vivo*. *Genes and Development*, **10**, 948-62.

Holzman, L.B., Merritt, S.E. and Fan, G. 1994. Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein kinase that defines a second subfamily of mixed lineage kinases. *Journal of Biological Chemistry*, **269**, 30808-17.

Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. and Matsuda, M. 1995. Bordetella bronchiseptica dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. *Journal of Cell Science*, **108**, 3243-51.

Horii, Y., Beeler, J.F., Sakaguchi, K., Tachibana, M. and Miki, T. 1994. A novel oncogene, ost, encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways. *EMBO Journal*, **13**, 4776-86.

Hunt, D.F., Yates, J.R., Shabanowitz, J., Winston, S. and Hauer, C.R. 1986. Protein Sequencing by Tandem Mass Spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6233-6237.

Hunter, T. 1991. Cooperation between oncogenes. *Cell*, **64**, 249-70.

Iemura, A., Tsai, M., Ando, A., Wershil, B.K. and Galli, S.J. 1994. The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *American Journal of Pathology*, **144**, 321-8.

Iritani, B.M., Alberola-Ila, J., Forbush, K.A. and Perimutter, R.M. 1999. Distinct signals mediate maturation and allelic exclusion in lymphocyte progenitors. *Immunity*, **10**, 713-22.

Irving, B.A., Alt, F.W. and Killen, N. 1998. Thymocyte Development in the Absence of Pre-T Cell Receptor Extracellular Immunoglobulin Domains. *Science*, **280**, 905-908.

Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y. and Narumiya, S. 1997. p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Letters*, **404**, 118-24.

Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T. and Narumiya, S. 1999. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nature Medicine*, **5**, 221-5.

Iwashima, M., Irving, B.A., van Oers, N.S.C., Chan, A.C. and Weiss, A. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*, **263**, 1136-1139.

Izquierdo, M., Leevers, S.J., Marshall, C. and Cantrell, D.A. 1993. p21ras couples the T cell antigen receptor to Extracellular Signal-regulated Kinase 2 in T lymphocytes. *Journal of Experimental Medicine*, **178**, 1199-1208.

Jahner, D. and Hunter, T. 1991. The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Molecular & Cellular Biology*, **11**, 3682-90.

Jain, J., Nalefski, E.A., McCaffrey, P.G., Johnson, R.S., Spiegelman, B.M., Papaioannou, V. and Rao, A. 1994. Normal peripheral T-cell function in c-Fos-deficient mice. *Molecular & Cellular Biology*, **14**, 1566-1574.

Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H. 1994. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *Journal of Cell Biology*, **126**, 801-10.

Ji, Q.S., Winnier, G.E., Niswender, K.D., Horstman, D., Wisdom, R., Magnuson, M.A. and Carpenter, G. 1997. Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proc. Natl. Acad. Sci. USA* , **94**, 2999-3003.

Jiang, D., Lenardo, M.J. and Zuniga-Pflucker, C. 1996. p53 prevents maturation to the $CD4^{+}CD8^{+}$ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. *Journal of Experimental Medicine*, **183**, 1923-8.

Jiang, R. and Gridley, T. 1997. Gene targeting: things go better with Cre. *Current Biology*, **7**, R321-3.

Johnson, R.S., van Lingen, B., Papaioannou, V.E. and Spiegelman, B.M. 1993. A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes & Development*, **7**, 1309-17.

Just, I., Mohr, C., Schallehn, G., Menard, L., Didsbury, J.R., Vandekerckhove, J., van Damme, J. and Aktories, K. 1992. Purification and characterization of an ADP-ribosyltransferase produced by Clostridium limosum. *Journal of Biological Chemistry*, **267**, 10274-80.

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. and Aktories, K. 1995. Glucosylation of Rho proteins by Clostridium difficile toxin B. *Nature*, **375**, 500-3.

Katz, M.E. and McCormick, F. 1997. Signal transduction from multiple Ras effectors. *Current Opinion in Genetics & Development*, **7**, 75-9.

Kelly, K. and Siebenlist, U. 1995. Immediate-early genes induced by antigen receptor stimulation. *Current Opinion in Immunology*, **7**, 327-332.

Khosravi-Far, R., Solski, P.A., Clark, G.J., Kinch, M.S. and Der, C.J. 1995. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Molecular & Cellular Biology*, **15**, 6443-6453.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. 1996a. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*, **273**, 245-248.

Kirchgessner, C., Patil, C., Evans, J., Cuomo, C., Fried, L., Carter, T., Oettinger, M. and Brown, J. 1995. DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science*, **267**, 1178-.

Kirchhausen, T. and Rosen, F.S. 1996. Disease mechanism: unravelling Wiskott-Aldrich syndrome. *Current Biology*, **6**, 676-678.

Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Takai, Y. 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *Journal of Cell Biology*, **120**, 1187-95.

Kisielow, P., Bluthmann, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+CD8^+$ thymocytes. *Nature*, **333**, 742-746.

Kiyono, M., Satoh, T. and Kaziro, Y. 1999. G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). *Proc. Natl. Acad. Sci. USA*, **96**, 4826-31.

Koch, G., Norgauer, J. and Aktories, K. 1994. ADP-ribosylation of the GTP-binding protein Rho by Clostridium limosum exoenzyme affects basal, but not N-formyl-peptide-stimulated, actin polymerization in human myeloid leukaemic (HL60) cells. *Biochemical Journal*, **299**, 775-9.

Kondo, M., Akashi, K., Domen, J., Sugamura, K. and Weissman, I.L. 1997. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity*, **7**, 155-62.

Kosako, H., Goto, H., Yanagida, M., Matsuzawa, K., Fujita, M., Tomono, Y., Okigaki, T., Odai, H., Kaibuchi, K. and Inagaki, M. 1999. Specific accumulation of Rho-associated kinase at the cleavage furrow during cytokinesis: cleavage furrow-specific phosphorylation of intermediate filaments. *Oncogene*, **18**, 2783-8.

Kozma, R., Ahmed, S., Best, A. and Lim, L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Molecular & Cellular Biology*, **15**, 1942-52.

Kozma, R., Ahmed, S., Best, A. and Lim, L. 1996. The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Molecular & Cellular Biology*, **16**, 5069-80.

Krotkova, A., von Boehmer, H. and Fehling, H. 1997. Allelic Exclusion in pT-alpha-deficient Mice: No Evidence for Cell Surface Expression of Two TCR-Beta chains, but less efficient Inhibition of Endogeneous V-beta to (D)JBeta Rearrangements in the presence of a functional TCR-Beta Transgene. *Journal of Experimental Medicine*, **186**, 767 - 775.

Kuhn, R., Schwenk, F., Aguet, M. and Rajewsky, K. 1995. Inducible gene targeting in mice. *Science*, **269**, 1427-9.

Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y. and Narumiya, S. 1993. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. *Journal of Biological Chemistry*, **268**, 24535-8.

Kyriakis, J.M. and Avruch, J. 1996. Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays*, **18**, 567-77.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A. 1997. Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell*, **87**, 519-529.

Lancaster, C.A., Taylor-Harris, P.M., Self, A.J., Brill, S., van Erp, H.E. and Hall, A. 1994. Characterization of rhoGAP. A GTPase-activating protein for rho-related small GTPases. *Journal of Biological Chemistry*, **269**, 1137-42.

Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M. and Bertoglio, J. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO Journal*, **15**, 510-9.

Lang, P., Guizani, L., Vitte-Mony, I., Stancout, R., Dorseuil, O., Gacon, G. and Bertoglio, J. 1992. ADP-ribosylation of the ras-related, GTP-binding protein rhoA inhibits lymphocyte-mediated cytotoxicity. *Journal of Biological Chemistry*, **267**, 11677-11680.

Lantz, O., Sharara, L.I., Tilloy, F., Andersson, A. and DiSanto, J.P. 1997. Lineage relationships and differentiation of natural killer (NK) T cells: intrathymic selection and interleukin (IL)-4 production in the absence of NKR-P1 and Ly49 molecules. *Journal of Experimental Medicine*, **185**, 1395-401.

Laudanna, C., Campbell, J.J. and Butcher, E.C. 1996. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science*, **271**, 981-3.

Leevers, S.J. and Marshall, C.J. 1992. Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoproteins. *EMBO Journal*, **11**, 569-574.

Lemmon, M.A., Ferguson, K.M. and Schlessinger, J. 1996. PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, **85**, 621-4.

Leung, T., Chen, X.Q., Manser, E. and Lim, L. 1996a. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Molecular & Cellular Biology*, **16**, 5313-27.

Leung, T., Manser, E., Tan, L. and Lim, L. 1996b. A novel serine/threonine kinase binding the ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J.Biol.Chem.*, **270**, 29051-29054.

Levin, S.D., Anderson, S.J., Forbush, K.A. and Perlmutter, R.M. 1993. A dominant-negative transgene defines a role for p561ck in thymopoiesis. *EMBO Journal*, **12**, 1671-80.

Lowy, D.R. and Willumsen, B.M. 1993. Function and regulation of ras. *Annual Review of Biochemistry*, **62**, 851-91.

Lynch, E.D., Lee, M.K., Morrow, J.E., Welcsh, P.L., Leon, P.E. and King, M.C. 1997. Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the Drosophila gene diaphanous [see comments]. *Science*, **278**, 1315-8.

Ma, A., Pena, J.C., Chang, B., Margosian, E., Davidson, L., Alt, F.W. and Thompson, C.B. 1995. Bcl-x regulates the survival of double-positive thymocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 4763-4767.

Ma, L., Rohatgi, R. and Kirschner, M.W. 1998. The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. *Proc. Natl. Acad. Sci. USA*, **95**, 15362-7.

Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M. and Narumiya, S. 1993. A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. *Zygote*, **1**, 325-31.

MacDonald, H.R. 1995. NK1.1^{+} T cell receptor alpha/beta+ cells: New Clues to their Origin, Specificity, and Function. *Journal of Experimental Medicine*, **182**, 633-638.

Machesky, M. and Hall, A. 1996. Rho: a connection between membrane receptor signalling and the cytoskeleton. *TICB*, **6**, 304 - 310.

Mackarehtschian, K., Hardin, J.D., Moore, K.A., Boast, S., Goff, S.P. and Lemischka, I.R. 1995. Targeted Disruption of the flk2/flt3 gene Leads to Deficiencies in Primitive Hematopoietic Progenitors. *Immunity*, **3**, 147-161.

Madaule, P., Eda, M., Watanabe, N., Fujisawa, K., Matsuoka, T., Bito, H., Ishizaki, T. and Narumiya, S. 1998. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature*, **394**, 491-4.

Mahadevan, M.S., Amemiya, C., Jansen, G., Sabourin, L., Baird, S., Neville, C.E., Wormskamp, N., Segers, B., Batzer, M., Lamerdin, J. and et al. 1993. Structure and genomic sequence of the myotonic dystrophy (DM kinase) gene. *Human Molecular Genetics*, **2**, 299-304.

Maki, K., Sunaga, S., Komagata, Y., Kodaira, Y., Mabuchi, A., Karasuyama, H., Yokomuro, K., Miyazaki, J.I. and Ikuta, K. 1996. Interleukin 7 receptor-deficient mice lack gammadelta T cells. *Proc. Natl. Acad. Sci. USA*, **93**, 7172-7.

Manser, E., Chong, C., Zhao, Z.S., Leung, T., Michael, G., Hall, C. and Lim, L. 1995. Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family. *Journal of Biological Chemistry*, **270**, 25070-8.

Marais, R., Light, Y., Paterson, H. and Marshall, C. 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO Journal*, **14**, 3136-3145.

Marais, R., Wynne, J. and Treisman, R. 1993. The SRF accesory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*, **73**, 381-393.

Maraskovsky, E., O'Reilly, L.A., Teepe, M., Corcoran, L.M., Peschon, J. and Strasser, A. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor deficient mice but not in mutant rag-1 -/- mice. *Cell*, **89**, 1011-1019.

Mariathasan, S., Jones, R.G. and Ohashi, P.S. 1999. Signals involved in thymocyte positive and negative selection. *Seminars in Immunology*, **11**, 263-272.

Marshall, C.J. 1996. Ras effectors. *Current Opinion in Cell Biology*, **8**, 197-204.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO Journal*, **15**, 2208-2216.

Matthews, W., Jordan, C.T., Wiegand, G.W., Pardoll, D. and Lemischka, I.R. 1991. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell*, **65**, 1143-52.

McCormick, F. 1993. How receptors turn ras on. *Nature*, **363**, 15-16.

Melton, E., Sarner, N., Torkar, M., van der Merwe, P.A., Russell, J.Q., Budd, R.C., Mamalaki, C., Tolaini, M., Kioussis, D. and Zamoyska, R. 1996. Transgene-encoded human CD2 acts in a dominant negative fashion to modify thymocyte selection signals in mice. *European Journal of Immunology*, **26**, 2952-63.

Menard, L., Tomhave, E., Casey, P.J., Uhing, R.J., Snyderman, R. and Didsbury, J.R. 1992. Rac1, a low-molecular-mass GTP-binding-protein with high intrinsic GTPase activity and distinct biochemical properties. *European Journal of Biochemistry*, **206**, 537-46.

Miki, H., Suetsugu, S. and Takenawa, T. 1998. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO Journal*, **17**, 6932-41.

Miki, T., Smith, C.L., Long, J.E., Eva, A. and Fleming, T.P. 1993. Oncogene ect2 is related to regulators of small GTP-binding proteins. *Nature*, **362**, 462-5.

Minden, A., Lin, A., Claret, F., Abo, A. and Karin, M. 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, **81**, 1147-1157.

Mock, K.K., Sutton, C.W. and Cottrell, J.S. 1992. Sample immobilization protocols for matrix-assisted laser-desorption mass spectrometry. *Rapid Commun. Mass Spectrom.*, **6**, 233-238.

Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.-U., Veillette, A., Davidson, D. and Mak, T.W. 1992. Profound block in thymocyte development in mice lacking p56lck. *Nature*, **357**, 161-164.

Mombaerts, P., Anderson, S.J., Perlmutter, R.M., Mak, T.W. and Tonegawa, S. 1994. An activated lck transgene promotes thymocyte development in RAG-1 mutant mice. *Immunity*, **1**.

Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itohara, S., Lafaille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M.L. and Tonegawa, S. 1992a. Mutations in T-cell

antigen receptor genes α and β block thymocyte development at different stages. *Nature*, **360**, 4641-4653.

Mombaerts, P., Iacomini, J., Johnson, R.-S., Herup, K., `Tonegava, S. and Papaioannou, V.-E. 1992b. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*, **68**, 869-877.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N. and Kupfer, A. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*, **395**, 82-6.

Monks, C.R., Kupfer, H., Tamir, I., Barlow, A. and Kupfer, A. 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature*, **385**, 83-6.

Moore, T.A., von Freeden-Jeffry, U., Murray, R. and Zlotnik, A. 1996. Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7 -/- mice. *Journal of Immunology*, **157**, 2366-73.

Moore, T.A. and Zlotnik, A. 1995. T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood*, **86**, 1850-60.

Moore, T.A. and Zlotnik, A. 1997. Differential effects of Flk-2/Flt-3 ligand and stem cell factor on murine thymic progenitor cells. *Journal of Immunology*, **158**, 4187-92.

Moorman, J.P., Bobak, D.A. and Hahn, C.S. 1996. Inactivation of the small GTP binding protein Rho induces multinucleate cell formation and apoptosis in murine T lymphoma EL4. *Journal of Immunology*, **156**, 4146-4153.

Moreau, V., Galan, J.M., Devilliers, G., Haguenauer-Tsapis, R. and Winsor, B. 1997. The yeast actin-related protein Arp2p is required for the internalization step of endocytosis. *Molecular Biology of the Cell*, **8**, 1361-75.

Moreau, V., Madania, A., Martin, R.P. and Winson, B. 1996. The Saccharomyces cerevisiae actin-related protein Arp2 is involved in the actin cytoskeleton. *Journal of Cell Biology*, **134**, 117-32.

Morrison, D.K. and Cutler, R.E. 1997. The complexity of Raf-1 regulation. *Current Opinion in Cell Biology*, **9**, 174-9.

Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr. and Potter, S.S. 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell*, **65**, 677-89.

Mullins, R.D., Heuser, J.A. and Pollard, T.D. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA*, **95**, 6181-6.

Na, S., Chuang, T.H., Cunningham, A., Turi, T.G., Hanke, J.H., Bokoch, G.M. and Danley, D.E. 1996. D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. *Journal of Biological Chemistry*, **271**, 11209-13.

Nacht, M. and Jacks, T. 1998. V(D)J Recombination is Not Required for the Development of Lymphoma in p53-Deficient Mice. *Cell Growth and Development*, **9**, 131-138.

Narumiya, S., Ishizaki, T. and Watanabe, N. 1997. Rho effectors and reorganization of actin cytoskeleton. *FEBS Letters*, **410**, 68-72.

Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C. and Loh, D.Y. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*, **376**, 435-438.

Newton, K., Harris, A.W., Bath, M.L., Smith, K.G.C. and Strasser, A. 1998. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO Journal*, **17**, 706-18.

Nielsen, O., Davey, J. and Egel, R. 1992. The ras1 function of Schizosaccharomyces pombe mediates pheromone-induced transcription. *EMBO Journal*, **11**, 1391-5.

Nishiyama, T., Sasaki, T., Takaishi, K., Kato, M., Yaku, H., Araki, K., Matsuura, Y. and Takai, Y. 1994. rac p21 is involved in insulin-induced membrane ruffling and rho p21 is involved in hepatocyte growth factor- and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Molecular & Cellular Biology*, **14**, 2447-56.

Nobes, C. and Hall, A. 1995. Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. *Cell.*, **81**, 53-62.

Norman, J.C., Price, L.S., Ridley, A., Hall, A. and Koffer, A. 1994. Actin filament organisation on activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. *Journal of Cell Biology*, **126**, 1005-1015.

Nunes, J., Truneh, A., Olive, D. and Cantrell, D.A. 1996. Signal Transduction by CD28 ligands B7-1 and B7-2: CD28 regulation of tyrosine kinase adapter molecules. *Journal of Biological Chemistry*, **251**, 1591-1598.

O'Shea, C., Thornell, A., Rosewell, I., Hayes, B. and Owen, M. 1997. Exit of the pre-TCR from the ER/cis-Golgi is necessary for Signalling Differentiation, Proliferation, and Allelic Exclusion in Immature thymocytes. *Immunity*, **7**, 591-599.

O'Shea, C.C., Crompton, T., Rosewell, I.R., Hayday, A.C. and Owen, M.J. 1996. Raf regulates positive selection. *European Journal of Immunology*, **26**, 2350-2355.

O'Shea, J.J., Ashwell, J.D., Bailey, T.L., Cross, S.L., Samelson, L.E. and Klausner, R.D. 1991. Expression of v-src in a murine T-cell hybridoma results in constitutive T-cell receptor phosphorylation and interleukin-2 production. *Proc. Natl. Acad. Sci. USA*, **88**, 1741-1746.

Ohbo, K., Suda, T., Hashiyama, M., Mantani, A., Ikebe, M., Miyakawa, K., Moriyama, M., Nakamura, M., Katsuki, M., Takahashi, K., Yamamura, K. and Sugamura, K. 1996. Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor gamma chain. *Blood*, **87**, 956-67.

Olson, M.F., Ashworth, A. and Hall, A. 1995. An essential role for rho, rac, and cdc42 GTPases in cell cycle progression through G_1. *Science*, **269**, 1270-1272.

Olson, M.F., Paterson, H.F. and Marshall, C.J. 1998. Signals from Ras and Rho interact to regulate expression of p21waf1/Cip1. *Nature*, **394**, 295-299.

Page, D.M., Kane, L.P., Allison, J.P. and Hedrick, S.M. 1993. Two signals are required for negative selection of $CD4^+CD8^+$ thymocytes. *Journal of Immunology*, **151**, 1868-80.

Pappin, D.J.C., Hojrup, P. and Bleasby, A.J. 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Current Biology*, **3**, 327-332.

Pappin, D.J.C., Rahman, D., Hansen, H.F., Bartlet-Jones, M., Jeffery, W. and Bleasby, A.J. (1996) In A. L. Burlingame and S. A. Carr (eds.), *Chemistry, mass spectrometry and peptide-mass databases: evolution of methods for the rapid identification and mapping of cellular proteins.* Humana, Totowa, NJ, pp. 135-150.

Park, S.Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T. and Saito, T. 1995. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity*, **3**, 771-82.

Penninger, J., Kishihara, K., Molina, T., Wallace, V.A., Timms, E., Hedrick, S.M. and Mak, T.W. 1993. Requirement for tyrosine kinase p56lck for thymic development of transgenic gamma delta T cells. *Science*, **260**, 358-61.

Penninger, J.M. and Crabtree, G.R. 1999. The actin cytoskeleton and lymphocyte activation. *Cell*, **96**, 9-12.

Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R. and Lacal, J.C. 1997. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes & Development*, **11**, 463-75.

Peschon, J.J., Morrissey, P.J., Grabstein, K.H., Ramsdell, F.J., Maraskovsky, E., Gliniak, B.C., Park, L.S., Ziegler, S.F., Williams, D.E., Ware, C.B. and et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *Journal of Experimental Medicine*, **180**, 1955-1960.

Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F.W. and Geha, R.S. 1998. Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. *Cell*, **94**, 229-38.

Prendergast, G.C., Khosravi Far, R., Solski, P.A., Kurzawa, H., Lebowitz, P.F. and Der, C.J. 1995a. Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene*, **10**, 2289-2296.

Prendergast, G.C., Khosravi-Far, R., Solski, P.A., Kurzawa, H., Lebowitz, P.F. and Der, C.J. 1995b. Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene*, **10**, 2289-96.

Purdie, C.A., Harrison, D.J., Peter, A., Dobbie, L., White, S., Howie, S.E., Salter, D.M., Bird, C.C., Wyllie, A.H., Hooper, M.L. and et al. 1994. Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene*, **9**, 603-9.

Qiu, R.G., Abo, A., McCormick, F. and Symons, M. 1997. Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Molecular & Cellular Biology*, **17**, 3449-58.

Qiu, R.G., Chen, J., Kirn, D., McCormick, F. and Symons, M. 1995a. An essential role for Rac in Ras transformation. *Nature*, **374**, 457-459.

Qiu, R.G., Chen, J., McCormick, F. and Symons, M. 1995b. A role for Rho in Ras transformation. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 11781-11785.

Quilliam, L.A., Kato, K., Rabun, K.M., Hisaka, M.M., Huff, S.Y., Campbell-Burk, S. and Der, C.J. 1994. Identification of residues critical for Ras(17N) growth-inhibitory phenotype and for Ras interaction with guanine nucleotide exchange factors. *Molecular and Cellular Biology*, **14**, 1113-1121.

Quilliam, L.A., Lambert, Q.T., Mickelson Young, L.A., Westwick, J.K., Sparks, A.B., Kay, B.K., Jenkins, N.A., Gilbert, D.J., Copeland, N.G. and Der, C.J. 1996. Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *Journal of Biological Chemistry*, **271**, 28772-6.

Rankin, S., Morii, N., Narumiya, S. and Rozengurt, E. 1994. Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin. *FEBS Letters*, **354**, 315-9.

Rao, A. 1995. NFATp, a cyclosporin-sensitive transcription factor implicated in cytokine gene induction. *Journal of Leukocyte Biology*, **57**, 536-542.

Rao, A., Luo, C. and Hogan, P.G. 1997. Transcription factors of the NFAT family: regulation and function. *Annual Review of Immunology*, **15**, 707-747.

Rapp, U.R., Goldsborough, M.D., Mark, G.E., Bonner, T.I., Groffen, J., Reynolds, F.H., Jr. and Stephenson, J.R. 1983. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc. Natl. Acad. Sci. USA*, **80**, 4218-22.

Reibel, L., Dorseuil, O., Stancou, R., Bertoglio, J. and Gacon, G. 1991. A hemopoietic specific gene encoding a small GTP binding protein is overexpressed during T cell activation. *Biochemical & Biophysical Research Communications*, **175**, 451-8.

Reid, T., Furuyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P. and Narumiya, S. 1996. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *Journal of Biological Chemistry*, **271**, 13556-60.

Reif, K., Gout, I., Waterfield, M.D. and Cantrell, D.A. 1993. Divergent regulation of phosphatidylinositol 3-kinase p85α and p85β isoforms upon T cell activation. *Journal of Biological Chemistry*, **268**, 10780-10788.

Reif, K., Nobes, C.D., Thomas, G., Hall, A. and Cantrell, D.A. 1996. Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Current Biology*, **6**, 1445-1455.

Ren, X.D., Bokoch, G.M., Traynor Kaplan, A., Jenkins, G.H., Anderson, R.A. and Schwartz, M.A. 1996. Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Molecular Biology of the Cell*, **7**, 435-42.

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**, 389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekman, D. and Hall, A. 1992. The small GTP binding protein rac regulates growth factor induced membrane ruffling. *Cell*, **70**, 401-410.

Ridley, A.J., Self, A.J., Kasmi, F., Paterson, H.F., Hall, A., Marshall, C.J. and Ellis, C. 1993. rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities *in vitro* and *in vivo*. *EMBO Journal*, **12**, 5151-60.

Rincon, M., Whitmarsh, A., Yang, D.D., Weiss, L., Derijard, B., Jayaraj, P., Davis, R.J. and Flavell, R.A. 1998. The JNK pathway regulates the *in vivo* deletion of immature $CD4^+CD8^+$ thymocytes. *Journal of Experimental Medicine*, **188**, 1817-30.

Robinson, L.C., Gibbs, J.B., Marshall, M.S., Sigal, I.S. and Tatchell, K. 1987. CDC25: a component of the RAS-adenylate cyclase pathway in Saccharomyces cerevisiae. *Science*, **235**, 1218-21.

Rodewald, H.-R., Awad, K., Moingeon, P., D'Adamio, L., Rabinowitz, D., Shinkai, Y., Alt, F.W. and Reinherz, E.L. 1993. FcγRII/II and CD2 expression mark distinct subpopulations

of immature CD4-CD8- murine thymocytes: *in vivo* developmental kinetics and T cell receptor β chain rearrangement status. *Journal of Experimental Medicine*, **177**, 1072-1092.

Rodewald, H.R., Kretzschmar, K., Swat, W. and Takeda, S. 1995. Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes *in vivo*. *Immunity*, **3**, 313-9.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M.W. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell*, **97**, 221-31.

Ron, D., Zannini, M., Lewis, M., Wickner, R.B., Hunt, L.T., Graziani, G., Tronick, S.R., Aaronson, S.A. and Eva, A. 1991. A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr. *New Biologist*, **3**, 372-9.

Rosnet, O., Marchetto, S., deLapeyriere, O. and Birnbaum, D. 1991. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. *Oncogene*, **6**, 1641-50.

Roux, P., Gauthier-Rouviere, C., Doucet-Brutin, S. and Fort, P. 1997. The small GTPases Cdc42Hs, Rac1 and RhoG delineate Raf-independent pathways that cooperate to transform NIH3T3 cells. *Current Biology*, **7**, 629-37.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G. and et al. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*, **360**, 689-92.

Rubin, E.J., Gill, M.D., Bouquet, P. and Popoff, M.R. 1988. Functional modification of a 21 kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Molecular & Cellular Biology*, **8**, 418-426.

Rubin, G.M. 1991. Signal transduction and the fate of the R7 photoreceptor in Drosophila. *Trends in Genetics*, **7**, 372-7.

Rudd, C.E. 1996. Upstream-downstream: CD28 cosignaling pathways and T cell function. *Immunity*, **4**, 527-534.

Sahai, E., Alberts, A.S. and Treisman, R. 1998. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. *EMBO Journal*, **17**, 1350-61.

Sahai, E., Ishizaki, T., Narumiya, S. and Treisman, R. 1999. Transformation mediated by RhoA requires activity of ROCK kinases. *Current Biology*, **9**, 136-145.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H.J. and von Boehmer, H. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science*, **266**, 1208-12.

Samelson, L.E. and Klausner, R.D. 1992. Tyrosine kinases and tyrosine-based activation motifs. *Journal of Biological Chemistry*, **267**, 24913-24916.

Sasaki, T. and Takai, Y. 1998. The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control. *Biochemical & Biophysical Research Communications*, **245**, 641-5.

Schlessinger, J. 1993. How receptor tyrosine kinases activate Ras. *Trends in Biochemical Sciences*, **18**, 273-5.

Schmid, I., Uittenbogaart, C.H. and Giorgi, J.V. 1994. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry*, **15**, 12-20.

Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M. and Aktories, K. 1997. Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1. *Nature*, **387**, 725-9.

Seger, R. and Krebs, E.G. 1995. The MAPK signaling cascade. *FASEB Journal*, **9**, 726-35.

Sekine, A., Fujiwara, M. and Narumiya, S. 1989. Asparagine residue in the rho gene product is the modification site for the botulinum ADP ribosyltransferase. *Journal of Biological Chemistry*, **264**, 8602-8605.

Settleman, J., Narasimhan, V., Foster, L.C. and Weinberg, R.A. 1992. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. *Cell*, **69**, 539-549.

Sherman, N.E., Yates, N.A., Shabanowitz, J., Hunt, D.F., Jeffery, W.A., Bartlet-Jones, M. and Pappin, D.J.C. (1995) *A Novel N-terminal derivative designed to simplify peptide fragmentation.*, Atlanta, Georgia, pp. 626-627.

Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K.M., Loh, D.Y., Reinherz, E.L. and Alt, F.W. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science*, **259**, 822-5.

Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E.-M., Stewart, V., M., M., Charron, J., Datta, M., Young, F., Stall, A.-M. and Alt, F.-W. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangment. *Cell*, **68**, 855-867.

Shortman, K. and Wu, L. 1996. Early T lymphocyte progenitors. *Annual Review of Immunology*, **14**, 29-47.

Simon, M.A., Bowtell, D.D., Dodson, G.S., Laverty, T.R. and Rubin, G.M. 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell*, **67**, 701-16.

Small, J.V., Rottner, K. and Kaverina, I. 1999. Functional design in the actin cytoskeleton. *Current Opinion in Cell Biology*, **11**, 54-60.

Stein, P.L., Lee, H.-M., Rich, S. and Soriano, P. 1992. p59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell*, **70**, 741-750.

Stern, J.B. and Smith, K.A. 1986. Interleukin-2 induction of T-cell G1 progression and c-myb expression. *Science*, **233**, 203-206.

Stevenson, B.J., Ferguson, B., De Virgilio, C., Bi, E., Pringle, J.R., Ammerer, G. and Sprague, G.F., Jr. 1995. Mutation of RGA1, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast Saccharomyces cerevisiae. *Genes & Development*, **9**, 2949-63.

Stokoe, D., MacDonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. 1994. Activation of raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463-1467.

Stowers, L., Yelon, D., Berg, L.J. and Chant, J. 1995. Regulation of the polarizati9on of T cells towards antigen-presenting cells by Ras-related GTPase Cdc42. *Proc.Nat.Acad.Sci.*, **92**, 5027-5031.

Suda, T. and Zlotnik, A. 1991. IL-7 maintains the T cell precursor potential of $CD3^-CD4^-CD8^-$ thymocytes. *Journal of Immunology*, **146**, 3068-73.

Suetsugu, S., Miki, H. and Takenawa, T. 1999. Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochemical & Biophysical Research Communications*, **260**, 296-302.

Sugamura, K., Asao, H., Kondo, M., Tanaka, N., Ishii, N., Ohbo, K., Nakamura, M. and Takeshita, T. 1996. The interleukin-2 receptor-gamma chain - its role in the multiple cytokine receptor complexes and T-cell development in XSCID. *Annual Review of Immunology*, **14**, 179-205.

Sugawara, T., Moriguchi, T., Nishida, E. and Takahama, Y. 1998. Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity*, **9**, 565-74.

Sun, Y., Yamauchi, J., Kaziro, Y. and Itoh, H. 1999. Activation of c-fos promoter by Gbetagamma-mediated signaling: involvement of Rho and c-Jun N-terminal kinase. *Journal of Biochemistry*, **125**, 515-21.

Sutton, C.W., Pemberton, K.S., Cottrell, J.S., Corbett, J.M., Wheeler, C.H., Dunn, M.J. and Pappin, D.J. 1995. Identification of myocardial proteins from two-dimensional gels by peptide mass fingerprinting. *Electrophoresis*, **16**, 308-316.

Suwa, H., Ohshio, G., Imamura, T., Watanabe, G., Arii, S., Imamura, M., Narumiya, S., Hiai, H. and Fukumoto, M. 1998. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. *British Journal of Cancer*, **77**, 147-52.

Suzuki, H., Duncan, G.S., Takimoto, H. and Mak, T.W. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *Journal of Experimental Medicine*, **185**, 499-505.

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 p85α subunit of phosphoinositide 3-kinase. *Science*, **283**, 390-2.

Swat, W., Ignatowica, L., von Boehmer, H. and Kisielow, P. 1991. Clonal deletion of immature $CD4^+CD8^+$ thymocytes in suspension culture by extrathymic antigen presenting cells. *Nature*, **351**, 150-153.

Swat, W., Shinakai, Y., Cheng, H.-L., Davidson, L. and Alt, F.W. 1996. Activated Ras signals differentiation and expansion of CD4+8+ thymocytes. *Proc.Nat.Acad.Sci.USA*, **93**, 4683-4687.

Symons, M., Derry, J.M., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U. and Abo, A. 1996. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell*, **84**, 723-734.

Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S. and Takai, Y. 1997. Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *Journal of Biological Chemistry*, **272**, 23371-5.

Takahashi, M., Mukai, H., Toshimori, M., Miyamoto, M. and Ono, Y. 1998. Proteolytic activation of PKN by caspase-3 or related protease during apoptosis. *Proc. Natl. Acad. Sci. USA*, **95**, 11566-71.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M.S., Gibbs, J.B., Matsumoto, K., Kaziro, Y. and Toh-e, A. 1990. S. cerevisiae genes IRA1 and IRA2 encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. *Cell*, **60**, 803-807.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K. and Tybulewicz, V.L.J. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature*, **374**, 467-470.

Taylor, D., Badiani, P. and Weston, K. 1996. A dominant interfering Myb mutant causes apoptosis in T cells. *Genes and Development*, **10**, 2732-44.

Teramoto, H., Coso, O.A., Miyata, H., Igishi, T., Miki, T. and Gutkind, J.S. 1996. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *Journal of Biological Chemistry*, **271**, 27225-8.

Thomis, D.C., Gurniak, C.B., Tivol, E., Sharpe, A.H. and Berg, L.J. 1995. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. *Science*, **270**, 794-7.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-80.

Thomson, F.J., Jess, T.J., Moyes, C., Plevin, R. and Gould, G.W. 1997. Characterization of the intracellular signalling pathways that underlie growth-factor-stimulated glucose transport in Xenopus oocytes: evidence for ras- and rho-dependent pathways of phosphatidylinositol 3-kinase activation. *Biochemical Journal*, **325**, 637-43.

Tibbles, L.A., Ing, Y.L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J.R. and Lassam, N.J. 1996. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO Journal*, **15**, 7026-35.

Toksoz, D. and Williams, D.A. 1994. Novel human oncogene lbc detected by transfection with distinct homology regions to signal transduction products. *Oncogene*, **9**, 621-8.

Tolias, K., Cantley, L. and Carpenter, C. 1995. Rho-family GTPases bind to phosphoinositide kinases. *Journal of Biological Chemistry*, **270**, 17656-17659.

Tolias, K.F., Couvillon, A.D., Cantley, L.C. and Carpenter, C.L. 1998. Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. *Molecular & Cellular Biology*, **18**, 762-70.

Trahey, M. and McCormick, F. 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science*, **238**, 542-5.

Treisman, R. 1990. The SRE: a growth factor responsive transcriptional regulator. *Seminars in Cancer Biology*, **1**, 47-58.

Treisman, R. 1994. Ternary complex factors: growth factor regulated transcriptional activators. *Current Opinion in Genetics and Development*, **4**, 96-101.

Turner, H. and Cantrell, D.A. 1997. Distinct Ras effector pathways are involved in FcεR1 regulation of the transcriptional activity of Elk-1 and NFAT in mast cells. *Journal of Experimental Medicine*, **185**, 43-57.

Turner, M., Mee, P.J., Walters, A.E., Quinn, M.E., Mellor, A.L., Zamoyska, R. and Tybulewicz, V. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity*, **7**, 451-460.

Ullman, K.S., Northrop, J.P., Verweij, C.L. and Crabtree, G.R. 1990. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annual Review of Immunology*, **8**, 421-452.

Van Aelst, L., Joneson, T. and Bar-Sagi, D. 1996. Identification of a novel Rac1-interacting protein involved in membrane ruffling. *EMBO Journal,* **15**, 3778-86.

Van Leeuwen, F.N., Van der Kammen, R.A., Habets, G.G. and Collard, J.G. 1995. Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. *Oncogene*, **11**, 2215-2221.

van Lohuizen, M., Verbeek, S., Krimpenfort, P., Domen, J., Saris, C., Radaszkiewicz, T. and Berns, A. 1989. Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. *Cell*, **56**, 673-82.

van Oers, N.S., Lowin Kropf, B., Finlay, D., Connolly, K. and Weiss, A. 1996. alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. *Immunity*, **5**, 429-36.

Veis, D.J., Sorenson, C.M., Shutter, J.R. and Korsmeyer, S.J. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*, **75**, 229-40.

Vincent, S., Jeanteur, P. and Fort, P. 1992. Growth-regulated expression of rhoG, a new member of the ras homolog gene family. *Molecular & Cellular Biology*, **12**, 3138-48.

Vincent, S. and Settleman, J. 1997. The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. *Molecular & Cellular Biology*, **17**, 2247-56.

von Boehmer, H. 1992. Thymic selection: a matter of life and death. *Immunology Today*, **13**, 454-458.

von Boehmer, H., Aifantis, I., Feinberg, J., Lechner, O., Saint-Ruf, C., Walter, U., Buer, J. and Azogui, O. 1999. Pleiotropic changes controlled by the pre-T-cell receptor. *Current Opinion in Immunology*, **11**, 135-42.

von Freeden-Jeffry, U., Solvason, N., Howard, M. and Murray, R. 1997. The earliest T lineage-commited cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity*, **7**, 147-154.

Von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E. and Murray, R. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *Journal of Experimental Medicine*, **181**, 1519-1526.

Walsh, C.M., Wen, B.G., Chinnaiyan, A.M., O'Rourke, K., Dixit, V.M. and Hedrick, S.M. 1998. A role for FADD in T cell activation and development. *Immunity*, **8**, 439-49.

Ward, S.G., June, C.H. and Olive, D. 1996. PI 3-kinase: a pivotal pathway in T cell activation. *Immunology Today*, **17**, 187-198.

Warne, P., Rodriguez-Viciana, P. and Downward, J. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature*, **364**, 352-355.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Marii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. 1996. Protein kinase N (PKN) and PKN-related protein Rhophilin as targets of small GTPase Rho. *Science*, **271**, 645-648.

Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S. 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganisation. *Nature Cell Biology*, **1**, 136-143.

Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B.M. and Narumiya, S. 1997. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO Journal*, **16**, 3044-3056.

Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell*, **73**, 209-212.

Weiss, A. and Littman, D.R. 1994. Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263-274.

Weston, K. 1998. Myb protein in life, death and differentiation. *Curr Op Gen Dev*, **8**, 76-81.

Westwick, J.K., Lambert, Q.T., Clark, G.J., Symons, M., Van Aelst, L., Pestell, R.G. and Der, C.J. 1997. Rac regulation of transformation, gene expression, and actin organization by multiple PAK-independent pathways. *Molecular and Cellular Biology*, **17**, 1324-1335.

Wilm, M. and Mann, M. 1996. Analytical properties of the Nanoelectrospray Ion Source. *Analytical Chemistry*, **68**, 1-8.

Woodrow, M., Rayter, S., Downward, J. and Cantrell, D.A. 1993. p21ras function is important for T cell antigen receptor and protein kinase C regulation of nuclear factor of activated cells. *Journal of Immunology*, **150**, 1-9.

Woodside, D.G., Wooten, D.K. and McIntyre, B.W. 1998. Adenosine diphosphate (ADP)-ribosylation of the guanosine triphosphatase (GTPase) rho in resting peripheral blood human T lymphocytes results in pseudopodial extension and the inhibition of T cell activation. *Journal of Experimental Medicine*, **188**, 1211-21.

Yablonski, D., Kane, L.P., Qian, D. and Weiss, A. 1998. A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK. *EMBO Journal*, **17**, 5647-57.

Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. 1993. ADP-ribosylation of the rhoA gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G1 phase of the cell cycle. *Oncogene*, **8**, 1449-1455.

Yamanashi, Y. and Baltimore, D. 1997. Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell*, **88**, 205-211.

Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M. and Flavell, R.A. 1998. Differentiation of $CD4^+$ T cells to Th1 cells requires MAP kinase JNK2. *Immunity*, **9**, 575-85.

Yoshida, H., Nishina, H., Takimoto, H., Marengere, L.E., Wakeham, A.C., Bouchard, D., Kong, Y.Y., Ohteki, T., Shahinian, A., Bachmann, M., Ohashi, P.S., Penninger, J.M., Crabtree, G.R. and Mak, T.W. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity*, **8**, 115-24.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R.P. and Samelson, L.E. 1998. LAT: The ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*, **92**, 83-92.

Zhang, W., Sommers, C.L., Burshtyn, D.N., Stebbins, C.C., DeJarnette, J.B., Trible, R.P., Grinberg, A., Tsay, H.C., Jacobs, H.M., Kessler, C.M., Long, E.O., Love, P.E. and Samelson, L.E. 1999. Essential role of LAT in T cell development. *Immunity*, **10**, 323-32.

Zheng, Y., Cerione, R. and Bender, A. 1994. Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. *Journal of Biological Chemistry*, **269**, 2369-72.

Zheng, Y., Hart, M.J. and Cerione, R.A. 1995a. Guanine nucleotide exchange catalyzed by dbl oncogene product. *Methods in Enzymology*, **256**, 77-84.

Zheng, Y., Olson, M.F., Hall, A., Cerione, R.A. and Toksoz, D. 1995b. Direct involvement of the small GTP-binding protein Rho in lbc oncogene function. *Journal of Biological Chemistry*, **270**, 9031-4.

Zhumabekov, T., Corbella, P., Tolaini, M. and Kioussis, D. 1995. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *Journal of Immunological Methods*, **185**, 133-40.

Zornig, M., Hueber, A.O. and Evan, G. 1998. p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. *Current Biology*, **8**, 467-70.