Signalling Mechanisms Regulating Proliferation and Apoptosis in Immature and Mature B Cells

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d'i nostri sensi ch'è del rimanente,
non vogliate negar l'esperienza,
di retro al sol, del mondo sanza gente

Consider ye the seed from which ye sprang;
Ye were not made to live like unto brutes,
But for pursuit of virtue and of knowledge.

The Divine Comedy, Dante Alighieri

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Abbreviations

Ab	Antibody
ADCC	Antibody dependent cell mediated cytotoxicity
Ag	Antigen
APC	Antigen-presenting cell
ATP	Adenosine 5'-triphosphate
BAP	BCR-associated protein
BCR	B cell receptor
BLNK	B cell linker protein
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
cAMP	adenosine-3',5'-cyclic monophosphate
CDK	Cyclin dependent kinase
CFSE	Carboxy-fluorescein diacetate succinimidyl ester
Con A	Concanavalin A
COX	Cyclooxygenase
CTLA	Cytotoxic T-lymphocyte antigen
DAG	Diacylglycerol
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
DOK	Downstream of kinase
DTT	1,4-dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β–aminoethylether)tetraaceticacid
ER	Endoplasmic reticulum
ErkMAPKinase	Extracellular signal-regulated protein kinase
ES	Excretory-secretory
$F(ab')_2$	Fragment antigen binding
Fc	Fragment crystallisable
FcαR	Receptor for the constant region of IgA
FcδR	Receptor for the constant region of IgD
FcεR	Receptor for the constant region of IgE
FcγR	Receptor for the constant region of IgG
FcμR	Receptor for the constant region of IgM
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluoroscein isothiocyanate
G-protein	GTP-binding protein
GAP	GTPase activating protein
GC	Germinal centre
GDP	Guanine 5'-diphosphate

GEF	Guanine exchange factor
GPI	Glycosyl phophatidyl inositol
Grb2	Growth factor Receptor Binding protein 2
GSK-3	Glycogen synthase kinase 3
GTP	Guanine 5'-triphosphate
HEL	Hen-egg lysosyme
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IgA/D/E/G/M	Immunoglobulin A/D/E/G/M
I-κβ	Inhibitor-κB
IKK	I-κB kinase
IL	Interleukin
$InsP_3$ or IP_3	Inositol trisphosphate
$InsP_4$ or IP_4	Inositol tetrakisphosphate
IP	Immune precipitate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun N terminal kinase
KIR	Killer inhibitory receptor
LAB	Linker for activation of B cells
LAT	Linker for activation of T cells
LIR	Leukocyte inhibitory receptor
LY	LY294002
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MEK or MKK	MAPKinase kinase
MEKK or MKKK	MAPKinase kinase kinase
MHC	Major Histocompatibility Complex
$\Delta\psi m$	Mitochondrial transmembrane potential
mIgM	Membrane immunoglobulin M
MLB	Magnesium lysis buffer
mRNA	Messenger ribonucleic acid
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear Factor -κB
NIK	NF-κB-inducing kinase
NK	Natural killer
OD	Optical density
PA	Phosphatidic acid
PAC	Phosphatase of activated cells
PAK	p21 activated kinase
PALS	Periarteriolar lymphoid sheath
PBS	Phosphate buffered saline

PC	Phosphatidylcholine
PDK	PIP_3 dependent kinase
PHA	Phytohemagglutinin
PH domain	Pleckstrin homology domain
PI-3-K	Phosphatidyl inositol-3 kinase
PKA/PKB/PKC	Protein kinase A/B/C
PLA/PLC/PLD	Phospholipase A/C/D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PTB domain	Phosphotyrosine binding domain
PtdIns(4,5)P_2 or PIP_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3 or PIP_3	Phosphatidylinositol 3,4,5-bisphosphate
PTK	Protein tyrosine kinase
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
SHIP	SH2 domain containing inositol phosphatase
SHP	SH2 domain containing protein tyrosine phosphatase
sIg	Surface immunoglobulin
SLP-65	SH2 domain containing linker protein – 65 kDa
Sos	Son of sevenless
T1	Immature-transitional B cell
TBE	Tris-Borate/EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris buffered saline containing Tween-20
T_C	Cytotoxic T lymphocyte
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
T_H	Helper T lymphocyte
TNF	Tumour necrosis factor
TRAF	TNF-Receptor-associated protein
Tris	Tris (hydroxymethyl) methylamine
tRNA	Transfer ribonucleic acid
U	Units

Summary

Ligation of the antigen receptor (BCR) on B lymphocytes results in differential biological outcomes depending upon the maturation stage of the cell. Thus, mature B lymphocytes become activated and proliferate (clonal expansion) in response to antigen receptor cross-linking, whilst immature B cells either become unresponsive, alter the specificity of their BCR, or undergo apoptosis (clonal deletion). These biological responses to antigen are well defined, and many of the membrane proximal signalling events activated upon BCR ligation have been delineated and appear to be similar, despite their differential biological outcomes. The precise molecular events downstream of BCR signalling that are responsible for these distinct responses therefore remain to be established. This project has therefore centred upon identifying the important signalling mechanisms linking BCR ligation with regulation of the cell cycle and the induction of apoptosis in immature and mature B lymphocytes.

The murine B cell lymphoma cell line WEHI-231 is widely used as a model for clonal deletion of immature B lymphocytes. This is because it has the cell surface phenotype of an immature B lymphocyte, and as such responds to BCR ligation by undergoing growth arrest and apoptosis, mimicking the processes of clonal deletion. Moreover, WEHI-231 cells can be rescued from BCR-mediated apoptosis by co-stimulation via CD40, conditions mimicking T cell driven rescue of useful clonatypes. This cell line was therefore utilised to further examine the signalling events important for anti-Ig induced growth arrest and apoptosis, and CD40-mediated rescue, in immature B lymphocytes. In particular, this investigation has explored the differential roles of the Ras/Erk-MAPKinase pathway and a number of specific PKC isoforms in the regulation of survival of immature B cells.

Initial studies examined the activation of the Erk-MAPKinase in both response to apoptotic signalling via the BCR and under conditions of CD40-mediated rescue. These studies indicated that Erk-MAPKinase plays a dual role in WEHI-231 cells, being required both for BCR induced growth arrest and apoptosis and CD40-

mediated rescue. The outcome of Erk-MAPKinase activity is determined by the kinetics of activation, with an early, strong, yet transient signal required for BCR induced apoptosis, whilst CD40 signalling induces a more sustained, cycling pattern of Erk-MAPKinase activation. The role of Erk-MAPKinase was further addressed in these cells by expression of constitutively active mutants of the small GTPase, Ras, a key upstream regulator of Erk-MAPKinase as well as PI-3-kinase. Interestingly, cells expressing a mutant form of Ras capable of directly coupling to the activation of PI-3-kinase, but not Erk-MAPKinase (RasV12-C40), was found to be better at rescuing the cells from growth arrest than the inverse mutant, which can couple to the Erk-MAPKinase, but not PI-3-kinase pathway (RasV12-S35). This appears to be due, at least in part, to the fact that in addition to activating PI-3-kinase dependent cell survival pathways, RasV12-C40 expressing cells can additionally activate Erk-MAPKinase, presumably downstream of PI-3-kinase activation. Cells expressing the RasV12-C40 mutation additionally highlighted a role for PI-3-kinase in the CD40-mediated survival of WEHI-231 cells. This study also highlighted a key downstream role for p27 in the induction of growth arrest in immature B cells in response to BCR ligation, as the expression of p27 correlates with the induction of growth arrest.

One hypothesis for the different biological responses to antigen between immature and mature B cells is that immature B cells exhibit reduced PKC activity in response to antigen, compared to mature B cells. Indeed, stimulation of immature B cells with phorbol esters, which activate the conventional and novel PKC isozymes, can overcome anti-Ig induced apoptosis. As PKC isoforms from all three subclasses have been implicated in the regulation of Erk-MAPKinase activity, the role of a variety of PKC family members in BCR signalling in immature B cells was investigated by transfection of either catalytically active or kinase dead mutants. Expression of the PKC isoforms PKCα, PKCδ, PKCε, and PKCζ all appear capable of impairing BCR driven growth arrest to a certain extent. Indeed, the novel PKC, PKCε, appears to alter the response of WEHI-231 cells to BCR ligation, as proliferation in these cells was slightly enhanced. PKCα also seems to enhance CD40-mediated rescue of anti-Ig induced growth arrest

and apoptosis, whilst the novel PKC, PKCδ, seems to play a negative role in proliferation in response to stimulation via CD40, as WEHI-231 cells expressing a kinase dead form of PKCδ appear to display enhanced proliferation in response to stimulation with anti-Ig and anti-CD40. Examination of candidate downstream effector molecules failed to identify the mechanisms employed by these PKC isoforms to mediate their effects.

In contrast to immature B cells, mature B lymphocytes become activated and proliferate in response to antigen receptor cross-linking. Efficient B cell responses generally require interactions with helper T cells (T_H) that are specific for the same antigen, responses being modulated by both positive and negative co-receptors, which can lower the threshold of signalling required for a positive response, or down-modulate BCR signalling, respectively. As Erk-MAPKinase proved to play such key roles in both the BCR induced growth arrest and apoptosis, and CD40 mediated proliferation in immature B cells, the functions of Erk-MAPKinase in the response of mature B cells to BCR ligation was addressed. These studies highlighted a key role for the Erk-MAPKinase in the proliferative response of mature B cells to antigen, particularly sustained Erk-MAPKinase activity akin to that found in immature B cells undergoing CD40-mediated proliferation. A major negative regulator of BCR signalling in mature B cells is the FcγRIIb receptor, which was observed to mediate its effects partly by inhibiting the sustained Erk-MAPKinase activity that is required for B cell proliferation. Other key targets of FcγRIIb mediated inhibition of BCR driven proliferation in mature B cells appear to include the tumour suppressor proteins Rb and p53, as FcγRIIb ligation leads to inhibition of phosphorylation of Rb, a process required for cells to enter the cell cycle, whilst the tumour suppressor protein p53 appears to be activated in response to FcγRIIb ligation. The activation of p53 provides a mechanism by which FcγRIIb ligation can lead to the apoptosis of mature B cells, as Bax, a pro-apoptotic member of the Bcl-2 family and a target of p53, is upregulated in response to co-ligation of the BCR with FcγRIIb.

Chapter 1 – General Introduction

1.1 The immune system

The immune system is the defence mechanism that has evolved to protect the host from pathogens such as parasites, bacteria or viruses. The cells and molecules that comprise the immune system and mediate an immune response collaborate in a complex manner, through a finely balanced network of interactions. This network enables the host to detect the presence of the foreign agent, co-ordinate a specific attack, and finally, once the agent has been successfully removed, quench that specific response. The immune system also has a memory for agents that have been successfully cleared in the past, allowing a more rapid and effective response upon subsequent encounters. The immune system comprises two functionally distinct but interacting arms, the innate and the adaptive immune responses.

The innate immune response is the first line of defence a host has against infection, and as such is non-specific in nature as it functions regardless of the foreign substance or pathogen. The innate response is mainly driven by phagocytes, cells that engulf microorganisms before exposing them to an array of killing mechanisms. The phagocytic cells of innate immunity are derived from myeloid progenitor cells, and can be broadly divided into two families, monocytes and granulocytes, of which the latter can be further sub-divided into eosinophils, neutrophils and basophils. The internalisation process fundamental for innate immunity relies primarily upon cell surface receptors and secreted proteins produced by these cells that recognise conserved microbial structures such as carbohydrate, lipid, protein and DNA components. Upon internalisation these cells use a variety of mechanisms to destroy the ingested material, including the generation of reactive oxygen intermediates such as superoxide anions (O_2^-), nitric oxide production, proteolytic enzymes such as lysozyme and acid

hydrolyases, cationic proteins or changes in intracellular pH. Other myeloid-derived cells are induced to release pro-inflammatory molecules, known as cytokines, which promote an immune response and assist in clearance of the pathogen. However, innate immunity relies on the infectious agent carrying common surface molecules that can be recognised by phagocytes, therefore many infectious agents have evolved mechanisms by which to evade detection by the innate immune system. As a result, the adaptive immune response evolved to provide an improved detection mechanism and a more versatile means of defence.

The adaptive immune response is comprised of cells derived from lymphoid progenitor cells, which give rise to two major types of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells). Adaptive immunity relies upon the ability of lymphocytes to recognise antigens and respond in a highly specific and selective manner, as each lymphocyte will only recognise a unique antigen. B cells generate and secrete antibodies, molecules that are specific for each unique antigen, and are important for combating infection by extracellular pathogens. These antibodies circulate in the bloodstream and permeate other body fluids. When an antibody molecule encounters its antigen and binds to it, it protects the host via one of three key processes, neutralisation, opsonisation or complement activation. Neutralisation is the process by which antigen-antibody complexes prevent antigen binding to receptors on host cells and causing pathology, for instance by preventing entry of viral particles or bacterial toxins into cells. Opsonisation is the process by which the antibody coating allows the antigen to be recognised as foreign by phagocytic cells, leading to its ingestion and destruction. The third mechanism involves the recruitment of a system of plasma proteins known as complement by antibody-antigen complexes, particularly when antibodies coat a bacterial cell. The antibodies form a receptor for the C1 component of complement, leading to activation of the system, which enhances opsonisation of the antigen, or can lead to direct lysis of some bacteria.

T cells are involved in a wide range of activities, and are required to combat infections by intracellular pathogens, which cannot be detected by antibodies. T cells are comprised of two main classes, $CD8^+$ T cells, and $CD4^+$ T cells. $CD8^+$ T cells are capable of directly eliminating host cells that have become infected by viruses or other pathogens, therefore they are also known as cytotoxic T cells (T_C). Some $CD4^+$ T cells are capable of modulating the functions of other immune cells, such as B cells, macrophages and cytotoxic T cells, hence they are also known as helper T cells (T_H). Upon activation by antigen, T_H cells can differentiate into either T_H1 or T_H2 effector T cells, and the outcome of this differentiation can be critical for determining whether the pathogen is eliminated or evades the host defence. This decision is influenced by the repertoire of cytokines present early in the immune response, and as such effector T cells can influence the adaptive immune response towards either a cellular (T_H1) or humoral (T_H2) response. There is also a small population (~ 10%) of $CD4^+$ T cells that are $CD25^+$, and these are associated with the suppression of harmful immunopathological responses to self or foreign antigens. These naturally occurring suppressor T cells, now regarded as regulatory T cells (T_R), are capable of inhibiting the proliferation of other T cell populations both *in vitro* and *in vivo*. One interesting feature of the adaptive immune response is that, unlike the innate response, it improves with each successive encounter with the same pathogen. The adaptive immune response is therefore important for the generation of life-long immunity to a particular infection.

There is considerable interaction between the innate and adaptive arms of the immune system throughout the course of an immune response. T cells are only capable of recognising antigens presented to them in a particular way, and this is a key role of the phagocytes of the innate immune system. Cells such as dendritic cells display antigen via MHC molecules to T cells in a process known as antigen presentation, resulting in the activation of the T cell and the induction of a specific cell-mediated response.

The cells responsible for mediating the cellular and humoral responses have become major targets of biomedical research. This has resulted in the acquisition of a great deal of knowledge of both the development and effector functions of key cell types. However, much remains to be elucidated. One area that has received much interest, yet has still to yield much more information, is the molecular and biochemical mechanisms underlying B cell development and differentiation, and the differential responses that depend on maturation status. One receptor is responsible for the transduction of antigen encounter throughout B cell development, yet the biological response varies depending on the developmental stage. The study of these differential responses will further our understanding not only of normal B cell development, but also the role of B lymphocyte dysfunction in a number of disease states, including many autoimmune diseases.

1.2 The role of B cells in the adaptive immune response

1.2.1 Generation and selection of B cells

B cells are the principal cellular mediators of the specific humoral response to infection by bacteria, viruses and parasites as they produce antigen-specific antibodies. The processes controlling development of B cells are tightly regulated to ensure a constant supply of B cells expressing antigen receptors of distinct specificity, enabling identification of any encountered antigen, yet at the same time avoiding generation of autoreactive B cells that recognise 'self' antigens. This complex process involves the integration of numerous signals at a number of stages, including antigen, soluble mediators and accessory cells.

In mammals, B cell development begins with the commitment of haematopoietic stem cells (HSCs) to the B cell lineage, a process that occurs in the foetal liver, then after birth and into adult life in the bone marrow (**Figure 1.1**). Commitment of HSCs to the B cell lineage is dependent on the expression of the paired box

transcription factor Pax5 (Cory, 1999). Pax5 acts to promote the expression of B cell lineage genes whilst suppressing those genes responsible for T cell, erythroid or myeloid cell development, other lineages HSCs are capable of differentiating into. Once committed, the precursor B cells pass through a number of developmental stages marked by a series of changes in location and in the expression of genes, intracellular proteins and cell surface markers. Encounter with antigen in the bone marrow, however, leads to death by apoptosis, or anergy, a process by which the B cell becomes unresponsive to future encounters with its particular antigen. The B cells that emerge into the periphery are termed immature B cells, and these migrate to the secondary lymphoid organs, such as the spleen and lymph nodes. It is here, in association with specialised antigen-presenting cells and stromal cells, that B cell recognition of antigen can lead to one of several developmental pathways: (1) anergy and/or apoptosis, (2) activation, proliferation and differentiation into high rate antibody secreting plasma cells, or (3) differentiation into memory B cells (Tarlinton, 1994).

The stages of B cell development can be broadly divided into two quite distinct phases; antigen-independent and antigen-dependent (Rolink and Melchers, 1991). The antigen-independent phase is completed in the bone marrow, and involves the production of a repertoire of immature B cells bearing functional antigen receptors (B cell receptor, BCR).

The production of a functional BCR relies on the completion of a complex pattern of immunoglobulin gene rearrangements to produce one functional heavy chain, followed by one functional light chain (Coleclough *et al.*, 1981). This rearrangement is under the control of the protein products of the recombination-activating genes, RAG-1 and RAG-2, which are highly expressed at the pro- and pre-B cell stages of differentiation and are essential for rearrangement (Spanopoulou *et al.*, 1994). The immunoglobulin heavy chain variable region is encoded by V (variable) and J (joining) gene segments, with additional diversity provided by the D (diversity) gene segment. Rearrangement of the heavy chain

gene begins in the early pro-B stage with the joining of D_H to J_H. Cells are allowed to progress to the next stage provided a productive rearrangement is achieved. Progression to the late pro-B cell stage is accompanied by the joining of a V_H gene to the pre-formed DJ_H complex. Although no functional immunoglobulin is expressed in late pro-B cells, recent studies have shown the expression of components of the mature BCR on their surface, namely the accessory Ig-α/Ig-β heterodimers in association with calnexin (Nagata *et al.*, 1997) (**Figure 1.2**). These accessory molecules have been shown to be essential for the continuing development of the pro-B cells to the pre-B cell stage (Gong and Nussenzweig, 1996; Minegishi *et al.*, 1999). Indeed, it has been shown that mice deficient in Ig-β exhibit a complete block in B cell development before V_H to D_HJ_H rearrangement. It was therefore been suggested that signalling through the Ig-α/Ig-β-calnexin receptor on pro-B cells may be required for successful initiation of V_H to D_HJ_H gene rearrangement (Nagata *et al.*, 1997). However, it has recently been shown that V_H to D_HJ_H recombination can still take place in pro-B cells from mice lacking either Igα or Igβ (Pelanda *et al.*, 2002).

A successful first rearrangement results in the production and transient expression of intact μ heavy chains in an immunoglobulin-like "pre-BCR" complex with the surrogate light chains, λ5 and VpreB (Karasuyama *et al.*, 1994). λ5 bears close similarity to the known constant (C) λ light-chain domains, whilst VpreB resembles a variable (V) domain but bears an extra N-terminal protein sequence. If this first rearrangement is unsuccessful, a second rearrangement is undertaken. Pro-B cells in which both rearrangements are unsuccessful are unable to produce a pre-B cell receptor. Surface expression of a pre-BCR is known to be important for instructing the cell to stop further V_H gene rearrangements (Neuberger, 1997) by inhibiting recombination at the heavy chain locus, a process described as allelic exclusion (Benschop and Cambier, 1999). Pre-B cell receptor expression also drives the transition to the large pre-B cell stage and induces proliferation in addition to signalling to the cell that gene rearrangements to the immunoglobulin light chain should begin. Thus, a lack of

pre-B cell receptor expression results in a block in B cell development at the pre-B cell stage. This signalling to the cell is apparently in the absence of any specific ligand or cooperating cells, and indeed has recently been shown to be dependent upon the non-immunoglobulin portion of the surrogate light chain molecule, λ5 (Ohnishi and Melchers, 2003). This study showed that deletion or mutation of the N-terminal region of λ5 resulted in a failure to initiate pre-BCR signalling, leading to the hypothesis that the pre-BCR might serve as its own ligand with oppositely charged amino acids in λ5 and VpreB binding the two proteins together, resulting in autonomous self-cross-linking of neighbouring pre-BCRs.

Maturation of pre-B cells to immature B cells involves the rearrangement of immunoglobulin light chain genes to generate a conventional light chain (κ or λ), with appropriate constant and variable regions. Once a light chain gene has been rearranged successfully, a BCR consisting of μ heavy chain, conventional light chains and accessory Ig-α/Ig-β molecules is expressed by the immature B cells. This intact surface IgM is the first BCR to exhibit antigen specificity (Reth, 1992) and thus the B cell enters the antigen-dependent stage of development. It has been estimated that about 10^8 B lineage precursors are generated every day in the murine bone marrow, which in turn give rise to about 2×10^7 sIgM-expressing immature B cells (Osmond, 1986). Thus, it is clear that the majority of B cells (80%) maturing in the bone marrow undergo a process of negative selection. Indeed, ligation of the antigen receptors on the vast majority of immature B cells leads ultimately to anergy, a state of non-responsiveness to antigen (Goodnow *et al.*, 1988) or deletion via apoptosis (programmed cell death) (Hartley *et al.*, 1991; Hasbold and Klaus, 1990; Nemazee and Burki, 1989).

The inactivation of self-reactive B cell clones by deletion or inactivation (anergy) is important for the maintenance of self-tolerance by the immune system. Immature B cells that are capable of recognising self-antigens are eliminated or inactivated, preventing them from developing further and secreting antibodies that bind to host cells or tissues. Self-reactive immature B cells may also be

rescued from deletion by undergoing receptor editing, where the autoreactive receptor is replaced with the product of a further rearrangement event (Radic *et al.*, 1993). Immature B cells isolated from the bone marrow undergo apoptosis when cultured *in vitro* in the presence of anti-Ig antibodies, but these cells are purified, therefore not in their physiological context (Norvell *et al.*, 1995). In contrast, when immature B cells are cocultured with whole syngeneic bone marrow they do not respond to anti-Ig by undergoing apoptosis but by re-expressing *RAG-2*, permitting receptor editing (Sandel and Monroe, 1999). These studies suggest that the consequences of an immature B cell recognising its antigen are dictated by the site of antigen encounter, with the bone marrow microenvironment providing signals that promote receptor editing. If the immature B cell first encounters its antigen in the periphery, the absence of these signals results in commitment to apoptosis.

By maintaining high level expression of mIgM, transitional-immature (T1) B cells entering the periphery remain sensitive to antigen deletion for a number of days (Allman *et al.*, 1993). This is especially important for the development of tolerance, since not all self-antigens are expressed within the bone marrow (Monroe, 2000; Sandel and Monroe, 1999). To promote their survival and migration to the spleen, these cells require T cell-dependent help. Typically, of the 2×10^7 sIgM$^+$ B cells that develop daily in murine bone marrow only 10% will reach the spleen and only 1-3% will survive and develop to the next stage of maturation (Allman *et al.*, 1993). The development into a transitional (T2) B cell is accompanied by the surface expression of IgD and requires constant BCR-derived signals for progression, resulting in a IgM^{high} IgD^{high} phenotype (Carsetti *et al.*, 1995; Neuberger, 1997; Sandel and Monroe, 1999). In addition, stimulation via cytokines or co-receptor ligation is thought to help shape the BCR repertoire and signalling thresholds (Craxton *et al.*, 1999). As the B cells migrate into the primary follicles of the spleen they are finally regarded as "mature" IgM^{low} IgD^{high} B cells.

In stark contrast to the immature B cell, antigen-receptor ligation leads to the activation of mature B cells. The activated B cell can develop into an IgM antibody-secreting plasma cell or undergo isotype switching and V region somatic mutation to become a memory B cell, in the presence of the correct T cell-derived cytokines and cell-cell contacts (Cushley and Harnett, 1993; Liu *et al.*, 1992). Following re-exposure to the same antigen and affinity maturation, whereby the affinity of the cell for its particular antigen improves by a process of somatic hypermutation, the memory B cell can evolve into an IgG secreting plasma cell. The formation of memory is critical for mounting a rapid, specific secondary immune response and the ability of the immune system to generate these memory B cells forms the basis of effective vaccination.

The generation of memory B cells occurs in germinal centres (GCs), which are formed during primary immune responses to T cell-dependent antigens in lymphoid follicles (**Figure 1.3**). B cells activated by T cell-dependent antigens enter the primary lymphoid follicles where they undergo proliferation in areas rich in follicular dendritic cells (FDCs), which fix unprocessed antigen on their surface for presentation to newly-formed B cells. Following their expansion, the B cell blast population migrates from the centre of the follicle to form the dark zone of the GC, where they continue to proliferate and lose sIg expression, becoming centroblasts. Centroblasts undergo somatic mutation of the immunoglobulin variable region genes and subsequently migrate to the light zone of the GC, which is rich in FDCs, and are now termed centrocytes, which express the mutated antigen receptors. In the light zone, centrocytes with the highest affinity antigen receptors are selected and return to the dark zone for further rounds of mutation and selection, whilst those with lower affinities undergo apoptosis. As centrocytes are intrinsically programmed to undergo apoptosis unless they are actively rescued, the level of B cell apoptosis in the light zone is very high. Rescue of centrocytes from apoptosis is driven by two signals. The first is generated by FDC-displayed antigen resulting in ligation of the high-affinity surface immunoglobulin, the second by CD40 ligation on the surface of

centrocytes, suggesting a role for helper T cell interactions in promoting centrocyte survival (Craxton *et al.*, 1999). This process results in the selection of high affinity B cell clones as redundant and low affinity receptors are selected against. Positively selected centrocytes then go on to establish the memory B cell pool in the apical light zone, providing the precursors for plasma cells that will produce antibodies of high affinity.

The developmental stages described in this section give rise to a population of "conventional" B cells. However a second population of B cells exists that appear to arise from distinct stem cells early on in development (Hayakawa and Hardy, 2000). These latter cells are known as B-1 B cells, to distinguish them from the conventional B-2 B cells described above. This subset of B cells was first distinguished from conventional B cells by the surface expression of CD5 (Hayakawa *et al.*, 1983). Stem cells arising from the foetal liver give rise to B cell progenitors that have little or no terminal deoxynucleotidyl transferase (TdT), which in turn give rise to B-1 cells. A lack of TdT activity in these B-1 cells results in limited immunoglobulin heavy chain rearrangements and the expression of low-affinity antigen receptors for multivalent self-antigens. The restricted diversity of B-1 cell antigen-receptors includes broad specificities in antigen recognition, particularly to common bacterial polysaccharides such as lipopolysaccharide (LPS) and phosphorylcholine (PC), as well as phosphatidyl-choline, immunoglobulins and DNA. B-1 B cells are particularly important in newborns, as they are presumed to be responsible for natural immunity prior to the production of conventional B-2 B cells in adult life.

Predominantly located in the peritoneal cavity, the self-renewing population of mature B-1 cells express sIgM with little or no surface expression of IgD. Little is known about the normal function of B-1 cells, however, the propensity of B-1 cells to produce low-affinity autoreactive antibodies implicates a role for these cells in autoimmune disease. Indeed, B-1 B cell populations that produce

autoantibodies are expanded in autoimmune disease models such as SHP-$1^{-/-}$ (motheaten) and Lyn-deficient mice (Hayakawa and Hardy, 2000).

Another B cell subset is the naïve, marginal zone (MZ) B cell population, which is enriched primarily in the marginal zone of the spleen (Oliver *et al.*, 1997). These cells are involved at an early stage of an immune response, and seem to have a lower threshold for activation, proliferation and differentiation into plasma cells. The MZ B cell population remains poorly characterised compared to the 'conventional' B2 subset, however, it has been shown that MZ B cells are excellent antigen presenting cells, and are among the first cells to encounter blood-borne antigen in T cell dependent immune responses. This suggests that they may participate, along with lymphoid dendritic cells, in the early stages of T cell activation, though this remains to be seen.

1.2.2 BCR structure

The B cell antigen receptor (BCR) is a multiprotein structure, comprising a clonatypic antigen binding component (sIg) produced from the rearrangement of immunoglobulin heavy and light chain genes, which are non-covalently bonded with disulfide-linked Igα (CD79a) and Igβ (CD79b) heterodimers (Pao *et al.*, 1997) (**Figure 1.4**). In contrast to the T cell receptor for antigen, which only recognises processed antigen associated with MHC molecules, the BCR recognises antigens in solution or on cell surfaces in a native conformation. B cell activation by antigen leads to antigen uptake by the BCR, processing and presentation to T cells. The BCR is essential for B cell development, the specific response to antigen, and B cell survival (reviewed by Niiro and Clark, 2002).

There are five classes of immunoglobulin, IgM, IgD, IgG, IgA and IgE, and all of these can be produced either in secreted or membrane-bound receptor form. However, only IgM and IgD are expressed on the surface of mature naive B cells. The ligand-binding unit of the BCR consists of Ig heavy chain homodimers, each

linked by disulphide bonds to an Ig light chain, forming a symmetrical four chain structure with two antigen binding sites. A transmembrane region of 25 amino acids anchors it to the cell surface. The sIg subunit lacks any intrinsic enzymatic activity within its cytoplasmic domains; thus it associates with accessory signal transducing molecules. Encoded by the *mb1* and *b29* genes respectively (Reth, 1995), Ig-α (34 kDa) and Ig-β (38 kDa) possess single, immunoglobulin extracellular domains and exist as disulphide linked heterodimers.

Like mIgM and mIgD, Igα and Igβ do not contain any intrinsic catalytic activity. However, they do possess consensus sequence motifs responsible for the initiation of signal transduction. This motif originally designated as TAM, ARAM or RETH, is now termed the immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995a; Cambier, 1995b). This motif, spaced over 18 amino acids, is composed of a twice-repeated YxxL sequence flanking seven variable residues. Antigen-ligation of the BCR promotes the phosphorylation of the two tyrosine residues within each ITAM (four tandem motifs per BCR) by non-receptor protein tyrosine kinases (PTKs) (**Figure 1.4**). Phosphorylation of the tyrosine residues is essential to the function of the motif and is deemed as being necessary and sufficient to initiate signal transduction (Cambier, 1995a). Many immunoreceptors utilise this mechanism for initiation of cellular signalling events. Other ITAM containing membrane proteins include the CD3 and ζ-chains of the T cell receptor (TCR) and several receptors for the Fc domain of immunoglobulin (FcRs).

1.2.3 BCR signalling

The downstream signalling pathways that couple surface Ig ligation to B cell activation and differentiation have become a major area of biomedical research with a view to understanding, and perhaps one day artificially modulating, the immune response. As such, the signalling pathways activated immediately following BCR ligation have been well elucidated. The first signalling event

mediated by the BCR is the recruitment and concomitant activation of non-receptor protein tyrosine kinases (PTKs) (Campbell, 1999; Kurosaki, 1999). Recruitment and activation of three distinct types of PTKs are known, namely the Src- (Lyn, Blk, Fyn), Syk- and Tec- (Bruton's tyrosine kinase, Btk) PTKs (Cambier, 1995a; Kurosaki, 1999) (**Figure 1.4**). Prior to BCR ligation, the Src family kinases are associated with the BCR in an inactive conformation, due to intramolecular interactions involving their SH2 domains. Following BCR ligation, these Src family PTKs become activated and phosphorylate the key tyrosine residues within the ITAMs of the Igα and Igβ accessory molecules. The phosphorylated ITAMs create binding sites for the tandem SH2 domains of Syk, which, once recruited, itself becomes tyrosine phosphorylated and activated. One substrate of Syk is the adaptor protein BLNK (B cell linker protein), whose phosphorylation provides docking sites for Btk, allowing Btk to be brought into close proximity with, and subsequently become activated by phosphorylation by, Syk (Baba *et al.*, 2001). These kinases subsequently phosphorylate and activate downstream targets in various signalling cascades.

These early signalling events result in the aggregation and activation of a number of important downstream enzymes and adaptor proteins that, in turn, trigger three major signalling pathways (**Figure 1.5**) (Cushley and Harnett, 1993). The first pathway involves the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2 or PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3 or IP_3) by phospholipase C-γ2 (PLC-γ2). The second pathway results in the generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3) following phosphorylation of PtdIns(4,5)P_2 by phosphatidylinositol 3 kinase (PI-3-K). The third pathway is the classical Ras/Raf/Erk-MAPKinase pathway. These three pathways have recently been shown to interact at a variety of levels, increasing the complexity of BCR signalling.

1.2.3.1 Activation of PLC-γ

The PLC-γ pathway was one of the first downstream pathways shown to be activated by the BCR (Bijsterbosch *et al.*, 1985). The PLC family comprises three homologous groups of enzymes (PLC-β, γ and δ), all of which contain PH domains. In tyrosine kinase dependent signalling, including that initiated by most immune receptors, one or both of the two isoforms of PLC-γ, PLC-γ1 and PLC-γ2, are activated. In addition to the PH domain, PLC-γ isoforms also contain two SH2 domains and an SH3 domain (Lee and Rhee, 1995). The SH2 domain aids the recruitment and phosphorylation of PLC-γ by the PTKs Btk and Syk, whilst the PH domain permits docking to the inner plasma membrane via a PtdIns(3,4,5)P_3 dependent mechanism (Campbell, 1999).

The translocation of PLC-γ to the cell membrane and its subsequent PTK-mediated tyrosine phosphorylation are essential for its activation. Indeed, a deficiency in either Btk or Syk results in ablation of PLC-γ activation and Ins(1,4,5)P_3 production. PtdIns(3,4,5)P_3 plays a role in activating Btk by binding to its PH domain, allowing active Btk to proceed to phosphorylate and activate PLC-γ. In addition, PtdIns(3,4,5)P_3 may directly activate PLC-γ via PH domain binding interactions. Although the SH2 domain of PLC-γ can bind to Syk *in vitro*, reconstitution experiments indicated that additional factors were required to initiate a BCR-mediated calcium response (Kelly and Chan, 2000). More recently, the B cell specific adaptor protein, BLNK (B cell linker protein), also termed SLP-65, has been identified as the additional factor required for coupling Syk to activation of PLC-γ (Fu *et al.*, 1998; Ishiai *et al.*, 1999a). Indeed, studies using the chicken DT40 cell line have shown that BLNK gene disruption abolishes PLC-γ phosphorylation (Ishiai *et al.*, 1999b). BLNK is also thought to play a role in the recruitment and activation of Vav, Grb2 and Nck, serving to focus these signalling effectors at the plasma membrane for phosphorylation by Syk.

PLC-γ1 deficient mice are embryonic lethal and fail to mobilise calcium (Ji *et al.*, 1997). Whilst PLC-γ2 insufficiency is not lethal (Wang *et al.*, 2000), PLC-γ2 deficient B cells have signalling defects similar to that seen in Btk knockouts (Khan *et al.*, 1995) or the naturally occurring *xid* immunodeficient mice (Rawlings *et al.*, 1993), which possess a mutation in the PH domain of Btk preventing its recruitment and concomitant activation by PtdIns(3,4,5)P_3. The effects of PLC-γ2 deficiency have recently been shown to be due to a block in B cell maturation at the transitional 2 to follicular B cell transition. There is a dramatic increase in BCR-induced apoptosis in all subsets of splenic B cells from PLC-γ2-deficient mice, due to a failure to increase the expression of the pro-survival proteins A1 and Bcl-2 (Wen *et al.*, 2003).

The generation of second messengers, DAG and Ins(1,4,5)P_3, has been shown to be important for the activation of protein kinase C (PKC) isoforms, the release of intracellular calcium stores and the nuclear translocation of NF-κB (Berridge, 1993; Berridge and Irvine, 1989; Lee and Rhee, 1995). DAG activates conventional and novel protein kinase C (PKC) isoforms at the plasma membrane (Parekh *et al.*, 2000), whilst Ins(1,4,5)P_3 binds to receptors (InsP_3R) in the endoplasmic reticulum. InsP_3R activation leads to a process of capacitative calcium entry, by which the depletion of intracellular stores activates calcium influx across the plasma membrane (Parekh and Penner, 1997).

Members of the PKC superfamily are serine/threonine kinases, and to date 11 PKC isoforms have been identified, which are subdivided into three groups depending on their structure and cofactor regulation (Mellor and Parker, 1998). Conventional PKCs (α, β_I, β_{II} and γ) require both calcium and DAG for activation, novel PKCs (δ, ε, η, θ) are calcium independent but require DAG whilst, atypical PKCs (ξ, λ/ι) are calcium independent and are not activated by DAG. Several studies have highlighted the role for PKCs in modulating the BCR-mediated response in a maturation stage-dependent manner. Studies in knockout or depletion models of PKC isoforms have demonstrated that a lack of DAG-

responsive PKC isoform activation, in particular PKCβ, may be responsible for BCR-induced apoptosis in immature B cells (King *et al.*, 1999; Leitges *et al.*, 1996). Thus, apoptosis can be overcome by stimulating immature B cells with phorbol esters (e.g. PMA), which bypass the BCR and activate the conventional and novel PKC isoenzymes directly (King *et al.*, 1999; Nishizuka, 1992). In mast cells, stimulation via FcεRI leads to phosphorylation of both PKCα and PKCβ_I by Syk, resulting in the generation of a binding site for the SH2 domain of Grb-2. The concomitant recruitment of the Grb-2/Sos complex contributes to the full activation of Ras/Erk-MAPKinase cascade, demonstrating a link between PKC activation and the Ras/Erk-MAPKinase cascade (Kawakami *et al.*, 2003).

The recently isolated member of the CDC25 family of Ras guanyl nucleotide exchange factors (GEFs), RasGRP3, is preferentially expressed in B cells. It has a C1 domain that binds DAG, suggesting it could be responsible for some of the DAG signalling events that have previously been attributed to PKC. Upon stimulation of Ramos B cells, RasGRP3 associates with the membrane, and is phosphorylated in a PKC-dependent manner. *In vitro*, PKCθ and PKCβ_I are capable of phosphorylating RasGRP3 (Teixeira *et al.*, 2003). The kinetics of RasGRP3 phosphorylation correlate with the levels of both active Ras and Erk-MAPKinase, consistent with the idea that phosphorylation of RasGRP3 is related to its functional activity, the activation of Ras by exchanging GDP for GTP. Interestingly, RasGRP3 from stimulated cells does not react with phospho-tyrosine antibodies, suggesting that it contains phospho-threonine or phospho-serine, supporting the idea it is a substrate for PKC.

1.2.3.2 Activation of PI-3-Kinase

Phosphatidylinositol 3-kinase (PI-3-Kinase) catalyses the phosphorylation of the inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 on the 3' position of the inositol ring to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively (Fry, 1994). Three classes of PI-3-Kinase exist, which are classified

according to their *in vitro* substrate specificities. Class I PI-3-Kinases are capable of phosphorylating all three forms of inositol phospholipid and can be further subdivided into Class IA and Class IB. Class IA comprise a p110 catalytic subunit (α, β or δ) and a regulatory SH2/SH3-domain containing p85 family adaptor subunit. The Class IA isoforms are normally activated by PTK coupled receptors. Class IB isoforms consist of a p110γ catalytic subunit and a p101 adaptor subunit and are activated following signalling via G-protein coupled receptors. Class II isoforms phosphorylate PI and PtdIns(4)P whilst Class III phosphorylates PI only.

PI-3-Kinase activity, and the concomitant generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, are known to be important for BCR-mediated B cell proliferation, differentiation (Aagaard-Tillery and Jelinek, 1996) and survival (Campbell, 1999). Indeed, inhibition of PI-3-Kinase activity in actively cycling B cells results in apoptosis (Craxton *et al.*, 1999). However, a negative role for PI-3-Kinase has been shown in a human RL B cell line where BCR-induced growth-arrest can be blocked by inhibiting PI-3-Kinase activity (Craxton *et al.*, 1999). The interaction of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with SH2 and PH domain containing proteins, such as PLC-γ, Btk, Vav, PDK1/2 and Akt/PKB permits their recruitment to the plasma membrane (Aagaard-Tillery and Jelinek, 1996; Alessi *et al.*, 1997; Falasca *et al.*, 1998; Franke and Cantley, 1997). PI-3-Kinase activation following BCR ligation was thought to be solely dependent on PTK activity. However, studies have demonstrated the essential requirement for upstream activation of the GTPase Ras (Kodaki *et al.*, 1994), which then binds the p110 catalytic sub-unit of the p85-dependent PI-3-Kinase (Genot and Cantrell, 2000). This mechanism establishes a link between the PI-3-Kinase pathway and the pathways driven by GTP binding proteins, such as the Ras/MAPK pathway.

Recently, it has been demonstrated that PI-3-Kinase activity is required for the BCR-induced phosphorylation and activation of Erk-MAPKinase. If murine splenic B cells are pretreated with the PI-3-Kinase inhibitors LY294002 or wortmannin, Erk-MAPKinase activation in response to BCR-ligation is greatly

reduced (Jacob *et al.*, 2002). Such inhibition of PI-3-Kinase has no effect on Ras activity, but does block Raf and MEK activity, placing PI-3-Kinase between Ras and Raf in the pathway. This is somewhat surprising, as Ras is capable of directly interacting with Raf through its effector domain.

PI-3-Kinase activity is also required for B cell development, particularly of B1 cells in the peritoneum and marginal zone (MZ) B cells. Mice deficient in the p85α regulatory subunit demonstrate a partial block in B cell development at the pro-B cell stage, and have a reduction in the number of B cells in the spleen, as well as a reduction in the number of B1 cells (Fruman *et al.*, 1999) (Suzuki *et al.*, 1999). p110δ-deficient mice display a very similar phenotype, with B cell development blocked at the pro-B cell stage and a reduction in the number of splenic B cells (Okkenhaug *et al.*, 2002). Interestingly, these mice lack B1 and MZ B cells, as do mice lacking CD19, one of the key regulators of PI-3-Kinase activity in B cells (Martin and Kearney, 2000), suggesting CD19 and PI-3-Kinase play important roles in the development or maintenance of MZ and B1 B cells.

1.2.3.3 Activation of the Ras/MAPKinase pathway

The mitogen-activated protein (MAP) kinases are a family of serine-threonine protein kinases that have been widely conserved throughout the evolution of eukaryotic cells. They are activated by a wide range of extracellular stimuli and are able to mediate a wide range of cellular functions ranging from proliferation and activation to growth arrest and cell death (**Figure 1.6**). The MAPKinase family is subdivided into three groups; the classical extracellular signal-regulated kinases (Erk-MAPKinase), the c-Jun N-terminal kinases, also known as the stress activated protein kinases (JNK/SAPK) and the p38 MAPKinases (Dhanasekaran and Premkumar Reddy, 1998; Elion, 1998). Activation of each group is determined by distinct upstream MAPKinase kinases (MEKs) and MAPKinase kinase kinases (MEKK). MAPKs are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-X-Y motif,

where X is different in each group. Following MAPKinase activation, activation of a number of downstream transcription factors occurs; Erk-MAPKinase activates Elk-1 and c-myc, JNK activates c-Jun and ATF-2 and p38 MAPKinase activates ATF-2 and MAX. The phosphorylation and activation of these transcriptional regulators enables the MAPKinase families to regulate gene expression and hence, cellular responses.

In B cells, coupling of the BCR to the Erk-MAPKinase pathway relies on the formation of adaptor protein scaffolds, which are recruited by activated protein tyrosine kinases. These complexes then facilitate the recruitment of downstream guanine nucleotide exchange factors (GEFs) and kinase cassettes of the Erk-MAPKinase pathway. Following BCR ligation, the adaptor protein Shc binds to the phosphorylated BCR ITAMs via its SH2 domain, and is in turn phosphorylated by the PTK Syk. Activated Shc then recruits Grb2-SoS complexes to the phosphorylated ITAMs (Harmer and DeFranco, 1997; Li *et al.*, 1993). In conjunction with a GTPase activating protein (RasGAP), the GEF SoS (son of sevenless) regulates the activities of the guanine nucleotide binding protein Ras. Ras activation can also occur in a Shc-independent manner due to the utilisation of the linker protein BLNK (Kelly and Chan, 2000). Whilst already implicated as an intermediate adaptor in the membrane recruitment and activation of PLC-γ by Syk, BLNK is known to associate with Grb2 and SoS to form a BLNK/Grb2/SoS complex, highlighting the ability of different adaptor molecules to mediate BCR-induced signalling.

Ras is a member of a family of small GTPases, which cycle between inactive GDP-bound and active GTP-bound states, functioning as signal relays linking membrane receptors to signal transduction pathways. They exist in their inactive GDP-bound state until they associate with GEFs, which promote their activation by inducing the exchange of GDP for GTP. Active, GTP-bound GTPases then interact with a variety of effector molecules, activating downstream signalling pathways. They are returned to their inactive GDP-bound state via their intrinsic

GTPase activity, which is accelerated by association with GAPs (Henning and Cantrell, 1998). Thus, following BCR ligation the Grb2-SoS complex promotes the exchange of GDP for GTP on Ras to generate an active GTP-bound form. Ras is then able to regulate a wide range of downstream cellular processes, from cytoskeletal reorganisation through to modulation of transcription (Vojtek and Der, 1998). One effector of Ras is Raf, a serine/threonine kinase, which active Ras can bind and derepress, resulting in the activation of MAPKinase kinases (MEK) and the subsequent downstream activation of the Erk-MAPKinases (Kolch, 2000). Active Ras also promotes cell survival by direct interactions with PI-3-kinase, resulting in the production of the second messengers, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, and the activation of the serine/threonine protein kinase Akt, a key mediator of survival .

Ras is not the only small GTPase that can be activated in response to BCR-ligation. Co-recruitment of the GEF Vav by BLNK (Fu *et al.*, 1998) can in turn activate the Rho-family of GTPases (Rac, RhoA and Cdc42) (Crespo *et al.*, 1997). These GTPases provide a critical link for the activation of the other MAPKs, JNK/SAPK and p38 MAPK, by specific MEKKs (MEKK1-4). Rho-GTPases are also able to activate PI-5-kinase, which phosphorylates inositol phospholipids on the 5' position of the inositol ring, leading to the activation of PLC-γ and the generation of Ins(3,4,5)P_3 and calcium mobilisation (Campbell, 1999).

Alternative pathways following GEF activation may also occur due to the structural similarities between the effector binding domains of the GTPases Ras and Rap-1. The Rap-1 GEF, C3G, interacts with Crk, a Grb2 like adaptor molecule, to form a C3G-Crk complex that associates with phosphorylated receptor tyrosine kinases. Activation of Rap-1 by C3G may lead to the sequestration of the downstream effector of Ras, Raf, resulting in the down-regulation of Erk-MAPKinase activation, as Rap-1 can bind Raf without activating it. However, in some cases, Rap-1 activation may actually occur in a Crk/C3G

independent manner, as formation of a stable complex between Rap-1 and B-Raf promotes the activation of Erk-MAPKinase in nerve growth factor treated PC12 cells, suggesting that Rap-1 activity can positively regulate receptor-mediated responses in some cell types (York *et al.*, 1998). Indeed, transient Ras-dependent Erk-MAPKinase activation in PC12 cells results in proliferation, whilst sustained Rap-1-dependent MAPKinase activation induces differentiation (York *et al.*, 1998). Utilisation of this alternative pathway may be facilitated by the different cellular localisation of these two GTPases; Ras is predominantly plasma membrane bound whilst Rap-1 is located in endocytic and lysosomal vesicles. It has recently been demonstrated that, in the WEHI-231 and A20 cell lines, Rap activation negatively regulates BCR-induced phosphorylation of Akt on key activating sites, but has no effect on Erk-MAPKinase activation (Christian *et al.*, 2003). This strongly suggests that BCR-induced Rap activation functions to limit the ability of the BCR to activate Akt. Indeed, blocking Rap activation in WEHI-231 cells enables them to survive prolonged treatment with anti-IgM antibodies, presumably as a result of increased Akt activation, as expression of a constitutively active Akt in WEHI-231 cells has been shown to reduce anti-IgM induced cell death (Banerji *et al.*, 2001).

Another adaptor protein recruited to the plasma membrane upon BCR ligation is Bam32. Its recruitment requires the activity of PI-3-Kinase, and it is a component in a novel pathway that leads from the BCR to the Erk-MAPKinase cascade, as well as JNK. A key initiator of this pathway is haematopoietic progenitor kinase 1 (HPK1), a MAPKKKK activated downstream of both the BCR and TCR. HPK1 interacts directly with Bam32, possibly allowing localisation of HPK1 to the plasma membrane where it can activate MEKK1. This novel pathway is required for normal BCR-mediated proliferation, but not for B cell survival (Han *et al.*, 2003). In humans, Bam32 expression is largely restricted to haematopoietic cells with the exception of T cells, and its expression is thought to be enhanced in germinal centre B cells (Marshall *et al.*, 2000). In mice, the expression pattern is very similar, except there is no increase in germinal centre B cells, or activated

splenic B cells, but rather a decrease. B cells develop normally in Bam32$^{-/-}$ mice, except for B-1 B cells, which are slightly reduced. Bam32$^{-/-}$ mice have normal T cell-dependent immune responses, but defective T cell-independent immune responses.

MAPKinase pathways ultimately regulate cell cycle machinery, thus controlling the fate of a cell and dictating whether it progresses through to rounds of proliferation or enters into growth arrest or apoptosis. One recently discovered target of Erk-MAPKinase is the RNA polymerase III machinery, whose output is tightly linked to growth (Larminie *et al.*, 1998) and subject to cell cycle regulation (White *et al.*, 1995). RNA pol III is responsible for the synthesis of tRNAs, as well as other small RNAs including the 5S ribosomal RNA subunit. Erk-MAPKinase phosphorylates BRF1, a component of the RNA pol III transcription factor TFIIIB, resulting in increased transcription of RNA pol III target genes (Felton-Edkins *et al.*, 2003). Erk-MAPKinase also stimulates transcription of large ribosomal RNAs by RNA pol I (Stefanovsky *et al.*, 2001). As ribosomes require an equimolar ratio of rRNAs, it seems likely that Erk-MAPKinase plays a role in co-ordinating production of ribosomes, which are necessary for growth and cell cycle progression (Stefanovsky *et al.*, 2001).

In B cells, the Erk-MAPKinase pathway has been shown to promote proliferation by inducing the expression of cyclin D1 (Lavoie *et al.*, 1996). The cyclins are pivotal in controlling successful passage of cells through the cell cycle (see below). Phosphorylation of certain Ribosomal S6 Kinase isoforms (p90RSK1-3) by Erk-MAPKinase has also been postulated to be important for the regulation of translation (Frodin and Gammeltoft, 1999). In contrast, activation of the JNK/SAPK and p38 MAPKinases induces growth arrest and apoptosis in a human immature B cell line following BCR-ligation (Graves *et al.*, 1996).

1.2.5 Role of co-receptors in B cell signalling

A key feature of the immune response is the maintenance of equilibrium between antigen reactivity and cellular quiescence, as strict regulation and control over a potentially inappropriate response is vital. Recent studies into inhibitory cell surface receptors have highlighted the requirement for the balance of activatory and inhibitory pathways to maintain this control. Indeed, loss of suitable inhibitory signalling is frequently associated with the development of inflammatory responses and, in some cases, autoimmunity.

This balance of control is exemplified by the fine-tuning of B cell responses to antigen, resulting in different biological responses. A number of factors can alter BCR-mediated signalling, including the concentration of the antigen, the avidity with which it is bound, the timing and duration of antigen encounter and co-stimulation by other cells, receptors or antigenic sources (Healy and Goodnow, 1998). Similarly, the differential expression of signalling molecules at different B cell maturation stages or the use of alternative signalling pathways by the recruitment of different adaptor molecules leads to the generation of a full repertoire of responses.

Another mechanism that regulates the BCR signalling threshold for B cell responses is the involvement of co-receptors. B cell co-receptors and their modulation of the cellular response have been widely studied in recent years (Tsubata, 1999). Molecules such as CD19, CD22, CD72 and the Fcγ receptor, FcγRIIb, appear to associate with the BCR either constitutively or inducibly, thus altering the signalling threshold of the BCR either by facilitating positive signalling or by downmodulating BCR function.

1.2.5.1 CD19

CD19 is a B cell restricted surface glycoprotein, which forms a non-covalent complex with CD21 (complement receptor type 2, CR2), CD81 (TAPA-1) and Leu-13. Co-ligation of this complex with the BCR results in a reduction of the signalling threshold required for B cell activation, such that CD19-deficient mice are hyporesponsive to stimulation via the BCR (Engel *et al.*, 1995). CD19-BCR co-ligation results in an increase in the release of intracellular calcium, DNA synthesis, Erk-MAPKinase activation and antibody production (Tedder *et al.*, 1997). CD19 is expressed throughout B cell development, until plasma cell differentiation, with an increase in expression shown by mature B cells. Alteration in the levels of CD19 expression has little effect on B cell development until maturity, where an overexpression of CD19 by immature B cells results in a significant reduction of conventional B cells in the periphery (Engel *et al.*, 1995). This reduction is presumably as a result of hyper-responsiveness of immature B cells to antigen receptor ligation, leading to enhanced negative selection in the bone marrow. In some cases, CD19-overexpression can lead to hyper-gammaglobulinema and autoimmune disease, which is believed to be due to CD19-mediated lowering of the threshold for BCR-mediated signalling. Thus, overexpression of CD19 may permit autoreactive B cells to overcome their anergic state. Consistent with this, CD19-deficient mature B cells have also been reported to have reduced responses to T-cell dependent antigens and subsequent lack of germinal centre formation and decreased affinity maturation of serum antibodies (Fujimoto *et al.*, 1999b). This suggests that CD19-signalling is important for T-cell dependent B cell responses.

Following BCR-ligation, the PTK Lyn phosphorylates the cytoplasmic tail of CD19, creating SH2 binding sites facilitating the recruitment and activation of various downstream signal-transducing molecules (Fujimoto *et al.*, 1999a). Src-family PTKs, PLC-γ, PI-3-kinase, the GEF Vav and the adaptor molecule, Shc, have all been implicated in direct binding to activated CD19 (Tuveson *et al.*,

1993; Weng *et al.*, 1994). Studies into these complex associations have led to the belief that CD19 may be responsible for many of the initial early signalling events following BCR co-ligation, as absence of CD19 leads to a reduction in the tyrosine phosphorylation of multiple effector molecules downstream of the BCR following BCR activation (Fujimoto *et al.*, 1999b). The association of adaptor molecules with CD19 has also helped to explain the ability of CD19 to promote events normally associated with positive BCR signalling. Thus, CD19 can regulate BCR coupling to PLC-γ and sustained calcium influx via amplification of the Src-family PTK signal. The phosphorylation of CD19 by Lyn initiates additional rounds of PTK activation including the activation of Syk and Btk, leading to the recruitment of the BLNK/PLC-γ/calcium pathway. This amplification of PTK activity may be a key role of CD19, implying that loss of this amplification could be the main reason for the hypo-responsiveness of BCRs in $CD19^{-/-}$ mice. CD19 can also interact with other regulators of BCR signalling such as CD22, which is a B cell specific protein that appears to be both a negative and a positive regulator of B cell activation (Sato *et al.*, 1996).

1.2.5.2 CD22

CD22 is a cell surface glycoprotein restricted to the B cell lineage, whose ligand is a glycosylated sialic acid residue expressed at high levels on lymphocytes and inflamed endothelial cells. CD22 expression levels are low during the early stages of B cell development, but increase as B cells approach maturity in the periphery (Craxton *et al.*, 1999). In contrast to CD19, CD22 activation results in the specific suppression of Erk-MAPKinase and the modulation of JNK activity, increasing the threshold for BCR activation. Thus, CD22-deficient mice show enhanced B cell responses, including augmented intracellular calcium mobilisation. Following BCR ligation, CD22 is tyrosine phosphorylated via the PTK Lyn, permitting recruitment of multiple SH2 domain containing effector molecules to its cytoplasmic ITIM, including the tyrosine phosphatase SHP-1. SHP-1 dephosphorylates a number of proteins, including Syk, BLNK, and SH2

domain-containing leukocyte protein 76 (SLP-76) (Maeda *et al.*, 1999) (Binstadt *et al.*, 1998). SHP-1 is a strong candidate for mediating the negative effects of CD22, as there are increased levels of SHP-1 recruited by CD22 following BCR ligation. Indeed, recent data suggests that CD22 may function as a molecular "scaffold" that specifically coordinates the docking of multiple effector molecules, in addition to SHP-1, in a context necessary for BCR-dependent JNK stimulation. (Poe *et al.*, 2000).

A role for CD22 in the induction of autoimmunity has also been suggested since the gene for CD22 maps within a region associated with the development of autoimmune disease in certain strains of mice. The CD22 protein expressed in these autoimmune mice is markedly underphosphorylated and binds very little SHP-1 (Cornall *et al.*, 1998). However, the disease phenotype displayed is milder than that of SHP-1 deficient mice, confirming that SHP-1 may also regulate B cell signalling by associating with other inhibitory receptors. The transmembrane protein tyrosine phosphatase CD45 has also been implicated in the regulation of CD22, since CD45 deficient mice display increased tyrosine phosphorylation of CD22, prior to BCR ligation. Not only that, they also exhibit enhanced recruitment of SHP-1 following BCR ligation. Consistent with this, cross-linking of CD45 leads to a decrease in tyrosine phosphorylation of CD22 and subsequent SHP-1 recruitment (Greer and Justement, 1999).

1.2.5.3 CD72

CD72, a type II membrane protein, is also thought to negatively regulate B cell activation via the recruitment of SHP-1 (Parnes and Pan, 2000). However, the interaction of SHP-1 with CD72 is thought to have a different function than that displayed by other SHP-1 dependent inhibitory receptors, which recruit SHP-1 to dephosphorylate key mediators of BCR signalling. The tyrosine phosphorylation of CD72 in response to BCR ligation correlates strongly with BCR-induced growth arrest and/or apoptosis in B cell lines and primary B cells. Thus, CD72

acts to send a signal resulting in cell death by decreasing the activation threshold level of the BCR in immature B cells. However, if CD72 is pre-ligated its tyrosine phosphorylation levels are decreased and B cell tolerance levels are increased, preventing BCR mediated apoptosis. Thus, SHP-1 acts to keep CD72 dephosphorylated, thus preventing BCR signalling from causing cell death.

1.3 CD40

CD40 and its ligand, CD40L (CD154), are known to play crucial roles in the regulation of the humoral immune response, including cell activation, proliferation, immunoglobulin isotype switching, formation of germinal centres and induction of memory B cells (Durie *et al.*, 1994). In addition to having roles in mediating B cell biology during normal development, recent studies have also suggested an important role for CD40 in antitumour strategies against B lymphocyte malignancies (Costello *et al.*, 1999). These studies have indicated that CD40 stimulation leads to the up-regulation of adhesion and/or costimulatory molecules on the cell surface of tumour cells, which aids the recognition of such cells by the immune system. In addition, CD40 stimulation of tumour cells has also been shown to induce the release of anti-tumour cytokines by the tumour itself. However, the effectiveness of CD40 as a viable therapy against some B lymphocyte malignancies is in doubt, due to the anti-apoptotic/proliferative signals delivered by CD40-ligation in some low-grade B lymphocyte malignancies. Therefore, current research into CD40-mediated tumour therapy is focused on avoiding pro-tumourigenic effects of CD40 stimulation whilst understanding the role of CD40 in tumour recognition and the stimulation of the immune system.

1.3.1 CD40 structure and expression

The CD40 receptor is a 48-kDa transmembrane glycoprotein and is a member of the TNF receptor (TNFR) superfamily (van Kooten and Banchereau, 1997) (**Figure 1.7**). Structurally, human CD40 consists of a 193 amino-acid extracellular domain, a 22 amino-acid transmembrane domain and a 62 amino-acid cytoplasmic domain. The extracellular domain consists of four homologous, repeating, cysteine-rich extracellular domains characteristic of TNFR family motifs (Kehry, 1996). It has been shown that murine CD40 shares approximately 60% homology to human CD40, with the greatest homology (78%) to the human form in the cytoplasmic domain (Gray, 1997). This cytoplasmic domain contains no sequence of known protein tyrosine kinase activity. Indeed, the cytoplasmic tail of human CD40 contains no tyrosine residues, whilst only one exists in the murine form. Nevertheless, the cytoplasmic domain of CD40 is constitutively phosphorylated, presumably on serine and/or threonine residues, in which Thr-234 has been shown to be crucial for signal transduction (Gray, 1997).

Although CD40 was originally thought to be a B cell specific receptor, it has since been shown to be expressed on a variety of human and mouse cells, including dendritic cells, monocytes, macrophages, mouse fibroblasts and human smooth muscle cells (Grewal and Flavell, 1998). Although CD40 has been reported to be expressed on virtually all B cells, the level of CD40 expression varies depending on the maturation stage of the cell. Murine CD40 is expressed at low levels on 30-40% of pre-B cells, at intermediate levels on 80% of immature B cells and on essentially all mature B cells, suggesting that CD40 expression may be more important in later stages of B cell maturation (Hasbold *et al.*, 1994).

1.3.2 CD40 signal transduction

Like both the p55 and p70 isoforms of the TNF receptor, CD40 has no intracellular kinase domain and no consensus sequence for binding kinases (van

Kooten and Banchereau, 1997). However, CD40 ligation is known to rapidly activate the protein tyrosine kinases Lyn and Syk (Faris *et al.*, 1994), whilst also inducing the tyrosine phosphorylation of PI-3-kinase and PLC-γ2 (van Kooten and Banchereau, 1997) and activating serine/threonine kinases (Durie *et al.*, 1994). However, like all signalling mechanisms, it is important to be careful when comparing data from different cell types. Consequently, studies highlight quite distinct differences in proximal CD40 signalling events in B cells depending on whether the cells used were resting, activated or EBV-transformed (Faris *et al.*, 1994; Gray *et al.*, 1994). In addition, some studies suggest that instead of phosphorylating and activating a number of protein tyrosine kinases, CD40 engagement de-phosphorylates the Src family PTKs or syk (Craxton *et al.*, 1999).

As well as regulating protein tyrosine kinase activity, CD40 is known to associate with intracellular proteins termed TNF receptor-associated proteins (TRAFs). TRAF2, TRAF3 and TRAF 5 are known to associate with specific regions in the cytoplasmic domain of CD40 (Kehry, 1996) (**Figure 1.7**). TRAF3, or CD40 receptor-associated factor (CRAF1), is specific to CD40 signal transduction and does not bind to other membrane proteins such as p70 TNFRII (Gray, 1997). One fascinating feature of the TRAFs is their ability to bind both membrane elements (such as CD40) and also DNA. This latter function is due to the five zinc ring finger motifs encoded in the N-terminal domain of TRAF 2 and 3. In addition, TRAF proteins share homology in a C-terminal TRAF binding domain. This allows the various TRAFs to form homo- or hetero-dimers following CD40 ligation (Kehry, 1996). These complexes (much like the STAT family of proteins) may then translocate to the nucleus to function as transcriptional activators. Indeed, TRAF2 is predicated to play a crucial role in CD40-mediated activation of the transcription factor NF-κB.

1.4 The role of FcγRIIb in B cells

Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system by binding the Fc domain of antibodies. A separate FcR exists for each of the five classes of immunoglobulin; FcαR (IgA), FcδR (IgD), FcεR (IgE), FcγR (IgG), and FcμR (IgM) (Ravetch and Kinet, 1991). The major function of most Fc receptors is the activation of the accessory cell they are found on, for example ligation of the FcεRI receptor on mast cells activates a biochemical cascade leading to the secretion of inflammatory mediators.

The FcγRs are specific for the Fc domain of IgG, and comprise a family of structurally homologous, yet distinct, receptors. Three classes of FcγR exist, FcγRI, FcγRII, and FcγRIII (Ravetch and Kinet, 1991). These three classes are defined by their cellular distribution, structure and affinity for the IgG subclasses (Hulett and Hogarth, 1994). FcγRI is a high affinity receptor, which is capable of binding monomeric IgG at physiological concentrations (Allen and Seed, 1989). In contrast, FcγRII and FcγRIII are low affinity receptors that can only bind IgG that is complexed to multivalent soluble antigen as immune complexes (Hulett and Hogarth, 1994). The only FcγR found on B cells is the FcγRIIb receptor (CD32), where it functions to inhibit signalling through the BCR upon cross-linking of the two receptors by IgG containing immune complexes, courtesy of a 13 amino acid inhibitory ITIM motif in its cytoplasmic domain (Muta *et al.*, 1994).

1.4.1 Mechanisms of FcγRIIb-mediated negative regulation

Negative regulation of BCR-mediated activation was first recognised when passively administered antigen-specific IgG antibodies were shown to inhibit *in vivo* primary responses to that antigen (Chan and Sinclair, 1971). A molecular basis for this inhibition was revealed following the cloning of the genes for murine low-affinity IgG receptors, FcγRIIb and FcγRIII (Ravetch *et al.*, 1986). Inhibition

by IgG-containing immune complexes required the co-ligation of the BCR with the Fc receptor, FcγRIIb (van de Winkel and Capel, 1993). Although the mechanisms underlying the negative regulation of BCR signalling are poorly understood, several of the well characterised cellular responses of BCR stimulation; phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and immunoglobulin secretion are all inhibited following co-engagement of FcγRIIb.

Early *in vitro* studies on FcγRIIb indicated that recruitment of the tyrosine phosphatases, SHP-1 and SHP-2, mediated the inhibitory effect of this receptor in B cells (D'Ambrosio *et al.*, 1995). However, SHP-1 was shown to be dispensable for FcγRIIb-mediated inhibition of B cell antigen receptor activation (Nadler *et al.*, 1997). Thus, *in vivo,* FcγRIIb may not recruit SHP-1 or SHP-2 as the primary effectors, but rather the inositol 5' phosphatase, SHIP (Liu *et al.*, 1998; Ono *et al.*, 1997). Studies with dominant negative SHIP mutants and knockout models have confirmed the inhibitory role of SHIP in mediating the FcγRIIb negative signal (Gupta *et al.*, 1997; Huber *et al.*, 1998; Liu *et al.*, 1998). Recruitment of SHIP to the tyrosine-phosphorylated ITIM of FcγRIIb, following co-ligation with the BCR, leads to a drastic reduction in levels of phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and immunoglobulin secretion. FcγRIIb abrogation of BCR activation by the hydrolysis of PtdIns(3,4,5)P_3 disrupts PH domain phosphoinositol lipid interactions and prevents the association of Btk and PLC-γ with the plasma membrane (Bolland *et al.*, 1998). The deletion of SHIP increases PtdIns(3,4,5)P_3 levels, resulting in increased Btk membrane association and hyper-responsive BCR signalling. The negative effects of SHIP on Btk recruitment can also be overcome by the expression of Btk as a membrane-associated chimera. Thus, one key role of SHIP-1 is to inhibit the pathways leading to calcium mobilisation by interrupting PLC-γ recruitment to the membrane.

Overall, the recruitment and activation of SHIP by FcγRIIb results in the inhibition of multiple B cell signalling pathways and the modulation of both BCR-induced B cell activation and antigen internalisation (**Figure 1.8**). In addition, ligation of FcγRIIb alone on B cells has been found to be capable of generating an apoptotic signal (Ashman *et al.*, 1996; Ono *et al.*, 1997). Studies in the B cell line, DT40, demonstrated that this response was independent of SHIP recruitment, suggesting that FcγRIIb may directly couple to an apoptotic pathway in the absence of BCR-ligation. Additional work by Pearse and colleagues has shown that failure to recruit SHIP, either by deletion of SHIP or mutation of FcγRIIb, resulted in enhanced FcγRIIb-triggered apoptosis (Pearse *et al.*, 1999). Further studies demonstrated that this SHIP-independent pathway lead to the Btk-dependent activation of JNK. Overall, these studies suggest that aggregation of FcγRIIb in B cells results in a stress response that leads to apoptosis and that SHIP recruitment following co-ligation with the BCR 'rescues' the cells from apoptosis. Interestingly, SHIP-1 may mediate this effect by recruiting the p85 subunit of PI-3-kinase upon BCR-ligation and could act to regulate downstream events such as B cell activation-induced apoptosis (Gupta *et al.*, 1999).

1.5 Cell cycle and apoptosis

Signalling via the BCR plays a pivotal role in the activation of B cells, leading to various distinct cellular responses, including proliferation, differentiation, anergy and cell death. Despite numerous investigations, it is still not entirely clear how the biochemical signalling events can be translated into these responses. What is clear is that any cellular response is regulated by a number of enforced checkpoints. Checkpoints serve a critical role in the control of cell cycle progression and the damage response system, as they provide the cell with an opportunity to assess the situation and initiate an appropriate response. Therefore a cellular response is the integration of biochemical signals that promote or inhibit the progression of the cell cycle.

The cell cycle is the co-ordinated series of events required for cell growth and division. This complex process relies on the assembly of nuclear proteins that integrate signals and programme cell cycle progression (**Figure 1.9**) (O'Connor *et al.*, 2000). There are four main stages of the cycle, during which a cell must duplicate its contents and divide. G_1 is characterised by gene expression and protein synthesis, resulting in an increase in cell size and production of all the proteins required for DNA synthesis. This is the main phase of the cell cycle that is regulated primarily by extracellular signals, as after a cell exits G_1 it is generally committed to completing a full cycle. DNA duplication occurs in the S phase (synthesis). After chromosome replication a second growth period, G_2, allows the cell to monitor DNA integrity and cell growth prior to M phase (mitosis) when the cell finally divides. The resulting daughter cells either immediately enter G_1 and may go through the full cycle again, or alternatively stop cycling temporarily and enter the G_0 phase (quiescence) (Planas-Silva and Weinberg, 1997). A number of checkpoints operate at various stages of the cell cycle to ensure events are completed properly and in the correct order.

Progression through the cell cycle is mainly driven by two essential cellular components, cyclin dependent kinases (CDKs) and their cyclin partners. Cyclins were first identified in sea urchin eggs as proteins whose levels oscillated with the cell cycle (Evans *et al.*, 1983). It is now known that each cell contains many different cyclins, each expressed at different stages of the cell cycle. The kinases include CDK4 and CDK6, which associate with the D-type cyclins, and CDK2, which associates with cyclin E (Pavletich, 1999). At the G_1 checkpoint cells have to decide whether or not to commit to DNA synthesis. Here, provided they receive the correct signals, D-type cyclins bind to CDKs 4 or 6 and the resulting complex promotes G_1/S transition by initiating the sequential phosphorylation of the retinoblastoma protein, pRb^{105}, with the cyclin E-CDK2 complex completing the phosphorylation, thus releasing the braking effect of Rb (Harbour *et al.*, 1999). Hypophosphorylated Rb actively blocks cycling by sequestering the

transcription factor, E2F, thus blocking expression of necessary S-phase genes (Dyson, 1998). Once phosphorylated by the cyclin-CDK complexes, E2 F is released and S phase genes are transcribed (**Figure 1.9**).

In response to cellular stress or damage, various external signals and inhibitory proteins can halt the progression of the cycle at the G_1/S boundary and promote growth-arrest and/or apoptosis (see below). Extracellular regulators include the transforming growth factor-β (TGF-β) and interferon-α (IFN-α) (Sangfelt *et al.*, 2000). These pleiotropic factors act in part to suppress Rb phosphorylation, through the inhibition of CDKs and the recruitment of CDK inhibitors (**Figure 1.10**) (Sherr and Roberts, 1999). The INK4 (p15, p16, p18 and p19) and the WAF1 (p21, p27 and p57) families of CDK inhibitors act to block CDK activity at various stages of the cell cycle. p15, $p16^{INK4A}$ and $p27^{Kip1}$ have all been demonstrated to inhibit the Cyclin D-CDK 4/6 complex *in vitro*, whilst $p19^{ARF}$ and $p21^{Waf1}$ are thought to induce cell cycle arrest through interactions with the tumour suppressor gene, p53. However, the picture is not as simple as this, as some CKIs have been shown to be required for cycling by acting as cyclin-CDK assembly factors. Although $p27^{Kip1}$ can inhibit recombinant cyclin D-CDK complexes *in vitro*, it is a far more effective inhibitor of cyclin E-CDK complexes (Toyoshima and Hunter, 1994). Indeed, $p27^{Kip1}$ immunoprecipitates contain a kinase activity with a strong preference for Rb, but not histone H1, a key indicator of cyclin D-CDK activity (Soos *et al.*, 1996). Assembly of cyclin D-CDK complexes is impaired in mouse embryo fibroblasts from mice deficient in $p21^{Waf1}$, $p27^{Kip1}$, or both (Cheng *et al.*, 1999a).

Activation of the tumour suppresser gene, p53, in response to DNA damage from both endogenous and exogenous sources, results in cell cycle arrest in the G_1 phase. This is presumably to allow an opportunity for DNA repair to occur before replication or mitosis (Hartwell and Kastan, 1994). However, in some cell types, including immature B cells, p53 activation results in apoptosis. Anti-Ig treatment of WEHI-231 cells increases the levels of p53 protein, as well as $p21^{WAF1/CIP1}$ (Wu

et al., 1998). The increase in protein levels of $p21^{WAF1/CIP1}$, a downstream transcriptional target of p53, was delayed relative to p53 induction, consistent with the notion that functionally active p53 induces $p21^{WAF1/CIP1}$ expression. This study also showed that ectopic expression of p53 in WEHI-231 cells leads to an induction of apoptosis, in the absence of any further stimulation.

The final outcome of p53 activation appears to depend on the action of a variety of downstream effector genes transactivated by p53. The protective role of p53 is highlighted by the fact that around 50% of all cancers possess an inactive form of p53, or have lost p53 all together. Indeed, the inactivation of various cell cycle proteins including Rb^{105}, $p16^{INK4A}$ and p15 has been implicated in the progression of human cancers, the overall effect of their loss being deregulation of the cell cycle and subsequent excessive proliferation of the cell.

The growth and proliferation of cells is tightly regulated to prevent the production of excessive cell numbers. Programmed cell death, or apoptosis, provides a mechanism for the disposal of "unwanted" cells in a co-ordinated manner (Hengartner, 2000). This mechanism also protects the organism by enabling the destruction of damaged or potentially harmful cells. The classical morphological features of apoptosis include the condensation of chromatin, protein and DNA fragmentation and the formation of apoptotic bodies. Thus, biologically activated apoptosis is quite distinct from necrosis, where cellular death arises due to chemical or physical injury. Most of the observed changes associated with apoptosis (i.e. DNA/protein cleavage, nuclear shrinking, loss of cell shape) are implemented by a set of cysteine proteases, caspases, which were at first thought to be specifically activated in apoptotic cells (Thornberry and Lazebnik, 1998).

The caspases are a family of proteases that possess an active-site cysteine and cleave substrates after aspartic residues. The four residues amino-terminal to the cleavage site determines the distinct substrate specificity of different caspases

(Thornberry *et al.*, 1997). Proteolytic cleavage by caspases can lead to diverse results depending on the nature of the substrate and the exact cleavage site position. Thus, caspases are able to mediate both activation and inactivation of downstream target proteins. Most caspases are activated by proteolytic cleavage of an inactive pro-caspase form. Each pro-caspase contains in its prodomain a protein-protein interaction module, which allows it to bind and associate with upstream regulators. Initiator caspases-8 and -10 contain a death-effector domain (DED), permitting interaction with CD95 and the adaptor molecule FADD, whilst the effector caspases-2 and -9 contain a caspase activation and recruitment domain (CARD). Activation can occur simply by exposure to another previously activated caspase, resulting in a 'caspase cascade' of activation.

Many of the functions of the caspase cascade appear to converge on mitochondria, where the induction of apoptosis has been shown to result in a rapid loss of mitochondrial membrane potential and organelle swelling. However, the fate of a cell is often decided through the activity of the Bcl-2 family of apoptosis regulator proteins (**Figure 1.11**) (Jacobson, 1997). Homo- or heterodimers of pro- and anti-apoptotic Bcl-2 members act at the surface of the mitochondria and compete to regulate cytochrome c release, which, in association with the adaptor Apaf-1, has been implicated in the activation of caspase-9 in the cytosol. This complex results in the activation of caspase-3, another effector caspase, and can be antagonised by the expression of inhibitors-of-apoptosis proteins (IAPs). However, the Smac/DIABLO protein, also released from the mitochondria, negatively regulates IAP family members ensuring that they do not inhibit caspase activation once a cell is committed to apoptosis.

Cleavage of the pro-apoptotic Bcl-2 family member, Bid, by caspase-8 releases an active, truncated form (tBid) which translocates to the mitochondria and promotes the release of cytochrome c (Porter, 1999) (**Figure 1.12**). In addition, interaction of the pro-apoptotic regulator Bad with anti-apoptotic regulators Bcl-2

or Bcl-X_L at the mitochondrial surface promotes apoptosis. In contrast, phosphorylation of Bad by the survival protein Akt promotes the association of Bad with the phospho-serine binding protein, 14-3-3 and its removal from the mitochondria. The blocking of the interaction of Bad with other Bcl-2 family members thus promotes cell survival (Franke and Cantley, 1997).

The role of caspases and other, non-caspase, execution effector proteases has had to be reassessed recently with the observation that, in dense human tonsillar B cells, activation of caspase-8 and caspase-6 are required for proliferation in response to a number of stimuli, including anti-CD40. Indeed, inhibition of caspase-6 with specific inhibitors blocks the transcription of cyclin D and CDK4. In contrast, the activation of caspase-3 is reduced upon stimulation of the cells with proliferative stimuli. These data strongly suggest that a distinct pattern of caspase activation, different from that involved in apoptosis, is induced by proliferative stimuli, with caspase-8 and caspase-6 playing positive roles in proliferation, while caspase-3 plays a negative role in proliferation (Olson *et al.*, 2003).

Support for a negative role of caspase-3 in B cell proliferation comes from studies in caspase-3-deficient mice (Woo *et al.*, 2003). B cells deficient in caspase-3 hyperproliferate, resulting in splenomegaly, though they display no defect in apoptosis. Hyperproliferation of caspase-3-deficient B cells depends upon the CDK inhibitor $p21^{Cip1/Waf1}$. This at first appears paradoxical, as it would be assumed that an increase in the expression of a CDK inhibitor would lead to cell cycle inhibition. However, there is a concomitant increase in the levels of PCNA, which associates with p21 to promote mitosis. Interestingly, caspase-3 cleaves p21 at its C-terminal PCNA binding site, abolishing the interaction between p21 and PCNA (Gervais *et al.*, 1998). Thus, it is clear that apoptosis and proliferation are intimately coupled.

1.6 The role of lipid rafts in immune cells

Immune receptors and components of their signalling cascades are spatially organised, and this spatial organisation plays a key role in the initiation and regulation of signalling. This spatial organisation is due to cholesterol- and sphingolipid-rich plasma membrane microdomains, or lipid rafts. Lipid rafts have been implicated in signal transduction, membrane trafficking and internalisation and other functions initiated at the plasma membrane through their ability to concentrate or exclude proteins and lipid mediators. Signalling molecules found in lipid rafts include dually acylated src family tyrosine kinases, heterotrimeric G protein subunits, adaptor proteins, PIP_2 and lipid kinases and phosphatases.

Lipid rafts are estimated to represent a significant portion of immune cell membranes, greater than 40% by measurements of fluorescence anisotropy of the lipid order in plasma membranes and lipid rafts. In resting cells rafts appear to be highly dynamic, submicroscopic structures (50 nm in diameter) containing only thousands of lipids and a small number of proteins. Upon cross-linking of receptors associated with rafts, lipid rafts become larger, microscopic (100s of nm to μm in diameter), and more stable structures, often attached to the actin cytoskeleton.

In resting B cells the BCR is excluded from lipid rafts. Following cross-linking either by Ig-specific antibodies or antigen, the BCR associates with lipid rafts, and a number of components of the BCR signalling pathways are recruited to rafts (Cheng *et al.*, 1999b). The association of the BCR with lipid rafts is dependent on membrane cholesterol, but it does not require a signalling competent receptor or active Src kinases and is not dependent on the actin cytoskeleton. Disruption of rafts by cholesterol sequestration blocks BCR redistribution but enhances BCR-mediated calcium mobilisation, which suggests that rafts play a role in both enhancing and suppressing B cell responses (Petrie *et al.*, 2000). Indeed, the inositol phosphatase SHIP that inhibits BCR signalling

has been shown to be transiently recruited to lipid rafts following BCR cross-linking (Petrie *et al.*, 2000). The association of the BCR with rafts is transient; by 15 to 30 minutes after cross-linking, the BCR is no longer isolated in rafts. The association of the BCR with rafts is even less stable and more transient when the BCR is unable to initiate signalling or to attach to the actin cytoskeleton, suggesting that signalling and clustering are necessary to stabilise the BCR in rafts (Cheng *et al.*, 2001). The maintenance of lipid rafts in B cells also requires a recently identified protein, Raftlin, the absence of which reduces the quantity of lipid rafts, resulting in an impairment of growth and signalling in Raftlin-deficient B cells (Saeki *et al.*, 2003).

In immature B cells, which respond to antigen by undergoing growth arrest and/or apoptosis, the BCR appears to be excluded from lipid rafts upon antigen ligation (Sproul *et al.*, 2000) (Chung *et al.*, 2001). This is similar to anergic B cells, which also do not undergo proliferation in response to antigen, as the BCR fails to efficiently associate with lipid rafts upon antigen stimulation (Weintraub *et al.*, 2000). This strongly suggests that the failure of the BCR to associate with lipid rafts may contribute to the different biological outcomes of BCR ligation in immature and anergic B cells, compared to mature B cells.

The association of the BCR with rafts is one consequence of BCR ligation that can be regulated by both positive and negative co-receptors. The association of the BCR with lipid rafts is more stable and less transient when the CD19/CD21 co-receptor complex is co-ligated to the BCR via the binding of C3d-tagged antigens (Cherukuri *et al.*, 2001). In contrast, when co-ligated in mature B cells both the BCR and FcγRIIb1 associate with lipid rafts, where FcγRIIb1 recruits the inositol phosphatase SHIP and blocks BCR signalling (Aman *et al.*, 2001). This results in a more transient association of the BCR with rafts.

1.7 Aims and objectives of this study

The signalling events proximal to the BCR initiated in response to BCR ligation are becoming well established, with three key signalling pathways now known to be the major targets. The biological outcomes of BCR ligation at various stages of maturation have also been well established, with immature cells undergoing growth arrest or apoptosis, while mature B cells undergo proliferation and differentiation. The links between these proximal signalling events and the resulting biological response are less well known.

One of the key signalling pathways activated by the BCR is the Erk-MAPKinase pathway. This pathway is controlled by the small GTPase Ras, a key regulator of cell growth in all eukaryotic cells. This study aims to investigate the role of the Ras signalling pathway in the response of immature-transitional B cells to BCR ligation and CD40-mediated rescue of BCR-induced growth arrest and apoptosis. To achieve this, three constitutively active Ras mutants, along with a portion of $p62^{Dok}$, a negative regulator of Ras, and mutants of SHIP, a negative regulator of PI-3-Kinase, will be introduced into the WEHI-231 cell line, and their effects on BCR-induced growth arrest and apoptosis investigated.

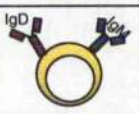
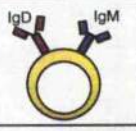
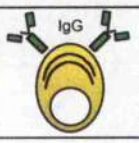
Another key signalling pathway initiated by the BCR is the PLCγ pathway, which results in the production of the second messengers IP_3 and DAG. Key downstream effectors of PLCγ are the PKC family of kinases, two sub-groups of which are activated by DAG. A further aim of this study was to utilise mutants of PKC family members to elucidate the roles of individual PKC family members in immature B cell signalling.

Mature B cells respond to BCR ligation in a very different manner, by undergoing proliferation and differentiation as opposed to growth arrest and apoptosis. It was therefore planned to investigate the initiation and maintenance of proliferation in

primary splenic B cells in response to a variety of signals, including mitogenic stimuli and T cell derived 'help' signals. The key pathways activated by these stimuli will also be examined, both by using specific inhibitors and by recruiting a physiologically relevant negative regulator of BCR signalling, FcγRIIb. Finally, the elements important for BCR-mediated regulation of the cell cycle machinery and the recruitment of nuclear transcription components required for proliferation shall also be investigated.

Figure 1.1 Summary of the development of conventional B cells

The stages of B cell development are marked by a series of changes in location and in the expression of immunoglobulin heavy and light genes, intracellular proteins, and surface markers. B cell development starts in the bone marrow (or foetal liver) with the commitment of haematopoietic stem cells (HSCs) to the B cell lineage. Rearrangement of the heavy chain locus genes begins in the early pro-B stage. Cells are allowed to progress to the next stage if a productive rearrangement has been achieved. Although no functional immunoglobulin is expressed in late pro-B cells, expression of accessory Ig-α/Ig-β heterodimers on the surface in association with calnexin has been demonstrated (Gong and Nussenzweig, 1996; Nagata *et al.*, 1997). The antigen-independent stage continues within the bone marrow, where pre-B cells express a pre-BCR consisting of cytoplasmic μ chain in combination with a surrogate light chain, V_{preB} and λ5. Successful light-chain gene rearrangements result in the surface expression of a complete IgM molecule. The immature B cells then enter the antigen-dependent stage of B cell development where recognition of self-antigen can lead to clonal deletion, receptor editing or clonal inactivation (anergy). Once in the periphery, the mature B cells migrate to the lymphoid follicles and following further selection stages, enter the mature B cell pool until they encounter antigen. Upon interacting with their specific antigen in conjunction with co-stimulatory signals from T_H cells, the B cell is activated. Depending on the nature of the signals, the mature B cell gives rise to antibody generating plasma cells or long-lived memory cells which contribute to lasting protective immunity

B cells		Heavy-chain genes	Light-chain genes	Intra-cellular proteins	Surface Marker proteins
Stem cell		Germline	Germline		Sca1 CD117^{+}
Early pro-B cell		D-J rearranged	Germline	RAG-1 RAG-2 TdT λ5, VpreB	CD117^{+} CD19^{-} CD43^{+} CD45R^{+} CD24^{-}
Late pro-B cell		V-DJ rearranged	Germline	TdT λ5, VpreB	CD117^{-} CD19^{+} CD24^{+} CD25^{-} CD40 CD43^{+} CD45R^{+}
Large pre-B cell		VDJ rearranged	Germline	RAG-1 RAG-2 μ λ5, VpreB	CD43^{-} CD25^{+} CD19^{+} CD24^{++} CD40 CD45R^{+}
Small pre-B cell		VDJ rearranged	V-J rearranged		CD19^{+} CD24^{++} CD25^{+} CD40 CD45R^{+}
Immature B cell		VDJ rearranged μ heavy chain	V-J rearranged		IgM^{++} CD19^{+} CD24^{++} CD25^{-} CD40 CD45R^{+}
T1 B cell		VDJ rearranged μ heavy chain	V-J rearranged		IgM^{+++} CD19^{+} CD24^{++} CD40 CD45R^{+}
T2 B cell		VDJ rearranged μ heavy chain	V-J rearranged		IgM^{++} IgD^{+} CD19^{++} CD24^{+++} CD40 CD45R^{++}
Mature naive B cell		VDJ rearranged μ heavy chain	V-J rearranged		IgM^{+} IgD^{++} CD19^{++} CD23^{+} CD24^{+} CD40 CD45R^{++}
Lympho-blast		VDJ rearranged secreted μ chains	V-J rearranged	IgM	CD19^{++} CD40 CD45R^{++}
Memory B cell		Isotype Switch to Cγ, Cα or Cε hypermut^{n}	V-J rearranged Somatic hypermut^{n}		IgG, IgA CD19^{++} CD40 CD45R^{++}
Plasma cell		Secreted γ, α or ε chains	V-J rearranged	Ig	Plasma cell antigen -1

ANTIGEN INDEPENDENT

ANTIGEN DEPENDENT

BONE MARROW

PERIPHERY

Figure 1.2 The structure of the B cell receptor (BCR) throughout B cell development

The progression of B cells from the pro-B cell stage to the mature B cell is accompanied by changes in the surface expression of the BCR accessory molecules, Ig-α/Ig-β, and associated molecules such as the chaperone molecule, calnexin, and the immunoglobulin heavy chain, μm. Progression through the developmental stages is driven by incremental signalling through these Ig-α/Ig-β-containing receptors, therefore the expression of these molecules as part of receptor complexes is required for normal B cell development. Successful B cell development is dictated by (1) expression of Ig-α/Ig-β in association with calnexin at the late pro-B cell stage. (2) Intact μ heavy chains expressed in an immunoglobulin-like "pre-BCR" complex with surrogate light chains, λ5 and VpreB at the pre-B cell stage. (3) A mature BCR consisting of μ heavy chain, conventional light chains and accessory Ig-α/Ig-β molecules from the immature B cell stage onwards.

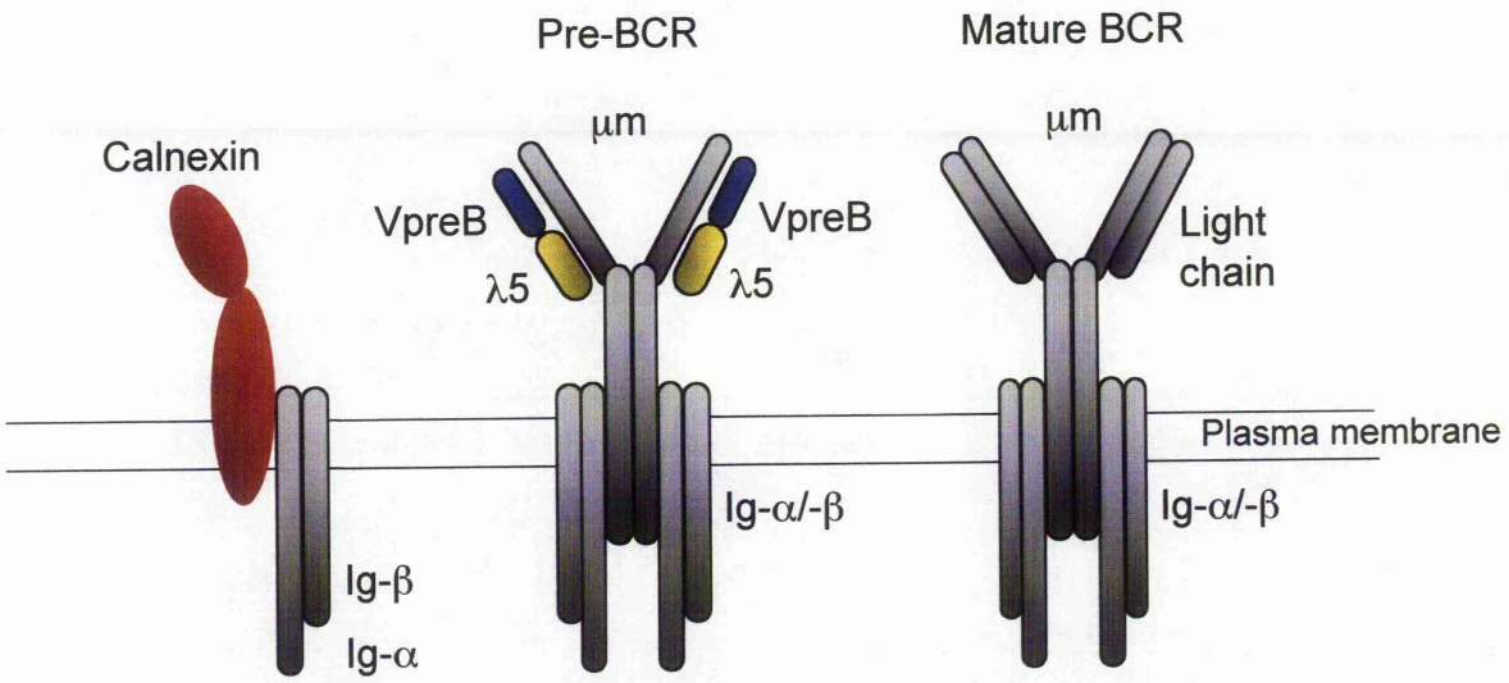

Pre-BCR
Mature BCR
μm
μm
Calnexin
VpreB
VpreB
λ5
λ5
Light
chain
Plasma membrane
Ig-α/-β
Ig-α/-β
Ig-β
Ig-α

Figure 1.3 B cell activation and selection in germinal centres

Following T cell dependent activation (1) B cells migrate from the follicular mantle into the primary lymphoid follicles and form germinal centres. Here, B cells undergo proliferation (2) and differentiate into centroblasts (3) where they form the dark zone of the germinal centre. The rapidly dividing centroblasts undergo somatic hypermutation of their immunoglobulin variable-domain genes before differentiating into centrocytes (4). Within the light zone of the germinal centre, the small, non-dividing centrocytes are programmed to die unless they interact with follicular dendritic cells (FDC) that display complexed antigen on their cell surface. Positive selection of centrocytes is dependent on the affinity of their mutated antigen receptors. Centrocytes with low affinity or autoreactive antigen receptors undergo spontaneous apoptosis. The positively selected centrocytes move to the outer edge of the light zone and interact with CD40 ligand expressing T cells (5). Here the centrocytes may undergo CD40-mediated isotype switching, become protected from Fas-induced apoptosis and finally differentiate into either memory B cells (6) or plasma cells (7).

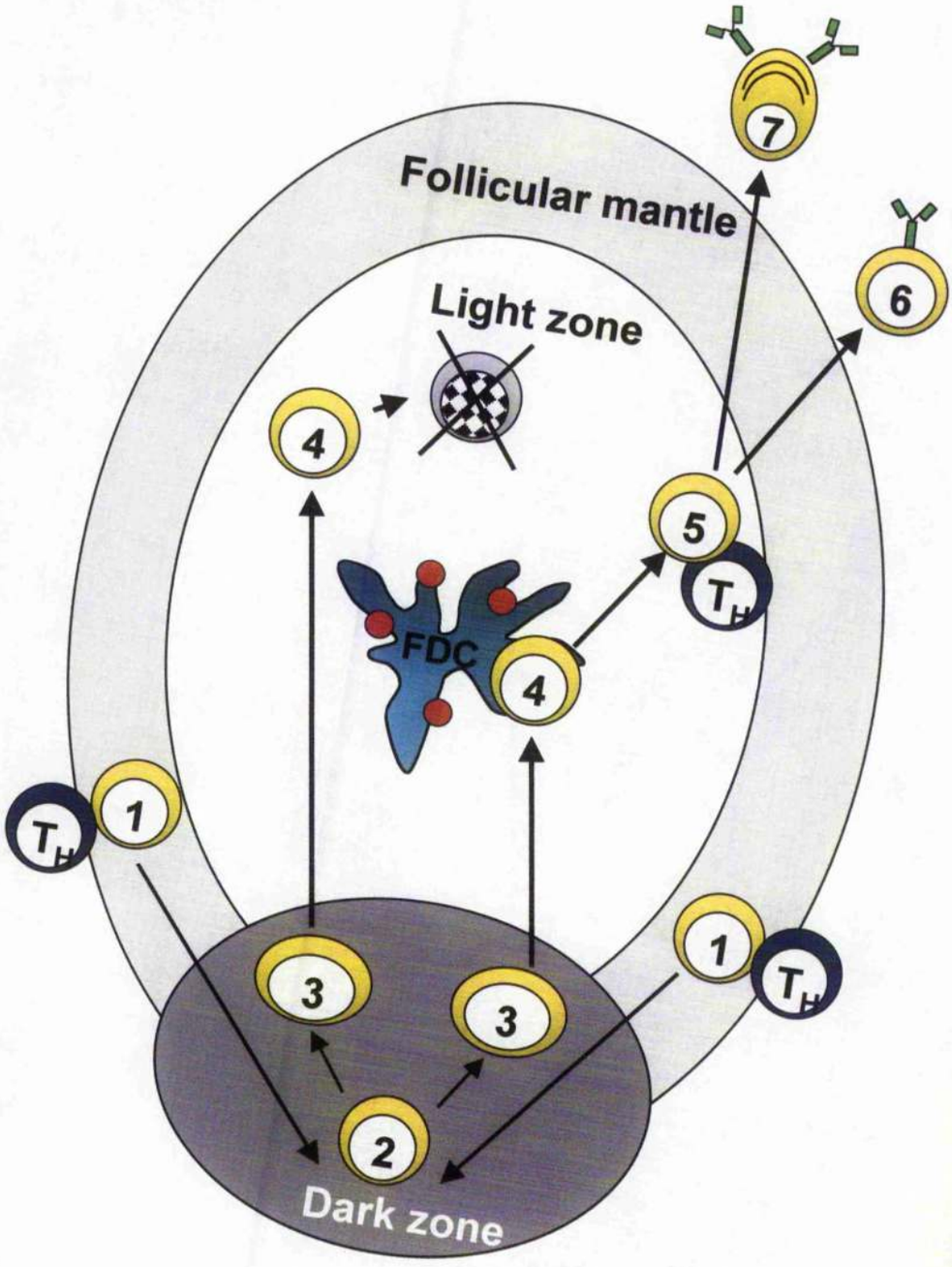

Follicular mantle
Light zone
Dark zone
FDC
7
6
5
4
4
3
3
2
1
1
T_H
T_H
T_H

Figure 1.4 The mature B cell receptor

The mature B cell receptor for antigen (BCR) is functionally divided into the immunoglobulin molecule (sIg), which is responsible for ligand binding, and the Ig-α (CD79a) and Ig-β (CD79b) accessory molecule heterodimers, which are responsible for signal transduction. Conserved immunoreceptor tyrosine-based activation motifs (ITAMs), present in the cytoplasmic domains of the accessory molecules, are essential for the signal transducing capacity of the receptor. The ITAMs aid the recruitment and activation of three distinct types of non-receptor protein tyrosine kinases (PTKs).

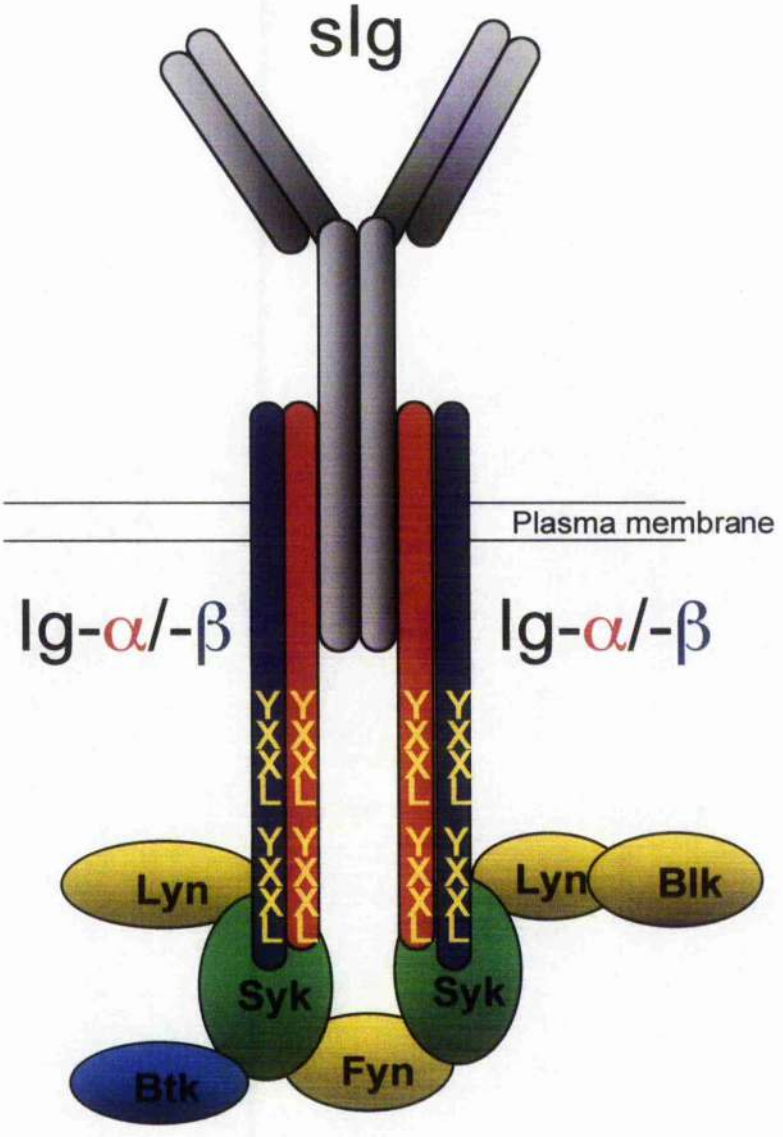

sIg
Plasma membrane
Ig-α/-β
Ig-α/-β
Lyn
Lyn
Blk
Syk
Syk
Fyn
Btk

Figure 1.5 Signal transduction pathways initiated by ligation of the BCR

A schematic representation of the parallel cascades initiated following ligation of the B cell receptor (BCR) on mature B cells. The tyrosine phosphorylation of conserved ITAMs, present in the cytoplasmic domains of the sIg accessory molecules Ig-α and Ig-β, results in the recruitment of sIg associated PTKs. These include the Src-PTK family (Blk, Fyn, Lck, and Lyn), Syk, and Btk. Following activation of these kinases three parallel, but potentially cross-regulatory, pathways are recruited to the activated BCR complex. The phospholipase C-γ (PLC-γ) pathway results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2), to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$). The phosphatidylinositol 3-kinase (PI-3-Kinase) pathway generates phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P_3) whilst the classical Ras/MAPKinase cascade leads to the activation of Erk-MAPKinase. These pathways converge on the nucleus to initiate a cellular response.

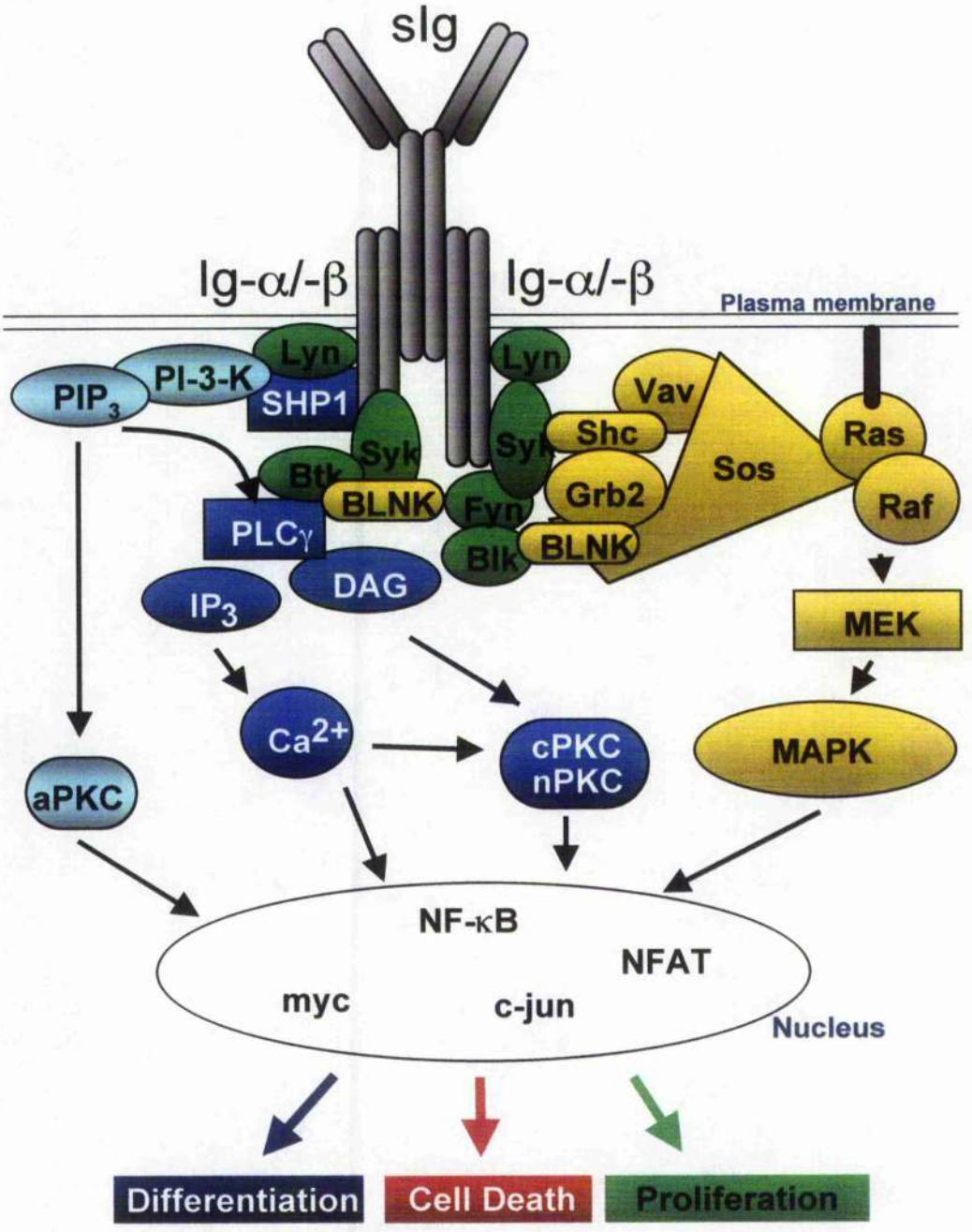

sIg
Ig-α/-β
Ig-α/-β
Plasma membrane
Lyn
PI-3-K
PIP3
SHP1
Lyn
Vav
Shc
Ras
Syk
Syk
Sos
Btk
BLNK
Fyn
Grb2
Raf
PLCγ
Blk
BLNK
IP3
DAG
MEK
Ca2+
cPKC
nPKC
MAPK
aPKC
NF-κB
NFAT
myc
c-jun
Nucleus
Differentiation
Cell Death
Proliferation

Figure 1.6 Mitogen-activated protein kinase (MAPKinase) signalling pathways

The mitogen-activated protein (MAP) kinases are a family of serine-threonine protein kinases that have been widely conserved throughout evolution. They are activated by a wide range of extracellular stimuli and are able to mediate a wide range of cellular functions ranging from proliferation and activation to growth arrest and cell death. The MAPKinase family is subdivided into three groups; the classical extracellular signal-regulated kinases (Erk-MAPKinase), the c-Jun N-terminal kinases, also known as the stress activated protein kinases (JNK/SAPK) and the p38 MAPKinases. Activation of each group is determined by distinct upstream MAPKinase kinases (MEKs) and MAPKinase kinase kinases (MEKK). MAPKs are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-X-Y motif, where X is different in each group. Following MAPKinase activation, activation of a number of downstream transcription factors occurs; Erk-MAPKinase activates Elk-1 and c-myc, JNK activates c-Jun and ATF-2 and p38 MAPKinase activates ATF-2 and MAX. The phosphorylation and activation of these transcriptional regulators enables the MAPKinase families to regulate gene expression and hence, cellular responses.

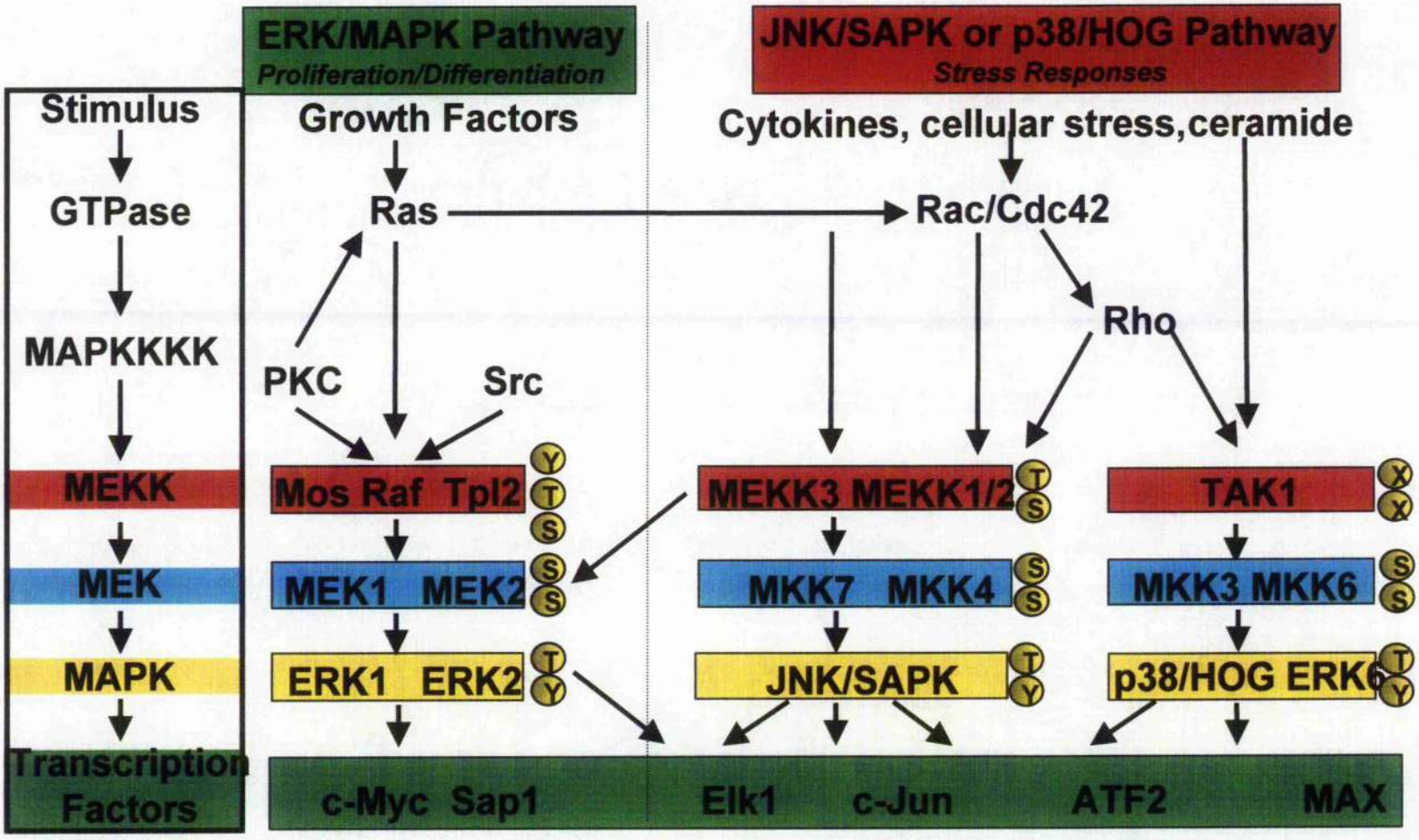

ERK/MAPK Pathway
Proliferation/Differentiation
JNK/SAPK or p38/HOG Pathway
Stress Responses
Stimulus
GTPase
MAPKKKK
MEKK
MEK
MAPK
Transcription Factors
Growth Factors
Cytokines, cellular stress,ceramide
Ras
Rac/Cdc42
Rho
PKC
Src
Mos Raf Tpl2
MEK1 MEK2
ERK1 ERK2
MEKK3 MEKK1/2
MKK7 MKK4
JNK/SAPK
TAK1
MKK3 MKK6
p38/HOG ERK6
c-Myc Sap1
Elk1
c-Jun
ATF2
MAX

Figure 1.7 Structure of CD40

The CD40 receptor is a 48-kDa transmembrane glycoprotein and is a member of the TNF receptor (TNFR) superfamily. Structurally, human CD40 consists of a 193 amino-acid extracellular domain, a 22 amino-acid transmembrane domain and a 62 amino-acid cytoplasmic domain. The extracellular domain consists of four homologous, repeating, cysteine-rich extracellular domains characteristic of TNFR family motifs. CD40 is known to associate with intracellular proteins termed TNF receptor-associated proteins (TRAFs). TRAF2, TRAF3 and TRAF 5 are known to associate with a specific region in the cytoplasmic domain of CD40.

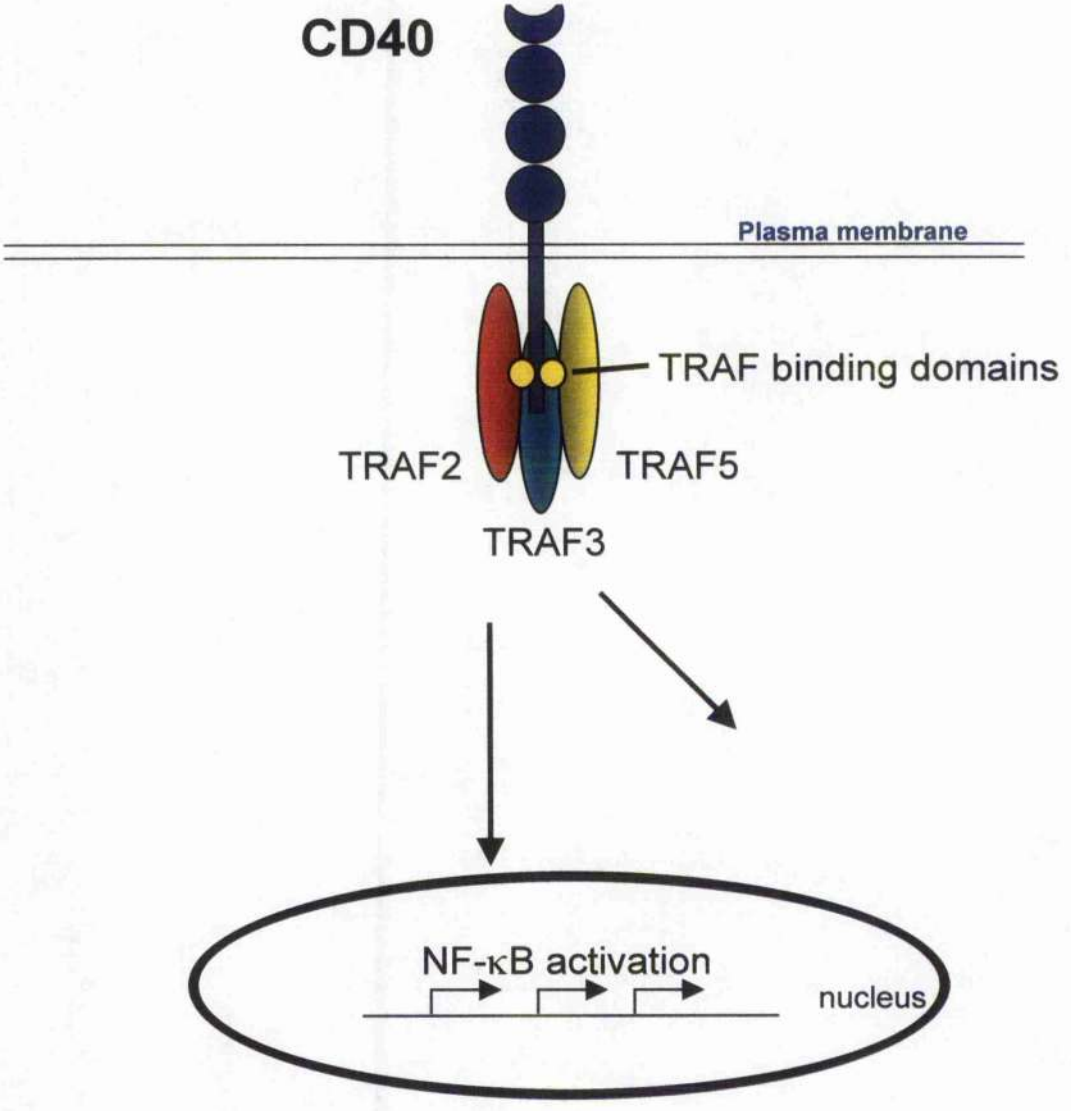

CD40
Plasma membrane
TRAF binding domains
TRAF2
TRAF5
TRAF3
NF-κB activation
nucleus

Figure 1.8 Molecular basis for the inhibitory activity of FcγRIIb

FcγRIIb (CD32) is a single chain, low affinity receptor for the Fc domain of IgG molecules, and as such can only interact with IgG in the form of immune complexes. It is the only Fcγ receptor found on B cells, and contains a 13 amino acid inhibitory ITIM motif in its cytoplasmic domain that is responsible for its inhibitory effects on BCR signalling. Co-ligation of the BCR and FcγRIIb by cognate antigen-antibody complexes leads to tyrosine phosphorylation of the ITIM by the Src-family kinase Lyn, and subsequent recruitment of the protein phosphatases SHP-1, SHP-2 and the inositol phosphatase SHIP. The overall outcome of the recruitment of these molecules is the abrogation of the 3 key signalling pathways activated upon BCR ligation.

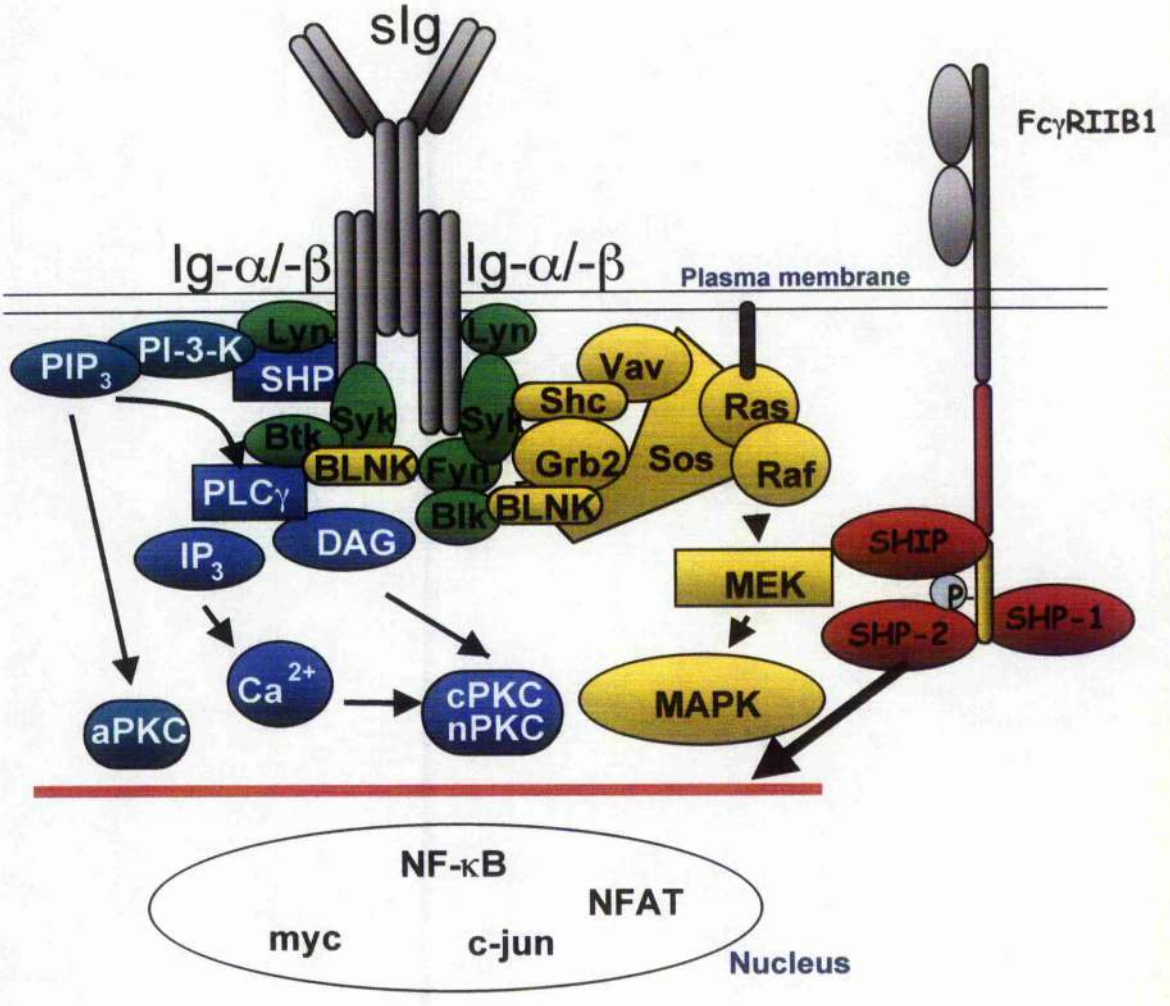

sIg
FcγRIIB1
Ig-α/-β
Ig-α/-β
Plasma membrane
PIP3
PI-3-K
Lyn
SHP
Lyn
Vav
Shc
Ras
Syk
Syk
Btk
BLNK
Fyn
Grb2
Sos
Raf
PLCγ
Blk
BLNK
IP3
DAG
SHIP
MEK
P
SHP-2
SHP-1
Ca2+
cPKC
nPKC
aPKC
MAPK
NF-κB
NFAT
myc
c-jun
Nucleus

Figure 1.9 The cell cycle

The cell cycle is the co-ordinated series of events required for cell growth and division. There are four main stages of the cycle, during which a cell must duplicate its contents and divide. G_1 is characterised by gene expression and protein synthesis, resulting in an increase in cell size and production of all the proteins required for DNA synthesis. DNA duplication occurs in the S phase (synthesis). After chromosome replication a second growth period, G_2, allows the cell to monitor DNA integrity and cell growth prior to M phase (mitosis) when the cell finally divides. The resulting daughter cells either immediately enter G_1 and may go through the full cycle again, or alternatively stop cycling temporarily and enter the G_0 phase (quiescence). Cyclins and their activation of cyclin-dependent kinases (CDKs) are essential for the regulation of the cell cycle. At the G_1 checkpoint cells have to decide whether to commit to DNA synthesis. Here, D-type cyclins bind to CDKs 4 or 6 and the resulting complex promotes G_1/S transition by initiating phosphorylation of the retinoblastoma protein, pRb^{105}, before cyclin E and CDK2 catalyse further phosphorylation events, thus releasing the braking effect Rb applies. Hypophosphorylated Rb actively blocks cycling by sequestering the transcription factor, E2F, thus blocking expression of necessary S-phase genes. Once phosphorylated by the cyclin-CDK complexes, E2-F is released and S phase genes are transcribed.

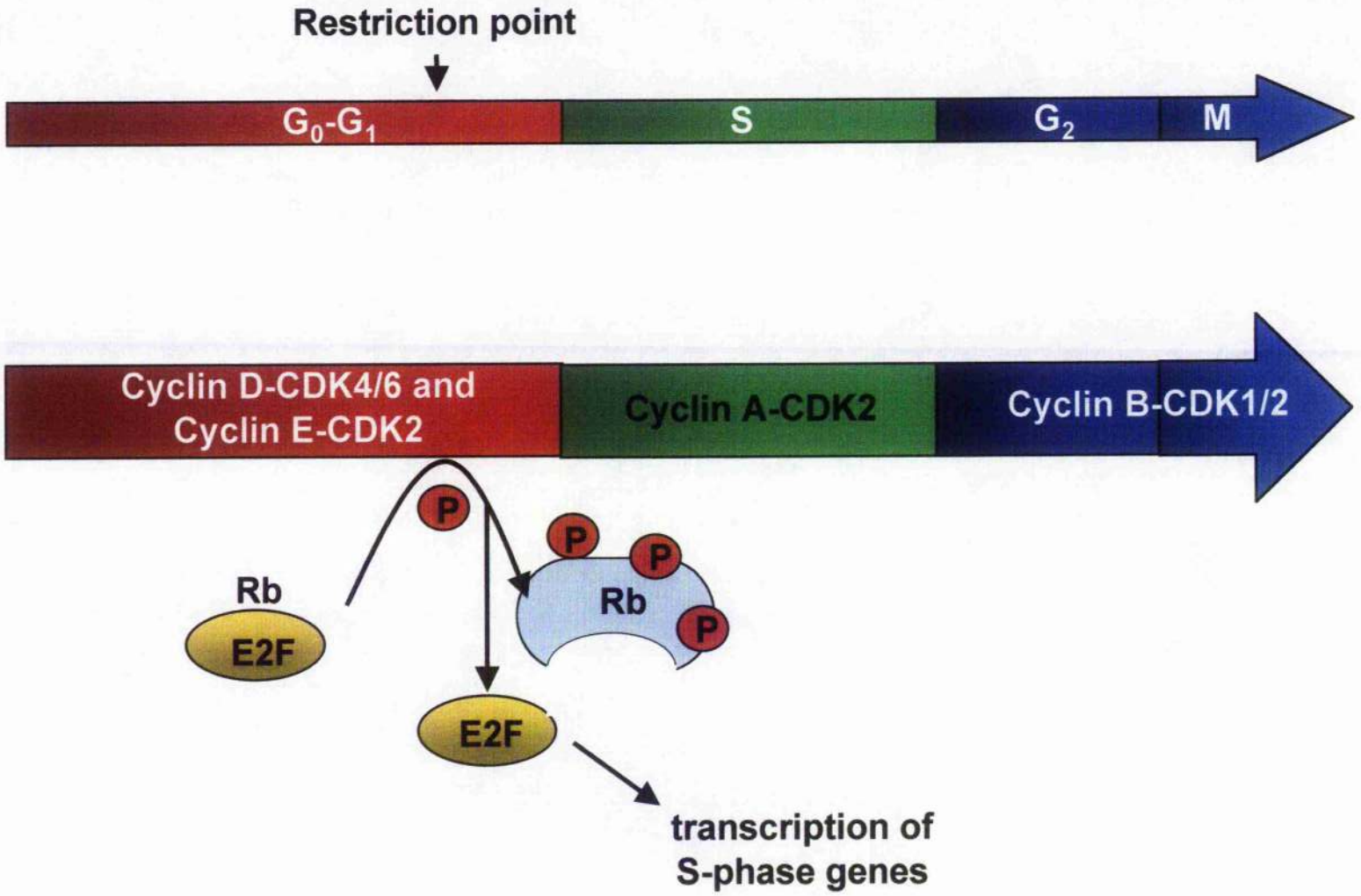

Restriction point
G0-G1
S
G2
M
Cyclin D-CDK4/6 and
Cyclin E-CDK2
Cyclin A-CDK2
Cyclin B-CDK1/2
P
P
P
P
Rb
E2F
Rb
E2F
transcription of
S-phase genes

Figure 1.10 The role of cyclin-dependent kinase inhibitors

During G_1 phase of the cell cycle, D-type cyclins bind to CDKs 4 or 6 and the resulting complex phosphorylates key residues of the retinoblastoma protein, pRb^{105}, allowing CDK2-cyclin E complexes to phosphorylate further key residues. Once Rb is phosphorylated by the cyclin-CDK complexes, E2-F is released and S phase genes are transcribed. The activity of the cyclin-CDK complexes can be inhibited by CDK inhibitory proteins of the INK4 (p15, p16, p18 and p19) and the WAF1 (p21, p27 and p57) families, blocking entry into S phase. These CDK inhibitors (CKIs) can be induced by a number of factors, one of which is the tumour suppressor protein, p53. Activation of p53, in response to DNA damage from both endogenous and exogenous sources, results in cell cycle arrest in the G_1 phase as a consequence of the induction of the CKIs p21 and p27.

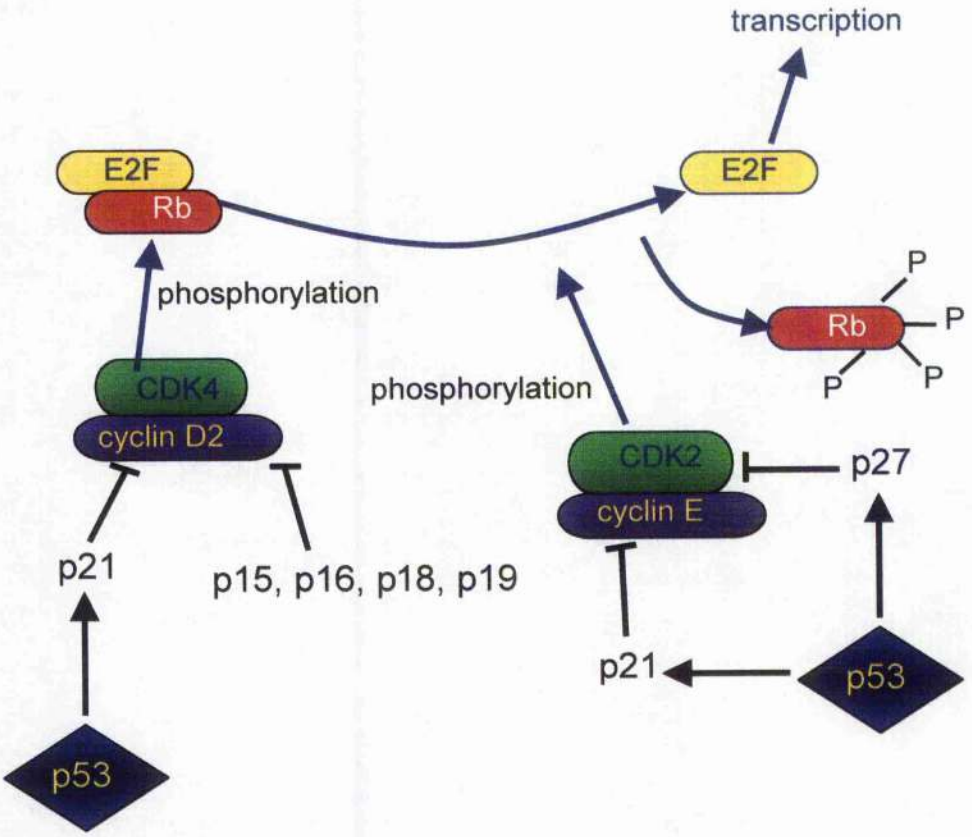
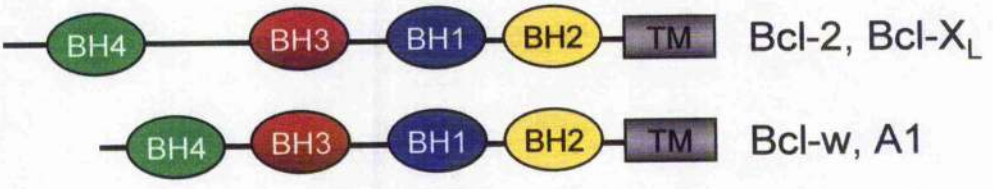

transcription
E2F
Rb
E2F
phosphorylation
P
P
P
P
Rb
CDK4
cyclin D2
phosphorylation
CDK2
cyclin E
p27
p21
p15, p16, p18, p19
p21
p53
p53

Figure 1.11 The Bcl-2 family of apoptosis regulators

The Bcl-2 family of apoptotic regulators is comprised of over a dozen proteins, which have been classified into three functional groups. Bcl-2 family members are recognised due to the presence of one or more conserved Bcl-2 homology (BH) domains. Group I members all possess anti-apoptotic activity, thus protect cells from death, and contain one each of the four BH domains, as well as a transmembrane domain allowing their insertion into the mitochondrial membrane. Members of group II and III promote cell death, hence are known as pro-apoptotic. Pro-apoptotic Bcl-2 family members have fewer BH domains, indeed some contain only a single BH3 domain. Many family members can homodimerise, but more importantly, pro- and anti-apoptotic members can form heterodimers to antagonise each other's activity. For example, pro-apoptotic Bax can heterodimerise with the anti-apoptotic protein, Bcl-2. This interaction blocks the anti-apoptotic capabilities of the Bcl-2 protein and induces a pro-apoptotic response.

Anti-apoptotic Bcl-2 family members (Group I)



Pro-apoptotic Bcl-2 family members (Groups II/III)

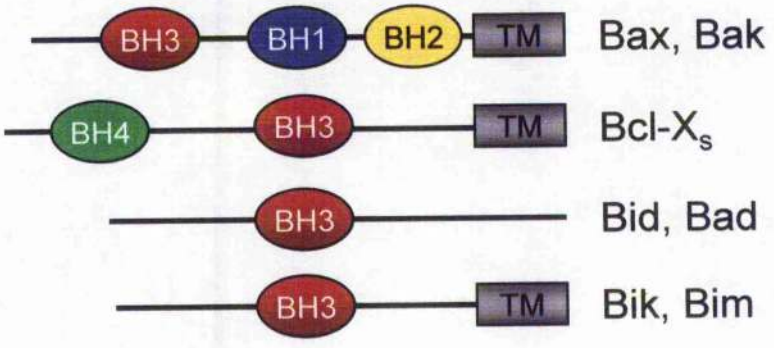
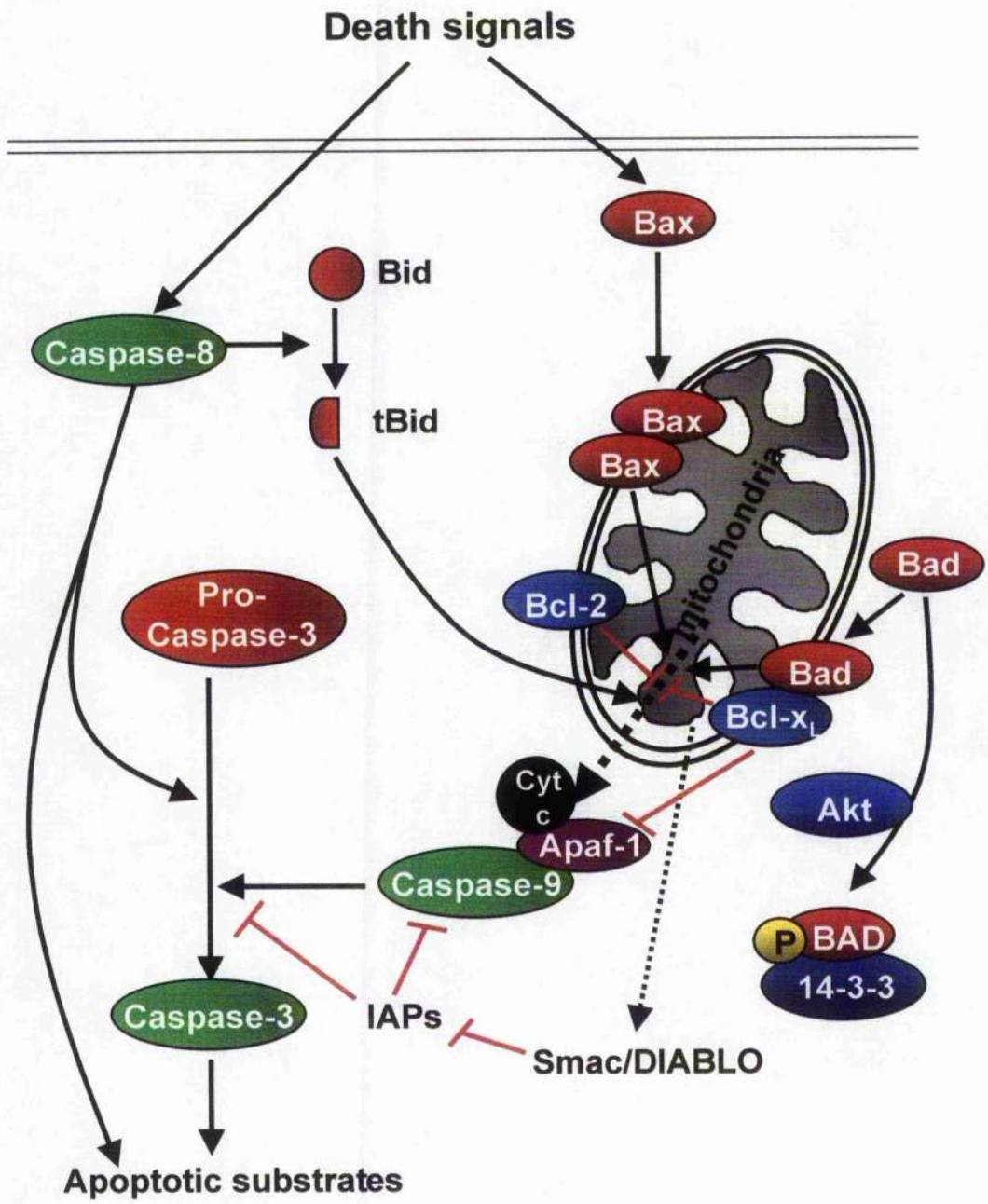


Figure 1.12 The role of the mitochondria during apoptosis

Many cell death-inducing signals appear to converge on the mitochondria, which plays a pivotal role in the decision of a cell's fate. The Bcl-2 family proteins form homo- or heterodimers and integrate competing pro- and anti-apoptotic signals at the surface of the mitochondria and other sites within the cell. The pro-apoptotic members of the Bcl-2 family have been proposed to act by inactivating dimers of anti-apoptotic Bcl-2 family members, which act to preserve mitochondrial integrity. For example, following apoptotic signals, the pro-apoptotic regulator Bax, is believed to heterodimerise with the anti-apoptotic regulator Bcl-2. This interaction blocks the anti-apoptotic capabilities of the Bcl-2-like proteins. Similarly, the pro-apoptotic regulator Bad is believed to interact with Bcl-2 or Bcl-X_L, thus promoting apoptosis. Phosphorylation of Bad by the survival factor Akt promotes the association of Bad with the phospho-serine binding protein, 14-3-3 and its removal from the mitochondria. The blocking of the interaction of Bad with its other family members thus promotes cell survival. Pro-apoptotic signals also result in the cleavage of Bid by caspase-8, which releases an active, truncated form (tBid). tBid then translocates to the mitochondria, where it is involved in promoting the release of cytochrome c. The released cytochrome c associates with the adaptor Apaf-1 and together they activate caspase-9 in the cytosol. The death and mitochondrial pathways converge at the level of caspase-3 activation. Caspase 3 activation and activity is antagonised by the expression of inhibitors-of-apoptosis (IAPs), which in turn are inhibited by Smac/DIABLO proteins, also released from the mitochondria.


Death signals
Bax
Bid
Caspase-8
tBid
Bax
Bax
Mitochondria
Bad
Pro-
Caspase-3
Bcl-2
Bad
Bcl-x$_L$
Cyt
c
Akt
Apaf-1
Caspase-9
P BAD
14-3-3
Caspase-3
IAPs
Smac/DIABLO
Apoptotic substrates

Chapter 2 - Materials and Methods

2.1 Cell culture reagents and antibodies

All cell culture reagents were purchased from Invitrogen Life Technologies. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated, and were of the highest grade available. See **Tables 2.1** and **2.2**.

2.2 Animals

Male Balb/c mice, at least 7 weeks of age, were used unless otherwise stated. These mice were maintained at the Central Research Facility (CRF), University of Glasgow. Mice, arriving from Harlan UK Ltd, were quarantined for 7 days prior to commencing any experiment.

2.3 Purification of murine splenic B cells

Primary B cells were prepared from murine spleens using the CD43-magnetic bead negative-selection method of Miltenyi Biotec (Deehan *et al.*, 2002). All procedures were performed at 4°C. A single cell suspension was prepared by mashing the spleens through a wire mesh (Sigma-Aldrich), in RPMI-1640 media. The resultant suspension was centrifuged (400 x g, 7 min, 4°C) and the pellet resuspended in 9 ml of red blood cell removal buffer (0.168 M NH_4Cl, pH 7.2). The suspension was carefully layered over 1 ml heat-inactivated foetal calf serum (FCS) and incubated on ice for 7 min to permit red blood cell lysis and lipid precipitation. The supernatant was removed, carefully layered over 1 ml FCS in a fresh tube and centrifuged again (400 x g, 7 min, 4°C).

The cell pellet was resuspended in 9 ml dead cell removal buffer (HEPES-buffered, mouse tonicity, balanced salt solution (BSS) supplemented with 0.12 M Sorbitol, 20 mM Glucose) (Shortman *et al.*, 1972; von Boehmer and Shortman, 1973). The suspension was immediately filtered through two prepared dead cell removal columns (absorbent cotton wool plugged, short-form, glass pipettes, wetted with 1 ml RPMI/5% FCS). Cells were recovered from the column into a 15 ml tube on ice containing 1 ml RPMI/5% FCS. The cells were centrifuged (400 x g, 7 min, 4°C) and resuspended in 50 ml ice-cold MACS buffer (phosphate buffered saline (PBS), 0.5% BSA, 2 mM EDTA) counted by Trypan blue exclusion, and pelleted by centrifugation (400 x g, 7 min, 4°C).

The cells were resuspended in ice-cold MACS buffer (2×10^8 cells/ml) and passed through gauze, to produce a single cell suspension for labelling. Cells were incubated for 25 min at 4°C with anti-CD43 (Ly-48) beads (100 µl CD43+ beads/2×10^8 cells). Labelled cells were passed through gauze again and applied to a CS-type negative selection magnetic column (Miltenyi Biotec) in a strong magnetic field. Purified mature B cells (CD43-) were eluted from the column by washing with 50 ml ice-cold MACS buffer. The cells were centrifuged (400 x g, 7 min, 4°C), resuspended in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine, counted and assessed for purity by FACS analysis. Typically, around 40 % of the mononuclear cells were B cells, with a purity >98 % B220 positivity after negative selection (as assessed by flow cytometry – **Figure 2.1 C and D**).

2.4 Purification of anti-CD40 antibody from the FGK45 hybridoma and anti-IgM antibody from the B7.6 hybridoma

Anti-CD40 antibody (rat anti-mouse) was purified from the FGK45 hybridoma, and anti-IgM antibody (rat anti-mouse) was purified from the B7.6 hybridoma, as described previously (Gilbert *et al.*, 1998). FGK45 and B7.6 hybridoma cells were

maintained in RPMI-1640 medium containing 5 % foetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), and 2-mercaptoethanol (50 μM) (RPMI complete) at 37°C in 5 % CO_2. Culture supernatants were collected for purification of the appropriate antibody, and filter sterilised immediately prior to use. A protein G sepharose column was washed twice with binding buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, pH 7 at 25°C) and the appropriate antibody supernatant solution passed through the column overnight at 4°C to allow the immunoglobulin to bind to the column. The column was then washed twice with binding buffer, before eluting the bound antibody with elution buffer (0.1 M glycine, pH2.7). Eluted antibody was collected in 1.5 ml microcentrifuge tubes containing 0.5 ml 1 M Tris buffer, and the protein concentration of each aliquot assessed by spectrometry. Fractions whose protein concentration was greater than 0.1 mg/ml were pooled, and dialysed for 48 hours at 4°C against 1 x PBS. The purity of the antibody was assessed by SDS-PAGE, final protein concentration measured by spectroscopy and the antibody filter sterilised prior to being aliquoted and stored at -20°C.

2.5 Cell Culture

The murine B cell lymphoma WEHI-231 has the surface phenotype of an immature B lymphocyte (membrane (m) IgM^+, $mIgD^{-/low}$, FcR^{low}, Fas^{low}, and MHC class II^{low}), and was obtained from the European Collection of Cell Cultures (ECACC; CAMR, Porton Down, U.K.). WEHI 231 cells were cultured in RPMI-1640 medium containing 5 % foetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), and 2-mercaptoethanol (50 μM) (RPMI complete) at 37°C in 5 % CO_2. All cell culture reagents were obtained from Invitrogen Life Technologies.

2.6 [^{3}H]-Thymidine DNA synthesis assay

Proliferation of WEHI-231 cells and primary cells was assessed by the tritiated thymidine uptake assay to evaluate DNA synthesis. Briefly, WEHI-231 cells (1 x 10^4 cells/well) or purified B cells (2 x 10^5 cells/well) were cultured in triplicate in round bottomed microtitre plates in RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-Mercaptoethanol (2-Me). Cells were stimulated in the presence of the appropriate agonists in a final well volume of 200 μl. Cells were cultured for 48 hours at 37°C in a 5% (v/v) CO_2 atmosphere at 95% humidity.

DNA synthesis was assessed by pulsing with 0.5 μCi/well [6-^{3}H]-thymidine (Amersham Pharmacia Biotech) for the last four hours of culture, to allow incorporation into cellular DNA. Cells were harvested onto glass fibre filter mats (Wallac) using a Betaplate 96-well harvester (Amersham Pharmacia Biotech). Incorporated label was assessed by liquid scintillation counting and results are expressed as counts per minute (c.p.m) incorporated ± SD.

2.7 Cell stimulation and preparation of cell lysates

WEHI-231 cells or splenic B cells (1 x 10^7) were stimulated for the times indicated at 37°C. Control stimulations were carried out in the presence of medium alone (RPMI-1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-Mercaptoethanol (2-Me)). Following incubation, cells were lysed by the addition of ice-cold, modified RIPA buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 2% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate plus 0.5 mM

phenylmethylsulfonyl fluoride, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml antipain and 10 μg/ml pepstatin). Whole cell lysates were incubated on ice for 30 min to permit protein extraction. Cellular debris was removed by centrifugation (20000 x g, 30 min, 4°C) and the supernatants transferred to fresh tubes, either for immediate use or stored at -20°C until required.

2.8 Immunoprecipitation

A BCA Protein Assay kit (Pierce PerBio) was used to assess the protein concentration of whole cell lysates. Samples were normalised to 1 mg/ml with 2 x modified RIPA buffer and then pre-cleared with 10 μl of a 50% protein-G-sepharose:RIPA slurry for 30 min at 4°C with constant rotation. Following centrifugation (13,000 x g, 15 min, 4°C), the pre-cleared supernatant was transferred to a fresh tube and incubated with the appropriate immunoprecipitating antibody (1-2 μg/sample) overnight at 4°C with constant rotation.

The protein-antibody complex was pulled down with 10 μl of protein G-sepharose for 2 hours at 4°C with constant rotation. Protein-sepharose complexes were harvested by centrifugation (13,000 x g, 30 min, 4°C) and washed three times in ice-cold modified RIPA buffer. The samples were then dissociated from the sepharose beads by boiling in 50 μl of 2 x sample loading buffer (100 mM Tris pH 6.8, 4% (v/v) bromophenol blue, 0.2% SDS and 20% v/v glycerol) containing 5% 2-Me for 10 min at 100°C, followed by pulse centrifugation to pellet the sepharose beads. Supernatants were resolved by SDS-PAGE.

2.9 SDS-Polyacrylamide gel electrophoresis

Cell lysates or immunoprecipitates were resolved by Tris-glycine, SDS-PAGE (Laemlli, 1970). Pre-cast NuPAGE® 10% bis-tris gels were purchased from Invitrogen Life Technologies and assembled into the gel apparatus according to the manufacturer's instructions, and each well rinsed with 1 x MOPS (3-(N-morpholino) propane sulfonic acid) electrophoresis buffer (50 mM MOPS, 50 mM Tris, 3.5 mM SDS, 1 mM EDTA) prior to sample loading. Equal protein loadings of cell lysates or immunoprecipitates were resuspended in an appropriate volume of NuPAGE® LDS sample buffer (Invitrogen Life Technologies). Immediately prior to loading, NuPAGE® reducing agent was added to each sample (0.5 mM dithiothreitol, DTT) and samples denatured by heating to 70°C for 10 minutes. Samples were loaded into individual wells, along with the appropriate positive control proteins into one or two wells, and molecular weight markers into one well to elucidate the molecular weights of unknowns. The samples were electrophoresed into the stacking gel at 100V and then run at 200V through the resolving gel for approximately 60 minutes.

2.10 Western blotting

SDS-PAGE resolved proteins were transferred to nitrocellulose using the Invitrogen Life Technologies apparatus. The foot and the wells of the gel were removed and the gel transferred to two sheets of filter paper, pre-wetted with NuPAGE® transfer buffer (25 mM bicine, 25 mM bis-Tris, 1 mM EDTA, 50 μM chlorobutanol) (Invitrogen Life Technologies), then placed on top of two sponges in the blotting module. The nitrocellulose (Amersham Pharmacia Biotech) was placed on top of the gel and a further two sheets of pre-wetted filter paper placed on top. Any air bubbles were rolled out before completing the gel/filter sandwich, and the blotting module sealed and placed in the running tank. The blotting

module was topped up with transfer buffer and surrounded with distilled water to act as a coolant. The proteins were transferred at a constant voltage (30V) for 90 minutes.

The nitrocellulose membranes were then incubated in modified TBS buffer I (TBS, pH 7.4, containing 5% non-fat milk and 0.1% Tween-20) for at least 1 hour at room temperature to block non-specific protein binding. Primary antibodies were diluted, at concentrations recommended by the manufacturer, in TBS buffer I or TBS buffer II (TBS, pH 7.4, containing 5% BSA and 0.1% Tween-20) according to the manufacturer's instructions, and incubated with the membranes overnight at 4°C. Membranes were washed four times in TBS buffer (TBS, pH 7.4, and 0.1% Tween-20) before incubating with the appropriate HRP-conjugated secondary antibody in modified TBS buffer I for 1 hour at room temperature. The membranes were then washed six times in TBS buffer and the immuno-reactive protein bands visualised using the Enhanced ChemiLuminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech) and exposed to X-OMAT film (Kodak).

2.11 Flow cytometric analysis

2.11.1 Cell surface markers

Cells (0.5-1.0 x 10^6) were washed twice in 5 ml tubes (Becton Dickinson) with 2 ml cold FACS buffer (PBS, 1% BSA, 0.1% sodium azide) at 400 x g for 5 min, 4°C. Cells were resuspended in 50 µl of an anti-Fc receptor blocking solution (supernatant from the 2.4G2 rat anti-mouse FcγRII/III hybridoma, this is required as B cells express the FcγRII receptor which has affinity for the constant region of IgG molecules) or 50 µl of FACS buffer for 10 min on ice. Cells were incubated with 50 µl of primary antibody, at a concentration previously determined by titration of optimum binding, for 20 min on ice. Cells were washed with 2 ml FACS buffer (400 x g, 5 min, 4°C) and then resuspended in 300 µl FACS buffer if

the antibody was directly conjugated to a fluorescent molecule. For non-directly conjugated primary antibodies, the staining procedure was repeated with the addition of the secondary antibody. Immediately prior to data acquisition 50 μg/ml propidium iodide (Calbiochem) was added to enable exclusion of dead cells from the analyses. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and analysed using Cell Quest software (Becton Dickinson) or FlowJo software (Tree Star, Inc.) (**Figure 2.1**).

2.11.2 Cell cycle analysis

To determine the percentage of cells at a particular stage of the cell cycle, propidium iodide (PI) staining of cells was undertaken. Propidium iodide intercalates into DNA in a stoichiometric fashion, enabling DNA content to be assessed as this will be proportional to fluorescence. Primary B cells (5×10^5) were washed twice in ice-cold FACS buffer then fixed with 100 μl ice-cold 70% ethanol for 15 min at 4°C. The cells were washed in FACS buffer and then incubated with propidium iodide (250 μg/ml) plus Ribonuclease A (50 μg/ml) for 35 min at room temperature.

WEHI-231 cells (5×10^5) were washed twice in ice-cold FACS buffer. Cells were resuspended in 200 μl ice cold PI hypotonic buffer (0.1% (w/v) sodium(tri) citrate, 0.1% Triton X-100 (v/v), 50 μg/ml propidium iodide, and 50 μg/ml Ribonuclease A) and incubated for 45 minutes at room temperature. WEHI-231 cells were stained by a different method as it was found that they displayed a high level of auto-fluorescence after treatment with ethanol. The PI fluorescence of the stained cells was measured at an excitation wavelength of 488 nm. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibur™ flow cytometer and analysed using Cell Quest software.

To determine the percentage of cells in G_0/G_1 phase, S phase, G_2/M phase of the cell cycle, or containing a sub-diploid DNA content, linear FL-3 voltage settings

were used (**Figure 2.2**). The FL-3 voltage settings were altered until the G_1 peak (2N DNA) was approximately 300 fluorescence units, and a marker was set at either side of the peak. The upper and lower 2N DNA peak values were then doubled to produce the upper and lower 4N DNA peak values and markers set. Sub-diploid DNA or apoptotic cells were determined by setting markers below the 2N peak, whilst cells in the S phase were taken as those between the 2N and 4N peaks.

2.11.3 Mitochondrial membrane potential

Incorporation of the cationic lipophilic dye $DiOC_6(3)$ into the mitochondria is proportional to the mitochondrial transmembrane potential, $\Delta\psi m$ (Zamzami *et al.*, 1995). Cells were stained for surface markers, as previously described, and then washed with FACS buffer. Cells were then incubated with 2.5 μM $DiOC_6(3)$ (Molecular Probes) for 30 min at room temperature, washed once with FACS buffer, and resuspended in 300 μl FACS buffer. Fluorescence data was acquired using a Becton Dickinson FACSCalibar™ flow cytometer and results were analysed using Cell Quest software (**Figure 2.3**).

2.12 Laser scanning cytometry

2.12.1 Cell cycle analysis by LSC

The laser scanning cytometer (LSC) is a microscope based cytofluorometer that combines advantages of flow cytometry and image analysis (Kamentsky *et al.*, 1997a; Kamentsky *et al.*, 1997b). Fluorescence of individual cells is measured rapidly, with high sensitivity and accuracy comparable to that of flow cytometry. Cell analysis on slides eliminates cell loss, which generally occurs during repeated centrifugations in sample preparation for flow cytometry. The data generated by LSC are equivalent to data generated by flow cytometers, however,

LSC can measure more than just total fluorescence per cell. As LSC measurements are slide based, it is possible to measure each cell at a number of locations on the cell to generate additional low resolution morphologic features, and to record the position of each cell on the slide as a feature so that cells can be relocated after measurements are completed and re-examined visually or by image analysis with or without restaining. To distinguish cells from background, contours are drawn round each event by the LSC software using a threshold set by the user, and a number of parameters for each event recorded. Some key parameters recorded by the LSC are the integral (the sum of pixel values in the data contour), max pixel (the maximum pixel value within the data contour), and area (the area of the thresholding contour) (**Figure 2.4**).

One application of LSC is to determine the DNA content of cells using stoichiometric fluorescent DNA specific dyes, such as PI or 4', 6'-diamidino-2-phenylindole (DAPI). The parameters required for such analyses are the integral and the max pixel of the cells. In this instance, the integral value corresponds to the DNA content of the cell, whilst the max pixel gives and indication of the chromatin density of the cell. Cellular DNA is more concentrated in mitotic cells, so the max pixel value is brighter in these cells than other cells in interphase. Therefore, mitotic cells can be distinguished from other cells in G_2 as they have a higher max pixel (**Figure 2.5**), and newly formed daughter cells have the same DNA content (integral) as other G_0/G_1 cells, but they have higher max pixel values than the other G_0/G_1 cells.

After stimulation by the desired agonists, cells were attached to microscope slides by cytocentrifugation at 600 rpm for 4 minutes in a Shandon Cytospin centrifuge (Shandon Co.), then fixed in 4% formaldehyde in PBS for 10 minutes at room temperature. The slides were washed with PBS, and the cells permeabilised with 2% FCS, 2mM EDTA pH 8.0, 0.01% w/v saponin for 5 minutes at room temperature. The slides were then washed 3 x with PBS and incubated with blocking buffer (10% goat serum, 1% BSA, 0.02% sodium azide,

and 1 x PBS) for 10 minutes. Subsequently, the cells were incubated for 30 minutes with propidium iodide (5 μg/ml) and RNase A (200 μg/ml). The slides were then washed 3 x 3 minutes with PBS/1% BSA, allowed to dry, and mounted in Vectashield without DAPI (Vector Labs). Slides were stored at 4°C in the dark until required.

2.12.2 Analysis of intracellular staining by LSC

The LSC can also be used for analysis of intracellular proteins, for example in order to determine the activation status of proteins activated by phosphorylation, using phospho-specific proteins. Cells were stimulated as required, then attached to microscope slides by cytocentrifugation at 600 rpm for 4 minutes in a Shandon Cytospin centrifuge (Shandon Co.), then fixed in 4% formaldehyde in PBS for 10 minutes at room temperature, washed with PBS, and permeabilised with 2% FCS, 2mM EDTA pH 8.0, 0.01% w/v saponin for 5 minutes at room temperature. The slides were then washed 3 times with PBS and incubated with a blocking solution containing 10% goat serum, 1% BSA, and 0.02% sodium azide in PBS for 10 minutes. Subsequently, a 50μl aliquot of a 1% BSA/PBS solution containing a 1:250 dilution of anti-phospho p44/42 MAPK antibody (Cell Signaling Technology) was placed atop the site with the attached cells on the microscope slide and incubated for 30 minutes. The sites were washed 3 x 3 minutes with 1% BSA/PBS and incubated with a 50μl aliquot of a 1% BSA/PBS solution containing a 1:100 dilution of anti-rabbit FITC conjugated secondary antibody in 5μg/ml Propidium iodide containing RNase A (200μg/ml) for 30 minutes. Cells were washed a further 3 x 3 minutes in 1% BSA/PBS then allowed to dry in the dark before being mounted in Vectashield without DAPI and left in the dark at 4°C until data acquisition on the LSC (CompuCyte Corp), and data analysed using WinCyte software (CompuCyte Corp).

2.12.3 Image analysis using the LSC

The LSC also has image analysis capabilities, permitting localisation studies to be carried out. This was utilised to investigate the activity of Erk-MAPKinase, and co-localisation of activated Erk MAPK with lipid rafts in primary B cells. Activated Erk MAPK was detected via a commercially available monoclonal phospho-specific antibody, whilst cholera toxin B subunit was used to detect the location of lipid rafts. Cholera toxin B subunit attaches to cells by binding to ganglioside G_{M1}, a ubiquitous glycolipid cell surface receptor that is a component of lipid rafts.

Cells were stimulated as indicated, attached to slides by cytocentrifugation at 600 rpm for 4 minutes in a Shandon Cytospin centrifuge (Shandon Co.), then fixed in 4% formaldehyde in PBS for 10 minutes at room temperature. The slides were washed with PBS, then incubated in Blocking Reagent (Molecular Probes) for 30 minutes. If required, biotin-labelled cholera toxin B subunit (Sigma) was added (1 μg/ml) in TNB (50 mM Tris, 75 mM NaCl, 0.5% Blocking Reagent) to the cells and incubated for 30 minutes. The slides were washed 3 x 5 minutes with TNT wash buffer (10 mM Tris, 15 mM NaCl, 0.05% Tween 20), then Streptavidin-Alexa Fluor 647 (Molecular Probes) added, diluted 1:500 in TNB, and incubated for 30 minutes. Slides were washed a further 3 x 5 minutes with TNT wash buffer.

To facilitate intracellular staining following the surface staining, cells were incubated with permeabilisation buffer (2% FCS, 2 mM EDTA pH 8.0, 0.1% w/v saponin) for 10 minutes, then washed 3 x 5 minutes with PBS. Cells were incubated for 15 minutes with Blocking Reagent, then for 30 minutes with anti-phospho p44/42 MAPK monoclonal antibody (Cell Signaling Technology), diluted 1:200 in 1% Blocking Reagent, 0.1% w/v saponin. Cells were washed 3 x 5 minutes with TNT wash buffer, and anti-mouse IgG HRP conjugate (Amersham Pharmacia Biotech) added, diluted 1:100 in 1% Blocking Reagent, 0.1 % w/v saponin), and incubated with the cells for 30 minutes. Cells were washed a

further 3 x 5 minutes with TNT wash buffer, and incubated with Alexa Fluor 488-labelled tyramide in Amplification Buffer (Molecular Probes) containing 0.015% H_2O_2 for 10 minutes. Following a further 3 x 5 minute washes, slides were allowed to dry and mounted with Vectashield containing DAPI (Vector Labs), permitting visualisation of the nuclei. Slides were stored at 4°C in the dark until image analysis took place.

2.13 Generation of WEHI-231 mutant cell lines by retroviral transfection

2.13.1 Constructs for retroviral transfection

The murine stem cell virus (MSCV)-based MIEV.CMV retroviral vector was constructed by replacing the phosphoglycerate kinase (PKG) promoter (P_{PKG}) in MIEV (Leung *et al.*, 1999) with the CMV promoter from the pcDNA3.1 (Invitrogen Life Technologies) backbone (Michie *et al.*, 2001) (**Figure 2.6**). The key features of this vector are the ampicillin resistance gene to allow selection of the vector in bacterial culture, the 5' and 3' long terminal repeats (LTR) that are required for integration into the host genome and initiate a full length viral transcript, the CMV promoter to drive translation of the gene of interest, the multiple cloning site (MCS) for insertion of the gene of interest, the internal ribosome entry site (IRES) allowing the bicistronic expression of the gene of interest and the enhanced green fluorescent protein (eGFP), and the eGFP gene itself. The green fluorescence protein allows for detection or sorting of infected cells, and should be expressed at around one tenth of the levels of the gene of interest. All genes of interest were inserted into the MCS, and all vectors were purified by maxi-prep, following the manufacturer's instructions (Invitrogen Life Technologies).

2.13.2 Generation of stable retroviral packaging lines

The retroviral vector MIEV.CMV contains all the *cis* acting viral sequences necessary for transmission, but does not contain the structural genes required for production of an infectious virus particle (*gag, pol, env*). A cell line known as the packaging cell line must supply the products of these genes. To generate stable retroviral packaging lines, the vector is first transiently transfected into the PT67 cell line (Clontech). The PT67 cell line is an NIH/3T3-based packaging line that expresses the 10A1 viral envelope. Virus packaged from PT67 cells can be used to infect a broad range of mammalian cells because the virus can enter cells via two different surface molecules, the amphotropic retrovirus receptor and the GALV receptor. Virus packaged by transfected PT67 cells was then used to infect the ecotropic packaging cell line, GP+E-86. The GP+E-86 cell line is an NIH/3T3-based packaging line produced by cotransfecting a plasmid containing the *gag* and *pol* genes and a plasmid containing the *env* gene of the Moloney murine leukaemia virus into NIH/3T3 cells (Markowitz *et al.*, 1988).

PT67 cells were transiently transfected using the Effectene™ transfection reagent, according to the manufacturer's instructions (Qiagen). Briefly, the cells were seeded at a density of 1 x 10^5 cells/well in RPMI complete in a 6 well plate and incubated overnight at 37°C. 0.4 μg DNA was diluted with DNA-condensation buffer, buffer EC, to a final volume of 100 μl, 3.2 μl enhancer added and the solution mixed by vortexing for 1 second. After incubation at room temperature for 5 minutes 10 μl effectene transfection reagent was added to the DNA-enhancer mixture, the solution mixed by vortexing for 10 seconds, and incubated at room temperature for 10 minutes. The cells were washed once with PBS and fresh RPMI complete added to the cells. Medium (600 μl) was added to the tube containing the transfection complexes, mixed, then added drop-wise to the cells. The cells were then incubated for 24 hours, an additional 0.5 ml media added and the cells incubated for a further 24 hours to permit production of virus particles.

The GP+E-86 cell line was transfected using the supernatant of transiently transfected PT67 cells. Briefly, GP+E-86 cells were seeded at a density of 1 x 10^5 cells/well in RPMI complete in a 6 well plate 24 hours prior to transfection and incubated overnight at 37°C. Polybrene™ (5 μg/ml) was added to the cells and incubated for 4 hours. Polybrene™ (Hexadimethrine Bromide) is a small, positively charged molecule that binds to cell surfaces and neutralises surface charge, apparently allowing the viral glycoproteins to bind more efficiently to their receptors, because it reduces the repulsion between sialic acid-containing molecules. After 4 hours the supernatant taken from PT67 cells transfected 48 hours previously was filter sterilised to remove any cells, the media removed from the GP+E-86 cells, and 1.5 ml of the sterilised PT67 supernatant added to the GP+E-86 cells, along with 5 μg/ml polybrene™. The cells were then centrifuged, in the 6 well plate, at 550 x g for 45 minutes at room temperature, incubated overnight at 37°C, and 0.5 ml media supplemented with polybrene™ (5 μg/ml) added. The cells were then incubated for a further 24 hours to allow expansion before selection of successful transfectants by cell sorting.

2.13.3 Selection of stable retroviral packaging lines

Stably transfected GP+E-86 cells were selected on the basis of expression of green fluorescent protein by means of a cell sorter. Briefly, 48 hours after transfection the cells were trypsinised, washed once in sterile sorting buffer (HBSS, 1% BSA) and resuspended in sterile sorting buffer. The cells were filtered through a 70 μm mesh, and incubated on ice for transportation to the cell sorter. GFP^+ cells were sorted into 2 ml media with a FACSVantage™ SE cell sorter (Becton Dickinson), harvested by centrifugation (400 x g, 5 minutes, 4°C), resuspended in RPMI complete and incubated at 37°C until colonies were formed. After approximately 14 days, cells were sorted once more to ensure there were no GFP^- cells in the culture.

2.13.4 Transfection of WEHI-231 cells using stable retroviral packaging lines

Stably transfected GP+E-86 cells were used to infect WEHI-231 cells with the appropriate MIEV vector by co-culture. Stably transfected GP+E-86 cells that had been sorted twice to ensure GFP positivity were grown to 75% confluency in a 6 well plate. WEHI-231 cells were pretreated for 4 hours with polybrene (5 μg/ml) prior to transfection. 3×10^6 WEHI-231 cells in media containing 5 μg/ml polybrene were added to the wells containing the appropriate stable GP+E-86 transfectants, and incubated at 37°C for 16 hours. After this time, the WEHI-231 cells were removed by careful aspiration and incubated for a further 4 hours, before a sample was removed for FACS analysis to assess the percentage of transfected cells by their green fluorescence.

2.14 Generation of WEHI-231 mutant cell lines by electroporation

2.14.1 Feasibility of transfection of WEHI-231 cells by electroporation

Transfection of mammalian cells by electroporation relies upon transient membrane pore formation to allow passage of DNA molecules into the cell. A slight drawback with electroporation is that it requires large numbers of cells, due to the fact that a high proportion of cells do not survive. As such, optimisation is required to balance transfection efficiency against cell death. In order to optimise electroporation of WEHI-231 cells, pCMV-GFP was transfected at a constant capacitance (960 μFarads) with increasing voltages, 200 V, 250 V, 270 V, and 300 V. Efficiency of electroporation was assessed by flow cytometry to detect the proportion of GFP^+ cells, and the proportion of dead cells, 24 hours after transfection (**Figure 2.7**).

Selection of WEHI-231 cells successfully transfected by electroporation with pcDNA3.1 constructs was facilitated by resistance to the antibiotic G418, which is conferred by pcDNA3.1 (**Figure 2.8**). The concentration of G418 used has to be sufficient to prevent any wild type cells from growing, but not at such a level that growth of transfected cells is inhibited in any way. In order to determine the concentration of G418 required to eliminate wild type WEHI-231 cells, cells were cultured in RPMI complete containing G418 at a variety of concentrations for up to 72 hours. The concentrations used were 0 μg/ml, 250 μg/ml, 500 μg/ml, 750 μg/ml, 1 mg/ml, and 1.25 mg/ml. The minimum concentration required to kill all wild type cells was found to be 500 μg/ml, as assessed by microscopy and trypan blue exclusion (results not shown).

2.14.2 Constructs for transfection by electroporation

The pUC18 based vector pcDNA3.1 (Invitrogen Life Technologies) was used as the vector for the introduction of specific genes to WEHI-231 cells by electroporation. The key features of this vector are ampicillin resistance for selection in bacterial cultures, the CMV promoter to drive translation of the gene of interest, the multiple cloning site (MCS) for insertion of the gene of interest, the BGH polyadenylation sequence to ensure proper modification of the mRNA, and the neomycin resistance gene, to allow for selection of cells successfully transfected using G418 (**Figure 2.8**).

2.14.3 Preparation of vectors for transfection by electroporation

In order for DNA to be integrated into the genome of mammalian cells following transfection, it has to be linear before transfection takes place. For all of the constructs transfected into WEHI-231 cells by electroporation, this was achieved by digestion with *Pvu* I, as there is only one *Pvu* I site in pcDNA3.1 and none in any of the genes of interest. 10 μg of each of the vectors to be digested were incubated with 1 unit of *Pvu* I for 1 hour, with 0.5 units *Pvu* I added and incubated

for a further 1 hour, at 37°C to ensure complete digestion. Following digestion, the fragments were separated by electrophoresis in a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) (**Figure 2.9**), and the appropriately sized fragment for each construct excised. Fragments were extracted from the agarose slice by means of the QIAquick™ gel extraction kit, following the manufacturer's protocol (Qiagen). Briefly, the gel slices were weighed, 3 volumes of buffer QG added to 1 volume of gel and the gel dissolved by incubating at 50°C for 10 minutes, with vortexing every 3 minutes. Once the gel was dissolved 1 gel volume of isopropanol was added and the sample mixed. Samples were added to QIAquick spin columns and centrifuged for 1 minute at maximum speed in a microcentrifuge to allow binding of DNA to the columns. The flow through was discarded, and the column washed by adding 750 µl buffer PE and centrifuging for 1 minute, as before. The flow through was again discarded and the column centrifuged for a further 1 minute, before being placed in a fresh, clean 1.5 ml tube. DNA was eluted from the column by adding 20 µl dH_2O to the centre of the membrane, standing for 1 minute, followed by centrifugation for 1 minute. Purified DNA was transferred to ice and introduced to the cells by electroporation immediately.

2.14.4 Transfection of WEHI-231 cells by electroporation

24 hours prior to transfection cells were split to ensure they would be in log growth phase at the time of transfection to maximise efficiency. 5×10^6 cells per electroporation were washed in electroporation media (RPMI-1640 + 20%FCS) and resuspended in electroporation media at 2×10^7 cells/ml. Linearised DNA recovered from an agarose gel was added to a cuvette and cooled on ice for 5 minutes. 5×10^6 cells were added and the cuvettes tapped lightly to mix, before being incubated on ice for a further 10 minutes. Cells were then electroporated at 960 µFarads, 220 Volts (the optimum conditions for electroporation of WEHI-231 cells, **Figure 2.7**), and incubated on ice for a further 10 minutes. After this time the cells were removed from the cuvettes by pipetting and placed into RPMI-

complete media. The cells were incubated for 48 hours at 37°C in 5% CO_2, before addition of the antibiotic G418 at 500 μg/ml (concentration high enough to kill wild type WEHI-231 cells – see **Section 2.14.1**) to select for transfected cells. After 48 hours, cells were transferred to a 15 ml tube, underlaid with 1 ml FCS and centrifuged at 400 g for 7 minutes to remove debris. Cells were resuspended in RPMI complete supplemented with G418 (500 μg/ml) and incubated at 37°C to allow for expansion of successfully transfected cells.

2.15 Total cellular RNA preparation

Total RNA was extracted using RNAzol™ B (Biogenesis) as described (Chomczynski and Sacchi, 1987). Briefly, cells (5 x 10^6) were washed twice with ice-cold PBS (2000 x g, 5 min at 4°C) and the cellular pellet was resuspended in RNAzol™ B (0.2 ml/10^6 cells). Samples were extracted for 5 min on ice and the suspension was transferred to a fresh tube. Chloroform was added (0.1 ml /1 ml of RNAzol™) and the solution mixed by vigorous shaking for 15 seconds. The samples were incubated on ice for a further 5 min.

Following centrifugation (13,000 x g, 15 min, 4°C) the colourless upper phase was removed to a fresh tube (approximately 50% of the initial RNAzol™ volume) and an equal volume of isopropanol was added. RNA was precipitated by incubation on ice for 30 min and separated by centrifugation (13,000 x g, 15 min, 4°C). The RNA pellet was washed with ice-cold 75% ethanol (1 ml/ 1 ml RNAzol™ volume), briefly dried for 10 min at room temperature and finally resuspended in 20-50μl of sterile, distilled water. RNA samples were incubated at 68°C for 5 min in order to denature any double strands, prior to concentration assessment by UV spectroscopy. Absorbance readings of diluted samples (1:50) were taken at $A_{260/280}$ and the concentration of RNA calculated (40 μg/ml RNA = A_{260} of 1).

2.16 Ribonuclease protection assay (RPA)

Expression levels of a group of genes involved in cell cycle control or apoptosis were examined by means of a RiboQuant™ ribonuclease protection assay (Pharmingen). The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantification of mRNA species. Radio-labelled anti-sense RNA probes are generated from a cDNA fragment of interest, and hybridised with target RNA from samples of interest. Free probe and other single-stranded RNA are digested with RNases, the remaining RNase-protected probes are purified, resolved on denaturing polyacrylamide gels, and quantified by autoradiography or phosphorimaging (**Figure 2.10**). As undigested probes are resolved by electrophoresis, a number of RNA probes can be analysed provided they are of distinct length and each represents a unique sequence in a distinct mRNA species.

The protocol supplied with the ribonuclease protection assay kit was followed. Briefly, the RPA template set was incubated for one hour at 37°C with T7 RNA polymerase in the presence of ribonucleotides, including [α-^{32}P]-UTP (Amersham Pharmacia Biotech). The reaction was terminated by the addition of DNase, to remove the template, with a further 30 minute incubation at 37°C. The RNA probe was extracted by phenol:chloroform extraction, then precipitated by incubation with 100% ethanol for 30 minutes at -70°C. Following centrifugation at 13000g for 15 minutes at 4°C the RNA pellet was washed with ice-cold 90% ethanol then allowed to dry briefly. The pellet was resuspended in hybridisation buffer, then the labelling assessed by scintillation counting. Probes had a typical yield of ~2 x 10^6 Cherenkov counts/μl (measurement of cpm/μl without the presence of scintillation fluid).

RNA samples to be hybridised with the probe were prepared using RNAzol™ B as above, and 10μg of each sample was dried in a vacuum evaporator

centrifuge. Each sample was solubilised by the addition of hybridisation buffer, to which was added the probe at the optimal probe concentration, according to the data sheet. The samples were placed in a heat block pre-heated to 90°C and the temperature was immediately turned down to 56°C to allow for annealing of complimentary strands, and allowed to incubate for 16 hours. Samples were then incubated with RNase at 30°C for 45 minutes to digest single stranded RNA. RNase was inactivated by incubation with proteinase K at 37°C for 15 minutes. RNA was extracted by phenol:chloroform extraction and precipitated with ethanol, as before. Protected probes were resolved on a 5% acrylamide gel at 50 watts for around 3 hours in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8.3). The gel was dried on a vacuum drier and the levels of radioactivity of protected probes quantified by phosphorimaging using a Storm phosphorimager.

2.17 Suppliers addresses

Amersham Pharmacia Biotech
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA

BD Biosciences
21 Between Towns Road
Cowley
Oxford OX4 3LY

Biogenesis
Technology Road
Poole BH17 7DA

Calbiochem
c/o CN Biosciences
Boulevard Industrial Park
Padge Road
Beeston
Nottingham NG9 2JR

Cell Signalling Technology
New England Biolabs (UK) Ltd
73 Knowl Piece, Wilbury Way
Hitchin
Hertfordshire SG4 0TY

CompuCyte Corp, c/o
Genetic Research Instrumentation
Gene House
Queenborough Lane
Rayne, Braintree
Essex CM77 6TZ

European Collection of Cell
Cultures (ECACC), c/o CAMR
Porton Down
Salisbury
Wiltshire SP4 0JG

Harlan UK Ltd
Shaw's Farm, Blackthorne
Bicester
Oxon OX25 1TP

Invitrogen Life Technologies
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF

Jackson Immunoresearch Laboratories
c/o Stratech Scientific
61-63 Dudley Street
Luton
Bedfordshire LU2 0NP

Kodak Ltd
Kodak House
Station Road
Hemel Hempstead
Hertfordshire HP1 1JU

Miltenyi Biotec
Almac House
Church Lane
Bisley
Surrey GU24 9DR

Molecular Probes
c/o Cambridge Bioscience
24-25 Signet Court
Newmarket Road
Cambridge CB5 8LA

Perbio Science UK Ltd
Centruy House
High Street, Tattenhall
Cheshire CH3 9RJ

Pharmingen
c/o BD Biosciences
see above

QIAGEN Ltd
Boundary Court
Gatwick Road, Crawley
West Sussex RH10 9AX

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset BH12 4QH

Shandon Inc
93-96 Chadwick Road
Astmoor, Runcorn,
Cheshire WA7 1PR

Tree Star Inc
340 A Street Bd. 1#203
Ashland
OR 97520, USA

Vector Laboratories Ltd
3 Accent Park
Bakewell Road
Orton Southgate
Peterborough
PE2 6XS

Wallac
c/o PE Life Sciences
(Applera UK)
Kelvin Close
Birchwood Science Park North
Warrington
Cheshire WA3 7PB

Table 2.1 Other Reagents

2-mercaptoethanol	2-Me	Invitrogen
3.3'-dihexyloxacarbocyanine iodide	$DiOC_6$ (3)	Mol. Probes
5-/6-carboxyfluorescindiacetate, succinimidyl ester	CFSE	Calbiochem
[α-^{32}P]-uridine tri phosphate	[α-^{32}P]-UTP	
Effectene transfection reagent		Qiagen
Enhanced ChemiLuminescence Kit	ECL	Amersham
Foetal Calf Serum	FCS	Invitrogen
Geneticin antibiotic	G418	
Glutamine	Glutamine	Invitrogen
Hexadimethrine Bromide	Polybrene	Sigma
MEM Non-essential amino acids	Amino acids	Invitrogen
MicroBCA or Coomassie Protein Assay	Protein Assay	Pierce
NuPage pre-cast gels		Invitrogen
Penicillin/Streptomycin	Pen/Strep	Invitrogen
Propidium Iodide	PI	Calbiochem
*Pvu*I restriction enzyme		Roche
Ribonuclease A	RNase A	Sigma
RPMI-1640 culture media	RPMI-1640	Invitrogen
Sodium Pyruvate	Sodium Pyruvate	Invitrogen
[6-^{3}H]-thymidine (5 Ci/mmol)	[3H]- thymidine	Amersham
Vectashield		VectorLabs

Table 2.2 Antibodies

anti-mouse IgG + IgM $F(ab')_2$		G, R	S	Jackson Immunoresearch Labs
anti-mouse IgG + IgM Intact		G, R	S	Jackson Immunoresearch Labs
anti-mouse IgM (μ chain) $F(ab')_2$		G	S	Jackson Immunoresearch Labs
anti-IgG Mouse, Rabbit	HRP	V	W	Cell Signalling Technology
anti-IgG Goat	HRP	V	W	Jackson Immunoresearch Labs
B220	PE		F	Pharmingen
CD3	FITC		F	In House
CD4	APC		F	Pharmingen
CD8	APC		F	Pharmingen
CD19	Biotin		F	Pharmingen
FcγRII/II (2.4G2)	FITC	Ra		In House
phospho-cdc2 (Tyr 15)		R	W	Cell Signalling Technology
Cyclin D1/D2		M	W	Upstate
p27		R	W	Pharmingen
p44/42 Erk-MAPK		R	W	Cell Signalling Technology
phospho-p44/42 Erk-MAPK		M	W	Cell Signalling Technology
phospho-p44/42 Erk-MAPK		R	W	Cell Signalling Technology
p53		R	W, IP	Santa Cruz
phospho-p53 (ser 15)		R	W	Cell Signalling Technology
Retinoblastoma (Rb)		M	W	Pharmingen
phospho-Rb (Ser807/811)		R	W	Cell Signalling Technology
CD43	Magnetic beads	Ra	Pure	Miltenyi Biotech

[1]Host: R = Rabbit, G = Goat, Ra = Rat, M = Mouse, V = Various.
[2]Use: WB = Western Blotting, IP = Immunoprecipitation, F = FACS, S = Stimulation (*in vitro*), PD = Pull Down, Pure = Purification

Figure 2.1 FACS analysis of cell surface marker staining

A A dot plot of forward scatter versus side scatter, showing the population of cells deemed healthy by their position in this plot. Forward scatter gives a measure of the size of the cells, whilst side scatter gives a measure of their granularity.

B A dot plot of forward scatter versus propidium iodide (PI) staining, showing the population of cells deemed viable by their lack of PI fluorescence. PI is excluded from viable cells, but can enter cells with porous membranes, such as dead cells, whereupon it binds the cellular DNA in a stoichiometric manner.

C Dot plots of murine splenic mononuclear cells stained with anti-B220-PE, anti-CD4-APC, anti-CD8-APC, and anti-CD19-biotin-streptavidin-FITC. Dead cells were excluded from analysis by adding propidium iodide (50 μg/ml) immediately prior to data collection, as healthy cells should not accumulate PI. The positions of gates were decided by including unstained cells and cells stained for single surface markers, then examining their fluorescence on a histogram. This allows a marker to be positioned between positively stained cells, and unstained cells, typically between 20 and 50 fluorescence units.

D Dot plots of murine splenic B cells, after purification with with anti-CD43 (Ly-48) magnetic beads, stained with anti-B220-PE, anti-CD4-APC, anti-CD8-APC, and anti-CD19-biotin-streptavidin-FITC. Analysis was carried out as above.

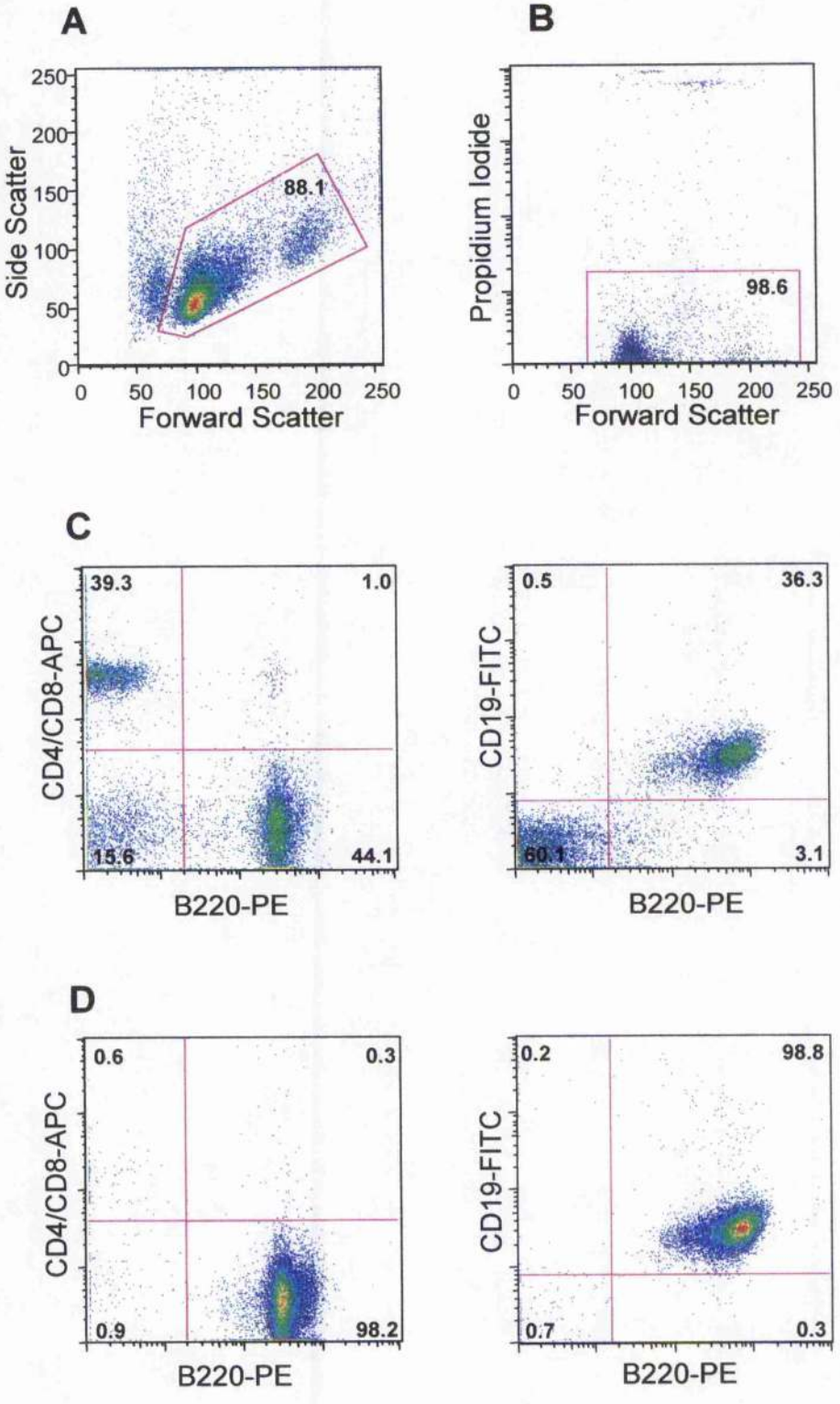
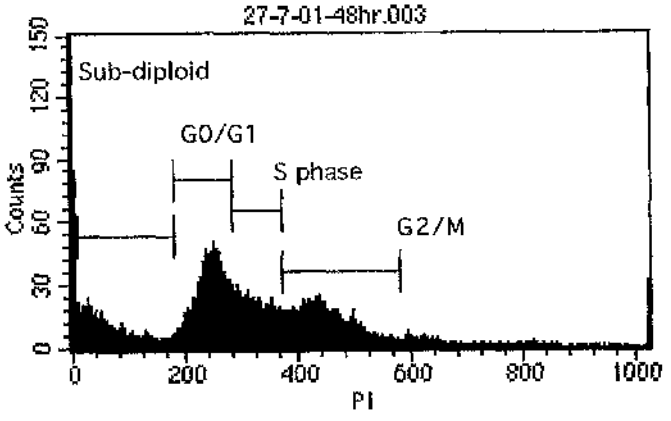

A
Side Scatter
Forward Scatter
88.1
B
Propidium Iodide
Forward Scatter
98.6
C
CD4/CD8-APC
B220-PE
39.3
1.0
15.6
44.1
CD19-FITC
B220-PE
0.5
36.3
60.1
3.1
D
CD4/CD8-APC
B220-PE
0.6
0.3
0.9
98.2
CD19-FITC
B220-PE
0.2
98.8
0.7
0.3

Figure 2.2 FACS histogram of DNA content analysis

A histogram plot of cells stained with propidium iodide (50 μg/ml for WEHI-231 cells, 250 μg/ml for primary splenic B cells). Histogram markers determine the percentage of cells in each stage of the cell cycle. The G_1 peak (2N DNA) is set around 300 fluorescence units on the FL-3 x-axis and the G_2/M peak (4N DNA) calculated accordingly. Cells exhibiting sub-diploid DNA content (representing apoptotic cells) are marked as those below the 2N peak, whilst cells in S phase are determined as those between the 2N and 4N peaks.



Histogram Statistics

File: 27-7-01-48hr.003
Sample ID: 48 Hrs Control
Acquisition Date: 27-Jul-1
Total Events: 10000

Log Data Units: Linear Valu
Patient ID: a-Ig + aCD40
Gated Events: 10000
X Parameter: FL3-H PI (Line

Marker	Left, Right	Events	% Total	Mean	Median
All	0, 1023	10000	100.00	225.02	243.00
Sub-diploid	9, 181	1022	10.22	66.56	55.00
G0/G1	181, 284	2466	24.66	243.69	246.00
S phase	284, 372	1454	14.54	324.82	323.00
G2/M	372, 581	1931	19.31	446.80	439.00

Figure 2.3 FACS histogram of the mitochondrial membrane potential of cells

A histogram of cells stained with the cationic lipophilic dye $DiOC_6(3)$ (2.5 μM) for analysis of mitochondrial membrane potential. Histogram markers determine the proportion of cells with low or high $DiOC_6(3)$ fluorescence on the FL-1 x-axis. Cells with low $DiOC_6(3)$ fluorescence are judged to represent the population that are committed to apoptosis, having decreased their mitochondrial membrane potential. Anti-IgM induces apoptosis in WEHI-231 cells, therefore the histogram pertaining to cells treated with anti-IgM displays a far greater proportion of cells with low mitochondrial membrane potential.

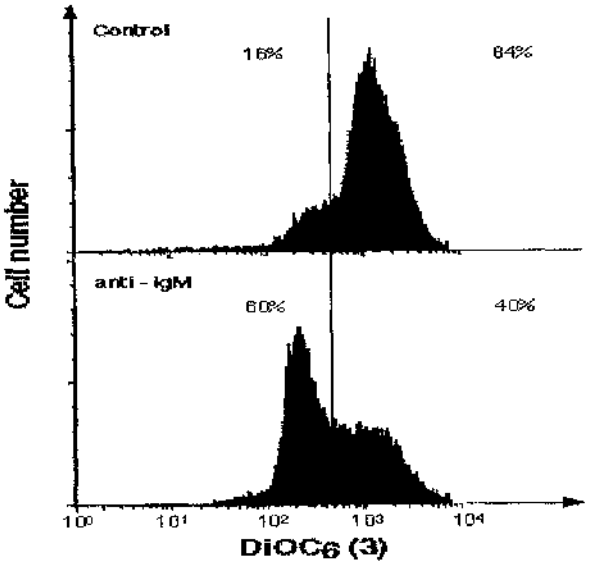
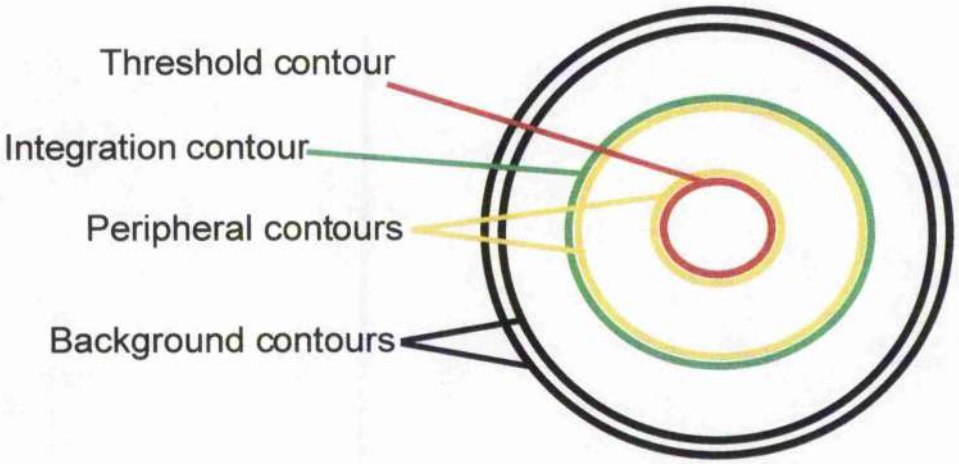
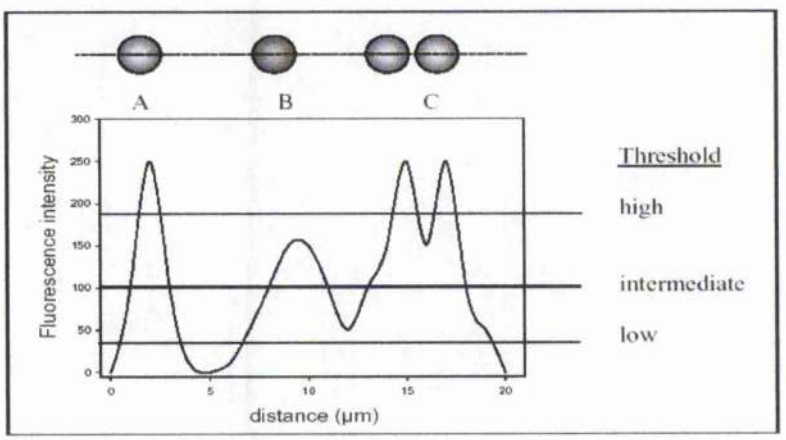

Control
16%
84%
anti - IgM
60%
40%
Cell number
10⁰
10¹
10²
10³
10⁴
DiOC6 (3)

Figure 2.4 The detection of cells by the laser scanning cytometer (LSC)

To distinguish cells from background, contours are drawn round each event by the LSC software using a threshold set by the user on data from one sensor, typically the particular nuclear stain used. These contours are the threshold contour, integration contour, peripheral contours, and the background contours. The same contour locations are used when acquiring data for each event from all other sensor data.





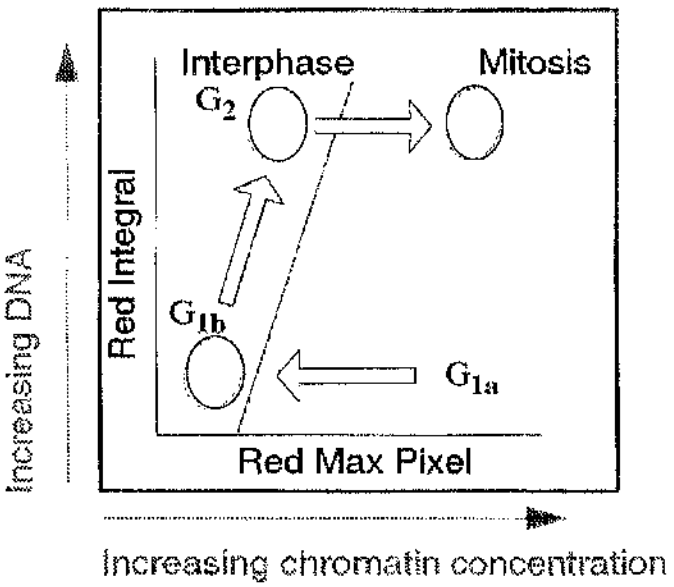
A: High threshold - can pick up mainly individual events.

B: Intermediate. threshold -will pick up two cells or more as one event.

C: Low threshold - will pick up multiple cells as one event.

Figure 2.5 Cell cycle analysis by laser scanning cytometry (LSC)

Analysis of the cell cycle by LSC. Cells are stained with a stoichiometric fluorescent DNA specific dye (*eg* PI or DAPI), and data acquired by means of the LSC. Max pixel versus Integral plot visualises cells at various cell cycle stages, with varying DNA content (Integral) and varying chromatin concentration (Max pixel) permitting assignment of cell cycle stage to cell populations.



G1a - newly formed daughter cells

G1b - resting cells

G2 - cells in S-phase

Mitosis - Dividing cells

Figure 2.6 Map of the MIEV.CMV retroviral vector

The MIEV.CMV vector was used to introduce various RasV12 mutants into WEHI-231 cells. This vector was constructed by replacing the phosphoglycerate kinase (PKG) promoter (P_{PKG}) in MIEV (Leung *et al.*, 1999) with the CMV promoter from the pcDNA3.1 (Invitrogen Life Technologies) backbone (Michie *et al.*, 2001).

Amp^R: ampicillin resistance
5' and 3' LTR: long terminal repeats
P_{CMV}: cytomegalovirus promoter
MCS: multiple cloning site
IRES: internal ribosome entry site
eGFP: enhanced green fluorescent protein

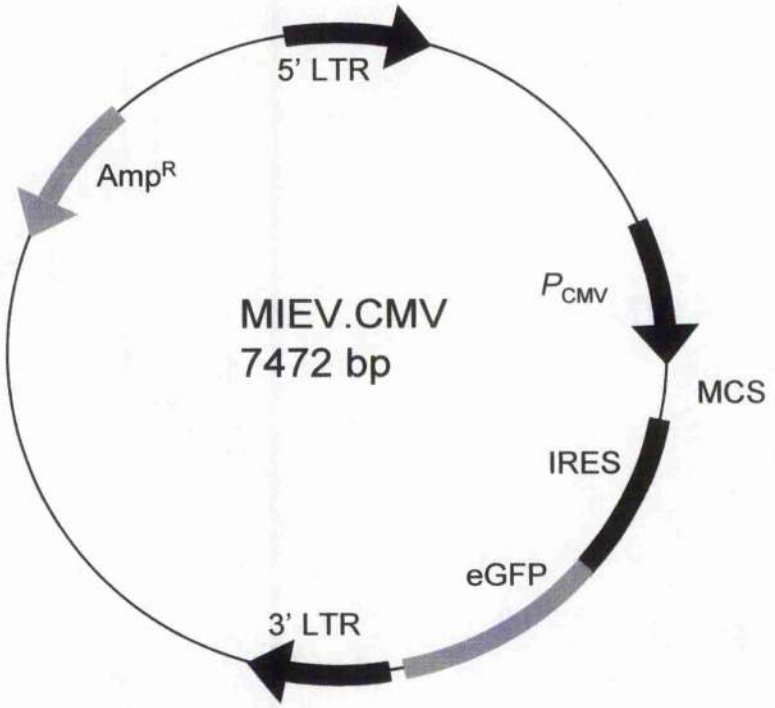

5' LTR
Amp^R
MIEV.CMV
7472 bp
P_CMV
MCS
IRES
eGFP
3' LTR

Figure 2.7 Analysis of efficiency of transfection of WEHI-231 cells by electroporation

In order to optimise electroporation of WEHI-231 cells, pCMV-GFP was transfected into cells at a constant capacitance (960 μFarads) with increasing voltages, 200 V, 250 V, 270 V, and 300 V. 24 hours after transfection, cells were harvested, washed twice in FACS buffer, and immediately prior to acquisition propidium iodide was added to each tube (50 μg/ml) to enable the proportion of dead cells to be assessed. Viable cells were selected from the dot plot on the basis that propidium iodide is excluded from viable cells, whilst successful transfectants were visualised on the basis that they should express the enhanced green fluorescent protein (eGFP), therefore display fluorescence in the FL-1 channel.

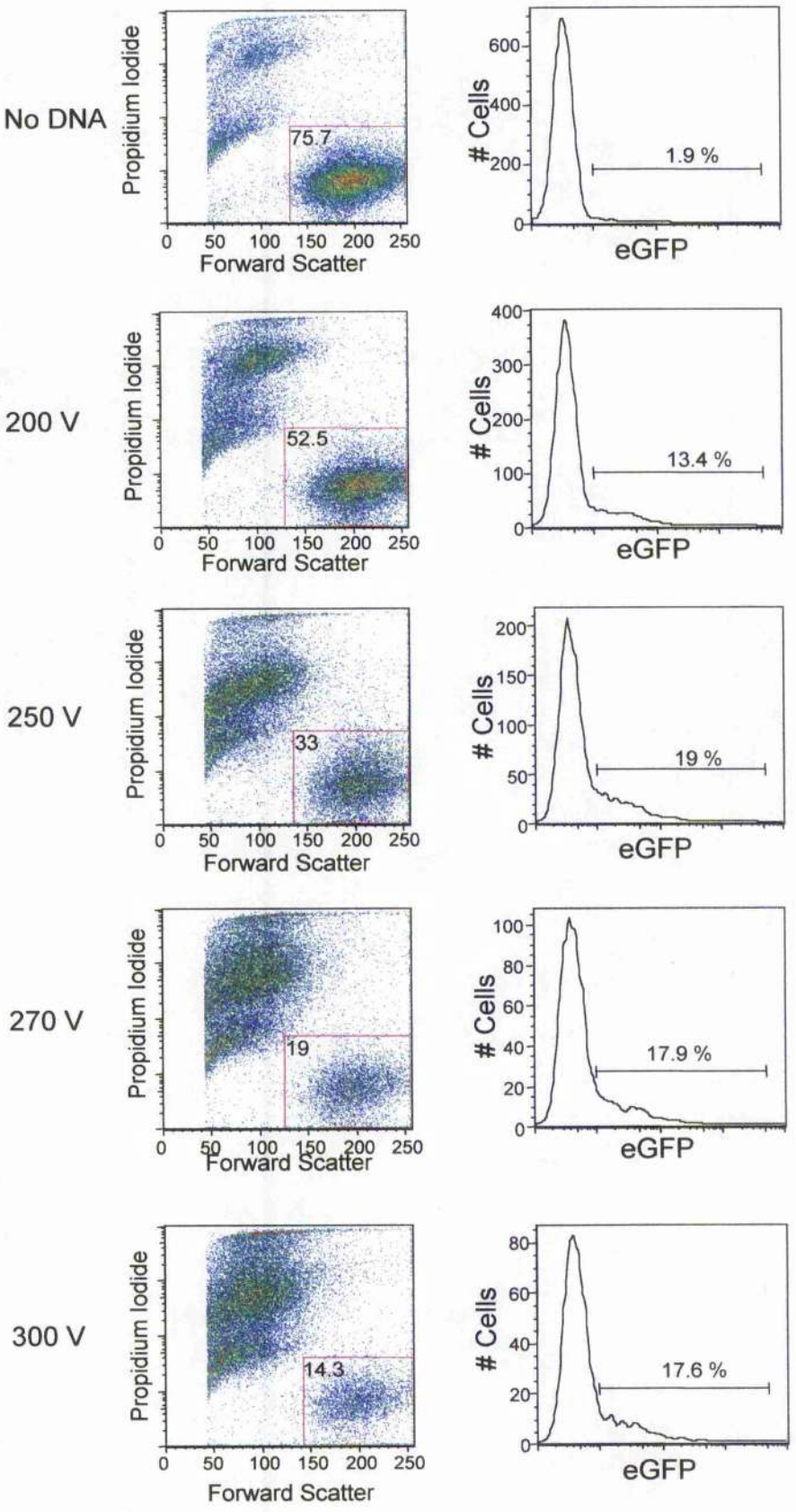

No DNA
Propidium Iodide
75.7
Forward Scatter
Cells
1.9 %
eGFP
200 V
52.5
13.4 %
250 V
33
19 %
270 V
19
17.9 %
300 V
14.3
17.6 %

Figure 2.8 Map of the pcDNA3.1 vector

The pcDNA3.1 (+) vector (Invitrogen Life Technologies) was used to introduce a variety of mutant PKC isotypes into the WEHI-231 cell line.

Ampicillin: ampicillin resistance gene (*bla*)
P_{CMV}: cytomegalovirus promoter
BGH pA: BGH polyadenylation sequence
f1 ori: origin of replication from the f1 phage
SV40 ori: SV40 early promoter and origin
Neomycin: neomycin resistance gene
SV40 pA: SV40 polyadenylation sequence
pUC ori: pUC origin

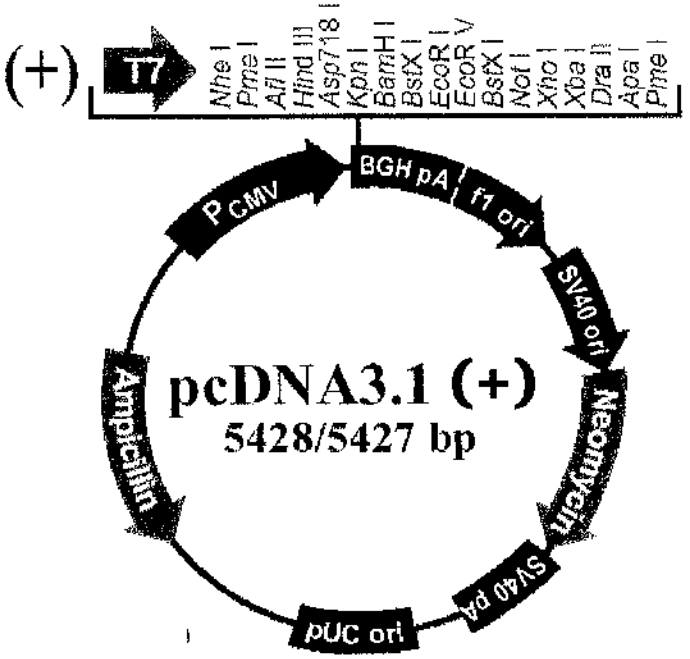

(+)
T7
Nhe I
Pme I
Afl II
Hind III
Asp718 I
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Dra III
Apa I
Pme I
P CMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
pUC ori
Ampicillin
pcDNA3.1 (+)
5428/5427 bp

Figure 2.9 Agarose gel electrophoresis of linearised vectors

Constructs comprising pcDNA3.1 containing various PKC isotypes were completely digested with *Pvu* I. Following digestion, the fragments were separated by electrophoresis in a 0.8% agarose gel. Contents of the lanes are as follows, along with the expected size of the linearised vector:

1: 1 kb DNA ladder	
2: PKC-δ KR	7.9 kb
3: PKC-δ CAT	6.4 kb
4: PKC-ε KR	7.6 kb
5: PKC-ε CAT	6.4 kb
6: PKC-ζ KR	7.17 kb
7: PKC-ζ CAT	6.4 kb
8: pcDNA3.1	5.4 kb

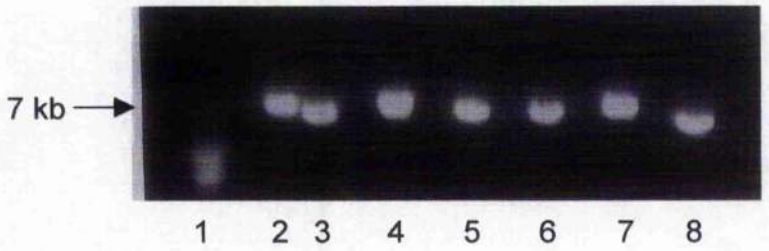
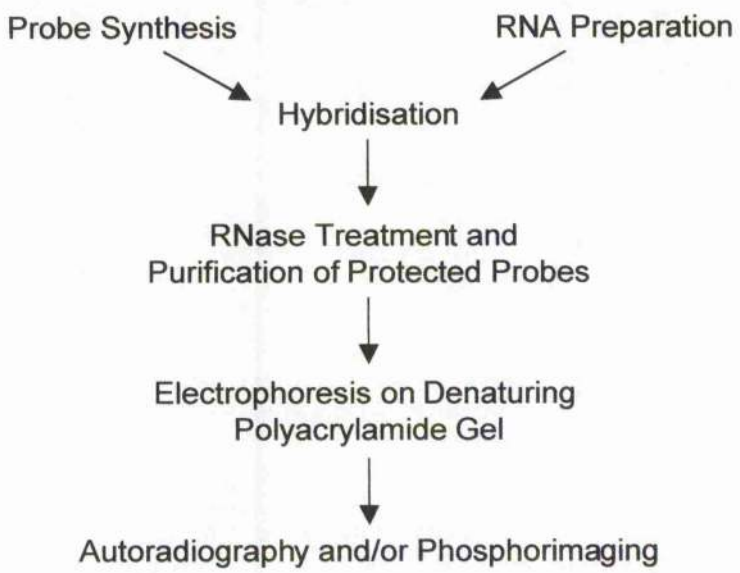

7 kb
1
2
3
4
5
6
7
8

Figure 2.10 Ribonuclease protection assay

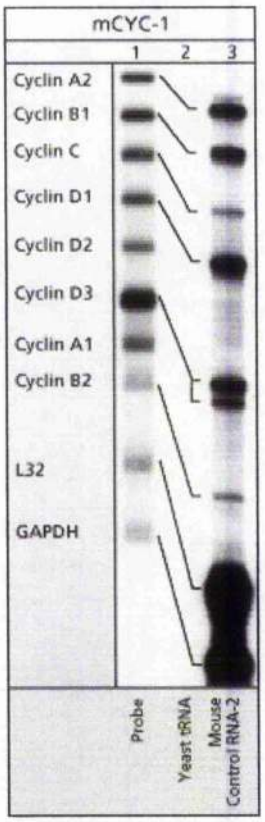
A An overview of the ribonuclease protection assay (RPA) protocol (adapted from the RiboQuant™ instruction manual).

B Sample data for mouse cell cycle regulator multiprobe template set (taken from RiboQuant™ instruction manual). The protected probes of the various genes vary in size by at least 10 nucleotides from each other, allowing them to be separated in a denaturing polyacrylamide gel. The housekeeping gene probes, L32 and GAPDH, are included for normalising samples to permit comparison of individual mRNA species between samples. Undigested probes are run on the gel as markers, though protected probes are approximately 29 nucleotides shorter due to the fact that the unprotected probe includes flanking sequences derived from the multiple cloning site of the vector.

A



B



	Probe	Protected
Cyclin A2	315	286
Cyclin B1	282	253
Cyclin C	255	226
Cyclin D1	231	202
Cyclin D2	210	181
Cyclin D3	189	160
Cyclin A1	174	145
Cyclin B2	162	133
L32	141	112
GAPDH	125	97

Chapter 3 – The role of the Ras/Erk-MAPKinase pathway in the survival and proliferation of WEHI-231 cells

3.1 Introduction

During the generation of a functional B lymphocyte repertoire it is necessary to regulate the maturation of immature B cells to prevent the emergence of cells that bind self-antigen and which are therefore potentially auto-reactive. This major checkpoint in B lymphocyte development not only occurs in the bone marrow, but also in the periphery in cells termed transitional immature B cells (Monroe, 2000). These cells arise as a result of immature B cells maintaining their immature phenotype for several days after their exit into the periphery (Allman *et al.*, 1992). In contrast to mature B lymphocytes which generally undergo a process of activation following antigen encounter, transitional and immature B lymphocytes undergo a process of negative selection that may involve deletion, anergy or replacement of the self-reactive receptor with a non-self-reactive BCR (Choi *et al.*, 1995). The fate of the immature B cell upon antigen encounter is dependent upon a number of factors, including the affinity of the antigen and which additional, external signals they receive. One signal that has been shown to play an important role in immature B lymphocyte fate is that via CD40, which has been implicated in the rescue of these cells from apoptosis due to T-cell dependent interactions (Monroe, 1997). This would enable such cells to be recruited to an ongoing immune response in which T cell help has already been triggered.

3.1.1 The WEHI-231 B cell lymphoma: a cell line model for immature B cell selection.

The murine B cell lymphoma cell line WEHI-231 is widely used as a model for immature B lymphocyte clonal deletion. This is because it has a cell surface phenotype of an immature B lymphocyte (membrane (m) IgM^{+}, $mIgD^{-/low}$, FcR^{low},

Fas^{low}, and MHC class II^{low}), and as such responds to BCR ligation by undergoing growth arrest and apoptosis (Choi *et al.*, 1995; Wiesner *et al.*, 1997) and can be rescued from BCR-mediated growth arrest and apoptosis by costimulation via CD40. The signalling mechanisms responsible for such growth arrest, apoptosis and rescue remain to be precisely defined.

As stated in **Chapter 1**, the first signalling event mediated by the BCR is the recruitment and concomitant activation of non-receptor protein tyrosine kinases (PTKs) (Campbell, 1999; Kurosaki, 1999). Recruitment and activation of three distinct types of PTKs are known, namely the Src- (Lyn, Blk, Fyn), Syk- and Tec- (Bruton's tyrosine kinase, Btk) PTKs (Cambier, 1995; Kurosaki, 1999) (**Figure 1.4**). Following BCR ligation, the Src family PTKs become activated and phosphorylate the key tyrosine residues within the ITAMs of the Igα and Igβ accessory molecules. The phosphorylated ITAMs create binding sites for the tandem SH2 domains of Syk, which, once recruited, itself becomes tyrosine phosphorylated and activated. One substrate of Syk is the adaptor protein BLNK (B cell linker protein), whose phosphorylation provides docking sites for Btk, allowing Btk to be brought into close proximity with, and subsequently become activated by phosphorylation by, Syk (Baba *et al.*, 2001). These kinases subsequently phosphorylate and activate downstream targets in various signalling cascades.

These early signalling events result in the aggregation and activation of a number of important downstream enzymes and adaptor proteins that, in turn, trigger three major signalling pathways (Cushley and Harnett, 1993). The first pathway involves the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2 or PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3 or IP_3) by phospholipase C-γ2 (PLC-γ2). The second pathway results in the generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3) by phosphorylation of PIP_2 by phosphatidylinositol 3 kinase (PI-3-K). The third pathway is the classical Ras/Raf/Erk-MAPKinase pathway. These seemingly

independent pathways have recently been shown to interact at a variety of levels, increasing the complexity of BCR signalling. It is not clear how these three pathways interact to induce proliferation in mature B cells, but growth arrest and apoptosis in WEHI-231 cells.

BCR-induced apoptosis in the WEHI-231 cell line has been shown to involve the regulation of the c-*myc* oncogene, as well as the tumour suppressor p53 and one of its transcriptional targets, the cyclin-dependent-kinase inhibitor $p21^{WAF1/CIP1}$. In certain cell types, overexpression of c-*myc* can induce apoptosis (Evan *et al.*, 1992), however, inhibition of c-*myc* expression induces apoptosis in WEHI-231 cells (Wu *et al.*, 1996). Indeed, during BCR-mediated apoptosis in WEHI-231 cells there is a marked decrease in c-*myc* at both the mRNA and protein level, following an initial, rapid elevation (Schauer *et al.*, 1996). This down-regulation is a consequence of decreases in the binding of Rel/NF-κB to DNA, activity vital for maintaining c-*myc* levels and promoting cell survival (Wu *et al.*, 1998). The transcription factor NF-κB has been shown to be important for B lymphocyte activation and development, and its inhibition using protease inhibitors (which prevent receptor-mediated degradation of the NF-κB inhibitor, IκB) has been shown to induce apoptosis in a number of B lymphocyte cell lines (Baichwal and Baeuerle, 1997). It has also been found that anti-CD40 stimulation is able to rescue WEHI-231 cells from anti-Ig mediated apoptosis by inducing NF-κB activity, consequentially elevating and maintaining c-*myc* RNA levels (Schauer *et al.*, 1996). Anti-Ig stimulation of WEHI-231 cells enhances the stability of IκB-α, leading to accumulation of IκB-α in both the cytosol and nucleus, and a concomitant decrease in the nuclear expression of the Rel/NF-κB family member c-Rel (Ku *et al.*, 2000).

As stated above, the tumour suppressor protein p53 has also been implicated in WEHI-231 cell death, with anti-Ig treatment of WEHI-231 cells increasing the levels of p53 protein, as well as $p21^{WAF1/CIP1}$ (Wu *et al.*, 1998). The increase in protein levels of $p21^{WAF1/CIP1}$, a downstream transcriptional target of p53, was

delayed relative to p53 induction, consistent with the notion that functionally active p53 induces $p21^{WAF1/CIP1}$ expression. This study also showed that ectopic expression of p53 in WEHI-231 cells leads to an induction of apoptosis, in the absence of any further stimulation.

The same group have also demonstrated that the induction of the cyclin dependent kinase inhibitor $p27^{Kip1}$ following anti-Ig treatment promotes apoptosis, as well as cell cycle arrest (Wu *et al.*, 1999). Microinjection of an antisense c-*myc* expression vector induces apoptosis in WEHI-231 cells, and this can be rescued by co-microinjection of an antisense p27 expression vector, or an anti-p27 antibody to ablate p27 levels. This c-*myc* pathway is independent of the p53/p21 pathway as inhibition of the p53 pathway has no effect on the anti-Ig mediated drop in c-*myc* levels or the elevation of p27 levels (Wu *et al.*, 1999). WEHI-231 cells are not the only cell type in which the induction of p27 induces apoptosis, this phenomenon has also been shown in lung fibroblasts and HeLa cells (Katayose *et al.*, 1997; Wang *et al.*, 1997).

3.1.2 Ras signalling

As stated above, the membrane proximal signalling events initiated in response to BCR ligation are becoming fairly well elucidated, with 3 key signalling pathways now known to be the major targets, one of these pathways being the Erk-MAPKinase pathway. A key regulator of the Erk-MAPKinase pathway is the small GTPase, Ras, which regulates cell growth in all eukaryotic cells. When serum-starved NIH-3T3 cells that are arrested in G_0 are exposed to serum, they undergo a period of intense signalling lasting 30-60 minutes, and this starts to decline as receptors are internalised and degraded. Serum or growth factors are required for a further 8-10 hours however, if the cells are to enter the cell cycle. In cells continuously exposed to growth factors in this manner, Ras signalling is required in at least two phases of the G_0 to S phase transition, and these two phases of Ras signalling require different effector molecules. ERK is activated in

the early stages of Ras activation, but its activity is not detected at later stages of G_1 progression, even though Ras is still active. PI-3-Kinase is another effector molecule of Ras signalling important for G_0 to S phase progression. PI-3-Kinase is active during the G_0/G_1 transition and in mid to late G_1 phase (Jones *et al.*, 1999). Inhibition of PI-3-Kinase activity during the G_0/G_1 transition has no effect on cell cycle progression of HepG2 cells, however inhibiting the mid to late G_1 phase PI-3-Kinase activity prevented cells from entering S phase (Jones *et al.*, 1999). In WEHI-231 cells, short term inhibition of PI-3-Kinase activity has no effect on spontaneous proliferation, anti-Ig induced growth arrest or CD40-mediated rescue. However, prolonged inhibition (up to 30 hours) of PI-3-Kinase results in growth inhibition of unstimulated cells, suggesting that PI-3-Kinase activity may be important for the basal proliferation of WEHI-231 cells. Prolonged inhibition also abrogated CD40-mediated rescue of anti-Ig induced growth arrest, but this may be due to the fact that PI-3-Kinase inhibition blocks basal proliferation of these cells (Gauld, 2001).

RasV12 is a constitutively active form of Ras, the consequence of a point mutation that results in the substitution of valine for glycine at position 12. This mutation disables the intrinsic GTPase activity of Ras, therefore once Ras binds GTP and becomes active it is unable to deactivate itself by hydrolysing GTP to GDP. RasV12-S35 contains a further point mutation in the effector domain of the protein, resulting in the substitution of serine for threonine. This mutation prevents Ras from binding the p110α subunit of PI-3-Kinase, an interaction that is known to lead to the activation of PI-3-Kinase (Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). By contrast, RasV12-C40 contains a point mutation in the effector domain resulting in the substitution of cysteine for tyrosine. This mutation abrogates the interaction between Ras and Raf, preventing Ras from activating the Erk-MAPKinase pathway.

Another tool for investigating the role of Ras is the $p62^{Dok}$ protein, a negative regulator of Ras activity. The Dok proteins are a family of adaptor proteins that

are phosphorylated by a wide range of protein tyrosine kinases. The first Dok protein to be isolated was $p62^{dok}$, originally identified as a 62 kD tyrosine-phosphorylated protein associated with Ras-GAP (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), a negative regulator of Ras (Bollag and McCormick, 1991). $p62^{dok}$ is rapidly tyrosine phosphorylated in response to a wide range of stimuli, including ligation of the BCR (Gold *et al.*, 1993) and FcγRIIB1 ligation (Vuica *et al.*, 1997), and once phosphorylated interacts with a number of signalling molecules including Ras-GAP (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), Nck (Tang *et al.*, 1997), and Csk (Vuica *et al.*, 1997) via their SH2 domains. In addition to SH2 domain-binding sites, $p62^{dok}$ also contains a pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain, and potential SH3 domain-binding sites. Initially, $p62^{dok}$ was suspected of playing a positive role in mitogenic signalling as it was first identified as a substrate for the $p210^{bcr\text{-}abl}$ oncoprotein (Carpino *et al.*, 1997), as well as a target of v-Abl in v-Abl transformed B cells (Yamanashi and Baltimore, 1997). However, evidence is accumulating that suggests $p62^{dok}$ actually plays a negative role in Erk-MAPKinase activation and proliferation. Cells from mice deficient in $p62^{dok}$ demonstrate increased proliferation in response to growth factors, and such cells exhibit prolonged Erk-MAPKinase and Ras activation in response to growth factors (Di Cristofano *et al.*, 2001). Inactivation of $p62^{dok}$ also enhances the transforming ability of $p210^{bcr\text{-}abl}$, accelerating the onset of the chronic myelogenous leukaemia (CML)-like disease triggered by expression of the chimeric protein (Di Cristofano *et al.*, 2001). Reintroduction of $p62^{dok}$ into mouse embryo fibroblast (MEF) cells from $p62^{dok\text{-}/\text{-}}$ mice results in a return to a normal level of proliferation in response to PDGF, rather than the enhanced proliferative response seen in $p62^{dok\text{-}/\text{-}}$ MEFs, and this was concomitant with an abrogation of the sustained Ras and Erk-MAPKinase activation seen in $p62^{dok\text{-}/\text{-}}$ cells (Zhao *et al.*, 2001). In B cells, $p62^{dok}$ negatively regulates Erk-MAPKinase activation and cell proliferation mediated by the BCR, by abrogating Ras activation (Yamanashi *et al.*, 2000), and is phosphorylated in response to BCR-FcγRIIB co-ligation, enhancing its binding of Ras-GAP, implicating $p62^{dok}$ as a key mediator of

FcγRIIB inhibition of BCR-mediated Ras activation (Tamir *et al.*, 2000). The phosphorylation of $p62^{dok}$, and subsequent association with Ras-GAP, is dependent on phosphorylation of the ITIM of FcγRIIB, and the concomitant recruitment of SHIP to the ITIM, as $p62^{dok}$ does not have a domain with which to interact with the ITIM, and has been shown to interact directly with SHIP (Tamir *et al.*, 2000).

3.1.3 CD40 mediated rescue of apoptosis

As mentioned above, not all immature B lymphocytes undergo apoptosis in response to antigen. This reflects the importance of extrinsic signals upon the fate of B lymphocytes, and one such key signal is generated through CD40. The ability of CD40 to rescue both immature B lymphocytes from BCR-mediated apoptosis and prevent the spontaneous death of mature B cells is well documented (Choi *et al.*, 1995; Monroe, 2000). However, the signalling mechanisms utilised by CD40 to achieve these outcomes are only just coming to light, particularly the downstream events. The initial signalling mechanisms, however, have been partially elucidated in recent years. For example, it has been shown that CD40 ligation results in the activation of the Erk-MAPKinase, JNK, and p38 stress-activated kinases, depending on the maturation stage of the cell, making dissection of the signalling mechanisms downstream of CD40 complicated (Berberich *et al.*, 1996; Purkerson and Parker, 1998; Sutherland *et al.*, 1996).

Studies in our laboratory have shown no evidence of Erk-MAPKinase activation in WEHI-231 cells in response to CD40 stimulation alone, however, anti-CD40 co-stimulation was shown to alter the kinetics of ERK-MAPKinase activation in anti-Ig treated WEHI-231 cells (Gauld, 2001). In contrast, p38 is activated in response to either anti-Ig or anti-CD40 stimulation alone in WEHI-231 cells, with slightly different kinetics, though maximal activation is seen within 15 minutes in both cases (Craxton *et al.*, 1998).

3.2 Aims and objectives

The membrane proximal signalling events initiated in response to BCR ligation are becoming fairly well elucidated, with 3 key signalling pathways now known to be the major targets. The biological responses that are a consequence of BCR ligation have also been well established, with immature B cells undergoing growth arrest and/or apoptosis, while mature B cells undergo proliferation and differentiation. Some major effector molecules of these events have also been well characterised, with NF-κB and c-myc known to be major players in B cell response to antigen. However, the links between the activation of the 3 major signalling pathways and the key effector molecules are less well known. The aims of this research project are to further elucidate the downstream signalling pathways involved in linking BCR ligation with regulation of the cell cycle and induction of apoptosis in immature B lymphocytes. This study aims to investigate the role of Erk-MAPKinase in cell cycle progression of immature B lymphocytes, using the WEHI-231 cell line as a model. In particular, it aims to address:

1. The role of the small GTPase Ras in BCR-mediated signalling, using the constitutively active Ras mutant, RasV12.
2. The role of the effector molecules that Ras interacts with, using mutant forms of RasV12 that are unable to interact with key effector molecules.
3. The use of negative regulators of Ras (the $p62^{Dok}$ protein) and PI-3-Kinase (the inositol phosphatase SHIP) to corroborate Ras effector mutant data.
4. How these pathways link with the cell cycle machinery.

3.3 Results

3.3.1 Anti-Ig induces growth arrest of WEHI-231 cells, and this is rescued by co-stimulation with anti-CD40

It is well established that treatment of WEHI-231 immature B lymphocytes with anti-Ig antibodies inhibits DNA synthesis and induces apoptosis (Hasbold and Klaus, 1990). Thus, as shown in **Figure 3.1**, treatment of WEHI-231 cells with anti-Ig (B7.6) causes a dose-dependent increase in growth arrest, as assessed by the [^{3}H]-thymidine uptake assay. In addition, co-stimulation of WEHI-231 cells through CD40 is known to protect the cells from such anti-Ig induced growth arrest (Choi *et al.*, 1995). Thus, **Figure 3.1** also shows that co-stimulation with anti-CD40 (10 μg/ml) restores DNA synthesis, as measured by [^{3}H]-thymidine uptake, back to the levels observed in unstimulated cells.

3.3.2 Anti-Ig induces apoptosis in WEHI-231 cells, and this is rescued by co-stimulation with anti-CD40

The above experiments confirmed that BCR-ligation induces growth arrest in WEHI-231 cells, and that this growth arrest can be abrogated by co-stimulation with anti-CD40. BCR-ligation is also known to induce apoptosis in WEHI-231 cells (Hasbold and Klaus, 1990), but thymidine uptake experiments give no indication as to the levels of apoptosis. To investigate the levels of apoptosis induced by anti-Ig stimulation, and the protection from apoptosis afforded by co-stimulation with anti-CD40, the number of cells in a population displaying sub-diploid DNA content, as measured by flow cytometry following staining with the DNA intercalating dye propidium iodide, was measured. **Figure 3.2** shows the effect of BCR-ligation on the DNA content profile of WEHI-231 cells, and the effect co-stimulation with anti-CD40 has on anti-Ig-mediated apoptosis. The results confirm the ability of BCR-ligation to induce apoptosis in WEHI-231 cells, and the ability of stimulation via CD40 to protect WEHI-231 cells from anti-Ig

induced apoptosis. Anti-Ig also induces a substantial increase in the proportion of cells in G_1 phase of the cell cycle, with a concomitant decrease in the proportion of cells in both S phase and G_2/M. Co-stimulation with anti-Ig and anti-CD40 leads to restoration of the proportion of cells in S phase and G_2/M, corroborating the data from the previous section that stimulation via CD40 overcomes anti-Ig induced growth arrest (**Figure 3.1**). By 72 hours, unstimulated cells, as well as cells stimulated with the combination of anti-Ig and anti-CD40, begin to accumulate in G_1. This is most likely due to depletion of essential nutrients in the culture, as the cells are not dying, just ceasing to proliferate.

3.3.3 Anti-Ig treatment of WEHI-231 cells results in the long term down regulation of Erk-MAPKinase activity, and this is restored during CD40-mediated rescue

Studies to investigate the signalling mechanisms underlying the growth arrest and apoptosis induced by anti-Ig treatment of WEHI-231 cells, and the CD40-mediated rescue of anti-Ig mediated effects, initiated in this laboratory by Gauld and Harnett identified potential dual roles for Erk-MAPKinase signalling in apoptosis and cellular proliferation in these cells. For example, in untreated WEHI-231 cells, there is a cyclical activation of Erk-MAPKinase over a 48 hour period (**Figure 3.3**). Stimulation with anti-Ig, however, enhances the early activation of Erk-MAPKinase, particularly at 2 hours post stimulation, but completely abolishes the sustained Erk-MAPKinase activity exhibited by unstimulated cells beyond 4 hours (**Figure 3.3**). Treatment of cells with the combination of anti-Ig and anti-CD40 prevents this down-regulation of Erk-MAPKinase activity, and indeed re-establishes and strengthens the cyclical pattern of Erk-MAPKinase activitation in WEHI-231 cells (**Figure 3.3**). Whilst anti-CD40 alone does not stimulate Erk-MAPKinase activity at early time points, the cyclical pattern of Erk-MAPKinase activation is still observed, with the levels peaking at 8 and 48 hours, with a lower level at 24 hours post stimulation. These findings suggest a key role for Erk-MAPKinase activity in the spontaneous

proliferation of WEHI-231 cells, and that the abrogation of this activity, due to BCR ligation, may be sufficient to induce growth arrest and apoptosis. The restoration of Erk-MAPKinase activity induced by anti-CD40 treatment further underscores the potential significance of Erk-MAPKinase activity in the proliferation of WEHI-231 cells.

3.3.4 Erk-MAPKinase activity contributes to spontaneous proliferation and CD40-mediated rescue of growth arrest in WEHI-231 cells

Due to the sustained, but cyclical, activity of Erk-MAPKinase in untreated WEHI-231 cells and the ability of CD40 stimulation to restore Erk-MAPKinase activity in BCR stimulated cells, it was decided to study more closely the role Erk-MAPKinase plays in WEHI-231 B cell responses. Thus, MEK inhibitors were used to dissect the role Erk-MAPKinase plays in the proliferation of WEHI-231 cells. To ensure maximal inhibition of Erk-MAPKinase, two MEK inhibitors, PD98059 and U0126, were tested in combination. These two inhibitors have different mechanisms for inhibiting MEK, suggesting they might work synergistically. For example, PD98059 inhibits MEK by binding to it and preventing its phosphorylation and concomitant activation by Raf, whereas U0126 directly inhibits the catalytic activity of MEK.

WEHI-231 cells were pre-treated with the MEK1/2 inhibitors PD98059 and U0126 (both at 1 μM), or no inhibitor, and stimulated with anti-Ig, either alone or in combination with anti-CD40. Control cells received no stimulating antibodies. Cells treated with the MEK inhibitors were treated with an additional dose (1 μM) every 4 hours for a total of 32 hours due to the inherent instability of these inhibitors in WEHI-231 cells (Gauld *et al.*, 2002). Proliferation was assessed after 48 hours by means of the [^{3}H]-thymidine incorporation assay (**Figure 3.4**). Following a stimulation time of 48 hours, the inhibition of MEK in this manner was effective at inducing growth arrest of spontaneously proliferating cells, as well as enhancing anti-Ig induced growth arrest. Inhibition of MEK was also effective at

abrogating CD40-mediated rescue from growth arrest. These results suggest a key role for Erk-MAPKinase in both the spontaneous proliferation of WEHI-231 cells and in the rescue of WEHI-231 cells from anti-Ig mediated growth arrest by CD40, reinforcing earlier observations.

3.3.5 Effect of cell cycle inhibitors on WEHI-231 cells

The observations outlined above strongly suggest that Erk-MAPKinase plays a major role in the proliferation of WEHI-231 cells. To further investigate how Erk-MAPKinase promotes WEHI-231 cell proliferation, we decided to examine the role Erk-MAPKinase plays at various stages of the cell cycle. In order to achieve this, cell cycle inhibitors were utilised to arrest WEHI-231 cells at various stages of the cell cycle, with the intention of synchronising the cells at a particular stage then releasing them from the inhibition. As we intended to study the function of Erk-MAPKinase at various cell cycle stages, a number of cell cycle inhibitors were assessed for their suitability at arresting the cell cycle at either G_0/G_1, S phase, or G_2/M.

Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) is a purine derivative that acts as a competitive inhibitor of cyclin dependent kinases (CDKs) and induces G_1 arrest (see **Figure 1.9**) (Abraham *et al.*, 1995). In contrast, aphidicolin inhibits DNA synthesis by binding to DNA polymerase α, therefore inducing arrest in S phase (Mutomba and Wang, 1996). Nocodazole is an antimitotic agent that disrupts microtubules by binding to β-tubulin, inhibiting microtubule dynamics, disrupting mitotic spindle function and fragmenting the Golgi apparatus, reportedly resulting in G_2/M arrest in a number of cell types.

To determine the efficiency of the cell cycle inhibitors olomoucine, aphidicolin, and nocodazole at inducing cell cycle arrest in WEHI-231 cells, cells were treated with each inhibitor over a period of 48 hours, and the cell cycle position of the cell

populations determined at various time points by DNA content analysis by flow cytometry after staining with propidium iodide (50 μg/ml).

As **Figure 3.5** shows, olomoucine and aphidicolin induce arrest in WEHI-231 cells in the appropriate phase of the cell cycle. Olomoucine results in a dramatic increase in the proportion of cells in G_0/G_1 compared to control cells, whilst aphidicolin greatly increases the proportion of cells in S phase, as would be expected. Nocodazole, however, appeared to be less efficient than the other two, as the proportion of cells in G_2/M is very similar to that of the control cells. This failure could be explained by the accompanying increase in the proportion of sub-diploid cells, which is perhaps unsurprising as nocodazole is known to induce apoptosis in a number of normal and tumour cell lines.

Using these inhibitors, it was only possible to attain enriched populations of each phase, as opposed to fully synchronised populations. As olomoucine inhibits the cdc2 (also known as CDK1) and CDK2 kinases, this is possibly due to the fact that the G_2/M transition will be blocked slightly as well as the G_1/S transition (see **Figure 1.9**), resulting in most cells arresting in G_1, but a small percentage also arresting in G_2. Too high a concentration, or treatment for an extended period of time, resulted in a vast increase in the percentage of WEHI-231 cells undergoing apoptosis, particularly following treatment with aphidicolin and nocodazole. The optimum conditions to induce reversible arrest in WEHI-231 cells were found to be 50 μM olomoucine for 40 hours, and 5 μg/ml aphidicolin for 24 hours. As nocodazole failed to significantly enrich the G_2/M population without causing an increase in apoptosis, it was decided not to pursue use of this inhibitor in further studies.

3.3.6 A role for cyclical Erk-MAPKinase activation in cell cycle progression of WEHI-231 cells

To investigate how cycling Erk activation correlates with cell cycle progression, the activity of Erk in WEHI-231 cells was analysed after release of cells from either G_1 or S phase arrest, induced by olomoucine and aphidicolin respectively. WEHI-231 cells were therefore incubated with either 50 μM olomoucine for 40 hours, or 5 μg/ml aphidicolin for 24 hours to induce arrest, then washed twice with fresh media to remove the cell cycle blockers. Arrested cells were then stimulated with anti-Ig, a combination of anti-Ig and anti-CD40, or left unstimulated in media for up to 48 hours, and whole cell lysates made at the appropriate time points. Erk activity was examined by western blotting of cell lysates with an anti-phospho-Erk antibody (**Figure 3.6**).

As with asynchronous cells, treatment with anti-Ig alone induces inhibition of Erk activity by 16-24 hours, following an earlier, transient increase. Co-stimulation with anti-CD40 induces strong cycling Erk activation during this later time period, particularly in cells released from olomoucine-induced growth arrest (G_1). This supports the hypothesis that anti-Ig induces growth arrest in WEHI-231 cells by suppressing sustained Erk activity associated with cell cycle progression, and that anti-CD40 rescues cells by reinstating such Erk activity, promoting cell cycle progression. The pattern of Erk activation in response to anti-Ig and anti-CD40 co-stimulation is slightly different between the cells arrested at different phases, most strikingly at 16 hours. The cells released from olomoucine arrest appear to demonstrate a strong Erk signal at this time. This time point could correlate with the G_1/S phase transition as these cells were previously arrested in G_1, suggesting a key role for Erk-MAPKinase activity somewhere around the G_1/S phase boundary. Overall, it appears that Erk activity follows a cyclical pattern regardless of where in the cell cycle the cells are at the outset, and that BCR ligation is capable of inhibiting Erk activity at any cell cycle stage, suggesting that it is unlikely to be associated with a single phase of the cell cycle. To address

more directly the role of Erk-MAPKinase in driving cell cycle progression, intracellular staining of phospho-Erk was analysed at distinct cell cycle phases using the Laser Scanning Cytometer (LSC).

WEHI-231 cells were incubated in the presence of olomoucine (50 μM) for 40 hours, or aphidicolin (5 μg/ml) for 24 hours, then washed to remove the cell cycle blocker. Arrested cells were then stimulated with anti-Ig, a combination of anti-Ig and anti-CD40, or left unstimulated in media for up to 48 hours. Cells were attached to slides at the appropriate time points, fixed and permeabilised, then stained for phospho-Erk and the DNA content assessed by staining with propidium iodide, as described in **Section 2.12.2**. Following staining, cells were scanned by LSC.

The majority of cells transiting the cell cycle were found to exhibit phospho-Erk staining, whereas fewer than 50% of newly formed daughter cells demonstrate phospho-Erk staining (**Figure 3.7**). Growth arrest-inducing treatments (olomoucine or anti-Ig) reduced the percentage of cells expressing phospho Erk, indicating a strong link between Erk activity and cell cycle progression. This suggests that a late Erk signal is required by WEHI-231 cells to transit from G_1 to S phase. Indeed, in each cell cycle phase a high proportion of the cells display phospho-Erk staining, strongly suggesting that Erk-MAPKinase activity is required by WEHI-231 cells at every stage of the cell cycle.

3.3.7 Transfection of WEHI-231 cells with RasV12 constructs

The above results suggest that Erk-MAPKinase plays an important role in the spontaneous proliferation of WEHI-231 cells, as well as in CD40-mediated rescue of anti-Ig induced growth arrest. One of the key upstream regulators of Erk-MAPKinase activity is the small GTPase Ras, a key regulator of growth in all eukaryotic cells. To complement the above studies, we wished to see if over-

expression of a constitutively active Ras mutant, RasV12, could rescue WEHI-231 cells from anti-Ig induced growth arrest and/or apoptosis.

The first system for transfection we utilised was retroviral transfection, whereby the DNA of interest is packaged into a retroviral particle, which enters the cells via a specific receptor. DNA encoding RasV12 was inserted into the MIEV vector (**Figure 2.7**) using the *Eco* RI restriction enzyme sites present in the vector. This vector contains an internal ribosome entry site between the gene of interest and an enhanced green fluorescent protein gene (eGFP). This permits the identification of transfected cells on the basis that the eGFP is expressed along with the protein of interest, therefore transfected cells can be identified by flow cytometry and selected using a fluorescence activated cell sorter. The vector containing the RasV12 gene was first transiently transfected, as described in **Section 2.13.2**, into the PT67 cell line, an NIH/3T3-based packaging cell line that expresses the 10A1 viral envelope, permitting the vector DNA to be packaged into a form that is capable of transfecting cells expressing either the amphotropic retrovirus receptor or the GALV receptor. The supernatant from PT67 cells transiently transfected with MIEV.RasV12 was then used to transfect the GP+E-86 cell line, another NIH/3T3-based packaging cell line that contains the *gag*, *pol*, and *env* genes of MMLV, and generates viral particles capable of infecting rat or murine cells (ecotropic). The *gag*, *pol*, and *env* genes are required for production of virus particles as the MIEV vector has had the structural genes removed to allow for insertion of the gene of interest. Successfully transfected GP+E-86 cells were selected on the basis of their green fluorescence using a FACSVantage™ cell sorter (Becton Dickinson) as described in **Section 2.13.3**.

Stably transfected GP+E-86 cells were then used to infect WEHI-231 cells with the MIEV.RasV12 vector by co-culture, as described in **Section 2.13.4**. After co-culture with the stably-transfected GP+E-86.MIEV.RasV12 retroviral packaging line, **Figure 3.8** shows that at least 80% of the WEHI-231 population were transfected, as measured by green fluorescence. The MIEV vector alone was

also transfected into WEHI-231 cells in the same manner, and **Figure 3.8** also shows that approximately 60% of cells were transfected with empty vector.

To complement the retroviral transfection studies, we decided to introduce the RasV12 coding sequence into WEHI-231 cells via electroporation also. To facilitate this, the RasV12 coding sequence was also inserted into the pcDNA3.1 vector (**Figure 2.9**), again using the *Eco* RI restriction enzyme sites. This vector was introduced to WEHI-231 cells by electroporation, as described in **Section 2.14.** Successfully transfected cells were selected by the addition of G418 (500 μg/ml) 48 hours after electroporation, as pcDNA3.1 confers resistance to G418 upon WEHI-231 cells.

3.3.8 Effect of RasV12 transfection on response to anti-Ig mediated growth arrest in WEHI-231 cells

Wild type WEHI-231 cells exhibit a pronounced growth arrest in the G_1 phase of the cell cycle in response to anti-Ig stimulation, as demonstrated in **Figure 3.1.** To investigate the effects of the constitutively active Ras mutant, RasV12, on anti-Ig mediated growth arrest in WEHI-231 cells, WEHI-231 cells transfected with this form of Ras, either retrovirally or by electroporation, were stimulated with various concentrations of anti-Ig for 48 hours, and proliferation assessed by the [^{3}H]-thymidine uptake assay. When WEHI-231 cells retrovirally transfected with the constitutively active Ras mutant, RasV12, are stimulated with anti-Ig the resulting growth arrest is less pronounced than that exhibited by wild type cells, particularly with 10 μg/ml anti-Ig (**Figure 3.9A**).

WEHI-231 cells that have been transfected with pcDNA3.1.RasV12 by electroporation demonstrate an even more pronounced abrogation of anti-Ig induced growth arrest (**Figure 3.9B**). In neither case is the alteration in anti-Ig induced growth arrest a result of the vector, as WEHI-231 cells transfected with either empty vector behave in the same manner as wild type WEHI-231 cells,

when stimulated with anti-Ig (**Figure 3.9A** and **3.9B**). As the RasV12 mutant is constitutively active these results suggest that activation of Ras is sufficient to prevent, or at least inhibit, anti-Ig mediated growth arrest, resulting in promotion of proliferation. Indeed, in most cell types, RasV12 is capable of inducing transformation by circumventing the requirement for growth factor signals.

3.3.9 Effect of RasV12 transfection on response to anti-Ig mediated apoptosis in WEHI-231 cells

To investigate whether the effect of RasV12 transfection on anti-Ig induced growth arrest was a result of a concomitant decrease in anti-Ig mediated apoptosis, DNA content analysis by flow cytometry following staining with propidium iodide was performed, following anti-Ig stimulation. Retroviral transfection of WEHI-231 cells with RasV12 inhibits anti-Ig-induced apoptosis, as there are far fewer WEHI-231 cells expressing RasV12 with sub-diploid DNA content than wild type cells (**Figure 3.10A**). This decrease in the proportion of cells with a sub-diploid DNA content in response to anti-Ig is accompanied by an increase in the proportion of cells in both S phase and G_2/M, which is rather surprising as the [^{3}H]-thymidine uptake assay suggested that these cells were still susceptible to anti-Ig induced growth arrest, though not to quite the same extent as those containing the empty vector (**Figure 3.9A**). As before, this effect is not a direct consequence of the introduction of the MIEV vector to the cells, as WEHI-231 cells transfected with the MIEV empty vector respond in the same manner as wild type cells to anti-Ig, with the levels of apoptosis doubling when cells are treated with anti-Ig (**Figure 3.10A**).

WEHI-231 cells that have been transfected with pcDNA3.1.RasV12 by electroporation do not display the same inhibition of anti-Ig induced apoptosis, but rather have a higher percentage of cells with a sub-diploid DNA content compared to those transfected with the empty pcDNA3.1 vector (**Figure 3.10B**). This is accompanied by an increase in the percentage of cells in G_0/G_1, and a

decrease in the percentage of cells in S phase and G_2/M, in response to anti-Ig. This is surprising as the [^{3}H]-thymidine assay suggested that these cells were protected from anti-Ig induced growth arrest (**Figure 3.9B**), yet when the DNA content is analysed it appears as though anti-Ig induced growth arrest is not affected.

3.3.10 Effect of RasV12 transfection on CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis in WEHI-231 cells

To investigate the effects that RasV12 has on CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis, transfected cells were stimulated with anti-Ig alone, or a combination of anti-Ig and anti-CD40. Following stimulation for 48 hours, proliferation was assessed by the [^{3}H]-thymidine incorporation assay (**Figure 3.11**), and apoptosis was assessed by DNA content analysis by flow cytometry following staining with propidium iodide (**Figure 3.10**). The effect of RasV12 on CD40-mediated rescue of anti-Ig induced growth arrest appears to vary greatly depending on the transfection method (**Figure 3.11**). Retrovirally transfected RasV12 appears to completely abrogate CD40-mediated rescue of anti-Ig induced growth arrest, as the levels of proliferation are similar to that of cells stimulated with anti-Ig alone. In contrast, cells transfected by electroporation with RasV12 exhibit greater proliferation in response to anti-Ig and anti-CD40 stimulation than cells transfected with the vector alone. As RasV12 induces growth and transformation of most cell types, increased CD40-mediated proliferation is not an unexpected phenomenon. What is unexpected, however, is the difference in consequences depending on the transformation method.

DNA content analysis of WEHI-231 cells retrovirally transfected with RasV12 suggests that CD40-mediated rescue of anti-Ig induce growth arrest and apoptosis is slightly enhanced by the expression of RasV12, particularly as there are over twice as many cells in G_2/M compared to cells transfected with the empty vector alone (**Figure 3.10A**). This appears to contradict the [^{3}H]-thymidine

assay data, where anti-CD40 did not appear to overcome anti-Ig induced growth arrest (**Figure 3.11**). However, the [^{3}H]-thymidine assay specifically measures cells in S phase of the cell cycle, therefore as there are a large percentage in G_2/M these would not be detected in that assay. In contrast, DNA content analysis of WEHI-231 cells containing the pcDNA3.1.RasV12 vector corroborates the [^{3}H]-thymidine results, with cells exhibiting a strong rescue from anti-Ig induced apoptosis, as there is a decrease in the proportion of apoptotic cells and a concomitant increase in the proportion of cells in S phase or G_2/M phases upon anti-Ig and anti-CD40 co-stimulation, compared to cells stimulated with anti-Ig alone (**Figure 3.10B**).

3.3.11 Effect of the p62^{Dok} PH-PTB domain on anti-Ig mediated growth arrest and apoptosis in WEHI-231 cells

The Dok proteins are a family of adaptor proteins that are phosphorylated by a wide range of protein tyrosine kinases. The first Dok protein to be isolated was p62^{dok}, originally identified as a 62 kD tyrosine-phosphorylated protein associated with Ras-GAP (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), a negative regulator of Ras (Bollag and McCormick, 1991). In B cells, p62^{dok} negatively regulates Erk-MAPKinase activation and cell proliferation mediated by the BCR, by abrogating Ras activation (Yamanashi *et al.*, 2000). To complement the above studies, we decided to use the amino terminal portion of Dok, which contains pleckstrin homology (PH) and phosphotyrosine binding domains (PTB). This portion of the protein should not be able to abrogate Ras, and concomitant Erk-MAPKinase, activation, as these domains are not involved in Ras-GAP binding, therefore will not be able to induce the GTPase activity of Ras. WEHI-231 cells overexpressing the first 258 amino acids of Dok (Dok-PH/PTB a kind gift, along with cells expressing the empty retroviral vector, pMXI, of Stephen Gauld, Idan Tamir and John Cambier, Denver, CO, USA) were used to determine the role p62^{dok} plays, if any, in the anti-Ig mediated growth arrest and apoptosis of WEHI-231 cells.

To investigate the effects this portion of $p62^{dok}$ has on anti-Ig induced growth arrest, cells expressing this domain were stimulated with anti-Ig and proliferation assessed after 48 hours by the [3H]-thymidine uptake assay, and apoptosis assessed by DNA content analysis following staining with propidium iodide. WEHI-231 cells expressing Dok-PH/PTB exhibit no significant difference in growth arrest induced by anti-Ig, or in anti-CD40-mediated rescue when compared to cells transfected with the empty vector alone (**Figure 3.12A**). The DNA content analysis following propidium iodide staining further demonstrates this, with 80% of Dok-PH/PTB expressing cells arrested in G_0/G_1 in response to anti-Ig stimulation, compared to 75% of those cells containing the empty vector (**Figure 3.12B**). Interestingly, despite the proliferation assay suggesting CD40-mediated rescue occurs as normal, there appears to be slightly fewer cells in S and G_2/M phases upon stimulation of these cells with anti-Ig and anti-CD40, and a greater proportion displaying a sub-diploid DNA content. This is not what would be anticipated, as cells lacking $p62^{dok}$ demonstrate sustained Ras activation (Zhao *et al.*, 2001), and as the Dok-PH/PTB domain is thought to act in a dominant negative fashion, it would be expected that Ras would be activated and the cells more prone to proliferation. However, recent studies in our laboratory have suggested that BCR ligation in WEHI-231 cells increases the association of the Erk-MAPKinase specific phosphatase, Pac-1, with Erk-MAPKinase (Gauld, 2001). This could result in BCR-induced Erk-MAPKinase activity being switched off downstream from Ras and MEK, so perhaps this overrides any effects $p62^{Dok}$ may have had on Erk-MAPKinase activity upon BCR ligation.

3.3.12 Transfection of the WEHI-231 cell line with RasV12 effector mutants

Ras has a number of effector pathways involved in an array of biological outcomes, two of the most important being the ERK-MAPK pathway and the PI-3-K pathway. To address the question of whether these two pathways are involved in the inhibition of anti-Ig-induced growth arrest, two RasV12 effector

mutants were transfected into the WEHI-231 cell line. These two mutants were RasV12-S35 and RasV12-C40, both of which contain the V12 point mutation, along with one further point mutation each. RasV12-S35 contains a point mutation that results in a substitution of serine for threonine at position 35, in the heart of the domain of Ras responsible for interacting with its effector molecules. This mutation renders Ras incapable of interacting with the p110α subunit of PI-3-Kinase, or Ral.GDS, but it is still capable of interacting with Raf-1, therefore this Ras mutant can only activate the ERK-MAPK pathway (Rodriguez-Viciana *et al.*, 1997). RasV12-C40 is the consequence of a point mutation that results in the substitution of cysteine for tyrosine at position 40. This substitution abrogates the interaction between Ras and its effectors Raf-1 and Ral.GDS, but not PI-3-Kinase, resulting in a form of active Ras that can only activate the PI-3-Kinase pathway (Rodriguez-Viciana *et al.*, 1997).

As with the RasV12 mutant, the effector mutants were introduced to the cells via two different methods, retroviral transfection and electroporation. To facilitate retroviral transfection, DNA encoding each effector mutant was inserted into the MIEV vector (**Figure 2.7**), and these vectors were used to generate stably transfected GP+E-86 retroviral packaging lines, as above. These packaging lines were then used to infect WEHI-231 cells in the same manner as the MIEV.RasV12 vector. **Figure 3.13** shows that approximately 65% of the population of WEHI-231 cells were successfully transfected with the RasV12-S35 construct, whilst approximately 61% were successfully transfected with the RasV12-C40 construct, as determined by green fluorescence. The coding sequences for RasV12-S35 and RasV12-C40 were also inserted into the pcDNA3.1 vector, and introduced to cells by electroporation, as described above. Successful transfectants were selected using the antibiotic G418.

3.3.13 Effect of the RasV12 effector mutant RasV12-S35 on WEHI-231 response to anti-Ig mediated growth arrest

To determine the effect that the RasV12-S35 mutant has on the response of WEHI-231 cells to anti-Ig and anti-CD40 stimulation, transfected cells were stimulated as before and their biological responses assessed. WEHI-231 cells retrovirally transfected with the RasV12-S35 construct (WEHI-231 MIEV.RasV12-S35) exhibit no great difference in their response to anti-Ig compared to cells transfected with the empty vector (**Figure 3.14A**). This is not the case when RasV12-S35 is introduced to the cells by electroporation, as the growth arrest in response to anti-Ig in these cells is not as great as those transfected with the empty vector alone, and is not significantly different from cells expressing RasV12 (**Figure 3.14B**). CD40-mediated rescue from anti-Ig induced growth arrest is not affected by RasV12-S35, as cells transfected by both methods exhibit a robust proliferative response upon stimulation by anti-Ig and anti-CD40 (**Figure 3.14A** and **B**). This is in contrast to cells retrovirally transfected with RasV12, which are not refractive to CD40-mediated rescue of anti-Ig induced growth arrest (**Figure 3.14A**).

3.3.14 Effect of the RasV12-S35 effector mutant on anti-Ig mediated apoptosis in WEHI-231 cells

To determine the effects of the RasV12-S35 effector mutant on BCR-mediated apoptosis, the percentage of sub-diploid cells was investigated as before, by DNA content analysis following staining with propidium iodide, after stimulation with anti-Ig alone, or in combination with anti-CD40. WEHI-231 MIEV.RasV12-S35 cells have a similar proportion of cells displaying a sub-diploid DNA content in response to anti-Ig, when compared to cells transfected with the empty vector (**Figure 3.15A**). These cells show an alteration in their response to co-stimulation with anti-Ig and anti-CD40, as anti-CD40 does not afford the same level of protection from apoptosis, as there are a similar proportion of sub-diploid cells

after co-stimulation with anti-Ig and anti-CD40 as there are when the cells were stimulated with anti-Ig alone (**Figure 3.15A**). This is in stark contrast to cells transfected with MIEV.RasV12, as a very low percentage of these cells display a sub-diploid DNA content when they are treated with anti-Ig and anti-CD40. These results suggest that Ras activation of the PI-3-Kinase pathway may play a role in the CD40-mediated reversal of anti-Ig induced cell death in WEHI-231 cells, as the RasV12-S35 mutant is unable to activate PI-3-Kinase. The reversal of growth arrest is not affected as measured by the [^{3}H]-thymidine assay (**Figure 3.14A**), and the DNA content analysis appears to corroborate this as the proportion of RasV12-S35 expressing cells in S phase and G_2/M in response to anti-Ig and anti-CD40 costimulation is similar to that of cells expressing the empty vector alone (**Figure 3.15A**). This suggests that Ras induced PI-3-Kinase activity is required for CD40-mediated protection from anti-Ig induced apoptosis, but not anti-Ig induced growth arrest. As PI-3-Kinase has been widely suggested to play a role in cell survival, a role for it in the CD40-mediated protection from apoptosis and not growth arrest is plausible.

Cells expressing the RasV12-S35 mutant from the pcDNA3.1 vector (WEHI-231 pcDNA3.1.RasV12-S35) behave in a slightly different fashion. The percentage of sub-diploid cells is quite low in response to anti-Ig in these experiments, though the FACS data correlates with the proliferation data in that there is an increase in the number of cells in G_0/G_1 upon anti-Ig stimulation, compared to unstimulated cells, hence cell cycle arrest is still taking place in response to anti-Ig (**Figure 3.15B**). The proportion of sub-diploid cells decreases upon co-stimulation with anti-CD40, in stark contrast to the retrovirally transfected cells, where it appeared as though CD40-mediated inhibition of anti-Ig induced apoptosis was abrogated.

3.3.15 Effect of the RasV12-C40 effector mutant on anti-Ig mediated growth arrest in WEHI-231 cells

To determine the effect that the RasV12-C40 mutant has on the response of WEHI-231 cells to anti-Ig and anti-CD40 stimulation, transfected cells were stimulated as before and their proliferation assessed by the [3H]-thymidine uptake assay. WEHI-231 cells transfected with MIEV.RasV12-C40 exhibit a similar extent of anti-Ig mediated growth arrest to cells transfected with the empty vector alone, and cells transfected with RasV12, when 10 μg/ml anti-Ig is used, however, at lower concentrations anti-Ig has little effect (**Figure 3.16A**). CD40-mediated rescue from anti-Ig induced growth arrest does not appear to be altered by expression of RasV12-C40, as these cells exhibit a robust proliferative response to anti-Ig and anti-CD40 stimulation, particularly compared to cells expressing the RasV12 mutant (**Figure 3.16A**).

Cells that have been transfected with RasV12-C40 by electroporation demonstrate a low level of growth arrest in response to a wide range of concentrations of anti-Ig, from 0.1 to 10 μg/ml (**Figure 3.16B**). This strongly suggests that RasV12-C40 affords some level of protection against anti-Ig induced growth arrest in WEHI-231 cells, as cells expressing RasV12-C40 display around 80% of the proliferation of untreated cells when stimulated with 10 μg/ml anti-Ig. Cells transfected with the empty vector alone on the other hand, display only around 40% of the proliferation of untreated cells when stimulated with anti-Ig, ruling out the vector as a possible explanation for this observation (**Figure 3.16B**). Co-stimulation of cells expressing RasV12-C40 with anti-Ig and anti-CD40 results in proliferation levels higher than untreated cells, but as the growth arrest was much less in response to anti-Ig, this is a less than surprising observation (**Figure 3.16B**). Indeed, the levels of proliferation of RasV12-C40 expressing cells appear to be greater than those of cells containing the empty vector by a consistent amount when cells are stimulated with anti-Ig alone or in combination with anti-CD40.

3.3.16 Effect of the RasV12-C40 effector mutant on anti-Ig mediated apoptosis in WEHI-231 cells

To determine the effects of the RasV12-C40 effector mutant on BCR-mediated apoptosis, the percentage of sub-diploid cells was investigated as before by DNA content analysis following propidium iodide staining. WEHI-231 MIEV.RasV12-C40 cells have a reduced apoptotic response to anti-Ig, demonstrating a lower proportion of cells with a sub-diploid DNA content compared to cells transfected with the empty vector, though still higher than cells transfected with RasV12 (**Figure 3.17A**). The reasons for this are undoubtedly manifold. We have recently shown that anti-Ig induced apoptosis requires an early (≤ 2 hours) Erk signal, which activates a phospholipase A_2 pathway that mediates arachidonic acid-mediated collapse of $\Delta\psi_m$ and depletion of cellular ATP, resulting in cathepsin B execution of apoptosis. When the activation of Erk-MAPKinase is inhibited by specific MEK1/2 inhibitors, anti-Ig induced apoptosis is significantly decreased, almost to basal levels (Gauld *et al.*, 2002). As this Ras mutant is unable to interact with and activate the Erk-MAPKinase pathway, this could provide one explanation for the decrease in anti-Ig induced apoptosis, though endogenous Ras should still be able to activate Erk-MAPKinase. Another possible explanation is that the PI-3-Kinase pathway is increasing the activity of PKB/Akt, a powerful mediator of pro-survival signals capable of suppressing apoptosis induced by a wide range of stimuli (Datta *et al.*, 1997). As there was a decrease in anti-Ig induced apoptosis, it is difficult to draw any conclusions concerning the effect of RasV12-C40 upon anti-CD40-mediated rescue of apoptosis, though the levels of apoptosis are very similar when cells are stimulated with anti-Ig in the presence or absence of anti-CD40 (**Figure 3.17A**).

By contrast, WEHI-231 cells containing the pcDNA3.1.RasV12-C40 vector exhibit a greater proportion of cells with a sub-diploid DNA content in response to anti-Ig, compared to unstimulated cells, though the percentage of unstimulated cells displaying a sub-diploid DNA content is very low to begin with (**Figure 3.17B**).

Co-stimulation with anti-CD40 inhibits anti-Ig induced apoptosis, as the proportion of cells with a sub-diploid DNA content drops. This is accompanied by an increase in the proportion of cells displaying an S phase or G_2/M DNA content, supporting the [^{3}H]-thymidine data that showed these cells demonstrate increased proliferation when co-stimulated with anti-Ig and anti-CD40.

3.3.17 Effect of a catalytically inactive SHIP mutant and the SHIP SH2 domain on anti-Ig mediated growth arrest in WEHI-231 cells

To further investigate the role of PI-3-Kinase signalling, we decided to use cells expressing mutant versions of the 5' inositol phosphatase SHIP, a negative regulator of PI-3-Kinase signalling. SHIP antagonises the functions of PI-3-Kinase by dephosphorylating the 5' position of PtdIns(3,4,5)P_3, a product of PI-3-Kinase that is important for the membrane localisation of many PH domain containing proteins. WEHI-231 cells retrovirally transfected so that they overexpress either a catalytically inactive mutant of SHIP (SHIP-CI) or the SH2 domain alone (SHIP-SH2, both a kind gift of Stephen Gauld, Idan Tamir, and John Cambier, Denver, CO, USA) were used to determine the role SHIP plays, if any, in the anti-Ig mediated growth arrest and apoptosis of WEHI-231 cells. Overexpression of these mutants should elicit a dominant negative effect, by preventing endogenous SHIP interacting with the appropriate proteins, thus its activity. To investigate the effects expression of these SHIP mutants has on anti-Ig induced growth arrest in WEHI-231 cells, cells expressing them were stimulated with anti-Ig, either alone or in combination with ani-CD40, and proliferation assessed after 48 hours by the [3H]-thymidine uptake assay.

WEHI-231 cells expressing either of these SHIP mutants do not appear to exhibit any significant difference in anti-Ig induced apoptosis when compared to cells containing the empty vector (**Figure 3.18**). CD40-mediated rescue does not appear to be affected by expression of either of these SHIP mutants either, as

cells transfected with either of these mutants display similar levels of proliferation in response to anti-Ig and anti-CD40 as cells containing the empty vector.

3.3.18 Effect of a catalytically inactive SHIP mutant and the SHIP SH2 domain on anti-Ig mediated apoptosis in WEHI-231 cells

To investigate the effects expression of these SHIP mutants has on anti-Ig induced apoptosis in WEHI-231 cells, cells expressing them were stimulated with anti-Ig, either alone or in combination with anti-CD40 for 48 hours. Apoptosis was assessed via DNA content analysis by flow cytometry, following staining with propidium iodide.

WEHI-231 cells expressing SHIP-CI appear to be more prone to anti-Ig mediated apoptosis than cells containing the empty vector after 48 hours treatment, as over 20% of the cells exhibit a sub-diploid DNA content, as assessed by propidium iodide staining and FACS analysis, in response to anti-Ig (**Figure 3.19**). Upon anti-Ig stimulation only around 1% of cells containing the empty vector exhibit sub-diploid DNA content, though a vast proportion (almost 80%) display a diploid (G_1 phase) quantity of DNA. WEHI-231 SHIP-SH2 cells behave in a similar manner to the empty vector-containing cells, with the vast majority arrested in G_0/G_1. All 3 cell types display an increase in the proportion of cells in S phase and G_2/M upon co-stimulation with anti-Ig and anti-CD40, supporting the above results that demonstrated that these cells all display CD40-mediated rescue from anti-Ig induced growth arrest (**Figure 3.18**).

3.3.19 Effect of RasV12 mutants on expression of $p27^{Kip1}$ in response to anti-Ig and anti-CD40 stimulation

The cell cycle is the co-ordinated series of events required for cell growth and division. This complex process relies on the assembly of nuclear proteins that integrate signals and programme cell cycle progression (see **Section 1.5**)

(**Figure 1.9**). In order to elucidate the effects of the expression of the various mutant proteins at a molecular level, as opposed to a biological level, we decided to look at the expression profiles of key cell cycle proteins in response to anti-Ig and anti-CD40 stimulation. One such key regulator of the cell cycle is the cyclin dependent kinase inhibitor (CKI) $p27^{Kip1}$, an inhibitor of the CDK2-cyclin E complex during G_1, whose accumulation is associated with G_1 arrest (**Figure 1.9**).

Whole cell lysates from WEHI-231 cells expressing the empty pcDNA3.1 vector, pcDNA3.1RasV12, pcDNA3.1RasV12-S35 or pcDNA3.1RasV12-C40 were examined by western blotting following the appropriate stimulation. Anti-Ig treatment of WEHI-231 cells transfected with the empty pcDNA3.1 vector induces an increase in $p27^{Kip1}$ levels over time (**Figure 3.20**). Stimulation via CD40, both on its own and in combination with anti-Ig, induces a decrease in $p27^{Kip1}$ levels after an initial, transient, increase. WEHI-231 cells expressing the constitutively active RasV12 mutant exhibit a similar profile of $p27^{Kip1}$ expression in response to anti-Ig, with an increase over time. However, upon stimulation with anti-CD40 or the combination of anti-Ig and anti-CD40 p27 expression drops to barely detectable levels.

Overexpression of the RasV12-S35 mutant results in a dramatic increase in the level of expression of $p27^{Kip1}$ in response to anti-Ig stimulation, and this increase is particularly evident after 24 and 48 hours of stimulation (**Figure 3.20**). This increase is repressed by co-stimulation with anti-CD40. In response to anti-Ig, cells overexpressing the RasV12-C40 mutant demonstrate a slight increase in $p27^{Kip1}$ expression but not to the same degree as cells expressing the RasV12-S35 mutant (**Figure 3.20**). Stimulation with anti-CD40 alone induces a decrease in $p27^{Kip1}$ levels over time, whilst co-stimulation with anti-Ig and anti-CD40 induces a decrease after 8 hours, with a modest increase over 24 and 48 hours.

These results correlate fairly well with the proliferation assay results (**Figures 3.14B** and **3.16B**), as RasV12-S35 expressing cells demonstrated a more pronounced anti-Ig-induced growth arrest than the RasV12 or RasV12-C40 expressing cells, or cells containing the empty vector alone, possibly explained by a greater induction of p27 in response to anti-Ig.

3.3.20 Effect of RasV12 mutants on Tyr15 phosphorylation of the cdc2 protein in response to anti-Ig and anti-CD40 stimulation

A key regulator of the G2/M phase transition is the cyclin dependent kinase cdc2, whose cyclin partner is cyclin B. Entry of all eukaryotic cells into M-phase of the cell cycle is regulated by activation of cdc2. Activation of cdc2 is controlled at several steps, including cyclin binding and phosphorylation at threonine 161. However, the critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation at tyrosine 15 and threonine 14 (Atherton-Fessler *et al.*, 1993). Phosphorylation at Tyr15 and inhibition of cdc2 is carried out by Wee1 and Myt1 protein kinases, while Tyr15 dephosphorylation and activation of cdc2 is carried out by the cdc25 phosphatase.

To assess the effects of the RasV12 mutants on the activity of cdc2 under different conditions, whole cell lysates were subjected to western blot analysis using an anti-phospho-cdc2 (Tyr15) antibody. WEHI-231 cells transfected with the empty pcDNA3.1 vector display relatively low levels of Tyr15 phosphorylated (inactive) cdc2, which increase from 1 to 8 hours, then decrease again between 24 and 48 hours (**Figure 3.21**). Stimulation of these cells with anti-Ig, which induces G_1 arrest (**Figure 3.9**), leads to an increase in the levels of Tyr15 phosphorylated cdc2 at 1 hour compared to unstimulated cells, followed by a marked decrease over the time course (**Figure 3.21**). This is rather surprising, as during G_1 cdc2 would be expected to be inactive, hence Tyr15 phosphorylated. It would be expected that under mitogenic conditions (treatment with anti-CD40, either alone or in combination with anti-Ig) there would be a decrease in Tyr15

phosphorylation as cells progress through the cell cycle, yet there appears to be more Tyr15 phosphorylation under these conditions, particularly at 24 hours (**Figure 3.21**).

This is not the case with cells transfected with RasV12, as the levels of phosphorylated Tyr15 in response to anti-Ig and anti-CD40 co-stimulation are barely detectable, particularly at the later time points, suggesting a large proportion of cells in mitosis. The levels are also low in response to anti-Ig treatment alone, and as anti-Ig failed to induce much growth arrest in WEHI-231 pcDNA3.1.RasV12 cells, this is perhaps hardly surprising. Cells expressing either RasV12-S35 or RasV12-C40 exhibit a drop in phospho-cdc2 (Tyr15) levels when treated with anti-Ig and anti-CD40 in combination, compared to anti-CD40 alone (**Figure 3.21**). The levels displayed by cells treated with anti-Ig alone are comparable to the co-stimulated cells for both types, and again anti-Ig failed to induce growth arrest to the same levels as in cells containing the empty vector, so the low levels of phospho-cdc2 (Tyr15) could indicate more cells passing through mitosis.

3.3.21 Effect of RasV12 mutants on phosphorylation of the retinoblastoma protein, Rb, in response to anti-Ig and anti-CD40 stimulation

Another key regulator of the cell cycle is the retinoblastoma tumour suppressor protein (Rb). Rb inhibits progression from G_1 to S phase of the cell cycle by binding to the E2F family of transcription factors and preventing transcription of key genes required for cell cycle progression (Dyson, 1998) (**Figure 1.9**). The interaction between Rb and E2F is abrogated by phosphorylation of Rb by CDKs at a number of sites in its C-terminal domain, allowing E2F to activate transcription of a number of genes. Therefore, Rb phosphorylation is a measure of cell cycle progression, from G_1 to S phase. To investigate the phosphorylation state of Rb, an antibody against phosphorylated Rb (Ser^{807} and Ser^{811}) was used in western blot analysis. Surprisingly, this antibody failed to detect phospho-Rb in

whole cell lysates from WEHI-231 cells containing the RasV12 mutant. However, phospho-Rb was detected in cells expressing both RasV12 effector mutants (**Figure 3.22**).

WEHI-231 cells containing the empty pcDNA3.1 vector exhibit low levels of Rb phosphorylation in the absence of any stimulation, and the levels drop to undetectable after 48 hours. Stimulation with anti-Ig accelerates the disappearance of detectable Rb phosphorylation, with no detectable levels at 24 hours, as opposed to 48 hours. This correlates very well with the anti-Ig induced growth arrest of these cells (**Figure 3.9**)

Cells expressing RasV12-S35 display a low level of Rb phosphorylation over the time period looked at when they are not stimulated. Stimulation with anti-Ig results in a reduction in Rb phosphorylation, after 8 hours, with levels barely detectable after 24 and 48 hours. Stimulation with anti-CD40 alone sees a slight decrease in the level of Rb phosphorylation up to 24 hours, with levels at 48 hours barely detectable. Co-stimulation with anti-CD40 sees a large amount of Rb phosphorylation at 8 hours, but this drops by 24 hours and remains low at 48 hours. Cells expressing the RasV12-C40 mutant display a very high basal level of Rb phosphorylation prior to any stimulation, and anti-Ig stimulation induces a decrease over time. Cells stimulated with anti-CD40 alone exhibit a basal level of Rb phosphorylation slightly lower than that displayed by unstimulated cells, however, the levels do remain constant up to 24 hours then appear to decrease at 48 hours. Co-stimulation with anti-Ig and anti-CD40 results in a slight increase in Rb phosphorylation by 8 hours, followed by a decrease at 24 hours with the levels remaining low after 48 hours.

3.3.22 Effect of Dok PH-PTB domain and SHIP mutants on expression of $p27^{Kip1}$ in response to anti-Ig and anti-CD40 stimulation

To assess the effect of the overexpression of the Dok PH-PTB domain and the expression of SHIP mutants in WEHI-231 cells on the protein levels of $p27^{Kip1}$, whole cell lysates of unstimulated, anti-Ig stimulated, anti-CD40 stimulated and anti-Ig plus anti-CD40 stimulated WEHI-231 cells expressing the appropriate constructs were probed by western blotting with an anti-$p27^{Kip1}$ antibody (**Figure 3.23**). In cells containing the empty pMXI vector, anti-Ig stimulation results in an increase in $p27^{Kip1}$ expression compared to unstimulated cells, concomitant with anti-Ig induced cell cycle arrest and apoptosis. Co-stimulation with anti-CD40 causes a reduction in the expression of $p27^{Kip1}$, concomitant with the re-instatement of proliferation induced in these cells by co-stimulation with anti-Ig and anti-CD40. WEHI-231 cells expressing the Dok PH-PTB domain have a similar profile of p27 expression levels under these conditions, though at the later time points anti-CD40 appears less efficient at abrogating anti-Ig induced up-regulation of p27 expression as the levels are slightly higher than those detected in cells containing the empty vector.

Expression of both SHIP-CI and SHIP-SH2 seems to decrease the upregulation of $p27^{Kip1}$ induced by anti-Ig stimulation compared to cells transfected with the empty vector alone (**Figure 3.23**). This is despite the fact that these cells demonstrate a similar level of growth arrest as cells expressing the empty vector in response to anti-Ig treatment (**Figure 3.18**). Anti-CD40 stimulation of WEHI-231 SHIP-SH2 cells induces an increase in p27 levels after 1 and 8 hours, but the levels drop again at 24 and 48 hours. This increase is not as evident in cells expressing SHIP-CI, though they do seem to have a slightly increased level of p27 in response to anti-CD40 compared to unstimulated cells. Anti-Ig and anti-CD40 co-stimulation in these cells does seem to result in a decrease in $p27^{Kip1}$ expression, compared to the unstimulated cells, and as the proliferation data

suggests CD40 ligation can rescue anti-Ig induced growth arrest, lower levels of $p27^{Kip1}$ would be expected.

3.3.23 Effect of the Dok PH-PTB domain and SHIP mutants on Tyr15 phosphorylation of the cdc2 protein in response to anti-Ig and anti-CD40 stimulation

To assess the effects of the Dok PH-PTB domain and the SHIP mutants on the activity of cdc2 under different conditions, whole cell lysates were subjected to western blot analysis using an anti-phospho-cdc2 (Tyr15) antibody. Unstimulated cells containing the empty vector seem to have fairly high levels of phosphorylated (inactive) cdc2, particularly at 8 and 24 hours, and anti-Ig stimulation appears to decrease the Tyr15 phosphorylation of cdc2, suggesting they have more active cdc2 than unstimulated cells (**Figure 3.24**). Stimulation of these cells with anti-CD40 alone results in an increase in the Tyr15 phosphorylation at 24 hours, though this drops again by 48 hours, whilst cells co-stimulated with anti-Ig and anti-CD40 have lower levels of phosphorylated cdc2 than cells treated with anti-Ig alone. The expression of the Dok PH-PTB domain does not alter the pattern of cdc2 phosphorylation under these conditions, as it is very similar to the pattern exhibited by empty vector containing cells.

This is also the case with cells transfected with either of the SHIP mutants, as again, the pattern does not change significantly. Anti-Ig stimulation appears to decrease the amount of phosphorylation of cdc2 in both cell types, despite the fact that both demonstrate cell cycle arrest in response to anti-Ig treatment. Again, the levels are highest after 24 hours treatment with anti-CD40, indicative of cells in G_1, S phase or early to mid G_2. Perhaps the majority of these cells pass through mitosis between 8 and 20 hours, resulting in them containing inactive cdc2 at 24 hours.

3.3.24 Effect of Dok PH-PTB domain and SHIP mutants on expression of D-type cyclins in response to anti-Ig and anti-CD40 stimulation

A key cyclin responsible for G_1 progression in B cells is cyclin D2 (Solvason *et al.*, 2000), the cyclin partner for CDK 4 which is responsible for phosphorylation of the retinoblastoma protein and hence G_1 to S phase progression. To investigate the effects of the Dok PH-PTB domain and the SHIP mutants on the levels of cyclin D2, whole cell lysates were subjected to western blot analysis using an anti-cyclin D1/2 antibody. Expression of this cyclin is low in WEHI-231 cells containing the empty vector when they are not stimulated, and levels are barely detectable upon stimulation of these cells with anti-Ig (**Figure 3.25**). Stimulation with anti-CD40 alone induces an increase in cyclin D expression, yet cells co-stimulated with anti-Ig and anti-CD40 have barely detectable levels of cyclin D1/2 except at 1 hour. WEHI-231 cells expressing the Dok PH-PTB domain have similar expression of cyclin D2 compared to cells containing the empty vector, except upon co-stimulation with anti-Ig and anti-CD40. Co-stimulation induces an increase compared to unstimulated cells over the time period looked at, not seen in cells containing the empty vector. However, this increase is less than that induced by anti-CD40 treatment alone.

Both WEHI-231 SHIP-CI and SHIP-SH2 cells have a much higher basal level of cyclin D2 expression when unstimulated, compared to cells containing the empty vector. Stimulation of both WEHI-231 SHIP-CI and SHIP-SH2 cells with anti-Ig induces a decrease in cyclin D2 expression to undetectable levels, as would be expected as anti-Ig induces growth arrest very effectively in these cells (**Figure 3.25**). Rather surprisingly, co-stimulation of either cell type with anti-Ig and anti-CD40 does not induce much of an increase in cyclin D2 expression, though WEHI-231 SHIP-CI cells do exhibit some cyclin D2, unlike cells containing the empty vector. This is slightly surprising as co-stimulation restores proliferation very efficiently in cells expressing either SHIP construct.

3.4 Discussion

Immature B cells generally respond to antigen by undergoing negative selection, though *in vivo* the presence of other, extrinsic signals can lead to alternative fates. One of these signals is mediated through CD40, and this can induce rescue from BCR-mediated growth arrest and apoptosis. This is most likely to occur in the periphery rather than the bone marrow, as B cells maintain the immature B cell phenotype for several days after exit into the periphery to ensure any B cells that are specific for self-antigens expressed in the periphery and not the bone marrow are negatively selected (Carsetti *et al.*, 1995). The ability of CD40 to mediate rescue from BCR-induced growth arrest and apoptosis would allow for recruitment of antigen-specific immature B cells into an ongoing immune response where T cell help already exists (Monroe, 2000). The results obtained at the beginning of this chapter showing that WEHI-231 cells undergo BCR-mediated growth arrest and apoptosis, and that this is rescued by CD40-mediated signals, confirm the WEHI-231 cell line represents a good model for the study of BCR-mediated growth arrest and apoptosis, and CD40-mediated rescue from BCR-induced growth arrest and apoptosis.

The early signalling mechanisms activated upon ligation of the BCR under conditions leading to proliferation in mature B cells or growth arrest and apoptosis in immature B cells appear to be very similar, with phospholipase C, PI-3 kinase and Erk-MAPKinase all being activated under both circumstances. However, we have shown that although BCR ligation of WEHI-231 cells results in early stimulation of Erk-MAPKinase activity, in the long term it leads to down-regulation of the sustained, cycling Erk-MAPKinase activity observed in proliferating WEHI-231 cells. This is in contrast to what we see in mature B cells, where BCR ligation leads to sustained, cycling Erk-MAPKinase activity (see **Section 5.3.6**). Co-stimulation of WEHI-231 cells with anti-Ig and anti-CD40 re-establishes the sustained, cycling Erk activity, driving proliferation of these cells (**Figure 3.26**). This is evidenced by the fact that sustained inhibition of MEK, a

key activator of Erk-MAPKinase, leads to growth arrest of unstimulated WEHI-231 cells, as well as abrogating CD40-mediated rescue of BCR-driven growth arrest of WEHI-231 cells. Cell cycle inhibitor studies suggested that the requirement for Erk-MAPKinase activity was not associated with a single cell cycle checkpoint. Indeed, the requirement for Erk-MAPKinase throughout cell cycle progression of WEHI-231 cells is further demonstrated by the observation that, following release from olomoucine induced G_1 arrest, Erk-MAPKinase activity is exhibited in a large proportion of cells that are traversing the cell cycle, whereas it is not found in the majority of newly formed daughter cells or those undergoing growth arrest. Taken together, these results strongly suggest that sustained, cycling Erk-MAPKinase activity is required for proliferation of WEHI-231 cells, and that the BCR abrogates this sustained activation to induce growth arrest, whilst signalling via CD40 restores this sustained, cycling Erk-MAPKinase activity, allowing the cells to proliferate.

The early, transient, activation of Erk-MAPKinase in response to BCR ligation in WEHI-231 cells is important for the induction of apoptosis, as is evidenced by the fact that inhibition of early Erk-MAPKinase activity by pharmacological inhibitors of MEK can suppress BCR-induced apoptosis (Gauld *et al.*, 2002). Therefore, Erk-MAPKinase activity in WEHI-231 cells is capable of mediating diverse outcomes depending upon the timing and duration of the Erk-MAPKinase activity, with strong early transient Erk-MAPKinase activity leading to apoptosis, whilst sustained, cycling activation leads to proliferation. A similar phenomenon of differential biological outcomes depending on the kinetics, amplitude and duration of Erk-MAPKinase activity has also been demonstrated in PC12 cells, where transient Erk-MAPKinase activation results in proliferation, whilst sustained Erk-MAPKinase activation causes the cells to differentiate (York *et al.*, 1998).

One of the key upstream regulators of Erk-MAPKinase activity is the small GTPase Ras, a key regulator of growth in all eukaryotic cells. To further

investigate the mechanisms involved in regulating the sustained Erk-MAPKinase activity in WEHI-231 cells, the role of Ras and its downstream effectors was explored, utilising constitutively active mutants of Ras with varying degrees of affinity for two of its key effectors, Raf and PI-3-Kinase. Expression of these constitutively actively Ras mutants provided partial protection to WEHI-231 cells from anti-Ig induced growth arrest and apoptosis, especially when transfected via electroporation. This was particularly evident with the RasV12-C40 mutant, which can only interact with PI-3-Kinase and not Raf, as anti-Ig only reduced [^{3}H]-thymidine uptake by around 20%, compared to 60% in cells containing the empty vector (**Figure 3.16**). The converse mutant, RasV12-S35, which can only interact with Raf, not PI-3-Kinase, also protects WEHI-231 cells from anti-Ig induced growth arrest, but not to quite the same extent as [^{3}H]-thymidine uptake is reduced by around 40%. Indeed, cells expressing the RasV12-C40 construct exhibited greater proliferation in response to co-stimulation with anti-Ig and anti-CD40. Expression of constitutively active Ras mutants might be expected to result in the constitutive activation of Erk-MAPKinase, but this is not the case in WEHI-231 cells. In studies performed in collaboration with Catriona Ford, in unstimulated cells, there is no evidence to suggest that either RasV12 or RasV12-S35 constitutively activate Erk-MAPKinase as the activity of Erk-MAPKinase follows a cyclical pattern, although levels are higher than in cells containing the empty vector. Rather surprisingly, this is also true of those cells transfected with the RasV12-C40 vector, suggesting that Erk-MAPKinase can be additionally activated downstream of PI-3-Kinase as reported elsewhere for B cells and other systems (Han *et al.*, 2003; Jacob *et al.*, 2002; Li and Carter, 2000; Von Willebrand *et al.*, 1996) or alternatively that PI-3-Kinase signalling can abrogate/antagonise the negative regulatory signals acting on Erk-MAPKinase. Upon ligation of the BCR, all of the Ras mutants, but particularly RasV12-C40 prevent anti-Ig induced inhibition of sustained, cyclical Erk-MAPKinase activity. However, because they do not induce constitutive Erk-MAPKinase signalling these results strongly suggest that BCR-induced suppression of Erk-MAPKinase activity involves Erk-dependent negative regulatory mechanisms that function

downstream of Ras (Catriona Ford, personal communication). This proposal is consistent with studies in this laboratory that have demonstrated that ligation of the BCR in WEHI-231 cells leads to an abrogation in Erk-MAPKinase activity not by uncoupling the upstream regulators of Erk-MAPKinase (Ras, Raf, MEK), but rather by upregulating the expression of the protein phosphatase Pac-1 and promoting its interaction with Erk-MAPKinase (Gauld *et al.*, 2002).

Ras signalling has been shown to be required in at least two phases of the G_0 to S phase transition in fibroblasts, and these two periods of Ras signalling require different effector molecules, therefore this may explain the slightly different effects of the RasV12-S35 (which couples to Raf) and RasV12-C40 (which couples to PI-3-Kinase and indirectly to Erk) mutants on WEHI-231 cells. Fibroblasts that are continually exposed to growth factors exhibit Erk-MAPKinase activity in the early stages of Ras activation, but its activity is not detected at later stages of G_1 progression, despite the fact Ras is still active. PI-3-Kinase is active in mid to late G_1, and this activity is required for entry into S phase (Jones *et al.*, 1999). In WEHI-231 cells, inhibition of PI-3-Kinase activity for a prolonged period (up to 30 hours) results in growth arrest of unstimulated cells, suggesting that PI-3-Kinase plays an important role in proliferation of WEHI-231 cells (Gauld, 2001). Our results suggest that activation of both PI-3-Kinase and Erk-MAPKinase by RasV12 or RasV12-C40 is capable of overcoming anti-Ig induced growth more efficiently than Ras induced Erk-MAPKinase activity alone (resulting from transfection with RasV12-S35).

The results of the experiments on cells expressing the constitutively active RasV12 mutant rather surprisingly varied slightly depending upon the transfection method. Cells transfected with pcDNA3.1.RasV12 exhibit an abrogation in their growth arrest in response to anti-Ig, whilst cells transfected with MIEV.RasV12 also demonstrate reduced growth arrest, but to a lesser extent (**Figure 3.9**). These results suggest that the RasV12 mutant is capable of overcoming anti-Ig induced growth arrest, which is unsurprising as RasV12

induces transformation in a number of cell types (**Figure 3.27**) and we have shown sustained, cycling Erk-MAPKinase activity to be important for WEHI-231 B cell proliferation. The most intriguing difference between transfection methods involves the rescue of anti-Ig induced growth arrest by CD40 ligation. The MIEV.RasV12 transfected cells do not exhibit a great deal of DNA synthesis in response to co-stimulation after 48 hours, whereas the pcDNA3.1.RasV12 cells do. The reasons for this are unclear, however, one possibility may simply reflect a limitation of the [^{3}H]-thymidine incorporation assay in that, if these cells are proliferating at a faster rate, the majority may have already passed through S phase by 48 hours. This idea is supported by the flow cytometry analysis, which shows that, after 48 hours, a greater proportion of MIEV.RasV12 transfected cells are in G_2/M as opposed to S phase. If the cells are proliferating at a faster rate, it is also plausible that they may have exhausted the nutrients in the media by 48 hours, therefore they are unable to continue proliferation through additional rounds of cell division, hence the drop in [^{3}H]-thymidine incorporation. It would be interesting to investigate the number of rounds of cell division RasV12 expressing cells undergo compared to those containing the empty vector, by labelling the cells with carboxy-fluorescein diacetate succinimidyl ester (CFSE) and measuring the successive halving of the fluorescence. Initial attempts to investigate this found that CFSE is highly toxic to B cells, therefore requires a great deal of optimisation.

Investigation of the downstream events regulating cell cycle progression did not provide clear-cut answers as to how the Ras mutants were able to reduce anti-Ig-mediated growth arrest. Nevertheless, it was clear that when cells containing the empty vector are stimulated with anti-Ig, an increase in the levels of the cyclin dependent kinase inhibitor p27 occurs, concomitant with growth arrest. Consistent with this, the mechanism by which the RasV12-C40 mutant is able to inhibit anti-Ig induced growth arrest appears, at least in part, to involve modulation p27 levels. Thus, cells expressing RasV12-C40, which show reduced anti-Ig induced growth arrest, do not increase their expression levels of p27. This

correlates well with recent observations that inhibition of PI-3-Kinase activity in mouse embryo fibroblasts results in G_1 arrest, concomitant with p27 up-regulation (Collado *et al.*, 2000). Other recent findings suggest that, rather than interfering with the transcription or translation of p27, Akt, a major effector of PI-3-Kinase, impairs nuclear import of p27, thus abrogating the ability of p27 to induce G_1 arrest resulting from interactions with cyclin E/CDK2 complexes in the nucleus (Fujita *et al.*, 2002; Liang *et al.*, 2002; Shin *et al.*, 2002). This finding underlines the important role p27 plays in anti-Ig mediated growth arrest of WEHI-231 cells, as demonstrated previously (Ezhevsky *et al.*, 1996), and that the effects of the RasV12-C40 mutant can be explained, at least in part, by the repression of p27 expression. The regulation of p27 expression is highly complex, and involves both translational control and regulation of the stability of the protein, with various mechanisms of degradation being described, such as ubiquitin-dependent degradation by the proteasome (Montagnoli *et al.*, 1999) and degradation by calpain (Patel and Lane, 2000). The translation of p27 is negatively regulated by Rho, another small GTPase, as inhibition of Rho increases the translational efficiency of p27 mRNA. A dominant negative mutant of Rho, $RhoA^{N19}$, suppresses the transformation ability of RasV12, but not in cells deficient in p27 (Vidal *et al.*, 2002), so it would be interesting to investigate whether co-expression of this Rho mutant with these Ras mutants in WEHI-231 cells would alter the response to anti-Ig. It might be expected that WEHI-231 cells co-expressing RasV12-C40 and $RhoA^{N19}$ would undergo growth arrest in response to anti-Ig, as Rho would be inhibited, therefore p27 levels should increase.

By contrast, the increase in p27 levels in anti-Ig stimulated cells expressing RasV12-S35, relative to empty vector control, is rather unexpected as these cells displayed reduced growth arrest. However, this apparent contradiction may be explained by recent findings that the role of p27 is not as simple as cyclin dependent kinase inhibitor. For example, although $p27^{Kip1}$ can inhibit recombinant cyclin D-CDK complexes *in vitro*, it is a far more effective inhibitor of

cyclin E-CDK complexes (Toyoshima and Hunter, 1994). Indeed, assembly of cyclin D-CDK complexes is impaired in mouse embryo fibroblasts from mice deficient in $p21^{Waf1}$, $p27^{Kip1}$, or both (Cheng *et al.*, 1999), implying a role for p27 as an assembly factor for cyclin D-CDK complexes. The assembly functions and subsequent sequestration of p27 in cyclin D-CDK4 complexes can titrate the amount of p27 from cyclin E-CDK2, the key target for p27 inhibition, allowing cells to progress through G_1 and enter S phase (Tsutsui *et al.*, 1999). It would be interesting to determine the subcellular localisation of p27 in these cells, as in the cytoplasm p27 interacts with cyclin D/CDK complexes and does not inhibit cell cycle progression, but when it is present in the nucleus it interacts with cyclin E/CDK2 complexes and induces G_1 arrest (Shin *et al.*, 2002). The Western blots investigating p27 expression were performed on whole cell lysates, therefore any differences in subcellular localisation would not be detected. This could be achieved either with the imaging software attached to the laser scanning cytometer, or by confocal microscopy, or by subcellular fractionation, followed by western blotting of individual fractions.

The investigation of other key regulators of the cell cycle produced unusual results, particularly the phosphorylation at tyrosine 15 of cdc2, the key regulator of the G_2/M transition. The critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation at tyrosine 15 (Atherton-Fessler *et al.*, 1993), yet the mitogenically stimulated cells appeared to possess more cdc2 phosphorylated at tyrosine 15 than cells stimulated via the BCR, which induces growth arrest. The reasons for this are unclear, but perhaps as the cells were an asynchronous population the dephosphorylation of cdc2 occurs at different times, resulting in the apparent absence of any effects. It is possible that synchronisation of the cells prior to stimulation would result in the majority of the population passing the G_2/M transition around the same time, thus the dephosphorylation of cdc2 would become more evident.

To corroborate these data on the role of Ras and its downstream effectors in regulating immature B cell apoptosis, survival and proliferation we also transfected WEHI-231 cells with mutants of $p62^{Dok}$, a negative regulator of Ras. The Dok proteins are a family of adaptor proteins that are phosphorylated by a wide range of protein tyrosine kinases. The first Dok protein to be isolated was $p62^{dok}$, originally identified as a 62 kD tyrosine-phosphorylated protein associated with Ras-GAP (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), which is a negative regulator of Ras (Bollag and McCormick, 1991). Indeed, in B cells, $p62^{dok}$ has been shown to negatively regulate Erk-MAPK activation and cell proliferation mediated by the BCR, by abrogating Ras activation (Yamanashi *et al.*, 2000). The behaviour of the Dok-PH/PTB mutants in this study, however, appears at first to be rather odd, as the Dok-PH/PTB domain should act in a dominant negative fashion, leading to sustained activation of Ras, as demonstrated by cells lacking $p62^{Dok}$ (Zhao *et al.*, 2001). This might be expected to result in the same biological outcomes as transfection with the RasV12 mutant, however, WEHI-231 cells transfected with the Dok-PH/PTB domain respond in much the same manner as wild type cells, undergoing BCR-induced growth arrest. Despite this apparent absence of an effect at the biological level, inhibition of Dok has an effect at the molecular level as Erk-MAPKinase activity in WEHI-231 cells expressing the Dok-PH/PTB domain is significantly enhanced and, perhaps rather surprisingly given the Ras mutant data, is constitutively active even upon stimulation of the cells with anti-Ig (Catriona Ford, personal communication). Therefore, despite the fact that these cells demonstrate sustained Erk-MAPKinase activation in the presence of anti-Ig, they still undergo anti-Ig induced growth arrest and apoptosis, and do not exhibit enhanced proliferation, as might be expected. These data suggest that Dok may play a role in mediating the negative regulation of Erk-MAPKinase in response to BCR ligation, as inhibition of Dok abrogates anti-Ig induced down regulation of Erk-MAPKinase activity. This implies that Dok utilises mechanisms other than the recruitment of RasGAP to regulate the activity of Erk-MAPKinase, as expression of a constitutively active Ras mutant did not have the same effect on Erk-

MAPKinase activity, yet one of the expected consequences of inhibiting Dok would be that Ras would be more active, as Dok is a negative regulator of Ras via RasGAP. This is consistent with recent findings that Dok can exert its negative effect on Erk-MAPKinase activity independently of its ability to associate with RasGAP and another of its effectors, Nck (Zhao *et al.*, 2001). Given our findings that sustained Erk-MAPKinase activity is associated with proliferation of WEHI-231 cells, it was rather surprising that cells expressing the Dok-PH/PTB domain undergo anti-Ig induced growth arrest. However, the fact that WEHI-231 cells expressing the Dok-PH/PTB domain undergo anti-Ig induced growth arrest is consistent with the observation that they upregulate the CDK inhibitor $p27^{Kip1}$, whose accumulation has been linked to anti-Ig induced cell cycle arrest in WEHI-231 cells (Ezhevsky *et al.*, 1996).

Analysis of WEHI-231 cells expressing the RasV12-C40 construct, along with previous work undertaken in this laboratory, suggested a key role for PI-3-Kinase in the proliferation and survival of WEHI-231 cells. To further investigate this, we utilised mutants of an antagonist of PI-3-Kinase activity, the SH2-containing 5'-inositol phosphatase, SHIP. SHIP antagonises the functions of PI-3-Kinase by dephosphorylating the 5' position of PtdIns(3,4,5)P_3, a product of PI-3-Kinase that is important for the membrane localisation of many PH domain containing proteins. However, our results using two distinct mutant forms of SHIP which interfere with SHIP activity by either competing for SH2 domain interactions (SHIP-SH2) or competing for SH2 interactions as well as substrate binding (SHIP-CI) suggest that SHIP does not play a significant role in the regulation of anti-Ig induced growth arrest or apoptosis in WEHI-231 cells. In contrast, in mature B cells SHIP has been shown to play a major role in the FcγRIIb mediated inhibition of BCR-induced proliferative signalling (Liu *et al.*, 1998). We have recently demonstrated a role for the 3'-inositol phosphatase, PTEN, in FcγRIIb mediated inhibition of BCR-induced proliferative signalling, suggesting that B cells are capable of antagonising PI-3-Kinase activity via the recruitment of both 3'- and 5'-inositol phosphatases (Brown *et al.*, 2004). Therefore, perhaps in

WEHI-231 cells, inhibition of SHIP by the overexpression of a catalytically inactive mutant or its SH2 domain does not lead to an observable effect as the levels of PIP_3 can still be reduced by the 3'-inositol phosphatase activity of PTEN. It would be very interesting to assess the activity of PTEN in WEHI-231 cells under different conditions, and expressing different constructs, to determine whether or not it plays a role in regulating responses in WEHI-231 cells.

Overall, these results demonstrate that Erk-MAPKinase activity is required for anti-Ig induced growth arrest and apoptosis in WEHI-231 cells, as well as for CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis (**Figure 3.26**). The small GTPase, Ras, and its effector molecules, also appear to play a key role in BCR-mediated responses (**Figure 3.27**). The Ras/PI-3-Kinase pathway seems to play a role in CD40-mediated rescue of anti-Ig induced apoptosis of WEHI-231 cells, as cells overexpressing a constitutively active Ras mutant unable to interact with PI-3-Kinase are refractory to CD-40 mediated rescue of anti-Ig induced apoptosis. Anti-Ig induced growth arrest in WEHI-231 cells is accompanied by an increase in the expression of the cyclin dependent kinase inhibitor $p27^{Kip1}$ (**Figure 3.28**).

Figure 3.1 Anti-Ig treatment induces growth arrest in WEHI-231 cells which is overcome by co-treatment with anti-CD40 antibodies

WEHI-231 cells (1×10^4 cells/well) were cultured in the presence of anti-Ig (0.1, 1, 10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least 4 other independent experiments.

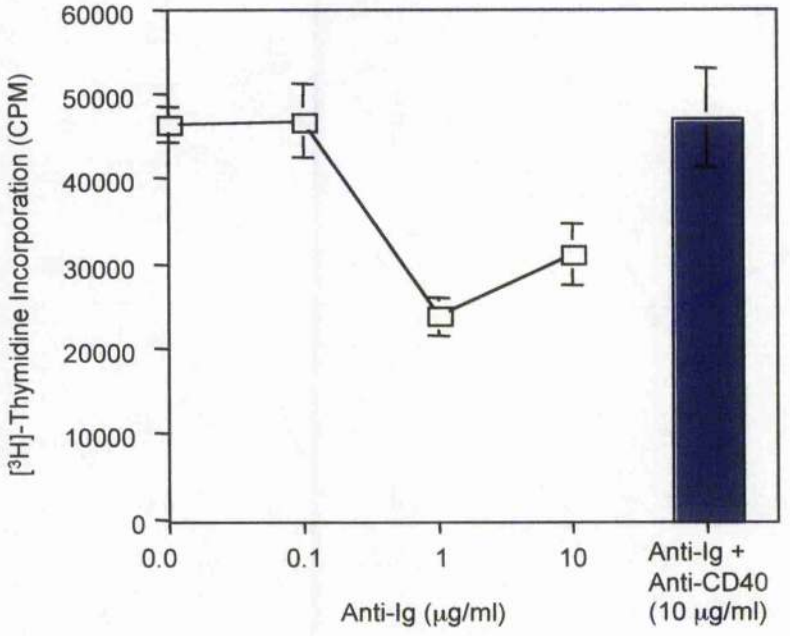

60000
50000
40000
30000
20000
10000
0
[3H]-Thymidine Incorporation (CPM)
0.0
0.1
1
10
Anti-Ig (μg/ml)
Anti-Ig +
Anti-CD40
(10 μg/ml)

Figure 3.2 Anti-Ig treatment induces apoptosis in WEHI-231 cells which is overcome by co-treatment with anti-CD40 antibodies

WEHI-231 cells (5×10^5 cells/ml) were cultured in the presence of anti-Ig (10 μg/ml), with or without anti-CD40 (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining to assess DNA content, and FACS analysis after 24, 48 and 72 hours, as described in Materials and Methods.

A DNA profiles of WEHI-231 cells treated with the appropriate agonists, from a single experiment, representative of at least four other independent experiments.

B Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least four other independent experiments.

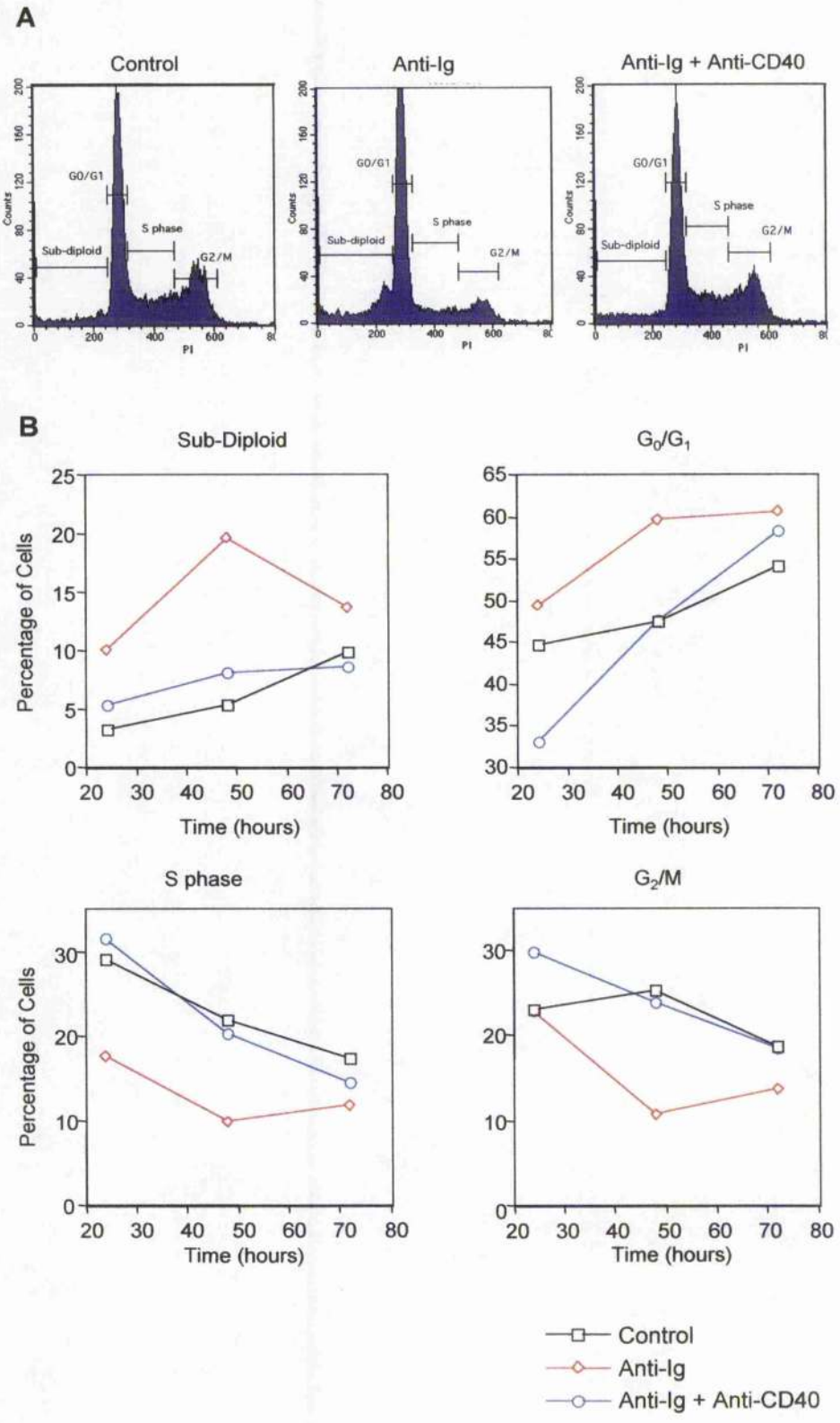

A
Control
Anti-Ig
Anti-Ig + Anti-CD40
G0/G1
S phase
G2/M
Sub-diploid
Counts
PI
B
Sub-Diploid
G_0/G_1
S phase
G_2/M
Percentage of Cells
Time (hours)
Control
Anti-Ig
Anti-Ig + Anti-CD40

Figure 3.3 Differential phosphorylation of Erk-MAPKinase in response to anti-Ig and anti-CD40 co-stimulation in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) were stimulated in the presence of 10 μg/ml anti-Ig, either alone or in combination with 10 μg/ml anti-CD40, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with phospho-specific Erk-MAPK antibody. Equal protein loading was confirmed with a whole Erk-MAPKinase antibody.

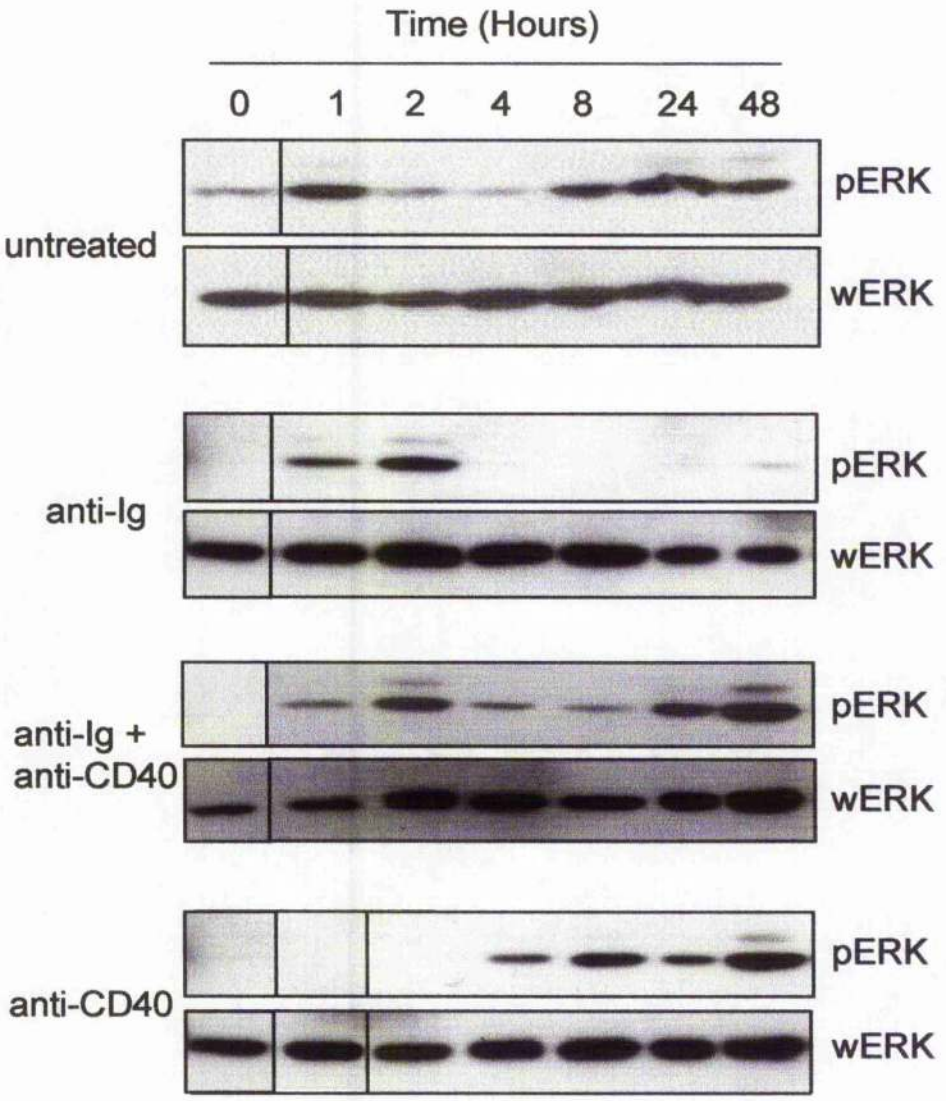

Time (Hours)
0
1
2
4
8
24
48
untreated
pERK
wERK
anti-Ig
pERK
wERK
anti-Ig +
anti-CD40
pERK
wERK
anti-CD40
pERK
wERK

Figure 3.4 Sustained Erk-MAPKinase inhibition prevents normal cell proliferation but also inhibits CD40-mediated rescue from anti-Ig induced growth arrest

WEHI-231 cells (10^4 cells/well) were pre-incubated for 3 hours with PD98059 and U0126 (both 1 μM). Cells were then treated with 10μg/ml anti-Ig, either alone or in combination with 10μg/ml anti-CD40. Control cells received no stimulating antibody. Cells treated with PD98059 and U0126 were treated with an additional dose (both at 1 μM) every 4 hours for a total of 32 hours. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of quadruplicate measurements taken from a single experiment, representative of at least two other independent experiments.

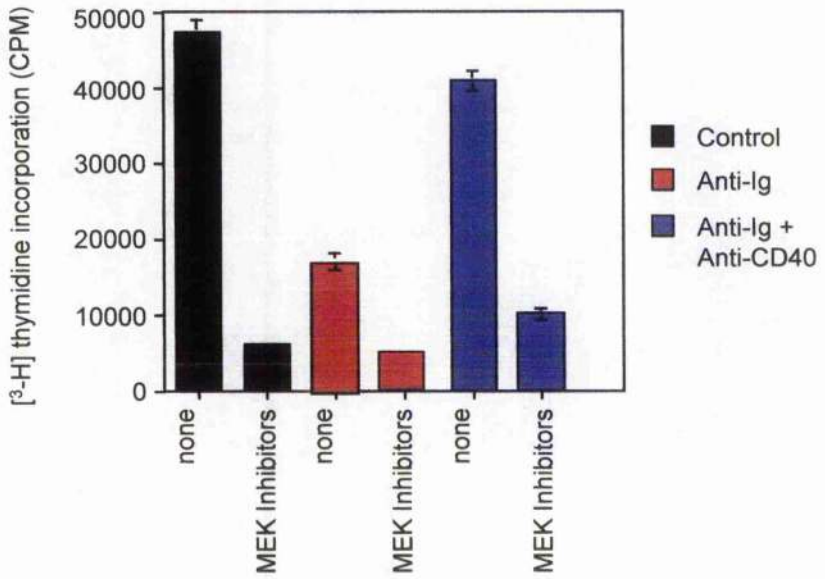

[3-H] thymidine incorporation (CPM)
50000
40000
30000
20000
10000
0
none
MEK Inhibitors
none
MEK Inhibitors
none
MEK Inhibitors
Control
Anti-Ig
Anti-Ig +
Anti-CD40

Figure 3.5 Effects of the cell cycle inhibitors olomoucine and aphidicolin on the WEHI-231 cell cycle

WEHI-231 cells (5×10^5 cells/ml) were treated with the cell cycle inhibitors olomoucine (50 μM), aphidicolin (5 μg/ml), or nocodazole (10 μg/ml) for 48 hours. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining to assess DNA content, followed by FACS analysis, as described in Materials and Methods. Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least two other independent experiments.

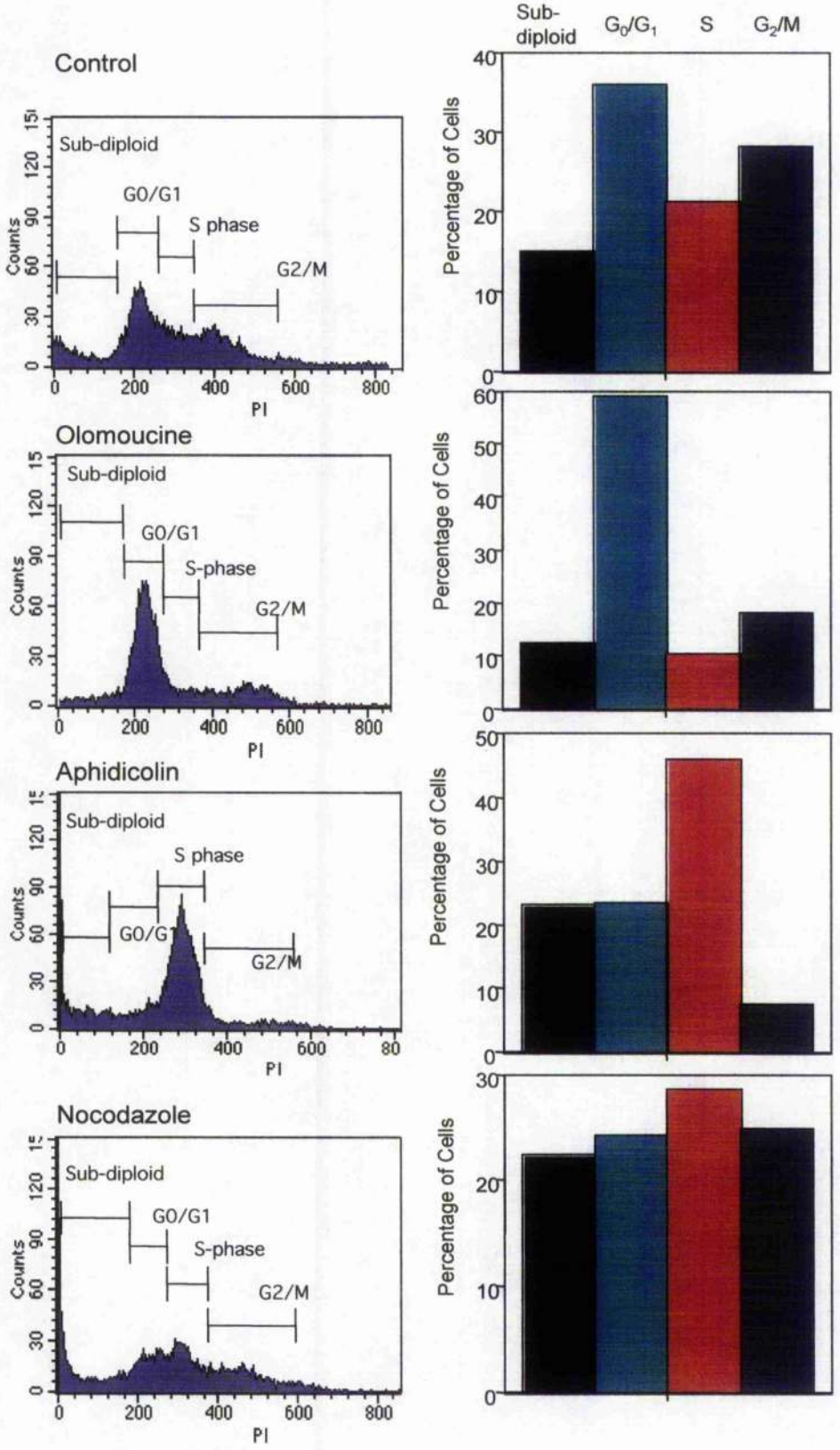
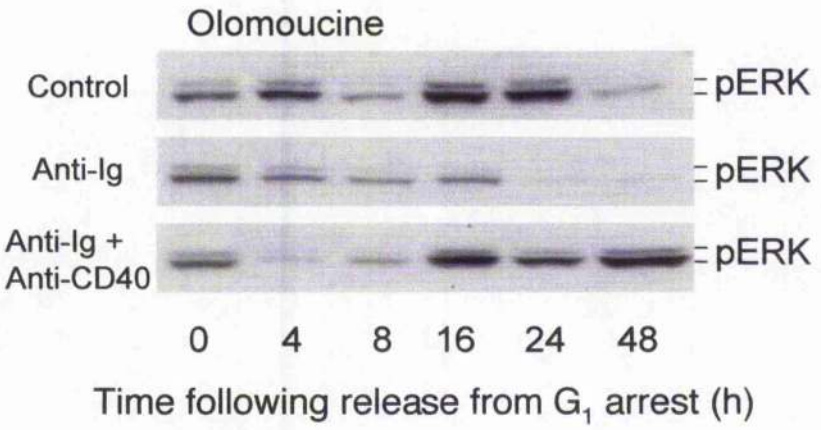

Control
Sub-diploid
G0/G1
S phase
G2/M
Counts
PI
Olomoucine
Sub-diploid
G0/G1
S-phase
G2/M
Counts
PI
Aphidicolin
Sub-diploid
S phase
G0/G1
G2/M
Counts
PI
Nocodazole
Sub-diploid
G0/G1
S-phase
G2/M
Counts
PI
Sub-diploid
G_0/G_1
S
G_2/M
Percentage of Cells
Percentage of Cells
Percentage of Cells
Percentage of Cells

Figure 3.6 Effects of the cell cycle inhibitors olomoucine and aphidicolin on the phosphorylation of Erk-MAPKinase in WEHI-231 cells in response to anti-Ig and anti-CD40 co-stimulation

A WEHI-231 cells were treated with olomoucine (50 μM) for 40 hours to induce G_1 arrest. Cells were washed twice in media to remove the olomoucine, then stimulated (1×10^7 cells) in the presence of anti-Ig (10 μg/ml) either alone or in combination with anti-CD40 (10 μg/ml) for the times indicated. Cells incubated in the presence of medium alone following removal of olomoucine were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with a phospho-specific Erk-MAPK antibody.

B WEHI-231 cells were treated with aphidicolin (5 μg/ml) for 24 hours to induce S phase arrest. Cells were washed twice in media to remove the aphidicolin, then stimulated (1×10^7 cells) in the presence of anti-Ig (10 μg/ml) either alone or in combination with anti-CD40 (10 μg/ml) for the times indicated. Cells incubated in the presence of medium alone following removal of aphidicolin were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with a phospho-specific Erk-MAPK antibody.

A


Olomoucine
Control
Anti-Ig
Anti-Ig +
Anti-CD40
pERK
pERK
pERK
0 4 8 16 24 48
Time following release from G1 arrest (h)

B

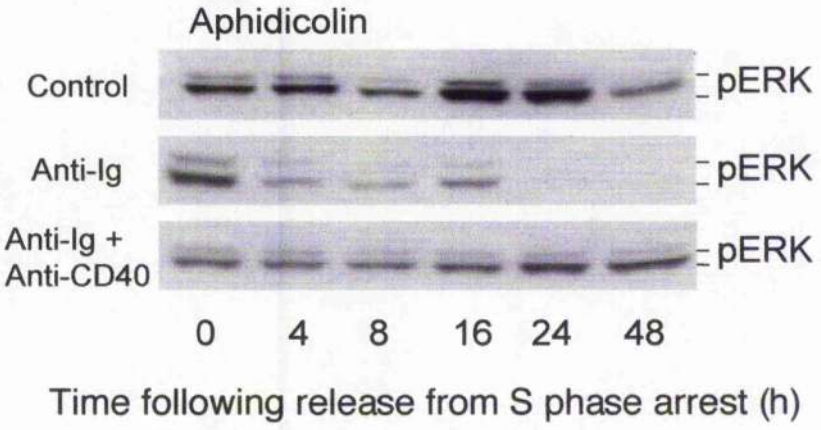
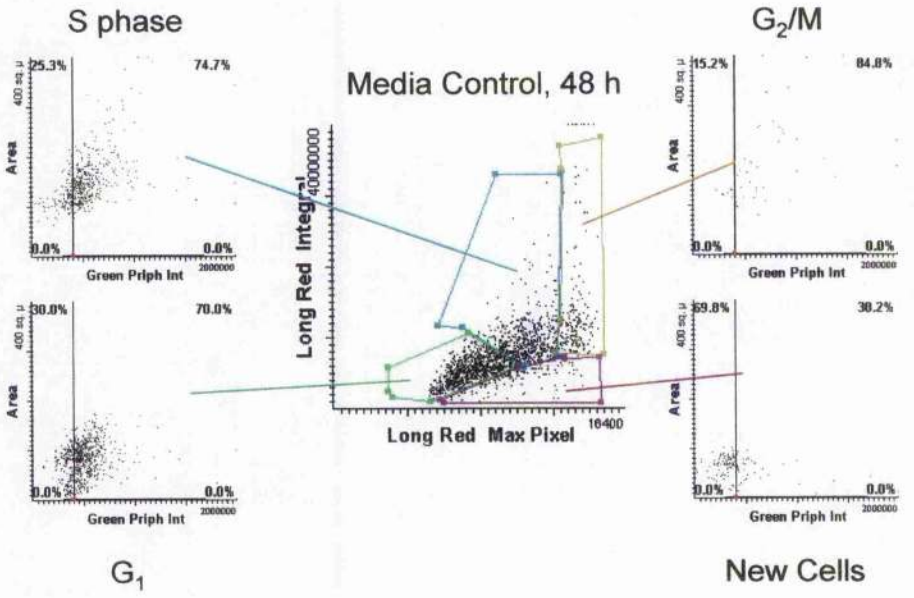

Aphidicolin
Control
Anti-Ig
Anti-Ig +
Anti-CD40
pERK
pERK
pERK
0 4 8 16 24 48
Time following release from S phase arrest (h)

Figure 3.7 Effects of cell cycle inhibitors on Erk-MAPKinase activation

WEHI-231 cells were incubated in the presence of olomoucine (50 μM) for 40 hours, or left untreated, then washed twice in fresh media to remove the olomoucine. Arrested cells were then stimulated with anti-Ig (10 μg/ml), a combination of anti-Ig and anti-CD40 (both 10 μg/ml), or left unstimulated in media for up to 48 hours. Cells were attached to slides at the appropriate time points, fixed and permeabilised, then stained using an anti-phospho-Erk antibody, along with propidium iodide to assess DNA content. Following staining, cells were scanned using the LSC. The percentage of cells in each cell cycle phase is shown, along with the proportion of those cells that were positive for phospho-Erk.

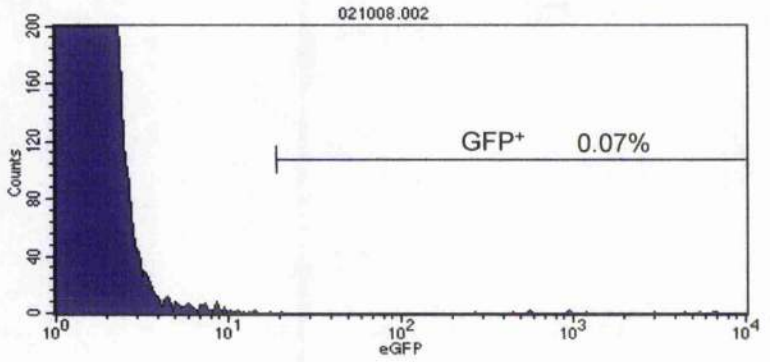


Treatment	% in G_1 % pErk	% in S % pErk	% in G_2/M % pErk	New Cells % pErk
Media Ctrl 48 h	46 70	30.8 74.7	11.6 68.6	9.2 30.2
Media a-Ig 48 h	68.9 35.6	12.8 66.8	3.1 84.8	11.8 27.1
Olomoucine Ctrl 24 h	75.1 35.8	18.4 57.1	0.9 92.9	0.8 46.2
Olomoucine Ctrl 48 h	0.2 66.7	6.3 76.8	72 85.2	13.1 48
Olomoucine a-Ig + aCD40 48 h	31.5 52	29 81.1	26.3 79.7	8 40.5

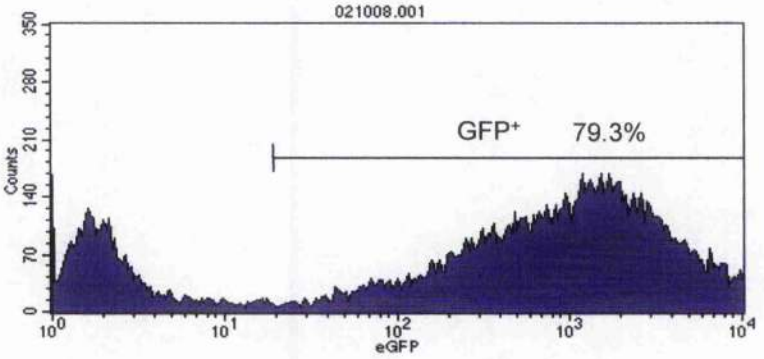
Figure 3.8 Retroviral transfection of WEHI-231 cells with the MIEV.RasV12 vector

WEHI-231 cells were co-cultured in the presence of the retroviral packaging lines GP+E.86.MIEV (the empty vector) or GP+E.86.MIEV.RasV12 (the MIEV vector carrying the DNA encoding the RasV12 mutant). Stably transfected GP+E-86 were grown to 75% confluency in a 6 well plate. WEHI-231 cells were pretreated for 4 hours with polybrene (5 μg/ml) prior to transfection. 3×10^6 WEHI-231 cells in media containing 5 μg/ml polybrene were added to the wells containing the appropriate stable GP+E-86 transfectants, and incubated at 37°C for 16 hours. After this time, the WEHI-231 cells were removed by careful aspiration and incubated for a further 4 hours. Efficiency of transfection was assessed by flow cytometry to detect green fluorescent protein.

WEHI-231



WEHI-231 MIEV.RasV12



WEHI-231 MIEV

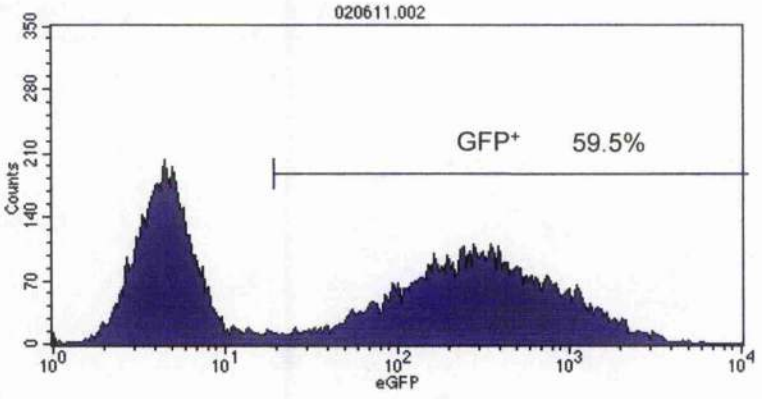
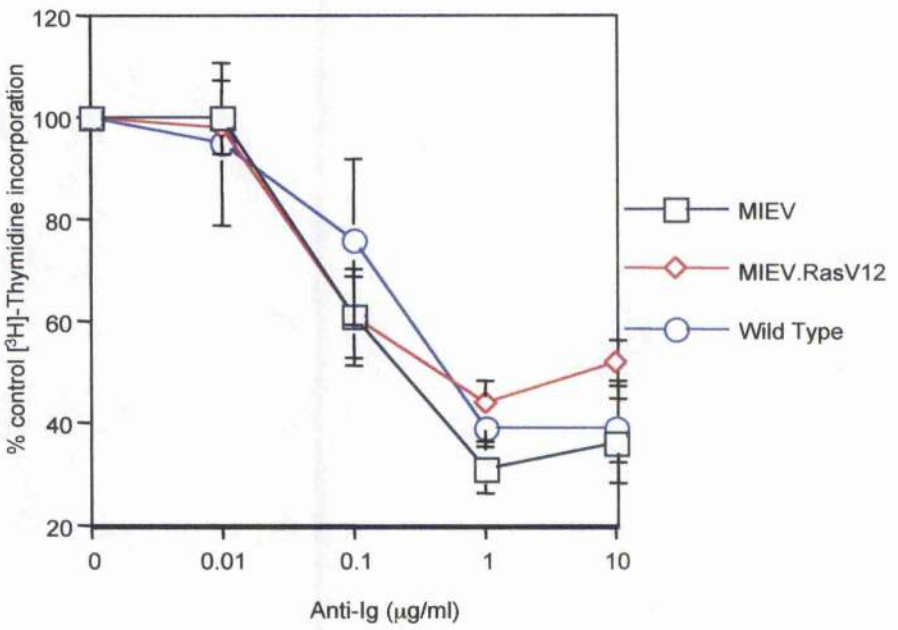


Figure 3.9 Effects of overexpression of the RasV12 mutant on anti-Ig-induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing MIEV.RasV12 or the MIEV vector were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig (0.01, 0.1, 1, 10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 4 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing pcDNA3.1.RasV12 or the pcDNA3.1 vector were cultured and proliferation assessed as in **A**. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 4 independent experiments were then pooled and expressed as means ± sem.

A


120
100
80
60
40
20
% control [3H]-Thymidine incorporation
0
0.01
0.1
1
10
Anti-Ig (μg/ml)
MIEV
MIEV.RasV12
Wild Type

B

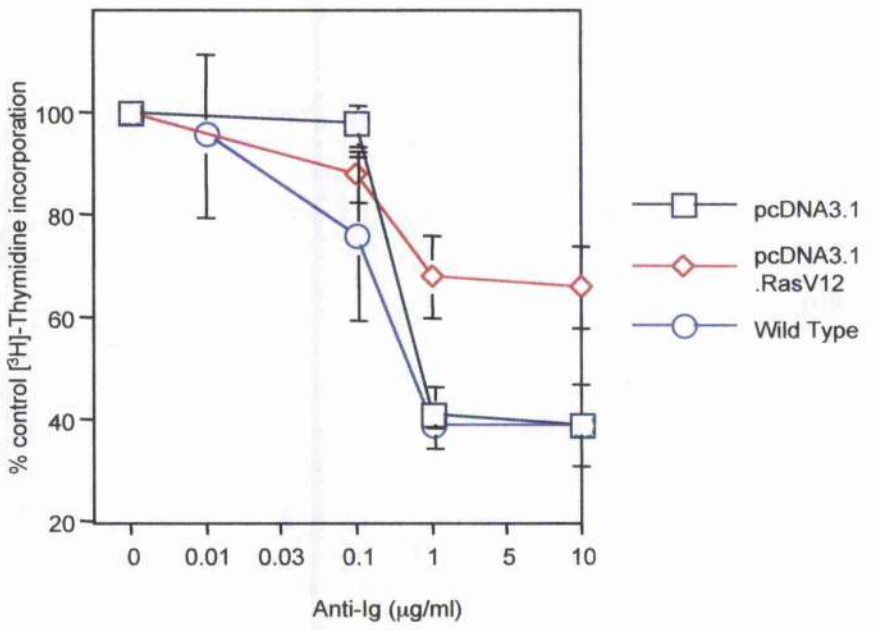

100
80
60
40
20
% control [3H]-Thymidine incorporation
0
0.01
0.03
0.1
1
5
10
Anti-Ig (μg/ml)
pcDNA3.1
pcDNA3.1
.RasV12
Wild Type

Figure 3.10 Effects of overexpression of the RasV12 mutant on anti-Ig-induced apoptosis in WEHI-231 cells

WEHI-231 cells (5×10^5 cells/ml) containing MIEV.RasV12, the MIEV vector, pcDNA3.1.RasV12, or the pcDNA3.1 vector were cultured in the presence of anti-Ig (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 48 hours, as described in Materials and Methods. Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least three other independent experiments.

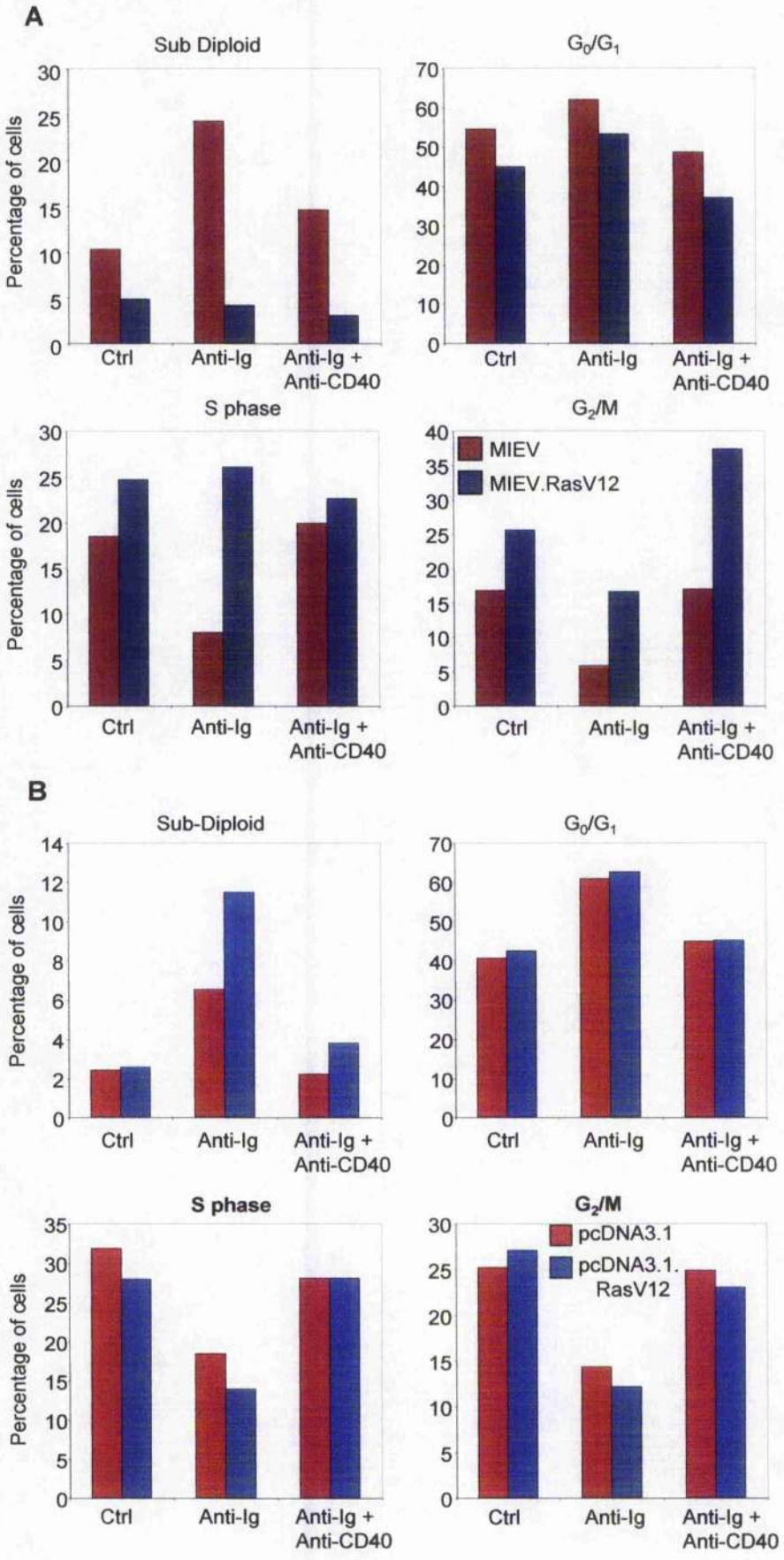

A
Sub Diploid
G_0/G_1
S phase
G_2/M
Percentage of cells
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40
MIEV
MIEV.RasV12
B
Sub-Diploid
G_0/G_1
S phase
G_2/M
pcDNA3.1
pcDNA3.1. RasV12

Figure 3.11 Effect of overexpression of the RasV12 mutant on CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis in WEHI-231 cells

WEHI-231 cells (1 x 10^4 cells/well) containing MIEV.RasV12, the MIEV vector, pcDNA3.1.RasV12, or the pcDNA3.1 vector were cultured, in triplicate, in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 4 independent experiments were then pooled and expressed as means ± sem.

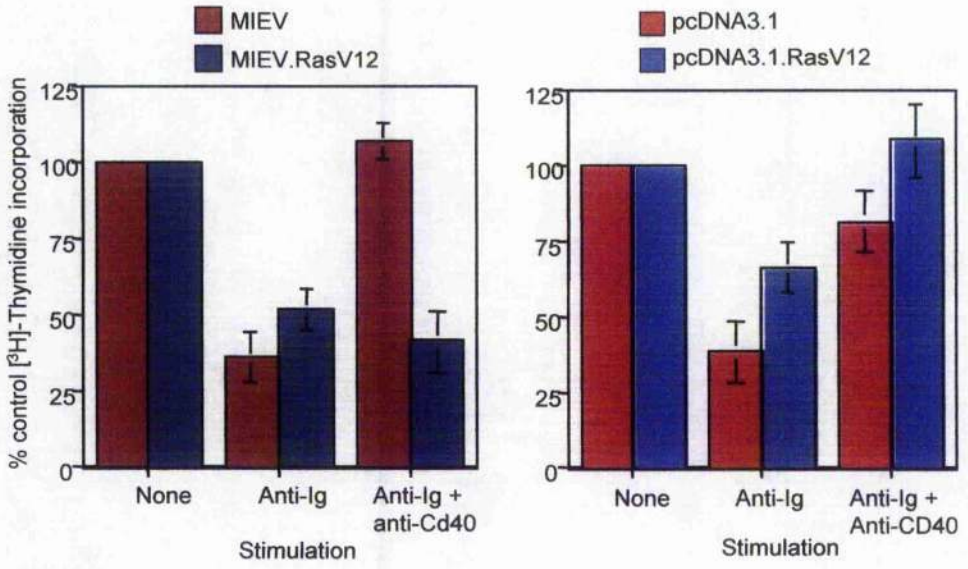

MIEV
MIEV.RasV12
% control [3H]-Thymidine incorporation
125
100
75
50
25
0
None
Anti-Ig
Anti-Ig + anti-Cd40
Stimulation
pcDNA3.1
pcDNA3.1.RasV12
125
100
75
50
25
0
None
Anti-Ig
Anti-Ig + Anti-CD40
Stimulation

Figure 3.12 Effect of overexpression of the Dok-PH/PTB domain on anti-Ig induced growth arrest and apoptosis in WEHI-231 cells

A WEHI-231 cells containing the pMXI.Dok-PH/PTB vector or the empty pMXI vector (1×10^4 cells/well) were cultured, in triplicate, in the presence of various concentrations of anti-Ig (0.01, 0.1, 1, 10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 7 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells containing the pMXI.Dok-PH/PTB vector or the empty pMXI vector (5×10^5 cells/ml) were cultured in the presence of anti-Ig (10 μg/ml), with or without anti-CD40 (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis were determined by propidium iodide staining and FACS analysis to assess DNA content after 48 hours, as described in Materials and Methods. Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least three other independent experiments.

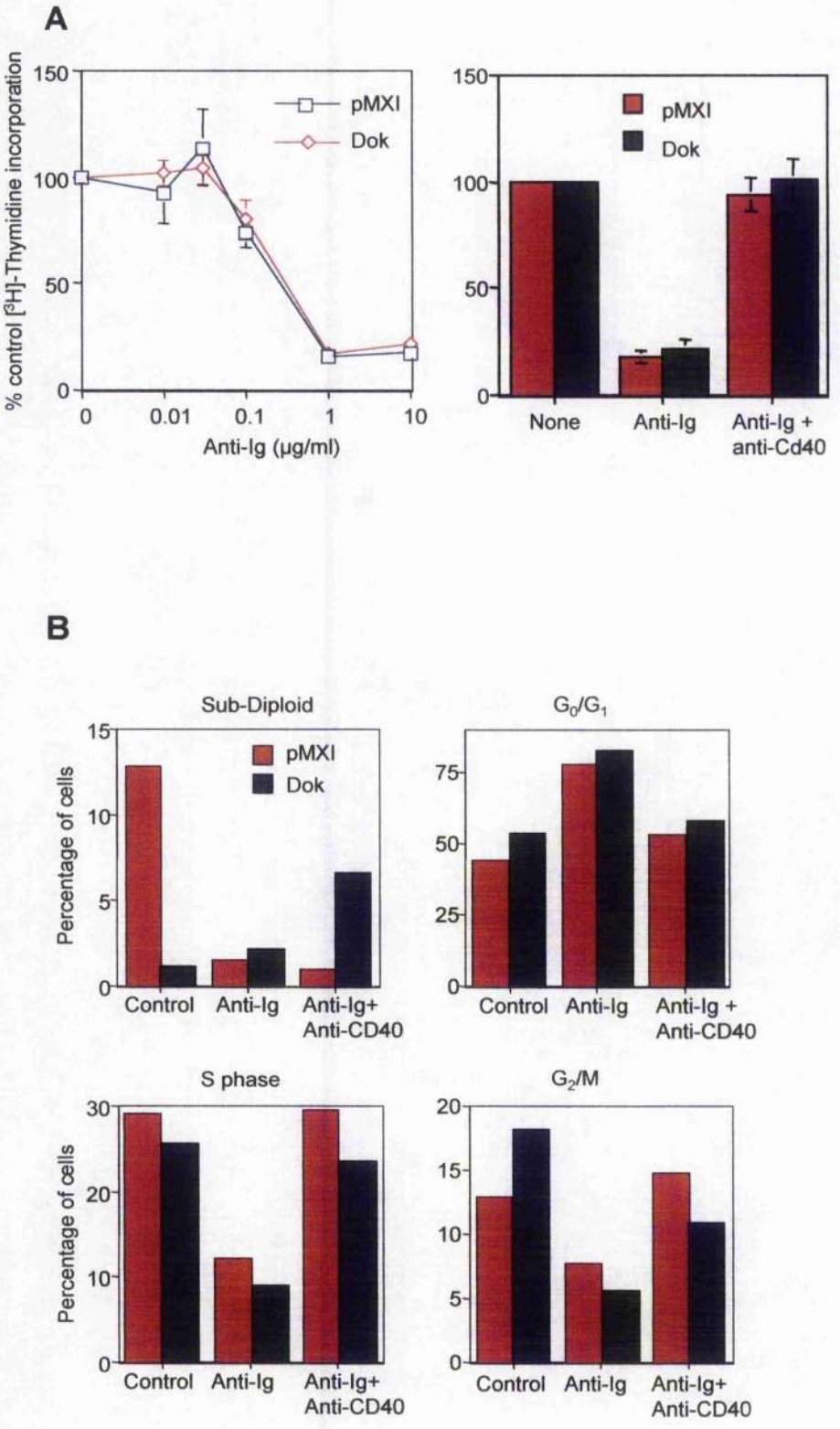
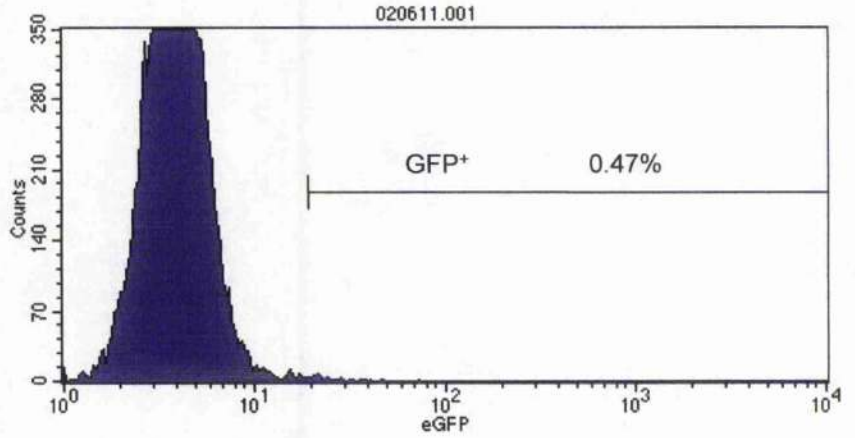

A
% control [3H]-Thymidine incorporation
150
100
50
0
pMXI
Dok
0
0.01
0.1
1
10
Anti-Ig (µg/ml)
150
100
50
0
pMXI
Dok
None
Anti-Ig
Anti-Ig + anti-Cd40
B
Sub-Diploid
Percentage of cells
15
10
5
0
pMXI
Dok
Control
Anti-Ig
Anti-Ig+ Anti-CD40
G0/G1
75
50
25
0
Control
Anti-Ig
Anti-Ig + Anti-CD40
S phase
Percentage of cells
30
20
10
0
Control
Anti-Ig
Anti-Ig+ Anti-CD40
G2/M
20
15
10
5
0
Control
Anti-Ig
Anti-Ig+ Anti-CD40

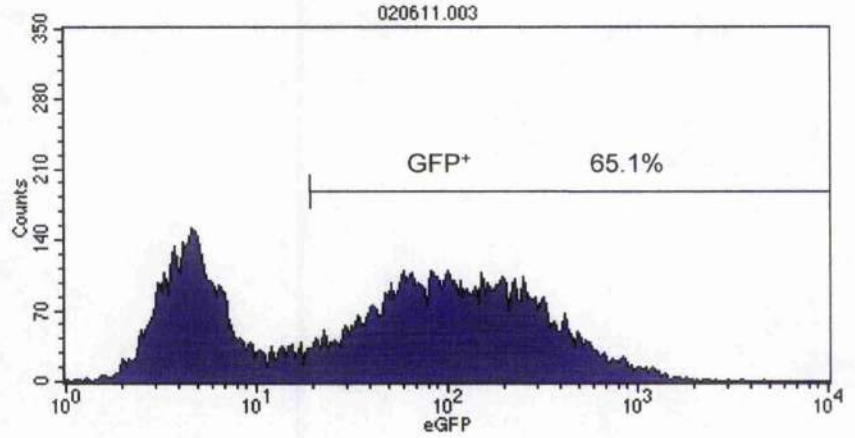
Figure 3.13 Retroviral transfection of WEHI-231 cells with the RasV12 effector mutants RasV12-S35 and RasV12-C40

WEHI-231 cells were co-cultured in the presence of the retroviral packaging lines GP+E.86.MIEV.RasV12-S35 or GP+E.86.MIEV.RasV12-C40. Stably transfected GP+E-86 were grown to 75% confluency in a 6 well plate. WEHI-231 cells were pretreated for 4 hours with polybrene (5 μg/ml) prior to transfection. 3×10^6 WEHI-231 cells in media containing 5 μg/ml polybrene were added to the wells containing the appropriate stable GP+E-86 transfectants, and incubated at 37°C for 16 hours. After this time, the WEHI-231 cells were removed by careful aspiration and incubated for a further 4 hours. Efficiency of transfection was assessed by flow cytometry to detect green fluorescent protein.

WEHI-231



WEHI-231 MIEV.RasV12-S35



WEHI-231 MIEV.RasV12-C40

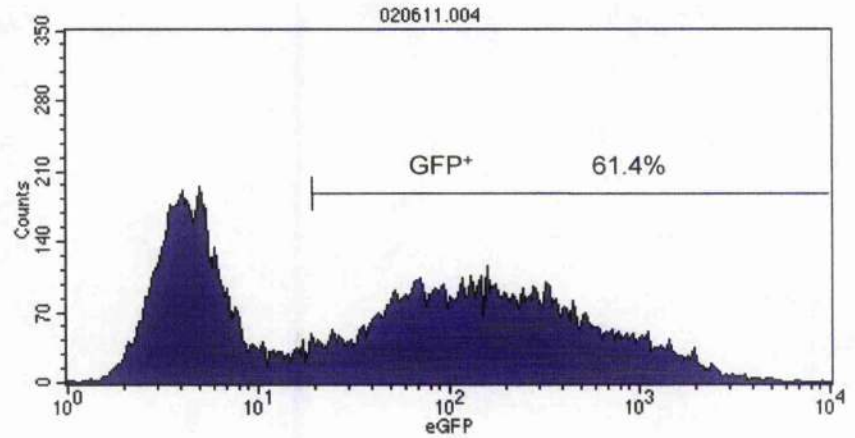
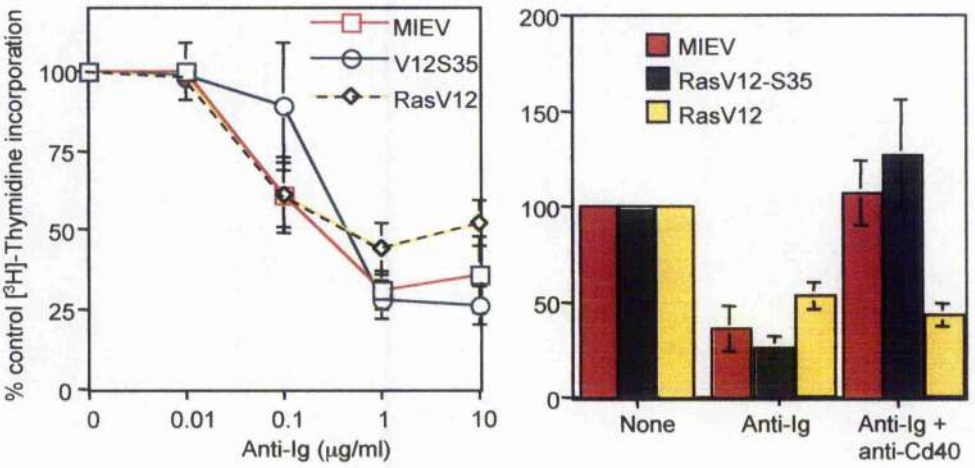


Figure 3.14 Effects of overexpression of the RasV12 effector mutant RasV12-S35 on anti-Ig-induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the MIEV, MIEV.RasV12, or MIEV.RasV12-S35 vectors were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig (0.01, 0.1, 1, 10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] - thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the empty pcDNA3.1 vector, pcDNA3.1.RasV12, or pcDNA3.1.RasV12-S35 vectors were cultured and proliferation assessed as in **A**. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 14 independent experiments were then pooled and expressed as means ± sem.

A


% control [3H]-Thymidine incorporation
MIEV
V12S35
RasV12
Anti-Ig (μg/ml)
MIEV
RasV12-S35
RasV12
None
Anti-Ig
Anti-Ig + anti-Cd40

B

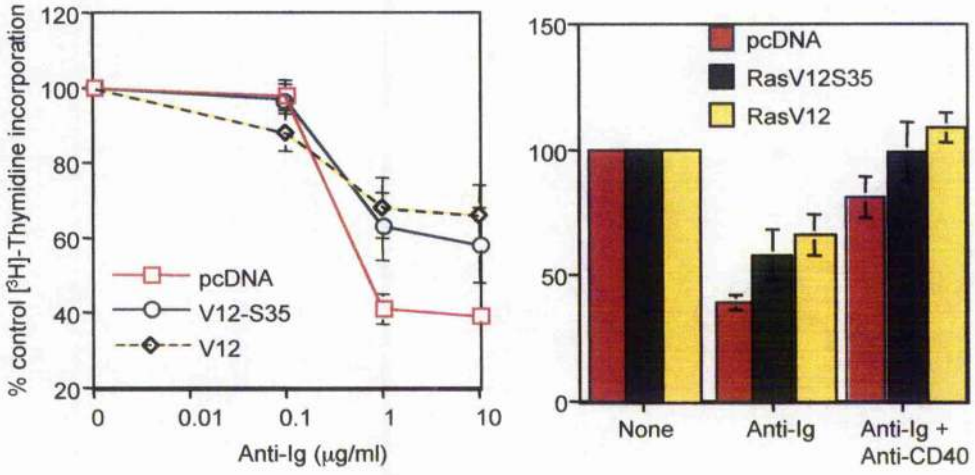

% control [3H]-Thymidine incorporation
pcDNA
V12-S35
V12
Anti-Ig (μg/ml)
pcDNA
RasV12S35
RasV12
None
Anti-Ig
Anti-Ig + Anti-CD40

Figure 3.15 Effects of overexpression of the RasV12 effector mutant RasV12-S35 on anti-Ig-induced apoptosis in WEHI-231 cells

A WEHI-231 cells (5 x 10^5 cells/ml) containing the MIEV, MIEV.RasV12, or MIEV.RasV12-S35 vectors were cultured in the presence of anti-Ig (10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Control cells were cultured in the presence of medium alone. Levels of apoptosis were determined by propidium iodide staining and FACS analysis to assess DNA content after 48 hours, as described in Materials and Methods. Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least three other independent experiments.

B WEHI-231 cells (5 x 10^5 cells/ml) containing the empty pcDNA3.1 vector, pcDNA3.1.RasV12, or pcDNA3.1.RasV12-S35 vectors were cultured and apoptosis assessed as in **A**. Data represents each cell cycle phase expressed as a percentage of the total cells analysed from a single experiment, representative of at least three other independent experiments.

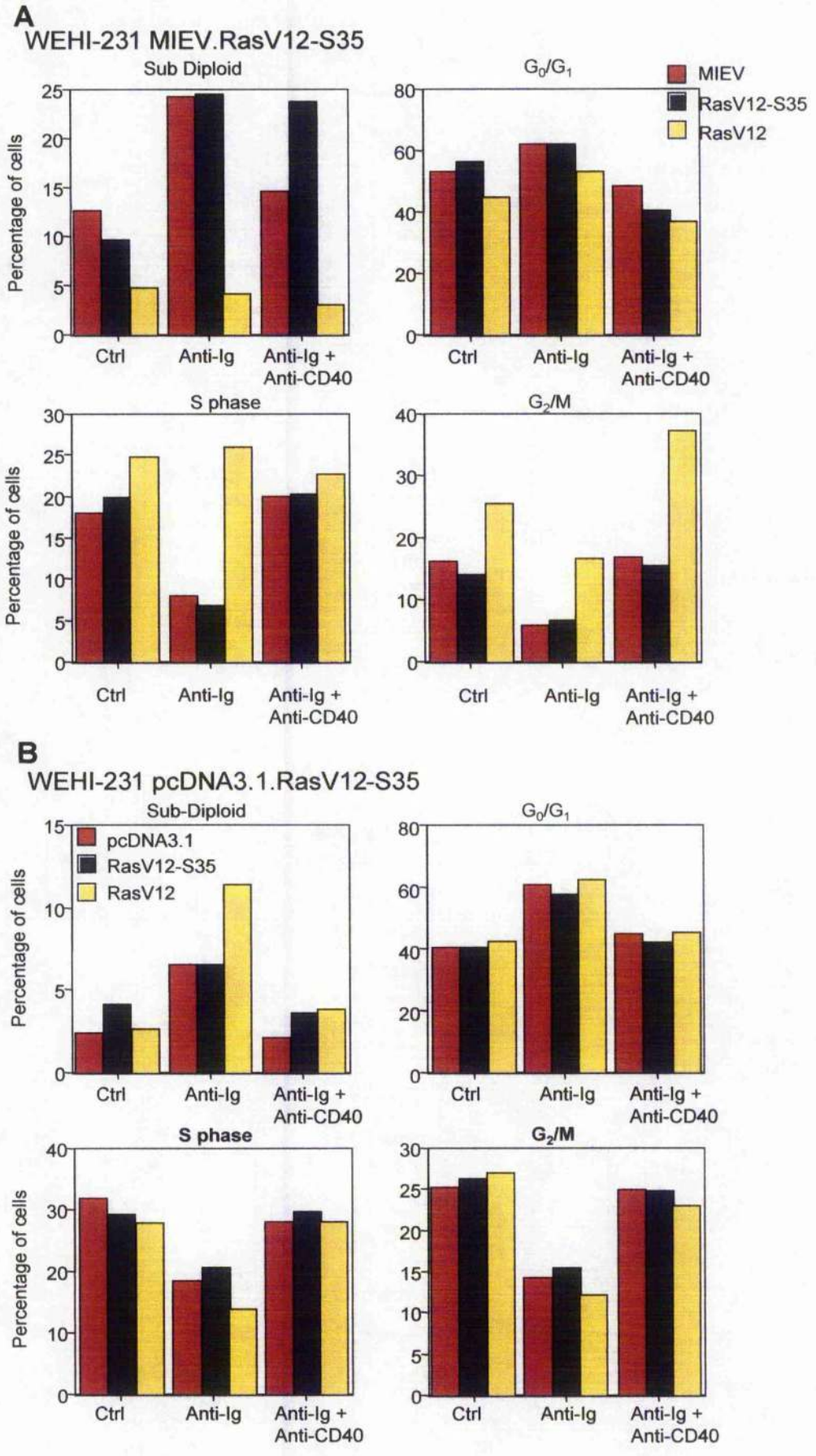
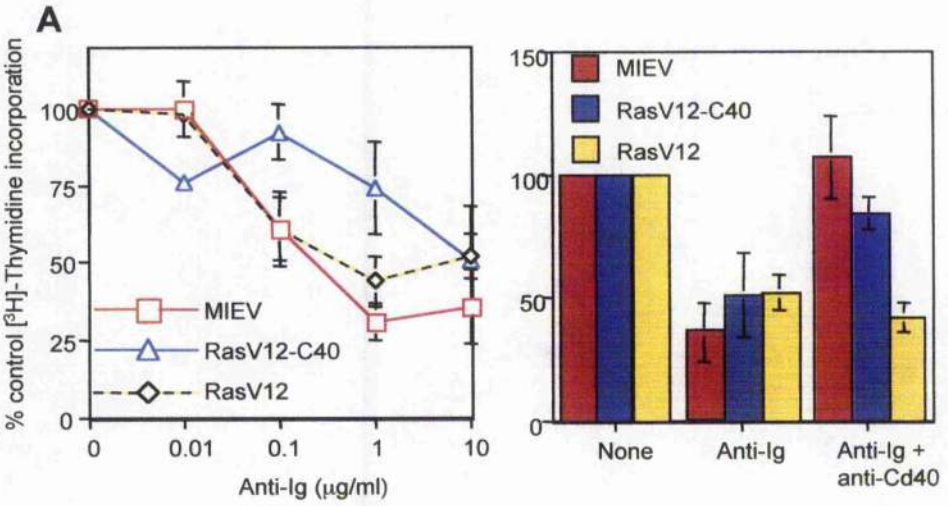

A
WEHI-231 MIEV.RasV12-S35
Sub Diploid
G_0/G_1
MIEV
RasV12-S35
RasV12
Percentage of cells
S phase
G_2/M
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40
B
WEHI-231 pcDNA3.1.RasV12-S35
Sub-Diploid
pcDNA3.1
RasV12-S35
RasV12

Figure 3.16 Effects of overexpression of the RasV12 effector mutant RasV12-C40 on anti-Ig-induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the MIEV, MIEV.RasV12, or MIEV.RasV12-C40 vectors were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig (0.01, 0.1, 1, 10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the empty pcDNA3.1 vector, pcDNA3.1.RasV12, or pcDNA3.1.RasV12-C40 vectors were cultured and proliferation assessed as in **A**. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 14 independent experiments were then pooled and expressed as means ± sem.


A
% control [3H]-Thymidine incorporation
100
75
50
25
0
0
0.01
0.1
1
10
Anti-Ig (μg/ml)
MIEV
RasV12-C40
RasV12
150
100
50
0
None
Anti-Ig
Anti-Ig +
anti-Cd40

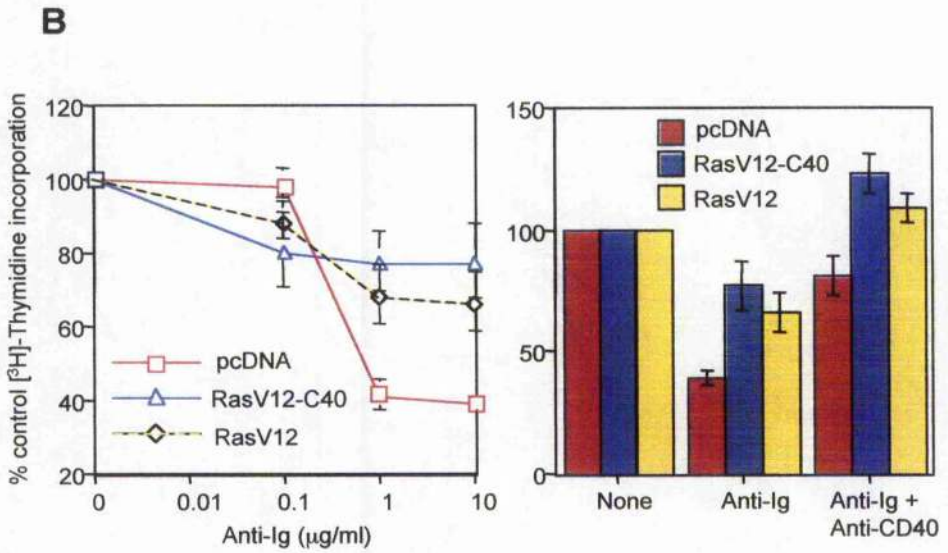

B
% control [3H]-Thymidine incorporation
120
100
80
60
40
20
0
0.01
0.1
1
10
Anti-Ig (μg/ml)
pcDNA
RasV12-C40
RasV12
150
100
50
0
None
Anti-Ig
Anti-Ig +
Anti-CD40

Figure 3.17 Effects of overexpression of the RasV12 effector mutant RasV12-C40 on anti-Ig-induced apoptosis in WEHI-231 cells

A WEHI-231 cells (5 x 10^5 cells/ml) containing the MIEV, MIEV.RasV12, or MIEV.RasV12-C40 vectors were cultured in the presence of anti-Ig (10 μg/ml), or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Control cells were cultured in the presence of medium alone. Levels of apoptosis were determined by propidium iodide staining and FACS analysis to assess DNA content after 48 hours, as described in Materials and Methods.

B WEHI-231 cells (5 x 10^5 cells/ml) containing the empty pcDNA3.1 vector, pcDNA3.1.RasV12, or pcDNA3.1.RasV12-C40 vectors were cultured and apoptosis assessed as in **A**.

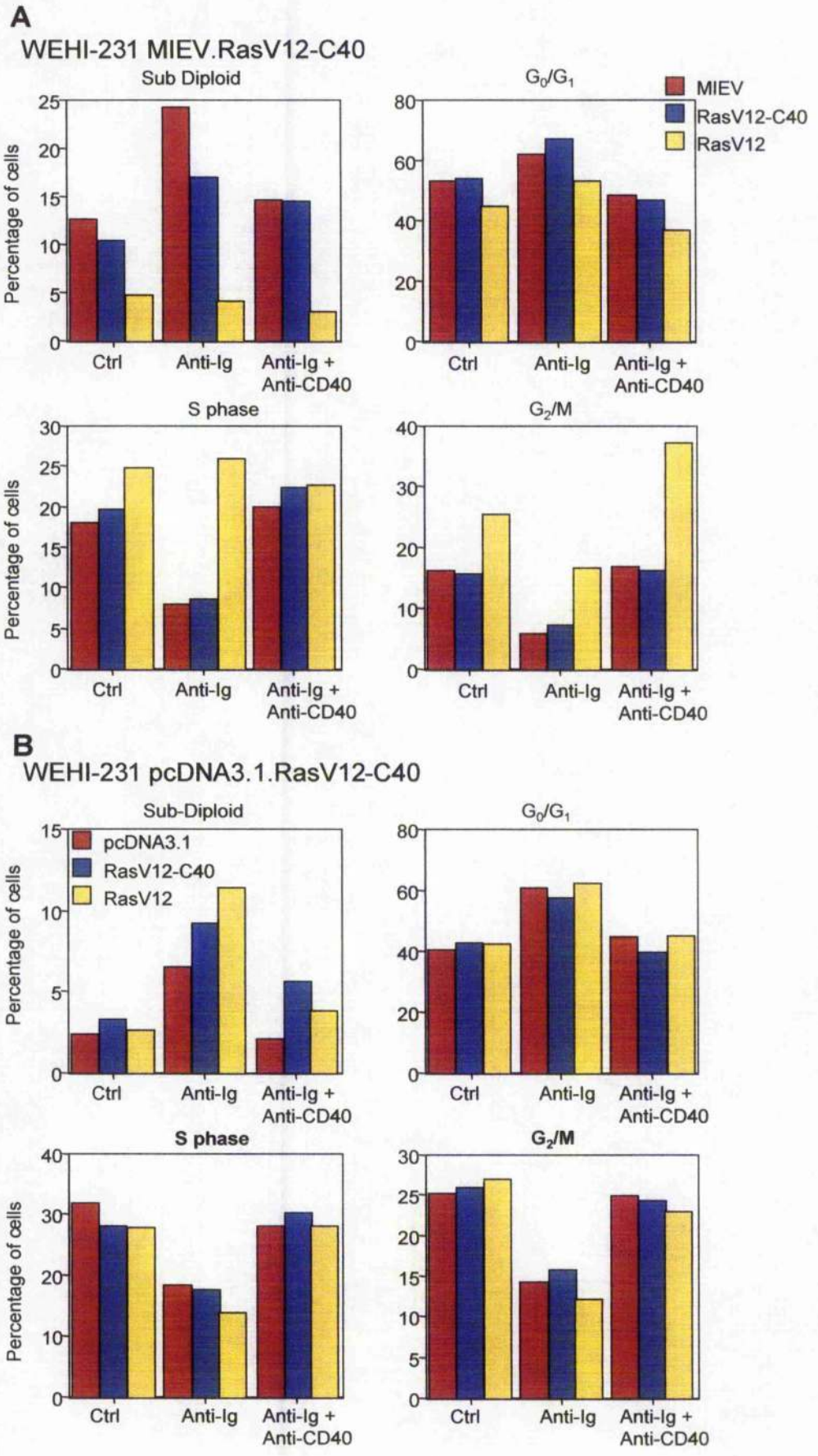
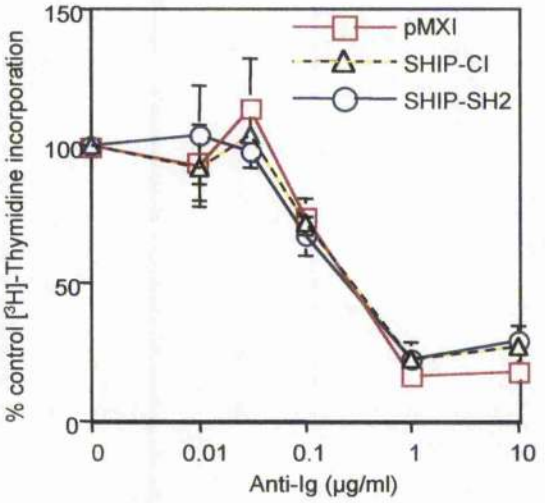

A
WEHI-231 MIEV.RasV12-C40
Sub Diploid
G_0/G_1
MIEV
RasV12-C40
RasV12
Percentage of cells
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40
S phase
G_2/M
B
WEHI-231 pcDNA3.1.RasV12-C40
Sub-Diploid
pcDNA3.1
RasV12-C40
RasV12
S phase
G_2/M

Figure 3.18 Effects of overexpression of a catalytically inactive SHIP mutant and the SHIP-SH2 domain on anti-Ig induced growth arrest and CD40-mediated rescue in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the empty pMXI, pMXI.SHIP-CI or pMXI.SHIP-SH2 vectors were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.01, 0.1, 1, 10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 7 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the empty pMXI, pMXI.SHIP-CI or pMXI.SHIP-SH2 vectors were cultured in the presence of anti-Ig (10 μg/ml), either alone or in combination with anti-CD40 (10 μg/ml), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 7 independent experiments were then pooled and expressed as means ± sem.

A


150
100
50
0
% control [3H]-Thymidine incorporation
pMXI
SHIP-CI
SHIP-SH2
0
0.01
0.1
1
10
Anti-Ig (µg/ml)

B

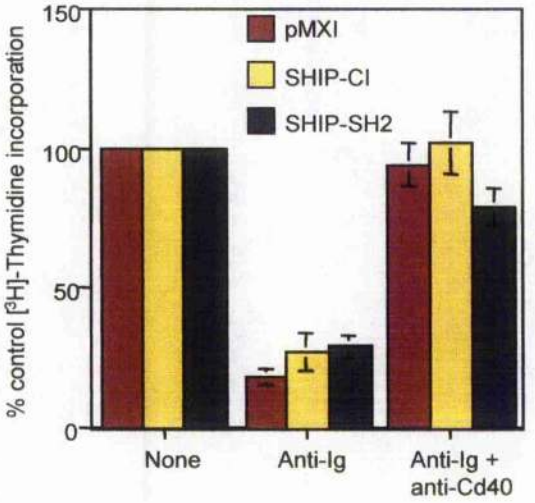

150
100
50
0
% control [3H]-Thymidine incorporation
pMXI
SHIP-CI
SHIP-SH2
None
Anti-Ig
Anti-Ig +
anti-Cd40

Figure 3.19 Effects of overexpression of a catalytically inactive SHIP mutant and the SHIP-SH2 domain on anti-Ig induced apoptosis in WEHI-231 cells

WEHI-231 cells (5 x 10^5 cells/ml) containing the empty pMXI, pMXI.SHIP-CI or pMXI.SHIP-SH2 vectors were cultured in the presence of anti-Ig (10 μg/ml), with or without anti-CD40 (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 48 hours, as described in Materials and Methods.

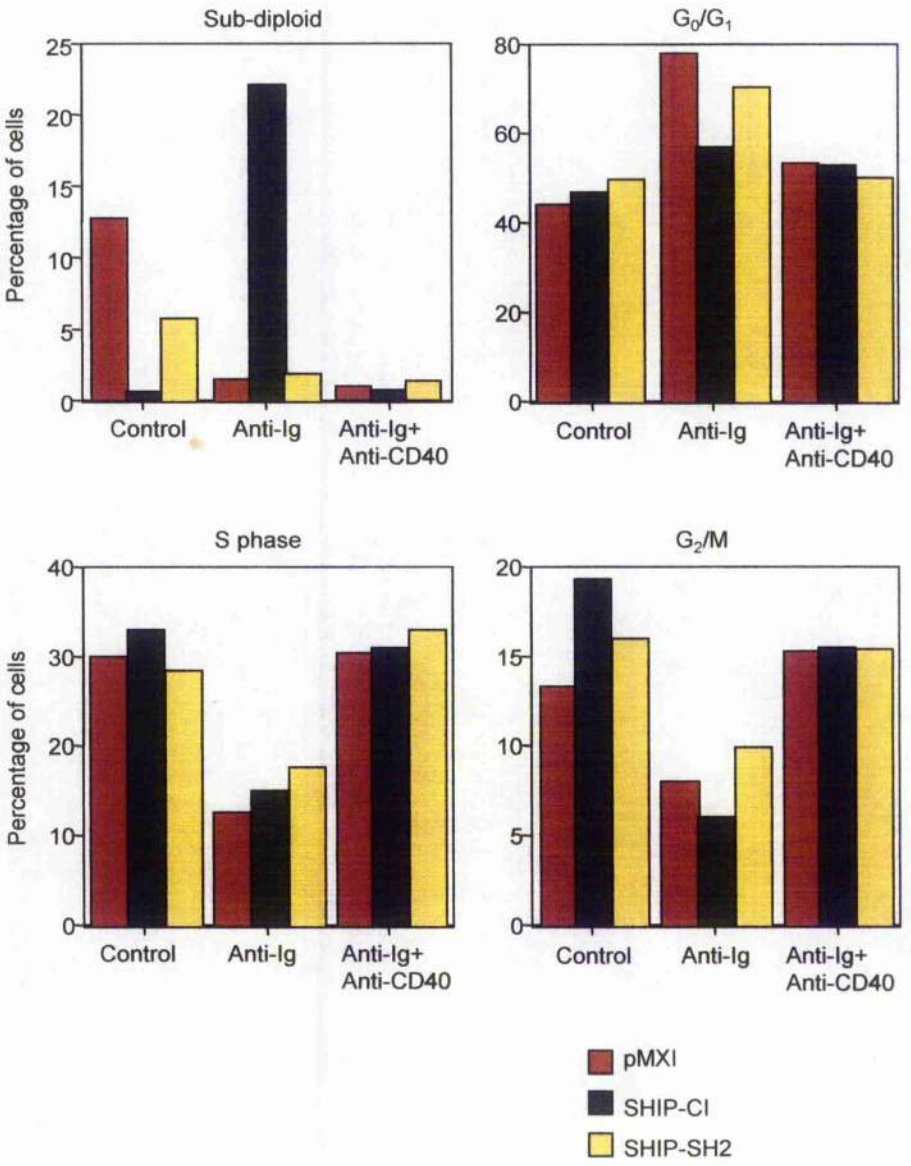

Sub-diploid
G_0/G_1
S phase
G_2/M
Percentage of cells
25
20
15
10
5
0
80
60
40
20
0
40
30
20
10
0
20
15
10
5
0
Control
Anti-Ig
Anti-Ig+
Anti-CD40
pMXI
SHIP-CI
SHIP-SH2

Figure 3.20 Effects of the overexpression of RasV12, RasV12-S35 and RasV12-C40 on the expression of $p27^{Kip1}$ in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) containing the pcDNA3.1, pcDNA3.1.RasV12, pcDNA3.1.RasV12-S35 or pcDNA3.1.RasV12-C40 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-$p27^{Kip1}$ antibody.

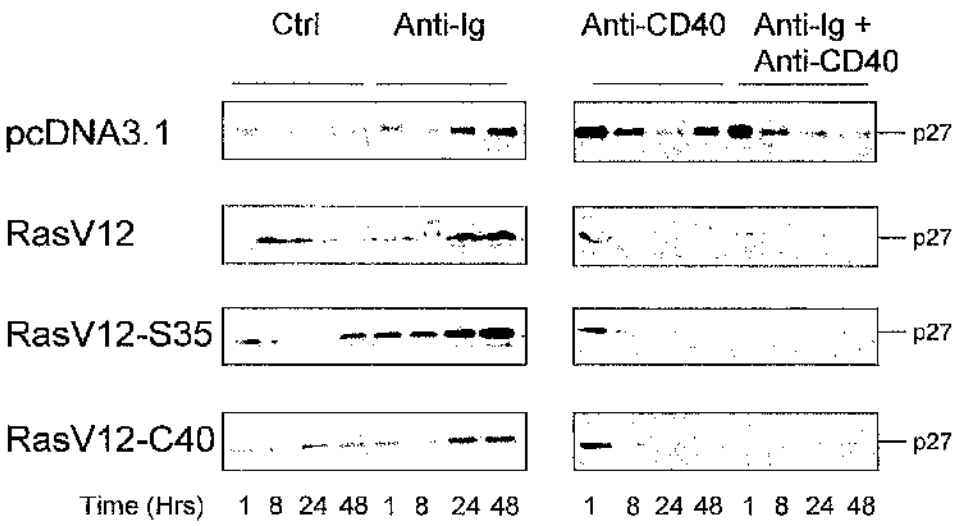

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
RasV12
RasV12-S35
RasV12-C40
p27
p27
p27
p27
Time (Hrs) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48

Figure 3.21 Effects of the overexpression of RasV12, RasV12-S35 and RasV12-C40 on the regulation of the cdc2 protein in WEHI-231 cells

WEHI-231 cells (1 x 10^7 cells) containing the pcDNA3.1, pcDNA3.1.RasV12, pcDNA3.1.RasV12-S35 or pcDNA3.1.RasV12-C40 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-phospho-cdc2 (Tyr^{15}) antibody.

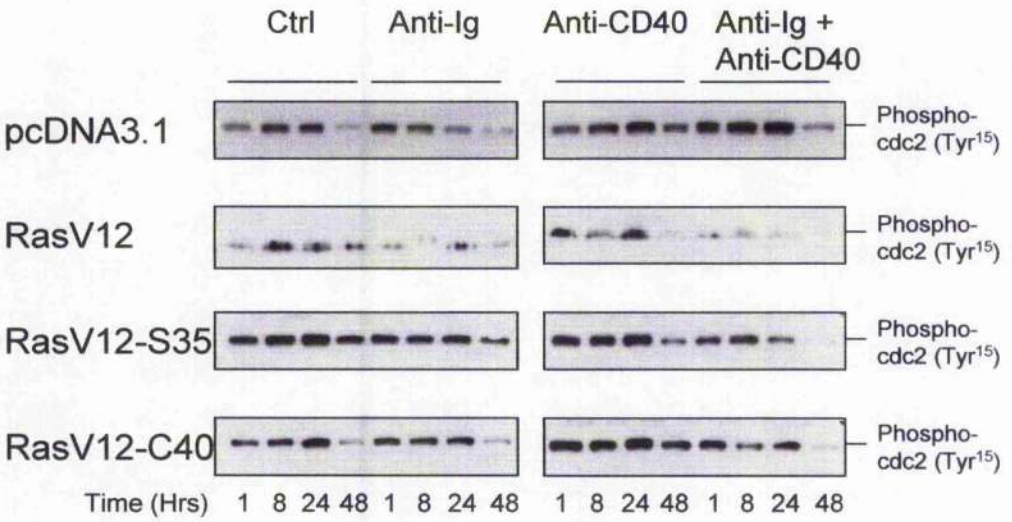

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
RasV12
RasV12-S35
RasV12-C40
Phospho-
cdc2 (Tyr15)
Time (Hrs) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48

Figure 3.22 Effects of the overexpression of RasV12, RasV12-S35 and RasV12-C40 on the regulation of the retinoblastoma protein in WEHI-231 cells

WEHI-231 cells (1 x 10^7 cells) containing the pcDNA3.1, pcDNA3.1.RasV12, pcDNA3.1.RasV12-S35 or pcDNA3.1.RasV12-C40 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-phospho-Rb ($Ser^{807/811}$) antibody.

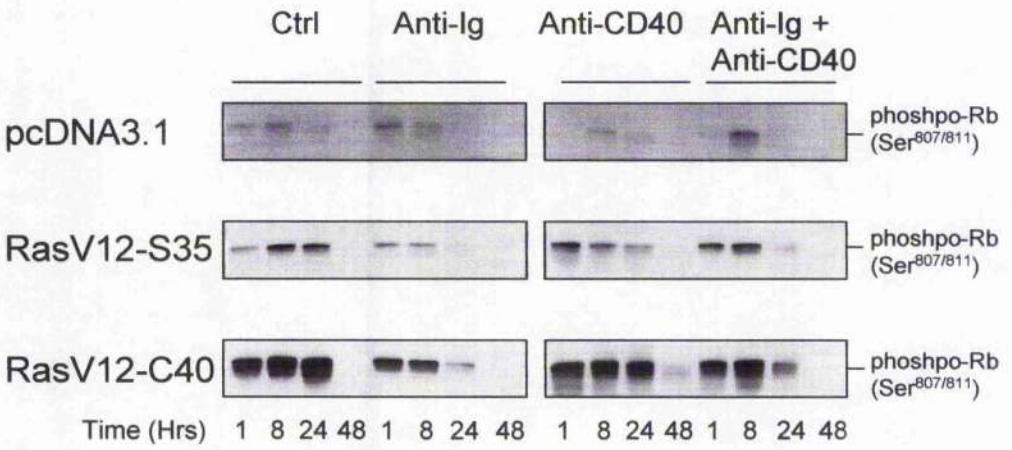

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
RasV12-S35
RasV12-C40
phoshpo-Rb
(Ser807/811)
phoshpo-Rb
(Ser807/811)
phoshpo-Rb
(Ser807/811)
Time (Hrs) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48

Figure 3.23 Effects of the overexpression of Dok PH-PTB, SHIP-CI, and SHIP-SH2 on the expression of $p27^{Kip1}$ in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) containing the pMXI, pMXI Dok PH-PTB, pMXI SHIP-CI or pMXI SHIP-SH2 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-$p27^{Kip1}$ antibody.

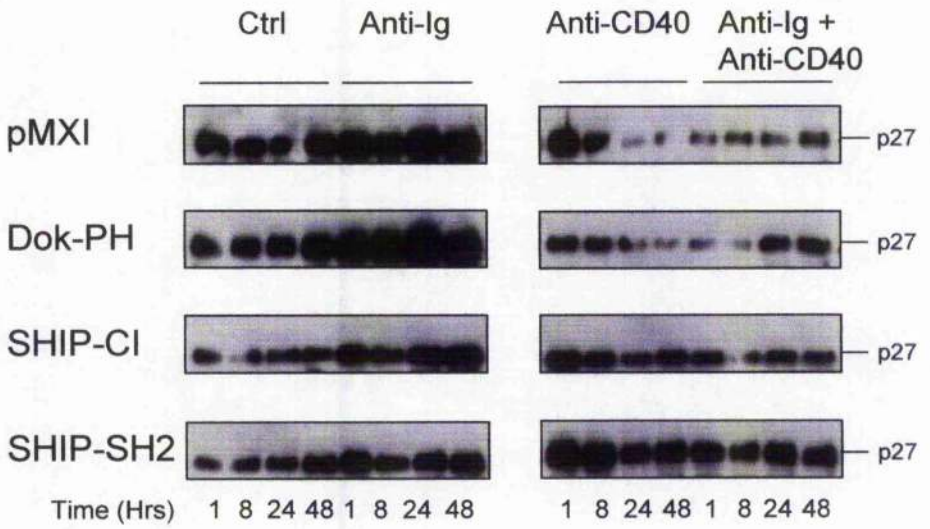

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pMXI
Dok-PH
SHIP-CI
SHIP-SH2
p27
p27
p27
p27
Time (Hrs)
1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 3.24 Effects of the overexpression of Dok PH-PTB, SHIP-CI, and SHIP-SH2 on the regulation of the cdc2 protein in WEHI-231 cells

WEHI-231 cells (1 x 10^7 cells) containing the pMXI, pMXI Dok PH-PTB, pMXI SHIP-CI or pMXI SHIP-SH2 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-phospho-cdc2 (Tyr^{15}) antibody.

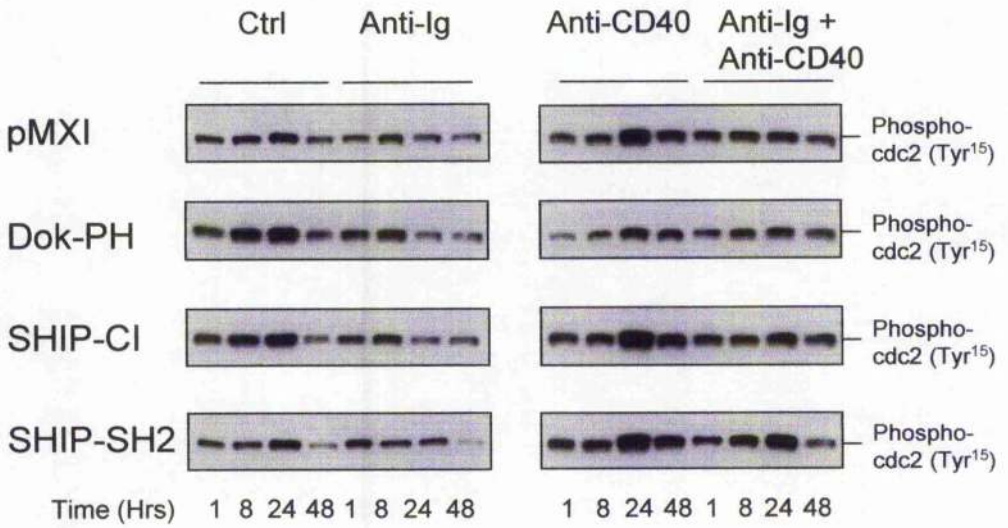

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pMXI
Dok-PH
SHIP-CI
SHIP-SH2
Phospho-
cdc2 (Tyr15)
Phospho-
cdc2 (Tyr15)
Phospho-
cdc2 (Tyr15)
Phospho-
cdc2 (Tyr15)
Time (Hrs) 1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 3.25 Effects of the overexpression of Dok PH-PTB, SHIP-CI, and SHIP-SH2 on the expression of D type cyclins in WEHI-231 cells

WEHI-231 cells (1 x 10^7 cells) containing the pMXI, pMXI Dok PH-PTB, pMXI SHIP-CI or pMXI SHIP-SH2 vectors were stimulated in the presence of 10 μg/ml anti-Ig or 10 μg/ml anti-CD40, alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-cyclin D1/2 antibody.

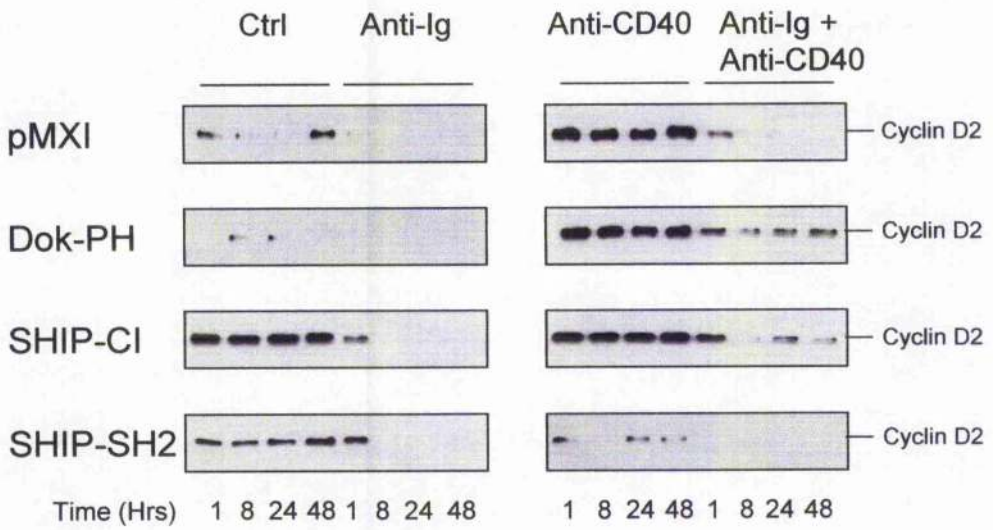

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pMXI
Dok-PH
SHIP-CI
SHIP-SH2
Cyclin D2
Cyclin D2
Cyclin D2
Cyclin D2
Time (Hrs) 1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 3.26 Erk-MAPKinase plays a dual role in WEHI-231 cells

Ligation of the BCR on the WEHI-231 immature B cell line leads to growth arrest and apoptosis. Growth arrest and apoptosis is preceded by an essential, but transient, activation of Erk-MAPKinase. WEHI-231 cells are responsive to T cell derived signals, permitting rescue of anti-Ig induced growth arrest and apoptosis by T cell derived signals such as CD40 ligand (or anti-CD40 antibodies). CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis also requires Erk-MAPKinase activity, but in this instance it is a sustained, cyclical activity, as opposed to transient activity. This demonstrates a dual role for the Erk-MAPKinase in WEHI-231 cells, with the kinetics of the activation controlling the biological response.

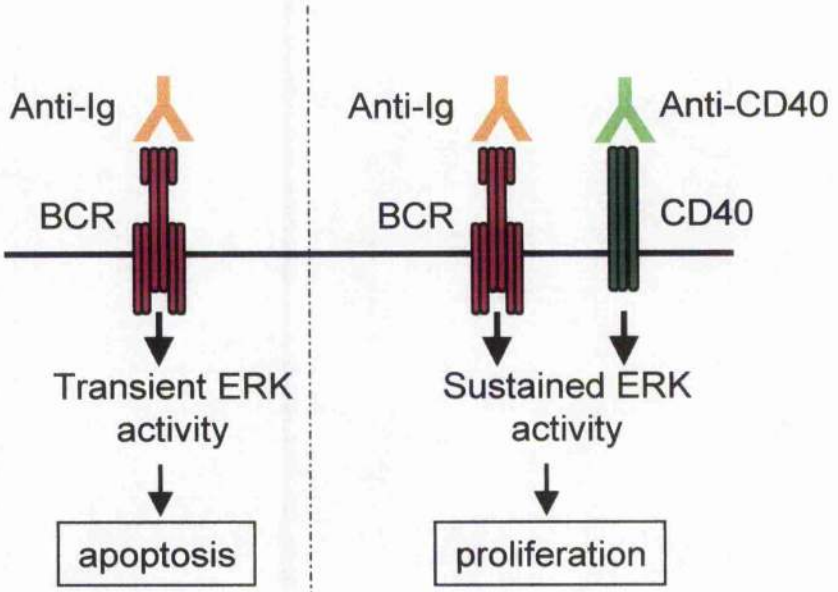
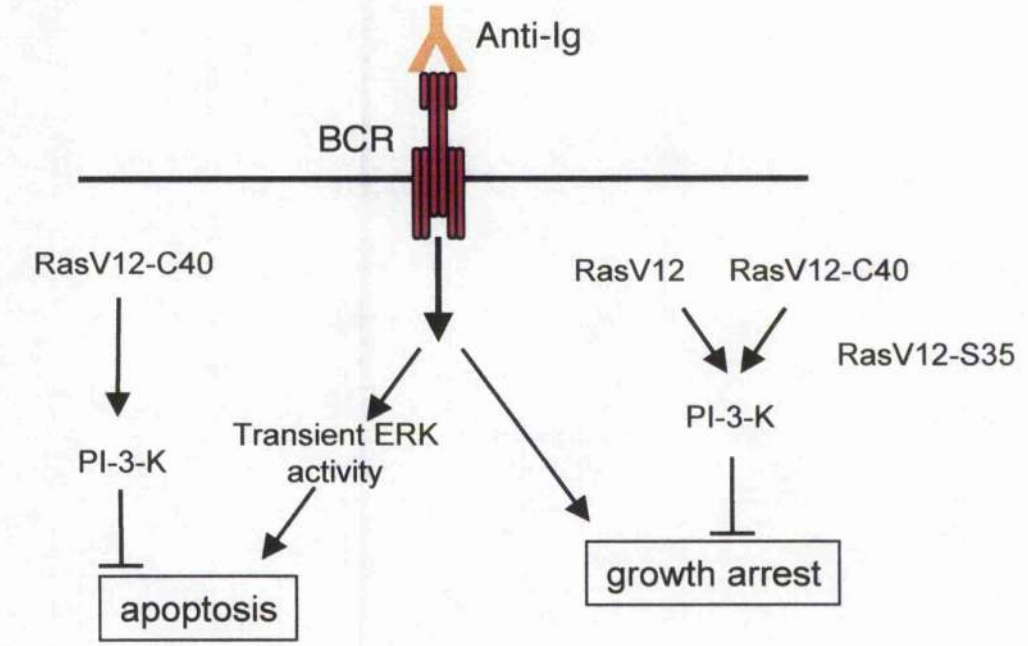

Anti-Ig
BCR
Transient ERK activity
apoptosis
Anti-Ig
Anti-CD40
BCR
CD40
Sustained ERK activity
proliferation

Figure 3.27 Role of the small GTPase, Ras, in anti-Ig induced growth arrest and apoptosis, and CD40 mediated rescue, in WEHI-231 cells

Overexpression of the constitutively active Ras mutant, RasV12, in WEHI-231 cells results in the cells being less susceptible to anti-Ig induced growth arrest, though the effects on anti-Ig induced apoptosis are unclear as this varies greatly depending upon the transfection method. The constitutively active mutant RasV12-C40, which can only activate the PI-3-Kinase pathway and not the Erk-MAPKinase pathway directly, though it does activate Erk-MAPKinase indirectly, provides a measure of protection from both anti-Ig induced growth arrest and apoptosis, unsurprising as PI-3-Kinase signalling is widely held to promote survival of cells. This is further evidenced by the observation that RasV12-C40 expressing cells display enhanced CD40-mediated protection from anti-Ig induced apoptosis, whilst RasV12-S35 expressing cells are refractory to CD40-mediated rescue from anti-Ig induced apoptosis, though, surprisingly, not anti-Ig mediated growth arrest. RasV12-S35 expressing cells are protected from anti-Ig induced growth arrest to a certain extent, but not as well as by the mutants that induce both PI-3-Kinase and Erk-MAPKinase.


Anti-Ig
BCR
RasV12-C40
RasV12
RasV12-C40
RasV12-S35
PI-3-K
Transient ERK activity
PI-3-K
apoptosis
growth arrest

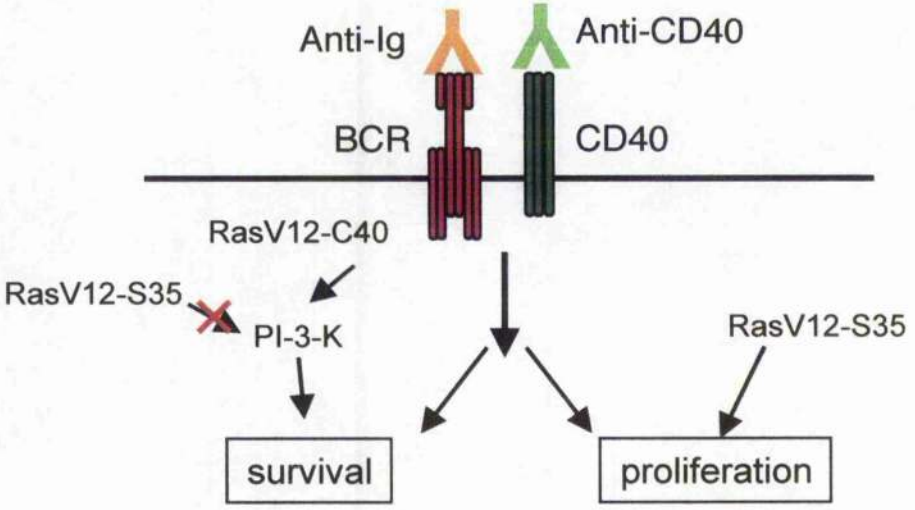
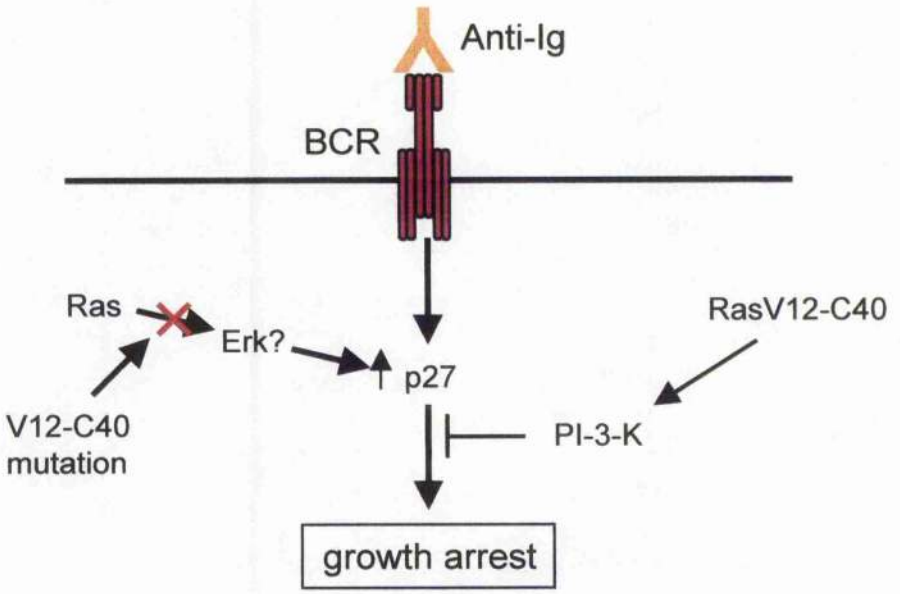

Anti-Ig
Anti-CD40
BCR
CD40
RasV12-C40
RasV12-S35
PI-3-K
RasV12-S35
survival
proliferation

Figure 3.28 Role of the cyclin dependent kinase inhibitor $p27^{Kip1}$ in anti-Ig induced growth arrest of WEHI-231 cells

Anti-Ig induced growth arrest of WEHI-231 cells is accompanied by an increase in the levels of expression of the cyclin dependent kinase inhibitor p27. This may be, at least in part, a consequence of Erk-MAPKinase activation, as cells expressing RasV12-C40 do not respond to anti-Ig by undergoing growth arrest, nor by increasing expression of p27. This is in contrast to RasV12-S35 expressing cells, as these cells increase the level of expression of p27 more than cells containing the empty vector, despite not demonstrating an increase in growth arrest compared to cells containing the empty vector.


Anti-Ig
BCR
Ras
Erk?
p27
RasV12-C40
V12-C40
mutation
PI-3-K
growth arrest

Chapter 4 – The role of PKC isoforms in the regulation of survival and proliferation of WEHI-231 cells

4.1 Introduction

Protein kinase C (PKC) was initially identified and characterised as a proteolytically activated kinase and named protein kinase M in the late 1970s (Inoue *et al.*, 1977; Takai *et al.*, 1977). Members of the PKC superfamily are serine/threonine kinases and are downstream effectors of multiple signalling pathways. To date, 11 isoenzymes have been identified, and these can be subdivided into three groups on the basis of their enzymatic properties, though they all require association with acidic phospholipids such as phosphatidylserine (PS). The conventional (sometimes known as classical) PKCs (cPKCs; α, β_I, β_{II}, γ) are Ca^{2+}-dependent and are activated by diacylglycerol (DAG); the novel PKCs (nPKCs; δ, ε, η, θ) are Ca^{2+}-independent but are also activated by DAG; the atypical PKCs (aPKCs; ζ, λ/ι – λ being the murine isoform, ι the human isoform) are calcium-independent and are not activated by DAG. Each PKC isoform is likely to have different substrates and therefore unique functions.

Activation of the cPKCs depends primarily on activation of the phospholipase C (PLC) pathway that results in the generation of DAG as well as inositol 1,4,5-trisphosphate which causes increases in cytoplasmic Ca^{2+} concentrations. The nPKCs can also be activated by the PLC pathway and presumably by other reactions that produce DAG including phosphatidylcholine hydrolysis and the dephosphorylation of phosphatidic acid to DAG. The full activation of nPKC isoforms also requires phosphorylation by other kinases such as PDK1 and PKCζ which are downstream targets of phosphatidylinositol 3-kinase (PI-3-kinase). The aPKCs are both downstream targets of PI-3-kinase signalling, therefore, as BCR ligation leads to the activation of both the PLC and PI-3-Kinase pathways (Campbell, 1999), it seems highly likely that multiple PKC isoforms participate in BCR signalling.

4.1.1 Expression of PKC isoforms in B lymphocytes

To elucidate the functions of PKCs in BCR signalling it is important to first determine which PKCs are activated following BCR ligation. Seven members of the PKC family have been detected in B cells; PKCα, PKCβ, PKCδ, PKCε, PKCζ, PKCη, and PKCθ (Bras *et al.*, 1997; Krappmann *et al.*, 2001; Mischak *et al.*, 1991; Morrow *et al.*, 1999). Of the novel PKC isoforms, PKCδ and PKCε are expressed at much higher levels in B cells than T cells, while PKCθ is expressed at higher levels in T cells (Krappmann *et al.*, 2001). Of these seven members that have been detected in B cells, most have shown to be involved in B cell signalling in some way or another.

4.1.2 PKCα

PKCα was the first member of the PKC family to be identified and cloned, being isolated from a brain cDNA library over 15 years ago (Parker *et al.*, 1986). It has since been found to have a wide tissue distribution, and as such has been implicated in the control of a number of major cellular functions, including proliferation, differentiation, apoptosis and mobility. In some cell types, overexpression of PKCα is sufficient to induce proliferation, and it has recently been shown that expression of constitutively active PKCα in NIH-3T3 cells results in increased expression of cyclins D1 and E, and increased proliferation rates (Soh and Weinstein, 2003).

As well as promoting proliferation, accumulating evidence suggests a role for PKCα in the inhibition of apoptosis. Reducing the level of PKCα results in apoptosis in a number of cell types, including U937 cells (Whelan and Parker, 1998) and glioma cells (Dooley *et al.*, 1998). PKCα has also been shown to phosphorylate the survival promoting protein Bcl-2 on a site that enhances the antiapoptotic properties of Bcl-2 (Ito *et al.*, 1997; Ruvolo *et al.*, 1998). Furthermore, the induction of apoptosis by ceramide involves the inhibition of

PKCα by dephosphorylation (Lee *et al.*, 1996). Perhaps pertinently, PKCα has also been shown to play an essential role in the survival of the Ramos human B cell line (Keenan *et al.*, 1999).

4.1.3 PKCβ

The PKCβ gene encodes two proteins, $PKC\beta_I$ and $PKC\beta_{II}$, which are generated by alternative splicing of the C-terminal exons, resulting in two proteins differing in only the final 50 ($PKC\beta_I$) or 52 ($PKC\beta_{II}$) amino acids, the V5 region. $PKC\beta_I$ appears to play an important role in B cell survival, at least in WEHI-231 cells. WEHI-231 cells expressing the C-terminal half of leukocyte-specific protein 1 (LSP1) display enhanced apoptosis in response to anti-IgM (Jongstra-Bilen *et al.*, 1999). This increase in anti-IgM-induced apoptosis correlates with failure to translocate $PKC\beta_I$ to the plasma membrane, but not $PKC\beta_{II}$ or PKCα, and a decrease in activation of ERK2 (Cao *et al.*, 2001).

Interestingly, $PKC\beta^{-/-}$ mice have significantly fewer B1 lymphocytes as well as B-1a cells in the peritoneal cavity, compared to wild type mice. The proportion and absolute number of pre-B and mature B cells in the bone marrow of $PKC\beta^{-/-}$ mice and wild type mice are essentially identical, and the proportion of mature B cells in the spleen and lymph nodes is also similar, suggesting that PKCβ is not absolutely required for B cell maturation. However, the absolute number of splenic B cells is slightly reduced (Leitges *et al.*, 1996). The T cell compartment of $PKC\beta^{-/-}$ mice is comparable to that of wild type mice, with the thymus of $PKC\beta^{-/-}$ mice being of normal size and containing $CD4^+CD8^+$ double-positive cells and $CD4^+$ or $CD8^+$ single-positive cells at normal ratios. $PKC\beta^{-/-}$ mice have a reduced primary response to T cell-dependent antigens, as well as a decreased response to T cell-independent type II antigens (Leitges *et al.*, 1996). The phenotype of $PKC\beta^{-/-}$ mice is very similar to that of *xid* mice, which exhibit a mild immunodeficiency and a slight reduction in the number of peripheral B cells, particularly mature B cells, as a result of a point mutation in the PH domain of Btk

(Kurosaki, 1999). Further similarities between PKCβ$^{-/-}$ and *xid* mice are demonstrated by the *in vitro* responses to various stimuli. B cells from both types of mice exhibit significantly less proliferation in response to anti-IgM, anti-CD40 or LPS. This reduction in antigen receptor-mediated proliferation in the absence of PKCβ is restricted to B cells, as the *in vitro* response of splenic T cells and thymocytes to anti-CD3 antibody from PKCβ$^{-/-}$ mice is unchanged.

PKCβ$^{-/-}$ transitional 2 and mature follicular B cells fail to activate NF-κB or up-regulate Bcl-x_L in response to BCR stimulation, resulting in increased apoptosis. This failure to activate NF-κB is a consequence of an inability to induce the degradation of IκB as a result of IKK not being recruited to lipid rafts, and hence not becoming activated, as in wild type cells PKCβ is rapidly recruited to lipid rafts upon BCR ligation (Su *et al.*, 2002). This is reminiscent of PKCθ$^{-/-}$ mice, where T cell stimulation fails to induce NFκB activation (Sun *et al.*, 2000). In wild type T cells, TCR signalling induces recruitment of PKCθ and IKK into lipid rafts (Khoshnan *et al.*, 2000).

Interestingly, and in marked contrast to this, PKCβ activity is required for HLA-DR-mediated B cell death (Guo *et al.*, 2003). However, HLA-DR-induced PKCβ activation does not require its translocation to lipid rafts, indeed, the majority of HLA-DR-activated PKCβ exists outwith lipid rafts. Moreover, cell activation state plays a role in MHC class II signalling, as resting murine B cells undergo MHC class II-mediated cell death, whereas activated cells are insensitive to MHC class II-mediated cell death (Newell *et al.*, 1993). The opposite is true in human B cells, with resting human B cells insensitive to MHC class II-mediated death, whilst activated cells are highly susceptible to HLA-DR-mediated death (Garban *et al.*, 1998). Interestingly, the signalling pathways regulating these distinct functional outcomes appear to be the same, as activated murine B cells and resting human B cells exhibit PTK activation and high Ca^{2+} flux, whereas resting murine B cells

and activated human B cells demonstrate elevated cAMP levels and PKC activation.

Although PKCβ$^{-/-}$ mice exhibit an immunodeficiency similar to that seen in *xid* mice (that arise from a point mutation in Btk) the overall tyrosine phosphorylation of Btk is significantly enhanced in PKCβ-deficient B cells. It has recently been shown that PKCβ actually acts as a negative feedback inhibitor of Btk by phosphorylating Btk. This results in a conformational change in the PH domain of Btk abrogating its interaction with PIP_3, hence disrupting its membrane localisation, which is required for its activity. Mutation of the phosphorylation site creates a mutant Btk that is resistant to PKCβ-mediated inhibition and enhances BCR-dependent calcium signalling. The inhibition of Btk by PKCβ is likely to play a key role in setting and maintaining the BCR signalling threshold (Kang *et al.*, 2001).

4.1.4 PKCδ

PKCδ is expressed at high levels in B cells (Mischak *et al.*, 1991) and has been shown to become tyrosine phosphorylated in response to BCR ligation (Barbazuk and Gold, 1999), suggesting that PKCδ could be an important mediator of BCR signalling. This phosphorylation in response to BCR ligation occurs in the WEHI-231 immature B cell line, the BAL17 mature B cell line, and in small resting murine splenic B cells, suggesting that this process is not dependent on the developmental stage of the B cell. Tyrosine phosphorylation of PKCδ in response to BCR ligation in WEHI-231 cells correlates with its translocation to the membrane, hence its activation, as PKCs must bind membrane lipids in order to become activated. Activation of PLC, and the subsequent production of DAG, is both necessary and sufficient for this membrane recruitment of PKCδ, and necessary but not sufficient for its BCR-induced phosphorylation.

The consequences of PKCδ activation appear to vary depending on cell type. Overexpression of PKCδ in fibroblasts can induce activation of the Raf/MEK/ERK signalling cascade, resulting in activation of the transcription factor AP-1 (Hirai *et al.*, 1994; Ueda *et al.*, 1996). However, PKCδ is also thought to participate in apoptosis, as it is cleaved by caspases (Emoto *et al.*, 1995), producing a constitutively active form of the enzyme that is sufficient to induce apoptosis when expressed in human myeloid leukaemia cells (Ghayur *et al.*, 1996). This catalytic fragment inhibits DNA-dependent protein kinase and contributes to DNA damage-induced apoptosis. It has also been demonstrated that levels of PKCδ expression decrease as cells progress through G_1 and enter S phase of the cell cycle, and increase above initial levels as cells re-enter G_1 (Srivastava *et al.*, 2002), suggesting that PKCδ acts as an inhibitor of cell cycle progression.

PKCδ knockout mice have a greater percentage of B lymphocytes in the spleen and lymph nodes compared to wild type mice, and indeed the absolute number of B lymphocytes in spleen and lymph nodes of PKCδ$^{-/-}$ mice is greater than from wild type animals (Miyamoto *et al.*, 2002). This expansion of the B cell population occurs in the periphery, as there are no significant differences in the bone marrow of PKCδ$^{-/-}$ mice compared to that of wild type mice. The proliferative response of PKCδ-deficient B cells stimulated with anti-IgM and anti-CD40 in combination, or LPS alone was significantly greater than that of B cells from wild type mice, whilst the response of T cells was unchanged, indicating that the effect of loss of PKCδ is B cell specific (Miyamoto *et al.*, 2002). Another group generated PKCδ-deficient mice independently at the same time (Mecklenbrauker *et al.*, 2002), and they claim that loss of PKCδ does not affect BCR-mediated B cell activation *in vitro* or *in vivo*. However, this paper does not assess proliferation of stimulated B cells by means of a DNA synthesis assay as Miyamoto, but by staining with CFSE. An alternative interpretation of the CFSE data is that B cells from PKCδ$^{-/-}$ mice undergo more than 1 cell division, whereas B cells from wild type mice only undergo 1 cell division, in concurrence with Miyamoto. It was also

found that B cells from PKCδ$^{-/-}$ are refractory to tolerance, permitting maturation and terminal differentiation of self-reactive B cells in the presence of the tolerance-inducing antigen. The serum of PKCδ$^{-/-}$ mice also contained autoreactive anti-DNA and anti-nuclear antibodies (Mecklenbrauker *et al.*, 2002) and PKCδ$^{-/-}$ mice are prone to autoimmune disease (Miyamoto *et al.*, 2002), further demonstrating a likely role for PKCδ in the induction of self antigen-induced B cell tolerance. Despite its proposed role as a tumour suppressor, due to its ability to inhibit cell cycle progression, there was no increase in cancer-induced death in the PKCδ$^{-/-}$ animals compared to control animals, suggesting that the lack of functional PKCδ does not lead to predisposition towards cancer. The phenotype seen in B cells of PKCδ$^{-/-}$ mice contrasts markedly with that seen in PKCβ$^{-/-}$ mice, as PKCβ$^{-/-}$ animals develop an immunodeficiency as a result of decreased proliferation of B cells (Leitges *et al.*, 1996), suggesting that the activation of PKCβ is required for the proliferation of stimulated B cells, whilst PKCδ plays a role in negatively regulating the proliferation of stimulated B cells.

Overexpression of PKCδ leads to a decrease in the expression of cyclin D1 in primary bovine smooth muscle cells, and overexpression of a dominant negative PKCδ results in an increase in cyclin D1 expression (Page *et al.*, 2002). It has also recently been shown that expression of a constitutively active PKCδ mutant inhibits expression from the cyclin D1 promoter in NIH3T3 cells, but there was no increase in activity of the cyclin D1 promoter in response to a PKCδ dominant negative mutant (Soh and Weinstein, 2003).

4.1.5 PKCε

PKCε is thought to be a positive regulator of cell survival and proliferation. PKCε has been shown to activate the Raf-1/ERK pathway (Hamilton *et al.*, 2001), thus promoting the activation of the two key survival factors NF-κB (Tojima *et al.*, 2000) and Akt (Matsumoto *et al.*, 2001). PKCε may also promote cell survival by

contributing to the activation of Akt, a protein kinase that mediates PI-3-kinase survival signals, as expression of a catalytically inactive form of PKCε has been shown to inhibit the activation of Akt by insulin (Matsumoto *et al.*, 2001). PKCε appears to play a role in cell proliferation also, as it is activated in response to a variety of mitogens (Moriya *et al.*, 1996; Olivier and Parker, 1994). In certain cell types, overexpression of PKCε results in transformation, a consequence of PKCε inducing the phosphorylation of Raf-1 and the resulting activation of the ERK-MAPK signalling pathway (Cacace *et al.*, 1996; Cai *et al.*, 1997; Perletti *et al.*, 1998). More recently, it has been shown that expression of a constitutively active mutant of PKCε in NIH3T3 cells activates the cyclin D1 promoter (Soh and Weinstein, 2003). However, overexpression of PKCε in NIH-3T3 cells increases radiation-induced cell death, as a result of increased Erk1/2 activation (Lee *et al.*, 2003). Overexpression of a constitutively active PKCε caused an even greater increase in radiation-induced cell death, whereas overexpression of a dominant negative PKCε returned cell death to control levels.

PKCε is highly expressed in B lineage cells, and ligation of the BCR of the A20 murine B cell line induces its translocation from the cytoplasm to cellular membranes, and this translocation coincides with PI-3-Kinase-dependent phosphorylation of PKCε, the two steps required for maximal activation of PKCε (Ting *et al.*, 2002). Translocation of PKC enzymes from the cytoplasm to the membrane allows them to bind their lipid activators and is taken as a measure of PKC activation. The role of PKCε in BCR signalling remains to be elucidated, however, in other cell types activation of PKCε has been strongly linked to cell survival and proliferation. PKCε activation inhibits both TNF- and TRAIL-induced apoptosis (Mayne and Murray, 1998; Shinohara *et al.*, 2001), so it would seem likely that PKCε activation contributes to BCR-induced survival signals. The BCR-induced survival signal is thought to involve, at least in part, NF-κB, and it has been shown that PKCε promotes the activation of NF-κB (Tojima *et al.*, 2000).

Further support for the idea that PKCε activation could be involved in B cell proliferation comes from the fact that PKCε was found to be constitutively phosphorylated in a number of B lymphoma cell lines that are constitutively proliferating, but such phosphorylation was only induced in mature splenic B cells by BCR ligation (Ting *et al.*, 2002).

4.1.6 PKCζ

PKCζ has been implicated in the control of Erk-MAPKinase and NF-κB signalling pathways. In PKCζ$^{-/-}$ mice the composition of the B cell compartment in lymphoid tissue is abnormal. PKCζ$^{-/-}$ mice have a higher percentage of immature B cells in Peyer's Patches at all ages examined, and in the lymph nodes at 2 weeks. The difference in percentage between wild type and knockout mice was less pronounced in lymph nodes at 7-8 weeks, though there was still a slightly higher percentage of immature cells in the PKCζ-deficient mice, as assessed by B220 and MHC expression. In PKCζ$^{-/-}$ mice, proliferation of purified splenic B cells activated via the BCR is severely impaired, however, this is not the case when the cells are stimulated with LPS or anti-CD40 (Martin *et al.*, 2002). This attenuation of proliferation in response to BCR stimulation correlates with a significant decrease in activation of MEK, and a concomitant decrease in activation of Erk-MAPKinase, as assessed by western blotting with anti-phospho-MEK and -ERK antibodies respectively (Martin *et al.*, 2002).

4.2 Aims and Objectives

Many of the seemingly contradictory results whereby PKC activation led to apoptosis or survival in different cell types stems from the use of phorbol esters as activators of PKCs. Phorbol esters can stimulate both cPKC and nPKC isoforms, but prolonged treatment of cells with these agents results in PKC down regulation and cellular depletion. However, generally speaking, conventional and

atypical PKCs are considered to be predominantly anti-apoptotic, being principally involved in promoting cell survival and proliferation. The novel PKCs, however, generally have a tumour suppressor function and are regarded as pro-apoptotic proteins. PKCα and PKCδ are two of the most well characterised PKCs with respect to their roles in preventing and promoting apoptosis, respectively. It is interesting that these two have been demonstrated as having key roles in regulating apoptosis, as these isoforms are present in the majority of B cell stages.

To further elucidate the roles of individual PKC isoforms in signalling in immature B cells, mutant forms of four PKC isoforms were transfected into the WEHI-231 immature B cell line. The isoforms were chosen on the basis that knockout studies imply that they have roles in B cells. This chapter aims to address:

1. The role of the conventional PKC, PKCα, in BCR-mediated signalling, using the constitutively active PKCα-CAT mutant and the kinase dead PKCα-KR mutant.
2. The role of the novel PKC, PKCδ, in BCR-mediated signalling, using the constitutively active PKCδ-CAT mutant and the kinase dead PKCδ-KR mutant.
3. The role of the novel PKC, PKCε, in BCR-mediated signalling, using the constitutively active PKCε-CAT mutant and the kinase dead PKCε-KR mutant.
4. The role of the atypical PKC, PKCζ, in BCR-mediated signalling, using the constitutively active PKCζ-CAT mutant and the kinase dead PKCζ-KR mutant.
5. The effects all of these mutants have on key regulators of the cell cycle in WEHI-231 cells.

4.3 Results

4.3.1 Transfection of WEHI-231 cells with PKC mutants

To investigate the roles of the four PKC family members PKCα, PKCδ, PKCε, and PKCζ, two mutant forms of each were inserted into the pcDNA3.1 vector (**Figure 2.9**). The mutant forms used were either a constitutively active catalytic domain (CAT) or full-length kinase dead as the result of a lysine – arginine substitution (KR) (**Figure 4.1**). All eight mutants were transfected into the WEHI-231 cell line by electroporation, as described in **Section 2.14**. Successfully transfected cells were selected by the addition of G418 (500 μg/ml) 48 hours after electroporation and continued culture of the cells with 500 μg/ml G418.

4.3.2 Effect of PKCα mutants on anti-Ig mediated growth arrest in WEHI-231 cells

Wild type WEHI-231 cells exhibit a pronounced growth arrest in G_1 phase of the cell cycle in response to anti-Ig, as shown in **Figure 3.1**. To investigate the effects of expression of a constitutively active PKCα (PKCα-CAT) or a kinase dead form of PKCα (PKCα-KR) on anti-Ig mediated growth arrest in WEHI-231 cells, transfected cells were stimulated with various concentrations of anti-Ig, and proliferation assessed by the [^{3}H]-thymidine uptake assay at 24 and 48 hours (**Figure 4.2**). Cells transfected with the empty vector were used as a control.

Even at 24 hours, WEHI-231 cells transfected with the empty pcDNA3.1 vector exhibit a pronounced growth arrest in response to anti-Ig, with thymidine uptake levels only approximately 60% of those of unstimulated cells in response to 1 μg/ml anti-Ig, and only about 50% in response to 10 μg/ml anti-Ig (**Figure 4.2A**). By 48 hours, both concentrations of anti-Ig have reduced levels of thymidine uptake to around 40% of those of the unstimulated cells (**Figure 4.2B**). In contrast to this, cells transfected with the PKCα mutants do not show signs of

growth inhibition in response to 10 μg/ml anti-Ig at 24 hours. If anything, PKCα-KR cells actually exhibit slightly increased levels of proliferation after 24 hours stimulation with anti-Ig, particularly 0.1 μg/ml and 10 μg/ml. Cells expressing PKCα-CAT demonstrate slightly lower levels of proliferation in response to low doses of anti-Ig (0.1 and 1 μg/ml), but the levels increase in response to 10 μg/ml (**Figure 4.2A**). After 48 hours treatment cells expressing either PKCα mutant do demonstrate growth arrest in response to anti-Ig at 1 or 10 μg/ml, but not quite to the same extent as cells containing the empty vector alone (**Figure 4.2B**). The most surprising thing about this result is the fact that both mutants confer the same properties upon WEHI-231 cells, despite the fact that one should be constitutively active and the other dominant negative.

4.3.3 Effect of PKCα mutants on anti-Ig mediated apoptosis in WEHI-231 cells

To investigate the effects of expression of PKCα-CAT and PKCα-KR on anti-Ig mediated apoptosis in WEHI-231 cells, transfected cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml) for up to 72 hours. Cells receiving no stimulating antibodies were used as a control. At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase.

At each time point, cells containing the empty vector display an increase in the proportion of cells displaying a sub-diploid DNA content, along with an increase in cells in G_0/G_1 and a concomitant decrease in cells in S phase or G_2/M in response to anti-Ig stimulation (**Figure 4.3**). WEHI-231 cells expressing the PKCα-CAT mutant display a similar pattern, with an increase in sub-diploid cells and cells in G_0/G_1, and a decrease in cells in S phase and G_2/M. The increase in apoptosis is much greater than that shown by cells containing the empty vector

at 48 hours, with PKCα-CAT expressing cells demonstrating a 6-fold increase in apoptosis, compared to approximately 3-fold for the vector containing cells at the same time point. On the contrary, the PKCα-KR mutant does not appear to alter the apoptotic response of WEHI-231 cells to anti-Ig, as the increase in apoptosis induced by anti-Ig is of a similar magnitude to cells containing the empty vector.

4.3.4 Effect of PKCα mutants on CD40-mediated rescue from anti-Ig mediated growth arrest and apoptosis in WEHI-231 cells

To investigate the effects of the PKCα mutants on CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis, cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Following stimulation for 48 hours, proliferation was assessed by the [^{3}H]-thymidine incorporation assay. At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase.

Expression of PKCα-CAT enhances CD40-mediated rescue of anti-Ig induced growth arrest at 48 hours compared to cells carrying the empty vector (**Figure 4.4**). This is not as evident when the proportion of cells in each cell cycle phase is assessed by flow cytometry, as the PKCα-CAT expressing cells have a proportion of cells in each cell cycle phase intermediate between that of unstimulated cells and those stimulated with anti-Ig alone. However, there is a dramatic increase in the proportion of S phase cells at 48 hours compared to cells stimulated with anti-Ig alone, though this does not reach the proportion of unstimulated cells in S phase (**Figure 4.3**). PKCα-KR expressing cells exhibit a very similar level of CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis as cells containing the empty vector.

4.3.5 Effect of PKCδ mutants on anti-Ig mediated growth arrest in WEHI-231 cells

To investigate the effects of expression of a constitutively active PKCδ (PKCδ-CAT) or a kinase dead form of PKCδ (PKCδ-KR) on anti-Ig mediated growth arrest in WEHI-231 cells, transfected cells were stimulated with increasing concentrations of anti-Ig, and proliferation assessed by the [^{3}H]-thymidine uptake assay at 24 and 48 hours (**Figure 4.5** and results not shown). Cells transfected with the empty vector were used as a control.

After 24 hours of stimulation with anti-Ig, WEHI-231 cells expressing either PKCδ mutant show no signs of growth arrest, even at the maximum concentration of anti-Ig, whereas cells containing the empty vector exhibit only 50% of the [^{3}H] thymidine uptake of unstimulated cells (**Figure 4.5A**). By 48 hours of treatment, 10 μg/ml has induced growth arrest of a similar magnitude to cells containing the empty vector in cells expressing either PKCδ mutant (**Figure 4.5B**).

4.3.6 Effect of PKCδ mutants on anti-Ig mediated apoptosis in WEHI-231 cells

To investigate the effects of expression of PKCδ-CAT and PKCδ-KR on anti-Ig mediated apoptosis in WEHI-231 cells, transfected cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml) for up to 72 hours. Cells receiving no stimulating antibodies were used as a control. At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase.

After 24 hours of treatment, WEHI-231 cells expressing PKCδ-CAT or PKCδ-KR do not exhibit an increase in apoptosis in response to anti-Ig, nor do they appear

to demonstrate any growth arrest as there is a high proportion of cells in S phase and G_2/M (**Figure 4.6**), corroborating the [^{3}H]-thymidine data (**Figure 4.5A**). By 48 hours however, both mutants exhibit very similar patterns to cells carrying the empty vector, with anti-Ig stimulation inducing an increase in the proportion of cells with sub-diploid DNA content and a huge increase in the proportion of cells in G_0/G_1. As expected there is a concomitant decrease in cells in S phase and G_2/M. By 72 hours the proportion of apoptotic cells is still greater in cells stimulated with anti-Ig compared to unstimulated cells, but unstimulated cells are starting to accumulate in G_0/G_1, probably as a result of exhaustion of essential nutrients in the culture.

4.3.7 Effect of PKCδ mutants on CD40-mediated rescue from anti-Ig mediated growth arrest and apoptosis in WEHI-231 cells

To investigate the effects of the PKCδ mutants on CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis, cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Following stimulation for 48 hours, proliferation was assessed by the [^{3}H]-thymidine incorporation assay (**Figure 4.7**). Apoptosis was assessed at 24, 48 and 72 hours by flow cytometry following propidium iodide staining, to determine the proportion of cells with sub-diploid DNA content as well as the proportion of cells in each cell cycle phase (**Figure 4.6**).

Expression of PKCδ-CAT does not appear to affect CD40-mediated rescue of anti-Ig induced growth arrest as [^{3}H] thymidine incorporation is not significantly different from control cells in response to anti-Ig alone or the combination of anti-Ig and anti-CD40 (**Figure 4.7**). PKCδ-KR appears to have a slight positive effect on CD40-mediated rescue, as cells expressing PKCδ-KR appear to have enhanced growth in response to anti-Ig and anti-CD40 treatment, compared to control cells (**Figure 4.7**). This is supported by the DNA content analysis as PKCδ-KR expressing cells display a larger proportion of cycling cells (S phase or

G_2/M) upon co-stimulation than in the population of cells stimulated with anti-Ig alone at 48 hours (**Figure 4.6**). Neither PKCδ-CAT nor PKCδ-KR appear to affect CD40-mediated rescue from anti-Ig induced apoptosis as the proportion of cells with sub-diploid DNA content is greatly reduced by co-stimulation with anti-Ig and anti-CD40, compared to anti-Ig treatment alone (**Figure 4.6**).

4.3.8 Effect of PKCε mutants on anti-Ig mediated growth arrest in WEHI-231 cells

To investigate the effects of expression of a constitutively active PKCε (PKCε-CAT) or a kinase dead form of PKCε (PKCε-KR) on anti-Ig mediated growth arrest in WEHI-231 cells, transfected cells were stimulated with increasing concentrations of anti-Ig over a period of 48 hours, and proliferation assessed by the [^{3}H]-thymidine uptake assay at 24 and 48 hours (**Figure 4.8**). Cells transfected with the empty vector were used as a control.

WEHI-231 PKCε-CAT cells appear to increase their proliferation upon stimulation with anti-Ig for 24 hours, in a dose–dependent manner (**Figure 4.8A**). WEHI-231 PKCε-KR cells do not undergo growth arrest in response to anti-Ig after 24 hours, but nor do they increase their proliferation. By 48 hours, however, anti-Ig (1 and 10 μg/ml) has induced growth arrest in both these cell types, to the same level as the empty vector control (**Figure 4.8B**).

4.3.9 Effect of PKCε mutants on anti-Ig mediated apoptosis in WEHI-231 cells

To investigate the effects of expression of PKCε-CAT and PKCε-KR on anti-Ig mediated apoptosis in WEHI-231 cells, transfected cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml) for up to 72 hours. Cells receiving no stimulating antibodies were used as a control. At 24, 48 and 72 hours the DNA content was assessed by flow cytometry

following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase.

WEHI-231 PKCε-CAT cells exhibit an abrogation of anti-Ig induced growth arrest and apoptosis as there is only a slight increase in the proportion of cells with sub-diploid DNA content or in cells in G_0/G_1, whereas there is no great decrease in cells in S phase or G_2/M (**Figure 4.9**). This corroborates the [^{3}H] thymidine data, which suggested that PKCε-CAT confers a level of resistance to anti-Ig mediated growth arrest at 24 hours. However, by 48 and 72 hours, anti-Ig induces a substantial increase (6-fold at 48, 5-fold at 72) in the proportion of WEHI-231 PKCε-CAT cells with sub-diploid DNA content relative to unstimulated cells. This is accompanied at 48 hours by an increase in cells arrested in G_0/G_1, and a decrease in cells in both S phase and G_2/M. PKCε-KR also appears to confer resistance to anti-Ig induced growth arrest and apoptosis at 24 hours, as there is very little increase in the proportion of cells with a sub-diploid DNA content and in G_0/G_1, and no great decrease in the proportion of cells in S phase or G_2/M, compared to cells containing the empty vector. This effect again is not sustained as by 48 hours there is a dramatic increase in the proportion of cells with a sub-diploid DNA content and arrested in G_0/G_1, and this remains the case at 72 hours.

4.3.10 Effect of PKCε mutants on CD40-mediated rescue from anti-Ig mediated growth arrest and apoptosis in WEHI-231 cells

To investigate the effects of the PKCε mutants on CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis, cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Following stimulation for 48 hours, proliferation was assessed by the [^{3}H] thymidine incorporation assay (**Figure 4.10**). At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine

the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase (**Figure 4.9**).

Neither PKCε-CAT or PKCε-KR seem to affect CD40-mediated rescue of anti-Ig induced growth arrest or apoptosis, as the results of the proliferation assay (**Figure 4.10**) and DNA content analysis (**Figure 4.9**) do not differ significantly from control cells. This is somewhat surprising, given the increase in anti-Ig mediated apoptosis induced by expression of PKCε-CAT.

4.3.11 Effect of PKCζ mutants on anti-Ig mediated growth arrest in WEHI-231 cells

To investigate the effects of expression of a constitutively active PKCζ (PKCζ-CAT) or a kinase dead form of PKCζ (PKCζ-KR) on anti-Ig mediated growth arrest in WEHI-231 cells, transfected cells were stimulated with increasing concentrations of anti-Ig, and proliferation assessed by means of the [^{3}H]-thymidine uptake assay at 24 and 48 hours (**Figure 4.11**). Cells transfected with the empty vector were used as a control.

After 24 hours stimulation with 0.1 μg/ml anti-Ig, WEHI-231 cells expressing either PKCζ construct display a decrease in proliferation compared to the empty vector, but when stimulated with 1 or 10 μg/ml anti-Ig WEHI-231 cells expressing these constructs exhibit a minimal decrease in proliferation (**Figure 4.11A**). After 48 hours stimulation, there is no great difference in the growth arrest induced by 10 μg/ml upon WEHI-231 cells expressing either PKCζ mutant compared to cells containing the empty vector (**Figure 4.11B**). Similar to the PKCα mutants, the behaviour of the PKCζ mutants is fairly similar at the level of the biological response to anti-Ig.

4.3.12 Effect of PKCζ mutants on anti-Ig mediated apoptosis in WEHI-231 cells

To investigate the effects of expression of PKCζ-CAT and PKCζ-KR on anti-Ig mediated apoptosis in WEHI-231 cells, transfected cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml) for up to 72 hours. Cells receiving no stimulating antibodies were used as a control. At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase.

Interestingly, at 24 hours, cells transfected with either PKCζ mutant display a modest increase in the proportion of sub-diploid cells upon stimulation with anti-Ig compared to cells containing the empty vector (**Figure 4.12**). Indeed, they still display relatively large proportions of cells in S phase and G_2/M, corroborating the [^{3}H]-thymidine data, which suggested that these cells are refractory to anti-Ig induced growth arrest at 24 hours (**Figure 4.11**). Transfection with the PKCζ-CAT mutant results in a substantial increase in apoptosis upon 48 hours of treatment with anti-Ig, with a 7-fold increase in the proportion of sub-diploid cells compared to unstimulated cells, whilst cells containing the empty vector display a more modest 3-fold increase (**Figure 4.12**). This is accompanied by a large decrease in the proportion of cells in either S phase or G_2/M, corroborating the results yielded by the [^{3}H]-thymidine incorporation assay (**Figure 4.11**). In contrast, PKCζ-KR expressing cells exhibit only a 3-fold increase in apoptosis upon stimulation with anti-Ig for 48 hours, compared to unstimulated cells. However, there is a substantial decrease in the proportion of cells in either S phase or G_2/M, again corroborating the [^{3}H]-thymidine data (**Figure 4.11**).

4.3.13 Effect of PKCζ mutants on CD40-mediated rescue from anti-Ig mediated growth arrest and apoptosis in WEHI-231 cells

To investigate the effects of the PKCζ mutants on CD40-mediated rescue from anti-Ig induced growth arrest and apoptosis, cells were stimulated with anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) and anti-CD40 (10 μg/ml). Following stimulation for 48 hours, proliferation was assessed by the [^{3}H]-thymidine incorporation assay (**Figure 4.13**). At 24, 48 and 72 hours the DNA content was assessed by flow cytometry following staining with propidium iodide, to determine the proportion of cells with a sub-diploid DNA content, as well as the proportion of cells in each cell cycle phase (**Figure 4.12**).

Neither PKCζ-CAT or PKCζ-KR seem to affect CD40-mediated rescue of anti-Ig induced growth arrest or apoptosis, as the results of the proliferation assay (**Figure 4.13**) and DNA content analysis (**Figure 4.12**) do not differ significantly from control cells.

4.3.14 Effect of PKC mutants on p27^{Kip1} expression levels in response to anti-Ig and anti-CD40 stimulation

To assess the effects of the PKC mutants on the protein levels of key cell cycle regulators in response to anti-Ig and anti-CD40 stimulation, whole cell lysates from WEHI-231 cells expressing each mutant were examined by Western blotting following the appropriate stimulation. One key regulator of the cell cycle is the cyclin dependent kinase inhibitor p27^{Kip1}. An anti-p27 antibody was used to detect levels of p27^{Kip1} following Western blotting (**Figure 4.14**).

Unstimulated WEHI-231 cells containing the empty vector display very low levels of p27 throughout the 48 hour period. Stimulation with anti-Ig antibodies leads to an increase in p27 levels over time, concomitant with induction of growth arrest. Stimulation of these cells with anti-CD40 antibodies leads to a transient increase

in the levels of p27, though they drop by 24 hours, before increasing again slightly at 48 hours. Co-stimulation with anti-Ig and anti-CD40 antibodies also leads to a transient increase in p27 expression, though the levels fall quickly and remain low.

WEHI-231 PKCα-CAT cells display a very similar pattern of p27 expression to cells containing the empty vector, with anti-Ig inducing a substantial increase over time. The same is true of WEHI-231 PKCα-KR cells, though perhaps there is also a slight increase in p27 levels in unstimulated cells at 48 hours, probably as a result of exhaustion of the nutrients in the media.

WEHI-231 PKCδ-CAT cells have a slightly different p27 expression profile from WEHI-231 pcDNA3.1 cells, as anti-Ig fails to induce an increase in p27 levels in these cells. In contrast, anti-CD40 stimulation of WEHI-231 PKCδ-CAT cells appears to induce an increase in p27 levels. WEHI-231 PKCδ-KR cells also have a different pattern of p27 expression, almost exactly the opposite of WEHI-231 PKCδ-CAT cells. Unstimulated WEHI-231 PKCδ-KR cells, and those stimulated with anti-Ig, exhibit fairly high levels of p27 expression compared to WEHI-231 pcDNA3.1 cells. Stimulation of these cells with anti-CD40, either alone or in combination with anti-Ig, results in a decrease in p27 expression.

WEHI-231 PKCε-CAT cells display a largely similar pattern of p27 expression, with anti-Ig inducing an increase over time, even though the basal levels appear to be slightly higher. Stimulation with anti-CD40, either alone or in combination with anti-Ig, leads to a transient increase in levels, followed by a steady decrease. WEHI-231 PKCε-KR cells also have fairly high basal levels of p27 expression, but anti-Ig doesn't appear to induce an increase. Stimulation with anti-CD40 does, however, lead to the expected decrease in p27 levels, following a transient increase.

WEHI-231 PKCζ-CAT cells and WEHI-231 PKCζ-KR cells exhibit a similar pattern of p27 expression to WEHI-231 pcDNA3.1 cells, albeit with a higher basal level in untreated cells. Stimulation with anti-Ig leads to an increase in p27 expression levels over time, whilst stimulation with anti-CD40, either alone or in combination with anti-Ig, leads to a decrease over time.

The PKC mutant that induces the greatest difference in $p27^{Kip1}$ expression levels in response to anti-Ig and anti-CD40 stimulation is PKCδCAT. In all the other cell types, anti-Ig induces a vast increase in $p27^{Kip1}$ expression at 24 and 48 hours, but this is not evident in the WEHI-231 PKCδ-CAT cells. These cells, however, did not exhibit anti-Ig induced growth arrest at 24 hours, though by 48 hours they had succumbed to growth arrest (**Figure 4.5**). Surprisingly, stimulation with anti-CD40 alone, or a combination of anti-Ig and anti-CD40 induces an increase in $p27^{Kip1}$ levels, compared to unstimulated cells. This is entirely unexpected as $p27^{Kip1}$ is a key inhibitor of the cell cycle, so upregulation of its expression would not be conducive to increased proliferation.

4.3.15 Effect of PKC mutants on D-type cyclin expression levels in response to anti-Ig and anti-CD40 stimulation

A key cyclin responsible for G_1 progression in B cells is cyclin D2 (Solvason *et al.*, 2000), the cyclin partner for CDK 4 which is responsible for phosphorylation of the retinoblastoma protein and hence G_1 to S phase progression. To investigate the effects of the PKC mutants on cyclin D2, an anti-cyclin D1/2 antibody was used (**Figure 4.15**). Cyclin D2 could not be detected in samples from PKCδ or PKCζ expressing cells.

Unstimulated WEHI-231 pcDNA3.1 cells express a fairly constant level of cyclin D2 over the time points investigated, with perhaps a slight increase at 24 hours. Stimulation with anti-Ig antibodies leads to a decrease in cyclin D2 levels over time, which would be expected as the cells are undergoing growth arrest. Cells

stimulated with anti-CD40 alone also display a relatively constant level of cyclin D2 expression, and, somewhat surprisingly, co-stimulation with anti-Ig and anti-CD40 induces a decrease in cyclin D2 protein levels over time, despite an increase in proliferation.

WEHI-231 PKCα-CAT and WEHI-231 PKCαKR cells demonstrate a largely similar profile of cyclin D2 expression to cells containing the empty vector, with anti-Ig, either alone or in combination with anti-CD40, inducing a decrease in expression. WEHI-231 PKCε-CAT and WEHI-231 PKCεKR cells also exhibit a largely similar profile of cyclin D2 expression to cells containing the empty vector, with anti-Ig, either alone or in combination with anti-CD40, inducing a decrease in expression. Therefore it appears as though none of these PKC mutants can prevent the anti-Ig mediated down-regulation of expression of cyclin D2.

4.3.16 Effect of PKC mutants on phosphorylation of the retinoblastoma protein, Rb, in response to anti-Ig and anti-CD40 stimulation

Another key regulator of the cell cycle is the retinoblastoma tumour suppressor protein (Rb). Rb inhibits progression from G_1 to S phase of the cell cycle by binding to the E2F family of transcription factors and preventing transcription of key genes required for cell cycle progression (Dyson, 1998) (**Figure 1.9**). The interaction between Rb and E2F is abrogated by phosphorylation of Rb by CDKs at a number of sites in its C-terminal domain, allowing E2F to activate transcription of a number of genes. Therefore, Rb phosphorylation is a measure of cell cycle progression, from G_1 to S phase. To investigate the effects of PKC mutants on the phosphorylation state of Rb, an antibody against phosphorylated Rb (Ser^{807} and Ser^{811}) was used in western blot analysis (**Figure 4.16**).

The phosphorylation of Rb at $Ser^{807/811}$ in WEHI-231 pcDNA3.1 cells does not appear to vary much between different stimulations. Unstimulated cells have a low level, which decreases by 24 hours and is undetectable at 48 hours.

Stimulation with anti-Ig accelerates the decrease, with phospho-Ser$^{807/811}$ undetectable by 24 hours. Cells stimulated with anti-CD40, either alone or in combination with anti-Ig, only appear to have detectable Ser$^{807/811}$ phosphorylation at 8 hours.

WEHI-231 PKCα-CAT cells and WEHI-231 pcDNA3.1 cells display a generally similar pattern when unstimulated, but upon stimulation with anti-Ig Ser$^{807/811}$ phosphorylation of Rb appears to increase in WEHI-231 PKCα-CAT cells. Stimulation with anti-CD40, alone or in combination with anti-Ig, leads to a transient increase in phosphorylation that diminishes to undetectable levels by 48 hours. Unstimulated WEHI-231 PKCα-KR cells increase their levels of phospho-Ser$^{807/811}$ Rb with time, whilst cells stimulated with anti-Ig have barely detectable levels except at 24 hours, where there is a substantial increase. WEHI-231 PKCα-KR cells stimulated with anti-CD40, alone or in combination with anti-Ig, have barely detectable levels throughout the time course.

WEHI-231 PKCδ-CAT cells have a very similar pattern of Rb phosphorylation to WEHI-231 PKCα-CAT cells, particularly when co-stimulated with anti-Ig and anti-CD40, though not when stimulated with anti-Ig alone. WEHI-231 PKCδ-KR cells also have a very similar pattern of Rb phosphorylation to WEHI-231 PKCα-CAT cells, this time particularly when stimulated with anti-Ig alone, suggesting that PKCδ-CAT provides some measure of protection against anti-Ig mediated down regulation of phosphorylation of Rb.

Unstimulated WEHI-231 PKCε-CAT cells and those stimulated with anti-Ig exhibit an increase in Rb phosphorylation levels compared to cells containing the empty vector. Phosphorylated Rb could not be detected in whole cell lysates from WEHI-231 PKCε-CAT cells stimulated with either anti-CD40 or a combination of anti-Ig and anti-CD40. WEHI-231 PKCε-KR cells display greater levels of Rb phosphorylation at Ser$^{807/811}$ under all conditions, suggesting that these cells may demonstrate enhanced proliferation, as phosphorylated Rb is an indication of

cells leaving G_1. In particular, these cells do not display the anti-Ig induced down regulation of Rb phosphorylation displayed by the cels containing the empty vector.

Unstimulated WEHI-231 PKCζ-CAT cells have high levels of Rb phosphorylation at 1 hour, but the levels drop to barely detectable at 8, 24 and 48 hours, slightly different from cells containing the empty vector. With all other treatments, WEHI-231 PKCζ-CAT cells show a very similar pattern of Rb phosphorylation to cells containing the empty vector. WEHI-231 PKCζ-KR cells do not exhibit any significant differences in terms of the pattern of Rb phosphorylation compared to that of cells containing the empty vector.

4.3.17 Effect of PKC mutants on Tyr15 phosphorylation of the cdc2 protein in response to anti-Ig and anti-CD40 stimulation

Entry of all eukaryotic cells into M-phase of the cell cycle is regulated by activation of cdc2. Activation of cdc2 is controlled at several steps, including cyclin binding and phosphorylation of threonine 161. However, the critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation of tyrosine 15 and threonine 14. Phosphorylation at Tyr15 and inhibition of cdc2 is carried out by Wee1 and Myt1 protein kinases, while Tyr15 dephosphorylation and activation of cdc2 is carried out by the cdc25 phosphatase. The antibody used is specific for Phospho-Tyr15 cdc2, the inactive form of the kinase (**Figure 4.17**).

All cell types tested exhibit a broadly similar pattern of cdc2 phosphorylation at Tyr^{15}, including cells containing the empty vector. Unstimulated cells exhibit a fairly steady increase in levels to 24 hours, before dropping by 48 hours. Anti-Ig appears to induce a decrease in cdc2 phosphorylation over time, which would not be expected as the unphosphorylated form of the protein is active, therefore capable of promoting entry to mitosis. Stimulation with anti-CD40, either alone or

in combination with anti-Ig, results in a similar pattern to unstimulated cells, with cdc2 phosphorylation increasing to 24 hours before dropping at 48 hours. As all the cell types display similar patterns, it appears as though none of the PKC mutants tested alter the role of cdc2 in WEHI-231 cells.

4.4 Discussion

Upon ligation by antigen the B cell receptor activates a number of signalling pathways to mediate a response. One of the first pathways shown to be activated by the BCR was phospholipase C, which results in the generation of the second messenger DAG, a key regulator of both conventional PKCs and novel PKCs. This suggests a key role for PKC family members in BCR signalling. Indeed, BCR cross-linking on mature B cells leads to strong activation of PKCs, whereas ligation of the BCR on immature B cells does not, providing a possible explanation for the differential responses to BCR cross-linking in mature and immature B cells (Sarthou *et al.*, 1989). As a consequence, it has been proposed that BCR uncoupling from PKC activation may be a major factor in anti-Ig induced growth arrest and apoptosis in immature B cells. Consistent with this, treatment of WEHI-231 cells with PMA, a potent activator of most PKC isoforms, protects them from anti-Ig induced growth arrest and apoptosis (Gold and DeFranco, 1987). Moreover, PKC isoforms from all three subclasses have been implicated in the regulation of Erk-MAPKinase, which we have shown to be important for apoptosis and proliferation, through a number of mechanisms. For instance, PKCα and PKCε have both been implicated as directly activating Raf, an upstream regulator of Erk-MAPKinase, in NIH-3T3 and COS cells (Cai *et al.*, 1997). Other studies have demonstrated that conventional and atypical PKCs, but not novel PKCs, can induce dissociation of Raf from RKIP (Raf Kinase Inhibitory Protein) by phosphorylation of RKIP at a key serine residue (Corbit *et al.*, 2003). In addition, it has recently been demonstrated that RasGRP3, an activator of Ras selectively expressed in B cells, is phosphorylated in a PKC dependent fashion, and that this phosphorylation is required for its optimal activation (Teixeira *et al.*, 2003). Furthermore, a novel mechanism for PKC mediated activation of Erk-MAPKinase has recently been identified in mast cells where FcεRI driven phosphorylation of PKCβI and PKCα by Syk generates binding sites for Grb2, thus recruiting Grb2/Sos to the plasma membrane and contributing to the full activation of Ras (Kawakami *et al.*, 2003). All these studies

suggest that at least some of the key roles that PKCs play in a number of cellular processes may be due to the regulation of Erk-MAPKinase activity. As Erk-MAPKinase activity plays important roles in apoptotic and proliferative signalling via the BCR in immature B cells, we investigated the roles of key PKC isoforms that had previously been shown to be involved in B cell signalling in regulating these differential functions.

The conventional PKC, PKCα, has been proposed to play a positive role in proliferation and an anti-apoptotic role in a number of cell types. For instance, overexpression of a catalytically active PKCα in NIH-3T3 cells results in increased expression of cyclins D1 and E and a concomitant increase in proliferation (Soh and Weinstein, 2003). Consistent with this, expression of a catalytically active form of PKCα in WEHI-231 cells appears to protect cells from anti-Ig induced growth arrest and apoptosis at 24 hours (**Figure 4.2A** and **4.3**). However, at 48 hours, these cells do exhibit signs of growth arrest and apoptosis, suggesting that PKCα perhaps alters the kinetics of anti-Ig induced growth arrest and apoptosis, perhaps by slowing the onset of apoptosis (**Figure 4.2B** and **4.3**). It is also possible, however, that, if PKCα enhances proliferation then the cells may divide at a faster rate, resulting in the depletion of essential nutrients in the media, slowing growth and making cells more susceptible to apoptosis at later timepoints. Overexpression of PKCα-CAT also led to an enhancement of anti-CD40 mediated rescue from anti-Ig induced growth arrest (**Figure 4.4**). Interestingly, previous, unpublished studies in this laboratory using anti-sense oligonucleotides generated results which corroborate these, as anti-sense PKCα oligonucleotides abrogated anti-CD40 mediated rescue of anti-Ig induced growth arrest in WEHI-231 cells. However, expression of a kinase dead PKCα mutant does not lead to an enhancement of anti-Ig induced growth arrest or apoptosis, nor a defect in CD40-mediated rescue. Indeed, the kinase dead PKCα mutant had a very similar effect on the cells as the PKCα-CAT mutant, protecting cells from anti-Ig induced growth arrest and apoptosis. Taken together, these results suggest that PKCα may not play a significant role in the normal proliferation and

anti-CD40 mediated rescue from anti-Ig induced apoptosis in WEHI-231 cells, but that overexpression of a constitutively active form of PKCα can override anti-Ig induced signals which induce growth arrest, at least for 24 hours. Consistent with this, PKCα-CAT cells display sustained, cycling Erk-MAPKinase activity in the presence of anti-Ig, whilst PKCα-KR has no effect on Erk-MAPKinase activity (Catriona Ford, personal communication). PKCα-CAT expression also results in general up regulation of Bcl-X_L, even in the presence of anti-Ig, unlike cells containing the empty vector. Again, PKCα-KR had no effect (Catriona Ford, personal communication). These findings strongly suggest that PKCα-CAT plays both proliferative and pro-survival roles in WEHI-231 cells.

WEHI-231 cells expressing either PKCδ mutant do not exhibit anti-Ig induced growth arrest after 24 hours, while cells containing the empty vector do. After 48 hours cells expressing both PKCδ mutants do display growth arrest, as assessed by the [^{3}H]-thymidine incorporation assay. The propidium iodide data supports this, as after 24 hours there is still a high proportion of cells in S phase and G_2/M after anti-Ig stimulation, and no increase in the proportion of cells with a sub-diploid DNA content. Indeed, the proportion of cells with a sub-diploid DNA content is considerably less than that exhibited by cells containing the empty vector. The expression of the PKCδ-CAT mutant appeared to have no effect on CD40-mediated rescue from growth arrest or apoptosis, though PKCδ-KR cells appear to have an enhanced proliferative response to anti-Ig and anti-CD40. This is in agreement with studies in PKCδ$^{-/-}$ mice where B cells exhibit a greater proliferative response upon stimulation with anti-Ig and anti-CD40 (Miyamoto *et al.*, 2002). This suggests that PKCδ plays a negative role in proliferation of B cells, a hypothesis supported by the observation that PKCδ$^{-/-}$ mice have an increased number of splenic and lymph node B cells (Miyamoto *et al.*, 2002). This is also in accordance with the fact that levels of PKCδ expression decrease as fibroblasts progress through G_1 and enter S phase of the cell cycle, and increase above initial levels as cells re-enter G_1 (Srivastava *et al.*, 2002),

suggesting that PKCδ acts as an inhibitor of cell cycle progression (**Figure 4.18**). Interestingly, expression of PKCδ-CAT did not induce apoptosis in unstimulated cells, nor enhance anti-Ig induced apoptosis, yet it has previously been shown that expression of a catalytically active fragment of PKCδ induces apoptosis in a number of cell types, including human myeloid leukaemia cells (Emoto *et al.*, 1995). Interestingly, expression of PKCδ-CAT abrogates anti-Ig induced down regulation of Erk-MAPKinase activity, as anti-Ig stimulated cells display sustained, cycling Erk-MAPKinase activity, whilst PKCδ-KR has no effect on Erk-MAPKinase activity, as these cells have a very similar pattern to wild type cells under all stimulatory conditions (Catriona Ford, personal communication).

Neither WEHI-231 PKCε-CAT nor WEHI-231 PKCε-KR cells demonstrate anti-Ig induced growth arrest or apoptosis at 24 hours (**Figures 4.8A** and **4.9**). Interestingly, WEHI-231 PKCε-CAT cells actually display increased proliferation, as assessed by [^{3}H]-thymidine uptake, in response to anti-Ig, in a dose dependent manner. This is supported by the DNA content analysis, which shows that a high proportion of these cells are in S phase and G_2/M in response to anti-Ig at 24 hours. However, this protection form anti-Ig induced growth arrest and apoptosis is lost by 48 hours, as both cell types exhibit anti-Ig induced growth arrest and apoptosis after 48 hours stimulation (**Figures 4.8B** and **4.9**). Indeed, PKCε-CAT expressing cells exhibit high levels of apoptosis in response to anti-Ig at 48 and 72 hours, suggesting that the protective effects can be subverted by prolonged exposure to antigen. When PKCε is overexpressed in NIH-3T3 cells, these cells become more susceptible to radiation-induced cell death, 48 hours after exposure, as a result of increased Erk1/2 activation (Lee *et al.*, 2003). Perhaps in WEHI-231 cells PKCε-CAT is enhancing the extent of anti-Ig induced apoptosis, though slowing the kinetics. It would be interesting to assess the number of divisions PKCε-CAT expressing cells undergo in response to anti-Ig compared to cells containing the empty vector, as it might be expected that they have undergone more divisions as the [^{3}H]-thymidine incorporation is much greater at 24 hours. Taken together, these results suggest that PKCε might play

a role in anti-Ig mediated apoptosis in WEHI-231 cells, though the kinetics are slowed compared to wild type cells (**Figure 4.18**). Interestingly, despite the fact that PKCε has been implicated as a direct activator of Raf in fibroblasts (Cai *et al.*, 1997), expression of PKCε-CAT or PKCε-KR did not lead to an overall increase in Erk-MAPKinase activity in WEHI-231 cells, and only led to a very slight increase in activity of Erk-MAPKinase in anti-Ig treated cells (Catriona Ford, personal communication).

Overexpression of PKCζ-CAT or PKCζ-KR has a slight effect on anti-Ig induced growth arrest of WEHI-231 cells, as cells expressing these constructs do not display the same level of anti-Ig induced growth arrest at 24 hours as cells containing the empty vector. By 48 hours, however, cells expressing these constructs behave in a very similar manner to those containing the empty vector. This is slightly surprising, as PKCζ-deficient mice have a higher percentage of immature B cells, suggesting that PKCζ plays a role in negative selection of immature B cells (Leitges *et al.*, 2001). This implies that PKCζ positively regulates apoptosis in immature B cells, and consistent with this PKCζ has recently been suggested to play a role in negatively regulating the pro-survival kinase Akt (Doornbos *et al.*, 1999). Indeed, B cells from PKCζ deficient mice display accelerated apoptosis in culture, strongly suggesting that PKCζ plays an important role in the survival of B cells (Martin *et al.*, 2002). Interestingly, this is a similar phenotype to that observed in RelA and c-Rel deficient mice (Grossmann *et al.*, 2000), and PKCζ deficient mice also display impaired activation of transcription of NF-κB-dependent genes, though the nuclear translocation of NF-κB is not inhibited, placing PKCζ upstream of NF-κB in B cells (Martin *et al.*, 2002). It would be interesting to assess the ability of WEHI-231 cells expressing PKCζ mutants to activate NF-κB-mediated transcription, as anti-Ig induced apoptosis involves down-regulation of NF-κB activity, whilst one mechanism by which CD40 mediates survival is through the induction of NF-κB activity (Schauer *et al.*, 1996). Both PKCζ isoforms inhibit anti-Ig induced down regulation of Erk-

MAPKinase activity as sustained, cycling Erk-MAPKinase phosphorylation is exhibited in these cells (Catriona Ford, personal communication).

The analysis of expression of key cell cycle regulators to determine the mechanisms underlying the effects PKC isoforms have on WEHI-231 cells did not always produce results apparently consistent with the biological data, given our current understanding. For example, most cell types exhibit a substantial increase in p27 expression in response to anti-Ig at 24 hours, yet do not exhibit any growth arrest. The most interesting mutant with respect to p27 expression is the PKCδ-CAT, as cells expressing this construct do not exhibit an increase in p27 levels in response to anti-Ig, yet still display anti-Ig induced growth arrest after 48 hours. Similarly, in all cells in which cyclin D2 was detected (PKCα CAT and KR, and PKCε CAT and KR), anti-Ig induces a down regulation of cyclin D2, yet there was proliferation in all of these cells at 24 hours. This is somewhat surprising, as overexpression of PKCδ has been shown to lead to a decrease in cyclin D1 expression in bovine smooth muscle cells, whilst over expression of a dominant negative PKCδ results in an increase in cyclin D1 expression (Page *et al.*, 2002). Cyclins D2 and D3 have been detected in primary murine B cells, but not cyclin D1 (Solvason *et al.*, 1996), yet no cyclin D expression was detected in cells expressing either PKCδ mutant. The antibody used was specific for cyclins D1 and D2, so perhaps in immature B cells cyclin D3 plays an important role in the regulation of proliferation. This would be consistent with the observation that B cell development is not impaired in cyclin $D2^{-/-}$ mice (Solvason *et al.*, 2000).

One puzzling aspect to emerge from this study was the similarity in biological responses between the CAT and KR mutants, one supposedly catalytically active, the other dominant negative. One possible explanation comes from the structure of these mutants in relation to the structure of native PKCs (**Figure 4.1**). The regulatory domains of PKCs are responsible for the binding of receptors for activated C kinases (RACKs), a family of anchoring proteins that determine the ultimate sub-cellular locations of PKCs after activation (Schechtman and Mochly-

Rosen, 2001). The CAT mutants used in this study lack their regulatory domains, therefore it is possible that they are not targeted to their correct sub-cellular locations. This could result in lack of phosphorylation of the appropriate targets, or even phosphorylation of inappropriate targets, as the constructs are not able to interact with the correct substrates, or are not targeted to the same area within the cell as their intended substrates. If the appropriate substrates are not phosphorylated, this could easily explain the absence of an effect. This highlights disadvantages of using overexpression methods and mutant constructs to investigate the roles of signalling molecules, as sometimes the regulation of pathways may be circumvented. To compliment these studies the use of inhibitors specific for individual PKC isoforms could be utilised, though this also raises issues of specificity of inhibitors.

Another possibility is that the PKC signalling events taking place here are independent of kinase activity. This is not without precedent, for example, in IFNγ-primed U937 cells, PKCα assists the activation of PLD1 following ligation of FcγRI by directly binding to PLD1 in a PKCα kinase-independent manner. Indeed, inhibition of PKCα kinase activity does not prevent FcγRI-mediated induction of PLD1 whereas downregulation of PKCα levels does impair PLD1 activation. FcγRI-dependent induction of PLD1 thus requires the recruitment of PKCα but PKCα kinase activity is not necessary (Melendez *et al.*, 2001), strongly suggesting that PKCα can perform important signalling functions independently of its kinase activity, possibly through protein-protein interactions via the regulatory domain. However, this is unlikely to be the case for all isoforms studied, indeed, PKCα-CAT and PKCα-KR did not have the same impact on either Erk-MAPKinase or Bcl-X_L expression in response to anti-Ig. PKCα-CAT suppressed anti-Ig mediated down-regulation of Erk-MAPKinase activity and induced a general increase in Bcl-X_L expression, yet PKCα-KR had no effect on either (Catriona Ford, personal communication). To further explore the requirement for the kinase and regulatory domains of PKCs, it would be interesting to transfect WEHI-231 cells with PKC-CAT-KR constructs, which

encode the catalytic domain of PKC only, but with a point mutation that abolishes kinase activity, and with full length constitutively active constructs, to compare the effects they have on WEHI-231 cell responses with the effects of the catalytic fragment only.

Another puzzling aspect is the differential responses to anti-Ig observed at 24 and 48 hours, particularly in the [^{3}H]-thymidine incorporation assay. Thus, some cell types exhibit no growth arrest in response to anti-Ig at 24 hours, indeed those expressing PKCε-CAT display enhanced proliferation in response to anti-Ig at this time point, yet at 48 hours they exhibit a level of growth arrest at least equal to that of the cells containing the empty vector. The reasons for this are unclear at present, but merit further investigation. Perhaps the protection afforded by these mutants can be overcome by prolonged signalling via the BCR, resulting in delayed onset of growth arrest and apoptosis. It may also be that prolonged activity of these enzymes results in the induction of negative regulators of PKC activity, enabling the constitutively active mutants to be switched off. It would be interesting to assess [^{3}H]-thymidine incorporation at time points between 24 and 48 hours, both in cells containing the empty vector and cells expressing PKC constructs, to further gauge the kinetics of growth arrest. It should be noted, however, that anti-Ig induced growth arrest of WEHI-231 cells containing the empty vector is not as pronounced at 24 as 48 hours, and it has been reported that the negative signals generated following BCR-ligation in immature B lymphocytes progress in an ordered kinetic manner (Monroe, 2000). Perhaps the cells containing the PKC constructs take longer to pass through the cell cycle, therefore their kinetics of response to anti-Ig are altered accordingly. It might be interesting to determine the number of divisions cells undergo in 48 hours, utilising a dye like CFSE, which binds to cellular proteins and is then equally divided between daughter cells, allowing divisions to be counted in terms of the halving of fluorescence.

Overall, the results presented in this chapter demonstrate that overexpression of different PKC family members in WEHI-231 alter the response of WEHI-231 cells to stimulation via the BCR. All isoforms studied provided protection from anti-Ig induced growth arrest and apoptosis after 24 hours stimulation, most noticeably PKCε, as WEHI-231 PKCε-CAT cells actually demonstrated a dose dependent increase in proliferation in response to anti-Ig, a response more suited to a mature B cell, not immature transitional B cells. However, by 48 hours, WEHI-231 cells expressing all PKC constructs examined appear to exhibit growth arrest. Examination of key cell cycle regulatory proteins failed to reveal a clear molecular basis for the alterations in response to anti-Ig.

Figure 4.1 Structure of constitutively active and dominant-negative mutants of specific isoforms of protein kinase C

Schematic diagrams of structures of PKC mutants. PKC-CAT constructs encode a truncated protein in which the catalytic domain is expressed but the entire regulatory domain has been deleted. PKC-KR constructs encode a full-length PKC with a point mutation that abolishes ATP binding ability.

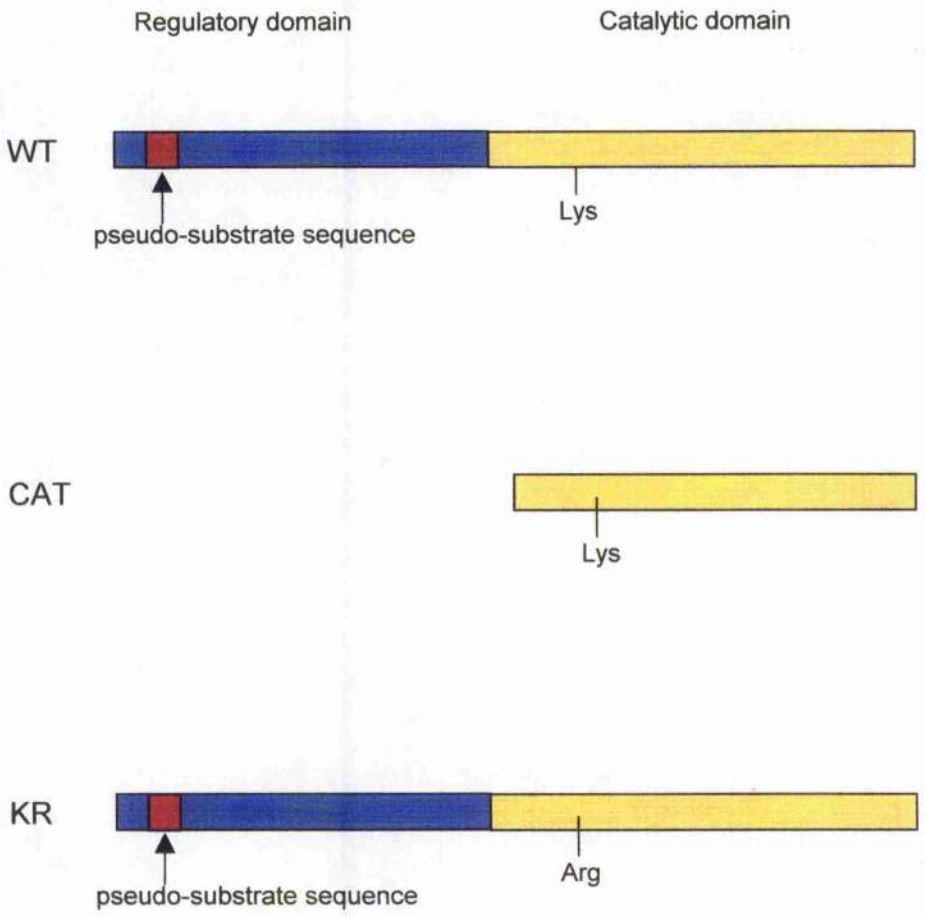
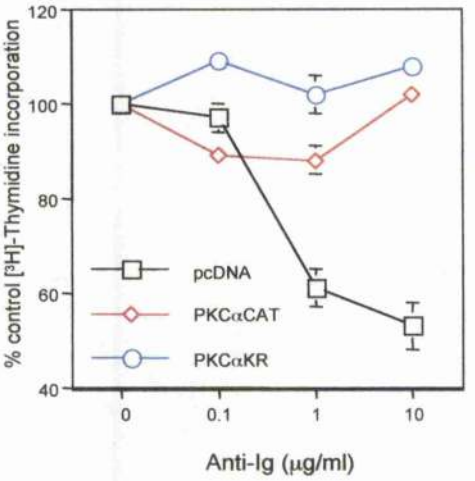

Regulatory domain
Catalytic domain
WT
pseudo-substrate sequence
Lys
CAT
Lys
KR
pseudo-substrate sequence
Arg

Figure 4.2 Effects of expression of PKCα mutants on anti-Ig induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCα-CAT, or pcDNA3.1 PKCα-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 24 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCα-CAT, or pcDNA3.1 PKCα-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

A


120
100
80
60
40
% control [3H]-Thymidine incorporation
pcDNA
PKCαCAT
PKCαKR
0
0.1
1
10
Anti-Ig (μg/ml)

B

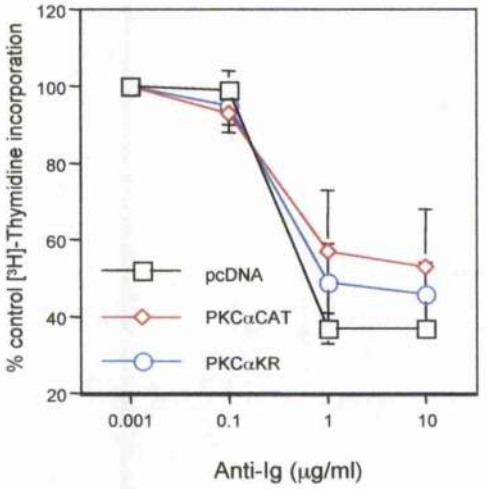

120
100
80
60
40
20
% control [3H]-Thymidine incorporation
pcDNA
PKCαCAT
PKCαKR
0.001
0.1
1
10
Anti-Ig (μg/ml)

Figure 4.3 Effects of expression of PKCα mutants on anti-Ig induced apoptosis in WEHI-231 cells

WEHI-231 cells (5 x 10^5 cells/ml) containing the pcDNA3.1 vector, pcDNA3.1 PKCα-CAT, or pcDNA3.1 PKCα-KR were cultured in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 24, 48 and 72 hours, as described in Materials and Methods. Data are representative of three independent experiments.

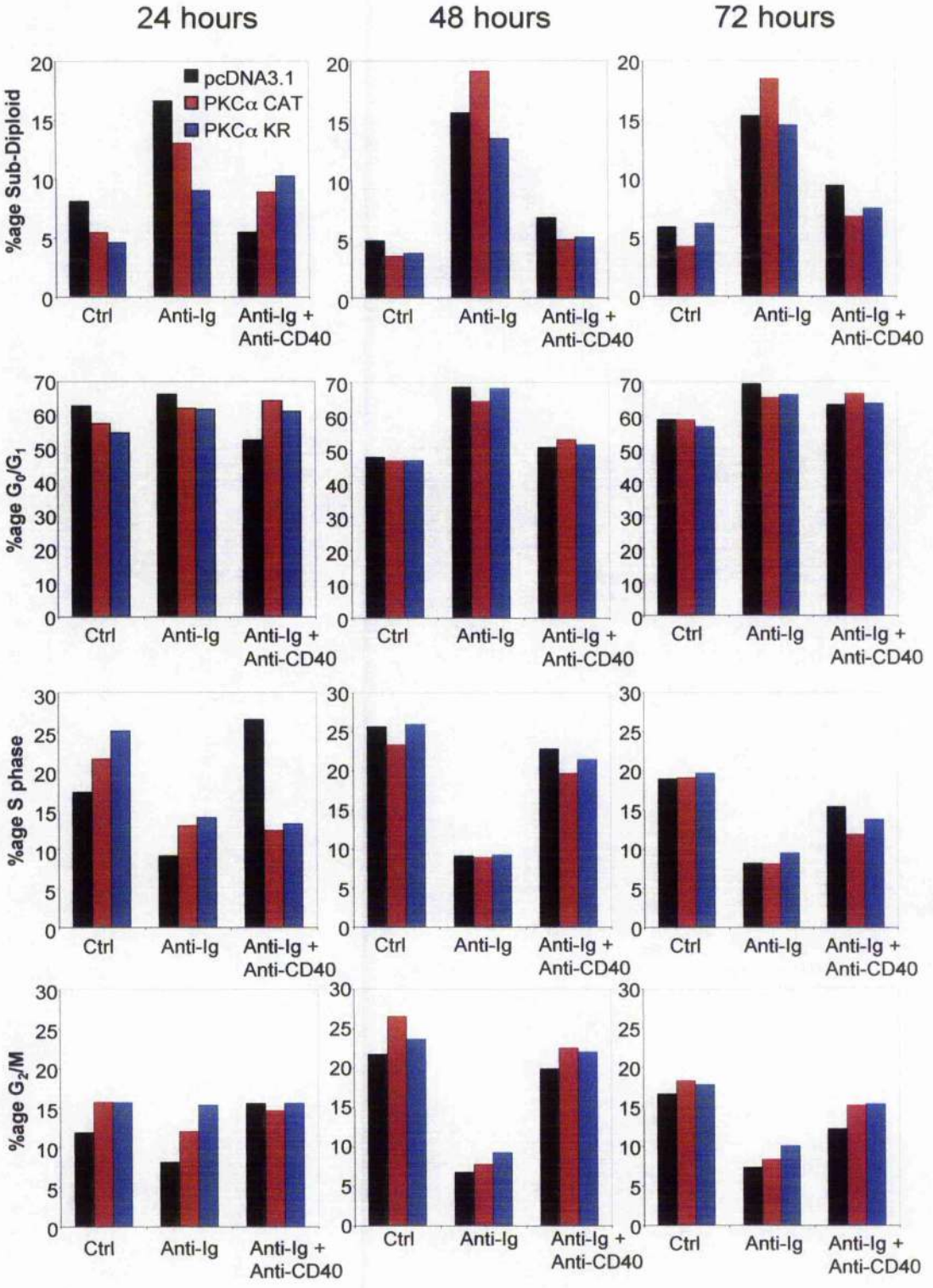

24 hours
48 hours
72 hours
pcDNA3.1
PKCα CAT
PKCα KR
%age Sub-Diploid
%age G0/G1
%age S phase
%age G2/M
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40

Figure 4.4 Effects of expression of PKCα mutants on CD40-mediated rescue of anti-Ig induced growth arrest in WEHI-231 cells

WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCα-CAT, or pcDNA3.1 PKCα-KR were cultured, in triplicate, in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

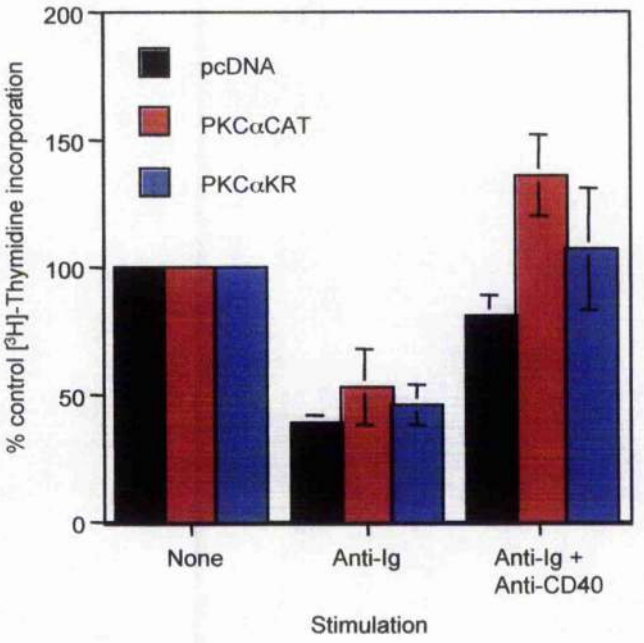
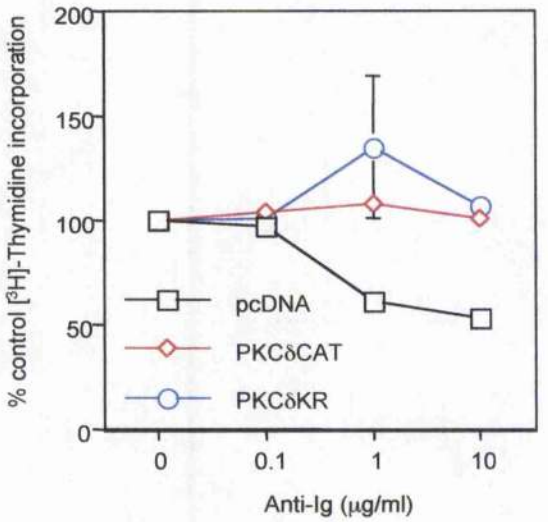

200
150
100
50
0
% control [³H]-Thymidine incorporation
pcDNA
PKCαCAT
PKCαKR
None
Anti-Ig
Anti-Ig +
Anti-CD40
Stimulation

Figure 4.5 Effects of expression of PKCδ mutants on anti-Ig induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCδ-CAT, or pcDNA3.1 PKCδ-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 24 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCδ-CAT, or pcDNA3.1 PKCδ-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

A


200
150
100
50
0
% control [³H]-Thymidine incorporation
pcDNA
PKCδCAT
PKCδKR
0
0.1
1
10
Anti-Ig (μg/ml)

B

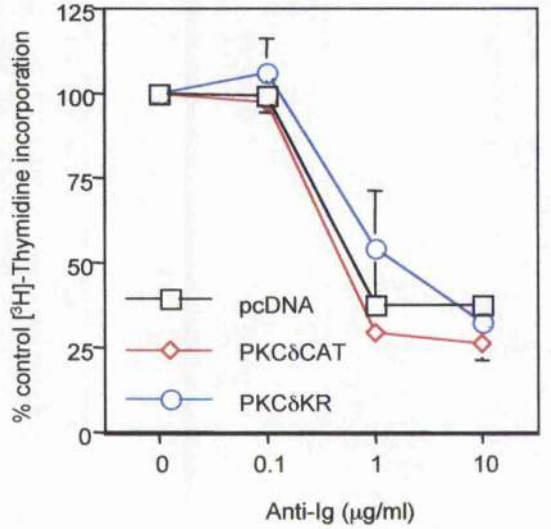

125
100
75
50
25
0
% control [³H]-Thymidine incorporation
pcDNA
PKCδCAT
PKCδKR
0
0.1
1
10
Anti-Ig (μg/ml)

Figure 4.6 Effects of expression of PKCδ mutants on anti-Ig induced apoptosis in WEHI-231 cells

WEHI-231 cells (5×10^5 cells/ml) containing pcDNA3.1 PKCδ-CAT, pcDNA3.1 PKCδ-KR, or the pcDNA3.1 vector were cultured in the presence of anti-Ig (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 24, 48 and 72 hours, as described in Materials and Methods. Data are representative of three independent experiments.

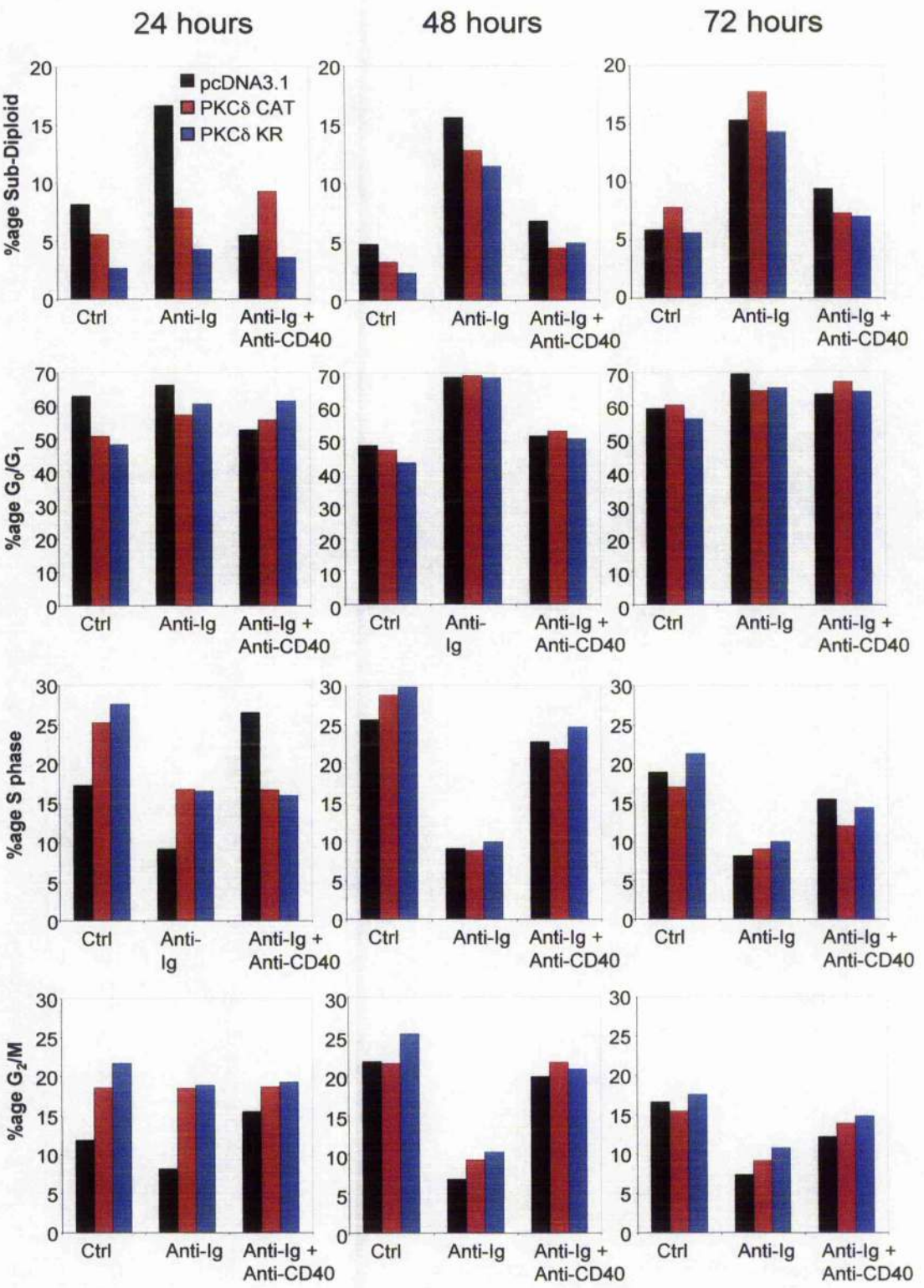

24 hours
48 hours
72 hours
pcDNA3.1
PKCδ CAT
PKCδ KR
%age Sub-Diploid
%age G0/G1
%age S phase
%age G2/M
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40

Figure 4.7 Effects of expression of PKCδ mutants on CD40-mediated rescue of anti-Ig induced growth arrest in WEHI-231 cells

WEHI-231 cells (1×10^4 cells/well) containing pcDNA3.1 PKCδ-CAT, pcDNA3.1 PKCδ-KR, or the pcDNA3.1 vector were cultured, in triplicate, in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

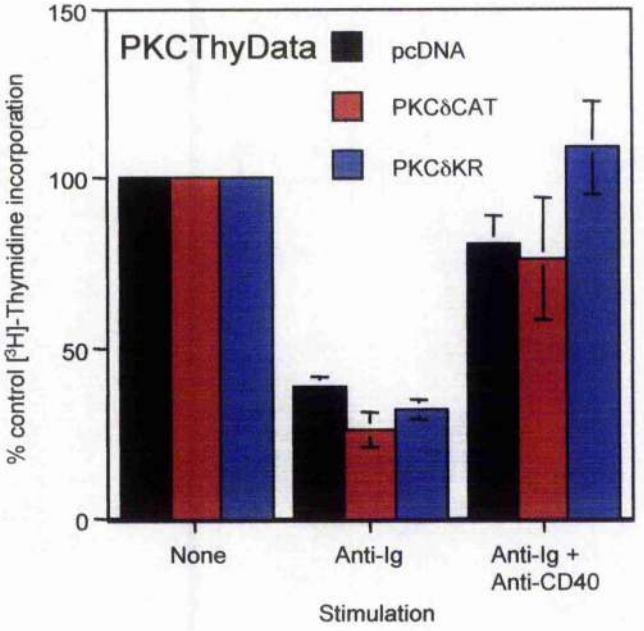
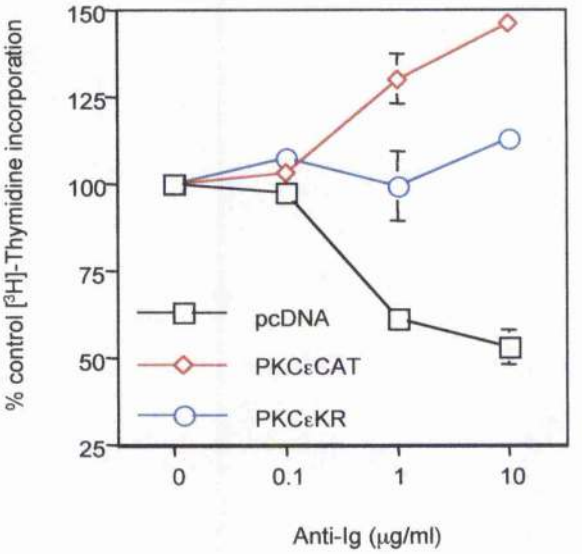

PKCThyData
pcDNA
PKCδCAT
PKCδKR
150
100
50
0
% control [3H]-Thymidine incorporation
None
Anti-Ig
Anti-Ig +
Anti-CD40
Stimulation

Figure 4.8 Effects of expression of PKCε mutants on anti-Ig induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCε-CAT, or pcDNA3.1 PKCε-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 24 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCε-CAT, or pcDNA3.1 PKCε-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

A


150
125
100
75
50
25
% control [3H]-Thymidine incorporation
pcDNA
PKCεCAT
PKCεKR
0
0.1
1
10
Anti-Ig (μg/ml)

B

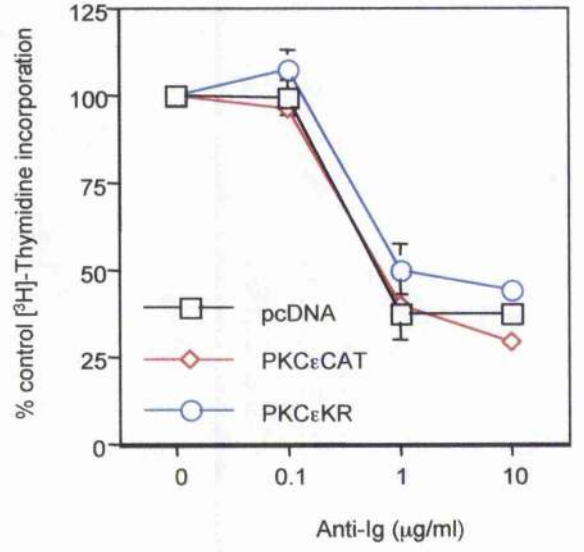

125
100
75
50
25
0
% control [3H]-Thymidine incorporation
pcDNA
PKCεCAT
PKCεKR
0
0.1
1
10
Anti-Ig (μg/ml)

Figure 4.9 Effects of expression of PKCε mutants on anti-Ig induced apoptosis in WEHI-231 cells

WEHI-231 cells (5×10^5 cells/ml) containing pcDNA3.1 PKCε-CAT, pcDNA3.1 PKCε-KR, or the pcDNA3.1 vector were cultured in the presence of anti-Ig (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 24, 48 and 72 hours, as described in Materials and Methods. Data are representative of three independent experiments.

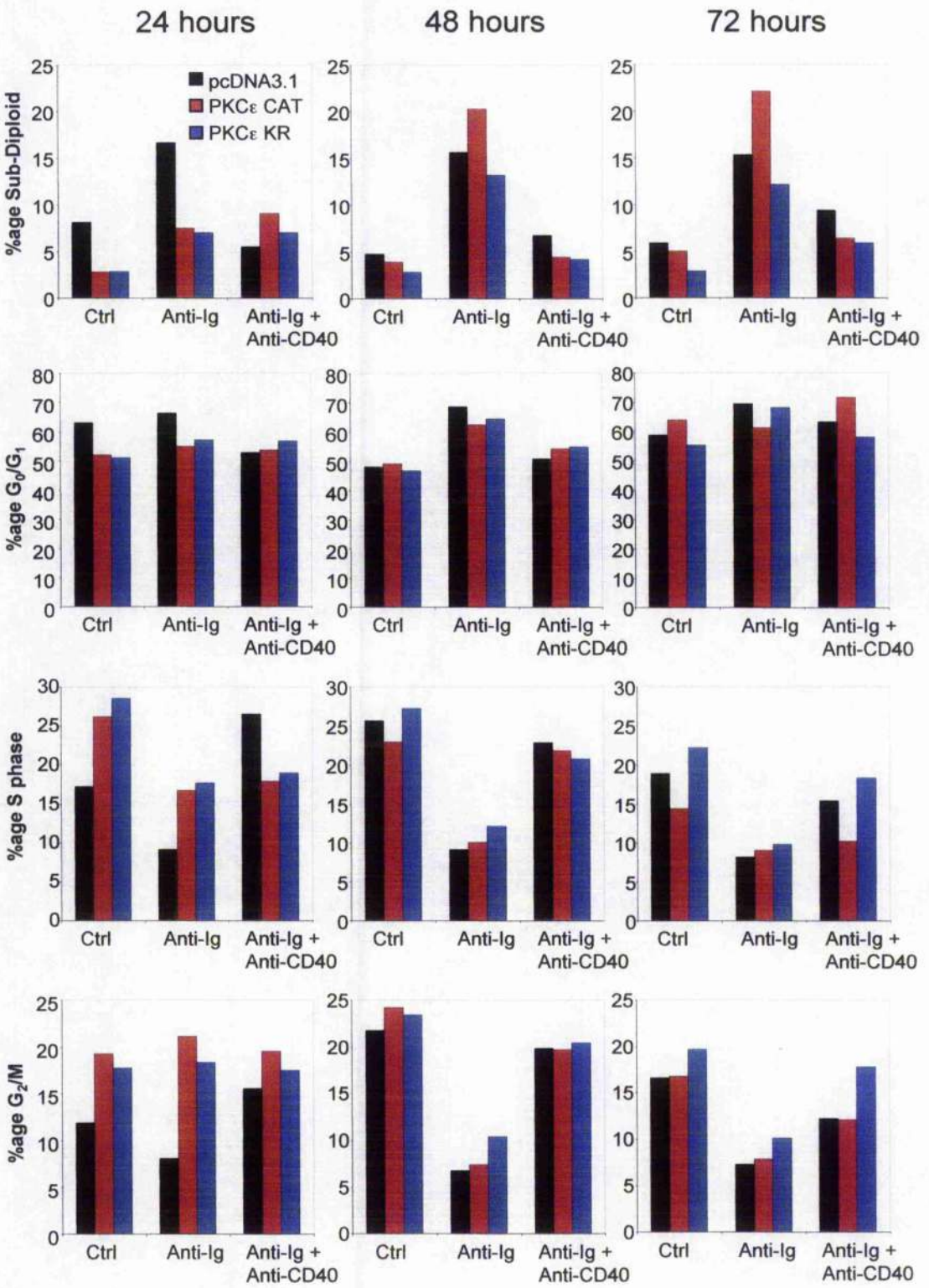

24 hours
48 hours
72 hours
pcDNA3.1
PKCε CAT
PKCε KR
%age Sub-Diploid
%age G0/G1
%age S phase
%age G2/M
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40

Figure 4.10 Effects of expression of PKCε mutants on CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis in WEHI-231 cells

WEHI-231 cells (1×10^4 cells/well) containing pcDNA3.1 PKCε-CAT, pcDNA3.1 PKCε-KR, or the pcDNA3.1 vector were cultured, in triplicate, in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

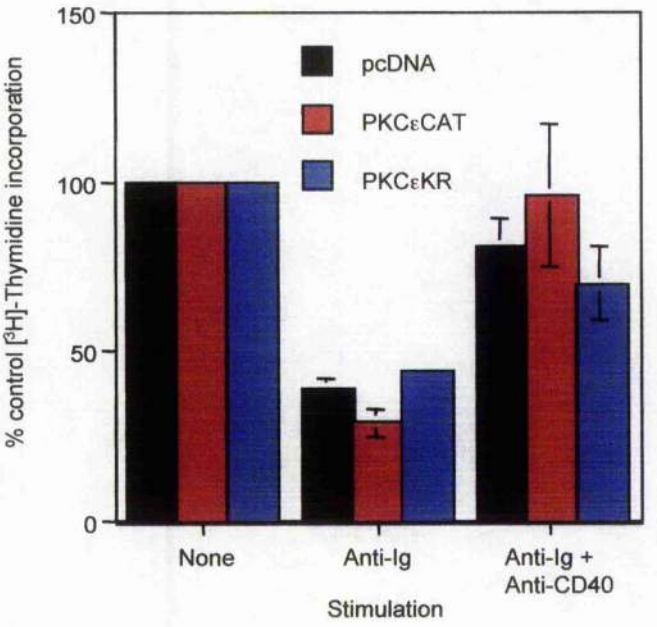
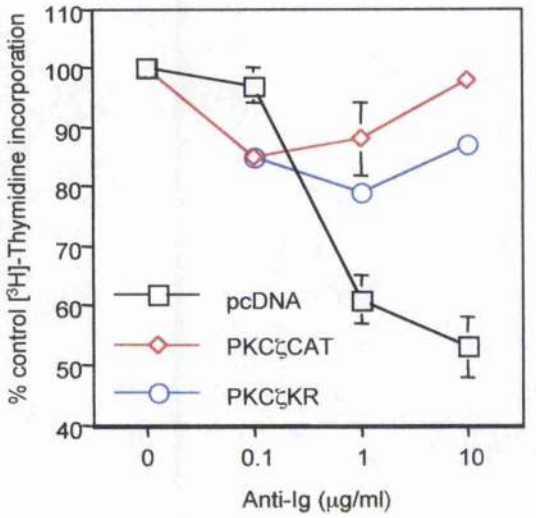

150
100
50
0
% control [³H]-Thymidine incorporation
pcDNA
PKCεCAT
PKCεKR
None
Anti-Ig
Anti-Ig +
Anti-CD40
Stimulation

Figure 4.11 Effects of expression of PKCζ mutants on anti-Ig induced growth arrest in WEHI-231 cells

A WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCζ-CAT, or pcDNA3.1 PKCζ-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 24 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

B WEHI-231 cells (1 x 10^4 cells/well) containing the pcDNA3.1 vector, pcDNA3.1 PKCζ-CAT, or pcDNA3.1 PKCζ-KR were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig antibodies (0.1, 1, 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from 3 independent experiments were then pooled and expressed as means ± sem.

A


% control [3H]-Thymidine incorporation
110
100
90
80
70
60
50
40
0
0.1
1
10
Anti-Ig (μg/ml)
pcDNA
PKCζCAT
PKCζKR

B

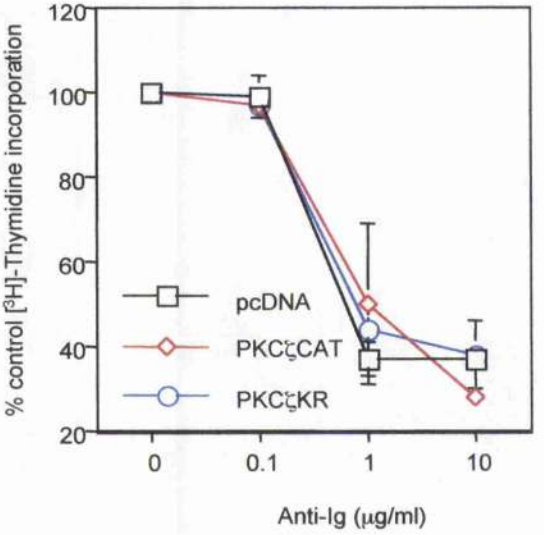

% control [3H]-Thymidine incorporation
120
100
80
60
40
20
0
0.1
1
10
Anti-Ig (μg/ml)
pcDNA
PKCζCAT
PKCζKR

Figure 4.12 Effects of expression of PKCζ mutants on anti-Ig induced apoptosis in WEHI-231 cells

WEHI-231 cells (5 x 10^5 cells/ml) containing pcDNA3.1 PKCζ-CAT, pcDNA3.1 PKCζ-KR, or the pcDNA3.1 vector were cultured in the presence of anti-Ig (10 μg/ml). Cells cultured in the presence of medium alone were included as a control. Levels of apoptosis and the proportion of cells in each cell cycle phase were determined by propidium iodide staining followed by FACS analysis to assess DNA content after 24, 48 and 72 hours, as described in Materials and Methods. Data are representative of three independent experiments.

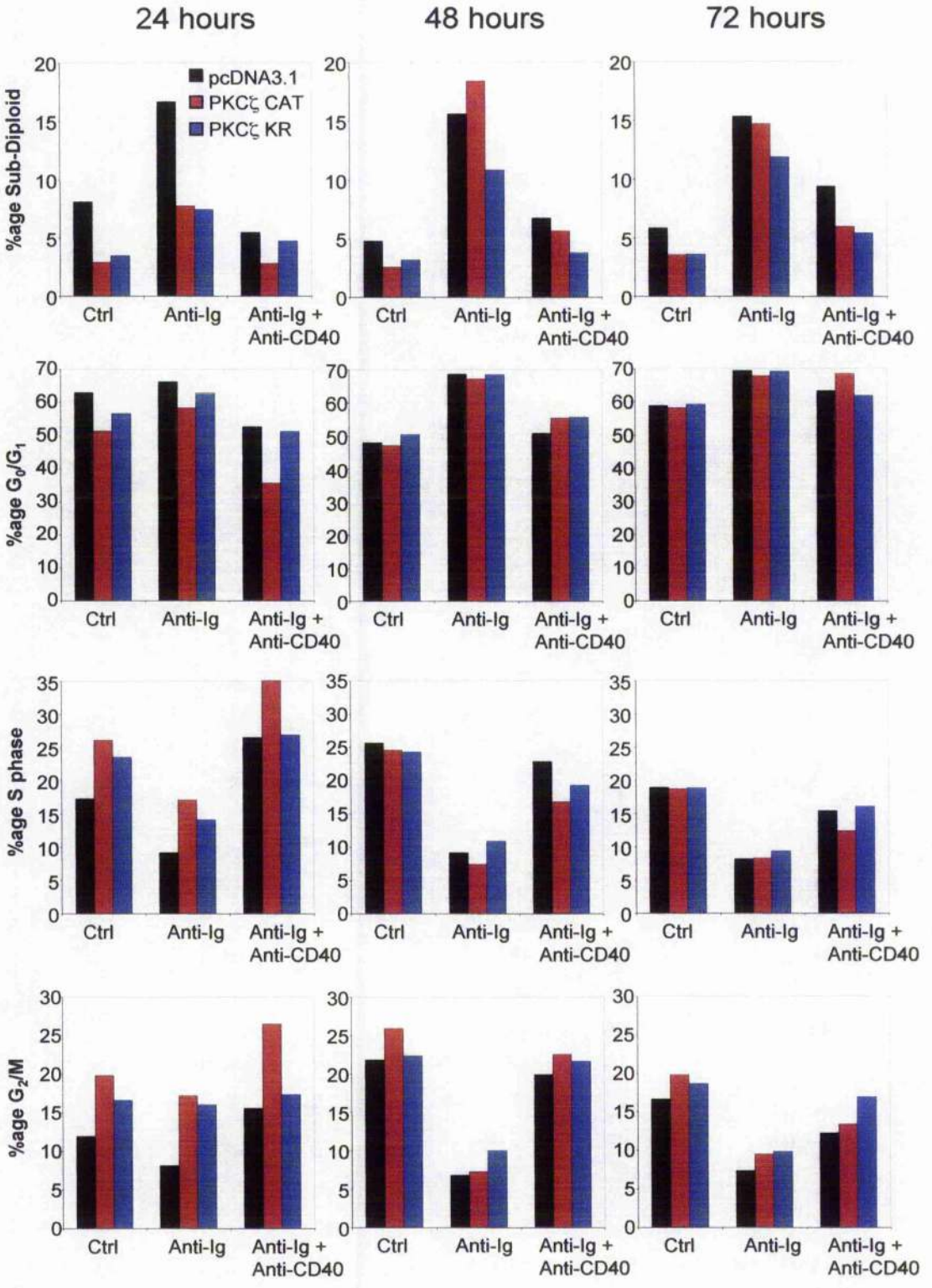

24 hours
48 hours
72 hours
pcDNA3.1
PKCζ CAT
PKCζ KR
%age Sub-Diploid
%age G0/G1
%age S phase
%age G2/M
Ctrl
Anti-Ig
Anti-Ig + Anti-CD40

Figure 4.13 Effects of expression of PKCζ mutants on CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis in WEHI-231 cells

WEHI-231 cells (1 x 10^4 cells/well) containing pcDNA3.1 PKCζ-CAT, pcDNA3.1 PKCζ-KR, or the pcDNA3.1 vector were cultured, in triplicate, in the presence of anti-Ig (10 μg/ml) or a combination of anti-Ig and anti-CD40 (both 10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H] -Thymidine (0.5 μCi/well) for 4 hours prior to harvesting at 48 hours and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of treated cells as a percentage of those obtained with control cell cultures. The normalised values from 4 independent experiments were then pooled and expressed as means ± sem.

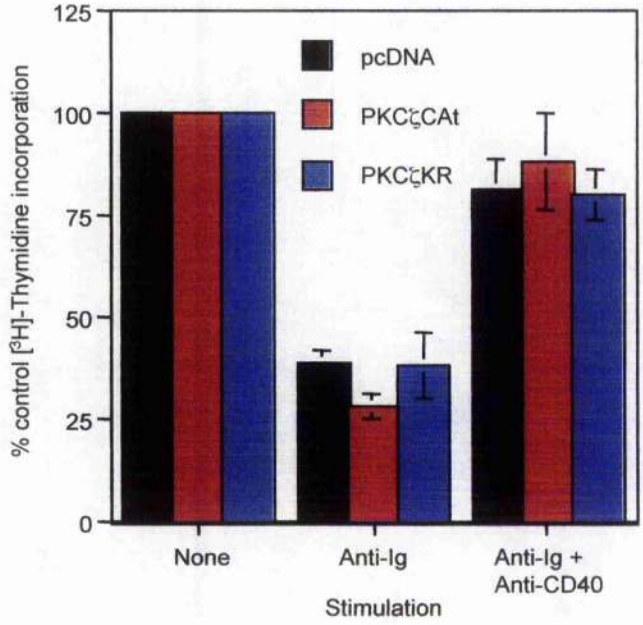

125
100
75
50
25
0
% control [3H]-Thymidine incorporation
pcDNA
PKCζCAt
PKCζKR
None
Anti-Ig
Anti-Ig +
Anti-CD40
Stimulation

Figure 4.14 Effects of the expression of PKC mutants on the expression of $p27^{Kip1}$ in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) containing the individual PKC constructs or the empty pcDNA3.1 vector were stimulated in the presence of anti-Ig (10 μg/ml) or anti-CD40 (10 μg/ml), alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-$p27^{Kip1}$ antibody.

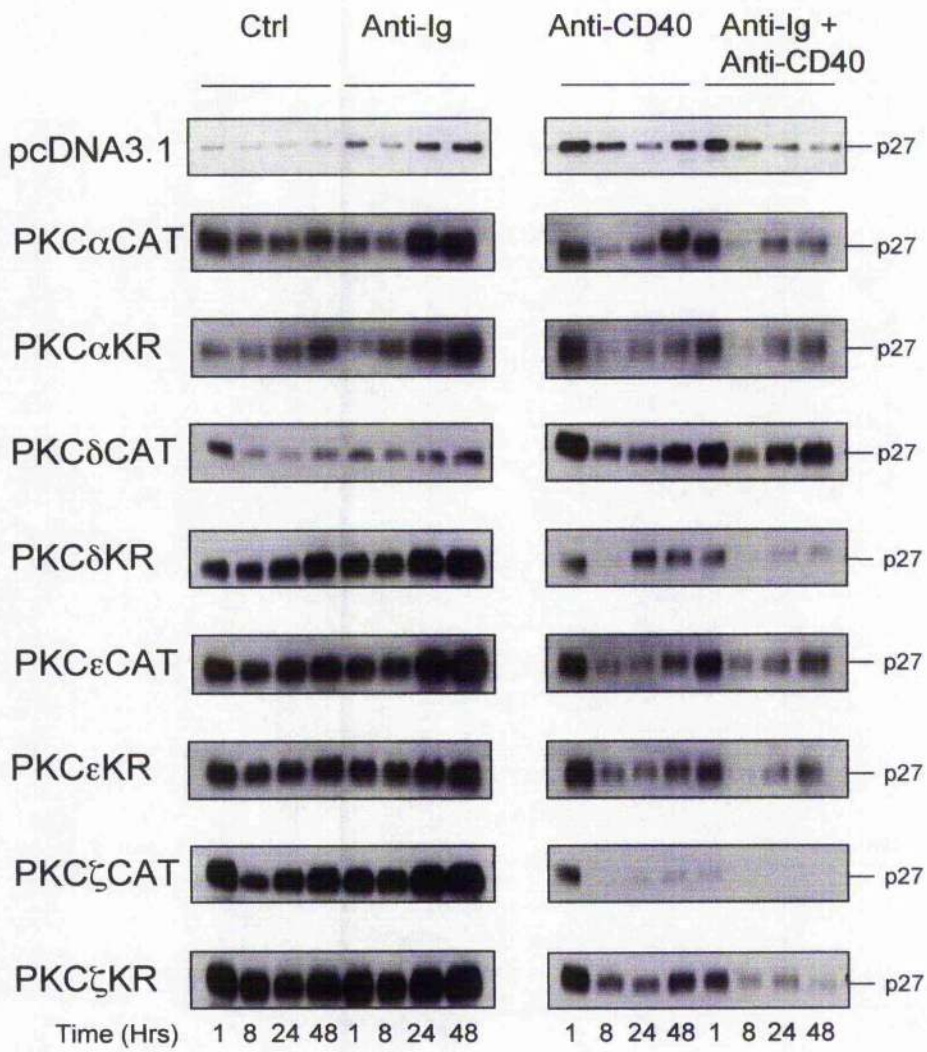

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
PKCαCAT
PKCαKR
PKCδCAT
PKCδKR
PKCεCAT
PKCεKR
PKCζCAT
PKCζKR
p27
Time (Hrs) 1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 4.15 Effects of the expression of PKC mutants on the expression of D-type cyclins in WEHI-231 cells

WEHI-231 cells (1 x 10^7 cells) containing the individual PKC constructs or the empty pcDNA3.1 vector were stimulated in the presence of anti-Ig (10 μg/ml) or anti-CD40 (10 μg/ml), alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-cyclin D1/2 antibody.

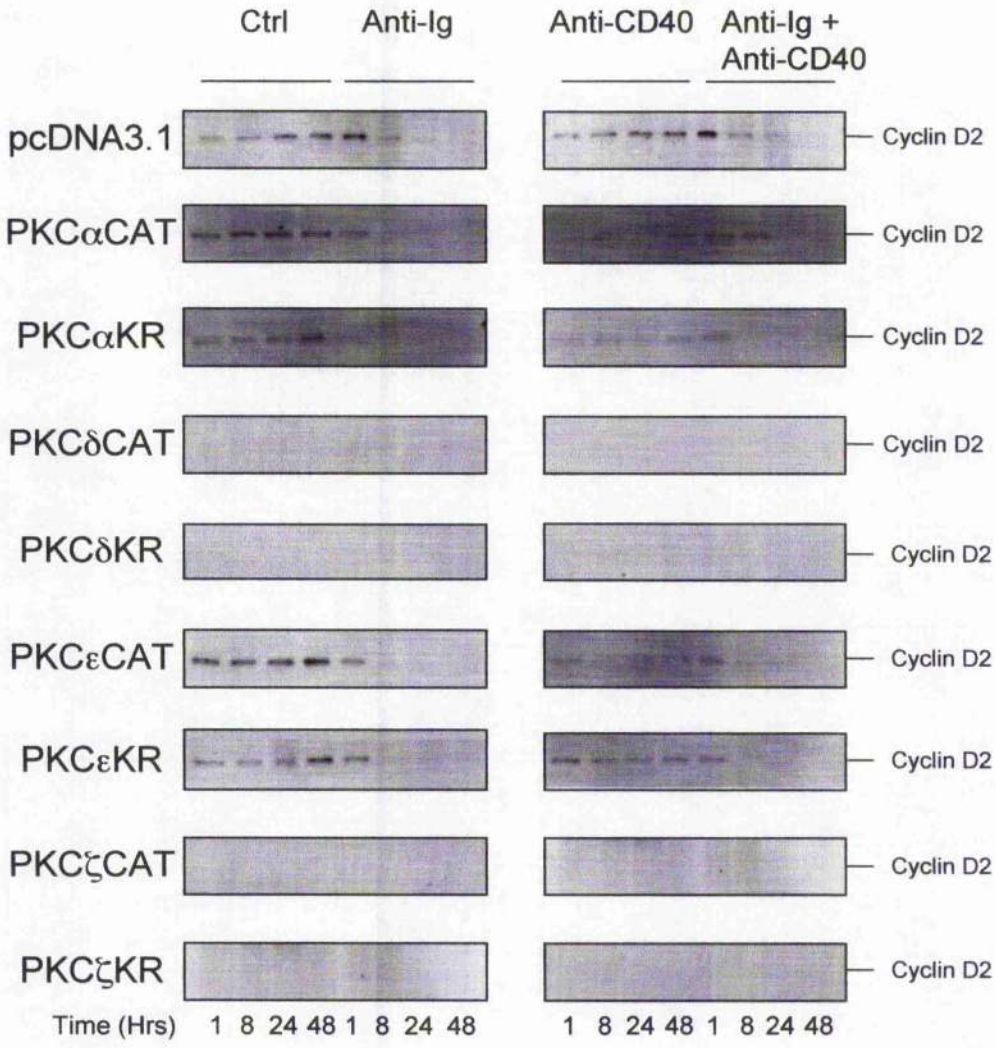

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
PKCαCAT
PKCαKR
PKCδCAT
PKCδKR
PKCεCAT
PKCεKR
PKCζCAT
PKCζKR
Cyclin D2
Time (Hrs)
1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 4.16 Effects of the expression of PKC mutants on the regulation of the retinoblastoma protein, Rb, in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) containing the individual PKC constructs or the empty pcDNA3.1 vector were stimulated in the presence of anti-Ig (10 μg/ml) or anti-CD40 (10 μg/ml), alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-phospho-Rb ($Ser^{807/811}$) antibody.

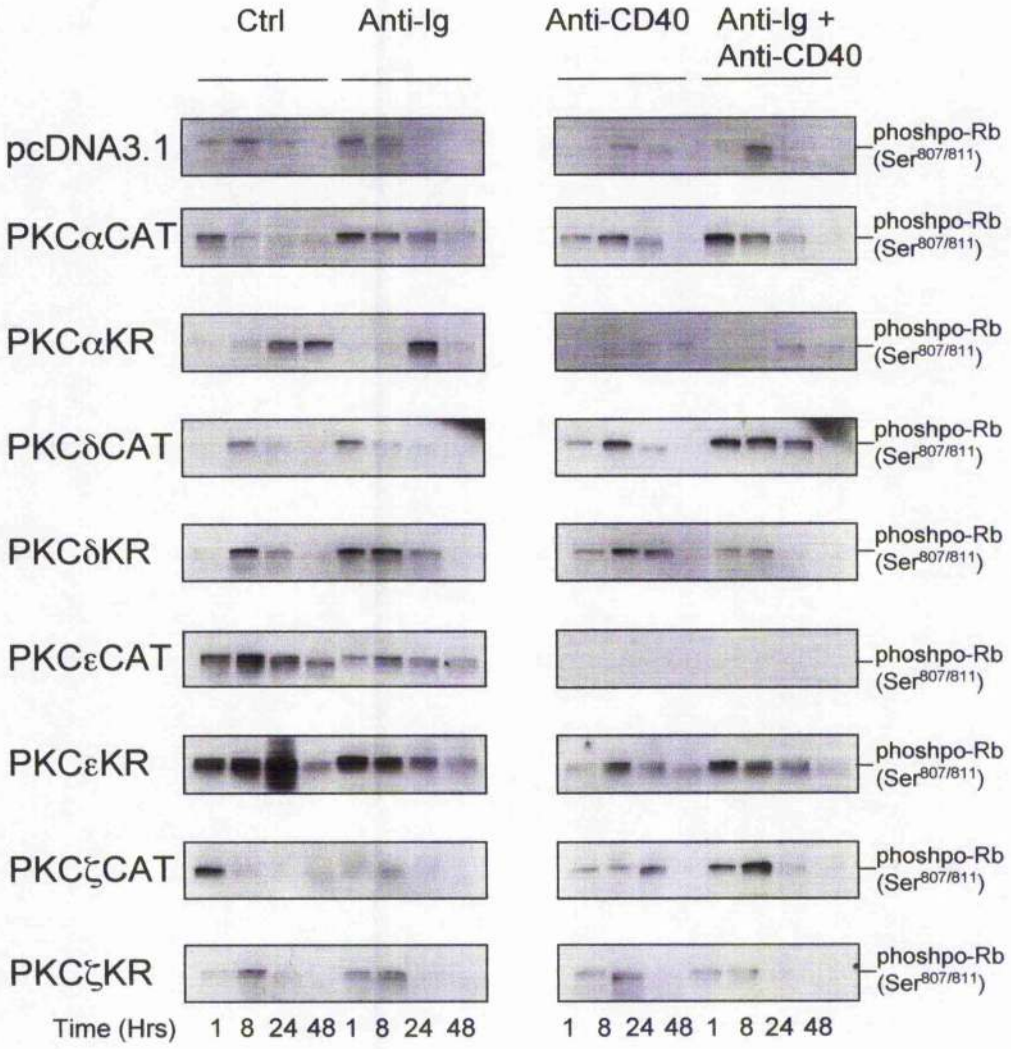

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig + Anti-CD40
pcDNA3.1
PKCαCAT
PKCαKR
PKCδCAT
PKCδKR
PKCεCAT
PKCεKR
PKCζCAT
PKCζKR
phoshpo-Rb (Ser807/811)
Time (Hrs) 1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 4.17 Effects of the expression of PKC mutants on the regulation of the cdc2 protein in WEHI-231 cells

WEHI-231 cells (1×10^7 cells) containing the individual PKC constructs or the empty pcDNA3.1 vector were stimulated in the presence of anti-Ig (10 μg/ml) or anti-CD40 (10 μg/ml), alone and in combination, for the times indicated. Cells incubated in the presence of medium alone were included as a control. Whole cell lysates were prepared and subjected to Western blot analysis with anti-phospho-cdc2 (Tyr^{15}) antibody.

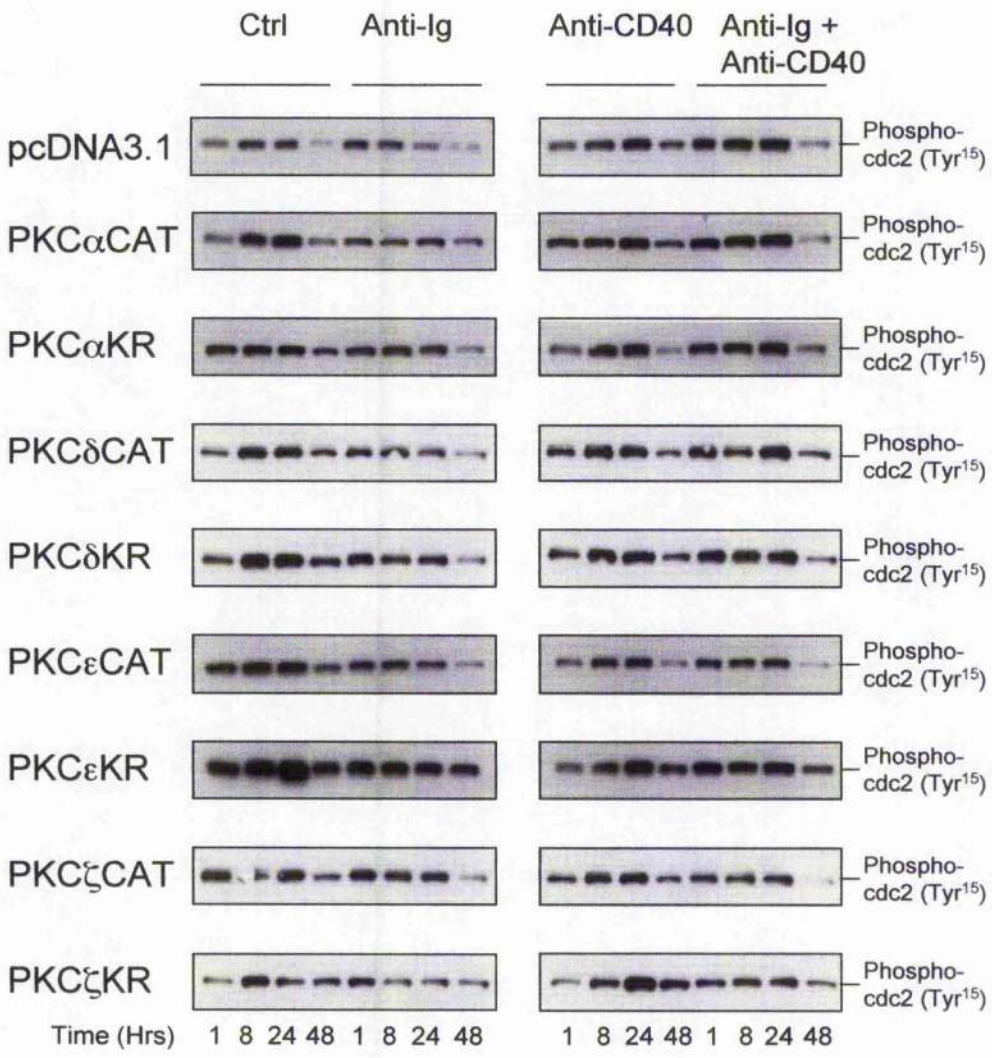
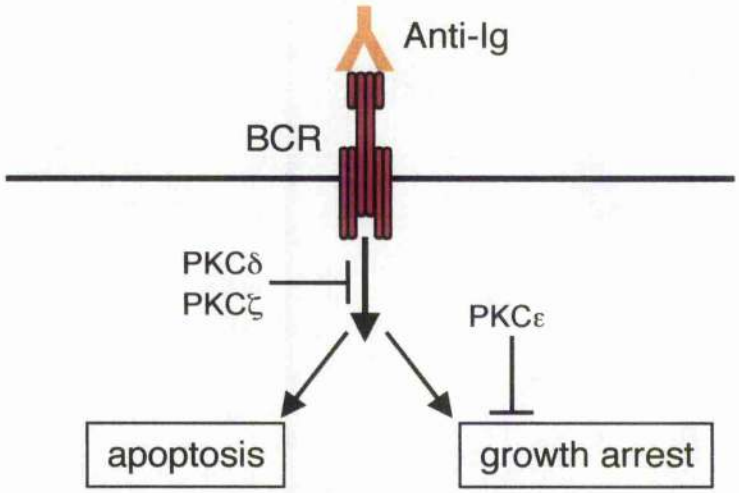
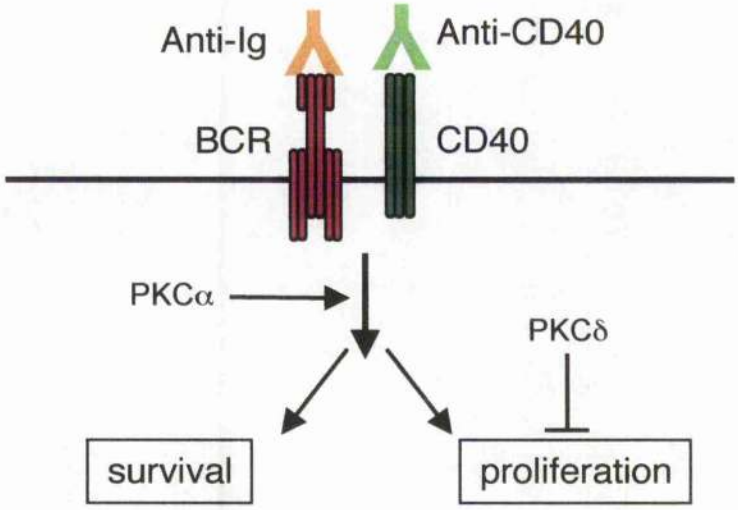

Ctrl
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pcDNA3.1
PKCαCAT
PKCαKR
PKCδCAT
PKCδKR
PKCεCAT
PKCεKR
PKCζCAT
PKCζKR
Phospho-
cdc2 (Tyr15)
Time (Hrs)
1 8 24 48 1 8 24 48
1 8 24 48 1 8 24 48

Figure 4.18 Role of PKC family members in anti-Ig induced growth arrest and apoptosis, and CD40-mediated rescue, in WEHI-231 cells

The novel PKC, PKCε, abrogates anti-Ig induced growth arrest in WEHI-231 cells. PKCα seems to play a role in promoting CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis, whilst the novel PKC, PKCδ, appears to negatively regulate CD40-mediated proliferation. The atypical PKC, PKCζ, protects WEHI-231 cells from anti-Ig induced growth arrest and apoptosis to a certain extent.


Anti-Ig
BCR
PKCδ
PKCζ
PKCε
apoptosis
growth arrest


Anti-Ig
Anti-CD40
BCR
CD40
PKCα
PKCδ
survival
proliferation

Chapter 5 – Regulation of BCR-mediated responses by the Erk-MAPKinase in mature B cells

5.1 Introduction

Regulation of the B cell response to antigen requires the maintenance of appropriate levels of signalling through the B cell antigen receptor (sIg/BCR). To maintain this control, B cells employ a variety of positive and negative co-receptors such as CD19, CD22, CD72, and FcγRIIb that modulate the signals that are transduced by the BCR. Signals generated by these molecules appear to alter the signalling threshold of the BCR either by facilitating the positive signalling or by down modulating its function. Furthermore, efficient B cell responses generally require interactions with helper T cells (T_H) that are specific for the same antigen. Activated T cells can provide assistance to B cells via both membrane-bound molecules such as CD40-ligand (CD40L) and secreted molecules such as IL-4.

IL-4 was first described in 1982 as a cofactor in the proliferation of resting B cells stimulated through the cross-linking of their membrane IgM by anti-IgM (Howard and Paul, 1982). IL-4 is the hallmark of the T_H2 subset of $CD4^+$ helper T cells, though it can also be produced by activated mast cells and basophils, as well as some $CD8^+$ T cells. IL-4 is secreted by T_H2 cells directly towards the interacting B cell, ensuring that it acts selectively on the antigen-specific target B cell. IL-4 exerts different effects on B cells at different stages of the cell cycle. For example, on resting B cells, IL-4 acts as an activating factor, inducing them to enlarge in size and increase class II MHC expression. Following activation by an antigen, IL-4 acts as a growth factor, driving DNA replication in the B cells. In the case of proliferating B cells, IL-4 acts as a differentiation factor by regulating class switching to IgE and IgG1 isotypes. CD40L plays a crucial role in the regulation of the humoral immune response, including cell activation,

proliferation, immunoglobulin isotype switching, formation of germinal centres and induction of memory B cells (Durie *et al.*, 1994).

The necessity for a balance of activation and inhibitory pathways has been highlighted by observations that a loss of suitable inhibitory signalling can lead to inappropriate stimulation and activation of B cells (O'Keefe *et al.*, 1996), resulting in a variety of chronic inflammatory processes, including autoimmune disease.

5.1.1 FcγRIIb modulation of BCR activated signalling pathways

Fc receptors (FcRs) provide a critical link between the humoral and cellular arms of the immune system by binding the Fc domain of antibodies. The FcγRs are specific for the Fc domain of IgG, and comprise a family of structurally homologous, yet distinct, receptors. The only FcγR found on B cells is the low affinity FcγRIIb receptor, where it functions to inhibit signalling through the BCR upon cross-linking of the two receptors by IgG containing immune complexes. This down regulation of the BCR response to antigen provides a valuable negative feedback mechanism under physiological conditions, whereby immune complex formation results in aggregation of FcγRIIb with the BCR, leading to growth arrest and/or apoptosis. This may be of importance for preventing the appearance of autoreactive B cells in the periphery, in addition to the negative selection of immature B cells, by promoting the deletion of low affinity, self-reactive B cells in germinal centres and by the induction of IgG-mediated peripheral B cell tolerance.

FcγRIIb signalling appears to inhibit B cell activation by modulating three key signalling pathways activated by the BCR. These are the phospholipase C-γ (PLC-γ) pathway which is responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns-(4,5)P_2 or PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3 or IP_3), the phosphatidylinositol 3-kinase (PI-3-Kinase) pathway which is responsible for the generation of phosphatidylinositol-

3,4,5-trisphosphate (PtdIns-(3,4,5)P_3 or PIP_3), and the Ras/MAPKinase pathway. Signals derived from these pathways converge at the nucleus where they regulate key transcription factors that dictate the fate of the B cell.

The elevation of cytosolic calcium levels is a major downstream event of B cell activation via the BCR. This is the result of an initial release of calcium from intracellular stores followed by a lower, but sustained, rise in intracellular calcium that requires the presence of extracellular calcium. The generation of the second messenger IP_3, by PLC-γ, has been shown to be essential for the release of calcium by binding to IP_3 receptors on the endoplasmic reticulum (Parekh and Penner, 1997). Emptying of the BCR-sensitive intracellular stores activates a plasma membrane calcium channel with L-type characterisitics, a phenomenon known as capacitative calcium entry (Grafton *et al.*, 2003). In co-operation with DAG, the second PLC-γ product, calcium regulates several key B cell signalling events including activation of some PKC isoforms and the nuclear localisation and activity of NF-κB (Berridge, 1993). The activation of PKC isoforms is one of the earliest events in the cascade, leading to a variety of cellular responses including gene expression and proliferation (Dekker and Parker, 1994; Nishizuka, 1992). Induction of expression of genes such as *c-fos* seems to require both PKC activation and calcium mobilisation, whereas the induction of *egr-1* and *c-myc* appear to require only PKC activation. Furthermore, the induction of *Ets-1* requires calcium but is independent of PKC activation. PKC activation can also play a role in the regulation of the Erk-MAPK pathway, as PKCα is known to be a potent activator of Raf-1 (Schonwasser *et al.*, 1998).

Activation of PI-3-Kinase and the subsequent generation of PIP_2 and PIP_3 are known to be important for BCR-mediated proliferation, differentiation and survival (Campbell, 1999). The generation of PIP_3 by PI-3-Kinase has been shown to activate the survival promoting factor Akt (also known as protein kinase B, PKB) via the recruitment of the protein serine/threonine kinases, PDK1 and PDK2 to the plasma membrane. PDK1 and PDK2 activate Akt by phosphorylating it, within

its activation loop, on Thr^{308} and Ser^{473}, respectively. Akt promotes survival by phosphorylating and compromising multiple components of the cell death pathway, including the pro-apoptotic Bcl-2 family member Bad (del Peso *et al.*, 1997) and the initiator caspase, caspase-9 (Cardone *et al.*, 1998). In addition to regulating cell survival, Akt may regulate cell proliferation by phosphorylation of other target molecules. Recently, two separate reports demonstrated that Akt phosphorylates the cyclin dependent kinase inhibitor, $p27^{Kip1}$, at threonine 157 in breast cancer cells. This residue lies within the nuclear localisation signal of $p27^{Kip1}$, so as a result of this phosphorylation event nuclear import is inhibited, enabling the cdk2/cyclin E complex in the nucleus to become activated, inducing cell cycle progression (Liang *et al.*, 2002; Shin *et al.*, 2002).

The mitogen-activated protein kinases (MAPKs) are activated by a wide range of external stimuli, and as such are able to mediate a wide range of cellular responses from proliferation and activation to growth arrest and cell death. MAPK signalling cascades, consisting of an upstream serine/threonine kinase (MAPKKK), a middle dual-specificity kinase (MAPKK) and a downstream serine/threonine kinase (MAPK) (Dhanasekaran and Premkumar Reddy, 1998) are common to all eukaryotic cells, from yeast to mammals. Activation of the extracellular signal-regulated kinase (Erk) MAPK pathway has been shown to act on a wide range of downstream molecules and transcription factors controlling growth and differentiation. In B cells, initiation of this signalling cascade requires recruitment of the adaptor molecule, Shc, to the phosphorylated cytoplasmic tail of the activated BCR. The phosphorylation of Shc promotes its interaction with the adaptor molecule, Grb2, in an SH2 domain-dependent manner. Along with Shc, a recently identified adaptor protein that localises to lipid rafts, LAB (Linker for Activation of B cells), and the linker protein BLNK, appear to play key roles in the activation of the Erk-MAPKinase pathway via the recruitment of Grb2 to the BCR (Janssen *et al.*, 2003) (Kelly and Chan, 2000). Grb2 is already associated with the Ras guanine nucleotide exchange factor (GEF), Sos, via SH3 domain interactions, bringing Sos into close proximity with Ras, promoting the conversion

of Ras from its GDP-bound inactive form into its active GTP-bound form (Henning and Cantrell, 1998; Li *et al.*, 1993). Active Ras then recruits the serine/threonine kinase Raf-1, an upstream regulator of Erk-MAPKinase activity, to the plasma membrane, where it becomes phosphorylated. Phosphorylated Raf-1 then activates the dual specificity kinases MEK1 and MEK2 (MAPKinase Kinases 1 and 2), which go on to activate Erk1 (p44) and Erk2 (p42) MAPKinases by phosphorylating key threonine and tyrosine residues. Activated Erk-MAPKinases proceed to phosphorylate and activate a number of cytoplasmic effectors, such as cytosolic phospholipase A_2 ($cPLA_2$), as well as translocating to the nucleus where it phosphorylates a number of transcription factors, including Elk-1 and Stat-3. The 90 kDa Ribosomal S6 kinase (p90RSK) is also phosphorylated by active ErkMAPKinase and regulates gene expression through the phosphorylation of the Creb and c-fos transcription factors.

5.1.2 Mechanisms of FcγRIIb negative regulation of BCR signalling

Following the discovery of the ITIM motif within the cytoplasmic domain of FcγRIIb and other inhibitory receptors, distinct pathways of inhibition have been described (Gupta *et al.*, 1997; Scharenberg and Kinet, 1998). The signalling pathways responsible involve the tyrosine phosphorylation of the ITIM by protein tyrosine kinases and the recruitment of SH2 domain-containing proteins to the phosphorylated ITIM. The protein tyrosine phosphatases, SHP-1 and SHP-2 (Burshtyn *et al.*, 1999) and the inositol 5' phosphatase, SHIP (Ono *et al.*, 1996) have been shown to be the major proteins recruited by ITIM containing inhibitory receptors such as FcγRIIb, CD22 and CD72 in B cells, CTLA-4 in T cells and the family of Leukocyte Inhibitory Receptors (LIRs) (Daeron, 1997; Unkeless and Jin, 1997; Vely *et al.*, 1997). These SH2-domain containing phosphatases act to dephosphorylate key signalling components downstream of the inhibiting receptors, thereby inhibiting normal cellular responses.

Early *in vitro* studies on FcγRIIb indicated that recruitment of the tyrosine phosphatases, SHP-1 and SHP-2, mediated the inhibitory effect of this receptor in B cells (D'Ambrosio *et al.*, 1995). SHP-2 has a broad expression profile and has been shown to mediate both positive and negative regulatory effects. Its association with the platelet-derived growth factor β (PDGF-β) receptor, leads to the recruitment of the Grb-2-SoS complex and MAPKinase activation (Bennett *et al.*, 1994). However, in human B cells, SHP-2 is thought to mediate FcγRIIb inhibition of the Ras/MAPKinase and PI3-kinase pathways by dephosphorylating its potential substrates SHIP and Shc (Koncz *et al.*, 1999). In contrast, SHP-1 expression appears to be limited to cells of the haematopoietic lineage. SHP-1 is widely accepted to act as a negative regulator of many immunoreceptors, including the B cell receptor, CD22, CD72, and the natural killer cell inhibitory receptor (KIR) (Daeron, 1997; Vely *et al.*, 1997).

The negative regulatory role of SHP-1 was highlighted in SHP-1 deficient motheaten (*me*) and motheaten viable (me^v) mice. These mice, which are SHP-1 null or express a catalytically inactive form of SHP-1, respectively (Tsui and Tsui, 1994) display a phenotype of widespread autoimmunity as a result of multiple haematopoietic cell defects (Shultz *et al.*, 1997). In particular, B cells from *me* and me^v mice are hyper-responsive to stimulation via the BCR. This suggests that by removing SHP-1, the threshold of activation of B cells is substantially lowered, thus the threshold of apoptosis in immature B cells is similarly decreased. Hence, SHP-1 deficient mice possess a reduced number of bone marrow $B220^+$ progenitor B cells and a decreased resting B cell population. A high percentage of the residual peripheral B cells are also spontaneously activated with a skewing towards the B-1 subset. As B-1 B cells are associated with autoantibody production and are often expanded under conditions of autoimmunity (Hayakawa and Hardy, 2000), this would explain the observation that these mice exhibit many autoreactive features such as hyper-gammaglobulinemia, increased autoantibody titres and immune complex deposition.

However, SHP-1 was shown to be dispensable for FcγRIIb-mediated inhibition of B cell antigen receptor activation (Nadler *et al.*, 1997). Thus, *in vivo*, FcγRIIb does not necessarily recruit SHP-1 or SHP-2 but rather recruits the inositol 5' phosphatase, SHIP (Liu *et al.*, 1998; Ono *et al.*, 1997). Interestingly, the binding of SHP-1 to the phosphorylated ITIM of FcγRIIb seems to be correlated with the amount of receptor aggregation (Lesourne *et al.*, 2001; Sato and Ochi, 1998). Thus, in conditions of low receptor aggregation SHIP appears to be preferentially utilised. However, when there are high levels of immune complexes and superclustering of receptors both SHIP and SHP-1 are recruited to FcγRIIb. Thus, FcγRIIb-associated SHP-1 activity may be involved in downregulating BCR signalling, for example by deactivating the Src-family PTK, Lyn. It is possible, therefore, that the conditions of BCR-FcγRIIb co-ligation determine the selectivity of these phosphatases and that SHP-1 may act to enhance the downstream inhibitory effects of SHIP.

Studies with dominant negative SHIP mutants and knockout models have confirmed the inhibitory role of SHIP in mediating the FcγRIIb negative signal (Gupta *et al.*, 1997; Huber *et al.*, 1998; Liu *et al.*, 1998). SHIP is a 145 kDa, SH2-domain containing, 5'-inositol-polyphosphate phosphatase. The catalytic domain bears homology to several 5'-inositol phosphatases, whilst the c-terminal region contains several PTB (phospho-tyrosine binding) domains and a proline rich region. Post-translational C-terminal truncated forms of SHIP, 135-, 125- and 110-kDa in size, have recently been identified (Damen *et al.*, 1998). The 110 kDa form possesses the same inositol 5' phosphatase activity as the 145 kDa form. It is therefore possible that different forms of SHIP are generated *in vivo* and perform distinct functions within haematopoietic cells.

Recruitment of SHIP to the tyrosine-phosphorylated ITIM of FcγRIIb, following co-ligation with the BCR, leads to a drastic reduction in levels of phosphoinositide hydrolysis, influx of extracellular calcium, cellular proliferation and

immunoglobulin secretion. FcγRIIb abrogation of BCR activation by the hydrolysis of PtdIns(3,4,5)P_3 disrupts PH domain phosphoinositol lipid interactions and prevents the association of Btk and PLC-γ with the plasma membrane (Bolland *et al.*, 1998). The deletion of SHIP increases PtdIns(3,4,5)P_3 levels, resulting in increased Btk membrane association and hyper-responsive BCR signalling. The negative effects of SHIP on Btk recruitment can also be overcome by the expression of Btk as a membrane-associated chimera. Thus, one key role of SHIP-1 is to inhibit the pathways leading to calcium mobilisation by interrupting PLC-γ recruitment to the membrane. Another substrate of SHIP-1 is Ins(1,3,4,5)P_4, which is hydrolysed to Ins(1,3,4)P_3, a form of IP_3 unable to bind to receptors in the endoplasmic reticulum, preventing release of calcium from intracellular stores (Scharenberg and Kinet, 1998), providing another mechanism for the abrogation of BCR-induced calcium signalling.

Through its catalytic phosphatase domain, SHIP-1 also results in the partial inhibition of the survival factor Akt/PKB by hydrolysis of PtdIns(3,4,5)P_3 (Aman *et al.*, 1998). However, the hydrolysis product, PtdIns(3,4)P_2 has also been shown to be involved in the positive regulation of Akt activity (Downward, 1998). Thus, the inhibition of Akt may be as a result of upstream deregulation by the alternative inositol phosphatase, PTEN (Cantley and Neel, 1999), which is able to dephosphorylate the 3' position of its inositol substrates (Maehama and Dixon, 1998). Indeed, a study by Stambolic , 1998 confirmed that PTEN can negatively regulate Akt/PKB by dephosphorylating PtdIns(3,4,5)P_3. Furthermore, PTEN has also been shown to dephosphorylate PtdIns(3,4)P_2 suggesting that PTEN may directly counteract PI3-kinase activation of Akt/PKB (Haas-Kogan *et al.*, 1998).

FcγRIIb ligation also inhibits BCR-mediated activation of the RasMAPK pathway. However, there is a lack of consensus as to the mechanism underlying this downstream effect. There are currently three proposed models; the first concerns the GTPase exchange factors (GEFs), SOS and Vav. SOS and Vav interact with PtdIns(3,4,5)P_3 at the plasma membrane via PH domain interactions. As

discussed above, by hydrolysing PtdIns(3,4,5)P_3, SHIP-1 may prevent Ras activation by preventing the translocation of PH-domain containing exchange factors to the plasma membrane (Bolland *et al.*, 1998).

The second and most popular of the models involves the ability of SHIP-1 to also act as an adaptor protein and engage in PTB binding domain interactions. Phosphorylated SHIP-1 can recruit the adaptor molecules Shc (p52/46) and Grb-2 and the association of SHIP-1 with Shc is enhanced upon BCR-FcγRIIb coligation (Chacko *et al.*, 1996). Thus, SHIP may indirectly inhibit RasMAPK activation by competing with SOS/Grb2 complexes for Shc binding (Tridandapani *et al.*, 1997). However, this interaction is not considered sufficient to mediate the inhibitory action of SHIP-1 on the RasMAPK pathway as multiple stimuli, including BCR aggregation and cytokine activation, can induce the association between SHIP and Shc, possibly via PTB binding domain interactions (Lamkin *et al.*, 1997).

Thirdly, a Ras GTPase activating protein (RasGAP) has been shown to be recruited by the adaptor protein, $p62^{Dok}$, in a SHIP-dependent manner following FcγRIIb co-ligation with the BCR (Tamir *et al.*, 2000). By activating RasGAP via p62Dok, FcγRIIb may enhance the conversion of active Ras-GTP to inactive Ras-GDP, thus preventing activation of the ErkMAPK pathway.

Overall, the recruitment and activation of SHIP by FcγRIIb results in the inhibition of multiple B cell signalling pathways and the modulation of both BCR-induced B cell activation and antigen internalisation (**Figure 1.8**). In addition, ligation of FcγRIIb alone on B cells has been found to be capable of generating an apoptotic signal (Ashman *et al.*, 1996; Ono *et al.*, 1997). Studies in the B cell line, DT40, demonstrated that this response was independent of SHIP recruitment, suggesting that FcγRIIb may directly couple to an apoptotic pathway in the absence of BCR-ligation. Additional work by Pearse , 1999 demonstrated that failure to recruit SHIP, either by deletion of SHIP or mutation of FcγRIIb, resulted

in enhanced FcγRIIb-triggered apoptosis. Further studies demonstrated that this SHIP-independent pathway led to the Btk-dependent activation of JNK. Overall, these studies suggest that aggregation of FcγRIIb in B cells results in a stress response that leads to apoptosis and that SHIP recruitment following co-ligation with the BCR 'rescues' the cells from apoptosis. Interestingly, SHIP-1 may mediate this effect by recruiting the p85 subunit of PI3-kinase upon BCR-ligation and could act to regulate downstream events such as B cell activation-induced apoptosis (Gupta *et al.*, 1999).

Another mechanism by which FcγRIIb can negatively regulate BCR signalling is by disrupting its association with lipid rafts. In resting B cells the BCR is excluded from lipid rafts, but following cross-linking either by Ig-specific antibodies or antigen, the BCR associates with lipid rafts, and a number of components of the BCR signalling pathways are recruited to rafts (Cheng *et al.*, 1999). The association of the BCR with rafts is one consequence of BCR ligation that can be regulated by both positive and negative co-receptors. The association of the BCR with lipid rafts is more stable and less transient when the CD19/CD21 co-receptor complex is co-ligated to the BCR via the binding of C3d-tagged antigens (Cherukuri *et al.*, 2001). In contrast, when co-ligated in mature B cells both the BCR and FcγRIIb1 associate with lipid rafts, where FcγRIIb1 recruits the inositol phosphatase SHIP and blocks BCR signalling (Aman *et al.*, 2001). This results in a more transient association of the BCR with rafts.

5.2 Aims and Objectives

Effective B cell proliferation requires not only ligation of the BCR by antigen, but signals derived from helper T cells also, such as IL-4 and CD40-L. The signalling mechanisms activated immediately after BCR ligation are becoming well established, but the processes underlying sustained proliferation in response to BCR ligation are less well defined. This is similar to the role of FcγRIIb in

terminating a B cell response, as previous studies on FcγRIIb signalling have focused on the early signalling events following BCR-FcγRIIb co-ligation. However, recent studies have indicated that FcγRIIb ligation can lead to distinct cellular responses of growth arrest and/or apoptosis in B cells, dependent on the levels and type of aggregation. This finding has important implications for the selection and modulation of B cell activity by immunocomplexes during B cell development.

This study aims to investigate the signalling mechanisms responsible for the initiation and, more importantly, the maintenance of proliferation, and for FcγRIIb inhibition of BCR-mediated proliferative signalling in primary splenic B cells, and how these affect the key controllers of the cell cycle. In particular, it aims to address:

1. The kinetics of initiation and maintenance of proliferation in response to BCR ligation and T cell derived signals.
2. Identification of the signalling molecules involved, utilising specific inhibitors of key kinases.
3. The role of phosphodiesterases, particularly PDE4 and PDE7, in the B cell response to antigen, using specific PDE4 or PDE7 inhibitors.
4. The role of Erk-MAPKinase in BCR signalling, using the feedback inhibition mechanism provided by the FcγRIIb receptor.
5. The effects of BCR ligation and FcγRIIb inhibition on key cell cycle regulatory molecules, and key regulators of apoptosis.

5.3 Results

Efficient B cell responses generally require an interaction with helper T cells (T_H) that are specific for the same antigen. Activated T cells can provide assistance to B cells via both membrane-bound molecules such as CD40-ligand (CD40L) and secreted molecules such as IL-4. In order to examine the signals involved in the ongoing responses of B cells to a variety of stimuli, it was first important to assess the activation of B cells in response to a variety of mitogenic factors.

5.3.1 The effects of mitogenic signalling on RNA synthesis in primary splenic B cells

Experimentally, $F(ab')_2$ fragments of anti-Ig are used to stimulate B cells as they possess exactly the same binding characteristics as a whole antibody molecule, but are unable to interact with BCR co-receptors, allowing the BCR effects to be assayed directly. In contrast, intact antibodies that ligate the BCR are poor polyclonal B cell activators, in comparison to $F(ab')_2$ fragments, because the Fc portion of the intact antibody is able to interact with the inhibitory Fc receptor expressed by B cells, FcγRIIb. One measure of activation of B cells is the level of RNA synthesis. Small, resting B cells do not require much in the way of RNA synthesis, as there is very little protein synthesis going on. Before any proliferation can occur, mitogenically stimulated B cells must first increase their size, therefore increase both their RNA and protein synthesis rates.

The effects of $F(ab')_2$ fragments of anti-Ig, either alone or in combination with increasing concentrations of anti-CD40 or recombinant IL-4 on RNA synthesis in primary murine splenic B cells was investigated by means of the [^{3}H]-uridine assay. Stimulation of primary murine splenic B cells for 24 hours with increasing concentrations of $F(ab')_2$ fragments of anti-Ig leads to a dose dependent increase in RNA synthesis, as measured by [^{3}H]-uridine incorporation (**Figure 5.1A**). This is greatly enhanced by co-stimulation with anti-CD40, mimicking B cell - helper T

cell interactions. The effects of anti-CD40 on $F(ab')_2$ fragment of anti-Ig induced RNA synthesis are also dose dependent, with a maximal effect afforded by 10 μg/ml (**Figure 5.1B**). Co-stimulation with IL-4, mimicking T_H2 cytokine production, also enhances $F(ab')_2$ anti-Ig induced RNA synthesis, again in a dose dependent manner, with 10 U/ml IL-4 providing the maximum response (**Figure 5.1C**).

5.3.2 The effects of mitogenic signalling on DNA synthesis in primary splenic B cells

Another measure of B cell activation is DNA synthesis, as activated mature B cells proliferate. It is well established that ligation of the murine BCR with $F(ab')_2$ fragments of anti-mouse Ig antibodies leads to B cell proliferation (Klaus *et al.*, 1985; Klaus *et al.*, 1984). Using the [^{3}H]-thymidine assay as a measure of DNA synthesis, **Figure 5.2A** shows that stimulation of murine splenic B cells via the BCR with $F(ab')_2$ fragments of anti-Ig results in a dose-dependent increase in DNA synthesis. The effects of co-stimulation with $F(ab')_2$ fragments of anti-Ig and either anti-CD40 or recombinant IL-4 on DNA synthesis in primary murine splenic B cells was also investigated by means of the [^{3}H]-thymidine assay (**Figure 5.2B** and **5.2C**). Co-stimulation with anti-CD40 enhances $F(ab')_2$ anti-Ig induced proliferation in a dose dependent manner, again with 10 μg/ml providing the maximal enhancement. As with the induction of RNA synthesis, co-stimulation with IL-4 also enhances $F(ab')_2$ anti-Ig induced proliferation, but to a lesser extent than co-stimulation with anti-CD40.

To assess the longevity of the proliferative response, the proliferation of murine splenic B cells was assessed over a period of 168 hours, by means of the [^{3}H]-thymidine incorporation assay, in response to $F(ab')_2$ fragments of anti-Ig plus either anti-CD40 (**Figure 5.3A**) or IL-4 (**Figure 5.3B**). These data show that, upon stimulation with $F(ab')_2$ fragments of anti-Ig alone, the proliferation of B cells peaks around 48 to 72 hours, then drops significantly. Upon co-stimulation with anti-CD40, the kinetics are altered slightly. The peak of proliferation (as assessed

by [^{3}H]-thymidine incorporation) shifts to 72 hours, and though the levels drop there is still considerable proliferation ongoing at 120 hours (**Figure 5.3A**). IL-4 co-stimulation produces a more dramatic increase in proliferation at 120 hours, with the levels around the same as the levels exhibited at 72 hours (**Figure 5.3B**). At 48 hours, the levels of proliferation are very similar whether cells are stimulated with F(ab')$_2$ fragments of anti-Ig alone or co-stimulated via CD40 or with IL-4. This clearly demonstrates the importance of T cell derived signals in the sustained B cell response to antigen.

5.3.3 Effects of specific kinase inhibitors on BCR-mediated proliferation

Ligation of the BCR leads to activation of, amongst other molecules, three major signalling pathways, the PLC-γ, PI3-Kinase, and Erk-MAPKinase pathways. As demonstrated in Chapter 3, the Erk-MAPKinase plays a key role in BCR-mediated signalling in immature and transitional B cells. To determine which signals are important for BCR-mediated proliferation in primary splenic B cells, specific kinase inhibitors were used.

Primary splenic B cells were incubated with the MEK inhibitor PD98059, another MEK inhibitor, U0126, the p38 inhibitor SB203580, or the PI-3-Kinase inhibitor LY294002 and stimulated with F(ab')$_2$ fragments of anti-Ig. After 48 hours of stimulation proliferation was assessed by the [^{3}H]-thymidine incorporation assay. Treatment of primary splenic B cells with F(ab')$_2$ fragments of anti-Ig, in the absence of any inhibitor, induces proliferation (**Figure 5.4**). F(ab')$_2$ anti-Ig induced proliferation is completely abrogated by treatment of the cells with either of the MEK inhibitors, PD98059 or U0126, suggesting that Erk-MAPKinase activity plays a major role in F(ab')$_2$ anti-Ig induced proliferation. Inhibition of p38 activity with the p38 inhibitor, SB203580, inhibits F(ab')$_2$ anti-Ig induced proliferation to a certain extent, but not as effectively as inhibition of MEK. Inhibition of PI-3-Kinase with the inhibitor LY294002 also reduces F(ab')$_2$ anti-Ig induced proliferation, to a similar extent as inhibition of p38. These results

suggest that Erk-MAPKinase plays a major role in the proliferative response of mature B cells to antigen, and that PI-3-Kinase, and to a lesser extent, p38, may play minor roles in the proliferation of mature B cells. It was decided to concentrate on the effects of Erk-MAPKinase on the mitogenic response of mature B cells in light of the results indicating the importance of Erk-MAPKinase in the WEHI-231 immature B cell line.

5.3.4 The effects of PDE7 inhibitors on B cell proliferative response to antigen

Another key signalling pathway that is involved in controlling a wide range of events in different cell types is the cyclic AMP (cAMP) pathway. Recently it has been demonstrated that the cAMP pathway and the Erk-MAPKinase pathway are interlinked at a number of distinct points (Houslay and Kolch, 2000). The effects elevated levels of cAMP have on Erk-MAPKinase activity vary between cell types, depending on which Raf isoform is expressed. Raf-1 can be inhibited by PKA, which is activated by elevated levels of cAMP. Conversely, B-Raf is activated in response to elevated levels of cAMP, hence leading to Erk-MAPKinase activation.

Cyclic nucleotide phosphodiesterases (PDEs) are responsible for regulating the cyclic nucleotide signalling system by converting adenosine-3',5'-cyclic monophosphate (cAMP) into adenosine-5'-monophosphate (5'-AMP), switching off pathways downstream from cAMP. PDEs are a diverse and complex group of enzymes, with at least 10 isoforms described to date. They are responsible for the regulation of a wide variety of responses, from platelet aggregation to cardiac muscle inflammation. When T cell receptors are activated by antigen, cAMP levels drop and proliferation occurs. Previous studies in our laboratory had shown that PDE4 family members were activated following mitogenic stimulation via the BCR (Harnett, unpublished observations), and in WEHI-231 cells, stimulation via the BCR, which induces growth arrest, inhibits PDE4 activity,

leading to an increase in cAMP (Gauld, 2001). Other PDE family members are known to play roles in regulation of proliferation in lymphocytes, for instance, it has been shown that PDE7 mRNA and protein levels are up-regulated when human T cells become activated, and that blocking the PDE7 induction blocked the activation of the cells (Li *et al.*, 1999). These results strongly suggest that suppressing levels of cAMP could enhance proliferation in lymphocytes. To determine if PDE7 is also important in B cell activation, PDE7 activity was inhibited with a variety of compounds.

Primary splenic B cells were stimulated, in the presence or absence of a variety of PDE7 inhibitors (10 μM), with $F(ab')_2$ fragments of anti-Ig, either alone or in combination with anti-CD40 or recombinant IL-4, or with LPS. Following 48 hours treatment proliferation was assessed by the [^{3}H]-thymidine uptake assay. As **Figure 5.5** shows, B cells receiving no stimulation incorporate very little [^{3}H]-thymidine as they are resting and not undergoing proliferation. In response to stimulation with $F(ab')_2$ fragments of anti-Ig, cells receiving no PDE7 inhibitors display an increase in their thymidine incorporation, indicating increased proliferation in response to BCR ligation. B cells stimulated with $F(ab')_2$ fragments of anti-Ig in the presence of the PDE7 inhibitor PD0329870 also demonstrate a massive increase in thymidine incorporation, indicating that this inhibitor has no effect on BCR-induced proliferation. Indeed, this inhibitor fails to alter the response of B cells to any of the stimuli used. In contrast to this, PD0322408 inhibits B cell proliferation in response to all stimuli used, more effectively than the other inhibitors. This is most obvious in response to LPS, where the other inhibitors only marginally inhibit B cell proliferation, whilst PD0322408 reduces it by approximately 75%. The other 3 inhibitors all appear to have very similar effects on B cell proliferation, in that they inhibit anti-Ig induced proliferation, even in the presence of IL-4, but not in the presence of anti-CD40.

PD0322408 and PD0326875 both have effects on B cell proliferation, so to further characterise these inhibitors, B cells were stimulated with either the

combination of LPS and IL-4, or the combination of $F(ab')_2$ fragments of anti-Ig, IL-4 and anti-CD40 in the presence of increasing concentrations of these two PDE7 inhibitors. The PDE4 specific inhibitor, rolipram, was also used to compare the effects of PDE7 and PDE4 inhibition on B cell responses. Proliferation was assessed after 48 hours by means of the [^{3}H]-thymidine incorporation assay.

These data demonstrate that the effects of PD0322408 on LPS induced proliferation are reduced slightly by the presence of IL-4, as the levels of proliferation in response to LPS are reduced to about two thirds in the presence of 10 μM PD0322408 (**Figure 5.6**). In contrast, the effects on anti-Ig induced proliferation are not as striking in the presence of IL-4 and anti-CD40, again demonstrating the importance of T cell derived signals in B cell activation. The inhibitor PD326875 does not significantly alter the proliferative response to any of the stimuli used, nor indeed does rolipram. These results suggest that neither PDE4 or PDE7 play significant roles in mitogen induced proliferation.

5.3.5 The effects of PDE7 inhibitors on B cell apoptosis

The decrease in [^{3}H]-thymidine incorporation induced by these PDE7 inhibitors could be due to non-specific cytotoxic effects, rather than as a specific result of PDE7 inhibition. To assess the effects of the various PDE7 inhibitors on B cell viability, primary splenic B cells were stimulated, in the presence or absence of a variety of PDE7 inhibitors (10 μM), with $F(ab')_2$ fragments of anti-Ig, either alone or in combination with anti-CD40, or with LPS. Following 48 hours treatment, DNA content was assessed by flow cytometry following propidium iodide staining. **Figure 5.7** shows the percentage of cells in each cell cycle phase.

Reflecting their resting state, B cells receiving no inhibitor or agonists are mainly in G_0/G_1 phase, and treatment with an agonist induces an increase in the percentage of cells in either S phase or G_2/M. Stimulation with $F(ab')_2$ fragments of anti-Ig appears to induce a slight increase in the percentage of cells displaying

a sub-diploid DNA content. The PDE7 inhibitor, PD0322408, induces a rather large increase in apoptosis in the absence of any agonist, and this is enhanced when cells are treated with the combination of inhibitor and $F(ab')_2$ fragments of anti-Ig. This could account for the decrease in proliferation demonstrated by cells treated with this inhibitor in the [^{3}H]-thymidine incorporation assay (**Figure 5.5**). Treatment with LPS or the combination of $F(ab')_2$ fragments of anti-Ig and anti-CD40 provide protection from apoptosis induced by PD0322408, though the inhibitor still manages to inhibit proliferation induced by these agonists, as there are fewer cells in S phase and G_2/M compared to cells not treated with inhibitor.

Corroborating the results from the [^{3}H]-thymidine incorporation assay, the inhibitor PD0323590 prevents anti-Ig induced proliferation as there is a lower percentage of cells in S phase or G_2/M in response to anti-Ig compared to cells stimulated in the absence of inhibitor. This appears to be mostly due to an increase in apoptosis, rather than an increase in growth arrest, as there is a greater proportion of cells with a sub-diploid DNA content.

5.3.6 MEK inhibition blocks BCR-mediated Erk-MAPKinase activation

In the WEHI-231 immature B cell line, the kinetics of Erk-MAPKinase signalling determine the specific outcome. For example, ligation of the BCR with anti-Ig leads to a transient Erk-MAPKinase activation that terminates within 4 hours, whilst CD40-mediated rescue of anti-Ig induced growth arrest and apoptosis leads to a sustained, cyclical pattern of Erk-MAPKinase activation, with activity still detectable after 24 and 48 hours (**Section 3.3.3**) (Gauld *et al.*, 2002). In mature B cells, BCR ligation also leads to the activation of Erk-MAPKinase, within minutes of antigen binding (Campbell, 1999). The activity of Erk-MAPKinase in response to BCR ligation over a longer period of time remains to be fully elucidated. It seems highly likely that Erk-MAPKinase activity is required for B cell responses to antigen, as inhibition of Erk-MAPKinase with the specific MEK inhibitor PD98059 blocks $F(ab')_2$ anti-Ig mediated proliferation (**Figure 5.4**).

To assess the longer term effects of BCR ligation on Erk-MAPKinase phosphorylation, primary splenic B cells were stimulated with $F(ab')_2$ fragments of anti-Ig in the presence or absence of the MEK inhibitor PD98059 for the times indicated. The presence of PD98059 abrogates the phosphorylation of Erk-MAPKinase at 6 hours, and dramatically reduces it at 17 and 20 hours (**Figure 5.8A**). By 24 hours, PD98059 is still effective at blocking the BCR-mediated activation of Erk-MAPKinase (**Figure 5.8B**).

To assess the localisation of this phosphorylated Erk-MAPKinase, primary splenic B cells were stimulated for 16 hours with $F(ab')_2$ fragments of anti-Ig in the presence or absence of the MEK inhibitor PD98059. Cells were adhered to slides, and the activity of Erk-MAPKinase assessed by staining with an anti-phospho Erk1/2 antibody, and staining visualised using the image analysis abilities of the LSC, as described in **Section 2.12.3**. The nucleus was visualised via DAPI staining. Stimulation via the BCR leads to phosphorylation of Erk-MAPKinase, even after 16 hours (**Figure 5.8A**). It appears as though this phospho-Erk localises to an area adjacent to the nucleus. Treatment of cells stimulated via the BCR with the MEK inhibitor, PD98059, abrogates this phosphorylation of Erk-MAPKinase.

5.3.7 FcγRIIb ligation abrogates antigen receptor mediated B cell proliferation

The above data show that, upon mitogenic stimulation via the BCR, Erk-MAPKinase becomes activated, and that inhibition of this Erk-MAPKinase activity by specific inhibitors blocks BCR-induced proliferation. This is analogous to the physiological negative regulation of the BCR by the FcγRIIb receptor, so it was decided to utilise the FcγRIIb receptor to further investigate the role of Erk-MAPKinase in mature B cells under more physiologically relevant conditions.

It is well established that ligation of the murine BCR with $F(ab')_2$ fragments of anti-mouse Ig antibodies leads to B cell proliferation, whilst BCR-FcγRIIb co-ligation using intact anti-Ig antibodies inhibits BCR-mediated DNA synthesis (Klaus *et al.*, 1985; Klaus *et al.*, 1984). Using the [^{3}H]-thymidine assay as a measure of DNA synthesis, **Figure 5.9A** shows that stimulation of murine splenic B cells via the BCR ($F(ab')_2$ anti-Ig) results in a dose-dependent increase in DNA synthesis. Following stimulation with intact antibody alone over a range of similar concentrations, no DNA synthesis is detected (**Figure 5.9B**). Co-ligation of the BCR with FcγRIIb using a combination of $F(ab')_2$ fragments and intact anti-Ig antibodies together results in a dose-dependent decrease in BCR-induced DNA synthesis (**Figure 5.9C**) and provides an experimental model for negative feedback inhibition of ongoing B cell responses. Maximal inhibition of BCR-induced DNA synthesis was achieved using intact anti-Ig antibodies at a concentration of 75 μg/ml, i.e. at a concentration which is equimolar to the concentration of $F(ab')_2$ fragments of anti-Ig required for maximum DNA synthesis (**Figure 5.9C**).

To confirm that co-ligation of the BCR with FcγRIIb is required for the inhibitory effect of intact anti-Ig antibody on BCR-mediated proliferation, an anti-FcγRII/RIII antibody (2.4G2) was used to block BCR-FcγRIIb co-ligation (**Figure 5.9D**). Co-stimulation of murine splenic B cells with $F(ab')_2$ fragments and intact anti-Ig antibodies in the presence of 2.4G2 resulted in the almost total restoration of levels of DNA synthesis observed when cells were stimulated via the BCR alone, consistent with previously published observations (Unkeless, 1979). Together, these results confirm that FcγRIIb co-ligation can reduce or prevent BCR-mediated proliferation of murine splenic B cells, demonstrating that this receptor plays a key role in negative feedback regulation of B cells.

5.3.8 Ligation of FcγRIIB on splenic B cells induces apoptosis

The above results confirmed that co-ligation of the BCR with FcγRIIb is able to inhibit BCR-induced DNA synthesis, a process that could be reversed by an anti-FcγRII/III blocking antibody. To investigate whether the FcγRIIb-mediated inhibition of BCR-induced DNA synthesis was a result of growth arrest, or a consequence of apoptosis, cells were stimulated with $F(ab')_2$ fragments of anti-Ig, intact anti-Ig, alone and in combination. Unstimulated cells were used as a control. The DNA content of the cells was determined by flow cytometry analysis following propidium iodide staining (**Figure 5.10**). These results confirm the ability of BCR stimulation to promote the entry of murine splenic B cells into the S and G_2/M phases of the cell cycle, consistent with the increased levels of DNA synthesis seen in **Figure 5.9**. Co-ligation of the BCR with FcγRIIb resulted in an increase in the proportion of B cells arrested in G_0/G_1 phases of the cell cycle, and an increase in apoptosis (sub-diploid DNA content) compared to the basal level.

5.3.9 FcγRIIb inhibits Erk-MAPKinase phosphorylation

Ligation of the BCR leads to activation of Erk-MAPKinase following the formation of adaptor protein scaffolds, which are recruited by protein tyrosine kinases (Campbell, 1999). Inhibition of Erk-MAPKinase activity with the specific MEK inhibitor PD98059 blocks BCR-mediated proliferation very effectively (**Figure 5.4**), as does co-ligation of the BCR with FcγRIIb (**Figure 5.9**). Ligation of FcγRIIb results in the activation of the phosphatases SHIP and SHP-1, which are known to dephosphorylate and deactivate key BCR-coupled signalling molecules, abrogating BCR-induced proliferative signalling.

To investigate the effect of FcγRIIb ligation on Erk-MAPKinase activation, primary splenic B cells were stimulated with $F(ab')_2$ fragments of anti-Ig alone, or a combination of $F(ab')_2$ fragments and intact anti-Ig antibodies. Unstimulated cells

were used as a control. Whole cell lysates were prepared and probed for phospho-Erk expression using a phospho-Erk1/2 antibody. Unstimulated B cells exhibit no detectable Erk phosphorylation by Western blot analysis, reflecting the resting state of the cells (**Figure 5.11**) Stimulation of B cells via the BCR alone results in a rapid upregulation of Erk-MAPKinase phosphorylation, sustained for at least 30 minutes. Co-ligation of the BCR with FcγRIIb results in a more transient increase in Erk-MAPKinase phosphorylation, which is terminated by 30 minutes.

5.3.10 Localisation of active Erk-MAPkinase in response to BCR ligation and FcγRIIb co-ligation

Immune receptors and components of their signalling cascades are spatially organised, and this spatial organisation plays a key role in the initiation and regulation of signalling. This spatial organisation is due to cholesterol- and sphingolipid-rich plasma membrane microdomains, or lipid rafts. Lipid rafts are known to be important for signalling via both the BCR and FcγRIIb. The BCR associates with lipid rafts following antigen binding, and upon co-ligation with the BCR, FcγRIIb also localises to lipid rafts, where it can recruit SHIP and abrogate BCR signalling. One of the key roles of lipid rafts is thought to be to enable the co-localisation of key signalling molecules.

To investigate the effects of BCR ligation and BCR and FcγRIIb co-ligation on the localisation of active Erk, primary splenic B cells were stimulated with $F(ab')_2$ fragments of anti-Ig, or intact anti-Ig, either alone or in combination, in the presence and absence of the MEK inhibitor PD98059. Lipid rafts were visualised by staining with a biotin labelled chlera toxin B subunit, which attaches to cells by binding to ganglioside G_{M1}, a ubiquitous glycolipid cell surface receptor that is a component of lipid rafts. Dually phosphorylated Erk-MAPKinase was detected with a monoclonal, phospho-specific antibody, whilst DNA was stained with

DAPI, as described in **Section 2.12.3**. In **Figure 5.12** lipid rafts are shown in red, the nucleus in blue, and phospho-Erk-MAPKinase as green.

Stimulation of primary splenic B cells via the BCR with $F(ab')_2$ fragments of anti-Ig results in a dramatic increase in the presence of phospho-Erk, hence Erk-MAPKinase activity (**Figure 5.12**). This Erk-MAPKinase activity appears to be heavily polarised, with most cells displaying a single point of intense Erk-MAPKinase activity, which appears to co-localise with lipid rafts at earlier time points. Co-ligation of the BCR with FcγRIIb abrogates this Erk-MAPKinase activity. Interestingly, on its own PD98059 does not appear to fully abrogate $F(ab')_2$ anti-Ig-induced Erk-MAPKinase activation at 1 hour as there are still some cells displaying a low level of Erk-MAPKinase activity. However, when cells are stimulated with the combination of $F(ab')_2$ fragments and intact anti-Ig antibodies, and treated simultaneously with PD98059, Erk-MAPKinase activity is completely abolished. After 20 hours of treatment with $F(ab')_2$ fragments of anti-Ig and PD98059 Erk-MAPKinase activity is abolished completely. Treatment of cells with intact anti-Ig antibodies alone for 20 hours appears to alter the integrity or composition of lipid rafts, as the staining appears more punctate compared to control cells at this time.

5.3.11 FcγRIIb modulates the expression of key cell-cycle regulator proteins

The retinoblastoma protein,Rb, is a tumour suppressor protein that controls the progression of cells through the G_1 restriction point, the key regulatory step prior to S phase entry. Rb inhibits progression into S phase by binding to and sequestering the E2F family of transcription factors, which are required for transcription of key genes involved in DNA synthesis and cell cycle progression. This interaction is abrogated by phosphorylation of Rb by Cdk4/6 and Cdk2 complexes, allowing E2F to induce transcription of its target genes (Dyson, 1998). Both immature and mature B cells show BCR-induced increases in Cyclin D and Cdk4 levels, which are essential for early G_1 phase progress and transition

through the G_1 restriction point. This may be due to Erk-MAPKinase dependent activation of the Cyclin D gene via the transcription of MAPKinase responsive genes.

Since FcγRIIb signalling has been demonstrated to inhibit B cell G_1-S phase progression following co-ligation with the BCR the effect of FcγRIIb recruitment on the expression of key cell-cycle regulator proteins was investigated. The expression profile of Rb was investigated in whole cell lysates of splenic B cells stimulated via the BCR or BCR-FcγRIIb for 72 hours. The existence of multiple forms of Rb observed during Western blotting is indicative of Rb phosphorylation; with hyper-phosphorylated Rb exhibiting a greater apparent molecular weight in comparison to hypo- or unphosphorylated Rb. **Figure 5.14A** shows that Rb appears to be differentially phosphorylated in splenic B cells following BCR and/or FcγRIIb stimulation. Correlating with entry into the S and G_2/M phases of the cell cycle, BCR stimulation over 72 hours results in the hyper-phosphorylation of Rb, as indicated by the predominance of the higher molecular weight form. In contrast, co-ligation of FcγRIIb results in a Rb phosphorylation profile similar to that of basal cells, with a large percentage of cells demonstrating hypo-phosphorylated Rb. Thus, FcγRIIb actively inhibits entry in the proliferative phases of the cell cycle by preventing the hyper-phosphorylation and deactivation of Rb.

Following mitogenic stimulation of cells, two events controlling the G_1/S transition are thought to occur. Firstly Cyclin D levels are increased and secondly, the levels of p27, a Cyclin-dependent kinases inhibitor (CKI), are reduced (Sherr and Roberts, 1999). Together with another CKI, p21, p27 inhibits Cyclin-E/Cdk2 complexes whilst acting as an assembly factor for Cyclin D and Cdk4/6 (Cheng *et al.*, 1999). By binding to the Cyclin/Cdk complexes, these CKIs lead to the inhibition of Cdk activity and thus prevent the phosphorylation of Rb and cell cycle progression. Whilst p27 is constitutively expressed, p21 levels are induced during periods of stress by p53.

The tumour suppresser protein, p53, plays a major role in the cellular response to DNA damage and other genomic aberrations. Importantly, the activation of p53 by MAPK mediated phosphorylation on multiple residues, can either lead to cell cycle arrest and DNA repair, or apoptosis (Levine, 1997; Meek, 1994). p53 is normally held in an inactive complex with a negative regulator, MDM2. MDM2 inhibits the accumulation of p53 by targeting it for ubiquination and proteasomal degradation (Chehab *et al.*, 1999). DNA damage or apoptotic signals induce the phosphorylation of p53 at residues serine 15 and serine 20. These phosphorylation events impair the MDM2-p53 interaction thus promoting both the accumulation and functional activation of p53.

To investigate whether p53 was functionally activated by FcγRIIb ligation, whole cell lysates from BCR and/or FcγRIIb co-stimulated splenic B cells were Western blotted for the expression of phospho-p53 Ser^{15}. **Figure 5.14B** clearly demonstrates that FcγRIIb induces the phosphorylation of p53 on the critical serine residue over 72 hours, compared to control or BCR stimulated cells. Thus, the activation of p53 may represent an important pro-apoptotic mechanism initiated by FcγRIIb following ligation on splenic B cells.

Cyclin dependent kinases (Cdks) play a major role in the phosphorylation of Rb during G_1 (Zarkowska and Mittnacht, 1997). Cyclin D-Cdk4/6 complexes and Cyclin E-Cdk2 complexes contribute to the activation of Rb. As Cdk4 is required for the phosphorylation of Rb, the expression levels of Cdk4 were investigated in whole cell lysates of BCR and BCR-FcγRIIb stimulated cells. **Figure 5.14C** shows that BCR stimulation for 1 hour results in a downregulation in Cdk4 expression levels followed by a return to basal levels over time. A similar pattern of slight Cdk4 suppression followed by a return to basal levels is shown following FcγRIIb co-ligation with the BCR. The lack of significant modulation of Cdk4 levels following stimulation may be due to the short stimulation times chosen and an investigation of later times in the cell cycle may highlight differential

expression or activation of Cdk4 by FcγRIIb and the BCR. However, consistent with our results, recent studies in murine B cells over 48 hours have shown that FcγRIIb ligation does not inhibit the expression of Cdk4 but acts to block the assembly and stabilisation of Cyclin D-Cdk4/6 complexes (Tanguay *et al.*, 1999).

5.3.12 FcγRIIb modulates the expression of key cell-cycle regulatory genes

The transcription of a further group of cell cycle regulatory proteins was also assessed by means of the ribonuclease protection assay (RPA). The RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species. The multi-probe system developed by Pharmingen allows the quantitation of several mRNA species in a single sample of total RNA due to the fact that each template is a distinct length and represents a sequence in distinct mRNA species. The multi-probe template set utilised here is shown in **Table 5.1**.

To investigate the mRNA levels of the key cell cycle regulatory genes purified B cells were stimulated with $F(ab')_2$ fragments of anti-Ig, either alone or in combination with intact anti-Ig antibodies. At the times indicated, whole RNA was isolated from each sample (1×10^6 cells) as described in Materials and Methods (**Section 2.15**). The assay was carried out as per the manufacturer's instructions (see **Section 2.16**), and the RNA levels quantitated using a phosphorimager.

As **Figure 5.14** shows, the levels of cyclin D2 did not alter much under any condition, and the same was true for levels of cdk2 mRNA, though these were slightly higher upon antibody stimulation that in the control cells. Somewhat surprisingly, the levels of cyclin D3 were increased by co-stimulation with $F(ab')_2$ fragments and intact anti-Ig, which normally does not lead to proliferation, but not by $F(ab')_2$ fragments alone, conditions which normally do lead to proliferation (**Figure 5.9**). A further surprising result was the increase in $p27^{Kip1}$ expression induced by $F(ab')_2$ fragments of anti-Ig alone after 16 hours of stimulation. This is possibly the opposite of what would be expected as an increase in the levels of

p27 protein is associated with G_1 arrest, yet $F(ab')_2$ fragments of anti-Ig induce proliferation.

5.3.13 Ligation of FcγRIIB on splenic B cells induces a collapse of the mitochondrial membrane potential

The tumour suppressor protein p53 possesses two distinct functions, the ability to induce cell cycle arrest and the ability to induce apoptosis. These functions are separable, as evidenced by the isolation of structural p53 mutants that possess defects in apoptosis induction, but not cell cycle arrest (Ryan and Vousden, 1998). One of the mechanisms by which p53 is able to induce apoptosis is the modulation of mitochondrial function, perhaps as a result of its ability to translocate to the mitochondria (Marchenko *et al.*, 2000). An early indication of apoptosis is the reduction in mitochondrial membrane potential ($\Delta\psi_m$), as this constitutes an early, obligatory step of apoptosis in lymphocytes (Zamzami *et al.*, 1995). This reduction in $\Delta\psi_m$ can be detected using $DiOC_6(3)$, a cationic lipophilic dye whose uptake by cells correlates with their $\Delta\psi_m$. To investigate whether FcγRIIb-induced apoptosis in murine splenic B cells correlated with a reduction in $\Delta\psi_m$, cells were stimulated via the BCR alone, the FcγRIIb alone, or by co-ligation of the two receptors. Unstimulated cells were included as a control. Mitochondrial membrane potential was analysed by flow cytometry following staining of the cells with $DiOC_6(3)$ (**Figure 5.13**). These data clearly show that co-ligation of the BCR and FcγRIIb results in an increased percentage of primary splenic B cells with a low $DiOC_6(3)$ fluorescence intensity, indicating apoptosis (**Figure 5.13**). These results confirm that FcγRIIb ligation on B cells can initiate mechanisms that lead to the disruption of mitochondrial stability.

5.3.14 FcγRIIb modulates the expression of key apoptosis regulatory genes

Apoptosis is a highly regulated process, and as such a number of different molecules are involved in the control of apoptosis, from Bcl-2 family members to

the caspases. In order to further investigate the induction of apoptosis by FcγRIIb, the transcription of some apoptosis regulatory genes was also assessed by RPA. The protocol was exactly as described in **Section 5.3.12**, and the multi-probe template set is shown in **Table 5.1**.

The expression levels of p53, bax, and Bcl-X_L were all assessed by means of the RPA (**Figure 5.16**). These results show that stimulation of B cells with the combination of $F(ab')_2$ fragments and intact anti-Ig antibodies leads to an increase in the transcription of p53, as well as Bak, albeit to a lesser extent. This is unsurprising, as Bak is a known transcriptional target of p53, and we had previously shown that the p53 protein is activated in response to ligation of FcγRIIb (**Figure 5.13**). Interestingly, co-ligation of the BCR with FcγRIIb also led to the increased transcription of Bcl-X_L, an anti-apoptotic member of the Bcl-2 family. In the WEHI-231 immature B cell line, Bcl-X_L plays a key role in protecting the cells from anti-Ig induced apoptosis (Choi *et al.*, 1995), so as co-ligation of the BCR with FcγRIIb leads to apoptosis, it might be expected that Bcl-X_L levels would fall.

5.4 Discussion

Regulation of the B cell response to antigen requires the maintenance of appropriate levels of signalling through the B cell antigen receptor. The signalling mechanisms activated immediately after BCR ligation are becoming well established, but the processes underlying sustained proliferation in response to BCR ligation are less well defined. Experimental models have developed, however, which potentially allow dissection of the key regulatory events. For example, stimulation of the BCR with $F(ab')_2$ fragments of anti-Ig antibodies results in B cell activation and proliferation. In contrast, by using intact anti-Ig antibodies to mimic physiological antibody-antigen complexes, it is possible to co-ligate the BCR with FcγRIIb, inhibiting the response to mitogenic $F(ab')_2$ anti-Ig antibodies. Thus, BCR signalling and negative feedback regulation of BCR signalling by FcγRIIb can be investigated *in vitro*. This study utilised this approach, in conjunction with the use of specific inhibitors of key kinases, to investigate the signalling mechanisms involved in the initiation and maintenance of BCR-mediated proliferation, and how these affect key cell cycle regulatory proteins.

In general, maximal B cell responses require an interaction with helper T cells (T_H) that are specific for the same antigen. Such signals include the soluble cytokine IL-4, and a molecule on the T cell surface, CD40-ligand (CD40L), which interacts with CD40 on the surface of B cells. Experimentally, the CD40 – CD40L interaction can be mimicked using anti-CD40 antibodies, which bind CD40 and activate the signalling pathways downstream of the receptor. The induction of RNA synthesis by B cells is maximal when the cells were stimulated with the combination of $F(ab')_2$ fragments of anti-Ig and anti-CD40, with the level almost double that of the combination of $F(ab')_2$ anti-Ig plus IL-4, and around five times that of $F(ab')_2$ anti-Ig alone. This is also the case for DNA synthesis, with the combination of $F(ab')_2$ fragments of anti-Ig and anti-CD40 inducing far more DNA

synthesis than $F(ab')_2$ anti-Ig alone or in combination with IL-4. This underlines the physiological importance of T cell derived signals in the activation of B cells.

Not only are T cell derived signals important for the induction of B cell activation, they also play a major role in the ongoing B cell response to antigen, as evidenced by the duration of proliferation when T cell derived signals are present. Stimulation with $F(ab')_2$ fragments of anti-Ig alone results in proliferation of B cells, but not to the same levels as when IL-4 or anti-CD40 are present, and the duration is greatly reduced in the absence of T cell derived signals. Even by 72 hours, the response to $F(ab')_2$ fragments of anti-Ig alone is diminishing, however, in the presence of IL-4, the response stays high until 120 hours, before falling by 168 hours. Again, this underlines the importance of the help signals produced by T cells in the activation of B cells. It would be very interesting to assess the number of divisions B cells undergo over this period. This was attempted using the CFSE fluorescent molecule, which can be incorporated into cells prior to stimulation, and the decrease in fluorescence in successive cell divisions assessed by flow cytometry. This was attempted, but encountered difficulties due to cytotoxic effects of CFSE on B cells, as well as variable uptake of the dye by the cells prior to stimulation. The uptake conditions require optimisation for B cells to ensure minimal cytotoxicity, and it may also be useful to select a population of cells with a narrow range of fluorescence with a cell sorting machine prior to stimulation. This would help ensure that defined peaks can be observed after each subsequent division, as another of the difficulties arose from the lack of distinct peaks after stimulation, as there was a large range of fluorescence at the outset.

In order to identify the relative roles of the various signalling pathways involved in B cell proliferation, we used specific inhibitors to prevent activation of key kinases, then assessed the effects on B cell proliferation by means of the [^{3}H]-thymidine incorporation assay. The MEK inhibitors, PD98059 and U0126, completely abrogated $F(ab')_2$ anti-Ig induced proliferation of B cells,

demonstrating the importance of Erk-MAPKinase signalling in the B cell proliferative response to antigen. Both the p38 inhibitor, SB203580, and the PI-3-Kinase inhibitor, LY294002, inhibited BCR induced proliferation to a certain extent, but not to anywhere near the level of the MEK inhibitors, further underscoring the importance of Erk-MAPKinase in B cell activation. The ability of the stimulation via BCR to induce sustained activation of Erk-MAPKinase activity strongly suggest that, as in WEHI-231 immature B cells, sustained Erk-MAPKinase activity plays a role in the proliferation of mature B cells. The apparent translocation of Erk-MAPKinase to an area adjacent to the nucleus upon sustained activation is rather intriguing, and is similar to findings in other systems whereby sustained activation of Erk-MAPKinase is associated with translocation to the nucleus (Shah *et al.*, 2003). However, in contrast with our findings that sustained Erk-MAPKinase activity is associated with proliferation, in a number of other systems transient Erk-MAPKinase activity usually leads to proliferation, whereas sustained Erk-MAPKinase activity often elicits differentiation (Kao *et al.*, 2001; Liang *et al.*, 2002; York *et al.*, 1998).

Erk-MAPKinase activity can be modulated by many signalling elements, including cyclic AMP (cAMP). cAMP is generated at the plasma membrane by adenylate cyclases, a large family of enzymes, and is degraded is through the action of various cyclic nucleotide phosphodiesterases (PDEs), of which several families also exist. The importance of PDEs in many cellular processes including the regulation of Erk-MAPKinase activity (Houslay and Kolch, 2000) has been reinforced recently with the emergence of inhibitors of these enzymes, particularly the PDE4 family, as having potential therapeutic value in treating a number of inflammatory diseases, such as asthma, as well as possessing anti-depressant properties (Houslay and Baillie, 2003). Recently, it has been demonstrated that a novel PDE family member, PDE7, plays a key role in T cell activation, as PDE7 mRNA levels are up-regulated upon activation of human T cells, and blocking the induction of PDE7 abrogates activation of the cells (Li *et al.*, 1999). A role for PDE7 in B cells has been suggested due to the fact that

PDE7 is expressed in human B cells, and is upregulated by elevation of intracellular cAMP levels (Lee *et al.*, 2002). Indeed, it appears as though PDE7 may also play a role in regulating B cell proliferation, as $F(ab')_2$ anti-Ig induced proliferation was reduced when cells were treated with PDE7 inhibitors. The inhibitors used in this study had widely different efficacies, with PD0322408 producing the most dramatic effect (**Figure 5.5**), though only PD0329870 did not greatly inhibit BCR-mediated proliferation. This inhibition appears largely to be the result of the induction of apoptosis. Due to the highly variable efficacies of the inhibitors, however, it was decided not to follow up the role of PDE7 in regulating B cell proliferation by Erk-MAPKinase signalling. Nevertheless, IL-4 was unable to overcome this inhibition, however, CD40-mediated signals did appear to provide some measure of protection. This suggests that some signalling pathway activated in response to CD40 ligation, but not ligation of the IL-4 receptor, provides a signal that can override inhibition of PDE7.

The low affinity receptor for IgG, FcγRIIb, negatively regulates BCR-mediated proliferative signalling, and as such provides a negative feedback mechanism whereby co-crosslinking of FcγRIIb with the BCR by antigen-antibody complexes inhibits antigen-driven responses and may act to prevent progression or induction of certain autoimmune diseases (Ravetch and Bolland, 2001). The understanding of the mechanisms underlying FcγRIIb-mediated suppression of BCR signalling are beginning to be fairly well elucidated, at least in terms of the initial signalling events. How these events impinge on downstream targets, such as the cell cycle machinery, is less well understood. Experimentally this can be demonstrated by stimulating B cells with a combination of $F(ab')_2$ fragments of anti-Ig and intact anti-Ig antibodies to cross-link the BCR and FcγRIIb, as the presence of intact anti-Ig antibodies leads to inhibition of $F(ab')_2$ anti-Ig-induced proliferation. One of the ways this is achieved is by the rapid inactivation of Erk-MAPKinase, as assessed by Western blotting using a phospho-Erk antibody. This was further evidenced by imaging studies, where we demonstrate that co-ligation of FcγRIIb with the BCR produces an effect very similar to that induced

by the MEK inhibitor PD98059 in that sustained Erk-MAPKinase signalling is abolished (**Figure 5.12**). FcγRIIb recruitment of SHIP is proposed to disrupt Ras/MAPKinase mediated proliferative signalling by sequestering Shc, one of the adaptor molecules responsible for coupling the BCR to the Ras/MAPKinase pathway (Tridandapani *et al.*, 1997), and by the recruitment of the RasGAP binding protein $p62^{Dok}$, to promote inactivation of Ras (Tamir *et al.*, 2000). It has also been recently demonstrated by this laboratory that FcγRIIb recruits the nuclear phosphatase, Pac-1, which is capable of dephosphorylating and inactivating Erk-MAPKinase (Brown *et al.*, 2004). Thus, FcγRIIb utilises multiple mechanisms to abrogate Erk-MAPKinase activity, both by inhibiting the upstream activation of Erk and terminating any ongoing Erk-MAPKinase activity (**Figure 5.17**).

We investigated the effects of signalling via the BCR and/or FcγRIIb on a number of potential downstream targets of Erk-MAPKinase that might impact on growth arrest, such as cyclin D and $p27^{Kip1}$. The results of the ribonuclease protection assay (RPA) investigating the expression patterns of such key cell cycle regulators at the mRNA level at first appear very contradictory (**Figure 5.14**). Despite inducing proliferation, it appears as though stimulation with $F(ab')_2$ fragments of anti-Ig does not increase the expression of either cyclin D2 or cyclin D3, which is surprising as cyclin D2 has been reported to be essential for BCR-mediated proliferation in that cyclin $D2^{-/-}$ B cells fail to proliferate in response to anti-Ig stimulation (Solvason *et al.*, 2000). Interestingly, development of conventional B cells in these mice was normal, however, their B-1 B cell compartment was significantly reduced, perhaps indicating a particular role for cyclin D2 in the development of this population of B cells, which are self-renewing (Solvason *et al.*, 2000). The RPA only assesses mRNA levels, however, therefore the protein levels of cyclin D2 may increase without there being a detectable increase in the levels of the mRNA due to instability of the mRNA. Similarly, the apparent increase in $p27^{Kip1}$ expression in $F(ab')_2$ stimulated cells after 16 hours also appears counter intuitive, as $p27^{Kip1}$

accumulation is associated with G_1 arrest. However, the regulation of p27 is highly complex, involving both translational control and regulation of the stability of the protein, so an increase in the transcription does not necessarily lead to an increase in the protein levels. Furthermore, although $p27^{Kip1}$ is a potent inhibitor of cyclin E/Cdk2 complexes, it is also an essential assembly factor for cyclin D/CDK4 complexes, so this increase in expression could be required for the assembly of the cyclin D/CDK4 complex, allowing the initiation of phosphorylation of Rb. Further work would have to include the use of antibodies specific for key phosphorylated residues of Rb, such as Ser^{807} and Ser^{811}, to assess fully the phosphorylation status of Rb in response to stimulation with $F(ab')_2$ fragments of anti-Ig, both alone and in combination with intact anti-Ig.

A key regulator of the mammalian cell cycle involved in the regulation of cyclin dependent kinase inhibitor expression and activity is the tumour suppressor protein, p53 (Levine, 1997). The observed activation of the p53 protein induced by ligation of FcγRIIb (**Figure 5.13B**) suggests that one of the mechanisms by which FcγRIIb induces growth arrest is via the p53 pathway. p53 plays a key role in cellular responses to many types of stress, including DNA damage and inappropriate proliferative signals, and has the ability to induce cell cycle arrest, DNA repair, or apoptosis, depending on a number of factors, including cell type (Bates and Vousden, 1996). This activation of p53 may play a role in the down-regulation of Erk-MAPKinase activity, as it has recently been shown that the dual specificity protein phosphatase MKP1, which is capable of inactivating Erk-MAPKinase, is a transcriptional target of p53 (Li *et al.*, 2003). Another transcriptional target of p53 is the phosphatase, Pac-1, which is also capable of inactivating Erk-MAPKinase (Yin *et al.*, 2003), and which has recently been shown by this laboratory to be activated by FcγRIIb, resulting in rapid dephosphorylation and inactivation of Erk-MAPKinase, uncoupling Erk-MAPKinase activity from the BCR (Brown *et al.*, 2004).

Another functional outcome of p53 activation is apoptosis, which can also be induced by co-ligation of the BCR with FcγRIIb. One of the earliest, non-reversible steps in apoptosis is the depolarisation of the mitochondrial membrane potential ($\Delta\psi_m$), which can be detected using cationic lipophilic dyes such as $DiOC(6)_3$ (Zamzami *et al.*, 1995). It appears as though the apoptosis induced by FcγRIIb follows this mitochondrial pathway, as stimulation of B cells with intact antibody, either alone or in combination with $F(ab')_2$ fragments of anti-Ig, leads to reduction in $\Delta\psi_m$. This may be linked to the activation of p53, as p53 has been associated with mitochondrial function, as it has been shown to translocate to the mitochondria (Marchenko *et al.*, 2000), where it may induce a rise in oxidative species and degradation of mitochondrial components (Polyak *et al.*, 1997). p53 is also capable of inducing the expression of Bax, a pro-apoptotic member of the Bcl-2 family (Miyashita and Reed, 1995), as well as negatively regulating the expression of Bcl-2, an anti-apoptotic member of the family (Miyashita *et al.*, 1994). The results of the RPA assay investigating the expression of apoptotic regulators agrees with this, as p53 expression is greatly increased, and the western blotting studies show p53 is activated in response to FcγRIIb ligation by phosphorylation on serine 15, abrogating the interaction between MDM2 and p53, presumably leading to the observed increase in the transcription of Bax (**Figure 5.16**). This strongly suggests that one mechanism by which FcγRIIb induces apoptosis is by the activation of p53, which in turn leads to the increased expression of Bak which can then proceed to disrupt mitochondrial function (**Figure 5.17**).

Overall, the results obtained in this chapter further demonstrate the importance of T cell derived signals in the initiation and maintenance of a B cell response to antigen. The importance of sustained Erk-MAPKinase in promoting B cell proliferation is also underlined, both by studies utilising MEK inhibitors and studies using the FcγRIIb receptor to inhibit BCR-mediated proliferation. Finally, the ability of FcγRIIb to induce B cell anergy, growth arrest and/or apoptosis can

be explained, at least in part, by its modulation of BCR-activated cell cycle regulatory proteins and important apoptotic factors.

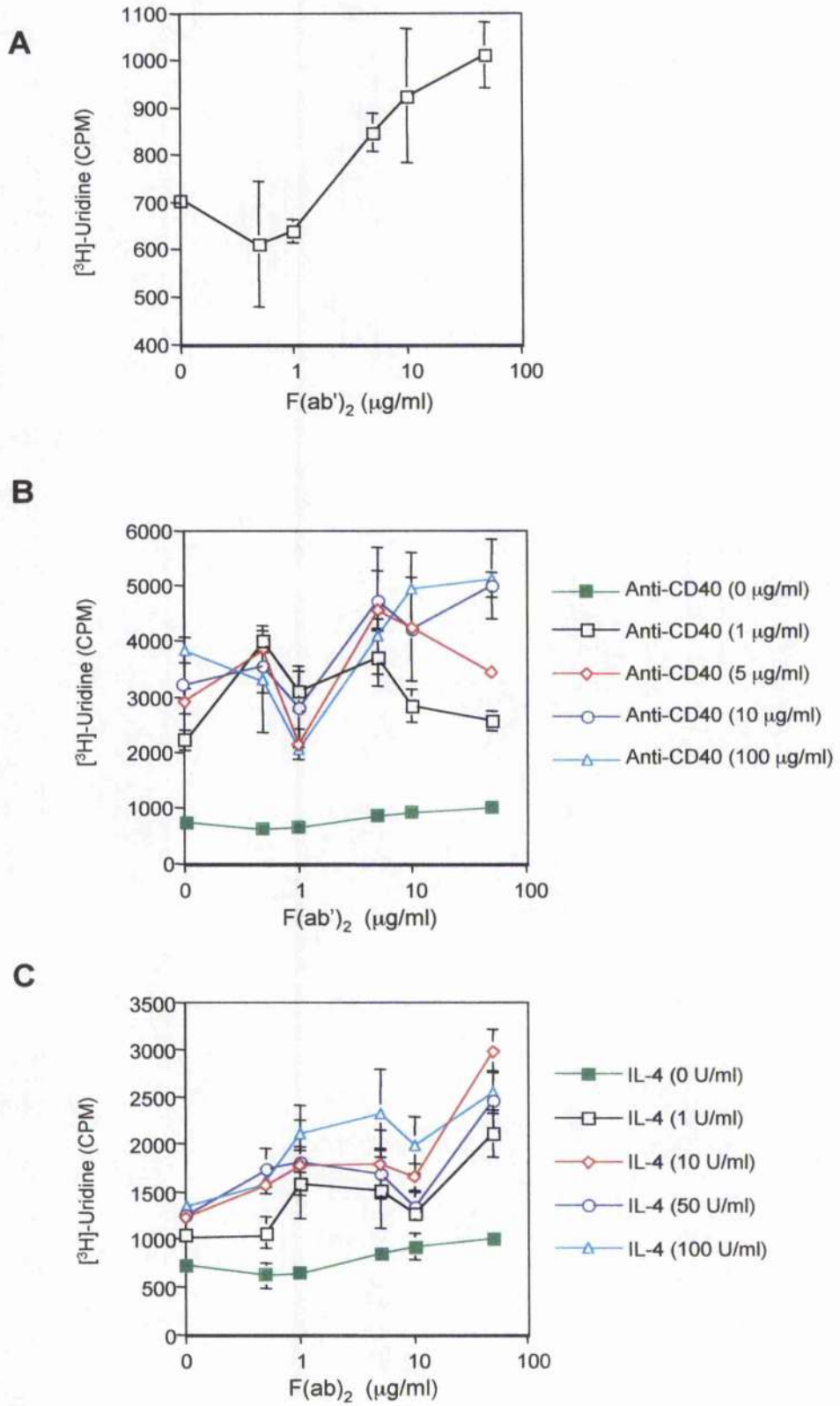
Figure 5.1 Ligation of the BCR induces RNA synthesis in primary splenic B cells, and this is enhanced by helper T cell signals

Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 24 hours in the presence of the indicated agonists. Culture wells were pulsed with [^{3}H]-uridine (1 μCi/well) 4 hours prior to harvesting and [^{3}H]-uridine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from the same experiment, representative of at least two other independent experiments.

A Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific).

B Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of anti-CD40 antibody (FGK45).

C Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of recombinant IL-4.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1000
900
800
700
600
500
400
[3H]-Uridine (CPM)
0
1
10
100
F(ab')2 (μg/ml)
B
6000
5000
4000
3000
2000
1000
0
[3H]-Uridine (CPM)
0
1
10
100
F(ab')2 (μg/ml)
Anti-CD40 (0 μg/ml)
Anti-CD40 (1 μg/ml)
Anti-CD40 (5 μg/ml)
Anti-CD40 (10 μg/ml)
Anti-CD40 (100 μg/ml)
C
3500
3000
2500
2000
1500
1000
500
0
[3H]-Uridine (CPM)
0
1
10
100
F(ab)2 (μg/ml)
IL-4 (0 U/ml)
IL-4 (1 U/ml)
IL-4 (10 U/ml)
IL-4 (50 U/ml)
IL-4 (100 U/ml)

Figure 5.2 BCR induced proliferation is enhanced by signals emanating from helper T cells

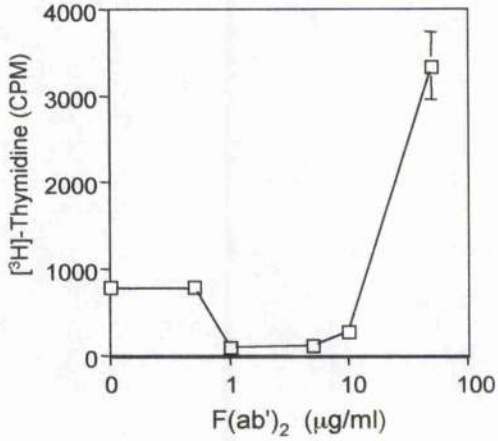
Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours in the presence of the indicated agonists. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from the same experiment, representative of at least two other independent experiments.

A Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific).

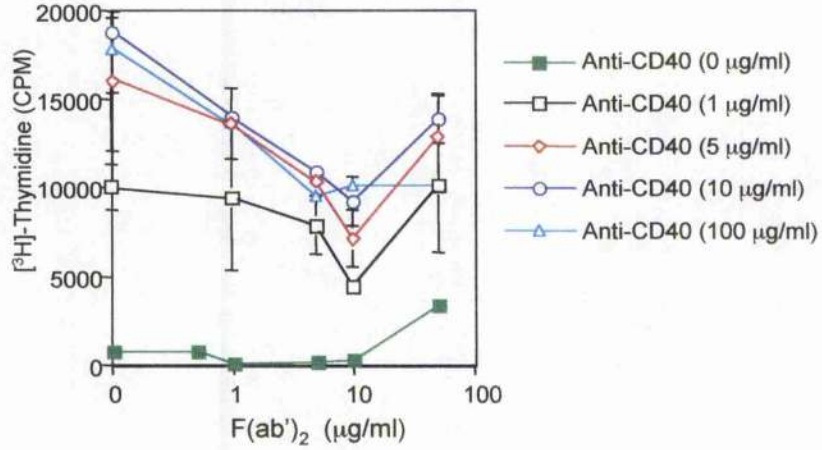
B Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of anti-CD40 antibody (FGK45).

C Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of recombinant IL-4.

A


4000
3000
2000
1000
0
[3H]-Thymidine (CPM)
0
1
10
100
F(ab')2 (μg/ml)

B


20000
15000
10000
5000
0
[3H]-Thymidine (CPM)
0
1
10
100
F(ab')2 (μg/ml)
Anti-CD40 (0 μg/ml)
Anti-CD40 (1 μg/ml)
Anti-CD40 (5 μg/ml)
Anti-CD40 (10 μg/ml)
Anti-CD40 (100 μg/ml)

C

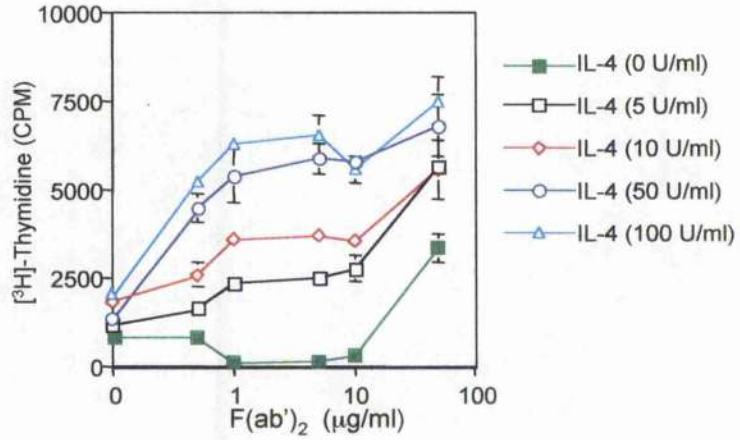

10000
7500
5000
2500
0
[3H]-Thymidine (CPM)
0
1
10
100
F(ab')2 (μg/ml)
IL-4 (0 U/ml)
IL-4 (5 U/ml)
IL-4 (10 U/ml)
IL-4 (50 U/ml)
IL-4 (100 U/ml)

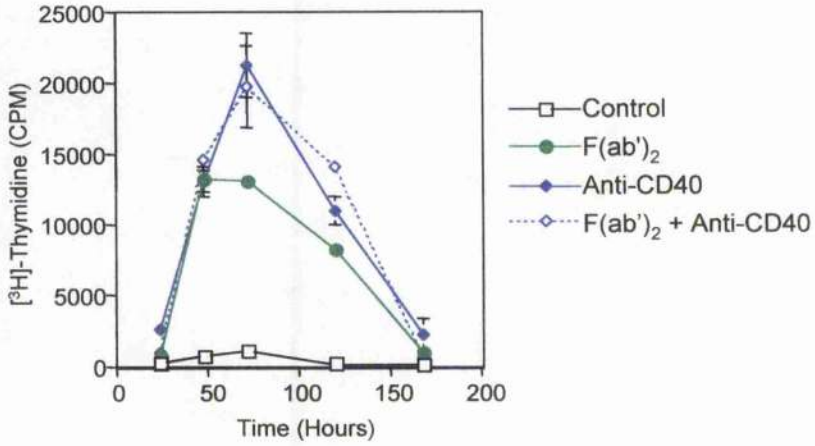
Figure 5.3 BCR induced proliferation is sustained by signals emanating from helper T cells

Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for up to 168 hours in the presence of the indicated agonists. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting at 24, 48, 72, 120 or 168 hours, and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from the same experiment, representative of at least two other independent experiments.

A Cells stimulated with F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml), anti-CD40 antibody (FGK45) (10 μg/ml), or a combination of both.

B Cells stimulated with F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml), recombinant IL-4 (10 U/ml), or a combination of both.

A


25000
20000
15000
10000
5000
0
[3H]-Thymidine (CPM)
0
50
100
150
200
Time (Hours)
Control
F(ab')2
Anti-CD40
F(ab')2 + Anti-CD40

B

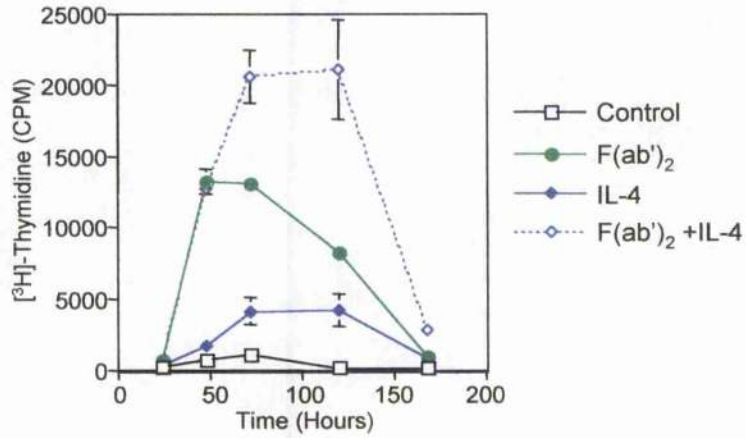

25000
20000
15000
10000
5000
0
[3H]-Thymidine (CPM)
0
50
100
150
200
Time (Hours)
Control
F(ab')2
IL-4
F(ab')2 +IL-4

Figure 5.4 Specific kinase inhibitors can abrogate BCR-mediated proliferation of murine primary splenic B cells

Purified splenic B cells from Balb/c mice (2 x 10^5 cells/well) were stimulated with 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), either alone or in the presence of the MEK inhibitor PD98059 (10 μM), the MEK inhibitor U0126 (1 μM), the PI-3-Kinase inhibitor LY294002 (1 μM), or the p38 inhibitor SB203580 (5 μM), for 48 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from the same experiment, representative of at least two other independent experiments.

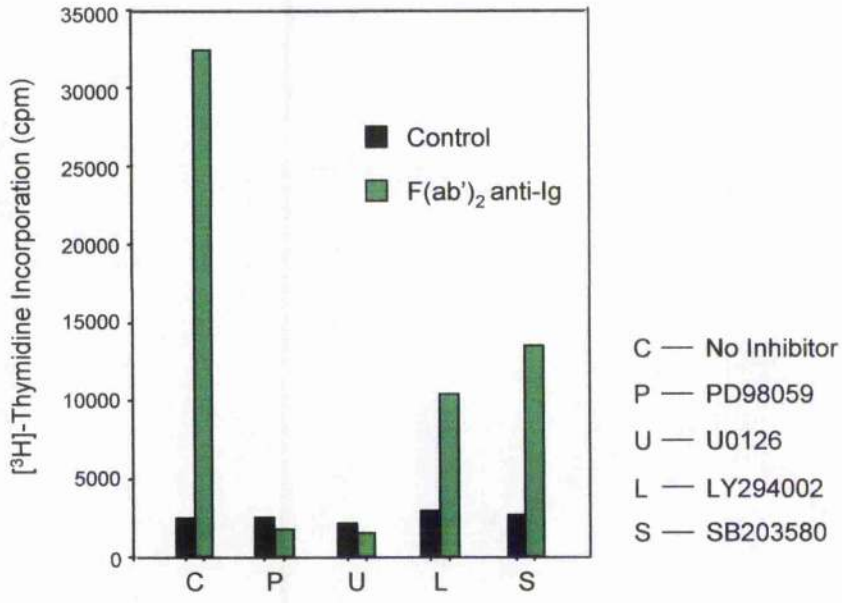

35000
30000
25000
20000
15000
10000
5000
0
[3H]-Thymidine Incorporation (cpm)
Control
F(ab')2 anti-Ig
C
P
U
L
S
C — No Inhibitor
P — PD98059
U — U0126
L — LY294002
S — SB203580

Figure 5.5 The effects of inhibition of PDE7 on the proliferative response of B cells to stimulation

Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours in the presence of the indicated agonists. The concentrations of the agonists were $F(ab')_2$ fragments of goat anti-mouse IgM (50 μg/ml), IL-4 (10 U/ml), Anti-CD40 (10 μg/ml), LPS (50 μg/ml). Control cells were cultured in the presence of medium alone. In addition, cells were cultured in the presence of a variety of specific PDE7 inhibitors (10 μM), as a control some cells received no PDE7 inhibitor. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least one other independent experiment.

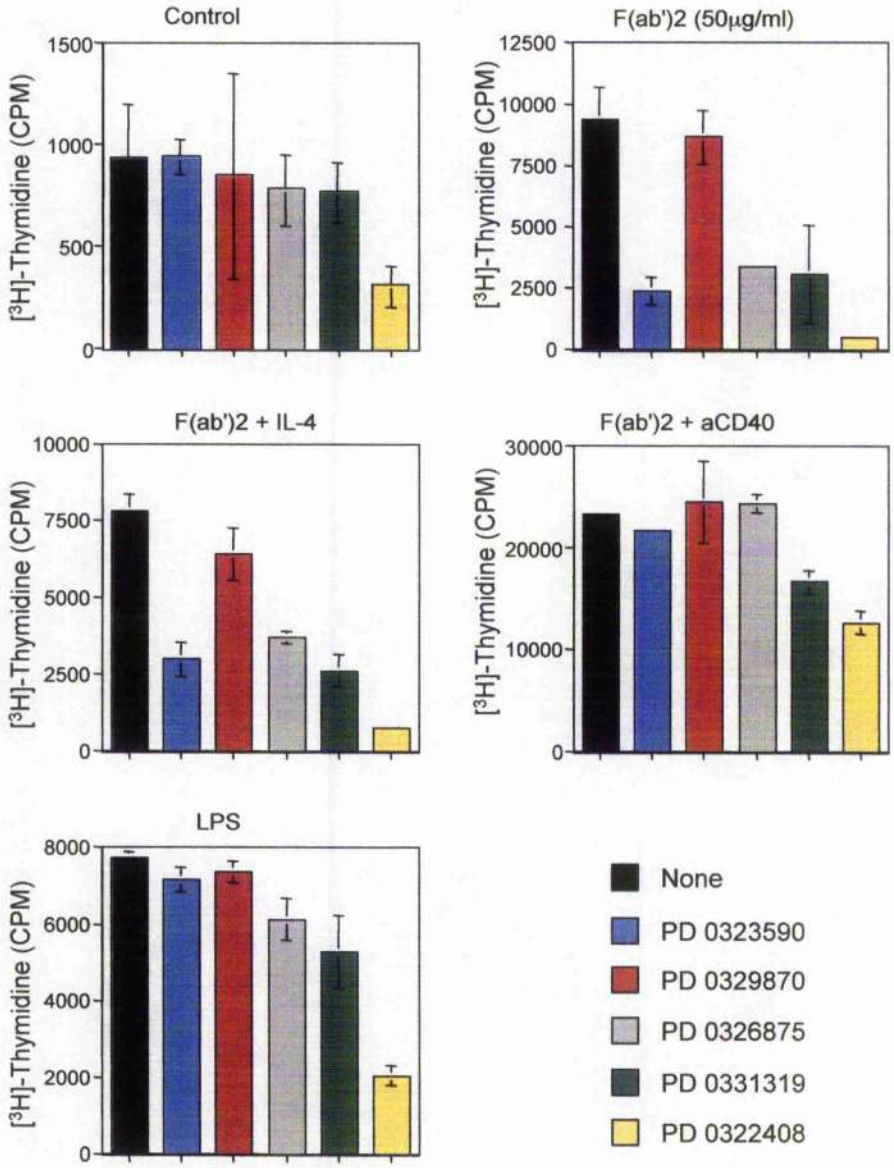
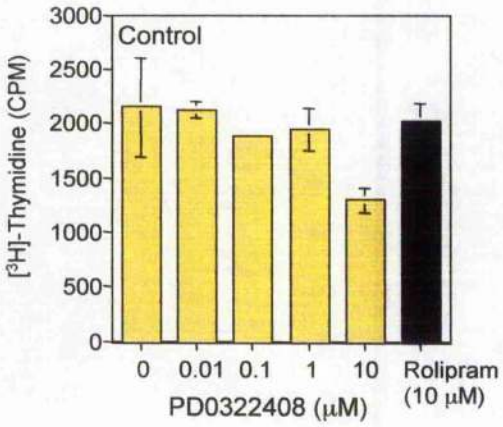
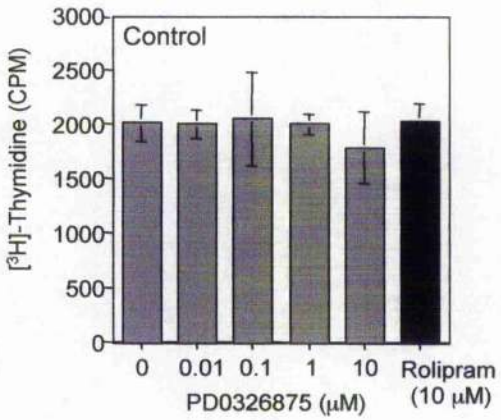
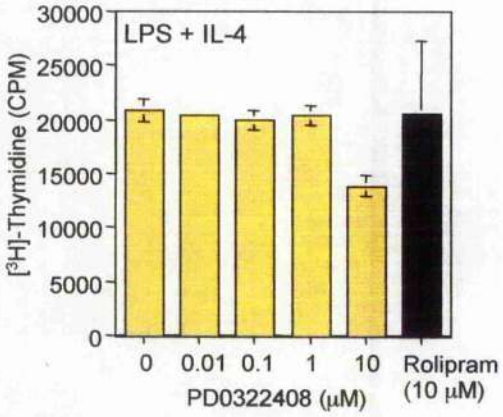

Control
F(ab')2 (50μg/ml)
F(ab')2 + IL-4
F(ab')2 + aCD40
LPS
[3H]-Thymidine (CPM)
0
500
1000
1500
2500
5000
7500
10000
12500
20000
30000
2000
4000
6000
8000
None
PD 0323590
PD 0329870
PD 0326875
PD 0331319
PD 0322408

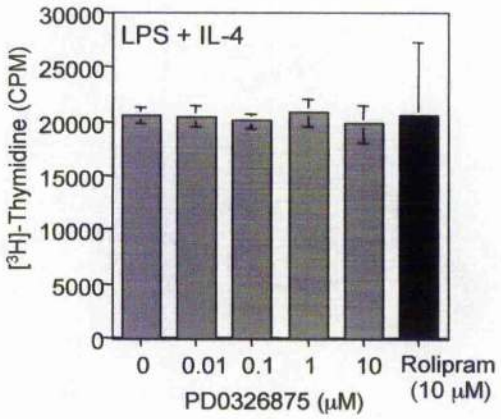
Figure 5.6 The effects of the PDE7 inhibitors PD0322408 and PD0326875 on the proliferative responses of B cells to stimulation

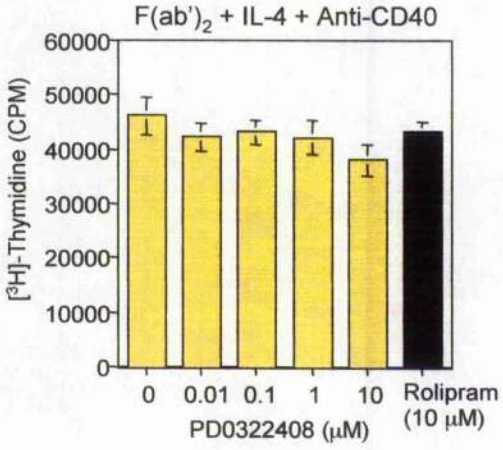
Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours in the presence of the indicated agonists. The concentrations of the agonists were $F(ab')_2$ fragments of goat anti-mouse IgM (50 μg/ml), IL-4 (10 U/ml), Anti-CD40 (10 μg/ml), LPS (50 μg/ml). Control cells were cultured in the presence of medium alone. In addition, cells were cultured in the presence of a the specific PDE7 inhibitors PD0322408 or PD0326875 over a range of concentrations (0.01, 0.1, 1, and 10 μM). As a control some cells received no PDE7 inhibitor, whilst others received the specific PDE4 inhibitor, rolipram (10 μM). Culture wells were pulsed with [^{3}H]-Thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least one other independent experiment.


Control
[3H]-Thymidine (CPM)
3000
2500
2000
1500
1000
500
0
0 0.01 0.1 1 10 Rolipram
PD0322408 (μM) (10 μM)


Control
[3H]-Thymidine (CPM)
3000
2500
2000
1500
1000
500
0
0 0.01 0.1 1 10 Rolipram
PD0326875 (μM) (10 μM)


LPS + IL-4
[3H]-Thymidine (CPM)
30000
25000
20000
15000
10000
5000
0
0 0.01 0.1 1 10 Rolipram
PD0322408 (μM) (10 μM)


LPS + IL-4
[3H]-Thymidine (CPM)
30000
25000
20000
15000
10000
5000
0
0 0.01 0.1 1 10 Rolipram
PD0326875 (μM) (10 μM)


F(ab')2 + IL-4 + Anti-CD40
[3H]-Thymidine (CPM)
60000
50000
40000
30000
20000
10000
0
0 0.01 0.1 1 10 Rolipram
PD0322408 (μM) (10 μM)

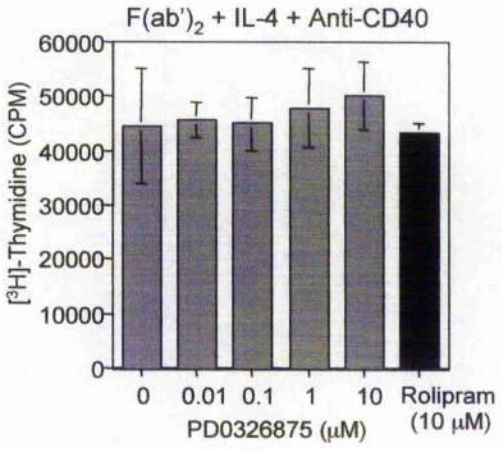

F(ab')2 + IL-4 + Anti-CD40
[3H]-Thymidine (CPM)
60000
50000
40000
30000
20000
10000
0
0 0.01 0.1 1 10 Rolipram
PD0326875 (μM) (10 μM)

Figure 5.7 The effects of inhibition of PDE7 on the viability of B cells in response to antigenic stimulation

Purified splenic B cells from Balb/c mice (1 x 10^6 cells/well) were stimulated for 48 hours with 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific), either alone (2) or in combination with anti-CD40 (10 μg/ml) (4), or with LPS alone (50μg/ml) (3), in the presence of various specific PDE7 inhibitors (10 μM). Control cells were cultured in the presence of medium alone (1). DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total cells analysed from a single experiment, representative of at least one other independent experiment.

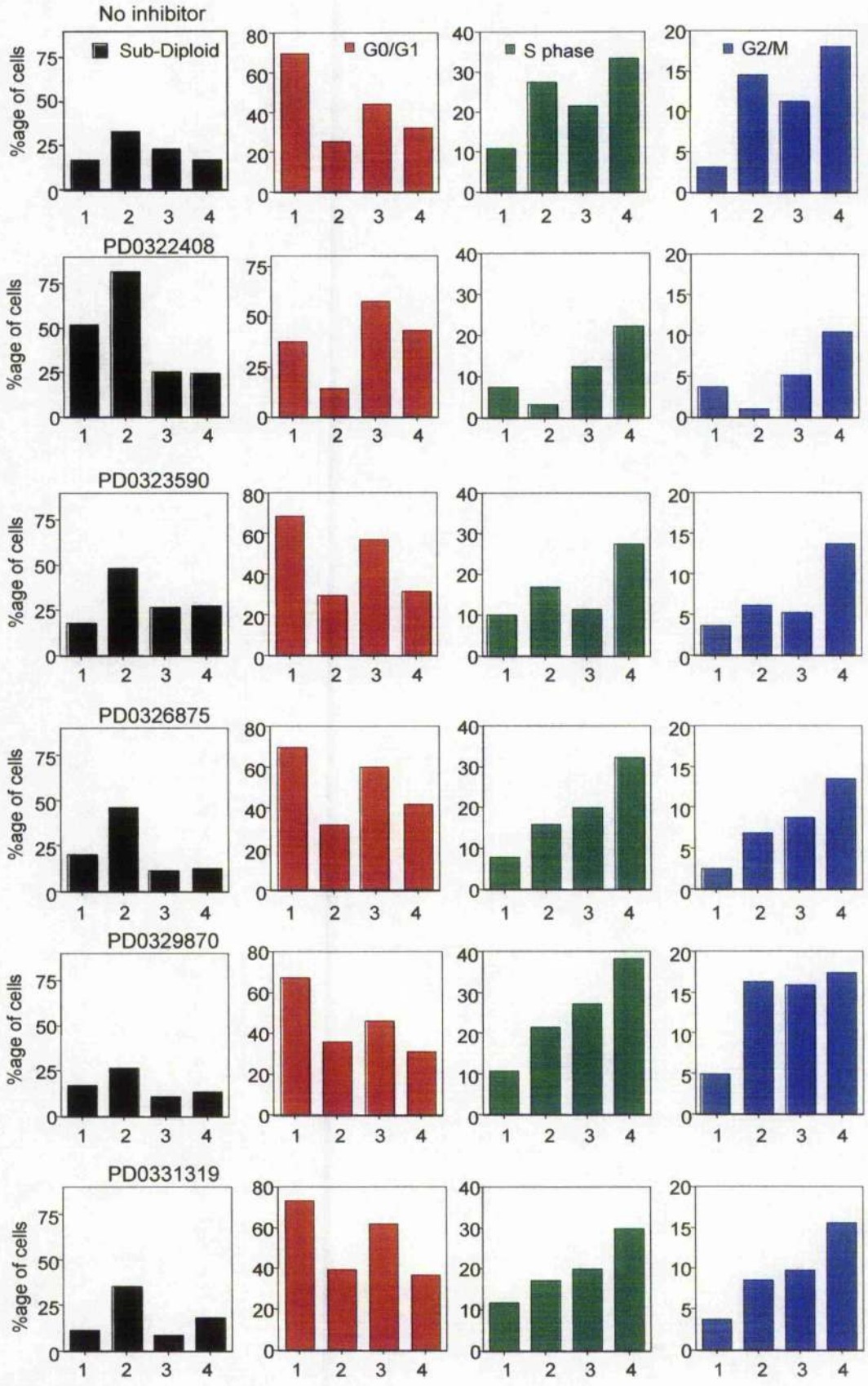

No inhibitor
Sub-Diploid
G0/G1
S phase
G2/M
%age of cells
PD0322408
PD0323590
PD0326875
PD0329870
PD0331319

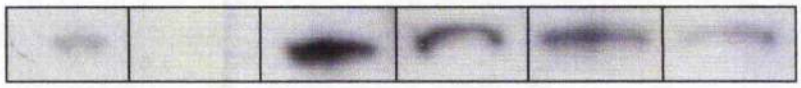
Figure 5.8 Effect of prolonged BCR and FcγRIIb co-ligation and the MEK inhibitor PD98059 on the phosphorylation of Erk-MAPKinase

A Purified splenic B cells from Balb/c mice (2 x 10^5 cells/well) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), in the presence or absence of the MEK inhibitor PD98059 (10 μM), for the times indicated. Control cells were cultured in the presence of medium alone. Whole cell lysates were subjected to Western blot analysis with phospho-specific Erk-MAPKinase antibody.

B Purified splenic B cells from Balb/c mice (2 x 10^5 cells/well) were stimulated in the presence or absence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), in the presence or absence of the MEK inhibitor PD98059 (10 μM), for 24 hours. Control cells were cultured in the presence of medium alone. Whole cell lysates were subjected to Western blot analysis with phospho-specific Erk-MAPKinase antibody.

C Purified splenic B cells from Balb/c mice (2 x 10^6 cells/ml) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), in the presence or absence of the MEK inhibitor PD98059 (10 μM), for 16 hours. Control cells were cultured in the presence of medium alone. Cells were adhered to slides, fixed and permeabilised, then stained for phospho-Erk and the nucleus stained with DAPI, as described in Materials and Methods. Images were acquired using the LSC with Openlab 3.0.9 software (Improvision, Coventry, U.K.). Phospho-Erk appears as green, the nucleus appears as blue.

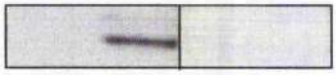
A



PD98059 – + – + – +

Time (Hours) 6 17 20

B



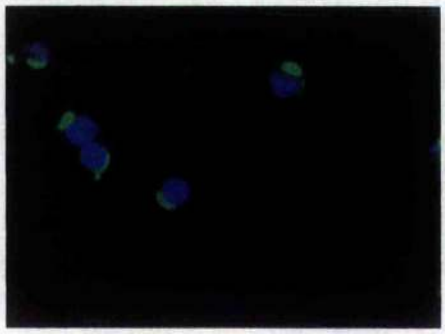
24 Hours

$F(ab')_2$ - + - +

PD98059 - - + +

C

$F(ab')_2$



$F(ab')_2$ + PD98059

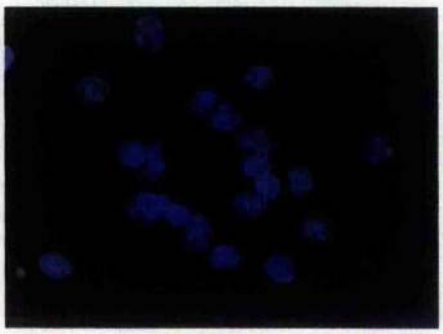


Figure 5.9 FcγRIIb co-ligation inhibits BCR-mediated B cell proliferation, which can be restored in the presence of a blocking anti-FcγRII/III antibody

Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours in the presence of the indicated antibodies. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least two other independent experiments.

A Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM(μ-chain specific).

B Cells stimulated with increasing concentrations of intact rabbit anti-mouse IgG/IgM.

C Cells stimulated with increasing concentrations of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in combination with increasing concentrations of intact rabbit anti-mouse IgG/IgM.

D Cells stimulated with 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM or 75 μg/ml intact rabbit anti-mouse IgG/IgM or 50 μg/ml anti-FcγRII/III (2.4G2) antibody either alone, or in combination, as indicated.

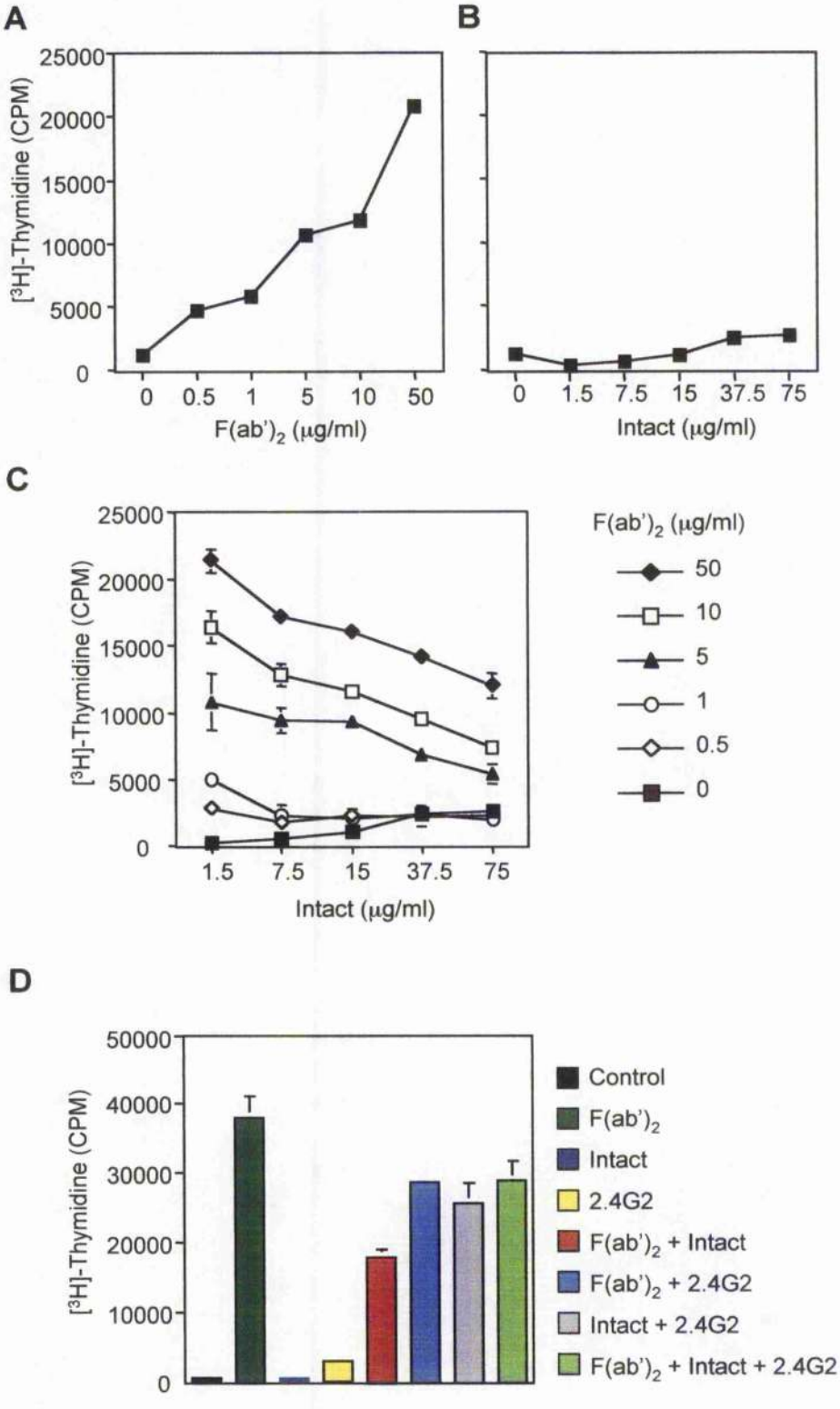

A
B
C
D
[3H]-Thymidine (CPM)
25000
20000
15000
10000
5000
0
0 0.5 1 5 10 50
F(ab')2 (μg/ml)
0 1.5 7.5 15 37.5 75
Intact (μg/ml)
F(ab')2 (μg/ml)
50
10
5
1
0.5
0
1.5 7.5 15 37.5 75
Intact (μg/ml)
50000
40000
30000
20000
10000
0
T
Control
F(ab')2
Intact
2.4G2
F(ab')2 + Intact
F(ab')2 + 2.4G2
Intact + 2.4G2
F(ab')2 + Intact + 2.4G2

Figure 5.10 Effects of BCR and FcγRIIb co-ligation on the DNA content of splenic B cells from Balb/c mice

Purified splenic B cells from Balb/c mice (1×10^6 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. As a control, an aliquot of cells was removed prior to any stimulation to permit DNA content analysis of unstimulated cells. DNA content was determined by propidium iodide staining and FACS analysis, as described in Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total cells analysed from a single experiment, representative of at least two other independent experiments.

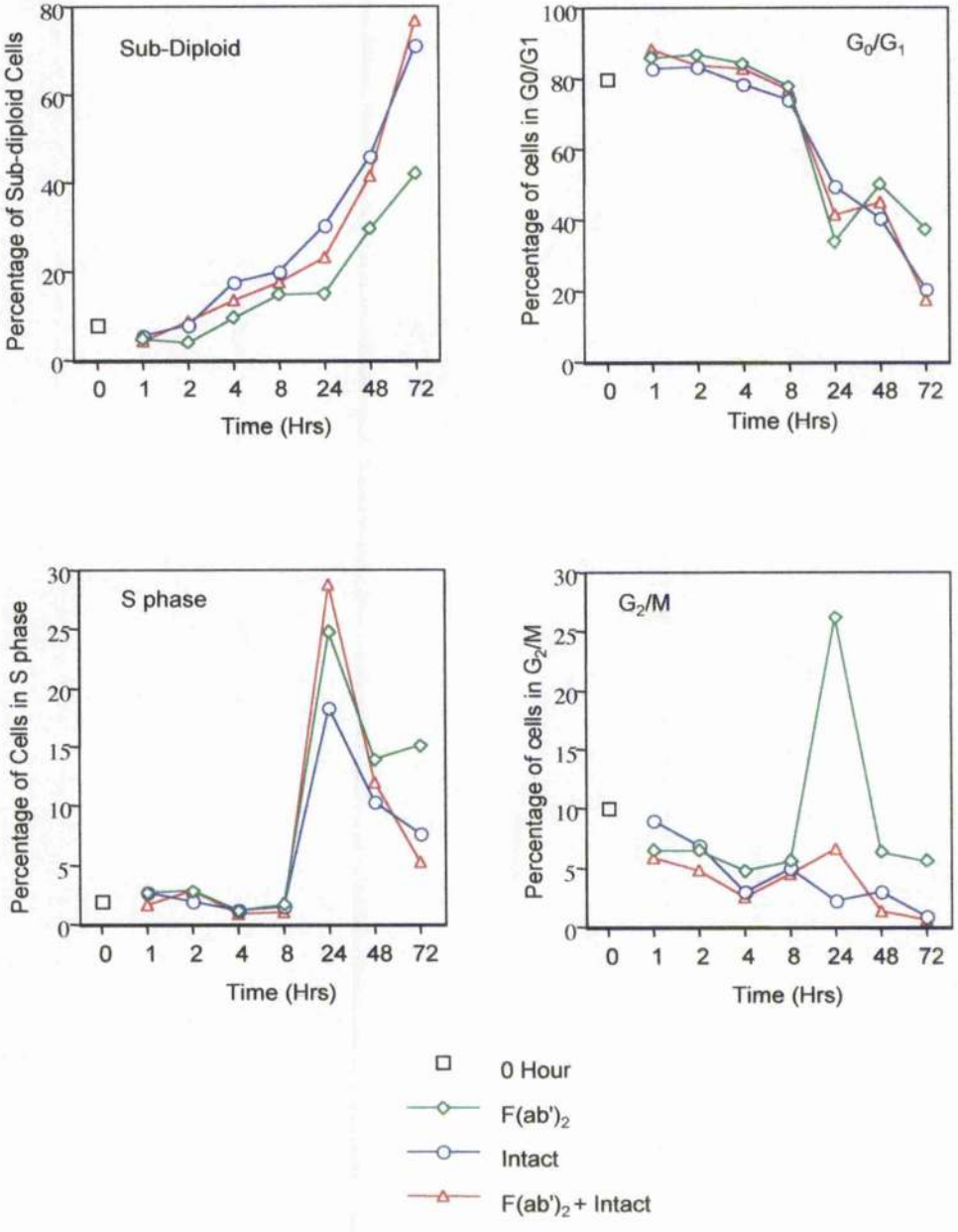

Sub-Diploid
Percentage of Sub-diploid Cells
Time (Hrs)
G_0/G_1
Percentage of cells in G0/G1
S phase
Percentage of Cells in S phase
G_2/M
Percentage of cells in G_2/M
0 Hour
$F(ab')_2$
Intact
$F(ab')_2$ + Intact

Figure 5.11 Effect of BCR and FcγRIIb co-ligation on the phosphorylation of Erk-MAPKinase

Purified splenic B cells from Balb/c mice (1×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), either alone or in combination with 75 μg/ml intact rabbit anti-mouse IgG/IgM, for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Whole cell lysates were subjected to Western blot analysis with phospho-specific Erk-MAPKinase antibody (upper panel). Blots were stripped and reprobed with total Erk-MAPKinase antibody (lower panel).

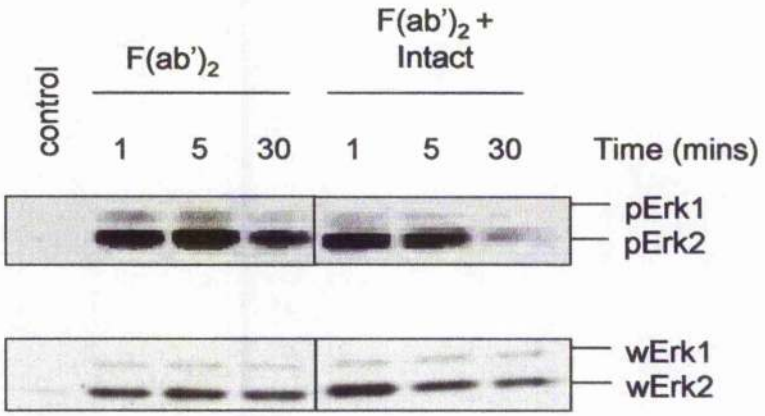

control
F(ab')2
F(ab')2 + Intact
1
5
30
1
5
30
Time (mins)
pErk1
pErk2
wErk1
wErk2

Figure 5.12 Effect of BCR and FcγRIIb co-ligation on the localisation of Erk-MAPKinase

Purified splenic B cells from Balb/c mice (2 x 10^6 cells/ml) were stimulated in the presence of 50 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific), either alone or in combination with 75 μg/ml intact rabbit anti-mouse IgG/IgM, in the presence or absence of the MEK inhibitor PD98059 (10 μM), for the times indicated. Control cells were cultured in the presence of medium alone. Cells were adhered to slides, fixed and permeabilised, then stained for ganglioside G_{M1}, phospho-Erk (with a monoclonal phospho-Erk antibody) and the nucleus stained with DAPI, as described in Materials and Methods. Images were acquired using the LSC with Openlab 3.0.9 software (Improvision, Coventry, U.K.). Ganglioside G_{M1} appears as red, phospho-Erk appears as green, and the nucleus appears as blue.

i Unstimulated, no PD908059
ii F(ab')$_2$ fragments of anti-Ig, no PD98059
iii F(ab')$_2$ fragments of anti-Ig + Intact antibody, no PD98059
iv Unstimulated, plus PD908059
v F(ab')$_2$ fragments of anti-Ig, plus PD98059
vi F(ab')$_2$ fragments of anti-Ig + Intact antibody, plus PD98059

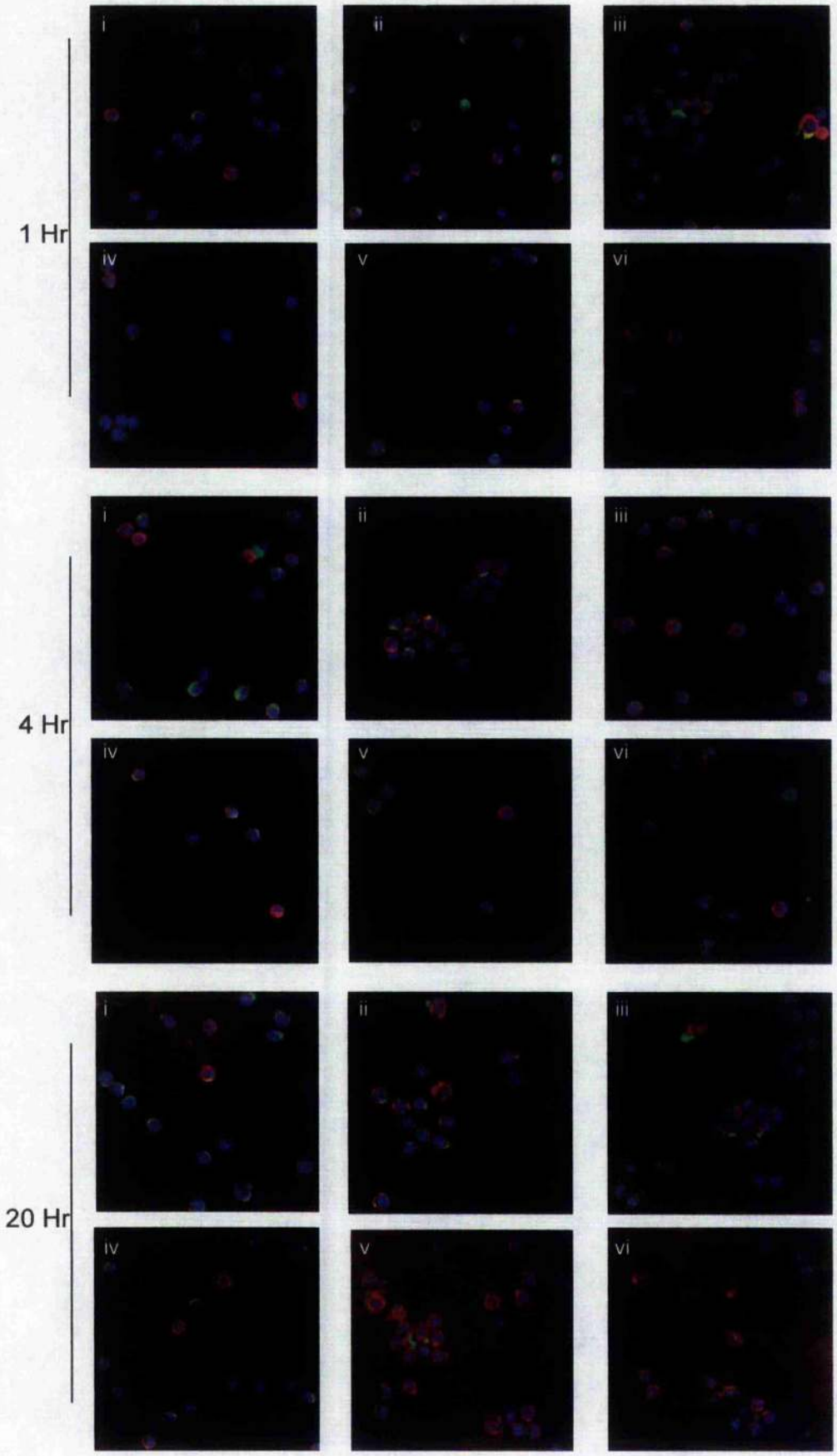

1 Hr
4 Hr
20 Hr
i
ii
iii
iv
v
vi

Figure 5.13 FcγRIIb ligation modulates BCR-mediated phosphorylation of Rb and p53.

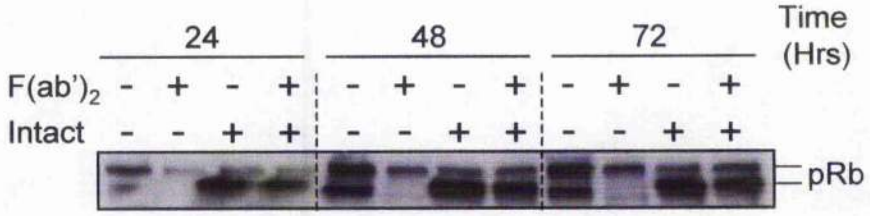
Purified splenic B cells from Balb/c mice (1×10^7 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM either alone, or in combination, for the times indicated. Cells stimulated in the presence of medium alone were included as a control.

A Whole cell lysates (50 μg/lane) were subjected to Western blot analysis with an anti-Rb antibody.

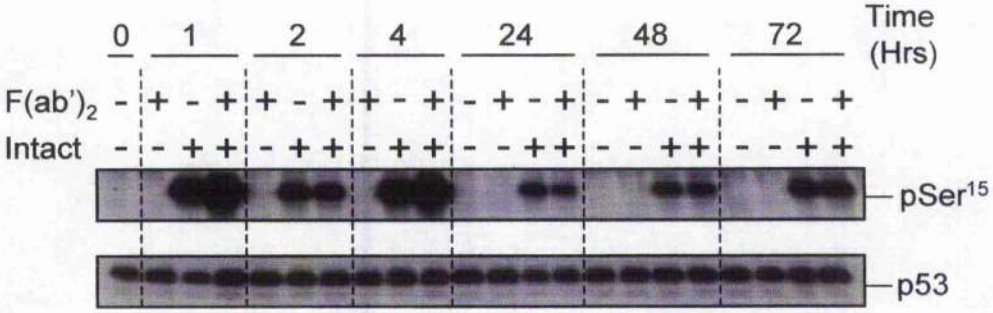
B Anti-p53 immune precipitates were subjected to Western blot analysis with a monoclonal anti-phospho-p53 (ser15) antibody, stripped and reprobed with an anti-p53 antibody.

C Whole cell lysates (50 μg/lane) were subjected to Western blot analysis with an anti-Cdk4 antibody.

A


24
48
72
Time (Hrs)
F(ab')2 - + - + - + - + - + - + -
Intact - - + + - - + + - - + +
pRb

B


0
1
2
4
24
48
72
Time (Hrs)
F(ab')2 - + - + + - + + - + - + - + - + - + - + - +
Intact - - + + - + + - + + - - + + - - + + - - + +
pSer15
p53

C

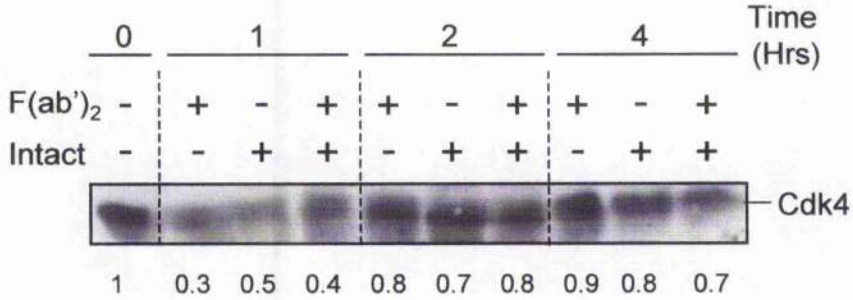

0
1
2
4
Time (Hrs)
F(ab')2 - + - + + - + + - +
Intact - - + + - + + - + +
Cdk4
1 0.3 0.5 0.4 0.8 0.7 0.8 0.9 0.8 0.7

Table 5.1 The multi-probe template set used in the RPA analysis

A list of the genes included in the mutli-probe template set used in the ribonuclease protection assay, including the size, in nucleotides, of the probe, and the size of the protected fragments after digestion.

Gene	Probe	Protected
CDK1	459	430
CDK2	417	388
FasL	381	352
p53	345	316
Bcl-X_L	299	272
Bak	231	202
Cyclin D2	210	181
Cyclin D3	189	160
p27	172	143
L32	141	112
GAPDH	125	97

Figure 5.14 Expression profiles of key cell cycle regulatory genes alter in response to BCR ligation and co-ligation with FcγRIIb

Purified splenic B cells from Balb/c mice (1×10^7 cells) were stimulated in the presence of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml), either alone or in combination with intact rabbit anti-mouse IgG/IgM (75 μg/ml), for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Total RNA was isolated from the cells at the times indicated, and the expression of key proteins assessed by means of the ribonuclease protection assay (RPA), as described in Materials and Methods (**Section 2.16**). The multi-probe template set used is shown in **Table 5.1**. Data were quantified using a Storm phosphorimager.

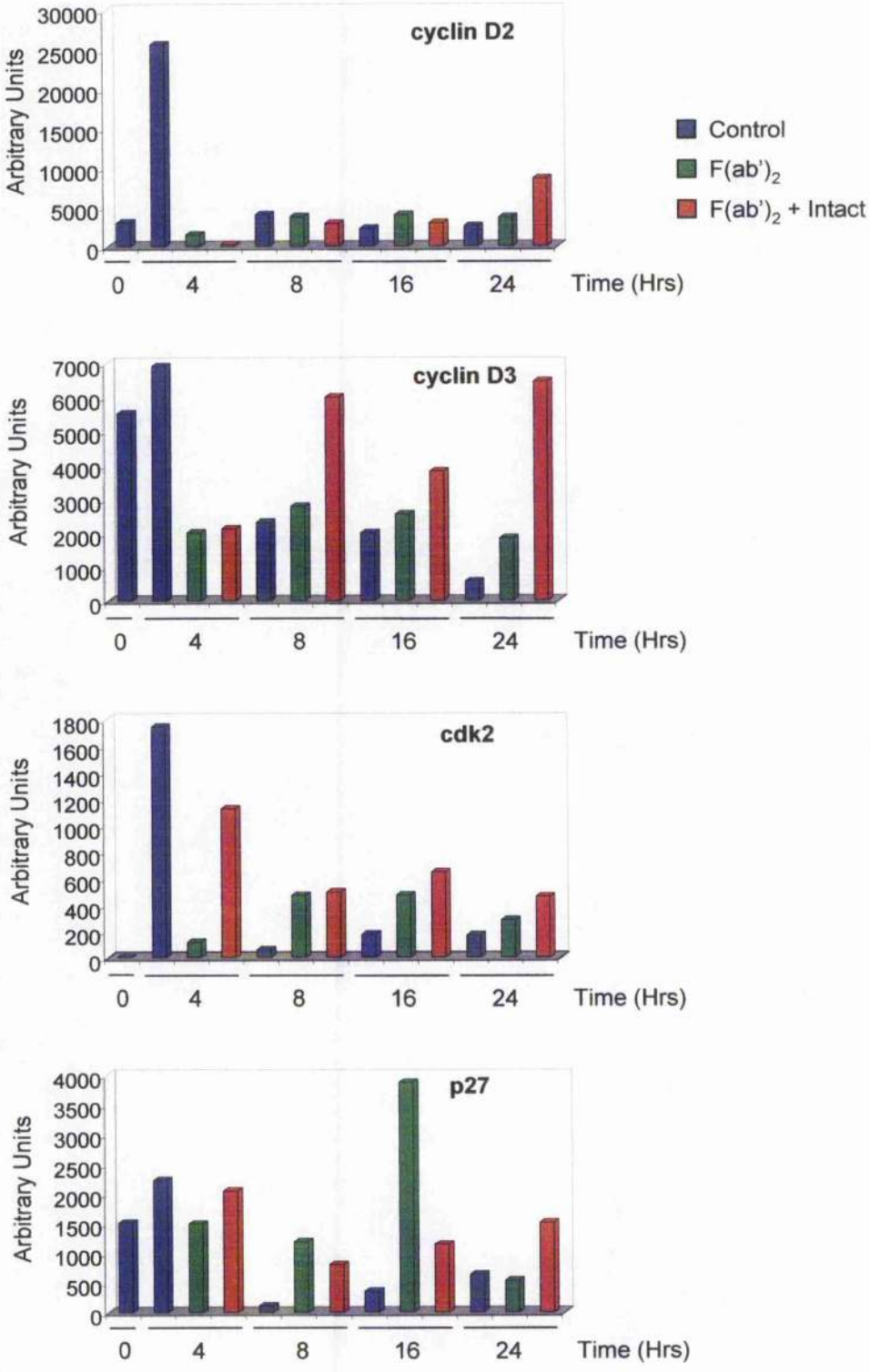

cyclin D2
Arbitrary Units
30000
25000
20000
15000
10000
5000
0
0
4
8
16
24
Time (Hrs)
Control
F(ab')2
F(ab')2 + Intact
cyclin D3
Arbitrary Units
7000
6000
5000
4000
3000
2000
1000
0
0
4
8
16
24
Time (Hrs)
cdk2
Arbitrary Units
1800
1600
1400
1200
1000
800
600
400
200
0
0
4
8
16
24
Time (Hrs)
p27
Arbitrary Units
4000
3500
3000
2500
2000
1500
1000
500
0
0
4
8
16
24
Time (Hrs)

Figure 5.15 FcγRIIb-mediated mechanisms of B cell inhibition result in a decrease in mitochondrial membrane potential

Purified splenic B cells from Balb/c mice (1×10^6 cells) were stimulated in the presence of 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) or 75 μg/ml intact rabbit anti-mouse IgG/IgM alone, or in combination as indicated for 72 hours. Cells stimulated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in Materials and Methods. Data represents the $DiOC_6(3)^{LOW}$ cell population, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total cells analysed of a single experiment and representative of at least two other independent experiments.

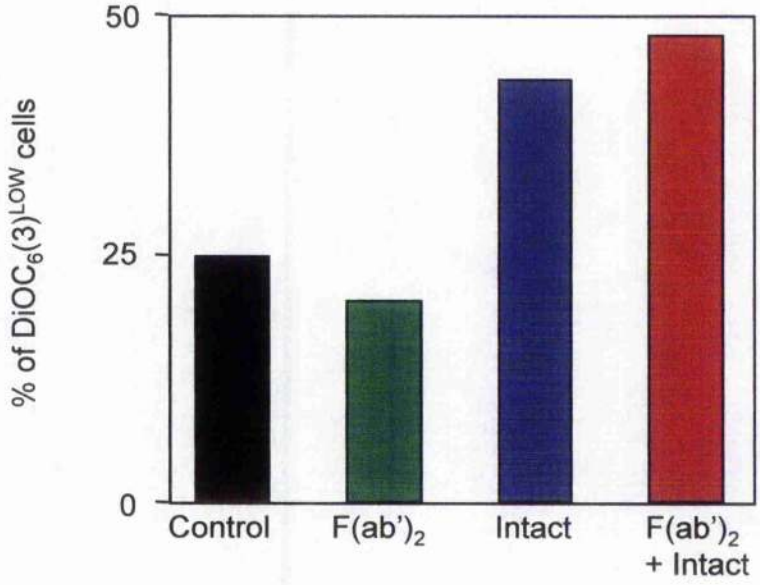
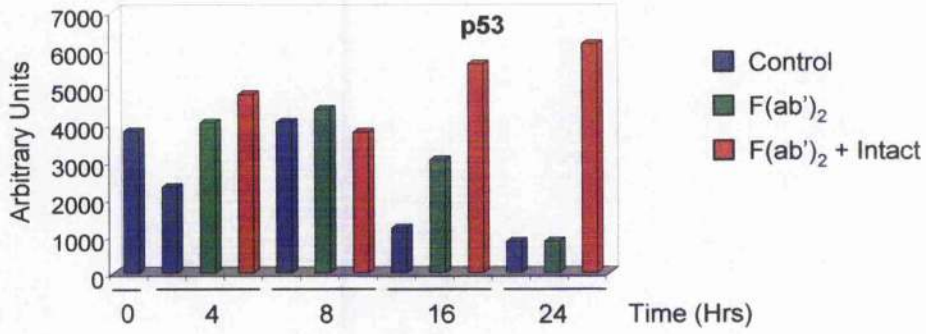
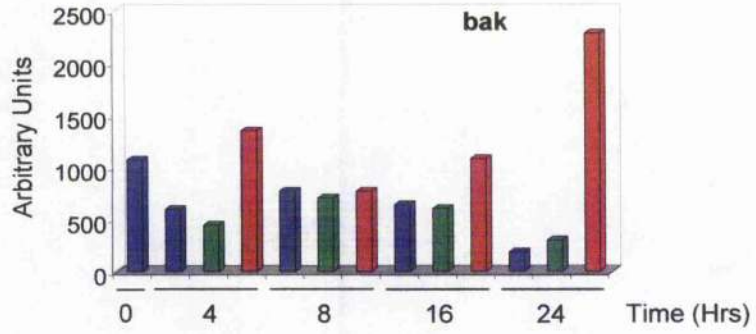

50
25
0
% of DiOC6(3)LOW cells
Control
F(ab')2
Intact
F(ab')2
+ Intact

Figure 5.16 Expression profiles of key apoptosis regulatory genes alter in response to BCR ligation and co-ligation with FcγRIIb

Purified splenic B cells from Balb/c mice (1×10^7 cells) were stimulated in the presence of $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml), either alone or in combination with intact rabbit anti-mouse IgG/IgM (75 μg/ml), for the times indicated. Cells stimulated in the presence of medium alone were included as a control. Total RNA was isolated from the cells at the times indicated, and the expression of key proteins assessed by means of the ribonuclease protection assay (RPA), as described in Materials and Methods (**Section 2.16**). The multi-probe template set used is shown in **Table 5.1**. Data were quantified using a Storm phosphorimager.


p53
Arbitrary Units
7000
6000
5000
4000
3000
2000
1000
0
0
4
8
16
24
Time (Hrs)
Control
F(ab')2
F(ab')2 + Intact


bak
Arbitrary Units
2500
2000
1500
1000
500
0
0
4
8
16
24
Time (Hrs)

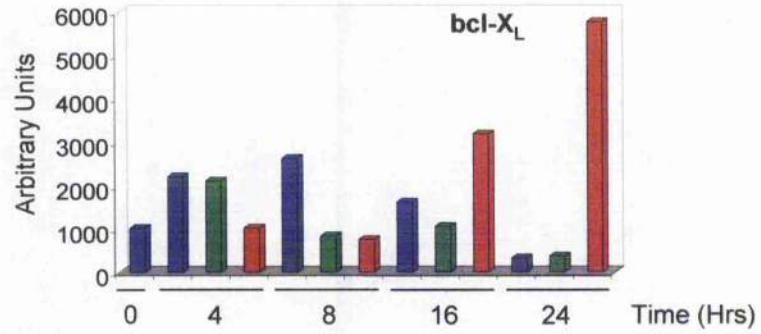
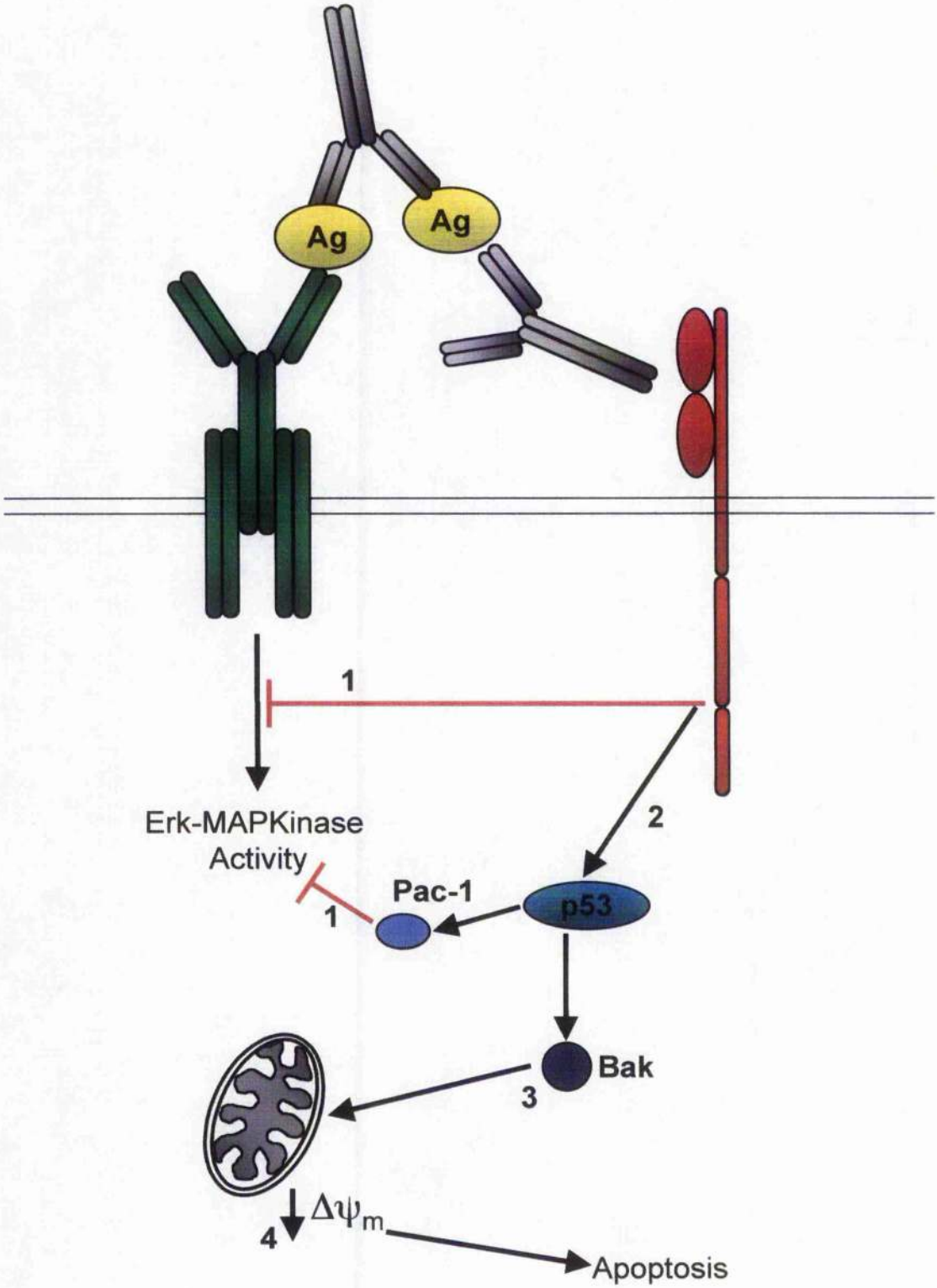

bcl-XL
Arbitrary Units
6000
5000
4000
3000
2000
1000
0
0
4
8
16
24
Time (Hrs)

Figure 5.17 Effect of BCR and FcγRIIb co-ligation on the activity of p53 and Bak, and the consequences upon the mitochondria

Co-ligation of the BCR with FcγRIIb results in the inhibition of BCR-induced Erk-MAPKinase activity (**1**), and an increase in the activity of the tumour suppressor protein p53 (**2**). The inhibition of BCR-induced Erk-MAPKinase activity is dual-pronged, with both the recruitment of $p62^{Dok}$ to inhibit Ras activity, and the induction of expression of a transcription target of p53, the phosphatase Pac-1, which dephosphorylates and inactivates Erk-MAPKinase directly. The increase in p53 activity may also lead to an increase in expression of the pro-apoptotic Bcl-2 family member, Bak (**3**), and a concomitant decrease in mitochondrial membrane potential (**4**), an early, irreversible step in the apoptosis pathway.


Ag
Ag
1
Erk-MAPKinase
Activity
1
Pac-1
2
p53
Bak
3
4
$\Delta\psi_m$
Apoptosis

Chapter 6 - General Discussion

B cells are the principal cellular mediators of the specific humoral response to infection by bacteria, viruses and parasites as they are the cells that produce antigen-specific antibodies. During B cell development, however, the response of B cells to antigen encounter changes drastically. Immature B cells generally respond to antigen by undergoing negative selection, whereas mature B cells are usually activated by antigen. Immature B cells do not always undergo negative selection, however, as *in vivo* the presence of other, extrinsic signals can lead to alternative fates. One of these signals is mediated through CD40, which can induce rescue from BCR-mediated growth arrest and apoptosis. This is most likely to occur in the periphery rather than the bone marrow, as B cells maintain the immature B cell phenotype for several days after exit into the periphery to ensure any B cells that are specific for self-antigens expressed in the periphery and not the bone marrow are negatively selected (Carsetti *et al.*, 1995). The ability of CD40 to mediate rescue from BCR-induced growth arrest and apoptosis would allow for recruitment of antigen-specific immature B cells into an ongoing immune response where T cell help already exists (Monroe, 2000).

The work presented in this thesis has focused on identifying the important signalling mechanisms linking BCR ligation with regulation of the cell cycle and induction of apoptosis in immature and mature B lymphocytes. In particular, investigations in Chapter 3 focused on the role of the Erk-MAPKinase pathway in anti-Ig induced growth arrest and apoptosis of immature B cells, and CD40-mediated rescue of these effects, by investigating the function of the small GTPase, Ras. Chapter 4 set out to ascertain the roles individual PKC isoforms, known to regulate Erk-MAPKinase activity in a number of systems, might play in the regulation of growth arrest and apoptosis in immature B lymphocytes, by utilising constitutively active or kinase dead mutants of individual PKC family members. Finally, in Chapter 5, the emphasis switched to mature B cells, and the signalling mechanisms important for the initiation and maintenance of a

proliferative response in mature B cells, as well as the key signalling events involved in FcγRIIb mediated inhibition of BCR-mediated proliferation and how these affect the key controllers of the cell cycle.

6.1 The role of the small GTPase Ras in WEHI-231 cells

The early signalling mechanisms activated upon ligation of the BCR under conditions leading to proliferation or apoptosis appear to be very similar, with phospholipase C, PI-3 kinase and Erk-MAPKinase all being activated under both circumstances. However, we have shown that although BCR ligation of WEHI-231 cells results in early stimulation of Erk-MAPKinase activity, in the long term it leads to down-regulation of Erk-MAPKinase activity. In contrast, co-stimulation with anti-Ig and anti-CD40 re-establishes sustained, cycling Erk activity, driving proliferation of the cells. This temporal regulation of Erk-MAPKinase activity to induce differential responses is becoming evident in a number of other systems, for example in PC12 cells transient activation of Erk-MAPKinase results in proliferation, whilst sustained MAPKinase activation induces differentiation (York *et al.*, 1998). Furthermore, the location of the Erk-MAPKinase activity is also thought to play a role in determining the outcome of the signal, though generally speaking this is linked to the duration of the signal as sustained activation is associated with translocation to the nucleus (Liang *et al.*, 2002). It would be very interesting to ascertain the intracellular location of the both the transient Erk-MAPKinase activity and the sustained Erk-MAPKinase activity in WEHI-231 cells, in order to establish whether the location of the signal, as well as the duration, is playing a role in determining the outcome. As WEHI-231 cells are rather small, confocal microscopy would be a better method for determining this than LSC, as the resolution of confocal microscopy is much greater.

It is interesting to speculate as to the nature of the targets of Erk-MAPKinase activity in B cells under the differential functional outcomes of apoptosis versus prolfieration. The Erk-MAPKinase has been shown to promote proliferation by

inducing the expression of cyclin D1 (Lavoie *et al.*, 1996). However, it has been suggested that cyclin D1 is not expressed in B cells, and that cyclin D2 is the major D type cyclin involved in the regulation of proliferation of B cells (Solvason *et al.*, 2000). Other proliferative targets for Erk-MAPKinase are numerous, and include both the RNA pol I and RNA pol III transcription machinery (Felton-Edkins *et al.*, 2003; Stefanovsky *et al.*, 2001). Transcription by RNA pol I and pol III have to be tightly linked, as ribosomes require an equimolar ratio of rRNAs, so it seems likely that Erk-MAPKinase plays a role in co-ordinating production of ribosomes, which are necessary for growth and cell cycle progression (Stefanovsky *et al.*, 2001). It would be interesting to assess ribosome production in both anti-Ig and anti-Ig plus anti-CD40 stimulated WEHI-231 cells, to determine if the different Erk-MAPKinase signals affect transcription by RNA pols I and III in different manners. Phosphorylation and concomitant activation of certain ribosomal S6 kinase isoforms (RSK1-3) by Erk-MAPKinase has also been postulated to be important for the regulation of translation (Frodin and Gammeltoft, 1999). Moreover, one substrate of RSK, and Erk-MAPKinase, is Sos, with phosphorylation of Sos leading to dissociation of the Grb2-Sos-Ras complex, perhaps a negative feedback loop. Investigation of the activation of RSK may also provide clues as to the differential targets of the transient and sustained Erk-MAPKinase activity, as anti-Ig induced Erk-MAPKinase may activate RSK, leading to the down-regulation of Erk-MAPKinase activation by the dissociation of the Grb2-Sos-Ras complex. This does not appear to be the case in WEHI-231 cells, as results in this laboratory have demonstrated that BCR ligation abrogates Erk-MAPKinase activity not by affecting upstream regulators, but rather by activating phosphatases such as Pac-1, to inactivate Erk-MAPKinase directly (Gauld *et al.*, 2002).

The small GTPase Ras, a key regulator of Erk-MAPKinase and PI-3-kinase, also seems to play an important role in both anti-Ig induced growth arrest and apoptosis, and CD40-mediated rescue. For example, a constitutively active Ras mutant, RasV12, appears to reduce anti-Ig induced growth arrest, enabling

WEHI-231 cells to continue proliferating in the presence of anti-Ig, presumably because it can restore, at least to a certain extent, sustained, cycling Erk-MAPKinase activity. Interestingly, a constitutively active mutant of Ras that can activate the PI-3-kinase pathway but not the Erk-MAPKinase pathway, RasV12-C40, also reduces anti-Ig induced growth arrest. Interestingly, these cells also display sustained, cycling Erk-MAPKinase activity, suggesting that Erk-MAPKinase can be activated downstream of PI-3-Kinase, as reported elsewhere for B cells and other systems (Jacob *et al.*, 2002). One of the major substrates of PI-3-kinase is the serine/threonine protein kinase Akt (also known as PKB), which has been shown to be a powerful mediator of growth factor-induced survival and is capable of suppressing apoptosis induced by a wide range of stimuli (Datta *et al.*, 1997). PI-3-kinase activity has also been shown to be required for progression from G_1 phase of the cell cycle to S phase in fibroblasts (Jones *et al.*, 1999), and previous studies in our laboratory suggest a positive role for PI-3-kinase in proliferation of unstimulated WEHI-231 cells, as inhibition of PI-3-kinase induces growth arrest (Gauld, 2001). This strongly suggests that the PI-3-kinase pathway plays a role in the proliferation and CD40-mediated survival of WEHI-231 cells.

The data presented in Chapter 3 showed that the Ras mutants only partially rescued anti-Ig mediated growth arrest. This may simply reflect that although the Ras mutants generate constitutively active Ras signals, this was not reflected in terms of constitutive Erk-MAPKinase activity. However, sustained Erk-MAPKinase activation was partially restored (Catriona Ford, personal communication), thus it would be interesting to ascertain the kinetics of anti-Ig induced effects, or lack thereof, in WEHI-231 cells expressing these RasV12 mutants, in light of the observations that WEHI-231 cells transfected with PKC mutants exhibit different responses at 24 and 48 hours (**Chapter 4**). Indeed, preliminary experiments suggest that, at 24 hours, all three RasV12 mutants abrogate anti-Ig induced growth arrest more efficiently than at 48 hours, as these cells exhibit similar levels of proliferation in the absence or presence of anti-Ig,

even at 10 μg/ml (**Figure 6.1**). This suggests that the kinetics of anti-Ig induced growth arrest are altered by expression of these constructs, as anti-Ig does induce growth arrest in these cells at 48 hours, yet in cells containing the empty vector growth arrest is demonstrated as early as 24 hours. It would be interesting to study the proliferation of these cells at a number of time points between 24 and 48 hours, as well as the number of divisions these cells undergo.

The down regulation of sustained, cycling Erk-MAPKinase activity by anti-Ig stimulation of WEHI-231 cells does not seem to depend upon position of the cell in the cell cycle, as anti-Ig can induce growth arrest and inhibit Erk activity following arrest and release of cells in either G_1 or S phase. It would, however, be interesting to synchronise WEHI-231 cells transfected with the various RasV12 constructs to further investigate whether the effects these molecules have on anti-Ig mediated growth arrest and apoptosis are dependent upon cell cycle stage. In particular, it would be very interesting to determine if the cells can undergo apoptosis from any cell cycle phase, or if they have to transit to G_1 in order to apoptose. For example, when B cells are infected with the protozoan apicomplexan parasite, *Theileria*, they require parasite survival for the observed oncogenic transformation. Thus, when the parasite is killed, the cells apoptose, but they can only do so from G_1, whereas T cells in the same circumstances can undergo apoptosis at any stage of the cell cycle (Gordon Langsley, Institut Pasteur, Paris, personal communication). It would also be interesting to determine whether the cellular localisation of p27 differs as the cells proliferate, as the localisation of p27 determines its function. In the cytoplasm, p27 interacts with cyclin D/CDK complexes, acting as an essential assembly factor, whereas in the nucleus, p27 interacts with cyclin E/CDK2, inhibiting the kinase activity and blocking cell cycle progression. It has recently been proposed that Akt, a major effector of PI-3-kinase, impairs nuclear import of p27, thus abrogating the ability of p27 to induce G_1 arrest (Liang *et al.*, 2002).

Thus, this study has demonstrated a key role for the Erk-MAPKinase in anti-Ig induced growth arrest and apoptosis, as well as CD40-mediated rescue of anti-Ig induced effects in WEHI-231 cells. It has also established the ability of constitutively active Ras mutants to alter the normal biological response of WEHI-231 cells to antigen, as well as indicating a role for PI-3-kinase in CD40-mediated rescue of anti-Ig induced apoptosis. This study has also suggested that the 5' inositol phosphatase, SHIP, does not play a significant role in the regulation of anti-Ig induced effects in WEHI-231 cells, unlike in mature B cells where SHIP is thought to be a major negative regulator of anti-Ig induced proliferation.

6.2 The role of PKC isoforms in WEHI-231 cells

Upon ligation with antigen the B cell receptor activates a number of signalling pathways to mediate a response. One of the first pathways shown to be activated by the BCR was phospholipase Cγ, which results in the generation of the second messenger DAG, a key regulator of both the conventional PKCs and the novel PKCs. This suggests a key role for PKC family members in BCR signalling. Indeed, BCR cross-linking on mature B cells leads to strong activation of PKCs, whereas ligation of the BCR on immature B cells does not, providing a possible explanation for the differential responses to BCR cross-linking in mature and immature B cells (Sarthou *et al.*, 1989). As a consequence, it has been proposed that BCR uncoupling from PKC activation may be a major factor in anti-Ig induced growth arrest and apoptosis in immature B cells. This is consistent with the finding that treatment of WEHI-231 cells with PMA, a potent activator of most PKC isoforms, protects them from anti-Ig induced growth arrest and apoptosis (Gold and DeFranco, 1987).

The activation of PKCs is also linked to the upstream activity of PI-3-kinase, as PI-3-kinase activity produces PIP_3, which recruits Btk to the plasma membrane where it can proceed to activate PLCγ, resulting in the production of DAG, which

is required for activation of the conventional and novel PKCs. The importance of PI-3-kinase and Btk in PLCγ2 activation are evidenced by the similarity in phenotype of p110δ-deficient mice (the major PI-3-kinase catalytic subunit found in B cells), PLCγ2 deficient mice and *xid* immunodeficient mice, which have a mutation in the PIP_3 binding domain of Btk, preventing its recruitment and concomitant activation of PLCγ (Okkenhaug *et al.*, 2002; Rawlings *et al.*, 1993; Wang *et al.*, 2000). The downstream targets of PKC isoforms are manifold, and include the Erk-MAPKinase pathway, via both Raf, which is upstream of MEK (Cacace *et al.*, 1996), and the Grb-2/Sos adaptor complex, which is involved in Ras activation (Kawakami *et al.*, 2003).

Expression of most of the PKC isoforms in WEHI-231 B cells led to an increase in phosphorylation of Erk-MAPKinase. A recently isolated member of the CDC25 family of Ras GEFs, RasGRP3, which is also recruited to the plasma membrane by DAG, is preferentially expressed in B cells, and has been proposed to be a downstream target of PKC isoforms (Teixeira *et al.*, 2003). It would be interesting to investigate the role of RasGRP3 in WEHI-231 cells transfected with these PKC constructs, as one of the major roles of RasGRP3 is to activate Ras. Indeed, PKC inhibitors that block phosphorylation of RasGRP3 also attenuate Ras/Erk-MAPKinase signalling in Ramos B cells (Teixeira *et al.*, 2003).

With reference to the individual PKC isoforms, PKCα has been proposed to play a positive role in proliferation and an anti-apoptotic role in a number of cell types. Thus, overexpression of a catalytically active PKCα in NIH-3T3 cells results in increased expression of cyclins D1 and E and a concomitant increase in proliferation (Soh and Weinstein, 2003). However, it has been reported that cyclin D1 is not expressed in B lymphocytes, cyclin D2 being the predominant isoform in B cells (Solvason *et al.*, 1996), yet there was no evidence of an increase in cyclin D2 expression in WEHI-231 cells expressing a constitutively active PKCα mutant (**Figure 3.16**). It has recently been reported, however, that cyclin $D2^{-/-}$ mice display normal conventional B cell development, suggesting

perhaps that cyclin D3 is more important than cyclin D2 for B cell development, or can at least fulfil the role of cyclin D2 in the absence of cyclin D2 (Solvason *et al.*, 2000). It would be interesting to investigate the expression patterns of cyclin D3 in WEHI-231 cells.

The results obtained did not highlight a clear cut unifying role for a particular element in the regulation of immature B cell apoptosis and proliferation. However, a number of PKC isoforms have been implicated in the regulation of NF-κB, a key regulator of survival in B lymphocytes (Gerondakis and Strasser, 2003). Therefore, it would be interesting to assess the ability of WEHI-231 cells expressing PKC mutants to activate NF-κB-mediated transcription, as anti-Ig induced apoptosis involves down-regulation of NF-κB activity, whilst one mechanism by which CD40 mediates survival is through the induction of NF-κB activity (Schauer *et al.*, 1996). It must be noted, however, that the results obtained in this study do not fully support the hypothesis that the differential responses of immature and mature B cells are a result of uncoupling of the BCR from PKC activation in immature B cells, though expression of most of these PKC constructs did provide a level of protection from anti-Ig induced growth arrest at 24 hours.

6.3 Maintenance of activation of mature B cells

Mature B cells generally respond quite differently to BCR ligation than immature B cells. Upon ligation of the BCR on mature B cells, they undergo proliferation, which can lead to differentiation to effector memory or plasma cells. Under physiological conditions, an optimal B cell response generally requires signals from helper T cells, such as IL-4 and CD40L, which provide positive signals to the cell to enhance proliferation. In contrast, there are also factors that negatively regulate B cell proliferation, such as those signals emanating from the FcγRIIb receptor, a low affinity IgG receptor. Co-ligation of the BCR and FcγRIIb leads to the inhibition of BCR-triggered B cell activation and cell cycle arrest. This

mechanism is important for quenching a specific B cell response at an appropriate time. Thus, the immune system is capable of terminating a response once the pathogenic agent has been cleared.

Besides the key roles Erk-MAPKinase plays in the responses of immature B cells to antigen, as evidenced by its importance in the WEHI-231 cell line, Erk-MAPKinase also plays a major role in the response of mature B cells to antigen, despite the fact that these two responses are very different. The fact that inhibition of MEK abrogates $F(ab')_2$ induced proliferation strongly suggests that Erk-MAPKinase is a major player in initiation and maintenance of proliferation in these cells. The importance of Erk-MAPKinase in BCR-induced proliferation in mature B cells is further underlined by the actions of the FcγRIIb receptor, which negatively regulates BCR-mediated proliferative signalling. One of the mechanisms by which FcγRIIb achieves this is the rapid inactivation of Erk-MAPKinase activity. Not only is the initial inactivation of Erk-MAPKinase activity rapid, it is prolonged, with Erk-MAPKinase activity remaining undetectable after 20 hours. In contrast, cells stimulated with $F(ab')_2$ fragments of anti-Ig antibodies exhibit a sustained activation of Erk-MAPKinase activity, in much the same way that proliferating WEHI-231 cells exhibit sustained activation of Erk-MAPKinase. Indeed, when WEHI-231 cells are stimulated with anti-Ig, which induces growth arrest, the sustained nature of the Erk-MAPKinase activation is lost, comparable to co-ligation of the BCR with the FcγRIIb in mature B cells, demonstrating a key role for sustained Erk-MAPKinase activity in promoting B cell survival and proliferation in both immature and mature B cells. It would be interesting to further study the localisation of this sustained Erk-MAPKinase activity in mature B cells, to determine whether, as in HEK293 cells, sustained activation of Erk-MAPKinase is associated with translocation of Erk to the nucleus (Shah *et al.*, 2003). The LSC is not the ideal instrument for this, as the resolution is not as good as that afforded by confocal microscopy, and mature B cells are very small in size, so high resolution is required for an accurate assessment of intracellular localisation of proteins.

The effects that BCR ligation and co-ligation of the BCR with FcγRIIb have on the expression patterns of key cell cycle regulatory proteins potentially downstream of Erk-MAPKinase signalling are very interesting. Thus, the pattern of Rb phosphorylation correlates with the observed biological responses of the cells at each time point, with BCR ligation seemingly inducing hyper-phosphorylation of Rb, which is required for proliferation. On the other hand, cells that have their BCRs co-ligated with FcγRIIb, which results in inhibition of B cell proliferation, possess hypo-phosphorylated Rb, which correlates with growth arrest. It also appears that co-ligation of the BCR with FcγRIIb results in the activation of the tumour suppressor protein p53, which is known to possess both apoptotic and growth inhibitory functions. These results suggest that Rb and p53 may play key roles in the mechanisms by which FcγRIIb exerts its growth arrest and apoptotic effects.

Another key regulator of the cell cycle is the CKI, p27, which induces G_1 arrest by inhibiting the cyclin E/CDK2 complex. The role of p27 in regulating B cell responses appears to be non-classical, however. Thus, stimulation of B cells with $F(ab')_2$ fragments of anti-Ig, which induces proliferation, also induces a strong increase in the transcription of the $p27^{Kip1}$ gene. As stated above, the regulation of p27 expression is highly complex, and involves both translational control and regulation of the stability of the protein. The translation of p27 is negatively regulated by Rho (Vidal *et al.*, 2002), and the stability of the protein is regulated by both ubiquitin-dependent degradation by the proteasome (Montagnoli *et al.*, 1999) and degradation by calpain (Patel and Lane, 2000). Thus, an increase in the translation of the mRNA may not necessarily lead to an increase in the levels of protein, therefore one future experiment would be to assess the levels of the p27 protein. If the protein levels also show an increase, it could be that in B cells p27 plays an important role in the assembly of cyclin D/CDK4 complexes, as opposed to the inhibition of cyclin E/CDK2 complexes. As mentioned previously, the subcellular localisation of p27 also regulates its activity, therefore if the

protein levels do show a rise, it would be important to assess the location within the cell, either by western blotting of specific subcellular fractions, or by confocal microscopy.

It seems highly likely that p53 plays a role in FcγRIIb mediated apoptosis of mature B cells, as not only is it strongly upregulated at the mRNA level, but it is also activated by phosphorylation on serine 15, a key indication of activation, in response to ligation of FcγRIIb. Moreover, transcription of one of its downstream targets, the pro-apoptotic Bcl2 family member Bak, is also induced. This suggests that one mechanism utilised by FcγRIIb to initiate apoptosis is the activation of p53, and the subsequent increase in the levels of Bak. This proceeds to induce a drop in the mitochondrial membrane potential, and subsequently apoptosis.

Though interesting, the RPA results require a degree of validation, as they represent a single experiment. This would preferably be repeated, and perhaps also supported with western blotting analysis to assess protein levels, as mRNA need not necessarily directly reflect the amount of protein, particularly of key cell cycle regulatory proteins which are strictly regulated at the mRNA level as well as protein stability, such as p27 (Montagnoli *et al.*, 1999; Vidal *et al.*, 2002).

Overall, this study has further demonstrated the importance of T cell derived signals in the initiation and maintenance of a B cell response to antigen. The importance of the Erk-MAPKinase in mature B cells, as well as immature B cells, is also underlined, as proliferation of both immature and mature B cells is associated with sustained activation of Erk-MAPKinase. It has also suggested a link between FcγRIIb, p53, Bak and apoptosis.

6.4 Concluding remarks

This project has examined the differential roles of Erk-MAPKinase signalling in immature and mature B cells. In particular, the roles of differential Ras signals and the impact of specific PKC isoforms on these pathways have been investigated in the response of immature transitional B cells to BCR ligation and CD40 mediated rescue of BCR-induced growth arrest and apoptosis. These studies demonstrated that the differential regulation of Ras signals by the BCR and CD40 is important for determining the functional biological outcome of ligation of these receptors. For example, the Ras effector, PI-3-Kinase, appears to play a major role in B cell proliferation and survival, perhaps through its modulation of p27 activity. A key role for sustained, cycling Erk-MAPKinase activity in the proliferation of immature B cells was also demonstrated. Consistent with this, the study also highlighted a key role for sustained Erk-MAPKinase activity in the proliferation of mature B cells, along with downstream roles for the tumour suppressor proteins Rb and p53 in the regulation of proliferation and survival of mature B cells, particularly with respect to FcγRIIb-mediated negative feedback inhibition of BCR signalling.

Figure 6.1 Effects of overexpression of RasV12 mutants on anti-Ig-induced growth arrest in WEHI-231 cells

WEHI-231 cells (1×10^4 cells/well) transfected with either pcDNA3.1.RasV12, pcDNA3.1.RasV12-S35, pcDNA3.1.RasV12-C40 or the pcDNA3.1 vector were cultured, in triplicate, in the presence of increasing concentrations of anti-Ig (0.01, 0.1, 1, 10 μg/ml), for 24 hours. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data from individual experiments were normalised by expressing the mean [^{3}H]-thymidine uptake values of anti-Ig treated cells as a percentage of those obtained with control cell cultures. The normalised values from at least 2 independent experiments were then pooled and expressed as means ± sem.

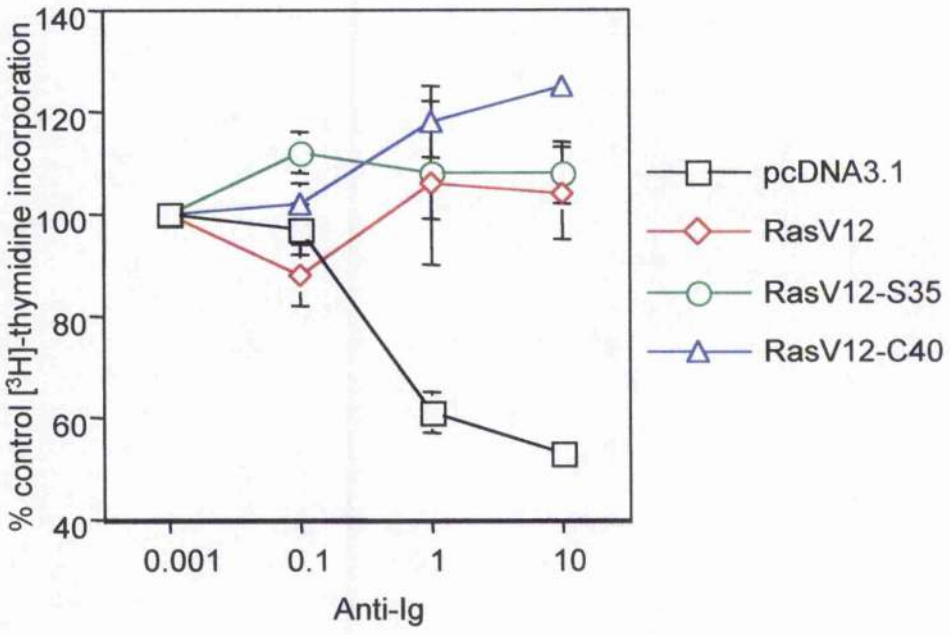

% control [3H]-thymidine incorporation
140
120
100
80
60
40
0.001
0.1
1
10
Anti-Ig
pcDNA3.1
RasV12
RasV12-S35
RasV12-C40

Figure 6.2 Integrated signalling mechanisms responsible for the induction of apoptosis and growth arrest, and CD40-mediated rescue, in WEHI-231 cells

The differential responses of immature B cells to BCR ligation in the presence and absence of T cell derived signals are mediated by a complex network of signalling pathways enabling the growth arrest and apoptosis, or proliferation and survival decision of WEHI-231 cells to be carefully regulated. Stimulation via the BCR leads to a strong, but transient activation of Erk-MAPKinase followed by desensitisation of sustained, cycling Erk-MAPKinase activity, possibly due to the induction of the phosphatase Pac-1, which results in growth arrest and apoptosis. Expression of PKCδ or PKCζ appear to enable stimulation via the BCR alone to lead to sustained activation of Erk-MAPKinase, which normally leads to proliferation (**1**). Constitutive activation of the small GTPase Ras can provide some measure of protection from anti-Ig induced growth arrest, mediated, at least in part, by PI-3-Kinase and the subsequent induction of the anti-apoptotic Bcl-2 family member, Bcl-X_L. Interestingly, constitutive activation of Ras does not result in constitutive activation of Erk-MAPKinase, possibly as a consequence of the actions of phosphatases, including Pac-1, which can deactivate Erk-MAPKinase by removing key phosphoryl residues.

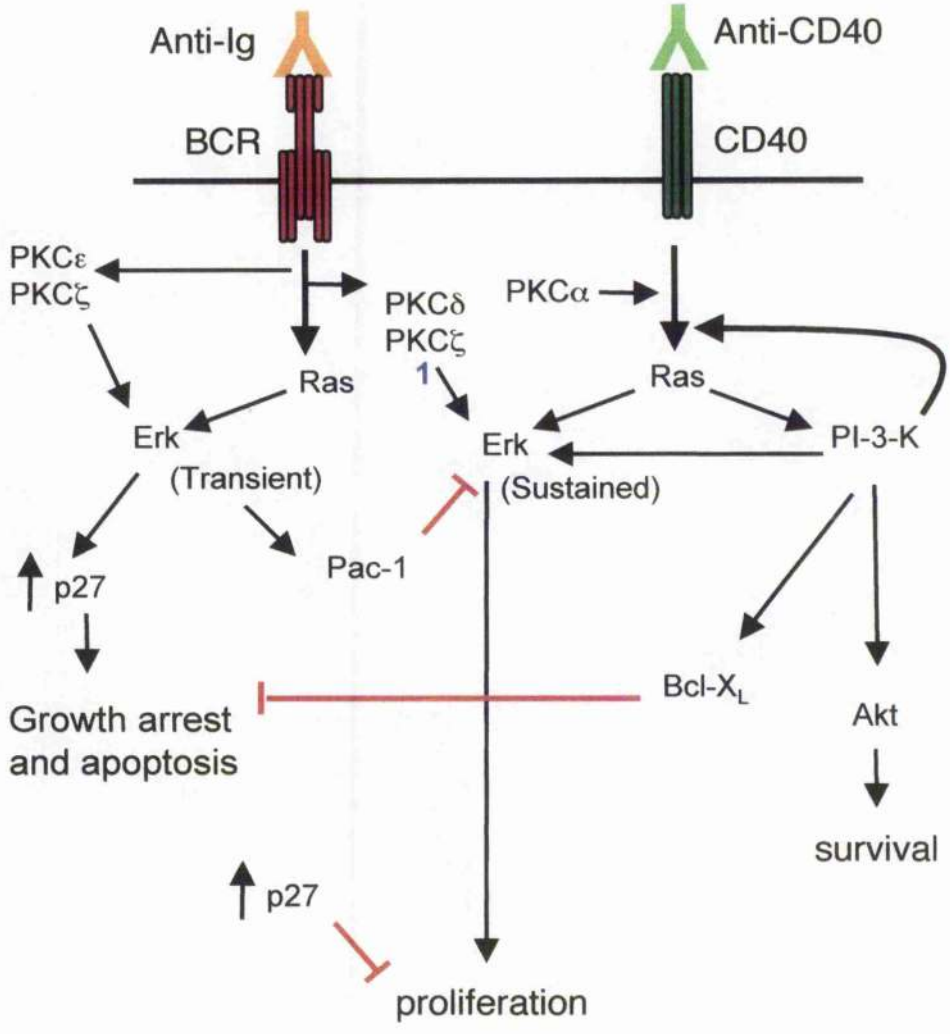
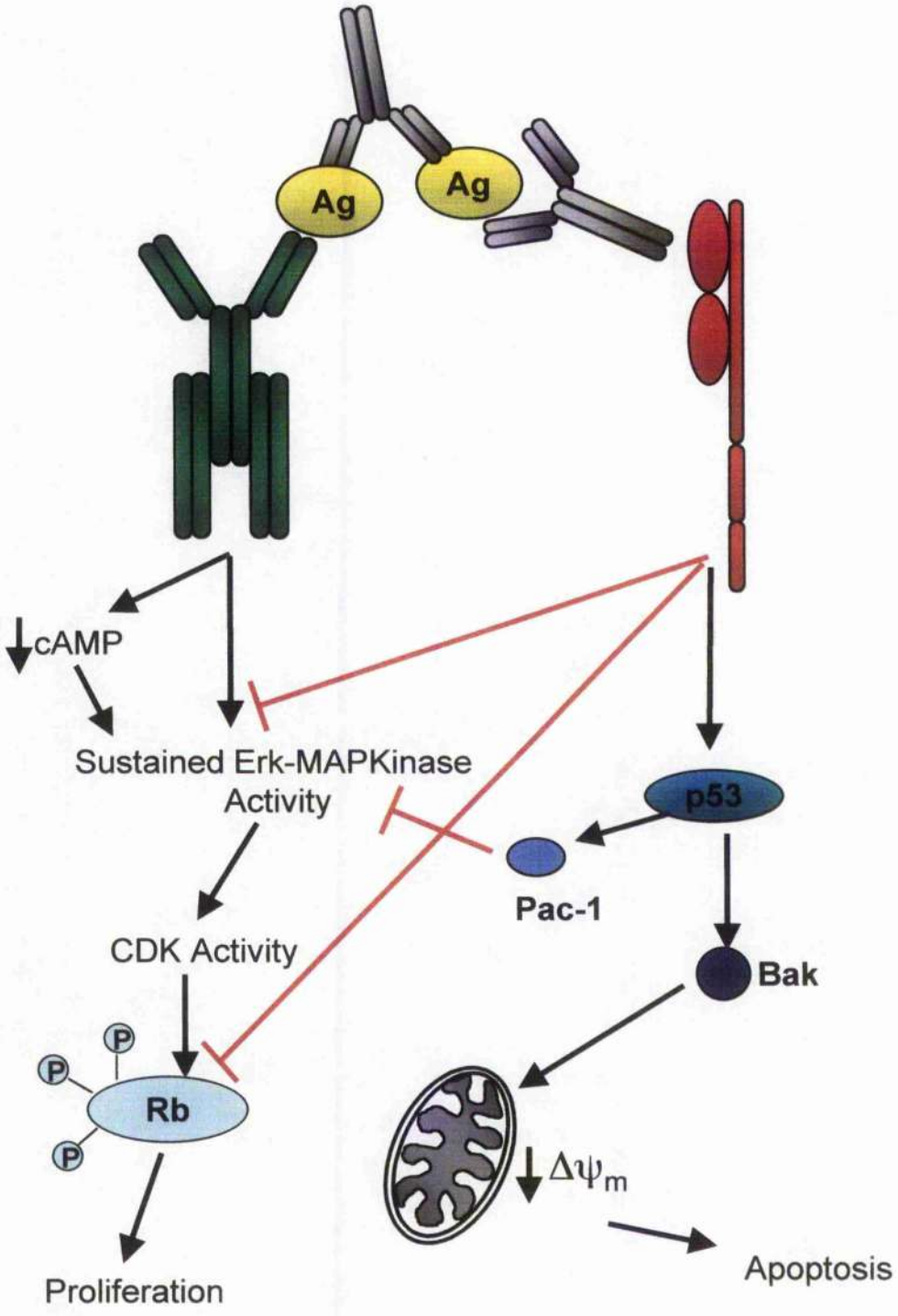

Anti-Ig
Anti-CD40
BCR
CD40
PKCε
PKCζ
PKCδ
PKCζ
1
PKCα
Ras
Ras
Erk
(Transient)
Erk
(Sustained)
PI-3-K
p27
Pac-1
Growth arrest
and apoptosis
Bcl-X$_L$
Akt
survival
p27
proliferation

Figure 6.3 Inhibition of BCR induced proliferation by FcγRIIb mediated signals

Ligation of the BCR on mature B cells leads to sustained activation of the Erk-MAPKinase, concomitant with increased phosphorylation of the retinoblastoma protein, Rb, and proliferation. Co-ligation of the BCR with the inhibitory FcγRIIb receptor abrogates this sustained activation of Erk-MAPKinase, in part by inducing the interaction between Erk and the nuclear phosphatase, Pac-1. The phosphorylation of the retinoblastoma protein is also abrogated by co-ligation of the BCR with FcγRIIb. The tumour suppressor protein, p53, is activated in response to BCR and FcγRIIb co-ligation, and this is concomitant with increased expression of Bak, a collapse in the mitochondrial membrane potential and apoptosis.


Ag
Ag
cAMP
Sustained Erk-MAPKinase Activity
p53
Pac-1
CDK Activity
Bak
P
P
P
Rb
$\Delta\psi_m$
Proliferation
Apoptosis

Appendix – Modulation of B cell responses by parasitic products

A.1 Introduction

Filarial nematodes are a large group of arthropod-transmitted vertebrate parasites that mediate lifelong infection. Moreover, individual parasites are capable of surviving in the host for in excess of five years. They are known to cause a range of debilitating diseases known collectively as filariasis, which includes lymphatic filariasis and onchocerciasis. Clinical manifestations of the disease can include blindness, swollen limbs, and severe damage to the kidneys and lymphatic system, much of this pathology resulting as a consequence of the host immune response. The general success of the parasites, however, reflects the fact that the majority of infected individuals exhibit much less severe symptoms as a consequence of their ability to modulate or suppress the host immune response. Indeed, filarial infection has been demonstrated to modulate both parasite-specific and general immune cell mediated responses (King and Nutman, 1991).

It is reasonable to postulate that the longevity of parasitic nematodes is dependent on molecular secretions that interact with the host immune system. Consistent with this, studies have shown that the rodent filarial nematode *Acanthocheilonema viteae* secretes a protein, ES-62 (Harnett *et al.*, 1989) that possesses many immunomodulatory properties (Harnett and Harnett, 2001). For example, ES-62 is found to reduce murine lymphocyte proliferation and polarise immune responses towards an anti-inflammatory phenotype. For example, exposure to ES-62 not only renders B and T cells anergic to subsequent stimulation via the antigen receptors but also polarises immune responses to a T_H2 phenotype by inhibiting pro-inflammatory cytokine production and driving differentiation of DCs to a DC2, T_H2-promoting phenotype (Harnett and Harnett, 1999).

Extensive studies in lymphocytes have shown that ES-62 mediates its anti-proliferative effects by uncoupling the antigen receptors from key downstream proliferative signalling pathways. In particular, by selectively downregulating certain PKC isoforms and by desensitising protein tyrosine kinase-dependent coupling of the antigen receptors to PI-3-kinase and the Ras/ErkMAPKinase signalling cascades (**Figure A.1**). Thus, this uncoupling results in a novel mechanism by which ES-62 can inhibit antigen receptor-mediated proliferative responses and subvert the normal immune response of the host.

Similarities between the inhibitory signalling mechanisms of ES-62 and FcγRIIb on B cells suggested that they could share similar effector pathways. Specifically, ES-62 has been shown to desensitise the BCR-mediated activation of ErkMAPK by the recruitment of the tyrosine phosphatase SHP-1 and the MAPKinase phosphatase, Pac-1 (Deehan *et al.*, 2001) (**Figure A.2**). Moreover, the ability of ES-62 to initiate a polarised T_H2 response may represent a novel therapeutic strategy for the treatment of inflammatory-type autoimmune diseases, since the generation of a T_H1-type immune response by collagen-specific $CD4^+$ T cells, is crucial for the development of arthritis in the collagen induced arthritis model in mice. Thus, the ability of ES-62 to suppress B cell activation and antibody production was of interest when investigating the role of FcγRIIb in the regulation of CIA.

It has also been shown that ES-62 contains a post-translational modification, the covalent addition of phosphorylcholine (PC) to an N-type glycan (Harnett and Harnett, 2001). This PC group provides a major contribution to the immunomodulatory properties of ES-62 (Harnett and Harnett, 1999; Harnett and Harnett, 2001). The addition of PC to carbohydrate appears to be a feature of the nematode phylum, extending to a number of proteins and, in addition, some glycosphingolipids (Lochnit *et al.*, 2000). The immunomodulatory properties of these latter molecules are largely unexplored but two PC-containing glycosphingolipids of *Ascaris suum* that induce secretion of TNF-α, IL-1 and IL-6

from human peripheral blood mononuclear cells have recently been described (Lochnit *et al.*, 1998a). This activity was shown to be due to PC as the removal of PC from the molecules eliminates this activity.

A.2 Aims and objectives

Previous studies in this laboratory have investigated the mechanisms by which ES-62 modulates the proliferative response of lymphocytes to antigen, in order to identify key pathways involved in both the positive and negative regulation of proliferation. The aim of this study was to further investigate the immunomodulatory properties of the PC-containing glycosphingolipids produced by the filarial nematode *Ascaris suum*, specifically repeating a number of the experiments previously undertaken in this laboratory to examine ES-62, with the aim of further elucidating the mechanisms utilised by filarial nematodes to regulate the host immune response.

In particular, this study aims to address:

1. The effects these molecules have on the proliferative response of B cells upon stimulation via the BCR.
2. The specific role the PC moiety plays, if any, in the modulation of B cell responses.
3. The ability of synthetically produced mimetics to induce the same effects as the natural products on B cell responses.

A.3 Results

A.3.1 Modulation of the B cell proliferative response by *Ascaris suum* glycosphingolipids

To investigate the effects of the PC-containing glycosphingolipids produced by *Ascaris suum* on B cells, B cell responses to a number of stimuli were investigated in the presence and absence of these molecules. Primary splenic B cells were incubated with native *Ascaris suum* glycosphingolipids or *Ascaris suum* glycosphingolipids that had had the PC moiety chemically removed, and stimulated with either $F(ab')_2$ fragments of anti-Ig (50 μg/ml), or LPS (50 μg/ml). Unstimulated cells were included as a control. After 48 hours stimulation proliferation was assessed by means of the [^{3}H]-thymidine incorporation assay. As expected, in the absence of *Ascaris suum* glycosphingolipids both LPS and $F(ab')_2$ induced a substantial increase in proliferation of the mature B cell (**Figure A.3**). The presence of the *Ascaris suum* glycosphingolipids (10 μg/ml) significantly reduces proliferation induced by either of these stimuli. In the case of $F(ab')_2$ anti-Ig induced proliferation, the glycosphingolipid induced reduction is not dependent on the PC moiety, as $F(ab')_2$ anti-Ig induced proliferation is reduced by a similar amount by PC-free glycosphingolipid and whole glycosphingolipid. However, LPS induced proliferation is dependent upon the PC moiety as PC-free glycosphingolipid is unable to inhibit LPS induced proliferation.

A.3.2 Modulation of the B cell proliferative response by synthetic mimetics of *Ascaris suum* glycosphingolipids

To dissect the mechanism by which these *Ascaris suum* glycosphingolipids modulate the B cell proliferative response, and to attempt to establish the nature of the non-PC immunomodulatory moiety within the molecules, a number of chemically synthesised homologues were produced. A native form of the whole glycosphingolipid molecule, a PC-free molecule, and a ceramide free molecule were all generated (Dr Rudi Geyer and Dr Gunther Lochnit, Giessen, Germany). To assess the effectiveness of these synthetic molecules as immunomodulators,

primary splenic B cells were incubated with each of them individually, or the ceramide component alone, or a p120 glycoprotein containing demethylamino ethanol instead of PC from *L. carinii* (all at 10 μg/ml), and stimulated with either $F(ab')_2$ fragments of anti-Ig or LPS (both 50 μg/ml). Proliferation was assessed after 48 hours by means of the [^{3}H]-thymidine incorporation assay.

Of the molecules tested, the ceramide component has by far the greatest effect on the B cell response to LPS (**Figure A.4**). However, this is likely due to cytotoxicity rather than specific effects on the response to LPS, as ceramide abolishes proliferation in the absence of any other stimuli. We have previously shown that ceramide induces apoptosis in the WEHI-231 immature B cell line (Katz *et al.*, 2001). Of the other molecules, none have any significant effect on LPS-induced proliferation of B cells except for the PC-free molecule, which slightly inhibits LPS-induced proliferation of primary splenic B cells (p value <0.05). In terms of the effect of these molecules on anti-Ig-induced proliferation, again ceramide produced the largest effect, due to its cytotoxicity. Of the other molecules studied, none produced any significant effects on anti-Ig-induced proliferation. Taken together, these results suggest that the synthetically produced molecules do not accurately mimic the effects of the molecules produced by *Ascaris suum*. 'Natural' *Ascaris suum* glycosphingolipids can inhibit anti-Ig-induced proliferation of primary splenic B cells in a PC-independent manner, whereas the inhibition of LPS-induced proliferation is entirely dependent upon PC. However, the chemically synthesised molecules have no effect on anti-Ig-induced proliferation, whilst the PC-free molecule inhibits LPS-induced proliferation. As for the *L.carinii* molecule, it appears that this has no effect on the proliferation of B cells in response to either LPS or anti-Ig.

A.3.3 *Ascaris suum* glycosphingolipids inhibit anti-Ig-induced B cell proliferation by inducing an increase in apoptosis

The above results showed that the glycosphingolipids from *Ascaris suum* are capable of inhibiting anti-Ig induced B cell proliferation in a PC-independent

manner. To investigate whether this was a result of growth arrest or an increase in apoptosis, the DNA content of the cells was analysed by propidium iodide staining. B cells were stimulated with $F(ab')_2$ fragments of anti-Ig in the presence or absence of *Ascaris* glycosphingolipids for 48 hours, and the DNA content assessed by flow cytometry following propidium iodide staining (**Figure A.5**). Co-stimulation of B cells with $F(ab')_2$ anti-Ig and *Ascaris* glycosphingolipids results in a substantial increase in the proportion of cells with a sub-diploid DNA content, compared to unstimulated cells, and cells stimulated with $F(ab')_2$ fragments of anti-Ig or glyco-sphingolipid alone. As expected, this increase in the proportion of sub-diploid cells is accompanied by a decrease in the proportion of cells either in S phase or G_2/M. On the other hand, B cells stimulated with $F(ab')_2$ anti-Ig alone demonstrate an increase in the proportion of cells in S phase, consistent with the $[^3H]$-thymidine incorporation data that showed that $F(ab')_2$ stimulated cells increase their proliferation (**Figure A.3**).

To corroborate the above findings that co-stimulation of B cells with $F(ab')_2$ fragments of anti-Ig and *Ascaris* glycosphingolipids induces apoptosis, the mitochondrial membrane potential was assessed. Primary splenic B cells were stimulated with $F(ab')_2$ fragments of anti-Ig in the presence or absence of *Ascaris* glycosphingolipids for 20 hours, and the mitochondrial potential assessed by flow cytometry following $DiOC_6(3)$ staining. Co-stimulation of B cells with $F(ab')_2$ fragments of anti-Ig and *Ascaris* glyco-sphingolipids induces a substantial increase in the proportion of cells displaying a shift to low $DiOC_6(3)$ uptake, hence a reduction in mitochondrial membrane potential (**Figure A.6**). $F(ab')_2$ fragments of anti-Ig or glycosphingolipid alone do not induce a collapse of mitochondrial membrane potential, consistent with the DNA content analysis data. These data strongly suggest that one mechanism by which *Ascaris suum* glycosphingolipids inhibit anti-Ig induced B cell proliferation is by increasing the level of apoptosis.

A.4 Discussion

Previous work in our laboratory has shown that two PC-containing "molecules", the filarial nematode glycoprotein ES-62 (reviewed in Harnett and Harnett, 2001) and a glycosphingolipid fraction of *A. suum* (Lochnit *et al.*, 1998a), possess immunomodulatory properties. The latter were found by bioassay to induce release of the proinflammatory cytokines, IL-1, IL-6 and TNF-α when co-cultured with human peripheral blood mononuclear cells. That this effect was likely to be due to the PC moiety was revealed when the selected glycosphingolipids lost their biological activity following removal of PC by HF treatment (Lochnit *et al.*, 1998b). Whereas these experiments targetting human PBMC represent our only investigation of the immunomodulatory properties of the glycosphingolipids to date, ES-62 has been subject to analysis for almost a decade (reviewed in Harnett and Harnett, 2001). Based mainly on studies on mice, it has been shown to affect a number of cells of the immune system, with an overall tendency to suppress pro-inflammatory immune reactivity and hence produce a phenotype that is essentially anti-inflammatory. Although only one study has been conducted on PC-depleted ES-62 (on how removal of PC influences antibody responses to other epitopes present on this molecule (Houston *et al.*, 2000)), many of ES-62's effects can be mimicked by PC alone or conjugated to BSA (reviewed in Harnett and Harnett, 1999). Taking all of this into account, it was assumed that the glycosphingolipids would mirror ES-62 with respect to their immunomodulatory properties and hence we set out to test the PC-containing *Ascaris* glycosphingolipids in some of the assays in which ES-62 had previously been tested.

As predicted, PC-containing *Ascaris* glycosphingolipids were found to inhibit activation of resting splenic B cells induced via the antigen receptor. This form of immunomodulation represents the first ever observed with ES-62 and was considered to be due to PC as it could be mimicked by PC-conjugated to BSA or even PC alone (Harnett and Harnett, 1993). It was therefore a surprise to find

that inhibition of anti-Ig-mediated B cell proliferation could also be inhibited by PC-free glycosphingolipids (**Figure A.3**). This result indicates that the glycosphingolipids contain a non-PC immunomodulatory component. Additionally of interest, however, was the finding that the PC-depleted glycosphingolipids inhibited B cell proliferation at least as well as the native *Ascaris* material. This result suggests that either the PC component of the glycosphingolipids is not active in this system or that the two immunomodulatory components are active in a way that is not additive.

Previous studies in our laboratory have shown that the ability of ES-62 to inhibit anti-Ig-mediated B cell proliferation is not due to apoptosis (M. Harnett, personal communication). The results shown here indicate that the inhibitory effect induced by the glycosphingolipids was however associated with increased apoptosis (**Figure A.5 and Figure A.6**). This result underscores the idea initially invoked by the difference in PC-dependence, that ES-62 and the *Ascaris suum* glycosphingolipids inhibit B cell proliferation by different mechanisms. Of the two non-PC components of glycosphingolipids - carbohydrate and ceramide, the latter is perhaps more likely to be the immunomodulatory moiety. This is because ceramide has been shown to be an apoptotic agent in many cell types, including, B cells (Katz *et al.*, 2001). The finding that treatment with *Ascaris suum* glycosphingolipids on their own does not induce apoptosis but that it requires costimulation with anti-Ig, however, suggests that crosstalk between signals elicited by the ceramide moiety and the BCR are required for induction of cell death, presumably via an activation-induced cell death mechanism.

The PC-containing *Ascaris* glycosphingolipids were also found to inhibit LPS-induced activation of B cells, and this effect appears to be PC-dependent (**Figure A.3**). This would suggest that the inhibitory effect results from either PC targetting the LPS receptor or from cross talk following ligation of the LPS and PC receptors. BiaCore and Far Western analysis has indicated that ES-62 binds to lymphocyte and monocyte membrane fractions with receptor-like affinity in a PC-dependent manner (M. Harnett, personal communication). This binding could

reflect the PC-receptor previously identified on B cells (Bach *et al.*, 1983). Alternatively, as PC has been conserved by pathogens throughout evolution and can be detected in a wide range of prokaryotic and eukaryotic pathogens (Harnett and Harnett, 1999), it is possible that PC-containing pathogen products are recognised via binding of PC to pattern-recognition receptors, such as the recently identified Toll-like receptors (TLRs). TLRs have been proposed to initiate and direct innate and adaptive immune responses by recognising conserved "Danger signals" such as LPS on pathogens (Kaisho and Akira, 2000). It would prove very interesting to study the effects of these glycosphingolipid molecules in mice deficient in members of the TLR family.

Overall, these results show that the glycosphingolipid molecules produced by *Ascaris suum* are able to modulate the response of B cells to both anti-Ig and LPS. These effects are not both due to the PC moiety, as modulation of anti-Ig induced proliferation occurred in the absence of the PC moiety, whereas inhibition of LPS-induced proliferation proved to be PC-dependent. This inhibitory effect can be partly explained by an induction of apoptosis. This apoptosis-inducing effect is not necessarily an intrinsic property of these molecules, as alone they do not induce apoptosis, only when anti-Ig is also present.

Figure A.1 ES-62 desensitises coupling of the BCR to key proliferative signalling cascades.

Exposure of murine B cells to ES-62 selectively desensitises BCR-mediated proliferation by targeting key proliferative signalling pathways. Whilst, early sIg-coupled mediators such as the PTKs Lyn, Syk and Blk are relatively unaffected the subsequent PTK-mediated activation of downstream PI3-K and Ras-MAPK pathways is targeted. The nematode product also modulates the activity of certain PKC isoforms. The BCR is uncoupled from those pathways represented to the right of the arrow.

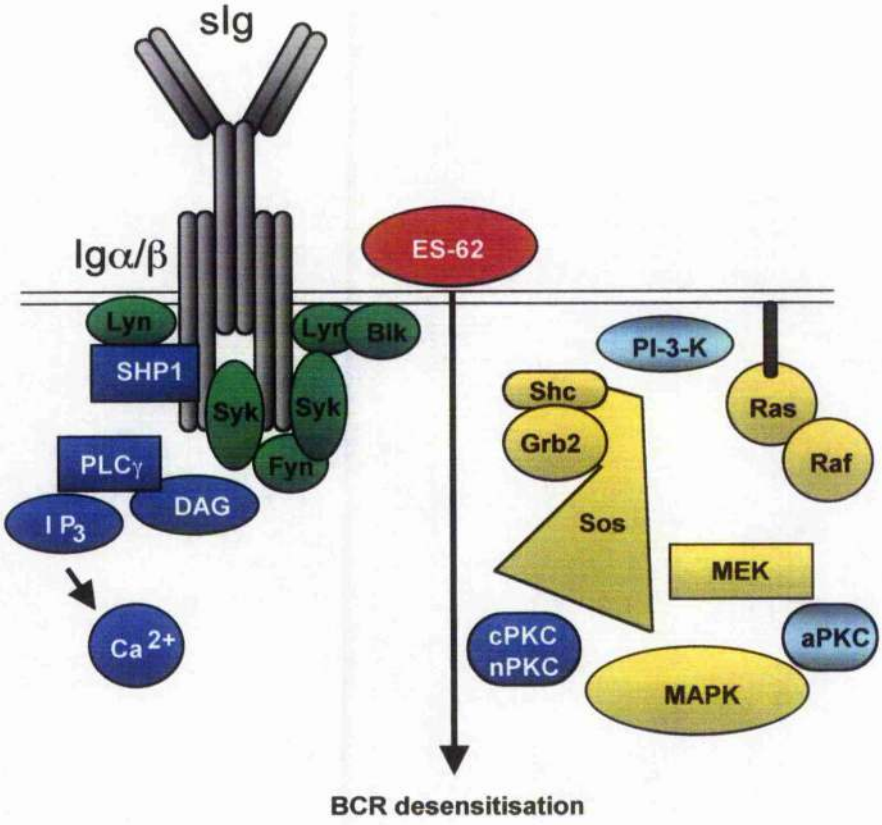

sIg
Igα/β
ES-62
Lyn
Lyn
Blk
SHP1
Syk
Syk
Fyn
PLCγ
DAG
I P3
Ca 2+
PI-3-K
Shc
Grb2
Sos
Ras
Raf
MEK
cPKC
nPKC
aPKC
MAPK
BCR desensitisation

Figure A.2 ES-62 uncouples the BCR from Erk-MAPKinase activation by priming B cells for BCR-mediated recruitment of SHP-1 and Pac-1.

ES-62 uncouples the BCR from Erk-MAPKinase by (1) promoting the BCR recruitment of Lyn-SHP-1 protein tyrosine phosphatase complexes. (2) the dephosphorylation of Ig-β, preventing the recruitment of Shc-SOS complexes. (3) the formation of Erk-MAPK/Pac-1 complexes.

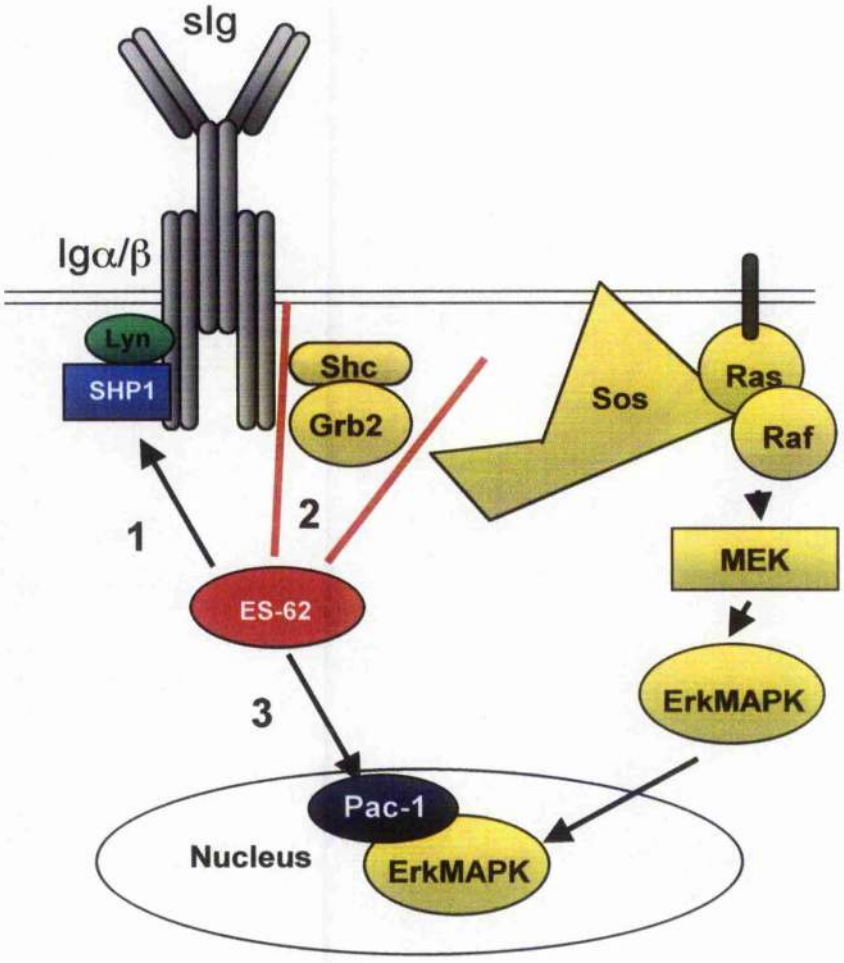
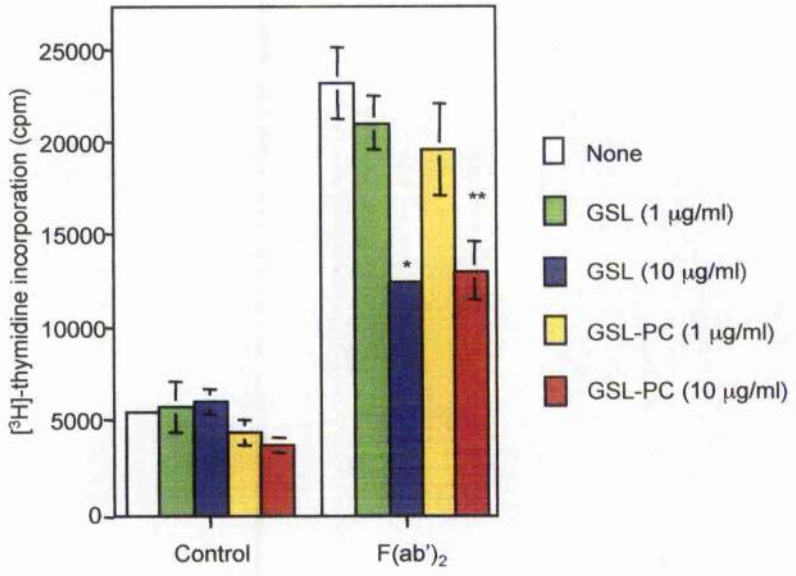

sIg
Igα/β
Lyn
SHP1
Shc
Grb2
Sos
Ras
Raf
MEK
ErkMAPK
1
2
3
ES-62
Pac-1
Nucleus
ErkMAPK

Figure A.3 *Ascaris* glycosphingolipids inhibit anti-Ig- and LPS-induced proliferation of primary splenic B cells

A Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours with 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific), in the presence or absence of native (GSL) or PC-free (GSL-PC) *Ascaris suum* glycosphingolipids at the indicated concentrations. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least two other independent experiments.

B Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours with LPS (50 μg/ml), in the presence or absence of native or PC-free *Ascaris suum* glycosphingolipids at the indicated concentrations. Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least two other independent experiments.

A


25000
20000
15000
10000
5000
0
[3H]-thymidine incorporation (cpm)
*
**
Control
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GSL (10 μg/ml)
GSL-PC (1 μg/ml)
GSL-PC (10 μg/ml)

B

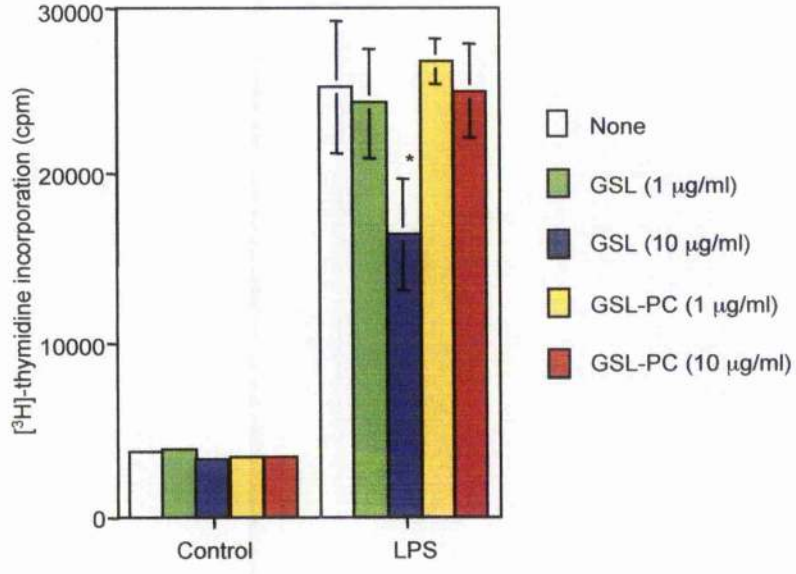

30000
20000
10000
0
[3H]-thymidine incorporation (cpm)
*
Control
LPS
None
GSL (1 μg/ml)
GSL (10 μg/ml)
GSL-PC (1 μg/ml)
GSL-PC (10 μg/ml)

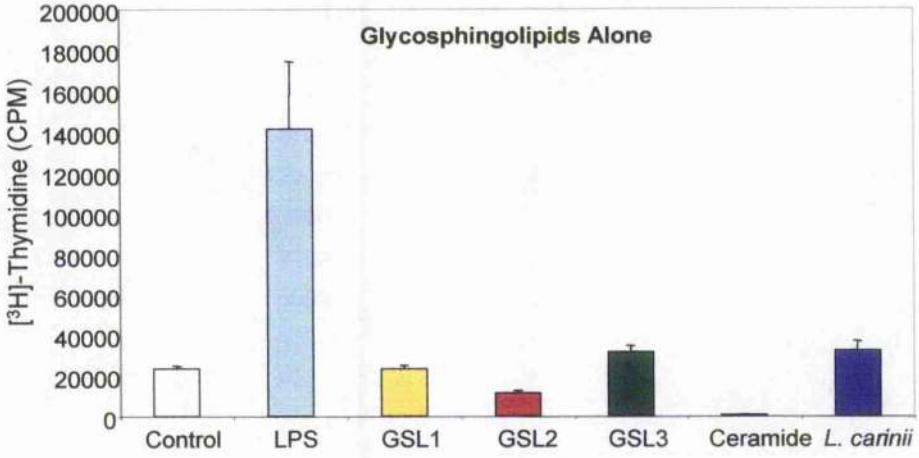
Figure A.4 Synthetic mimetics of *Ascaris suum* glycosphingolipids fail to exert the same effect on anti-Ig- and LPS-induced proliferation of primary splenic B cells

A Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were cultured for 48 hours in the presence of three chemically synthesised components of *Ascaris suum* glycosphingolipids (10 μg/ml) (GSL1, GSL2, GSL3), the ceramide component (10 μg/ml), or natural *L. carinii* glycosphingolipids (10 μg/ml). Control cells were cultured in the presence of medium alone. Culture wells were pulsed with [^{3}H]-thymidine (0.5 μCi/well) 4 hours prior to harvesting and [^{3}H]-thymidine incorporation was assessed by liquid scintillation counting. Data are the mean ± standard deviation of triplicate measurements from a single experiment, representative of at least two other independent experiments.

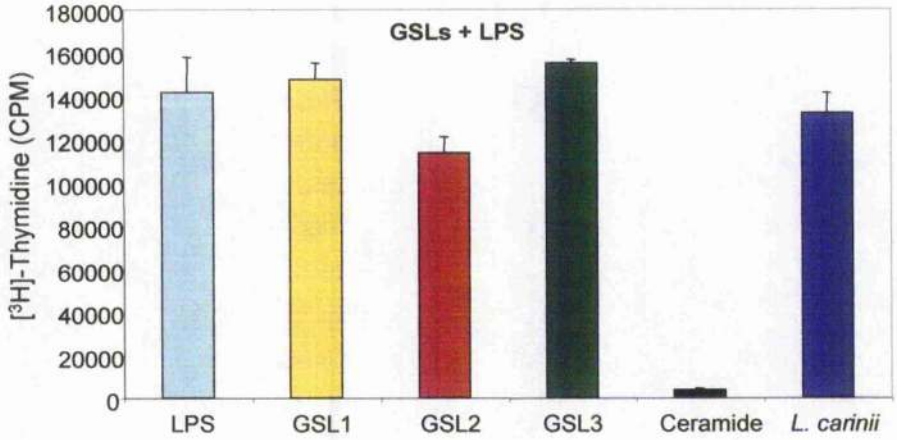
B Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours with LPS (50 μg/ml) in the presence or absence of glycosphingolipid components, and DNA synthesis assessed as above.

C Purified splenic B cells from Balb/c mice (2×10^5 cells/well) were stimulated for 48 hours with F(ab')$_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml) in the presence or absence of glycosphingolipid components, and DNA synthesis assessed as above.

A


Glycosphingolipids Alone
[3H]-Thymidine (CPM)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Control
LPS
GSL1
GSL2
GSL3
Ceramide
L. carinii

B


GSLs + LPS
[3H]-Thymidine (CPM)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
LPS
GSL1
GSL2
GSL3
Ceramide
L. carinii

C

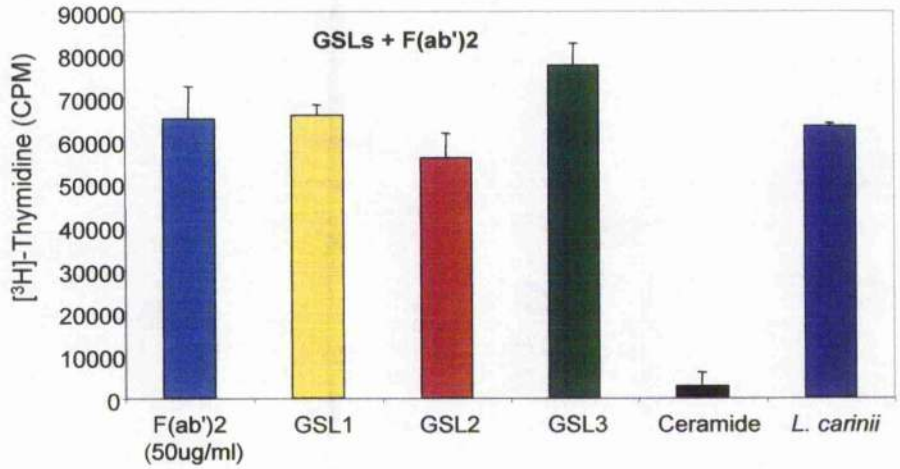

GSLs + F(ab')2
[3H]-Thymidine (CPM)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
F(ab')2
(50ug/ml)
GSL1
GSL2
GSL3
Ceramide
L. carinii

Figure A.5 *Ascaris suum* glycosphingolipids inhibit anti-Ig-induced B cell proliferation by inducing an increase in apoptosis

Purified splenic B cells from Balb/c mice (1×10^6 cells) were stimulated with 50 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) in the presence or absence of ES-62 (2 μg/ml) or native *Ascaris suum* glycosphingolipid (GSL) (5 μg/ml). Cells incubated in the presence of medium alone were included as a control. DNA content was determined by propidium iodide (PI) staining and FACS analysis, as described in Materials and Methods. Data represents each cell cycle stage expressed as a percentage of the total cells analysed from a single experiment, representative of at least one other independent experiment.

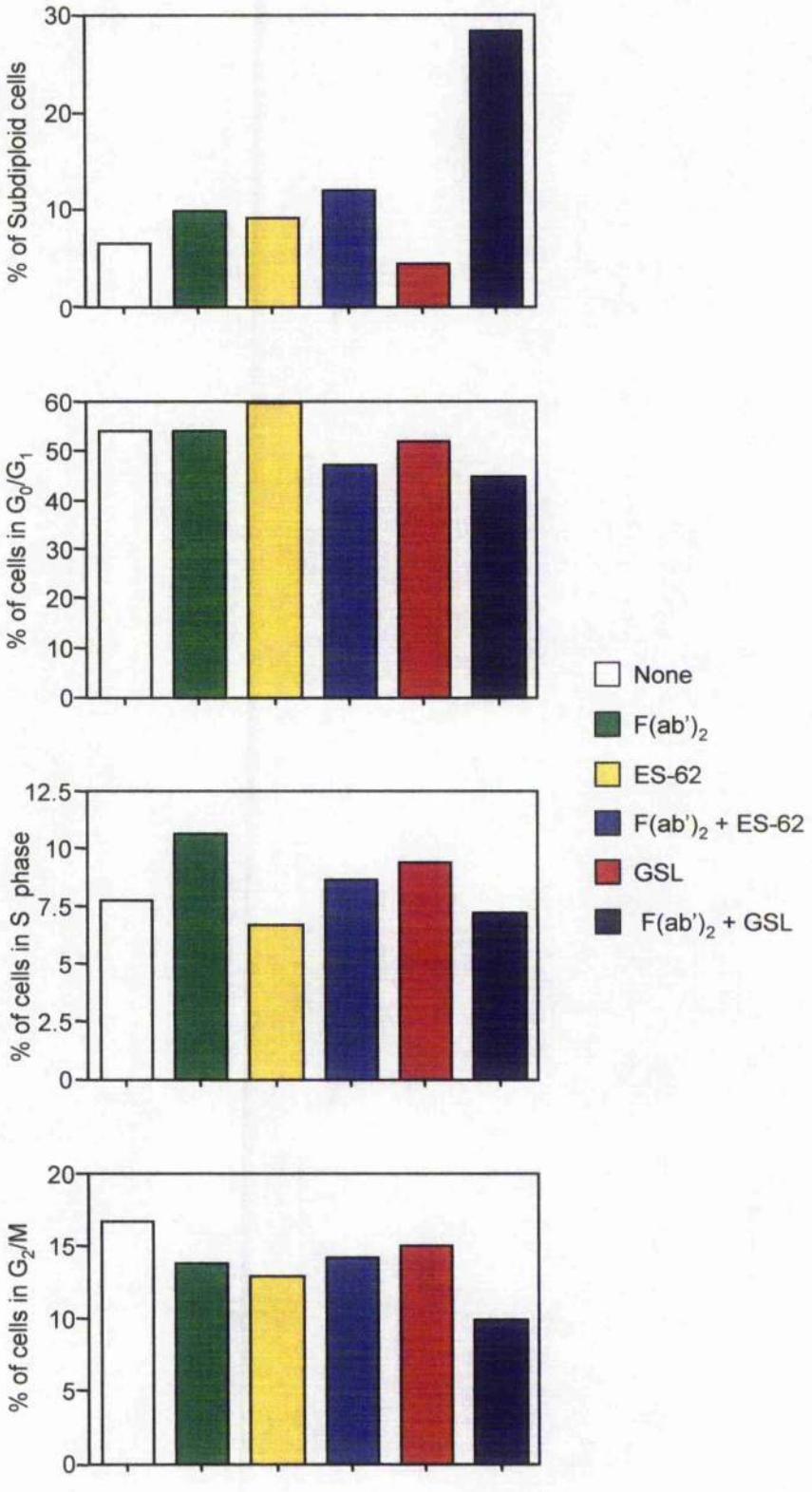
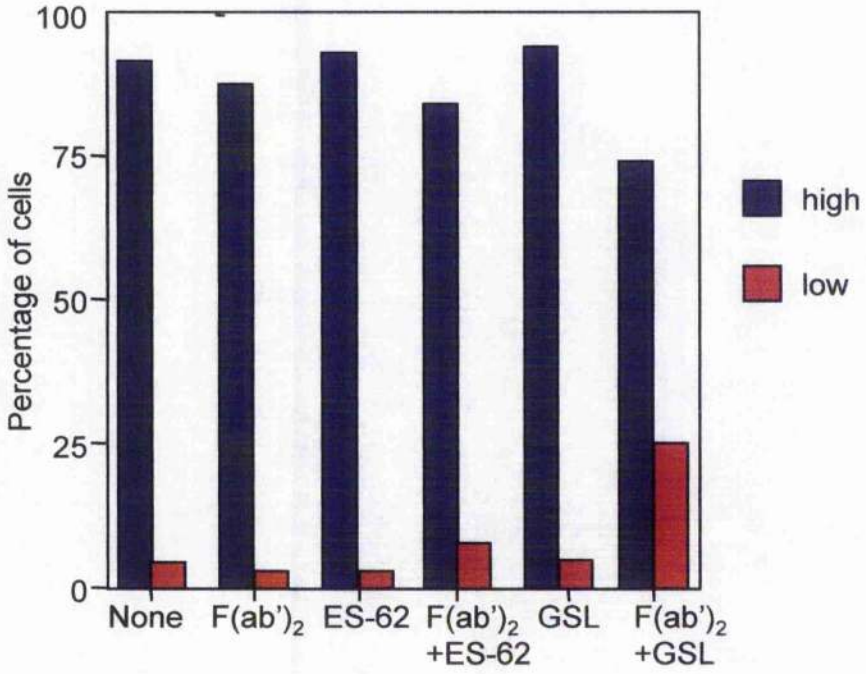

% of Subdiploid cells
30
20
10
0
% of cells in G_0/G_1
60
50
40
30
20
10
0
% of cells in S phase
12.5
10
7.5
5
2.5
0
% of cells in G_2/M
20
15
10
5
0
None
F(ab')$_2$
ES-62
F(ab')$_2$ + ES-62
GSL
F(ab')$_2$ + GSL

Figure A.6 *Ascaris suum* glycosphingolipid-mediated increase in apoptosis involves a collapse of the mitochondrial membrane potential

Purified splenic B cells from Balb/c mice (1×10^6 cells) were stimulated for 20 hours with $F(ab')_2$ fragments of goat anti-mouse IgM (μ-chain specific) (50 μg/ml) in the presence or absence of ES-62 (2 μg/ml) or native *Ascaris suum* glycosphingolipid (GSL) (5 μg/ml). Cells incubated in the presence of medium alone were included as a control. The mitochondrial membrane potential was determined by $DiOC_6(3)$ staining and FACS analysis, as described in Materials and Methods. Data represents the $DiOC_6(3)$ high (non-apoptotic) or $DiOC_6(3)$ low (apoptotic) cell populations, as determined on a logarithmic FL-1 axis, and expressed as a percentage of the total cells analysed in a single experiment, and representative of at least two other independent experiments.


100
75
50
25
0
Percentage of cells
high
low
None
F(ab')2
ES-62
F(ab')2 +ES-62
GSL
F(ab')2 +GSL

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