Differential regulation of Erk-MAPK in the control of survival and proliferation of WEHI-231 immature B cells

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SUMMARY

Ligation of the B cell antigen receptor (BCR) on B lymphocytes has divergent effects including anergy, apoptosis, proliferation or differentiation depending on the developmental state of the cell. Immature B cells are sensitive to the process of negative selection enabling the removal of self-reactive B lymphocytes. Consequently, ligation of the BCR on immature B cells induces growth arrest and apoptosis. However, co-ligation of CD40 can rescue immature B cells and enable them to survive, mature and proliferate. The murine B cell lymphoma cell line WEHI-231 is widely used as a model of clonal deletion of immature B lymphocytes. This investigation has explored the signalling mechanisms used by the BCR and CD40 to differentially regulate the survival and proliferation of WEHI-231 immature B cells. In particular, the role of extracellular signal regulated kinase-mitogen-activated protein kinase (Erk-MAPK) in controlling these processes was examined.

The activity of Erk-MAPK in WEHI-231 cells was investigated by Western blotting. WEHI-231 cells were shown to undergo a transient activation (≤2 hr) of Erk that was associated with the induction of the BCR-driven mitochondrial death pathway. However, a second, more sustained (12-48 hr) Erk signal was also identified and this appears to be critical for the proliferation of WEHI-231 cells. This sustained Erk signal was suppressed by ligation of the BCR and this seems to be mediated by the induction of MAPK phosphatases rather than a downregulation of upstream activators of Erk. However, co-ligation of CD40 restored sustained and cyclic activation of Erk to WEHI-231 cells and thus enabled them to undergo cell cycle progression and proliferation, as shown by measuring the uptake of [^{3}H] thymidine. A dual role for Erk-MAPK in BCR-driven apoptosis and CD40-mediated rescue of WEHI-231 cells was thus identified.

The mechanisms used to regulate the activity of Erk-MAPK in WEHI-231 cells were further examined using WEHI-231 cells that were transfected with constitutively active forms of Ras. These studies showed that Ras is an important upstream activator of Erk in WEHI-231 cells and it can induce Erk1/2 activation via Raf-1 and/or phosphatidylinositol-3-kinase (PI-3-K) pathways. The induction of sustained and cyclic Erk signals and hence proliferation of WEHI-231 cells therefore requires active Ras and stimulation of its downstream effectors. In addition to stimulating sustained Erk signals, the Ras-dependent

activation of PI-3-K was shown to induce the pro-survival Bcl-2-family member, Bcl-x_L and the induction of Bcl-x_L by CD40 appears to be critical for suppressing BCR-driven apoptosis.

A key event in the stimulation of BCR-driven apoptosis in WEHI-231 cells is the induction of early (≤2 hr) Erk signals since this appears to contribute to the activation of cytosolic phospholipase A_2 ($cPLA_2$). Previous studies have shown that following the activation of $cPLA_2$, arachidonic acid is produced at the mitochondria, leading to a disruption of mitochondrial function and the stimulation of cathepsin B. However, CD40 signalling has widely been reported to rescue WEHI-231 cells from BCR-driven apoptosis. In this study, evidence is presented to show that the induction of Bcl-x_L by CD40 is critical for mediating this rescue. Indeed, analysis of the sub-diploid DNA content of WEHI-231 cells showed that the expression of Bcl-x_L in these cells impaired BCR-driven apoptosis. Furthermore, the mechanism of Bcl-x_L-dependent survival was implicated by its ability to prevent mitochondrial PLA_2 activity, disruption of mitochondrial function, cathepsin B activation and hence post-mitochondrial execution of the cell. Moreover, expression of Bcl-x_L protected WEHI-231 cells from arachidonic acid-induced apoptosis suggesting it acts downstream of $cPLA_2$ to impair BCR-driven apoptosis, presumably by protecting the integrity of the mitochondrial membranes. Nonetheless, Bcl-x_L was also shown to prevent arachidonic acid-mediated activation of $cPLA_2$ independently of its regulation of mitochondrial membrane potential and this is likely to further suppress the BCR-driven mitochondrial death pathway. The induction of Bcl-x_L by CD40 is thus critical for enabling the survival of BCR-stimulated WEHI-231 cells.

However, expression of Bcl-x_L did not prevent BCR-driven growth arrest and Western blotting for Erk-MAPK indicated that this was due to the fact that Bcl-x_L was insufficient to restore sustained and cyclic activation of Erk. By contrast, metabolites of arachidonic acid were implicated as mediators of CD40-dependent rescue from BCR-driven growth arrest. In particular, ligation of CD40 was shown to induce cyclooxygenase 2 enabling arachidonic acid to be metabolised to prostaglandin E_2. The metabolism of arachidonic acid impaired BCR-driven apoptosis not only by reducing the levels of arachidonic acid but also because eicosanoids appear to have a role in promoting sustained Erk signals and hence proliferation of WEHI-231 cells.

Additional regulators of Erk-MAPK have also been identified in these investigations. In particular, the protein kinase C (PKC) isoforms PKCα, PKCδ, PKCε and PKCζ appear to be capable of promoting the induction of sustained Erk signals and hence impair BCR-driven growth arrest of WEHI-231 cells. Nonetheless, PKC may also promote proliferation of WEHI-231 cells independently of inducing Erk since expression of kinase-dead mutants of PKCα and PKCδ were unable to enhance Erk signalling despite promoting proliferation of WEHI-231 cells. Specific PKC isoforms therefore appear to promote proliferation of WEHI-231 cells and this is likely to be mediated by several mechanisms.

PKCα and PKCδ were also implicated as having a role in regulating the induction of Bcl-x_L in WEHI-231 cells. Indeed, Western blotting for Bcl-x_L indicated that expression of these isoforms of PKC increased Bcl-x_L levels and impaired BCR-driven apoptosis, as indicated by analysing the sub-diploid DNA content of the cells. Similarly, sphingosine-1-phosphate was identified as an important mediator of CD40-dependent induction of Bcl-x_L since inhibition of sphingosine kinase reduced the levels of Bcl-x_L in WEHI-231 cells. Nonetheless, ligation of CD40 enhanced the survival of Bcl-x_L-overexpressing WEHI-231 cells that were cultured in the presence of sphingosine kinase inhibitors suggesting CD40 may also induce Bcl-x_L via sphingosine kinase-independent mechanisms or CD40 may promote survival of WEHI-231 cells via Bcl-x_L-independent mechanisms. Consistent with this, expression of PKCζ was found to increase the survival of WEHI-231 cells without inducing Bcl-x_L suggesting PKCζ may act downstream of Bcl-x_L or in parallel to Bcl-x_L.

This investigation has thus demonstrated that the differential regulation of Erk-MAPK signals and Bcl-x_L by the BCR and CD40 is critical for determining the survival and proliferation of WEHI-231 immature B cells. Much of this investigation has focused on the temporal regulation of Erk1/2 and Bcl-x_L in WEHI-231 cells and the mechanisms used by the BCR and CD40 to control these signalling molecules. Indeed, a complex network of signalling pathways has been shown to regulate the activity of Erk-MAPK and the expression of Bcl-x_L and this is likely to ensure the survival and proliferation of immature B cells is carefully regulated.

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DECLARATION

This work represents original work carried out by the author and has not been submitted in any form to any other University. Where use has been made of materials provided by others, due acknowledgement has been made.

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ABBREVIATIONS

AA	arachidonic acid
Ag	antigen
Akt	protein kinase B (PKB)
aPKC	atypical PKC
BAFF	B cell activating factor
BAFF-R	B cell activating factor receptor
BCR	B cell antigen receptor
BH	Bcl-2 homology
BLNK	B cell linker protein
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
cAMP	cyclic adenosine monophosphate
CARD	caspase activation and recruitment domain
CD40-L	CD40 ligand
Cdk	cyclin dependent kinase
CLP	common lymphoid progenitor
Cox	cyclooxygenase
cPKC	conventional PKC
$cPLA_2$	cytosolic phospholipase A_2
cpm	counts per minute
CREB	cAMP response element binding protein
Csk	C-terminal Src kinase
DAG	diacylglycerol
DED	death effector domain
DMS	N, N-Dimethyl-D-erythro-sphingosine
EBF	early B cell factor
EDG	endothelial differentiation gene
EGF	epidermal growth factor
Erk	extracellular signal regulated kinase
FADD	Fas-associated death domain
FDC	follicular dendritic cell
FLIP	FLICE-like inhibitory protein
Gab-1	Grb-2-associated binder-1

GFP	green fluorescence protein
HRP	horse radish peroxidase
HSC	hematopoietic stem cell
IAP	inhibitor of apoptosis proteins
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INK	inhibitor of Cdk4
IP_3	inositol-1, 4, 5-trisphosphate
IP_4	inositol-1, 3, 4, 5-tetraphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun N-terminal kinase
LAB	linker for activation of B cells
LOI	ethyl-3,4-dihydroxybenzylidenecyanoacetate
Lox	lipoxygenase
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
NF-AT	nuclear factor for activated T cells
nPKC	novel PKC
NS-398	N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide
PA	phosphatidic acid
PAK	p21-activated ser/thr kinases
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
pErk	phospho-Erk
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

PGH_2	prostaglandin H_2
PH	pleckstrin homology
PI	propidium iodide
PI-3-K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol-4, 5-bisphosphate
PIP_3	phosphatidylinositol-3, 4, 5-trisphosphate
PKA	protein kinase A (cAMP-dependent protein kinase)
PKB	protein kinase B (Akt)
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
pMEK	phospho-MEK
PP2A	protein phosphatase 2A
pSAkt	phospho-serine^{473} Akt
pTAkt	phospho-threonine^{308} Akt
PTB	phospho-tyrosine binding
PTEN	phosphatase and tensin homologue
RACK	receptor for activated C kinase
RasGAP	Ras GTPase activating protein
Rb	retinoblastoma protein
RKIP	Raf kinase inhibitor protein
sem	standard error of the mean
SH2	Src homology 2
SH3	Src homology 3
SHIP	SH2 domain-containing inositol 5-phosphatase
SHP-1	SH2 domain-containing protein tyrosine phosphatase-1
SHP-2	SH2 domain-containing protein tyrosine phosphatase-2
sIg	surface immunoglobulin
SOS	Son of Sevenless
SPP	sphingosine-1-phosphate
tBid	truncated Bid
TBS	Tris buffered saline
TCR	T cell antigen receptor
TdT	terminal deoxynucleotide transferase

TNF	tumor necrosis factor
TRAF	TNF-receptor-associated factor
WASP	Wiscott-Aldrich syndrome protein
zVAD.fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

CHAPTER 1 - General Introduction

1.1 The immune system

The immune system protects the host from a diverse range of pathogens including bacteria, viruses and parasites. Protection is enabled by a series of non-specific and specific mechanisms of defence. The non-specific (innate) immune response forms the initial line of defence against invading pathogens. It is rapidly activated and can effectively eliminate infections. Two families of phagocytic cells, the monocytes/macrophages and the granulocytes, are responsible for mediating innate immunity. Phagocytes express receptors for a variety of molecules commonly found on the surface of pathogens and activation of these receptors leads to the internalisation of the pathogen where it is exposed to a variety of killing mechanisms including proteolytic enzymes, reactive oxygen species, pH changes, nitric oxide and cationic proteins. In addition to their role in killing pathogens, phagocytes also amplify the immune response by releasing pro-inflammatory cytokines and assisting the activation of a specific immune response.

The specific (adaptive) immune response is initiated more slowly than the non-specific response but it is specifically adapted to target the invading pathogen. Mediators of the adaptive immune response, lymphocytes, specifically recognise individual pathogens. There are two types of lymphocytes: B cells and T cells. B lymphocytes are the key cellular mediators of the humoral immune response and produce antigen-specific antibodies. Antibodies are particularly important for fighting extracellular pathogens including bacteria and parasites. T lymphocytes have multiple roles in the specific immune response. Helper ($CD4^+$) T cells co-ordinate the immune response by regulating B cell development and proliferation and by interacting with phagocytes to aid the destruction of internalised pathogens. Cytotoxic ($CD8^+$) T cells are particularly important for fighting intracellular pathogens since they can directly destroy infected host cells. The co-ordinated action of cells of the adaptive immune response therefore provides the body with comprehensive protection against both extracellular and intracellular pathogens. Furthermore, the adaptive immune response can remember previous infections and the

quality of the specific immune response improves with each successive encounter with the same pathogen.

1.2 B cell development

B lymphocytes are the key cellular mediators of the humoral immune response and produce antigen-specific antibodies. The development of B cells has two basic criteria: firstly, a large repertoire of B cells must be generated so that the pool of B lymphocytes within the body can recognise a wide variety of antigens, enabling effective protection from pathogens; secondly, non-productive and self-reactive B cells must be eliminated to ensure a successful humoral immune response can be generated without attacking the body itself (1). In order to fulfil these criteria B cell development is a continuous (2) and yet wasteful process.

The B cell antigen receptor (BCR) (reviewed in (3)) is critical for B cell development and function. It contains surface immunoglobulin non-covalently bound to the accessory molecules Igα and Igβ (figure 1.1). Surface immunoglobulin (sIg) is composed of two identical heavy chains and two identical light chains that come together to form antigen-recognition sites. The cytoplasmic domain of sIg is not large enough to directly activate signal transduction pathways but signals can be activated via Igα and Igβ (4, 5). The binding of antigen to sIg induces the tyrosine phosphorylation of Igα and Igβ at specific regions called immunoreceptor tyrosine-based activation motifs (ITAMs). The resultant phospho-tyrosine residues provide anchoring sites for the activation of intracellular signalling cascades that can regulate the survival, activity and function of the B lymphocyte.

1.2.1 Generation of a diverse range of BCRs

Each B cell expresses a single type of BCR and this will differ to the BCR expressed on other newly generated B cells. Different BCRs are created by somatic rearrangement of heavy and light chain immunoglobulin genes. Each chain has a constant and a variable segment with the variable segment being involved in antigen-recognition. The variable region of the heavy chain is generated by two random recombination events; firstly the D_H and the J_H chains are joined then the V_H chain is joined to the D_HJ_H segment (figure 1.2A). There

are about 300 variable (V_H) genes, 30 diversity (D_H) genes and 6 joining (J_H) genes therefore there is the potential to make more than 50,000 possible heavy chains via somatic recombination. Further diversity is generated by TdT (terminal deoxynucleotide transferase), an enzyme that can randomly add nucleotides into the heavy chain genes without following a template. The variable region of the light chain is also generated by somatic recombination but it lacks a D segment and is created via a single recombination event (figure 1.2B). Two possible genes, kappa or lambda encode the light chain of the BCR. Both genes encode multiple V and J segments allowing the potential for several hundred different variable light chain regions.

Surface immunoglobulin of the BCR is generated by the coming together of rearranged heavy and light chains therefore somatic rearrangement of the variable segments of these chains enables a huge repertoire of BCRs to be generated. However, the recombination process is not always successful and non-productive rearrangements, for example out-of-frame joining of the genes encoding the immunoglobulin variable segments, are common. B cell development contains a series of checkpoints to eliminate developing B cells with unsuccessful heavy and light chain gene rearrangements. Another potential problem associated with the generation of a huge repertoire of B cells is the risk of creating self-reactive BCRs therefore checkpoints also exist to eliminate these cells.

1.2.2 Antigen-independent B cell development

In mammals, B lymphocytes develop in the liver of the fetus and in the bone marrow after birth. Newly formed B cells leave the bone marrow and further mature in the periphery and lymph nodes. B cells therefore develop through a series of well-defined stages starting with B-lineage commitment in the bone marrow and finishing with fully immunocompetent B lymphocytes in the periphery. The initial stages of B cell development occur independently of antigen (Ag) whilst later stages are shaped by exposure to antigen (figure 1.3). Studies using knockout mice have provided valuable information to help characterise the different stages of B cell development. Each of the different stages can be distinguished by their surface phenotype (figure 1.4) and the expression of B-lineage genes, specific transcription factors, the mu (μ) heavy

chain and the kappa (κ) light chain (6-9).

The pluripotent hematopoietic stem cell (HSC) is the progenitor of all cells of the immune system including lymphocytes. HSCs give rise to common lymphoid progenitor cells (CLP), which in turn can either form T or B lymphocytes. Early stages of B-lineage commitment require the expression of at least three transcription factors, E2A, EBF (early B cell factor) and Pax5 (10, 11). For example, heterozygous $E2A^{+/-}EBF^{+/-}$ mice undergo B cell developmental arrest at the early pro-B cell stage and $pax5^{-/-}$ mice undergo a similar developmental arrest (12-14). E2A and EBF regulate the expression of proteins that are required in the recombination process including TdT and the recombinase enzymes RAG1 and RAG2. Rearrangement of the immunoglobulin μ heavy chain gene begins at the intermediate pro-B cell stage with D_H-J_H joining, followed by V_H-D_HJ_H joining in late pro-B cells. Following rearrangement of the μ heavy chain, it is expressed on the surface of the developing B cell in conjunction with the surrogate light chain (λ5, VpreB) and Igα/Igβ to form the pre-BCR complex. Expression of the μ heavy chain causes RAG1/2 to be downregulated to prevent further rearrangement of the heavy chain gene. This is necessary for allelic exclusion of the heavy chain locus that is it ensures only one type of heavy chain is expressed in any one particular developing B lymphocyte.

Like the BCR complex of immature and mature B lymphocytes, the immunoglobulin of the pre-BCR complex couples to the accessory signal transduction molecules Igα and Igβ. Igα and Igβ are first expressed on the surface of late pro-B cells in association with the integral membrane protein and molecular chaperone calnexin (figure 1.4) (7, 15) and they are necessary for pre-BCR signal transduction. Unlike the BCR complex of immature and mature B cells no antigen has been identified for the pre-BCR. It has been proposed that ligand-independent basal signals generated through the ITAMs of Igα and Igβ are sufficient for promoting pro-B cell to pre-B cell development with the functional pre-BCR serving to anchor Igα/β to the plasma membrane (16). Furthermore, it has recently been suggested that the induction of signals by the pre-BCR involves the ligand-independent aggregation of components of the pre-BCR (17). Alternatively, it has been suggested that a bone marrow stromal factor such as galectin-1 may bind to and activate the pre-BCR leading to the

phosphorylation of ITAMs in Igα/β (18).

The successful rearrangement of the immunoglobulin μ heavy chain and the expression of this chain as a functional pre-BCR complex is necessary for the development of pre-B cells since the pre-BCR complex initiates signals, including the Erk (extracellular signal regulated kinase)-MAPK (mitogen-activated protein kinase) cascade, that are essential for the survival and proliferation of the developing B cell (19). It is likely that the pre-BCR complex uses similar intracellular signalling molecules to the mature BCR complex. For example, the protein tyrosine kinase Btk (Bruton's tyrosine kinase) is essential for pre-BCR signalling and a deficiency in Btk impairs B cell development at the pre-B cell stage, as indicated by the diseases X-linked agammaglobulinaemia in humans or the less severe X-linked immunodeficiency in mice (15, 20). Furthermore, mice deficient in the tyrosine kinase Syk have impaired pre-BCR signalling leading to a disruption in B cell development at the pro-B to pre-B cell transition (21, 22). IL-7 is also a survival factor for late pro-B cells and large pre-B cells since IL-7, like the pre-BCR, can activate Erk in these cells (19, 23). Signals activated downstream of the pre-BCR thus promote survival and proliferation of the pre-B cells. Cells that have been unsuccessful in rearranging their immunoglobulin heavy chain and cannot express a functional pre-BCR do not undergo proliferation and cannot progress to the next stage of development.

The pre-BCR complex is only expressed transiently on the surface of large pre-B cells and following a limited number of cell division cycles the pre-BCR is lost from the surface of the cells. The resultant small pre-B cells are non-proliferative and they express the recombinase machinery (RAG1 and RAG2) allowing the rearrangement of the light chain genes. This initially occurs at the kappa gene locus but should this be unsuccessful the lambda gene will be rearranged. Successful rearrangement of the light chain allows it to be expressed with the μ heavy chain to form the BCR.

1.2.3 Antigen-dependent B cell development

The mature BCR is first expressed on the surface of immature B cells. Immature B cells are generated in the bone marrow from small pre-B cells but they migrate to the periphery as transitional B cells where they can survive for 2-4 days. Cells that express the BCR undergo antigen-dependent development.

Immature and transitional B cells are largely exposed to self-antigen therefore these cells undergo negative selection in order to prevent the survival of auto-reactive B lymphocytes. In negative selection, ligation of the BCR complex has three possible out comes: anergy, receptor editing or apoptosis (9, 24). Soluble antigens or low affinity interactions with the BCR generally stimulate anergy or receptor editing. Anergic cells are non-responsive and short-lived. Receptor editing involves the activation of the recombinase machinery and editing of the light chain by using a different J gene or the heavy chain by using a new V gene (25). Receptor editing is sometimes able to rescue self-reactive B cells. High affinity interactions with the BCR induce programmed cell death (apoptosis) leading to the clonal deletion of self-reactive B lymphocytes. The strength and nature of the interaction between self-antigen and BCR of immature and transitional B cells therefore directs the fate of the cell.

Additional factors also influence negative selection including the microenvironment of the immature B cell and contact with other cells. Immature B cells from bone marrow or periphery (spleen) of mice undergo apoptosis within 16 hr of treatment of these cells with anti-BCR antibodies *in vitro* (26). However, within the microenvironment of the bone marrow, ligation of the BCR on immature B cells does not stimulate apoptosis but causes RAG2 to be upregulated, allowing receptor editing. Cell-to-cell contact is important for the activation of receptor editing. The microenvironment of the bone marrow thus delays apoptosis of self-reactive immature B cells, allowing them time to edit the BCR (27). Once the immature B cells leave the bone marrow they travel to the spleen through the vascular system. The microenvironment of the spleen does not favour receptor editing therefore ligation of the BCR on these transitional immature B cells induces the default pathway of apoptosis (27). However, co-stimulation by T cells can rescue these B cells from apoptosis. The fate of immature B cells therefore depends upon ligation of the BCR and additional co-stimuli that can be provided by the microenvironment of the cell.

The surface phenotype of immature and transitional B lymphocytes is also thought to have some role in determining the sensitivity of these cells to antigen-induced apoptosis (7, 28-30). Immature B cells express surface IgM but do not express surface IgD. The sIgM allows antigen detection and antigen binding tends to favour the induction of receptor editing over apoptosis.

Immature B cells are largely located within the bone marrow but they form transitional T1 cells and these can be found in the bone marrow, blood and spleen (30-32). In the spleen, T1 transitional cells are located in the outer periarteriolar sheet, close to the primary follicle (8). T1 B cells are very sensitive to BCR-driven apoptosis and a 20 min exposure to anti-Ig (anti-immunoglobulin) is adequate to stimulate death signals. T1 cells die within 16 hr of BCR-stimulation unless they receive T cell-derived rescue signals such as CD40 stimulation or IL-4 (29, 33-35). T1 transitional cells express higher levels of sIgM and do not express sIgD. The high levels of sIgM are thought to increase the sensitivity of these cells to antigen-induced apoptosis and ligation of sIgM on T1 cells does not readily activate recombinase so is unlikely to induce receptor editing.

T1 cells that do not undergo apoptosis develop into T2 transitional B cells ($IgM^{high}IgD^{high}$), which are less sensitive to antigen-induced apoptosis. T2 cells are located inside the follicle of the spleen. During the transition between T1 and T2 cells, the ligand BAFF (B cell activating factor) binds to its receptor (BAFF-R) and drives the formation of T2 transitional cells (36). A deficiency in either BAFF or BAFF-R blocks B cell development at the T1 stage (37). Ligation of the BAFF-R enhances B cell survival by activating the MAPK cascade and NF-κB leading to an upregulation of pro-survival Bcl-2 family members (Bcl-2 and Bcl-x_L) and a decrease in the expression of the pro-apoptotic protein Bak (38, 39). The interaction of BAFF and the BAFF-R cannot overcome B cell negative selection therefore it does not promote the survival of autoreactive B lymphocytes (40). BAFF is expressed on the surface of cells of the myeloid lineage including monocytes and dendritic cells, it may be expressed on the surface of some T cells and it can exist in a soluble form. The formation and survival of T2 transitional B cells is thus enhanced by the presence of myeloid cells and T lymphocytes that express BAFF (37).

T2 transitional B cells can develop into naïve mature B lymphocytes ($IgM^{low}IgD^{high}$). Mature B lymphocytes express the BAFF-R and activation of this receptor promotes survival of these cells (36, 37). However, the formation of naïve mature B cells also requires signalling through the BCR (8). Naïve mature B cells have low levels of sIgM but have high sIgD expression. Some signals activated downstream of the BCR in mature B cells appear to be different to

those activated in immature/transitional B cells (41, 42). In particular ligation of the BCR on mature B cells does not induce growth arrest and apoptosis but can stimulate proliferation and activation of the cell (7, 33, 35, 43). Activation of mature B cells with a relatively high affinity antigen at the BCR and with appropriate co-stimulation initiates a humoral immune response (44, 45). Activated mature B cells undergo proliferation and can differentiate into either plasma cells or memory B cells (46). Plasma cells are antibody-secreting cells that mediate the primary immune response. Memory B cells are required for the induction of a secondary immune response.

1.2.4 Formation of memory B cells

Memory B cells are formed during primary immune responses to T cell-dependent antigens. The generation of memory B cells takes place in specialised regions known as germinal centres (figure 1.5). Following stimulation of a B cell by a T cell-dependent antigen, B cell blasts undergo exponential growth in areas rich in follicular dendritic cells (FDCs). FDCs are able to stimulate helper T cells and they retain unprocessed antigen on their surface that can be used later in the selection of high affinity memory B cells (47). The expanded population of B cell blasts migrates into the centre of the follicle, forming the dark zone of the germinal centre. In the dark zone B cell blasts continue to proliferate but no longer express sIgM and are now called centroblasts. Centroblasts undergo somatic hypermutation of the V regions of the BCR. This is a random process that will generate B cells with BCRs with either increased or decreased affinity for the activating antigen (25). Following hypermutation the centroblasts stop proliferating, migrate to the light zone of the germinal centre and express the new BCR on the cell surface. The cells, now called centrocytes, are particularly sensitive to apoptosis. FDCs within the light zone present antigen to the newly formed centrocytes. The availability of antigen is limited therefore only centrocytes expressing high affinity BCRs can bind to antigen. Survival of centrocytes also requires a second signal that can be provided by ligation of CD40, suggesting a role for helper T cells in the selection of centrocytes (48). Positively selected centrocytes form a pool of memory B cells that can act as precursors for plasma cells with improved antibody affinity (47-49).

1.3 Cell Cycle

The cell cycle ensures DNA is replicated and the cellular mass is doubled prior to cell division. There are four basic stages to the cell cycle (figure 1.6A). Initially cells undergo a period of growth (G1 phase) before replicating their DNA (S phase). DNA replication is followed by a second growth phase (G2) that generally passes more quickly than the G1 phase since the cell has more ribosomes by the G2 phase allowing faster protein synthesis. The final stage of the cell cycle is mitosis (M phase) in which the cell divides to form two daughter cells.

The cell cycle is a very tightly regulated process with two major checkpoints (figure 1.6B). Firstly at the G1-S boundary where cells commit to DNA synthesis and secondly at the G2-M transition where cells commit to mitotic cell division. Progression through these checkpoints is dependent upon the activity of cyclin-dependent kinases (Cdks) and their regulators, the cyclins (figure 1.6B). Progression through the early stages of the G1 phase of the cell cycle requires the activation of Cdk4 and Cdk6. These are cyclin D kinases and are activated by cyclin D1, D2 or D3. Cdk2 and its activator cyclin E are required during the late stages of G1 and for progression into S phase. The Cdk-cyclin complexes of G1 phase promote cell cycle progression by phosphorylating the retinoblastoma (Rb) pocket domain protein (50). Hypophosphorylated Rb binds to transcription factor III B and to upstream binding factor so preventing transcription mediated by RNA polymerase III. RNA polymerase III is required for the generation of transfer RNA and the small ribosomal subunit and hence protein synthesis and cell growth. Phosphorylation of Rb allows RNA polymerase III-mediated transcription and hence cellular growth. Hypophosphorylated Rb also binds to the transcription factor E2F and prevents it from forming dimers with DP transcription factors. Dimerisation with a DP family member is essential for E2F to activate transcription therefore Rb prevents E2F-dependent transcription. Phosphorylation of Rb by Cdks causes Rb to dissociate from E2F, allowing transcription to proceed. Activation of E2F is necessary for the G1-S phase transition since it regulates the expression of genes required for DNA synthesis and cell cycle progression including DNA polymerase alpha, thymidine synthetase, cyclin D3, cyclin E and cyclin A (33, 50-54).

DNA is replicated during S phase of the cell cycle and this phase is sustained by cyclins A and E and Cdk2. On completion of DNA synthesis the cells enter the second period of growth, the G2 phase. During G2 the Cdk2-cyclin B complex accumulates (55) and Cdc25, a protein phosphatase that is only expressed once DNA replication is complete, can activate this complex. Phosphorylation of various target proteins by the active Cdk2-cyclin B complex allows cells to enter mitosis. Cdk2-cyclin B can also phosphorylate and inhibit itself thus forming a negative feedback loop.

The ability of Cdk-cyclin complexes to allow cell cycle progression is regulated by two structurally distinct families of Cdk inhibitors (figure 1.6B). Firstly the WAF1 family (p21, p27 and p57) of proteins and secondly the INK4 (inhibitor of Cdk4) family (p15, p16, p18 and p19). The inhibitors p15 and p16 are specific for cyclin D kinases therefore they are particularly important regulators of Rb and hence can suppress progression through G1 phase and entry into S phase of the cell cycle. The inhibitor p27 suppresses Cdk2-cyclin A (56). Several Cdk-cyclin complexes can be inhibited by p21 including Cdk4-cyclin D1, Cdk2-cyclin E, Cdk2-cyclin A and Cdc2-cyclin A (51) Thus, the activation of p21 can inhibit DNA synthesis and induce cell cycle arrest at both G1 and G2 phases (57). Expression of the gene encoding p21 can be stimulated by p53, the "guardian of the genome" (58, 59). The levels of p53 within a cell are increased during cellular stress or damage and the activity of p53 can be enhanced by $p19^{INK4}$, which stimulates the degradation of MDM2, an inhibitor of p53 (60, 61). By stopping DNA synthesis and cell cycle progression, p53 is able to prevent the replication of faulty DNA or a damaged cell (51). Following the activation of p53 and cell cycle arrest, proteins involved in DNA-repair are induced and minor damage to DNA is repaired. The expression of MDM2 is regulated by p53, creating a negative feedback loop that enables cell cycle progression to proceed once DNA damage has been repaired.

In addition to its role in regulating the cell cycle, p53 is also a mediator of apoptosis. Indeed, thymocytes and immature B cells that lack p53 are resistant to apoptosis induced by specific stimuli (51, 58). One mechanism by which p53 induces apoptosis is by increasing the expression of the pro-apoptotic protein Bax (59, 62-64). The ability of p53 to induce apoptosis is regulated by multiple

factors. For example, treatment of rat embryo fibroblasts with the adenovirus E1A protein induces p53-dependent apoptosis but the adenovirus normally expresses E1B proteins that impair p53-dependent transcription and p53-mediated apoptosis (51).

1.4 Apoptosis

At least two distinct types of cell death have been characterised: necrosis and apoptosis. Necrosis is induced following acute injury of the cell and the distinctive features are cytoplasmic swelling and membrane rupturing without significant alteration of the nucleus. Necrotic cells release their contents into the surrounding area and this may trigger an inflammatory response. In contrast, apoptosis, the process of programmed cell death, does not affect neighbouring cells and is unlikely to induce inflammation. Apoptosis is an active process whilst necrosis is passive. The characteristic features of apoptosis are swelling and disruption of intracellular organelles, cytoskeletal collapse, membrane blebbing, chromatin condensation, DNA fragmentation and cellular disassembly. Apoptosis is essential for the development and maintenance of healthy organisms. For example, in mammals apoptosis is used for limb modelling and neurological development. Furthermore, in the immune system apoptosis is required to eliminate self-reactive cells and to terminate an immune response (65, 66).

1.4.1 Bcl-2 family proteins - regulators of apoptosis

Members of the Bcl-2 family are important regulators of apoptosis in eukaryotic cells. The Bcl-2 family can be split into three groups based on their expression of Bcl-2 homology (BH) domains (figure 1.7) (67). Two groups of Bcl-2 family proteins promote apoptosis and the third group favours survival. The pro-survival group of Bcl-2 family proteins e.g. Bcl-2 and Bcl-x_L have all four BH domains (BH1-4). The pro-apoptotic groups of Bcl-2 family proteins have either 3 BH domains (BH1-3) e.g. Bax and Bak, or the BH3 domain alone e.g. Bid and Bik. The BH1, BH2 and BH3 domains of a Bcl-2 family protein can come together to form a hydrophobic pocket. The BH3 domain of another Bcl-2 family protein can bind to this pocket to form a dimer. Using this mechanism homo- or hetero-dimers are formed and the nature of these dimers determines

the fate of the cell.

Bcl-2 family proteins are largely regulated at the transcriptional level and the fate of a cell depends upon the balance of expression of pro-survival and pro-apoptotic members of the Bcl-2 family (67). Post-translational modification can further regulate the dimerisation and hence function of Bcl-2 family members. For example, in the interleukin-3 (IL-3)-dependent FL5.12 cell line, IL-3 induces the serine phosphorylation of the pro-apoptotic protein Bad causing it to dissociate from Bcl-x_L and to bind to 14-3-3 proteins. This sequesters Bad and prevents it from promoting apoptosis thus enabling Bcl-x_L to mediate its pro-survival functions (68, 69). Additional mechanisms of regulation have also been observed for Bcl-2 proteins that only contain a BH3 domain. In particular, some of these proteins are constitutively expressed but can be sequestered and inactivated by binding to the cytoskeleton (70). Bid is also regulated by proteolysis; activation of caspase 8 results in the cleavage of the inactive 22 kD Bid to form an active 15 kD tBid (truncated Bid) that is targeted to the mitochondria by N-myristoylation where it can promote apoptosis (71, 72).

Pro-survival members of the Bcl-2 family normally bind to the membranes of organelles, especially the mitochondria, and maintain the integrity of the membrane to prevent the contents of the organelle leaking into the cytoplasm (73). In apoptosis, pro-apoptotic Bcl-2 family proteins are upregulated and they can form dimers with pro-survival Bcl-2 family members to suppress their guardian function. Alternatively, pro-apoptotic Bcl-2 proteins can dimerise with other pro-apoptotic Bcl-2 family proteins to promote apoptosis. In particular, dimers of pro-apoptotic Bcl-2 family proteins induce apoptosis by forming pores in the outer mitochondrial membrane resulting in a loss of mitochondrial integrity. Loss of mitochondrial function has three particularly significant effects. Firstly, ATP can no longer be produced therefore the regular functions of the cell cannot be sustained. Secondly, the loss of mitochondrial integrity increases the production of reactive oxygen species and these are toxic to the cell. Thirdly, and perhaps most importantly, the formation of pores in the outer mitochondrial membrane causes the contents of the mitochondria to be released into the cytoplasm, providing co-factors for the activation of caspases, an important group of proteins that can mediate apoptosis.

1.4.2 Caspase-dependent apoptosis

Key mediators of apoptosis are the caspases, a family of evolutionary conserved cysteine proteases. Caspases were first studied in *C. elegans* (figure 1.8A) where the caspase CED3 mediates all somatic cell death. CED3 is activated by a single protein, CED4 and the activity of CED4 is regulated by the proteins CED9 and EGL-1. CED9 physically blocks the association of CED4 and CED3 so is a negative regulator of these proteins whilst EGL-1 inhibits CED9, allowing the activation of CED4 and CED3 (74, 75).

Mammalian cells express homologues of the *C. elegans* apoptotic machinery and can undergo apoptosis using a similar mechanism. However, apoptosis in mammalian cells is more complex than in *C. elegans* since they express multiple regulators, initiators and effectors of apoptosis (figure 1.8A). Indeed, more than fourteen mammalian caspases have been identified and they have been divided into three groups based on their substrate specificity. Substrates of caspases are cleaved at the C-terminal side of an aspartic acid residue. The four amino acids to the N-terminal side of the Asp residue determine the substrate specificity of a particular caspase (67). The three categories of mammalian caspases, DEXDases, WEHDases and (IVL)EXDases, require different tetrapeptide sequences. The WEHDases can function as inflammatory mediators whilst members of the other two classes of caspases regulate apoptosis either as initiator or effector caspases.

All caspases are expressed as zymogens with an N-terminal pro-domain, a central p20 domain and a C-terminal p10 domain (67). Activation of caspases requires two proteolysis reactions firstly to remove the pro-domain and secondly to cleave between the p20 and p10 subunits allowing two of each of these subunits to come together to form a catalytically active tetramer (figure 1.8B). Caspases can either be activated by autocleavage or by proteolysis catalysed by an upstream caspase. In general, initiator caspases are activated by autocleavage and effector caspases are stimulated downstream of initiator caspases.

Initiator and effector caspases of mammals can be physically distinguished by the length the their pro-domain (67). Initiator caspases (caspases 2, 8, 9 and 10) have long pro-domains whereas effector caspases (caspases 3, 6 and 7) have short pro-domains. The pro-domains not only

function to inhibit the caspase when it is not required but they also provide docking sites that help to stimulate the caspase under apoptotic conditions. For example, the long pro-domains of initiator caspases include protein-protein interaction domains, allowing them to bind to upstream regulators. Caspases 2 and 9 have caspase activation and recruitment domains (CARD) whereas caspases 8 and 10 have death effector domains (DED). CARD domains generally associate with other CARD domains. The CED4 homologue Apaf-1 is a particularly important CARD domain-containing protein that activates pro-caspase 9 (figure 1.9). In healthy cells, Apaf-1 is associated with the mitochondria using pro-survival Bcl-2 family proteins. However, Apaf-1 is displaced from the mitochondria during apoptosis due to the increased levels of pro-apoptotic Bcl-2 family proteins. Furthermore, the pro-apoptotic Bcl-2 family proteins disrupt the integrity of the mitochondria resulting in the contents of the mitochondria being released into the cytoplasm and the cytoplasmic levels of cytochrome c, ATP and Ca^{2+} become elevated. Cytochrome c binds to the WD40 repeats in the C-terminal region of Apaf-1, opening the conformation and allowing ATP to bind to the nucleotide binding domain of Apaf-1. Adjacent to the nucleotide-binding domain of Apaf-1 is an N-terminal CARD domain that can then bind to the CARD domain of pro-caspase 9. This complex of cytochrome c, ATP, Apaf-1 and pro-caspase 9 is known as the Apaf-1 apoptosome and it is likely to form multimeric complexes with other Apaf-1 apoptosomes. These complexes function as holoenzymes since caspase 9 is most active when it is associated with Apaf-1 (67, 75-77). Caspase 9 induces apoptosis of the cell via the activation of effector caspases including caspase 3 (67).

Caspase 3 and other effector caspases can also be activated via DED domain-containing caspases. These are stimulated in a slightly different way to CARD domain-containing caspases. DED domains can physically interact with other DED domains or death domains. Death domains can be found in association with many pro-apoptotic receptors including CD95 (Fas). Ligation of Fas induces oligomerisation of the receptor and any associated proteins including FADD (Fas-associated death domain) which can bind to the DEDs of pro-caspases 8 and 10 (figure 1.10). This creates a high local concentration of protease enabling the low intrinsic activity of the pro-caspase to mediate autocleavage and activation of caspases 8 and 10. These initiator caspases can

then cleave and activate pro-caspase 3 to induce apoptosis of the cell (67, 74, 78). Furthermore, caspase 8 can cleave and activate Bid, which can dimerise with Bax to cause damage to the mitochondria resulting in the activation of CARD domain-containing caspases and additional effector caspases (figure 1.10) (71, 72).

Effector caspases mediate apoptosis of the cell via the proteolysis of multiple target proteins (67, 79). For example, caspase-activated DNase is stimulated by caspase-mediated proteolysis and it can then cleave the DNA between nucleosomes to cause DNA laddering (80). Effector caspases also cleave nuclear lamins resulting in the shrinkage of the nucleus and cytoskeletal proteins like fodrin and gelsolin to induce a loss of the shape of the cell (67). Furthermore, in Jurkat T cells that are undergoing Fas-induced apoptosis, PAK2, a member of the p21-activated kinase family, is constitutively activated by effector caspase-mediated proteolysis. PAK2 alters the morphology of the cell by phosphorylating myosin and has also been implicated in stimulating JNK (c-Jun N-terminal kinase) MAPK to further promote the death response of the cell (81, 82).

Caspase-mediated apoptosis is a carefully regulated process. In non-apoptotic cells pro-survival Bcl-2 family members protect the mitochondria and other intracellular organelles to ensure there is no release of caspase-activating co-factors into the cytoplasm. Furthermore, IAPs (inhibitor of apoptosis proteins) are present in the cytoplasm to suppress the activation of caspases. For example, XIAP can bind to and inhibit the protease activity of caspase 9, preventing its stimulation and subsequent activation of caspase 3. Fas-dependent activation of caspase 8 is also suppressed in healthy cells by the FADD-inhibitor FLIP (FLICE-like inhibitory protein) (83). During an apoptotic response pro-apoptotic Bcl-2 family members are upregulated and activated leading to a loss of mitochondrial integrity. As previously described, disruption of the mitochondria is necessary for the formation of the Apaf-1 apoptosome and the activation of caspases. Disruption of the mitochondria also releases the pro-apoptotic protein Smac (also known as DIABLO) into the cytoplasm (84). Smac promotes apoptosis by associating with the Apaf-1 apoptosome and inhibiting IAPs resulting in an increased activation of caspases (67, 78, 84). The induction of caspases is therefore a tightly regulated process that ensures

caspases are suppressed in healthy cells but are potently induced in apoptotic cells enabling rapid and efficient execution of the cell.

1.4.3 Apoptosis-independent functions of caspases

Caspases are undoubtedly important mediators of apoptosis. However, they also perform non-apoptotic functions; for example, caspase 8 can interact with the I-κB kinase signalosome resulting in an increased kinase activity and the potential to enhance NF-κB activity (79). Caspases have also been implicated in regulating the activities of JNK and p38 MAPKs (81, 85, 86). Furthermore, FADD and caspases have been implicated in promoting proliferation of T and B lymphocytes (reviewed in (79)). For example, thymocytes and peripheral T cells that are deficient in FADD not only have impaired death receptor-mediated apoptosis but they also have defective T cell antigen receptor (TCR)-driven proliferation (87). These cells have abnormal regulation of cell cycle proteins including increased expression of the cell cycle inhibitor p21 and constitutive activation of Cdks 2 and 6, and this causes the cells to undergo growth arrest at the G1 to S phase transition. These observations suggest FADD promotes proliferation of antigen-stimulated T cells. Furthermore, caspase 8-deficient humans have defects in their activation of B lymphocytes, T lymphocytes and natural killer cells (88).

There have also been several reports suggesting caspases are activated downstream of the TCR without inducing apoptosis. Indeed, caspases have been shown to cleave multiple proteins involved in regulation of the cell cycle. For example, in endothelial cells the cell cycle inhibitors p21 and p27, which normally suppress cyclin-Cdk2 complexes to prevent G1 to S phase transition, can be cleaved by caspase 3 to prevent the inhibitors binding to cyclin-Cdk2 complexes and hence the activity of Cdk2 is increased (89). Furthermore, caspase-mediated cleavage of p27 has been observed in non-apoptotic proliferating lymphoid cells suggesting caspases may promote proliferation of lymphocytes (90).

The differential regulation of apoptosis and proliferation of cells by caspases may depend on the intracellular location of the activated caspases. Furthermore, post-translational modifications of substrates are likely to determine their susceptibility to cleavage by caspases (79).

1.4.4 Caspase-independent apoptosis

Caspases are unlikely to be essential for all forms of apoptosis (74, 91). Initial reports of caspase-independent apoptosis came from the observation that in specific systems caspase inhibitors are unable to prevent all the symptoms of apoptosis. For example, in Jurkat T cells apoptosis is characterised by a loss of cell viability, cell shrinkage, potassium efflux, altered mitochondrial membrane potential and DNA fragmentation. Cell shrinkage and the change in mitochondrial membrane potential are observed prior to the other symptoms of apoptosis. Treatment of the Jurkat cells with inhibitors of caspases (DEVD to inhibit caspase 3 or IETD to inhibit caspase 8) prevented the damage to DNA but were unable to protect the cells from the other symptoms of apoptosis. Furthermore, treatment of the cells with calcium ionophores prevented cell shrinkage, potassium efflux and the change in mitochondrial membrane potential but they did not prevent DNA damage. These observations suggest that cell shrinkage, potassium efflux and changes in the mitochondrial membrane potential in Jurkat cells can occur separately to DNA degradation and via pathways that are largely independent of caspases (65). A similar situation was observed in Rat-1 fibroblast cells treated with zVAD.fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) or BD.fmk (pan-caspase inhibitors). Inhibition of caspases protected these cells from DNA damage that would normally occur under apoptotic conditions but other characteristics of apoptosis such as cell shrinkage and membrane blebbing proceeded in the presence of caspase inhibitors (92). These observations imply caspases are critical mediators of nucleosomal laddering and DNA damage in apoptosis but other symptoms of apoptosis including cell shrinkage, membrane blebbing and loss of mitochondrial function can be performed by caspase-independent pathways.

Bcl-2 family members are potential mediators of caspase-independent apoptosis (74). For example, in Jurkat T cells and cerebellar granule cells, overexpression of Bax can induce apoptosis that is not prevented by zVAD.fmk despite this inhibitor being able to suppress caspase 3 and protect the cells from nuclear fragmentation (93, 94). Indeed, studies in knockout mice have indicated Bax, Bak and Bim are important mediators of negative selection to remove autoreactive thymocytes (95). Under apoptotic conditions Bax rapidly

translocates to the mitochondria and inserts into the outer mitochondrial membrane causing a disruption in mitochondrial function and inducing cell death (76, 77). Several pro-survival factors protect the cell from Bax indicating the importance of this Bcl-2 family protein in apoptosis. For example, overexpression of Bcl-x_L during rod photoreceptor apoptosis prevents Bax from translocating to the mitochondria and inhibits subsequent apoptosis (96). The small peptide humanin also protects cells from apoptosis by binding to Bax, preventing Bax from undergoing the conformational change that is required for it to translocate to the mitochondria (97). These observations strongly suggest Bax can mediate apoptosis independently of caspases and a critical stage of this process is the translocation of Bax to the mitochondria and subsequent disruption of mitochondrial function. Other pro-apoptotic proteins including Bak, Bik and Bim are known to be essential for apoptosis of specific cell types (95, 98-100) therefore Bax may not be the only Bcl-2 family protein that can mediate apoptosis in the presence of caspase inhibitors.

In the classical apoptotic pathway, disruption of the mitochondria leads to the activation of caspases and these mediate the execution of the cell (figure 1.9 and 1.10). However, proteases other than caspases have also been implicated in mediating apoptosis (74, 91). For example, disruption of the mitochondria causes the cytoplasmic levels of calcium to be elevated and Ca^{2+} can activate the calpain group of proteases. Calpains have been implicated as mediators of irradiation or dexamethasone-driven apoptosis in thymocytes (74). Calpains may also help regulate clonal deletion in immature B cells since ligation of the BCR in these cells can stimulate calpains whilst co-stimulation of CD40 causes calpastatin to be upregulated resulting in the inhibition of calpains (101). Calpains can cleave the cytoskeletal protein fodrin therefore they can mediate disruption of the cytoskeleton leading to a loss of cell shape and membrane blebbing. The cathepsins are another group of proteases that have been implicated in caspase-independent apoptosis of lymphocytes (102). For example, sphingosine-induced apoptosis of Jurkat T cells requires cathepsin D and possibly cathepsins B and L and the activation of these cathepsins seems to precede the disruption of the mitochondrial membrane (103). Indeed, in human T cells, mitochondrial-dependent apoptosis can be induced by cathepsin D since this mediates the activation of Bax resulting in the formation of pores in

the outer mitochondrial membrane (104). The lysosomal protease cathepsin D can be induced in the apoptotic response of HeLa cells following exposure to interferon (IFN) gamma or Fas and in U937 cells treated with TNF (tumor necrosis factor) α (105). Furthermore, in TNF-induced apoptosis of WEHI-S fibrosarcoma cells cathepsin B is the main executioner protease (106).

It is important to note that several caspase inhibitors are also capable of inhibiting cathepsins and calpains. For example, the general caspase inhibitor zVAD.fmk, which is widely used to inhibit caspases and caspase-dependent apoptosis, can also inhibit additional cellular proteases including cathepsins B and H and calpains (74, 91, 106, 107). Caspase inhibitors must thus be used at relatively low concentrations when investigating the mechanism of apoptosis and the use of caspase-inhibitors is not necessarily adequate proof that caspases are essential for apoptosis.

1.5 The B cell antigen receptor complex

The BCR complex (reviewed in (3)) is a multiprotein structure that is held together by disulphide bonds (figure 1.1). It includes sIg, which is required for antigen-recognition. There are five classes of immunoglobulin, IgM, IgD, IgG, IgE and IgA. All five can be expressed on the surface of B cells depending on the maturation state of the cell with IgM being the major form found on the surface of immature B lymphocytes (108). The majority of the sIg structure is extracellular with only a small section of it being found in the cytoplasm. The precise length of the cytoplasmic region may depend on the class of immunoglobulin (3) but it is generally agreed that the cytoplasmic tail of sIg is too short to possess any intrinsic catalytic activity, implying accessory molecules are required for signal transduction.

In 1988 two accessory molecules were discovered and cloned (4, 5). Encoded by the *mb-1* and *B29* genes respectively, Igα (CD79α) and Igβ (CD79β) exist as disulphide-linked heterodimeric complexes with extracellular and intracellular domains (3, 109, 110). Igα/Igβ are essential for the transport and surface expression of the BCR (111) and are necessary for activating signals downstream of the BCR. Igα and Igβ do not have intrinsic catalytic activity but they do contain conserved ITAMs. These motifs have the consensus sequence -YXXL/I-X_{6-8}-YXXL/I- where X is any amino acid. Ligation of the BCR

complex by antigen or anti-Ig antibodies causes receptor aggregation and subsequent phosphorylation of tyrosine residues in the ITAMs of Igα/Igβ. This occurs mainly at the membrane-proximal tyrosine and creates phospho-tyrosine motifs that can recruit other signalling molecules using their SH2 (Src homology 2) domains. Nonetheless, both tyrosine residues of ITAMs can be phosphorylated and this provides a specific binding site for the two SH2-domains of the tyrosine kinase Syk (110). Phosphorylation of ITAMs of Igα/Igβ is essential for the propagation of signals downstream of sIg. Mutagenesis of the cytoplasmic domain of Igα or Igβ has shown that these molecules are not functionally redundant with Igα being particularly important for pre-B cell development and Igβ being specifically required for progression beyond the immature B cell stage (112-114).

1.6 BCR signalling in mature B lymphocytes

Ligation of the BCR on B lymphocytes leads to three possible outcomes, activation, anergy or apoptosis, depending on the developmental state of the cell. The signalling pathways activated downstream of the BCR have largely been studied in mature B lymphocytes although similar signalling networks appear to be stimulated by the BCR of immature B cells.

1.6.1.1 Activation of non-receptor tyrosine kinases

Following ligation of the BCR complex, three different families of non-receptor tyrosine kinases are activated: the Src-family (Lyn, Fyn and Blk), Syk and the Tec-family (Btk). Experiments performed using knockout mice have revealed that each of these families has important roles in BCR signalling and in B cell development (115). Mice deficient in Lyn have an increased number of immature B cells but have fewer peripheral B cells indicating Lyn is required for the immature to mature B cell transition. The early events of BCR signalling are delayed in splenic B cells of $Lyn^{-/-}$ mice indicating Lyn has a role in initiating BCR signalling but functional redundancy means BCR signalling can proceed, albeit at a slower rate, in the absence of Lyn (116). Syk is also an important component of the BCR complex and of other signalling complexes. Syk is especially required for pre-BCR signalling since irradiated mice reconstituted

with Syk-deficient fetal liver display a B cell developmental block at the pro-B to pre-B cell transition. Syk is also required for the production or maintenance of mature B cells (21, 22). The Tec-family tyrosine kinase Btk is another essential component of pre-BCR signalling since a deficiency in Btk impairs B cell development at the pre-B cell stage, as indicated by the diseases X-linked agammaglobulinaemia (humans) and X-linked immunodeficiency (mice). Mice lacking Tec and Btk have impaired peripheral B cell activation indicating these kinases are also required for BCR signalling (15, 20).

The activation of Src-family, Syk and Tec-family tyrosine kinases downstream of the BCR complex is thus necessary for normal B cell development and function. However, the precise mechanisms underlying the initial activation of these kinases following aggregation of the BCR complex are poorly understood. Kinetic analysis experiments suggest Src family kinases are activated before Syk and Btk (117). The activity of Src-family kinases is dependent on the phosphorylation state of two key residues: Tyr^{416} and Tyr^{527}. Tyr^{416} is located in the catalytic domain of the enzymes and phosphorylation of this residue, often catalysed by autophosphorylation, can increase the activity of the kinase. However, in unstimulated cells Src-family kinases are autoinhibited by the phosphorylation of Tyr^{527} (Tyr^{508} of Lyn). Tyr^{527} is located in the C-terminus of the kinase and when phosphorylated this residue can interact with the N-terminal SH2 domain of the kinase to prevent substrate binding. In BCR signalling, Csk (C-terminal Src kinase) is particularly important for mediating the phosphorylation of the C-terminal tyrosine residue of Src-family kinases resulting in a suppression of their kinase activity (117-119). Activation of Src-family kinases requires the dephosphorylation of the inhibitory tyrosine residue or the competitive binding of a phospho-tyrosine-containing protein to the SH2 domain of the kinase (120). In BCR signalling, CD45, a transmembrane tyrosine phosphatase, appears to be responsible for mediating the dephosphorylation of the C-terminal inhibitory tyrosine residue of Src-family kinases. Indeed, CD45-deficient B cells display hyperphosphorylation of Lyn at Tyr^{508} and a resultant decrease in the recruitment and activation of Lyn following BCR ligation. This impairs the activation of additional signals downstream of the BCR complex (3, 117, 121). CD45 is thus required for efficient activation of Lyn following ligation of the BCR complex.

Once activated, Lyn can phosphorylate the tyrosine residues of ITAMs of Igα/Igβ. In particular, Lyn phosphorylates the proximal tyrosine residues of ITAMs (122) to create phospho-tyrosine residues that can bind to SH2 domain-containing proteins including additional molecules of Lyn and other Src-family kinases. The binding of Lyn and other Src-family kinases to the ITAMs via their SH2 domains leads to their activation by inducing a conformational change that allows substrates to bind to the catalytic domain of the kinase (123). Furthermore, it has been reported that Lyn and Fyn Src-family kinases can associate with both phosphorylated and non-phosphorylated cytoplasmic domains of Igα/Igβ. However, the region of the Src-family kinase involved in the interaction depends on the phosphorylation-state of Igα/Igβ: the N-terminal 10 residues of Src-family kinases interact with non-phosphorylated Igα/Igβ whilst the SH2 domain of the kinases binds to phosphorylated ITAMs. The activation of Src-family kinases therefore may require the phospho-tyrosines of Igα/Igβ to induce a change in orientation of the associated kinase that can alter its activity (124).

In addition to increasing the activity of Src-family kinases, the induction of Lyn at the BCR complex leads to the stimulation of Syk. Syk has two SH2 domains therefore it requires two phospho-tyrosine residues in the ITAMs in order to bind to Igα/Igβ. It has been proposed that the initial recruitment of Syk to the BCR complex is mediated by the binding of the SH2 domains of Syk to phospho-tyrosine residues of other tyrosine kinases including Lyn (125). Syk is an allosteric enzyme that is potently stimulated by binding to the phospho-tyrosines of ITAMs in Igα/Igβ (122). Syk can then catalyse the phosphorylation of both tyrosine residues of ITAMs leading to further recruitment and activation of Syk and other tyrosine kinases. For example, the Tec-family kinase Btk is recruited to tyrosine phosphorylated ITAMs of Igα/Igβ following ligation of the BCR complex. At the BCR complex, Btk is stimulated by phosphorylation of the activation loop at Tyr^{551} by Src-family kinases or Syk, followed by autophosphorylation at Tyr^{223} (110, 126).

Ligation of the BCR complex therefore leads to the recruitment and activation of multiple tyrosine kinases. The kinases can phosphorylate tyrosine residues of ITAMs leading to further activation of non-receptor tyrosine kinases. Furthermore, the phospho-tyrosine residues of Igα/Igβ and of the tyrosine

kinases themselves provide docking sites for additional SH2-domain-containing proteins and the recruitment of these molecules is critical for stimulating downstream signalling pathways. Ultimately the activation of these signalling pathways regulates the activity, proliferation and survival of the B lymphocyte.

1.6.1.2 CD22

Appropriate B cell function requires careful regulation of signals stimulated downstream of the BCR complex. In particular, the activity of non-receptor tyrosine kinases must be regulated since their activation represents one of the earliest events of BCR signal transduction. CD22 is a co-receptor of B lymphocytes that can both positively and negatively regulate BCR signalling by targeting the early signals activated downstream of the BCR complex. CD22 binds to ligands with α-2,6-sialic acid residues (127, 128) and is rapidly phosphorylated at tyrosine residues following ligand binding or BCR-activation (129). Lyn can catalyse the phosphorylation of CD22 although maximal phosphorylation also requires Syk (130, 131).

Lyn and Syk phosphorylate the cytoplasmic domain of CD22 at three specialised regions known as ITIMs (immunoreceptor tyrosine-based inhibitory motifs). These are characterised by the amino acid sequence -V/IXYXXL- where X is any amino acid. Phosphorylation of ITIMs on CD22 creates docking sites for SH2 domain-containing proteins including the protein tyrosine phosphatase SHP-1 (SH2 domain-containing phospho-tyrosine phosphatase-1).

In unstimulated cells, SHP-1 is autoinhibited by the binding of the SH2 domains of SHP-1 to its phospho-tyrosine phosphatase domain (132, 133). Stimulation of CD22 and subsequent phosphorylation of ITIMs causes SHP-1 to be recruited via its SH2 domain, which induces a conformational change in SHP-1 that activates its phosphatase activity (128, 129). SHP-1 is a negative regulator of BCR signalling (132) and this was demonstrated using mice with deficiencies in this enzyme. For example, SHP-1 deficiencies are largely responsible for the symptoms observed in mice homozygous for the *motheaten* (*me/me*) or the allelic *viable motheaten* (me^{v}/me^{v}) mutations (134). *Motheaten* mice do not express SHP-1 and *motheaten viable* mice express catalytically inactive SHP-1. B cells from these mice have a reduced threshold for B cell activation and this contributes to the development of severe autoimmunity and

inflammation in these mice (134). SHP-1 therefore is a very important negative regulator of BCR signalling.

CD22$^{-/-}$ mice have a similar phenotype to *motheaten* mice but with less severe symptoms since SHP-1 is also required downstream of immunomodulatory receptors other than CD22 (132, 135). Nonetheless, CD22-mediated activation of SHP-1 is important for appropriate B cell activation and function since SHP-1 can dephosphorylate and inhibit Igα/Igβ and BCR-associated tyrosine kinases including Lyn and Syk and their downstream targets including the adaptor protein BLNK (B cell linker protein) and phospholipase Cγ (PLCγ). This leads to a suppression of further BCR-regulated signals including MAPK activation and calcium signalling. CD22-associated SHP-1 also suppresses BCR signalling by inhibiting the co-receptor CD19, which amplifies BCR-activated signals. The activation of SHP-1 by CD22 therefore inhibits B cell activation (131, 134, 136-138).

SHP-1 is believed to act in concert with CD45. Mice deficient in SHP-1 have enhanced BCR signalling and CD45-deficient mice have impaired BCR-driven tyrosine phosphorylation. The B cells of mice deficient in both CD45 and SHP-1 have appropriate induction of protein kinase activity, MAPK activation and BCR-driven proliferation. This suggests that CD45 and SHP-1 are important regulators of BCR signalling and they have opposing effects on the regulation of specific signalling molecules and pathways downstream of the BCR (139).

However, CD22 can recruit signalling molecules in addition to SHP-1 including Lyn, Syk, PLCγ, PI-3-K (phosphatidylinositol-3-kinase) and Grb-2. The significance of these interactions is controversial but it has been suggested that their association with CD22 may lower the threshold for BCR-stimulation (127, 132, 136). Different phospho-tyrosine residues in the cytoplasmic tail of CD22 are used to bind to SHP-1 and Grb-2 (130) therefore the ability of CD22 to suppress or enhance BCR signalling may be regulated by the differential phosphorylation of specific tyrosine residues in the cytoplasmic tail.

1.6.2 Recruitment of adaptor proteins

Following activation of non-receptor tyrosine kinases, additional signalling molecules are recruited to the BCR complex. Ultimately, this results in the

stimulation of four key signalling pathways: Erk-MAPK, PLCγ, PI-3-K and the Rho-family of GTPases (figure 1.11). Activation of these pathways requires adaptor proteins to couple the BCR to the upstream components of these signalling cascades. The BCR does not activate isolated signalling cascades but instead it stimulates a complex network of signals that have many points of "cross-talk" between the pathways. Activation of signals downstream of the BCR regulates the activity and the fate of the cell leading ultimately to proliferation, differentiation or apoptosis (figure 1.11).

Adaptor proteins are molecules that mediate protein-to-protein and protein-to-lipid interactions via modular interaction domains. Adaptor proteins often have multiple interaction domains enabling them to bring several signalling molecules into close proximity. Different modular interaction domains have distinct properties. For example, SH2 domains are regions of approximately 100 amino acids that form three-dimensional structures that can bind to phospho-tyrosine residues. The amino acids that surround the phospho-tyrosine confer specificity for distinct SH2 domains (120, 140, 141); for instance, the SH2 domain of the adaptor protein Grb-2 recognises the motif pYXNX (142, 143).

Many SH2 domain-containing proteins also have SH3 (Src homology 3) domains. These are regions of approximately 50 amino acids that form a structure that can bind to proline-rich regions to mediate protein-to-protein interactions (120). Adaptor proteins can also mediate protein-to-lipid interactions; for example, PH (pleckstrin homology) domains can bind to 3-phosphorylated phosphatidylinositol lipids including phosphatidylinositol-3, 4, 5-trisphosphate (PIP_3). This interaction is important for enabling membrane anchorage of specific proteins. Modular interaction domains thus direct the formation of complexes of multiple signalling molecules and they can determine the intracellular location of these complexes.

Several adaptor proteins have been identified as mediators of BCR signalling and each of these adaptors can be characterised by their modular interaction domains (figure 1.12) (110, 126, 144-151). The SH2 domains enable specific adaptor proteins to be recruited to phosphorylated ITAMs of Igα/Igβ. One such adaptor protein, BLNK (also known as SLP-65), forms an essential component of the BCR signalling network and is required during B cell

development. Deficiencies in BLNK cause B lymphocyte development to arrest at the pro-B cell to pre-B cell transition (152-154). In peripheral B lymphocytes, BLNK is phosphorylated downstream of the BCR by Syk (146), creating multiple phospho-tyrosine residues that can recruit additional signalling molecules via their SH2 domains (figure 1.13). Amongst the BLNK-associated proteins is the tyrosine kinase Btk and one of its substrates, PLCγ. PLCγ is activated by phosphorylation catalysed by Syk and Btk therefore BLNK acts as a scaffold for PLCγ-activation (146, 155-157). Furthermore, the substrate of PLCγ is a component of the plasma membrane therefore BLNK also brings PLCγ into close proximity to its substrate. Phospho-tyrosine residues on BLNK can recruit additional signalling molecules including Vav, a guanine nucleotide exchange factor of Rho-family G-proteins. The simultaneous activation of PLCγ and Rho-family G-proteins is required for the stimulation of JNK and p38 MAPK (146) therefore BLNK serves as a scaffold for the activation of multiple signalling pathways.

The adaptor protein Nck can also bind to phospho-tyrosine residues on BLNK using its SH2 domain (110, 149). Nck can interact with p21-activated ser/thr kinases (PAKs) and the Wiscott-Aldrich syndrome protein (WASP), both of which are involved in regulating the cytoskeletal structure of cells. PAK can also regulate MAPKs in several different cellular systems including Jurkat T cells, where PAK is required for Fas-mediated activation of JNK (81, 82, 158-162).

BLNK can also couple the BCR to Erk-MAPK via the recruitment of the adaptor protein Grb-2. Grb-2 has two SH3 domains separated by a single SH2 domain. It binds to BLNK via the SH2 domain and can then associate with the guanine nucleotide exchange factor SOS (Son of Sevenless) using its SH3 domains. The recruitment of SOS to the membrane complex enables SOS to activate the monomeric G protein Ras, which can then lead to the stimulation of several signalling molecules including Erk-MAPK.

LAB (linker for activation of B cells) is a recently identified adaptor protein of BCR signalling that can interact with similar molecules to BLNK (figure 1.13). Ligation of the BCR induces the tyrosine-phosphorylation of LAB leading to the recruitment and activation of Grb-2/SOS and hence Ras/Erk. LAB can also bind to PLCγ to enhance calcium signalling (150, 151). Deficiencies in BLNK can

impair B cell development and BCR signalling indicating BLNK and LAB are not functionally redundant (152-154). The BCR thus uses several adaptor proteins to stimulate specific signalling cascades presumably to allow careful regulation of these signalling pathways.

In addition to LAB and BLNK, the BCR can stimulate the Grb-2/SOS pathway via the adaptor protein Shc (figure 1.13) (163). The recruitment and phosphorylation of Shc at the BCR complex requires both Src-family and Syk tyrosine kinases since Src-family kinases contribute to the activation of Syk and Syk can directly associate with and phosphorylate Shc (123, 163). Shc can also associate with the adaptor protein Gab-1 (Grb-2-associated binder-1), which is phosphorylated at tyrosine residues following ligation of the BCR. The phospho-tyrosine residues of Gab-1 can interact with multiple proteins including Shc, PI-3-K, SHP-2 and possibly Crk-L (figure 1.13) (147, 164). Both Shc and SHP-2 can bind to Grb-2 thus linking Gab-1 to the activation of Erk-MAPK (110, 117, 132, 165). The association of Gab-1 and SHP-2/Grb-2 has also been observed downstream of the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and this interaction was specifically associated with the sustained activation of Erk1/2 (166). This suggests that the signalling networks generated by specific adaptor proteins can influence the kinetics of activation of particular signalling cascades. Furthermore, the co-localisation of SHP-2 and PI-3-K at Gab-1 can co-ordinate the activation of Erk by these two signalling molecules (167).

The association of Gab-1 and PI-3-K leads to the activation of PI-3-K therefore regulation of this interaction can determine the activity of PI-3-K. For example, in immortalised mIMCD-3 epithelial cells treated with hepatocyte growth factor, Erk phosphorylates threonine residues on Gab-1 to enhance the interaction of PI-3-K and Gab-1 and to increase PI-3-K activity. However, treatment of these cells with EGF leads to the Erk-mediated recruitment of SHP-2 and subsequent dephosphorylation of tyrosine residues on Gab-1 resulting in a decreased association of Gab-1 and PI-3-K (168). Regulation of the association of Gab-1and PI-3-K can thus determine the activity of PI-3-K. In Ramos B cells, Gab-1 and its associated signalling molecules can be found in the membrane-enriched particulate fraction therefore another function of Gab-1 is to recruit signalling molecules to cellular membranes (164). PI-3-K catalyses

the phosphorylation of phosphatidylinositol lipids therefore the Gab-1-mediated recruitment of PI-3-K to cellular membranes brings PI-3-K into close proximity with its substrates.

The BCR can also recruit and stimulate PI-3-K via the adaptor protein Cbl (figure 1.13). Ligation of the BCR induces Syk-mediated tyrosine phosphorylation of Cbl. The p85 subunit of PI-3-K binds to phospho-tyrosine residues of Cbl, inducing a conformational change in PI-3-K that greatly enhances its kinase activity often leading to relatively slow but sustained activation of PI-3-K (169). The phospho-tyrosines of Cbl can also bind to Crk-L and Crk-II bringing these molecules to the plasma membrane. Crk can function as an adaptor protein since it has two SH3 domains in addition to its SH2 domain. For example, in human embryonic kidney 293T cells Pyk2 can stimulate JNK MAPK via Crk (170). Crk is also an upstream regulator of Erk-MAPK since the SH3 domains of Crk can bind to SOS, leading to the activation of Ras and Erk, and to C3G a guanine nucleotide exchange factor for Rap1 (171, 172). In B cells C3G is constitutively associated with Crk (171). C3G activates Rap1 by exchanging GDP for GTP and Rap1 can regulate Erk-MAPK both positively and negatively since Rap1 inhibits the kinase Raf-1 leading to a suppression of Erk but activates B-Raf to stimulate Erk. The outcome of Rap1 activation therefore depends on the relative amounts of Raf-1 and B-Raf in the cell (173).

In addition to regulating PI-3-K and Crk, Cbl has also been found to play a significant role in suppressing BCR signals. A loss of Cbl-b in mature B cells prolongs BCR-signalling resulting in sustained activation of PLCγ, Erk and JNK (174). Cbl has a RING finger domain, a proline-rich region and a N-terminal tyrosine kinase-binding region that includes a SH2 domain, a four-helix bundle and calcium-binding EF-hand motif (figure 1.12) (148). The tyrosine kinase-binding region enables Cbl to bind to non-receptor tyrosine kinases and the RING finger domain allows Cbl to interact with ubiquitin conjugating enzymes. Downstream of the BCR, Cbl associates with Syk using the tyrosine kinase-binding domain and this leads to the ubiquitylation and subsequent degradation of Syk (174, 175). In B cells, Cbl is thus a negative regulator of signalling mediated by Syk and given the importance of Syk in initiating signals at the BCR complex it is possible that Cbl helps to set the threshold for BCR signalling

(174). Similarly in T cells, Cbl can drive the ubiquitylation and degradation of Fyn and Lck tyrosine kinases (176). Cbl also functions to exclude Lck from lipid rafts to further inhibit TCR signalling (177).

Adaptor proteins therefore have several functions. Firstly, they couple the BCR to multiple downstream signalling molecules via their different modular interaction domains. Secondly, they co-localise specific signalling molecules to enable their efficient regulation. Thirdly, adaptor proteins localise signalling molecules at a particular intracellular location and this can bring the signalling molecule into close proximity with its substrate. Finally, adaptor proteins can act as a platform for mediating the inhibition of specific signalling molecules.

1.6.3 Signalling pathways activated by the BCR complex

Ligation of BCR results in the stimulation of non-receptor tyrosine kinases and the phosphorylation of specific substrates including the adaptor proteins. Adaptors proteins enable the efficient regulation of four main signalling pathways: PI-3-K (figure 1.14), PLCγ (figure 1.15), Rho-family of G-proteins (figure 1.16) and Erk-MAPK (figure 1.17).

1.6.3.1 PI-3-K

PI-3-K enzymes are lipid and protein kinases. They have been divided into four classes (I_A, I_B, II and III) based on their structural characteristics and substrate-specificity (reviewed in (178)). Class I_A enzymes are heterodimers of a regulatory subunit (p85α, p55α, p50α, p85β or p55γ) and a catalytic subunit (p110α, β or δ). The regulatory subunits have two SH2 domains and the region separating these domains is constitutively associated with the catalytic subunit. Class I_A regulatory subunits also have an SH3 domain, at least one proline-rich domain and a Bcr Homology domain that can bind to Rac-1/Cdc42. Only one member of the class I_B PI-3-K enzymes is known. It is a heterodimer of p101 regulatory subunit and p110γ catalytic subunit. The p101 regulatory subunit lacks many of the structural domains of class I_A PI-3-K regulatory subunits and the p110γ subunit cannot bind to regulatory subunits of class I_A PI-3-K enzymes. Class I_A and I_B enzymes therefore have the same substrate-specificity but are regulated by different mechanisms. There are three known

members of the class II PI-3-K family and they are ubiquitously expressed. The C-termini of these enzymes have two distinctive domains: a phox homology domain to bind 3-phosphorylated phosphatidylinositol lipids, probably to enable membrane-anchorage and a C2 domain of unknown function. Class II PI-3-K enzymes are activated downstream of receptors for integrins, chemokines and some growth factors. Only one mammalian class III PI-3-K enzyme is known and it is constitutively active *in vivo*. It appears to regulate the trafficking of proteins through lysosomes (178).

It is difficult to study the function of specific PI-3-K enzymes in any system since no isoform-specific inhibitors are commercially available. Knockout mice therefore have been used to elucidate the function of specific PI-3-K enzymes (reviewed in (178)). However, knockout mice of class II and class III PI-3-K enzymes have not been generated and hence the function of these PI-3-K enzymes is poorly understood. Nonetheless, knockout mice of class I_A and I_B PI-3-K enzymes have been generated. Mice lacking either p110α or p110β die *in utero* but p110$\delta^{-/-}$ or p110$\gamma^{-/-}$ mice develop normally suggesting embryonic development has specific requirements for p110α and p110β. The regulatory subunits p85α, p55α and p50α are all generated from the same gene but mice lacking p85α are viable whilst mice lacking all three gene products die after birth suggesting an essential role for p55α or p50α in the development of the mouse. Mice lacking p85β are viable and develop a functional immune system indicating this regulatory subunit is not required to mediate an immune response.

The function of class II and III PI-3-K enzymes in the immune system is poorly understood. However, class 1_A and 1_B enzymes are required in cells of the immune system including B cells. B lymphocyte development is impaired at the pro-B cell to pre-B cell transition in mice that lack either p110δ or p85α (178, 179). Furthermore, p110$\delta^{-/-}$ mice have a reduced number of marginal zone B cells and the mice have impaired antibody production in response to T cell-dependent and T cell-independent antigens (179). In addition, the splenic B cells of mice deficient in p110δ or p85α undergo a poor proliferative response following stimulation at the BCR or CD40 indicating the activation of PI-3-K is required for BCR-driven proliferation (179-181).

The stimulation of PI-3-K downstream of the BCR is dependent on both

Ras and protein tyrosine kinases (110). Both class 1_A and 1_B PI-3-K enzymes can interact with Ras in a GTP-dependent manner enabling Ras to activate these enzymes (182). In quiescent cells the C-terminal region of p85 prevents Ras-GTP from activating class I_A p110 enzymes. Activation of protein tyrosine kinases leads to the production of tyrosine-phosphorylated peptides that bind to the p85 subunit, inducing a conformational change that allows Ras-GTP to activate p110. The active p110 can be inhibited by the recruitment of SHP-1 to the plasma membrane via the p85 subunit (183). The activation of class I_B PI-3-K also requires Ras-GTP to bind to the catalytic subunit of the enzyme. The regulation of this process is not fully understood but the Ras-dependent stimulation of p110γ appears to involve the induction of a conformational change since relocation of p110γ to the plasma membrane is insufficient to activate the enzyme (182).

The SH3 domain of the tyrosine kinase Lyn can bind to the proline-rich domain of p85 PI-3-K but Lyn-deficient mice do not have impaired stimulation of PI-3-K downstream of the BCR indicating Lyn is not essential for the activation of PI-3-K (156). Syk tyrosine kinase has also been implicated in coupling the BCR to p85 PI-3-K activation and BCR-mediated tyrosine phosphorylation of the catalytic subunit of PI-3-K is abolished in Syk-deficient DT40 cells (156). Indeed, Syk can phosphorylate the multidomain signalling protein Cbl to create a phospho-tyrosine residue that is recognised by the SH2 domains of the p85 subunit. Dominant negative Syk prevents the tyrosine phosphorylation of Cbl and blocks the association of Cbl and PI-3-K. The binding of the p85 subunit of PI-3-K to the phospho-tyrosine residue of Cbl induces a conformational change in PI-3-K that greatly enhances its kinase activity often leading to relatively slow but sustained activation of PI-3-K. Syk and Cbl are located at the plasma membrane therefore the newly activated PI-3-K is in close proximity to its lipid substrate (169). The role of Ras in the activation of PI-3-K via Cbl has yet to be established suggesting the BCR may stimulate PI-3-K via more than one mechanism.

PI-3-K phosphorylates phosphatidylinositol lipids at the 3' position of the inositol ring generating PI-3, 4, 5-P_3 (PIP_3) from PI-4, 5-P_2 (PIP_2) and PI-3, 4-P_2 from PI-4-P. Phosphatidylinositol lipids with 3' phosphorylation are recognised by proteins with PH domains. The action of PI-3-K therefore enables specific

proteins and complexes of proteins to be recruited to the cellular membranes (figure 1.14).

The activation of PI-3-K is necessary for the stimulation of multiple proteins including protein kinase B (PKB/Akt). Akt and its activating kinase PDK-1 (phosphoinositide-dependent kinase-1) are recruited to PIP_3 at the plasma membrane via their PH domains. PDK-1 phosphorylates Akt at Thr^{308} and this contributes to the activation of Akt but Akt must also be phosphorylated on Ser^{473} for efficient stimulation of the kinase. The enzyme responsible for phosphorylating Ser^{473} is an unknown kinase termed PDK-2. PIP_3 is essential for Akt stimulation but phosphorylation of Ser^{473} also requires PI-3, 4-P_2 suggesting PDK-2 may be recruited to the plasma membrane via PI-3, 4-P_2 (184). Protein phosphatase 2A (PP2A) can inhibit Akt by catalysing the dephosphorylation of Thr^{308} and Ser^{473} indicating the importance of these residues in regulating the activity of this kinase (185). Furthermore, heat shock protein 90 can prolong Akt activation by preventing its dephosphorylation by PP2A (186). However, Tyr^{474} of Akt has also been proposed to be a regulatory amino acid since a 55% reduction in Akt activity can be observed when this residue is mutated to Phe to prevent its phosphorylation (187). The activation of Akt may thus be dependent on multiple protein kinases.

Akt is a serine/threonine protein kinase that promotes cell survival (186). Akt protects cells from apoptosis by phosphorylating and inhibiting capase-9, Bad and the family of forkhead-related transcription factors (188). Akt also helps to retain Bax in the cytoplasm to prevent it from causing damage to the mitochondria (189) and Akt phosphorylates MDM2 to increase its translocation to the nucleus where it can inhibit p53 (186). In $CD4^+$ T lymphocytes, CD28-mediated activation of Akt can also prevent Fas-induced apoptosis (190) suggesting Akt can suppress caspases 8 and 3. However, in IL-3-dependent 32D cells withdrawal of cytokine causes Fas-activated caspases to cleave Akt resulting in its degradation (191). The balance of Akt and caspase activity can thus determine the survival of a cell.

Akt is an important mediator of B and T lymphocyte survival. This is illustrated by the observation that many B and T cell malignancies have high levels of the *TCL1 (T cell leukemia 1)* gene product, which can activate Akt by enhancing its concentration and phosphorylation at the plasma membrane

(192). Furthermore, B cells of mice that are deficient in the p110δ subunit of PI-3-K are prone to apoptosis, possibly reflecting their inability to activate Akt (179, 180). In splenic B cells, the activation of PI-3-K/Akt downstream of the BCR can promote cell survival by stimulating NF-κB, which can increase the transcription of pro-survival factors including c-Myc (193). Akt can activate NF-κB by catalysing the phosphorylation of I-κB and this can lead to further stimulation of Akt since NF-κB is able to increase the expression of Akt (188, 194). BCR-mediated activation of Akt also enhances the activity of NF-AT (nuclear factor for activated T cells) by inhibiting glycogen synthase kinase 3. NF-AT activates AP-1-like transcription factors to regulate gene expression and this may contribute to the survival of the B cell (195). The mammalian target of rapamycin and $p70^{S6}$ kinase have also been implicated as mediators of PI-3-K-dependent proliferation of B cells downstream of the BCR (196). Akt, PDK-1 and PKC (protein kinase C) ζ can all contribute to the phosphorylation and activation of $p70^{S6}$ kinase (197).

PDK-1 and Akt are not the only PH-domain containing proteins involved in B cell signalling. For example, the recruitment of PKCζ to the plasma membrane via PIP_3 enhances the activation of this atypical PKC enzyme by Ras (198). B lymphocytes from $PKC\zeta^{-/-}$ mice undergo spontaneous apoptosis and have impaired activation and antibody production suggesting PKCζ has an important role in promoting B cell survival and activation (199). The production of PIP_3 downstream of the BCR can also induce Vav leading to changes in the cytoskeleton and the activation of MAPK enzymes (178).

Furthermore, the B cells of mice that are deficient in the p110δ subunit of PI-3-K have impaired BCR-driven activation of Btk, PLCγ and calcium mobilisation suggesting PI-3-K can regulate each of these signalling molecules. Mice lacking p110δ have impaired proliferation suggesting these molecules can promote proliferation of B cells downstream of the BCR (179, 180). Btk has a PH domain therefore the production of PIP_3 can bring Btk to the plasma membrane and the BCR signalling complex. This increases the activity of Btk and brings it into close proximity to its substrates including PLCγ which can also be recruited via its PH domains (20, 169). The stimulation of PLCγ results in increased calcium signalling and the activation of downstream molecules including PKC (178).

1.6.3.2 PLCγ

There are three families of phospholipase C enzymes (figure 1.15A). The PLCγ family has two members, PLCγ1 and PLCγ2. Both PLCγ enzymes have a similar structure that includes two PH domains separated by two SH2 domains and a SH3 domain (figure 1.15A). These domains have important functions in the regulation of PLCγ activity. B lymphocytes predominantly express PLCγ2 and it is stimulated downstream of the BCR (117). The activation of PLCγ2 downstream of the BCR is impaired in cells that are deficient in the adaptor protein BLNK, or the tyrosine kinases Syk and Btk indicating all three molecules are required for efficient stimulation of PLCγ (126, 146, 155, 199). Following ligation of the BCR the tyrosine kinase Syk is stimulated and Syk phosphorylates BLNK at multiple tyrosine residues (126). Both Btk and PLCγ are recruited to the BCR complex by binding to the tyrosine phosphorylated BLNK using their SH2 domains (figure 1.15B). The translocation of Btk to the BCR complex is assisted by the presence of 3-phosphorylated phosphatidylinositol lipids since these enable Btk to associate with the plasma membrane via its PH domain. PLCγ also has a PH domain and this may assist the translocation of PLCγ to the BCR complex or it may help to maintain its localisation at the BCR complex (126). Activation of PLCγ at the plasma membrane requires phosphorylation of Tyr^{735} and Tyr^{759} of PLCγ. Btk has a far greater ability than Syk to phosphorylate these residues indicating the principal role of Syk in the activation of PLCγ is to phosphorylate BLNK and enable the PLCγ-BLNK-Btk complex to be formed (157). The close proximity of Btk and PLCγ in this complex enables Btk to phosphorylate and activate PLCγ (155).

B lymphocytes that lack BLNK have impaired but not abolished PLCγ2 activation suggesting the BCR can also stimulate PLCγ via a BLNK-independent pathway. The adaptor protein LAB is a likely candidate for mediating the BLNK-independent pathway since LAB is phosphorylated downstream of the BCR and this enables it to bind to PLCγ (126, 150).

In addition to assisting the activation of PLCγ, the recruitment of PLCγ to the plasma membrane via LAB/BLNK brings the enzyme into close proximity with its substrate PI-4, 5-P_2. PLCγ cleaves the lipid PIP_2 to generate diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP_3) (figure 1.15B). IP_3 is

a cytosolic second messenger that can bind to IP_3 receptors on the endoplasmic reticulum and induce the release of calcium from intracellular stores (200, 201). The elevation of cytosolic calcium levels activates downstream signalling molecules including calcineurin (a Ca^{2+}-sensitive protein phosphatase), conventional PKC isoforms and JNK MAPKs (117, 202, 203).

DAG is a lipid soluble second messenger therefore it is membranous. DAG can activate phorbol ester-sensitive isoforms of PKC and hence it stimulates conventional PKC isoforms (α, βI, βII and γ) and novel PKC isoforms (δ, ε, θ, η, ν) (204-208). The functions of PKC enzymes in cells of the immune system are incompletely understood but the activation of PKC can enhance the activity of MAPKs especially Erk1/2 (202). For example, in IL-3-stimulated murine hematopoietic cells phorbol ester-sensitive isoforms of PKC can directly phosphorylate and activate Raf-1 (209).

There are many different types of PKC enzymes and they are likely to have specific functions within particular cell types. For example, $PKC\beta^{-/-}$ mice have impaired humoral immune responses and their B cells respond poorly to BCR-stimulation (210) indicating PKCβ provides an important activating signal in B lymphocytes. The phenotype of $PKC\beta^{-/-}$ mice is similar to that of Btk- or PLCγ-deficient cells suggesting PKCβ functions in the same signalling pathway as the PLCγ-BLNK-Btk complex (reviewed by (199)). The PLCγ-BLNK-Btk complex is required for BCR-dependent activation of the transcription factor NF-κB and this in turn regulates the expression of the genes encoding the pro-survival proteins Bcl-x_L and Bcl-2 (199, 211). BCR-stimulated B cells from $PKC\beta^{-/-}$ mice have impaired activation of the Bcl-x_L and Bcl-2 but cyclin D2 levels are unaffected suggesting PKCβ is more involved in regulating survival than proliferation. The function of T lymphocytes is not impaired in $PKC\beta^{-/-}$ mice suggesting this enzyme is specifically required in B cells. However, PKCθ is required for TCR-mediated activation of NF-κB and no defects in B cell NF-κB activation have been observed in $PKC\theta^{-/-}$ mice. Furthermore, the pre-BCR can stimulate NF-κB via PKCλ indicating the function of particular PKC isoforms will depend on the type of cell and developmental stage of that cell (212).

Moreover, PKCδ is required in addition to PKCβ for appropriate function of mature B cells. However, B cells of $PKC\delta^{-/-}$ mice have enhanced B cell proliferation, elevated antibody production and are prone to autoimmunity

suggesting PKCδ is normally a negative regulator B cell activation (199). The different PKC isoforms can thus differentially regulate B cell activation and survival.

1.6.3.3 Rho-family of GTPases

The Rho-family of GTPases is activated downstream of the BCR via the guanine nucleotide exchange factor Vav. Vav has one SH2 domain and two SH3 domains and is recruited to the BCR complex via BLNK. At the BCR complex Vav is tyrosine phosphorylated and activated, probably by Syk, enabling it to stimulate the Rho-family of G-proteins including Rac1, RhoA and Cdc42 (144). Rac1 is a mediator of actin polymerisation and can alter the cytoskeleton of the cell. Rearrangement of the actin cytoskeleton is required for capping of the antigen receptor and for optimal calcium signalling in lymphocytes (110, 213). Rac1/Cdc42 also contributes to the stimulation of PLCγ2 and PI-3-K downstream of the antigen receptors of B cells (figure 1.16) and T cells and these can enhance calcium mobilisation and activate additional signalling molecules including Akt (146, 178, 213-216). Furthermore, the activation of Vav/Rho downstream of the BCR can stimulate phosphatidylinositol-4-phosphate-5-kinase (figure 1.16). This lipid kinase is responsible for maintaining the levels of PI-4, 5-P_2 in cellular membranes and thus ensures there is a constant supply of substrate for PLCγ (110).

In B and T lymphocytes Rac1/Cdc42 can also contribute to the activation of MAPK proteins, especially those of the JNK and p38 families (144, 146). For example, in DT40 B cells Rac1/Cdc42 activates JNK-MAPK via the stimulation of the serine/threonine protein kinases MEKK-1 or PAK1 and the dual-specificity kinases MKK4 or MKK7 (figure 1.16) (3, 217). Furthermore, in HeLa cells Rac-1/Cdc42 can activate Erk-MAPK via the stimulation of PAK1/3, which phosphorylates and activates Raf-1 (162). In addition to JNK and p38 MAPK, the BCR may thus couple to Erk-MAPK via Rac.

1.6.3.4 Erk-MAP kinase cascade

The classical mechanism of activating Erk-MAPK (figure 1.17) is via the adaptor protein Grb-2. The SH3 domains of Grb-2 bind to the guanine nucleotide exchange factor SOS. SOS activates the monomeric G-protein Ras

by exchanging GDP for GTP. Ras-GTP can then bind and activate the serine/threonine protein kinase Raf-1, leading to the stimulation of the dual-specificity protein kinases MEK1 and MEK2. MEK1/2 activates Erk-MAPK by phosphorylating the threonine-glutamate-tyrosine (T-E-Y) motif of the activation loop and Erk1/2 can phosphorylate a wide range of substrates including cytoplasmic molecules such as phospholipase A_2 (218) and nuclear substrates including the transcription factors Elk-1 and c-Myc (219). The particular substrates of Erk-MAPK will depend on the duration of Erk-activation, the intensity of the Erk signal and the presence of additional signalling complexes. For example, in fibroblasts a transient Erk signal is insufficient to stimulate the transcription factor c-Fos but a prolonged Erk signal can mediate the phosphorylation and stabilisation of c-Fos to promote AP-1-mediated transcription (220). The activation of Erk-MAPK in B cells is associated with a variety of cellular responses including proliferation and apoptosis.

The BCR complex can couple to Grb-2 via additional adaptor proteins including Shc (123, 163), BLNK (110, 153) and LAB (150). Furthermore, the BCR-associated adaptor Gab-1 can bind to Shc and SHP-2 and these can both bind to Grb-2/SOS (110, 117, 132, 165). The association of Gab-1 and SHP-2/Grb-2 has also been observed downstream of the receptors for EGF and PDGF and this interaction was specifically associated with sustained activation of Erk1/2 (166). The ability of the BCR to couple to Grb-2/SOS via multiple adaptor proteins may therefore regulate the kinetics of Erk-MAPK activation.

The BCR can further couple to the MAPK cascade via the multidomain protein Cbl (figure 1.17) since ligation of the BCR causes Syk to phosphorylate Cbl. The resultant phospho-tyrosine residues can bind to PI-3-K leading to its activation (169) and to Crk, bringing it to the plasma membrane (171). Crk can bind to SOS, leading to the activation of Ras and Erk and to C3G leading to the activation of Rap1 (171, 172). Rap1 has the same effector domain as Ras therefore it competes with Ras for substrate binding. Rap1 is both a negative and a positive regulator of Erk-MAPK since Rap1 inhibits Raf-1 but activates B-Raf. The outcome of Rap1 activation therefore depends on the relative amounts of Raf-1 and B-Raf in the cell. This is illustrated by the differential regulation of Erk in PC12 cells. Differentiation of PC12 cells following exposure to nerve growth factor requires sustained activation of Erk and this is mediated by

Crk/C3G/Rap-1 and B-Raf. A transient Erk signal stimulates proliferation of PC12 cells and this is mediated by Ras/Raf-1-dependent activation of Erk (173). Furthermore, in DT40 B cells ligation of the BCR can stimulate Erk-MAPK via Raf-1 or B-Raf but the distinct pathways of activation result in different intensities and durations of Erk activation. Consequently, either pathway can induce immediate early genes but only B-Raf-stimulated Erk can activate NF-AT (221). The differential regulation of Erk-MAPK by Raf-1 and B-Raf can thus determine the duration of Erk-stimulation and the substrates that become phosphorylated by Erk.

Rap1 stimulation has been observed downstream of the BCR in primary splenic B cells and in various B cell lines but the precise mechanism of Rap1-activation is incompletely understood. In Ramos B cells stimulation of the BCR has been shown to induce the tyrosine phosphorylation of Cbl leading to the recruitment of Crk and the activation of C3G and Rap1 (171). However, it has also been proposed that the BCR may activate Rap1 via a Crk-independent pathway that requires activation of PLCγ and the production of DAG. The mechanism of DAG-dependent stimulation of Rap-1 is incompletely understood but it may be mediated by DAG-activated isoforms of PKC (172).

1.6.3.5 Additional signalling pathways activated by the BCR

In mature B lymphocytes, ligation of the BCR can activate phospholipase D (PLD), a lipid hydrolysing enzyme. Two types of phosphatidylcholine-PLD have been cloned, PLD1 and PLD2. PLD1 is largely located in the Golgi and endoplasmic reticulum whereas PLD2 is predominantly found at the plasma membrane. PLD enzymes are stimulated by conventional isoforms of PKC, the Arf-family of monomeric G-proteins and Rho-family G-proteins. Furthermore, PI-4, 5-P_2 is a cofactor that is required for their activation. Both PLD enzymes can hydrolyse phospholipids to generate phosphatidic acid (PA). PA can function as a second messenger that activates phosphatidylinositol-4-phosphate-5-kinase, PKCη and PKCζ. Moreover, PA can be hydrolysed to DAG, to generate a sustained DAG signal that can activate conventional and novel PKC isoforms. However, PA can also be converted to lysophosphatidic acid and when secreted, lysophosphatidic acid can induce mitogenesis via a G-protein-coupled receptor (222).

Mature B lymphocytes undergo at least two different types of PLD signalling. Indeed, ligation of the BCR stimulates a non-phosphatidylcholine-hydrolysing PLD enzyme leading to the production of DAG and the stimulation of mitogenesis. However, extracellular ATP can inhibit anti-Ig-induced proliferation of mature B cells via the stimulation of P_2-purinoreceptors. Ligation of P_2-purinoreceptors induces a phosphatidylcholine-hydrolysing PLD, resulting in the production of PA and the induction of growth arrest. Furthermore, ceramide and sphingosine can also stimulate phosphatidylcholine-PLD leading to a suppression of B cell proliferation. The differential regulation of distinct types of PLD therefore can either induce growth arrest or promote B cell proliferation (222, 223). Interestingly, the BCR on immature B lymphocytes is not coupled to the early induction of either of these PLD pathways. The induction of growth arrest and apoptosis downstream of the BCR in immature B cells therefore is not mediated by early signalling via phosphatidylcholine-PLD or inhibition of non-phosphatidylcholine-PLD (223).

1.6.4 Integration of signalling pathways

Ligation of the BCR induces the activation of four main pathways: PI-3-K, PLCγ, Rho GTPases and Erk-MAPK cascade. However, these signalling cascades are not activated in isolation and cross-talk between the pathways is common (224). This is an important mechanism of regulating these signals and controlling the activation of specific downstream targets.

For example, the Erk-MAPK cascade and the PI-3-K pathway frequently interact since Ras can activate both Erk-MAPK and PI-3-K (225-227). Furthermore, PI-3-K can negatively regulate the Erk-MAPK cascade via the activation of Akt since Akt can inhibit Raf-1 by phosphorylating Ser^{259} and inhibit B-Raf by phosphorylating an N-terminal regulatory domain (228-230). Moreover, Erk can also regulate the activity of PI-3-K in both a positive and a negative manner by determining the phosphorylation state of the adaptor protein Gab-1 and hence affecting the ability of PI-3-K to associate with Gab-1 (168). Consequently, the relative activity of Erk and PI-3-K can determine the activity and fate of a cell (226, 231). For example, in vascular smooth muscle cells PDGF induces a strong and prolonged activation of PI-3-K/Akt leading to the inhibition of Raf-1/Erk and to the induction of mitogenesis. In contrast,

thrombin stimulates a transient and weak PI-3-K/Akt signal in these cells enabling strong and prolonged activation of Erk and this causes the cells to undergo differentiation (232).

Erk and PI-3-K can also co-operate to regulate the activity of specific downstream targets. For example, in the Burkitt's lymphoma cell line ST486 the simultaneous activation of Erk-MAPK and PI-3-K leads to the stimulation of $p90^{S6}$ ribosomal kinase. The activation of $p90^{S6}$ ribosomal kinase enhances the survival of the cells by phosphorylating and inhibiting Bad (233). Furthermore, in NIH-3T3 cells the simultaneous activation of Erk-MAPK and PI-3-K is necessary for the induction of DNA synthesis since Erk can stimulate the transcription factors Fra1 and Fra2 whilst PI-3-K induces cyclin D1 and both of these factors are required for DNA synthesis (234).

Moreover, in B lymphocytes BCR-dependent activation of cyclin D2 and subsequent cell cycle progression requires the induction of BLNK, PI-3-K and Btk (33). Indeed, the stimulation of cyclin D2 is impaired in BLNK-deficient mice (199) and B cells from mice lacking the p85α subunit of PI-3-K or Btk (181). However, the precise mechanism by which BLNK, PI-3-K and Btk mediate the induction of cyclin D2 to promote B cell proliferation is incompletely understood.

The survival of B lymphocytes is also regulated by multiple signalling cascades. For example, the activation of the transcription factor NF-κB can promote mature B cell survival since NF-κB can induce the transcription of pro-survival factors including c-Myc, Bcl-x_L and Bcl-2 (193, 199, 211). Moreover, the induction of NF-κB is impaired in B lymphocytes from various types of knockout mice including mice deficient in the p85α subunit of PI-3-K, Btk, BLNK, PLCγ or PKCβ (181, 199). This suggests that BCR-dependent activation of NF-κB in mature B cells requires the activation of PI-3-K and PLCγ. Indeed, Akt can phosphorylate and inhibit I-κB suggesting a potential mechanism of PI-3-K-dependent induction of NF-κB (194). Furthermore, B cells of $PKC\beta^{-/-}$ mice have defective activation of I-κB kinase leading to impaired inhibition of I-κB (199) suggesting PKCβ induces NF-κB by activating I-κB kinase. However, the observations from the knockout mice suggest that the efficient induction of NF-κB downstream of the BCR complex requires the co-ordinated stimulation of PI-3-K and PLCγ-regulated pathways.

Similarly, PI-3-K and PLCγ signals can both regulate the transcription

factor NF-AT. For example, the production of IP_3 by PLCγ induces calcium mobilisation and this can stimulate the calcium-sensitive phosphatase calcineurin. Calcineurin can then dephosphorylate NF-AT causing it to translocate to the nucleus where it can induce gene expression via AP-1-like transcription factors. However, NF-AT is inhibited by glycogen synthase kinase 3 since phosphorylation of NF-AT induces a conformational change that reveals a nuclear export signal. Ligation of the BCR can promote NF-AT activity via the induction of PI-3-K and Akt since Akt can inhibit glycogen synthase kinase 3 (195). Furthermore, the Erk-MAPK cascade also contributes to the activation of NF-AT in B lymphocytes. For example, in DT40 B cells BCR-driven activation of NF-AT requires a strong and prolonged Erk signal that requires B-Raf activity (221).

1.7 Co-Receptors

Signals activated by ligation of the BCR complex can regulate the activity and the fate of B lymphocytes. However, B cells express additional surface receptors, known as co-receptors, that can activate or inhibit the signalling pathways regulated by the BCR. Co-receptors can determine the activation threshold of the BCR, the phenotype of the activated cell and the duration of BCR stimulation. Several significant co-receptors have been studied in B cells including CD19, FcγRIIB and CD40 (127).

1.7.1 CD19

CD19 is a co-receptor that can enhance BCR-signalling. It is expressed throughout B cell development until plasma cell differentiation. On the surface of B cells, CD19 is found in association with CD21 and CD81. CD21 is a type 2 complement receptor that recognises C3d-labelled antigen. The simultaneous activation of the BCR and CD21 by C3d-labelled antigen can significantly lower the threshold for B cell activation (3, 127, 235).

Following ligation of CD21 and/or the BCR, the cytoplasmic tail of CD19 becomes phosphorylated at tyrosine residues. However, Lyn-deficient B cells do not have impaired CD19 phosphorylation indicating Lyn is not essential for the function of CD19 (236). The phosphorylation of Tyr^{482} and Tyr^{513} of CD19 can directly recruit the p85 subunit of PI-3-K using the SH2 domains of PI-3-K

(237). This leads to the activation of PI-3-K and 3-phosphorylated phosphatidylinositol lipids are generated, providing anchoring points for PH domain-containing proteins (figure 1.18). This pathway occurs independently of Cbl (43, 126, 156, 169). The activation of PI-3-K by CD19 is necessary for efficient stimulation of Akt downstream of the BCR (238). However, CD19 also amplifies and prolongs the activation of Btk although it is not essential for BCR-mediated stimulation of Btk (239). The combined induction of PI-3-K and Btk by CD19 enhances the activity of PLCγ and its downstream signals including calcium mobilisation (165, 235).

B cells that are deficient in CD19 are hyporesponsive to antigenic-stimulation and consequently generate poor immune responses. However, inactivation of the lipid phosphatase PTEN (phosphatase and tensin homologue) can compensate for the CD19-deficiency. PTEN reduces the cellular levels of 3-phosphorylated phosphatidylinositol lipids by removing the 3' phosphate group from the lipids. PTEN therefore catalyses the conversion of PI-3, 4, 5-P_3 to PI-4, 5-P_2 and hence opposes the action of PI-3-K. The ability of PTEN inactivation to compensate for CD19-deficiency suggests the main signalling molecule activated by CD19 is PI-3-K (240).

However CD19 has also been found to bind to Vav via phospho-Tyr^{391} (figure 1.18). Vav may contribute to the activation of PLCγ and calcium signalling. Furthermore, Vav is known to activate JNK MAPK downstream of the BCR (figure 1.16) therefore CD19-mediated activation of Vav may amplify BCR-dependent activation of JNK. Tyr^{391} of CD19 has also been suggested to contribute to the activation of Erk-MAPK in a Vav-independent manner suggesting Tyr^{391} of CD19 can interact with several molecules to amplify BCR signalling (235, 236).

1.7.2 FcγRIIB

In the early stages of an immune response, ligation of the BCR leads to B cell activation including increased gene transcription, protein synthesis and antibody production. In the later stages of the immune response, FcγRIIB, the low affinity receptor for the Fc portion of IgG, is recruited to BCR-containing lipid rafts enabling immune complexes to co-ligate the BCR and FcγRIIB. Co-ligation of FcγRIIB suppresses BCR-mediated signalling including calcium mobilisation,

Akt activation and stimulation of MAPK. FcγRIIB thus inhibits BCR-driven proliferation ensuring the activation of B lymphocytes is a carefully regulated process (241, 242).

The co-aggregation of the BCR and FcγRIIB induces the tyrosine phosphorylation of a single ITIM located in the cytoplasmic tail of FcγRIIB (114, 127). Lyn is required for phosphorylation of FcγRIIB and B cells from Lyn-deficient mice display enhanced BCR-mediated induction of MAPK and proliferation reflecting the importance of Lyn in the negative regulation of BCR signalling (116). The main protein to be recruited to the phosphorylated ITIM of FcγRIIB is the phosphatase SHIP (SH2 domain-containing inositol 5-phosphatase) (figure 1.19). SHIP removes the 5' phosphate residue from PI-3, 4, 5-P_3 (PIP_3) and inositol-1, 3, 4, 5-tetraphosphate (IP_4). IP_4 is an activator of an endothelial membrane Ca^{2+} channel therefore the degradation of IP_4 by SHIP reduces the intracellular calcium flux (127, 241). The degradation of PIP_3 by SHIP impairs the ability of the BCR to recruit and activate PH domain-containing proteins. SHIP can thus decrease the calcium flux further by impairing the activation of PLCγ (131). Furthermore, the degradation of PIP_3 by SHIP can inhibit Akt and Btk thus promoting apoptosis (131, 241). Indeed, the activity of Akt is enhanced in bone marrow-derived mast cells from *SHIP*$^{-/-}$ mice (184). However, Akt can also be stimulated by PI-3, 4-P_2, the product of SHIP-mediated dephosphorylation of PIP_3, therefore SHIP could potentially activate Akt under conditions in which PI-3, 4-P_2 is allowed to accumulate (127). Nonetheless, FcγRIIB signalling is generally reported to be inhibitory of Akt (131, 241). Indeed, ligation of FcγRIIB in mature B cells can induce PTEN resulting in a depletion of 3'-phosphorylated phosphatidylinositol lipids and an antagonism of PI-3-K and Akt signalling (243).

The Erk-MAPK cascade can also be inhibited by FcγRIIB (figure 1.19) since SHIP and Grb-2 directly compete for the binding of phospho-tyrosine residues on Shc. Consequently, SHIP impairs the formation of Grb-2/SOS complexes, resulting in a suppression of the MAPK cascade (241). Furthermore, FcγRIIB and SHIP can suppress the MAPK cascade via the recruitment and activation of p62 Dok (62 kD protein downstream of tyrosine kinase), also known as Dok1 (figure 1.19). The N-terminal region of Dok1 has a PH domain to allow phospholipid/membrane-binding. Dok1 also has a phospho-

tyrosine-binding domain and a C-terminal pro/tyr-rich region that regulates the repertoire of proteins that can associate with Dok1 (figure 1.12). Co-aggregation of FcγRIIB and the BCR increases the tyrosine phosphorylation of SHIP enabling Dok1 to bind to SHIP using its phospho-tyrosine-binding domain. At the plasma membrane, Dok-1 becomes tyrosine phosphorylated allowing it to interact with and activate RasGAP (Ras GTPase activating protein). RasGAP enhances the intrinsic GTPase activity of Ras leading to the inhibition of Ras and its downstream effectors including Erk (114, 131, 244). FcγRIIB can also suppress BCR-mediated activation of Erk via the induction of the MAPK phosphatase Pac-1 leading to dephosphorylation and inhibition of Erk (243).

Ligation of FcγRIIB can thus impair PI-3-K signalling, PLCγ activation and the induction of MAPK. SHIP is the main mediator of FcγRIIB-driven downregulation of BCR signalling but under some circumstances FcγRIIB can also bind to SHP-1 and SHP-2 *in vitro*. The physiological relevance of SHP-1 and SHP-2 in FcγRIIB signalling is uncertain as it does not appear to be essential for FcγRIIB function (131, 245). SHP-1 is activated by binding to phospho-tyrosine residues of ITIMs and it generally functions to inhibit the signals activated by the BCR and CD19 (134). SHP-1 can bind to and inhibit the tyrosine kinases Lyn and Syk resulting in the suppression of early signals activated by the BCR complex and CD19. SHP-1 also associates with BLNK to block signals activated downstream of this adaptor protein. SHP-1 can thus negatively regulate PLCγ and Ca^{2+} signalling, MAPK activation and PI-3-K stimulation (134, 136, 137, 165). SHP-2 is structurally related to SHP-1 but SHP-2 is generally considered to be a positive regulator of cell signalling. For example, SHP-2 can bind to Grb-2 leading to the activation of Erk-MAPK (132, 165, 166).

1.7.3 CD40

1.7.3.1 Functions of CD40 in mature B lymphocytes

CD40 is a 45 kD transmembrane glycoprotein that is a member of the TNF receptor family. It is expressed on several cell types including B cells, macrophages and dendritic cells. CD40 can be activated by CD40 ligand (CD40-L), which is expressed on activated $CD4^+$ T helper cells and via this

interaction T cells can assist the stimulation of B cells (246). The expression of CD40-L on T cells is a carefully regulated process that is initiated by activation of the T cell (247).

Knockout studies have shown that CD40 and CD40-L are required for germinal centre formation and the activation of B cells by T cell-dependent antigens (246, 248). Furthermore, hyper-IgM syndrome is an X-linked immunodeficiency caused by a genetic alteration of CD40-L resulting in a reduced ability to generate T cell-dependent antibody responses and patients lack circulating IgG, IgA and IgE and are unable to form memory B cells (246). CD40 therefore has important functions in the activation of B cells by T cell-dependent antigens and in the maturation of the immune response including germinal centre formation and immunoglobulin isotype switching from IgM to high affinity antibodies.

Ligation of CD40 alone on splenic mature B cells is mitogenic but co-ligation of CD40 and the BCR reduces the threshold for B cell activation resulting in enhanced B cell proliferation and inhibition of apoptosis (249, 250). CD40 therefore acts as a co-receptor to enhance BCR-mediated activation of the cell. Furthermore, ligation of the BCR alone is insufficient to trigger isotype switching in B lymphocytes (251). However, stimulation of CD40 can induce the expression of genes involved in immunoglobulin class switching from IgM to IgG, IgA and IgE (249, 252). Isotype switching is also directed by cytokines since these can determine the nature of the immunoglobulin class switch (246, 253).

CD40 also plays a significant role in directing the maturation of naïve mature B lymphocytes. In particular, it is required for the formation of germinal centres, the affinity maturation of B lymphocytes and the development of memory B cells (254). Activation of naïve B cells by T cell-dependent antigens in T cell-enriched regions of lymphoid follicles can lead to the formation of B centroblasts in the germinal centre. The germinal centre is required for affinity maturation of B lymphocytes. Ligation of CD40 in the germinal centres stimulates the B cells to express particular germinal centre markers including Fas. The upregulation of Fas renders the maturing B cells sensitive to apoptosis. However, cells that express antibodies with a high affinity for antigen will also undergo activation of the BCR and this triggers the upregulation of pro-

survival genes and enables these cells to survive (255, 256). CD40 therefore assists the selection of B cells that produce high affinity antibodies in the germinal centre.

CD40 also directs the differentiation of B cells to plasma cells and memory B cells. The terminal differentiation of B cells to plasma cells requires a disruption in the CD40/CD40-L association. Consequently, prolonged activation of CD40 promotes the formation of memory B cells whilst a shorter exposure to CD40 favours differentiation to plasma cells. This may be one mechanism of ensuring antibody is not released in the germinal centres (48, 246, 257, 258).

1.7.3.2 CD40-dependent signalling in mature B lymphocytes

CD40 exists as a monomer but the binding of CD40-L causes CD40 to polymerise and this is necessary for the activation of intracellular signals. CD40 itself lacks kinase activity and any known consensus sequence for binding kinases but the cytoplasmic region of CD40 does have two binding sites for TRAF (TNF-receptor-associated factor) proteins. TRAFs have several functional domains including a zinc finger domain and a RING finger domain, which may mediate DNA-binding and a TRAF domain to enable protein-protein interactions. TRAFs 1, 2, 3, 5 and 6 and an as yet unidentified 23 kD transmembrane protein have been found in association with CD40 and they assist the transduction of signals downstream of CD40 (246, 248, 259, 260). TRAF 3 knockout mice do not have impaired B cell activation signals suggesting TRAF3 is not essential. In contrast, the RING finger domain of TRAF 2 is required for CD40-mediated activation of NF-κB. However, TRAF 5 can also activate NF-κB downstream of CD40 (246).

Ligation of CD40 on primary murine B cells leads to the activation of protein tyrosine kinases including Lyn and Syk and multiple downstream signalling cascades including JNK and p38 MAPK, PLCγ and PI-3-K (figure 1.20) (246, 254). The activation of these pathways is important for regulating the activity of specific transcription factors and hence the expression of particular genes. For example, CD40 induces the expression, activation and nuclear translocation of NF-κB. In particular, CD40 mediates the induction of RelB with minimal activation of c-Rel. This pathway cannot be stimulated by anti-IgM or LPS suggesting it is specifically regulated by CD40 (261, 262).

CD40-mediated activation of NF-κB can increase the transcription of γ1 gene to promote isotype switching to IgG1 (253). The induction of RelB is thus required for isotype switching of B lymphocytes.

CD40-mediated induction of NF-κB can also promote proliferation of mature B cells. Furthermore, it can induce proliferation in splenic B cells from X-linked immunodeficient mice despite these cells being unable to undergo BCR-driven proliferation (263). CD40 therefore enhances the proliferation of splenic B cells via the activation of NF-κB and independently of Btk. BCR mediated proliferation of splenic B cells involves the induction of the NF-κB component c-Rel and subsequent transcription of the *c-myc* gene (33, 193). RelB may also induce the transcription of this gene to promote proliferation of B cells. Moreover, CD40 can also increase the survival of mature splenic B cells. The inhibitor of apoptosis c-IAP can be found in association with CD40-coupled TRAFs suggesting a potential mechanism for the inhibition of apoptosis in mature B cells (260).

1.8 Lipid rafts

Lipid rafts are evolutionarily conserved membranous structures that are rich in cholesterol, sphingolipids and glycolipids. They have a relatively ordered structure compared to the surrounding glycerophospholipid bilayer. Proteins can bind to the extracellular and intracellular surface of lipid rafts and some transmembrane proteins can enter rafts. In particular, the abundance of cholesterol and glycosphingolipids in rafts attracts the acyl chains of glycosylphosphatidylinositol-anchored or acylated proteins including Src-family kinases, monomeric and heterotrimeric G-proteins, CD4, CD8 and LAT. However, the protein content of rafts is variable and this can assist signalling processes. Lipid rafts therefore form a compartment that can differentially segregate and hence regulate signalling molecules (264-267).

In unstimulated mature T cells lipid rafts help to suppress signals downstream of the TCR by excluding the receptor from the rafts. The rafts are rich in several components of TCR signalling including Src-family kinases, PIP_2, Grb-2 and Ras thus preparing the cell to respond rapidly to TCR stimulation. Activation of the TCR causes it to translocate into lipid rafts along with several other signalling molecules including PLCγ. Lipid rafts of active T cells thus

accumulate specific signalling molecules and help to propagate signals downstream of the TCR (266-269).

Signalling through the BCR is also initiated from within lipid rafts. In unstimulated mature B cells the BCR complex is excluded from lipid rafts but the rafts are rich in signalling molecules including Src-family kinases. Ligation of the BCR enables the BCR and additional signalling molecules to enter lipid rafts thus assisting BCR signalling (270). The translocation of the BCR into lipid rafts is usually a transient process but it can be prolonged by the activation of co-receptors including CD19/CD21 (267). CD40 is always excluded from lipid rafts of mature B cells but ligation of CD40 enhances the movement of the BCR and other signalling molecules to rafts. (270, 271). Furthermore, ligation of CD40 can promote the formation of lipid rafts by increasing the production of ceramide via the activation of acid sphingomyelinases (271, 272). This could assist the formation of signal transduction complexes at the plasma membrane.

Following the accumulation of signalling molecules in rafts, a clustering of rafts can be observed, presumably to bring signalling molecules of several different rafts into a single larger compartment (267, 273, 274). This can enhance BCR signalling by bringing components of the signalling complex into close proximity but it may also assist the termination of BCR signalling by bringing negative regulatory co-receptors into the same compartment as the BCR. For example, FcγRIIB is constitutively associated with rafts but it does not suppress BCR signalling under activating conditions. Inhibition of BCR signalling by FcγRIIB requires either an increased recruitment of FcγRIIB into lipid rafts or a fusion of lipid rafts containing the BCR with those that include FcγRIIB (242). Lipid rafts thus assist the formation of a BCR signalling complex, propagate the BCR signals and help to regulate the signalling process.

An additional function of lipid rafts in mature B cell signalling is to assist the internalisation of the BCR/antigen complex. This is necessary for processing and presentation of antigen on MHC class II complexes thus enhancing the activation of T cells (275, 276). Lipid raft-independent mechanisms of BCR/Ag internalisation are also known and it has been suggested that the different mechanisms of internalisation may affect the intracellular trafficking of the complex (276).

Lipids rafts can further regulate the composition of the plasma membrane

by assisting the translocation of the lipid phosphatidylserine from the intracellular leaflet of the plasma membrane to the exoplasmic side. This process is triggered by an influx of Ca^{2+} through plasma membrane calcium channels and this is promoted by the activation of Erk-MAPK. The redistribution of phosphatidylserine is often observed under apoptotic conditions and it is thought to assist the recognition of the cell by phagocytes thus enhancing its destruction and removal (277).

Lipid rafts can also contribute to the development of an anergic state in lymphocytes. In anergic B cells the BCR can still bind to antigen but this does not lead to the recruitment and activation of tyrosine kinases and other downstream signalling molecules (278). An important difference in BCR signalling of naive and tolerant B cells is ligation of the BCR on tolerant B cells does not induce the translocation of the BCR into lipid rafts. The BCR is thus physically excluded from the signalling molecules retained in lipid rafts and this may impair the activation of specific pathways that are important for B cell stimulation and proliferation (279).

A similar situation is observed in immature B and T cells. Ligation of the antigen receptor on these cells can cause receptor aggregation but it does not induce the translocation of the BCR/TCR into rafts. Exclusion of antigen receptors from lipid rafts in immature lymphocytes will segregate the activated receptors away from the signalling molecules located in the rafts. Consequently, the signalling pathways activated downstream of the antigen receptors of immature lymphocytes will be restricted compared to those of mature cells. For example, exclusion of the antigen receptors from lipid rafts in immature B cells might prevent them activating the proliferative signals observed in BCR-stimulated mature B cells. This is thought to be an important determinant of the different outcomes of ligation of the antigen receptors on immature and mature lymphocytes (267, 270, 280-282).

1.9 BCR signalling in immature B cells

Ligation of the BCR on immature and mature B lymphocytes has very different consequences. Whilst stimulation of the BCR on mature B cells leads to proliferation, activation and differentiation, ligation of the BCR on immature B cells induces growth arrest and apoptosis. This is necessary for negative

selection to remove autoreactive B lymphocytes.

1.9.1 WEHI-231 B cell lymphoma

The murine B cell lymphoma WEHI-231 is widely used as a model for immature B cell clonal deletion (283). The surface phenotype of WEHI-231 B cells, IgM^{+} $IgD^{+/-}$ MHC class II^{low} FcR^{low} Fas^{low}, is very similar to that of primary immature/transitional B cells (table 1.1). Furthermore, ligation of the BCR on WEHI-231 cells induces growth arrest and apoptosis (284, 285) whilst co-ligation of CD40 can rescue the cells from these BCR-driven effects (286, 287). The signalling mechanisms underlying BCR-driven apoptosis and CD40-mediated rescue are incompletely understood.

1.9.2 Signalling pathways activated by the BCR complex of immature B cells

Ligation of the BCR on immature and mature B cells leads to the induction of very similar signalling elements including PI-3-K, PLCγ, Rho GTPases and the Erk-MAPK cascade and the downstream signalling molecules of these complexes (see section 1.6.3). However, ligation of the BCR on immature B cells leads to growth arrest and apoptosis whereas BCR-stimulation on mature B cells induces proliferation, activation and differentiation indicating there are important differences in the signals activated downstream of these BCRs. Indeed, there are likely to be many points of divergence including differences in the kinetics of activation of particular pathways and the intracellular location of the signalling molecules. Furthermore, some significant differences in the components of BCR signalling of mature and immature B cells have been observed.

For example, immature B cells and thymocytes express cytosolic phospholipase A_2 ($cPLA_2$) and it is activated under conditions that lead to clonal deletion. In contrast, mature B and T cells do not express $cPLA_2$ and expression cannot be induced following culture with a variety of different cytokines (218, 222, 288). Ligation of the BCR on immature B cells activates $cPLA_2$ via the Erk-MAPK cascade and calcium signals (222, 289). Cytosolic PLA_2 is the major form of intracellular PLA_2 and this 85 kD protein specifically hydrolyses lipids with a polyunsaturated fatty acid at the sn-2 position including

phosphatidylcholine. Activation of $cPLA_2$ in immature B cells leads to the production of arachidonic acid (218, 222) and arachidonic acid can potently induce apoptosis of the cells (102). The coupling of Erk-MAPK and calcium signalling to the stimulation of $cPLA_2$ and arachidonic acid production in immature B cells is thus an important pro-apoptotic signalling pathway. The absence of BCR-driven $cPLA_2$ activity in mature B lymphocytes suggests that they are unable to initiate this pathway.

In addition to these differences in pro-apoptotic signals, mature and immature B lymphocytes also have divergent proliferative and pro-survival signals. For example, at least two different types of PLD signalling have been found in mature B cells (section 1.6.3.5) that cannot be detected in immature B cells. One of these pathways is activated downstream of the BCR and it involves a non-phosphatidylcholine-hydrolysing PLD that can increase the intracellular levels of DAG to promote proliferation. The absence of this pathway in immature B cells is likely to contribute to the lack of proliferation observed following stimulation of the BCR in these cells. The second PLD pathway involves a phosphatidylcholine-hydrolysing PLD that is activated downstream of P_2-purinoreceptors and promotes growth arrest of the mature B lymphocytes. The absence of this early signalling pathway in immature B cells indicates BCR-driven growth arrest does not require early coupling to phosphatidylcholine-hydrolysing PLD (222, 223).

As well as having impaired PLD signalling, immature B cells have reduced PKC activity. In mature B cells BCR-ligation leads to PIP_2 hydrolysis, calcium mobilisation and stimulation of PKC (see section 1.6.3.2). In immature B cells BCR-ligation can mobilise calcium but PIP_2 hydrolysis and subsequent PKC activation are significantly reduced. PKC is an activator of PLD therefore the suppression of PKC signalling in immature B cells may contribute to the lack of PLD signalling in these cells. PLD is also a potential regulator of PKC since PLD can generate phosphatidic acid, a second messenger that stimulates PKCη and PKCζ. Mature B lymphocytes from $PKC\zeta^{-/-}$ mice undergo spontaneous apoptosis and have impaired activation, including reduced ability to produce antibody. This suggests that PKCζ promotes survival and activation of mature B cells therefore a suppression of this signal in immature B cells is likely to prevent them from inducing antibody production and increases their

sensitivity to apoptosis (199).

In addition to stimulating PKCη and PKCζ, PA can be converted to DAG, enabling the activation of phorbol ester-sensitive PKC isoforms. One such phorbol ester-sensitive PKC enzyme is PKCβ. This PKC isoform seems to participate in the activation of mature B cells since PKCβ$^{-/-}$ mice have reduced humoral immune responses and their B lymphocytes have impaired activation downstream of the BCR (210). More specifically, mature B lymphocytes from PKCβ$^{-/-}$ mice are less able to induce Bcl-x_L and Bcl-2 and hence are prone to apoptosis (199, 290). The expression of pro-survival Bcl-2 family members is also an important survival signal in immature B cells since induction of these proteins can protect them from BCR-driven apoptosis (290-293). The impaired activation of PKC in BCR-stimulated immature B cells therefore could increase their sensitivity to apoptosis by reducing the levels of pro-survival Bcl-2 family proteins. Indeed, treatment of primary splenic immature B cells or WEHI-231 B cells with phorbol ester can prevent BCR-driven apoptosis strongly suggesting the diminished activation of conventional and novel PKC isoforms in immature B cells contributes to their cell death (294, 295).

Furthermore, in mature B cells BCR-dependent PKC activity can induce NF-κB and this increases the survival of the cells since NF-κB can regulate the transcription of pro-survival factors including c-Myc, Bcl-x_L and Bcl-2 (193, 199, 211). Ligation of the BCR on immature B cells cannot sustain NF-κB activation, perhaps due to their defective stimulation of PKC. The reduction in NF-κB activity in BCR-stimulated immature B cells is likely to contribute to their death since NF-κB signalling is enhanced by conditions that favour cell survival, including CD40 co-stimulation (261, 296, 297).

1.9.3 CD40 signalling in immature B cells

Ligation of the BCR on immature B cells induces growth arrest and apoptosis and this is necessary for the clonal deletion of self-reactive B cells. This process can be regulated by the presence of T lymphocytes since ligation of CD40 protects immature B cells from BCR-driven growth arrest and apoptosis (286, 287). Consequently, enforced and prolonged expression of CD40-L can result in the production of autoantibodies (298, 299).

The structure of CD40 on immature B cells is essentially the same as on

mature B lymphocytes (see section 1.7.3.2) and the propagation of signals downstream of CD40 requires the recruitment of TRAFs. Following the multimerisation of TRAFs, several signalling cascades are activated including the MAPK, PI-3-K and PLCγ pathways. These cascades can then regulate additional signalling molecules to influence the survival and proliferation of the immature B cells (figure 1.21).

As in mature B cells, ligation of CD40 stimulates the activation of NF-κB but in immature B cells c-Rel complexes are particularly induced by CD40 (297). CD40 mediates the activation of NF-κB in immature B cells by inducing the sustained degradation of I-κBα. NF-κB can then regulate the expression of specific target proteins including protooncogene *pim-1*. The induction of Pim-1 correlates with increased survival and proliferation of B lymphocytes (300). Furthermore, CD40-mediated activation of NF-κB is likely to induce the expression of *c-myc* since the activation of NF-κB downstream of the BCR and CD40 correlates with the changes in expression of the *c-myc* gene in WEHI-231 immature B cells. Indeed, ligation of the BCR induces a transient activation of NF-κB and *c-myc* gene expression whilst co-ligation of CD40 stimulates a sustained NF-κB signal and prolonged expression of *c-myc* (301). The induction of c-Myc is important for CD40-mediated rescue from BCR-driven loss of immature B cell viability (302).

CD40 can also regulate the survival of immature B cells by controlling the expression of Bcl-2-family proteins. For example, ligation of CD40 or overexpression of Bcl-2 can protect WEHI-231 cells from apoptosis induced by concanamycin A, an inhibitor of vacuolar ATPases (303). Furthermore, ligation of CD40 on WEHI-231 cells induces the expression of the pro-survival protein A1 and expression of A1 can partially protect immature B cells from BCR-driven apoptosis but not growth arrest (302, 304).

Moreover, in WEHI-231 immature B cells ligation of CD40 can greatly enhance the expression of the *bcl-x*$_L$ gene without increasing the expression of *bcl-2* or *bax* (293). Ligation of CD40 also increases the protein levels of Bcl-x$_L$ whilst stimulation of WEHI-231 cells with anti-IgM or anti-IgD does not induce Bcl-x$_L$ (292). The use of antisense oligonucleotides for *bcl-x*$_L$ can partially block CD40-driven rescue from BCR-mediated apoptosis suggesting the upregulation of Bcl-x$_L$ is required for rescue from apoptosis (293). Furthermore,

overexpression of Bcl-x_L, but not Bcl-2, in WEHI-231 cells can protect them from BCR-driven apoptosis in the absence of CD40 stimulation (292). Bcl-x_L transfectants also display increased resistance to ceramide-driven apoptosis (305). Bcl-x_L is therefore an important mediator of CD40-dependent rescue from BCR-driven apoptosis. However, the signalling pathways used by CD40 to upregulate Bcl-x_L are incompletely understood. Nonetheless, CD40-stimulation is also known to activate p38 and JNK MAPKs but inhibition of these MAPKs does not impair the ability of CD40 to protect immature B cells from BCR-driven apoptosis suggesting they are not necessary for CD40-mediated survival (306-310).

Bcl-x_L appears to be specifically involved in preventing apoptosis of immature B cells since overexpression of Bcl-x_L, unlike ligation of CD40, does not rescue WEHI-231 cells from BCR-driven growth arrest. CD40 thus regulates the survival and proliferation of immature B cells via distinct signalling pathways (311). Indeed, mutagenesis of CD40 has shown that distinct regions of CD40 are required to induce Bcl-x_L and proteins involved in cell cycle progression (312). However, the mechanism of CD40-dependent rescue from BCR-driven growth arrest is poorly understood. WEHI-231 cells that overexpress Bcl-x_L can be made resistant to BCR-dependent growth arrest by inducing the overexpression of either E2F1 or the viral oncoprotein E1A. The activity of E2F is negatively regulated by hypophosphorylated retinoblastoma (Rb) protein but ligation of CD40 induces the phosphorylation of Rb, releasing E2F and allowing the transcription of genes required for DNA synthesis and cell cycle progression (52). CD40 can also upregulate the activity of the cyclin D kinases Cdk4 and Cdk6 (312) and Rb is a known substrate of these kinases therefore CD40 may induce E2F to allow entry into S phase by enabling cyclin D kinases to phosphorylate and inhibit Rb (50).

1.10 Regulation of apoptosis and proliferation in immature B lymphocytes

1.10.1 Regulation of apoptosis in immature B cells

1.10.1.1 Bcl-2 family proteins in immature B lymphocytes

The Bcl-2 family of proteins can regulate the survival of many different cell types including immature B lymphocytes (67). The viability of a cell is critically

dependent on the relative abundance of pro-survival and pro-apoptotic proteins of the Bcl-2 family. Conditions that elevate pro-survival proteins will favour survival by retaining the integrity of the membranes of intracellular organelles including the mitochondria. However, signals that induce pro-apoptotic proteins result in a loss of mitochondrial integrity and the activation of apoptotic mediators including the executioner proteases (70, 76, 78, 313).

Initial studies on immature B lymphocytes recognised that certain apoptotic stimuli can decrease the cellular levels of pro-survival Bcl-2 family proteins. For example, prolonged ligation of the BCR can decrease the cellular levels of Bcl-2 in WEHI-231 cells (292) and vaccinia virus can induce apoptosis of these cells by downregulating the intracellular levels of Bcl-2 (314). This suggests that decreasing the levels of pro-survival Bcl-2 family proteins in these cells assists the induction of apoptosis. However, several strains of WEHI-231 cells that are resistant to BCR-driven apoptosis appear to have normal levels of Bcl-2 indicating protection from apoptosis requires the upregulation of alternative pro-survival Bcl-2 family members (315, 316).

Indeed, immature B cells can be protected from specific apoptotic stimuli by the induction of other pro-survival Bcl-2 family proteins. For example, treatment of WEHI-231 cells with immunosuppressants (rapamycin, cyclosporin A or FK-506), an inhibitor of protein synthesis (emetine) or an inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum (thapsigargin) can induce apoptosis that is prevented by overexpression of the pro-survival protein Bcl-x_L. These agents also induce growth arrest of the WEHI-231 B cells but this cannot be rescued by overexpression of Bcl-x_L suggesting survival and proliferation are differentially regulated in immature B cells (291, 292).

Furthermore, BCR-stimulated growth arrest and apoptosis of immature B cells can be prevented by co-ligation of CD40. CD40 induces an upregulation of Bcl-x_L without increasing the expression of Bcl-2 or Bax (290, 292, 293) and overexpression of Bcl-x_L in WEHI-231 cells protects the cells from BCR-driven apoptosis (292, 311). The upregulation of Bcl-x_L can thus protect immature B cells from BCR-driven apoptosis. Moreover, mutagenesis of CD40 suggested that the same region (amino acids 246-269) of CD40 is required to stimulate Bcl-x_L and to protect WEHI-231 cells from BCR-driven apoptosis (312).

However, additional anti-apoptotic Bcl-2 family members, especially A1 and Mcl-1, may also contribute to the survival of immature B cells (302). For example, stimulation of WEHI-231 cells at CD40 can upregulate the expression of the *a1* gene and overexpression of A1 can partially protect the cells from BCR-driven apoptosis (304). However, A1 seems to protect the immature B cells by inhibiting caspase 9 and subsequent activation of caspase 7 enabling A1 to protect the cells from BCR-induced DNA laddering (317).

1.10.1.2 Caspase-dependent apoptosis in immature B cells

The signalling networks used to mediate BCR-driven apoptosis are complex and are likely to involve caspase-dependent and caspase-independent mechanisms (102, 317-319). Initial reports of caspase-dependent apoptosis involved the use of caspase inhibitors such as the general caspase inhibitor zVAD.fmk, which was reported to suppress BCR-driven apoptosis (53). Furthermore, Doi *et al.* observed that inhibition of caspases in BCR-stimulated WEHI-231 cells prevents some of the characteristics of apoptosis without giving protection against others. In particular, zVAD.fmk prevented BCR-driven cleavage of poly(ADP-ribose) polymerase (PARP), nuclear fragmentation, hypodiploidy and DNA fragmentation. However, it only partially suppressed BCR-driven changes in cellular oxidation-reduction potential and movement of phosphatidylserine to the outer leaflet of the plasma membrane. Furthermore, zVAD.fmk was unable to prevent BCR-driven disruption of the plasma membrane and loss of the mitochondrial membrane potential (318). These observations suggest that caspase-dependent signals are involved in mediating damage to the nucleus and DNA of BCR-stimulated WEHI-231 cells but additional characteristics of apoptosis, including disruption of the mitochondrial membrane potential, can occur independently of caspases. Nonetheless, overexpression of Bcl-x_L protects the WEHI-231 cells from all the symptoms of apoptosis suggesting it is able to prevent caspase-dependent and caspase-independent apoptosis (318).

The initiation and execution of caspase-dependent cell death downstream of the BCR in immature B cells is incompletely understood. However, ligation of the BCR is known to activate a neutral sphingomyelinase leading to the production of ceramide and ceramide can activate caspases and induce the

cleavage of PARP (102, 320). Caspase-dependent apoptosis therefore may be initiated by the production of ceramide downstream of the BCR. Overexpression of Bcl-x_L does not prevent the production of ceramide but it can protect the cells from ceramide-induced cell death (305).

A mechanism of caspase-dependent apoptosis has been proposed for human immature and mature B cell lines (319). It has been suggested that BCR-driven caspase-dependent apoptosis involves the stimulation of caspases 2 and 3 downstream of calcineurin. Caspase 9 activity was also observed and this may serve to amplify the caspase signals but caspases 1 and 8 were not found to be active (319). This is a novel caspase-dependent pathway that may be exclusive to human cell lines since inhibition of caspases 2 and 3 in murine WEHI-231 B cells does not prevent BCR-driven apoptosis (53, 319).

However, BCR-driven caspase 9 activity has been detected in WEHI-231 cells and it leads to the induction of caspase 7 (317). Furthermore, co-stimulation of the cells at CD40 can suppress caspase 7 activity by upregulating the pro-survival Bcl-2 family protein A1. Overexpression of A1 in these cells can also partially suppress BCR-driven apoptosis suggesting caspase 7 may be a mediator of this apoptotic process and the inhibition of this signal by CD40 may promote cell survival (302, 317). However, BCR-driven apoptosis is not suppressed by the specific inhibition of caspase 7 (53), suggesting BCR-driven apoptosis can use alternative caspase-dependent mechanisms of apoptosis and/or caspase-independent apoptotic pathways.

1.10.1.3 Caspase-independent apoptosis in immature B cells

A caspase-independent mechanism of BCR-driven apoptosis has been elucidated in immature WEHI-231 B cells (figure 1.22) (102). Ligation of the BCR on immature B cells activates $cPLA_2$ via the Erk-MAPK cascade and calcium signals. Calcium causes $cPLA_2$ to translocate from the cytosol to membranes of the nucleus, endoplasmic reticulum and mitochondria thus bringing $cPLA_2$ into close proximity to its lipid substrates (289). MAPK activates $cPLA_2$ by phosphorylation of Ser^{505} (222). Treatment of immature B cells with IL-4 reduces BCR-mediated activation of $cPLA_2$ and IL-4 also protects the cells from BCR-driven apoptosis suggesting $cPLA_2$ is a component of the pro-apoptotic pathway activated by the BCR (288). Indeed, activation of $cPLA_2$ in

immature B cells leads to the production of the pro-apoptotic molecule arachidonic acid (218, 222).

The mechanism of arachidonic acid and BCR-mediated apoptosis has been investigated in WEHI-231 cells (figure 1.22) (102). Ligation of the BCR or treatment of WEHI-231 cells with exogenous arachidonic acid induces a loss of mitochondrial membrane potential and subsequent depletion of cellular ATP levels. However, stabilisation of the mitochondrial membrane potential using the mitochondrial inhibitors antimycin or oligomycin can maintain a normal mitochondrial membrane potential and protect the WEHI-231 cells from BCR-driven apoptosis. Furthermore, the mitochondrial membrane potential is stabilised and cellular levels of ATP are retained by co-ligation of CD40 and it is widely recognised that CD40 can protect immature B cells from BCR-driven apoptosis. The loss of mitochondrial membrane potential is therefore a critical stage in the induction of apoptosis by the BCR and arachidonic acid.

However the loss of mitochondrial integrity does not induce the release of the caspase co-factor, cytochrome c and caspase 3 activity is not stimulated by ligation of the BCR. Furthermore, treatment of BCR-stimulated WEHI-231 cells with caspase inhibitors does not protect these cells from BCR-driven apoptosis. This suggests that the classical caspase-dependent pathway of apoptosis is not activated by ligation of the BCR on immature B cells (102). Indeed, caspase-independent cell death has also been observed in several other systems including glucocorticoid-induced and CD45-mediated death of thymocytes, death of hematopoietic cell lines following withdrawal of growth factors, Bax-mediated cell death and germinal centre B cell apoptosis (see section 1.4.4) (93, 104, 313, 321-324). Furthermore, many non-caspase proteases including the cathepsins and the calpains, can cleave some of the classical caspase substrates suggesting these alternative proteases can mimic the effects of caspases to mediate cell death (324). Indeed, ligation of the BCR in WEHI-231 cells does induce cathepsins and inhibition of cathepsin B protects the cells from BCR-driven apoptosis. This suggests that BCR-driven apoptosis of WEHI-231 cells is executed by cathepsins and the activation of these proteases is mediated by the disruption of mitochondrial integrity (102). The activation of $cPLA_2$ and subsequent production of arachidonic acid is the initiating step of this apoptotic pathway.

1.10.1.4 NF-κB

NF-κB is an important survival factor that is activated downstream of the BCR in mature B lymphocytes (193, 199, 211). However, ligation of the BCR on immature B cells is unable to sustain NF-κB activation and agents that inhibit NF-κB induce apoptosis of WEHI-231 cells (296). Furthermore, co-stimulation of CD40 induces NF-κB via the sustained degradation of I-κBα and overexpression of the pro-survival factor Bcl-x_L can protect WEHI-231 cells from apoptosis induced by agents that inhibit NF-κB. These observation suggest NF-κB is an important determinant of cell survival in WEHI-231 B cells (261, 297, 301, 312, 325).

However, the signalling pathways regulated by NF-κB in WEHI-231 cells are incompletely understood. NF-κB is a transcription factor and it can stimulate the transcription of genes that contain a NF-κB-regulatory region including *c-myc* (297). Indeed, NF-κB induces the transcription of *c-myc* in mature B lymphocytes (34, 211, 262) and in WEHI-231 immature B cells it is thought to be at least partially responsible for the induction of *c-myc* expression (301). Nonetheless, the transcription factor CTCF has also been implicated as a regulator of *c-myc* gene expression in WEHI-231 cells. However, CTCF appears to suppress *c-myc* gene expression downstream of the BCR to promote apoptosis of the cells. CTCF may also promote growth arrest of immature B cells by increasing the expression of regulators of the cell cycle including p21, p27, p53 and p19. (325).

1.10.2 Regulation of proliferation in immature B cells

Ligation of the BCR on immature B cells induces growth arrest and apoptosis that can be rescued by co-ligation of CD40. Survival and proliferation are likely to be mediated by distinct signalling pathways since they can be regulated independently of each other. For example, Bcl-x_L is a key mediator of CD40-dependent rescue from BCR-driven apoptosis but cannot prevent BCR-induced growth arrest (312).

In mature B cells, ligation of the BCR stimulates proliferation by inducing Cdk4 and cyclin D2 to allow progression through the early stages of G1 phase of the cell cycle and by enhancing the expression of cyclin E and Cdk2 to

enable the G1 to S phase transition. In contrast, ligation of the BCR on immature B cells enhances the levels of Cdk4 and cyclin D2 but does not increase the expression of cyclin E and Cdk2. Consequently, ligation of the BCR on immature B cells induces growth arrest at G1 phase. However, BCR-induced growth arrest can be overcome by co-culture of immature B cells with IL-4 since IL-4 enhances the expression of Cdk2 and cyclin E (33).

A potential mediator of these effects is c-Myc since the expression of *c-myc* is elevated in immature and mature B cells under conditions that allow proliferation but it is not expressed during growth arrest (302). Furthermore, the expression of *c-myc* can be reduced in WEHI-231 cells by BCR-dependent induction of CTCF and under these conditions there is an increase in the expression of cell cycle regulators including p21, p27, p53 and p19 (325). The induction of these proteins promotes growth arrest and apoptosis. In addition, BCR-ligation induces Rb to inhibit E2F and decrease the levels of cyclin A thus preventing entry into S phase. In contrast, ligation of CD40 on WEHI-231 cells inhibits Rb and rescues the cells from growth arrest (53, 56, 58). Proliferation of WEHI-231 cells is thus dependent on the differential regulation of Rb and E2F by the BCR and CD40.

1.11 Aims and objectives

Immature B cells are sensitive to the process of negative selection therefore ligation of the BCR on these cells induces growth arrest and apoptosis. However, co-ligation of CD40 can rescue the immature B cells and enable them to survive and proliferate. The aim of this investigation was to explore the signalling mechanisms used by the BCR and CD40 to differentially regulate survival and proliferation of immature B cells. In particular, the role of Erk-MAPK in controlling these processes was examined using the WEHI-231 immature B cell line.

The specific aims and objectives of this investigation were:

1. to investigate the dual role for Erk-MAP kinase in BCR-driven apoptosis and CD40-mediated rescue of WEHI-231 immature B cells and to explore the mechanisms regulating Erk activity in this system

2. to explore the role of arachidonic acid in mediating the induction of apoptosis downstream of Erk and to investigate the role of metabolites of arachidonic acid in regulating survival and proliferation of WEHI-231 cells

3. to further explore the mechanism of CD40-dependent rescue of WEHI-231 cells by determining the role of Bcl-x_L in rescuing the cells from BCR-driven growth arrest and apoptosis

4. to investigate other potential mediators of CD40-dependent rescue by analysing the role of sphingosine kinase in regulating survival and proliferation of WEHI-231 cells and by examining the mechanism of sphingosine-1-phosphate (SPP) signalling in this system

5. to investigate the observation that immature B cells have impaired induction of PKC signalling compared to mature B cells by exploring the role of PKC in regulating the survival and proliferation of WEHI-231 cells and by examining the signalling mechanisms used by PKC to regulate these processes.

Figure 1.1. The B cell antigen receptor. The BCR complex consists of surface immunoglobulin non-covalently bound to accessory signalling molecules Igα and Igβ. Surface immunoglobulin contains two identical heavy chains and two identical light chains that come together to form the antigen binding site. The binding of antigen to sIg results in the phosphorylation of Igα and Igβ at tyrosine residues located within ITAMs. The resultant phospho-tyrosine residues are required to initiate intracellular signalling cascades that ultimately determine the survival, proliferation and activity of the B lymphocyte.

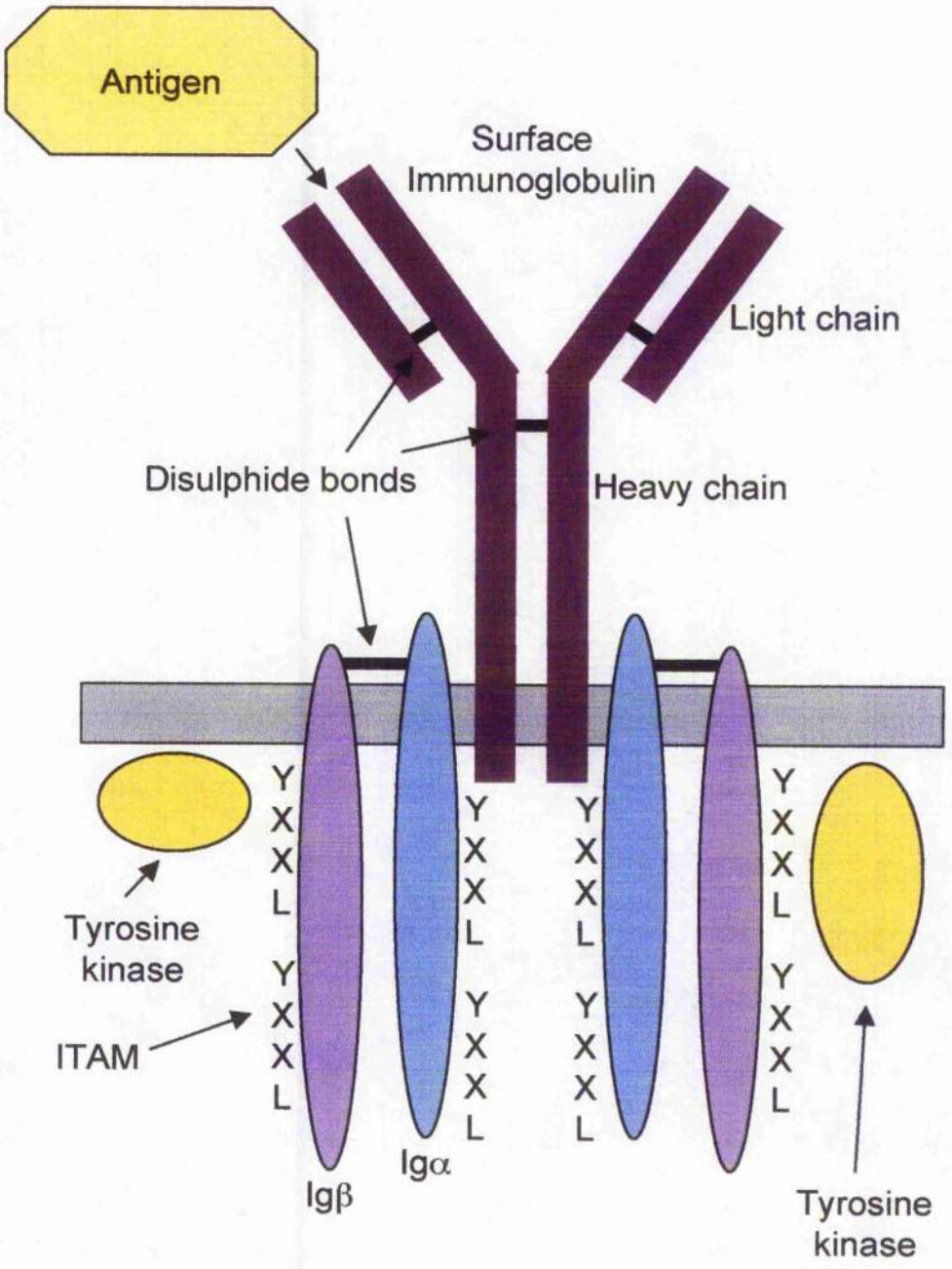
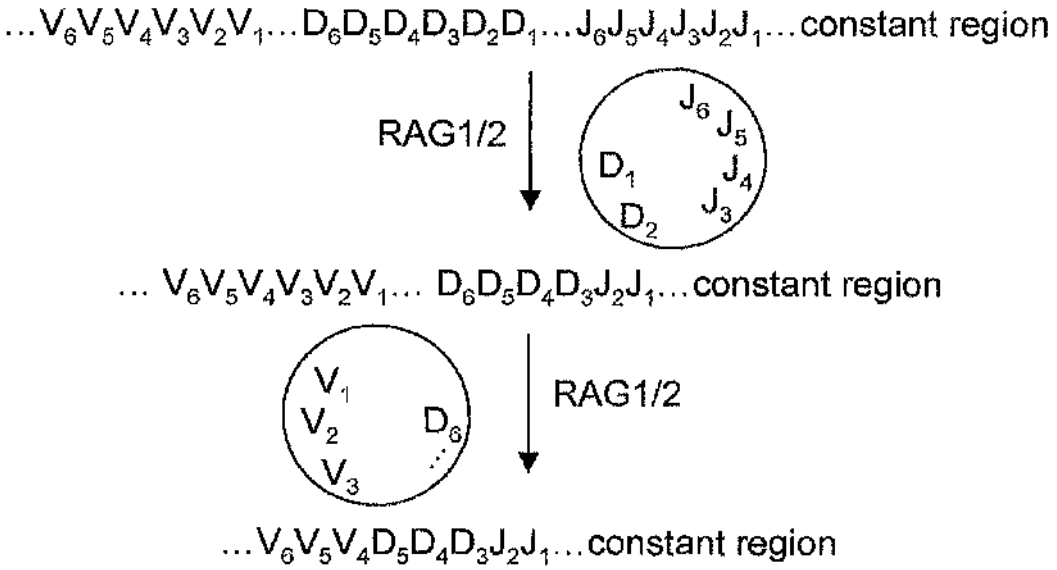

Antigen
Surface Immunoglobulin
Light chain
Disulphide bonds
Heavy chain
Tyrosine kinase
ITAM
Y X X L
Y X X L
Y X X L
Y X X L
Y X X L
Y X X L
Igβ
Igα
Tyrosine kinase

Figure 1.2. Somatic recombination of immunoglobulin heavy and light chains. **(A)** The variable region of the heavy chain is generated by two random recombination events; firstly the D_H and the J_H chains are joined then the V_H chain is joined to the D_HJ_H segment. **(B)** The variable region of the light chain is generated by a single random recombination event in which the V_L chain is joined to the J_L segment. Each somatic recombination event is catalysed by recombinase (RAG1 and RAG2).

(A)


...V6V5V4V3V2V1...D6D5D4D3D2D1...J6J5J4J3J2J1...constant region
RAG1/2
J6 J5
D1 J4
D2 J3
... V6V5V4V3V2V1... D6D5D4D3J2J1...constant region
V1
V2 D6
V3
RAG1/2
...V6V5V4D5D4D3J2J1...constant region

(B)

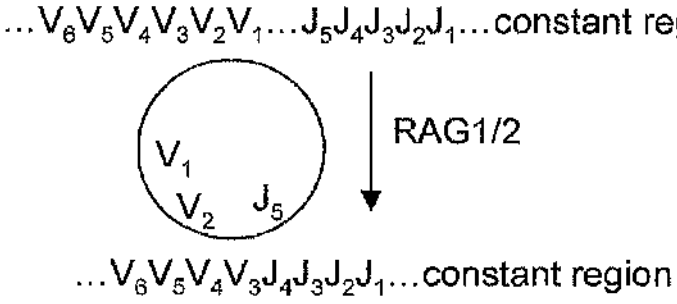

...V6V5V4V3V2V1...J5J4J3J2J1...constant region
V1
V2 J5
RAG1/2
...V6V5V4V3J4J3J2J1...constant region

Figure 1.3. Stages of B cell development. The initial stages of B cell development occur in the bone marrow and are antigen-independent. Pluripotent hematopoietic stem cells (HSC) give rise to common lymphoid progenitors (CLP) and these can form pro-B cells. There are three types of pro-B cells, early, intermediate and late. Immunoglobulin heavy chain gene rearrangement takes place at the pro-B cell stage, followed by positive selection of cells with a functional heavy chain expressed in the pre-B cell receptor. Expression of the pre-BCR enables late pro-B cells to form large pre-B cells. After a series of cell divisions large pre-B cells give rise to non-dividing small pre-B cells. Rearrangement of the immunoglobulin light chain takes place in small pre-B cells. Successful gene rearrangement leads to the expression of the BCR complex on immature B cells. Further B cell development is antigen-dependent. Ligation of the BCR on immature B cells leads to receptor editing or apoptosis thus eliminating self-reactive B cells. B cells that survive this process of negative selection are mature B cells and are located in the periphery where they can be used to generate a humoral immune response following ligation of the BCR by foreign antigen. Mature B cells can differentiate into antibody-secreting plasma cells or memory B cells.

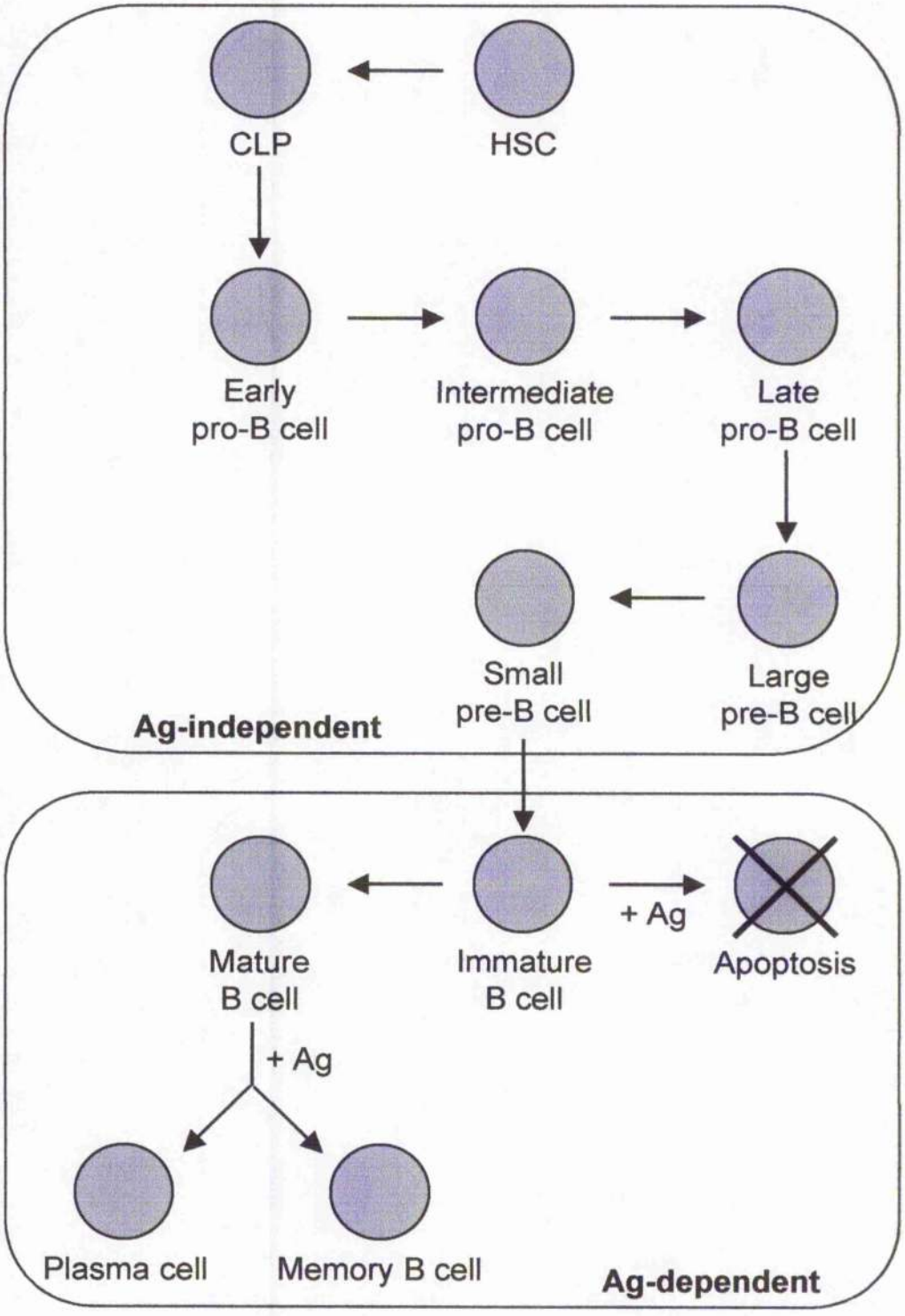
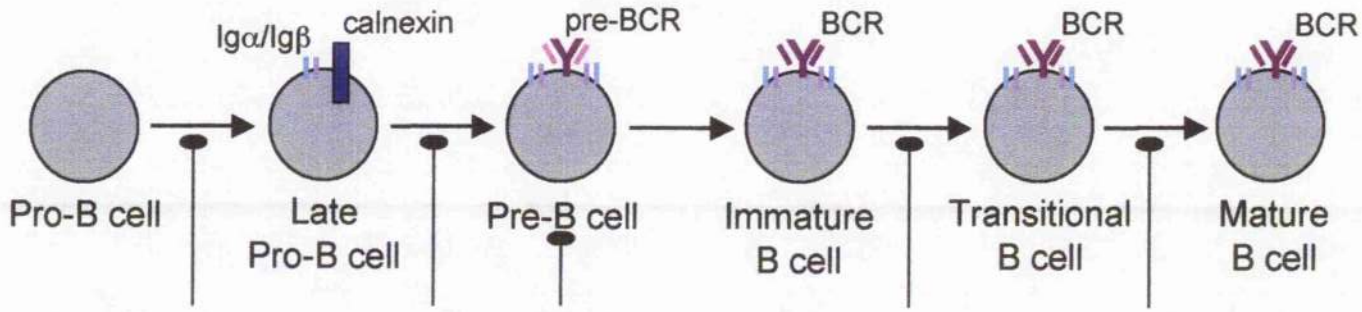

CLP
HSC
Early
pro-B cell
Intermediate
pro-B cell
Late
pro-B cell
Small
pre-B cell
Large
pre-B cell
Ag-independent
Mature
B cell
Immature
B cell
+ Ag
Apoptosis
+ Ag
Plasma cell
Memory B cell
Ag-dependent

Figure 1.4. Characterisation of the different stages of B cell development. Knockout models of particular proteins induce developmental arrest at a specific stage of B cell development indicating an essential role for that protein in progression beyond that stage. B cell development can be characterised by the changes in surface phenotype of the cells. CD45, also known as B220, is a protein phosphatase that promotes BCR signalling. CD19 and CD21 enhance BCR signals. CD23, also known as FcεRII is a surface receptor for the constant region of antibodies. CD40 is an important co-stimulus of BCR signalling in immature and mature B cells. Igα and Igβ are essential components of the pre-BCR and BCR complex. They are first expressed in conjunction with calnexin at the surface of the late pro-B cell. Expression of a functional pre-BCR is essential for the development of pre-B cells. IgM and IgD are mature BCRs. IgM is the main BCR of immature B cells whilst IgD is predominantly found in mature B lymphocytes.



Knockout models: RAG1/2$^{-/-}$ Pax5$^{-/-}$, Igβ$^{-/-}$, Syk$^{-/-}$ λ5$^{-/-}$, μm$^{-/-}$ Igα$^{-/-}$ Btk $^{-/-}$

Surface markers:

CD45	+	+	+	+	+	++
CD19	+	+	+	+	+	++
CD21	+	+	+	+	+	+
CD23	-	-	-	-	+/-	+
CD40	+	+	+	+	+	+
calnexin	-	+	-	-	-	-
pre-BCR	-	-	+	-	-	-
IgM	-	-	-	+	+	+
IgD	-	-	-	-	+/-	++

Figure 1.5. Mature B cell development in the germinal centre. Naïve mature B cells migrate into the follicular mantle (1) during a T cell-dependent immune response. B cells undergo extensive proliferation as centroblasts (2), cease to express surface IgM and form the dark zone. Centroblasts undergo somatic hypermutation in the dark zone then they migrate to the light zone as surface IgM-expressing centrocytes (3). Centrocytes are highly susceptible to apoptosis, enabling the elimination of cells expressing low affinity BCRs. Survival of the centrocytes requires ligation of the BCR by antigen and the provision of appropriate co-stimuli. Surviving B cells form a pool of memory B cells (4) or high-affinity antibody-producing plasma cells (5).

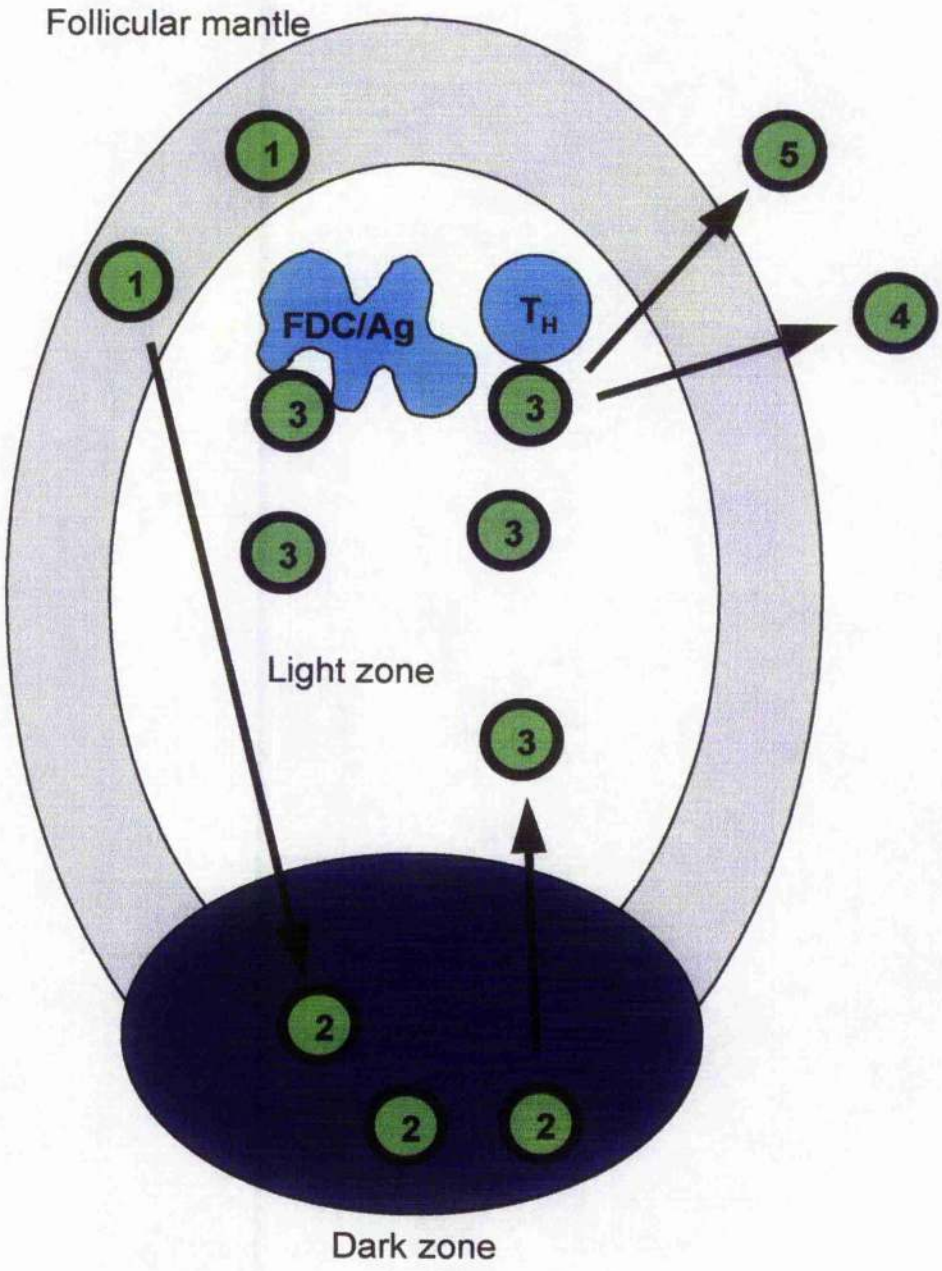

Follicular mantle
1
1
5
4
FDC/Ag
T_H
3
3
3
3
3
Light zone
2
2
2
Dark zone

Figure 1.6. The cell cycle. **(A)** The cell cycle contains several stages. Quiescent cells (G0) enter the cell cycle at G1 phase where cells undergo growth. On completion of G1 phase, cells undergo DNA synthesis (S phase), followed by another growth phase (G2) and then they undergo mitosis (M phase). **(B)** The cell cycle is carefully regulated with distinctive checkpoints at the end of each growth phase. Progression through the cell cycle is regulated by cyclin-Cdk complexes and by regulators of these complexes including Rb, p15, p16, p21, p27, p53 and p19.

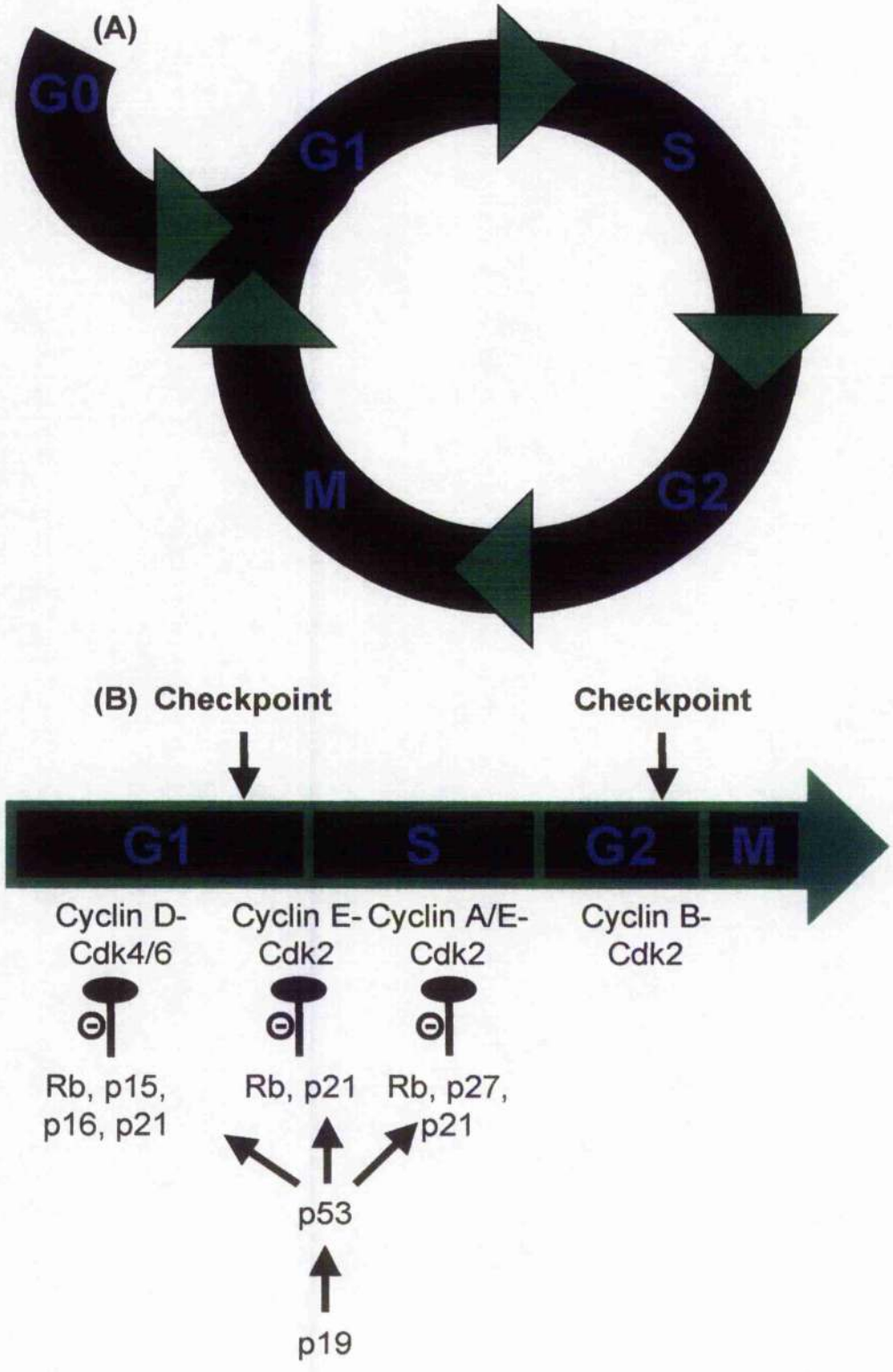

(A)
G0
G1
S
M
G2
(B) Checkpoint
Checkpoint
G1
S
G2
M
Cyclin D-Cdk4/6
Cyclin E-Cdk2
Cyclin A/E-Cdk2
Cyclin B-Cdk2
Rb, p15, p16, p21
Rb, p21
Rb, p27, p21
p53
p19

Figure 1.7. The Bcl-2 family can be split into three groups. Pro-survival Bcl-2 family proteins including Bcl-2 and Bcl-x_L have four BH (Bcl-2 homology) domains (BH1-4) and a C-terminal membrane attachment domain. Pro-apoptotic Bcl-2 family proteins such as Bax and Bak have three BH domains (BH1-3) and a C-terminal membrane attachment domain. A different group of pro-apoptotic Bcl-2 family proteins including Bid and Bik has only one BH domain (BH3) and may or may not have a membrane attachment region (67). BH1-3 can form alpha-helical structures that enable dimerisation between different Bcl-2 family proteins. BH3 only proteins can also bind to the alpha-helical cleft to form dimers with other groups of Bcl proteins. BH1-2 is required for the formation of a membrane-spanning pore.

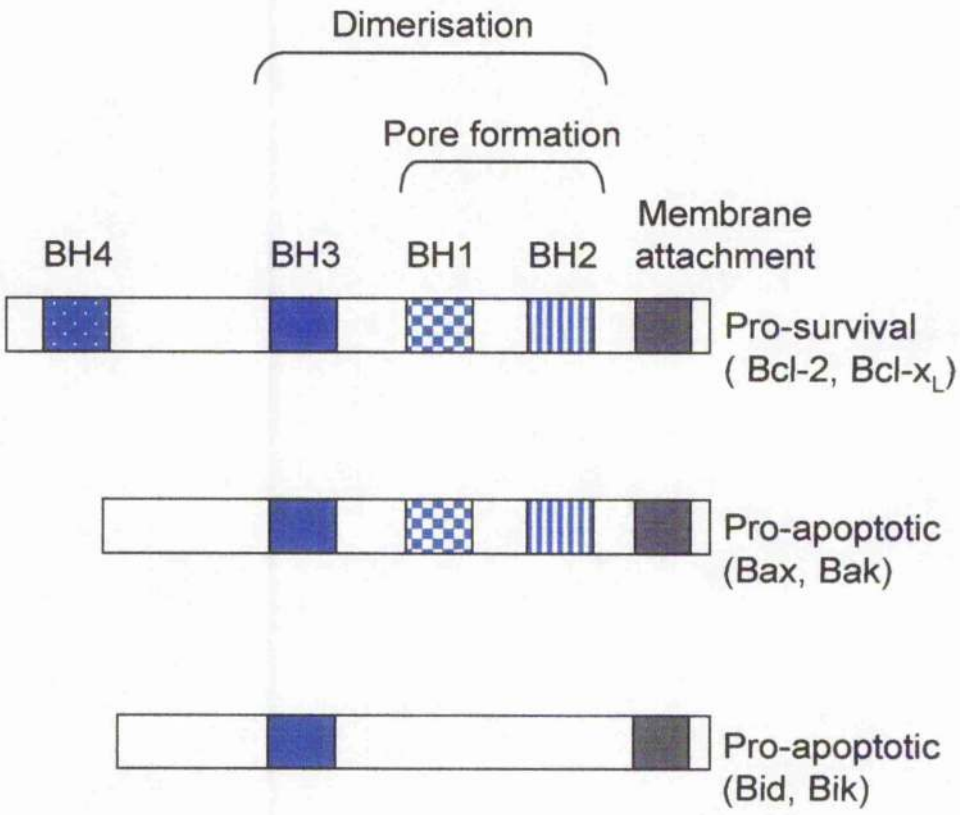
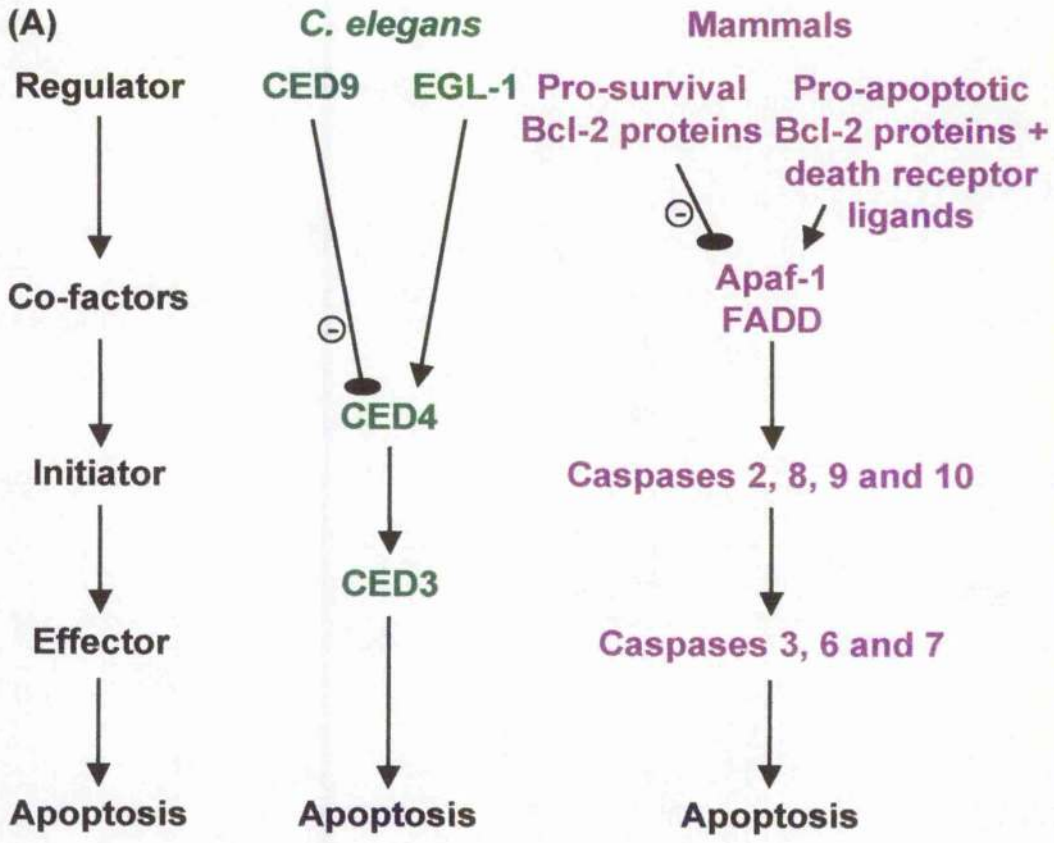

Dimerisation
Pore formation
Membrane attachment
BH4
BH3
BH1
BH2
Pro-survival
(Bcl-2, Bcl-x_L)
Pro-apoptotic
(Bax, Bak)
Pro-apoptotic
(Bid, Bik)

Figure 1.8. Caspase-dependent apoptosis and the activation of caspases. **(A)** Caspase-dependent apoptosis in *C. elegans* and mammals. Apoptosis in *C. elegans* is mediated by the caspase CED3. CED3 is activated by autocleavage following binding to CED4. The activity of CED4 is negatively regulated by CED9 and is stimulated by EGL-1. Caspase-mediated apoptosis in mammals is more complex than in *C. elegans*. Mammals have several effector caspases (caspases 3, 6 and 7) and their activity is regulated by multiple initiator caspases (caspases 2, 8, 9 and 10). The activation of caspases is regulated by mitochondrial co-factors including Apaf-1 and by death receptor-associated co-factors like FADD. These co-factors are regulated by members of the Bcl-2 family and by ligands of receptors containing associated DED domains. **(B)** Activation of caspases by proteolysis. Caspases are expressed as inactive zymogens that include a pro-domain, a p20 subunit and a p10 subunit. Activation of caspases requires proteolysis to remove the pro-domain and to separate the p20 and p10 subunits. Two p20 subunits combine with two p10 subunits to form a catalytically active tetramer.


(A)
C. elegans
Mammals
Regulator
CED9
EGL-1
Pro-survival Bcl-2 proteins
Pro-apoptotic Bcl-2 proteins + death receptor ligands
Co-factors
Apaf-1
FADD
CED4
Initiator
Caspases 2, 8, 9 and 10
CED3
Effector
Caspases 3, 6 and 7
Apoptosis
Apoptosis
Apoptosis

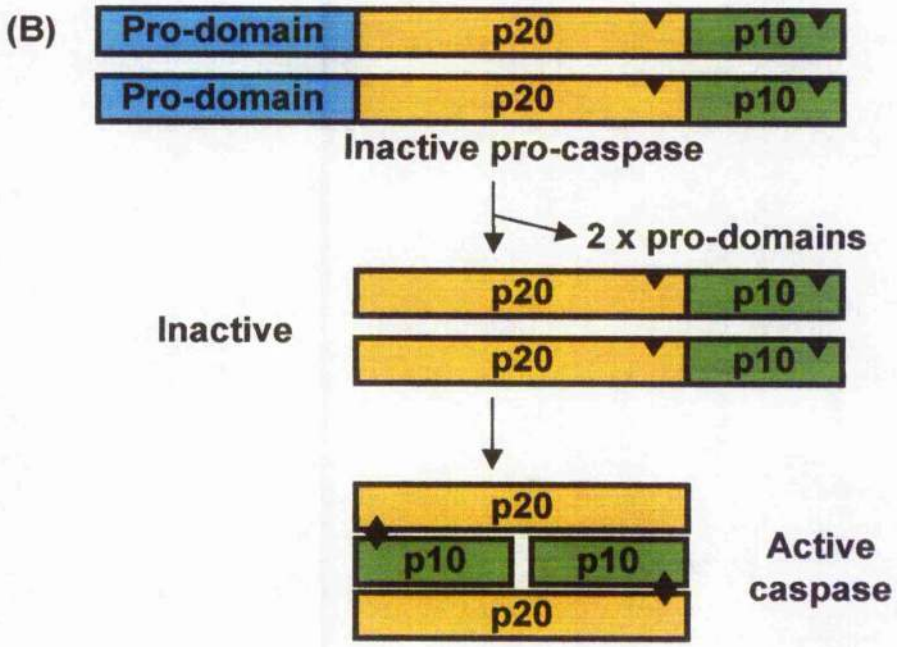

(B)
Pro-domain
p20
p10
Pro-domain
p20
p10
Inactive pro-caspase
2 x pro-domains
Inactive
p20
p10
p20
p10
p20
p10
p10
p20
Active caspase

Figure 1.9. Formation of the Apaf-1 apoptosome and activation of caspase 9. Under non-apoptotic conditions pro-survival members of the Bcl-2 family such as Bcl-2 protect the mitochondria. An apoptotic stimulus induces an upregulation of pro-apoptotic Bcl-2 family proteins including Bax. Bax and Bcl-2 form dimers on the surface of the mitochondria leading to the formation of a pore on the outer mitochondrial membrane. Mitochondria burst, releasing their contents including ATP, calcium and cytochrome c into the cytoplasm. Cytochrome c binds to Apaf-1 causing it to oligomerise. In the presence of ATP, cytochrome c and Apaf-1 form an Apaf-1 apoptosome, leading to the activation of pro-caspase 9 and subsequent stimulation of effector caspases (caspases 3, 6 and7). Effector caspases mediate apoptosis by cleaving multiple target proteins to induce DNA laddering, chromatin condensation, nuclear shrinkage, cytoskeletal collapse and membrane blebbing.

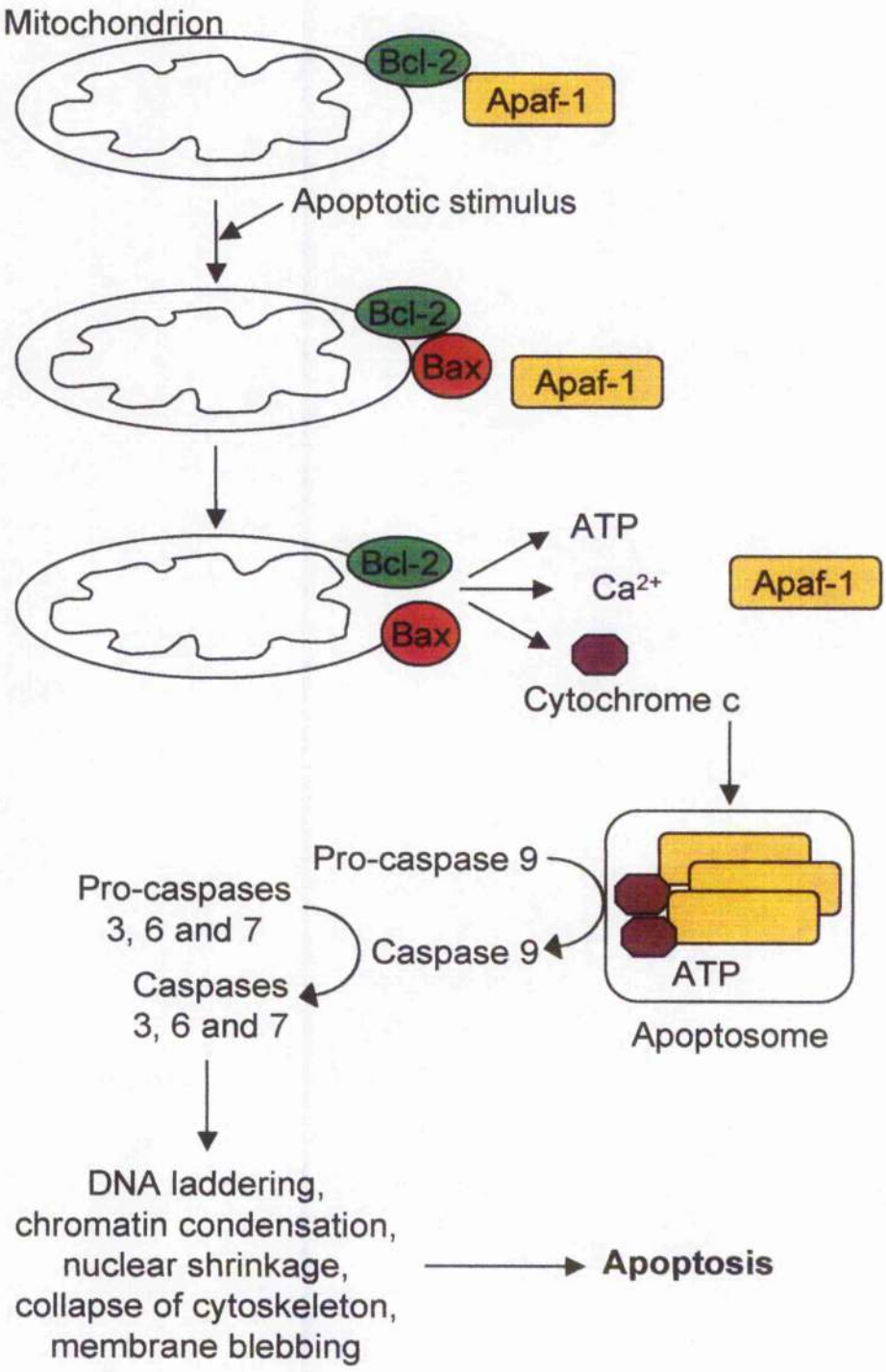

Mitochondrion
Bcl-2
Apaf-1
Apoptotic stimulus
Bcl-2
Bax
Apaf-1
ATP
Bcl-2
Ca2+
Apaf-1
Bax
Cytochrome c
Pro-caspase 9
Pro-caspases
3, 6 and 7
Caspase 9
ATP
Caspases
3, 6 and 7
Apoptosome
DNA laddering,
chromatin condensation,
nuclear shrinkage,
collapse of cytoskeleton,
membrane blebbing
Apoptosis

Figure 1.10. Caspase-mediated apoptosis in mammalian cells. Fas ligand induces the oligomerisation of Fas and the associated FADD proteins. Pro-caspase 8 associates with FADD via its DED domain resulting in an increased local concentration of the pro-caspase and it is activated by autocleavage. Caspase 8 can cleave and activate effector caspases resulting in the induction of apoptosis. Caspase 8 also cleaves and activates Bid and the resultant tBid promotes mitochondrial disruption by dimerising with pro-apoptotic Bcl-2 family proteins including Bax. Further disruption of the mitochondria is also promoted by Bcl-2/Bax dimers, which also form pores in the outer mitochondrial membrane. The contents of the mitochondria are released into the cytoplasm including Smac, an inhibitor of IAPs, thus preventing the inhibition of caspases. The mitochondria also release components of the Apaf-1 apoptosome resulting in the activation of pro-caspase 9 and the enhanced stimulation of effector caspases. Effector caspases mediate apoptosis by cleaving multiple target proteins to induce DNA laddering, chromatin condensation, nuclear shrinkage, cytoskeletal collapse and membrane blebbing.

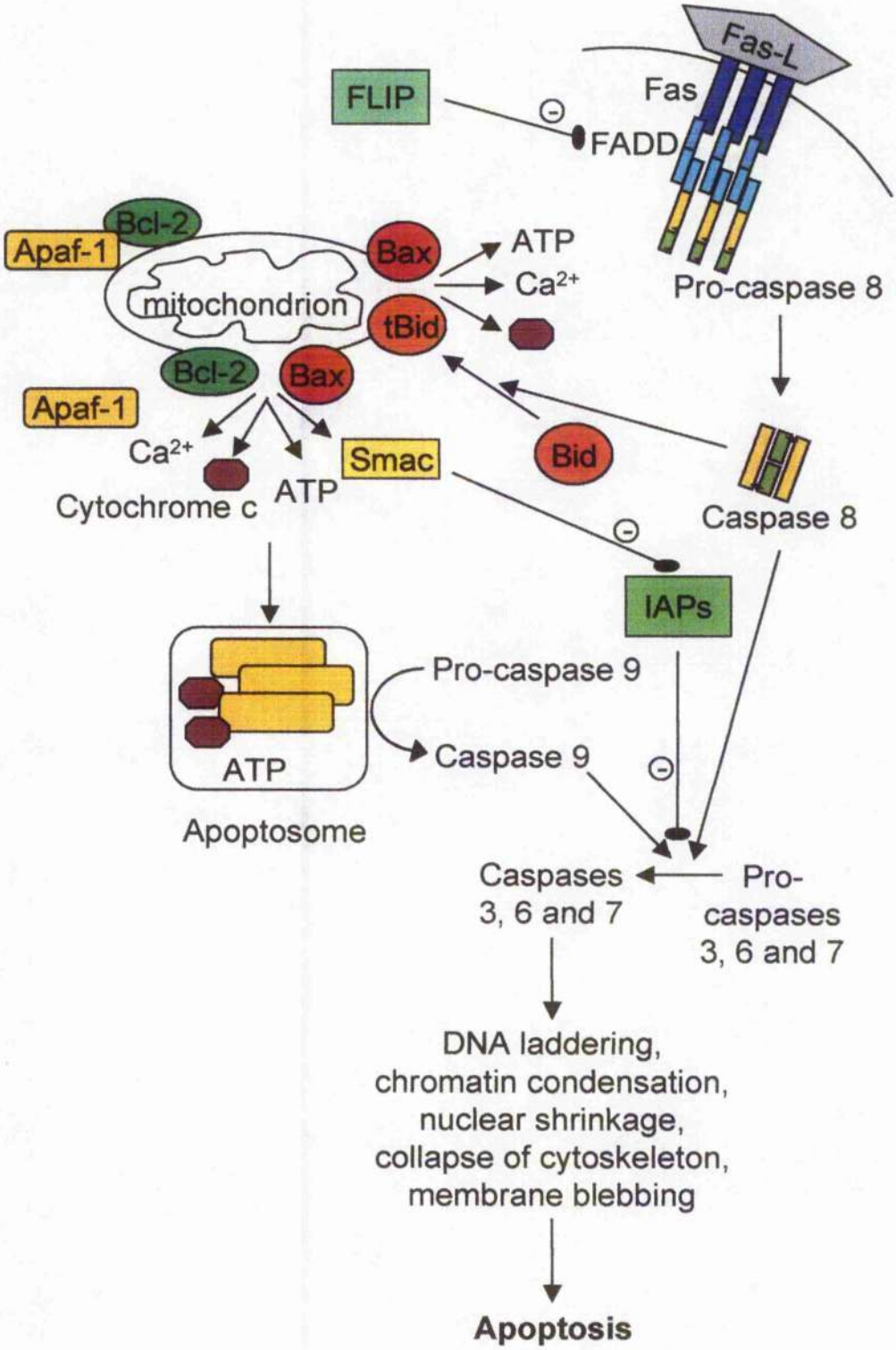

Fas-L
Fas
FLIP
FADD
Bcl-2
Apaf-1
Bax
ATP
Ca2+
mitochondrion
tBid
Pro-caspase 8
Bcl-2
Bax
Apaf-1
Ca2+
Smac
Bid
Cytochrome c
ATP
Caspase 8
IAPs
Pro-caspase 9
ATP
Caspase 9
Apoptosome
Caspases
3, 6 and 7
Pro-
caspases
3, 6 and 7
DNA laddering,
chromatin condensation,
nuclear shrinkage,
collapse of cytoskeleton,
membrane blebbing
Apoptosis

Figure 1.11. Signals activated downstream of the BCR complex. Ligation of the BCR induces the activation of four main pathways: PI-3-K, PLCγ, Vav/Rho and Erk-MAPK. Stimulation of these pathways regulates the expression of genes and can regulate the differentiation, apoptosis and proliferation of the cells.

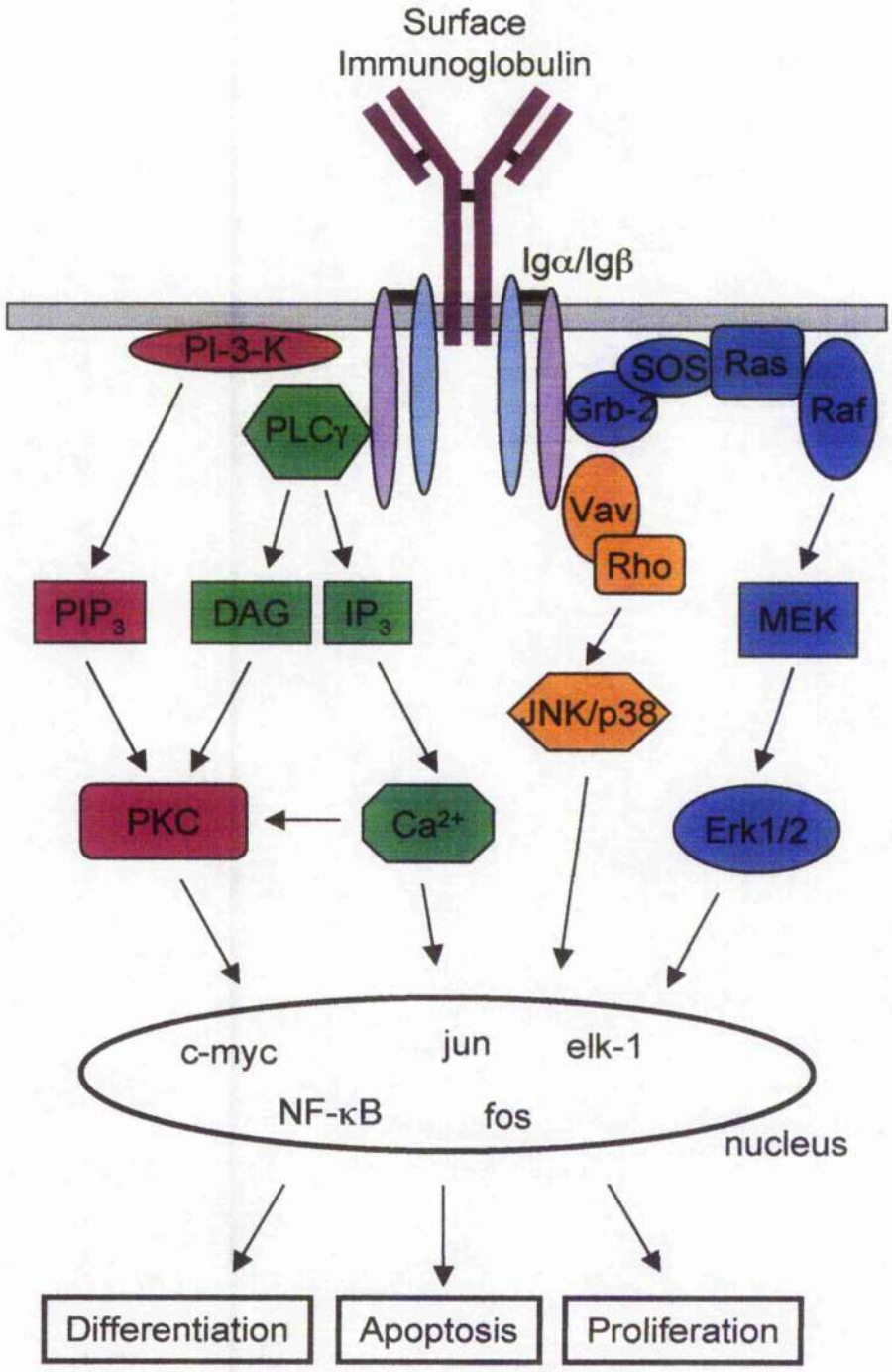
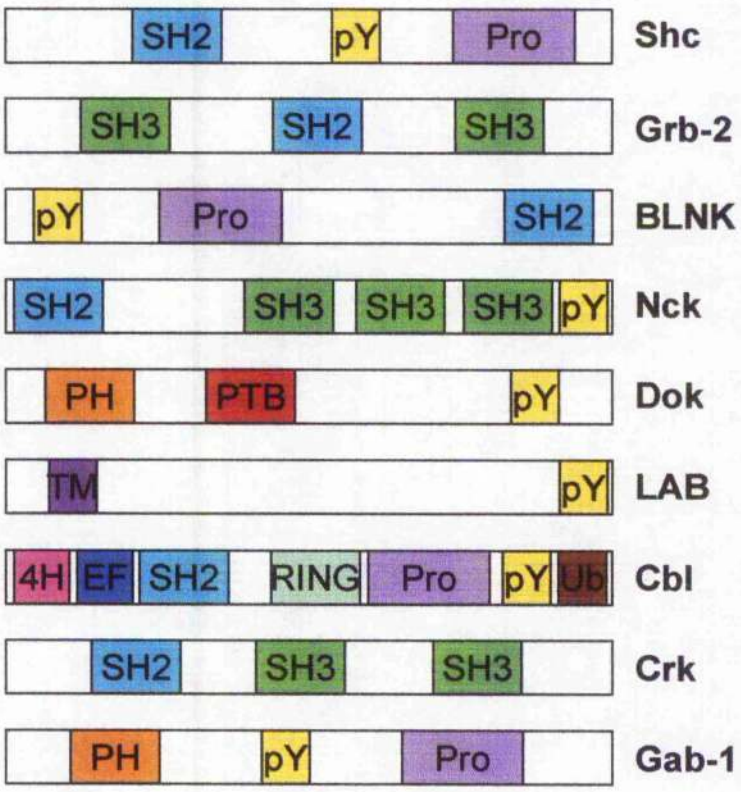

Surface
Immunoglobulin
Igα/Igβ
PI-3-K
PLCγ
SOS
Ras
Grb-2
Raf
Vav
Rho
PIP3
DAG
IP3
MEK
JNK/p38
PKC
Ca2+
Erk1/2
c-myc
jun
elk-1
NF-κB
fos
nucleus
Differentiation
Apoptosis
Proliferation

Figure 1.12. Adaptor proteins in BCR signalling. The BCR complex requires several adaptor proteins to regulate downstream signalling pathways. The different adaptor proteins are characterised by the presence of distinct modular interaction domains.



SH2 — SH2 domain

Pro — Proline-rich region

SH3 — SH3 domain

pY — Phospho-Tyr residues

PH — PH domain

PTB — Phospho-Tyr-binding domain

TM — Transmembrane region

4H — Four helix bundle

EF — EF hand motif

RING — RING finger domain

Ub — Ubiquitin-associated domain

Figure 1.13. Interaction of adaptor proteins with downstream signalling molecules. The adaptor proteins associated with the BCR complex couple the BCR to specific signalling pathways by mediating protein-to-protein and protein-to-lipid interactions via their modular interaction domains. Interactions between proteins are represented by a line with an arrow head at both ends whilst the inhibition of Syk by Cbl is represented by a line with a blunt end.

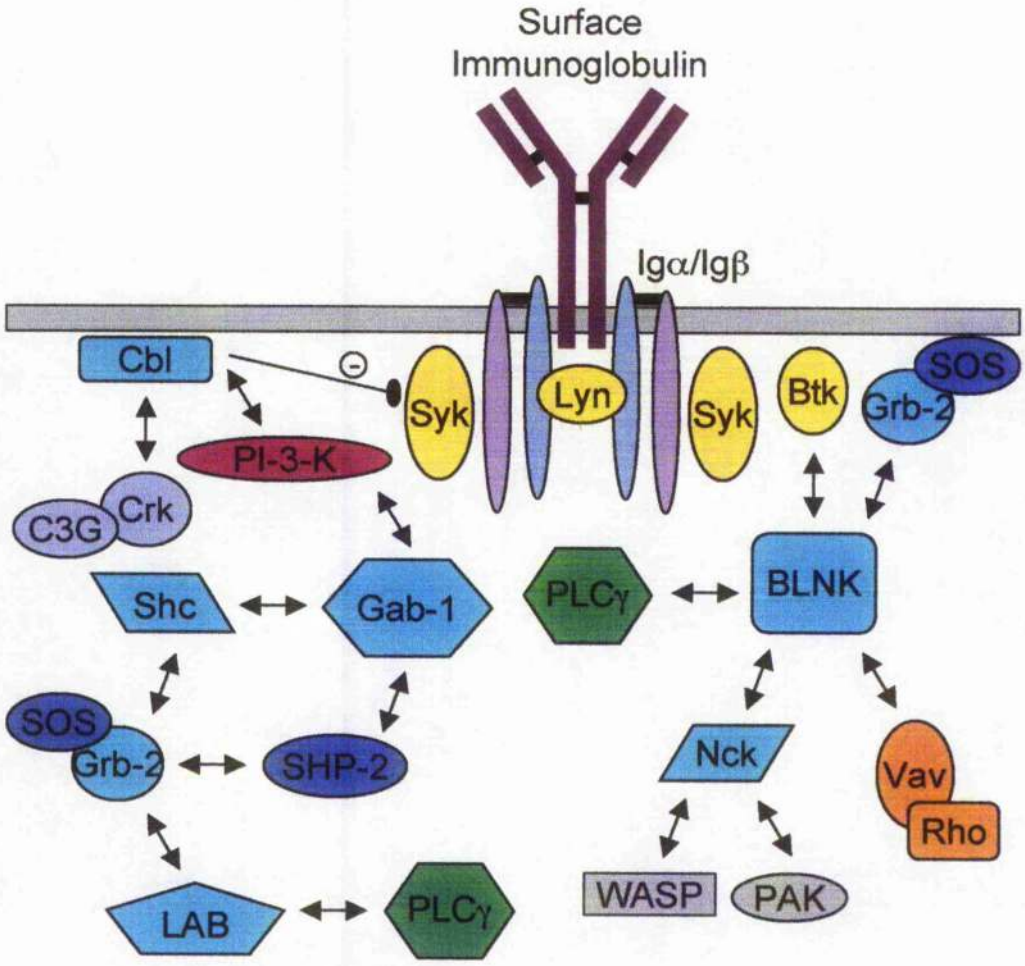

Surface
Immunoglobulin
Igα/Igβ
Cbl
Syk
Lyn
Syk
Btk
SOS
Grb-2
PI-3-K
C3G
Crk
Shc
Gab-1
PLCγ
BLNK
SOS
Grb-2
SHP-2
Nck
Vav
Rho
WASP
PAK
LAB
PLCγ

Figure 1.14. The activation of PI-3-K signals downstream of the BCR. PI-3-K can catalyse the phosphorylation of phosphatidylinositol lipids at the 3' position leading to the production of PI-3, 4, 5-P_3 and PI-3, 4-P_2. PH domain-containing proteins can interact with 3-phosphorylated phosphatidylinositol lipids. PH domain-containing proteins used downstream of the BCR complex include PDK1, PDK2, PKC, Btk and Vav. Akt is activated by PDK1/2 resulting in the inhibition of pro-apoptotic proteins and the activation of pro-survival signalling molecules. Protein-to-protein or protein-to-lipid interactions are represented by a line with an arrow head at both ends, the activation of signalling molecules is represented by a line with a single arrow head and the inhibition of signalling molecules is indicated by a line with a blunt end.

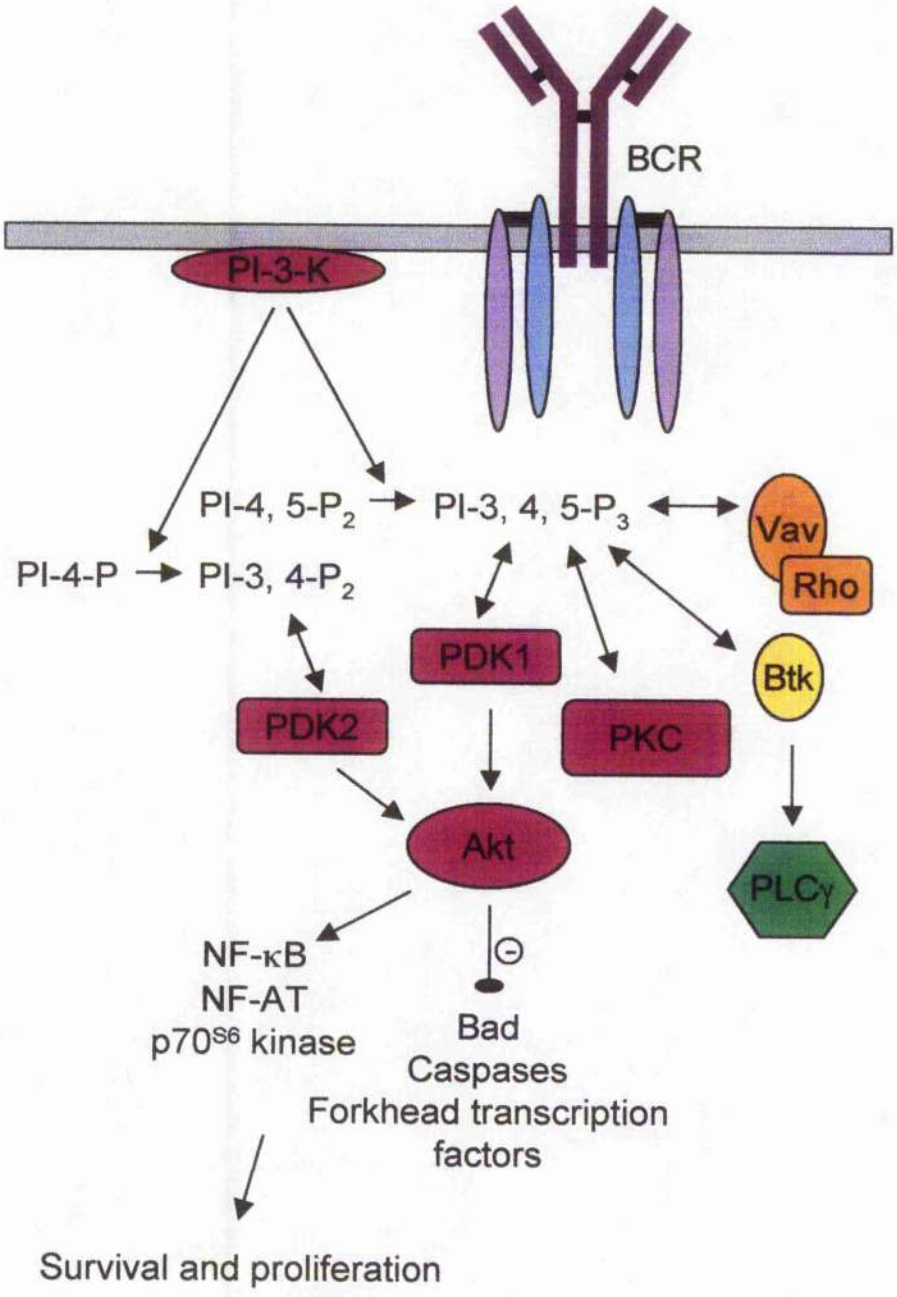
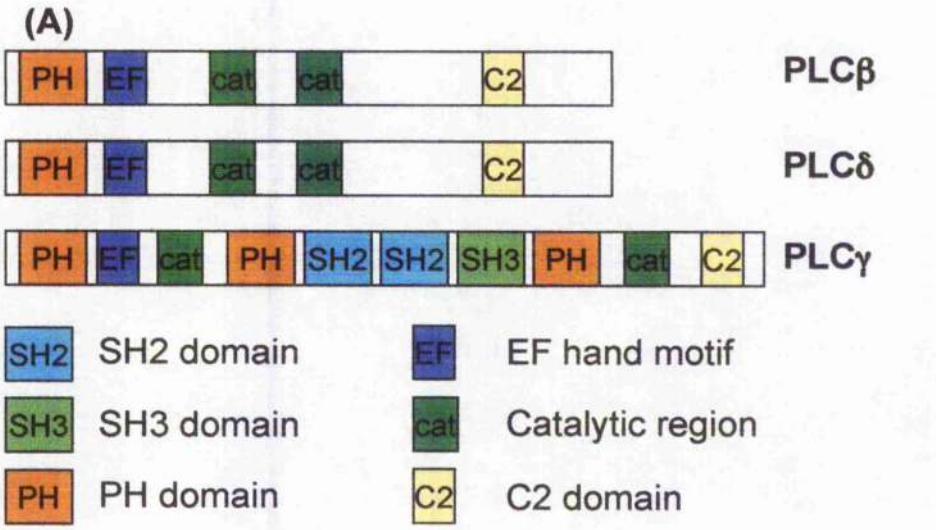

BCR
PI-3-K
PI-4, 5-P2
PI-3, 4, 5-P3
Vav
Rho
PI-4-P
PI-3, 4-P2
PDK1
Btk
PDK2
PKC
Akt
PLCγ
NF-κB
NF-AT
p70S6 kinase
Bad
Caspases
Forkhead transcription factors
Survival and proliferation

Figure 1.15. The structure of PLC and PLCγ signalling downstream of the BCR. **(A)** The structure of the PLC family. The three members of the PLC family can be distinguished by their different structures. In addition to the domains of PLCβ and PLCδ, PLCγ has two extra PH domains, two SH2 domains and an SH3 domain. **(B)** The activation of PLCγ signals downstream of the BCR complex. The activation of PLCγ requires the phosphorylation of BLNK by Syk and the formation of a PLCγ/BLNK/Btk complex. Both Btk and PLCγ can bind to PIP_3 via their PH domains to help to localise them at the BCR complex. Once activated, PLCγ catalyses the cleavage of PIP_2 to form DAG and IP_3. IP_3 induces the mobilisation of intracellular calcium stores contributing to the activation of PKC. DAG can also stimulate PKC enzymes. PKC in turn can regulate the activity of MAPK. Protein-to-protein or protein-to-lipid interactions are represented by a line with an arrow head at both ends and the activation of signalling molecules is represented by a line with a single arrow head.


(A)
PH
EF
cat
cat
C2
PLCβ
PH
EF
cat
cat
C2
PLCδ
PH
EF
cat
PH
SH2
SH2
SH3
PH
cat
C2
PLCγ
SH2 SH2 domain
SH3 SH3 domain
PH PH domain
EF EF hand motif
cat Catalytic region
C2 C2 domain

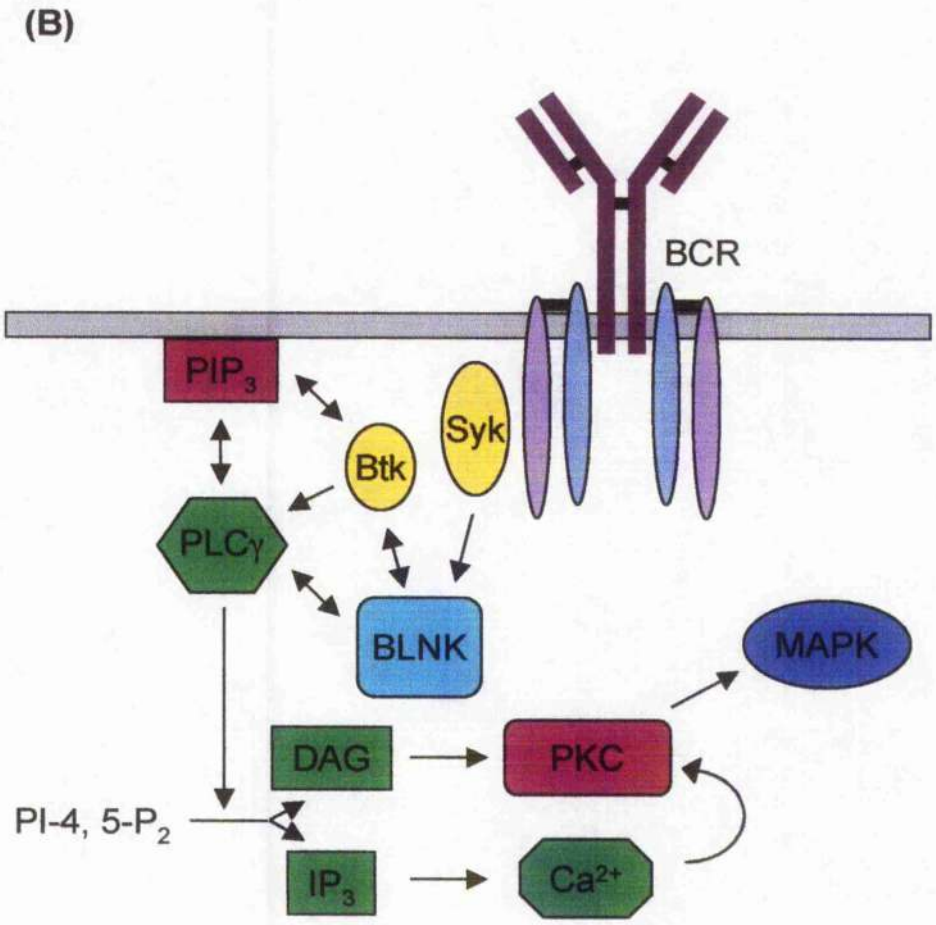

(B)
BCR
PIP3
Syk
Btk
PLCγ
BLNK
MAPK
DAG
PKC
PI-4, 5-P2
IP3
Ca2+

Figure 1.16. The activation of Vav/Rho signals downstream of the BCR complex. Vav/Rho can be recruited to the BCR complex via BLNK. Rho-family proteins can stimulate phosphatidylinositol-4-phosphate-5-kinase (PI-4-P-5-kinase) and PI-3-K leading to the production of PIP_2 and PIP_3, respectively. Rho-family proteins can also activate PLCγ and JNK/p38 MAPK. Protein-to-protein or protein-to-lipid interactions are represented by a line with an arrow head at both ends and the activation of signalling molecules is represented by a line with a single arrow head.

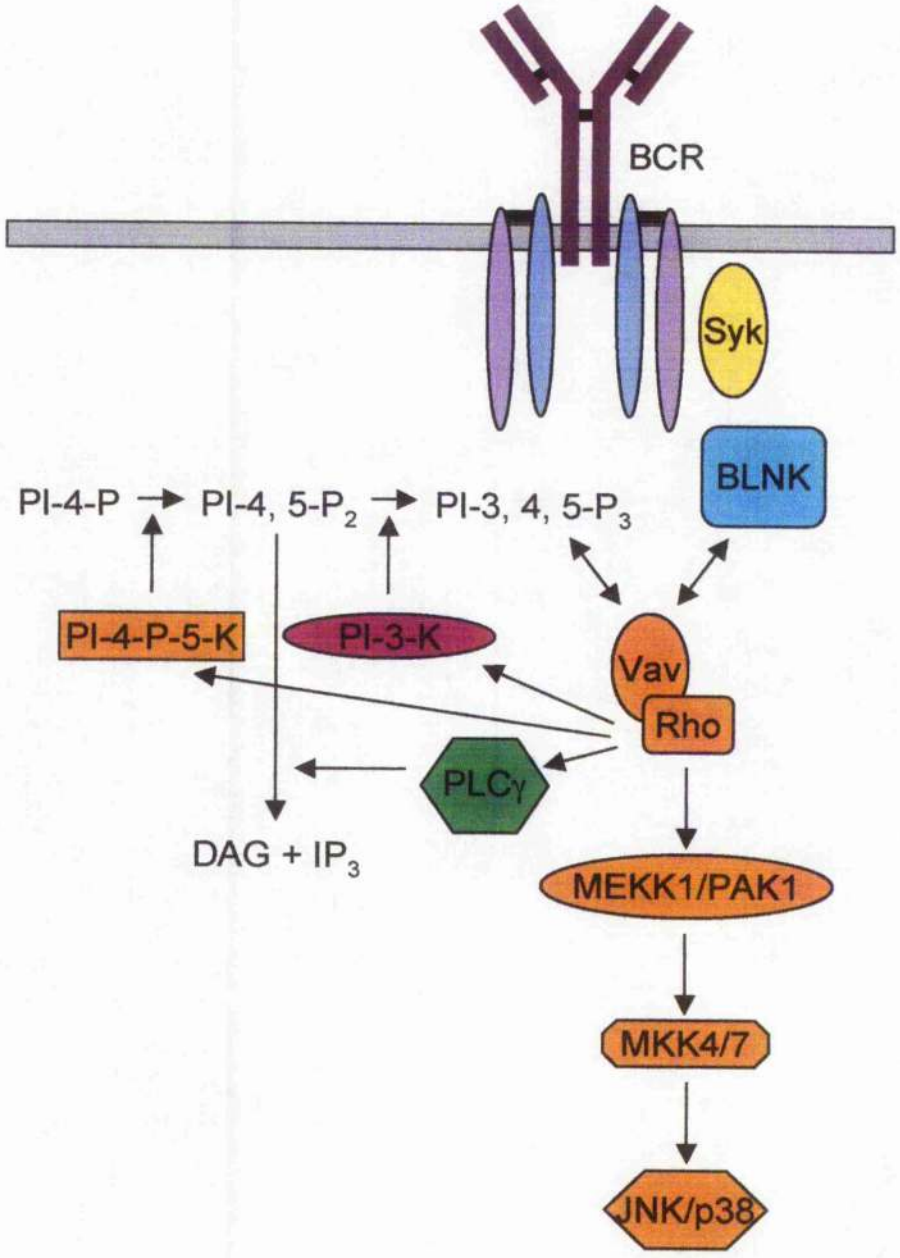

BCR
Syk
BLNK
PI-4-P
PI-4, 5-P2
PI-3, 4, 5-P3
PI-4-P-5-K
PI-3-K
Vav
Rho
PLCγ
DAG + IP3
MEKK1/PAK1
MKK4/7
JNK/p38

Figure 1.17. The activation of Erk-MAPK downstream of the BCR complex. Various adaptors can couple the BCR complex to Grb-2/SOS. SOS is an activator of Ras, which in turn leads to the stimulation of Raf-1, MEK and Erk1/2. The adaptor protein Cbl can also couple the BCR to Erk via the recruitment of Crk/C3G. C3G is an activator of Rap-1, which can inhibit Raf-1 to suppress Erk1/2 activity or it can activate B-Raf to stimulate Erk-MAPK. Protein-to-protein interactions are represented by a line with an arrow head at both ends, the activation of signalling molecules is represented by a line with a single arrow head and the inhibition of signalling molecules is indicated by a line with a blunt end.

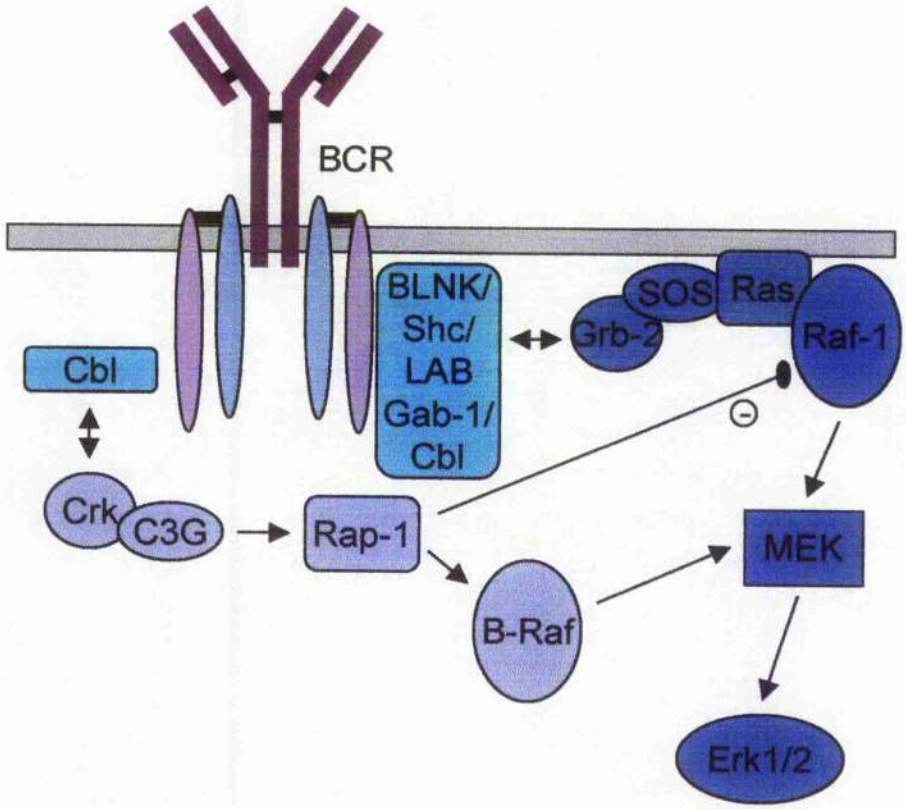

BCR
BLNK/
Shc/
LAB
Gab-1/
Cbl
SOS
Ras
Grb-2
Raf-1
Cbl
Crk
C3G
Rap-1
B-Raf
MEK
Erk1/2

Figure 1.18. Amplification of BCR-signalling by the co-receptor CD19. CD19 exists as a complex with CD81 and CD21. C3d-associated antigen simultaneously binds to CD21 and the BCR. Ligation of CD21 leads to the phosphorylation of tyrosine residues in the cytoplasmic region of CD19. The phospho-tyrosine residues of CD19 can then recruit and activate PI-3-K and Vav leading to the induction of downstream signalling molecules including Akt and Btk. CD19 amplifies BCR signalling and lowers the threshold for B cell activation.

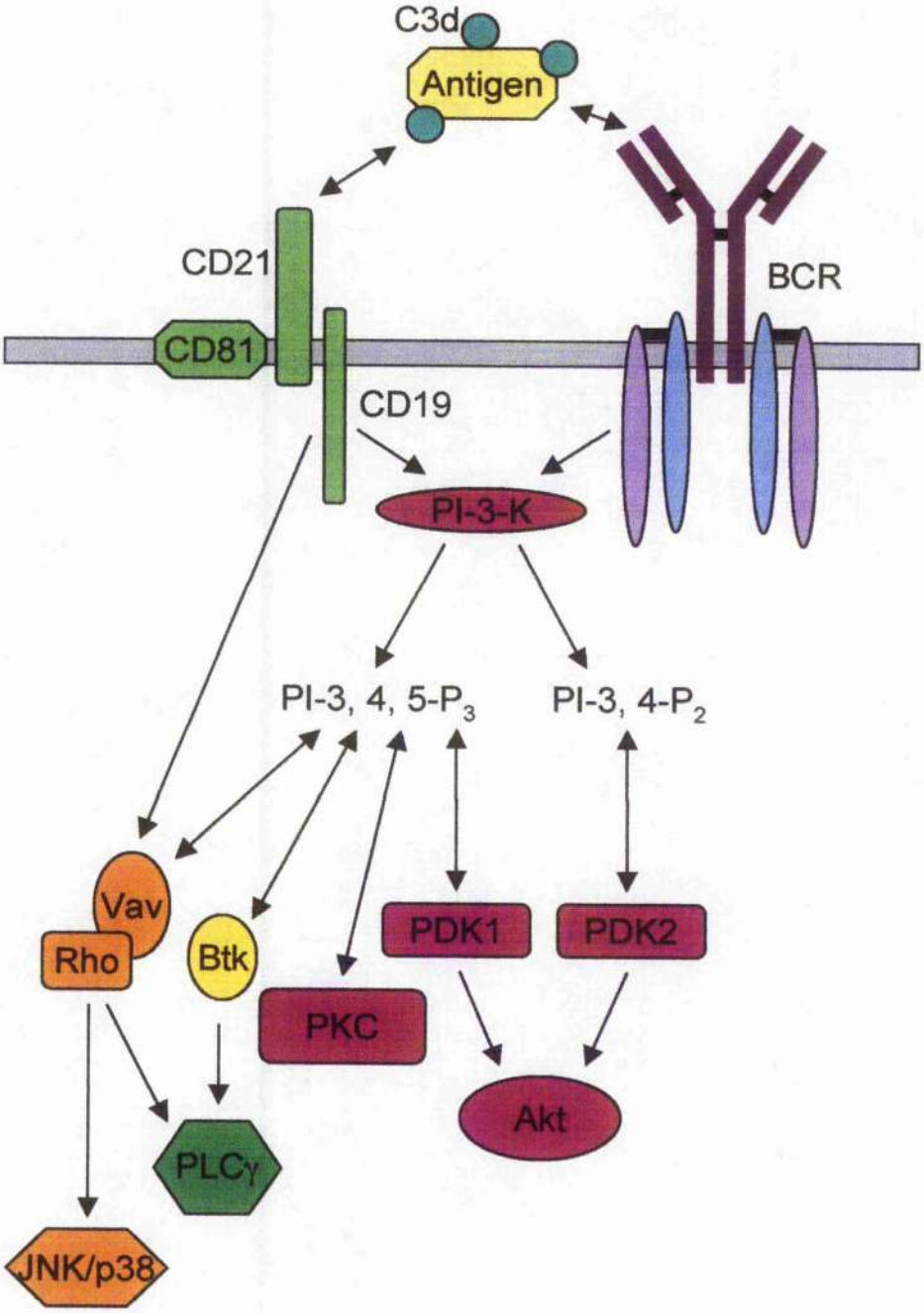

C3d
Antigen
CD21
CD81
CD19
BCR
PI-3-K
PI-3, 4, 5-P3
PI-3, 4-P2
Vav
Rho
Btk
PDK1
PDK2
PKC
Akt
PLCγ
JNK/p38

Figure 1.19. Inhibition of BCR signalling by FcγRIIB. FcγRIIB is a low affinity receptor for the Fc component of IgG therefore it can recognise antibody-bound antigen. Ligation of FcγRIIB causes Lyn to phosphorylate the single ITIM of the cytoplasmic region of FcγRIIB. The phospho-ITIM can recruit and activate the phosphatase SHIP. SHIP removes the 5' phosphate from PIP_3 leading to impaired recruitment and activation of PH domain-containing proteins. Furthermore, SHIP suppresses the BCR-mediated activation of MAPK by disrupting the association of Shc and Grb-2 and by recruiting and activating RasGAP via Dok-1. FcγRIIB can also impair the activation of Erk via the induction of Pac-1. Moreover, FcγRIIB induces the phosphatase PTEN to further impair PI-3-K signalling. FcγRIIB therefore suppresses the PI-3-K pathway and the Erk-MAPK cascade.

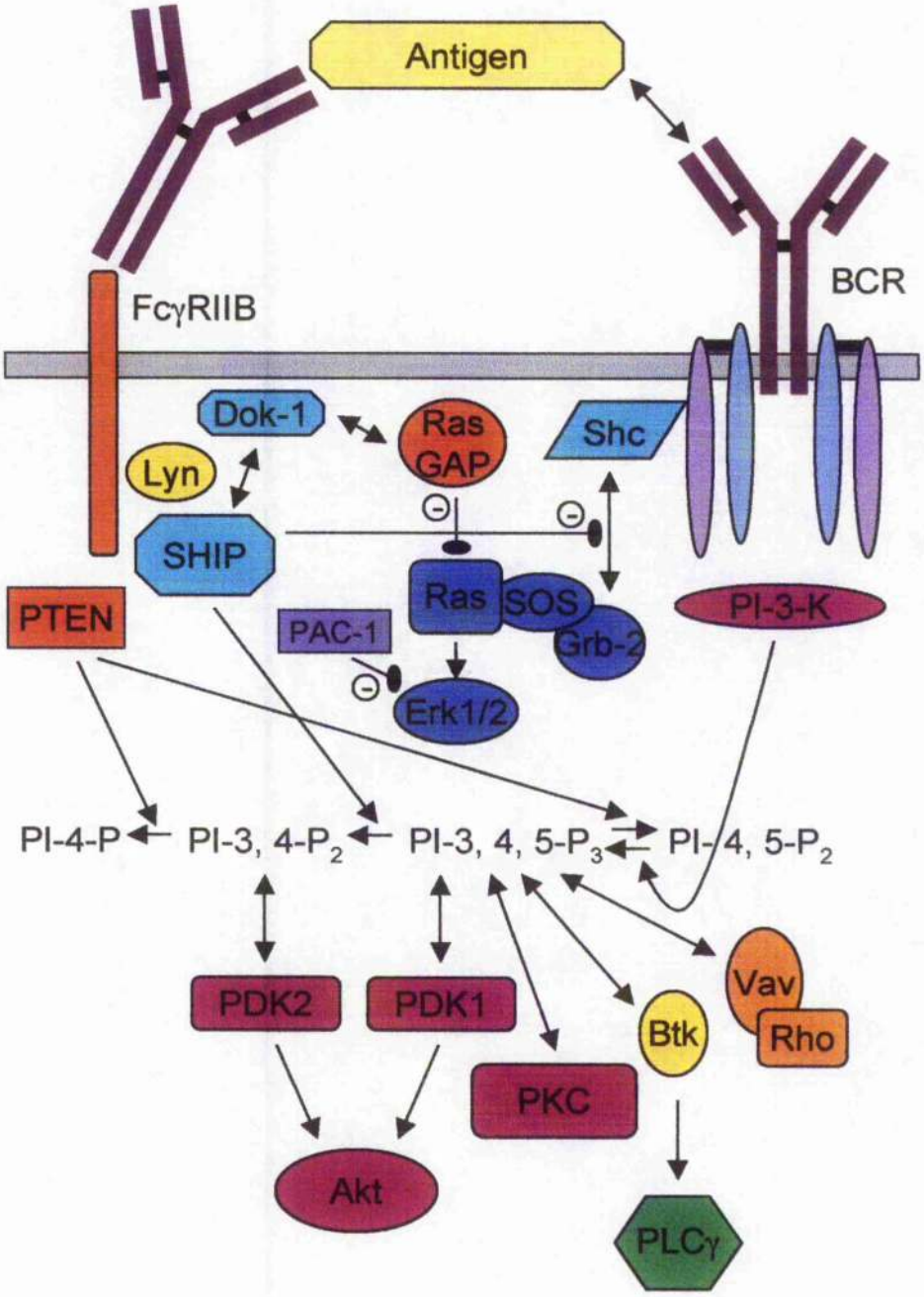

Antigen
BCR
FcγRIIB
Dok-1
Ras GAP
Shc
Lyn
SHIP
PTEN
Ras
SOS
Grb-2
PI-3-K
PAC-1
Erk1/2
PI-4-P
PI-3, 4-P2
PI-3, 4, 5-P3
PI-4, 5-P2
PDK2
PDK1
Vav
Rho
Btk
PKC
Akt
PLCγ

Figure 1.20. CD40 signalling in mature B lymphocytes. Ligation of CD40 induces the multimerisation of CD40-associated TRAFs including TRAFs 2, 3, 5 and 6. This leads to the activation of several intracellular signalling molecules including non-receptor tyrosine kinases, MAPK proteins, PI-3-K, PLCγ and NF-κB. These signalling molecules can regulate transcription factors, gene expression and the activity of signalling molecules to control various cellular functions including susceptibility to apoptosis.

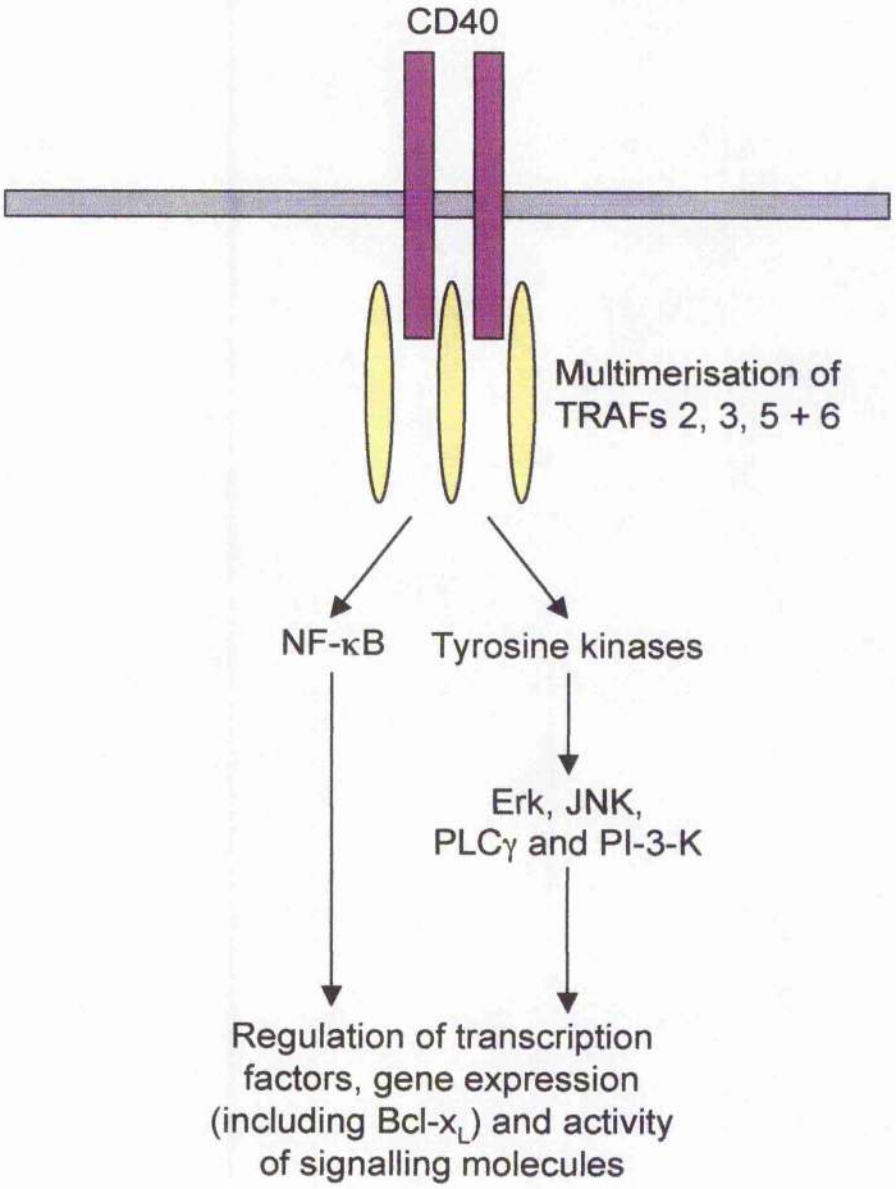

CD40
Multimerisation of
TRAFs 2, 3, 5 + 6
NF-κB
Tyrosine kinases
Erk, JNK,
PLCγ and PI-3-K
Regulation of transcription
factors, gene expression
(including Bcl-x_L) and activity
of signalling molecules

Table 1.1. Comparison of WEHI-231 cell line, primary immature B cells and primary transitional B cells. WEHI-231 cells are widely used as a model for clonal deletion of immature/transitional B cells. Indeed, WEHI-231 cells have very similar surface expression and behavioural characteristics to immature and transitional B cells.

Comparison of WEHI-231 cell line, primary immature B cells and primary transitional B cells.

	WEHI-231 cells	Immature B cells	Transitional B cells
IgM	+	+	+
IgD	+/-	-	+/-
CD40	+	+	+
CD23	+/-	-	+/-
Fas	+/-	-	+
MHC II	+/-	+/-	+/-
Receptor editing	-	+	-
Proliferation	+	-	-
BCR-driven apoptosis	+	+	+
CD40-dependent rescue	+	+	+

Figure 1.21. CD40 signalling in immature B lymphocytes. Ligation of CD40 induces the multimerisation of CD40-associated TRAFs. This leads to the activation of several intracellular signalling molecules including non-receptor tyrosine kinases, MAPK proteins, PI-3-K and PLCγ. These signalling molecules can then promote B cell survival by upregulating the pro-survival members of the Bcl-2-family and by activating NF-κB. CD40-mediated signals also promote proliferation of immature B cells by stimulating cyclin D kinases Cdk4 and Cdk6, leading to the phosphorylation and inactivation of retinoblastoma. This allows the activation of E2F and the induction of proliferation.

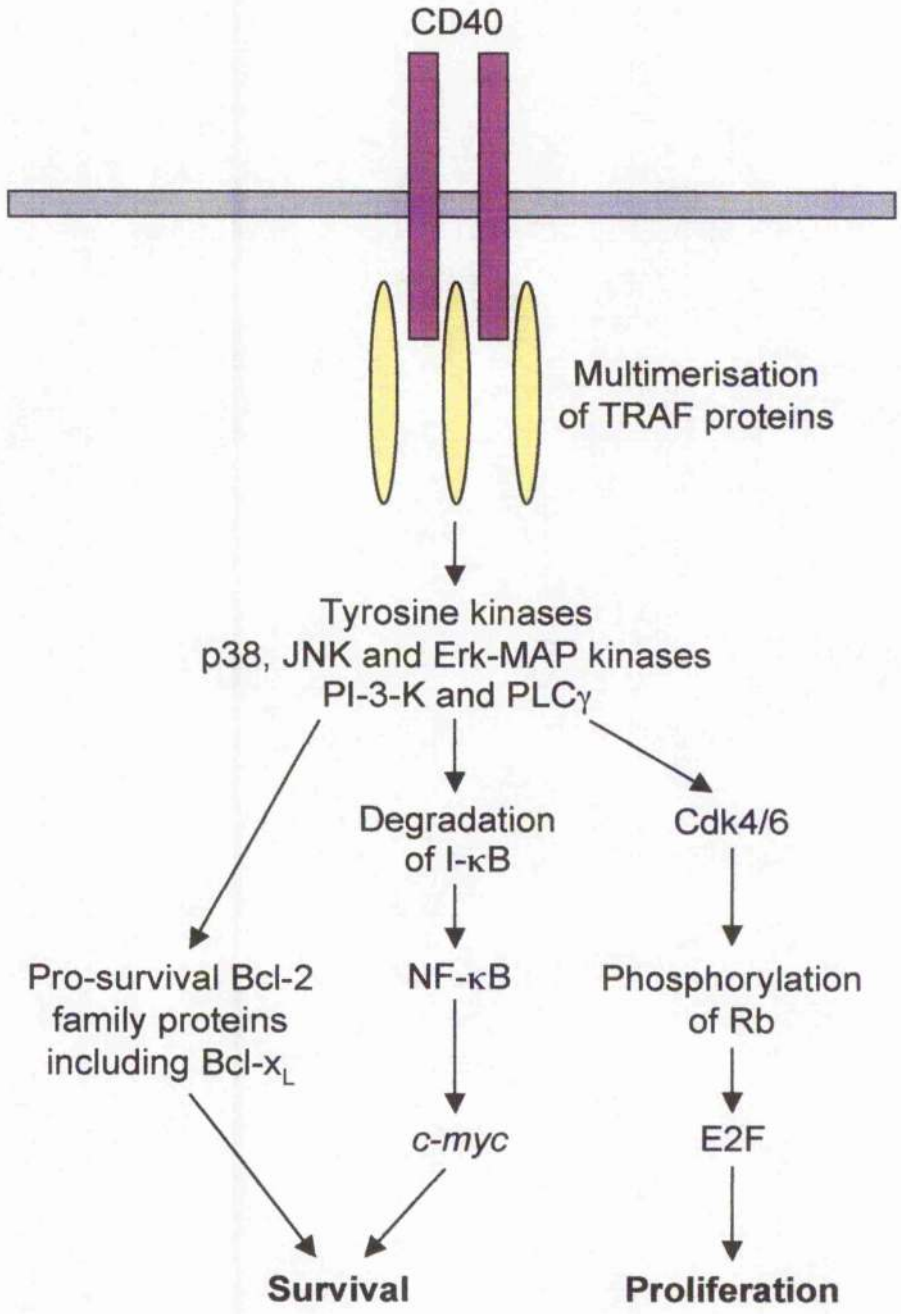
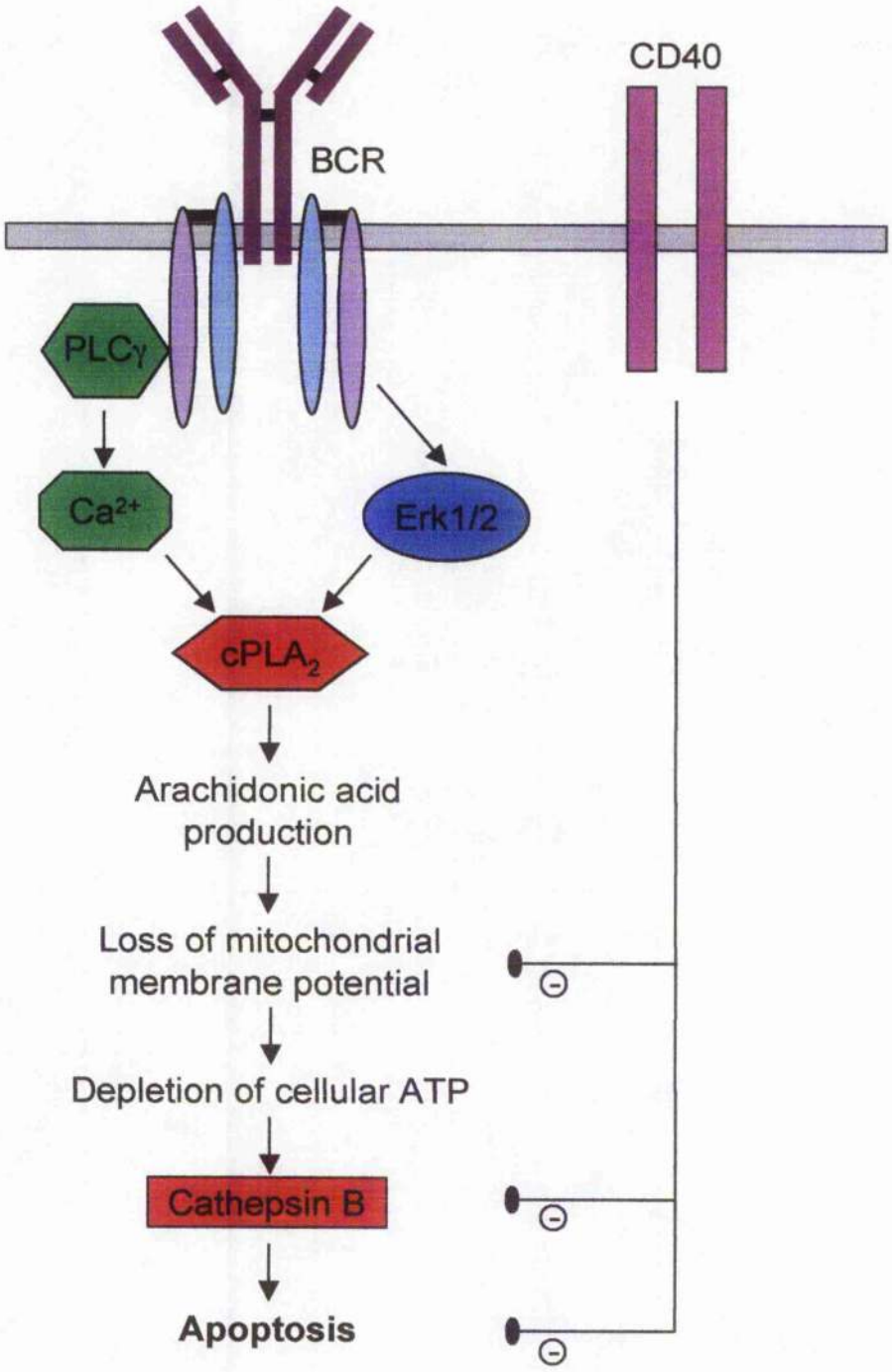

CD40
Multimerisation
of TRAF proteins
Tyrosine kinases
p38, JNK and Erk-MAP kinases
PI-3-K and PLCγ
Degradation
of I-κB
Cdk4/6
Pro-survival Bcl-2
family proteins
including Bcl-x$_L$
NF-κB
Phosphorylation
of Rb
c-myc
E2F
Survival
Proliferation

Figure 1.22. Induction of BCR-driven apoptosis in immature WEHI-231 cells. Ligation of the BCR on immature B cells leads to the activation of Erk-MAPK and calcium mobilisation. Calcium recruits $cPLA_2$ to the plasma membrane, where it can be activated by phosphorylation, catalysed by Erk1/2. Cytosolic PLA_2 hydrolyses membrane lipids to form arachidonic acid. Arachidonic acid induces apoptosis by disrupting the mitochondrial membrane potential, leading to a depletion of cellular ATP and the activation of cathepsin B. Cathepsin B is an executioner protease that mediates death of the immature B lymphocyte. Immature B cells can be protected from BCR-driven apoptosis by co-ligation of CD40. Stimulation of CD40 enables the cells to retain a normal mitochondrial membrane potential and consequently cellular ATP levels are not depleted and the cell does not undergo apoptosis.


CD40
BCR
PLCγ
Ca2+
Erk1/2
cPLA2
Arachidonic acid production
Loss of mitochondrial membrane potential
Depletion of cellular ATP
Cathepsin B
Apoptosis

2.1 Antibodies

2.1.1 Primary antibodies

Protein[1]	Supplier	Host	Clone or ID	Dilution	Usage[2]
Akt	New England Biolabs	Rabbit	9272	1:1000	WB
Phospho-Akt (pS^{473})	New England Biolabs	Rabbit	9271	1:1000	WB
Phospho-Akt (pT^{308})	New England Biolabs	Rabbit	9275	1:1000	WB
Bcl-$x_{L/S}$[3]	Santa Cruz Biotechnology	Rabbit	S-18	1:500 of 200 µg/ml stock	WB
CD40[5]	-	Rat	FGK 45	-	Stimulation
Erk1/2-MAPK[4]	New England Biolabs	Rabbit	9102	1:1000	WB IP
Phospho-Erk1/2 MAPK (pT^{202}/pY^{204})[3]	New England Biolabs	Rabbit	9101	1:1000	WB
Phospho-Erk1/2 MAPK (pT^{183})	Promega	Rabbit	V8081	1:4000	WB
Phospho-Erk1/2 MAPK (pY^{204})[3]	Santa Cruz Biotechnology	Rabbit	E-4	1:500 of 200 µg/ml stock	WB
Ig mu chain[5]	-	Rat	B7.6	-	Stimulation
MEK1/2[3]	New England Biolabs	Rabbit	9122	1:1000	WB IP

Protein	Supplier	Host	Clone or ID	Dilution	Usage
Phospho-MEK1/2 (pS^{217}/pS^{221})[3]	New England Biolabs	Rabbit	9121	1:1000	WB
PAC-1[3]	Santa Cruz Biotechnology	Goat	C-20	1:500 of 200 µg/ml stock	WB
SHP-1[3]	Santa Cruz Biotechnology	Rabbit	C-19	1:500 of 200 µg/ml stock	WB
SHP-2[3]	Santa Cruz Biotechnology	Rabbit	C-18	1:500 of 200 µg/ml stock	WB

[1] Antibodies were originally raised against the murine form of the protein unless otherwise indicated.

[2] Abbreviations: WB, Western blotting; IP, immunoprecipitation

[3] Antibodies were raised against the human form of the protein but are crossreactive with the mouse protein.

[4] Antibodies were raised against the rat form of the protein but are crossreactive with the mouse protein.

[5] Antibodies were from hybridoma cell lines, see antibody purification, section 2.5.

2.1.2 Secondary antibodies

Protein	Conjugate	Supplier	Host	Clone or ID	Dilution
Goat IgG	HRP	Jackson ImmunoResearch	Donkey	705-035-147	1:10000 of 0.8 mg/ml stock
Rabbit IgG	HRP	New England Biolabs	Goat	7074	1:2000

2.2 All other reagents and chemicals

Reagent	Supplier
Agarose	Roche Diagnostics Inc.
Antipain	Sigma-Aldrich Laboratories
Akt control cell extracts	New England Biolabs
Bovine serum albumin	Sigma-Aldrich Laboratories
Bromophenol blue	ICN Biomedicals Inc.
Chemiblocker	Chemicon International
Chymostatin	Sigma-Aldrich Laboratories
Disodium hydrogen orthophosphate (Na_2HPO_4)	BDH Laboratory Supplies
EDTA	Fisher Scientific
Effectine transfection reagent	Qiagen
Ethyl-3,4-dihydroxybenzylidenecyanoacetate (LOI)	Alexis Biochemicals
Fetal calf serum	Gibco-BRL
G418 antibiotic	Promega
Glycerol	BDH Laboratory Supplies
Glycine	Fisher Scientific
Hydrogen peroxide	Sigma-Aldrich Laboratories
Kodak X-ray film	Wolf Laboratories Ltd.
Indomethacin	ICN Biomedicals
Leupeptin	Sigma-Aldrich Laboratories
L-glutamine	Gibco-BRL
Luminol (3-aminophthalhydrazide)	Sigma-Aldrich Laboratories
MEK assay kit	TCS Biologicals
MEM non-essential amino acids	Gibco-BRL
2-Mercaptoethanol (for tissue culture)	Gibco-BRL
2-Mercaptoethanol (for all other uses)	BDH Laboratory Supplies
Methanol	BDH Laboratory Supplies
MicroBCA protein assay	Pierce
N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398)	Calbiochem

Reagent	Supplier
N, N-Dimethyl-D-erythro-sphingosine (DMS)	Alexis Biochemicals
Nitrocellulose	Amersham International
NP_{40}	Fisons Scientific Inc.
NuPAGE buffers and reagents	Invitrogen
p-Coumaric acid (4-hydroxycinnamic acid)	Sigma-Aldrich Laboratories
Penicillin	Gibco-BRL
Pepstatin A	Sigma-Aldrich Laboratories
PGE_2 competitive binding immunoassay kit	Cayman Chemical
Phenylmethylsulfonylfluoride	Sigma-Aldrich Laboratories
p42 MAP kinase recombinant control proteins	New England Biolabs
Polybrene	Sigma-Aldrich Laboratories
Potassium chloride (KCl)	BDH Laboratory Supplies
Potassium dihydrogen orthophosphate (KH_2PO_4)	Fisher Scientific
Propidium iodide (PI)	Calbiochem
Protein G-sepharose beads	Sigma-Aldrich Laboratories
RNase A	Sigma-Aldrich Laboratories
RPMI-1640 medium	Gibco-BRL
Sodium azide	Sigma-Aldrich Laboratories
Sodium chloride (NaCl)	Sigma-Aldrich Laboratories
Sodium dodecyl sulphate (SDS)	BDH Laboratory Supplies
Sodium deoxycholate	Sigma-Aldrich Laboratories
Sodium Dihydrogen orthophosphate (NaH_2PO_4)	BDH Laboratory Supplies
Sodium orthovanadate	Sigma-Aldrich Laboratories
Sodium pyruvate	Gibco-BRL
Sodium (tri) citrate	Sigma-Aldrich Laboratories
Streptomycin	Gibco-BRL
[6-^{3}H] thymidine (5 Ci/mmol)	Amersham International
Tris	Sigma-Aldrich Laboratories
Triton-X-100	Sigma-Aldrich Laboratories
Tween-20	Sigma-Aldrich Laboratories

2.3 Company and distributor addresses

Alexis Biochemicals, Nottingham, UK
Amersham International, Buckinghamshire, UK
BDH (VWR) Laboratory Supplies, Lutterworth, Leics, UK
BioRad, Hemel Hempstead, Hertfordshire, UK
Calbiochem, Cambridge, MA, USA
Cayman Chemical, Ann Arbor, MI, USA
Chemicon International, Temecula, CA, USA
Fisher Scientific, Loughborough, Leics, UK
Fisons Scientific Inc., Loughborough, Leics, UK
ICN Biomedicals, Aurora, Ohio, USA
Gibco-BRL, Paisley, Scotland, UK
Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA
Invitrogen, Paisley, UK
New England Biolabs, Hitchin, Herts, UK
Pierce, Rockford, IL, USA
Promega, Madison, WI, USA
Qiagen, Valencia, CA, USA
Roche Diagnostics Inc., Indianapolis, IN, USA
Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Sigma-Aldrich Laboratories, Poole, Dorset, UK
TCS Biologicals, Botolph Claydon, Buckinghamshire, UK
Wallac, Turku, Finland
Wolf Laboratories Ltd, Pocklington, York, UK

2.4 Cells

2.4.1 WEHI-231 immature B cells

The murine B cell lymphoma, WEHI-231 was cultured in RPMI-1640 medium supplemented with 5% (v/v) fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol (50 µM) (RPMI complete medium) at 37°C in 5% (v/v) CO_2 atmosphere at 95% humidity. All cell culture reagents were of the highest quality available.

2.4.2 Generation of Bcl-x_L WEHI-231 cells

WEHI-231.7 JM cells were transfected by electroporation with the pSFFV-Neo plasmid containing either the human *bcl-x_L* gene (Bcl-x_L WEHI-231) or no insert as control (Neo WEHI-231) (291). Stable transfectants were selected for the acquisition of neomycin resistance by growth in the presence of the antibiotic G418 (1 mg/ml) and were a gift from Dr. C. B. Thompson (University of Pennsylvania). Overexpression of Bcl-x_L was confirmed by Western blotting using an anti-Bcl-$x_{L/S}$ antibody.

2.4.3 Retroviral transfection of WEHI-231 cells

Retroviral constructs were generated by subcloning the gene of interest into the retroviral vector pMXI-egfp, 5' to the internal ribosomal entry site and green fluorescence protein (GFP) was encoded 3' to this site. Amphotropic phoenix cells were used as packaging cells for the retroviral transfection system. Pheonix cells were transfected using the effectine transfection reagent as per manufacturers instructions (Qiagen) with pMXI-egfp vectors containing no construct (empty vector control, pMXI-egfp), SHIP-CI construct, SHIP-SH2 construct or Dok-PH/PTB construct (see table 2.1A for details of constructs). Two days after transfection, the supernatants were collected, filtered (0.22 µm) and polybrene was added to a final concentration of 4 µg/ml. WEHI-231 B cells (5×10^5 cells/ml) were centrifuged in 12-well plates to promote adherence. Viral supernatants were added to adherent cells followed by centrifugation at 1,000 g for 2 hr at 32°C. Cells were then incubated at 32°C overnight before transferring them into 25 cm^3 flasks for expansion. Following expansion, cells were sorted for GFP-expression (Mo-Flo, Cytomation, Fort Collins, CO). Successful transfectants were a gift from Dr. S. B. Gauld (National Jewish Medical and Research Center, Denver, CO).

2.4.4 Transfection of WEHI-231 cells by electroporation

WEHI-231 cells (5×10^6 cells) undergoing logarithmic growth were washed and resuspended (2×10^7 cells/ml) in electroporation media (RPMI-1640 with 20% FCS). Linearised DNA (5 µg), recovered from an agarose gel, was chilled on ice for 5 min in an electroporation cuvette. WEHI-231 cells (5×10^6) were

added to the cuvette, gently mixed and chilled for 10 min on ice. Cells were electroporated at 960 µFarads at 220 Volts and were then chilled on ice for a further 10 min. Cells were then removed from the cuvette and were grown in RPMI complete medium for 48 hr at 37°C in 5% (v/v) CO_2 atmosphere at 95% humidity before selecting for successful transfectants using the antibiotic G418 (500 µg/ml). Electroporation of WEHI-231 B cells was used to generate several mutant WEHI-231 cells (table 2.1B) including an empty vector control, pcDNA3.1. Transfection of WEHI-231 cells by electroporation was performed in collaboration with Derek Blair, in this laboratory.

2.5 Purification of antibodies from hybridoma cell lines

2.5.1 Purification of anti-CD40 antibody

Anti-CD40 was purified from the FGK 45 hybridoma. FGK 45 cells were cultured in RPMI complete medium and the antibody-rich tissue culture supernatant was collected. Immunoglobulin was precipitated from the supernatant by mixing equal volumes of saturated ammonium sulphate solution and tissue culture supernatant (FGK 45) overnight at 4°C. The precipitated immunoglobulin was isolated by centrifugation and dialysed exhaustively in phosphate buffered saline (PBS) solution (160 mM NaCl, 3.2 mM KCl, 9.7 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.2). A column of 1 ml protein G-sepharose beads (immunoglobulin capacity >20 mg/ml) was washed with binding buffer (0.2 M $NaH_2PO_4.2H_2O$, 0.2 M $Na_2HPO_4.2H_2O$, pH 7.0) and the dialysed immunoglobulin solution was run through the column at 4°C. The column was washed with binding buffer and the immunoglobulin was eluted in 1 ml fractions using elution buffer (0.1 M glycine, pH 2.7). The protein concentration of each 1 ml fraction was determined using spectrophotometry to measure the absorbance at 280 nm (an optical density of 1.4 was approximately equivalent to 1 mg/ml of protein). The most protein-rich fractions were pooled and dialysed exhaustively in PBS. The resultant anti-CD40 was filter sterilised and stored at -20°C.

2.5.2 Purification of anti-Ig (anti-mouse μ-chain) antibody

Anti-Ig was purified from the B7.6 hybridoma. B7.6 cells were cultured in RPMI complete medium and the antibody-rich tissue culture supernatant was collected. Anti-Ig was purified using a protein G-sepharose column. The column of 1 ml protein G-sepharose beads (immunoglobulin capacity >20 mg/ml) was washed with binding buffer (0.2 M $NaH_2PO_4.2H_2O$, 0.2 M $Na_2HPO_4.2H_2O$, pH 7.0) then tissue culture supernatant (B7.6) was run through the column at 4°C. The column was washed with binding buffer then the immunoglobulin was eluted in 1 ml fractions using elution buffer (0.1 M glycine, pH 2.7). The protein concentration of each 1 ml fraction was determined using spectrophotometry to measure the absorbance at 280 nm (an optical density of 1.4 was approximately equivalent to 1 mg/ml of protein). The most protein-rich fractions were pooled and dialysed exhaustively in PBS. The resultant anti-Ig was filter sterilised and stored at -20°C.

2.6 Measurement of DNA synthesis

Proliferation of WEHI-231 cells was assessed by measurement of DNA synthesis. WEHI-231 cells (1×10^4 cells/well) were cultured in triplicate in 96-well round-bottomed microtitre plates in RPMI complete medium supplemented with 1% (v/v) MEM non-essential amino acids and sodium pyruvate (1 mM), in the presence of appropriate stimuli in a total volume of 200 μl. Cells were cultured at 37°C in a 5% (v/v) CO_2 atmosphere with 95% humidity for up to 72 hr. [6-^{3}H] thymidine (0.5 μCi/well) was added 4 hr prior to harvesting onto glass fibre filter mats using a betaplate 96-well automated cell harvester (Amersham International). Incorporated [^{3}H] thymidine was determined by liquid scintillation counting (Beta-plate counter, Wallac) and is represented as mean cpm incorporated ± standard deviation, n=3 or mean cpm of several experiments ± standard error of the mean (sem).

2.7 Stimulation of cells and preparation of whole cell lysates

WEHI-231 cells (1×10^7 cells) were stimulated as required in RPMI complete medium at 37°C in 5% (v/v) CO_2 atmosphere at 95% humidity. Reactions were terminated by centrifugation at 500 g for 7 min at 4°C followed

by resuspension of cells in ice cold lysis buffer (150 mM sodium chloride, 50 mM Tris pH 7.5, 2 % (v/v) NP_{40}, 0.25 % (w/v) sodium deoxycholate and 1 mM EDTA pH 8 with freshly added 10 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonylfluoride, 10 µg/ml chymostatin, 10 µg/ml leupeptin, 10 µg/ml antipain and 10 µg/ml pepstatin A). Lysates were solubilised for 30 min on ice followed by centrifugation at 13,000 g for 10 min at 4°C. The resultant supernatants (whole cell lysates) were transferred to fresh tubes and were stored at -20°C.

2.8 Immunoprecipitation

Whole cell lysates (100 µg total protein) were precleared with 5 µl protein G-sepharose beads in lysis buffer (50% slurry) for 1 hr at 4°C with constant rotation. Lysates were centrifuged at 13,000 g for 10 min at 4°C and the supernatants were transferred to a fresh tube. The proteins of interest were immunoprecipitated from the precleared lysates by the addition of an appropriate antibody (1-2 µg) for 5 hr at 4°C with constant rotation. The protein/antibody immunocomplex was captured using 10 µl protein G-sepharose beads in lysis buffer (50% slurry) overnight at 4°C with constant rotation. Immunocomplexes were isolated by centrifugation at 13,000 g for 10 min at 4°C and washed 3 times in lysis buffer. Immunocomplexes were resuspended in 30 µl 2 x sample loading buffer (100 mM Tris pH 6.8, 20% (v/v) glycerol, 0.2% (w/v) SDS and 4% (v/v) bromophenol blue with 5% 2-mercaptoethanol), boiled for 10 min at 100°C, pulse centrifuged and used in gel electrophoresis or stored at -20°C.

2.9 Gel electrophoresis and transfer

Equal protein loadings of whole cell lysates (determined by MicroBCA protein assay (Pierce)) or immunoprecipitates were resolved by gel electrophoresis. One of two gel systems was used. In the first system, lysates or immunoprecipitates were denatured in 2 x sample loading buffer (100 mM Tris pH 6.8, 20% (v/v) glycerol, 0.2% (w/v) SDS and 4% (v/v) bromophenol blue with 5% 2-mercaptoethanol) by heating for 10 min at 100°C. Following pulse centrifugation, the samples were resolved using 10% Tris-HCl pre-cast gels (BioRad) with electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1%

(w/v) SDS) in a BioRad gel electrophoresis kit. The resolved proteins were transferred onto nitrocellulose (Amersham) using transfer buffer (42 mM Tris, 39 mM glycine, 0.38% (w/v) SDS and 20% (v/v) methanol).

The second system of gel electrophoresis used the XCell *SureLock* Mini-Cell kit with NuPAGE Novex high-performance pre-cast Bis-Tris gels and NuPAGE buffers and reagents (all supplied by Invitrogen) as directed by the manufacturers instructions. In brief, equal protein loadings of lysates were diluted in lysis buffer to a constant final volume and the appropriate volume of 4 x NuPAGE LDS sample buffer and 10 x NuPAGE reducing agent were added prior to heating samples to 70°C for 10 min. Samples were resolved using NuPAGE Bis-Tris gels (10%) with NuPAGE MOPS running buffer (supplemented with NuPAGE antioxidant) and transferred onto nitrocellulose (Amersham) using NuPAGE transfer buffer with 20% (v/v) methanol.

2.10 Western blot analysis

Following transfer, nitrocellulose membranes were washed once in Tris buffered saline (TBS) (0.5 M NaCl and 20 mM Tris pH7.5) with 0.1% (v/v) Tween-20 (TBS/Tween) and blocked for 1 hr in TBS/Tween with either 5% non-fat milk or 5% bovine serum albumin (BSA). Alternatively, blots that were to be probed with an antibody generated in a goat were blocked for 1 hr in a 1 in 4 dilution of a non-animal blocking reagent, Chemiblocker (Chemicon International). Membranes were then incubated with the appropriate primary detection antibody overnight at 4°C. All antibodies were diluted in TBS/Tween with either 5% non-fat milk, 5% BSA or 1 in 4 dilution of ChemiBlocker. Following incubation with primary antibody nitrocellulose membranes were washed (6 x 5 minutes) with TBS/Tween and incubated in the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hr at room temperature. Nitrocellulose membranes were then washed (10 x 10 minutes) with TBS/Tween and protein bands were visualised using the ECL detection system. Nitrocellulose membranes were incubated in a mixture of equal volumes of ECL solution A (2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris pH8.5) and ECL solution B (0.002% hydrogen peroxide and 100 mM Tris pH8.5) for 1 min before exposing membranes to Kodak X-Ray film.

Nitrocellulose membranes were sometimes stripped and re-probed with a new primary antibody. Membranes were stripped at room temperature for 1-3 hr (depending on the strength of the previous Western blot signal) in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris pH6.7). Nitrocellulose membranes were washed thoroughly in TBS/Tween before re-starting the Western blotting protocol.

2.11 Dot blot analysis

Equal volumes of immunoprecipitates were heated for 10 min at 100°C followed by transfer onto nitrocellulose using a slot blotter (BioRad). Nitrocellulose membranes were washed once in TBS/Tween before being used in the Western blotting protocol (described in section 2.10).

2.12 Flow cytometry

2.12.1 Flow cytometry analysis of DNA content and cell cycle analysis

WEHI-231 cells (5 x 10^5 cells/well) were cultured in RPMI complete medium supplemented with 1% (v/v) MEM non-essential amino acids and sodium pyruvate (1 mM), in the presence of appropriate stimuli. Cells were cultured at 37°C in a 5% (v/v) CO_2 atmosphere with 95% humidity for up to 48 hr. WEHI-231 cells (5 x 10^5 cells) were harvested and washed twice in ice-cold FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Cells were resuspended in 100 µl propidium iodide (PI) stain (0.1% (w/v) sodium (tri) citrate, 0.1% (v/v) triton-X-100, 50 µg/ml propidium iodide and 200 µg/ml RNase A) for 45 min at room temperature. Cells were passed through nitex and analysed for PI fluorescence on a FACScalibur™ (Becton Dickinson) using CELLQuest™ software (Becton Dickinson).

Cell cycle analysis was used to determine the percentage of cells in the different phases of the cell cycle: sub-diploid (apoptotic), G0/G1, S phase or G2/M (figure 1.6). PI fluorescence was measured using both FL3 (linear scale) and FL2 (logarithmic scale) channels. When using FL3, the voltage was adjusted until the large G0/G1 peak was at 200-300 units. This voltage was kept constant whilst the data from any one experiment was acquired. Data was analysed by setting gates (figure 2.1A(i)). The G0/G1 peak represents 2N DNA

(50 units), the centre of this peak was identified and a gate was set to span 25 units either side of the centre point. The 2N DNA peak was doubled (representing 4N DNA) and markers were set 50 units either side of this point to form the G2/M gate. The S phase gate represents the cells between G0/G1 and G2/M phases. Cells located before the G0/G1 peak are the sub-diploid (apoptotic) cells. The sub-diploid peak starts at 5 units in order to eliminate non-specific fluorescence, as indicated in figure 2.1A(ii) in which cells were stained with a stain solution that lacked PI but were analysed in exactly the same way as PI-stained cells.

When using FL2, the voltage was adjusted until the large G0/G1 peak was at 10^2 units. This voltage was kept constant whilst the data from any one experiment was acquired. Data was analysed by setting gates (figure 2.1B(i)). The G0/G1 peak represents 2N DNA (30 units), the centre of this peak was identified and a gate was set to span 15 units either side of the centre point. The 2N DNA peak was doubled (representing 4N DNA) and markers were set 30 units either side of this point to form the G2/M gate. The S phase gate represents the cells between G0/G1 and G2/M phases. Cells located before the G0/G1 peak are the sub-diploid (apoptotic) cells. The sub-diploid peak starts at 1 unit in order to eliminate non-specific fluorescence, as indicated in figure 2.1B(ii) in which cells were stained with a stain solution that lacked PI but were analysed in exactly the same way as PI-stained cells.

2.12.2 Flow cytometry analysis of green fluorescence

Unstimulated retrovirally transfected cells (empty vector (pMXI-egfp), SHIP-CI, SHIP-SH2 and Dok-PH/PTB mutant WEHI-231 cells) were tested for successful transfection using FACS to detect the expression of the green fluorescent protein (GFP) marker. Cells (1×10^5) were washed twice in ice-cold FACS buffer, solubilised in FACS buffer with 7 µg/ml PI and passed through nitex. Green fluorescence was detected on a FACScalibur™ (Becton Dickinson) using CELLQuest™ software (Becton Dickinson).

Non-transfected WEHI-231 cells were used as a negative control since these cells do not contain the gene encoding GFP. GFP positive cells were rigorously gated by exclusion of the peak of cells observed in the negative control (figure 2.2). In the culture of empty vector control, pMXI-egfp, cells, 99%

were GFP positive but inclusion of a construct into the vector reduced the success rate of transfection. However, even with these rigorous gates, 71% of cells in the SHIP-CI culture were GFP positive and 83% of cells in the SHIP-SH2 culture expressed GFP. The Dok-PH/PTB WEHI-231 was the least homogeneous culture with 50% of the cells being GFP positive.

2.13 Measurement of intracellular prostaglandin E_2

Prostaglandin E_2 (PGE_2) concentration in WEHI-231 whole cell lysates was determined using a PGE_2 competitive binding immunoassay kit (Cayman Chemical) based on spectrophotometric detection (A_{405}), as described in the kit protocol. Briefly, whole cell lysates were prepared as described in section 2.7 except indomethacin (10 µg/ml), an inhibitor of cyclooxygenase, was added to the lysis buffer. Whole cell lysate (50 µg) was incubated with 50 µl PGE_2-Acetylcholinesterase conjugate plus 50 µl PGE_2 monoclonal antibody solution in a 96-well goat anti-mouse IgG coated plate for 2 hr at room temperature. The plate was washed three times before adding Ellman's reagent for 1 hr at room temperature. Ellman's reagent contains the substrate for acetylcholinesterase and forms a yellow-coloured product that absorbs strongly at 405-420 nm. The absorbance at 405 nm was measured using a microplate reader with wavelength correction set at 570 nm.

2.14 *In vitro* Erk-MAPK kinase (MEK) assay

Whole cell lysates were prepared from WEHI-231 B cells (1×10^7) stimulated with medium or anti-Ig (10 µg/ml) for up to 48 hr. MEK1/2-containing immune complexes were prepared from lysates (100 µg) using an anti-MEK1/2 antibody and protein G-sepharose beads. The MEK1/2 immunoprecipitates were assayed for MEK activity using the MEK Assay Kit (TCS Biologicals), and 0.5 U human activated MEK1 was used as the positive control sample. Briefly, the immune complex samples, or human activated MEK1, were incubated with assay buffer (20 mM MOPS pH7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT), Mg^{2+}-ATP mixture (75 mM magnesium chloride and 500 µM ATP in assay buffer) and 1 µg of inactive GST-p42 MAPK at 30°C for 30 min. Samples were then analysed by gel electrophoresis using pre-cast 10% Tris-HCl gels (BioRad). Western blotting

was performed using anti-phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}) (New England Biolabs) to detect phosphorylated GST-p42 MAPK (62 kD). The amount of GST-p42 MAPK is proportional to the activity of MEK1/2 in the sample. Blots were stripped and reprobed with anti-MEK1/2 (New England Biolabs) to check the loading of the gel.

Table 2.1. Constructs used in transfection of WEHI-231 cells. **(A)** Constructs used in retroviral transfection of WEHI-231 cells. Constructs were inserted into the pMXI-egfp vector and retroviral transfection was used to generate SHIP-CI, SHIP-SH2 and Dok-PH/PTB mutant WEHI-231 cells. Successful transfectants were selected based on their expression of GFP. **(B)** Constructs used in transfection by electroporation of WEHI-231 cells. Constructs were inserted into the pcDNA3.1 vector and electroporation was used to generate multiple PKC, Ras and MEKK3 mutant WEHI-231 cells. Successful transfectants were selected based on their resistance to G418 antibiotic.

(A) Constructs used in retroviral transfection of WEHI-231 cells

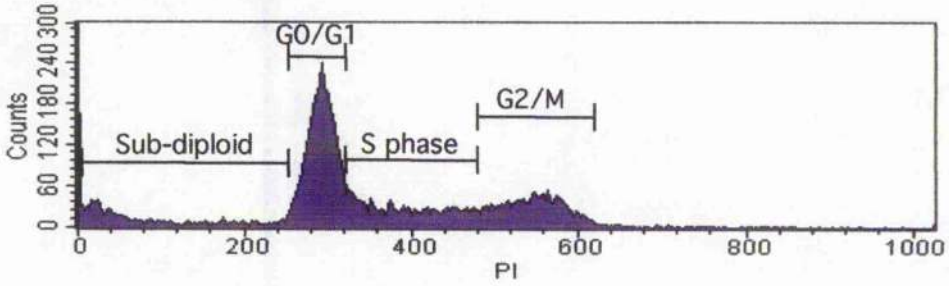
	Activity	**Mutation/ Coding Sequence**
SHIP-CI	phosphatase inactive	$D^{675}A$
SHIP-SH2	prevents SHIP-pITIM association	residues 1-114
Dok-PH/PTB	lacks pro/tyr-rich region	residues 1-258

(B) Constructs used in transfection by electroporation of WEHI-231 cells

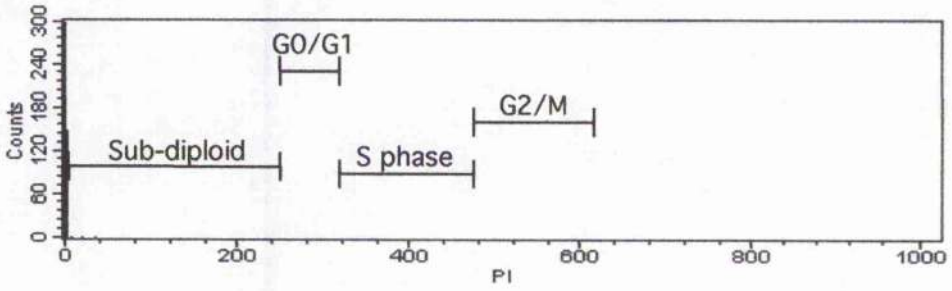
	Activity	**Mutation/ Coding Sequence**
PKCαKR	kinase inactive	residues 2-672 ($K^{368}R$)
PKCαCAT	constitutively active	residues 326-674
PKCδKR	kinase inactive	residues 2-674 ($K^{376}R$)
PKCδCAT	constitutively active	residues 334-674
PKCεKR	kinase inactive	residues 2-732 ($K^{437}R$)
PKCεCAT	constitutively active	residues 395-737
PKCζKR	kinase inactive	residues 2-592 ($K^{281}M$)
PKCζCAT	constitutively active	residues 239-592
$RasV^{12}$	constitutively active, interacts with all Ras effectors	V^{12}
$RasV^{12}S^{35}$	constitutively active, only interacts with Raf-1	$V^{12}S^{35}$
$RasV^{12}C^{40}$	constitutively active, only interacts with PI-3-K	$V^{12}C^{40}$
ΔMEKK3	constitutively active	residues 340-626

Figure 2.1. FACS analysis of PI-stained cells. WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr. Cells were prepared for FACS analysis by staining with either 50 µg/ml PI **(i)** or PI-free **(ii)** stain solution. FACS analysis with FL3 **(A)** or FL2 **(B)** fluorescence was used to analyse the populations of cells. Histograms show gates relating to apoptotic cells (sub-diploid DNA), cells in G0/G1 (2N DNA), S phase cells and cells in G2/M (4N DNA).

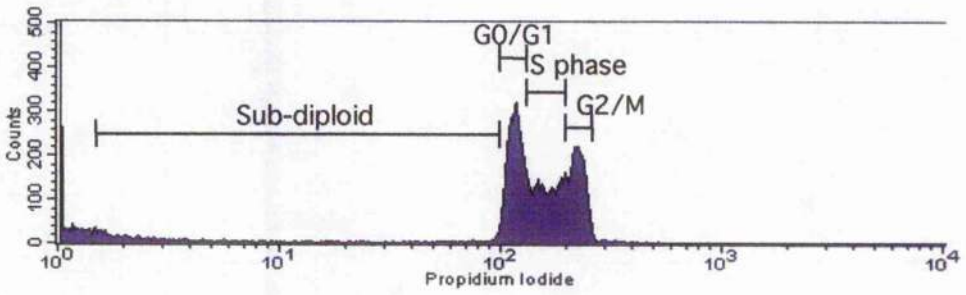
(A)(i) linear (FL3)



(ii)



(B)(i) log (FL2)



(ii)

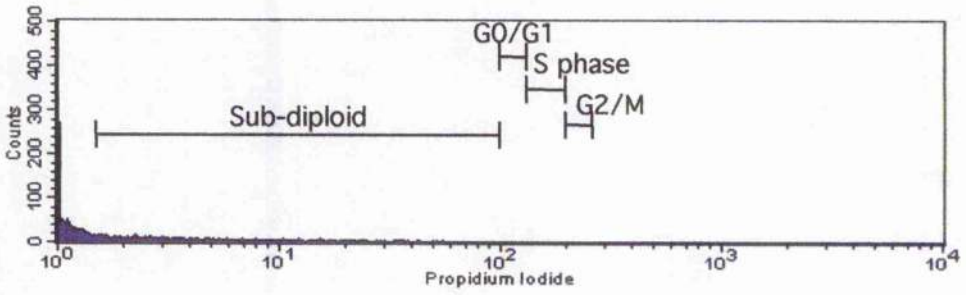
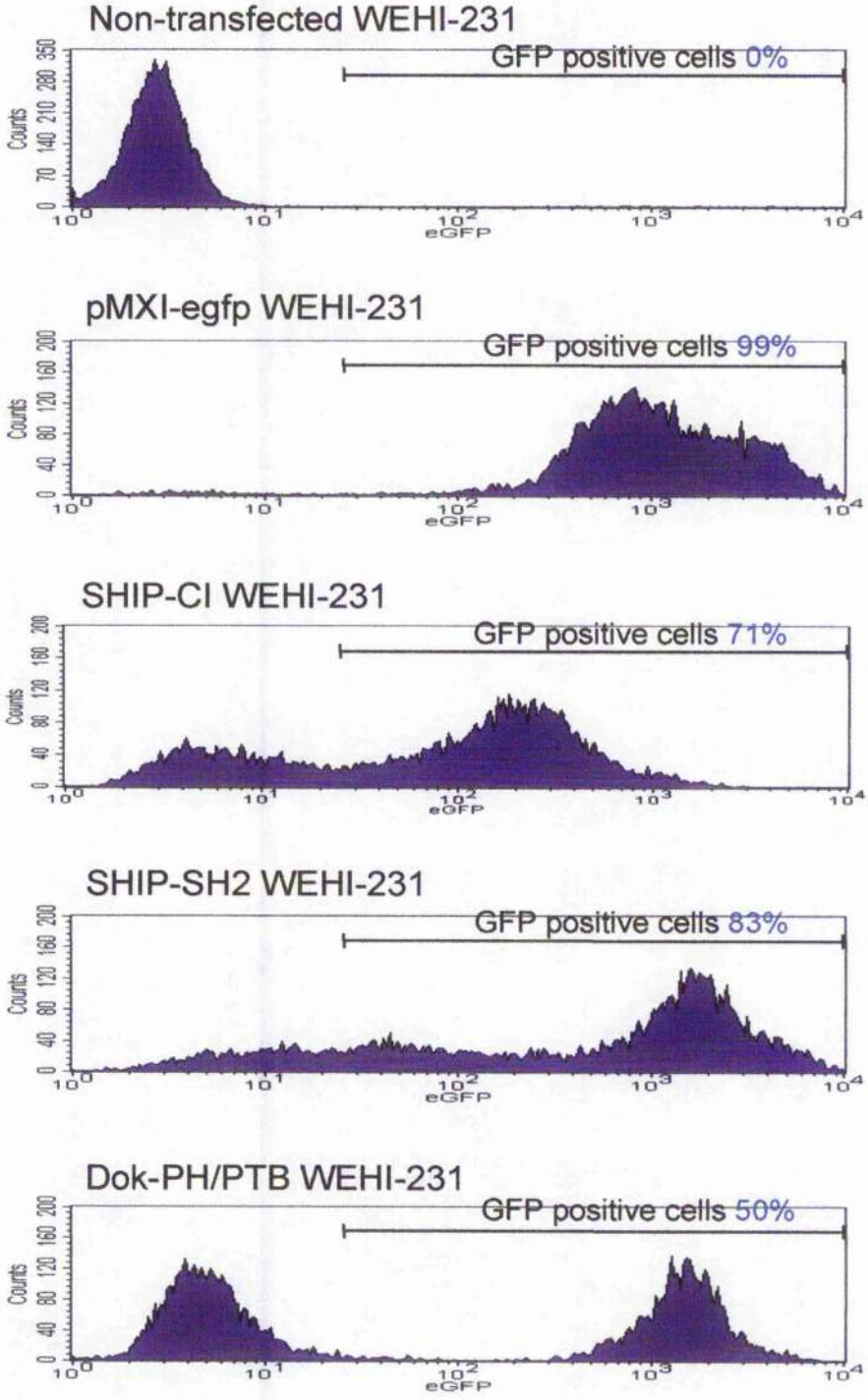


Figure 2.2. FACS analysis of retrovirally transfected WEHI-231 cells for expression of GFP. The expression of GFP in cultures of unstimulated retrovirally transfected WEHI-231 cells (1 x 10^5 cells/ml) was measured by FACS analysis. The pMXI-egfp vector contains the gene encoding GFP therefore successful transfectants express GFP whilst non-transfected cells are GFP-negative since they do not contain the gene encoding GFP. GFP-positive cells were gated by exclusion of the peak of cells observed in the non-transfected WEHI-231 culture.


Non-transfected WEHI-231
GFP positive cells 0%
pMXI-egfp WEHI-231
GFP positive cells 99%
SHIP-CI WEHI-231
GFP positive cells 71%
SHIP-SH2 WEHI-231
GFP positive cells 83%
Dok-PH/PTB WEHI-231
GFP positive cells 50%
Counts
eGFP

CHAPTER 3 - Dual role for Erk-MAP kinase in BCR-driven apoptosis and CD40-mediated rescue of WEHI-231 immature B cells

3.1 BCR signalling in B lymphocytes

Ligation of the BCR on B lymphocytes leads to three possible outcomes, activation, anergy or apoptosis, depending on the developmental state of the cell. The signalling pathways activated downstream of the BCR have largely been studied in mature B lymphocytes but similar signalling pathways have been observed in BCR-stimulated immature B cells. However, the divergent outcomes of BCR-ligation in mature B cells (activation) and immature B cells (growth arrest and apoptosis) suggests that there are important differences in the signals transduced from these receptors.

3.2 MAP kinases in lymphocyte signalling

It is well documented that MAPKs from the Erk, JNK and p38 families are activated downstream of the BCR and TCR (27, 219, 306, 326). Furthermore, ligation of CD40 on mature and immature B cells can stimulate MAPKs from all three families (219, 306, 307, 309). However, the response of a B cell to the differential regulation of MAPK by the BCR and CD40 depends on the developmental stage of the cell. In mature B cells the activation of Erk can stimulate proliferation (327). In contrast, BCR-dependent activation of Erk in immature B cells has been associated with the induction of apoptosis whilst CD40 has been shown to preferentially couple to JNK and p38 MAPK suggesting these kinases are more involved in mediating B cell survival (306-309). Nonetheless, recent reports have suggested that in immature B cells the BCR and CD40 can both induce Erk-MAPK and the kinetics of Erk1/2 activation are critical for determining the fate of the cell (219, 328). The differential regulation of Erk-MAPK by the BCR and CD40 plays a significant role in regulating the survival and proliferation of immature B cells (328).

3.3 MAP kinase family

The MAPK family is a large family of highly conserved serine/threonine protein kinases. There are at least three types of MAPK proteins, the Erk-

MAPKs, the JNK MAPKs, also known as stress-activated protein kinases, and the p38 MAPKs. Erk-MAPK can usually be activated by mitogenic stimuli including growth factors whilst JNK and p38 MAPKs are generally considered to be stimulated in response to cellular stress.

MAPKs are activated via a signalling network that is conserved from yeast to mammals (figure 3.1) (329-331). At each stage of the signalling network there is amplification. Stimulation of MAPK proteins requires the upstream activation of a MKK (MAPK kinase) and a MKKK (MAPK kinase kinase). MKKK are serine/threonine protein kinases that phosphorylate and activate MKKs. MKKs are dual-specificity protein kinases therefore they can phosphorylate serine/threonine and tyrosine residues. MKKs phosphorylate the threonine and tyrosine residues of a conserved T-X-Y motif of the activation loop of the MAPK. The X residue is different in each family of MAPKs: Erk has a T-E-Y motif, JNK has a T-P-Y motif and p38 has a T-G-Y motif. Phosphorylation of the T-X-Y motif activates the MAPKs allowing phosphorylation of a specific repertoire of cytoplasmic and nuclear proteins including various transcription factors. The substrate specificity of MAPKs is influenced by the X residue of the T-X-Y motif and by the amino acids surrounding this motif since this region can affect the availability or conformation of the substrate binding groove (326, 332-334).

MAPKs can regulate the activity and the fate of a cell therefore the MAPK cascade is carefully regulated. In yeast and mammals scaffolding proteins have been identified that couple MAPKs to their upstream regulators (329, 330, 335, 336). Moreover, each MAPK enzyme can be activated by a number of different upstream MKKs and MKKKs, allowing a broad range of stimuli to trigger the activation of MAPK (337). However, each MAPK, MKK and MKKK enzyme has unique properties regarding its regulation, substrate specificity and the kinetics of activation. Furthermore, the activity of specific proteins within the MAPK cascade can be regulated by many other signalling molecules including PI-3-K, PKC and protein kinase A (PKA). Finally, negative feedback mechanisms exist to ensure MAPK enzymes are not activated constitutively (338, 339). For example, MAPK can induce protein phosphatases that dephosphorylate and inhibit MAPK (340).

3.4 Erk-MAP kinase

3.4.1 The Erk-MAP kinase family

The Erk-family of MAPKs contains several members, the best studied being Erk1 and Erk2. Erk1 (p44) was initially purified and investigated as the regulator of microtubule-associated protein 2 kinase and ribosomal S6 kinase downstream of the insulin receptor (341). Soon after, Erk2 (p42) was cloned indicating several Erk proteins existed (342). Erk3 (62 kD) is ubiquitously expressed and constitutively localised to the nucleus. A serine residue in Erk3 replaces the threonine residue of the T-X-Y motif of Erk1/2. Despite its structural similarities to Erk2, Erk3 has a unique set of substrates (342, 343). Erk5 (also known as Big MAPK since it has 815 amino acids and is approximately 110 kD in size) is stimulated by MEK5 in the cytoplasm by phosphorylation of a T-E-Y activation motif. Activated Erk5 translocates to the nucleus to regulate transcription mediated by the MEF2 (myocyte enhancer factor 2) proteins. Erk5 may also target cytoskeletal elements within the cytoplasm of the cell. Erk5 and Erk1/2 have been reported to work together to mediate the activation of NF-κB and $p90^{S6}$ ribosomal kinase (344-346). Erk6 (45 kD) is required for differentiation of myoblasts to myotubules. Mutation of Tyr^{185} of the T-X-Y motif impairs Erk6 function suggesting it may be regulated by tyrosine phosphorylation (347). Erk7 (61 kD) is an autoactivated MAPK enzyme that has high basal activity and this is not increased by factors that normally activate Erk. The phosphorylation of the T-E-Y motif of Erk7 increases its activity but MEK-inhibitors do not prevent the activation of Erk7 and it is thought that Erk7 may autophosphorylate the T-E-Y motif (348). Erk8 (approximately 60 kD), like Erk7, is not inhibited by inhibitors of MEK1, 2 or 5 but Erk8 has low basal activity. Erk8 has two SH3 domains allowing it to bind to Src-family tyrosine kinases and this may be required for its activation (349).

3.4.2 Activation of Erk1/2

As stated above, Erk 1 and 2 are regulated by phosphorylation of a T-E-Y motif (350). The phosphorylation of the threonine and tyrosine residues can occur independently of each other and does not have to be in any particular order for Erk to be stimulated but both residues must be phosphorylated for full

activation of Erk (351). MEK1 and 2 are threonine/tyrosine kinases that catalyse the phosphorylation of the threonine and tyrosine residues of Erk1/2 and can fully activate Erk (352-354). MEK1/2 can be activated by phosphorylation of $Ser^{217/218}$ and Ser^{221}, catalysed by Raf-1. RKIP (Raf kinase inhibitor protein) can bind to Raf-1 to prevent its association with MEK thus inhibiting Erk (355, 356). Raf-1 is the major activator of MEK1/2 but MEK1/2 can also be stimulated by other protein kinases including Raf-B, c-Mos and MEKK1 (217, 357, 358).

Raf-1 is activated downstream of the monomeric G-protein Ras. Raf-1 binds to Ras-GTP via a Ras-binding domain but the full activation of Raf-1 also requires an adjacent cysteine-rich domain, which can enhance the interaction of Raf-1 and Ras. The mechanism of Raf-1 activation is complex and has several requirements including membrane translocation of Raf-1, phosphorylation of stimulatory residues and dephosphorylation of inhibitory residues (figure 3.2) (reviewed in (359)). Under unstimulated conditions, Raf-1 is suppressed by the binding of its amino terminal domain to its kinase region (360). Inactive Raf-1 is also phosphorylated at Ser^{259} and Ser^{621} allowing Raf-1 to bind to 14-3-3 proteins and this stabilises the inactive conformation of Raf-1. The activation of Raf-1 requires Raf-1 to translocate from the cytosol to membranes. Raf-1 can bind to phosphatidic acid and to Ras, both have been implicated in directing the membrane-translocation of Raf-1 (361, 362). The binding of Raf-1 to Ras displaces 14-3-3 proteins allowing PP2A to dephosphorylate Ser^{259}, and possibly Ser^{621} (363). The dephosphorylation of Ser^{259} enhances the association of Raf-1 to Ras and to MEK and is required for Raf-1 activation (362, 364). Phosphorylation of Ser^{336} and Tyr^{341} is also essential for activation of Raf-1 (365). Phosphorylation of Ser^{338} enhances the binding of Raf-1 to MEK whilst phosphorylation of Tyr^{341} has been suggested to enhance the phosphorylation of Ser^{338} and may remove the negative regulatory domain from the kinase domain of Raf-1 (366). Phosphorylation of Tyr^{341} also participates in the targeting of Raf-1 into lipid rafts where it can then bind to Ras (367). Raf-1 and Ras bind with high affinity. Phosphorylation of Ser^{43} by PKA reduces the affinity of Raf-1 for Ras resulting in an inhibition of Raf-1 (359, 368). Raf-1 can also be inhibited by phosphorylation of Ser^{259} catalysed by Akt (364).

The Ras family of GTPase proteins is encoded by three ubiquitously expressed genes generating H-Ras, K-Ras (4A and 4B) and N-Ras. All four

Ras proteins are structurally similar and include a C-terminal CAAX motif where C is cysteine, A is an aliphatic amino acid and X is serine or methionine. The CAAX motif is post-translationally modified to increase the hydrophobicity of the C-terminus allowing membrane attachment of Ras (369). The activity of Ras depends on its association with guanine nucleotides and this is regulated by guanine nucleotide exchange factors and GTPase-activating proteins (370, 371). Ras can be activated by the ubiquitously expressed guanine nucleotide exchange factor SOS, which is activated downstream of receptor tyrosine kinases, tyrosine kinase-coupled receptors and heterotrimeric G-protein-coupled receptors (370). SOS can be recruited to membrane-bound receptor complexes using adaptor proteins such as Grb-2.

3.5 Ras is involved in multiple signalling pathways

Ras-GTP has several effectors (370). The best-characterised effector is Raf-1 but it can also stimulate the related molecules B-Raf and A-Raf. Ras-GTP activates Raf-1 via a complex mechanism involving translocation of Raf to membranes, phosphorylation and dephosphorylation (figure 3.2) (359-361, 364-367). The activation of Raf by Ras leads to the stimulation of Erk1/2. Ras-GTP can also activate PI-3-K and the simultaneous activation of Erk and PI-3-K by Ras-GTP has been implicated in promoting BCR-driven activation of $p90^{S6}$ ribosomal kinase and subsequent inhibition of the pro-apoptotic protein Bad (233). However, the precise mechanism of Ras-dependent activation of PI-3-K is not fully understood and may involve Ras-dependent translocation of PI-3-K to the plasma membrane although the activation of PI-3-K is also dependent on tyrosine kinases. PI-3-K is a protein and a lipid kinase. It generates 3-phosphorylated phosphatidylinositol lipids and this contributes to the activation of Akt. A third effector of Ras-GTP is a family of guanine nucleotide exchange factors for Ral including RalGDS, Rgl1 and Rlf. The activation of Ral guanine nucleotide exchange factors results in the stimulation of the monomeric G-protein Ral and this may contribute to the oncogenic transformation of cells (370).

Mutant forms of Ras have been generated to restrict the repertoire of effectors that are activated downstream of Ras. Thus, $RasV^{12}$ is a constitutively active form of Ras that can activate Raf, PI-3-K and RalGDS. In contrast,

$RasV^{12}S^{35}$ can activate Raf-1 but has low affinity for PI-3-K and RalGDS and $RasV^{12}C^{40}$ can stimulate PI-3-K but has low affinity for Raf-1 and RalGDS. These mutants can be used to better understand the signals activated downstream of Ras to induce particular cellular functions (226, 227, 370).

3.6 Regulation of the Erk-MAP kinase cascade

The regulation of Erk-MAPK is a highly complex process that involves controlling the activity of the different components of the MAPK cascade. This can be mediated by other intracellular signalling molecules; for example, Raf-1 can be activated by Ras and PAK but is inhibited by Akt and PKA thus coupling Raf-1 to Ras, Rac, PI-3-K and cyclic nucleotide signalling pathways (372). However, Erk can also be regulated by positive and negative feedback loops. For example, MEK can phosphorylate and activate Raf-1 forming a positive feedback loop (340, 373). In contrast, Erk-MAPK can phosphorylate SOS leading to a dissociation of SOS and Grb-2 thus preventing further activation of Erk (374).

Furthermore, once activated, Erk1/2 can move into the nucleus and regulate the activity of several transcription factors including Elk-1, c-Jun, c-Fos and c-Myc. This results in the regulation of gene expression and the hence cellular functions. However, in addition to regulating the expression of genes required for the cell to respond to the stimulus that activated Erk, Erk1/2 can also enhance the expression of negative regulators of MAPK including MAPK phosphatases (340, 372). The Erk-MAPK cascade is thus regulated by several mechanisms including intrinsic negative feedback loops.

3.7 MAP kinase phosphatases

MAPKs contain distinct regions for binding to their upstream activating kinases, to their downstream substrates and to protein phosphatases (375, 376). MAPKs are activated by phosphorylation of their T-X-Y motif and full activity requires phosphorylation of both the threonine and the tyrosine residues. MAPKs can be inhibited by dephosphorylation of their T-X-Y motif and this can be catalysed by three different types of protein phosphatases: dual-specificity phosphatases, threonine phosphatases and tyrosine phosphatases (figure 3.3) (333, 377-379). For example, the MAPK phosphatases for Erk2

include, the dual-specificity phosphatase MKP3, the threonine phosphatase PP2A and the tyrosine phosphatase HePTP (380).

3.7.1 Dual-specificity protein phosphatases

Dual-specificity protein phosphatases form a family of proteins that can dephosphorylate both the threonine and tyrosine residues of the T-X-Y motif (378). The activity of dual-specificity phosphatases is largely regulated at the transcriptional level and the expression of specific protein phosphatases allows the selective inhibition of particular MAPK proteins since each phosphatase has a precise substrate-specificity (381). For example, PAC-1 dephosphorylates Erk and p38 whilst MKP-1 acts on all three families of MAPKs. In contrast, MKP-2 preferentially dephosphorylates Erk and JNK and MKP-M dephosphorylates JNK (382, 383). Indeed, each member of the dual-specificity phosphatase family has a unique set of properties including tissue distribution, subcellular localisation, regulation and substrate-specificity (378, 381). For example, PAC-1 is a dual-specificity phosphatase specifically located in the nucleus of hematopoietic cells. PAC-1 preferentially dephosphorylates Erk-MAPK and it acts on the tyrosine residue of the T-E-Y motif prior to dephosphorylating the threonine residue (384). The expression of PAC-1 in B and T cells is increased by various mitogenic stimuli since the transcription of the *pac-1* gene can be activated by transcription factors that are regulated by Erk (385). For example, ligation of the TCR in Jurkat T cells activates Erk, leading to the increased expression of PAC-1 and termination of the Erk signal (384).

The duration of an Erk signal can thus be regulated by controlling the expression of dual-specificity phosphatases and consequently, the expression of these proteins is dependent on multiple factors. For example, treatment of Rat-1 cells with EGF can lead to a biphasic activation of Erk. The activation of Erk can be inhibited by MKP-1 and the expression of MKP-1 is dependent on both Erk, to allow negative feedback, and on calcium signals, to enable sustained Erk signals to be generated (386). Dual-specificity phosphatases often have a short half-life to prevent excessive inhibition of MAPK (378). However, the degradation of dual-specificity phosphatases can also be regulated by MAPKs thus creating the potential for prolonged activation of Erk (387).

3.7.2 Serine/threonine protein phosphatases

There are several accounts of serine/threonine protein phosphatases regulating the activity of Erk, JNK and p38 by targeting upstream activators of these MAPKs (388-392). For example, PP2A can dephosphorylate and inhibit Raf-1 and MEK downstream of the PDGF receptor in NIH-3T3 cells leading to a suppression of Erk (393). However, Erk is also known to be a direct substrate of serine/threonine phosphatases and their action can suppress the activity of Erk (381). For example, in PC12 cells inactivation of an early transient Erk signal is mediated by the dephosphorylation of the threonine residue of T-E-Y by PP2A (394). Serine/threonine protein phosphatases are generally constitutively expressed and located in the cytoplasm of the cell therefore they are well suited to suppressing basal Erk activity in resting cells or to rapidly inhibiting newly activated cytoplasmic Erk (381).

3.7.3 Tyrosine protein phosphatases

Tyrosine phosphatases are also predominantly located in the cytoplasm of cells and may function to suppress cytoplasmic Erk. For example, the tyrosine phosphatase HePTP is expressed in hematopoietic cells and is activated downstream of the TCR resulting in an inhibition of Erk1/2 and p38 without affecting the activity of upstream activators of MAPK (333, 381). Another tyrosine phosphatase, PTP-SL, can inhibit Erk5 and prevent Erk5 from translocating into the nucleus of cells (395). Similarly, in Cos-7 and HEK-293 cells, the tyrosine phosphatases PTP-SL and STEP can bind to Erk2 to inactivate Erk2 by dephosphorylation of the T-E-Y motif and by preventing its nuclear translocation (396). Tyrosine phosphatases therefore inhibit MAPKs by dephosphorylation of the T-X-Y motif and by preventing nuclear translocation.

3.8 SHP-1 and SHP-2

The SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 are cytosolic tyrosine phosphatases with 60% sequence identity and similar structural features. Both SHP proteins have two N-terminal SH2 domains, a tyrosine phosphatase (catalytic) domain and a C-terminal regulatory region, which contains tyrosine residues that can be phosphorylated. The C-

terminal region of SHP-2 also has a proline-rich domain that is absent in SHP-1 (132). In the cytosol SHP-1 and SHP-2 are inactivated by autoinhibition via the binding of an N-terminal SH2 domain to a phospho-tyrosine residue of the C-terminal regulatory region of SHP. This blocks the catalytic domain and retains the enzyme in an inactive conformation (figure 3.4). The other N-terminal SH2 domain has greater flexibility and may be involved in the activation of SHP. SHP is activated by the binding of its SH2 domains to tyrosine phosphorylated ITIM motifs (figure 3.4) (133).

SHP-2 is ubiquitously expressed but SHP-1 is largely found in hematopoietic and epithelial cells. The function of SHP-1 has been investigated using SHP-1-deficient mice. *Motheaten* mice do not express SHP-1 and *motheaten viable* mice express catalytically inactive SHP-1. Both of these mutations cause premature death of the mice. SHP-1-deficient mice display multiple immunological defects including elevated numbers and inappropriate activation of myeloid cells, impaired differentiation of natural killer cells and hyper-responsiveness of peripheral B and T cells. SHP-1 deficiency therefore causes severe autoimmunity and inflammation. Furthermore, SHP-1-deficient B cells have a reduced threshold for B cell activation resulting in enhanced numbers of peripheral mature B cells, indicating SHP-1 is an important negative regulator of BCR signalling (132, 165).

In unstimulated B cells SHP-1 is constitutively, but weakly associated with the BCR complex and it helps to keep the BCR complex inactive by dephosphorylating the ITAMs of Igα/Igβ. Consistent with this, unstimulated B cells that are deficient in SHP-1 have impaired inhibition of the BCR complex resulting in a reduced threshold for activation. Ligation of the BCR normally causes SHP-1 to dissociate from the BCR complex, allowing ITAMs to be phosphorylated and the B cell to be activated. However, the activation of tyrosine kinases downstream of the BCR can lead to tyrosine phosphorylation of ITIMs of the co-receptors CD22, PIR-B, CD72 and FcγRIIB and SHP-1 can bind to these pITIMs leading to its stimulation. SHP-1 forms a negative feedback loop since its activation leads to an inhibition of signals stimulated by the BCR and CD19. For example, SHP-1 binds to and inhibits the tyrosine kinases Lyn and Syk thus inhibiting upstream signals of the BCR complex and CD19. SHP-1 also associates with BLNK to block signals downstream of this

adaptor. The binding of SHP-1 to these signalling molecules results in a downregulation of PLCγ and Ca^{2+} signalling, MAPK stimulation and PI-3-K activation. SHP-1 performs a similar role in the maintenance of resting T cells and in the inhibition of T cell activation following ligation of the TCR (134, 165).

SHP-2 is structurally very similar to SHP-1 and is retained in an inactive state in unstimulated cells by autoinhibition. Despite these similarities, SHP-1 and SHP-2 are functionally distinct, as indicated by the phenotype of *motheaten* mice since these mice lack SHP-1 but still express SHP-2. SHP-2 has been implicated as both a negative and a positive regulator of signalling initiated by the BCR and several tyrosine kinase-associated receptors. For example, SHP-2 can dephosphorylate and inhibit STAT1 downstream of the IFNγ receptor in 293T cells, resulting in a suppression of STAT1-mediated transcription (397). However, SHP-2 has also been implicated as a positive regulator of cell signalling pathways downstream of multiple receptors. In particular, the Erk-MAPK pathway can be stimulated by SHP-2 (167). For example, ligation of the BCR induces the tyrosine-phosphorylation of the adaptor protein Gab-1 and this creates a binding site for various proteins including SHP-2. SHP-2 binds to Gab-1 via its SH2 domain resulting in the activation of SHP-2. SHP-2 can then associate with Grb-2 leading to the recruitment of SOS and the stimulation of Erk-MAPK (110, 117, 132, 165). Furthermore, the association of Gab-1 and SHP-2/Grb-2 has been observed downstream of the receptors for EGF and PDGF and this interaction is required for the sustained activation of Erk1/2 (166). SHP-2, via its association with Grb2, can therefore be a positive regulator of Erk signalling.

3.9 SHIP

SHIP is a highly conserved cytosolic phosphatase that can be activated downstream of FcγRIIB in mature B lymphocytes where it functions as a negative regulator of BCR signalling (see section 1.7.2) (131, 184). SHIP can remove the 5' phosphate from PI-3, 4, 5-P_3 (PIP_3) resulting in the depletion of lipids that serve as anchors for PH domain-containing proteins. SHIP therefore antagonises the function of PI-3-K and can suppress the activation of Akt (127, 131, 184, 241).

SHIP is also a regulator of the Erk-MAPK pathway. SHIP binds to Shc using the same phospho-tyrosine residues as are required for the formation of the Grb2-Shc complexes so preventing the activation of SOS and the classical MAPK cascade (241). However, more recent reports have also suggested that tyrosine phosphorylation of Shc downstream of the BCR in mature B cells is SHIP-dependent and the formation of SHIP-Shc-Grb-2 complexes are necessary for mitogenic BCR signalling (398). Furthermore, tyrosine phosphorylated SHIP can bind to the adaptor protein p62 Dok (Dok1) via the phospho-tyrosine-binding domain of Dok1. This brings Dok1 into close proximity with tyrosine kinases and the C-terminal region of Dok1 becomes phosphorylated. Phosphorylation of p62 Dok increases its association with RasGAP (Ras GTPase activating protein) and Dok1 can regulate the activity of RasGAP. RasGAP enhances the intrinsic GTPase activity of Ras leading to the inhibition of Ras and the downstream effectors of Ras including Erk1/2 and PI-3-K (244).

3.10 Dok

The Dok family of proteins contains multiple modular interaction domains (figure 1.12) suggesting they can function as adaptor proteins. Dok proteins have an N-terminal PH domain to enable attachment to phospholipids and membranes and they have a central phospho-tyrosine-binding domain to allow binding to proteins with phospho-tyrosine residues. Furthermore, the C-terminal region of Dok proteins is rich in proline residues to allow binding to SH3 domains and in tyrosine residues that are potential sites of phosphorylation. The C-terminal region is the most variable section of Dok allowing distinct members of this family to bind to a different repertoire of proteins (399).

The first Dok protein to be identified was Dok1 (p62 Dok). Dok1 is a direct target of Bcr-Abl; the chimeric protein found in almost all patients with chronic myeloid leukemia. In hematopoietic progenitor cells isolated from patients with chronic myeloid leukemia, Dok1 is phosphorylated by Bcr-Abl at five tyrosine residues. Two of these phospho-tyrosine residues bind to RasGAP resulting in an inhibition of RasGAP and the prolonged activation of Ras and Erk. This is likely to contribute to the inappropriate cell division of these cells. The identity of the proteins that are recruited to the remaining three phospho-tyrosine residues

of Dok1 has not been established although several additional Dok1-interacting proteins have been identified. It is likely that some of these proteins will influence the activity of RasGAP at Dok1 since Dok1 is both a positive and a negative regulator of RasGAP (400, 401). For example, in B cells, co-ligation of the BCR and FcγRIIB leads to the phosphorylation of SHIP and Dok1 downstream of FcγRIIB. This increases the association of Dok1 with RasGAP and in this system Dok1 activates RasGAP leading to an inhibition of Ras and Erk (244). Similarly, in mast cells, the phosphorylation of Dok1 is enhanced following activation of FcεRI leading to an activation of RasGAP and an inhibition of Ras/Erk (402).

Several Dok1-associated proteins have been identified in addition to RasGAP. For example, in T cells Dok1 is tyrosine-phosphorylated by Lck and Fyn downstream of the CD2 receptor leading to the recruitment and activation of Crk-L (399, 403). Crk can function as an adaptor protein that binds to SOS and C3G (171, 172) leading to either the activation or inhibition of Erk-MAPK (see section 1.6.3.4). Crk is also an activator of JNK MAPK (170). CD2-mediated activation of Dok1 can suppress the activity of Erk1/2 but Dok1 can also inhibit PLCγ signalling and Ca^{2+} mobilisation (399). This suggests that additional proteins are still to be identified that can be recruited to the Dok1 complex to regulate these signalling cascades.

Dok1 is phosphorylated downstream of the insulin receptor leading to the recruitment and activation of RasGAP (404) and the adaptor protein Nck (110). Nck can then recruit additional signalling molecules to the Dok1 complex. For example, Nck can bind to PAK and WASP (110, 149). In hematopoietic cells, stem cell factor induces the phosphorylation of Dok1 by Btk and this leads to the recruitment of the adaptor proteins Grb2 and Grb7, the p85 subunit of PI-3-K and Src-family kinases (405). Dok1 can also bind to Csk, the kinase that controls the basal activity of Src-family kinases by phosphorylating the C-terminal inhibitory tyrosine residue leading to inhibition of Src-family kinases (151). The regulation of signalling pathways by Dok1 is therefore a very complex process involving many different signalling molecules that ultimately affect the activity of MAPK, PLCγ and PI-3-K.

The Dok-family of proteins has at least two additional members, p56 Dok (Dok2) and Dok3. Dok2 functions alongside Dok1 in mast cells to couple the

FcεRI receptor to Cas/Crk-L/Nck leading to the rearrangement of the cytoskeleton (402). Dok2 is also expressed in T cells and it functions to couple CD2 receptor to RasGAP and Nck (399). Dok1 and Dok2 therefore can regulate similar pathways. Dok3, also known as DokL, appears to be highly expressed in hematopoietic cells including B cells. It has been proposed that ligation of the BCR induces tyrosine phosphorylation of Dok3 by Src-family kinases. Dok3 cannot bind to RasGAP or Nck since it lacks the required YXXL motif (where X is any amino acid). However, Dok3 appears to directly bind to two negative regulators of BCR-signalling, SHIP and Csk, and this may result in a suppression of BCR-dependent activation of NF-AT and cytokine release (406, 407).

3.11 Aims and objectives

Recent reports have suggested that in immature B cells the BCR and CD40 can differentially regulate the activity of Erk-MAPK (219, 328). The kinetics of Erk1/2 activation appear to be critical for determining the survival and proliferation of immature B cells (328).

The aims of this investigation were:

1. to examine the effects of ligation of the BCR and CD40 on the survival and proliferation of WEHI-231 cells

2. to investigate the role of Erk-MAPK in the induction of apoptosis of BCR-stimulated WEHI-231 cells

3. to examine the role of Erk in regulating proliferation of WEHI-231 cells

4. to investigate the mechanisms used by the BCR to regulate Erk activity in WEHI-231 cells

5. to explore the role of Ras and PI-3-K in regulating the activity of Erk and other signalling pathways in WEHI-231 cells.

3.12 Results and Discussion

3.12.1 Ligation of the BCR on WEHI-231 cells induces growth arrest

Ligation of the BCR on immature B cells leads to the induction of growth arrest and apoptosis. This is necessary for the elimination of newly developed B lymphocytes that are self-reactive and hence have the potential to cause autoimmune diseases (9, 27, 29). However, immature B cells can be rescued from growth arrest and apoptosis by receiving T cell-derived signals including co-ligation of CD40 (27, 34).

The murine WEHI-231 cell line is a B cell lymphoma that is widely used as an *in vitro* model of negative selection in immature $IgM^{high}IgD^{+/-}$ B cells (see section 1.9.1) (30, 283-285). The BCR of WEHI-231 cells can be stimulated experimentally using anti-IgM antibodies (anti-Ig) whilst CD40 can be activated using anti-CD40 antibodies. Proliferation of WEHI-231 cells was measured by detecting the incorporation of [^{3}H] thymidine since this indicates the level of DNA synthesis.

Ligation of the BCR by treating WEHI-231 cells with anti-Ig induced growth arrest in a dose-dependent manner (figure 3.5A). Concentrations of anti-Ig greater than 0.01 µg/ml stimulated cell cycle arrest with a 60% decrease in DNA synthesis being observed between 0.01 and 0.1 µg/ml anti-Ig. Optimal growth arrest was obtained at 1-10 µg/ml anti-Ig. In addition, co-stimulation of immature B cells at CD40 is known to rescue the cells from BCR-driven growth arrest and apoptosis. Indeed, co-ligation of the BCR and CD40 completely rescued WEHI-231 cells from BCR-driven growth arrest (figure 3.5B) whilst treatment of WEHI-231 cells with anti-CD40 antibodies (10 µg/ml) alone had little effect on their proliferation.

3.12.2 Ligation of the BCR on WEHI-231 cells induces apoptosis

In addition to inducing growth arrest, ligation of the BCR on immature B cells can also stimulate apoptosis. The level of apoptosis in a population of WEHI-231 cells was determined by measuring the number of cells with sub-diploid DNA using the DNA-binding dye propidium iodide (PI) and FACS analysis. Asynchronous populations of cells were used in these experiments therefore populations of untreated WEHI-231 cells contained cells at each of the

four stages of the cell cycle (figure 3.6A and E). After 24 hr in culture, approximately 6% of these untreated cells were sub-diploid (apoptotic) (figure 3.6A) and after 48 hr 10% had sub-diploid DNA (figure 3.6E).

Treatment of WEHI-231 cells with anti-Ig (10 µg/ml) for 24 hr increased the percentage of sub-diploid cells from 6% to 8% (figure 3.6B) indicating a small amount of apoptosis had been induced. However, after 48 hr, anti-Ig increased the percentage of sub-diploid cells from 10% to 20% and hence doubled the number of cells with sub-diploid DNA (figure 3.6F). Ligation of the BCR therefore induces apoptosis of WEHI-231 cells. Furthermore, the induction of apoptosis by anti-Ig in WEHI-231 cells has previously been indicated by disruption of mitochondrial membrane potential, annexin V staining and DNA laddering (102, 285).

Analysis of the cell cycle of WEHI-231 cells also showed that anti-Ig induced growth arrest of WEHI-231 cells after 24 hr and 48 hr (figures 3.6B and F). This was indicated by anti-Ig increasing the number of cells in G0/G1 whilst decreasing the number of cells in S phase and G2/M. Anti-Ig therefore induces growth arrest at the G0/G1 phase of the cell cycle. These observations confirm the thymidine data, which showed that ligation of the BCR, induces growth arrest of WEHI-231 cells. Furthermore, previous studies by Carman *et al.* have shown that ligation of the BCR on immature B cells induces cell cycle arrest at the G1 to S phase transition since BCR-stimulation prevents the cells from inducing cyclin E and Cdk2 despite being able to increase the expression of cyclin D2 and Cdk4 (33). Moreover, after 24 hr there was a greater induction of growth arrest than apoptosis indicating the onset of anti-Ig-induced apoptosis takes longer than the induction of growth arrest in WEHI-231 cells.

Co-culture of the WEHI-231 cells with anti-Ig plus anti-CD40 for 24 hr or 48 hr replenished the populations of cells in S phase and G2/M. This is consistent with the thymidine data which showed anti-CD40 can protect WEHI-231 cells from anti-Ig-induced inhibition of DNA synthesis. Co-culture with anti-Ig plus anti-CD40 for 24 hr or 48 hr also rescued the cells from anti-Ig-induced apoptosis (figures 3.6C and G). Furthermore, rescue from apoptosis has previously been indicated by abrogation of loss of mitochondrial membrane potential, annexin V staining and DNA laddering (102). Anti-CD40-treatment alone had little effect on the death rate and the cell cycle progression of WEHI-

231 cells (figures 3.6D and H). Ligation of the BCR on WEHI-231 cells therefore induces growth arrest at G0/G1 phase of the cell cycle and apoptosis but co-ligation of CD40 enables the cells to survive and proliferate.

3.12.3 BCR-dependent induction of an early Erk-MAPK signal is associated with commitment to apoptosis of WEHI-231 cells

It is well documented that the BCR can couple to Erk-MAPK and in mature B lymphocytes the activation of Erk1/2 is associated with the stimulation of proliferation (327). In contrast, BCR-dependent activation of Erk in immature B cells has been associated with the induction of apoptosis whilst CD40 has been shown to preferentially couple to JNK and p38 MAPKs suggesting these proteins are more involved in mediating immature B cell survival (306-309).

Erk1/2-MAPK is regulated by controlling the phosphorylation state of the Thr-Glu-Tyr motif within its activation loop (352-354). The activity of Erk1/2 can thus be measured by Western blotting with an antibody that specifically recognises active (dual-phosphorylated) Erk1/2. Western blotting for total Erk (non-phosphorylated plus phosphorylated Erk) can be used to check gel loading since Erk is regulated post-translationally therefore total Erk levels are generally constant within a cell. The differential regulation of Erk-MAPK by the BCR and CD40 in immature B cells was thus investigated by Western blotting of WEHI-231 whole cell lysates for dual-phosphorylated, active Erk.

Work initially performed in collaboration with Stephen Gauld (328) indicated that treatment of WEHI-231 cells with increasing concentrations of anti-Ig for 30 min induced the activation of Erk2 with minimal induction of Erk1 (appendix 1A). The induction of Erk2 was observed between 5 and 25 µg/ml anti-Ig. Furthermore, analysis of the kinetics of activation indicated Erk2 was stimulated within 1 min of treatment with anti-Ig and maximal Erk activity was detected at 30 min (appendix 1B). In contrast, ligation of CD40 on WEHI-231 cells did not induce the activation of Erk over the 1 hr time course (appendix 1C). This is consistent with previous observations that suggest CD40 is less capable than the BCR at inducing early Erk signals (219, 306, 309). Co-culture of WEHI-231 cells with anti-Ig plus anti-CD40 did increase the activity of Erk but to a lesser extent than treatment with anti-Ig alone (appendix 1D). These observations show that the BCR and CD40 differentially regulate Erk1/2 in

WEHI-231 cells suggesting Erk1/2 is likely to be important for mediating the response of immature B cells to ligation of the BCR and CD40. In contrast, treatment of WEHI-231 cells with anti-Ig, anti-CD40 or a combination of anti-Ig plus anti-CD40 for 30 min in serum-supplemented conditions did not significantly increase the activity of p38 or JNK MAPKs above basal levels (328).

3.12.4 Inhibition of early Erk signals abrogates BCR-mediated apoptosis of WEHI-231 cells

The above observations therefore suggest that the BCR of WEHI-231 cells couples preferentially to early Erk signals implicating Erk as a potential mediator of BCR-driven growth arrest and apoptosis. Furthermore, the suppression of early Erk signals by CD40 may help to protect WEHI-231 cells from the effects of BCR ligation. In order to further investigate the importance of early Erk signals, pharmacological inhibitors of MEK (PD98059 and U0126) were employed to suppress Erk signals in anti-Ig-treated WEHI-231 cells (328).

Apoptosis of WEHI-231 cells treated with MEK inhibitors was investigated using PI-staining followed by FACS analysis. Pretreatment (2 hr) of WEHI-231 cells with UO126 (similar results were obtained using PD98059) induced a dose-dependent decrease in BCR-driven apoptosis with maximal effect being obtained at 1-5 μM UO126 (appendix 1E and results not shown). Indeed, 1 μM UO126 protected WEHI-231 cells from BCR-driven apoptosis without altering the extent of apoptosis in unstimulated cells. In contrast, treatment of WEHI-231 cells with a p38 inhibitor (SB203580) did not prevent BCR-driven apoptosis, which is consistent with the observation that ligation of the BCR did not significantly stimulate p38 MAPK (328). Furthermore, it has previously been reported that p38 MAPK is activated downstream of the BCR and TCR but inhibition of this does not impair antigen receptor-induced apoptosis in either of these cell types (308).

Inhibition of early Erk signals thus suppresses BCR-driven apoptosis. This is consistent with the observation that ligation of the BCR on immature B cells can induce apoptosis via the induction of $cPLA_2$, a MAPK-sensitive enzyme (102). Cytosolic PLA_2 is activated by Erk1/2 and Ca^{2+} downstream of the BCR in WEHI-231 cells and this leads to the production of arachidonic acid.

Arachidonic acid can disrupt the mitochondrial membrane potential leading to the activation of proteases and execution of the cell. Indeed, it has been observed that inhibition of early Erk1/2 activity in BCR-stimulated WEHI-231 cells can prevent anti-Ig-induced activation of $cPLA_2$, as indicated by measuring arachidonic acid production (328).

The BCR of WEHI-231 immature B cells thus couples to the early (≤1 hr) activation of Erk-MAPK and suppression of this signal can protect the cells from BCR-driven activation of $cPLA_2$ and subsequent apoptosis. BCR-driven apoptosis therefore requires the early stimulation of Erk and co-stimulation of CD40 can partially prevent BCR-mediated apoptosis by decreasing the early Erk signal. These observations are consistent with those made in T lymphocytes in which Erk signals have been shown to be required for negative selection of double positive thymocytes and also for activation-induced cell death of mature T cells (408-410).

3.12.5 Proliferating WEHI-231 cells undergo sustained and cyclic activation of Erk-MAP kinase

The above results implicate Erk-MAPK in the induction of apoptosis of anti-Ig-treated WEHI-231 cells. However, inhibition of this early Erk signal in WEHI-231 cells enhanced, rather than inhibited, BCR-driven growth arrest (appendix 1E). These observations specifically associate the early Erk signal with regulation of apoptosis and do not exclude the possibility that a more sustained proliferative Erk signal also exists in immature B cells. Indeed, there are numerous reports that associate Erk1/2 activity with the induction of proliferation (50, 331, 337, 411, 412). For example, BCR-driven proliferation of mature B lymphocytes requires the activation of Erk-MAPK (327). Moreover, the duration of Erk activation can affect the proliferation of particular cell types (173, 412, 413). For example, the induction of sustained Erk1/2 activity can enable entry into S phase of the cell cycle and promote proliferation of fibroblasts whereas a transient activation of Erk is insufficient to sustain proliferation of these cells (220). Furthermore, treatment of $CD4^+$ lymphoblast cell lines with IFNα2b inhibits proliferation via the suppression of 24-48 hr Erk signals (414).

The regulation of sustained Erk-MAPK signalling in WEHI-231 cells was thus investigated. In studies initiated by Stephen Gauld, Western blotting of

WEHI-231 whole cell lysates for dual-phosphorylated Erk1/2 showed proliferating (untreated or CD40-stimulated) WEHI-231 cells exhibit strong activation of Erk-MAPK between 8 hr and 48 hr (appendix 2A and D). The majority of active Erk in WEHI-231 cells was Erk2 (p42). This sustained activation of Erk does not merely reflect a constitutive activation of MAPK but instead reflects the prolonged and cyclic activation of Erk with peaks between 8-16 hr and 32-48 hr (appendix 2D and results not shown). Proliferating WEHI-231 cells therefore undergo sustained cyclic activation of Erk1/2.

3.12.6 Apoptotic signalling via the BCR suppresses sustained cyclic activation of Erk that can be re-established by CD40-mediated rescue

Treatment of WEHI-231 cells with anti-Ig altered the pattern of activation of Erk1/2-MAPK over a 48 hr time period (appendix 2B). Firstly, ligation of the BCR enhanced early (≤2 hr) Erk1/2 signals whilst co-ligation of the BCR and CD40 (appendix 2C), or stimulation with CD40 alone (appendix 2D), reduced these early Erk signals. This is consistent with the data presented in appendix 1 in which the BCR was shown to enhance early Erk signals leading to the induction of apoptosis in WEHI-231 cells.

In addition to enhancing early Erk1/2 signals, ligation of the BCR on WEHI-231 cells also inhibited sustained (>2 hr) cyclic activation of Erk-MAPK (appendix 2B). This suggests that sustained Erk signals may have a role in promoting survival or proliferation of WEHI-231 cells. Furthermore, treatment of WEHI-231 cells with a combination of anti-Ig plus anti-CD40 restored the sustained Erk signals (appendix 2C). These results show that WEHI-231 cells exhibit sustained cyclic activation of Erk1/2 under proliferative conditions and these Erk signals are suppressed in cells that are undergoing growth arrest and apoptosis.

3.12.7 Inhibition of sustained cyclic activation of Erk-MAPK can block basal proliferation and CD40-mediated rescue

Work performed in collaboration with Stephen Gauld and Derek Blair has demonstrated that the sustained cyclic activation of Erk is associated with proliferation of WEHI-231 cells (328). MEK inhibitors (UO126 and PD98059) were used to suppress Erk signals in WEHI-231 cells. However, a single

application of these inhibitors was only effective at suppressing Erk for ≤6 hr. Consequently, the application of MEK inhibitors (UO126 or PD98059) was repeated every 4 hr to inhibit sustained Erk signals in WEHI-231 cells. Repeated exposure of WEHI-231 cells to these inhibitors not only enhanced BCR-driven growth arrest but also blocked DNA synthesis of unstimulated, anti-CD40-treated or anti-Ig plus anti-CD40-stimulated WEHI-231 cells (appendix 3A). To ensure that these results reflected the inhibition of sustained Erk signals, the effects of a single dose of PD98059 or UO126 were investigated (appendix 3C-D). These results agreed with previous observations that inhibition of early Erk signals had minimal effect on the proliferation of WEHI-231 cells. These observations thus strongly imply that proliferation of WEHI-231 cells requires sustained cyclic activation of Erk-MAPK and inhibition of these signals using anti-Ig or pharmacological MEK inhibitors can induce growth arrest. Furthermore, ligation of CD40 was unable to restore proliferation to cells treated with MEK inhibitors indicating the induction of sustained cyclic Erk signals is critical for CD40-mediated restoration of proliferation.

To determine whether the observed inhibition of DNA synthesis following suppression of prolonged Erk signals reflected the induction of apoptosis, the sub-diploid DNA content was determined in cells exposed to PD98059 and UO126 (appendix 3B). These observations revealed that sustained inhibition of MEK and hence proliferation in unstimulated WEHI-231 cells induced apoptosis. This was not due to inhibitor toxicity since ligation of CD40 (in the presence and absence of anti-Ig) prevented this induction of apoptosis. This also indicates CD40 can initiate signals that enable WEHI-231 cells to survive in the presence of MEK inhibitors despite being unable to restore proliferation to these cells.

Work performed in collaboration with Derek Blair further implicated sustained and cyclic activation of Erk as being necessary for proliferation of WEHI-231 cells (328). Laser scanning cytometry was used to measure the levels of phospho-Erk in WEHI-231 cells at different stages of the cell cycle (G1, S phase and G2/M). These investigations showed that cycling WEHI-231 cells have high levels of phospho-Erk whilst <50% of newly divided cells displayed phospho-Erk staining (appendix 4A-B). Sustained Erk signals can be inhibited by the addition of anti-Ig or cell cycle blockers (olomoucine or aphidicolin) and treatment of WEHI-231 cells with these agents greatly reduced the amount of

phospho-Erk in growth arrested cells (appendix 4B and data not shown). However, within 48 hr of release from olomoucine-induced growth arrest, phospho-Erk levels in G1 cells were restored. Furthermore, the levels of phospho-Erk were restored by rescuing the cells from growth arrest via the addition of anti-CD40 (appendix 4B).

Collectively these results strongly suggest that WEHI-231 cells require sustained and cyclic activation of Erk-MAPK in order to proliferate and growth arrest can be induced by suppression of these Erk signals. In particular, these results show that inhibition of sustained Erk signals by ligation of the BCR is a critical stage for the induction of growth arrest whilst CD40-dependent rescue from growth arrest, but not apoptosis, requires prolonged Erk signals to be restored.

Erk can promote cell cycle progression in eukaryotic cells via the induction of cyclin D1/Cdk2 and cyclin E/Cdk2, leading to the phosphorylation and inhibition of Rb (50, 415). This allows cells to progress through the G1 to S phase transition. However, ligation of the BCR on immature B cells induces arrest of the cell cycle at the G1 to S phase transition. Furthermore, BCR-stimulation prevents the cells from inducing cyclin E/Cdk2 despite being able to increase the expression of cyclin D2/Cdk4 and hence impairs the phosphorylation of Rb (33, 53). Taken together, these observations suggest that sustained Erk signals may promote proliferation of WEHI-231 cells via the induction of cyclin E/Cdk2 leading to the phosphorylation and inhibition of Rb. However, BCR-ligation on WEHI-231 cells also stimulates other cell cycle inhibitors including p21, p27 and p53 therefore these proteins may also be targeted by Erk-MAPK to allow cell cycle progression (56, 58, 325, 416).

3.12.8 BCR-mediated inhibition of sustained Erk-MAPK signalling does not involve a dissociation of MEK from Erk or a suppression of MEK activity

Proliferating WEHI-231 cells therefore undergo sustained and cyclic activation of Erk-MAPK. Ligation of the BCR inhibits prolonged Erk signals and this is important for the induction of growth arrest whilst restoration of these signals can be mediated by co-ligation of CD40. To investigate the signalling

mechanism of BCR-dependent suppression of cycling Erk-MAPK activation, the effect of anti-Ig on the activity of MEK in WEHI-231 cells was determined.

MEK is a direct activator of Erk1/2 therefore the association of MEK and Erk was analysed by preparing immune complexes of Erk1/2 from whole cell lysates of WEHI-231 cells followed by Western blotting for MEK1/2 (figure 3.7A-D). In unstimulated cells, the interaction of MEK and Erk was observed throughout the 48 hr time period (figure 3.7A). However, the amount of MEK bound to Erk varied in a cyclic manner with the greatest MEK-Erk association being evident at 1 hr, 4-8 hr and 24-48 hr. The cyclic association of MEK and Erk was also evident in anti-CD40-treated cells (figure 3.7C). These observations are consistent with those of appendix 2 in which proliferating WEHI-231 cells were observed to undergo cyclic activation of Erk. The pattern of MEK-Erk association thus parallels that of Erk activation in proliferating WEHI-231 cells.

In contrast, treatment of WEHI-231 cells with anti-Ig did not induce a dissociation of MEK and Erk (figure 3.7B) despite these conditions being able to inhibit cycling Erk activity. Of particular interest, the levels of MEK1/2 were high in Erk1/2 immune complexes prepared between 24 hr and 48 hr suggesting that the BCR can inhibit the cyclic activation of Erk without dissociating Erk from its upstream activator MEK.

However, a significant dissociation of MEK and Erk was observed in early (≤8 hr) samples of cells treated with anti-Ig plus anti-CD40 (figure 3.7D). This implies that the ability of CD40 to rescue from BCR-dependent apoptosis may involve a downregulation of early Erk signals via a suppression of upstream activators of Erk-MAPK. However, the association of MEK and Erk was restored in 12-48 hr lysates prepared from these cells thus enabling the induction of sustained Erk signals. WEHI-231 cells therefore display high levels of association between MEK and Erk in 12-48 hr samples and this is not prevented by ligation of the BCR in the presence or absence of CD40 stimulation.

The inability of the BCR to dissociate MEK and Erk at times of low Erk activity suggests that inhibition of sustained cyclic activation of Erk by the BCR might not be mediated via the suppression of upstream activators of Erk. However, the results of figure 3.7A-D do not indicate the activity of MEK in

WEHI-231 cells. MEK is a component of the MAPK cascade therefore it is activated by phosphorylation of $Ser^{217/218}$ and Ser^{221}, catalysed by Raf-1 (372). The activity of MEK in WEHI-231 can thus be estimated by Western blotting using antibodies that are sensitive to the phosphorylation state of MEK. High levels of phospho-MEK were detected in 12-48 hr whole cell lysates of unstimulated WEHI-231 cells or cells treated with anti-Ig, anti-CD40 or a combination of anti-Ig plus anti-CD40 (figure 3.7E-H). These observations strongly suggest that MEK is highly active in both proliferating and growth arrested WEHI-231 cells and critically this can be observed at times when the activity of Erk1/2 is suppressed.

The mechanism by which the BCR suppresses cycling Erk activation in the induction of growth arrest was further investigated using an *in vitro* MEK kinase assay to determine the activity of MEK in anti-Ig-treated WEHI-231 cells. The MEK kinase assay determines the activity of MEK in MEK1/2 immune complexes prepared from WEHI-231 whole cell lysates. The activity of MEK in these complexes is indicated by its ability to phosphorylate an Erk-GST substrate *in vitro*. The activity of MEK in this assay is therefore directly proportional to the levels of dual phosphorylated Erk-GST. Levels of phospho-Erk-GST were determined by Western blotting using an antibody that specifically recognises dual-phosphorylated Erk.

Interestingly, despite the ability of the BCR to suppress the activity of cycling Erk, levels of phosphorylated Erk-GST were comparable in untreated and anti-Ig-treated WEHI-231 cells between 12 hr and 48 hr (figure 3.8A-B). Furthermore, the levels of phospho-MEK1/2 in the MEK immune complexes were also similar between 12 hr and 48 hr. In contrast, the levels of phospho-MEK appear to be depleted by anti-Ig in ≤8 hr samples despite there being no apparent loss of MEK activity at these times. This observation was surprising and suggests that the levels of phospho-MEK do not always correlate with MEK activity. Nonetheless, the results of figure 3.8 do agree with those of figure 3.7, which suggested that MEK1/2 was highly active between 12 hr and 48 hr despite the activity of Erk1/2 being inhibited by BCR ligation at these times. This data therefore indicates that the BCR does not uncouple Erk1/2 from upstream regulators of Erk but rather appears to induce negative feedback mechanisms that can inhibit Erk despite the ongoing activity of upstream activators.

3.12.9 Sustained cyclic activation of Erk-MAPK is inhibited by BCR-dependent activation of protein phosphatases

The presence of sustained MEK1/2 signals in anti-Ig-treated WEHI-231 cells suggests BCR-dependent suppression of cyclic activation of Erk is mediated by the direct inhibition of Erk1/2. The activity of Erk-MAPK can be inhibited by the action of three types of protein phosphatase: dual-specificity phosphatases, tyrosine phosphatases and serine/threonine phosphatases (see section 3.7) (380, 381). Indeed, work performed in collaboration with Stephen Gauld (328) has shown that treatment of WEHI-231 cells with anti-Ig increases the expression of PAC-1, a dual-specificity MAPK phosphatase (appendix 5A). Furthermore, ligation of the BCR increased the amount of PAC-1 detected in Erk immune complexes (appendix 5B). Co-ligation of CD40 did not appear to decrease the expression of PAC-1 but it did reduce the amount of PAC-1 detected in Erk immune complexes (results not shown and appendix 5B). These results implicate PAC-1 as a mediator of BCR and CD40 signalling and suggest that the BCR may, at least in part, suppress cyclic activation of Erk by upregulating PAC-1 and increasing its association with Erk. CD40 appears to prevent the association of PAC-1 and Erk thus suggesting one mechanism of CD40-dependent restoration of prolonged Erk signals in WEHI-231 cells. These results are consistent with previous observations in mature B lymphocytes in which the immunomodulatory products secreted by filarial nematodes can elicit B cell unresponsiveness by suppressing BCR-mediated Erk-MAPK activity via the induction of PAC-1 (417). Furthermore, PAC-1 can negatively regulate Erk-MAPK in T lymphocytes and the expression of PAC-1 is rapidly increased following ligation of the TCR on Jurkat cells (382, 384, 385).

The mechanism used by the BCR to induce PAC-1 in immature B cells is incompletely understood. However, Erk-MAPK has been associated with inducing the transcription of the *pac-1* gene in T cells and is a likely candidate for mediating *pac-1* expression in B cells (385). Most genes are regulated by multiple factors therefore additional mechanisms of inducing PAC-1 are likely to exist. One possible mediator is p53 since p53 is capable of stimulating the expression of *pac-1* in other systems and ligation of the BCR on WEHI-231 cells is known to induce p53 (64, 325). Furthermore, CD40 can suppress p53 in

BCR-stimulated WEHI-231 cells (325), and this could contribute to the restoration of sustained cyclic activation of Erk in proliferating cells.

However, it is important to recognise that there are many different protein phosphatases that are capable of regulating the activity of Erk-MAPK and in any particular system multiple phosphatases will control the activity of Erk. For example, it has been proposed that the cytosolic protein phosphatases HePTP, PP2A and PP2C can regulate the initial activation of Erk-MAPK in the cytosol of activated T cells. However, these phosphatases do not appear to enter the nucleus therefore nuclear MAPK must be suppressed by nuclear phosphatases including members of the dual-specificity phosphatase family (381, 418).

To further investigate the role of protein phosphatases in the regulation of Erk MAPK in WEHI-231 cells, the differential phosphorylation of the T-E-Y motif in the activation loop of Erk1/2 was explored by Western blotting using antibodies that are sensitive to the phosphorylation state of this motif (figure 3.9). The sensitivity of these antibodies was tested using recombinant Erk2 proteins supplied by New England Biolabs. Two recombinant Erk2 proteins were used: non-phosphorylated (T-E-Y) Erk2 and fully (dual)-phosphorylated (pT-E-pY) Erk2. Western blotting of these recombinant proteins indicated that the phospho-Erk1/2 (pT^{202}/pY^{204}) (pTpY-Erk) antibody exclusively bound to phosphorylated Erk since it was unable to recognise non-phosphorylated Erk2. The phospho-Erk1/2 (pY^{204}) (pY-Erk) antibody did not detect either of the recombinant Erk2 proteins indicating this antibody was unable to bind to non-phosphorylated or dual-phosphorylated Erk2. This is consistent with this antibody being specific for Erk that is only phosphorylated at the tyrosine residue of the T-E-Y motif. In contrast, the phospho-Erk1/2 (pT^{183}) (pT-Erk) was able to recognise dual-phosphorylated Erk2 indicating this antibody did not exclusively bind to the single-phosphorylated pT-Erk. However, this antibody did not recognise non-phosphorylated Erk2 therefore it was specific for phosphorylated Erk. Finally the total Erk1/2 (Erk) antibody recognised both recombinant forms of Erk indicating this antibody was able to bind to Erk regardless of the phosphorylation state of the activation loop.

Whole cell lysates prepared from untreated and anti-Ig-stimulated WEHI-231 cells were analysed by Western blotting using the phosphorylation-sensitive Erk1/2 antibodies. Western blotting for dual-phosphorylated Erk (pTpY-Erk)

showed that untreated proliferating WEHI-231 cells displayed sustained and cyclic activation of Erk-MAPK (figure 3.9A). Ligation of the BCR enhanced early Erk signals but suppressed prolonged MAPK activation (figure 3.9B). These results agree with previous observations (appendix 2) and indicate the cells used in this experiment were undergoing the regular pattern of Erk-MAPK activation.

Western blotting for pY-Erk indicated that the pattern of expression of pY-Erk paralleled that of pTpY-Erk (figure 3.9). Single-phosphorylated pY-Erk was therefore most abundant at ≤1 hr and 4-24 hr in untreated cells. In anti-Ig-treated cells pY-Erk was present at ≤4 hr but as with pTpY-Erk, sustained pY-Erk signals were suppressed by ligation of the BCR. This suggests that WEHI-231 cells contain a reservoir of pY-Erk at times when Erk is highly active. This pool of single-phosphorylated Erk may represent an intermediate step in the generation of dual-phosphorylated Erk. However, the kinetics of accumulation of pY-Erk and pTpY-Erk were not identical in WEHI-231 cells. For example, in untreated cells the level of pTpY-Erk was roughly equal in 24 hr and 48 hr samples whereas the levels of pY-Erk were significantly lower after 48 hr compared to levels at 24 hr (figure 3.9A). These observations suggest that pY-Erk may have a functional significance that is independent of its potential role as an intermediate step in the generation of dual-phosphorylated Erk. Indeed, recent reports have suggested that single-phosphorylated forms of Erk retain partial activity (379) therefore the pool of pY-Erk in WEHI-231 cells might represent a pool of partially active Erk that may be functionally distinct from dual-phosphorylated Erk. The ability of anti-Ig to induce a loss of pY-Erk in WEHI-231 cells suggests that the function of this species of Erk is antagonised by ligation of the BCR.

Single-phosphorylated pT-Erk was also detected in untreated and anti-Ig-treated WEHI-231 cells. Phospho-T-Erk, like pY-Erk, was detected at times when pTpY-Erk was abundant but this might reflect the ability of the pT-Erk antibody to bind to dual-phosphorylated Erk, as indicated by the recombinant dual-phosphorylated Erk2 control. Nonetheless, Western blotting for pT-Erk showed clearly that the expression of pT-Erk in WEHI-231 cells was very different to that of pY-Erk or pTpY-Erk. Indeed, a significant pT-Erk signal was detected in both untreated and anti-Ig-stimulated cells at times when dual-

phosphorylated Erk levels were low. For example, in untreated cells a strong pT-Erk signal was obtained at 2 hr and 48 hr despite the presence of very weak pY-Erk and pTpY-Erk signals at these times (figure 3.9A). This suggests that untreated WEHI-231 cells contain a reservoir of pT-Erk that is present when other phosphorylated forms of Erk are absent. The precise function of this pool of pT-Erk is not understood but, as with pY-Erk, pT-Erk is partially active (379) therefore it might represent a distinct reservoir of partially active Erk. Furthermore, treatment of WEHI-231 cells with anti-Ig did not diminish the levels of pT-Erk despite ligation of the BCR being able to suppress pY-Erk and pTpY-Erk signals (figure 3.9B). Indeed, high levels of pT-Erk were detected between 8 hr and 48 hr in anti-Ig-treated WEHI-231 cells indicating the function of pT-Erk is not suppressed by ligation of the BCR.

The results presented in figure 3.9 therefore indicate that WEHI-231 cells contain single-phosphorylated forms of Erk in addition to their dual-phosphorylated and non-phosphorylated forms. Since pT-Erk and pY-Erk are known to have kinase activities that are 2-3 orders of magnitude higher than non-phosphorylated Erk (379), these species of Erk are likely to have unique signalling functions in WEHI-231 cells. However, the activities of pT-Erk and pY-Erk are 1-2 orders of magnitude less than pTpY-Erk (379) therefore the formation of single-phosphorylated forms of Erk is likely to represent an alternative mechanism to PAC-1-mediated suppression of Erk activity in both proliferating and anti-Ig-stimulated WEHI-231 cells. For example, the abundance of pT-Erk at times when dual-phosphorylated Erk is barely detectable may be caused by the activation of tyrosine-specific protein phosphatases. Furthermore, the presence of high levels of pT-Erk in 8-48 hr samples of anti-Ig-treated WEHI-231 cells suggests that the synthesis of this single phosphorylated form of Erk may be activated by ligation of the BCR.

3.12.10 SHP tyrosine phosphatases interact with Erk1/2 in WEHI-231 cells

The tyrosine-specific protein phosphatases SHP-1 and SHP-2 are known mediators of BCR signalling in mature B lymphocytes (110, 117, 132, 134, 165) (see section 3.8). The function of these protein phosphatases in immature B cells is less well understood. The results of figure 3.9 implicated tyrosine phosphatases as potential mediators of Erk signalling in WEHI-231 cells. The

role of SHP proteins in regulating Erk-MAPK was thus investigated by determining the association of Erk and SHP in untreated and stimulated WEHI-231 cells. To do this, Erk1/2 immune complexes were prepared from WEHI-231 whole cell lysates and these were analysed by Western blotting for SHP-1 or SHP-2.

SHP-1 was found to be constitutively associated with Erk in untreated and anti-CD40-treated WEHI-231 cells over a period of 48 hr (figure 3.10A and D). This suggests that WEHI-231 cells can survive and proliferate when SHP-1 interacts with Erk. SHP-1 was also found to interact with Erk1/2 in anti-Ig-treated WEHI-231 cells (figure 3.10B). However, a dissociation of SHP-1 from Erk was observed in the 8 hr sample suggesting ligation of the BCR can regulate the interaction of these two proteins. Similarly, in cells treated with anti-Ig plus anti-CD40, SHP-1 was detected in Erk immune complexes except between 2 hr and 8 hr where there was a dissociation of SHP-1 and Erk (figure 3.10C). Co-ligation of CD40 therefore can also regulate the interaction of SHP-1 and Erk.

The results of figure 3.9 implicated a role for tyrosine phosphatases in BCR-mediated suppression of sustained Erk signals. Figure 3.10A-D shows that SHP-1 and Erk can indeed interact in WEHI-231 cells and this can be regulated by ligation of the BCR or CD40. However, unlike the binding of PAC-1 to Erk, the association of SHP-1 and Erk was not specifically enhanced by ligation of the BCR suggesting the interaction of SHP-1 and Erk may not mediate BCR-dependent inhibition of cyclic Erk signals. Furthermore, co-ligation of CD40 prevented the binding of PAC-1 and Erk suggesting a potential mechanism of CD40-mediated enhancement of Erk signals. However, at times when dual-phosphorylated Erk levels were most suppressed by treatment with anti-Ig (12-48 hr), anti-CD40 did not prevent SHP-1 and Erk interacting. It is thus unclear what precise purpose is served by the interaction of SHP-1 and Erk in WEHI-231 cells. One aspect of this interaction that requires further investigation is to determine the activity of SHP-1 in Erk immune complexes. In the cytosol, the activity of SHP-1 is normally suppressed by autoinhibition since an N-terminal SH2 domain of SHP binds to a C-terminal phospho-tyrosine residue thus blocking the catalytic domain (figure 3.4). SHP can be activated by the binding of its SH2 domains to tyrosine phosphorylated proteins (133). The

mechanism by which SHP-1 interacts with Erk is unclear and it is possible that activation of SHP-1 in this complex requires the SH2 domain of SHP-1 to interact with an additional protein to form a multicomponent complex. The interaction of SHP-1 with additional proteins may also serve to anchor SHP-1/Erk at a particular intracellular location. Furthermore, the influence of SHP-1 on Erk activity is unclear since Erk is not generally considered to be a substrate of SHP-1 (380, 381). However, it has recently been suggested that upstream activators, substrates and negative regulators of Erk-MAPK can compete with each other for binding to Erk-MAPK (376). The formation of Erk-SHP-1 complexes therefore might influence the activity of Erk by regulating the interaction of Erk and other proteins including additional regulators or substrates.

SHP-2 is structurally very similar to SHP-1 but is functionally distinct. For example, SHP-1 is generally considered to be a negative regulator of BCR signalling in mature B lymphocytes but SHP-2 can enhance BCR-mediated activation of Erk-MAPK via its interaction with Grb-2 (110, 117, 132, 165, 419). The role of SHP-2 in regulating Erk in WEHI-231 cells was investigated by detection of SHP-2-Erk complexes in WEHI-231 cells. The association of Erk1/2 with SHP-2 was studied by preparing immune complexes of Erk1/2 from WEHI-231 cell lysates and Western blotting with an anti-SHP-2 antibody. In untreated WEHI-231 cells, SHP-2 strongly associated with Erk1/2 especially between 0 and 1 hr although the association was also observed at later times including 4-8 hr and 24 hr (figure 3.10E). A sustained, cyclic pattern of SHP-2-Erk association was also observed in anti-CD40-treated cells (figure 3.10H).

In contrast, a strong association of SHP-2 and Erk in anti-Ig-treated WEHI-231 cells was exclusively observed between 0 and 1 hr suggesting that ligation of the BCR causes SHP-2 to dissociate from Erk (figure 3.10F). However, co-culture with anti-Ig plus anti-CD40 impaired the early binding of Erk1/2 and SHP-2 whilst enhancing the sustained interaction of these signalling molecules (figure 3.10G).

These observations show that SHP-2 can associate with Erk1/2 in WEHI-231 cells and this can be regulated by ligation of the BCR and CD40. Furthermore, the interaction of SHP-2 and Erk has distinct parallels with the pattern of Erk activation in WEHI-231 cells (appendix 2). Indeed, the SHP-2-Erk

complex was most abundant when the highest levels of pTpY-Erk could be detected. This suggests that SHP-2 does not mediate the dephosphorylation of pTpY-Erk but instead may increase the activity of Erk-MAPK.

The activation of Erk by SHP-2 has already been observed downstream of the BCR in mature B lymphocytes. However, in this system SHP-2 is recruited to the BCR complex via Gab-1 and SHP-2 can then associate with Grb-2 leading to the recruitment of SOS and the stimulation of Erk-MAPK (110, 117, 132, 165). A similar situation has been observed in Jurkat T cells downstream of the TCR (420). Furthermore, the association of Gab-1 and SHP-2/Grb-2 has been observed downstream of the receptors for EGF and PDGF and this interaction was specifically required for the sustained activation of Erk1/2 (166). SHP-2, via its association with Grb2, can therefore be a positive regulator of Erk signalling. However, it is not known if SHP-2 can also directly associate with Erk in these systems and hence in WEHI-231 cells it is unclear how the activity of Erk is regulated by the direct interaction of SHP-2 and Erk.

Furthermore, the intracellular location of the Erk-SHP-2 complex in WEHI-231 cells has not been established. The interaction between SHP-2 and Gab-1 occurs within the cytosol but SHP-2 can also enter the nuclei of cells and has been found in association with STAT-1 in the nuclei of A431 cells (397). Identification of the intracellular location of Erk-SHP-2 in WEHI-231 cells might indicate whether SHP-2 is a regulator of cytosolic or nuclear Erk-MAPK.

Taken together, these results show that Erk can bind either directly or indirectly via complexes to SHP-1 and SHP-2 tyrosine phosphatases in WEHI-231 cells. However, the kinetics of this interaction do not directly implicate SHP-1 as a regulator of Erk downstream of the BCR and CD40 and SHP-2 appears to be a positive mediator of Erk-MAPK signalling in WEHI-231 cells. The regulation of Erk-MAPK in WEHI-231 cells is thus incompletely understood. Protein phosphatases other than PAC-1 are likely to be involved in mediating BCR-driven suppression of cyclic Erk signals but further investigations are required to identify these phosphatases. Studies in T cells may prove useful in directing these additional studies since several protein phosphatases including PAC-1, MKP-1, MKP-2, MKP-3, MKP-6, VHR, VHX and HePTP are known to regulate the activity of Erk in these cells. For example, the tyrosine phosphatase HePTP and the dual-specificity phosphatase MKP-1 are cytosolic phosphatases

that suppress Erk activity in unstimulated T cells but ligation of the TCR stimulates Erk-MAPK and in turn Erk can induce PAC-1 leading to a termination of the Erk signal. However PAC-1 *de novo* synthesis is optimal 30-60 min after TCR stimulation and the suppression of later Erk signals requires the more sustained accumulation of additional MAPK phosphatases including VHR (382, 418).

3.12.11 Ras can stimulate Erk via Raf or PI-3-K in WEHI-231 cells

The regulation of Erk-MAPK in WEHI-231 cells is critical for determining the survival and proliferation of these cells. The mechanisms used by the BCR and CD40 to couple to Erk-MAPK are incompletely understood. The results presented above indicate that activation of an early Erk signal correlates with the presence of MEK-Erk complexes. However, sustained and cyclic activation of Erk can be regulated independently of upstream activators.

The classical Erk-MAPK cascade (figure 3.1) can be initiated by the monomeric G-protein Ras (see section 3.4.2). However, Ras has several effectors including Raf, PI-3-K and RalGDS (see section 3.5). The role of Ras in regulating the activity of Erk-MAPK in WEHI-231 cells was investigated using WEHI-231 cells transfected by electroporation with a pcDNA3.1 plasmid containing a $RasV^{12}$ construct ($RasV^{12}$ WEHI-231), a $RasV^{12}S^{35}$ construct ($RasV^{12}S^{35}$ WEHI-231), a $RasV^{12}C^{40}$ construct ($RasV^{12}C^{40}$ WEHI-231) or an empty plasmid (pcDNA3.1 WEHI-231) (see table 2.1B and section 3.5 for details of these mutations). To further investigate the importance of the Erk-MAPK cascade an additional mutant WEHI-231 cell line was generated by transfecting cells by electroporation with either a pcDNA3.1 plasmid containing a ΔMEKK3 construct (ΔMEKK3 WEHI-231) or an empty plasmid (pcDNA3.1 WEHI-231). The ΔMEKK3 construct encodes a constitutively active form of MEKK3 and was a generous gift from Dr. S. J. Cook (The Babraham Institute, Cambridge, UK). MEKK3 is a MAPKKK that can activate several different types of MAPK. MEKK3 can stimulate p38 via MKK3/6 and JNK via MKK4/7 (421, 422). MEKK3 also stimulates MEK1/2 and Erk1/2 and can activate Erk5 via MEK5 (423-425). Transfection of cells with ΔMEKK3 can be used to determine the effect of simultaneously activating multiple types of MAPK.

Expression of $RasV^{12}$, $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ induced a general increase in the activity of Erk in WEHI-231 cells indicating Ras is an important upstream activator of Erk in these cells (figure 3.11). Furthermore, Ras was able to stimulate Erk via at least two distinct pathways since $RasV^{12}S^{35}$ couples only to Raf whilst $RasV^{12}C^{40}$ only binds to PI-3-K. Ras can thus stimulate Erk via Raf-1 or PI-3-K in WEHI-231 cells. ΔMEKK3 WEHI-231 cells also had enhanced activation of Erk (figure 3.11E), which is consistent with MEKK3 being an activator of Erk1/2 (423).

Untreated or CD40-stimulated WEHI-231 cells transfected with the empty vector pcDNA3.1 underwent sustained cyclic activation of Erk-MAPK (figure 3.11A). Although transfection of these cells with $RasV^{12}$, $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ enhanced the activity of Erk (figure 3.11B-D), they still displayed cyclic activation of Erk over the 24 hr time period. This shows that the constitutive activation of Raf and/or PI-3-K by Ras was insufficient to sustain constitutively active Erk signals. Interestingly, untreated or CD40-stimulated WEHI-231 cells that were transfected with either $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ had greater Erk activity than cells transfected with $RasV^{12}$. This suggests that restricting the downstream effectors of Ras can actually increase the activation of Erk, further supporting the observation that Ras can stimulate Erk via Raf-1 or PI-3-K in WEHI-231 cells.

Ligation of the BCR on $RasV^{12}$ WEHI-231 cells enhanced the early (1 hr) Erk activity but suppressed the activation of Erk between 8 hr and 24 hr (figure 3.11B). Stimulation of the BCR therefore induces negative regulatory mechanisms that can suppress Erk activity even in the presence of constitutive activation of Raf-1 and PI-3-K by Ras. This is consistent with observations suggesting BCR-dependent inhibition of sustained and cyclic activation of Erk is mediated by the induction of MAPK phosphatases rather than the inhibition of upstream activators of Erk (figure 3.8 and appendix 5) and that these phosphatases may be Erk-dependent. Similarly, transfection of WEHI-231 cells with either $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ did not prevent BCR-driven inhibition of Erk (figure 3.11C-D).

Co-ligation of CD40 on BCR-stimulated pcDNA3.1 WEHI-231 cells restored cyclic activation of Erk, with optimal levels of dual-phosphorylated Erk being detected at 1 hr and 24 hr (figure 3.11A). Cyclic patterns of Erk activation

were also observed in $RasV^{12}$ transfected WEHI-231 cells that were treated with anti-Ig plus anti-CD40 (figure 3.11B). In contrast, $RasV^{12}S^{35}$ and $RasV^{12}C^{40}$ WEHI-231 cells treated with anti-Ig plus anti-CD40 displayed slightly impaired Erk activation at 24 hr (figure 3.11C-D). Indeed, these WEHI-231 cells had less dual-phosphorylated Erk than the equivalent pcDNA3.1 WEHI-231 cells. This suggests that CD40-mediated restoration of sustained Erk signals in BCR-stimulated WEHI-231 cells requires Ras-dependent activation of both Raf and PI-3-K.

3.12.12 Constitutive activation of Ras partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis

To investigate the functional significance of these observations, the DNA synthesis and apoptosis of $RasV^{12}$, $RasV^{12}S^{35}$, $RasV^{12}C^{40}$ and ΔMEKK3 WEHI-231 cells was measured. The level of DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine. After 24 hr, anti-Ig induced growth arrest of pcDNA3.1 WEHI-231 cells in a dose-dependent manner (figure 3.12A). However, WEHI-231 cells that were transfected with $RasV^{12}$, $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ were completely protected from anti-Ig-induced growth arrest after 24 hr. Expression of constitutively active Ras therefore protects WEHI-231 cells from anti-Ig-dependent growth arrest and it does so via the activation of Raf and/or PI-3-K. These observations agree with previous data that showed Erk-MAPK promotes proliferation of WEHI-231 cells (appendices 2-4). However, expression of these constitutively active forms of Ras did not substantially increase the levels of dual-phosphorylated Erk at 24 hr in anti-Ig-treated cells (figure 3.11) suggesting Ras might be promoting proliferation of WEHI-231 cells via an alternative mechanism.

Interestingly, expression of ΔMEKK3 was significantly less effective than the constitutively active forms of Ras at protecting WEHI-231 cells from BCR-driven growth arrest at 24 hr (figure 3.12A). This agrees with previous observations suggesting Erk-MAPK and not JNK or p38, is specifically required for proliferation of WEHI-231 cells (328) and suggests that JNK or p38 may have a role in suppressing proliferation of WEHI-231 cells.

DNA synthesis was also measured in $RasV^{12}$ WEHI-231 cell lines after 48 hr and 72 hr. In contrast to the protection observed after 24 hr, expression of

$RasV^{12}$, $RasV^{12}S^{35}$, $RasV^{12}C^{40}$ or ΔMEKK3 did not prevent anti-Ig-induced growth arrest after 48 hr or 72 hr (figure 3.12B-C). A small amount of protection was enabled by expression of $RasV^{12}$ or $RasV^{12}C^{40}$ suggesting that Ras-dependent activation of PI-3-K might have a greater role in promoting proliferation than Ras-dependent stimulation of Raf-1. Furthermore, CD40-mediated rescue was greatest in $RasV^{12}C^{40}$ WEHI-231 cells (figure 3.12D) although expression of each of the $RasV^{12}$ vectors induced a slight increase in proliferation compared to pcDNA3.1 WEHI-231 cells treated with anti-Ig plus anti-CD40. These observations were unexpected since CD40-mediated restoration of sustained Erk signals was slightly impaired in $RasV^{12}S^{35}$ and $RasV^{12}C^{40}$ WEHI-231 cells. This further suggests that Ras can induce proliferation of WEHI-231 cells via an alternative mechanism(s).

Expression of $RasV^{12}$ vectors therefore partially protects WEHI-231 cells from anti-Ig-induced growth arrest. To determine whether this was due to increased proliferation or merely reflected enhanced survival of the cells, the sub-diploid DNA content of these cells was analysed (figure 3.12E). After 48 hr, 10 μg/ml anti-Ig doubled the number of pcDNA3.1 WEHI-231 cells with sub-diploid DNA (figure 3.12E) but expression of vectors containing $RasV^{12}$, $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ reduced the ability of anti-Ig to induce apoptosis. This suggests that Ras can stimulate survival signals in WEHI-231 cells via the activation of Raf and/or PI-3-K. However, expression of $RasV^{12}S^{35}$ offered least protection indicating Ras-dependent activation of PI-3-K is more effective at rescuing WEHI-231 cells from BCR-driven apoptosis than Ras-dependent stimulation of Raf. These observations are consistent with the thymidine assay data suggesting Ras can promote survival and proliferation of WEHI-231 cells and this can be mediated by the activation of either Raf-1 or PI-3-K. Moreover, CD40-dependent rescue from BCR-driven apoptosis was slightly impaired in $RasV^{12}S^{35}$ WEHI-231 cells, confirming PI-3-K is the more effective mediator of Ras-dependent rescue. Constitutive activation of Ras can thus increase the survival of WEHI-231 cells and this may contribute to the inhibition of BCR-driven growth arrest observed in WEHI-231 cells expressing constitutively active forms of Ras.

BCR-driven apoptosis was also impaired in ΔMEKK3 WEHI-231 cells despite these cells being relatively sensitive to anti-Ig-induced growth arrest.

This suggests that the simultaneous activation of Erk1/2 with JNK, p38 or Erk5 may reduce pro-apoptotic signals in WEHI-231 cells. This is consistent with previous observations that have implicated JNK/p38 as mediators of immature B cell survival (306-309).

3.12.13 Ras-dependent activation of PI-3-K does not stimulate Akt

To investigate the mechanism of Ras-dependent and MEKK3-mediated survival of WEHI-231 cells, the activity of Akt in transfected WEHI-231 cells was determined since PI-3-K-dependent activation of Akt can promote the survival of many different cell types including mature splenic B lymphocytes (see section 1.6.3.1) (184, 186, 188, 189, 193-195). Furthermore, Akt has been implicated as an effector of Ras-dependent PI-3-K signalling downstream of TCR (426).

The activity of Akt is regulated by phosphorylation; in particular, Akt must be phosphorylated at Thr^{308} and Ser^{473} for activation (184). The activity of Akt in lysates prepared from $RasV^{12}$, $RasV^{12}S^{35}$, $RasV^{12}C^{40}$ and ΔMEKK3 WEHI-231 cells was determined by Western blotting with phosphorylation-sensitive antibodies that specifically recognise Akt with phospho-Thr^{308} (pTAkt) or phospho-Ser^{473} (pSAkt). Positive controls for phosphorylated and non-phosphorylated Akt (purchased from New England Biolabs) were included in all the Western blots.

In general, pcDNA3.1 WEHI-231 cells had very low levels of pTAkt and pSAkt indicating WEHI-231 cells do not normally have high Akt activity (figure 3.13A). Furthermore, ligation of the BCR and/or CD40 did not greatly enhance the phosphorylation of Akt and the only condition that appeared to induce phosphorylation of both Thr^{308} and Ser^{473} was treatment with anti-CD40. However, the levels of pTAkt and pSAkt in CD40-stimulated cells were very low and may not reflect a true Akt signal. These observations suggest WEHI-231 cells have low Akt activity and this is unlikely to be affected by ligation of the BCR and/or CD40.

Transfection of WEHI-231 cells with $RasV^{12}$, $RasV^{12}S^{35}$, $RasV^{12}C^{40}$ or ΔMEKK3 did not greatly enhance the activation of Akt (figure 3.13B-E). As with pcDNA3.1 cells, the only condition that induced phosphorylation of both Thr^{308} and Ser^{473} was CD40 stimulation. Surprisingly, the highest levels of phosphorylated Akt were observed in $RasV^{12}S^{35}$ WEHI-231 cells and this form

of Ras cannot couple to PI-3-K. This result must be treated with some caution since the levels of pTAkt were lower than or equal to those of the non-phosphorylated Akt control cell extract (figure 3.13C, anti-CD40-treatment, compare lane 5 to lanes 1-4). Overall, these observations suggest that the activity of Akt is low in WEHI-231 cells and Akt is unlikely to be a significant mediator of Ras-dependent or MEKK3-mediated survival and proliferation of these cells. Alternative effectors of PI-3-K thus appear to be responsible for enhancing the viability of $RasV^{12}C^{40}$ WEHI-231 cells.

3.12.14 Ras can induce Bcl-x_L in WEHI-231 cells especially via the activation of PI-3-K

To further investigate the enhanced survival potential of WEHI-231 cells transfected with constitutively active Ras mutants, the expression of Bcl-x_L was determined by Western blotting of lysates prepared from these cells. Bcl-x_L is known to be an important survival factor of WEHI-231 immature B cells that can be induced by CD40 to suppress BCR-dependent apoptosis (see section 1.9.3 and chapter 4 for further details) (292, 293, 305, 427). The mechanisms that regulate the expression of Bcl-x_L in WEHI-231 cells are incompletely understood.

Bcl-x_L levels were low in untreated and anti-Ig-stimulated pcDNA3.1 WEHI-231 cells but ligation of CD40, in the presence or absence of anti-Ig, greatly enhanced the expression of Bcl-x_L, especially between 8 hr and 48 hr (figure 3.14A). This is consistent with previous studies that have identified Bcl-x_L as a key mediator of CD40-driven survival (292, 293, 427).

Expression of $RasV^{12}$ or ΔMEKK3 induced a general increase in the levels of Bcl-x_L detected in WEHI-231 cells without altering the pattern of expression (figure 3.14B and E). Bcl-x_L was thus most abundant in CD40-stimulated cells and between 8 hr and 48 hr. Constitutive activation of Ras or MEKK3 therefore can overcome some of the negative regulatory mechanisms that prevent Bcl-x_L induction in unstimulated and anti-Ig-treated WEHI-231 cells to allow a slight increase in Bcl-x_L expression. This is likely to contribute to the increased survival of $RasV^{12}$ or ΔMEKK3 WEHI-231 cells observed in figure 3.12E.

However, expression of $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ substantially increased the levels of Bcl-x_L in untreated WEHI-231 cells (figure 3.14C-D). This suggests

Ras can induce Bcl-x_L in unstimulated cells via Raf or PI-3-K but this process was impaired by activation of both of these pathways (figure 3.14B). Interestingly, expression of $RasV^{12}C^{40}$ also increased the expression of Bcl-x_L in anti-Ig-stimulated cells suggesting Ras-dependent activation of PI-3-K can either induce Bcl-x_L expression or overcome the BCR-mediated negative regulation of Bcl-x_L expression. Furthermore, these observations are consistent with the results of figure 3.12, which showed $RasV^{12}C^{40}$ WEHI-231 cells were partially protected from BCR-driven apoptosis. Ras-dependent activation of Raf or PI-3-K can thus induce Bcl-x_L in unstimulated WEHI-231 cells. However, the activation of PI-3-K alone can increase Bcl-x_L levels in anti-Ig-stimulated cells. Interestingly, expression of constitutively active forms of Ras did not lead to the constitutive induction of Bcl-x_L in CD40-stimulated cells, suggesting signalling pathways other than Ras and its effectors are likely to contribute to CD40-dependent induction of Bcl-x_L. This is consistent with the observation that inhibition of MEK did not impair CD40-dependent survival (appendix 3B).

3.12.15 Inhibition of Dok can increase the activity of Erk in BCR-stimulated WEHI-231 cells

Dok-1 is an adaptor protein that has multiple functions including the regulation of Ras via RasGAP (see section 3.10). For example, in mature B cells Dok-1 is phosphorylated following co-ligation of the BCR and FcγRIIB and this leads to the activation of RasGAP and inhibition of Ras (244). To investigate the role of Dok1 in WEHI-231 cell signalling, WEHI-231 cells were retrovirally transfected with pMXI-egfp vectors containing Dok-PH/PTB (Dok-PH/PTB WEHI-231) or empty vector (pMXI WEHI-231). Expression of Dok-PH/PTB can suppress wild-type Dok1 since it prevents proteins from binding to the PH and PTB domains of cellular Dok1. Expression of Dok-PH/PTB therefore is expected to inhibit RasGAP in WEHI-231 cells and increase the activation of Ras.

The activity of Erk-MAPK in WEHI-231 cells transfected with Dok-PH/PTB was investigated by Western blotting. Expression of Dok-PH/PTB enhanced the activity of Erk in untreated and BCR-stimulated WEHI-231 cells (figure 3.15A-B). Of particular interest, inhibition of Dok appeared to prevent BCR-dependent suppression of sustained Erk signals, implicating Dok1 as a mediator of BCR-

driven suppression of Erk-MAPK. Interestingly, expression Dok-PH/PTB did not increase the levels of phospho-Erk in anti-CD40-treated WEHI-231 cells despite CD40 inducing Erk in pMXI WEHI-231 cells. This observation may reflect an ability of CD40 to suppress Dok1 in WEHI-231 cells.

Expression of Dok-PH/PTB did not substantially increase the activity of Akt in untreated or stimulated WEHI-231 cells (figure 3.15C-D). Furthermore, in contrast to the $RasV^{12}$ mutants, the expression of Bcl-x_L was slightly impaired in Dok-PH/PTB WEHI-231 cells (figure 3.15E-F). The phenotype of Dok-PH/PTB WEHI-231 cells reflected these observations since expression of Dok-PH/PTB did not significantly protect WEHI-231 cells from BCR-driven apoptosis after 24 hr or 48 hr (figure 3.16A-B). Furthermore, despite being able to increase the expression of Erk in untreated and BCR-stimulated WEHI-231 cells, expression of Dok-PH/PTB did not impair BCR-driven growth arrest of WEHI-231 cells after 24, 48 or 72 hr (figure 3.16C-E). This is likely to be due to these cells being susceptible to BCR-driven apoptosis. Expression of Dok-PH/PTB also did not affect CD40-dependent rescue from BCR-driven growth arrest (figure 3.16F).

Inhibition of Dok via the expression of Dok-PH/PTB in WEHI-231 cells therefore enhances Erk signalling. However, Dok-PH/PTB is unable to induce pro-survival proteins and consequently does not appear to enhance the survival or proliferation of WEHI-231 cells. Dok thus appears to be a regulator of Erk in WEHI-231 cells and it may be involved in mediating BCR-dependent suppression of sustained Erk signals. Precisely how Dok impairs Erk signalling in WEHI-231 cells is unknown but in other systems, Dok1 mediates its effects by regulating the activity of RasGAP (244). However, constitutive activation of Ras did not substantially increase sustained Erk signals in BCR-stimulated WEHI-231 cells (figure 3.11) suggesting Dok may inhibit Erk downstream of the BCR via an alternative effector. In T lymphocytes Crk is an effector of Dok and Crk can interact with C3G, an activator of Rap-1 (399, 403). The induction of Rap-1 can lead to the activation or inhibition of Erk-MAPK independently of Ras (figure 1.17). The role of Crk/C3G/Rap-1 in mediating Dok1 signalling in WEHI-231 cells has not been explored but preliminary data suggests that untreated WEHI-231 cells display cyclic activation of Rap1 (428).

3.12.16 Inhibition of SHIP increases Erk activation but cannot induce constitutive Erk signalling

Analysis of the $RasV^{12}C^{40}$ WEHI-231 cells indicated PI-3-K is an important effector of Ras. To further investigate the role of PI-3-K in WEHI-231 cell signalling, WEHI-231 cells were retrovirally transfected with pMXI-egfp vectors containing SHIP-CI (SHIP-CI WEHI-231), SHIP-SH2 (SHIP-SH2 WEHI-231) or empty vector (pMXI WEHI-231). SHIP-CI is a catalytically inactive form of SHIP whilst SHIP-SH2 encodes the SH2 domain of SHIP and hence it competes with wild-type SHIP to prevent its attachment to phospho-tyrosine residues. SHIP is a phosphatase that removes the 5' phosphate from the inositol ring, enabling it to reduce the levels of PI-3, 4, 5-P_3 and antagonise the function of PI-3-K (see sections 1.7.2 and 3.9). Transfection of WEHI-231 cells with dominant negative SHIP mutants therefore should enhance PI-3-K signalling.

The activity of Erk-MAPK in WEHI-231 cells transfected with empty vector (pMXI-egfp), SHIP-CI or SHIP-SH2 was investigated by Western blotting of lysates prepared from these cells. In general, the activity of Erk was reduced in retrovirally transfected WEHI-231 cells including pMXI WEHI-231 cells suggesting this method of transfection impairs Erk activation. Nonetheless, proliferating pMXI WEHI-231 cells underwent cyclic activation of Erk whilst BCR stimulation enhanced early (1 hr) Erk signals without elevating sustained levels of phospho-Erk and co-ligation of the BCR and CD40 restored sustained and cyclic activation of Erk (figure 3.17A).

Expression of SHIP-CI or SHIP-SH2-containing vectors did not greatly alter the pattern of Erk activation in untreated or stimulated cells (figure 3.17B-C) but consistent with the results of figure 3.11D, Erk signalling was slightly enhanced in these cells. These results further agree with the observations of figure 3.11 by showing enhanced PI-3-K function is insufficient to override the negative regulatory mechanisms that normally suppress Erk activity in WEHI-231 cells. For example, stimulation of SHIP-CI or SHIP-SH2 WEHI-231 cells with anti-Ig plus anti-CD40 induced a cyclic pattern of Erk activation with Erk being inhibited at 8 hr. Enhanced PI-3-K signalling therefore increases the activation of Erk at times of high Erk activity but cannot overcome the negatively regulatory mechanisms that normally suppress Erk signalling.

3.12.17 Expression of SHIP partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis

To further examine the role of PI-3-K signalling in WEHI-231 cells, the proliferation and apoptosis of SHIP-CI and SHIP-SH2 WEHI-231 cells was determined. Proliferation was investigated by measuring the DNA synthesis of populations of cells cultured for 24, 48 or 72 hr (figure 3.18A-C). Ligation of the BCR on pMXI WEHI-231 cells induced growth arrest in a dose-dependent manner. Furthermore, expression of SHIP-CI did not protect the cells from BCR-driven growth arrest. In contrast, anti-Ig-induced growth arrest was slightly impaired in SHIP-SH2 WEHI-231 cells, especially after 72 hr. For example, treatment of WEHI-231 cells with 1 μg/ml anti-Ig for 72 hr induced a 88% decrease in the percentage of DNA synthesis in pMXI WEHI-231 cells but the DNA synthesis was only decreased by 60% in SHIP-SH2 WEHI-231 cells (figure 3.18C). This agrees with the observations of figure 3.12 in which Ras-dependent activation of PI-3-K partially protected WEHI-231 cells from anti-Ig-induced growth arrest. Furthermore, the antagonism of PI-3-K by SHIP appears to require interactions mediated by the SH2 domain of SHIP. However, CD40-mediated rescue from anti-Ig-induced growth arrest was relatively unaffected by expression of SHIP-CI or SHIP-SH2 (figure 3.18D), suggesting SHIP does not normally impair CD40-dependent restoration of proliferation.

Apoptosis of SHIP-CI and SHIP-SH2 WEHI-231 cells was also measured. As with previous experiments, anti-Ig did not induce a significant amount of apoptosis after 24 hr (figure 3.18E). However, the 24 hr cultures do suggest that expression of SHIP-CI or SHIP-SH2 increased the basal level of survival of WEHI-231 cells, implicating PI-3-K activation as a pro-survival factor of proliferating cells. After 48 hr, anti-Ig induced apoptosis of pMXI WEHI-231 and SHIP-SH2 WEHI-231 cells (figure 3.18F). However, expression of SHIP-CI partially protected WEHI-231 cells from BCR-driven apoptosis. This was slightly unexpected since SHIP-CI did not appear to prevent BCR-driven proliferation and SHIP-SH2 did reduce the level of anti-Ig-induced growth arrest. CD40-mediated rescue from BCR-driven apoptosis was not significantly affected by expression of either SHIP mutant.

3.12.18 Inhibition of SHIP does not activate Akt but can induce Bcl-x_L

To further investigate the signals downstream of SHIP and PI-3-K, the activity of Akt was measured by Western blotting of lysates prepared from SHIP-CI and SHIP-SH2 WEHI-231 cells. However, no activation of Akt could be detected in unstimulated or stimulated pMXI WEHI-231, SHIP-CI WEHI-231 or SHIP-SH2 WEHI-231 cells (figure 3.19A-C). This is consistent with the observations of figure 3.13 and further suggests that Akt is unlikely to be a significant survival factor of unstimulated, BCR-activated or CD40-stimulated WEHI-231 cells.

However, expression of SHIP-CI and SHIP-SH2 did induce a slight increase in the expression of Bcl-x_L in untreated and BCR-stimulated WEHI-231 cells (figure 3.19D-F). This is likely to explain the general reduction in apoptosis observed in WEHI-231 cultures after 24 hr (figure 3.18E). Furthermore, these results agree with those of figure 3.14 in which Ras-dependent activation of PI-3-K induced Bcl-x_L in untreated WEHI-231 cells. Moreover, CD40-mediated induction of Bcl-x_L was not significantly affected by inhibition of SHIP.

Overall, the inhibition of SHIP appears to induce Bcl-x_L in untreated and possibly BCR-stimulated WEHI-231 cells and this contributes to the increased survival of these cells. These observations confirm those of the Ras mutants in which Ras-dependent activation of PI-3-K was implicated as an important survival factor of unstimulated or BCR-activated WEHI-231 cells via the induction of Bcl-x_L.

3.13 Conclusions

Ligation of the BCR on WEHI-231 cells induced growth arrest at G0/G1 phase of the cell cycle and apoptosis in a dose-dependent manner (figures 3.5-3.6). Co-ligation of CD40 rescued the cells from growth arrest and apoptosis. To investigate the signalling mechanisms used to regulate survival and proliferation of WEHI-231 cells, the activity of MAPK was determined. Anti-Ig induced early Erk signals with optimal stimulation at 30 min and this was reduced by co-ligation of CD40 (appendix 1). In contrast, ligation of the BCR or CD40 did not induce significant levels of p38 or JNK activity(328).

The functional significance of early Erk signals was investigated using pharmacological inhibitors of MEK to inhibit Erk activity. MEK inhibitors abrogated BCR-driven apoptosis (appendix 1E) and reduced the ability of the BCR to induce arachidonic acid production (328) in WEHI-231 cells. Previous data has suggested that the BCR induces apoptosis via the induction of $cPLA_2$ and arachidonic acid (102). Taken together, this suggests that ligation of the BCR induces early Erk signals that stimulate $cPLA_2$, leading to the production of arachidonic acid and the onset of apoptosis (figure 3.20A).

However, additional functions for Erk-MAPK were suggested by the observation that proliferating WEHI-231 cells undergo sustained and cyclic activation of Erk (appendix 2). Furthermore, ligation of the BCR disrupted cyclic activation of Erk by inhibiting sustained Erk signals whilst co-ligation of CD40 restored prolonged Erk signalling (appendix 2). The functional significance of these signals was investigated via the repeated application of MEK inhibitors to suppress Erk activation. Inhibition of sustained Erk signals inhibited basal and CD40-mediated proliferation and prevented CD40-dependent rescue from BCR-driven growth arrest (appendix 3). However, MEK inhibitors did not abrogate CD40-mediated rescue from BCR-driven apoptosis (appendix 3B) thus suggesting cyclic activation of Erk is particularly required for proliferation of WEHI-231 cells. Furthermore, laser scanning cytometry was used to show WEHI-231 cells undergoing cell cycle progression have high concentrations of phospho-Erk that is reduced by ligation of the BCR or the addition of cell cycle inhibitors but can be restored by CD40-dependent signals (appendix 4). Taken together, these observations strongly suggested that sustained and cyclic activation of Erk is required for proliferation of WEHI-231 cells. Disruption of these signals can be mediated by the BCR to induce growth arrest whilst CD40 can enable the cells to proliferate by restoring the sustained and cyclic Erk signals (figure 3.20A).

To investigate the mechanism of BCR-dependent inhibition of sustained Erk signals, the activity of MEK was determined. Ligation of the BCR did not dissociate MEK-Erk complexes and did not dephosphorylate key of residues of MEK that must be phosphorylated for activation of the kinase (figure 3.7). Furthermore, the activity of MEK was determined using an *in vitro* MEK assay and this showed that ligation of BCR does not inhibit sustained MEK signals

(figure 3.8). These observations indicate the BCR does not uncouple Erk from its upstream activators and hence the BCR must inhibit Erk via an alternative pathway.

The activation of Erk requires phosphorylation of a T-E-Y motif in the activation loop and dephosphorylation of these residues inhibits Erk (figure 3.3). Indeed, Erk can induce its own inhibition via the induction of the dual-specificity MAPK phosphatase PAC-1 (385). The role of PAC-1 in regulating Erk in WEHI-231 cells was investigated. Ligation of the BCR increased the expression of PAC-1 and enhanced the formation of PAC-1-Erk complexes (appendix 5). Furthermore, CD40 caused PAC-1 and Erk to dissociate. These observations strongly suggest that the BCR can inhibit Erk via the induction of PAC-1 enabling Erk activity to be suppressed despite the presence of active MEK (figure 3.20B). However, PAC-1 is unlikely to be the only inhibitor of Erk in WEHI-231 cells since WEHI-231 cells were found to contain pY-Erk and pT-Erk in addition to the dual-phosphorylated and non-phosphorylated forms (figure 3.9). This suggests that MAPK may also be regulated by threonine and tyrosine phosphatases.

The interaction of Erk with the tyrosine phosphatases SHP-1 and SHP-2 was thus investigated. Interestingly both phosphatases were found in association with Erk (figure 3.10). However, the kinetics of the association of SHP-1 and Erk did not directly implicate SHP-1 as a regulator of Erk downstream of the BCR and CD40 and further experiments are required to explore the functional significance of this interaction. In contrast, the association of SHP-2 and Erk did seem to be regulated by the BCR and CD40 and the SHP-2-Erk complexes were most abundant at times of high Erk activity suggesting a role for SHP-2 as a positive regulator of Erk in WEHI-231 cells.

The mechanism of Erk activation in WEHI-231 cells was further investigated by transfection of cells with constitutively active forms of Ras. Expression of active Ras induced a general increase in Erk activity in WEHI-231 cells (figure 3.11). However, constitutive activation of Ras did not lead to constitutive Erk activity, which is consistent with the activity of Erk being negatively regulated by MAPK phosphatases. Interestingly, mutagenesis of Ras to restrict its interaction with downstream effectors indicated Ras could stimulate Erk via the activation of Raf-1 and/or PI-3-K (figure 3.11C-D).

Furthermore, expression of active Ras partially protected WEHI-231 cells from anti-Ig-induced growth arrest and apoptosis and this was particularly evident in the $RasV^{12}C^{40}$ mutant which can only couple to PI-3-K (figure 3.12). Moreover, Ras was found to increase the expression of Bcl-x_L in untreated and anti-Ig-stimulated WEHI-231 cells (figure 3.14) and this was most effective when Ras was coupled to PI-3-K (figure 3.20B).

To confirm the importance of Ras and PI-3-K in promoting survival and proliferation of WEHI-231 cells, additional experiments were performed on cells expressing mutant forms of SHIP and Dok. Inhibition of SHIP supported the $RasV^{12}C^{40}$ data, which suggested PI-3-K has a role in regulating the survival and proliferation of WEHI-231 cells (figures 3.17-3.19). In particular, the induction of PI-3-K seems to be able to induce Bcl-x_L in unstimulated and anti-Ig-treated cells (figure 3.19D-F) although the mechanism of this has not been explored. Inhibition of Dok did not enhance Bcl-x_L expression but it did implicate Dok as an inhibitor of Erk in untreated and anti-Ig-stimulated WEHI-231 cells (figure 3.15). Dok is a known activator of RasGAP (244) but inhibition of Dok did not mimic constitutive activation of Ras suggesting the activity of Erk can be regulated by Ras-dependent and Ras-independent mechanisms in WEHI-231 cells.

The differential regulation of Erk-MAPK in WEHI-231 cells is thus critical for the survival and proliferation of WEHI-231 cells. Early Erk signals can induce a pro-apoptotic pathway involving arachidonic acid production whilst sustained Erk signals are critical for proliferation of WEHI-231 cells (figure 3.20A). Inhibition of sustained and cyclic Erk activation is critical for BCR-driven growth arrest and this is not mediated by uncoupling Erk from its upstream activators but instead requires the induction of MAPK phosphatases. However, the regulation of Erk in WEHI-231 cells is a complex process and is influenced by non-classical effectors of Erk including PI-3-K (figure 3.20B).

Figure 3.1. MAPKs are activated in response to a wide variety of stimuli via a conserved signalling network. MAP kinase proteins (Erk1/2, Erk5, JNK1-3 and p38) are activated via a conserved signalling cascade that is initiated by upstream signals that activate MKKKs (MAP kinase kinase kinases) leading to the stimulation of MKKs (MAP kinase kinases) which can then activate members of the MAPK family. MAP kinase proteins target both cytoplasmic and nuclear proteins including transcription factors.

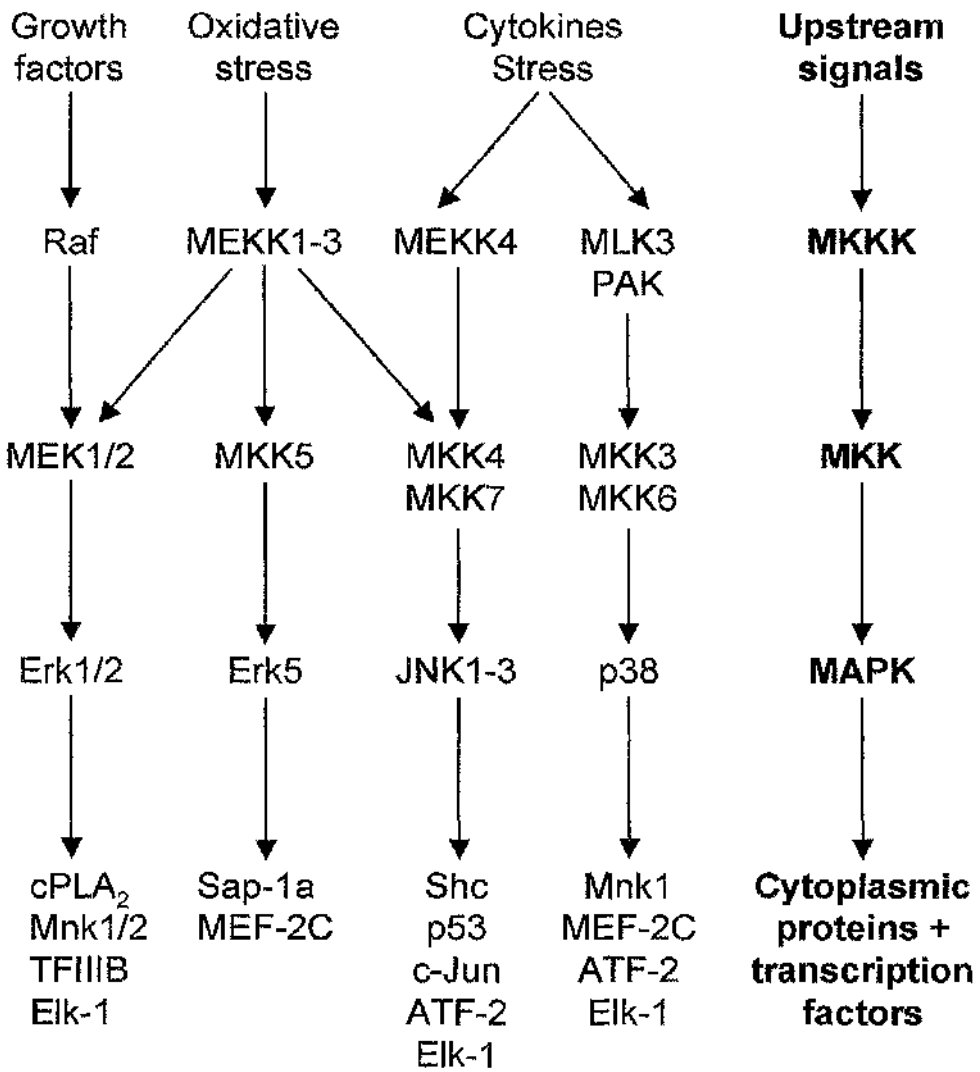

Growth factors
Oxidative stress
Cytokines Stress
Upstream signals
Raf
MEKK1-3
MEKK4
MLK3 PAK
MKKK
MEK1/2
MKK5
MKK4 MKK7
MKK3 MKK6
MKK
Erk1/2
Erk5
JNK1-3
p38
MAPK
cPLA2 Mnk1/2 TFIIIB Elk-1
Sap-1a MEF-2C
Shc p53 c-Jun ATF-2 Elk-1
Mnk1 MEF-2C ATF-2 Elk-1
Cytoplasmic proteins + transcription factors

Figure 3.2. Regulation of Raf-1 activity. **(1)** In unstimulated cells, Raf-1 is inhibited by the binding of its amino terminal to its kinase domain and this is stabilised by phosphorylation of Ser^{259} and Ser^{621}, which interact with 14-3-3 proteins. **(2)** Activation of Raf-1 requires it to translocate to the plasma membrane where it can associate with Ras-GTP. **(3)** PP2A dephosphorylates Ser^{259} causing 14-3-3 proteins to be displaced and enhancing the association of Raf-1 to Ras-GTP and MEK. Raf-1 is also phosphorylated at Ser^{338} and Tyr^{341} to assist the activation of Raf-1, perhaps by removing the N-terminal inhibitory region from the Raf-1 kinase domain. Phosphorylation of Ser^{338} and Tyr^{341} also increases the association of Raf-1 and MEK. Raf-1 can activate MEK by phosphorylation. **(4)** The inactivation of Raf-1 is mediated by phosphorylation of Ser^{43}, catalysed by protein kinase A, since this causes Raf-1 and Ras to dissociate. Akt can also catalyse the phosphorylation of Ser^{259}, providing a docking site for 14-3-3 proteins.

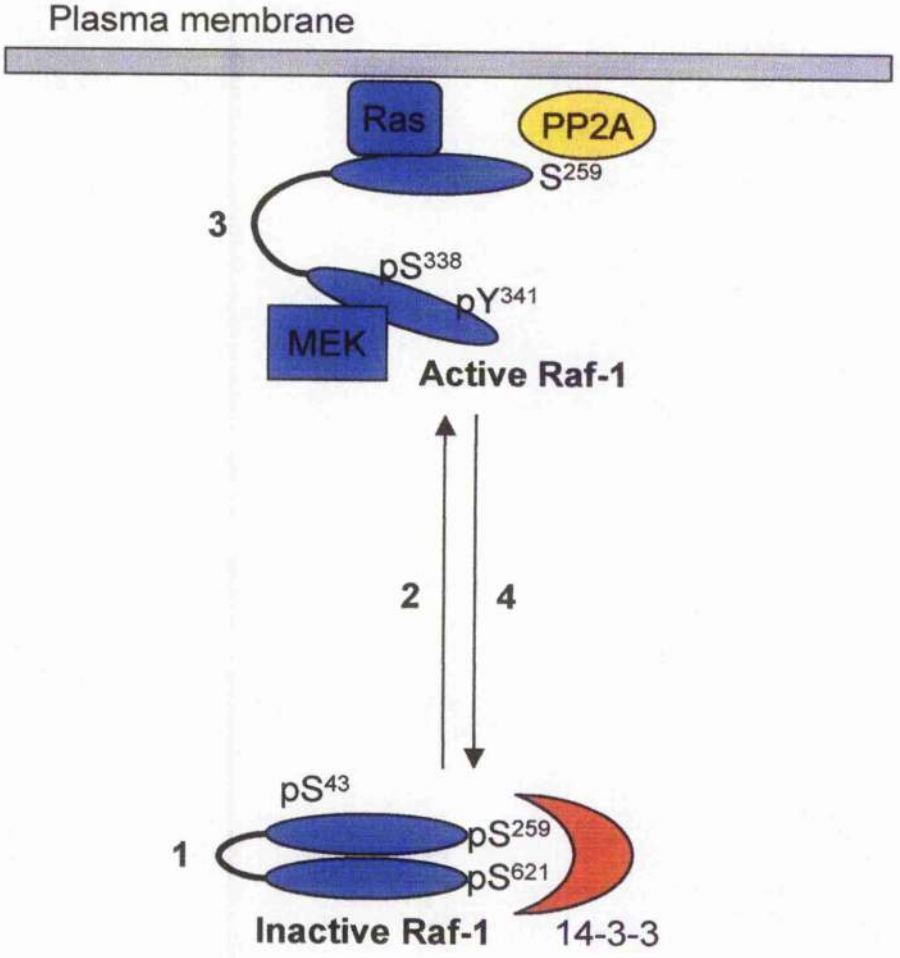

Plasma membrane
Ras
PP2A
S259
3
pS338
pY341
MEK
Active Raf-1
2
4
pS43
1
pS259
pS621
Inactive Raf-1
14-3-3

Figure 3.3. Inhibition of Erk-MAPK by protein phosphatases. MAPKs can be inactivated by the action of three different types of protein phosphatases: dual-specificity phosphatases, threonine phosphatases and tyrosine phosphatases. Erk1/2 that lacks phosphorylation of the T-E-Y motif is inactive. Erk is stimulated by phosphorylation of the threonine and tyrosine residues of the T-E-Y motif. Three different types of protein phosphatases can inhibit Erk. Dual-specificity phosphatases remove the phosphate from both the threonine and tyrosine residues of T-E-Y generating inactive Erk. Threonine phosphatases only dephosphorylate the threonine residue and tyrosine phosphatases only dephosphorylate the threonine residue. Both phosphatases can generate partially active forms of Erk.

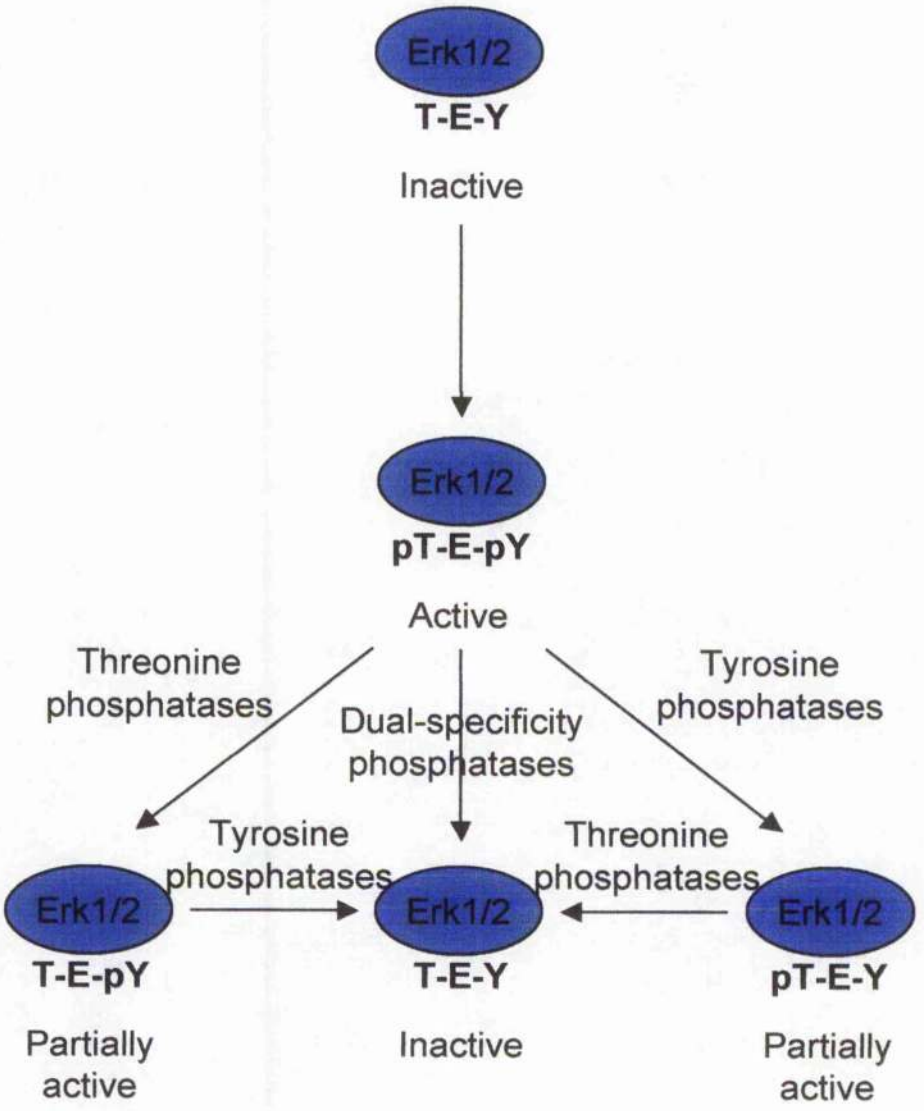

Erk1/2
T-E-Y
Inactive
Erk1/2
pT-E-pY
Active
Threonine phosphatases
Dual-specificity phosphatases
Tyrosine phosphatases
Tyrosine phosphatases
Threonine phosphatases
Erk1/2
T-E-pY
Partially active
Erk1/2
T-E-Y
Inactive
Erk1/2
pT-E-Y
Partially active

Figure 3.4. Activation of SHP. SHP tyrosine phosphatases are retained in an inactive conformation by the binding of their N-terminal SH2 domain to phospho-tyrosine residues in the C-terminal region of the protein. Autoinhibition is relieved by the binding of the SH2 domains of SHP to phosphorylated peptides including ITIM-containing proteins. This induces a conformational change in SHP that results in its activation.

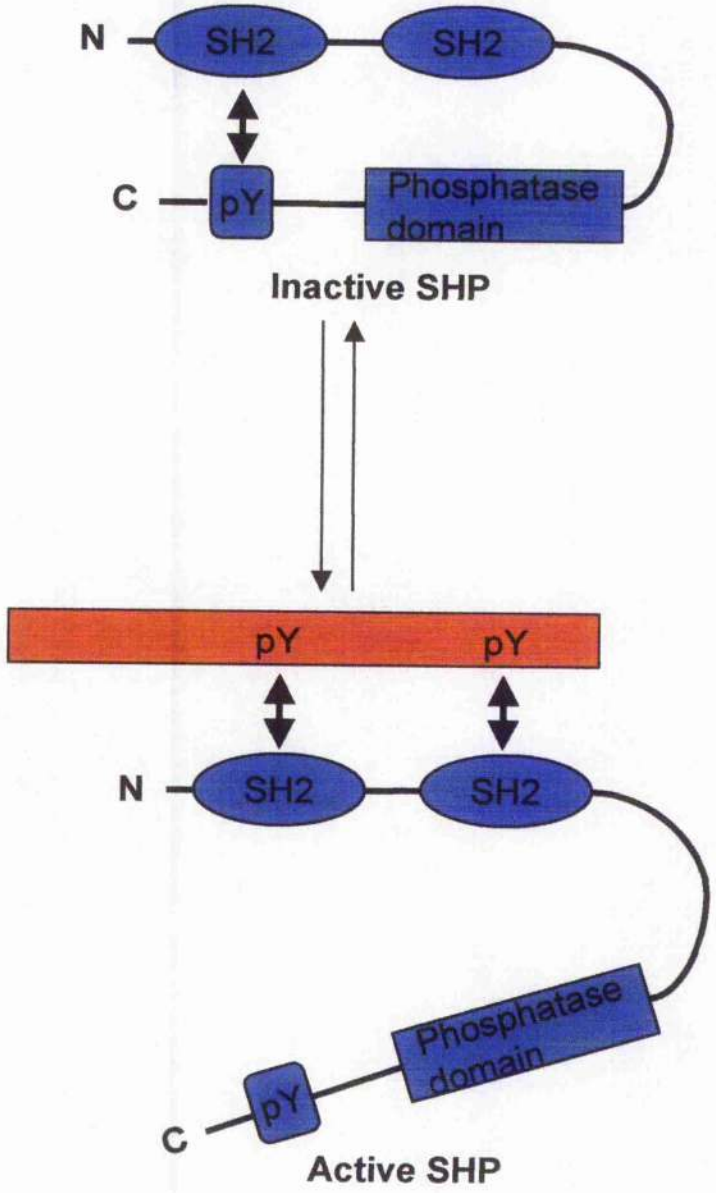
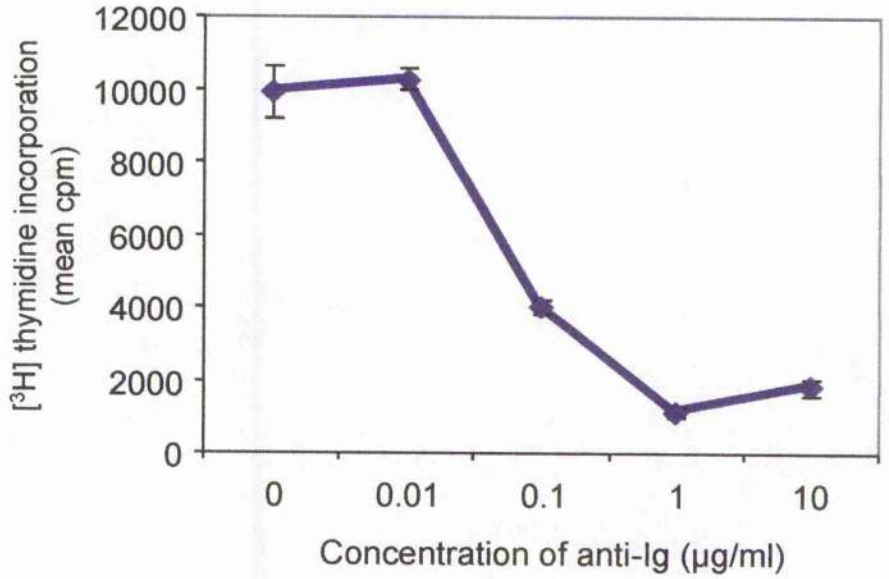

N
SH2
SH2
C
pY
Phosphatase domain
Inactive SHP
pY
pY
N
SH2
SH2
Phosphatase domain
C
pY
Active SHP

Figure 3.5. Ligation of the BCR on WEHI-231 cells induces growth arrest. **(A)** WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least twelve independent experiments. **(B)** WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of media (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least twelve independent experiments.

(A)


12000
10000
8000
6000
4000
2000
0
[3H] thymidine incorporation (mean cpm)
0
0.01
0.1
1
10
Concentration of anti-Ig (µg/ml)

(B)

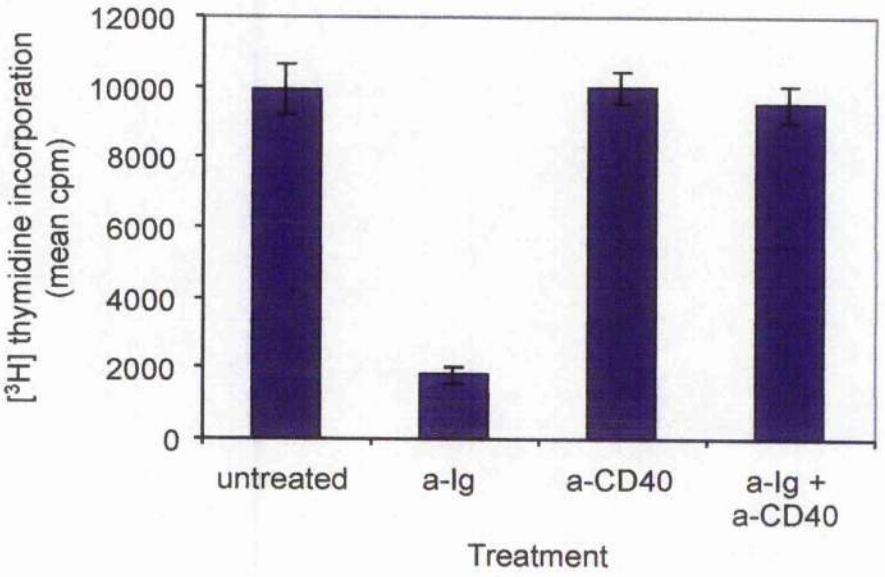
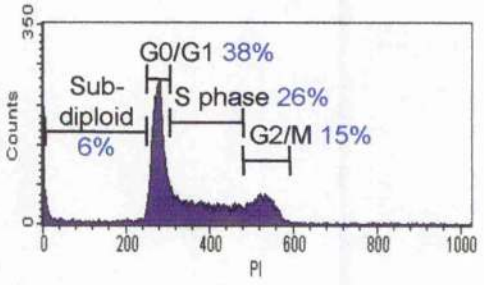
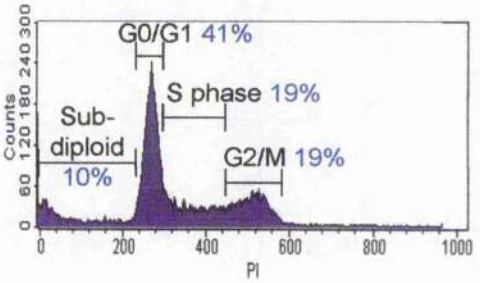

12000
10000
8000
6000
4000
2000
0
[3H] thymidine incorporation (mean cpm)
untreated
a-Ig
a-CD40
a-Ig + a-CD40
Treatment

Figure 3.6. Ligation of the BCR on WEHI-231 cells induces apoptosis. WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr with medium (untreated) **(A)**, anti-Ig (10 µg/ml) **(B)**, a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) **(C)** or anti-CD40 alone (10 µg/ml) **(D)**. Levels of apoptosis were indicated by measuring the number of cells with sub-diploid DNA and this was determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least ten independent experiments. WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated) **(E)**, anti-Ig (10 µg/ml) **(F)**, a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) **(G)** or anti-CD40 alone (10 µg/ml) **(H)**. Levels of apoptosis were indicated by measuring the number of cells with sub-diploid DNA and this was determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least ten independent experiments.

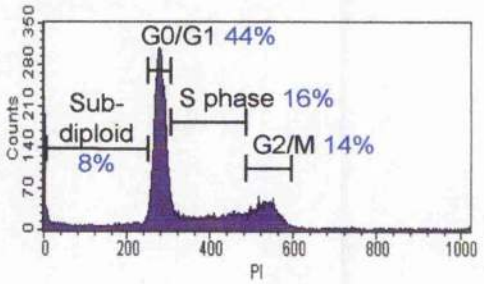
(A) Untreated 24 hr



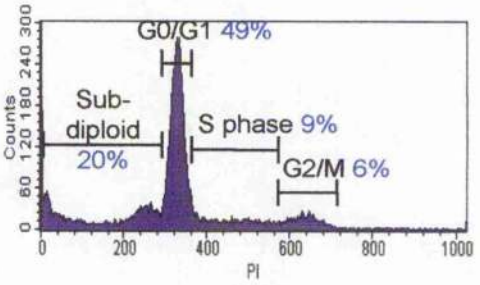
(E) Untreated 48 hr



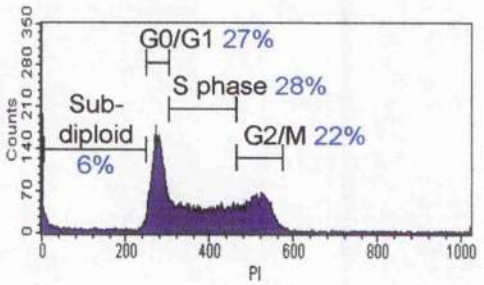
(B) Anti-Ig 24 hr



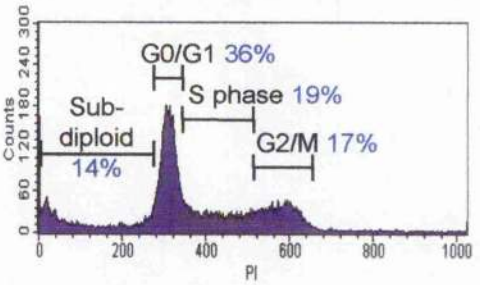
(F) Anti-Ig 48 hr



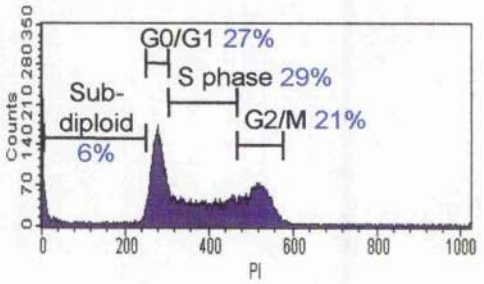
(C) Anti-Ig + anti-CD40 24 hr



(G) Anti-Ig + anti-CD40 48 hr



(D) Anti-CD40 24 hr



(H) Anti-CD40 48 hr

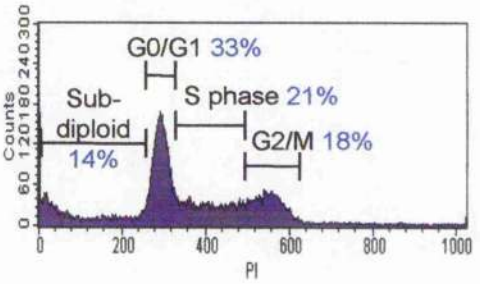


Figure 3.7. BCR-mediated downregulation of Erk activity does not involve a dissociation of MEK1/2 from Erk1/2. WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with **(A)** medium (untreated), **(B)** anti-Ig (10 µg/ml), **(C)** anti-CD40 (10 µg/ml) or **(D)** a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Erk1/2-containing immune complexes were prepared from 100 µg lysate and were analysed by Western blotting, using the NuPAGE system of gel electrophoresis, with anti-MEK1/2 antibody. Erk1/2 levels in the immune complexes were determined by dot blot analysis with Western blotting using the same anti-Erk1/2 antibody as was used to prepare the immune complexes. Ligation of the BCR does not decrease the levels of phospho-MEK1/2 in WEHI-231 cells. WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with **(E)** medium (untreated), **(F)** anti-Ig (10 µg/ml), **(G)** anti-CD40 (10 µg/ml) or **(H)** a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Tris-HCL gels using the BioRad system of gel electrophoresis. Levels of phospho-MEK (pMEK1/2) and total Erk (Erk1/2) were determined by Western blotting using phospho-MEK1/2 (pS^{217}/pS^{221}) and Erk1/2-MAPK antibodies, respectively.

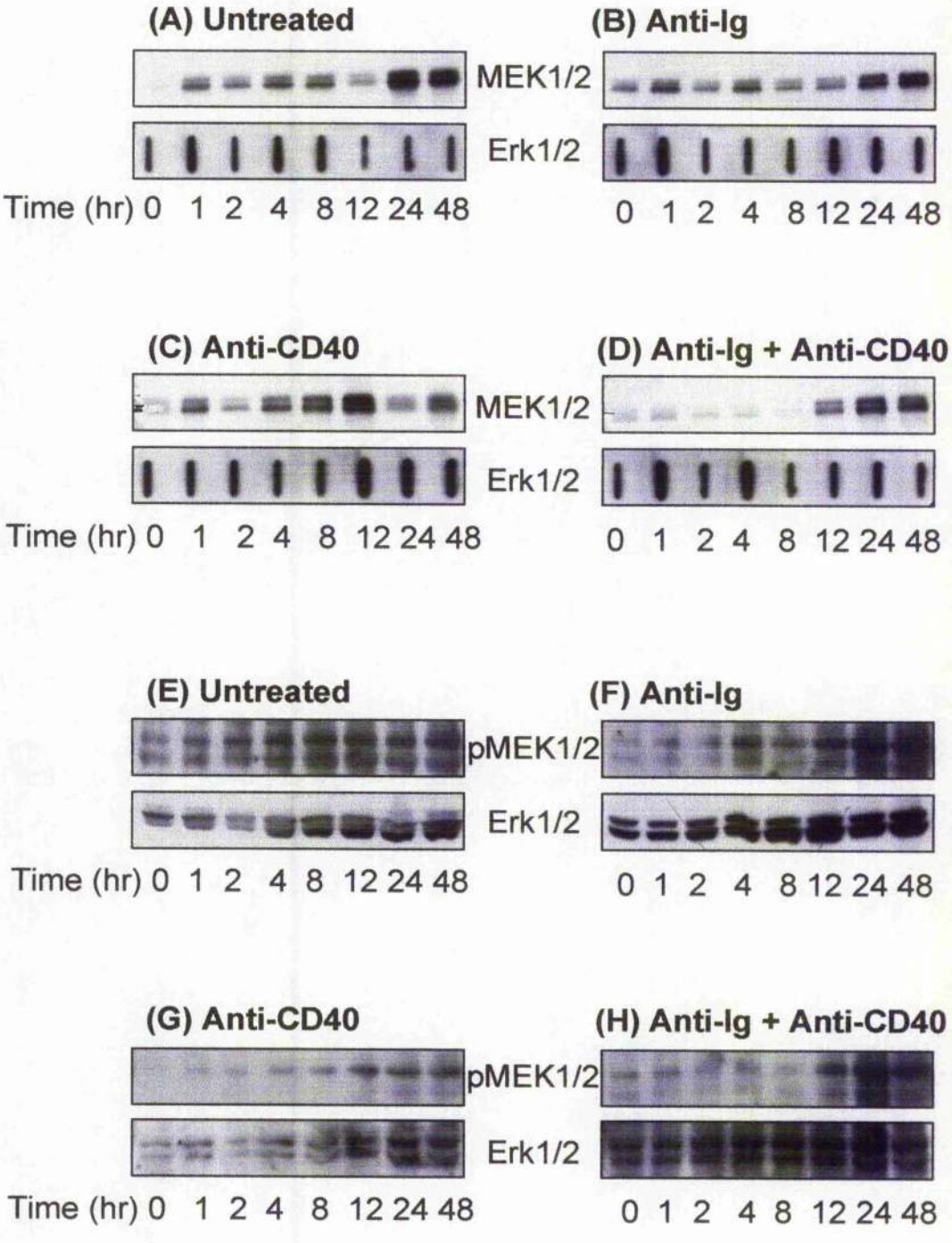
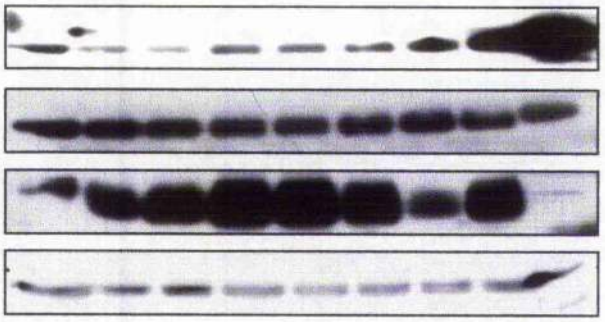

(A) Untreated
(B) Anti-Ig
MEK1/2
Erk1/2
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(C) Anti-CD40
(D) Anti-Ig + Anti-CD40
MEK1/2
Erk1/2
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(E) Untreated
(F) Anti-Ig
pMEK1/2
Erk1/2
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(G) Anti-CD40
(H) Anti-Ig + Anti-CD40
pMEK1/2
Erk1/2
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48

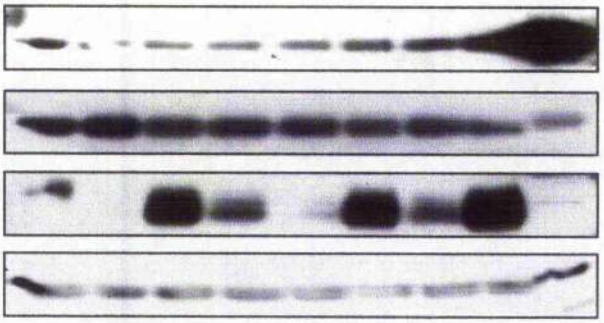
Figure 3.8. BCR-mediated downregulation of Erk activity does not reflect suppression of MEK activation. WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with either medium (untreated) **(A)** or 10 µg/ml anti-Ig **(B)** before preparing cell lysates. MEK-containing immune complexes were prepared from 100 µg lysate and *in vitro* MEK kinase assays were conducted using recombinant Erk-GST fusion protein as substrate and Western blotting using the BioRad electrophoresis system. A positive control for this assay is illustrated in lane *+ve* and reflected an *in vitro* kinase assay using recombinant human activated MEK1 (0.5 U) and the Erk-GST substrate. In addition, MEK kinase activity was supported by analysis of MEK activation by Western blotting using anti-phospho-MEK1/2 (pMEK1/2) antibodies that recognise the active form of MEK. Loading control for Erk-GST and MEK are also shown. Data are representative of at least two independent experiments.

(A) Untreated



Time (hr) 0 1 2 4 8 12 24 48 +ve

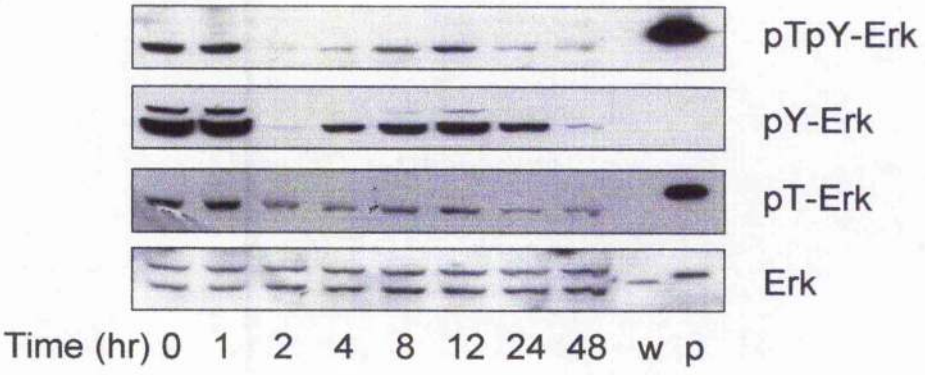
(B) Anti-Ig



Time (hr) 0 1 2 4 8 12 24 48 +ve

Figure 3.9. Erk is differentially phosphorylated at the T-E-Y motif of the activation loop. WEHI-231 cells (1 x 10^6 cells/ml) were stimulated with medium (untreated) **(A)** or 10 µg/ml anti-Ig **(B)** for up to 48 hr and whole cell lysates were prepared. Differential phosphorylation of the regulatory Thr and Tyr residues of Erk was determined by Western blotting of whole cell lysates (100 µg protein/lane), using 10% Bis-Tris gels on the NuPAGE electrophoresis system (Invitrogen) with recombinant non-phosphorylated Erk2 (w) and recombinant dual-phosphorylated Erk2 (p) control proteins. Phosphorylation-sensitive antibodies for Erk-MAP kinase were used in Western blotting. Single phosphorylated forms of Erk were detected using anti-phospho-Tyr^{204}-p44/p42 MAPK (pY-Erk) and anti-phospho-Thr^{183}-p44/p42 MAPK (pT-Erk) antibodies. The anti-phospho-Thr^{183} antibody detected some dual-phosphorylated Erk, as indicated by its ability to detect control protein p. Anti-phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}) (pTpY-Erk) and p44/p42 MAPK (Erk) antibodies were used to detect dual phosphorylated and total Erk levels, respectively.

(A) Untreated



(B) Anti-Ig

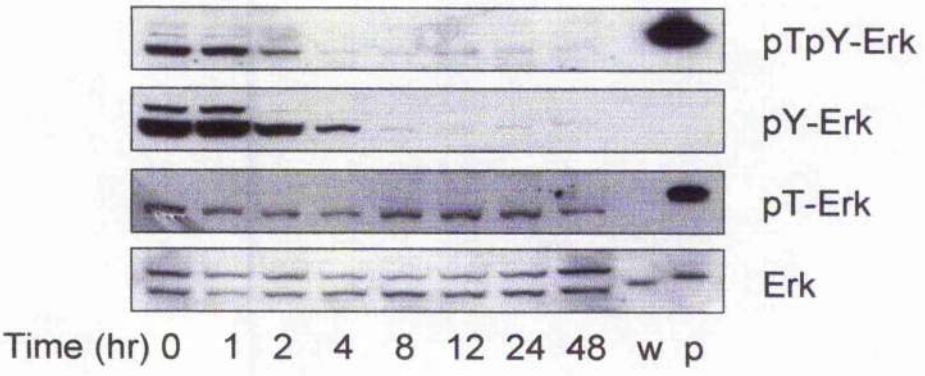


Figure 3.10. SHP-1 and SHP-2 interact with Erk1/2 in WEHI-231 cells. WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated) **(A)**, anti-Ig (10 µg/ml) **(B)**, anti-Ig plus anti-CD40 (both at 10 µg/ml) **(C)** or anti-CD40 (10 µg/ml) **(D)** before preparing cell lysates. Erk1/2-containing immune complexes were prepared from 100 µg lysate and were analysed by Western blotting, using the NuPAGE system of gel electrophoresis, with anti-SHP-1 antibody. Erk1/2 levels in the immune complexes were determined by dot blot analysis with Western blotting using the same anti-Erk1/2 antibody as was used to prepare the immune complexes. WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated) **(E)**, anti-Ig (10 µg/ml) **(F)**, anti-Ig plus anti-CD40 (both at 10 µg/ml) **(G)** or anti-CD40 (10 µg/ml) **(H)** before preparing cell lysates. Erk1/2-containing immune complexes were prepared from 100 µg lysate and were analysed by Western blotting, using the NuPAGE system of gel electrophoresis, with anti-SHP-2 antibody. Erk1/2 levels in the immune complexes were determined by dot blot analysis with Western blotting using the same anti-Erk1/2 antibody as was used to prepare the immune complexes.

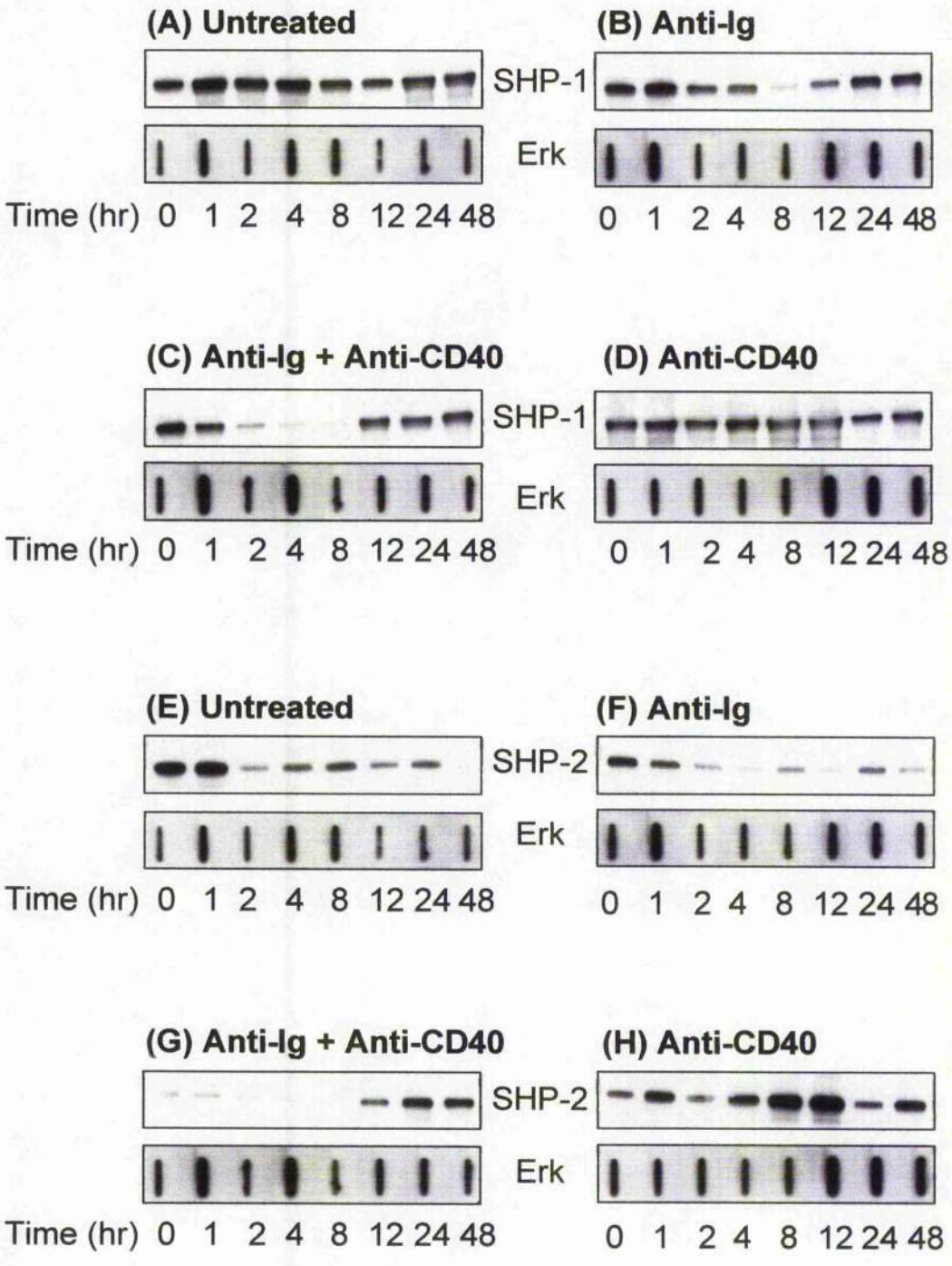

(A) Untreated
(B) Anti-Ig
SHP-1
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(C) Anti-Ig + Anti-CD40
(D) Anti-CD40
SHP-1
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(E) Untreated
(F) Anti-Ig
SHP-2
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
(G) Anti-Ig + Anti-CD40
(H) Anti-CD40
SHP-2
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48

Figure 3.11. Ras can stimulate Erk via Raf or PI-3-K in WEHI-231 cells. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pcDNA3.1 vector **(A)**, $RasV^{12}$-containing vector **(B)**, $RasV^{12}S^{35}$-containing vector **(C)**, $RasV^{12}C^{40}$-containing vector **(D)** or ΔMEKK3-containing vector **(E)** were cultured for up to 24 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting. All pErk Western blots were developed on the same piece of film and all Erk Western blots were developed on a single piece of film. The pErk signal was relatively weak in pcDNA3.1 cells therefore a longer exposure to photographic film has also been shown (upper pErk panel in figure 3.11A). Data are representative of at least two independent experiments.

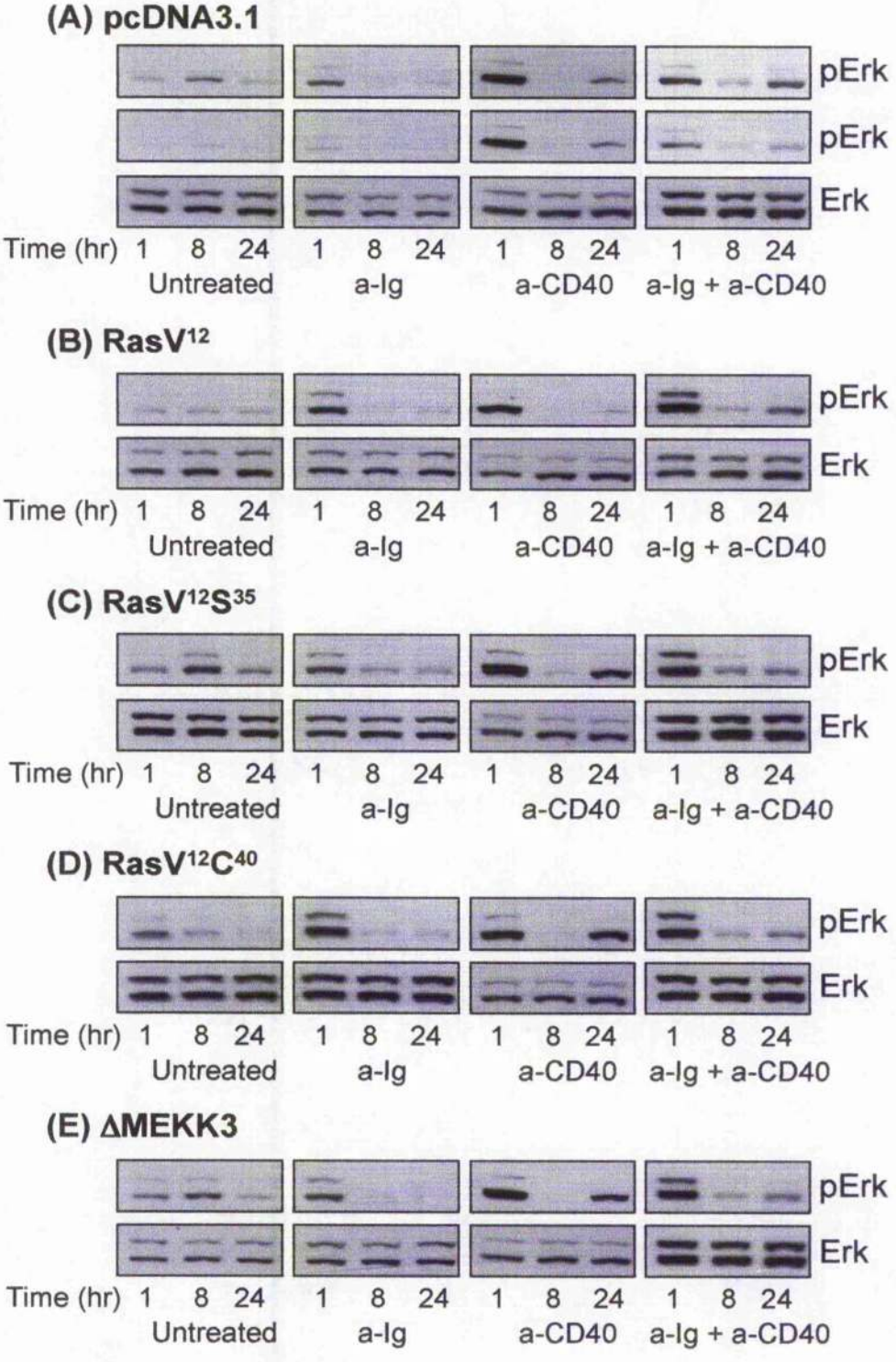
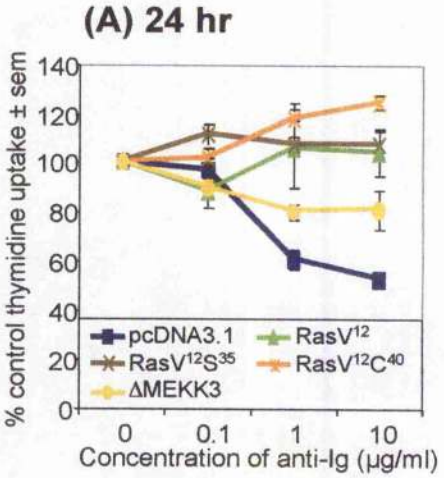
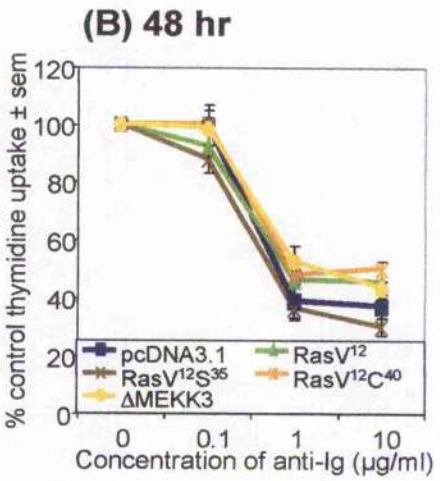
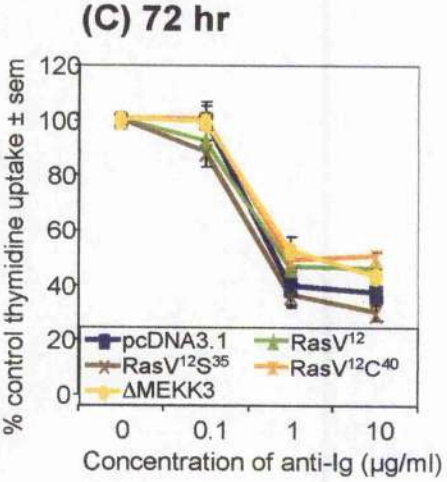
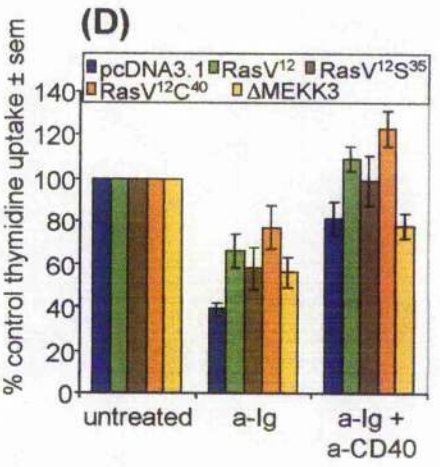

(A) pcDNA3.1
pErk
pErk
Erk
Time (hr) 1 8 24 1 8 24 1 8 24 1 8 24
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) RasV12
pErk
Erk
Time (hr) 1 8 24 1 8 24 1 8 24 1 8 24
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) RasV12S35
pErk
Erk
Time (hr) 1 8 24 1 8 24 1 8 24 1 8 24
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) RasV12C40
pErk
Erk
Time (hr) 1 8 24 1 8 24 1 8 24 1 8 24
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) ΔMEKK3
pErk
Erk
Time (hr) 1 8 24 1 8 24 1 8 24 1 8 24
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.12. Constitutive activation of Ras partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis. WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pcDNA3.1 vector, $RasV^{12}$-containing vector, $RasV^{12}S^{35}$-containing vector, $RasV^{12}C^{40}$-containing vector or ΔMEKK3-containing vector were cultured in the presence of increasing concentrations of anti-Ig (0-10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)**, 48 hr **(B)** or 72 hr **(C)**. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. **(D)** WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pcDNA3.1 vector, $RasV^{12}$-containing vector, $RasV^{12}S^{35}$-containing vector, $RasV^{12}C^{40}$-containing vector or ΔMEKK3-containing vector were cultured in the presence of media (untreated), anti-Ig (a-Ig, 10 μg/ml), or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. **(E)** WEHI-231 cells (5 x 10^5 cells/ml) transfected with empty pcDNA3.1 vector, $RasV^{12}$-containing vector, $RasV^{12}S^{35}$-containing vector, $RasV^{12}C^{40}$-containing vector or ΔMEKK3-containing vector were cultured for 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence).


(A) 24 hr
% control thymidine uptake ± sem
140
120
100
80
60
40
20
0
pcDNA3.1
RasV12
RasV12S35
RasV12C40
ΔMEKK3
0
0.1
1
10
Concentration of anti-Ig (μg/ml)


(B) 48 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
RasV12
RasV12S35
RasV12C40
ΔMEKK3
0
0.1
1
10
Concentration of anti-Ig (μg/ml)


(C) 72 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
RasV12
RasV12S35
RasV12C40
ΔMEKK3
0
0.1
1
10
Concentration of anti-Ig (μg/ml)


(D)
% control thymidine uptake ± sem
pcDNA3.1
RasV12
RasV12S35
RasV12C40
ΔMEKK3
140
120
100
80
60
40
20
0
untreated
a-Ig
a-Ig +
a-CD40

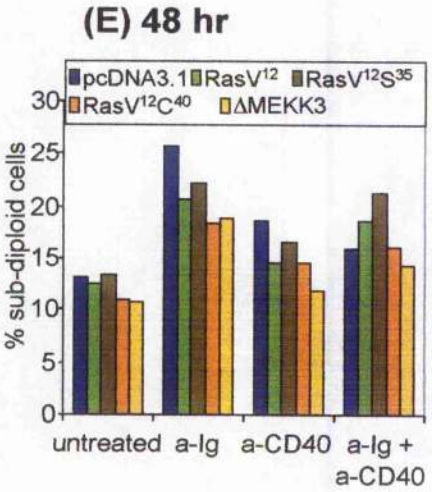

(E) 48 hr
pcDNA3.1
RasV12
RasV12S35
RasV12C40
ΔMEKK3
% sub-diploid cells
30
25
20
15
10
5
0
untreated
a-Ig
a-CD40
a-Ig +
a-CD40

Figure 3.13. Ras-dependent activation of PI-3-K does not stimulate Akt. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pcDNA3.1 vector **(A)**, $RasV^{12}$-containing vector **(B)**, $RasV^{12}S^{35}$-containing vector **(C)**, $RasV^{12}C^{40}$-containing vector **(D)** or ΔMEKK3-containing vector **(E)** were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1* 1 hr, *lane 2* 8 hr, *lane 3* 24 hr, *lane 4* 48 hr, *lane 5* non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002), *lane 6* phosphorylated Akt control (total cell extracts prepared from untreated Jurkat cells). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting. Data are representative of at least two independent experiments.

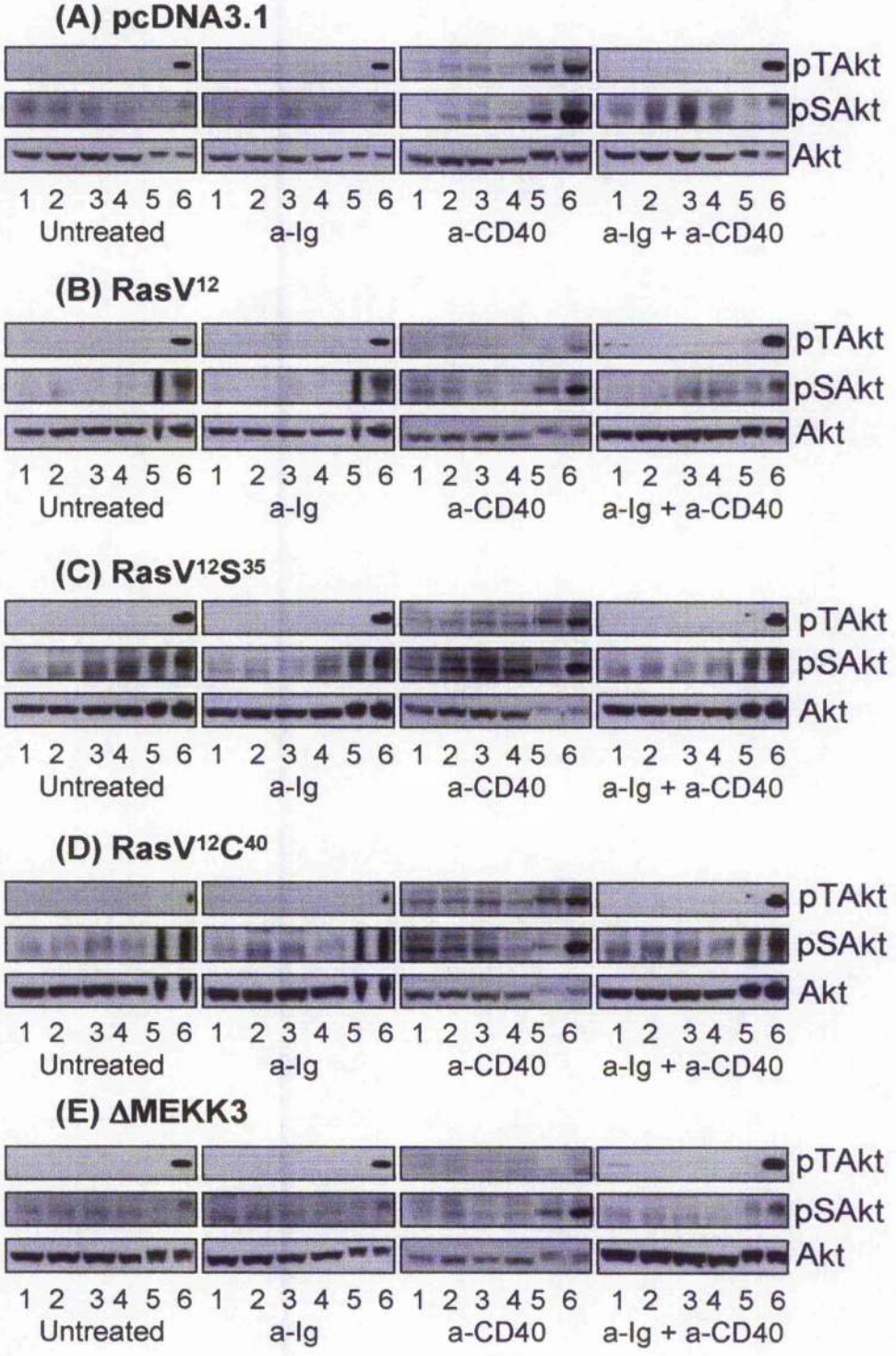

(A) pcDNA3.1
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) RasV12
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) RasV12S35
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) RasV12C40
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) ΔMEKK3
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.14. Ras can induce Bcl-x_L in WEHI-231 cells especially via the activation of PI-3-K. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pcDNA3.1 vector **(A)**, $RasV^{12}$-containing vector **(B)**, $RasV^{12}S^{35}$-containing vector **(C)**, $RasV^{12}C^{40}$-containing vector **(D)** or ΔMEKK3-containing vector **(E)** were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting. Total Erk was detected to check the gel loading. Data are representative of at least two independent experiments.

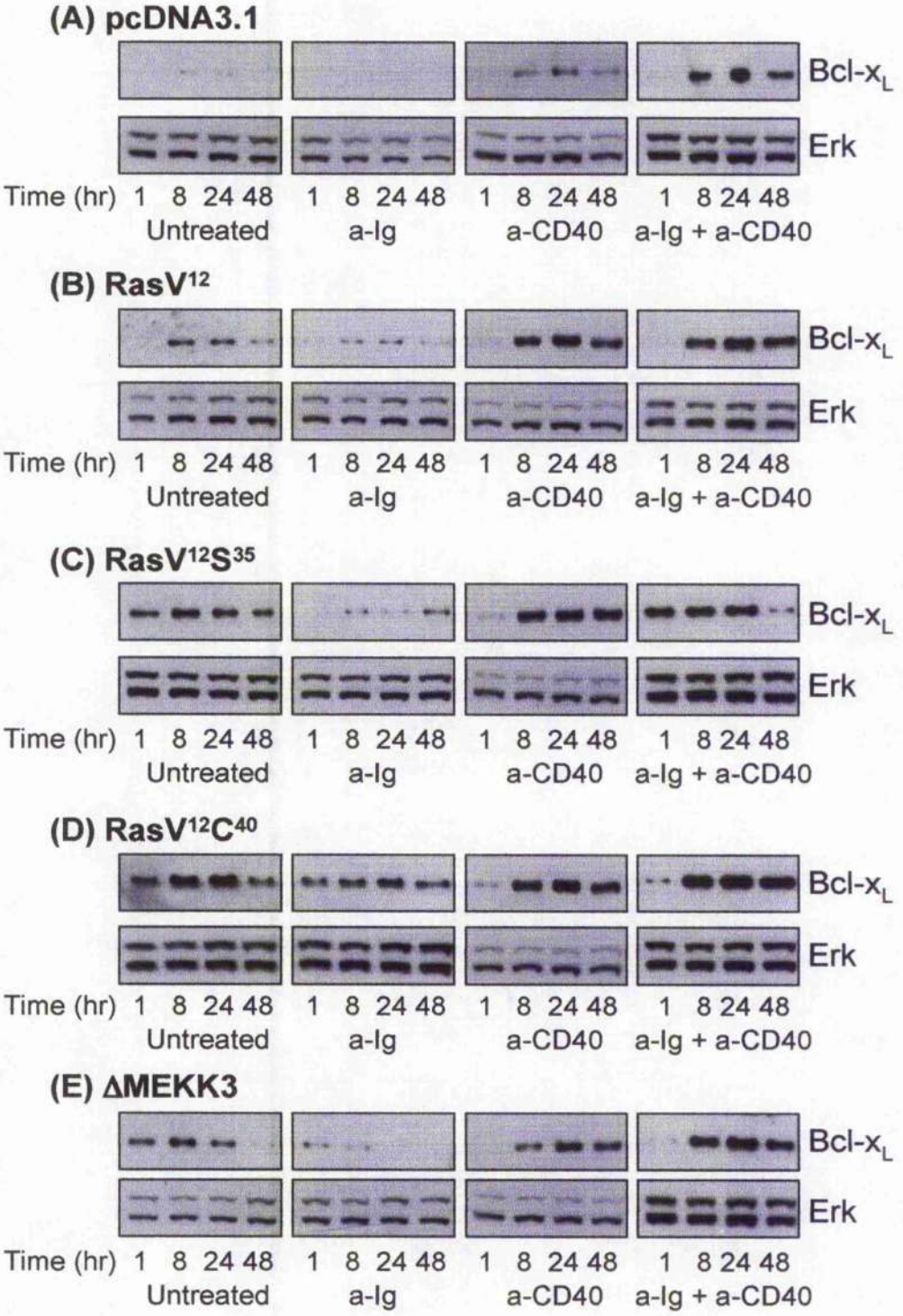

(A) pcDNA3.1
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) RasV^{12}
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) RasV^{12}S^{35}
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) RasV^{12}C^{40}
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) ΔMEKK3
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.15. Inhibition of Dok can increase the activity of Erk but does not stimulate Akt or Bcl-x_L in BCR-stimulated WEHI-231 cells. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(A)** or Dok-PH/PTB-containing vector **(B)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(C)** or Dok-PH/PTB-containing vector **(D)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1* 1 hr, *lane 2* 8 hr, *lane 3* 24 hr, *lane 4* 48 hr, *lane 5* phosphorylated Akt control (total cell extracts from untreated Jurkat cells), *lane 6* non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(E)** or Dok-PH/PTB-containing vector **(F)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting. Total Erk was detected to check the gel loading. Data are representative of at least two independent experiments.

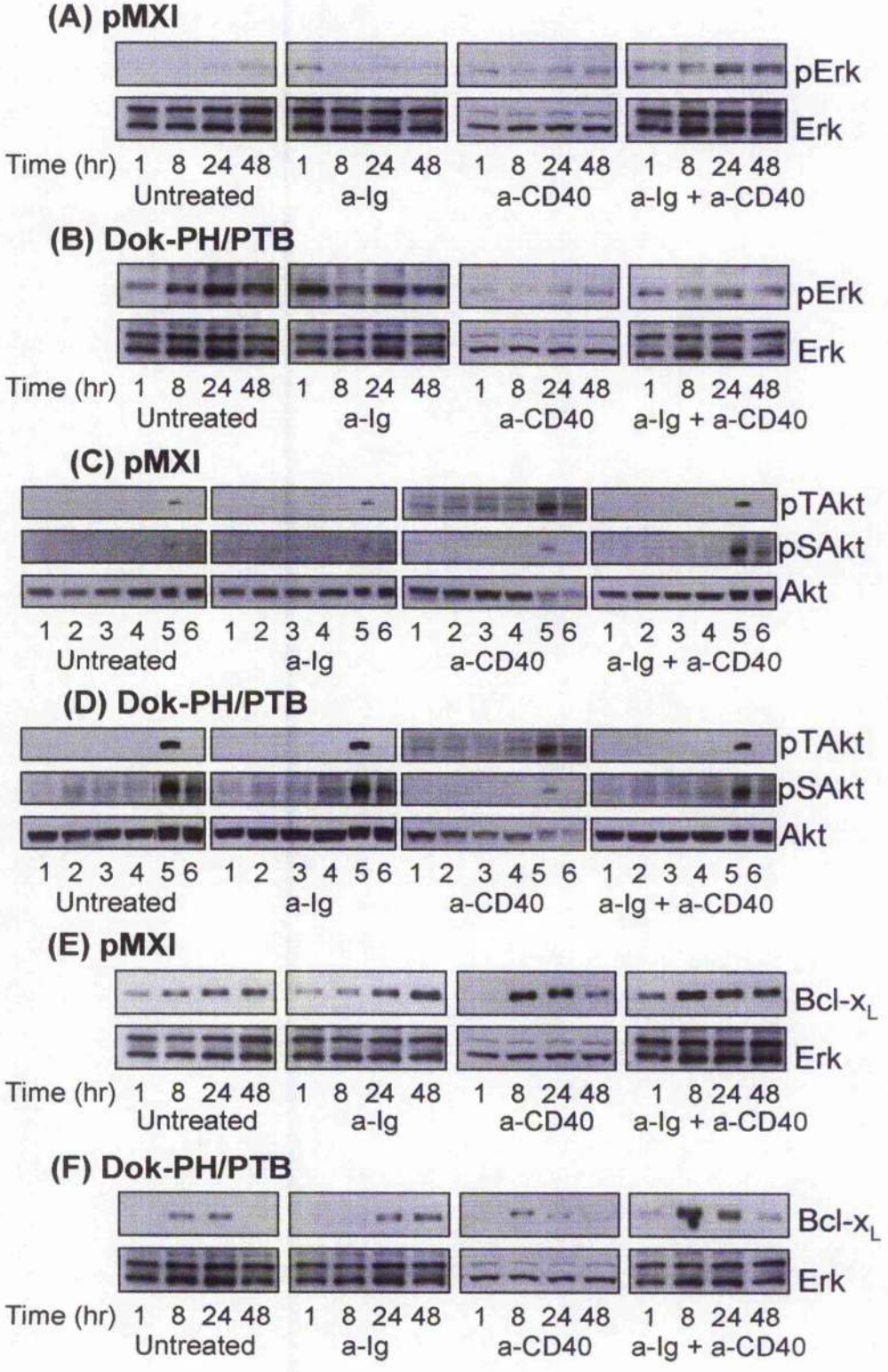

(A) pMXI
pErk
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) Dok-PH/PTB
pErk
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) pMXI
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) Dok-PH/PTB
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) pMXI
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) Dok-PH/PTB
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.16. Inhibition of Dok does not substantially protect WEHI-231 cells from BCR-driven growth arrest and apoptosis. WEHI-231 cells (5 x 10^5 cells/ml) transfected with empty pMXI-egfp vector or Dok-PH/PTB-containing vector were cultured for 24 hr **(A)** or 48 hr **(B)** with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence). WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pMXI-egfp vector or Dok-PH/PTB-containing vector were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(C)**, 48 hr **(D)** or 72 hr **(E)**. Values are the mean % control (pMXI-egfp WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. **(F)** WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pMXI-egfp vector or Dok-PH/PTB-containing vector were cultured in the presence of media (untreated), anti-Ig (10 µg/ml), or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pMXI-egfp WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate.

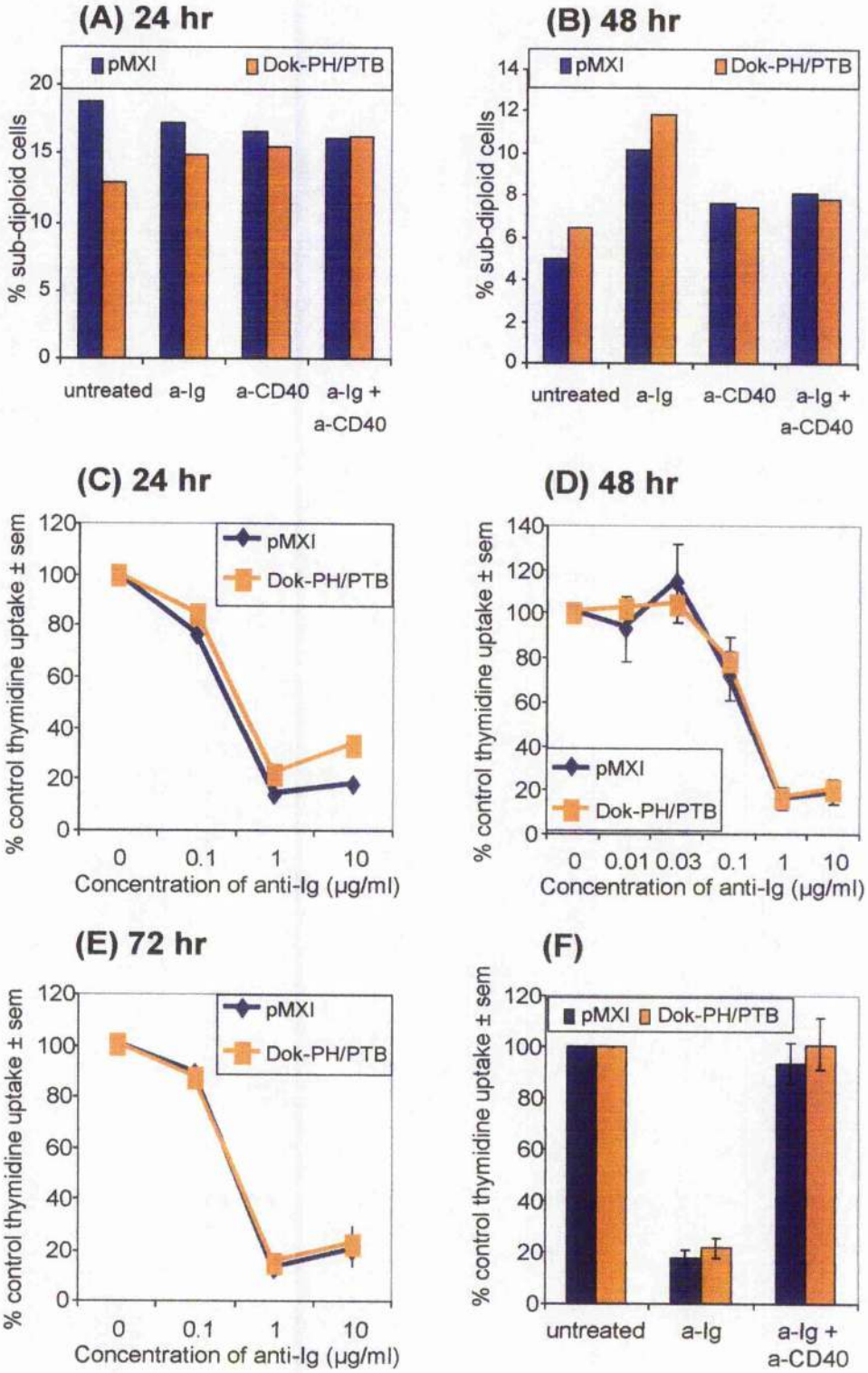
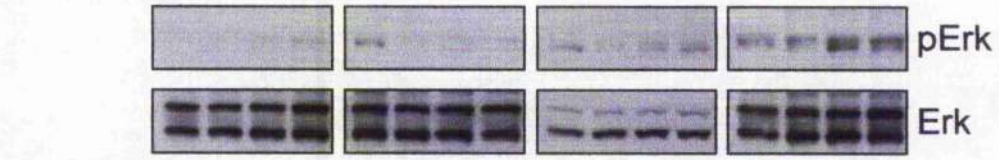

(A) 24 hr
pMXI
Dok-PH/PTB
% sub-diploid cells
0
5
10
15
20
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) 48 hr
pMXI
Dok-PH/PTB
% sub-diploid cells
0
2
4
6
8
10
12
14
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) 24 hr
pMXI
Dok-PH/PTB
% control thymidine uptake ± sem
0
20
40
60
80
100
120
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(D) 48 hr
pMXI
Dok-PH/PTB
% control thymidine uptake ± sem
0
20
40
60
80
100
120
140
0
0.01
0.03
0.1
1
10
Concentration of anti-Ig (µg/ml)
(E) 72 hr
pMXI
Dok-PH/PTB
% control thymidine uptake ± sem
0
20
40
60
80
100
120
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(F)
pMXI
Dok-PH/PTB
% control thymidine uptake ± sem
0
20
40
60
80
100
120
untreated
a-Ig
a-Ig + a-CD40

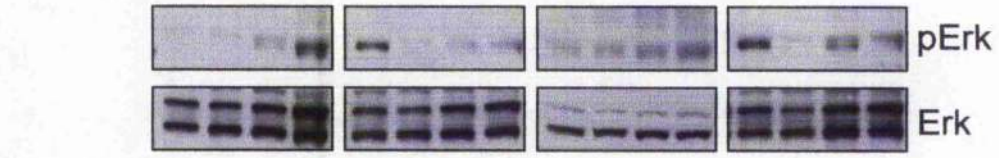
Figure 3.17. Inhibition of SHIP increases Erk activation but cannot induce constitutive Erk signalling. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(A)**, SHIP-CI-containing vector **(B)** or SHIP-SH2-containing vector **(C)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting. Data are representative of at least two independent experiments.

(A) pMXI



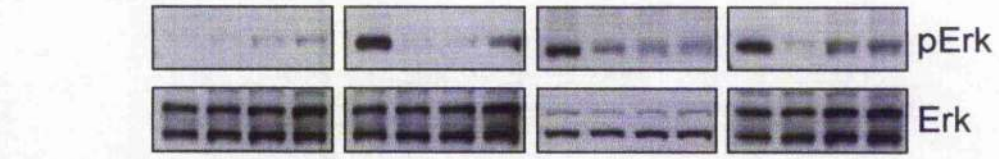
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

(B) SHIP-CI



Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

(C) SHIP-SH2



Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

Figure 3.18. Expression of SHIP partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis. WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pMXI-egfp vector, SHIP-CI-containing vector or SHIP-SH2-containing vector were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)**, 48 hr **(B)** or 72 hr **(C)**. Values are the mean % control (pMXI-egfp WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. **(D)** WEHI-231 cells (1 x 10^4 cells/well) transfected with empty pMXI-egfp vector, SHIP-CI-containing vector or SHIP-SH2-containing vector were cultured in the presence of media (untreated), anti-Ig (10 µg/ml), or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pMXI-egfp WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. WEHI-231 cells (5 x 10^5 cells/ml) transfected with empty pMXI-egfp vector, SHIP-CI-containing vector or SHIP-SH2-containing vector were cultured for 24 hr **(E)** or 48 hr **(F)** with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence).

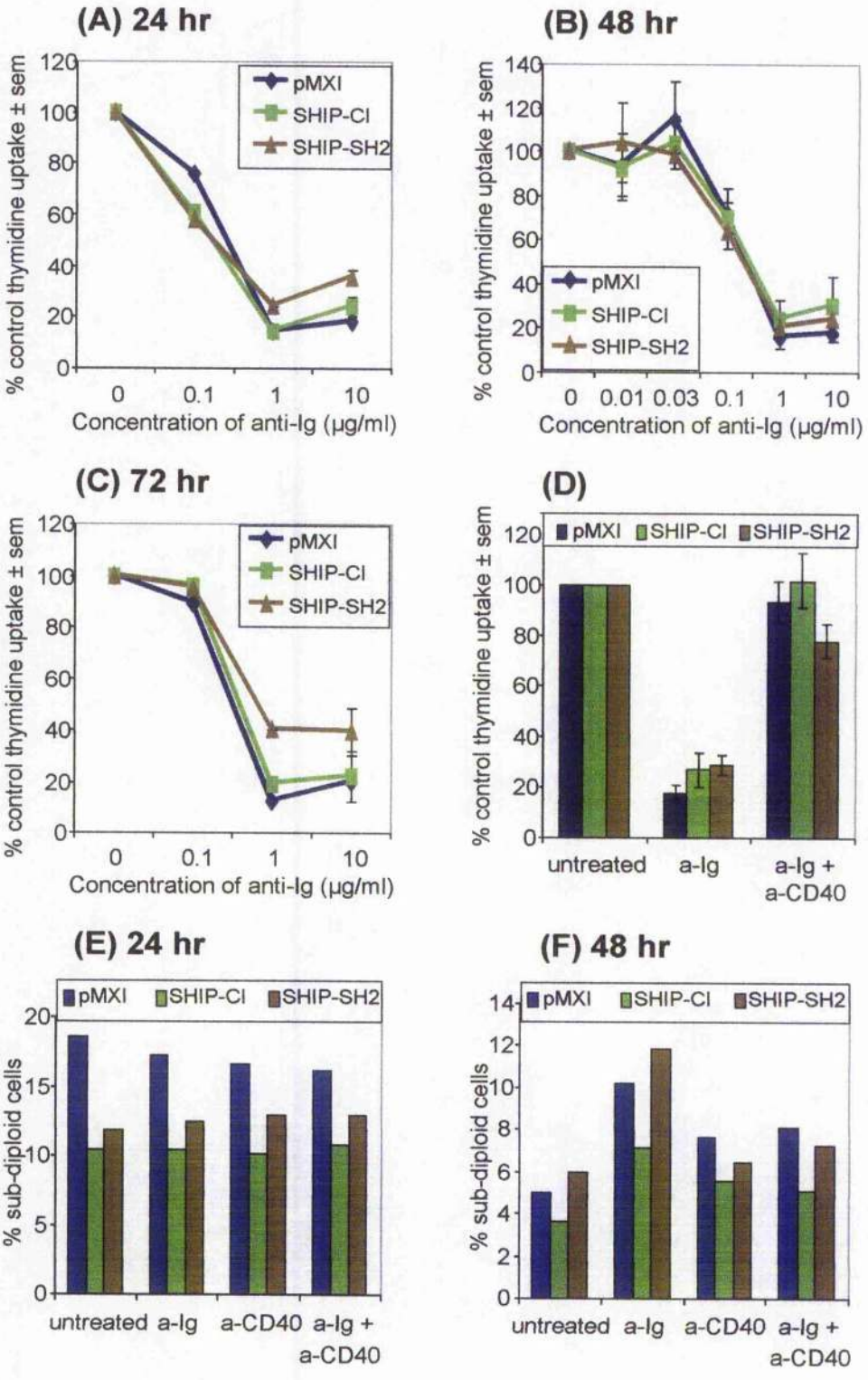

(A) 24 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pMXI
SHIP-CI
SHIP-SH2
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(B) 48 hr
% control thymidine uptake ± sem
140
120
100
80
60
40
20
0
pMXI
SHIP-CI
SHIP-SH2
0
0.01
0.03
0.1
1
10
Concentration of anti-Ig (µg/ml)
(C) 72 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pMXI
SHIP-CI
SHIP-SH2
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(D)
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pMXI
SHIP-CI
SHIP-SH2
untreated
a-Ig
a-Ig + a-CD40
(E) 24 hr
% sub-diploid cells
20
15
10
5
0
pMXI
SHIP-CI
SHIP-SH2
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) 48 hr
% sub-diploid cells
14
12
10
8
6
4
2
0
pMXI
SHIP-CI
SHIP-SH2
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.19. Inhibition of SHIP does not activate Akt but can induce Bcl-x_L. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(A)**, SHIP-CI-containing vector **(B)** or SHIP-SH2-containing vector **(C)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1* 1 hr, *lane 2* 8 hr, *lane 3* 24 hr, *lane 4* 48 hr, *lane 5* phosphorylated Akt control (total cell extracts from untreated Jurkat cells), *lane 6* non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting. Data are representative of at least two independent experiments. WEHI-231 cells (1 x 10^6 cells/ml) transfected with empty pMXI-egfp vector **(D)**, SHIP-CI-containing vector **(E)** or SHIP-SH2-containing vector **(F)** were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting. Total Erk was detected to check the gel loading. Data are representative of at least two independent experiments.

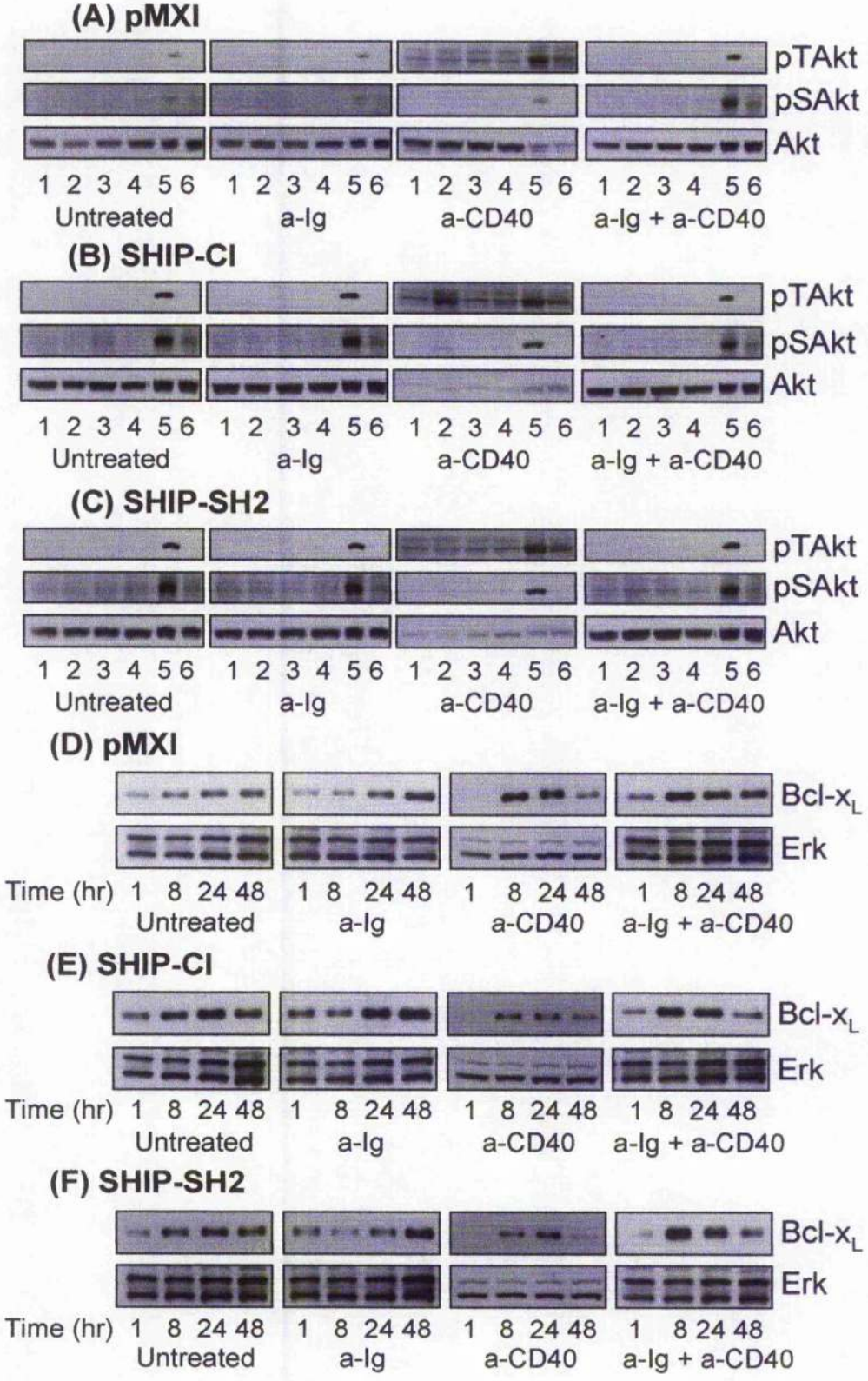
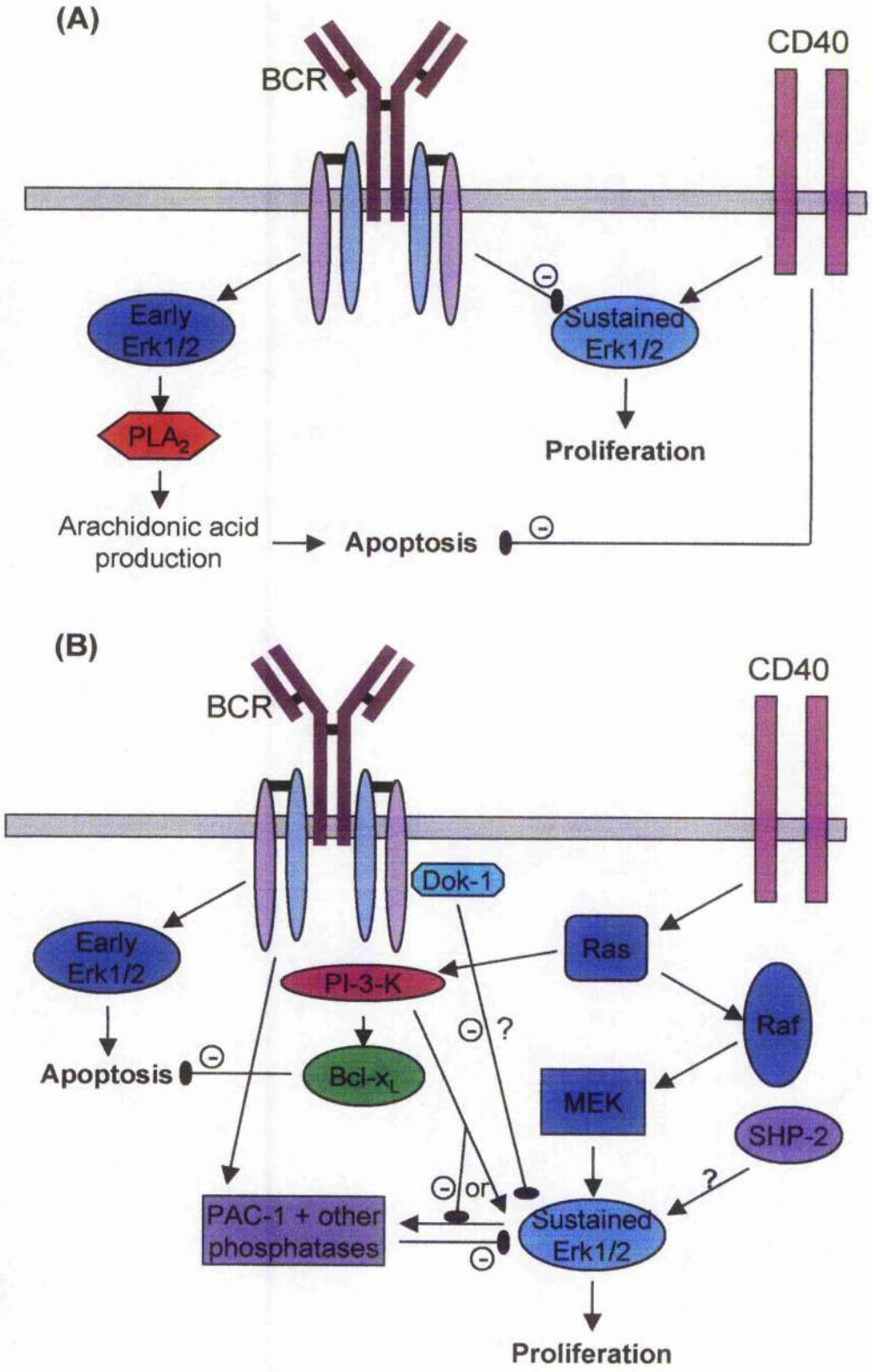

(A) pMXI
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) SHIP-CI
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) SHIP-SH2
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) pMXI
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) SHIP-CI
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) SHIP-SH2
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 3.20. Models of differential regulation of Erk by the BCR and CD40. **(A)** Ligation of the BCR induces early (≤4 hr) Erk signals that lead to the production of arachidonic acid and induction of apoptosis. WEHI-231 cells also undergo sustained and cyclic activation of Erk that is inhibited by the BCR but can be restored by co-ligation of CD40. These sustained Erk signals are necessary for proliferation of the cells. **(B)** The regulation of sustained Erk signals is a complex process. Ras can activate sustained Erk via Raf-1 or PI-3-K. The BCR does not inhibit sustained Erk signals by suppressing upstream regulators of Erk but instead induces PAC-1 and other MAPK phosphatases. PI-3-K might induce Erk directly or may prevent the induction of MAPK phosphatases. PI-3-K is also important for inducing Bcl-x_L to impair the BCR-driven apoptotic pathway. Additional molecules are likely to regulate the activity of Erk including Dok, which may be activated by the BCR to suppress Erk and SHP-2, which might enhance Erk activation.


(A)
BCR
CD40
Early Erk1/2
Sustained Erk1/2
PLA2
Proliferation
Arachidonic acid production
Apoptosis
(B)
BCR
CD40
Dok-1
Early Erk1/2
Ras
PI-3-K
Raf
Apoptosis
Bcl-xL
MEK
SHP-2
or
PAC-1 + other phosphatases
Sustained Erk1/2
Proliferation

CHAPTER 4 - Role of Bcl-x_L in CD40-mediated rescue from BCR-driven growth arrest and apoptosis

4.1 Regulation of apoptosis in immature B cells

Apoptosis plays a key role in the removal of self-reactive clones of newly developed B lymphocytes. WEHI-231 cells are widely used as a model of clonal deletion of immature B cells since ligation of the BCR induces growth arrest and apoptosis whilst cells can be rescued by T cell-dependent factors such as co-ligation of CD40 (108, 292, 305). However, the precise mechanisms of BCR-driven clonal deletion and CD40-mediated rescue are incompletely understood.

Recently (102) it has been shown that ligation of the BCR on immature B cells leads to the activation of mitochondrial PLA_2 and subsequent arachidonic acid-mediated disruption of mitochondrial membrane potential and function. This causes the cellular levels of ATP to be depleted and apoptosis to be induced. Moreover, WEHI-231 cells can be protected from BCR-driven apoptosis using stabilisers of the mitochondrial membrane potential or by CD40-mediated signalling (102).

It has also been shown that post-mitochondrial execution of such BCR-driven apoptosis is not dependent on caspase activation since BCR-driven disruption of the mitochondria does not cause cytochrome c to be released into the cytoplasm and effector caspases do not appear to be activated. Furthermore, BCR-driven loss of mitochondrial membrane potential and commitment to apoptosis can proceed in the presence of caspase inhibitors. In contrast, cathepsin B is stimulated by ligation of the BCR and inhibition of cathepsin B using EST prevents post-mitochondrial induction of apoptosis in WEHI-231 cells (102). Figure 1.22 summarises this pathway of BCR-driven apoptosis also see section 1.10.1 for further details.

4.2 Regulation of proliferation in immature B cells

As discussed in chapter 3, BCR-driven apoptosis of WEHI-231 cells requires the activation of early Erk signals that probably function to stimulate $cPLA_2$. In contrast, sustained and cyclic activation of Erk is observed in proliferating WEHI-231 cells. Ligation of the BCR suppresses sustained Erk

signals leading to the induction of growth arrest whilst co-ligation of CD40 restores these Erk signals and enables the cells to proliferate (328). Sustained and cyclic activation of Erk is thus necessary for proliferation of WEHI-231 B cells but precisely how these signals enable proliferation is incompletely understood.

As discussed in section 1.10.2, the BCR and CD40 can differentially regulate several proteins involved in controlling cell cycle progression. For example, in BCR-stimulated immature B cells Cdk4/cyclin D2 is induced, allowing the cells to progress through the early stages of G1 phase but these cells cannot induce Cdk2/cyclin E and hence are unable to progress through the G1 to S phase transition (33). Furthermore, ligation of the BCR on WEHI-231 cells increases the levels of p21 and p27 inhibitors of Cdk proteins and of p53, leading to growth arrest and promoting apoptosis. Moreover, Rb is hypophosphorylated in BCR-activated WEHI-231 cells, leading to the formation of Rb-E2F complexes and hence an inhibition of E2F. This causes cyclin A to be downregulated to further prevent S phase entry. However, co-ligation of CD40 results in the phosphorylation and inhibition of Rb and rescues the cells from BCR-driven growth arrest (53, 56, 58).

4.3 CD40-mediated rescue

CD40 can prevent BCR-driven growth arrest and apoptosis of immature B cells (102, 328). Although the mechanism of CD40-mediated rescue of BCR-stimulated WEHI-231 cells is incompletely understood, CD40-dependent survival is likely to involve the induction of pro-survival Bcl-2 family members (see sections 1.4.1 and 1.10.1.1). For example, CD40 induces an upregulation of Bcl-x_L without enhancing the expression of Bcl-2 or Bax (290, 292, 293) and overexpression of Bcl-x_L in WEHI-231 cells protects the cells from BCR-driven apoptosis (292, 311). The upregulation of Bcl-x_L can thus protect immature B cells from BCR-driven apoptosis. Furthermore, the region of CD40 that is required for the induction of Bcl-x_L is also necessary for the prevention of BCR-driven apoptosis (312).

However, additional anti-apoptotic Bcl-2 family members, especially A1 and Mcl-1, may also contribute to the survival of immature B cells (302). For example, stimulation of WEHI-231 cells at CD40 can upregulate the expression

of the *a1* gene and overexpression of A1 can partially protect the cells from BCR-driven apoptosis (304). However, A1 seems to protect the immature B cells by suppressing the activation of caspase 7 to impair BCR-dependent DNA laddering (317). A1 therefore may not prevent $cPLA_2$-mediated apoptosis in BCR-stimulated immature B cells.

4.4 Phospholipase A_2

The hydrolysis of membrane phospholipids is an important source of lipid second messengers. The phospholipase A_2 (PLA_2) family of enzymes hydrolyse phospholipids to generate free fatty acid. There are three types of PLA_2 enzymes: secretory PLA_2, calcium-independent PLA_2 and cytosolic PLA_2 ($cPLA_2$). Secretory PLA_2 is a 14 kD, ubiquitously expressed and calcium-dependent enzyme that can release several fatty acids including sn-2 arachidonic acid (429, 430). Calcium-independent PLA_2 also has broad substrate specificity but it does not require calcium for its activation. It exists as 26 kD and 86 kD forms. Cytosolic PLA_2 is an 85 kD enzyme that does not share any homology to secreted or calcium-independent PLA_2 enzymes and it preferentially hydrolyses sn-2 arachidonic acid from membrane phospholipids (429). Arachidonic acid is an important second messenger and is a precursor of eicosanoids.

The activation of $cPLA_2$ requires both calcium and Erk-MAPK-mediated phosphorylation. Calcium binds to the N-terminal region of $cPLA_2$, enabling it to translocate from the cytosol to the plasma membrane and other intracellular membranes (289). This brings $cPLA_2$ into close proximity to its lipid substrates but this alone is insufficient to promote prolonged activation of $cPLA_2$ and arachidonic acid production. Sustained activation of $cPLA_2$ requires Erk-MAPK-mediated phosphorylation of Ser^{505}. Indeed, phosphorylation of Ser^{505} and calcium binding synergistically mediate the activation of $cPLA_2$ (222, 289).

Additional mechanisms of regulation of $cPLA_2$ may also exist since protein kinase A (PKA) and PKC can also phosphorylate $cPLA_2$ *in vitro* although the functional significance of this is not fully understood. Furthermore, in the long term, $cPLA_2$ can be regulated at the transcriptional level. For example, glucocorticoids can suppress the transcription of $cPLA_2$ whilst in epithelial cells IFNγ can increase the expression of $cPLA_2$ (431).

Cytosolic PLA_2 had been proposed to be a ubiquitously expressed enzyme but it is not found in mature B and T cells and its expression cannot be induced following culture with a variety of different cytokines. In contrast, immature B cells and thymocytes do express $cPLA_2$ and it is activated under conditions that lead to clonal deletion (see section 4.1) (102, 218, 222, 288). Furthermore, IL-4 and CD40 can rescue immature B cells from BCR-driven apoptosis by reducing the activity of $cPLA_2$ (102, 288).

In addition to its pro-apoptotic functions, arachidonic acid is also a substrate for cyclooxygenase and lipoxygenase enzymes leading to the production of prostaglandins and leukotrienes, respectively. Prostaglandins and leukotrienes are eicosanoids and are important mediators of inflammation.

4.5 Cyclooxygenase

Cyclooxygenase (Cox) enzymes catalyse the rate-limiting step in the formation of prostaglandins and thromboxanes, collectively known as prostanoids, from arachidonic acid. Two isoforms of Cox have been characterised: Cox1 is constitutively expressed and is responsible for basal and constitutive prostanoid synthesis; Cox2 is an inducible enzyme that is encoded by an immediate early gene that is expressed in response to cytokines, mitogens and endotoxins (432, 433). The promoter of the Cox2 gene has binding sites for AP-1, NF-κB and cAMP response element binding protein (CREB) therefore the transcription of Cox2 can be regulated by several members of the MAPK family. For example, arachidonic acid production is stimulated in U937 cells treated with platelet microparticles leading to the activation of PI-3-K. PI-3-K then induces p38 and JNK MAPKs and PKC-dependent stimulation of Erk-MAPK. The activation of MAPKs leads to the induction of c-Jun and Elk-1 and the expression of Cox2 is increased resulting in the production of eicosanoids (434).

The initial stage of prostanoid generation involves the conversion of arachidonic acid to the common intermediate prostaglandin H_2 (PGH_2) and this is catalysed by PGH synthase. PGH synthase has peroxidase and Cox activity therefore prostanoid production is limited by the synthesis of PGH_2 (432, 433). PGH_2 is converted to prostanoids (prostaglandin E_2, prostaglandin D_2, prostaglandin $F_{2\alpha}$, 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2) by terminal

prostanoid synthase enzymes (figure 4.1) (435). For example, PGE synthase is a terminal prostanoid synthase that catalyses the synthesis of prostaglandin E_2 (PGE_2) from PGH_2. The differential expression of terminal prostanoid synthase enzymes determines the profile of prostanoid production in any particular cell and some terminal prostanoid synthase enzymes are inducible therefore extracellular ligands can determine which prostanoids are synthesised within a cell (433).

Prostanoids generally regulate cellular function by binding to specific G-protein-coupled receptors and the expression of these receptors depends on the cell type and the developmental stage of the cell. The different receptors for prostanoids are coupled to different G-proteins therefore prostanoids can mediate a broad range of effects. For example, the main prostanoid receptors expressed in B lymphocytes are EP1-4. These receptors all recognise PGE_2 but EP1 and one isoform of EP3 are coupled to Gi whilst EP2, the other isoform of EP3 and EP4 are coupled to Gs (433) therefore PGE_2 can mediate several functions in B cells. Indeed, in B lymphocytes PGE_2 has been associated with inducing growth arrest, especially at early stages of B cell development (436, 437). However, more recent evidence suggests exogenous PGE_2 can also function in synergy with various co-receptors including CD40, IL-4 and IL-10 to promote proliferation of B cells (438).

Prostanoids are mediators of inflammation, can regulate vascular function and have been associated with promoting cancer (433, 435, 439, 440). For example, in IL-4 and lipopolysaccharide-stimulated murine splenic B cells, PGE_2 promotes an inflammatory response by enhancing the production of IgE and IgG_1 whilst decreasing the synthesis of IgM and IgG_3 (441). Furthermore, overexpression of Cox2 in epidermal cells can elevate the levels of several prostaglandins including PGE_2, rendering the cells hypersensitive to the development of cancer upon exposure to carcinogens (439).

Indeed, Cox2 has been associated with promoting cell survival in several systems. For example, overexpression of Cox2 can protect human lung adenocarcinoma cells from apoptosis induced by anti-cancer drugs and UV radiation. It does so by increasing PGE_2 production, leading to the activation of PI-3-K and Akt, which then promote cellular survival (442). Cox2 can also suppress p53-mediated apoptosis possibly via increasing the expression of the

pro-survival protein Bcl-x_L (59). Furthermore, overexpression of Cox2 in epithelial cells leads to the production of PGE_2 and subsequent upregulation of Bcl-2 and stimulation of Erk-MAPK thus promoting cell survival and potentially inducing proliferation (443).

The action of Cox and the production of PGE_2 have thus been associated with several diseases including cancer, inflammation and heart disease. Manipulation of prostaglandin synthesis therefore has therapeutic potential for treating these disease states (440). For example, nonsteroidal anti-inflammatory drugs prevent prostaglandin biosynthesis by inhibiting Cox resulting in a suppression of inflammation and reduced risk of certain types of cancer (439, 444).

4.6 Lipoxygenase

Lipoxygenase (Lox) enzymes catalyse the oxidation of arachidonic acid to generate leukotrienes and other metabolites and these are important mediators of inflammation. At least three families of Lox enzymes have been characterised: 5-Lox, 12-Lox and 15-Lox. Each family of Lox enzymes oxidises arachidonic acid at a different position and so produces different metabolites of arachidonic acid (445).

5-Lox catalyses the initial stages in the production of leukotrienes and lipoxins, which are important inflammatory mediators. The activity of 5-Lox is enhanced by several protein kinases including Ca^{2+}/calmodulin kinase II, rendering the activity of 5-Lox sensitive to calcium, and PKA. However, 5-Lox can also be stimulated independently of calcium using unsaturated fatty acids including arachidonic acid. For example, arachidonic acid increases the ability of MK2, a p38 MAPK-activated protein kinase, to phosphorylate and stimulate 5-Lox (446). The activity of 5-Lox is also sensitive to the cellular redox status. For example, in B lymphocytes reactive oxygen species can stimulate 5-Lox by producing hydrogen peroxide and hydroperoxides. Once activated, 5-Lox can generate leukotriene B_4 and this can stimulate B cell activation, proliferation and differentiation (447). Furthermore, in acute pre-B lymphocytic leukemia, peripheral B lymphoblasts often have elevated expression of 5-Lox implicating the action of this enzyme in promoting cell survival and proliferation (448).

The action of 15-Lox enzymes can also regulate cell survival by determining the activity of Erk-MAPK and Akt. For example, in prostate cancer cells expression of 15-Lox-1 is often high whilst 15-Lox-2 is downregulated. 15-Lox-1 catalyses the release of 13-(S)-hydroxyoctadecadienoic acid leading to the activation of Erk and Akt. In contrast, 15-Lox-2 catalyses the release of 15-(S)-hydroxyeicosatetraenoic acid, which inhibits Erk1/2 and Akt (449). Different members of the 15-Lox family therefore have distinct functions. Furthermore, the action of 12-Lox has also been associated with promoting cancer. Cancerous cells that produce high levels of 12(S)-hydroxy-5, 8, 10, 14-eicosatetraenoic acid are prone to metastasis perhaps due to the ability of this metabolite to stimulate PKC resulting in the release of cathepsin B from malignant cells (445).

4.7 Aims and objectives

CD40 signalling can prevent BCR-driven induction of $cPLA_2$, arachidonic acid production, loss of mitochondrial function and ultimately apoptosis of WEHI-231 cells (102, 328). Ligation of CD40 on immature B cells induces Bcl-x_L and overexpression of Bcl-x_L in WEHI-231 cells can protect them from BCR-driven apoptosis (290, 292, 293, 311). The aim of this investigation was to further examine the role of Bcl-x_L in CD40-mediated rescue of WEHI-231 cells from BCR-driven mitochondrial death pathway.

The specific objectives of this investigation were:

1. to determine the effect of expression of Bcl-x_L on the survival and proliferation of BCR-stimulated WEHI-231 cells

2. to investigate the mechanisms of Bcl-x_L-dependent regulation of survival and proliferation of WEHI-231 cells and in particular, to analyse the effect of Bcl-x_L on the $cPLA_2$-mediated death pathway

3. to explore the function of arachidonic acid and its metabolites in regulating immature B cell survival and proliferation

4. to examine the signalling mechanisms used by metabolites of arachidonic acid to regulate the survival and proliferation of WEHI-231 cells.

4.8 Results and Discussion

4.8.1 Bcl-x_L is upregulated downstream of CD40 in WEHI-231 cells

Ligation of the BCR on WEHI-231 immature B cells leads to growth arrest and apoptosis that can be rescued by co-stimulation of CD40 (figures 3.5-3.6). An important mediator of CD40-dependent rescue is the pro-survival Bcl-2 family protein Bcl-x_L (290, 292, 293, 312). In collaboration with Elad Katz, Western blotting was used to measure the levels of Bcl-x_L in whole cell lysates prepared from WEHI-231 cells. In general, ligation of the BCR reduced the levels of Bcl-x_L in WEHI-231 cells over a 48 hr time period (Appendix 6). However, ligation of CD40 or co-ligation of the BCR plus CD40 restored levels to greater than or equal to the amount of Bcl-x_L found in untreated WEHI-231 cells (Appendix 6). Indeed, anti-CD40-treated WEHI-231 cells had high levels of Bcl-x_L especially between 4 hr and 48 hr (figure 4.2A). However, anti-Ig-treated WEHI-231 cells had substantially lower levels of Bcl-x_L throughout the 48 hr time period (figure 4.2B) indicating ligation of the BCR on WEHI-231 cells impaired the expression of Bcl-x_L. Nonetheless, co-ligation of the BCR and CD40 on WEHI-231 cells restored Bcl-x_L signalling and enabled Bcl-x_L to be upregulated between 8 hr and 48 hr (figure 4.2C). This is a very important time for CD40-mediated rescue since WEHI-231 cells can only be rescued from BCR-driven growth arrest and apoptosis if they are stimulated at CD40 within the first 8 hr of anti-Ig exposure (328). These observations suggest that Bcl-x_L is a mediator of CD40-dependent rescue of BCR-stimulated WEHI-231 cells. Indeed, CD40-mediated upregulation of Bcl-x_L has been observed previously and has been implicated in promoting immature B cell survival (290, 292, 293, 311).

4.8.2 Transfection of WEHI-231 cells with the *bcl-x_L* gene can mimic the upregulation of Bcl-x_L following ligation of CD40

The significance of Bcl-x_L as a mediator of CD40 signalling was studied using WEHI-231 cells transfected with the pSFFV-Neo plasmid containing the human *bcl-x_L* gene (Bcl-x_L WEHI-231) or empty plasmid as control (Neo WEHI-231). Western blot analysis of wild-type WEHI-231 cells treated with anti-CD40 or anti-Ig plus anti-CD40 showed expression of Bcl-x_L in CD40-stimulated

WEHI-231 cells was similar to that of untreated Bcl-x_L WEHI-231 cells (figure 4.2A and C). Bcl-x_L WEHI-231 cells therefore mimic CD40-mediated upregulation of Bcl-x_L.

4.8.3 Overexpression of Bcl-x_L protects WEHI-231 cells from BCR-driven apoptosis

To determine the function of CD40-mediated induction of Bcl-x_L, the effect of overexpression of Bcl-x_L on BCR-driven apoptosis was determined. Apoptosis of Neo and Bcl-x_L WEHI-231 cells was studied using PI-staining and FACS analysis. The BCR and CD40 were stimulated using anti-Ig and anti-CD40, respectively. To determine the optimal concentration of anti-Ig for BCR-mediated apoptosis, Neo and Bcl-x_L WEHI-231 cells were stimulated with increasing concentrations of anti-Ig and the % sub-diploid cells was measured. After 24 hr, anti-Ig induced apoptosis of Neo WEHI-231 cells with optimal levels of apoptosis being obtained between 1 and 10 µg/ml anti-Ig (figure 4.3A). However, after 48 hr, optimal apoptosis was not achieved in Neo WEHI-231 cells treated with 1 µg/ml anti-Ig, suggesting 10 µg/ml anti-Ig was a preferable concentration of anti-Ig for studying BCR-driven apoptosis (figure 4.3B). Furthermore, 10 µg/ml anti-CD40 was able to protect Neo WEHI-231 cells from apoptosis induced by 10 µg/ml anti-Ig after 24 hr or 48 hr indicating this was also a suitable concentration of anti-Ig to study CD40-mediated rescue.

Interestingly, overexpression of Bcl-x_L almost completely abrogated BCR-driven apoptosis of WEHI-231 cells at all concentrations of anti-Ig (0-10 µg/ml) after 24 hr and 48 hr (figure 4.3). Indeed, after 24 hr, anti-Ig (10 µg/ml) increased the percentage of Neo WEHI-231 cells with sub-diploid DNA from 6% to 18% indicating anti-Ig had induced apoptosis whilst co-culture with anti-CD40 (10 µg/ml) rescued the cells from apoptosis (figure 4.4A). In contrast, treatment of Bcl-x_L WEHI-231 with anti-Ig (10 µg/ml) for 24 hr only caused the percentage of sub-diploid cells to increase from 4% to 6% (figure 4.4B) indicating overexpression of Bcl-x_L can protect WEHI-231 cells from anti-Ig-induced apoptosis.

After 48 hr, the effects of anti-Ig and anti-CD40 on Neo and Bcl-x_L WEHI-231 cells were even more pronounced (figure 4.5). After 48 hr, anti-Ig increased the percentage of apoptotic Neo WEHI-231 cells from 10% to 40% (figure 4.5A).

In contrast, Bcl-x_L WEHI-231 cells remained resistant to anti-Ig-induced apoptosis after 48 hr since 10 µg/ml anti-Ig only increased the percentage of Bcl-x_L WEHI-231 cells with sub-diploid DNA from 6% to 11% (figure 4.5B). Expression of Bcl-x_L therefore protects WEHI-231 cells from BCR-driven apoptosis.

Interestingly, the ability of Bcl-x_L WEHI-231 cells to resist BCR-driven apoptosis was reproducibly slightly enhanced by ligation of CD40 (figures 4.3-4.5). For example, after 48 hr, co-ligation of CD40 on anti-Ig-treated Bcl-x_L WEHI-231 cells reduced the percentage of sub-diploid cells from 11% to 8% in this experiment (figure 4.5B). This suggests that although overexpression of Bcl-x_L is highly effective at preventing BCR-driven apoptosis, CD40 might initiate additional signals to further improve the survival of immature B cells. One possibility is ligation of CD40 on Bcl-x_L WEHI-231 cells might further increase the expression of Bcl-x_L in these cells to amplify this pro-survival signal.

4.8.4 Overexpression of Bcl-x_L does not protect WEHI-231 cells from BCR-driven growth arrest

Ligation of the BCR on WEHI-231 cells induces growth arrest and apoptosis of the cells that can be rescued by co-ligation of CD40. The results of figures 4.3-4.5 strongly implicate Bcl-x_L as a key mediator of CD40-dependent rescue from BCR-driven apoptosis. To further explore the role of Bcl-x_L in mediating CD40-dependent rescue, the effect of Bcl-x_L on the proliferation of WEHI-231 cells was examined by analysing the cell cycle of BCR-stimulated WEHI-231 cells using PI-staining and FACS analysis. Furthermore, the levels of DNA synthesis were determined by measuring the incorporation of [^{3}H] thymidine.

Neo and Bcl-x_L WEHI-231 cells were treated with increasing concentrations of anti-Ig to determine the optimal concentration of anti-Ig required for BCR-driven growth arrest. After 48 hr, DNA synthesis was measured in Neo and Bcl-x_L WEHI-231 cells. Ligation of the BCR induced complete growth arrest of Neo WEHI-231 cells with optimal growth arrest being observed between 1 and 10 µg/ml anti-Ig (figure 4.6A). Co-ligation of CD40 was able to restore DNA synthesis to Neo WEHI-231 cells treated with 10 µg/ml anti-

Ig indicating this concentration of anti-Ig was appropriate for the study of BCR-driven growth arrest and CD40-mediated restoration of proliferation.

To determine the role of Bcl-x_L in the regulation of immature B cell proliferation, the proliferation of Neo and Bcl-x_L WEHI-231 cells was compared. In untreated WEHI-231 cells, the level of DNA synthesis in Bcl-x_L WEHI-231 cells was consistently lower than that of untreated Neo WEHI-231 cells. A possible explanation for this is the increased survival of Bcl-x_L WEHI-231 cells results in a slower turnover rate of the cells and hence the level of DNA synthesis in a population of Bcl-x_L WEHI-231 cells is reduced. Interestingly, treatment of Bcl-x_L WEHI-231 cells with increasing concentrations of anti-Ig also suppressed DNA synthesis in a dose-dependent manner (figure 4.6A). This suggests Bcl-x_L cannot prevent BCR-driven growth arrest despite being able to protect WEHI-231 cells from BCR-dependent apoptosis. Furthermore, by measuring the DNA synthesis of WEHI-231 cells, Bcl-x_L WEHI-231 cells appear to be more sensitive to BCR-driven growth arrest than Neo WEHI-231 cells since 0.1 μg/ml anti-Ig was sufficient to induce optimal growth arrest of Bcl-x_L WEHI-231 cells (figure 4.6A). Overexpression of Bcl-x_L therefore did not appear to prevent BCR-driven growth arrest. Moreover, co-culture with anti-CD40 rescued both Neo and Bcl-x_L WEHI-231 cells from BCR-dependent growth arrest indicating signals other than Bcl-x_L are required for CD40-mediated induction of proliferation.

Previously it has been shown that ligation of the BCR on immature B cells induces growth arrest at G0/G1 phase of the cell cycle (figure 3.6) therefore the percentage of cells in G0/G1 phase of the cell cycle was determined by PI-staining and FACS analysis. After 24 hr, anti-Ig enhanced the G0/G1 population of Neo and Bcl-x_L WEHI-231 cells in a dose-dependent manner with optimal enrichment of G0/G1 cells being observed at 1-10 μg/ml anti-Ig (figure 4.6B). In agreement with the thymidine assay data, Bcl-x_L WEHI-231 cells were slightly more sensitive than Neo WEHI-231 cells to cell cycle arrest at G0/G1 (figure 4.6B). After 48 hr, the G0/G1 population of Bcl-x_L WEHI-231 cells was further enriched by anti-Ig; for example, 77% of cells treated with 10 μg/ml anti-Ig for 48 hr were in G0/G1 phase (figure 4.5B and 4.6C). However, in Neo WEHI-231 cells, exposure to 10 μg/ml anti-Ig for 48 hr was less effective at enriching the number of cells in G0/G1 phase, presumably reflecting the high degree of anti-

Ig-induced apoptosis in Neo WEHI-231 cultures (figure 4.5A). Nonetheless, co-ligation of CD40 reduced the number of Neo and Bcl-x_L WEHI-231 cells in G0/G1 phase indicating CD40 can efficiently protect these cells from BCR-driven cell cycle arrest (figures 4.4-4.6). Furthermore, this suggests that CD40 abrogates BCR-driven growth arrest via the induction of pathways other than Bcl-x_L.

Ligation of the BCR on WEHI-231 cells therefore potently induces growth arrest at G0/G1 phase of the cell cycle that cannot be prevented by overexpression of Bcl-x_L. The level of growth arrest can be further corroborated by examination of the percentage of cells in S phase and G2/M. After 24 hr, anti-Ig dramatically reduced the number of Neo WEHI-231 cells in S phase with optimal growth arrest being observed at 1-10 µg/ml anti-Ig (figure 4.7A). Furthermore, the number of Neo WEHI-231 cells in G2/M was reduced by anti-Ig in a dose-dependent manner with maximal growth arrest being induced by 10 µg/ml anti-Ig. For example, treatment of Neo WEHI-231 cells with 10 µg/ml anti-Ig for 24 hr, enhanced the percentage of cells in G0/G1 phase from 34% to 49% whilst reducing the number of S phase cells from 28% to 11% and G2/M phase cells from 24% to 15% (figure 4.4A). Ligation of the BCR therefore potently induced cell cycle arrest of Neo WEHI-231 cells at G0/G1 phase within 24 hr. However, co-ligation of CD40 completely protected Neo WEHI-231 cells from BCR-driven growth arrest (figures 4.4A and 4.7A).

Similarly, treatment of Bcl-x_L WEHI-231 cells with anti-Ig for 24 hr also reduced the number of cells in S phase in a dose-dependent manner (figure 4.7A). For example, 10 µg/ml anti-Ig increased the percentage of Bcl-x_L WEHI-231 cells in G0/G1 from 43% to 52% whilst reducing the S phase population from 30% to 16% (figure 4.4B). Overexpression of Bcl-x_L therefore was unable to prevent BCR-driven cell cycle arrest at G0/G1 phase. However, Bcl-x_L might offer some protection or delay the onset of growth arrest since treatment of Bcl-x_L WEHI-231 cells with 10 µg/ml anti-Ig for 24 hr did not diminish the number of cells in G2/M phase (figures 4.7A and 4.4B). Nonetheless, co-ligation of CD40 on Bcl-x_L WEHI-231 cells restored proliferation, as indicated by the reduction in the number of G0/G1 cells and the enrichment of the S phase population.

After 48 hr, similar observations were made although the levels of growth arrest were generally higher and 10 µg/ml anti-Ig was most effective at inducing

cell cycle arrest (figure 4.7B). For example, after 48 hr, 10 µg/ml anti-Ig potently induced growth arrest of Neo WEHI-231 cells since the percentage G0/G1 cells increased from 33% to 44%, the percentage of S phase cells decreased from 35% to 8% and the percentage G2/M cells fell from 22% to 3% (figure 4.5A). Bcl-x_L WEHI-231 cells were also highly sensitive to BCR-driven growth arrest after 48 hr. For example, after 48 hr, 77% of Bcl-x_L WEHI-231 cells treated with 10 µg/ml anti-Ig were in G0/G1 phase of the cell cycle and just 5% of cells were in G2/M phase indicating ligation of the BCR potently induced growth arrest of Bcl-x_L WEHI-231 cells at G0/G1 phase (figure 4.5B). Expression of Bcl-x_L therefore protects WEHI-231 cells from BCR-driven apoptosis but cannot prevent BCR-driven growth arrest.

Moreover, co-culture with anti-CD40 protected both Neo and Bcl-x_L WEHI-231 cells from BCR-driven growth arrest, even after 48 hr (figures 4.4-4.7). These results agree with previous observations that suggested overexpression of Bcl-x_L in WEHI-231 cells can protect them from anti-Ig-induced apoptosis but not growth arrest (292, 311). CD40-mediated rescue from BCR-driven apoptosis is thus likely to involve the upregulation of Bcl-x_L but CD40-dependent rescue from anti-Ig-induced growth arrest requires signals that are additional to Bcl-x_L.

4.8.5 Arachidonic acid induces apoptosis of WEHI-231 cells

Bcl-x_L is thus a key survival factor of WEHI-231 cells and overexpression of Bcl-x_L can abrogate BCR-driven apoptosis without preventing BCR-mediated growth arrest. The mechanism of BCR-driven apoptosis has previously been investigated in WEHI-231 cells (102, 218, 328) (see section 4.1) and this showed that ligation of the BCR induces an early Erk signal that contributes to the activation of $cPLA_2$ leading to the production of arachidonic acid at the mitochondria. The production of arachidonic acid precedes disruption of the mitochondrial membrane potential and death of the cell. Co-ligation of CD40 prevents BCR-dependent apoptosis by suppressing BCR-driven induction of $cPLA_2$ and subsequent production of arachidonic acid (102, 218).

To further investigate the role of Bcl-x_L in CD40-mediated rescue from BCR-driven apoptosis, the effect of Bcl-x_L on growth arrest and apoptosis induced by exogenous arachidonic acid was determined. The cell cycle of Neo

and Bcl-x_L WEHI-231 cells treated with exogenous arachidonic acid (AA) was investigated by PI-staining and FACS analysis. Treatment of Neo WEHI-231 cells with 10 µg/ml anti-Ig and/or 100 µM AA dramatically increased the number of cells with sub-diploid DNA indicating these agents potently induce apoptosis of Neo WEHI-231 cells (figure 4.8A). In contrast, overexpression of Bcl-x_L impaired both anti-Ig and AA-mediated apoptosis (figure 4.8A), suggesting Bcl-x_L targets the AA-mediated apoptotic pathway. However, Bcl-x_L was unable to completely prevent apoptosis induced by these agents especially when Bcl-x_L WEHI-231 cells were treated with a combination of 100 µM AA plus 10 µg/ml anti-Ig, suggesting the Bcl-x_L-mediated rescue pathway could be overwhelmed by a combination of these agents.

AA-dependent apoptosis was also impaired by ligation of CD40. Indeed, anti-CD40 and Bcl-x_L provided a similar level of protection from AA-mediated apoptosis. Furthermore, as with overexpression of Bcl-x_L, ligation of CD40 did not completely prevent AA-induced apoptosis (figure 4.8B). These observations suggest that Bcl-x_L and CD40 act via a common survival pathway. However, ligation of CD40 on Bcl-x_L WEHI-231 cells increased their ability to resist AA-mediated apoptosis suggesting CD40 signalling might further enhance the induction of Bcl-x_L or initiate Bcl-x_L-independent survival signals.

Furthermore, CD40 alone was unable to prevent apoptosis of Neo WEHI-231 cells treated with a combination of anti-Ig plus AA but CD40 stimulation of Bcl-x_L WEHI-231 cells impaired the ability of these agents to induce apoptosis (figure 4.8C). These observations are consistent with published data that implicates arachidonic acid as a key mediator of BCR-driven apoptosis (102). Moreover, they suggest that CD40 and Bcl-x_L both prevent apoptosis induced by anti-Ig and exogenous AA, which is consistent with Bcl-x_L being a key mediator of CD40-dependent survival. Furthermore, these results imply Bcl-x_L prevents BCR-driven apoptosis downstream of arachidonic acid. This is consistent with arachidonic acid-dependent apoptosis being mediated by inducing a loss of mitochondrial membrane potential (102) since Bcl-x_L is known to protect the integrity of intracellular organelles including the mitochondria (73).

4.8.6 Arachidonic acid induces growth arrest of WEHI-231 cells

To further investigate the role of Bcl-x_L and arachidonic acid in WEHI-231 cell signalling, the effect of Bcl-x_L on growth arrest induced by exogenous arachidonic acid was determined. Exogenous arachidonic acid was added to WEHI-231 cells and proliferation was assessed by measuring the DNA synthesis. The addition of exogenous AA to WEHI-231 cells for 48 hr induced growth arrest in a dose-dependent manner (figure 4.9A). This might reflect the ability of AA to induce apoptosis of WEHI-231 cells. Overexpression of Bcl-x_L slightly impaired but did not prevent AA-mediated cell cycle arrest in WEHI-231 cells (figure 4.9A). The apparent reduced sensitivity to apoptosis may have been due to the increased survival of Bcl-x_L WEHI-231 cells.

Proliferation of WEHI-231 cells treated with exogenous AA was further studied by staining the cells with PI followed by FACS analysis. Exogenous AA reduced the number of S phase cells to a similar extent as treatment of WEHI-231 cells with 10 µg/ml anti-Ig (figure 4.9B). Furthermore, AA and anti-Ig both depleted the G2/M population of Neo-WEHI-231 cells to a similar extent (figure 4.9B). Interestingly, overexpression of Bcl-x_L impaired AA and anti-Ig-mediated depletion of S phase cells. This may reflect the increased survival of these cells since anti-Ig and AA efficiently depleted the G2/M population of Bcl-x_L WEHI-231 cells (figure 4.9B). Overexpression of Bcl-x_L is thus inefficient at preventing BCR- or AA-mediated depletion of cycling WEHI-231 cells. However, ligation of CD40 partially protected Bcl-x_L WEHI-231 cells from AA-induced growth arrest and 10 µg/ml anti-CD40 raised the IC_{50} of AA from 38 µM to 55 µM (figure 4.9C). CD40 therefore protects WEHI-231 cells from apoptosis and cell cycle arrest induced by ligation of the BCR or exposure to exogenous AA. Bcl-x_L mimics CD40 in enabling the cells to survive in the presence of AA but it does not restore proliferation as efficiently as CD40, which is consistent with Bcl-x_L mediating CD40-dependent rescue from BCR-driven apoptosis but not growth arrest.

4.8.7 Overexpression of Bcl-x_L suppresses coupling of the BCR to mitochondrial PLA_2 activation

The finding that Bcl-x_L expression can impair AA-induced apoptosis of WEHI-231 cells suggested that this anti-apoptotic pathway was likely to act downstream of PLA_2 and presumably by protecting the integrity of the mitochondrial membranes (73). However, arachidonic acid is itself an activator of PLA_2 and can increase mitochondrial PLA_2 activity in untreated or anti-Ig-stimulated immature B cells (appendix 7A) (427) and this has also been reported by various other groups (429, 450). These observations raised the possibility that Bcl-x_L may also prevent BCR-driven apoptosis by suppressing mitochondrial PLA_2 activity. This possibility was investigated in collaboration with Elad Katz and Caroline Lord (427) by determining the activity of PLA_2 in isolated mitochondria of anti-Ig-stimulated Neo and Bcl-x_L WEHI-231 cells. Indeed, these investigations revealed Bcl-x_L does prevent AA-mediated activation of PLA_2 in WEHI-231 cells (appendix 7B). To eliminate the possibility that the observed PLA_2 activity was induced by mitochondrial dysfunction, the activity of PLA_2 was determined in mitochondria isolated from WEHI-231 cells treated with the mitochondrial stabilisers oligomycin or cyclosporin A. These stabilisers have previously been shown to prevent BCR-driven apoptosis by abrogating anti-Ig-mediated disruption of mitochondrial membrane potential and depletion of cellular ATP (appendix 7C and (102)). However, they did not prevent BCR-mediated induction of mitochondrial PLA_2 activity (appendix 7D) indicating the observed PLA_2 activity in Neo and Bcl-x_L WEHI-231 cells was not caused by disruption of the mitochondria. These observations therefore suggest Bcl-x_L suppresses mitochondrial PLA_2 activity independently of its regulation of mitochondrial membrane potential.

4.8.8 Overexpression of Bcl-x_L antagonises arachidonic acid- and BCR-mediated disruption of inner mitochondrial membrane potential

Previously it has been shown that treatment of WEHI-231 cells with exogenous arachidonic acid can mimic BCR-mediated disruption of mitochondrial membrane integrity thus committing the cell to later stages of apoptosis (102). To further investigate the mechanism of Bcl-x_L-mediated

protection of WEHI-231 cells from BCR- and AA-induced apoptosis, the effect of overexpression of Bcl-x_L on mitochondrial membrane potential was determined. In collaboration with Elad Katz, the mitochondrial membrane potential was measured by analysing the uptake of the lipophilic dye $DiOC_6$ since the uptake of this dye is directly proportional to the mitochondrial transmembrane potential. Treatment of Neo WEHI-231 cells with anti-Ig (10 µg/ml) or AA (100 µM) induced a profound loss of mitochondrial membrane potential that was maximal by 20-24 hr (appendix 7E). However, Bcl-x_L WEHI-231 cells were partially protected from anti-Ig- and AA-induced disruption of mitochondrial membrane potential. This is consistent with Bcl-x_L having roles in protecting the integrity of the mitochondria and suppressing apoptosis induced by these agents.

4.8.9 Bcl-x_L antagonises the post-mitochondrial induction of the executioner protease, cathepsin B in WEHI-231 cells

Previously it has been shown that ligation of the BCR mediates the post-mitochondrial induction of cathepsin B and this contributes to the executioner protease activity of apoptosis in WEHI-231 cells (102). In collaboration with Elad Katz and Caroline Lord, the effect of Bcl-x_L on cathepsin B activity was determined in WEHI-231 cells. In keeping with the ability of Bcl-x_L to prevent anti-Ig- or AA-induced collapse of mitochondrial membrane potential and induction of apoptosis, Bcl-x_L also abrogated BCR-driven activation of cathepsin B (appendix 7F). Bcl-x_L therefore impairs multiple stages of BCR- and AA-driven apoptosis including the activation of mitochondrial PLA_2, disruption of mitochondrial membrane integrity and the activation of effector caspases.

4.8.10 Overexpression of Bcl-x_L does not restore sustained cycling Erk activation required for proliferation in WEHI-231 cells

Although Bcl-x_L expression can mimic CD40-dependent rescue from BCR-driven apoptosis (figures 4.3-4.5), expression of Bcl-x_L cannot substitute for CD40-signalling in the reversal of anti-Ig-induced growth arrest of WEHI-231 cells (figures 4.4-4.7) (311, 312). The upstream mechanisms underlying CD40-dependent rescue of anti-Ig-induced growth arrest are incompletely understood but, as discussed in Chapter 3, a key event of CD40-mediated rescue is the restoration of sustained cyclic activation of Erk-MAPK, which is required for cell

cycle progression (328). To further investigate the function of Bcl-x_L in the rescue of BCR-stimulated WEHI-231 cells the effect of Bcl-x_L on Erk activity was determined.

The activation of Erk-MAPK in Neo and Bcl-x_L WEHI-231 cells was investigated using Western blotting with antibodies that specifically recognise dual-phosphorylated Erk1/2. In general, expression of Bcl-x_L did not alter the temporal pattern of Erk activation (figure 4.10). In particular, overexpression of Bcl-x_L did not restore sustained, cycling Erk activation to anti-Ig-treated WEHI-231 cells. In contrast, co-culture with anti-CD40 was able to restore sustained Erk signalling to Neo and Bcl-x_L WEHI-231 cells. The expression of Bcl-x_L is thus insufficient to prevent BCR-mediated inhibition of sustained Erk signalling. This is consistent with Bcl-x_L being unable to prevent BCR-driven growth arrest since restoration of this signal is necessary for proliferation of WEHI-231 cells. CD40-mediated restoration of sustained and cycling Erk activation and the induction of proliferation therefore requires signals in addition to Bcl-x_L. Furthermore, it has previously been shown that transient transfection of Bcl-x_L WEHI-231 cells with E2F1 or viral E1A can protect the cells from BCR-driven growth arrest (52). This suggests that a possible function of sustained Erk signals is to induce E2F, perhaps via the suppression of Rb. Rb is inhibited by phosphorylation catalysed by cyclin/Cdk complexes (50) and BCR-stimulated immature B cells particularly lack cyclin E/Cdk2 complexes (33) therefore sustained Erk signals might function to induce cyclin E/Cdk2 leading to a suppression of Rb. However, additional experiments must be performed to show a direct link between sustained Erk signalling and the induction of E2F.

4.8.11 Cox and Lox inhibitors implicate a key role for arachidonic acid in mediating the mitochondrial death pathway in WEHI-231 cells

Exogenous arachidonic acid can mimic BCR-driven disruption of mitochondrial function and subsequent induction of apoptosis. Furthermore, Bcl-x_L can suppress BCR-mediated activation of PLA_2, mitochondrial malfunction and apoptosis that is induced by either BCR-ligation or exogenous AA. These findings strongly imply that the BCR induces apoptosis via the activation of PLA_2 leading to the disruption of mitochondrial function. However, it has not been possible to show that PLA_2-mediated production of AA is actually the

cause of BCR-driven disruption of the mitochondria since inhibitors of $cPLA_2$ are non-metabolisable analogues of arachidonic acid and they themselves induce apoptosis of WEHI-231 cells (451). Consequently, it was necessary to regulate AA metabolism via an alternative mechanism involving inhibitors of cyclooxygenase (Cox) and lipoxygenase (Lox). AA is a substrate of Cox and Lox enzymes, leading to the production of prostaglandins and leukotrienes, respectively. Inhibitors of Cox2 (NS-398) and Lox (LOI) should therefore prevent AA metabolism and enhance AA signalling.

The Cox2 selective inhibitor, NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide) and the 12-Lox/15-Lox inhibitor, LOI (ethyl-3,4-dihydroxybenzylidenecyanoacetate) were used to explore the role of AA in BCR-mediated induction of the mitochondrial death pathway. Inhibition of Cox and Lox, enhanced BCR-driven apoptosis of Neo WEHI-231 cells, as indicated by the observed increase in the number of cells with sub-diploid DNA (figure 4.11). These inhibitors also enhanced disruption of the mitochondrial membrane potential (appendix 8). The BCR-driven mitochondrial death pathway of WEHI-231 cells is thus enhanced under conditions in which arachidonic acid cannot be metabolised to prostaglandins and leukotrienes. Furthermore, this enhancement of BCR-driven apoptosis was prevented by co-ligation of CD40, which is consistent with CD40 being able to suppress BCR- and AA-driven apoptosis (figure 4.11). Moreover, overexpression of Bcl-x_L mimicked CD40 in suppressing anti-Ig- and NS-398/LOI-driven apoptosis. Apoptosis was further suppressed by ligation of CD40 on Bcl-x_L WEHI-231 cells (figure 4.11), which is consistent with previous observations showing CD40 and Bcl-x_L can both protect WEHI-231 cells from BCR-driven apoptosis (figures 4.3-4.5). NS-398-mediated disruption of the mitochondrial membrane potential was also impaired by expression of Bcl-x_L (appendix 8). Enhancement of arachidonic acid signalling via the inhibition of Cox and Lox therefore induces apoptosis of unstimulated and anti-Ig-treated WEHI-231 cells that can be suppressed by ligation of CD40 or overexpression of Bcl-x_L. Taken together, these observations implicate a causal role for BCR-dependent arachidonic acid generation in mediating the mitochondrial death pathway and this pathway can be inhibited by induction of Bcl-x_L.

4.8.12 Inhibition of Cox and Lox impairs proliferation of WEHI-231 cells

The inhibition of Cox and Lox enhanced BCR-driven apoptosis suggesting the formation of prostaglandins and leukotrienes can inhibit apoptosis by reducing the cellular levels of AA. However, prostaglandins and leukotrienes can also function as signalling molecules and they have been associated with promoting inflammation, regulating vascular function and assisting carcinogenesis (433, 435, 439, 440). Inhibition of Cox2 and Lox in WEHI-231 cells therefore prevents prostaglandin and leukotriene signalling in addition to enhancing AA signals. To further explore the role of eicosanoids in WEHI-231 cells, the effect of Cox and Lox inhibitors on proliferation of WEHI-231 cells was determined.

Treatment of Neo WEHI-231 cells with NS-398, LOI or a combination of these two inhibitors suppressed DNA synthesis in a dose-dependent manner (figure 4.12A). Furthermore, treatment of Neo WEHI-231 cells with these inhibitors enhanced BCR-driven growth arrest in a concentration-dependent manner. NS-398 was less effective than LOI at inducing growth arrest and the combined exposure to NS-398 and LOI suppressed DNA synthesis more potently than treatment of the cells with a single inhibitor. Furthermore, PI-staining and FACS analysis of Neo WEHI-231 cells showed that NS-398 and LOI slightly enhanced BCR-driven depletion of S phase and G2/M cells (figure 4.13A-B). Inhibition of Cox and/or Lox therefore suppresses proliferation and enhances BCR-driven growth arrest.

Consistent with the observation that upregulation of Bcl-x_L does not prevent BCR-driven growth arrest (figures 4.4-4.7), expression of Bcl-x_L did not protect WEHI-231 cells from growth arrest induced by NS-398 and/or LOI (figure 4.12B). However, NS-398 and LOI also impaired CD40-mediated rescue from BCR-driven growth arrest (figure 4.13C-D) suggesting CD40-mediated restoration of proliferation might involve the activation of Cox2/Lox and hence metabolites of AA may promote proliferation of WEHI-231 cells. Indeed, inhibitors of Cox2 and Lox were found to promote growth arrest and apoptosis of WEHI-231 cells. Taken together, these observations suggest that the metabolism of arachidonic acid to produce prostaglandins and leukotrienes may play a role in switching off the AA-mediated death pathway and/or promoting proliferation of WEHI-231 cells.

4.8.13 Anti-Ig inhibits the production of prostaglandin E_2 in WEHI-231 cells

The observation that Cox2 and Lox inhibitors impaired proliferation of WEHI-231 cells and compromised CD40-mediated rescue from BCR-driven growth arrest suggested prostaglandins and leukotrienes might promote proliferation of WEHI-231 cells. The role of one particular eicosanoid, prostaglandin E_2 (PGE_2), in regulating the proliferation of WEHI-231 cells was investigated since PGE_2 has previously been associated with regulating proliferation of B lymphocytes. Early reports suggested that PGE_2 was associated with the induction of growth arrest of B lymphocytes, especially during early stages of B cell development (436, 437). However, more recent evidence suggests exogenous PGE_2 can function in synergy with various co-receptors including CD40, IL-4 and IL-10 to promote proliferation of B cells (438).

In studies initiated by Stephen Gauld, the levels of PGE_2 were measured in WEHI-231 cells using a PGE_2 competitive binding immunoassay kit (Cayman Chemical) (427). Ligation of the BCR reduced the levels of intracellular PGE_2 whilst co-ligation of CD40 partially restored PGE_2 levels (appendix 9). Such PGE_2 production appears to be exclusively intracellular since no PGE_2 could be detected in cell supernatants (data not shown). Moreover, CD40-dependent rescue of PGE_2 levels was independent of Bcl-x_L expression since BCR-signalling suppressed PGE_2 generation equally well in Neo and Bcl-x_L WEHI-231 cells (figure 4.14A).

The mechanisms regulating PGE_2 production in WEHI-231 cells are incompletely understood but the expression of Cox2 correlated with PGE_2 production (Western blot panel in appendix 9). Cox2 levels were thus depleted in BCR-activated cells but co-ligation of CD40 enabled Cox2 levels to be restored. Indeed, the expression of Cox2 and subsequent PGE_2 production has been observed downstream of CD40-ligation in lung fibroblast cells (452) suggesting CD40 can induce Cox2 in several systems.

4.8.14 Regulation of Erk-MAPK by arachidonic acid and its metabolites

The mechanism of prostaglandin/leukotriene-dependent regulation of WEHI-231 survival and proliferation is incompletely understood but eicosanoids

are known to regulate the activity of key signalling molecules such as MAPK to determine the survival, activity and proliferation of various types of cells. For example, Lox1 activity is enhanced in the PC3 prostate cancer cell line and this leads to increased release of 13-(S)-hydroxyoctadecadienoic acid, which can stimulate MAPK and Akt (449). Since the survival and proliferation of WEHI-231 cells is critically dependent on the differential regulation of Erk-MAPK, the effect of Cox and Lox inhibitors on the activity of Erk was investigated.

Treatment of proliferating Neo or Bcl-x_L WEHI-231 cells with NS-398/LOI suppressed sustained (8-48 hr) Erk signals (figure 4.14B). This is consistent with these signals being necessary for proliferation of WEHI-231 cells and with the observation that NS398/LOI can induce growth arrest of WEHI-231 cells. NS-398/LOI-dependent growth arrest may therefore be mediated by a suppression of sustained and cyclic Erk activation in WEHI-231 cells. Furthermore, coupled with the knowledge that the BCR and CD40 differentially regulate PGE_2 levels in WEHI-231 cells, these observations suggest a possible function of PGE_2 is to enhance sustained Erk signalling in WEHI-231 cells.

NS-398 and LOI also induced a general enhancement of early (≤ 4 hr) Erk-MAPK signals in Neo WEHI-231 cells (figure 4.15A). This may contribute to the ability of these inhibitors to induce apoptosis of WEHI-231 cells. Furthermore, these observations show that early Erk signals can be amplified under conditions in which AA is allowed to accumulate suggesting AA-dependent induction of $cPLA_2$ (appendix 7A) may be mediated by the activation of Erk. Moreover, NS-398 and LOI were less effective at enhancing early Erk signals in CD40-stimulated WEHI-231 cells. However, overexpression of Bcl-x_L did not mimic CD40 in protecting WEHI-231 cells from NS-398/LOI-dependent enhancement of early Erk signals (figure 4.15B) suggesting Bcl-x_L is acting downstream of Erk to impair AA-mediated induction of $cPLA_2$ and BCR-driven apoptosis. These results suggest that the accumulation of arachidonic acid may increase early Erk signals and this is impaired by ligation of CD40 but probably independently of Bcl-x_L expression.

To investigate this possibility further, WEHI-231 cells were cultured with exogenous arachidonic acid and the activity of Erk in lysates prepared from these cells was determined by Western blotting. In unstimulated and anti-Ig-treated Neo WEHI-231 cells, AA enhanced the activity of Erk between 0 and 4

hr, suggesting AA can induce Erk in WEHI-231 cells (figure 4.16A). In contrast, Erk signalling in CD40-stimulated WEHI-231 cells was not enhanced by exposure to AA, implying CD40 can impair AA-dependent activation of Erk (figure 4.16A). These results suggest AA can enhance early (0-4 hr) Erk signals, which is likely to contribute to AA-dependent activation of $cPLA_2$ but ligation of CD40 can protect WEHI-231 cells from AA-mediated induction of Erk. However, anti-CD40 was insufficient to prevent AA-mediated Erk activation in BCR-stimulated Neo WEHI-231 cells (figure 4.16A). This is consistent with the observations of figure 4.8C that showed CD40 was unable to prevent apoptosis induced by a combination of anti-Ig plus AA.

Furthermore, consistent with the results of figure 4.15, expression of Bcl-x_L only impaired the ability of AA to enhance early Erk signals when Bcl-x_L WEHI-231 cells were stimulated at CD40 (figure 4.16B). Taken together, these observations suggest that arachidonic acid can enhance early (0-4 hr) Erk activity in WEHI-231 cells and this is assisted by ligation of the BCR. However, ligation of CD40 can impair the ability of AA to induce Erk signalling and it seems to do so independently of Bcl-x_L. Conditions that increase the levels of AA therefore seem to promote early Erk signals and this could lead to an enhancement of the mitochondrial death pathway. Furthermore, the activation of Erk by AA might amplify arachidonic acid signalling since $cPLA_2$ can be stimulated by Erk and inhibitors of MEK are known to impair BCR-dependent arachidonic acid production (328).

4.9 Conclusions

Ligation of CD40 on WEHI-231 cells induced Bcl-x_L and this was mimicked by transfection of WEHI-231 cells with a pSFFV-Neo plasmid containing the human gene for Bcl-x_L (figure 4.2). Overexpression of Bcl-x_L protected WEHI-231 cells from BCR-driven apoptosis suggesting CD40-dependent survival is mediated by Bcl-x_L (figures 4.3-4.5). In contrast, overexpression of Bcl-x_L did not prevent BCR-driven growth arrest but ligation of CD40 on Bcl-x_L WEHI-231 cells restored proliferation to BCR-stimulated cells (figures 4.4-4.7). This suggests that CD40-dependent rescue from BCR-driven growth arrest requires signals that are additional to Bcl-x_L.

To further investigate the mechanism of Bcl-x_L-mediated rescue of BCR-stimulated WEHI-231 cells, the effect of expression of Bcl-x_L on the mitochondrial death pathway was explored. BCR-driven apoptosis can be mimicked by treatment of WEHI-231 cells with exogenous arachidonic acid (figure 4.8). Overexpression of Bcl-x_L or ligation of CD40 impaired AA-dependent apoptosis of WEHI-231 cells and protection was enhanced by a combination of Bcl-x_L expression and treatment with anti-CD40 (figure 4.8). These observations are consistent with AA being a key mediator of BCR-driven apoptosis and they show Bcl-x_L and CD40 can suppress apoptosis induced by ligation of the BCR or exposure to AA. This is consistent with Bcl-x_L being a key mediator of CD40-dependent survival. These observations also suggest that Bcl-x_L prevents BCR/AA-driven apoptosis downstream of arachidonic acid and this is likely to be mediated by Bcl-x_L-dependent protection of mitochondrial integrity.

However, AA can stimulate $cPLA_2$ activity to enhance arachidonic acid production in WEHI-231 cells. Overexpression of Bcl-x_L prevented AA-mediated induction of AA production and this was not impaired by mitochondrial stabilisers (appendix 7). Bcl-x_L therefore can suppress BCR/AA-dependent apoptosis by inhibiting AA-mediated induction of PLA_2. Moreover, Bcl-x_L protected WEHI-231 cells from BCR-driven disruption of mitochondrial membrane potential (appendix 7E), which is consistent with Bcl-x_L having a role in retaining the integrity of the mitochondria. Furthermore, BCR-dependent activation of cathepsin B was impaired by expression of Bcl-x_L (appendix 7F). Overexpression of Bcl-x_L therefore impaired the BCR-driven apoptotic pathway by suppressing AA-dependent induction of PLA_2 and protecting the mitochondria from AA-mediated disruption. Consequently, Bcl-x_L prevented BCR-dependent induction of effector proteases and resultant apoptosis.

In contrast, expression of Bcl-x_L did not prevent BCR- or AA-induced growth arrest (figures 4.4-4.7 and 4.9). Furthermore, expression of Bcl-x_L was unable to restore sustained and cyclic activation of Erk to BCR-stimulated WEHI-231 cells (figure 4.10). This is consistent with sustained Erk signalling being necessary for proliferation of WEHI-231 cells and with the observation that CD40-dependent proliferation requires signalling pathways other than Bcl-x_L.

To further understand the role of AA and its metabolites in determining the survival and proliferation of WEHI-231 cells the metabolism of AA was inhibited using Cox2 and Lox inhibitors. In this way intracellular AA levels were elevated, resulting in enhanced BCR-dependent disruption of mitochondrial membrane potential and BCR-driven apoptosis (figure 4.11 and appendix 8). The mitochondrial death pathway was thus amplified under conditions in which AA was able to accumulate. However, expression of Bcl-x_L or ligation of CD40 impaired these effects. Taken together, these observations implicate a causal role for BCR-dependent arachidonic acid production in mediating the mitochondrial death pathway and this can be inhibited by ligation of CD40 or induction of Bcl-x_L.

The metabolism of AA to prostaglandins and leukotrienes can thus impair BCR-driven apoptosis by reducing the levels of AA. However, a mitogenic role for metabolites of AA was also implicated using Cox2 and Lox inhibitors since these inhibitors impaired proliferation of WEHI-231 cells (figure 4.12). This was not rescued by expression of Bcl-x_L, which is consistent with Bcl-x_L being unable to restore proliferation to BCR-stimulated WEHI-231 cells. However, CD40-mediated rescue from BCR-driven growth arrest was also impaired by Cox2/Lox inhibitors (figure 4.13) suggesting proliferation of WEHI-231 cells might require the production of eicosanoids. Consistent with this, anti-Ig inhibited PGE_2 production in WEHI-231 cells but co-ligation of CD40 restored PGE_2 production (appendix 9). In contrast, Bcl-x_L was unable to restore PGE_2 production to anti-Ig-treated cells (figure 4.14A) thus suggesting PGE_2 is associated with promoting proliferation rather than survival of WEHI-231 cells. A possible mechanism for regulating PGE_2 production in WEHI-231 cells was implicated by the observation that Cox2 expression correlated with PGE_2 levels (appendix 9) indicating the BCR may promote growth arrest by downregulating Cox2 levels.

The significance of PGE_2 and other eicosanoids was investigated by determining the effect of Cox2/Lox inhibitors on the activity of Erk in WEHI-231 cells. Inhibition of Cox2/Lox suppressed sustained Erk signals and this is likely to contribute to the induction of growth arrest (figure 4.14B). Cox2/Lox inhibitors also enhanced early (≤4 hr) Erk signals (figure 4.15) suggesting a possible mechanism for NS-398/LOI-induced apoptosis since early Erk signals can

induce $cPLA_2$ to enhance AA-dependent disruption of mitochondrial function. Furthermore, treatment of unstimulated or BCR-activated WEHI-231 cells with exogenous AA enhanced Erk activity between 0 and 4 hr whereas ligation of CD40 impaired AA-mediated induction of Erk (figure 4.16). Conditions that increase the levels of AA therefore appear to promote early Erk signals and this might lead to the induction of $cPLA_2$ to further elevate AA levels leading to an enhancement of the mitochondrial death pathway.

Ligation of the BCR therefore induces early Erk signals that contribute to the activation of $cPLA_2$ and arachidonic acid production. Arachidonic acid can enhance early Erk signals, possibly leading to an induction of $cPLA_2$ and an elevation of AA levels. The accumulation of AA leads to a loss of mitochondrial membrane potential and commitment of the cell to apoptosis. However, BCR-driven apoptosis can be prevented by ligation of CD40. Bcl-x_L is a key mediator of CD40-dependent survival and it does so by suppressing AA-mediated induction of $cPLA_2$ activity and by protecting the mitochondria from AA-dependent disruption. The induction of Bcl-x_L by CD40 therefore impairs BCR-driven activation of cathepsin B and subsequent apoptosis. However, expression of Bcl-x_L cannot mimic CD40-dependent rescue from BCR-driven growth arrest. The precise mechanism of CD40-dependent proliferation is incompletely understood but it is likely to involve the induction of Cox2. This could impair BCR-driven apoptosis by diminishing the levels of arachidonic acid and could promote proliferation via the production of PGE_2. Indeed, PGE_2 and other eicosanoids may promote proliferation of WEHI-231 cells via the induction of sustained and cyclic activation of Erk. A model of these findings is presented in figure 4.17.

Figure 4.1. Generation of prostaglandins. Arachidonic acid is generated by $cPLA_2$ and is converted to the common intermediate prostaglandin H_2 (PGH_2) by prostaglandin H synthase. Prostaglandin H synthase has peroxidase and cyclooxygenase activity. PGH_2 is converted to prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, thromboxane B_2 and 6-keto $PGF_{2\alpha}$ by terminal prostanoid synthases. * The rate limiting step of prostaglandin synthesis is catalysed by Cox.

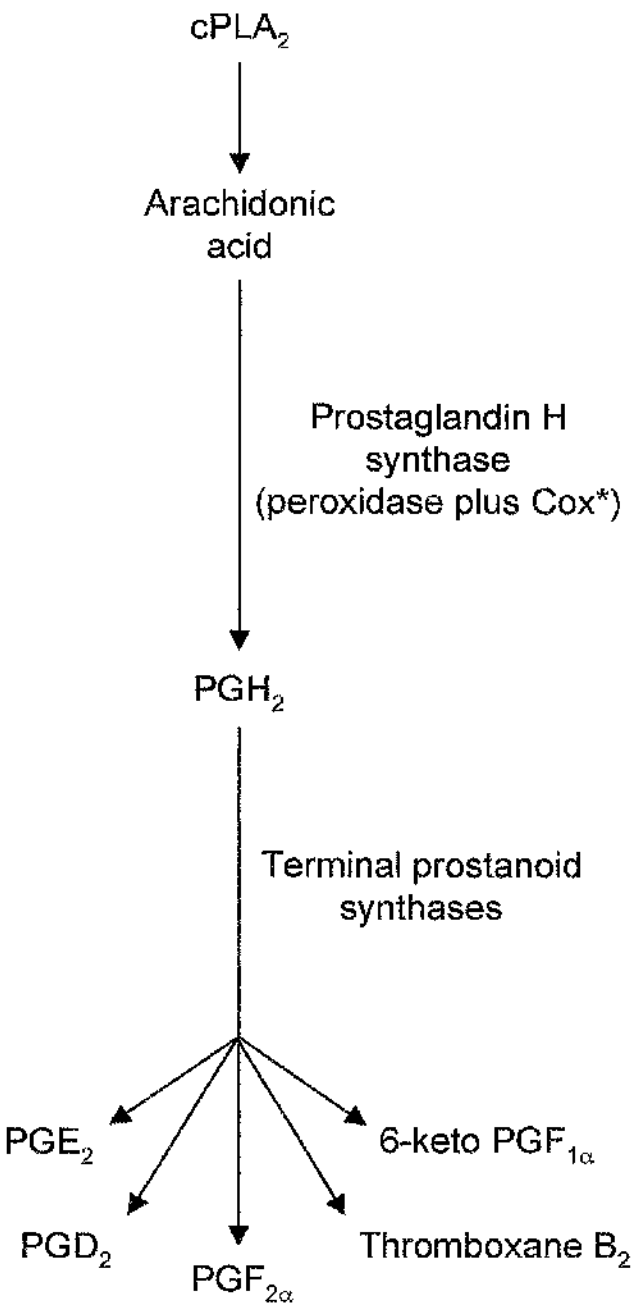
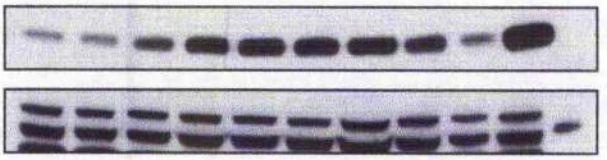

cPLA2
Arachidonic acid
Prostaglandin H synthase (peroxidase plus Cox*)
PGH2
Terminal prostanoid synthases
PGE2
6-keto PGF1α
PGD2
PGF2α
Thromboxane B2

Figure 4.2. Bcl-x_L is upregulated downstream of CD40 and this can be mimicked by transfection of WEHI-231 cells with the *bcl-x_L* gene. Wild-type WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with **(A)** anti-CD40 (10 µg/ml), **(B)** anti-Ig (10 µg/ml) or **(C)** a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates were analysed by Western blotting (50 µg/well), using the NuPAGE system of gel electrophoresis, with anti-Bcl-$x_{L/S}$ antibody. Expression of Bcl-x_L in anti-CD40-treated wild-type WEHI-231 cells was compared to that of unstimulated WEHI-231 cells transfected with empty vector (Neo) or overexpressing Bcl-x_L (Bcl). Recombinant p42 Erk2 was also loaded as an additional standard. Data are representative of at least three independent experiments.

(A) Anti-CD40

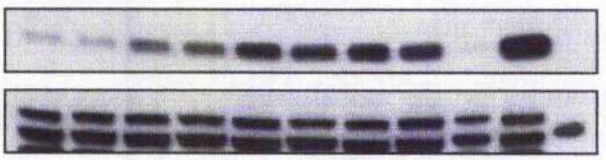


Time (hr) 0 1 2 4 8 12 24 48 Neo Bcl Erk

(B) Anti-Ig

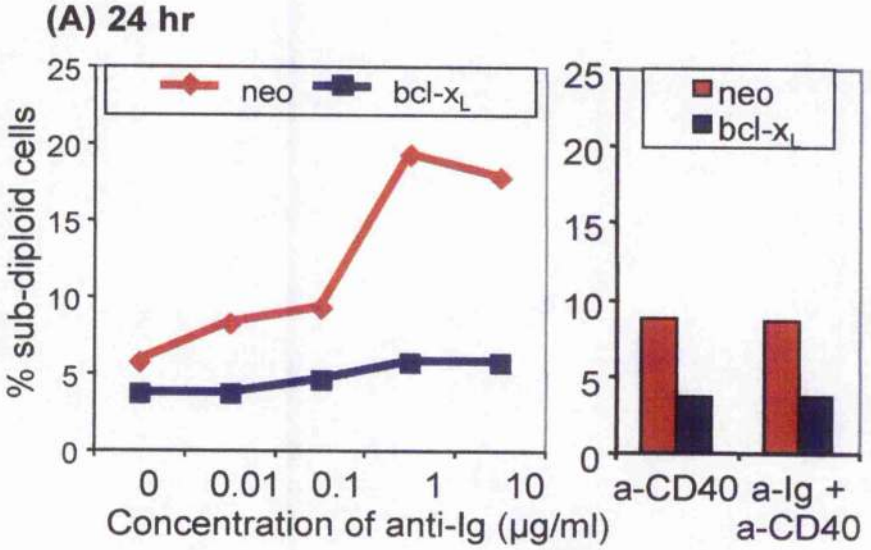
Time (hr) 0 1 2 4 8 12 24 48 Neo Bcl Erk

(C) Anti-Ig + anti-CD40



Time (hr) 0 1 2 4 8 12 24 48 Neo Bcl Erk

Figure 4.3. Overexpression of Bcl-x_L protects WEHI-231 cells from BCR-driven apoptosis. Neo or Bcl-x_L WEHI-231 B cells (5 x 10^5 cells/ml) were cultured for 24 hr **(A)** or 48 hr **(B)** with anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml). Levels of apoptosis were indicated by the number of cells with sub-diploid DNA and this was determined by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments.


(A) 24 hr
% sub-diploid cells
neo
bcl-x$_L$
0
5
10
15
20
25
0
0.01
0.1
1
10
Concentration of anti-Ig (μg/ml)
a-CD40
a-Ig + a-CD40

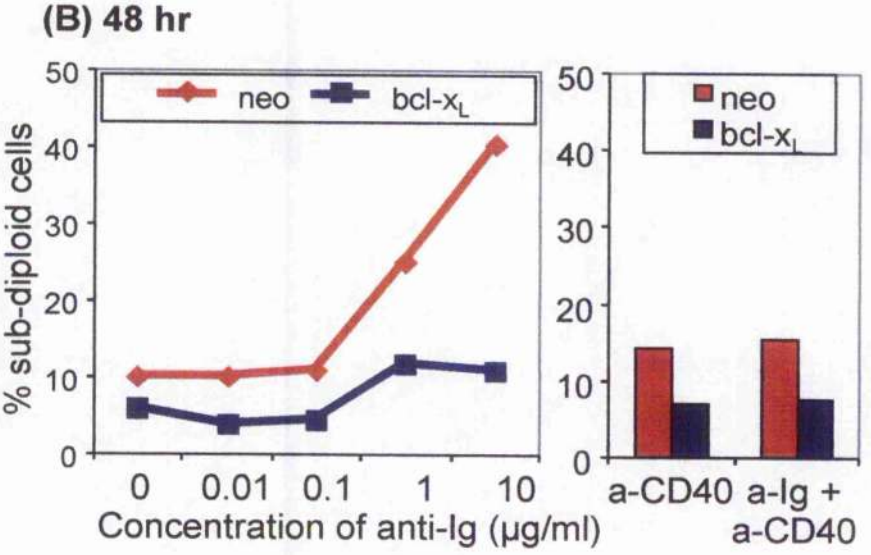

(B) 48 hr
% sub-diploid cells
neo
bcl-x$_L$
0
10
20
30
40
50
0
0.01
0.1
1
10
Concentration of anti-Ig (μg/ml)
a-CD40
a-Ig + a-CD40

Figure 4.4. Overexpression of Bcl-x_L protects WEHI-231 cells from BCR-driven apoptosis but not growth arrest after 24 hr. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 B cells (5 x 10^5 cells/ml) were cultured for 24 hr with medium (untreated), anti-Ig (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The cell cycle progression of WEHI-231 cells was analysed by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments.

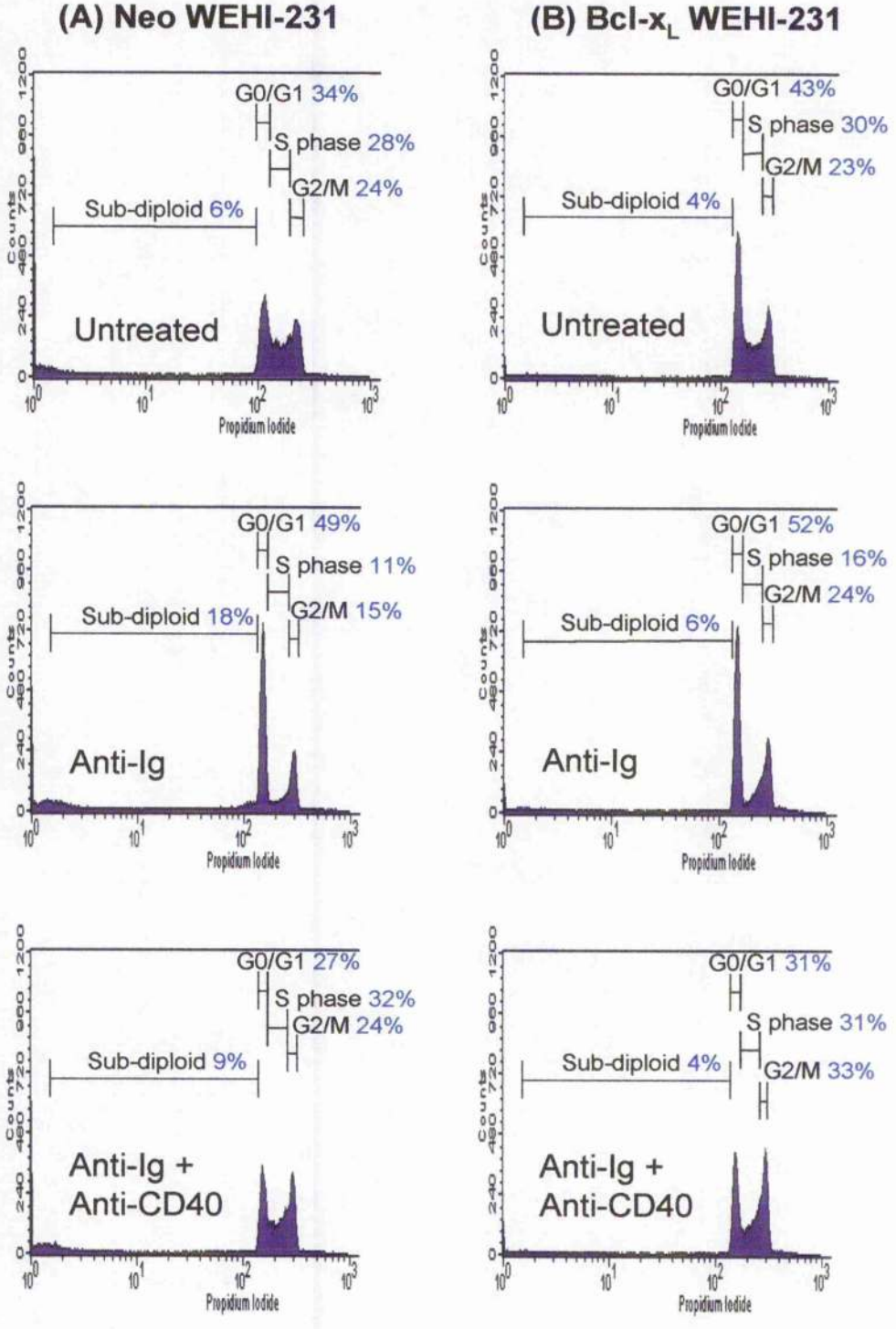

(A) Neo WEHI-231
(B) Bcl-x$_L$ WEHI-231
G0/G1 34%
S phase 28%
G2/M 24%
Sub-diploid 6%
Untreated
G0/G1 43%
S phase 30%
G2/M 23%
Sub-diploid 4%
Untreated
G0/G1 49%
S phase 11%
G2/M 15%
Sub-diploid 18%
Anti-Ig
G0/G1 52%
S phase 16%
G2/M 24%
Sub-diploid 6%
Anti-Ig
G0/G1 27%
S phase 32%
G2/M 24%
Sub-diploid 9%
Anti-Ig +
Anti-CD40
G0/G1 31%
S phase 31%
G2/M 33%
Sub-diploid 4%
Anti-Ig +
Anti-CD40
Counts
Propidium Iodide

Figure 4.5. Overexpression of Bcl-x_L protects WEHI-231 cells from BCR-driven apoptosis but not growth arrest after 48 hr. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 B cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), anti-Ig (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The cell cycle progression of WEHI-231 cells was analysed by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments.

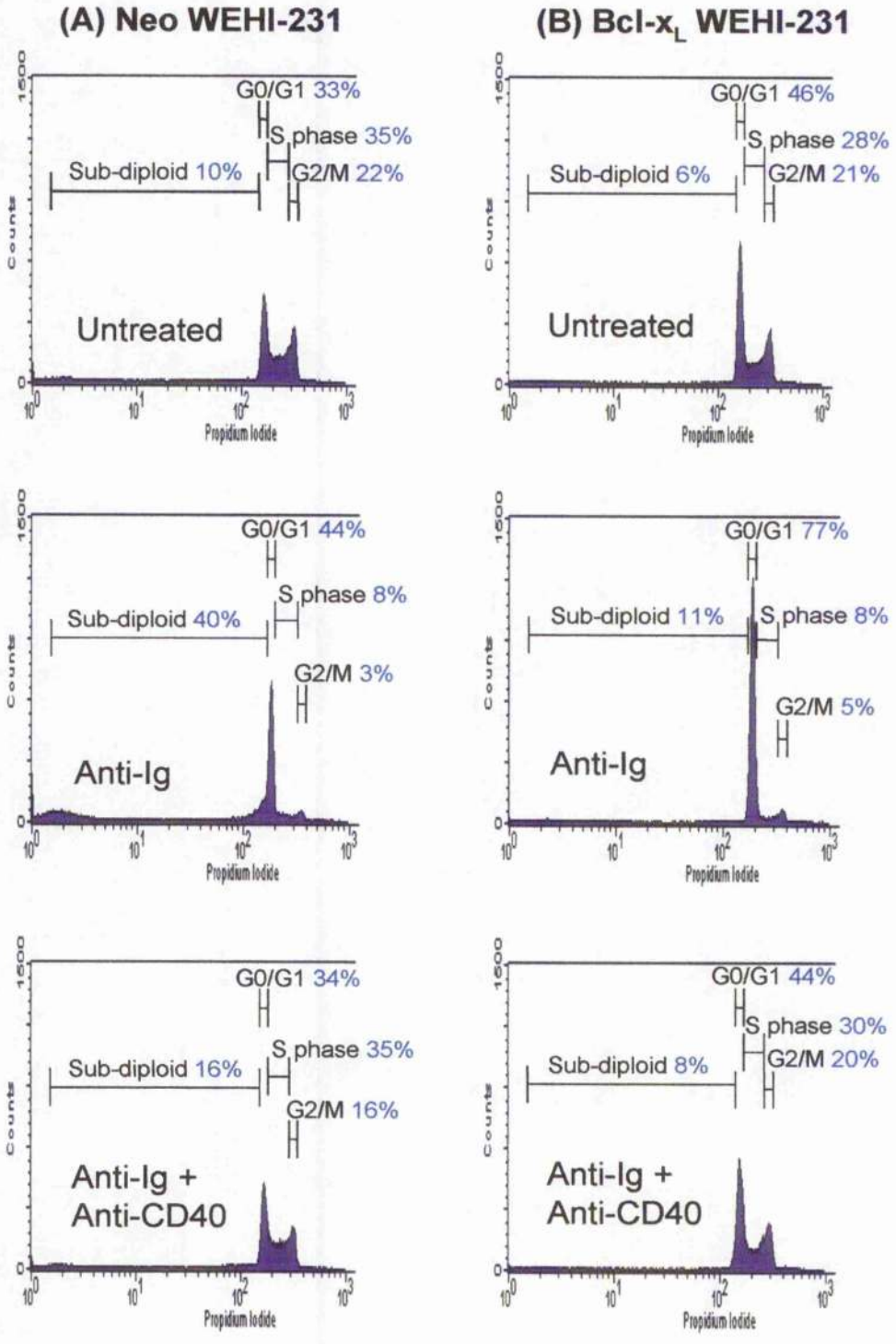

(A) Neo WEHI-231
(B) Bcl-x$_L$ WEHI-231
G0/G1 33%
S phase 35%
G2/M 22%
Sub-diploid 10%
Untreated
G0/G1 46%
S phase 28%
G2/M 21%
Sub-diploid 6%
Untreated
G0/G1 44%
S phase 8%
G2/M 3%
Sub-diploid 40%
Anti-Ig
G0/G1 77%
S phase 8%
G2/M 5%
Sub-diploid 11%
Anti-Ig
G0/G1 34%
S phase 35%
G2/M 16%
Sub-diploid 16%
Anti-Ig + Anti-CD40
G0/G1 44%
S phase 30%
G2/M 20%
Sub-diploid 8%
Anti-Ig + Anti-CD40
Counts
Propidium Iodide

Figure 4.6. Overexpression of Bcl-x_L does not protect WEHI-231 cells from BCR-driven growth arrest at G0/G1 phase. **(A)** Neo or Bcl-x_L WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (10 µg/ml) or anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least six independent experiments. **(B)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr with increasing concentrations of anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The number of cells in G0/G1 phase of the cell cycle was determined by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments. **(C)** Neo or Bcl-x_L WEHI-231 B cells (5 x 10^5 cells/ml) were cultured for 48 hr with increasing concentrations of anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The number of cells in G0/G1 phase of the cell cycle was determined by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments.

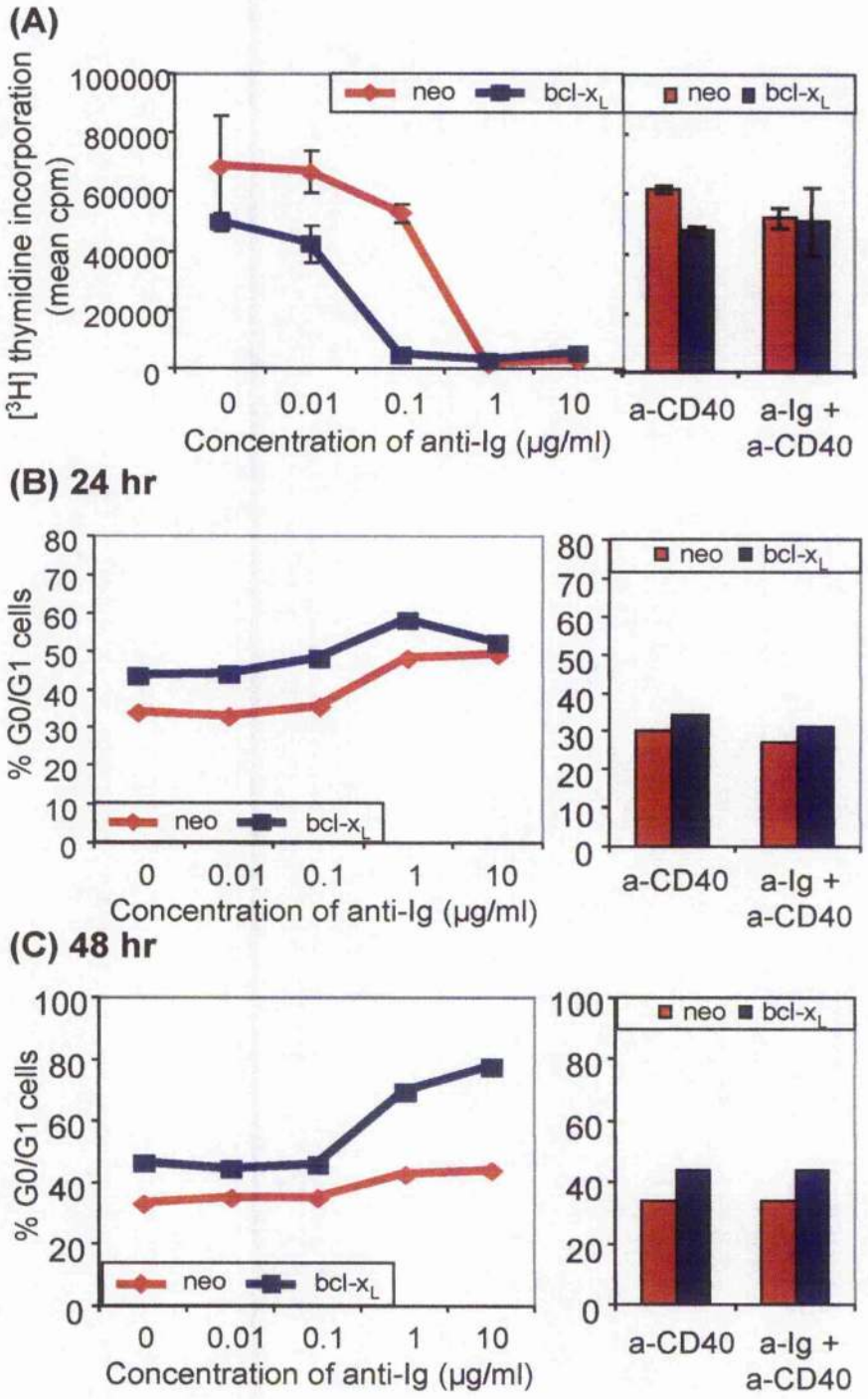
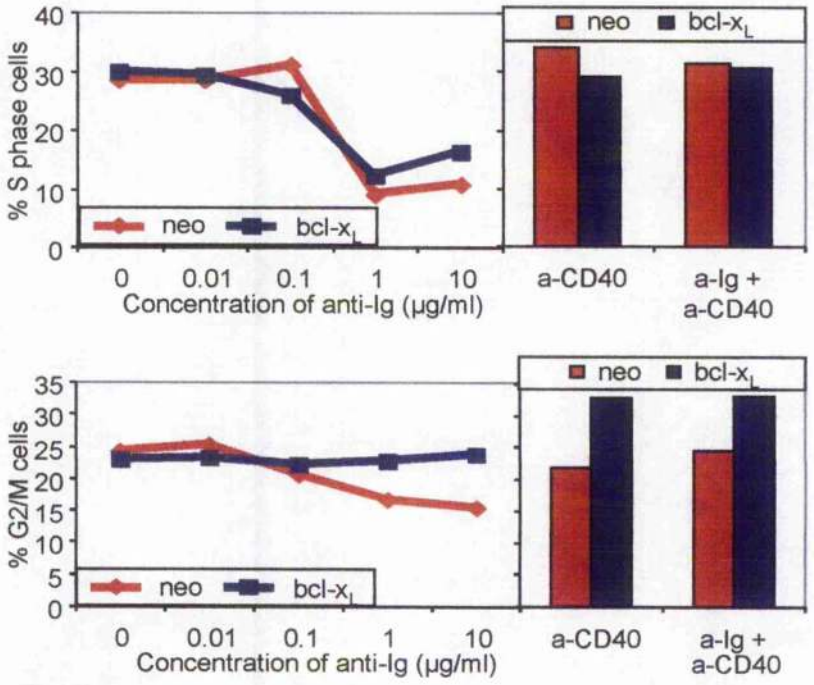

(A)
[3H] thymidine incorporation (mean cpm)
100000
80000
60000
40000
20000
0
neo
bcl-xL
0
0.01
0.1
1
10
Concentration of anti-Ig (μg/ml)
a-CD40
a-Ig + a-CD40
(B) 24 hr
% G0/G1 cells
80
70
60
50
40
30
20
10
0
(C) 48 hr
100
80
60
40
20
0

Figure 4.7. Overexpression of Bcl-x_L does not protect WEHI-231 cells from BCR-driven depletion of S phase and G2/M phase cells. **(A)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr with anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The number of cells in S phase and G2/M were determined by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments. **(B)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with anti-Ig (0-10 µg/ml, as indicated), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). The number of cells in S phase and G2/M were determined by PI-staining and FACS analysis (FL2 fluorescence). Data are representative of at least six independent experiments.

(A) 24 hr


% S phase cells
40
30
20
10
0
neo
bcl-x$_L$
0
0.01
0.1
1
10
Concentration of anti-Ig (μg/ml)
a-CD40
a-Ig + a-CD40
% G2/M cells
35
30
25
20
15
10
5
0

(B) 48 hr

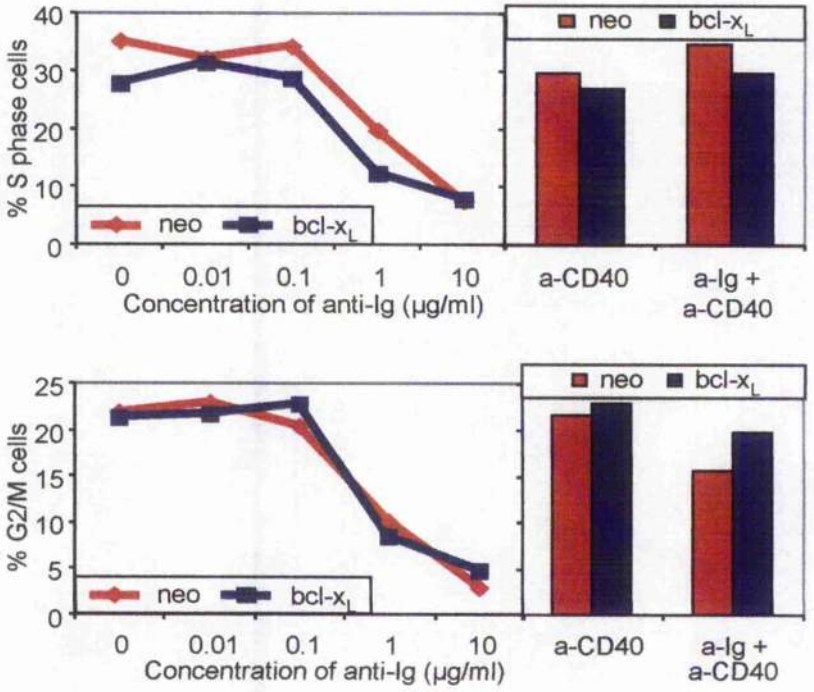
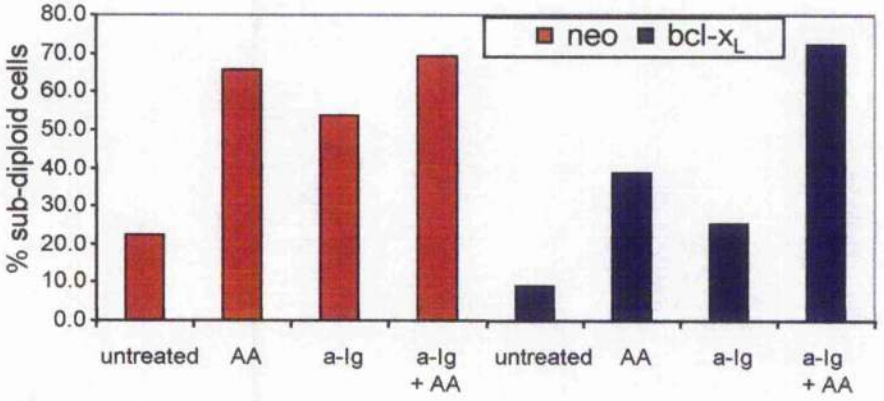
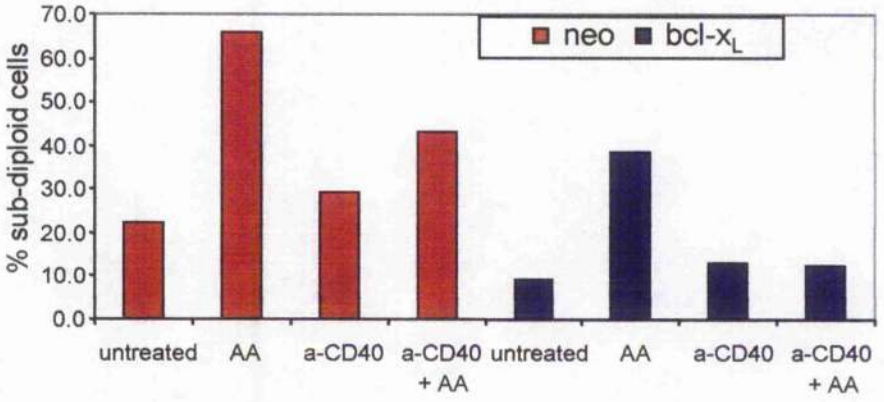

% S phase cells
40
30
20
10
0
neo
bcl-x$_L$
0
0.01
0.1
1
10
Concentration of anti-Ig (μg/ml)
a-CD40
a-Ig + a-CD40
% G2/M cells
25
20
15
10
5
0

Figure 4.8. Arachidonic acid induces apoptosis of WEHI-231 cells. **(A)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), 100 µM arachidonic acid (AA), 10 µg/ml anti-Ig (a-Ig) or a combination of 100 µM arachidonic acid plus 10 µg/ml anti-Ig (a-Ig + AA). Levels of apoptosis were determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments. **(B)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), 100 µM arachidonic acid (AA), 10 µg/ml anti-CD40 (a-CD40) or a combination of 100 µM arachidonic acid plus 10 µg/ml anti-CD40 (a-CD40 + AA). Levels of apoptosis were determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments. **(C)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), 100 µM arachidonic acid (AA), a combination of anti-Ig plus anti-CD40 (a-I/C, both at 10 µg/ml) or a combination of 100 µM arachidonic acid plus 10 µg/ml anti-Ig plus 10 µg/ml anti-CD40 (a-I/C + AA). Levels of apoptosis were determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments.

(A)


% sub-diploid cells
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
neo
bcl-x$_L$
untreated
AA
a-Ig
a-Ig + AA
untreated
AA
a-Ig
a-Ig + AA

(B)


% sub-diploid cells
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
neo
bcl-x$_L$
untreated
AA
a-CD40
a-CD40 + AA
untreated
AA
a-CD40
a-CD40 + AA

(C)

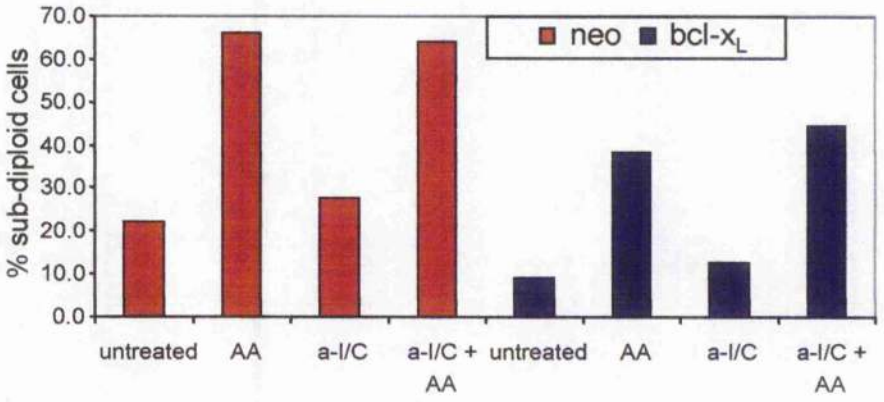

% sub-diploid cells
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
neo
bcl-x$_L$
untreated
AA
a-I/C
a-I/C + AA
untreated
AA
a-I/C
a-I/C + AA

Figure 4.9. Arachidonic acid induces growth arrest of WEHI-231 cells. **(A)** Neo or Bcl-x_L WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of arachidonic acid (0-100 μM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least three independent experiments. **(B)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), arachidonic acid (AA, 100 μM), anti-Ig (a-Ig, 10 μg/ml) or a combination of arachidonic acid (100 μM) plus anti-Ig (10 μg/ml) (a-Ig + AA). The number of cells in S phase and G2/M were determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments. **(C)** Bcl-x_L WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of arachidonic acid (0-100 μM, as indicated) with medium (no a-CD40), 1 μg/ml anti-CD40 or 10 μg/ml anti-CD40. Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least three independent experiments.

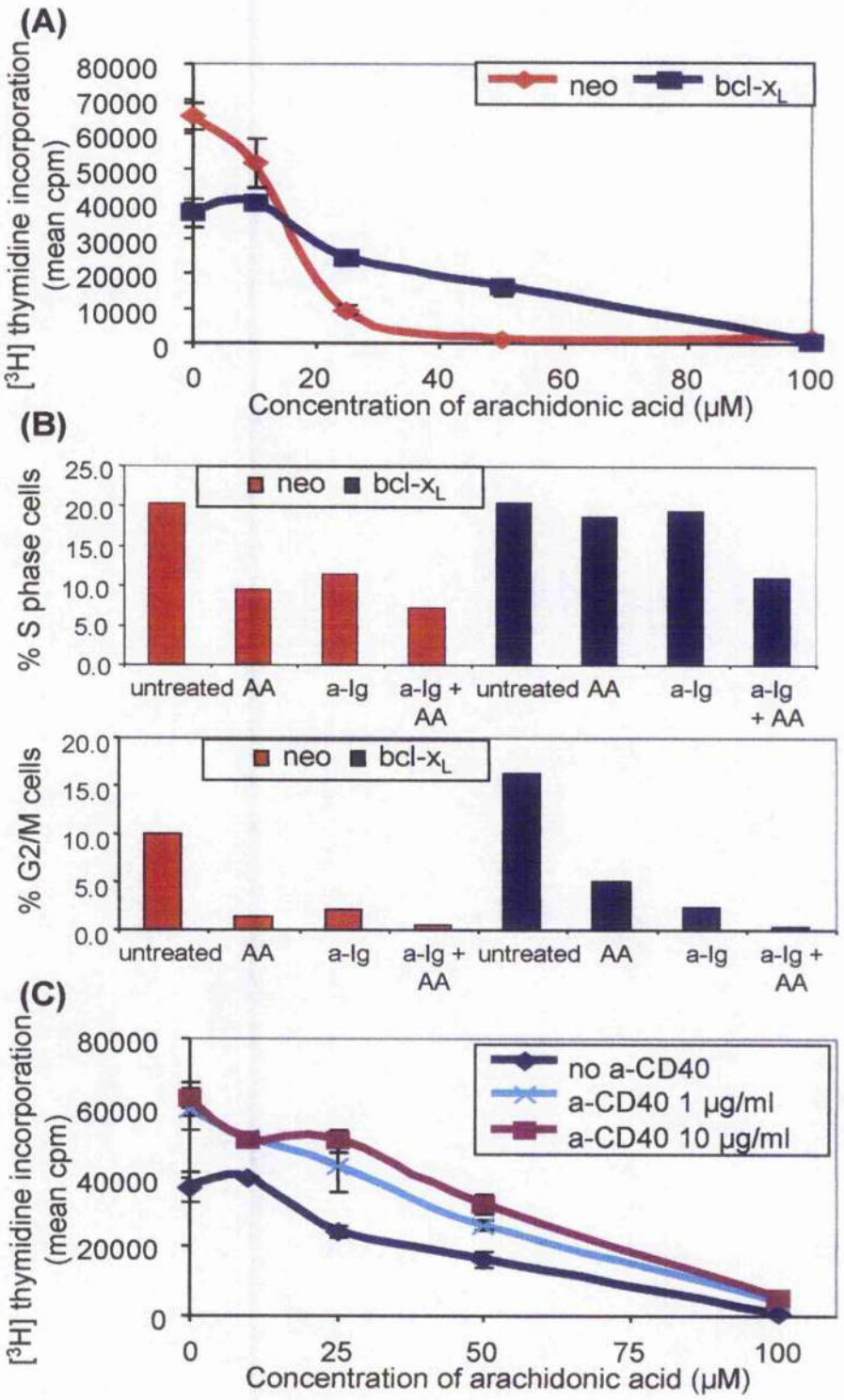

(A)
[3H] thymidine incorporation (mean cpm)
80000
70000
60000
50000
40000
30000
20000
10000
0
neo
bcl-xL
0
20
40
60
80
100
Concentration of arachidonic acid (μM)
(B)
% S phase cells
25.0
20.0
15.0
10.0
5.0
0.0
neo
bcl-xL
untreated
AA
a-Ig
a-Ig + AA
untreated
AA
a-Ig
a-Ig + AA
% G2/M cells
20.0
15.0
10.0
5.0
0.0
neo
bcl-xL
untreated
AA
a-Ig
a-Ig + AA
untreated
AA
a-Ig
a-Ig + AA
(C)
[3H] thymidine incorporation (mean cpm)
80000
60000
40000
20000
0
no a-CD40
a-CD40 1 μg/ml
a-CD40 10 μg/ml
0
25
50
75
100
Concentration of arachidonic acid (μM)

Figure 4.10. Overexpression of Bcl-x_L does not restore sustained cycling Erk activation required for proliferation in WEHI-231 cells. Neo or Bcl-x_L WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 24 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting. Data are representative of at least six independent experiments.

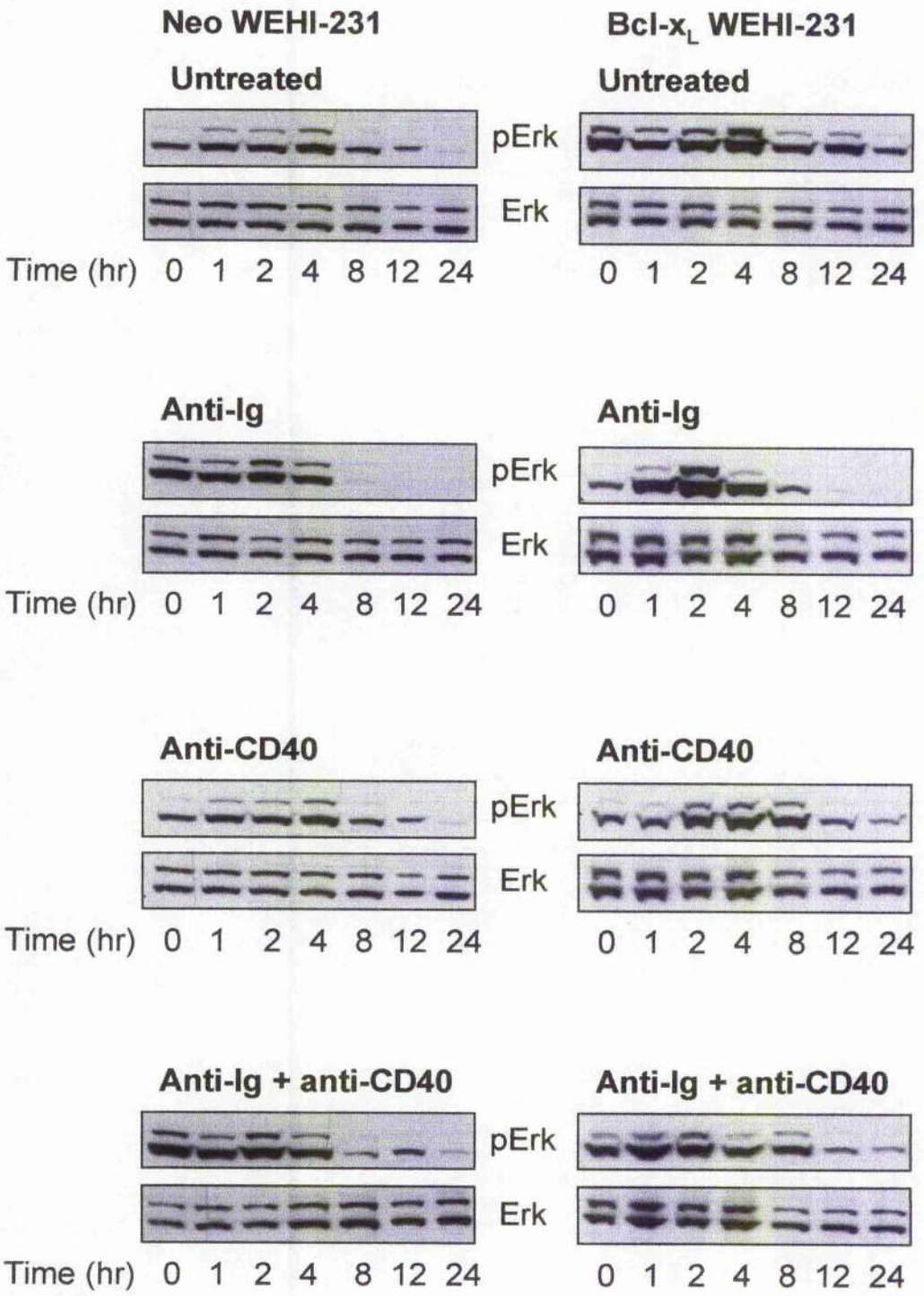

Neo WEHI-231
Bcl-x$_L$ WEHI-231
Untreated
Untreated
pErk
Erk
Time (hr) 0 1 2 4 8 12 24
0 1 2 4 8 12 24
Anti-Ig
Anti-Ig
pErk
Erk
Time (hr) 0 1 2 4 8 12 24
0 1 2 4 8 12 24
Anti-CD40
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4 8 12 24
0 1 2 4 8 12 24
Anti-Ig + anti-CD40
Anti-Ig + anti-CD40
pErk
Erk
Time (hr) 0 1 2 4 8 12 24
0 1 2 4 8 12 24

Figure 4.11. Cox and Lox inhibitors enhance BCR-mediated apoptosis of WEHI-231 cells. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated), a combination of NS-398 plus LOI (NS + LOI), anti-Ig (a-Ig), a combination of anti-Ig plus NS-398 plus LOI (a-Ig + NS + LOI), a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40) or a combination of anti-Ig plus anti-CD40 plus NS-398 plus LOI (a-Ig + a-CD40 + NS + LOI). The same concentrations of antibodies and inhibitors were used throughout: anti-Ig 10 µg/ml, anti-CD40 10 µg/ml, NS-398 10 µM and LOI 10 µM. Levels of apoptosis were determined by measuring the sub-diploid DNA content using PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments.

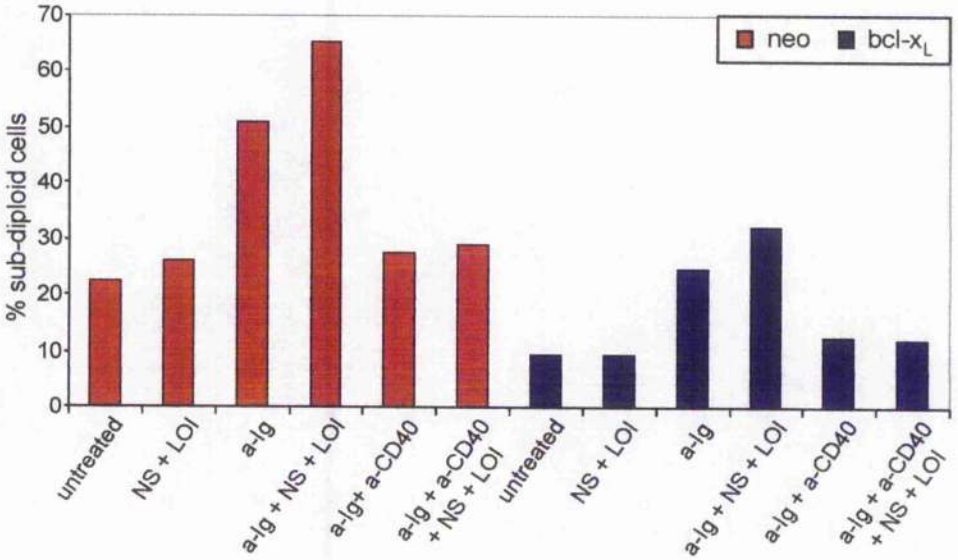

% sub-diploid cells
70
60
50
40
30
20
10
0
neo
bcl-x$_L$
untreated
NS + LOI
a-Ig
a-Ig + NS + LOI
a-Ig+ a-CD40
a-Ig + a-CD40 + NS + LOI
untreated
NS + LOI
a-Ig
a-Ig + NS + LOI
a-Ig + a-CD40
a-Ig + a-CD40 + NS + LOI

Figure 4.12. Inhibition of Cox and Lox impairs proliferation of WEHI-231 cells. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 cells were stimulated with increasing concentrations of anti-Ig (0-1 μg/ml, as indicated) plus NS-398 (0, 1 or 10 μM), LOI (0, 1 or 10 μM) or a combination of NS-398 plus LOI (both at 10 μM) for 48 hr. Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells and data are presented as % of control (Neo **(A)** or Bcl-x_L **(B)** WEHI-231 cells in the absence of anti-Ig and inhibitor) mean cpm ± standard deviation. Data are representative of at least three independent experiments.

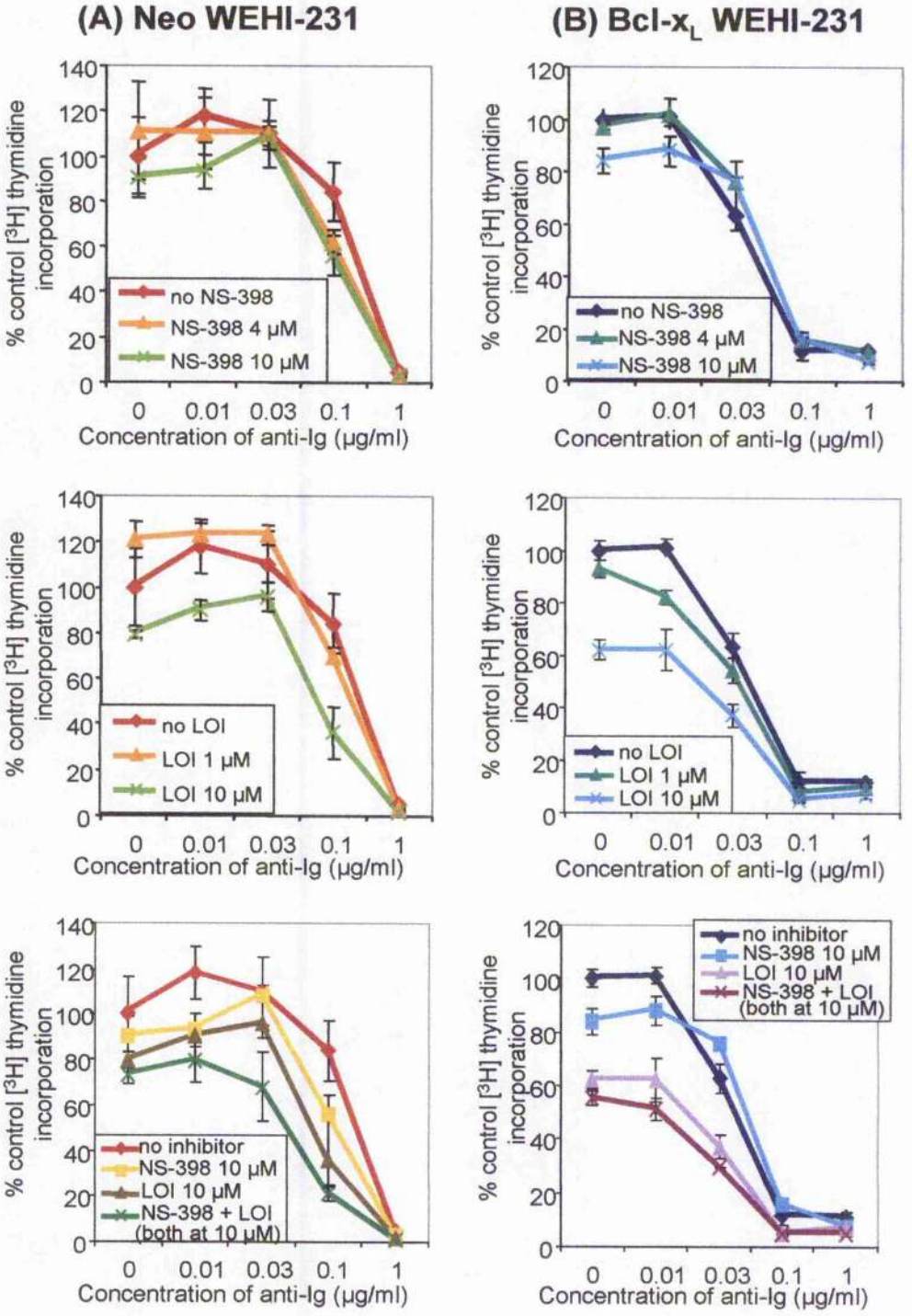

(A) Neo WEHI-231
(B) Bcl-x$_L$ WEHI-231
% control [^{3}H] thymidine incorporation
Concentration of anti-Ig (µg/ml)
0
0.01
0.03
0.1
1
no NS-398
NS-398 4 µM
NS-398 10 µM
no LOI
LOI 1 µM
LOI 10 µM
no inhibitor
NS-398 10 µM
LOI 10 µM
NS-398 + LOI
(both at 10 µM)

Figure 4.13. Inhibition of Cox and Lox impairs proliferation and CD40-mediated rescue of WEHI-231 cells. Neo WEHI-231 (5 x 10^6 cells/ml) were cultured for 48 hr with medium (untreated), a combination of NS-398 plus LOI (both at 10 μM), anti-Ig (10 μg/ml) or a combination of anti-Ig (10 μg/ml) plus NS-398 (10 μM) plus LOI (10 μM). The number of cells in S phase **(A)** or G2/M phase **(B)** were determined by PI-staining and FACS analysis (FL3 fluorescence). Data are representative of at least three independent experiments. **(C)** Neo WEHI-231 cells were stimulated for 48 hr with medium (untreated), anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or a combination of anti-Ig plus anti-CD40 (10 μg/ml) and for each of these conditions cells were also treated with medium (no inhibitor), NS-398 (10 μM), LOI (10 μM) or a combination of NS-398 plus LOI (both at 10 μM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells and data are presented as % of control (untreated Neo WEHI-231 cells in the absence of inhibitor) mean cpm ± standard deviation. Data are representative of at least three independent experiments. **(D)** Bcl-x_L WEHI-231 cells were stimulated for 48 hr with medium (untreated), anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or a combination of anti-Ig plus anti-CD40 (10 μg/ml) and for each of these conditions cells were also treated with medium (no inhibitor), NS-398 (10 μM), LOI (10 μM) or a combination of NS-398 plus LOI (both at 10 μM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells and data are presented as % of control (untreated Bcl-x_L WEHI-231 cells in the absence of inhibitor) mean cpm ± standard deviation. Data are representative of at least three independent experiments.

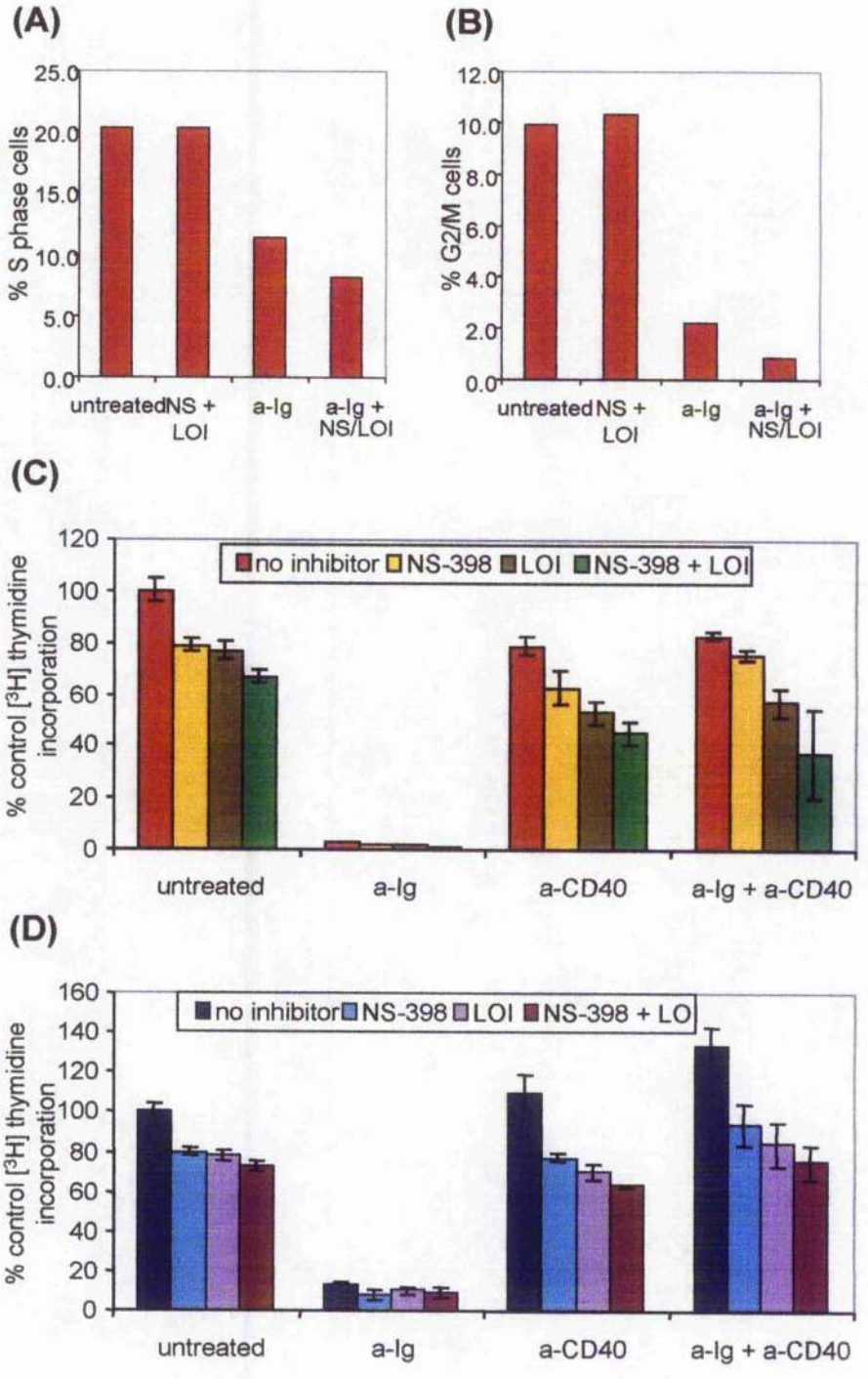

(A)
% S phase cells
25.0
20.0
15.0
10.0
5.0
0.0
untreated
NS + LOI
a-Ig
a-Ig + NS/LOI
(B)
% G2/M cells
12.0
10.0
8.0
6.0
4.0
2.0
0.0
untreated
NS + LOI
a-Ig
a-Ig + NS/LOI
(C)
% control [3H] thymidine incorporation
no inhibitor
NS-398
LOI
NS-398 + LOI
120
100
80
60
40
20
0
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D)
% control [3H] thymidine incorporation
no inhibitor
NS-398
LOI
NS-398 + LO
160
140
120
100
80
60
40
20
0
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 4.14. Bcl-x_L does not prevent anti-Ig-dependent inhibition of PGE_2 production in WEHI-231 cells and inhibition of Cox/Lox impairs CD40-mediated activation of sustained Erk signals. **(A)** Neo or Bcl-x_L WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr in the presence or absence of anti-Ig (1 µg/ml) before preparing cell lysates and determining the levels of PGE_2 using a PGE_2 competitive binding immunoassay kit (Cayman Chemical). Each anti-Ig-treated time point sample is expressed as a % of its unstimulated control and data are presented as means ± standard deviation, n=3. **(B)** Treatment of proliferating Neo or Bcl-x_L WEHI-231 cells with NS-398/LOI suppresses sustained (8-48 hr) Erk signals. Neo or Bcl-x_L WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with anti-CD40 (10 µg/ml) in the presence (NS-398/LOI) or absence (no inhibitor) of NS-398 plus LOI (both at 10 µM) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

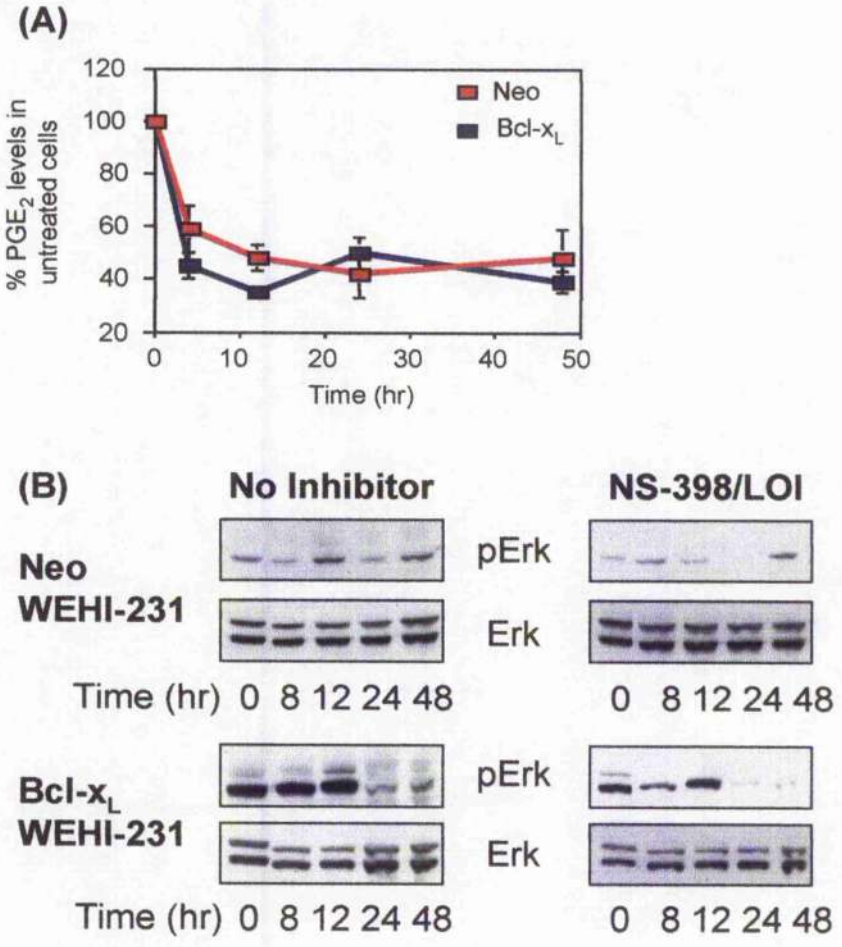

(A)
% PGE2 levels in untreated cells
120
100
80
60
40
20
0
10
20
30
40
50
Time (hr)
Neo
Bcl-xL
(B)
No Inhibitor
NS-398/LOI
Neo
WEHI-231
pErk
Erk
Time (hr) 0 8 12 24 48
0 8 12 24 48
Bcl-xL
WEHI-231
pErk
Erk
Time (hr) 0 8 12 24 48
0 8 12 24 48

Figure 4.15. Inhibition of Cox2 plus Lox enhances early (≤4 hr) Erk-MAPK signals in WEHI-231 cells. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 4 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) in the presence (NS-398/LOI) or absence (no inhibitor) of NS-398 plus LOI (both at 10 µM) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

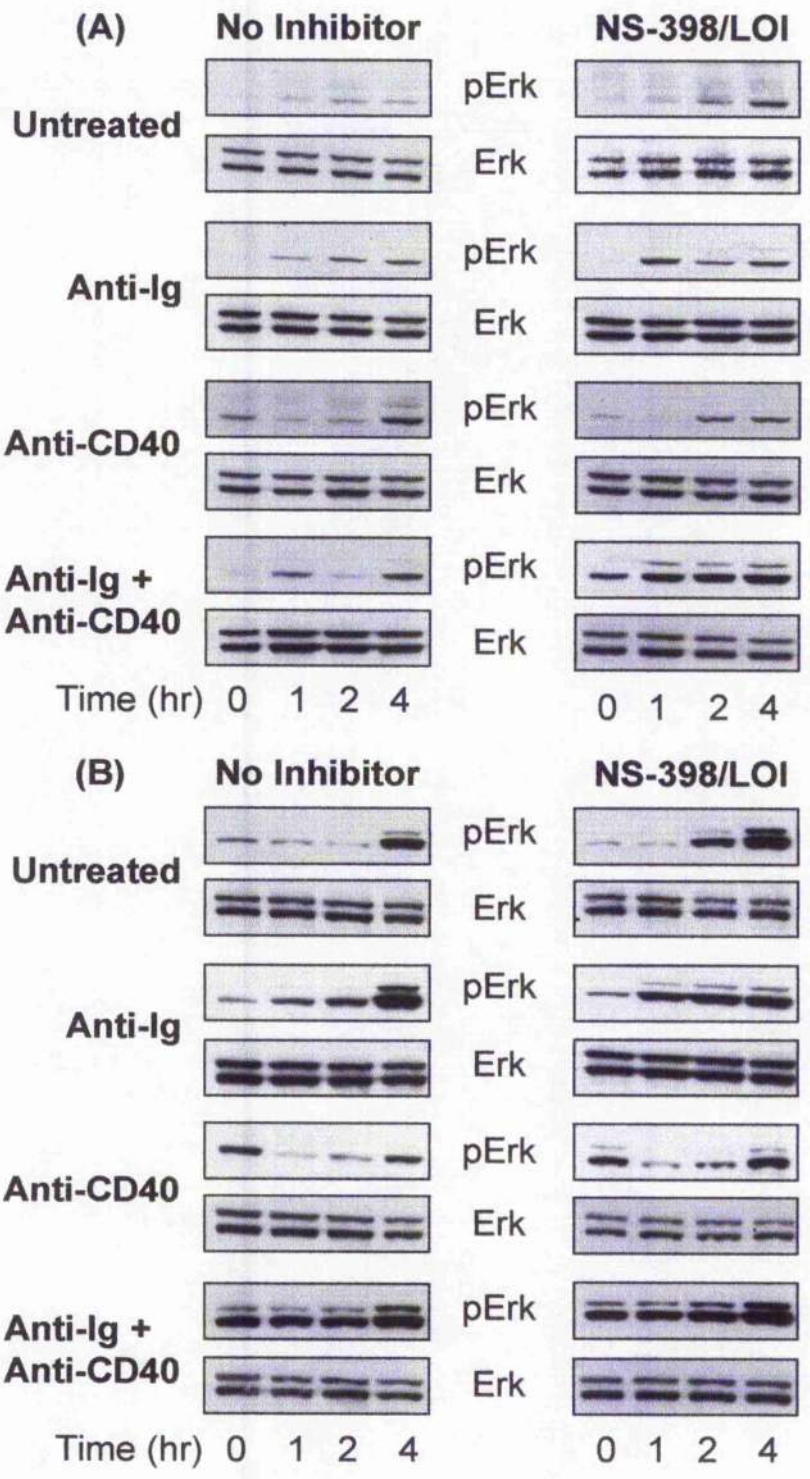

(A)
No Inhibitor
NS-398/LOI
Untreated
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4
0 1 2 4
(B)
No Inhibitor
NS-398/LOI
Untreated
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4
0 1 2 4

Figure 4.16. Arachidonic acid enhances the activity of Erk between 0 and 4 hr and this is impaired by ligation of CD40. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 4 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) in the presence (+ AA) or absence (- AA) of arachidonic acid (100 µM) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

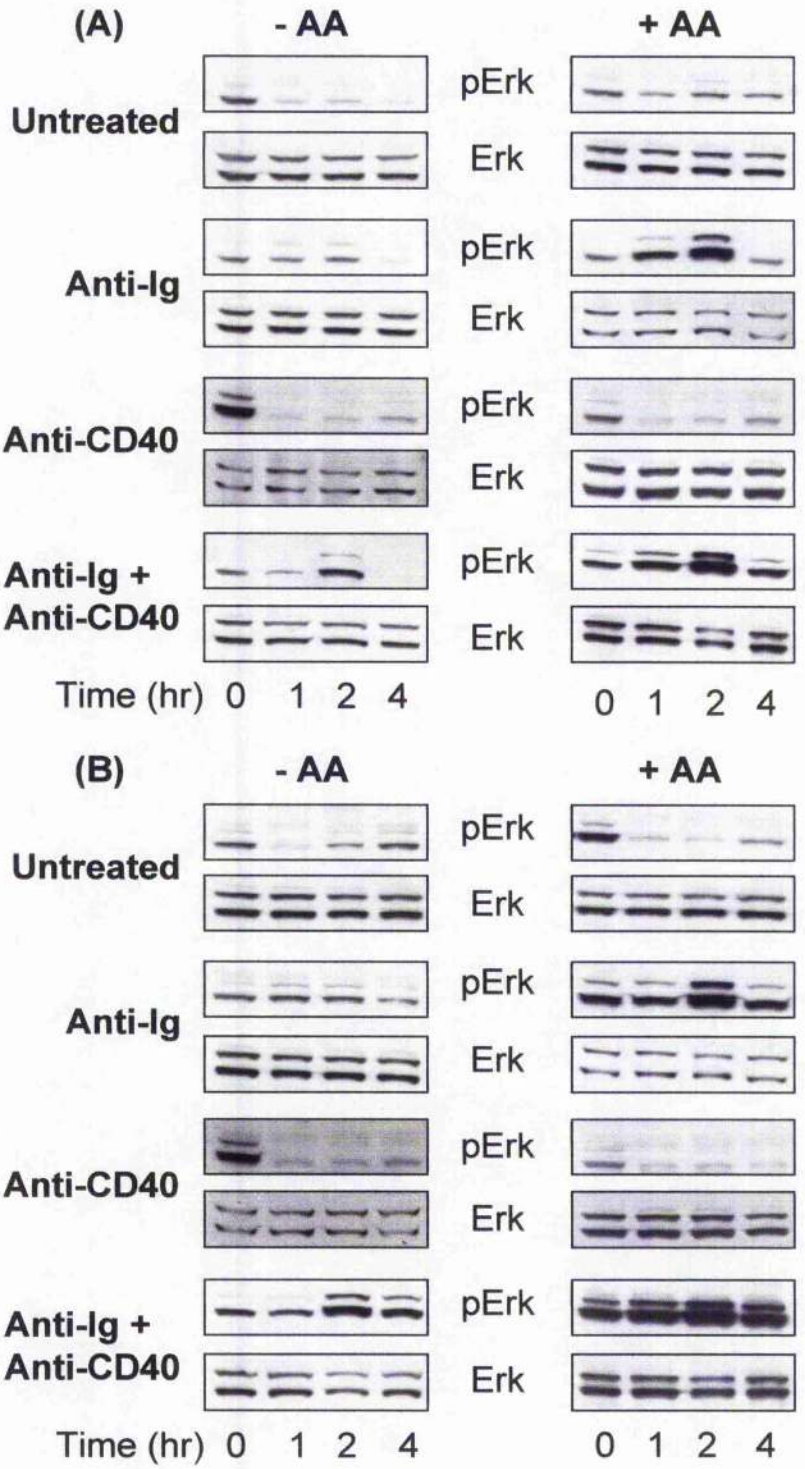
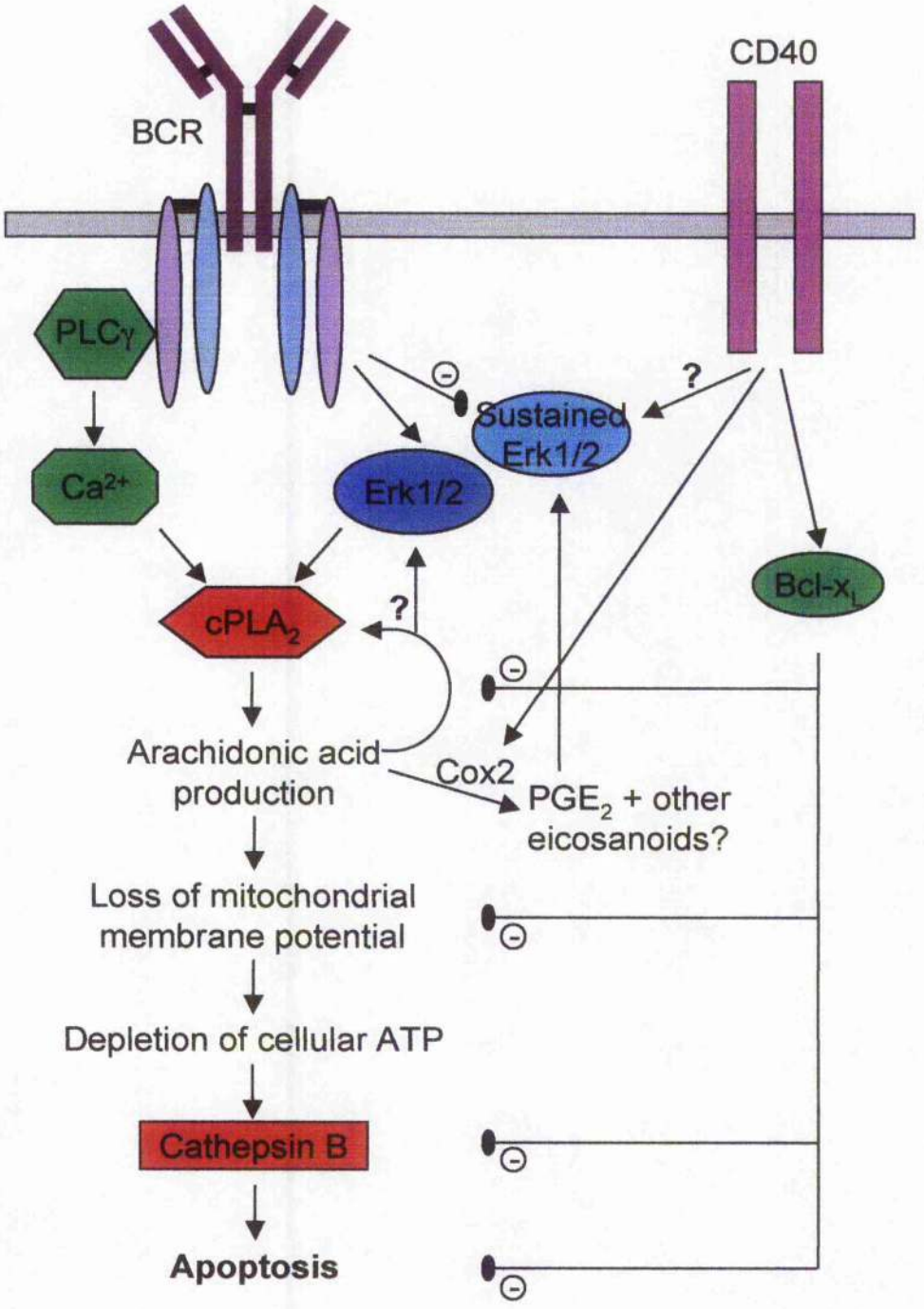

(A)
- AA
+ AA
Untreated
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4
0 1 2 4
(B)
- AA
+ AA
Untreated
Anti-Ig
Anti-CD40
Anti-Ig +
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4
0 1 2 4

Figure 4.17. Model of Bcl-x_L-dependent rescue of BCR-stimulated WEHI-231 cells. Ligation of the BCR induces early Erk signals that contribute to the activation of PLA_2 and arachidonic acid production. The accumulation of arachidonic acid leads to a loss of mitochondrial membrane potential and commitment of the cell to apoptosis. Ligation of CD40 prevents BCR-driven apoptosis via the induction of Bcl-x_L. Bcl-x_L is a key mediator of CD40-dependent survival and it does so by suppressing AA-mediated induction of $cPLA_2$ activity and by protecting the mitochondria from AA-dependent disruption. The induction of Bcl-x_L by CD40 therefore impairs BCR-driven activation of cathepsin B and subsequent apoptosis. However, expression of Bcl-x_L cannot protect WEHI-231 cells from BCR-driven growth arrest. The precise mechanism of CD40-dependent proliferation is incompletely understood but it involves the induction of sustained and cyclic Erk signals. The induction of Cox2 and subsequent production of PGE_2 downstream of CD40 appears to contribute to the activation of sustained Erk signals and hence proliferation. However, CD40 may also induce the production of additional eicosanoids to further promote proliferation of WEHI-231 cells.


CD40
BCR
PLCγ
Ca2+
Erk1/2
Sustained Erk1/2
?
cPLA2
?
Bcl-xL
Arachidonic acid production
Cox2
PGE2 + other eicosanoids?
Loss of mitochondrial membrane potential
Depletion of cellular ATP
Cathepsin B
Apoptosis

CHAPTER 5 - Role of sphingosine kinase in regulating survival and proliferation of WEHI-231 cells

5.1 Sphingolipids and ceramide

Sphingolipids are a diverse family of phospholipids and glycolipids that are characterised by their sphingoid backbone. The sphingoid backbone is based on the lipid ceramide. Ceramide can be generated by two distinct mechanisms: by *de novo* biosynthesis (figure 5.1A) or by the conversion of sphingolipids to ceramide using specific enzymes. For example, sphingomyelin can be converted to ceramide using sphingomyelinase enzymes. There are three classes of sphingomyelinases: acidic (lysosomal) sphingomyelinase (optimal pH of this enzyme is 5), magnesium-dependent sphingomyelinase (membrane-bound enzyme with neutral optimal pH) and magnesium-independent sphingomyelinase (cytosolic enzyme with neutral optimal pH). The activity of sphingomyelinases can be regulated by extracellular ligands: for example, TNFα elevates the cellular levels of ceramide by stimulating neutral sphingomyelinases and by inducing *de novo* biosynthesis (453).

De novo biosynthesis of ceramide (figure 5.1A) takes place in the endoplasmic reticulum and begins with the condensation of serine and palmitoyl CoA to form 3-ketosphinganine, which is then reduced to form dihydrosphingosine (sphinganine). Dihydrosphingosine can be converted to dihydroceramide via the attachment of a fatty acyl group using an amide link. Dihydroceramide can then be converted to ceramide using desaturase (453-455). Newly synthesised ceramide translocates from the endoplasmic reticulum to the Golgi in vesicles and the Golgi can then transport ceramide to other membranes within the cell (265).

Ceramide has several functions within a cell. Firstly, it is a structural component of membranes and is particularly enriched in lipid rafts (272). Secondly, ceramide can function as a second messenger especially via the activation of ceramide-activated protein kinases and protein phosphatases. Ceramide-activated protein phosphatases can induce growth arrest by dephosphorylating Rb and c-Jun. Ceramide-activated protein phosphatases also promote apoptosis by activating caspases and inhibiting PKCα and Bcl-2 (456). Ceramide-activated protein kinases may promote growth arrest and

apoptosis by inhibiting Ras and its downstream effectors (MAPK and PI-3-K) (453). For example, ceramide induces apoptosis in dendritic cells by inhibiting PI-3-K and the downstream effectors Akt, NF-κB and Bcl-x_L (457). Other targets of ceramide may include Raf-1, cathepsin D, $cPLA_2$, PKCζ and MEKK/JNK (458-460). Ceramide thus functions as a second messenger that promotes growth arrest and apoptosis.

A third function of ceramide is to act as a substrate for specific enzymes that can convert ceramide into other sphingolipids (figure 5.1A) (453). The simplest alteration of ceramide is its phosphorylation catalysed by ceramide kinase to form ceramide-1-phosphate (461). The reverse reaction is catalysed by ceramide phosphatase. By contrast, a series of chemical reactions are required to convert ceramide to glycosphingolipids, gangliosides and sulphatides. These lipids are involved in cell-to-cell interactions and may regulate the activities of receptor tyrosine kinases (462). Ceramide can also be converted to sphingomyelin using sphingomyelin synthase. Sphingomyelin is an important component of cellular membranes that differentially affects the properties of membranes depending on its fatty acyl composition. About 50% of cellular sphingomyelin is found in the plasma membrane and it is particularly abundant in lipid rafts.

Another important sphingolipid that can be formed from ceramide is sphingosine, which is the precursor for sphingosine-1-phosphate (SPP). The interconversion of ceramide, sphingosine and SPP, referred to as the *SPP rheostat*, can determine the fate of a cell (figure 5.1B). The reversible conversion of ceramide to sphingosine and sphingosine-1-phosphate is particularly important since sphingosine and sphingosine-1-phosphate can also function as signalling molecules. Ceramide is hydrolysed by ceramidase to form sphingosine. Sphingosine is generally considered to be a pro-apoptotic signalling molecule since it can mediate the activation of cathepsins and caspases (103). Sphingosine also inhibits PKC-dependent activation of Erk (463). In contrast to sphingosine and ceramide, SPP is generally considered to favour survival and proliferation of cells.

5.2 Sphingosine kinase

Sphingosine kinase catalyses the phosphorylation of sphingosine to

generate SPP (figure 5.1B). Two mammalian sphingosine kinases have been cloned and they have been found to be evolutionarily conserved lipid kinases that can be detected in multiple tissues with highest levels being found in the lung and spleen (464). Inside the cell, sphingosine kinase has been detected in both cytosolic and membrane fractions (465). The activity of sphingosine kinase can be regulated by multiple extracellular ligands. Indeed, sphingosine kinase has at least three consensus sequences for Ca^{2+}/calmodulin-binding indicating it is a calcium-sensitive enzyme. Sphingosine kinase also has multiple potential phosphorylation sites including one for PKA, two for casein kinase II and eight for PKC (464). Cellular levels of SPP can be depleted by converting SPP back to sphingosine using SPP phosphatase or by degrading SPP using SPP lyase to form palmitaldehyde and phosphoethanolamine (466, 467). However, the accumulation of SPP generally involves the activation of sphingosine kinase rather than the inhibition of degradation pathways (465).

5.3 Sphingosine-1-phosphate

SPP is thought to be a mediator of mitogenesis in multiple cellular systems in response to a wide variety of stimuli (463, 468). For example, constitutive expression of sphingosine kinase in NIH-3T3 cells enhances their proliferation and stimulates transformation (469). Furthermore, in C3H10T1/2 murine fibroblasts PDGF and fibroblast growth factor can both stimulate ceramidase and sphingosine kinase to elevate SPP levels and this is required for cellular survival, proliferation and differentiation (470).

However, an additional layer of diversity in SPP signalling was unravelled with the discovery that SPP can function as both an intracellular and an extracellular signalling molecule (463, 468, 471). As an extracellular ligand SPP is recognised by members of the EDG (endothelial-differentiation gene) family of G-protein-coupled receptors including EDG1, EDG3, EDG5, EDG6 and EDG8 (472). These receptors can couple SPP to several different types of G-proteins including Gi, Gq and $G_{12/13}$ (463).

Attempts have been made to attribute specific functions to different SPP-sensitive EDG receptors but these receptors are widely expressed in most cells and tissues and activation of any one of these receptors is likely to have multiple biological effects (471, 473). For example, in human T lymphoblastoma

cells SPP stimulates EDG3 or EDG5 and decreases the levels of Bax to prevent apoptosis (474). Furthermore, in airway smooth muscle cells SPP can stimulate EDG1, a Gi-coupled receptor, resulting in the activation of c-Src and Grb-2/PI-3-K leading to the stimulation of Erk1/2 and this can be enhanced by co-stimulation with PDGF resulting in increased DNA synthesis (475). Moreover, the SPP-mediated activation of EDG6, which is largely expressed in lymphoid and hematopoietic tissues, can activate Erk1/2 and PLC via Gi thus coupling extracellular SPP to MAPK and to calcium mobilisation (468). Calcium mobilisation is also observed downstream of EDG3 and EDG5 in C_2C_{12} myoblast cells (476). SPP therefore regulates multiple signalling networks downstream of EDG receptors including calcium mobilisation, PI-3-K and Erk-MAPK. In general, this leads to enhanced survival and proliferation of the cells. However, in human myofibroblasts SPP can suppress proliferation via the activation of EDG1, EDG3 and EDG5 (477). Furthermore, Rac/Cdc42-mediated stimulation of JNK has been implicated in coupling SPP-sensitive EDG receptors to the activation of caspases and apoptosis (463). The signals induced by extracellular SPP are thus diverse and can potentially regulate the survival, proliferation and differentiation of cells.

In addition to its role as an extracellular ligand, increasing evidence suggests SPP can function independently of EDG receptors as an intracellular second messenger (463, 468, 471). Initial studies suggesting SPP is an intracellular second messenger used pertussis toxin to inhibit Gi-coupled receptors but the discovery of Gq- and $G_{12/13}$-coupled EDG receptors showed that pertussis toxin-insensitive effects of SPP are not necessarily due to SPP functioning as an intracellular signalling molecule. More convincing evidence that SPP is an intracellular signalling molecule came from the use of dihydro-SPP, which can bind to and activate all SPP-sensitive EDG receptors but is unable to mimic all the effects of SPP. Furthermore, microinjection of SPP or caged SPP can elevate intracellular calcium levels and promote survival and proliferation of cells. Moreover, yeast lack G-protein-coupled receptors and yet phosphorylated sphingolipids and possibly SPP can mobilise calcium in yeast.

Intracellular SPP is believed to regulate a variety of signalling pathways including MAPK activation, Ca^{2+} signalling and PLD. For example, in C3H10T1/2 fibroblast cells ceramide activates apoptosis via JNK but the

conversion of ceramide to SPP stimulates Erk-MAPK and promotes survival, proliferation and differentiation of the cells (470). Furthermore, in U937 cells FcγRI activates sphingosine kinase resulting in the mobilisation of calcium independently of PLC stimulation and IP_3 production (478). SPP is also likely to amplify existing Ca^{2+} signals since sphingosine kinase has at least three Ca^{2+}/calmodulin-binding consensus sequences (463, 465). Moreover, ceramide can inhibit PLD but SPP is an activator of PLD so can increase the cellular levels of phosphatidic acid (479). Phosphatidic acid can stimulate PI-4-kinase, Ras and Raf and can be converted to DAG to stimulate phorbol ester-sensitive isoforms of PKC (480).

In general intracellular SPP promotes survival and proliferation of cells. For example, in U937 cells SPP can suppress apoptosis induced by TNFα, anti-Fas antibodies, serum deprivation and exogenous ceramide by suppressing the release of mitochondrial factors including cytochrome c and Smac/DIABLO and hence preventing the activation of executioner caspases. A similar protective effect can be observed in U937 cells that overexpress Bcl-x_L but SPP does not mediate its effects by increasing the expression of Bcl-x_L, Bcl-2 or Mcl-1 (84). Furthermore, in Swiss 3T3 cells PDGF can stimulate sphingosine kinase resulting in SPP production. SPP then promotes G1 to S phase transition to enhance proliferation of the cells. In this system sphingosine kinase and SPP are found in the nucleoplasm and nuclear envelope, indicating sphingosine kinase is a nuclear-associated enzyme that can mediate the production of SPP specifically at the nucleus. However, the signalling mechanisms used by nuclear SPP to promote cell cycle progression have not yet been deciphered (481).

5.4 SPP rheostat and sphingolipid signalling in B lymphocytes

The SPP rheostat describes the interconversion of ceramide, sphingosine and SPP (figure 5.1B). Each of these molecules regulates a complex network of signalling pathways but ceramide and sphingosine are generally associated with growth arrest and apoptosis whilst SPP favours survival and proliferation. The relative balance of these lipids can thus determine the survival and proliferation of a cell.

Under apoptotic conditions, ligation of the BCR on WEHI-231 cells can elevate the cellular levels of ceramide (305). The activity of sphingosine kinase can also be regulated by the BCR and by CD40. Indeed, ligation of the BCR on immature B cells has been shown to induce a rapid but transient (≤10 min) peak of sphingosine kinase activity whereas co-ligation of CD40 produces a more sustained activation of sphingosine kinase (428). The functional significance of the BCR-coupled transient peak of sphingosine kinase activity has not been explored but one possibility is it has a role in promoting calcium mobilisation to enhance PLA_2 activity and hence arachidonic acid production. Interestingly, the BCR of mature B lymphocytes is also coupled to SPP production but the kinetics are different to those of WEHI-231 cells. In particular, mature B cells display a delayed but prolonged accumulation of SPP (428). Since ligation of the BCR on mature B cells leads to proliferation and activation, prolonged SPP production may be more associated with promoting survival and proliferation of B cells.

Indeed, over a 48 hr time period, unstimulated proliferating WEHI-231 cells accumulate SPP. However, ligation of the BCR impairs SPP production in WEHI-231 cells whilst co-ligation of CD40 appears to restore SPP levels (428). Prolonged elevation of SPP therefore correlates with conditions that favour the survival and proliferation of WEHI-231 cells. Consistent with this, inhibition of sphingosine kinase impairs proliferation of WEHI-231 cells. Furthermore, SPP appears to function exclusively as an intracellular signalling molecule in WEHI-231 cells since no SPP can be detected in supernatants of these cells (428). The SPP rheostat therefore seems to be regulated by the BCR and CD40 in WEHI-231 cells and it is likely to affect the proliferation of the cells. However, the mechanism of SPP signalling in immature B cells is poorly understood.

5.5 Aims and objectives

Previous investigations have shown that ligation of the BCR and CD40 on WEHI-231 cells can regulate the activity sphingosine kinase and hence the cellular levels of SPP (428). However, the role of sphingosine kinase and SPP in regulating the survival and proliferation of WEHI-231 cells is incompletely understood.

The objectives of this investigation were:

1. to determine the effect of inhibition of sphingosine kinase on the survival and proliferation of WEHI-231 cells

2. to examine the effect of inhibition of sphingosine kinase on BCR-driven growth arrest and apoptosis

3. to investigate the signalling mechanisms used by SPP to determine the survival and proliferation of WEHI-231 cells by analysing the effect of inhibition of sphingosine kinase on the activity of Erk-MAPK and the levels of Bcl-x_L.

5.6 Results and Discussion

5.6.1 Inhibition of sphingosine kinase in WEHI-231 cells induces growth arrest and apoptosis

The cellular levels of SPP can be increased by various ligands and they generally do so by stimulating sphingosine kinase rather than by suppressing the degradation of SPP (465). However, the levels of sphingosine in mammalian cells are substantially lower than the levels of ceramide therefore the accumulation of SPP generally requires the simultaneous activation of sphingosine kinase and ceramidase (453, 460, 465, 470). Inhibition of sphingosine kinase can thus deplete the cell of SPP. DMS (N, N-Dimethyl-D-erythro-sphingosine) is a competitive inhibitor of sphingosine kinase 1 and a non-competitive inhibitor of sphingosine kinase 2 (482) with few non-specific toxic effects. WEHI-231 cells were treated with DMS to assess the role of sphingosine kinase and SPP in regulating survival and proliferation of immature B cells.

The effect of DMS on the cell cycle progression of WEHI-231 cells was determined using PI-staining and FACS analysis of DMS-treated cells. Treatment of WEHI-231 cells with DMS induced growth arrest and apoptosis after 24 hr and 48 hr in a dose-dependent manner (figure 5.2-5.3). Indeed, after 24 hr DMS enriched the G0/G1 population of cells (figure 5.3B) and diminished the number of cells in S phase (figure 5.3C) and G2/M (figure 5.3D), indicating DMS induces growth arrest at G0/G1 phase of the cell cycle. For example, after 24 hr, 2.5 μM DMS increased the percentage of G0/G1 cells from 34% to 50% whilst decreasing the percentage of cells in S phase from 28% to 6% and diminishing the G2/M population from 24% to 6% (figure 5.2A). After 48 hr, the number of cells in S phase and G2/M was decreased by DMS in a dose-dependent manner but DMS also depleted the G0/G1 population of cells (figure 5.3B-D). This is likely to reflect the high levels of apoptosis in the cultures of WEHI-231 cells treated with DMS for 48 hr. Indeed, DMS greatly enriched the sub-diploid population of cells, especially at 48 hr, indicating the inhibition of sphingosine kinase induces apoptosis of WEHI-231 cells (figure 5.3A). For example, 78% of WEHI-231 cells treated with 2.5 μM DMS for 48 hr had sub-diploid DNA. Interestingly, >1 μM DMS was required for the potent induction of

apoptosis whereas >0.25 μM DMS was adequate for significant induction of growth arrest of WEHI-231 cells (figure 5.3A-D). This suggests that the induction of growth arrest by DMS may not simply reflect the onset of apoptosis in these WEHI-231 cells. Treatment of WEHI-231 cells with DMS alone therefore induces growth arrest at G0/G1 phase of the cell cycle and causes the cells to undergo apoptosis.

To confirm the ability of DMS to induce growth arrest of WEHI-231 cells the effect of DMS on DNA synthesis of WEHI-231 cells was determined by measuring the incorporation of [^{3}H] thymidine after 48 hr. In agreement with the cell cycle data, DMS inhibited DNA synthesis in a dose-dependent manner with complete growth arrest being observed following exposure to 2.5 μM DMS for 48 hr (figure 5.3E). These observations therefore suggest that the conversion of ceramide and sphingosine to SPP by sphingosine kinase is important for the survival and proliferation of unstimulated WEHI-231 cells. This is consistent with published data that has associated these lipids with regulating survival and proliferation of multiple cell types (456, 463, 468). For example, SPP is required for cell cycle progression from G1 to S phase in PDGF-stimulated Swiss 3T3 cells (481).

5.6.2 Inhibition of sphingosine kinase enhances BCR-driven growth arrest

Previous investigations have suggested that the BCR of mature and immature B cells can regulate the activity of sphingosine kinase (428). To further explore the role of sphingosine kinase in BCR signalling, the effect of DMS on the cell cycle progression of anti-Ig-treated WEHI-231 cells was determined by PI-staining and FACS analysis.

After 24 hr and 48 hr, anti-Ig and DMS were both capable of inducing growth arrest of WEHI-231 cells in a dose-dependent manner (figure 5.4). However, treatment of WEHI-231 cells with a combination of anti-Ig plus DMS further enhanced the induction of growth arrest (figure 5.4). Furthermore, anti-Ig and/or DMS induced growth arrest at G0/G1 phase of the cell cycle since this population of cells was enriched by these agents whilst the number of cells in S phase and G2/M was diminished (compare figures 5.4A and D to 5.4B-C and 5.4E-F). This is consistent with the observations of figure 3.6 and figures 5.2-5.3 in which anti-Ig or DMS were shown to induce G0/G1 cell cycle arrest.

Moreover, analysis of DNA synthesis by measuring the incorporation of [^{3}H] thymidine after 48 hr also showed that DMS enhanced BCR-driven growth arrest (figure 5.5).

Furthermore, analysis of number of cells in S phase and G2/M suggested that anti-Ig and DMS synergistically induced growth arrest of WEHI-231 cells. For example, in the absence of DMS, treatment of WEHI-231 cells with 1 µg/ml anti-Ig for 48 hr reduced the percentage of S phase cells from 35% to 20% (15% difference) and in the absence of anti-Ig, 0.25 µM DMS reduced the number of S phase cells from 35% to 33% (2% difference). However, treatment of WEHI-231 cells with a combination of 1 µg/ml anti-Ig plus 0.25 µM DMS for 48 hr reduced the percentage of S phase cells from 35% to 9% (26% difference) (figure 5.4E). The effect of anti-Ig plus DMS was therefore greater than the sum of the effects of the two single inhibitors indicating anti-Ig and DMS synergistically reduced the number of cells in S phase. Furthermore, analysis of the G2/M population of cells also indicated anti-Ig and DMS were acting in synergy. For example, in the absence of DMS, treatment of WEHI-231 cells with 1 µg/ml anti-Ig for 48 hr reduced the number of cells in G2/M from 22% to 10% (12% difference) and in the absence of anti-Ig, 0.25 µM DMS reduced the percentage of cells in G2/M from 22% to 21% (1% difference) (figure 5.4F). However, treatment of WEHI-231 cells with 1 µM DMS plus 1 µg/ml anti-Ig for 48 hr reduced the percentage of G2/M cells from 22% to 4% (18% difference). These results show anti-Ig and DMS synergistically induce growth arrest implying both agents may affect the same signalling pathway to regulate proliferation of WEHI-231 cells. Furthermore, these observations suggest that BCR-driven growth arrest may involve the suppression of sphingosine kinase. Consistent with this, previous investigations have shown that ligation of the BCR on WEHI-231 cells can suppress sustained SPP signals (428).

5.6.3 Inhibition of sphingosine kinase enhances BCR-driven apoptosis

To further investigate the role of sphingosine kinase in BCR signalling, the effect of DMS on BCR-dependent apoptosis was determined by PI-staining of anti-Ig/DMS-treated WEHI-231 cells followed by FACS analysis. Both anti-Ig and DMS can induce apoptosis of WEHI-231 cells in a dose-dependent manner

(figure 3.6 and 5.2-5.3). Furthermore, treatment of WEHI-231 cells with DMS enhanced BCR-driven apoptosis after 24 hr and 48 hr (figure 5.6A-B). For example, after 24 hr, 1 µg/ml anti-Ig increased the percentage of cells with sub-diploid DNA from 6% to 19% (13% difference) and treatment with 2.5 µM DMS increased the percentage of sub-diploid cells from 6% to 31% (25% difference) (figure 5.6A). However, exposure of WEHI-231 cells to 1 µg/ml anti-Ig plus 2.5 µM DMS for 24 hr increased the percentage of apoptotic cells from 6% to 58% (52% difference) indicating anti-Ig and DMS synergistically induce apoptosis of WEHI-231 cells.

A similar situation was observed after 48 hr culture with anti-Ig and DMS. For example, after 48 hr, 1 µg/ml anti-Ig increased the percentage of apoptotic cells from 10% to 25% (15% difference) and 1 µM DMS increased the number of sub-diploid cells from 10% to 12% (2% difference) (figure 5.6B). However, co-culture with 1 µg/ml anti-Ig plus 1 µM DMS increased the number of cells with sub-diploid DNA from 10% to 58% (48% difference). Anti-Ig and DMS therefore synergistically induced apoptosis of WEHI-231 cells suggesting DMS can regulate the activation of the BCR-driven apoptotic pathway. Previously it has been shown that exogenous ceramide can induce apoptosis of WEHI-231 cells via the classical caspase-dependent pathway (102, 320) whilst BCR-driven apoptosis of WEHI-231 cells does not involve the release of cytochrome c from the mitochondria and is mediated by cathepsins (102). The observation that anti-Ig and DMS synergistically induced apoptosis of WEHI-231 cells suggests that DMS-mediated apoptosis may not be exclusively caused by the accumulation of ceramide.

Furthermore, previous investigations have shown that unstimulated WEHI-231 cells produce SPP over a 48 hr period with optimal levels being observed between 8 hr and 24 hr. Ligation of the BCR decreases the levels SPP throughout the 48 hr time period whilst co-ligation of CD40 can restore SPP levels (428). These observations show that SPP is a signalling molecule of WEHI-231 cells that can be differentially regulated by the BCR and CD40. Taken together, these observations suggest that BCR-dependent apoptosis may require the inhibition of sphingosine kinase and/or sphingosine kinase might be activated by CD40 to suppress BCR-driven apoptosis.

5.6.4 Ligation of CD40 partially protects WEHI-231 cells from DMS-driven apoptosis

To further investigate the role of sphingosine kinase in regulating BCR-driven apoptosis, the effect of DMS on CD40-mediated rescue from anti-Ig-induced apoptosis of WEHI-231 cells was examined. The sub-diploid DNA content of cells treated with anti-Ig, anti-CD40 and DMS was determined by PI-staining and FACS analysis.

As shown above, after 24 hr, DMS induced apoptosis of unstimulated WEHI-231 cells in a dose-dependent manner (figure 5.6C). Furthermore, DMS more potently induced apoptosis of anti-Ig-treated WEHI-231 cells. These observations are consistent with earlier data (figures 5.2-5.3 and 5.6A). However, after 24 hr, DMS (0-2.5 μM) did not induce apoptosis of anti-CD40 treated WEHI-231 cells, indicating ligation of CD40 can protect WEHI-231 cells from DMS-driven apoptosis (figure 5.6C). This is consistent with the theory that DMS enhances the BCR-driven apoptotic pathway since CD40 is known to protect WEHI-231 cells from BCR-driven apoptosis. Furthermore, after 24 hr, CD40 almost completely prevented apoptosis induced by a combination of anti-Ig plus DMS (figure 5.6C). Ligation of CD40 can therefore impair BCR- and DMS-driven apoptosis of WEHI-231 cells. This finding suggests that CD40-mediated survival may not require the activation of sphingosine kinase. However, after 48 hr, ligation of CD40 was unable to protect WEHI-231 cells from apoptosis induced by DMS, especially at 2.5 μM DMS (figure 5.6D). This suggests that inhibition of sphingosine kinase ultimately impairs CD40-mediated survival and hence implies that this kinase may have a role in promoting pro-survival signals or suppressing pro-apoptotic pathways in WEHI-231 cells.

5.6.5 CD40 partially protects WEHI-231 cells from DMS-induced growth arrest

To further investigate the role of sphingosine kinase in CD40-mediated rescue of BCR-stimulated WEHI-231 cells, the effect of DMS on CD40-dependent proliferation was explored. The level of DNA synthesis in DMS-treated WEHI-231 cells was determined by measuring the incorporation of [^{3}H] thymidine after 48 hr. In agreement with previous observations (figure 3.6 and

figures 5.2-5.5), anti-Ig and DMS both induced growth arrest of WEHI-231 cells (figure 5.7A). Furthermore, ligation of CD40 was inefficient at preventing DMS-mediated inhibition of DNA synthesis after 48 hr (figure 5.7A). For example, CD40 slightly impaired the inhibition of DNA synthesis by 1 μM DMS but was unable to prevent growth arrest induced by >1 μM DMS. Similarly, after 48 hr anti-CD40 was inefficient at preventing DMS-mediated growth arrest of BCR-activated WEHI-231 cells and DMS appeared to slightly impair CD40-mediated rescue from anti-Ig-induced growth arrest (figure 5.7A).

To further analyse the effect of DMS on CD40-mediated rescue from BCR-driven growth arrest, the cell cycle of WEHI-231 cells was analysed by PI-staining and FACS analysis. After 24 hr, ligation of CD40, in the presence or absence of BCR-stimulation, partially protected WEHI-231 cells from DMS-dependent depletion of S phase (figure 5.7B) and G2/M phase (figure 5.7C) populations of cells. Similarly, after 48 hr, anti-CD40 was able to partially protect WEHI-231 cells from DMS-driven depletion of S phase (figure 5.7D) and G2/M phase (figure 5.7E) cells. This was especially evident when cells were treated with 2.5 μM DMS. For example, after 48 hr, 2.5 μM DMS reduced the percentage of cells in S phase from 35% to 3% (32% difference) but in the presence of anti-CD40, 2.5 μM DMS only decreased the percentage of S phase cells from 35% to 11% (24% difference). Furthermore, after 48 hr, 2.5 μM DMS decreased the number of cells in G2/M from 22% to 1% (21% difference) but in CD40-stimulated cells, 2.5 μM DMS only reduced the percentage of G2/M cells from 22% to 6% (16% difference). Taken together, these observations suggest that ligation of CD40 can partially protect WEHI-231 cells from DMS-driven growth arrest but CD40 was unable to fully protect the cells.

5.6.6 Overexpression of Bcl-x_L protects WEHI-231 cells from DMS-driven apoptosis

Bcl-x_L seems to be a key mediator of CD40-dependent survival therefore Bcl-x_L WEHI-231 cells were used to further investigate the role of sphingosine kinase in CD40-mediated rescue of BCR-stimulated WEHI-231 cells. The effect of DMS on the sub-diploid DNA content of Neo and Bcl-x_L WEHI-231 cells was determined by PI-staining and FACS analysis. As shown previously (figures 5.2-5.3), DMS induced apoptosis of Neo WEHI-231 cells in a dose-dependent

manner, especially at >1 μM DMS (figure 5.8). In contrast, Bcl-x_L WEHI-231 cells were protected from DMS-driven apoptosis after 24 hr and 48 hr (figure 5.8A-B). For example, after 48 hr, 77% of Neo WEHI-231 cells treated with 2.5 μM DMS had sub-diploid DNA whilst 17% of Bcl-x_L WEHI-231 cells were apoptotic when exposed to the same conditions (figure 5.8B). Expression of Bcl-x_L therefore protects WEHI-231 cells from DMS-driven apoptosis. This is consistent with DMS amplifying BCR-driven apoptosis since Bcl-x_L has previously been shown to suppress this pro-apoptotic pathway (427).

Furthermore, expression of Bcl-x_L protected WEHI-231 cells from apoptosis induced by a combination of anti-Ig plus DMS (figure 5.9). For example, after 24 hr, 31% of Neo WEHI-231 cells treated with 2.5 μM DMS had sub-diploid DNA and co-culture with 10 μg/ml anti-Ig increased this percentage to 55%. In contrast, only 7% of Bcl-x_L WEHI-231 cells treated with 2.5 μM DMS had sub-diploid DNA and co-culture with 10 μg/ml anti-Ig increased this percentage to just 12% (figure 5.9A). Similarly, after 48 hr, overexpression of Bcl-x_L greatly reduced the level of apoptosis induced by a combination of anti-Ig plus DMS (figure 5.9B). Nonetheless, Bcl-x_L WEHI-231 cells treated with 2.5 μM DMS for 48 hr were slightly susceptible to anti-Ig-induced apoptosis suggesting these cells no longer had complete protection against these pro-apoptotic agents. This is consistent with previous observations where it was shown that the protective effects of Bcl-x_L could be overridden by amplification of the BCR-driven pro-apoptotic pathway (figure 4.8).

Taken together, this data strongly suggests that expression of Bcl-x_L can protect WEHI-231 cells from anti-Ig-induced and DMS-mediated apoptosis. This is consistent with Bcl-x_L being an important pro-survival factor of WEHI-231 cells. Interestingly, a comparison of CD40-dependent and Bcl-x_L-mediated rescue from DMS-driven apoptosis showed that expression of Bcl-x_L was more effective than ligation of CD40 at enabling WEHI-231 cells to survive in the presence of DMS (figure 5.9C). For example, 63% of Neo WEHI-231 cells treated with 2.5 μM DMS plus anti-CD40 for 48 hr had sub-diploid DNA. In contrast, only 16% of Bcl-x_L WEHI-231 cells were apoptotic when treated with 2.5 μM DMS in the absence of CD40. Bcl-x_L was thus significantly more effective than anti-CD40 at protecting WEHI-231 cells from DMS-induced

apoptosis. This implies that CD40-mediated survival is impaired by DMS and this can be overcome by expression of Bcl-x_L.

5.6.7 DMS impairs Bcl-x_L expression in WEHI-231 cells

To further investigate the mechanism of DMS-driven apoptosis, the effect of DMS on Bcl-x_L expression was examined since Bcl-x_L seems to be a key mediator of CD40-dependent survival (see chapter 4) and DMS appears to impair CD40-mediated survival in a manner that can be overcome by expression of Bcl-x_L. This suggests that DMS might impair CD40-mediated induction of Bcl-x_L in WEHI-231 cells and hence the expression of Bcl-x_L in DMS-treated WEHI-231 cells was investigated by Western blotting.

Bcl-x_L was detected in untreated WEHI-231 cells, especially in 24 hr and 48 hr lysates (figure 5.10A). However, treatment of these unstimulated WEHI-231 cells with DMS reduced the levels of Bcl-x_L in a dose-dependent manner. Inhibition of sphingosine kinase therefore impaired Bcl-x_L expression suggesting the activity of this kinase, which is cycling in untreated cells (428), contributes to the induction of Bcl-x_L in unstimulated WEHI-231 cells. Bcl-x_L is an important survival factor of WEHI-231 cells (292, 311) therefore this is likely to explain, at least in part, why DMS induces apoptosis of these cells.

The effect of DMS on CD40-mediated induction of Bcl-x_L was also examined since ligation of CD40 is known to upregulate Bcl-x_L in WEHI-231 cells (figure 4.2) and this is important for CD40-dependent survival. As with unstimulated WEHI-231 cells, treatment of anti-CD40-treated WEHI-231 cells with DMS reduced the levels of Bcl-x_L in the cells (figure 5.10B). Indeed, 0.5 μM DMS was able to reduce the levels of Bcl-x_L to a similar extent as exposure to 10 μg/ml anti-Ig. Moreover, Bcl-x_L levels were further reduced by treatment of WEHI-231 cells with a combination of anti-Ig plus DMS. This is consistent with the high levels of apoptosis observed in these cells (figure 5.6). Furthermore, WEHI-231 cells treated with a combination of anti-Ig plus anti-CD40 were less sensitive to DMS-dependent suppression of Bcl-x_L than cells treated with anti-Ig alone but they still had lower levels of Bcl-x_L than cells cultured in the absence of DMS (figure 5.10B). DMS therefore impaired the induction of Bcl-x_L in unstimulated and CD40-activated WEHI-231 cells and this is likely to contribute

to the observations of figure 5.6 in which CD40 was only able to partially protect Neo WEHI-231 cells from DMS-driven apoptosis.

These observations suggest that DMS-dependent apoptosis is likely to be mediated, at least in part, by reducing Bcl-x_L levels. This may reflect a mechanism used by the BCR to suppress pro-survival pathways since previous investigations have suggested that the BCR can impair SPP production (428). The BCR may therefore inhibit sphingosine kinase to reduce SPP levels and hence prevent the induction of Bcl-x_L. Alternatively, the BCR may impair Bcl-x_L signalling by allowing ceramide to accumulate since ceramide is known to decrease the expression of pro-survival Bcl-2-family members including Bcl-2 (456) and Bcl-x_L (457) in other cellular systems.

These observations can also account for the impaired CD40-mediated survival observed in DMS-treated cells (figure 5.6) since Bcl-x_L is an important mediator of CD40-dependent survival. Moreover, these observations implicate sphingosine kinase as a CD40-regulated enzyme whose action contributes to the upregulation of Bcl-x_L and hence to CD40-mediated survival.

5.6.8 Ligation of CD40 enhances the survival of DMS-treated Bcl-x_L WEHI-231 cells

To further explore the mechanism of CD40-mediated survival of anti-Ig- and DMS-treated WEHI-231 cells, the effect of ligation of CD40 on the survival of Bcl-x_L WEHI-231 cells was examined. Apoptosis of Bcl-x_L WEHI-231 cells was measured by determining their sub-diploid DNA content using PI-staining and FACS analysis.

In agreement with earlier observations (figures 4.3 and 5.8-5.9), overexpression of Bcl-x_L was found to impair anti-Ig and DMS-driven apoptosis (figure 5.11). However, Bcl-x_L WEHI-231 cells did display enhanced sub-diploid DNA content when treated with a combination of anti-Ig (10 µg/ml) plus high concentrations of DMS (2.5 µM), especially after 48 hr (figure 5.11). Interestingly, treatment of these cells with anti-CD40 impaired the ability of anti-Ig and DMS to induce apoptosis after 24 hr and 48 hr (figure 5.11A-B). Indeed, treatment with anti-CD40 enhanced the protective capacity of Bcl-x_L WEHI-231 cells and enabled full protection from apoptosis induced by a combination of 10 µg/ml anti-Ig plus 2.5 µM DMS. These observations indicate that despite DMS suppressing CD40-

mediated induction of Bcl-x_L, CD40 was able to improve the survival of anti-Ig/DMS-treated WEHI-231 cells. CD40 is therefore likely to stimulate DMS-insensitive pro-survival signals in addition to the DMS-sensitive pathway of inducing Bcl-x_L. The nature of these signals remains unknown but one possibility is CD40 can upregulate Bcl-x_L via sphingosine kinase-dependent and independent pathways. Alternatively, CD40 may induce Bcl-x_L-independent survival signals. However, these observations may also reflect the ability of DMS to promote cell death via more than one signalling pathway. For example, DMS may also promote caspase-dependent apoptosis by elevating ceramide levels (102).

5.6.9 Overexpression of Bcl-x_L does not protect WEHI-231 cells from DMS-induced growth arrest

To further examine the role of Bcl-x_L in protecting DMS-treated WEHI-231 cells, the proliferation of DMS-treated Neo and Bcl-x_L WEHI-231 cells was examined by measuring the incorporation of [^{3}H] thymidine and hence the level of DNA synthesis. Cell cycle progression of Neo and Bcl-x_L WEHI-231 cells was also analysed by PI-staining and FACS analysis.

Treatment of Neo WEHI-231 cells with DMS inhibited DNA synthesis in a dose-dependent manner and this was not prevented by the expression of Bcl-x_L (figure 5.12A). Furthermore, after 24 hr, Bcl-x_L was unable to prevent DMS-mediated depletion of S phase and G2/M phase populations of cells (figure 5.12B). For example, after 24 hr, in the absence of DMS 28% of Neo WEHI-231 cells were in S phase but treatment with 2.5 μM DMS reduced the number of cells in S phase to 6%. Similarly, in the absence of DMS 30% of Bcl-x_L WEHI-231 cells were in S phase but exposure to 2.5 μM DMS reduced the number of S phase cells to 5%. Likewise, after 48 hr expression of Bcl-x_L was unable to protect WEHI-231 cells from DMS-mediated depletion of S phase and G2/M phase cells (figure 5.12C). This suggests that inhibition of sphingosine kinase impairs proliferation of WEHI-231 cells and this cannot be prevented by expression of Bcl-x_L.

Furthermore, the ability of anti-Ig and DMS to co-ordinately suppress proliferation of WEHI-231 cells was not prevented by overexpression of Bcl-x_L (figure 5.13A-B). Bcl-x_L WEHI-231 cells were more sensitive to anti-Ig-induced

growth arrest but overexpression of Bcl-x_L did not alter the susceptibility of WEHI-231 cells to DMS-driven growth arrest. These observations suggest that sphingosine kinase may promote proliferation of WEHI-231 cells independently of its role in inducing Bcl-x_L-mediated survival. Similarly, distinct mechanisms appear to regulate the survival and proliferation of WEHI-231 cells downstream of the BCR and CD40 (102, 328, 427).

5.6.10 Overexpression of Bcl-x_L slightly enhances CD40-dependent rescue of DMS-driven growth arrest

To further investigate the regulation of proliferation of WEHI-231 cells by DMS, the effect of ligation of CD40 was explored. The number of cells in S phase was determined by PI-staining and FACS analysis of DMS-treated Neo and Bcl-x_L WEHI-231 cells. After 24 hr, DMS decreased the number of untreated Neo WEHI-231 cells in S phase in a dose-dependent manner (figure 5.14A). However, ligation of CD40 on Neo WEHI-231 cells impaired DMS-mediated growth arrest. In contrast, overexpression of Bcl-x_L did not protect WEHI-231 cells from DMS-driven growth arrest but ligation of CD40 on Bcl-x_L WEHI-231 cells did prevent DMS-mediated depletion of S phase cells. Analysis of the number of cells in G2/M gave similar results (data not shown). These observations suggest CD40 can protect WEHI-231 cells from DMS-driven growth arrest after 24 hr but this cannot be mimicked by expression of Bcl-x_L.

However, after 48 hr, CD40 was less effective at protecting Neo WEHI-231 cells from DMS-dependent growth arrest (figure 5.14B). Similarly, expression of Bcl-x_L was relatively ineffective at protecting WEHI-231 cells from DMS-mediated depletion of S phase cells. However, ligation of CD40 on Bcl-x_L WEHI-231 cells did impair DMS-mediated growth arrest. To further explore these observations, the level of DNA synthesis in WEHI-231 cells was determined by measuring the incorporation of [^{3}H] thymidine. Supporting the cell cycle data, the combination of overexpression of Bcl-x_L and ligation of CD40, albeit to a lesser degree than observed with cell cycle analysis, partially protected WEHI-231 cells from DMS-dependent inhibition of DNA synthesis after 48 hr (figure 5.14C).

These observations therefore show that after 24 hr, ligation of CD40 can impair DMS-dependent growth arrest whereas expression of Bcl-x_L is

insufficient to sustain proliferation of the cells. This indicates that CD40 signalling but not Bcl-x_L expression can prevent DMS-mediated growth arrest. However, after 48 hr CD40 was inefficient at preventing DMS-driven growth arrest unless Bcl-x_L was overexpressed. A possible explanation for this relates to the observation that DMS impaired CD40-mediated survival by suppressing the induction of Bcl-x_L. Ligation of CD40 alone therefore might be adequate to prevent DMS-mediated growth arrest but it was not able to prevent apoptosis and hence cell cycle progression was impaired. Moreover, expression of Bcl-x_L was unable to restore proliferation to DMS-treated WEHI-231 cells but it did protect the cells from apoptosis. Consequently, protection of WEHI-231 cells from DMS-induced growth arrest required the expression of Bcl-x_L to prevent apoptosis and CD40-mediated, Bcl-x_L-independent signals to prevent cell cycle arrest.

5.6.11 DMS does not prevent CD40-mediated rescue of WEHI-231 cells from BCR-driven growth arrest

Bcl-x_L WEHI-231 cells were treated with DMS to investigate the role of sphingosine kinase in CD40-mediated rescue from BCR-driven growth arrest. Bcl-x_L WEHI-231 cells were used since Bcl-x_L can protect WEHI-231 cells from DMS-driven apoptosis (figure 5.9). Proliferation was assessed by analysing the cell cycle using PI-staining and FACS analysis. Consistent with previous observations (figure 4.4-4.7) anti-Ig diminished the number of cells in S phase and G2/M indicating ligation of the BCR induced growth arrest of Bcl-x_L WEHI-231 cells after 24 hr and 48 hr (figure 5.15). However, co-ligation of CD40 replenished the S phase and G2/M phase populations of cells indicating CD40 can prevent BCR-driven growth arrest of Bcl-x_L WEHI-231 cells. Furthermore, the addition of DMS (0-2.5 μM) did not prevent CD40-mediated rescue from BCR-driven growth arrest after 24 hr or 48 hr (figure 5.15A-B). For example after 48 hr, in the absence of DMS, 20% of cells treated with anti-Ig plus anti-CD40 were in G2/M phase whereas 17% of cells treated with 2.5 μM DMS were in G2/M. DMS therefore slightly impaired but did not prevent CD40-mediated rescue from BCR-driven growth arrest. This suggests that CD40-mediated rescue from BCR-dependent growth arrest is not exclusively dependent on the activation of sphingosine kinase.

5.6.12 DMS does not alter sustained cyclic activation of Erk-MAPK in WEHI-231 cells

To investigate the mechanism of DMS-dependent growth arrest of WEHI-231 cells, the effect of DMS on Erk activity was determined since sustained activation of Erk is required for proliferation of WEHI-231 cells (328). Furthermore, it is well recognised that ceramide, sphingosine and SPP can differentially regulate the activity of MAPK in multiple systems (453, 458, 463, 471). For example, in C3H10T1/2 cells SPP is an activator of Erk-MAPK and this is believed to be important for the induction of proliferation (470). Conversely, in airway smooth muscle cells sphingosine can inhibit Erk1/2 and induce growth arrest (463). Furthermore, WEHI-231 cells appear to have similar patterns of SPP production and Erk activation. For example, over a 48 hr time period prolonged SPP production can be observed in unstimulated WEHI-231 cells with maximal levels being obtained after 8 hr. Anti-Ig suppresses the 8 hr peak of SPP production but anti-CD40 restores the signal (428). Similarly, unstimulated WEHI-231 cells have prolonged and cyclic activation of Erk over a 48 hr time period and this is suppressed by ligation of the BCR but is restored by co-ligation of CD40 (appendix 2). However, the signalling mechanisms used to regulate sustained Erk signals are incompletely understood. The regulation of Erk-MAPK by sphingosine kinase was thus investigated by Western blotting of lysates prepared from DMS-treated WEHI-231 cells.

Consistent with previous observations, unstimulated WEHI-231 cells displayed sustained cyclic activation of Erk-MAPK that was disrupted by ligation of the BCR but restored by co-ligation of CD40 (figure 5.16). However, treatment of WEHI-231 cells with DMS did not mimic anti-Ig in that it was unable to suppress sustained Erk signalling in untreated WEHI-231 cells (figure 5.16). This suggests that DMS-dependent growth arrest is not mediated by an inhibition of Erk-MAPK and sphingosine kinase does not appear to be required for sustained Erk signalling in WEHI-231 cells. Furthermore, inhibition of sphingosine kinase did not prevent CD40-mediated restoration of sustained and cyclic Erk signals in anti-Ig-treated WEHI-231 cells (figure 5.16). This implies that sphingosine kinase is not required for CD40-mediated activation of sustained Erk signals in WEHI-231 cells. This agrees with the observation that

DMS did not prevent CD40-dependent rescue from BCR-driven growth arrest (figure 5.15).

Taken together, this data shows that DMS does not induce growth arrest of WEHI-231 cells by suppressing sustained Erk signals and DMS does not impair CD40-dependent restoration of prolonged Erk signals in BCR-stimulated cells. This suggests that BCR-dependent suppression of sustained Erk signals is not mediated by an inhibition of sphingosine kinase and depletion of SPP levels. Furthermore, CD40-dependent restoration of sustained Erk signals does not require the induction of sphingosine kinase.

Previously it has been shown that WEHI-231 cells have both early and sustained SPP signals (428). The function of the early (<1 hr) signal is not known. The activity of Erk was measured in WEHI-231 cells in which the early SPP signal was permitted but the sustained (≥8 hr) SPP signal was inhibited by adding DMS to the WEHI-231 cultures after 4 hr. The inhibition of prolonged SPP production did not affect the activation of Erk-MAPK in unstimulated or anti-CD40-treated WEHI-231 cells (figure 5.17) supporting the proposal that sphingosine kinase is not necessary for inducing prolonged Erk signalling in WEHI-231 cells.

5.6.13 DMS does not alter the activity of MEK in WEHI-231 cells

Previous data has suggested that BCR-dependent inhibition of sustained Erk signalling does not require the inhibition of the upstream activator MEK (figures 3.7-3.8) but is instead mediated by MAPK phosphatases (appendix 5 and figure 3.9). To confirm the data of figure 5.16, which suggested sphingosine kinase was not required for BCR-dependent inhibition of Erk, the effect of DMS on the activity of MEK was determined in WEHI-231 cells. MEK is activated by phosphorylation of $Ser^{217/218}$ and Ser^{221}, catalysed by upstream kinases including Raf-1 (372) therefore the activity of MEK was determined by Western blotting using an antibody that only recognises MEK when it is phosphorylated at these residues.

In the absence of DMS (left hand side of figure 5.18) untreated WEHI-231 cells had high levels of phospho-MEK1/2 at late time points (8-48 hr) indicating the presence of sustained MEK activation. Stimulation of WEHI-231 cells with anti-Ig, anti-CD40 or a combination of anti-Ig plus anti-CD40 did not abolish

sustained MEK signalling. Furthermore, the addition of 0.5 μM DMS (right hand side of figure 5.18) did not alter the levels of phospho-MEK in unstimulated or stimulated WEHI-231 cells. This is consistent with the observation that DMS does not affect sustained Erk signalling (figure 5.16). The BCR and CD40 therefore do not require sphingosine kinase to regulate sustained MEK or Erk signals in WEHI-231 cells.

Nonetheless, inhibition of sphingosine kinase using DMS does enhance BCR-driven growth arrest. A potential role for sphingosine kinase in stimulating proliferation of WEHI-231 cells downstream of Erk is suggested by studies on NIH3T3 fibroblast cells. Transfection of NIH3T3 cells with $RasV^{12}$, a constitutively active form of Ras, induces cellular transformation. Inhibition of sphingosine kinase using DMS or overexpression of a kinase dead mutant of sphingosine kinase can impair $RasV^{12}$-dependent transformation: for example, 2.5 μM DMS reduced the cellular transformation by 41%. These results show that sphingosine kinase can signal downstream of Ras to promote cellular transformation and hence proliferation (469). Furthermore, it has recently been reported that sphingosine kinase can be directly phosphorylated by Erk1/2 and this enhances the activity of sphingosine kinase and promotes its translocation from the cytosol to the plasma membrane (483). Similarly, in WEHI-231 cells sphingosine kinase may function downstream of Erk-MAPK to increase SPP production and to promote proliferation since proliferation of WEHI-231 cells is known to be dependent on sustained Erk signals and these can be differentially regulated by the BCR and CD40 (328). To investigate this possibility further, the activity of sphingosine kinase could be measured in WEHI-231 cells treated with MEK inhibitors such as PD98059 and UO126.

5.6.14 DMS does not alter phospho-Erk signalling in Bcl-x_L WEHI-231 cells

To further examine the role of sphingosine kinase in CD40-mediated rescue from BCR-driven growth arrest the activity of Erk was measured in Bcl-x_L WEHI-231 cells treated with DMS. Bcl-x_L WEHI-231 cells were used since Bcl-x_L can prevent DMS-dependent apoptosis therefore these cells enable DMS-dependent growth arrest to proceed without the complicating factor of DMS-mediated apoptosis. The activation of Erk-MAPK was investigated in Neo and Bcl-x_L WEHI-231 cells using Western blotting for dual-phosphorylated Erk

(figure 5.19). In the absence of DMS, untreated Neo WEHI-231 cells had highest levels of phospho-Erk between 24 hr and 48 hr and these sustained Erk signals were inhibited by ligation of the BCR. However, co-ligation of CD40 restored Erk activation between 24 hr and 48 hr. Overexpression of Bcl-x_L in the absence of DMS did not alter the temporal pattern of Erk activation. This is consistent with the observations of figure 4.10 in which expression of Bcl-x_L was shown to be unable to restore sustained Erk signalling to BCR-stimulated WEHI-231 cells.

In general, the addition of 0.5 μM DMS (lanes 4-6 of figure 5.19) to Neo or Bcl-x_L WEHI-231 cells did not affect the temporal pattern or the intensity of phospho-Erk signalling in unstimulated cells or cells treated with anti-Ig in the presence or absence of anti-CD40. These observations show that 0.5 μM DMS does not affect Erk signalling in cells protected from DMS-mediated apoptosis. Taken together, the results of figures 5.16-5.19 indicate 0.5 μM DMS does not mimic anti-Ig in suppressing sustained Erk signalling and does not impair CD40-mediated restoration of sustained Erk signals.

However, exposure of Neo and Bcl-x_L WEHI-231 cells to 5 μM DMS (lanes 7-9 of figure 5.19) did cause a general suppression of phospho-Erk signalling, especially between 24 hr and 48 hr. This observation was not altogether surprising since high concentrations of DMS very potently induce growth arrest and apoptosis of WEHI-231 cells (figure 5.2) suggesting many of the cells used to prepare these lysates may have been undergoing growth arrest and apoptosis. Consequently, very few cells will have been cycling and hence signals associated with cell cycle progression such as sustained Erk signalling will have been suspended.

However, an interesting observation was made in the total Erk Western blots of the lysates prepared from Neo and Bcl-x_L WEHI-231 cells treated with 5 μM DMS (figure 5.19). The detection of total Erk (non-phosphorylated plus phosphorylated Erk) is often used as a loading control for phospho-Erk Western blotting but WEHI-231 cells treated with 5 μM DMS appeared to have reduced levels of total Erk. This was more evident in Neo WEHI-231 cells than in Bcl-x_L WEHI-231 cells and total Erk levels seemed to be enhanced by ligation of CD40, suggesting the depletion in total Erk levels correlated with increased cell death. Nonetheless, these observations are unlikely to be an artefact caused by

inadequate protein loading since Bcl-x_L levels were constant in all lysates prepared from Bcl-x_L WEHI-231 cells including those in which total Erk levels were depleted. Bcl-x_L WEHI-231 cells are transfected with a vector that causes them to constitutively express Bcl-x_L therefore Western blotting for Bcl-x_L can be used as a loading control for these cells.

These observations are representative of a single experiment and hence must be repeated to confirm these findings but they suggest that high concentrations of DMS may downregulate cellular levels of total Erk and this can be inhibited by anti-CD40, expression of Bcl-x_L or a combination of these two factors. Expression of Bcl-x_L and ligation of CD40 are both known to increase the survival of WEHI-231 cells therefore the reduced levels of total Erk observed in figure 5.19 are likely to be caused by the onset of apoptosis in these cells. However, these results also implicate a role for sphingosine kinase in increasing or sustaining total Erk levels in WEHI-231 cells, perhaps by preventing the degradation of Erk or by promoting the expression of the *erk* gene.

5.7 Conclusions

The role of sphingosine kinase in regulating survival and proliferation of WEHI-231 cells was investigated using the sphingosine kinase inhibitor DMS. DMS induced cell cycle arrest at G0/G1 phase and caused WEHI-231 cells to undergo apoptosis (figures 5.2-5.3). Furthermore, DMS and anti-Ig synergistically induced growth arrest and apoptosis (figures 5.4-5.6) suggesting DMS can regulate the pathways used by the BCR to induce growth arrest and apoptosis. This is consistent with previous observations, which showed that the BCR of WEHI-231 cells is coupled to sphingosine kinase and can suppress sustained SPP signals. Taken together, this suggests that the BCR of WEHI-231 cells is likely to be coupled to SPP signalling and may regulate SPP production to affect the survival and proliferation of the cells (figure 5.20).

Anti-Ig induced growth arrest and apoptosis can be prevented by co-ligation of CD40. However, CD40 only partially protected WEHI-231 cells from DMS-mediated growth arrest and apoptosis (figures 5.6-5.7). Nonetheless, overexpression of Bcl-x_L did protect WEHI-231 cells from apoptosis but not growth arrest induced by DMS and/or anti-Ig (figures 5.8-5.9). Indeed,

expression of Bcl-x_L was more effective than ligation of CD40 at preventing apoptosis of DMS-treated WEHI-231 cells (figure 5.9C) suggesting CD40-dependent survival was impaired by DMS and this could be overcome by expression of Bcl-x_L. Consistent with this, DMS was found to reduce the levels of Bcl-x_L in unstimulated WEHI-231 cells and cells treated with anti-Ig and/or anti-CD40 (figure 5.10). This suggests that sphingosine kinase is likely to contribute to the induction of Bcl-x_L in unstimulated and anti-CD40-treated WEHI-231 cells, enabling the BCR-driven mitochondrial death pathway to be suppressed (figure 5.20). However, ligation of CD40 enhanced the survival of DMS/anti-Ig-treated Bcl-x_L WEHI-231 cells (figure 5.11) suggesting CD40 may stimulate Bcl-x_L via sphingosine kinase-dependent and independent mechanisms or CD40 may promote survival of WEHI-231 cells via Bcl-x_L-independent mechanisms.

Expression of Bcl-x_L did not prevent anti-Ig- or DMS-dependent growth arrest (figures 5.12-5.13) but ligation of CD40 did impair DMS-mediated growth arrest of Bcl-x_L WEHI-231 cells (figures 5.14-5.15). This suggests that CD40 can stimulate mitogenic pathways that can overcome DMS-mediated growth arrest. To further investigate the mechanism of DMS-mediated growth arrest, the effect of DMS on sustained Erk signalling was explored since the BCR and CD40 differentially regulate Erk1/2 and this is important for determining the proliferation of WEHI-231 cells. This showed that DMS-dependent growth arrest was not mediated by an inhibition of sustained Erk signals since DMS did not mimic anti-Ig in suppressing these signals (figure 5.16). BCR-dependent suppression of sustained Erk signals therefore is unlikely to involve an inhibition of sphingosine kinase. Furthermore, DMS did not impair CD40-mediated restoration of sustained Erk signals to anti-Ig-treated WEHI-231 cells indicating sphingosine kinase is not required for CD40-dependent induction of prolonged Erk signals. However, recent reports suggest that sphingosine kinase may actually be regulated downstream of Erk1/2 therefore sphingosine kinase may promote proliferation of WEHI-231 cells downstream of Erk-MAPK (figure 5.20). Finally, high levels of DMS appeared to reduce the cellular levels of Erk1/2 (figure 5.19) suggesting sphingosine kinase may also have a role in ensuring constant levels of Erk can be found in WEHI-231 cells.

Figure 5.1. Sphingolipid biosynthesis and the SPP rheostat. **(A)** Sphingolipid biosynthesis. Ceramide is biosynthesised via a series of reactions catalysed by specific enzymes (enzymes are shown in blue). Ceramide forms the backbone of sphingolipids. Ceramide can be converted to sphingomyelin via the action of sphingomyelin synthase (1). This is a reversible reaction; sphingomyelin can be converted to ceramide using sphingomyelinase (2). Ceramide can form sphingosine using ceramidase (3) and the reverse reaction is catalysed by ceramide synthase (4). Ceramide can be phosphorylated by ceramide kinase (5) to form ceramide-1-phosphate and ceramide-1-phosphate is dephosphorylated by ceramide phosphatase (6) to form ceramide. Ceramide can also be converted to glycosphingolipids, gangliosides and sulphatides via a series of reactions catalysed by various enzymes. **(B)** The SPP rheostat. SPP rheostat describes the interconversion of ceramide, sphingosine and SPP. Ceramide can be formed from sphingomyelin using sphingomyelinase. Ceramide can then be converted to sphingosine by ceramidase and sphingosine can be phosphorylated to form SPP. Each of these reactions is reversible therefore SPP can be converted to sphingosine and sphingosine to ceramide. In general, ceramide and sphingosine are pro-apoptotic signalling molecules whilst SPP promotes survival and proliferation therefore the relative balance of ceramide/sphingosine and SPP can determine the survival and proliferation of the cell.

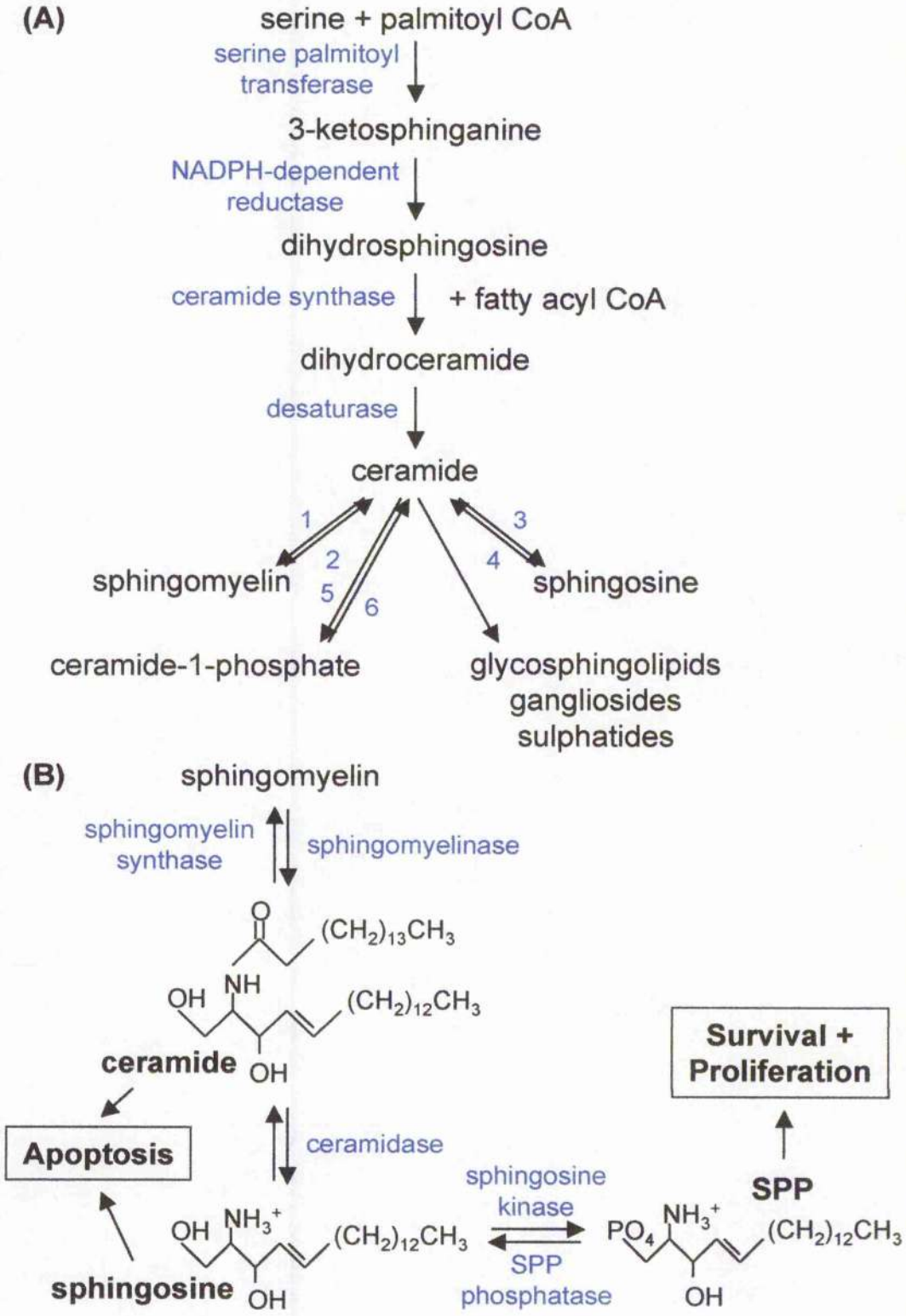

(A)
serine + palmitoyl CoA
serine palmitoyl transferase
3-ketosphinganine
NADPH-dependent reductase
dihydrosphingosine
ceramide synthase
+ fatty acyl CoA
dihydroceramide
desaturase
ceramide
1
2
3
4
5
6
sphingomyelin
sphingosine
ceramide-1-phosphate
glycosphingolipids
gangliosides
sulphatides
(B)
sphingomyelin
sphingomyelin synthase
sphingomyelinase
ceramide
Apoptosis
ceramidase
sphingosine
sphingosine kinase
SPP phosphatase
SPP
Survival + Proliferation

Figure 5.2. Inhibition of sphingosine kinase in WEHI-231 cells induces growth arrest and apoptosis. Neo WEHI-231 cells (5×10^5 cells/ml) were cultured for 24 hr or 48 hr with medium (no DMS) or increasing concentrations of DMS (0.25, 1 or 2.5 μM). The cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence). Data are representative of at least three independent experiments.

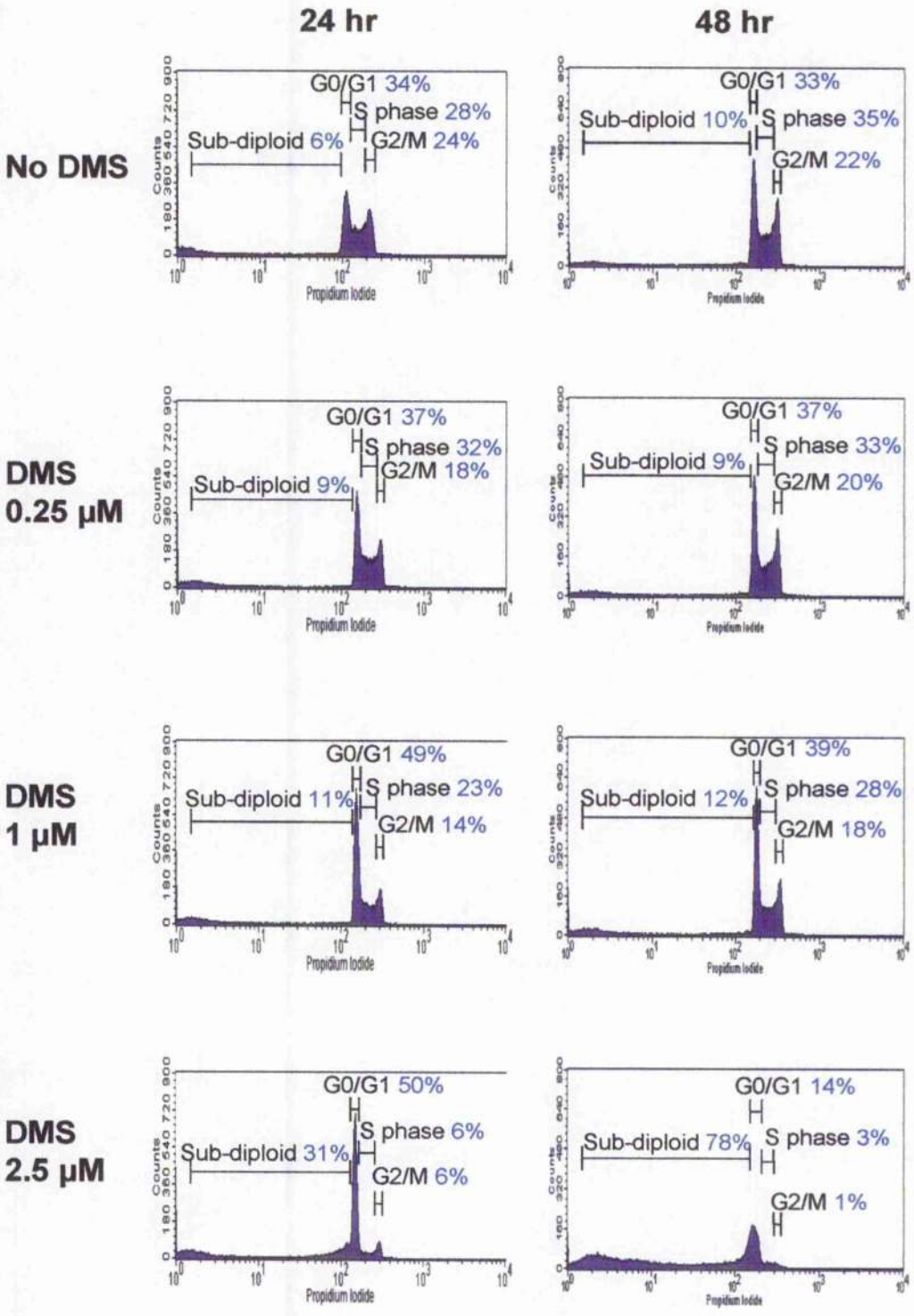

24 hr
48 hr
No DMS
G0/G1 34%
S phase 28%
Sub-diploid 6%
G2/M 24%
G0/G1 33%
Sub-diploid 10%
S phase 35%
G2/M 22%
DMS
0.25 µM
G0/G1 37%
S phase 32%
Sub-diploid 9%
G2/M 18%
G0/G1 37%
Sub-diploid 9%
S phase 33%
G2/M 20%
DMS
1 µM
G0/G1 49%
Sub-diploid 11%
S phase 23%
G2/M 14%
G0/G1 39%
Sub-diploid 12%
S phase 28%
G2/M 18%
DMS
2.5 µM
G0/G1 50%
Sub-diploid 31%
S phase 6%
G2/M 6%
G0/G1 14%
Sub-diploid 78%
S phase 3%
G2/M 1%
Counts
Propidium Iodide

Figure 5.3. Inhibition of sphingosine kinase in WEHI-231 cells induces growth arrest and apoptosis. Neo WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr or 48 hr with increasing concentrations of DMS (0-2.5 μM). Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells) **(A)** and the number of cells in G0/G1 phase **(B)**, S phase **(C)** and G2/M phase **(D)** of the cell cycle. Data are representative of at least three independent experiments. **(E)** Wild-type WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of DMS (0-5 μM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least three independent experiments.

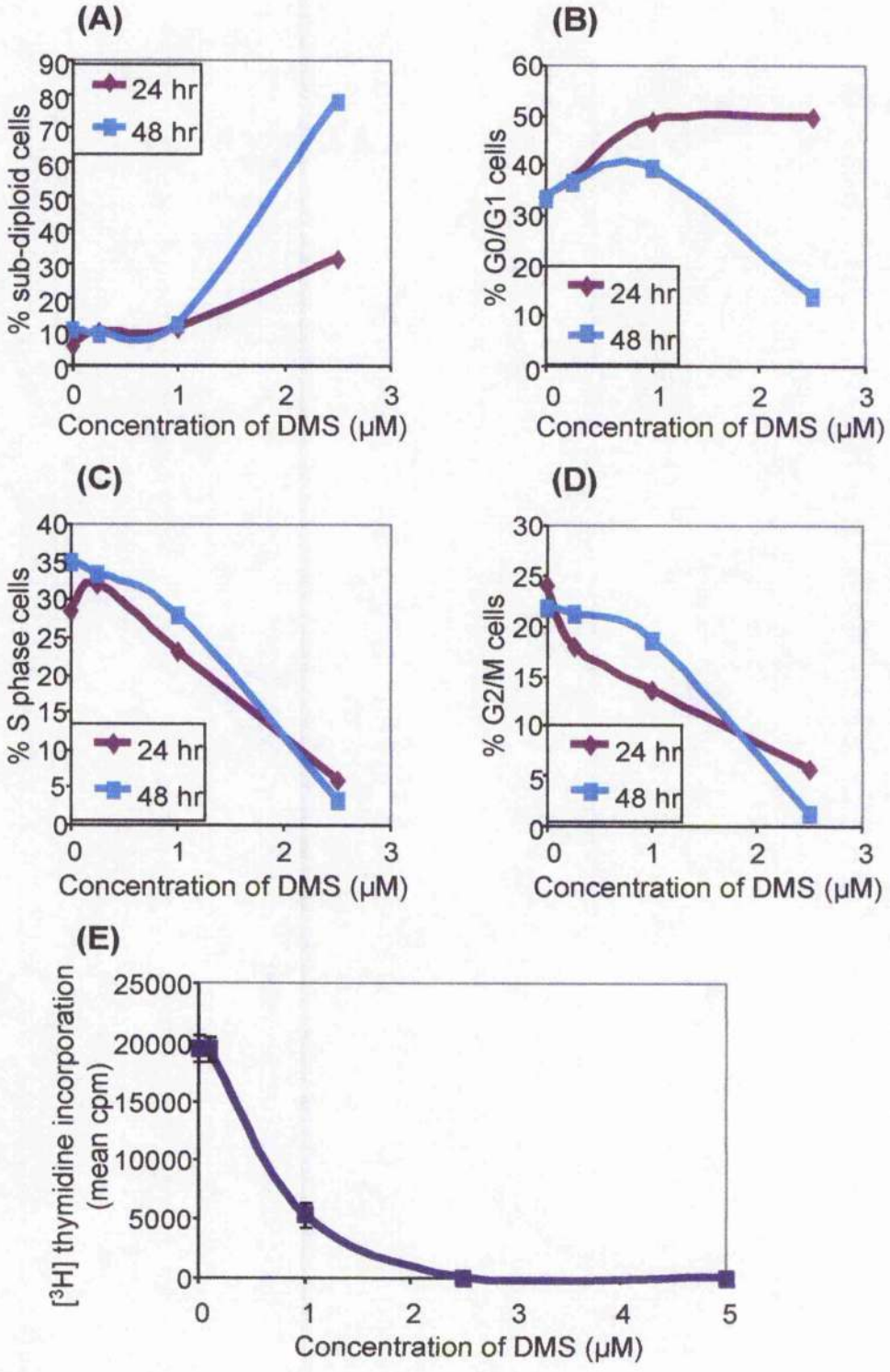

(A)
% sub-diploid cells
24 hr
48 hr
Concentration of DMS (μM)
(B)
% G0/G1 cells
24 hr
48 hr
Concentration of DMS (μM)
(C)
% S phase cells
24 hr
48 hr
Concentration of DMS (μM)
(D)
% G2/M cells
24 hr
48 hr
Concentration of DMS (μM)
(E)
[3H] thymidine incorporation (mean cpm)
Concentration of DMS (μM)

Figure 5.4. Inhibition of sphingosine kinase enhances BCR-driven growth arrest. Neo WEHI-231 cells (5 x 10^5 cells/ml) were cultured with increasing concentrations of anti-Ig (0-10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated) for 24 hr or 48 hr. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells in G0/G1 phase, S phase and G2/M phase of the cell cycle. **(A)** shows % G0/G1 cells after 24 hr, **(B)** is % S phase cells after 24 hr **(C)** is % G2/M cells after 24 hr, **(D)** is % G0/G1 cells after 48 hr, **(E)** is % S phase cells after 48 hr and **(F)** is % G2/M cells after 48 hr. Data are representative of at least three independent experiments.

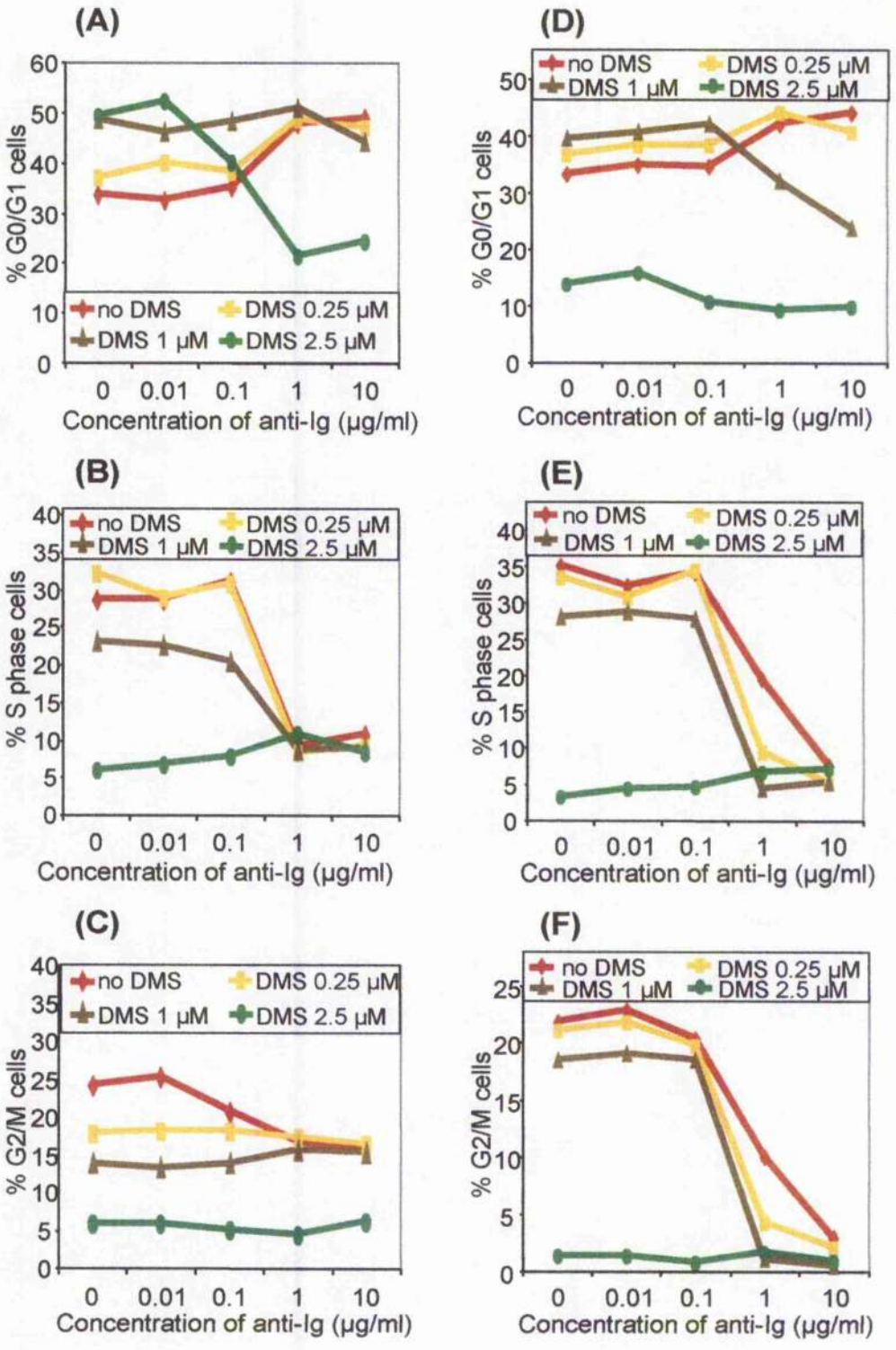

(A)
% G0/G1 cells
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
Concentration of anti-Ig (µg/ml)
(D)
% G0/G1 cells
Concentration of anti-Ig (µg/ml)
(B)
% S phase cells
Concentration of anti-Ig (µg/ml)
(E)
% S phase cells
Concentration of anti-Ig (µg/ml)
(C)
% G2/M cells
Concentration of anti-Ig (µg/ml)
(F)
% G2/M cells
Concentration of anti-Ig (µg/ml)

Figure 5.5. Inhibition of sphingosine kinase enhances BCR-driven growth arrest. Neo WEHI-231 cells (1×10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least three independent experiments.

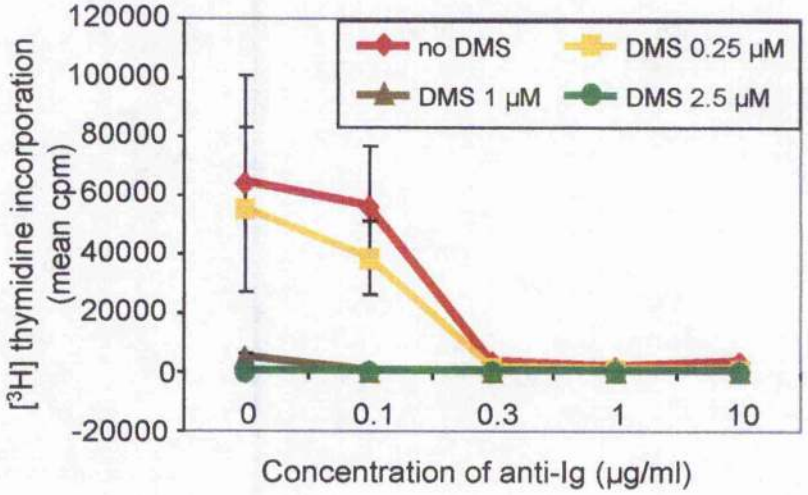

120000
100000
80000
60000
40000
20000
0
-20000
[3H] thymidine incorporation (mean cpm)
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
0
0.1
0.3
1
10
Concentration of anti-Ig (µg/ml)

Figure 5.6. Inhibition of sphingosine kinase enhances BCR-driven apoptosis but CD40-ligation partially protects WEHI-231 cells from DMS-driven apoptosis. Neo WEHI-231 cells (5 x 10^5 cells/ml) were cultured with increasing concentrations of anti-Ig (0-10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM) for 24 hr **(A)** or 48 hr **(B)**. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells). Neo WEHI-231 cells (5 x 10^5 cells/ml) were cultured with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM) for 24 hr **(C)** or 48 hr **(D)**. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells). Data are representative of at least three independent experiments.

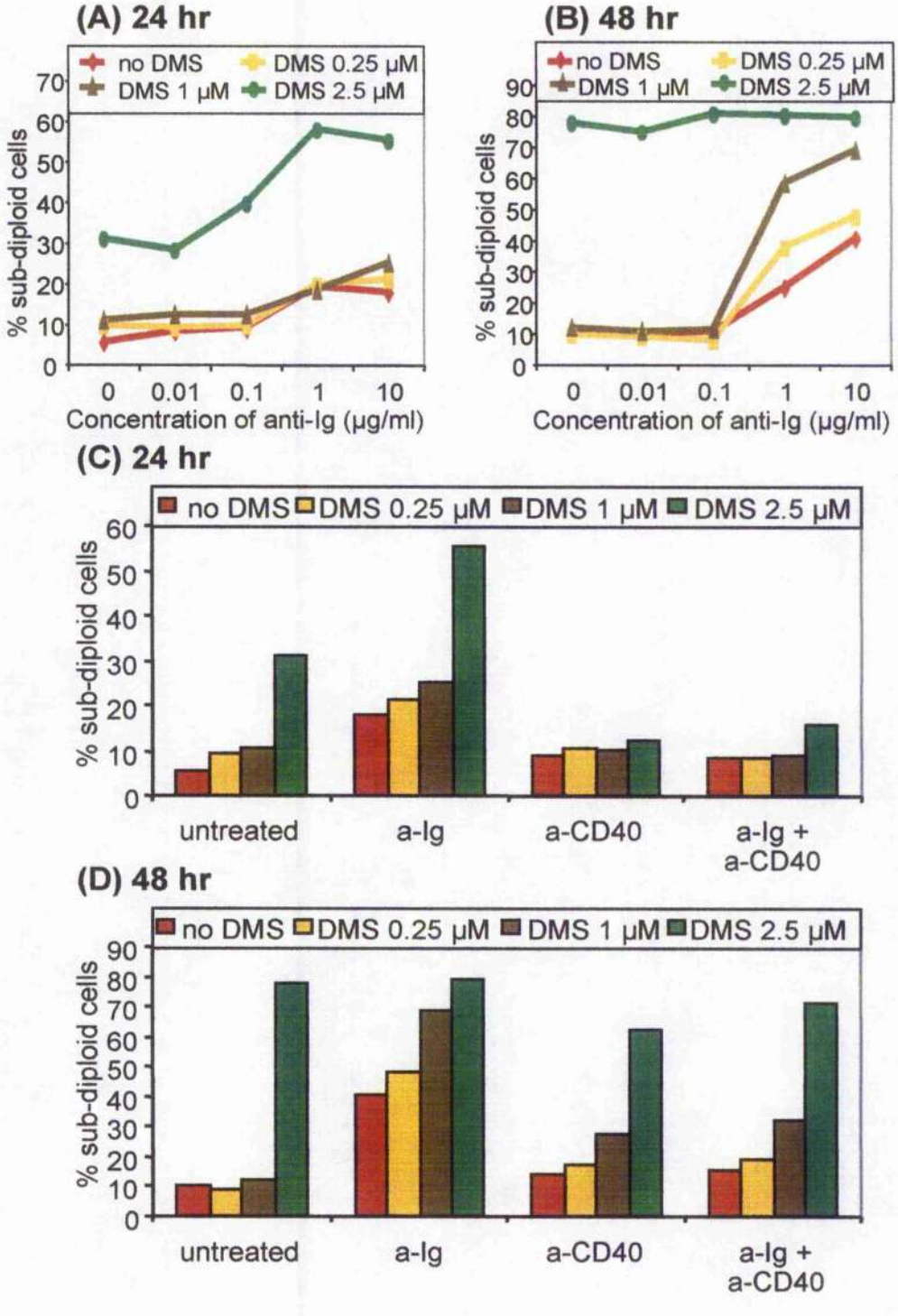

(A) 24 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
10
20
30
40
50
60
70
0
0.01
0.1
1
10
Concentration of anti-Ig (µg/ml)
(B) 48 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
10
20
30
40
50
60
70
80
90
0
0.01
0.1
1
10
Concentration of anti-Ig (µg/ml)
(C) 24 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
10
20
30
40
50
60
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) 48 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
10
20
30
40
50
60
70
80
90
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 5.7. CD40 partially protects WEHI-231 cells from DMS-induced growth arrest. **(A)** Neo WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of medium (untreated), anti-Ig (a-Ig, 1 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig (1 µg/ml) plus anti-CD40 (10 µg/ml) in the presence of increasing concentrations of DMS (0-5 µM, as indicated). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Data are representative of at least three independent experiments. Neo WEHI-231 cells (5 x 10^5 cells/ml) were cultured with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM) for 24 hr or 48 hr. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells in S phase and G2/M phase of the cell cycle. **(B)** shows the % S phase cells after 24 hr, **(C)** is % G2/M phase cells after 24 hr, **(D)** is % S phase cells after 48 hr and **(E)** is % G2/M phase cells after 48 hr. Data are representative of at least three independent experiments.

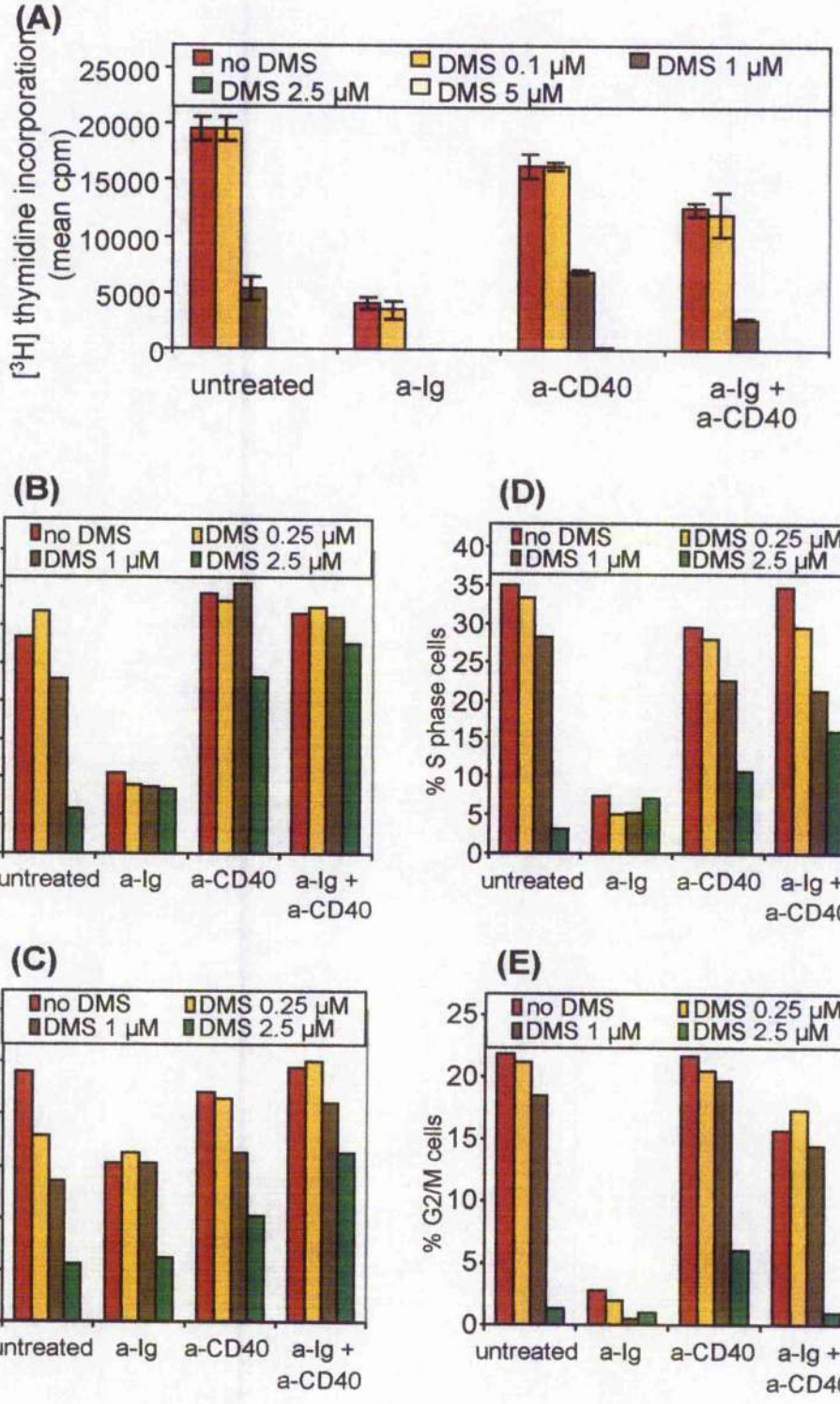
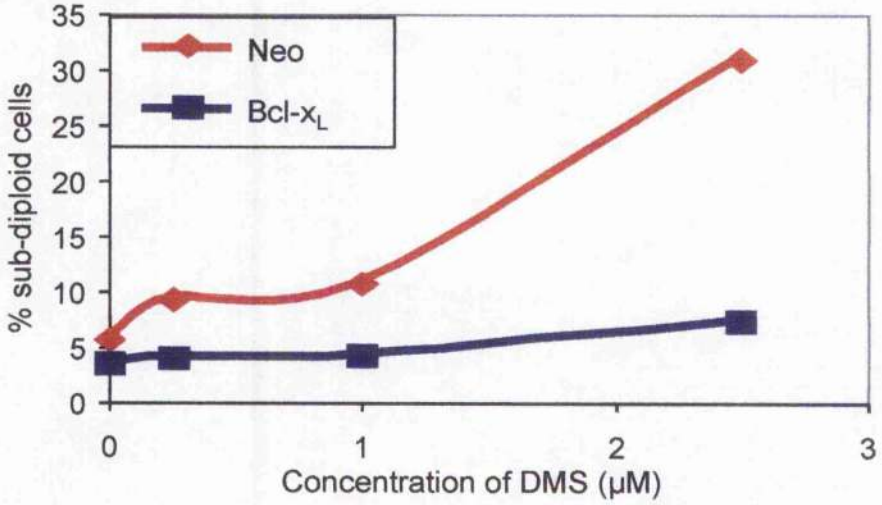

(A)
[3H] thymidine incorporation (mean cpm)
25000
20000
15000
10000
5000
0
no DMS
DMS 0.1 µM
DMS 1 µM
DMS 2.5 µM
DMS 5 µM
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B)
% S phase cells
40
35
30
25
20
15
10
5
0
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D)
% S phase cells
40
35
30
25
20
15
10
5
0
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C)
% G2/M cells
30
25
20
15
10
5
0
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E)
% G2/M cells
25
20
15
10
5
0
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 5.8. Overexpression of Bcl-x_L protects WEHI-231 cells from DMS-driven apoptosis. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(A)** or 48 hr **(B)** with increasing concentrations of DMS (0-2.5 μM). The cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells). Data are representative of at least three independent experiments.

(A) 24 hr


35
30
25
20
15
10
5
0
% sub-diploid cells
Neo
Bcl-x_L
0
1
2
3
Concentration of DMS (μM)

(B) 48 hr

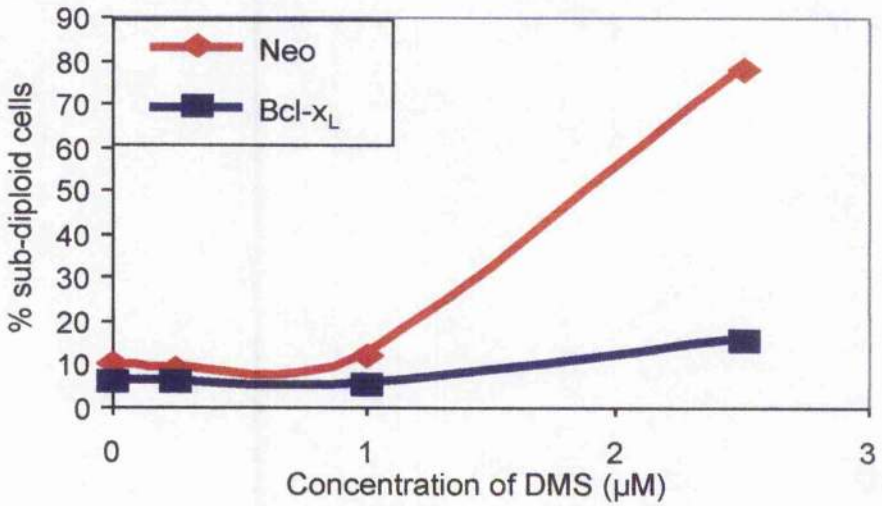

90
80
70
60
50
40
30
20
10
0
% sub-diploid cells
Neo
Bcl-x_L
0
1
2
3
Concentration of DMS (μM)

Figure 5.9. Overexpression of Bcl-x_L protects WEHI-231 cells from BCR- and DMS-driven apoptosis. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(A)** or 48 hr **(B)** with increasing concentrations of anti-Ig (0-10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated). The cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells). Data are representative of at least three independent experiments. **(C)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 48 hr with medium (untreated) or anti-CD40 (a-CD40, 10 µg/ml) plus increasing concentrations of DMS (0-2.5 µM, as indicated). The cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells).

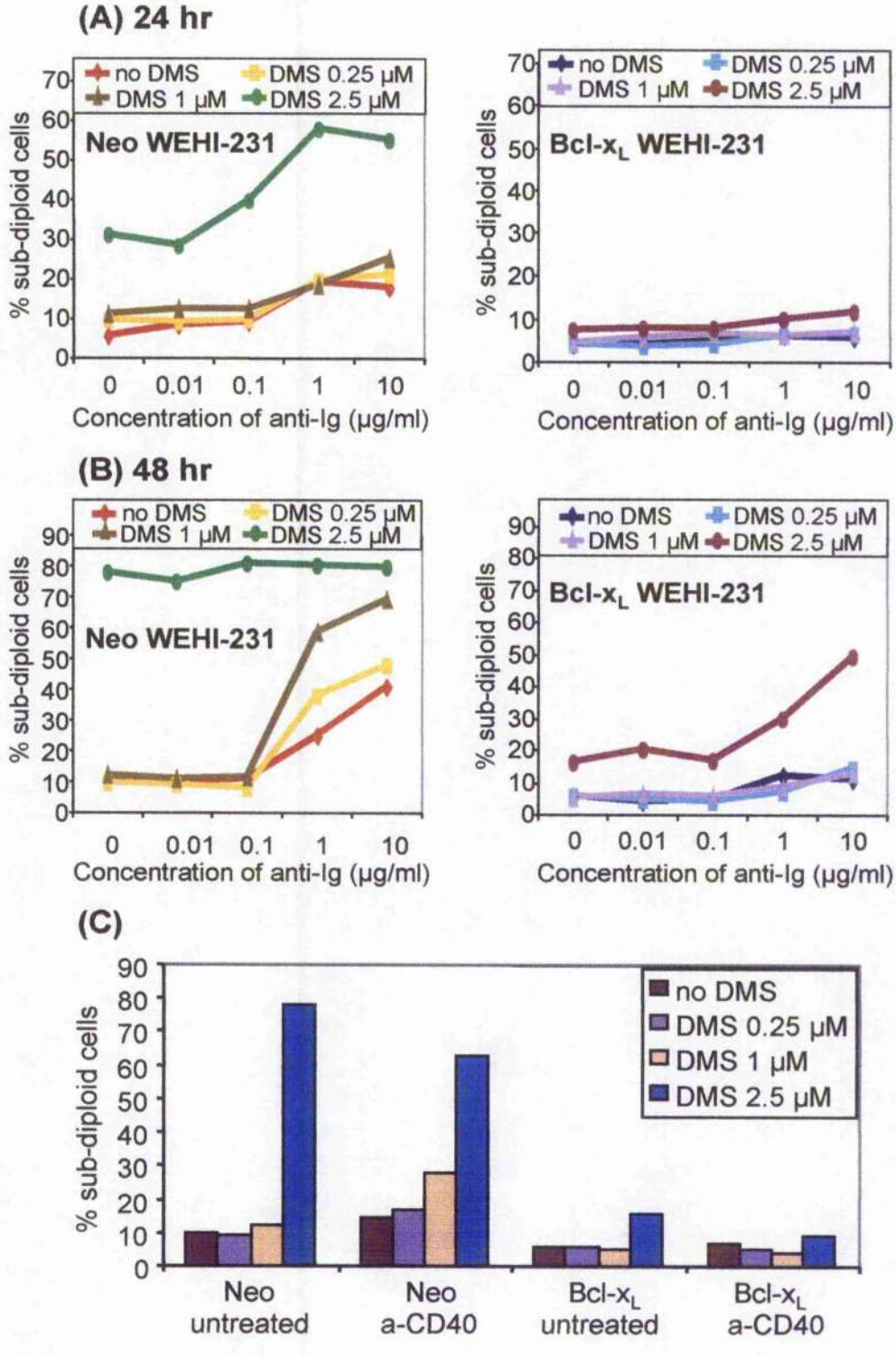
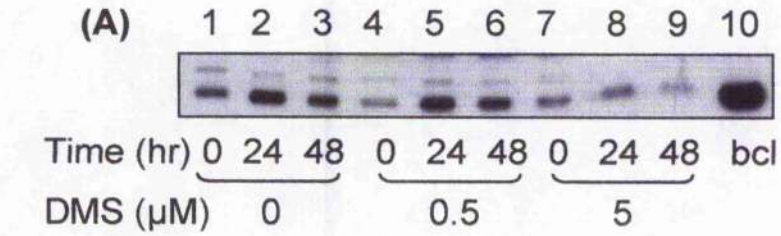

(A) 24 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
Neo WEHI-231
Bcl-x$_L$ WEHI-231
% sub-diploid cells
0 0.01 0.1 1 10
Concentration of anti-Ig (µg/ml)
(B) 48 hr
(C)
Neo untreated
Neo a-CD40
Bcl-x$_L$ untreated
Bcl-x$_L$ a-CD40

Figure 5.10. DMS impairs Bcl-x_L expression in WEHI-231 cells. **(A)** Neo WEHI-231 cells (1 x 10^6 cells/ml) were cultured for 0, 24 or 48 hr with several concentrations of DMS (0, 0.5 μM or 5 μM) before preparing cell lysates. Levels of Bcl-x_L were determined by Western blotting using the NuPAGE system of gel electrophoresis with 10% Bis-Tris gels. Gels were loaded (30 μg protein/lane) as follows: *lane 1*, no DMS time 0 lysate; *lane 2*, no DMS 24 hr lysate; *lane 3*, no DMS 48 hr lysate; *lane 4*, DMS 0.5 μM time 0 lysate; *lane 5*, DMS 0.5 μM 24 hr lysate; *lane 6*, DMS 0.5 μM 48 hr lysate; *lane 7*, DMS 5 μM time 0 lysate; *lane 8*, DMS 5 μM 24 hr lysate; *lane 9*, DMS 5 μM 48 hr lysate; *lane 10*, no DMS unstimulated time 0 Bcl-x_L WEHI-231 lysate (Bcl-x_L positive control). **(B)** Wild-type WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with anti-CD40 (10 μg/ml), anti-Ig (10 μg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 μg/ml) in the presence or absence of DMS (0.5 μM) before preparing cell lysates. Whole cell lysates (50 μg/lane) were run on 10% Tris-HCl gels using the BioRad system of gel electrophoresis. Bcl-x_L positive and negative controls were also included on the gels: *n* is no DMS unstimulated time 0 Neo WEHI-231 lysate (Bcl-x_L negative control) and *b* is no DMS unstimulated time 0 Bcl-x_L WEHI-231 lysate (Bcl-x_L positive control). Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting.


(A)
1 2 3 4 5 6 7 8 9 10
Time (hr) 0 24 48 0 24 48 0 24 48 bcl
DMS (μM) 0 0.5 5

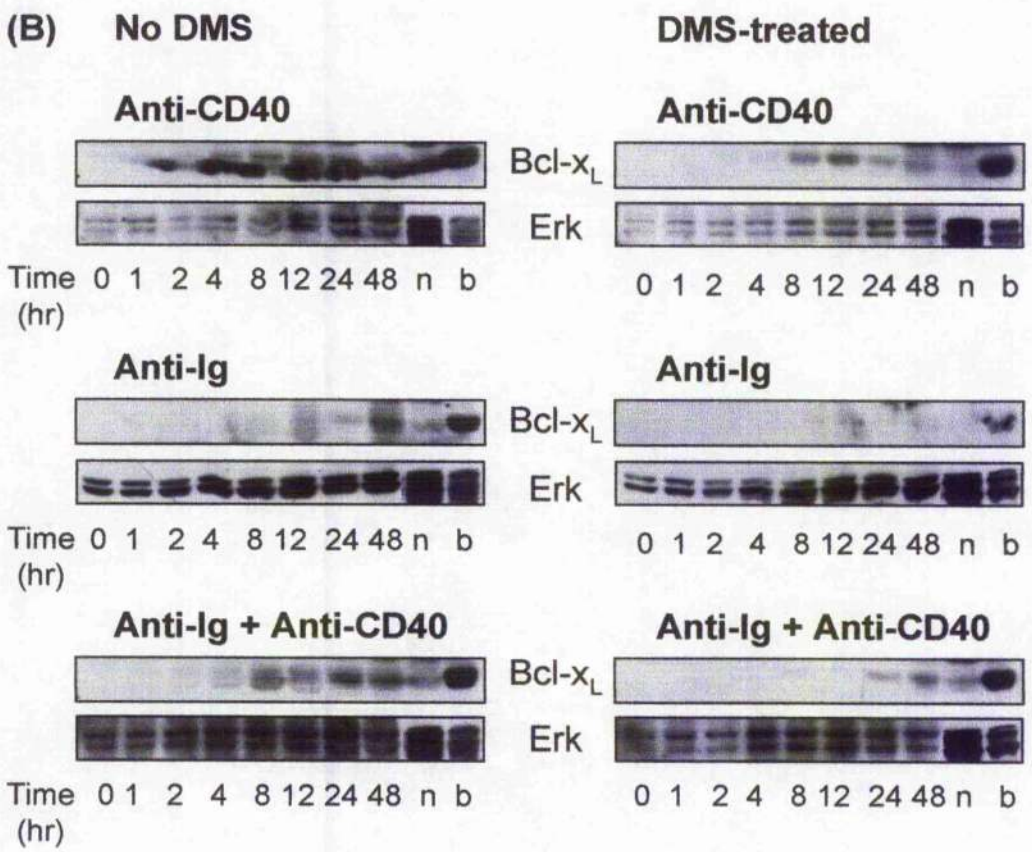

(B)
No DMS
DMS-treated
Anti-CD40
Anti-CD40
Bcl-xL
Erk
Time (hr) 0 1 2 4 8 12 24 48 n b
0 1 2 4 8 12 24 48 n b
Anti-Ig
Anti-Ig
Bcl-xL
Erk
Time (hr) 0 1 2 4 8 12 24 48 n b
0 1 2 4 8 12 24 48 n b
Anti-Ig + Anti-CD40
Anti-Ig + Anti-CD40
Bcl-xL
Erk
Time (hr) 0 1 2 4 8 12 24 48 n b
0 1 2 4 8 12 24 48 n b

Figure 5.11. Ligation of CD40 enhances the survival of DMS-treated Bcl-x_L WEHI-231 cells. Bcl-x_L WEHI-231 cells (5×10^5 cells/ml) were cultured for 24 hr **(A)** or 48 hr **(B)** with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated). The cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells with sub-diploid DNA (apoptotic cells). Data are representative of at least three independent experiments.

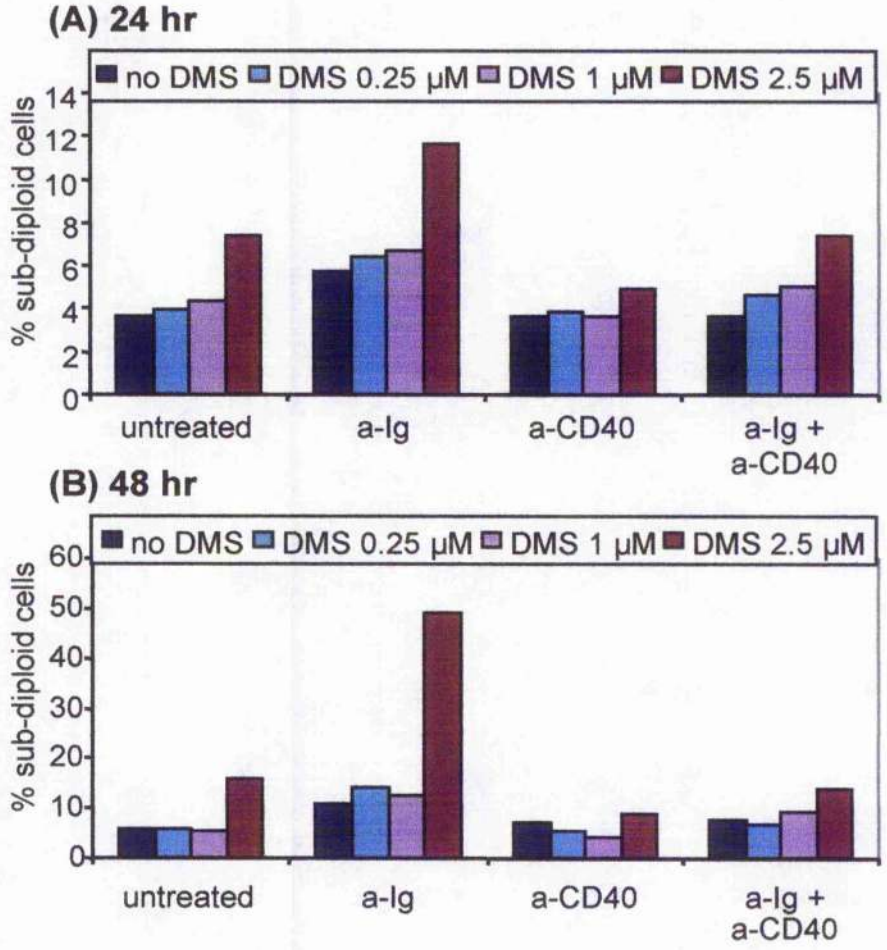
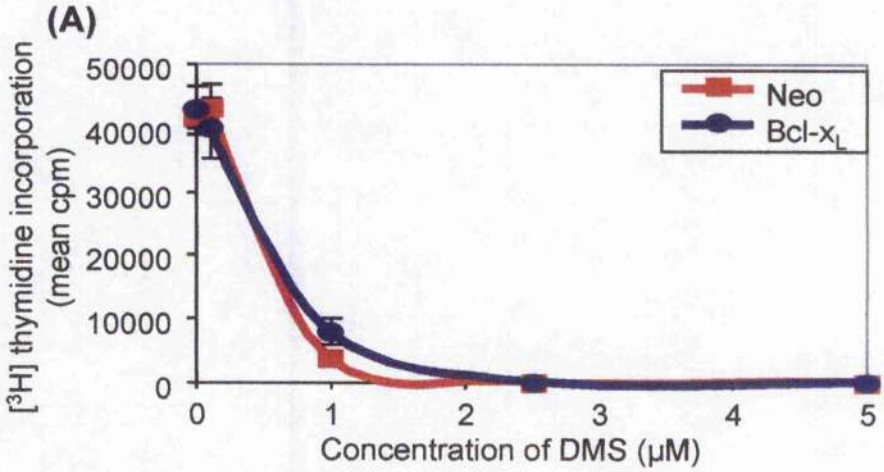
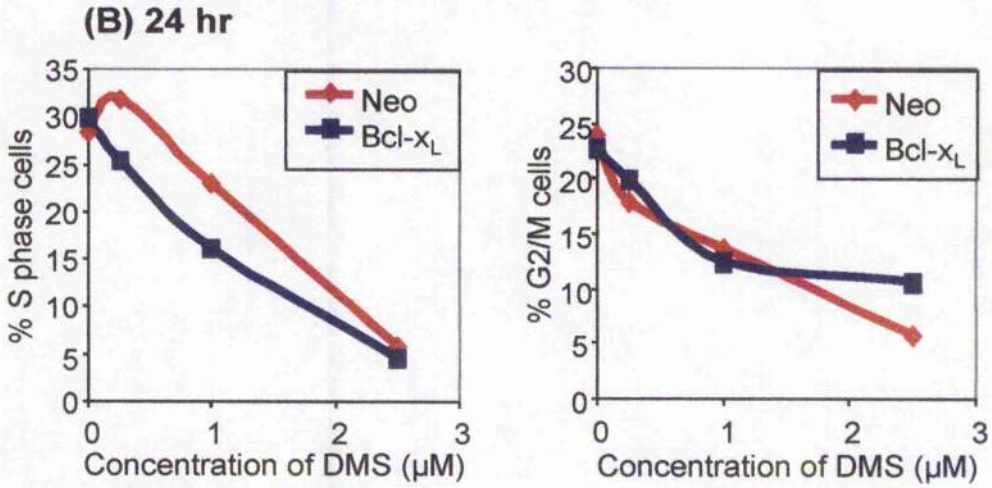

(A) 24 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
2
4
6
8
10
12
14
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) 48 hr
no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% sub-diploid cells
0
10
20
30
40
50
60
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 5.12. Overexpression of Bcl-x_L does not protect WEHI-231 cells from DMS-induced cell cycle arrest. **(A)** Neo or Bcl-x_L WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of DMS (0-5 μM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured with increasing concentrations of DMS (0-2.5 μM) for 24 hr **(B)** or 48 hr **(C)**. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells in S phase and G2/M phase of the cell cycle. Data are representative of at least three independent experiments.


(A)
[3H] thymidine incorporation (mean cpm)
50000
40000
30000
20000
10000
0
0
1
2
3
4
5
Concentration of DMS (μM)
Neo
Bcl-xL


(B) 24 hr
% S phase cells
% G2/M cells
Concentration of DMS (μM)
Neo
Bcl-xL

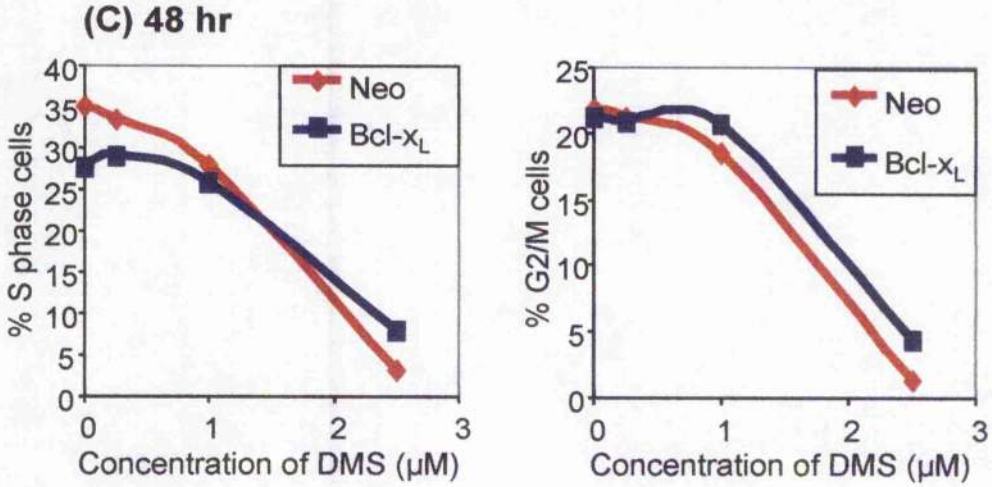
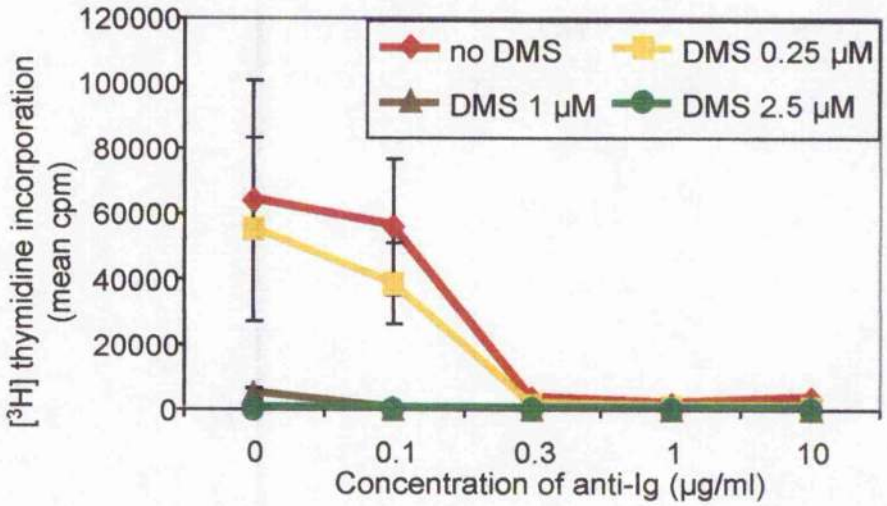

(C) 48 hr
% S phase cells
% G2/M cells
Concentration of DMS (μM)
Neo
Bcl-xL

Figure 5.13. Overexpression of Bcl-x_L does not protect WEHI-231 cells from DMS-induced growth arrest. Neo **(A)** or Bcl-x_L **(B)** WEHI-231 cells (1×10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells.

(A) Neo WEHI-231



(B) Bcl-x_L WEHI-231

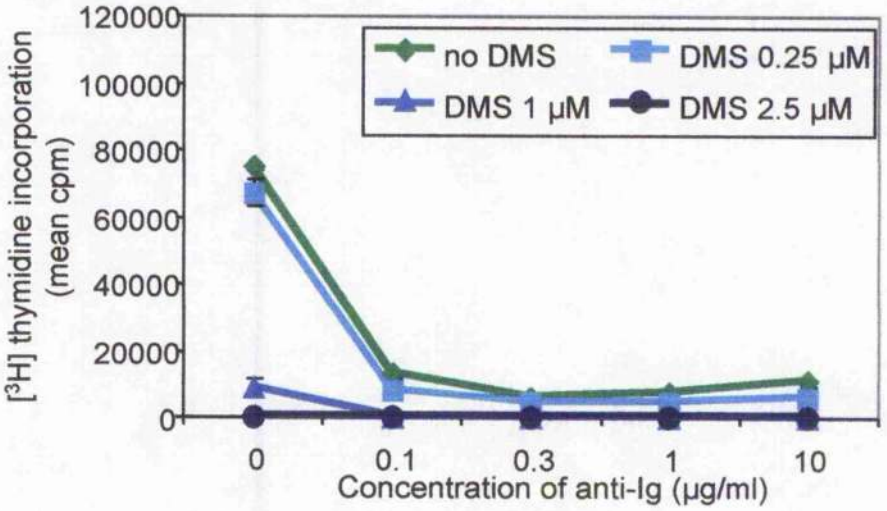
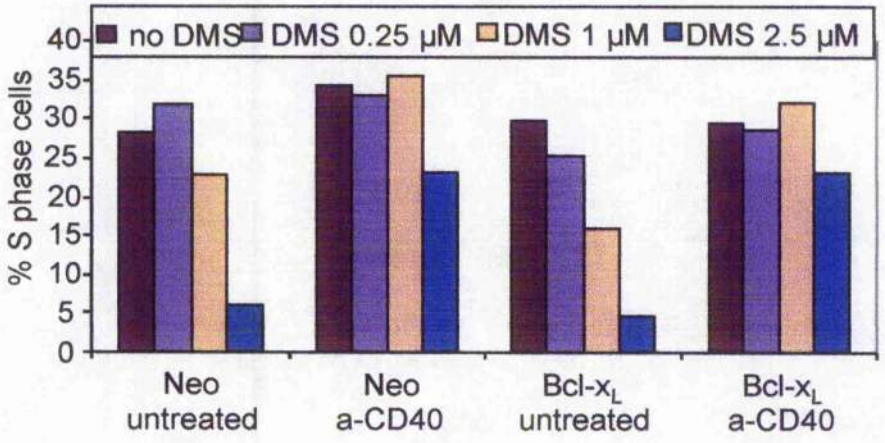
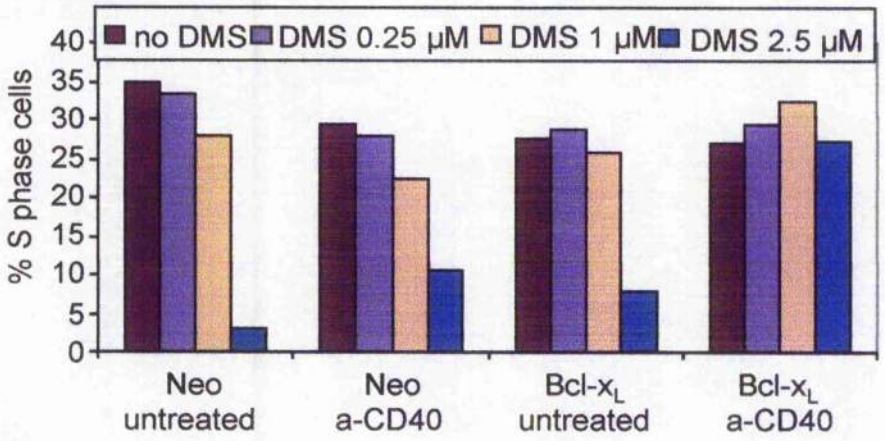


Figure 5.14. Overexpression of Bcl-x_L enhances CD40-dependent rescue of DMS-driven growth arrest. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured with medium (untreated) or anti-CD40 (a-CD40, 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated) for 24 hr **(A)** or 48 hr **(B)**. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells in S phase of the cell cycle. Data are representative of at least three independent experiments. **(C)** Neo or Bcl-x_L WEHI-231 cells (1 x 10^4 cells/well) were cultured with medium (untreated) or anti-CD40 (a-CD40, 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM, as indicated). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean counts per minute (cpm) ± standard deviation of triplicate wells.

(A) 24 hr



(B) 48 hr



(C)

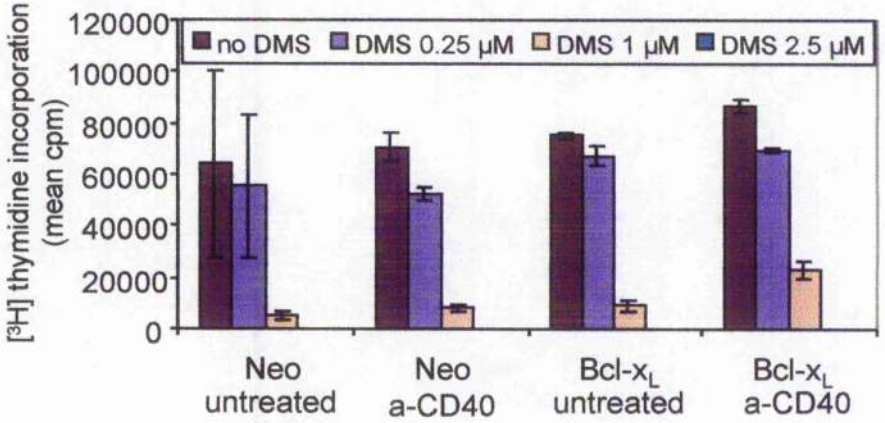
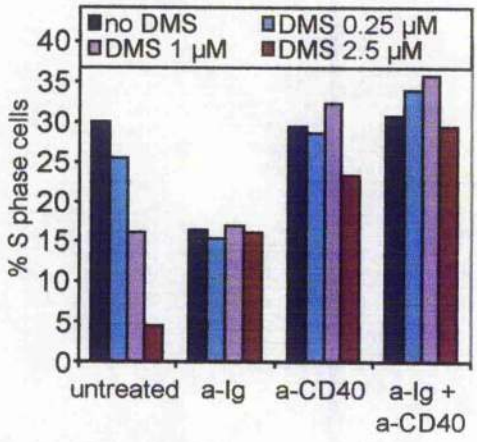
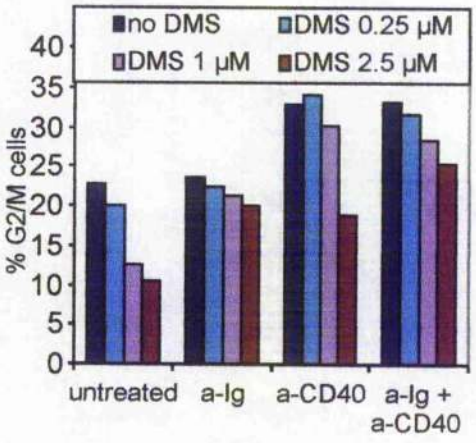


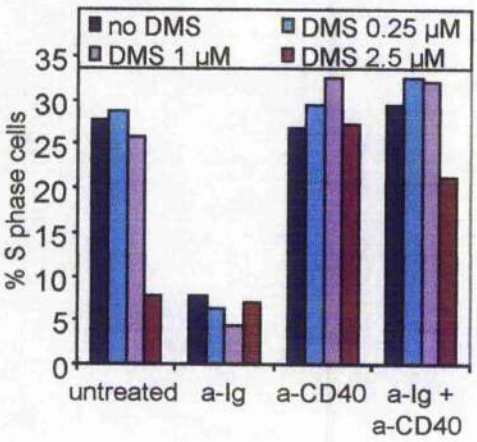
Figure 5.15. DMS does not prevent CD40-mediated rescue of WEHI-231 cells from BCR-driven growth arrest. Bcl-x_L WEHI-231 cells (5 x 10^5 cells/ml) were cultured with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) in the presence of increasing concentrations of DMS (0-2.5 µM) for 24 hr **(A)** or 48 hr **(B)**. Cells were then stained with PI and analysed by FACS analysis (FL2 fluorescence) to determine the number of cells in S phase and G2/M phase of the cell cycle. Data are representative of at least three independent experiments.

(A) 24 hr


no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% S phase cells
0
5
10
15
20
25
30
35
40
untreated
a-Ig
a-CD40
a-Ig + a-CD40


no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% G2/M cells
0
5
10
15
20
25
30
35
40
untreated
a-Ig
a-CD40
a-Ig + a-CD40

(B) 48 hr


no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% S phase cells
0
5
10
15
20
25
30
35
untreated
a-Ig
a-CD40
a-Ig + a-CD40

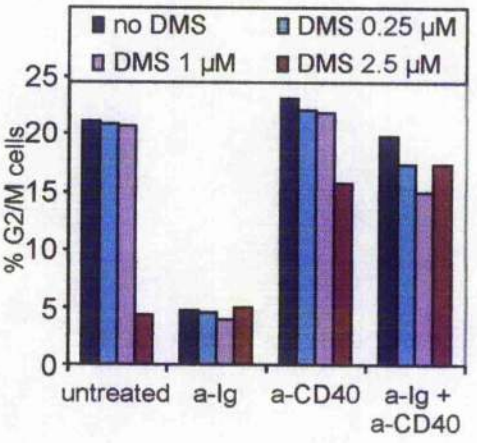

no DMS
DMS 0.25 µM
DMS 1 µM
DMS 2.5 µM
% G2/M cells
0
5
10
15
20
25
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 5.16. DMS does not alter sustained cyclic activation of Erk-MAPK in WEHI-231 cells. Wild-type WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) in the presence or absence of 0.5 µM DMS before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Tris-HCl gels using the BioRad system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

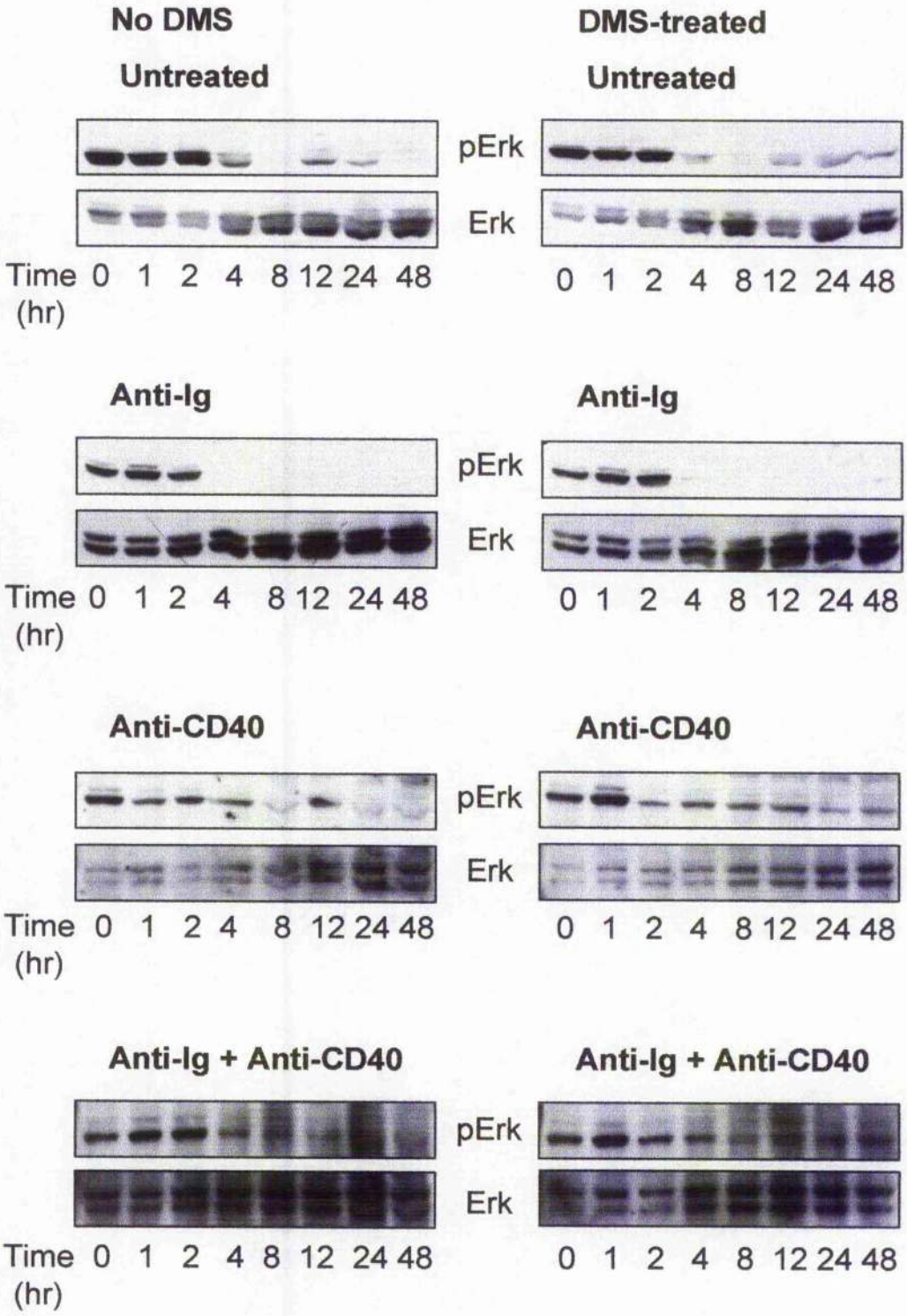

No DMS
Untreated
DMS-treated
Untreated
pErk
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-Ig
Anti-Ig
pErk
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-CD40
Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-Ig + Anti-CD40
Anti-Ig + Anti-CD40
pErk
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48

Figure 5.17. Inhibition of sphingosine kinase after 4 hr does not alter sustained cyclic activation of Erk-MAPK in WEHI-231 cells. Wild-type WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated) or anti-CD40 (10 µg/ml) and after 4 hr of culture cells were also treated with medium (no DMS) or DMS (0.5 µM) before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Recombinant non-phosphorylated Erk2 (w) and recombinant phosphorylated Erk2 (p) were used as positive controls. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

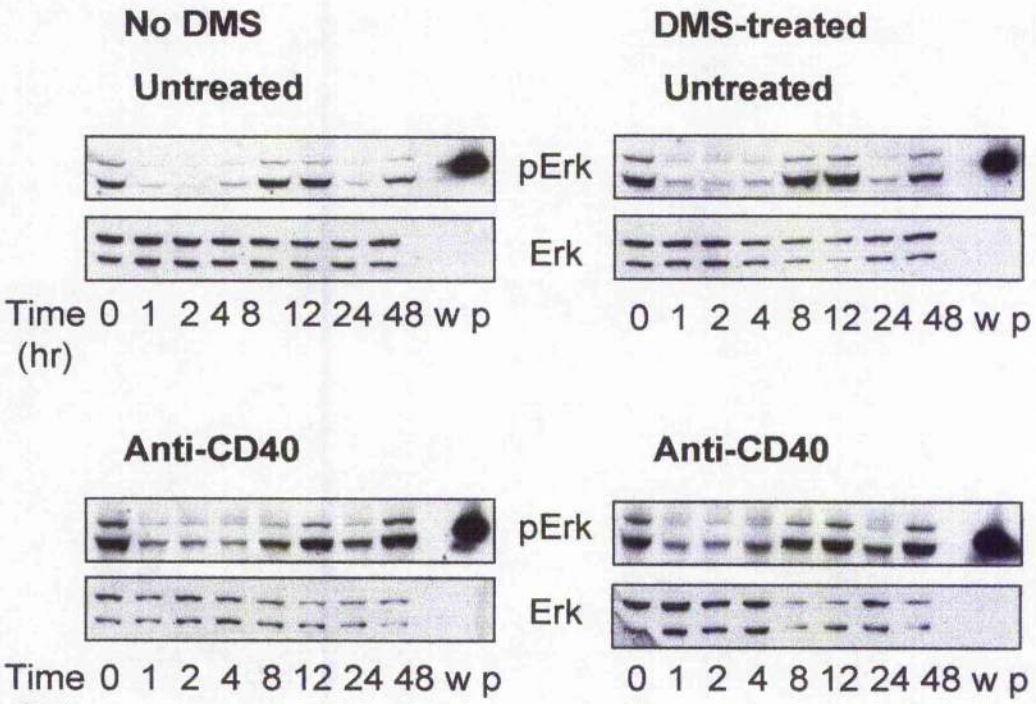

No DMS
Untreated
DMS-treated
Untreated
pErk
Erk
Time 0 1 2 4 8 12 24 48 w p
(hr)
0 1 2 4 8 12 24 48 w p
Anti-CD40
Anti-CD40
pErk
Erk
Time 0 1 2 4 8 12 24 48 w p
(hr)
0 1 2 4 8 12 24 48 w p

Figure 5.18. DMS does not alter the activity of MEK in WEHI-231 cells. Wild-type WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml) in the presence or absence of 0.5 µM DMS before preparing cell lysates. Whole cell lysates (50 µg/lane) were run on 10% Tris-HCl gels using the BioRad system of gel electrophoresis. Levels of phosphorylated MEK (pMEK) and total Erk (Erk) were determined by Western blotting.

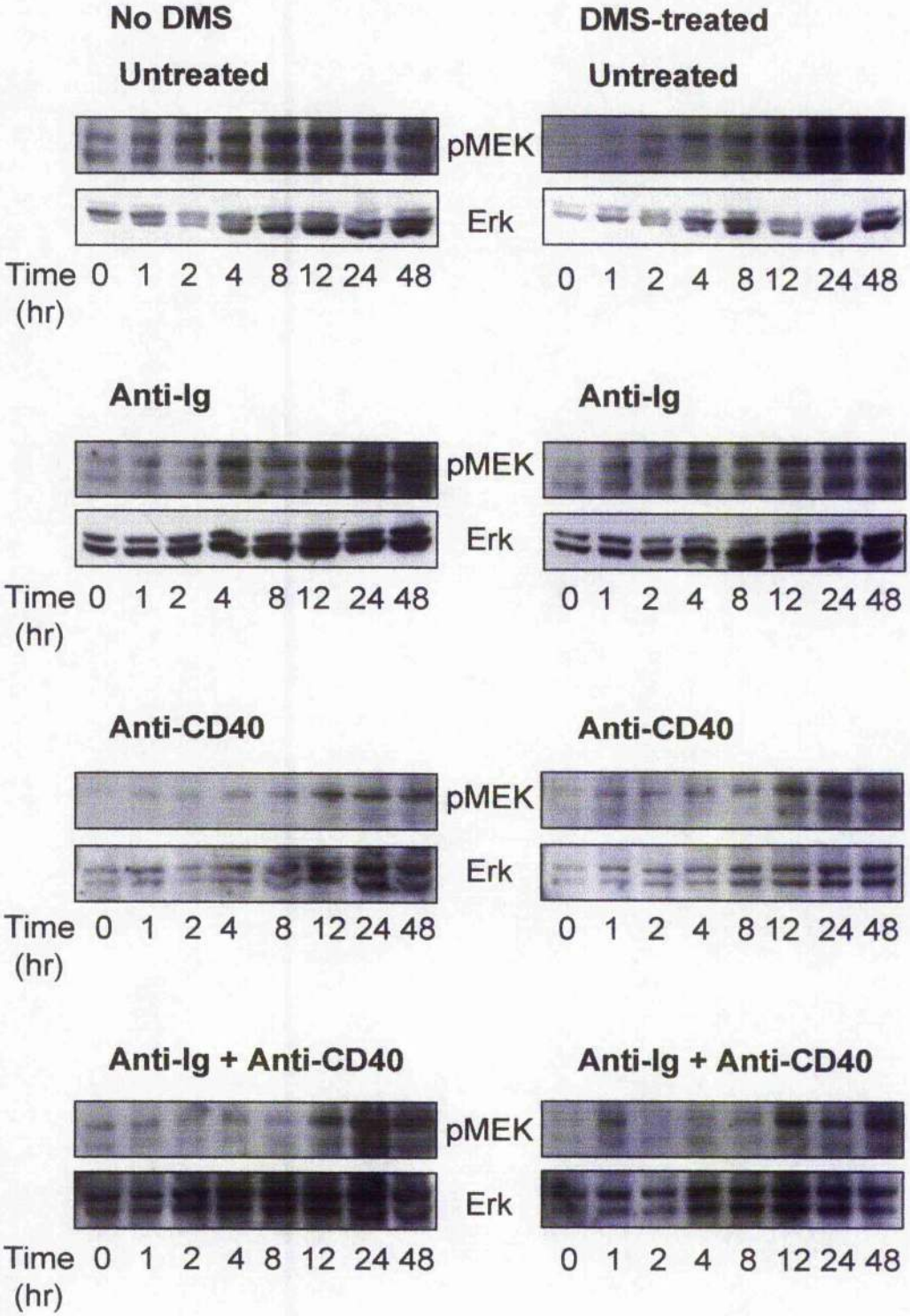

No DMS
Untreated
DMS-treated
Untreated
pMEK
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-Ig
Anti-Ig
pMEK
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-CD40
Anti-CD40
pMEK
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48
Anti-Ig + Anti-CD40
Anti-Ig + Anti-CD40
pMEK
Erk
Time (hr) 0 1 2 4 8 12 24 48
0 1 2 4 8 12 24 48

Figure 5.19. DMS does not alter phospho-Erk signalling in Bcl-x_L WEHI-231 cells. Neo or Bcl-x_L WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with DMS (0, 0.5 μM or 5 μM) and medium (untreated), anti-Ig (1 μg/ml) or a combination of anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) before preparing cell lysates. Whole cell lysates (30 μg/lane) were run on 10% Bis-Tris gels using the NuPAGE system of gel electrophoresis. Gels were loaded as follows: *lane 1*, no DMS time 0 lysate; *lane 2*, no DMS 24 hr lysate; *lane 3*, no DMS 48 hr lysate; *lane 4*, DMS 0.5 μM time 0 lysate; *lane 5*, DMS 0.5 μM 24 hr lysate; *lane 6*, DMS 0.5 μM 48 hr lysate; *lane 7*, DMS 5 μM time 0 lysate; *lane 8*, DMS 5 μM 24 hr lysate; *lane 9*, DMS 5 μM 48 hr lysate; *lane 10*, no DMS unstimulated time 0 Bcl-x_L WEHI-231 lysate (Bcl-x_L positive control); *lane 11*, recombinant phospho-Erk positive control (New England Biolabs). Levels of dual-phosphorylated Erk (pErk), total Erk (Erk) and Bcl-x_L were determined by Western blotting.

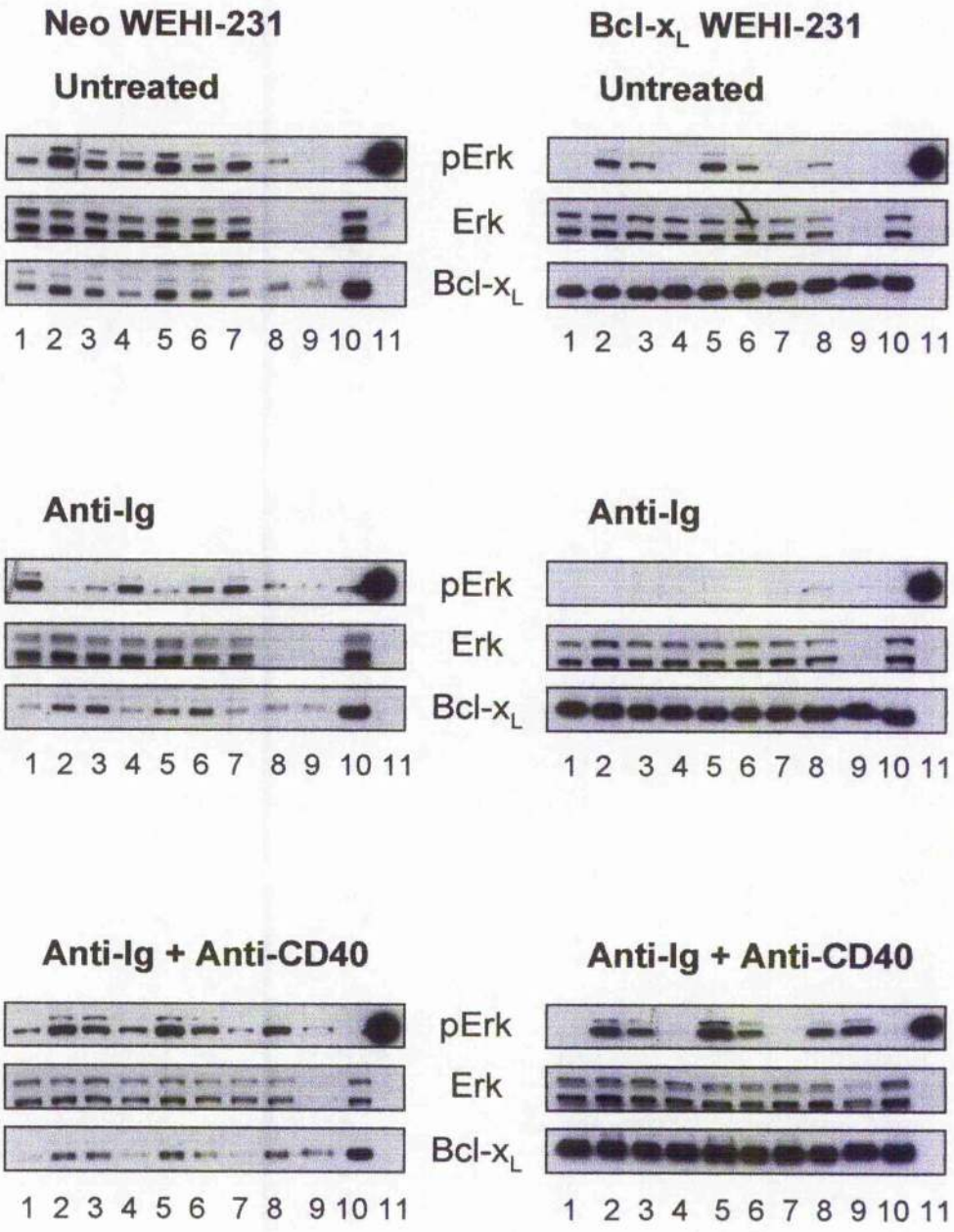
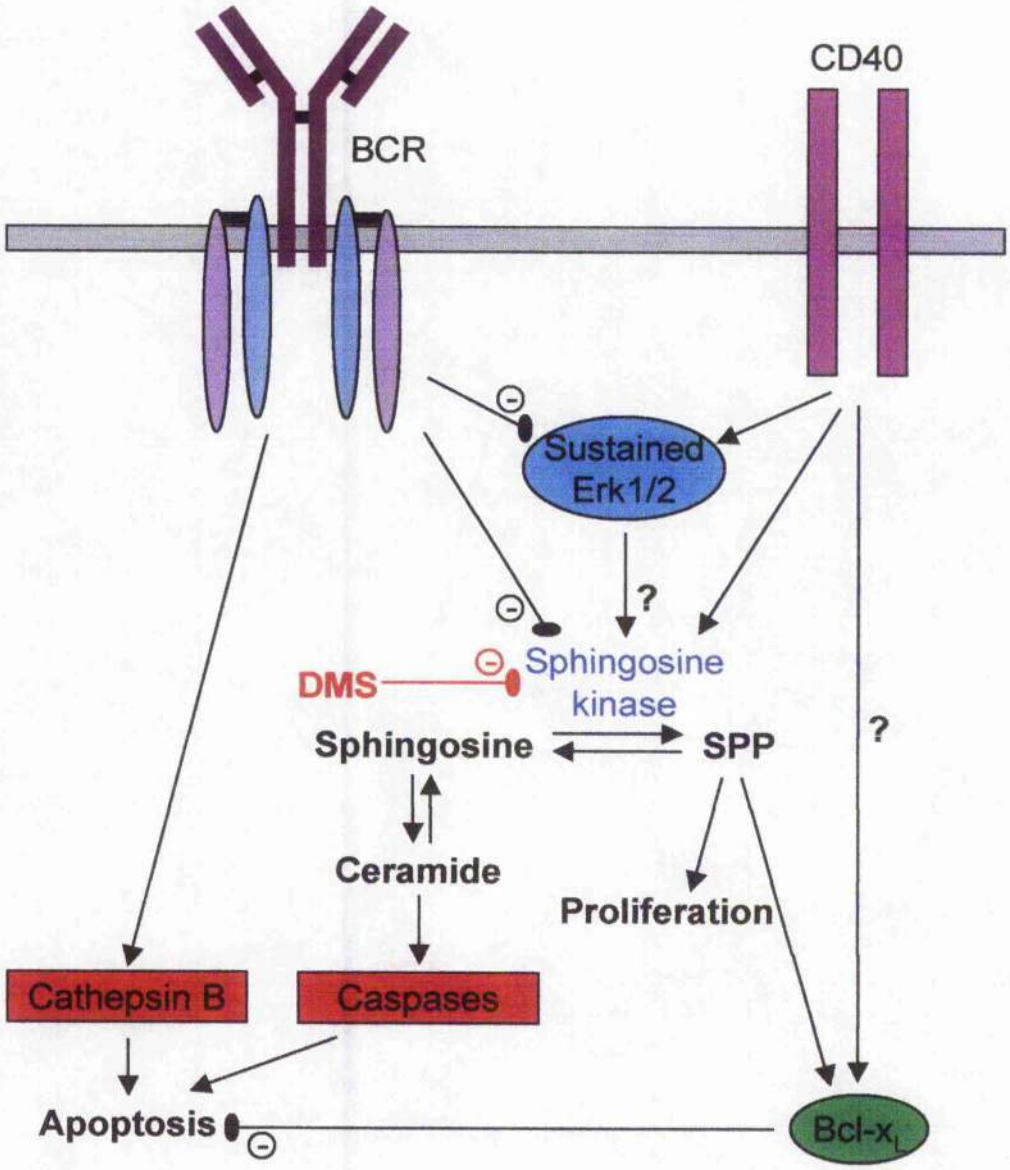

Neo WEHI-231
Untreated
Bcl-x$_L$ WEHI-231
Untreated
pErk
Erk
Bcl-x$_L$
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
Anti-Ig
Anti-Ig
pErk
Erk
Bcl-x$_L$
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
Anti-Ig + Anti-CD40
Anti-Ig + Anti-CD40
pErk
Erk
Bcl-x$_L$
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11

Figure 5.20. Model showing role of sphingosine kinase signalling in regulating survival and proliferation of WEHI-231 cells. DMS (shown in red) is an inhibitor of sphingosine kinase with few non-specific toxic effects therefore it prevents the formation of SPP from sphingosine. WEHI-231 cells were treated with DMS to assess the role of sphingosine kinase and SPP in regulating survival and proliferation of immature B cells. Sphingosine kinase and hence SPP production is differentially regulated by the BCR and CD40. SPP contributes to the induction of Bcl-x_L and hence can suppress BCR-driven induction of cathepsin B-mediated apoptosis. Additional pathways may also induce Bcl-x_L downstream of CD40. Inhibition of sphingosine kinase induces growth arrest of WEHI-231 cells suggesting SPP is also likely to promote proliferation of WEHI-231 cells. However, inhibition of sphingosine kinase did not alter Erk activity in WEHI-231 cells suggesting sphingosine kinase might be activated downstream of sustained Erk signals.


CD40
BCR
Sustained
Erk1/2
?
DMS
Sphingosine
kinase
Sphingosine
SPP
?
Ceramide
Proliferation
Cathepsin B
Caspases
Apoptosis
Bcl-x$_L$

CHAPTER 6 - Role of PKC in regulating the survival and proliferation of WEHI-231 cells

6.1 Protein kinase C

Protein kinase C (PKC) comprises a large family of kinases that can be categorised into three subclasses: conventional PKC (α, βI, βII and γ), novel PKC (δ, ε, θ, η, ν) and atypical PKC (ζ, ι/λ) (204, 208). Each PKC enzyme contains several conserved domains with specific functions (figure 6.1A). The C1 region is found in all PKC enzymes and includes a pseudosubstrate site that binds to the catalytic domain to inactivate the kinase. To activate PKC, the pseudosubstrate site must dissociate from the kinase domain. This can be mediated by the binding of PKC to membranes. The membrane attachment of conventional and novel PKC enzymes is enhanced by their association with DAG and phorbol ester. The DAG/phorbol ester-binding site of these subclasses of PKC enzymes is mediated by a region in the C1 domain that has two zinc-finger domains. Atypical PKC isoforms have a single zinc-finger domain and are unresponsive to DAG and phorbol ester.

The C2 domain of PKC enzymes also enhances their membrane attachment. For example, the C2 domain of conventional PKCs contains a calcium and lipid-binding domain that enables these enzymes to associate with phosphatidylserine in a Ca^{2+}-dependent manner. In contrast, the C2 domain of novel and atypical PKCs lacks one or more aspartate residues and cannot bind to calcium. However, the C2 domain of each subclass of PKC enzymes can bind to RACKs (receptor for activated C kinase). RACKs are not substrates of PKC but instead they are anchoring proteins that target PKC enzymes to particular intracellular locations. The intracellular location of PKC enzymes is also influenced by a V5 domain that is located at the C-terminus of PKC. The V5 domain is separated from the C1 and C2 domains by a hinge region and the kinase domain. The kinase domain contains binding sites for substrates and for ATP (204).

The different subclasses of PKC can be distinguished by their regulatory mechanisms and these are dependent on the structure of the enzymes. Conventional PKC (cPKC) isoforms are targeted to membranes by associating with phosphatidylserine in a Ca^{2+}-dependent manner. This contributes to their

activation but they also require DAG, which binds to the enzyme to increase its affinity for phosphatidylserine and calcium. Novel PKC (nPKC) isoforms are Ca^{2+}-insensitive but do require phosphatidylserine and DAG for activation. Atypical PKC (aPKC) isoforms are Ca^{2+}-insensitive and do not require DAG for stimulation (484). Phorbol ester can be used to mimic DAG and stimulate cPKC and nPKC isoforms.

The activity of all PKC enzymes can also be regulated by phosphorylation. In particular phosphorylation of a conserved threonine residue, Thr^{497} of PKCα (485), in the activation loop helps to align the activation loop for substrate binding. At the plasma membrane, this residue can be phosphorylated by PDK-1 (phosphoinositide-dependent kinase-1) (486). Additional sites of phosphorylation include a ser/thr residue, Thr^{641} of PKCβII (484), in a turn motif of the C-terminal segment of PKC. Phosphorylation of this residue stabilises the PKC enzyme. Conventional and novel PKC isoforms are further stabilised by the phosphorylation of a ser/thr residue flanked by a hydrophobic residue. In cPKCs and PKCε phosphorylation of the turn motif and the hydrophobic motif is mediated by autophosphorylation (484). PKC enzymes are also subject to tyrosine phosphorylation but the residues involved are specific to particular PKC isoforms.

PKC isoforms can also be regulated by proteolytic cleavage since this releases and activates the catalytic domain. PKCα, β and γ are cleaved by calpains whilst PKCδ, θ and ζ can be cleaved by caspases. The activation of PKC by proteolytic cleavage is one mechanism by which these cysteine proteases can promote apoptosis. For example, in the rat pituitary cell line GH3B6 genotoxic stress results in the stimulation of calpains and caspases and the active catalytic domains of PKCδ, PKCε and PKCα can accumulate at particular intracellular organelles including the mitochondria. At the mitochondria, the active catalytic domains of PKCδ and PKCε promote cytochrome c release and caspase 3 stimulation leading to enhanced apoptosis. The active catalytic domain of PKCα also promotes apoptosis but it does so independently of inducing cytochrome c release or caspase 3 stimulation (487).

PKC enzymes can further influence the survival and proliferation of cells by regulating additional intracellular signalling networks. For example, in HEK-

293 cells phorbol ester-sensitive isoforms of PKC can increase the production of SPP by promoting the translocation of sphingosine kinase to the plasma membrane leading to its phosphorylation and activation (488).

Furthermore, PKC enzymes from all three subclasses can regulate the activity of Erk-MAPK. However, the regulation of Erk1/2 by PKC is complex since PKC can target several signalling molecules in the MAPK cascade depending on the system being studied. For example, phorbol ester-sensitive PKC enzymes enhance the activity of Ras in growth factor-stimulated NIH3T3 and Swiss 3T3 fibroblasts and this is necessary for the activation of Erk. PKC-dependent activation of Ras in these systems can be suppressed by calmodulin suggesting cPKC enzymes are responsible for the induction of Ras (489). Furthermore, in IL-3-stimulated murine hematopoietic cells phorbol ester-sensitive isoforms of PKC can directly phosphorylate and activate Raf-1 (209). The indirect stimulation of Raf-1 by PKC has also been observed in immortalised embryonic rat hippocampal cells. In this system cPKC and aPKC isoforms can phosphorylate Ser^{153} of RKIP leading to a dissociation of RKIP and Raf-1 thus enhancing the ability of Raf-1 to stimulate MEK and Erk (490). In addition, transient transfection of Cos7 cells with members from all three subclasses of PKC enzymes indicated cPKC and nPKC isoforms can stimulate Raf-1 and hence Erk whilst aPKC enzymes activate MEK/Erk by a Raf-1-independent mechanism (491). Moreover, in erythroid cells Erk can be activated by MEK-dependent and MEK-independent mechanisms and this is necessary for erythropoietin-mediated proliferation. PKC is at least required for MEK-independent stimulation of Erk and PKC is essential for the induction of proliferation (492). PKC can also negatively regulate Erk-MAPK; for example, PKCε induces MKP-1 in lipopolysaccharide-stimulated macrophages leading to the inhibition of Erk1/2 (493). Different isoforms of PKC can thus positively or negatively regulate the Erk-MAPK cascade at multiple stages depending on the particular cellular system.

6.2 Protein kinase C in B lymphocytes

Immature B cells have diminished PKC signalling compared to mature B lymphocytes suggesting PKC may contribute to the differential response of distinct developmental stages of B cells to ligation of the BCR. For example,

ligation of the BCR on mature B cells leads to PIP_2 hydrolysis, Ca^{2+} mobilisation and stimulation of PKC. In contrast, ligation of the BCR on immature B cells can mobilise calcium but PIP_2 hydrolysis and subsequent PKC activation are significantly reduced.

Furthermore, PKCβ has been implicated as a mediator of mature B cell activation since $PKC\beta^{-/-}$ mice have reduced humoral immune responses and their B lymphocytes have impaired activation downstream of the BCR (210). More specifically, mature B lymphocytes from $PKC\beta^{-/-}$ mice are less able to activate Bcl-x_L and Bcl-2 and hence are prone to apoptosis (199, 290). The expression of pro-survival Bcl-2 family members is also an important survival signal in immature B cells (290-293, 317, 427) therefore the impaired activation of PKC in BCR-stimulated immature B cells could increase their sensitivity to apoptosis. Indeed, treatment of primary splenic immature B cells or WEHI-231 cells with phorbol ester can prevent BCR-driven apoptosis suggesting the diminished BCR-coupled activation of cPKC and nPKC isoforms in immature B cells contributes to their cell death (294, 295).

Moreover, PKC further promotes survival of mature B cells by inducing NF-κB since NF-κB can regulate the transcription of c-Myc, Bcl-x_L and Bcl-2 (193, 199, 211). In contrast, ligation of the BCR on immature B cells cannot sustain NF-κB activation, perhaps due to the defective stimulation of PKC. This is likely to contribute to cell death since NF-κB signalling is enhanced under conditions that favour cell survival, including CD40 co-stimulation (261, 296, 297).

Previous studies have shown that the BCR and CD40 on immature B cells can regulate the expression of multiple PKC isoforms. In particular, PKCα, PKCδ and PKCζ were highlighted as potential mediators of CD40-dependent rescue from anti-Ig-induced growth arrest. Furthermore, untreated WEHI-231 cells appeared to express multiple isoforms of PKC including, PKCα, PKCδ, PKCε and PKCζ (unpublished observations). Consequently, these four PKC isoforms were selected for further analysis.

6.3 PKCα

PKCα is an 82 kD cPKC isoform therefore it has a C1 domain that contains two zinc-finger domains to allow binding to DAG and phorbol ester. PKCα is activated synergistically by DAG and Ca^{2+} and hence can be stimulated downstream of PLCγ. Calcium binds to the C2 domain of PKCα since this is rich in aspartate residues. The activation of PKCα requires it to translocate to the membrane and this is enabled by PKCα binding to phosphatidylserine in a calcium-dependent manner (204).

PKCα is further regulated by phosphorylation of several residues. For example, Thr^{497} is located in the activation loop of PKCα and this residue must be phosphorylated for enzymic activity. Thr^{638} can also be phosphorylated although this is not directly required for activation of the enzyme but lack of phosphorylation causes PKCα to be hypersensitive to protein phosphatases and hence dephosphorylation and inhibition. Phosphorylation of Thr^{638} therefore can regulate the duration of PKCα activation (485). Similarly, the phosphorylation of Ser^{657} can protect PKCα from phosphatases and hence this residue also affects the duration of PKCα activation (494).

The activation of PKCα can induce apoptosis of cells by inhibiting Akt. For example, in LNCaP prostate cancer cells the induction of PKCα leads to the activation of PP2A, which can dephosphorylate and inhibit Akt (495). PKCα can also mediate its effects via the activation of Ras. For example, in mast cells, ligation of FcεRI leads to the induction of Syk, which can phosphorylate Tyr^{658} of PKCα and Tyr^{662} of PKCβ1. The resultant phospho-tyrosine residues can interact with the SH2 domain of Grb-2 and hence promote the formation of the Grb-2/SOS complex, leading to the induction of Ras and downstream effectors including Erk-MAPK (496). The activation of PKCα therefore leads to cell-type-specific effects.

6.4 PKCδ

PKCδ is a 78 kD nPKC isoform that is widely distributed among cells and tissues. The C-terminal half of PKCδ contains the catalytic domain and the regulatory region is in the N-terminal half. PKCδ has a C1 domain for DAG-

binding therefore it can be stimulated downstream of PLCγ. However, PKCδ lacks a classical C2 domain therefore it is regulated independently of Ca^{2+}. Nonetheless, the C2-like domain of nPKC enzymes can still bind to RACKs and this has important targeting functions. (204).

The activation of PKCδ is also promoted by binding to PIP_2 or PIP_3. Furthermore, PKCδ can be regulated by phosphorylation of Ser^{505} in the activation loop, Ser^{643} in the turn motif and Ser^{662} in the hydrophobic motif (497). Phosphorylation of Ser^{505} can be catalysed by PDK-1 and it is not necessary for activation of PKCδ but it functions to stabilise the enzyme. Ser^{643} of PKCδ can be autophosphorylated whilst Ser^{662} is phosphorylated by an upstream kinase. PKCζ is a component of the signalling cascade leading to the phosphorylation of Ser^{662} of PKCδ but it is not clear if PKCδ is a direct substrate of PKCζ. PKCδ can also be phosphorylated at tyrosine residues and this can be catalysed by various kinases including Src, Lyn, Fyn, Abl, PYK2, Lck and growth factor receptors. Tyrosine phosphorylation of PKCδ can increase its activity but DAG is required for full activation (497).

PKCδ can also be stimulated by caspase-dependent cleavage resulting in a free, active kinase domain. This is particularly important for promoting apoptosis (487). For example, in LNCaP prostate cancer cells apoptosis is mediated by a biphasic production of ceramide. Sustained ceramide production enables PKCδ-activation and translocation to the mitochondria where it can induce the release of cytochrome c resulting in the stimulation of caspase 9 (498, 499). In this system PKCδ also promotes apoptosis via the induction of p38 MAPK (495).

PKCδ has also been implicated as a negative regulator of B lymphocytes since $PKC\delta^{-/-}$ mice have enhanced mature B cell proliferation, elevated antibody production and are prone to autoimmunity. PKCδ therefore appears to suppress proliferation of mature B cells and may also assist negative selection of immature B cells (199, 497).

6.5 PKCε

PKCε is a 90 kD nPKC isoform that is regulated in a similar way to PKCδ. PKCε has a C1 domain that binds to DAG and other lipids but lacks a classical

C2 domain and hence is insensitive to calcium but can still interact with RACKs. The activation of PKCε is promoted by binding to PIP_2 and PIP_3. PKCε is also regulated by phosphorylation of Thr^{566} in the activation loop, Ser^{710} in the turn motif and Ser^{728} in the hydrophobic motif. Ser^{710} can be autophosphorylated. In addition, PKCε can be stimulated by proteolysis catalysed by caspase 3, caspase 7 or calpains. However, one unique feature of PKCε is it has an actin-binding motif and the binding of PKCε to actin is promoted by DAG and arachidonic acid. This has two effects: firstly, it anchors PKCε to a particular subcellular location and secondly, it can maintain PKCε in a catalytically active conformation (206).

PKCε is expressed in many different cells and tissues but is especially abundant in cells of the immune system and neuronal cells. For example, PKCε is required in macrophages therefore mice that lack PKCε have severely impaired macrophage survival and function resulting in an increased susceptibility to bacterial infection. A possible role for PKCε in promoting cell survival was suggested by the observation that PKCε can activate NF-κB (206).

PKCε is also a regulator of the Erk-MAPK cascade. For example, PKCε stimulates the MEK/Erk cascade in thrombin-stimulated Jurkat T cells (206). Furthermore, in cholecystokinin-stimulated pancreatic cells PLCγ-mediated activation of PKCε can enhance the Ras/Raf-1-mediated induction of Erk (500). Moreover, PKCε can mediate the activation of Erk by PI-3-K and this can promote mitogenesis of SW480 colon carcinoma cells (501). In addition, PKCε has Rap-1 guanine nucleotide exchange factor activity therefore it can stimulate Rap-1 to activate or inhibit Erk, depending on the cellular levels of B-Raf (502). PKCε has also been observed to negatively regulate Erk via the induction of MAPK phosphatases for example, in lipopolysaccharide-stimulated macrophages PKCε can suppress Erk by stimulating MKP-1 (493).

6.6 PKCζ

PKCζ is a 72 kD aPKC isoform that is activated independently of calcium and DAG. Alternative lipids mediate the translocation of PKCζ to membranes including phosphatidylinositol lipids such as PIP_3. PKCζ can thus be activated downstream of PI-3-K. PKCζ can also be targeted to specific subcellular

compartments via the binding of its C2 domain to RACKs. PKCζ is further regulated by phosphorylation but as with other aPKCs, PKCζ has only two sites of ser/thr phosphorylation since a glutamate residue replaces the phosphorylation site in the C-terminal hydrophobic motif (204).

In vascular smooth muscle cells PKCζ is activated downstream of ceramide leading to a direct inhibition of Akt and a suppression of proliferation (503). In contrast, PKCζ promotes cell survival in danorubicin-stimulated U937 cells since it mediates PI-3-K-dependent activation of Raf-1 and MEK/Erk (504). PI-3-K-dependent activation of PKCζ also increases Erk activity in lysophosphatidic acid-treated CHO cells (505). PKCζ can thus mediate PI-3-K-dependent activation of Erk in multiple systems suggesting it may also couple PI-3-K to Erk downstream of the BCR. Indeed, splenic B lymphocytes of PKCζ$^{-/-}$ mice have impaired BCR signalling including decreased activation of Erk and NF-κB (506). Mature B lymphocytes from PKCζ$^{-/-}$ mice therefore undergo spontaneous apoptosis and have defective activation, including reduced proliferation and impaired ability to produce antibody (199). PKCζ thus seems to have an important role in promoting survival and activation of mature B cells. Furthermore, the number of immature B cells in PKCζ$^{-/-}$ mice is greater than normal mice suggesting PKCζ may also be required for negative selection of immature B cells (506).

6.7 Aims and objectives

Several PKC isoforms have been associated with promoting survival and proliferation of mature B cells since they can induce pro-survival Bcl-2 family proteins and NF-κB (193, 199, 210, 211, 290). Immature B cells display impaired PKC signalling compared to mature B cells but treatment of primary splenic immature B cells or WEHI-231 cells with phorbol ester can prevent BCR-driven apoptosis suggesting the diminished activation of cPKC and nPKC isoforms in immature B cells contributes to their cell death (294, 295). Indeed, previous studies have shown that the BCR and CD40 on immature B cells can regulate the expression of multiple PKC isoforms. In particular, PKCα, PKCδ and PKCζ were highlighted as potential mediators of CD40-dependent rescue from anti-Ig-induced growth arrest (unpublished observations).

The aims of this investigation were:

1. to investigate the role of PKCα, PKCδ, PKCε and PKCζ in regulating the survival and proliferation of WEHI-231 cells

2. to explore the role of these PKC isoforms in regulating the activity of Erk-MAPK in WEHI-231 cells since PKC can regulate the activity of Erk in several other cellular systems (501, 504, 505) and, as discussed in previous chapters, Erk-MAPK signalling is important for determining the survival and proliferation of WEHI-231 cells

3. to examine the role of PKCα, PKCδ, PKCε and PKCζ in regulating the induction of the pro-survival proteins Bcl-x_L and Akt in WEHI-231 cells.

6.8 Results and Discussion

6.8.1 PKCα partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis

Previous studies using antisense oligonucleotides to PKCα showed that PKCα may help to promote proliferation of WEHI-231 cells (unpublished observations). To further investigate the role of PKCα in WEHI-231 cell signalling, WEHI-231 cells were transfected by electroporation with the pcDNA3.1 plasmid containing a constitutively active form of PKCα (PKCαCAT WEHI-231), a kinase inactive form of PKCα (PKCαKR WEHI-231) or an empty plasmid as control (pcDNA3.1 WEHI-231). The PKCαCAT construct encoded residues 326-674, which encompasses the kinase region of PKCα. The PKCαKR construct encoded most of PKCα (residues 2-672) but the kinase was inactivated via a point mutation, $K^{368}R$. Figure 6.1B illustrates the general structure of PKC-CAT and PKC-KR forms of PKC.

To determine the role of PKCα in regulating proliferation of WEHI-231 cells the DNA synthesis of cells transfected with PKCαCAT and PKCαKR was determined by measuring the incorporation of [^{3}H] thymidine. After 24 hr, anti-Ig induced growth arrest of pcDNA3.1 WEHI-231 cells in a dose-dependent manner (figure 6.2A). This is consistent with the observed response of wild-type WEHI-231 cells (figure 3.5) and shows that the empty pcDNA3.1 vector did not impair BCR-driven growth arrest of WEHI-231 cells. In contrast to pcDNA3.1 WEHI-231 cells, PKCαCAT WEHI-231 cells were protected from BCR-dependent inhibition of DNA synthesis after 24 hr (figure 6.2A). This suggests that PKCα prevents growth arrest and/or induces proliferation in WEHI-231 cells. This is consistent with earlier studies that implicated PKCα as a mediator of CD40-dependent rescue from anti-Ig-induced growth arrest (unpublished observations). However, expression of PKCαKR also protected WEHI-231 cells from anti-Ig-induced inhibition of DNA synthesis after 24 hr (figure 6.2A). This observation was unexpected and suggests that PKCα impairs BCR-driven growth arrest but PKCα catalytic activity does not appear to be essential.

After 48-72 hr, expression of PKCαCAT or PKCαKR in WEHI-231 cells provided partial protection from BCR-dependent inhibition of DNA synthesis

(figure 6.2B). Furthermore, expression of either PKCαCAT or PKCαKR may also slightly enhance CD40-mediated rescue from BCR-driven growth arrest after 48 hr (figure 6.2C). Taken together, these observations suggest that PKCα may have a role in promoting proliferation and/or in preventing growth arrest of WEHI-231 cells but the catalytic activity of PKCα does not seem to be essential for these effects.

To further investigate the role of PKCα in regulating proliferation of WEHI-231 cells, the cell cycle and apoptotic status of PKCαCAT and PKCαKR WEHI-231 cells was analysed by PI-staining and FACS analysis. The proliferation of WEHI-231 cells was measured by determining the number of cells in S phase plus G2/M since ligation of the BCR induces growth arrest at G0/G1 phase of the cell cycle and hence depletes the populations of S phase and G2/M phase cells (figure 3.6). After 24 hr and 48 hr, treatment of pcDNA3.1 WEHI-231 cells with anti-Ig increased the number of cells in G0/G1 phase but reduced the number of cells in S phase and G2/M whereas co-culture with anti-CD40 replenished these populations of cells (figure 6.2D-E and data not shown). Expression of PKCαCAT and PKCαKR did not have a dramatic effect on the number of cells in S phase and G2/M (figure 6.2D-E). Nonetheless, expression of PKCαCAT did partially protect WEHI-231 cells from BCR-dependent cell cycle arrest. For example, after 48 hr, treatment of pcDNA3.1 WEHI-231 cells with 10 μg/ml anti-Ig reduced the percentage of S phase plus G2/M phase cells from 41% to 17% and co-culture with 10 μg/ml anti-CD40 restored this population of cells to 41%. However, treatment of PKCαCAT WEHI-231 cells with 10 μg/ml anti-Ig only reduced the percentage of S phase plus G2/M phase cells to 24% and co-culture with anti-CD40 restored this population of cells to 40% (figure 6.2E). Similarly, expression of PKCαKR impaired BCR-driven growth arrest of WEHI-231 cells. For example, after 24 hr, 10 μg/ml anti-Ig reduced the percentage of pcDNA3.1 cells in S phase plus G2/M phase from 31% to 23% and co-culture with anti-CD40 restored this population of cells to 39%. However, 31% of PKCαKR WEHI-231 cells treated with 10 μg/ml anti-Ig were in S phase plus G2/M and co-ligation of CD40 increased this percentage to 46% (figure 6.2D). These observations therefore support the thymidine assay data, which suggested that PKCα can promote proliferation of WEHI-231 cells but this does not require the kinase to be active.

In addition, cell cycle analysis of PKCαCAT and PKCαKR WEHI-231 cells indicated that expression of both of these mutant forms of PKCα also slightly impaired anti-Ig-induced apoptosis after 24 hr and 48 hr (figure 6.2F-G). Moreover, expression of PKCαKR was slightly more effective than expression of PKCαCAT at preventing anti-Ig-induced apoptosis, indicating PKCα kinase activity was not essential for increasing the survival of WEHI-231 cells. Furthermore, expression of PKCαCAT or PKCαKR also slightly reduced the number of cells with sub-diploid DNA that had been cultured with anti-Ig plus anti-CD40 indicating PKCα may also improve CD40-mediated survival. PKCα therefore appears to increase the survival and the proliferation of BCR-stimulated and anti-CD40-treated WEHI-231 cells and PKCα kinase activity is not essential for these effects.

This is not an isolated example of PKCα signalling that is independent of kinase activity. 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 (507) indicating PKCα can perform important signalling functions independently of its kinase activity.

6.8.2 Expression of PKCαCAT increases sustained Erk signalling in WEHI-231 cells

To investigate the mechanism of PKCα-mediated enhancement of proliferation in WEHI-231 cells the effect of PKCα on Erk signalling was explored since the induction of sustained and cyclic Erk signals is important for proliferation of WEHI-231 cells. Furthermore, many PKC isoforms including PKCα can regulate the activity of Erk1/2 (see sections 6.1 and 6.3). The activity of Erk in PKCαCAT and PKCαKR WEHI-231 cells was determined by Western blotting using antibodies that recognise dual-phosphorylated Erk1/2.

Unstimulated pcDNA3.1 WEHI-231 cells displayed cycling Erk signals over a 48 hr time period (figure 6.3A). However, ligation of the BCR suppressed

sustained Erk signals whilst co-ligation of CD40 restored prolonged Erk signals (figure 6.3A). This is consistent with previous observations of Erk signalling in wild-type WEHI-231 cells (appendix 2). A similar pattern of Erk signalling was observed in PKCαKR WEHI-231 cells (figure 6.3B) indicating expression of PKCαKR did not increase the survival and proliferation of WEHI-231 cells by regulating the activity of Erk.

In contrast, expression of PKCαCAT did alter sustained Erk signals in WEHI-231 cells (figure 6.3C). In particular, ligation of the BCR on PKCαCAT WEHI-231 cells prevented anti-Ig-induced inhibition of sustained (48 hr) Erk signals. This is likely to contribute to the increased proliferation of PKCαCAT WEHI-231 cells. However, the mechanism of PKCαCAT-mediated induction of Erk1/2 has not been investigated. Nonetheless, in FcεRI-stimulated mast cells PKCα can induce Grb-2/SOS and hence Ras (496) therefore PKCαCAT may also regulate Erk in WEHI-231 cells by increasing the activation of Ras. However, expression of constitutively active Ras in BCR-stimulated WEHI-231 cells did not substantially increase sustained Erk signals (figure 3.11) suggesting PKCα may activate Erk via Ras-independent mechanisms such as inhibiting MAPK phosphatase activity.

Nonetheless, these observations indicate PKCαCAT and PKCαKR can increase the proliferation of WEHI-231 cells but they appear to do so via distinct mechanisms. Indeed, expression of PKCαCAT impairs BCR-mediated inhibition of sustained Erk signalling whilst expression of PKCαKR did not alter Erk signalling in WEHI-231 cells. The mechanism used by PKCαKR to increase the proliferation of WEHI-231 cells has not been determined but it may act downstream or in parallel to Erk and hence diminish the need for sustained Erk signals in proliferating cells.

6.8.3 Expression of PKCα can induce Bcl-x_L in WEHI-231 cells

To further explore the mechanism of PKCα-mediated survival and proliferation of WEHI-231 cells, the levels of Bcl-x_L in cells expressing PKCαKR or PKCαCAT were determined since, as discussed in chapter 4, Bcl-x_L is an important survival factor of WEHI-231 cells. Moreover, cPKCs have previously been associated with inducing Bcl-x_L in mature B lymphocytes since peripheral

B cells from PKCβ$^{-/-}$ mice are less able to activate Bcl-x_L and Bcl-2 (199, 290). Levels of Bcl-x_L in WEHI-231 cells were determined by Western blotting using anti-Bcl-$x_{L/S}$ antibodies.

Expression of PKCαKR or PKCαCAT enhanced the levels of Bcl-x_L in CD40-stimulated WEHI-231 cells (figure 6.4A-C). This is consistent with figure 6.2F-G in which CD40-stimulated PKCαKR and PKCαCAT WEHI-231 cells had a slightly lower sub-diploid DNA content than the equivalent pcDNA3.1 WEHI-231 cells. However, expression of PKCαCAT also enhanced the levels of Bcl-x_L in untreated and BCR-stimulated WEHI-231 cells (figure 6.4C) indicating PKCα can induce Bcl-x_L in the absence of CD40 stimulation. Furthermore, it is likely that this requires PKCα kinase activity since PKCαKR WEHI-231 cells did not display increased levels of Bcl-x_L in cells that had not been stimulated with CD40. This suggests that CD40 may enhance PKCα kinase activity to suppress BCR-driven apoptosis or the BCR may inhibit this kinase to enhance apoptosis of WEHI-231 cells.

However, expression of PKCαKR also increased the survival of untreated and BCR-stimulated WEHI-231 cells (figure 6.2F-G) suggesting PKCαKR can initiate additional pro-survival signals. The activity of Akt was thus investigated in PKCαKR and PKCαCAT WEHI-231 cells since Akt is an important survival factor of many cell types including mature splenic B lymphocytes (see section 1.6.3.1) (184, 186, 188, 189, 193-195). The activity of Akt was determined by Western blotting using phosphorylation-sensitive antibodies that specifically recognise Akt that is phosphorylated at Thr^{308} (pTAkt) or Ser^{473} (pSAkt). Positive controls for phosphorylated and non-phosphorylated Akt (purchased from New England Biolabs) were included in all the Western blots.

In general the activity of Akt was very low in WEHI-231 cells and pTAkt and pSAkt were not detected in unstimulated or anti-Ig-treated pcDNA3.1, PKCαKR or PKCαCAT WEHI-231 cells (figure 6.4D-F). This is consistent with the results of figures 3.13, 3.15 and 3.19 in which Akt was barely detectable in WEHI-231 cells even when they were expressing a constitutively active form of Ras that specifically couples to PI-3-K (figure 3.13D). However, expression of PKCαCAT or PKCαKR in cells treated with anti-Ig plus anti-CD40 did slightly increase the levels of pTAkt and pSAkt indicating PKCα might induce Akt in BCR and CD40 co-stimulated WEHI-231 cells (figure 6.4E-F). Furthermore, this

does not appear to require PKCα kinase activity since it was observed in cells expressing constitutively active and inactive forms of PKCα. Potentially the induction of Akt could increase the survival of WEHI-231 cells but it is not clear whether or not PKCα-mediated induction of Akt exists in wild-type WEHI-231 cells since this was only observed in cells that were constitutively expressing PKCα.

Taken together, these findings indicate there may be two distinct mechanisms of PKCα signalling: one that requires kinase activity and one that functions independently of active kinase. In WEHI-231 cells either mechanism of PKCα signalling appears to promote survival and proliferation of the cells but the signalling pathways that are regulated by PKCα depend on whether or not the kinase is active. In particular, PKCα kinase activity can induce Bcl-x_L in the presence or absence of anti-Ig and/or anti-CD40 whereas PKCα that lacks catalytic activity can only induce Bcl-x_L in CD40-stimulated cells. Additional PKCαKR-dependent survival pathways are thus likely to exist. Furthermore, PKCα kinase activity can enhance sustained Erk signals presumably to increase proliferation of WEHI-231 cells and impair BCR-driven growth arrest. However, expression of PKCαKR cannot enrich sustained Erk signals and hence must use alternative, as yet unidentified, signals to increase the proliferation of WEHI-231 cells.

6.8.4 PKCδ partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis

To further explore the use of PKC in WEHI-231 signalling, the role of the nPKC isoform PKCδ was investigated using WEHI-231 cells transfected by electroporation with the pcDNA3.1 plasmid containing a constitutively active form of PKCδ (PKCδCAT WEHI-231), a kinase inactive form of PKCδ (PKCδKR WEHI-231) or an empty plasmid as control (pcDNA3.1 WEHI-231). The PKCδCAT construct encoded residues 334-674, which encompasses the kinase region of PKCδ. The PKCδKR construct encoded most of PKCδ (residues 2-674) but the kinase was inactivated via a point mutation, $K^{376}R$.

The role of PKCδ in regulating the proliferation of WEHI-231 cells was examined by measuring the DNA synthesis of cells transfected with PKCδ

constructs. The level of DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine. After 24 hr, anti-Ig decreased the level of DNA synthesis in pcDNA3.1 WEHI-231 cells in a dose-dependent manner (figure 6.5A). However, expression of PKCδCAT prevented BCR-driven inhibition of DNA synthesis (figure 6.5A) suggesting PKCδ can protect WEHI-231 cells from BCR-driven growth arrest. This is consistent with previous antisense observations that implicated PKCδ as a mediator of CD40-dependent rescue from anti-Ig-induced growth arrest (unpublished observations). Furthermore, expression of PKCδKR also inhibited BCR-driven growth arrest and it appeared to slightly increase the amount proliferation above basal levels (figure 6.5A). These observations suggest that after 24 hr PKCδ can protect WEHI-231 cells from BCR-driven growth arrest but PKCδ kinase activity does not seem to be essential.

After 48-72 hr, expression of PKCδCAT did not prevent BCR-driven growth arrest and expression of PKCδKR only slightly impaired anti-Ig-induced growth arrest of WEHI-231 cells (figure 6.5B). This suggests that expression of PKCδ was insufficient to prevent growth arrest of WEHI-231 cells induced by prolonged exposure to anti-Ig. However, cell cycle analysis of PKCδCAT and PKCδKR WEHI-231 cells showed that expression of either of these constructs slightly impaired anti-Ig-induced depletion of S phase plus G2/M phase cells after 24 hr and 48 hr (figure 6.5D-E). Taken together, these observations implicate PKCδ as a mitogenic signalling molecule in WEHI-231 cells that can impair BCR-driven growth arrest. However, expression of PKCδCAT or PKCδKR only had slight effects on the proliferation of CD40-stimulated WEHI-231 cells (figure 6.5C-E).

Expression of PKCδKR did not affect the survival of unstimulated WEHI-231 cells or cells treated with anti-Ig and/or anti-CD40. Nonetheless, expression of PKCδCAT slightly reduced the number of cells with sub-diploid DNA (figure 6.5F-G). This suggests that PKCδ may improve the survival of WEHI-231 cells but this is likely to require PKCδ kinase activity. This observation is different to that of WEHI-231 cells expressing PKCα constructs since PKCα kinase activity was not essential for improving survival of WEHI-231 cells and this shows that different isoforms of PKC have distinct roles in WEHI-231 cell signalling.

Taken together, these observations suggest that PKCδ can promote the survival and proliferation of WEHI-231 cells. However, analysis of PKCδ$^{-/-}$ mice implicated PKCδ as a negative regulator of mature B cell proliferation (199, 497). Nonetheless, this is consistent with the observation that the BCRs of mature and immature B cells are generally coupled to the same signalling pathways despite having very different effects on the phenotype of the cell. It is possible that the amplitude, duration or intracellular location of PKCδ is dependent on the developmental stage of the cell.

6.8.5 Expression of PKCδCAT increases sustained Erk signalling in WEHI-231 cells

To investigate the mechanism of PKCδ-mediated enhancement of proliferation of WEHI-231 cells, the activity of Erk in PKCδCAT and PKCδKR WEHI-231 cells was determined by Western blotting with antibodies that specifically recognise dual-phosphorylated Erk1/2. In general, expression of PKCδCAT or PKCδKR slightly enhanced Erk signalling in cells treated with anti-Ig and/or anti-CD40 (figure 6.6), suggesting that expression of PKCδ favoured the activation of Erk. However, expression of PKCδKR was not able to prevent BCR-mediated inhibition of sustained Erk signals (figure 6.6B). PKCδKR therefore did not impair BCR-driven growth arrest by enhancing sustained Erk signals. In contrast, expression of PKCδCAT did enhance sustained Erk signals in anti-Ig-treated WEHI-231 cells (figure 6.6C) suggesting PKCδ can impair BCR-mediated inhibition of sustained Erk signals but this requires PKCδ kinase activity. PKCδCAT therefore is likely to increase the proliferation of WEHI-231 cells by enhancing sustained Erk1/2 signals. Furthermore, these observations suggest that the BCR may inhibit PKCδ kinase activity to suppress sustained Erk signals or CD40 might induce this kinase to enhance Erk signalling and hence regulate the proliferation of WEHI-231 cells.

However, in anti-CD40-treated WEHI-231 cells expression of either PKCδKR or PKCδCAT increased sustained Erk signals (figure 6.6) suggesting PKCδ can enrich Erk signals independently of PKCδ kinase activity in CD40-activated cells. Furthermore, expression of PKCδKR but not PKCδCAT increased sustained Erk signals in cells treated with anti-Ig plus anti-CD40. This

shows that PKCδ can stimulate sustained Erk signals independently of PKCδ kinase activity but only in CD40-activated WEHI-231 cells. Furthermore, the regulatory regions of PKCδ appear to be specifically required for the induction of sustained Erk signals in WEHI-231 cells treated with anti-Ig plus anti-CD40.

6.8.6 Expression of PKCδ can induce Bcl-x_L but cannot increase the activity of Akt

To investigate the mechanism of PKCδ-dependent survival, the activity of Akt was determined by Western blotting with phosphorylation-sensitive Akt antibodies. As with previous experiments, very low levels of pTAkt and pSAkt were detected in WEHI-231 cells. Furthermore, the activity of Akt was relatively unaffected by expression of PKCδKR or PKCδCAT suggesting PKCδ does not directly affect Akt activity in WEHI-231 cells (figure 6.7A-C). The induction of Akt therefore is unlikely to be responsible for the improved survival of PKCδCAT WEHI-231 cells.

To further investigate the signalling mechanism of PKCδ-mediated survival of WEHI-231 cells, the levels of Bcl-x_L were determined by Western blotting of lysates prepared from PKCδCAT and PKCδKR WEHI-231 cells (figure 6.7D-F). Expression of PKCδKR induced a slight increase in the levels of Bcl-x_L in untreated WEHI-231 cells but did not induce Bcl-x_L in anti-Ig-treated cells (figure 6.7E). This is consistent with the observations of figure 6.5F-G in which expression of PKCδKR was shown to be insufficient to increase the survival of WEHI-231 cells. In contrast, expression of PKCδCAT did slightly increase the survival of WEHI-231 cells. Furthermore, expression of PKCδCAT increased the expression of Bcl-x_L, especially in anti-Ig-treated WEHI-231 cells (figure 6.7F). This suggests that PKCδ can increase the survival of WEHI-231 cells, even in the presence of anti-Ig, via the induction of Bcl-x_L. Moreover, this requires PKCδ kinase activity although the substrates of PKCδ that are involved in this process have not been identified. These observations implicate PKCδ as an upstream mediator of Bcl-x_L signalling in WEHI-231 cells and suggest that ligation of the BCR might suppress this pro-survival signal by inhibiting PKCδ kinase activity.

6.8.7 PKCε partially protects WEHI-231 cells from BCR-driven growth arrest

To further investigate the use of PKC in WEHI-231 cell signalling, the role of the nPKC isoform PKCε was investigated using WEHI-231 cells transfected by electroporation with the pcDNA3.1 plasmid containing a constitutively active form of PKCε (PKCεCAT WEHI-231), a kinase inactive form of PKCε (PKCεKR WEHI-231) or an empty plasmid as control (pcDNA3.1 WEHI-231). The PKCεCAT construct encoded residues 395-737, which encompasses the kinase region of PKCε. The PKCεKR construct encoded most of PKCε (residues 2-732) but the kinase was inactivated via a point mutation, $K^{437}R$.

The role of PKCε in regulating proliferation of WEHI-231 cells was investigated by measuring the DNA synthesis and analysing the cell cycle of PKCεCAT and PKCεKR WEHI-231 cells. After 24 hr, expression of PKCεCAT prevented anti-Ig-dependent inhibition of DNA synthesis and actually induced proliferation of BCR-stimulated cells (figure 6.8A). Furthermore, expression of PKCεKR also prevented BCR-driven inhibition of DNA synthesis but it did not increase proliferation above basal levels. These observations show that PKCε can block BCR-driven growth arrest pathways and/or induce proliferative signals in WEHI-231 cells. This suggests that PKCε may be inhibited by the BCR to regulate proliferation of WEHI-231 cells. Furthermore, the kinase activity of PKCε is not essential for these effects but the presence of active kinase enables PKCε to induce proliferation more effectively.

However, after 48-72 hr, expression of PKCεCAT or PKCεKR was no longer able to protect WEHI-231 cells from anti-Ig-induced inhibition of DNA synthesis (figure 6.8B). The protective effects of PKCε can thus be overcome by prolonged exposure to anti-Ig. Furthermore, cell cycle analysis of PKCεCAT and PKCεKR WEHI-231 cells (figure 6.8D-E) showed that expression PKCε slightly increased the percentage of anti-Ig-treated cells in S phase and G2/M after 24 hr. However, after 48 hr protection was virtually abrogated. Moreover, in general, expression of PKCεCAT or PKCεKR did not greatly affect CD40-mediated rescue of WEHI-231 cells from BCR-driven growth arrest (figure 6.8C-E).

6.8.8 PKCε can induce Erk in BCR-stimulated WEHI-231 cells

To investigate the mechanism of PKCε-dependent rescue from BCR-driven growth arrest, the activity of Erk in PKCεCAT and PKCεKR WEHI-231 cells was determined by Western blotting. Expression of PKCεCAT, but not PKCεKR, in untreated WEHI-231 cells slightly increased the levels of phospho-Erk between 24 hr and 48 hr (figure 6.9) suggesting PKCε kinase activity may induce Erk in unstimulated cells. Furthermore, the expression of PKCεCAT or PKCεKR induced a general increase in Erk activity in CD40-stimulated WEHI-231 cells (figure 6.9).

Moreover, expression of either PKCεCAT or PKCεKR slightly increased the activity of Erk at 48 hr in anti-Ig-treated WEHI-231 cells (figure 6.9B-C). The presence of this signal has previously been associated with induction of proliferation in WEHI-231 cells (328) therefore the ability of PKCε to increase this signal is likely to contribute to PKCε-dependent protection from BCR-driven growth arrest. Furthermore, the observation that both PKCεCAT and PKCεKR can induce sustained Erk signals in BCR-activated WEHI-231 cells suggests that PKCε kinase activity was not essential. These observations are consistent with the thymidine assay data (figure 6.8A-C), which implicated PKCε as a mitogenic signal of WEHI-231 cells and suggests that the BCR may impair proliferation of WEHI-231 cells by inhibiting PKCε signalling.

PKCε can thus slightly increase sustained Erk signals in BCR-activated WEHI-231 cells presumably resulting in enhanced proliferation. The mechanism of PKCεCAT- or PKCεKR-mediated induction of Erk has not been explored. However, PKCε has been shown to regulate the activity of Erk via several different mechanisms in other cellular systems (see section 6.5). Of particular interest, PKCε has been shown to enhance the Ras/Raf-1-mediated induction of Erk and to couple PI-3-K to Erk (500, 501). Data presented in chapter 3 suggests that Ras is an important upstream activator of Erk in WEHI-231 cells and it can do so via Raf-1 and PI-3-K (see section 3.12.11) therefore PKCε may help to amplify Ras-dependent activation of Erk. To further investigate the role of PKCε in mediating Ras-dependent activation of Erk, WEHI-231 cells could be generated with mutations in both PKCε and Ras. Furthermore, BCR-dependent inhibition of sustained Erk signals can be mediated by the induction of MAPK

phosphatases and PKCε has been associated with regulating MAPK phosphatases in other systems. For example, in lipopolysaccharide-stimulated macrophages PKCε can induce MKP-1 to suppress Erk (493) suggesting PKCε may also regulate MAPK phosphatases in other systems, possibly even in a negative manner.

6.8.9 PKCε cannot induce Bcl-x_L or Akt and does not protect WEHI-231 cells from BCR-driven apoptosis

PKCε therefore can impair anti-Ig-induced growth arrest of WEHI-231 cells and this does not absolutely require PKCε kinase activity. In contrast, expression of PKCεCAT or PKCεKR did not appear to protect WEHI-231 cells from BCR-driven apoptosis since the number of cells with sub-diploid DNA was not reduced by expression of PKCε (figure 6.10A-B). These observations implicate PKCε as specifically affecting the proliferation of WEHI-231 cells.

Furthermore, analysis of the levels of Bcl-x_L in WEHI-231 cells showed that expression of PKCεCAT or PKCεKR did not substantially enhance the levels of Bcl-x_L in anti-Ig-treated WEHI-231 cells (figure 6.10C-E). Moreover, expression of PKCεCAT did not increase the activity of Akt, as indicated by Western blotting for pTAkt and pSAkt (figure 6.11A and C). However, the levels of pTAkt and pSAkt were slightly elevated by expression of PKCεKR in untreated and anti-Ig-treated WEHI-231 cells (figure 6.11B).

Taken together, these observations suggest that PKCε does not substantially modulate Bcl-x_L expression or Akt activity in WEHI-231 cells. Consequently, expression of PKCεCAT or PKCεKR did not increase the survival of WEHI-231 cells and did not prevent BCR-driven apoptosis. PKCε therefore appears to specifically regulate proliferation rather than survival of WEHI-231 cells. This is in contrast to the observed role of PKCδ in WEHI-231 cells despite PKCε and PKCδ both being nPKC isoforms and hence being sensitive to the same upstream activators. The functions of PKCε and PKCδ in WEHI-231 cells are thus likely to be distinct, which is consistent with analysis of these PKC isoforms in other systems. For example, in the androgen-dependent LNCaP cell line PKCδ and PKCα both promote apoptosis whereas PKCε can suppress apoptosis and induce proliferation (495). Different isoforms of the same

subclass of PKC enzymes can thus mediate distinct functions in several systems including WEHI-231 cells. One explanation for this is PKCδ and PKCε may have different intracellular locations in WEHI-231 cells since PKC enzymes can reside in particular intracellular locations via their interaction with RACKs. Furthermore, PKCε has an actin-binding motif that provides a unique anchoring point for this enzyme and the association of PKCε to actin is enhanced by arachidonic acid (206) therefore the production of arachidonic acid in WEHI-231 cells may influence the intracellular location of PKCε but not other PKC isoforms. However, the intracellular location of PKC enzymes has yet to be examined in WEHI-231 cells.

6.8.10 PKCζ partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis

To further explore the use of PKC in WEHI-231 signalling, the role of an aPKC isoform, PKCζ was investigated since PKCζ can be activated downstream of PI-3-K and Ras seems to be able to activate Erk via PI-3-K in WEHI-231 cells (section 3.12.11). Furthermore, PKCζ$^{-/-}$ mice have impaired mature B cell survival and activation and have elevated numbers of immature B cells suggesting PKCζ can regulate the survival of mature and immature B cells (199, 506). The role of PKCζ in WEHI-231 cells was explored using WEHI-231 cells transfected by electroporation with the pcDNA3.1 plasmid containing a constitutively active form of PKCζ (PKCζCAT WEHI-231), a kinase inactive form of PKCζ (PKCζKR WEHI-231) or an empty plasmid as control (pcDNA3.1 WEHI-231). The PKCζCAT construct encoded residues 239-592, which encompasses the kinase region of PKCζ. The PKCζKR construct encoded most of PKCζ (residues 2-592) but the kinase was inactivated via a point mutation, $K^{281}M$.

The role of PKCζ in regulating proliferation of WEHI-231 cells was examined by measuring the DNA synthesis of PKCζCAT and PKCζKR WEHI-231 cells and by analysing the cell cycle of these cells. Measurement of the DNA synthesis of WEHI-231 cells after 24 hr showed that expression of PKCζCAT or PKCζKR significantly reduced BCR-driven growth arrest (figure 6.12A). This suggests that PKCζ may be inhibited by the BCR to induce growth

arrest or may be stimulated by CD40 to enable proliferation of WEHI-231 cells. This is consistent with previous observations that implicated PKCζ as a mediator of CD40-dependent rescue from anti-Ig-induced growth arrest (unpublished observation). Moreover, PKCζ kinase activity did not appear to be necessary for preventing growth arrest of anti-Ig-treated WEHI-231 cells. In contrast, expression of PKCζCAT or PKCζKR was insufficient to protect WEHI-231 cells from BCR-driven inhibition of DNA synthesis after 48-72 hr indicating prolonged exposure to anti-Ig overcame the protection offered by PKCζ (figure 6.12B). Furthermore, expression of PKCζ did not appear to affect proliferation of cells treated with anti-Ig plus anti-CD40, suggesting PKCζ did not increase the proliferation of CD40-stimulated WEHI-231 cells (figure 6.12C-E).

Analysis of the cell cycle status of PKCζCAT and PKCζKR WEHI-231 cells suggested PKCζ might promote proliferation of anti-Ig-treated WEHI-231 cells (figure 6.12D-E). Furthermore, expression of PKCζCAT and PKCζKR appeared to be equally effective at promoting proliferation of WEHI-231 cells. Analysis of the proliferation of PKCζCAT and PKCζKR WEHI-231 cells therefore suggests that PKCζ might induce proliferation and/or inhibit BCR-driven growth arrest of WEHI-231 cells and the kinase activity of PKCζ does not seem to be essential for these effects.

In addition, cell cycle analysis of PKCζCAT and PKCζKR WEHI-231 cells also suggested that PKCζ might increase the survival of WEHI-231 cells since expression of either of these PKCζ enzymes slightly reduced the number of anti-Ig-treated WEHI-231 cells with sub-diploid DNA (figure 6.12F-G). Furthermore, expression of PKCζKR was more effective than expression of PKCζCAT at reducing the number of WEHI-231 cells with sub-diploid DNA. This suggests that PKCζ catalytic activity was not essential for PKCζ-mediated reduction in apoptosis of WEHI-231 cells. PKCζ can thus promote survival and proliferation of WEHI-231 cells but the kinase activity of PKCζ is not always essential for these processes.

6.8.11 PKCζ slightly increases the activity of Erk-MAPK in BCR-stimulated WEHI-231 cells

The activation of sustained and cyclic Erk signals is associated with the induction of proliferation in WEHI-231 cells. Furthermore, Ras is an important upstream activator of Erk and it can mediate the activation of Erk via Raf-1 or PI-3-K (discussed in chapter 3). Previous studies have shown that PKCζ can induce the Erk-MAPK cascade. In particular, PKCζ can mediate PI-3-K-dependent activation of Erk1/2. For example, PI-3-K-dependent activation of PKCζ increases Erk activity in lysophosphatidic acid-treated CHO cells and in danorubicin-stimulated U937 cells (504, 505).

To investigate the role of PKCζ in regulating the activity of Erk and hence the mechanism of PKCζ-dependent promotion of proliferation in WEHI-231 cells, the activity of Erk in PKCζCAT and PKCζKR WEHI-231 cells was determined by Western blotting using antibodies to dual-phosphorylated Erk1/2. Expression of either PKCζKR or PKCζCAT slightly increased the levels of dual-phosphorylated Erk at 48 hr in BCR-stimulated WEHI-231 cells (figure 6.13). This is consistent with expression of PKCζCAT and PKCζKR being able to partially protect WEHI-231 cells from BCR-driven growth arrest (figure 6.12). This suggests that PKCζ may promote proliferation of WEHI-231 cells by inducing sustained Erk signals but the kinase activity of PKCζ does not appear to be necessary for these effects.

6.8.12 Expression of PKCζ does not induce Bcl-x_L or Akt in WEHI-231 cells

To investigate the mechanism of PKCζ-dependent increase in survival of WEHI-231 cells, the effect of expression of PKCζCAT or PKCζKR on the levels of Bcl-x_L in WEHI-231 cells was determined since Bcl-x_L is an important survival factor of WEHI-231 cells. In untreated and anti-Ig-stimulated WEHI-231 cells, expression of PKCζCAT or PKCζKR did not affect the levels of Bcl-x_L (figure 6.14A-C), indicating the ability of PKCζ to impair BCR-driven apoptosis (figure 6.12G) was not mediated by enhancing Bcl-x_L levels. Expression of PKCζKR was more effective than expression of PKCζCAT at protecting WEHI-231 cells from BCR-driven apoptosis and yet expression of PKCζKR did not enhance

CD40-mediated induction of Bcl-x_L in WEHI-231 cells (figure 6.14B). PKCζKR therefore does not induce Bcl-x_L indicating PKCζKR increases the survival of WEHI-231 cells by acting downstream of Bcl-x_L or independently of Bcl-x_L.

In contrast, expression of PKCζCAT did induce a slight enhancement of Bcl-x_L levels in CD40-stimulated cells (figure 6.14C) suggesting active PKCζ kinase can promote the CD40-dependent pathway of inducing Bcl-x_L. However, the inability of PKCζCAT to induce Bcl-x_L in the absence of CD40-stimulation indicates PKCζ kinase activity is insufficient to directly induce Bcl-x_L signalling.

To further explore the mechanism of PKCζ-dependent survival of WEHI-231 cells, the effect of PKCζCAT and PKCζKR on Akt activity was determined since PKCζ can regulate the activity of Akt in other cellular systems (503). The activity of Akt was estimated by Western blotting with antibodies that were sensitive to the phosphorylation-state of Akt. However, as with previous experiments, the levels of pTAkt and pSAkt in WEHI-231 cells were exceptionally low and expression of PKCζCAT or PKCζKR did not enhance the activation of Akt (figure 6.14D-F). In particular, no pSAkt could be detected in PKCζCAT and PKCζKR WEHI-231 cells despite obtaining a strong signal in the positive control samples (figure 6.18E-F, *lane 5*). These observations suggest that PKCζ cannot induce Akt in WEHI-231 cells and hence PKCζ-dependent survival of these cells is not mediated by the induction of Akt.

6.9 Conclusions

Previous studies have suggested that PKCα, PKCδ and PKCζ have a role in CD40-mediated rescue of WEHI-231 cells from BCR-driven growth arrest (unpublished observations). Indeed, analysis of WEHI-231 cells expressing constitutively active or kinase inactive PKC isoforms showed that PKCα, PKCδ, PKCε and PKCζ can all impair BCR-driven growth arrest (figures 6.2, 6.5, 6.8 and 6.12). Furthermore, expression of constitutively active PKCε actually increased proliferation of anti-Ig-treated WEHI-231 cells. Moreover, each of these isoforms of PKC can promote proliferation when expressed as either a constitutively active catalytic domain (PKC-CAT) or when expressed as a complete PKC enzyme with a point mutation inactivating the kinase (PKC-KR).

These observations suggest that in WEHI-231 cells the catalytic activity of PKC is not always essential for PKC-mediated functions.

Furthermore, analysis of the activity of Erk in WEHI-231 cells that were expressing mutant forms of PKC showed that expression of PKCαCAT, PKCδCAT, PKCεCAT, PKCεKR, PKCζCAT or PKCζKR can all impair BCR-driven suppression of sustained (48 hr) Erk signals (figures 6.3, 6.6, 6.9 and 6.13). The increased proliferation observed in PKCαCAT, PKCδCAT, PKCεCAT, PKCεKR, PKCζCAT and PKCζKR WEHI-231 cells is thus likely to be caused, at least in part, by the enhancement of sustained Erk signals (figure 6.15). Furthermore, these observations suggest that PKCα, PKCδ, PKCε and PKCζ may be inhibited by the BCR and/or induced by CD40 to regulate Erk and hence proliferation of WEHI-231 cells. However, expression of PKCαKR or PKCδKR did not prevent BCR-mediated suppression of sustained Erk signals (figures 6.3 and 6.6) suggesting these isoforms of PKC impair BCR-driven growth arrest via a different mechanism although the details of this have not been explored. However, these observations show that PKC-CAT and PKC-KR mutants of the same PKC isoform are likely to use distinct signalling mechanisms to promote proliferation of WEHI-231 cells. To further explore the need for PKC kinase activity versus PKC regulatory regions it would be interesting to transfect WEHI-231 cells with additional PKC-containing constructs including mutants that encode the same region as the PKC-CAT mutants but with a point mutation to inhibit the kinase activity.

In addition to promoting proliferation of WEHI-231 cells, expression of some isoforms of PKC can also slightly improve the survival of the cells (figure 6.15). In particular, expression of PKCαCAT, PKCαKR, PKCδCAT, PKCζCAT or PKCζKR reduced the number of WEHI-231 cells with sub-diploid DNA (figures 6.2, 6.5 and 6.12). However, PKCε was unable to increase the survival of WEHI-231 cells (figure 6.10) suggesting this isoform is specifically involved in regulating proliferation. PKCδKR was also unable to increase the survival of WEHI-231 cells despite PKCδCAT reducing the level of apoptosis (figure 6.5). This further supports the theory that PKC-CAT and PKC-KR mediate their effects via distinct signalling mechanisms.

The mechanism of PKC-dependent survival was investigated by determining the effect of PKC on the induction of Bcl-x_L and Akt. None of the

PKC isoforms were able to substantially alter the activity of Akt (figures 6.4, 6.7, 6.11 and 6.14). This is consistent with the Akt studies in chapter 3, which showed that WEHI-231 cells have low Akt activity and it does not appear to be regulated by the BCR or CD40. However, a possible mechanism of PKC-dependent survival was suggested by the observation that specific PKC isoforms can induce Bcl-x_L in WEHI-231 cells (figure 6.15). Indeed, expression of either PKCαCAT or PKCαKR increased the levels of Bcl-x_L in CD40-stimulated WEHI-231 cells (figure 6.4). This suggests that PKCα-dependent survival is likely to be mediated, at least in part, by the induction of Bcl-x_L and that PKCα kinase activity is not essential for the induction of Bcl-x_L in CD40-stimulated WEHI-231 cells. However, PKCα kinase activity was essential for PKCα-dependent induction of Bcl-x_L in untreated and BCR-stimulated WEHI-231 cells (figure 6.4). This suggests that PKCα kinase activity can induce Bcl-x_L and hence survival of WEHI-231 cells implying this may represent a mechanism of CD40-dependent survival or this pathway may be inhibited by the BCR to promote apoptosis. These observations also indicate that PKCαCAT is either more effective at activating the pathway that increases Bcl-x_L levels or PKCαCAT and PKCαKR use distinct mechanisms for inducing Bcl-x_L. Furthermore, the inability of PKCαKR to induce Bcl-x_L in untreated and anti-Ig-stimulated WEHI-231 cells suggests PKCα may also promote survival via Bcl-x_L-independent mechanisms.

The levels of Bcl-x_L in WEHI-231 cells were also increased by expression of either PKCδKR or PKCδCAT (figure 6.7). However, as with PKCα-dependent induction of Bcl-x_L, PKCδ kinase activity was essential for upregulating Bcl-x_L in anti-Ig-treated WEHI-231 cells. PKCα and PKCδ-dependent survival of WEHI-231 cells thus seem to be mediated, at least in part, by the induction of Bcl-x_L. This suggests that the BCR may inhibit PKCα and/or PKCδ kinase activity to impair Bcl-x_L signalling or CD40 might increase the activity of these kinases to promote survival of WEHI-231 cells. Nonetheless, additional mechanisms of PKCα and PKCδ-dependent survival are likely to exist. Furthermore, expression of PKCζ was unable to induce Bcl-x_L (figure 6.14) suggesting PKCζ-dependent promotion of WEHI-231 cell survival is also mediated by signalling mechanisms that are independent of Bcl-x_L or function in parallel to Bcl-x_L.

Taken together, these observations show that expression of PKCα, PKCδ, PKCε and PKCζ can promote proliferation of WEHI-231 cells suggesting the suppression of these PKC enzymes may be induced by the BCR to promote growth arrest. Alternatively, these isoforms of PKC may be activated by CD40 to increase the proliferation of WEHI-231 cells. Furthermore, expression of each of these isoforms, except PKCε, can also increase the survival of WEHI-231 cells (figure 6.15) suggesting PKCα, PKCδ and PKCζ may be differentially regulated by the BCR and CD40 to determine the survival of WEHI-231 cells. Moreover, these effects can be mediated in a PKC-kinase dependent manner but some of them can also be performed in kinase inactive PKC mutants suggesting PKC also has signalling functions in WEHI-231 cells that do not require the phosphorylation of substrates.

Figure 6.1. Structure of PKC. **(A)** There are three subclasses of PKC enzymes, conventional PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC). They contain several conserved domains with specific functions. The C1 region of all PKCs contains a pseudosubstrate site and the C1 domain of conventional and novel PKC enzymes, but not atypical PKCs, also has two zinc-finger domains that can bind to DAG and phorbol ester. The C2 domain contains a calcium and lipid-binding domain that can bind to calcium in cPKCs and the C2 domain of all PKC enzymes can interact with RACKs. A hinge region of PKC attaches the C1 and C2 domains to the kinase domain. The kinase domain contains binding sites for substrates and for ATP. Attached to the kinase domain is a V5 domain that can determine the intracellular location of PKC enzymes. **(B)** Mutant forms of PKCα, δ, ε and ζ were generated to study the role of PKC in WEHI-231 cell signalling. PKC-CAT mutants are constitutively active and lack a regulatory region. PKC-KR mutants contain full length PKC but have a point mutation in the kinase domain to inactivate the PKC catalytic activity.

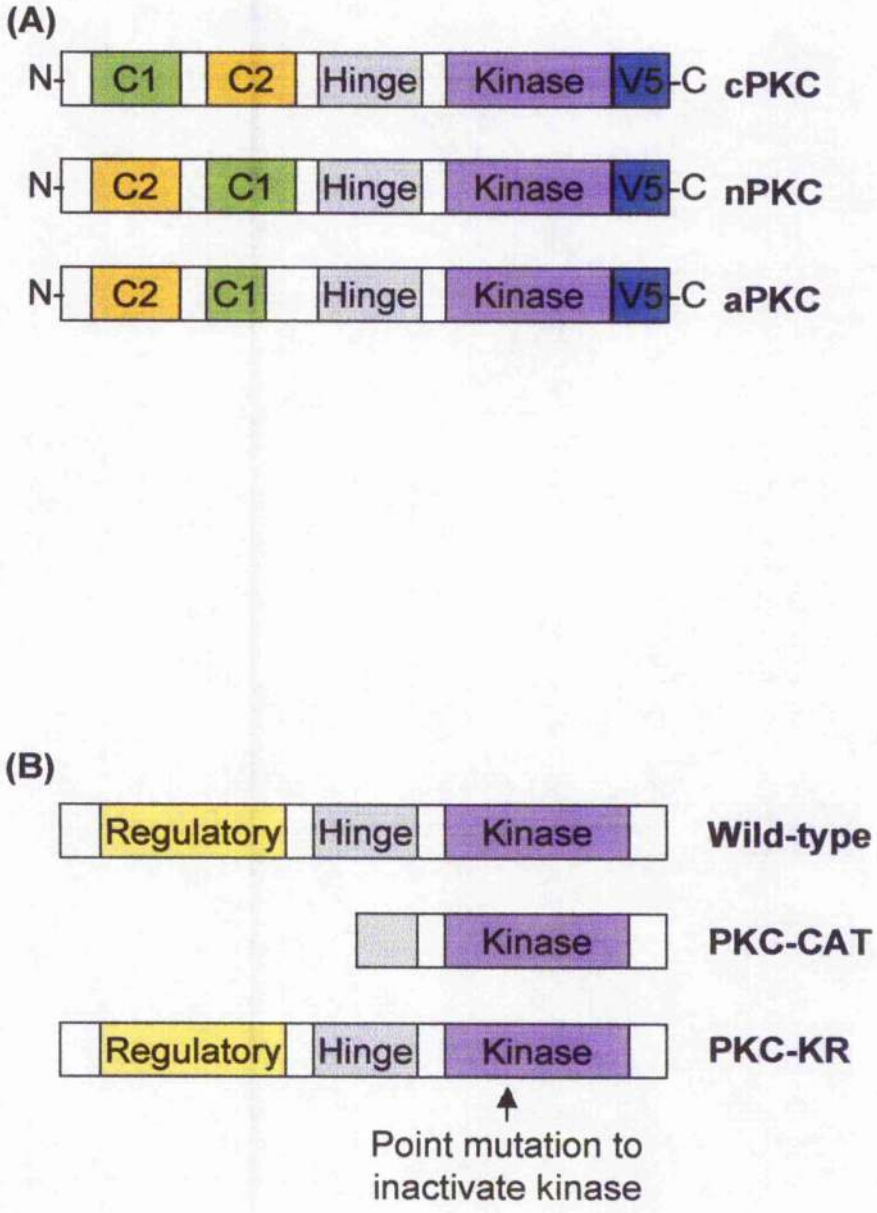

(A)
N
C1
C2
Hinge
Kinase
V5
C
cPKC
N
C2
C1
Hinge
Kinase
V5
C
nPKC
N
C2
C1
Hinge
Kinase
V5
C
aPKC
(B)
Regulatory
Hinge
Kinase
Wild-type
Kinase
PKC-CAT
Regulatory
Hinge
Kinase
PKC-KR
Point mutation to
inactivate kinase

Figure 6.2. PKCα partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis. PKCαCAT, PKCαKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)** or 48-72 hr **(B)**. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=3 individual experiments, each performed in triplicate. **(C)** PKCαCAT, PKCαKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of medium (untreated), anti-Ig (10 μg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. PKCαCAT, PKCαKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(D)** or 48 hr **(E)** with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). The number of mitogenic cells (S phase plus G2/M phase) was determined by PI-staining and FACS analysis (FL2 fluorescence). PKCαCAT, PKCαKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(F)** or 48 hr **(G)** with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence).

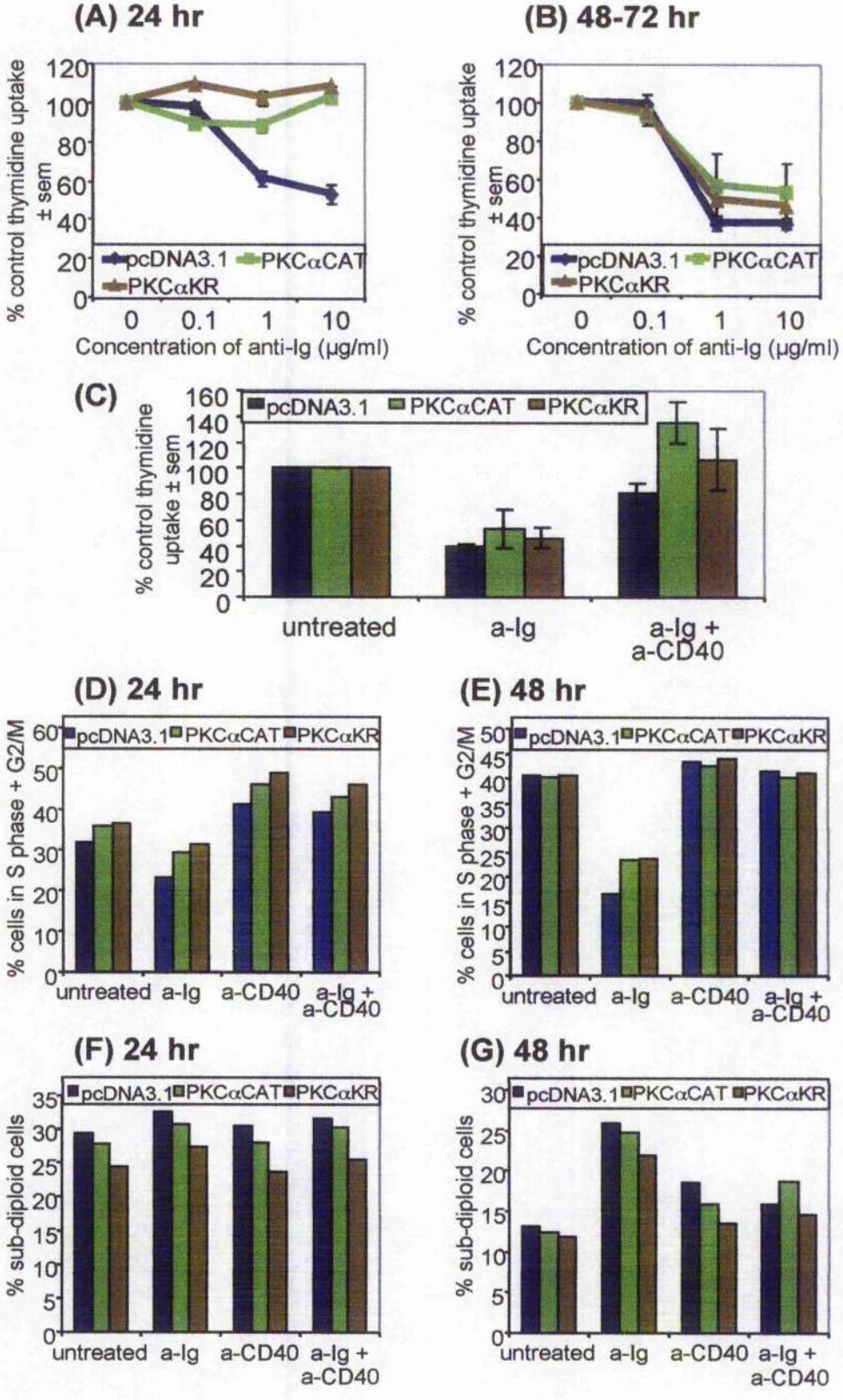

(A) 24 hr
(B) 48-72 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
PKCαCAT
PKCαKR
0
0.1
1
10
Concentration of anti-Ig (μg/ml)
(C)
% control thymidine uptake ± sem
160
140
120
100
80
60
40
20
0
pcDNA3.1
PKCαCAT
PKCαKR
untreated
a-Ig
a-Ig + a-CD40
(D) 24 hr
(E) 48 hr
% cells in S phase + G2/M
pcDNA3.1
PKCαCAT
PKCαKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) 24 hr
(G) 48 hr
% sub-diploid cells
pcDNA3.1
PKCαCAT
PKCαKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.3. Expression of PKCαCAT increases sustained Erk signalling in WEHI-231 cells. pcDNA3.1 **(A)**, PKCαKR **(B)** or PKCαCAT **(C)** WEHI-231 cells (1×10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

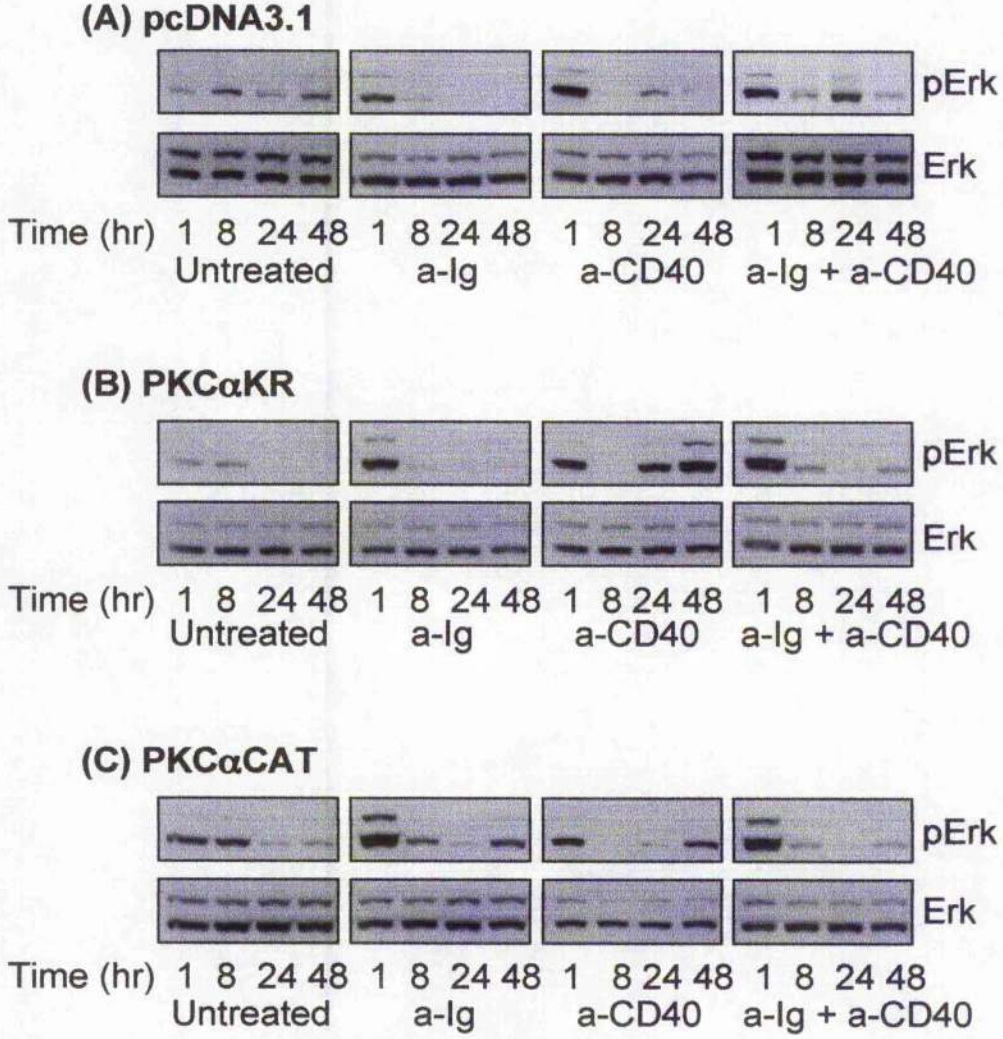

(A) pcDNA3.1
pErk
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) PKCαKR
pErk
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) PKCαCAT
pErk
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.4. Expression of PKCα can induce Bcl-x_L but does not activate Akt in WEHI-231 cells. pcDNA3.1 **(A)**, PKCαKR **(B)** or PKCαCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting. pcDNA3.1 **(D)**, PKCαKR **(E)** or PKCαCAT **(F)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1*, 1 hr; *lane 2*, 8 hr; *lane 3*, 24 hr; *lane 4*, 48 hr; *lane 5*, phosphorylated Akt control (total cell extracts from untreated Jurkat cells); *lane 6*, non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting.

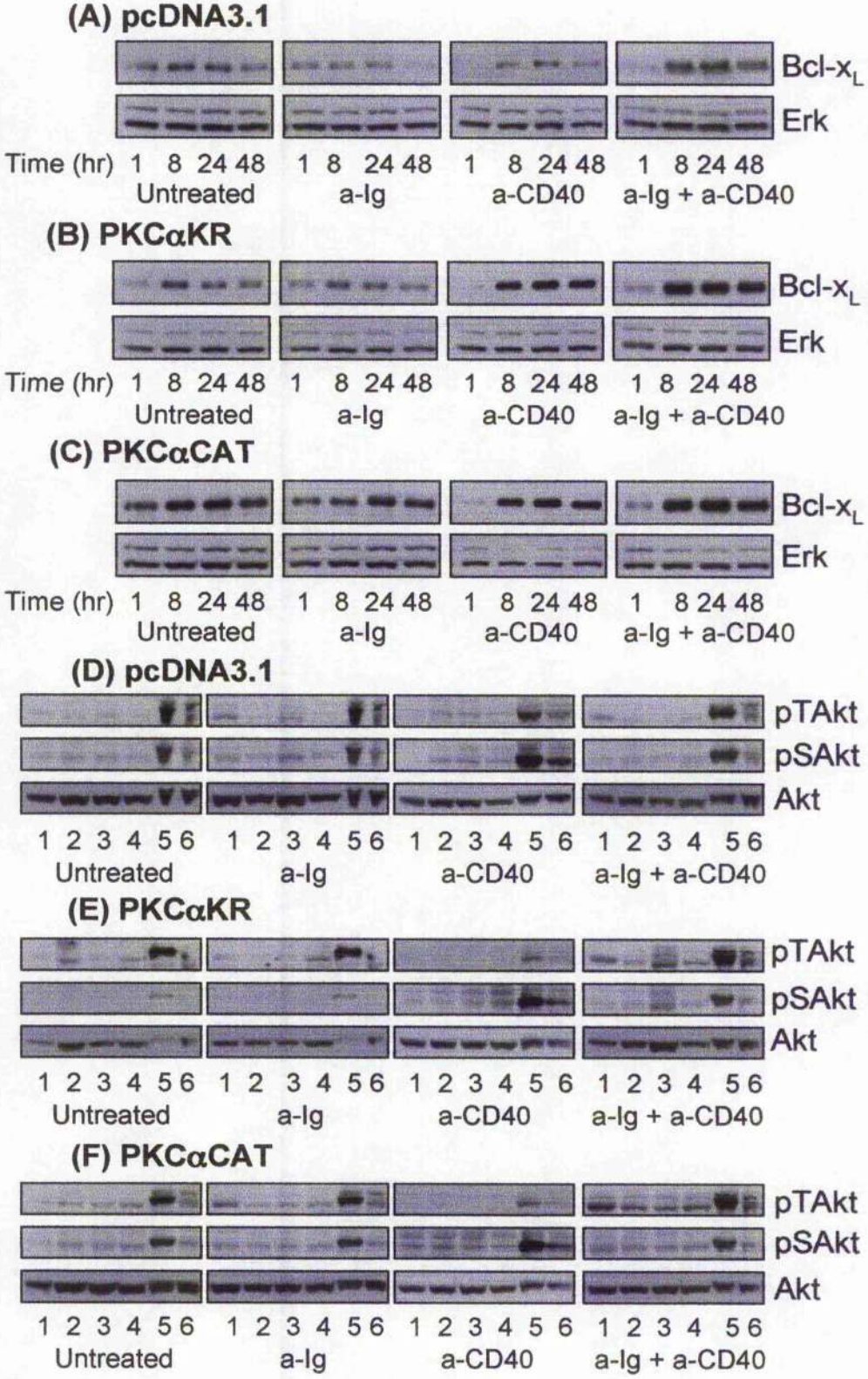

(A) pcDNA3.1
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) PKCαKR
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) PKCαCAT
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) pcDNA3.1
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) PKCαKR
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) PKCαCAT
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.5. PKCδ partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis. PKCδCAT, PKCδKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)** or 48-72 hr **(B)**. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=3 individual experiments, each performed in triplicate. **(C)** PKCδCAT, PKCδKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of medium (untreated), anti-Ig (10 μg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 μg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. PKCδCAT, PKCδKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(D)** or 48 hr **(E)** with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). The number of mitogenic cells (S phase plus G2/M phase) was determined by PI-staining and FACS analysis (FL2 fluorescence). PKCδCAT, PKCδKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(F)** or 48 hr **(G)** with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence).

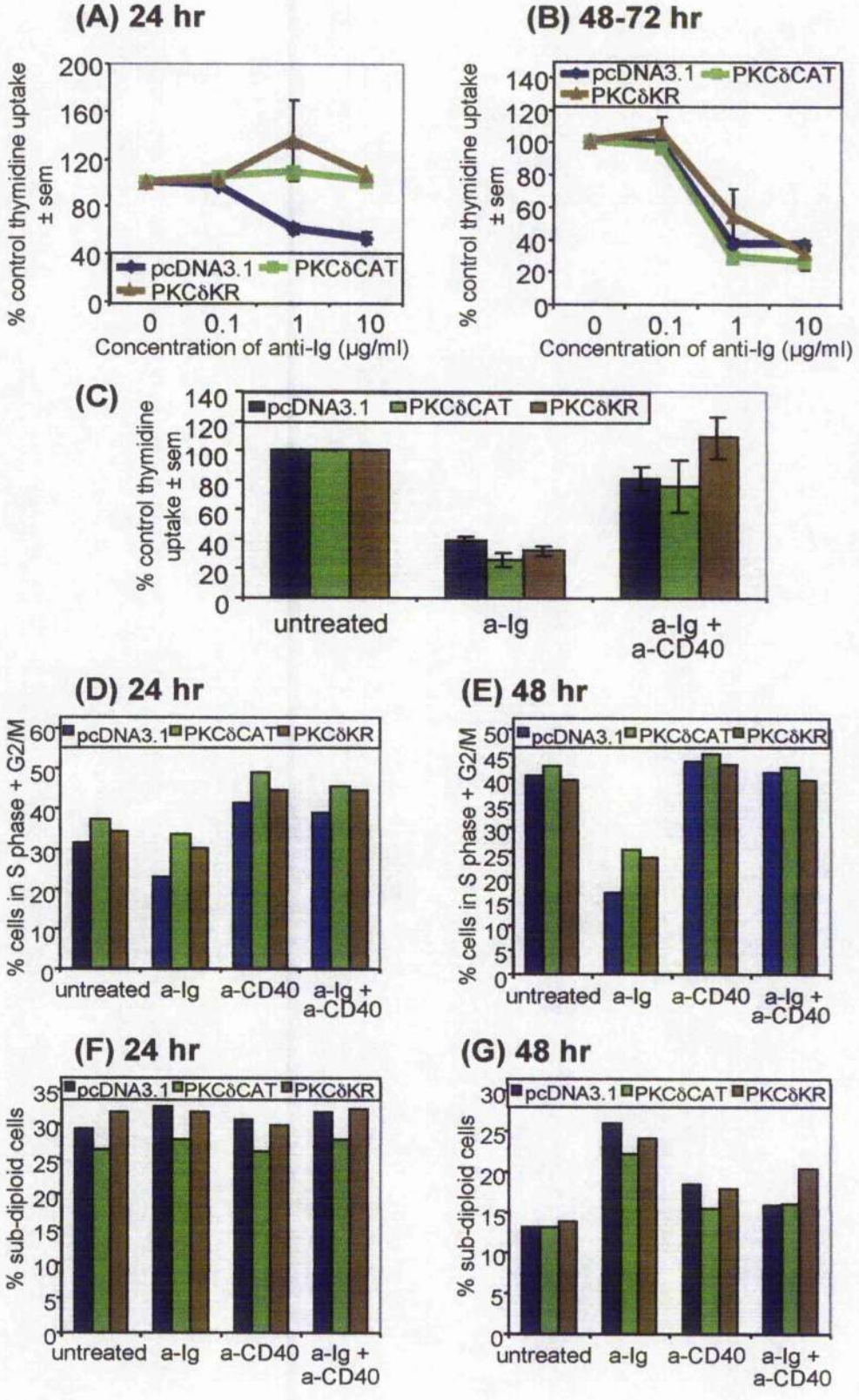
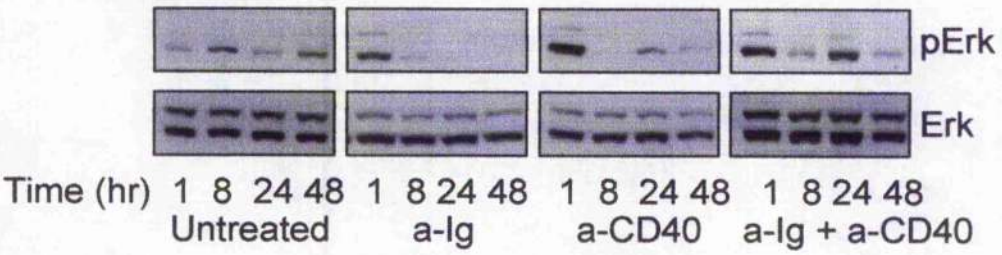
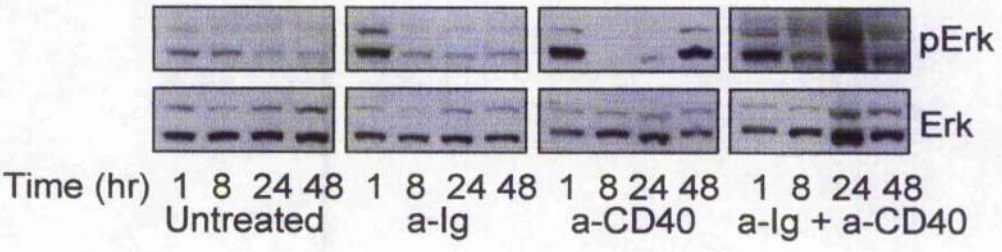

(A) 24 hr
% control thymidine uptake ± sem
200
160
120
80
40
0
pcDNA3.1
PKCδCAT
PKCδKR
0
0.1
1
10
Concentration of anti-Ig (μg/ml)
(B) 48-72 hr
% control thymidine uptake ± sem
140
120
100
80
60
40
20
0
pcDNA3.1
PKCδCAT
PKCδKR
0
0.1
1
10
Concentration of anti-Ig (μg/ml)
(C)
% control thymidine uptake ± sem
140
120
100
80
60
40
20
0
pcDNA3.1
PKCδCAT
PKCδKR
untreated
a-Ig
a-Ig + a-CD40
(D) 24 hr
% cells in S phase + G2/M
60
50
40
30
20
10
0
pcDNA3.1
PKCδCAT
PKCδKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) 48 hr
% cells in S phase + G2/M
50
45
40
35
30
25
20
15
10
5
0
pcDNA3.1
PKCδCAT
PKCδKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) 24 hr
% sub-diploid cells
35
30
25
20
15
10
5
0
pcDNA3.1
PKCδCAT
PKCδKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(G) 48 hr
% sub-diploid cells
30
25
20
15
10
5
0
pcDNA3.1
PKCδCAT
PKCδKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.6. Expression of PKCδCAT increases sustained Erk signalling in WEHI-231 cells. pcDNA3.1 **(A)**, PKCδKR **(B)** or PKCδCAT **(C)** WEHI-231 cells (1×10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

(A) pcDNA3.1



(B) PKCδKR



(C) PKCδCAT

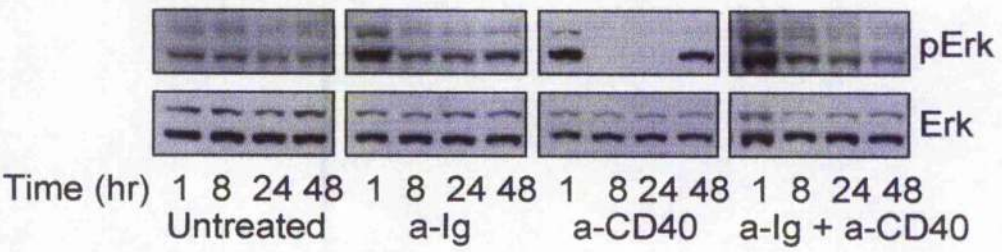


Figure 6.7. Expression of PKCδ cannot activate Akt but can induce Bcl-x_L in WEHI-231 cells. pcDNA3.1 **(A)**, PKCδKR **(B)** or PKCδCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1*, 1 hr; *lane 2*, 8 hr; *lane 3*, 24 hr; *lane 4*, 48 hr; *lane 5*, phosphorylated Akt control (total cell extracts from untreated Jurkat cells); *lane 6*, non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting. pcDNA3.1 **(D)**, PKCδKR **(E)** or PKCδCAT **(F)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting.

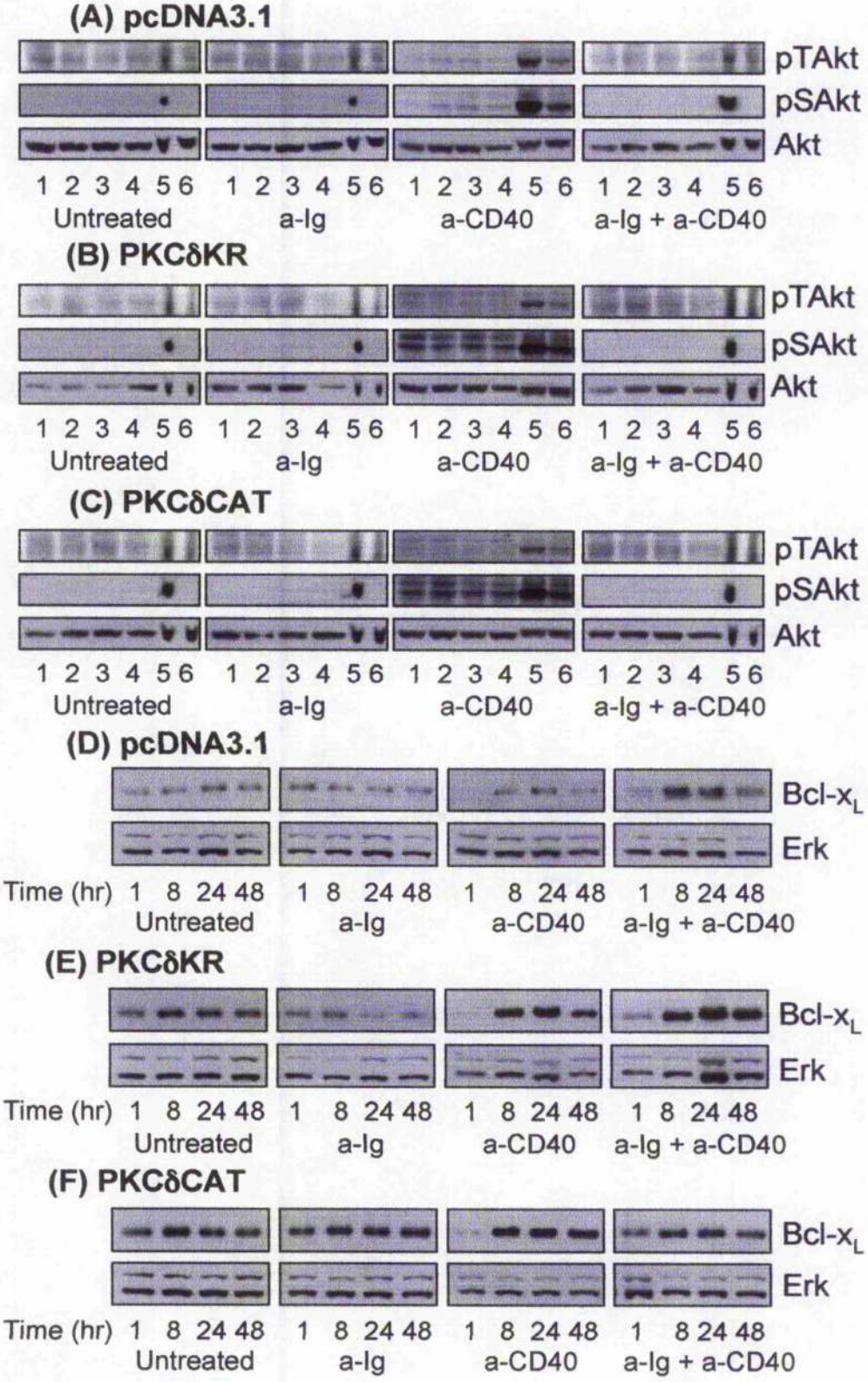

(A) pcDNA3.1
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) PKCδKR
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) PKCδCAT
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) pcDNA3.1
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) PKCδKR
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) PKCδCAT
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.8. PKCε partially protects WEHI-231 cells from BCR-driven growth arrest. PKCεCAT, PKCεKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)** or 48-72 hr **(B)**. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=3 individual experiments, each performed in triplicate. **(C)** PKCεCAT, PKCεKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of medium (untreated), anti-Ig (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. PKCεCAT, PKCεKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(D)** or 48 hr **(E)** with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml). The number of mitogenic cells (S phase plus G2/M phase) was determined by PI-staining and FACS analysis (FL2 fluorescence).

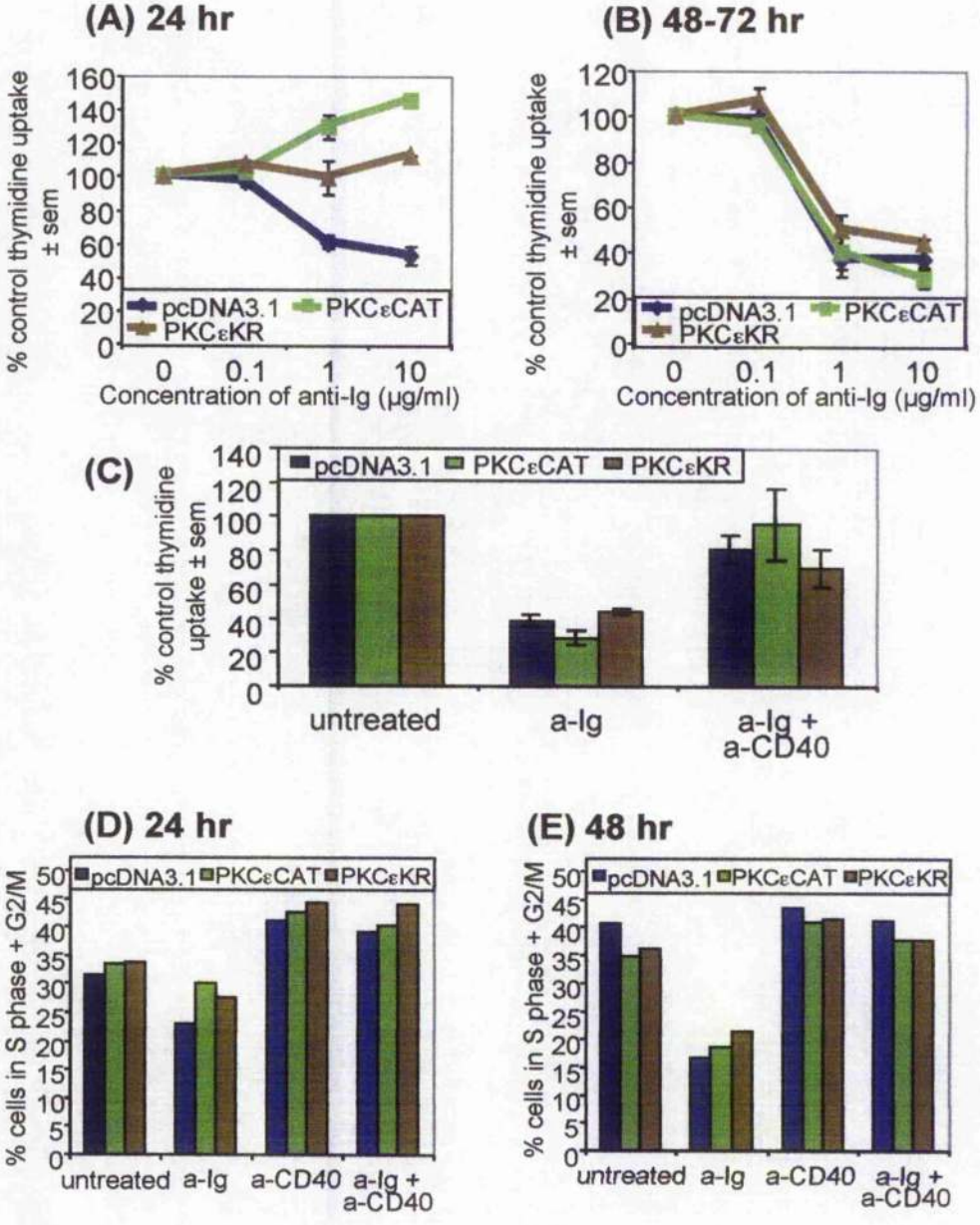
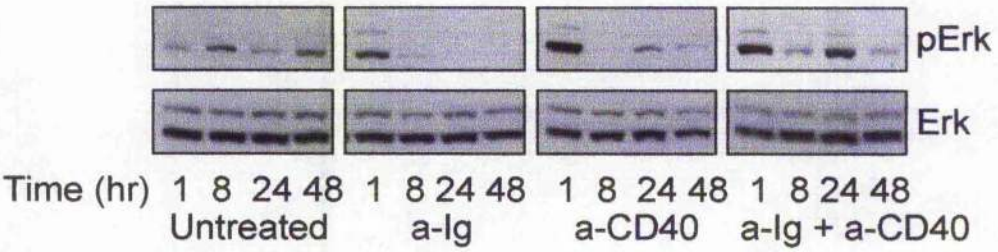
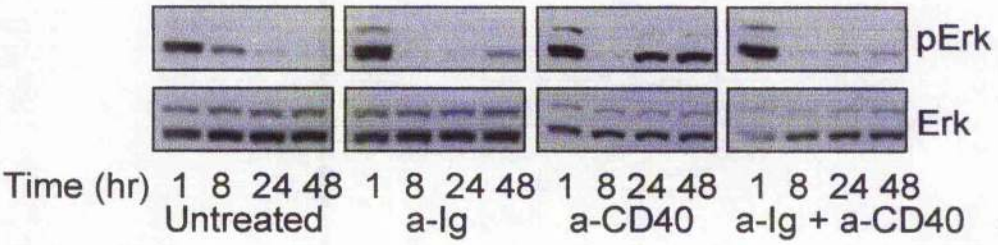

(A) 24 hr
% control thymidine uptake ± sem
160
140
120
100
80
60
40
20
0
pcDNA3.1
PKCεCAT
PKCεKR
0
0.1
1
10
Concentration of anti-Ig (μg/ml)
(B) 48-72 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
PKCεCAT
PKCεKR
0
0.1
1
10
Concentration of anti-Ig (μg/ml)
(C)
% control thymidine uptake ± sem
140
120
100
80
60
40
20
0
pcDNA3.1
PKCεCAT
PKCεKR
untreated
a-Ig
a-Ig + a-CD40
(D) 24 hr
% cells in S phase + G2/M
50
45
40
35
30
25
20
15
10
5
0
pcDNA3.1
PKCεCAT
PKCεKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) 48 hr
% cells in S phase + G2/M
50
45
40
35
30
25
20
15
10
5
0
pcDNA3.1
PKCεCAT
PKCεKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.9. PKCε can induce Erk in BCR-stimulated WEHI-231 cells. pcDNA3.1 **(A)**, PKCεKR **(B)** or PKCεCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

(A) pcDNA3.1



(B) PKCεKR



(C) PKCεCAT

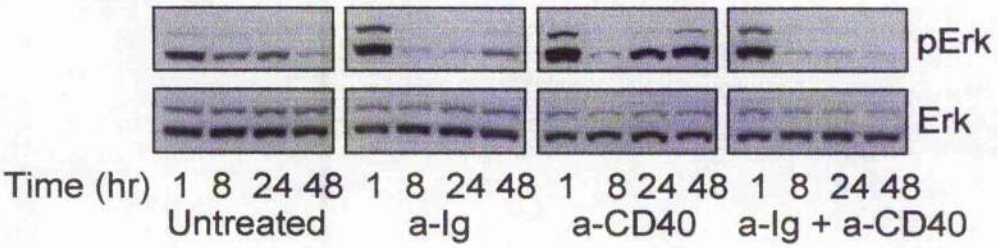


Figure 6.10. PKCε does not protect WEHI-231 cells from BCR-driven apoptosis and cannot induce Bcl-x_L. PKCεCAT, PKCεKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(A)** or 48 hr **(B)** with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence). pcDNA3.1 **(C)**, PKCεKR **(D)** or PKCεCAT **(E)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting.

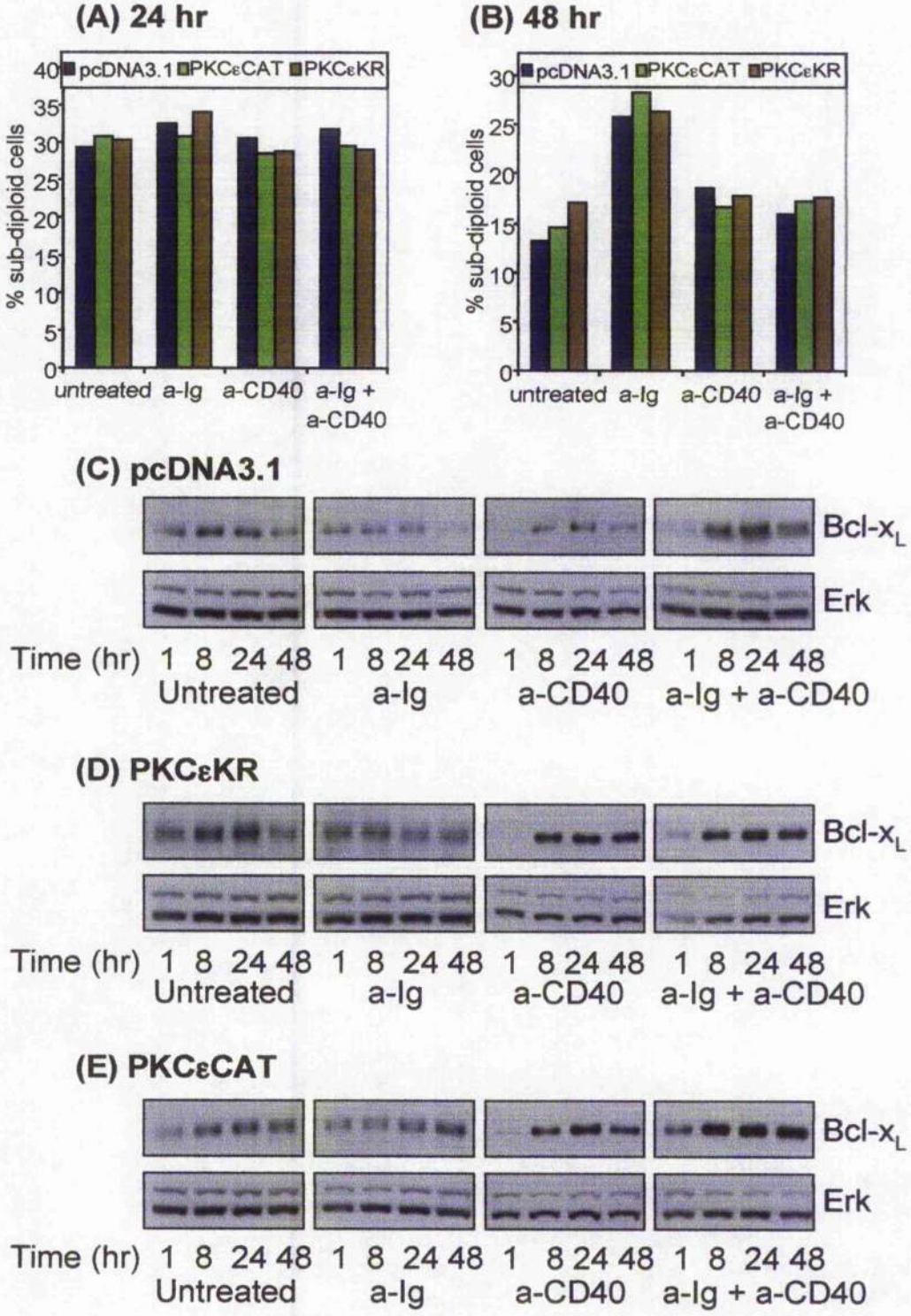
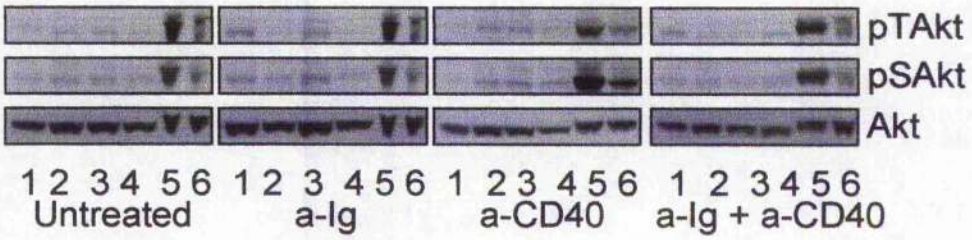
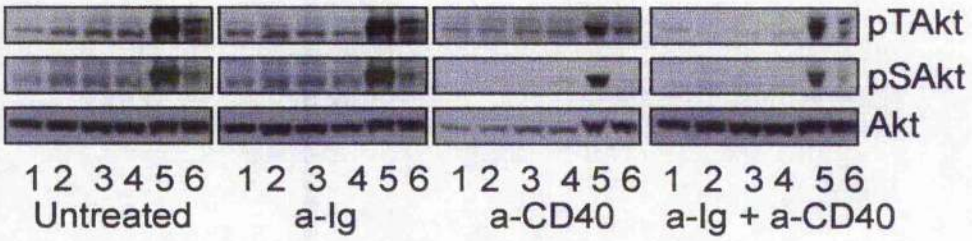

(A) 24 hr
pcDNA3.1 PKCεCAT PKCεKR
% sub-diploid cells
untreated a-Ig a-CD40 a-Ig + a-CD40
(B) 48 hr
pcDNA3.1 PKCεCAT PKCεKR
% sub-diploid cells
untreated a-Ig a-CD40 a-Ig + a-CD40
(C) pcDNA3.1
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40
(D) PKCεKR
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40
(E) PKCεCAT
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

Figure 6.11. PKCε cannot induce Akt in WEHI-231 cells. pcDNA3.1 **(A)**, PKCεKR **(B)** or PKCεCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-CD40 (a-CD40, 10 μg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 μg/ml) before preparing cell lysates. Whole cell lysates (50 μg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1*, 1 hr; *lane 2*, 8 hr; *lane 3*, 24 hr; *lane 4*, 48 hr; *lane 5*, phosphorylated Akt control (total cell extracts from untreated Jurkat cells); *lane 6*, non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting.

(A) pcDNA3.1



(B) PKCεKR



(C) PKCεCAT

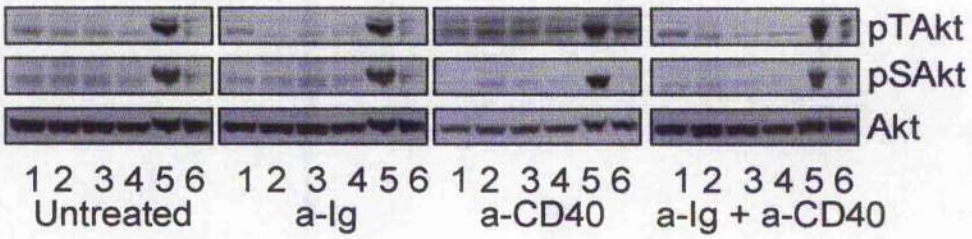


Figure 6.12. PKCζ partially protects WEHI-231 cells from BCR-driven growth arrest and apoptosis. PKCζCAT, PKCζKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of increasing concentrations of anti-Ig (0-10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 24 hr **(A)** or 48-72 hr **(B)**. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig) thymidine uptake ± sem, n=3 individual experiments, each performed in triplicate. **(C)** PKCζCAT, PKCζKR or pcDNA3.1 WEHI-231 cells (1 x 10^4 cells/well) were cultured in the presence of medium (untreated), anti-Ig (10 µg/ml) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine at 48 hr. Values are the mean % control (pcDNA3.1 WEHI-231 cells without anti-Ig or anti-CD40) thymidine uptake ± sem, n=4 individual experiments, each performed in triplicate. PKCζCAT, PKCζKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(D)** or 48 hr **(E)** with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml). The number of mitogenic cells (S phase plus G2/M phase) was determined by PI-staining and FACS analysis (FL2 fluorescence). PKCζCAT, PKCζKR or pcDNA3.1 WEHI-231 cells (5 x 10^5 cells/ml) were cultured for 24 hr **(F)** or 48 hr **(G)** with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml). Levels of apoptosis were indicated by the % sub-diploid cells and this was determined by PI-staining and FACS analysis (FL2 fluorescence).

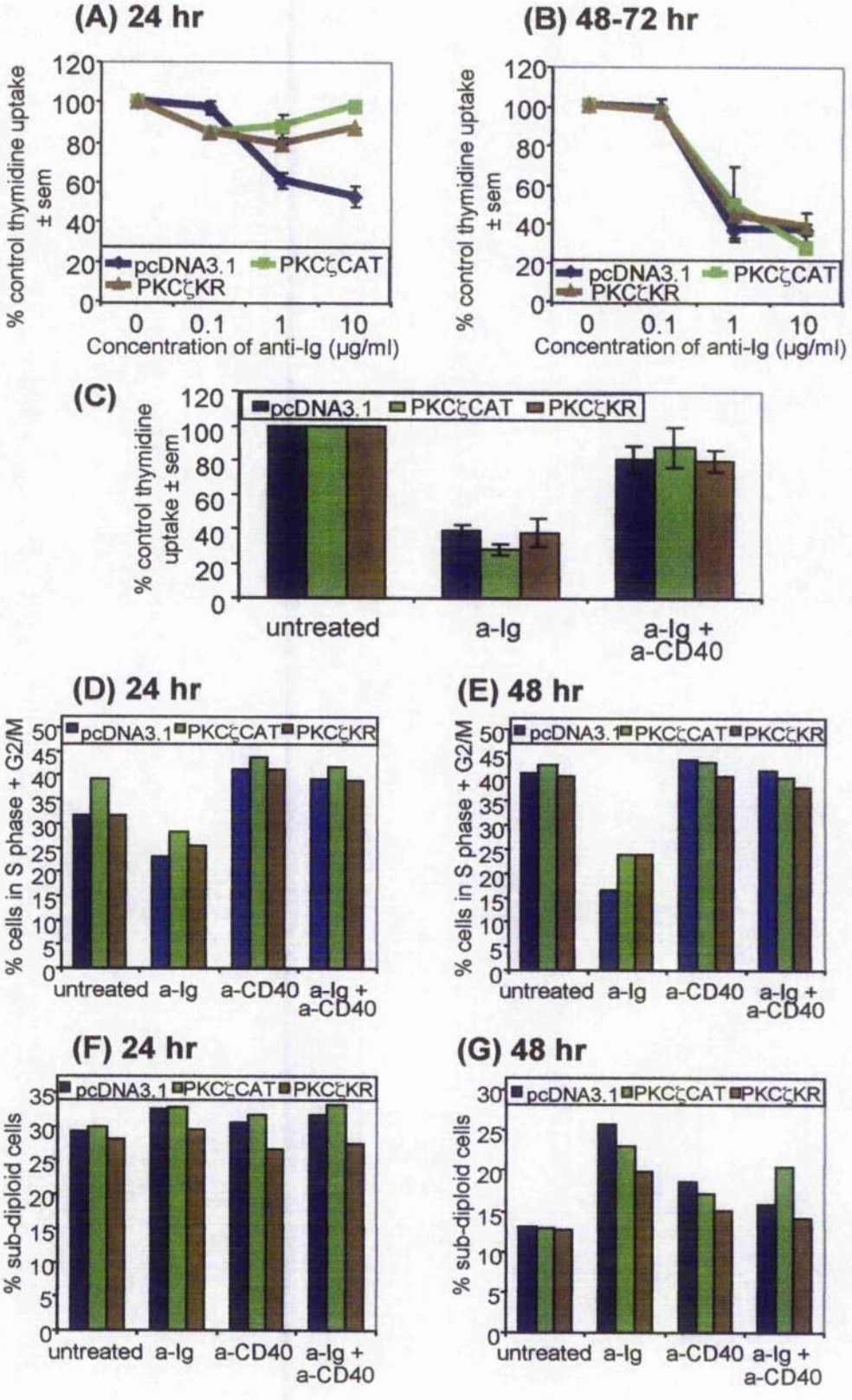
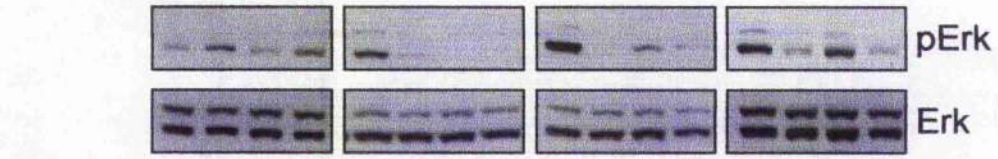

(A) 24 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
PKCζCAT
PKCζKR
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(B) 48-72 hr
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
PKCζCAT
PKCζKR
0
0.1
1
10
Concentration of anti-Ig (µg/ml)
(C)
% control thymidine uptake ± sem
120
100
80
60
40
20
0
pcDNA3.1
PKCζCAT
PKCζKR
untreated
a-Ig
a-Ig + a-CD40
(D) 24 hr
% cells in S phase + G2/M
50
45
40
35
30
25
20
15
10
5
0
pcDNA3.1
PKCζCAT
PKCζKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) 48 hr
% cells in S phase + G2/M
50
45
40
35
30
25
20
15
10
5
0
pcDNA3.1
PKCζCAT
PKCζKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) 24 hr
% sub-diploid cells
35
30
25
20
15
10
5
0
pcDNA3.1
PKCζCAT
PKCζKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40
(G) 48 hr
% sub-diploid cells
30
25
20
15
10
5
0
pcDNA3.1
PKCζCAT
PKCζKR
untreated
a-Ig
a-CD40
a-Ig + a-CD40

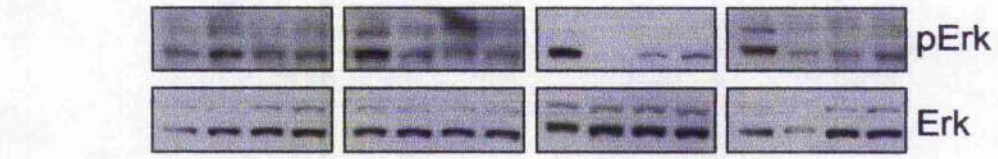
Figure 6.13. PKCζ slightly increases the activity of Erk-MAPK in BCR-stimulated WEHI-231 cells. pcDNA3.1 **(A)**, PKCζKR **(B)** or PKCζCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of dual-phosphorylated Erk (pErk) and total Erk (Erk) were determined by Western blotting.

(A) pcDNA3.1



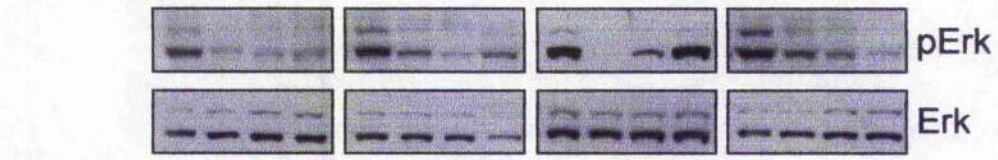
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

(B) PKCζKR



Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

(C) PKCζCAT



Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated a-Ig a-CD40 a-Ig + a-CD40

Figure 6.14. Expression of PKCζ does not induce Bcl-x_L or Akt in WEHI-231 cells. pcDNA3.1 **(A)**, PKCζKR **(B)** or PKCζCAT **(C)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Levels of Bcl-x_L and total Erk (Erk) were determined by Western blotting. pcDNA3.1 **(D)**, PKCζKR **(E)** or PKCζCAT **(F)** WEHI-231 cells (1 x 10^6 cells/ml) were cultured for up to 48 hr with medium (untreated), anti-Ig (a-Ig, 10 µg/ml), anti-CD40 (a-CD40, 10 µg/ml) or a combination of anti-Ig plus anti-CD40 (a-Ig + a-CD40, both at 10 µg/ml) before preparing cell lysates. Whole cell lysates (50 µg/lane) were analysed by Western blotting, using the NuPAGE system of gel electrophoresis. Gel loading was as follows: *lane 1*, 1 hr; *lane 2*, 8 hr; *lane 3*, 24 hr; *lane 4*, 48 hr; *lane 5*, phosphorylated Akt control (total cell extracts from untreated Jurkat cells); *lane 6*, non-phosphorylated Akt control (total cell extracts from Jurkat cells treated with PI-3-K inhibitor LY294002). Levels of phospho-Thr^{308} Akt (pTAkt), phospho-Ser^{473} Akt (pSAkt) and total Akt (Akt) were determined by Western blotting.

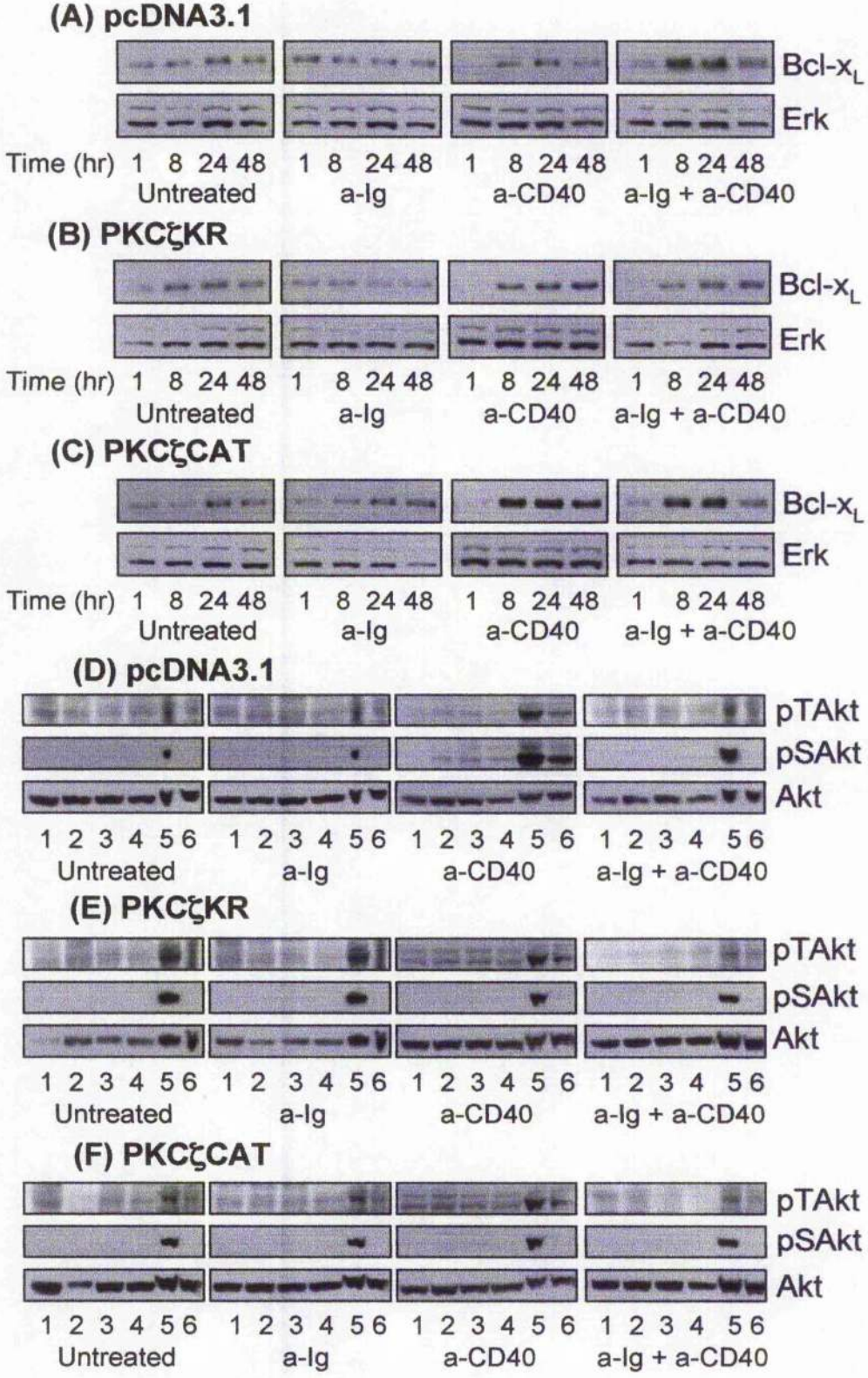
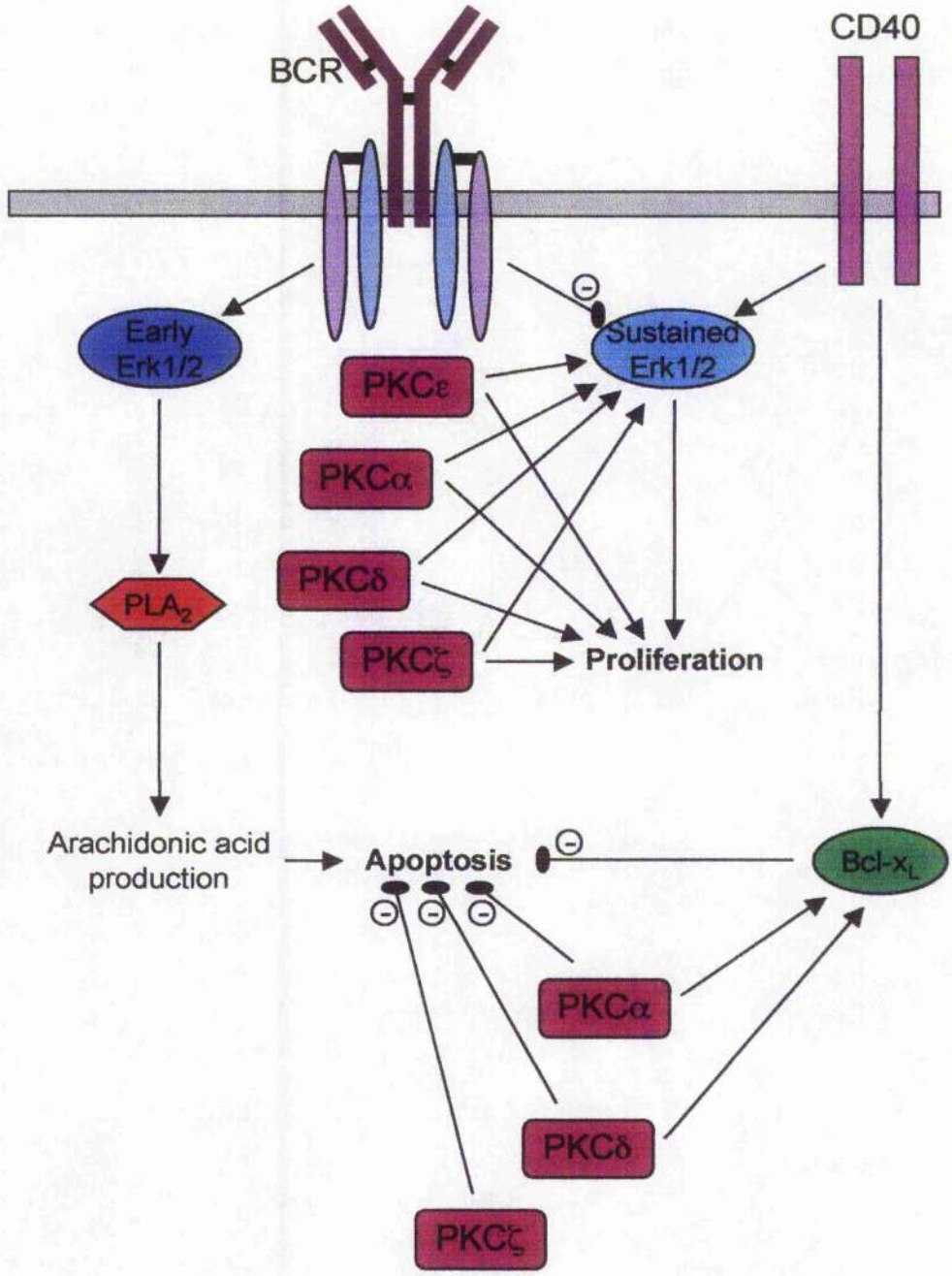

(A) pcDNA3.1
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(B) PKCζKR
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(C) PKCζCAT
Bcl-x$_L$
Erk
Time (hr) 1 8 24 48 1 8 24 48 1 8 24 48 1 8 24 48
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(D) pcDNA3.1
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(E) PKCζKR
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40
(F) PKCζCAT
pTAkt
pSAkt
Akt
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Untreated
a-Ig
a-CD40
a-Ig + a-CD40

Figure 6.15. Model showing the role of PKCα, PKCδ, PKCε and PKCζ in regulating survival and proliferation of WEHI-231 cells. Expression of PKCα, PKCδ, PKCε or PKCζ can increase the proliferation of WEHI-231 cells and impair BCR-driven growth arrest. Furthermore, expression of each of these PKC isoforms as constitutively active kinases can impair BCR-driven inhibition of sustained Erk signals. Expression of kinase inactivated forms of PKCε and PKCζ can also increase sustained Erk signals. The ability of certain PKC isoforms to impair BCR-driven inhibition of sustained Erk signals is likely to contribute to the enhanced proliferation observed in cells expressing these PKC isoforms. However, additional mechanisms of PKC-dependent induction of mitogenesis are likely to exist since expression of kinase inactivate forms of PKCα and PKCδ also increased proliferation of WEHI-231 cells but did not enhance sustained Erk signals. Expression of PKCα, PKCδ and PKCζ can also slightly increase the survival and impair BCR-driven apoptosis of WEHI-231 cells. PKCα and PKCδ can increase the levels of Bcl-x_L in WEHI-231 cells and this is likely to increase the survival of the cells. The mechanism of PKCζ-dependent survival has not been determined but it does not appear to induce Bcl-x_L or stimulate Akt in WEHI-231 cells.


BCR
CD40
Early Erk1/2
Sustained Erk1/2
PKCε
PKCα
PKCδ
PKCζ
PLA2
Proliferation
Arachidonic acid production
Apoptosis
Bcl-xL
PKCα
PKCδ
PKCζ

CHAPTER 7 - General Discussion

The immune system has developed mechanisms to protect against the generation of autoimmune B lymphocytes. Thus, immature B cells are programmed to be sensitive to the process of negative selection such that ligation of the BCR by antigen on these cells induces growth arrest and apoptosis. However, T cell-derived signals, generated in response to cognate antigen, such as co-ligation of CD40 can rescue the immature B cells and enable them to survive and proliferate. This investigation has explored the signalling mechanisms used by the BCR and CD40 to differentially regulate the survival and proliferation of immature B cells. In particular, the role of Erk-MAPK in controlling these processes was examined using the WEHI-231 immature B cell line.

7.1 Dual role for Erk-MAPK in regulating BCR-driven apoptosis and CD40-mediated rescue of WEHI-231 immature B cells

Evidence is presented in this investigation to show that the BCR and CD40 differentially regulate the activity of Erk-MAPK in WEHI-231 cells (appendices 1-2). In particular, the BCR couples to an early (≤ 2 hr) transient Erk signal that is impaired by ligation of CD40. This early Erk signal is important for the induction of apoptosis of WEHI-231 cells (328). Indeed, inhibition of early Erk signals using pharmacological inhibitors of MEK can suppress BCR-driven apoptosis (appendix 1E). This is consistent with the observation that ligation of the BCR on immature B cells can induce apoptosis via the induction of $cPLA_2$, a MAPK-sensitive enzyme (102). Cytosolic PLA_2 is activated by Erk1/2 and Ca^{2+} downstream of the BCR in WEHI-231 cells and this leads to the production of arachidonic acid. Arachidonic acid can disrupt the mitochondrial membrane potential leading to the activation of executioner proteases and apoptosis of the cell (102). Indeed, it has been observed that inhibition of early Erk1/2 activity in BCR-stimulated WEHI-231 cells can also prevent anti-Ig-induced activation of $cPLA_2$, as indicated by measuring the production of arachidonic acid (328). The BCR is thus coupled to an early (≤2 hr) Erk1/2 signal that plays a key role in committing WEHI-231 cells to apoptosis.

However, this investigation has also identified a second role for Erk-MAPK in promoting survival and proliferation of WEHI-231 cells. Indeed, in addition to the early (≤2 hr) pro-apoptotic Erk-MAPK signal, proliferating WEHI-231 cells display sustained (12-48 hr) and cyclic activation of Erk (appendix 2). Ligation of the BCR inhibits sustained Erk signals despite enhancing early pro-apoptotic Erk1/2 activity (appendix 2). However, co-ligation of CD40 can restore sustained Erk signals and data is presented in this study to suggest that this is important for CD40-dependent rescue from BCR-driven growth arrest.

Indeed, inhibition of sustained Erk signals using pharmacological inhibitors of MEK can induce growth arrest and apoptosis of proliferating WEHI-231 cells (appendix 3). In addition, they can impair CD40-dependent rescue from anti-Ig-induced growth arrest despite enabling CD40-mediated rescue from BCR-driven apoptosis. Furthermore, the combined analysis of cell cycle progression and intracellular phospho-Erk levels using laser scanning cytometry showed that cycling (G1, S and G2/M phase) WEHI-231 cells have high levels of phospho-Erk whereas newly formed daughter cells have significantly lower levels of phospho-Erk (appendix 4). Moreover, levels of phospho-Erk are low in WEHI-231 cells that have undergone cell cycle arrest following culture with cell cycle inhibitors or BCR-ligation. Taken together, these observations suggest that proliferation of WEHI-231 cells requires sustained and cyclic activation of Erk and the induction of growth arrest by the BCR involves a suppression of these signals whereas the restoration of these signals is important for CD40-dependent cell cycle progression.

The induction of Erk-MAPK in WEHI-231 cells therefore can mediate diverse functions since early Erk activation induces mitochondrial-dependent apoptosis whilst sustained and cyclic Erk signals promote proliferation of the cells. The response of WEHI-231 cells to Erk activity is thus dependent on the timing and duration of the Erk signals. Indeed, the temporal regulation of Erk signals is also important for determining the fate of other cell types. For example, ligation of the TCR induces early transient Erk signals and sustained Erk activation and these signals regulate distinct components of NF-κB (508). Consequently, sustained Erk signals are necessary for T cell activation and IL-2 production (508). Furthermore, in PC12 cells, transient Erk signals induce proliferation whereas sustained Erk signals cause the cells to differentiate (173).

One factor that is likely to contribute to the divergent effects of temporally distinct Erk signals is the intracellular location of the activated Erk. For example, treatment of GT1-7 cells with gonadotropin-releasing hormone induces a transient Erk signal that cannot translocate to the nucleus. By contrast, treatment of HEK293 cells with the same hormone activates a sustained Erk signal and such Erk signals accumulate in the nucleus (509). The duration of Erk signalling can thus determine the intracellular location of Erk and this will regulate the availability of substrates. For example, $cPLA_2$ is a cytoplasmic substrate whereas transcription factors are generally localised in the nucleus. To further examine the mechanism of Erk-dependent regulation of apoptosis and proliferation in WEHI-231 cells, the intracellular location of early and sustained Erk signals could be determined. Indeed, preliminary results have been obtained to suggest that phospho-Erk translocates to the nucleus in CD40-stimulated WEHI-231 cells but is largely retained in the cytoplasm of BCR-stimulated cells (328).

The specific targets of sustained Erk signals have not been identified but given the importance of this signal for the induction of proliferation in WEHI-231 cells, candidate molecules are likely to be involved in regulating the cell cycle. In particular, sustained Erk is likely to enable cells to undergo the G1 to S phase transition since BCR-stimulated WEHI-231 cells undergo growth arrest at G1 phase of the cell cycle (figure 3.6). Indeed, CD40-dependent proliferation requires the induction of the Cdk enzymes including cyclin D/Cdk4, cyclin D/Cdk6 and cyclin E/Cdk2 since these are required for progression through G1 phase of the cell cycle and for the G1 to S phase transition (33, 312). In other cellular systems, Erk can increase the expression of cyclin D1 suggesting Erk may contribute to the induction of cyclin D Cdks in WEHI-231 cells (50). However, it has been reported that ligation of the BCR on immature B cells can induce cyclin D/Cdk4 and cyclin D/Cdk6 but cannot stimulate cyclin E/Cdk2 expression or cell cycle progression (33). This suggests that sustained Erk signals may target cyclin E/Cdk2 or some other factor that is required for proliferation. Rb is a substrate of Cdk enzymes and cell cycle progression of immature B cells requires the phosphorylation and inhibition of Rb since Rb is a negative regulator of the transcription factor E2F (50, 52). E2F can regulate the expression of many genes required for DNA synthesis and cell cycle

progression including DNA polymerase alpha, thymidine synthetase, cyclin D3, cyclin E and cyclin A (33, 50-54). The importance of E2F in mediating cell cycle progression of WEHI-231 cells is indicated by studies on Bcl-x_L WEHI-231 cells since these cells are usually susceptible to BCR-driven growth arrest but expression of E2F can protect these cells from BCR-driven growth arrest (52). Sustained Erk signals therefore are likely to induce proliferation of WEHI-231 cells via the induction of Cdks, the inhibition of Rb and/or the stimulation of E2F.

7.2 Regulation of Erk-MAPK by the BCR and CD40 in WEHI-231 cells

To further investigate the induction of growth arrest by the BCR, the mechanism of BCR-dependent inhibition of sustained Erk signals was investigated. The ability of the BCR to suppress upstream activators of Erk was determined by comparing the activity of MEK in untreated and anti-Ig-treated WEHI-231 cells. Despite being able to suppress sustained (12-48 hr) Erk signals, ligation of the BCR did not inhibit sustained (12-48 hr) MEK activity (figures 3.7-3.8). This suggests that BCR-dependent suppression of Erk is not mediated by an inhibition of upstream activators of Erk. Indeed, an alternative mechanism of BCR-dependent suppression of sustained Erk signals was suggested by the observation that ligation of the BCR increases the cellular levels of PAC-1 and enhances its association with Erk (appendix 5). PAC-1 is a dual-specificity MAPK phosphatase therefore it inhibits Erk by dephosphorylating the threonine and tyrosine residues of the activation loop of Erk. Furthermore, it has previously been shown in B and T cells that mitogen-induced *pac-1* expression is mediated by Erk (384, 385) suggesting BCR-driven induction of PAC-1 may also represent an Erk-dependent negative feedback loop.

Dual-specificity MAPK phosphatases therefore appear to be key mediators of BCR-dependent inhibition of sustained Erk signalling. However, additional groups of protein phosphatases are likely to contribute to the regulation of Erk since single-phosphorylated forms of Erk (pT-Erk and pY-Erk) were detected in WEHI-231 cells (figure 3.9). In particular, pY-Erk was abundant at times of high Erk activity whilst pT-Erk was detected even when the levels of dual-phosphorylated Erk were low. The use of single-specificity phosphatases to regulate Erk1/2 activity in WEHI-231 cells adds an additional level of complexity

to the control of this kinase. Furthermore, recent studies have suggested that single-phosphorylated forms of Erk may have partial activity (379) therefore the generation of pT-Erk and pY-Erk in WEHI-231 cells may be functionally significant. To begin to explore the functional significance of single-phosphorylated Erk, the intracellular location of these forms of Erk should be determined since dual-phosphorylated Erk generally translocates to the nucleus but many single-specificity protein phosphatases are found in the cytoplasm of cells (381). Furthermore, the identity of the threonine and tyrosine phosphatases involved in generating pY-Erk and pT-Erk in WEHI-231 cells has not been fully explored. However, preliminary data has suggested that the association of PP2A and Erk in WEHI-231 cells is not regulated by the BCR and CD40 implying PP2A does not mediate BCR-dependent suppression of Erk (428).

Nonetheless, Erk was found in association with SHP-1 and SHP-2 tyrosine phosphatases in WEHI-231 cells but the kinetics of association did not immediately implicate these phosphatases as being involved in the production of pT-Erk downstream of the BCR and CD40 (figure 3.10). However, they did suggest that SHP-2 might be a positive regulator of Erk1/2 in WEHI-231 cells since the association of SHP-2 and Erk was regulated by ligation of the BCR and CD40 and was greatest when dual-phosphorylated Erk was most abundant. Moreover, SHP-2 has previously been implicated as a positive regulator of Erk in mature B cells (110, 117, 132, 165).

To further investigate the mechanisms controlling the induction of sustained Erk signals and proliferation of WEHI-231 cells, the role of Ras and its downstream effectors was explored. These studies suggested that Ras is an important activator of early and sustained Erk signals in WEHI-231 cells (figure 3.11). Furthermore, either Raf-1 or PI-3-K can mediate Ras-dependent induction of Erk. However, constitutive activation of these effectors by Ras did not lead to constitutive activation of Erk indicating they were insufficient to overcome BCR-dependent inhibition of sustained Erk signals (figure 3.11). This is consistent with the observation that the BCR inhibits sustained Erk by activating MAPK phosphatases in an Erk-dependent manner rather than suppressing upstream activators of Erk (figure 3.8 and appendix 5). However, the role of additional activators of Erk has yet to be explored in WEHI-231 cells.

For example, in DT40 B cells, B-Raf is a more effective activator of Erk than Raf-1 (221) suggesting prolonged activation of Erk in WEHI-231 cells may also be enhanced by B-Raf and this might represent a Ras-independent mechanism of inducing Erk. Indeed, preliminary data suggests that untreated WEHI-231 cells display cyclic activation of Rap1, an activator of B-Raf and this appears to be regulated by ligation of the BCR and CD40 (428).

To investigate the regulation of Ras by the BCR in WEHI-231 cells, the role of the adaptor protein Dok-1 was examined since Dok can differentially regulate the activity of RasGAP in various cellular systems (244, 400-402). Inhibition of Dok-1, by expressing Dok-PH/PTB in WEHI-231 cells, enhanced the activity of Erk in unstimulated and BCR-activated but not in CD40-stimulated cells (figure 3.15). This suggests that Dok-1 normally suppresses Erk in WEHI-231 cells and this may represent an additional mechanism of BCR-dependent suppression of Erk. In T cells, Dok-1 is tyrosine-phosphorylated downstream of the CD2 receptor leading to the recruitment and activation of Crk-L (399, 403). Crk can function as an adaptor protein that binds to SOS and C3G (171, 172) leading to either the activation or inhibition of Erk-MAPK. Similarly, the activity of Erk might be regulated in WEHI-231 cells via the Dok-1-mediated induction of Crk.

The functional significance of Ras activation in regulating the survival and proliferation of WEHI-231 cells was examined using WEHI-231 cells transfected with $RasV^{12}$, $RasV^{12}S^{35}$ and $RasV^{12}C^{40}$. These studies showed that expression of constitutively active forms of Ras can partially protect WEHI-231 cells from BCR-driven growth arrest and apoptosis. Indeed, after 24 hr, expression of $RasV^{12}$, $RasV^{12}S^{35}$ or $RasV^{12}C^{40}$ prevented BCR-driven growth arrest (figure 3.12). Furthermore, after 48 hr, BCR-driven apoptosis was impaired by expression of these constitutively active forms of Ras (figure 3.12). $RasV^{12}C^{40}$ was particularly effective at protecting WEHI-231 cells indicating the induction of PI-3-K by Ras is important for promoting survival and proliferation of the cells. In addition to being able to induce sustained Erk signals to enable proliferation of WEHI-231 cells, expression of $RasV^{12}C^{40}$ was able to induce Bcl-x_L presumably to enhance the survival of the cells (figure 3.14) since Bcl-x_L is an important survival factor of WEHI-231 cells (292, 293, 305, 427). Expression of $RasV^{12}$ and $RasV^{12}S^{35}$ also increased the levels of Bcl-x_L indicating Ras is likely to

induce Bcl-x_L via Raf-1 and/or PI-3-K. However, expression of $RasV^{12}C^{40}$, unlike $RasV^{12}$ or $RasV^{12}S^{35}$, was able to induce Bcl-x_L in BCR-activated WEHI-231 cells (figure 3.14) suggesting Ras-dependent activation of PI-3-K can impair BCR-driven apoptosis via the induction of Bcl-x_L. These observations were supported by enriching PI-3-K signalling via the inhibition of the phosphatase SHIP since inhibition of SHIP enhanced the activity of Erk and induced Bcl-x_L in WEHI-231 cells resulting in partial protection from BCR-dependent growth arrest and apoptosis (figures 3.17-3.19).

7.3 Bcl-x_L is a key mediator of CD40-dependent rescue from BCR-driven apoptosis

Previously it has been shown that a key event in the stimulation of BCR-driven apoptosis in WEHI-231 cells is the induction of early (≤2 hr) Erk signals (328) contributing to the activation of $cPLA_2$ and leading to the production of arachidonic acid and subsequent disruption of the mitochondrial membrane potential. The loss of mitochondrial function causes cellular levels of ATP to be depleted but cytochrome c does not seem to be released into the cytosol. Consistent with this, BCR-driven apoptosis does not appear to be mediated by caspases but instead the cathepsin family of proteases is responsible for execution of the cell (102). Coupling of the BCR to this apoptotic pathway can be abrogated by T cell-derived signals including exposure to IL-4 (218) and ligation of CD40 (102, 328). However, it has been widely reported that CD40 can upregulate the expression of Bcl-2 family members including Bcl-x_L therefore the role of Bcl-x_L in CD40-mediated rescue from BCR-driven growth arrest and apoptosis was examined in WEHI-231 cells. This investigation showed that Bcl-x_L is a key mediator of CD40-dependent rescue from BCR-driven apoptosis. Indeed, ligation of CD40 on immature B cells causes Bcl-x_L to be upregulated and this can be mimicked using WEHI-231 cells that are transfected with the pSFFV-Neo plasmid containing the human *bcl-x_L* gene (Bcl-x_L WEHI-231 cells) (figure 4.2). Bcl-x_L WEHI-231 cells are protected from BCR-driven apoptosis and have impaired apoptosis induced by exogenous arachidonic acid (figures 4.3-4.5 and 4.8). Bcl-x_L WEHI-231 cells are also protected from BCR- and AA-mediated disruption of mitochondrial membrane potential and have impaired BCR-dependent activation of cathepsin B

(appendix 7). Taken together, these observations suggest that Bcl-x_L mediates CD40-dependent survival by protecting WEHI-231 cells downstream of arachidonic acid, presumably by ensuring the mitochondrial membrane integrity is retained.

Nonetheless, expression of Bcl-x_L can also impair anti-Ig-induced activation of $cPLA_2$ and subsequent arachidonic acid production (appendix 7) suggesting Bcl-x_L not only protects the mitochondrial membrane integrity but also impairs BCR-dependent induction of $cPLA_2$. The mechanism of Bcl-x_L-dependent suppression of $cPLA_2$ activity is incompletely understood. Indeed, evidence is presented in this study showing that exogenous arachidonic acid might enhance early pro-apoptotic Erk signals and whilst ligation of CD40 can impair AA-mediated induction of transient Erk signals, overexpression of Bcl-x_L did not prevent arachidonic acid from inducing early Erk activity (figure 4.16). However, the activity of $cPLA_2$ is dependent on both calcium and Erk (222, 289) therefore an alternative potential mechanism of Bcl-x_L-dependent suppression of $cPLA_2$ is to impair calcium signalling. Indeed, Bcl-x_L has been reported to impair calcium signalling in murine T cells by decreasing the expression of IP_3 receptors (510). The ability of Bcl-x_L to regulate Ca^{2+} flux in WEHI-231 cells has yet to be explored.

Bcl-x_L is thus a very important survival factor of WEHI-231 cells that can suppress the BCR-activated mitochondrial death pathway to mediate CD40-dependent survival. Nonetheless, additional pro-survival Bcl-2 family members may also be induced downstream of the BCR in WEHI-231 cells. For example, ligation of CD40 can increase the expression of A1 and ectopic expression of A1 can partially protect WEHI-231 cells from BCR-driven apoptosis (302, 304, 317). However, A1 appears to impair caspase-dependent apoptosis rather than target the BCR-activated mitochondrial death pathway and disruption of the mitochondrial membrane integrity has been reported to be the major step that commits WEHI-231 cells to BCR-driven apoptosis (102) therefore inhibition of this stage is critical for CD40-induced survival. Taken together, this suggests that CD40-dependent rescue from BCR-driven apoptosis critically requires the induction of Bcl-x_L since this can prevent the mitochondrial death pathway. However, additional Bcl-2 family proteins may enhance the viability of the cells by impairing alternative apoptotic pathways. To further understand the

mechanism of CD40-dependent survival, it would be interesting to investigate the expression and intracellular location of pro-apoptotic bcl-2 family members since these are known antagonists of Bcl-x_L and other pro-survival Bcl-2 family proteins (67).

Although Bcl-x_L is a key mediator of CD40-dependent rescue from BCR-driven apoptosis, the expression Bcl-x_L does not protect WEHI-231 cells from BCR-driven growth arrest (figures 4.4-4.7 and 4.9) suggesting distinct mechanisms regulate survival and proliferation of these cells. Furthermore, expression of Bcl-x_L was unable to mimic CD40 and restore sustained Erk signals to BCR-stimulated WEHI-231 cells (figure 4.10). This is consistent with the suggestion that a key event in CD40-mediated rescue from BCR-driven growth arrest is the reversal of BCR-dependent abrogation of sustained and cyclic activation of Erk1/2. Taken together, these observations suggest that Bcl-x_L is a key mediator of CD40-dependent rescue from BCR-driven apoptosis but additional CD40-derived signals are required to restore proliferation to BCR-stimulated WEHI-231 cells.

To further investigate CD40-dependent rescue of WEHI-231 cells the mechanism of CD40-mediated induction of sustained Erk signals and hence proliferation was examined. Arachidonic acid is a key mediator of BCR-driven apoptosis but arachidonic acid is also a substrate for Cox and Lox enzymes and the products of arachidonic metabolism have been implicated in regulating survival and proliferation in various cellular systems. Inhibitors of Cox2 and Lox were thus used to investigate the importance of arachidonic acid metabolism in CD40-dependent rescue of WEHI-231 cells. Inhibition of Cox/Lox induced growth arrest and apoptosis of WEHI-231 cells (figures 4.11-4.12 and appendix 8). The induction of apoptosis is likely to be caused by the accumulation of arachidonic acid thus implicating a causal role for BCR-dependent arachidonic acid production in mediating the Bcl-x_L-sensitive mitochondrial death pathway.

However, the induction of growth arrest by Cox/Lox inhibitors also suggested that metabolites of arachidonic acid can promote CD40-dependent proliferation of WEHI-231 cells since these inhibitors impaired CD40-dependent rescue from BCR-driven growth arrest (figure 4.13). In particular, ligation of the BCR appears to reduce the levels of PGE_2 in WEHI-231 cells by decreasing the expression of Cox2 whilst co-ligation of CD40 can induce Cox2 and restore

PGE_2 levels (appendix 9). Expression of Bcl-x_L was unable to mimic CD40 and restore PGE_2 levels suggesting metabolites of arachidonic acid regulate proliferation rather than survival of WEHI-231 cells (figure 4.14A). Furthermore, inhibition of Cox/Lox impaired CD40-mediated activation of sustained Erk signals (figure 4.14B) suggesting metabolites of arachidonic acid can promote proliferation of WEHI-231 cells via the induction of Erk. Ligation of CD40 therefore seems to induce Cox2 and possibly other Cox/Lox enzymes to increase the production of PGE_2 and other prostaglandins/leukotrienes from arachidonic acid. This has two effects: firstly, it reduces the cellular levels of arachidonic acid and hence impairs the mitochondrial death pathway; secondly, it produces metabolites of arachidonic acid that can enhance sustained Erk signals and promote proliferation of the WEHI-231 cells.

Taken together, these results highlight two key mechanisms of CD40-dependent rescue of BCR-stimulated WEHI-231 cells. Firstly, ligation of CD40 induces Bcl-x_L resulting in a suppression of the BCR-driven mitochondrial death pathway. Secondly, CD40 induces Cox2 to increase the metabolism of arachidonic acid. This impairs the BCR-driven apoptotic pathway and enhances immature B cell proliferation via the production of PGE_2 and the induction of sustained Erk signals.

7.4 Role of PKC and sphingolipids in regulating WEHI-231 survival and proliferation

The results presented in chapter 3 implicated Ras as an important upstream activator of Erk. Expression of $RasV^{12}C^{40}$, which specifically couples to PI-3-K, was particularly effective at promoting survival and proliferation of WEHI-231 cells. In addition to promoting the activation of Erk1/2 (figure 3.11), Ras-dependent activation of PI-3-K also seems to induce Bcl-x_L (figure 3.14) and, as described in chapter 4, Bcl-x_L is an important pro-survival protein of WEHI-231 cells. Interestingly, the pro-survival protein Akt does not appear to be activated downstream of PI-3-K since no Akt activity was detected in WEHI-231 cells even when they were expressing constitutively active forms of Ras or mutant forms of SHIP (figures 3.13 and 3.19). Non-phosphorylated Akt was easily detected in WEHI-231 cells suggesting the activation of Akt was suppressed in these cells. This may have been due to compartmentalisation

separating Akt from its upstream activators or perhaps Akt inhibitors such as PP2A (185) were abundant.

In some respects this observation was surprising since Akt is activated downstream of PI-3-K in BCR-stimulated splenic B lymphocytes. However, the induction of Akt in mature B cells promotes survival by stimulating NF-κB and NF-AT (188, 193-195) and the BCR of immature B cells cannot sustain these signals (261, 296, 297). Nonetheless, ligation of CD40 on immature B cells is reported to induce NF-κB (297). The lack of Akt activity in CD40-stimulated WEHI-231 cells suggests that immature and mature B cells may use different mechanisms to induce NF-κB. A potential mediator of CD40-dependent activation of NF-κB in immature B cells is PKC since various PKC isoforms have been reported to contribute to the induction of NF-κB in lymphocytes. For example, PKCβ contributes to the activation of NF-κB in mature B cells (199), PKCθ in T cells and PKCλ in pre-B cells (212).

Indeed, several PKC isoforms have been associated with promoting survival and proliferation of mature B cells via the induction of pro-survival Bcl-2 family proteins and NF-κB (193, 199, 210, 211, 290). Immature B cells display impaired PKC signalling compared to mature B cells but treatment of primary splenic immature B cells or WEHI-231 cells with phorbol ester can prevent BCR-driven apoptosis suggesting the diminished activation of cPKC and nPKC isoforms in immature B cells contributes to their cell death (294, 295). Indeed, previous studies have shown that the BCR and CD40 on immature B cells can regulate the expression of multiple PKC isoforms and PKCα, PKCδ and PKCζ were highlighted by antisense studies as potential mediators of CD40-dependent rescue from anti-Ig-induced growth arrest (unpublished observations). The present study now shows that ectopic expression of PKCα, PKCδ, PKCε or PKCζ in WEHI-231 cells can affect the survival and proliferation of WEHI-231 cells. In particular, expression of PKCα, PKCδ and PKCζ slightly impaired BCR-driven apoptosis (figures 6.2, 6.5 and 6.12). PKCα and PKCδ appear to do so by inducing Bcl-x_L (figures 6.4 and 6.7) suggesting the expression of Bcl-x_L can be regulated downstream of these PKC isoforms in WEHI-231 cells. Furthermore, NF-κB might mediate the induction of Bcl-x_L by PKCα and PKCδ since PKC has been observed to induce NF-κB in lymphocytes and in mature B cells NF-κB induces Bcl-x_L (193, 199, 211).

However, PKCζ did not induce Bcl-x_L (figure 6.14) and is likely to either promote survival of WEHI-231 cells independently of Bcl-x_L or by acting downstream of this pro-survival protein.

Moreover, expression of PKCα, PKCδ, PKCε or PKCζ also promoted proliferation of WEHI-231 cells and each of these isoforms of PKC impaired BCR-driven growth arrest (figures 6.2, 6.5, 6.8 and 6.12). PKCα, PKCδ, PKCε and PKCζ appear to promote proliferation, at least in part, by enhancing sustained Erk signals in WEHI-231 cells (figures 6.3, 6.6, 6.9 and 6.13). Several isoforms of PKC can thus regulate the survival and proliferation of WEHI-231 cells via the induction of Bcl-x_L and Erk, respectively but also via additional, as yet unknown, mechanisms. Indeed, analysis of mutant forms of PKC showed that the kinase activity of PKCα, PKCδ, PKCε and PKCζ was not essential for all of these effects suggesting the regulatory regions of PKC enzymes can also mediate important signalling functions in WEHI-231 cells.

Sphingolipids are known regulators of specific isoforms of PKC including PKCα and PKCζ. The role of the sphingosine kinase and hence SPP in regulating the survival and proliferation of WEHI-231 cells was thus investigated. Previous evidence has suggested that ligation of the BCR reduces the levels of spontaneously cycling SPP in WEHI-231 cells whilst co-ligation of CD40 restores SPP levels (428). Indeed, inhibition of sphingosine kinase using DMS induced growth arrest and apoptosis of WEHI-231 cells and synergistically enhanced BCR-driven growth arrest and apoptosis (figures 5.2-5.7). However, DMS did not mimic anti-Ig in suppressing sustained Erk signals (figure 5.16) suggesting SPP acts downstream of Erk to promote proliferation of WEHI-231 cells. This suggests that SPP is not an upstream activator of PKCα, PKCδ or PKCε since the activation of each of these kinases impaired BCR-dependent suppression of sustained Erk signals. However, these observations do not eliminate the possibility of PKCζ being activated downstream of SPP to promote proliferation of WEHI-231 cells.

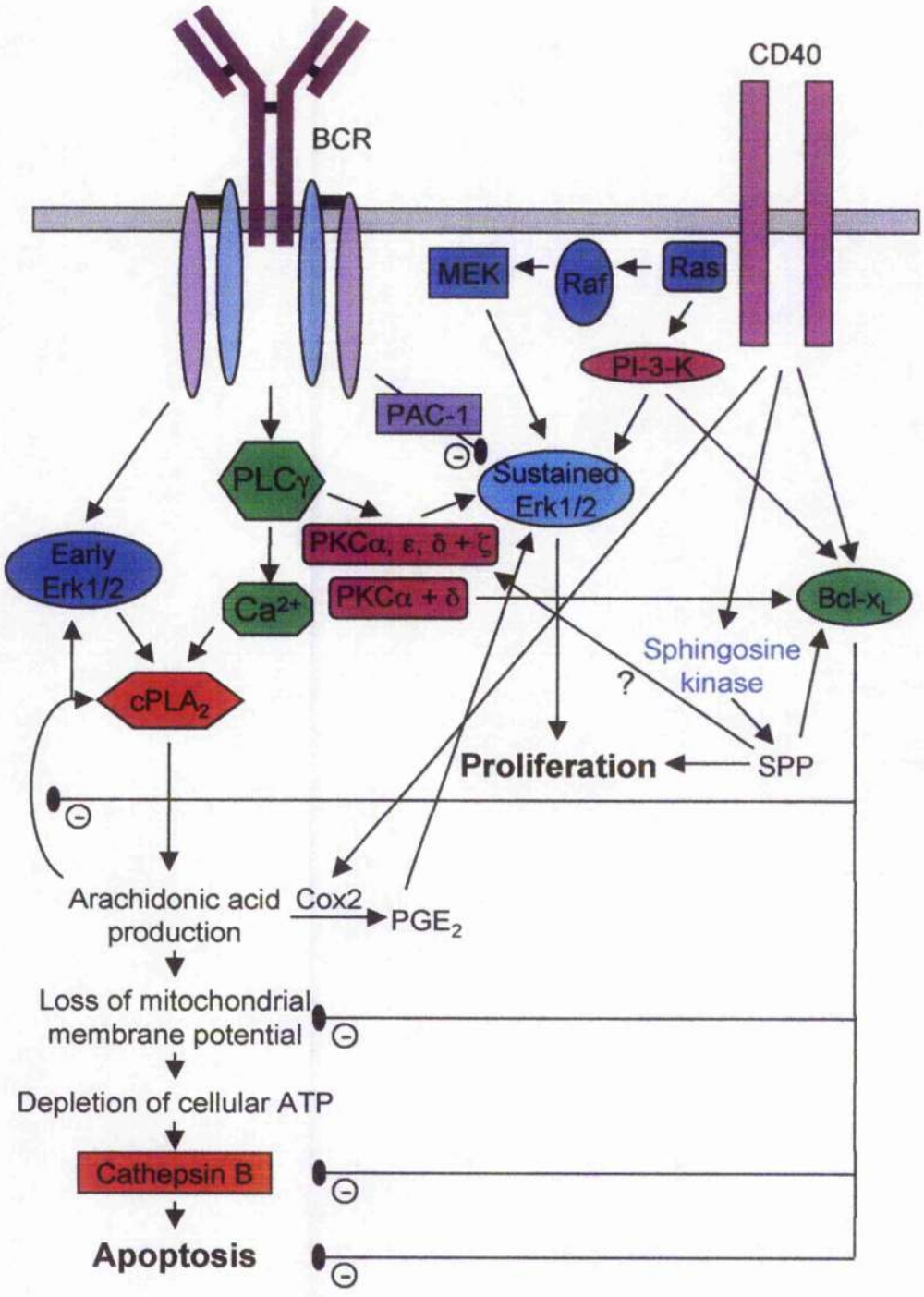
Ligation of CD40 partially protected WEHI-231 cells from DMS-induced growth arrest and apoptosis (figures 5.6-5.7). Furthermore, overexpression of Bcl-x_L protected WEHI-231 cells from DMS-driven apoptosis and was actually more effective than ligation of CD40 (figures 5.8-5.9). An explanation for this was provided by the observation that DMS impaired CD40-dependent induction

of Bcl-x_L (figure 5.10) suggesting one mechanism of CD40-dependent induction of Bcl-x_L is via the upregulation of SPP. Nonetheless, ligation of CD40 further enhanced the survival of Bcl-x_L WEHI-231 cells treated with DMS (figure 5.11). This suggests that CD40 may also induce Bcl-x_L via SPP-independent mechanisms. This is consistent with the $RasV^{12}$ data that implicated PI-3-K as an upstream mediator of Bcl-x_L (figure 3.14). Alternatively, CD40 might promote survival of WEHI-231 cells via additional Bcl-x_L-independent mechanisms. Indeed, CD40 has been found to increase the levels of Bcl-2, A1 and Mcl-1 in WEHI-231 cells (427).

7.5 Concluding remarks

This investigation has demonstrated that the differential regulation of Erk-MAPK signals and Bcl-x_L by the BCR and CD40 is critical for determining the survival and proliferation of WEHI-231 immature B cells. Much of this investigation has focused on the temporal regulation of Erk1/2 and Bcl-x_L in WEHI-231 cells and the mechanisms used by the BCR and CD40 to control these signalling molecules. Indeed, a complex network of signalling pathways (figure 7.1) has been shown to regulate the activity of Erk-MAPK and the expression of Bcl-x_L and this is likely to ensure the survival and proliferation of WEHI-231 cells is carefully regulated. However, future work into these signalling networks is expected to reveal additional levels complexity as new mediators of these pathways are discovered and as the intracellular compartmentalisation of signalling complexes is more closely examined.

Figure 7.1. The differential regulation of Erk and Bcl-x_L by the BCR and CD40 is mediated by a complex network of signalling pathways enabling the survival and proliferation of WEHI-231 cells to be carefully regulated.


CD40
BCR
MEK
Raf
Ras
PI-3-K
PAC-1
PLCγ
Sustained Erk1/2
PKCα, ε, δ + ζ
Early Erk1/2
Ca2+
PKCα + δ
Bcl-xL
Sphingosine kinase
?
cPLA2
Proliferation
SPP
Arachidonic acid production
Cox2
PGE2
Loss of mitochondrial membrane potential
Depletion of cellular ATP
Cathepsin B
Apoptosis

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PUBLICATIONS

Stephen B. Gauld, Derek Blair, **Catriona A. Moss**, Steven D. Reid and Margaret M. Harnett. Differential roles for extracellularly regulated kinase-mitogen-activated protein kinase in B cell antigen receptor-induced apoptosis and CD40-mediated rescue of WEHI-231 immature B cells. J Immunol 2002;168(8):3855-3864.

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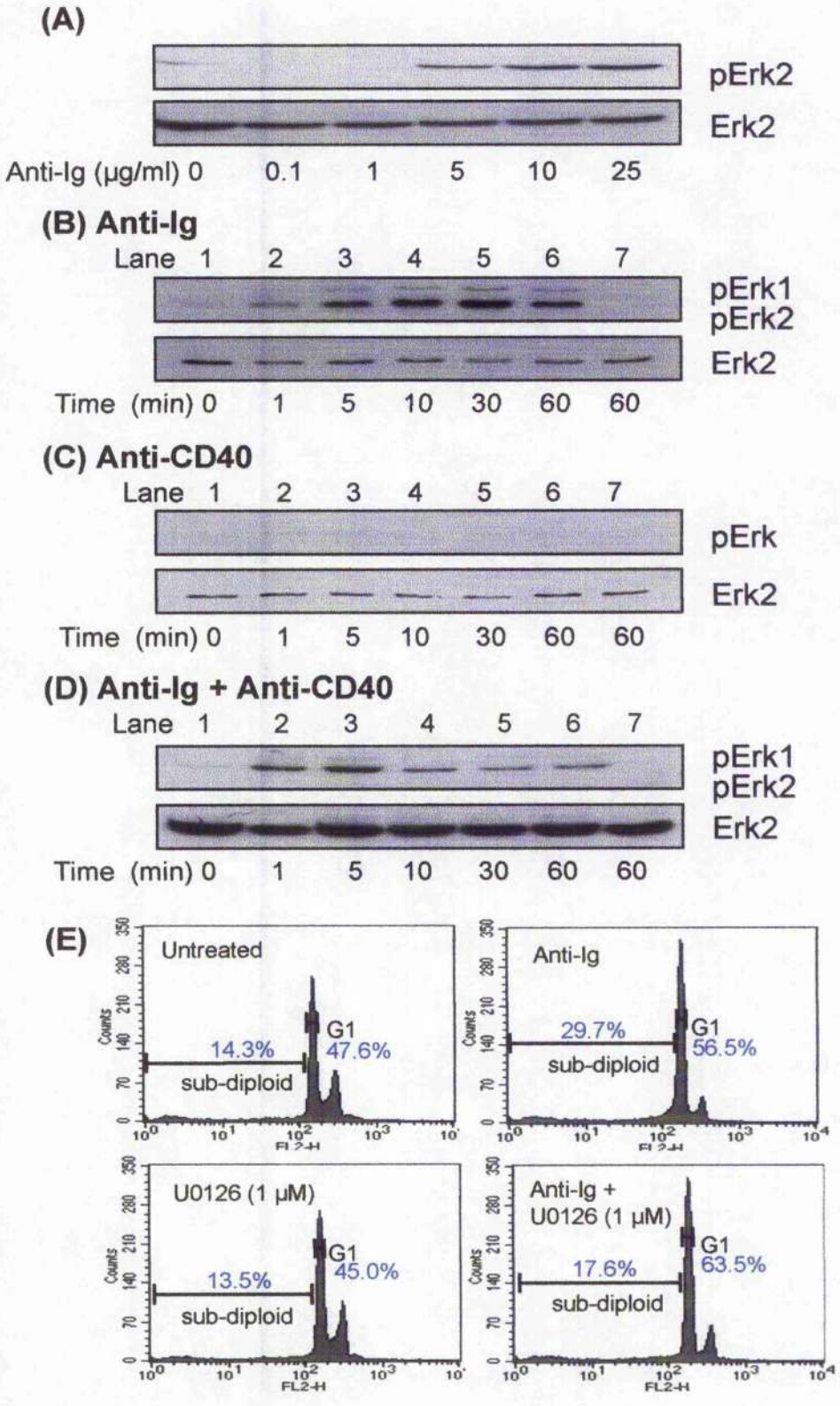
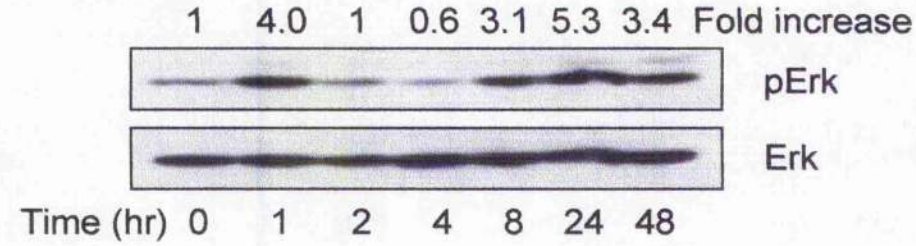
Appendix 1. BCR-mediated induction of an early Erk-MAPK signal is associated with commitment to apoptosis of WEHI-231 cells and inhibition of early Erk signals abrogates BCR-mediated apoptosis of WEHI-231 cells. BCR-mediated induction of an early Erk-MAPK signal is associated with commitment to apoptosis of WEHI-231 cells. **(A)** WEHI-231 cells (1 x 10^8/ml) were stimulated for 30 min with 0-25 µg/ml anti-Ig, as indicated. **(B)** WEHI-231 cells (1 x 10^8/ml) were stimulated with medium (lanes 1 and 7) or anti-Ig (10 µg/ml, lanes 2-6) for 0-60 min, as indicated. **(C)** WEHI-231 cells (1 x 10^8/ml) were stimulated with medium (lanes 1 and 7) or anti-CD40 (10 µg/ml, lanes 2-6) for 0-60 min, as indicated. **(D)** WEHI-231 cells (1 x 10^8/ml) were stimulated with medium (lanes 1 and 7) or a combination of anti-Ig plus anti-CD40 (both at 10 µg/ml, lanes 2-6) for 0-60 min, as indicated. Levels of pErk/Erk expression were determined by Western blotting (15 µg/lane). Densitometry indicates relative band density of phospho-p42 Erk-MAPK for anti-Ig-treated cells as: *lane 1*, 1; *lane 2*, 3; *lane 3*, 13; *lane 4*, 22; *lane 5*, 29; *lane 6*, 16; *lane 7*, 0.3. Densitometry indicates relative band density of phospho-p42 Erk-MAPK for anti-Ig plus anti-CD40-treated cells as: *lane 1*, 1; *lane 2*, 18; *lane 3*, 28; *lane 4*, 10; *lane 5*, 7; *lane 6*, 8; *lane 7*, 0.5. **(E)** Inhibition of early Erk signals abrogates BCR-mediated apoptosis of WEHI-231 cells. WEHI-231 cells (5 x 10^5) were preincubated in the absence (DMSO vehicle) or presence of 1 µM UO126 for 2 hr. Cells were then treated with medium (untreated) or anti-Ig (10 µg/ml) for 48 hr. Levels of apoptosis were determined by PI-staining and FACS analysis with FL2 fluorescence after 48 hr. This figure was taken from (328) and full details of materials and methods can be found in this reference.

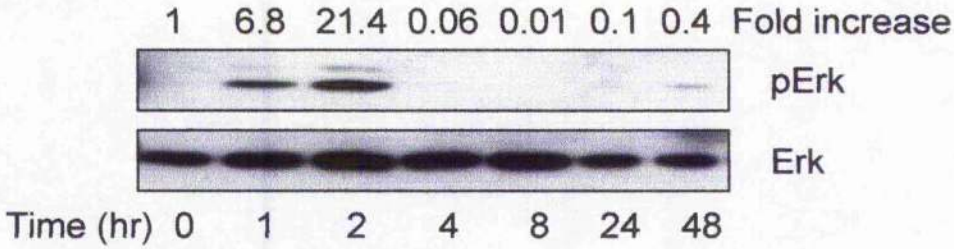

(A)
pErk2
Erk2
Anti-Ig (μg/ml) 0 0.1 1 5 10 25
(B) Anti-Ig
Lane 1 2 3 4 5 6 7
pErk1
pErk2
Erk2
Time (min) 0 1 5 10 30 60 60
(C) Anti-CD40
Lane 1 2 3 4 5 6 7
pErk
Erk2
Time (min) 0 1 5 10 30 60 60
(D) Anti-Ig + Anti-CD40
Lane 1 2 3 4 5 6 7
pErk1
pErk2
Erk2
Time (min) 0 1 5 10 30 60 60
(E)
Untreated
14.3%
sub-diploid
G1
47.6%
Anti-Ig
29.7%
sub-diploid
G1
56.5%
U0126 (1 μM)
13.5%
sub-diploid
G1
45.0%
Anti-Ig +
U0126 (1 μM)
17.6%
sub-diploid
G1
63.5%
Counts
FL2-H

Appendix 2. Apoptotic signalling via the BCR suppresses sustained cyclic activation of Erk that can be re-established by CD40-mediated rescue. WEHI-231 cells (5 x 10^5/ml) were stimulated with medium (untreated) **(A)**, 1 µg/ml anti-Ig **(B)**, 1 µg/ml anti-Ig plus 10 µg/ml anti-CD40 **(C)** or 10 µg/ml anti-CD40 **(D)** for 0-48 hr, as indicated. Levels of phospho-Erk/total Erk were determined by Western blotting (30 µg/lane). Densitometric analysis shows fold increase of p42 Erk-MAP kinase relative to the 0 hr samples for all treatments. These experiments were performed in collaboration with Stephen Gauld and this figure was taken from (328). This data is representative of at least eight independent experiments.

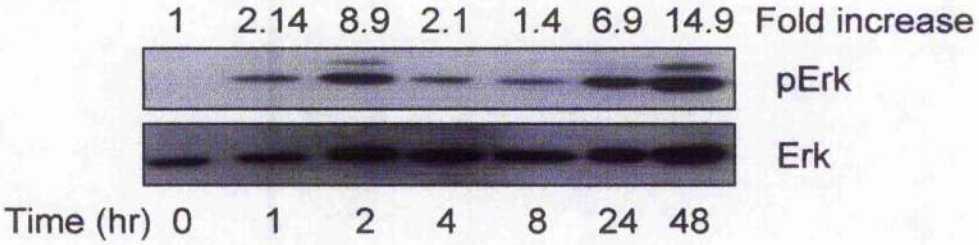
(A) Untreated



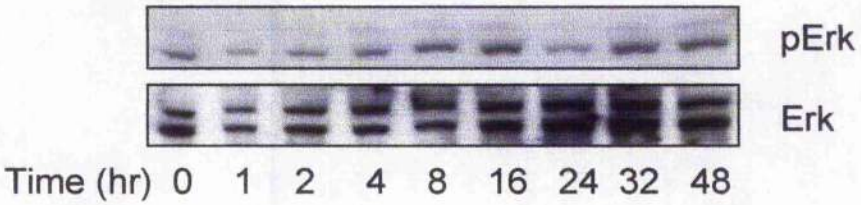
(B) Anti-Ig



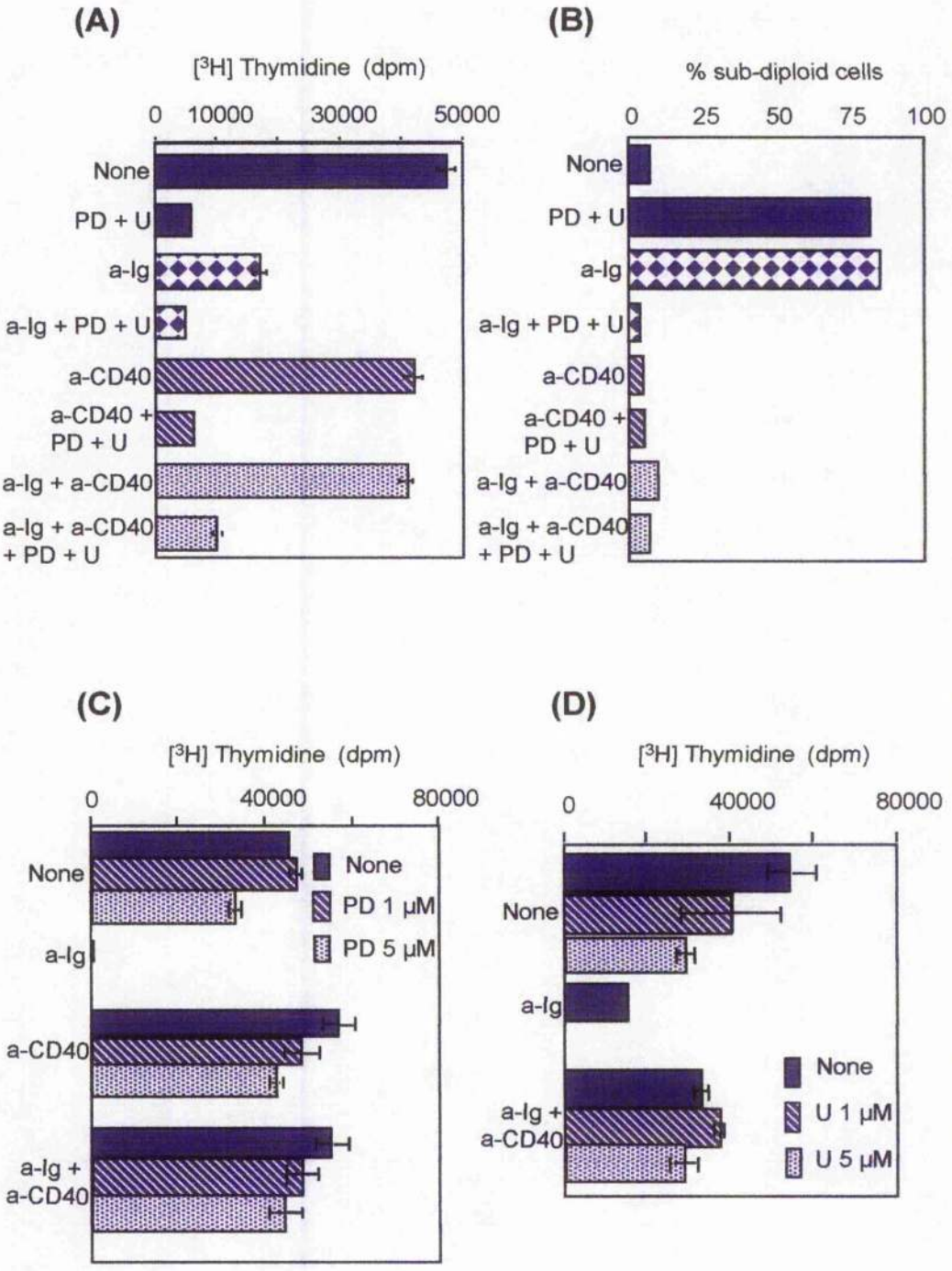
(C) Anti-Ig + Anti-CD40



(D) Anti-CD40

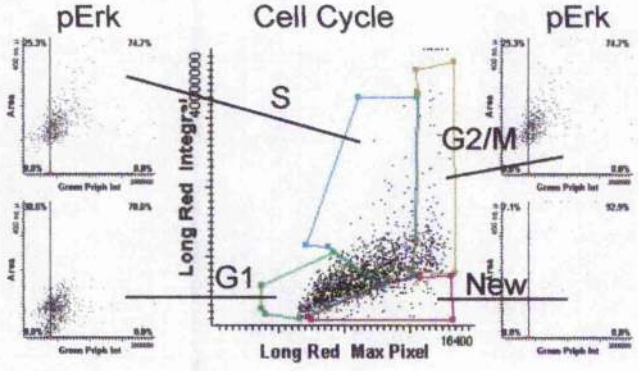


Appendix 3. Inhibition of sustained cyclic activation of Erk-MAPK can block basal proliferation and CD40-mediated rescue. **(A)** WEHI-231 cells (1 x 10^4 cells/well) were preincubated for 3 hr with DMSO vehicle or PD98059 plus U0126 (PD + U, both at 1 μM). Cells were then treated as indicated with 10 μg/ml anti-Ig (a-Ig), 10 μg/ml anti-CD40 (a-CD40), or a combination of both (a-Ig + a-CD40). Cells treated with PD98059 and UO126 were treated with an additional dose (1 μM or DMSO vehicle for cells lacking inhibitors) every 4 hr during 28 hr post-addition of anti-Ig and/or anti-CD40. Proliferation was assessed by measuring [^{3}H] thymidine uptake at 48 hr. Values are means ± standard deviation of quadruplicate wells. **(B)** WEHI-231 cells (5 x 10^5 cells/well) were preincubated with PD98059 (1 μM) plus UO126 (1 μM) or DMSO vehicle for 3 hr. Cells were then stimulated as indicated with 10 μg/ml anti-Ig, 10 μg/ml anti-CD40 or a combination of both. Cells originally treated with PD98059 plus UO126 were treated with additional doses (1 μM or DMSO vehicle for cells lacking inhibitors) every 4 hr for a total of 28 hr post-addition of anti-Ig and/or anti-CD40. Cells were harvested after 48 hr and DNA content was analysed by PI-staining and FACS analysis. **(C)** WEHI-231 cells (1 x 10^4 cells/well) were preincubated for 3 hr in PD98059 (1 and 5 μM) or DMSO vehicle (none) before stimulation with medium (none), anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml) or a combination of both. Proliferation was assessed by measuring [^{3}H] thymidine uptake at 48 hr. Values are means ± standard deviation of quadruplicate results. **(D)** WEHI-231 cells (1 x 10^4 cells/well) were preincubated for 3 hr in UO126 (1 or 5 μM) or DMSO vehicle before stimulation with medium, anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both at 10 μg/ml). Proliferation was assessed by measuring [^{3}H] thymidine uptake at 48 hr. Values are means ± standard deviation of quadruplicate wells. This figure was taken from (328) and full details of materials and methods can be found in this reference.


(A)
[3H] Thymidine (dpm)
0
10000
30000
50000
None
PD + U
a-Ig
a-Ig + PD + U
a-CD40
a-CD40 + PD + U
a-Ig + a-CD40
a-Ig + a-CD40 + PD + U
(B)
% sub-diploid cells
0
25
50
75
100
None
PD + U
a-Ig
a-Ig + PD + U
a-CD40
a-CD40 + PD + U
a-Ig + a-CD40
a-Ig + a-CD40 + PD + U
(C)
[3H] Thymidine (dpm)
0
40000
80000
None
a-Ig
a-CD40
a-Ig + a-CD40
None
PD 1 μM
PD 5 μM
(D)
[3H] Thymidine (dpm)
0
40000
80000
None
a-Ig
a-Ig + a-CD40
None
U 1 μM
U 5 μM

Appendix 4. Erk activation is associated with cell cycle progression and cell cycle arrest is associated with suppression of Erk signals. **(A)** WEHI-231 cells cultured for 48 hr in medium were analysed for PI-staining of DNA and intracellular staining of phospho-Erk. The distribution of cells in the phases of cell cycle and exhibiting phospho-Erk staining were, respectively: G1, 46% (70.1% phospho-Erk); S phase, 30.8% (74.8% phospho-Erk); G2/M 11.6% (68.7% phospho-Erk); and newly divided cells, 9.2% (30.2% phospho-Erk). **(B)** WEHI-231 cells were cultured in the absence (media) or presence of olomoucine (50 μM) for 40 hr. The cells were washed twice in sterile medium before being resuspended at 2 x 10^5 cells/ml and stimulated with anti-Ig (10 μg/ml), anti-Ig plus anti-CD40 (both at 10 μg/ml) or medium alone for up to 48 hr, as indicated. Cytospins were then fixed, permeabilised and stained for PI and phospho-Erk before analysis by LSC. This figure was taken from (328) and full details of materials and methods can be found in this reference.

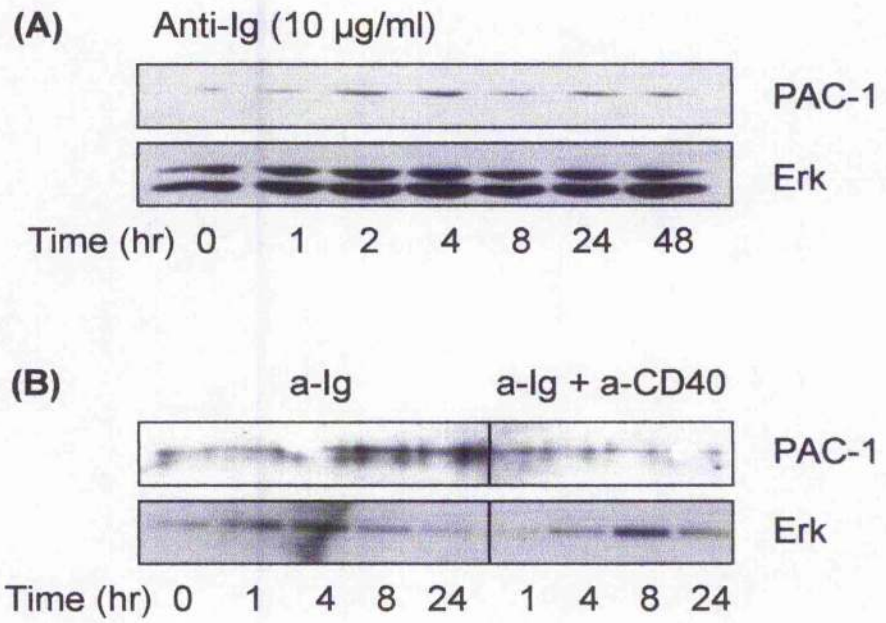
(A)



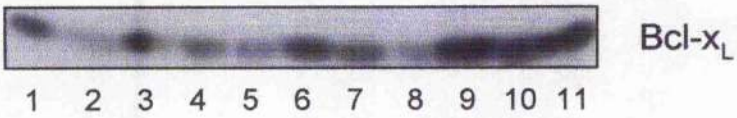
(B)

Culture conditions	%in G1 (% pErk)	% in S phase (% pErk)	% in G2/M (% pErk)	New Cells (% pErk)
Media/media 48 hr	46.0 (70.1)	30.8 (74.8)	11.6 (68.7)	9.2 (30.3)
Media/anti-Ig 48 hr	68.9 (35.6)	12.8 (66.9)	3.1 (84.9)	11.8 (27.2)
Olomoucine/media 24 hr	75.1 (35.9)	18.4 (57.2)	0.9 (92.9)	0.8 (46.3)
Olomoucine/media 48 hr	0.2 (66.7)	6.3 (76.9)	72 (85.3)	13.1 (48.0)
Olomoucine/anti-Ig + anti-CD40 48 hr	31.5 (52.2)	29.0 (81.2)	26.3 (79.8)	8.0 (40.6)

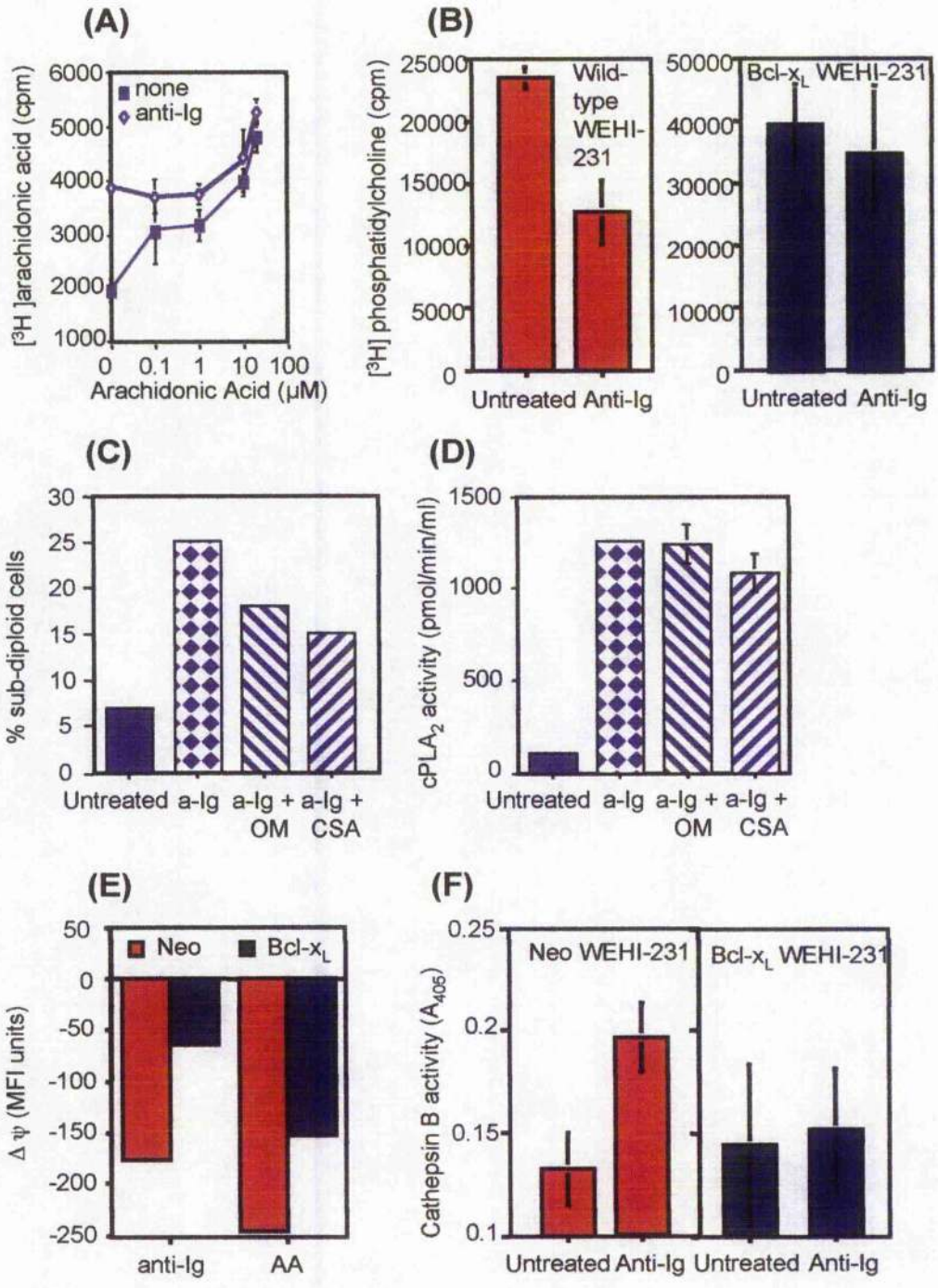
Appendix 5. BCR-mediated downregulation of Erk activity involves the induction of PAC-1. **(A)** WEHI-231 cells (1×10^7) stimulated with anti-Ig (10 μg/ml) for up to 48 hr were lysed and subjected to Western blot analysis of PAC-1 or Erk expression, as indicated. **(B)** Erk2-containing immune complexes derived from WEHI-231 cells stimulated with anti-Ig (10 μg/ml) or anti-Ig plus anti-CD40 (both at 10 μg/ml) for up to 24 hr, as indicated, were analysed for PAC-1 or Erk2 expression by Western blotting. This figure was taken from (328) and full details of materials and methods can be found in this reference.


(A)
Anti-Ig (10 μg/ml)
PAC-1
Erk
Time (hr) 0 1 2 4 8 24 48
(B)
a-Ig
a-Ig + a-CD40
PAC-1
Erk
Time (hr) 0 1 4 8 24 1 4 8 24

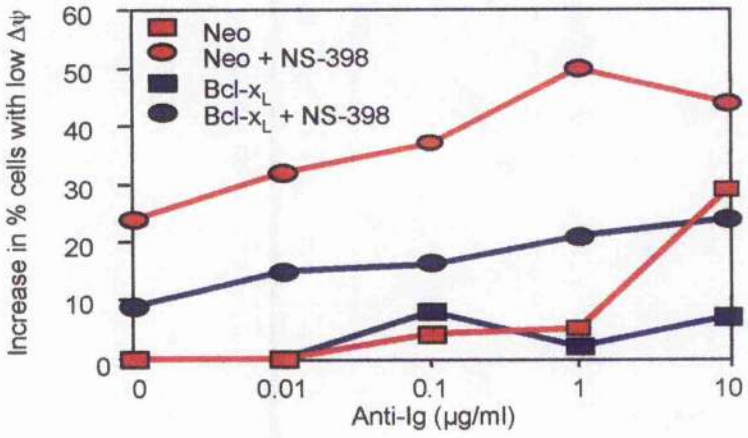
Appendix 6. Bcl-x_L is upregulated downstream of CD40. Wild-type WEHI-231 cells (1 x 10^7cells/lane) were cultured with medium, anti-Ig (5 µg/ml), anti-CD40 (10 µg/ml) or a combination of anti-Ig (5 µg/ml) plus anti-CD40 (10 µg/ml) for up to 48 hr. Cell lysates were prepared and Western blot analysis of Bcl-x_L expression was performed. Experimental conditions were as follows: *lane 1*, medium 0 hr; *lane 2*, anti-Ig 8 hr; *lane 3*, anti-CD40 8 hr; *lane 4*, anti-Ig plus anti-CD40 8 hr; *lane 5*, anti-Ig 24 hr; *lane 6*, anti-CD40 24 hr; *lane 7*, anti-Ig plus anti-CD40 24 hr; *lane 8*, anti-Ig 48 hr; *lane 9*, anti-CD40 48 hr; *lane 10*, anti-Ig plus anti-CD40 48 hr; *lane 11*, medium 48 hr. This figure was taken from (427) and full details of materials and methods can be found in this reference.


Bcl-x_L
1 2 3 4 5 6 7 8 9 10 11

Appendix 7. Overexpression of Bcl-x_L suppresses coupling of the BCR to mitochondrial PLA_2 activation. **(A)** Increasing concentrations (0-100 μM, as indicated) of AA induce PLA_2 activation and enhance anti-Ig (1 μg/ml)-stimulated PLA_2 activation as determined by the increased release (intracellular + extracellular) of [^{3}H] arachidonic acid from cellular [^{3}H] arachidonic acid-labelled phosphatidylcholine. This was measured by thin layer chromatography of cellular lipids in hexane: diethylether: formic acid (80: 20: 2, by volume). **(B)** Due to the loss of [^{3}H] arachidonic acid during the cellular fractionation process, anti-Ig (1 μg/ml)-stimulated mitochondrial PLA_2 activation was determined by the measurement of decreased levels of [^{3}H] arachidonic acid-labelled phosphatidylcholine in isolated mitochondria derived from wild-type and Bcl-x_L WEHI-231. Data are representative of at least three independent experiments. **(C)** WEHI-231 cells (5 x 10^5/ml) were cultured for 48 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-Ig (10 μg/ml) plus oligomycin (OM, 8 ng/ml) or anti-Ig (10 μg/ml) plus cyclosporin A (CSA, 1 nM) and the % apoptotic cells was measured by PI-staining of sub-diploid DNA content. **(D)** WEHI-231 cells (1 x 10^6/ml) were cultured for 3 hr with medium (untreated), anti-Ig (a-Ig, 10 μg/ml), anti-Ig (10 μg/ml) plus oligomycin (OM, 8 ng/ml) or anti-Ig (10 μg/ml) plus cyclosporin A (CSA, 1 nM) and isolated mitochondria were prepared. PLA_2 activity was assayed using a PLA_2 assay kit (Cayman). Data are presented as means ± standard deviation, where n=3 from single experiments that are representative of at least three independent experiments. **(E)** Neo or Bcl-x_L WEHI-231 cells (5 x 10^5/ml) were treated with anti-Ig (10 μg/ml) or AA (100 μM) for 24 hr and mitochondrial potential was assessed using $DiOC_6$-staining. The data are represented as the change in mean fluorescence intensity (MFI) of $DiOC_6$ staining following stimulation to demonstrate the extent of depolarisation. **(F)** Neo or Bcl-x_L WEHI-231 cells were stimulated for 24 hr with anti-Ig (10 μg/ml) and cell lysates were prepared. Cathepsin B activity as evidenced by the cleavage of the cathepsin B substrate, zRR-pNA (z-Arg-Arg-pNA, Calbiochem) was then assayed by measuring the absorbance at 405 nm. Data are representative of at least three independent experiments. This figure was taken from (427) and full details of materials and methods can be found in this reference. These experiments were performed by Elad Katz and Caroline Lord.


(A)
[3H]arachidonic acid (cpm)
6000
5000
4000
3000
2000
1000
none
anti-Ig
0 0.1 1 10 100
Arachidonic Acid (μM)
(B)
[3H] phosphatidylcholine (cpm)
25000
20000
15000
10000
5000
0
Wild-type WEHI-231
Untreated Anti-Ig
50000
40000
30000
20000
10000
0
Bcl-xL WEHI-231
Untreated Anti-Ig
(C)
% sub-diploid cells
30
25
20
15
10
5
0
Untreated a-Ig a-Ig + OM a-Ig + CSA
(D)
cPLA2 activity (pmol/min/ml)
1500
1000
500
0
Untreated a-Ig a-Ig + OM a-Ig + CSA
(E)
Δ ψ (MFI units)
50
0
-50
-100
-150
-200
-250
Neo
Bcl-xL
anti-Ig
AA
(F)
Cathepsin B activity (A405)
0.25
0.2
0.15
0.1
Neo WEHI-231
Bcl-xL WEHI-231
Untreated Anti-Ig Untreated Anti-Ig

Appendix 8. Inhibition of Cox2 enhances BCR-mediated loss of mitochondrial membrane potential in WEHI-231 cells. Neo or Bcl-x_L WEHI-231 cells (5 x 10^5/ml) were stimulated with 0-10 μg/ml anti-Ig in the absence and presence of 10 μM NS-398 for 24 hr before determining the % of cells exhibiting low mitochondrial membrane potential ($\Delta\psi$) using $DiOC_6$-staining. This figure was taken from (427) and full details of materials and methods can be found in this reference.


Increase in % cells with low Δψ
60
50
40
30
20
10
0
Neo
Neo + NS-398
Bcl-x$_L$
Bcl-x$_L$ + NS-398
0
0.01
0.1
1
10
Anti-Ig (µg/ml)

Appendix 9. Anti-Ig inhibits the production of prostaglandin E_2 in WEHI-231 cells. Wild-type WEHI-231 cells were stimulated with medium (untreated), anti-Ig (1 μg/ml), anti-CD40 (10 μg/ml) or a combination of anti-Ig (1 μg/ml) plus anti-CD40 (10 μg/ml) for 24 hr and cell lysates were prepared and PGE_2 levels estimated by competitive binding immunoassay kit. Data are presented as mean pg/ml PGE_2 ± standard deviation, n=3. In the insert, such lysates were probed for Cox2 expression by Western blot analysis. This figure was taken from (427) and full details of materials and methods can be found in this reference.