

**B CELL ANTIGEN RECEPTOR SIGNALLING:
REGULATION AND TARGETS OF THE PI3K/AKT PATHWAY**

by

SHERRI LYNN CHRISTIAN

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Abstract

The B cell receptor (BCR) is a major regulator of B cell development, activation, and cell death. The misregulation of these processes results in autoimmunity and B cell lymphoma. Engagement of the BCR activates multiple signalling pathways that are essential for normal B cell responses. However, the roles of individual signalling pathways in mediating these responses is not completely understood. Activation of the phosphatidylinositol-3-kinase (PI3K) pathway is important for the proper regulation of B cell survival and development. In this thesis I have investigated the regulation and targets of the PI3K pathway in B cells. A putative target of the PI3K pathway is β -catenin, a transcriptional activator with important roles in early development. I found that the BCR regulates β -catenin levels via the activation of the phospholipase-C/protein kinase C/glycogen synthase kinase-3 pathway and is partially dependent on PI3K. Signalling by the BCR also activates the Rap GTPases, putative antagonists of Ras-mediated signalling. Ras can activate both the Raf-1/ERK1/2/3 pathway and the PI3K/Akt pathway, pathways that can promote cell survival. I investigated whether Rap activation limits the activation of either of these pathways. I found that Rap activation had no effect on the BCR-induced activation of the Raf-1/ERK1/2/3 pathway. However, endogenous Rap limited the BCR-induced activation of the PI3K/Akt pathway, opposed the Akt-mediated inhibition of the FKHR/p27^{Kip1} pro-apoptotic module, and enhanced cell death. Therefore, Rap can oppose the pro-survival role of the PI3K pathway. BCR-induced activation of the PI3K effector Akt leads to changes in gene transcription through the Akt-mediated activation or inhibition of transcription factors which can promote cell survival. I used cDNA microarray technology to identify novel gene targets of Akt in B cells. I found that Akt activation resulted in changes

in expression of genes involved in the regulation of cell cycle progression, cell adhesion and apoptosis. In summary, I have identified novel targets and novel mechanisms of regulating the PI3K/Akt pathway in B cells. This work will hopefully contribute to the overall understanding of how the PI3K pathway affects the regulation of B cell development, activation, and survival.

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List of Abbreviations

4-HT	4-hydroxytamoxifen
A2-mER-Akt	non-myristoylated version of mER-Akt
Ab	antibody
ALLN	acetyl-leucine-leucine-norleucinol
api-1	apoptosis protein inhibitor-1
BAFF	B-cell activated factor of the tumour-necrosis-factor family
BCAP	B cell adaptor for PI3K
BCR	B cell antigen receptor
BIM I	bisindolylmaleimide I
BLNK	B cell linker protein
CDK	cyclin-dependent kinase
DAG	diacylglycerol
ERK1/2	extracellular signal-regulated kinase 1/2
FKHR/FOXO1	Forkhead-related transcription factor
FWD1	F-box/WD40 repeat protein 1
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GSK-3	glycogen synthase kinase-3
GST	glutathione S-transferase
GST-ECT	GST fused to the C-terminal portion of the cytoplasmic domain of E-cadherin
GST-RalGDS(RBD)	GST fusion protein containing the Rap1/2-binding domain of the RalGDS protein
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
IAP	inhibitor of apoptosis
I κ B	inhibitor of NF- κ B
Ig	immunoglobulin
ILK	integrin-linked kinase
IP ₃	inositol trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
mER-Akt	myristoylated Akt-estrogen receptor chimeric protein
MHC	major histocompatibility complex
mIg	membrane Ig
mTOR	mammalian target of rapamycin
MZ	marginal zone
NF-ATc	nuclear factor of activated T cells
p70 S6K	p70 S6 kinase
PCR	polymerase-chain reaction
PdBu	phorbol dibutyrate

PDK1	PI3K-dependent kinase 1
PDK2	PI3K-dependent kinase 2
PH	pleckstrin homology
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
PI(4)P	phosphatidylinositol 4-phosphate
PI(4,5)P ₂	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol-3-kinase
PIP ₃	phosphatidylinositol (3,4,5) trisphosphate
PKC	protein kinase C
PLC- γ	phospholipase C-gamma
PP2A	protein phosphatase-2A
PTK	protein tyrosine kinase
P-Tyr	phosphotyrosine
RBD	Rap1/2-binding domain
Ser473	serine 473
SH2	Src homology 2
SH3	Src homology 3
SHIP-1	Src homology 2 domain containing inositol phosphatase-1
SHP-1	SH2 domain-containing protein phosphatase 1
SNAP-25	soluble N-ethylmaleimide sensitive factor attachment protein 25
SNARE	soluble N-ethylmaleimide sensitive factor attachment receptor
TD	transactivation deficient
Thr308	threonine 308
TI-1	T-independent type 1 antigen
TI-2	T-independent type 2 antigen
TNFR	tumour necrosis factor receptor

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Chapter 1

Introduction

1.1 Role of B cells in the immune system

Antibodies produced by B lymphocytes (B cells) are an essential component of the adaptive immune system. Antibodies protect the body from pathogens by coating the pathogen and preventing it binding to host cells (neutralization), targeting the pathogen for uptake by phagocytic cells, and initiating the complement-mediated destruction of the pathogen.

B cells are produced and mature in the bone marrow and then migrate to the secondary lymphoid organs where they can be activated in response to pathogenic invasion. The process by which a B cell develops and differentiates into an antibody-producing plasma cell is controlled by signals from many different receptors. In particular, signals by the B cell antigen receptor (BCR) regulate many steps in B cell development, differentiation and activation. These include the survival and differentiation of pre B-cells, negative selection of immature B cells, tolerization of mature B cells, survival of mature B cells and the development of resting mature B cells into proliferating B cells that can differentiate into antibody-producing plasma cells (1-3).

1.2 Regulation of antibody production by B cells

Regulation of both negative selection and activation of B cells is necessary to prevent autoimmunity and to mount an antibody response in the event of infection by a pathogenic organism. An insufficient response will result in lack of protection from the pathogen and

potentially death of the host organism. In contrast, activation of B cells in the absence of infection can result in the generation of an auto-immune response that is destructive to the host. The context in which a B cell encounters antigen determines whether the B cell will be activated to become an antibody producing plasma cell or undergo negative selection by apoptosis or anergy.

Activation of B cells requires two signals, one signal through binding of the antigen by the BCR and a co-stimulatory signal. Activated T cells provide the co-stimulatory for T-dependent antigens in the form of CD40 ligand or cytokines such as IL-4 (4). T-independent antigens stimulate B cell activation in the absence of activated T cells and the co-stimulatory signal is provided by microbial products binding to Toll-like receptors (TLR) or cytokines such as IL-5 produced by cells other than T cells (5). T-independent antigens are generally multivalent antigens with highly repetitious epitopes that can cause extensive clustering of the BCR and induce high levels of BCR signalling. T-independent antigens can be divided into two types (5). T-independent type 1 (TI-1) antigens induce a polyclonal, non-antigen specific B cell response and include the gram-negative bacterial cell wall component lipopolysaccharide (LPS). T-independent type 2 (TI-2) antigens include repeating polysaccharides from bacterial or viral surfaces and protein polymers such as bacterial flagellin. In contrast, T-dependent antigens cross-link the BCR much less extensively than T-independent antigens and induce low levels of BCR activation. T-dependent antigens include soluble proteins and low molecular weight haptens bound to soluble proteins. While the response to T-dependent antigens results in the generation of high affinity IgG antibodies and memory B cells, the response to T-independent antigens is characterized by secretion of lower affinity IgM antibodies and does not result in the generation of memory B cells (6). T-independent Ab responses are mediated primarily by IgM antibodies produced by B-1 B cells

and play an essential role in the protection from Gram positive bacteria with polysaccharide capsules (7).

T-dependent and T-independent antigens differ in the types of co-stimulatory signals they require to activate B cells. Activation of B cells by T-dependent antigens requires a co-stimulatory signal through interaction with an activated T helper cell. Activated T helper cells express CD154 (CD40 ligand) which binds to CD40 on B cells. Engagement of both the BCR and CD40 receptor on the quiescent mature B cell will result in activation, proliferation and ultimately differentiation of the B cell into a plasma cell (8). In contrast, engagement of the BCR alone will result in apoptosis or anergy, while signalling via CD40 in the absence of a BCR signal will result in Fas-mediated cell death via interaction with an activated T cell expressing Fas-ligand (9). In addition to CD40, other co-stimulatory molecules can influence B cell survival and activation. Signals from the T-cell generated cytokine IL-4 can promote B cell activation by inhibiting Fas-mediated apoptosis (9). T-independent antigens do not require a second signal from activated T helper cells but it has been proposed that activation of B cells by TI-2 antigens requires a second signal that can be provided by microbial products that bind to Toll-like receptors such as LPS (at low concentrations) and CpG DNA (5).

The requirement for a B cell to receive two different signals to generate a response is an inherent protective mechanism, since it is unlikely that both these signals would be present in the absence of an infection in a normal, healthy individual.

1.3 Signal transduction pathways activated by the B cell antigen receptor

There are multiple signalling pathways that are activated by the BCR in response to engagement by antigen. Major pathways activated by the BCR include the

phosphatidylinositol-3-kinase (PI3K) pathway, the phospholipase C-gamma (PLC- γ) pathway and the Ras pathway. However the roles of individual signalling pathways in mediating responses to BCR engagement are not completely understood.

1.4 Initiation of B cell receptor signalling

The BCR is composed of four distinct polypeptides that together provide antigen recognition and the capability to initiate the activation of signal transduction pathways (Fig 1.1). The immunoglobulin (Ig) heavy and light chains are identical to secreted antibodies except the heavy chain of the membrane Ig (mIg) has an alternative carboxy terminus that provides it with a hydrophobic membrane spanning region. The Ig heavy and light chains are covalently linked by disulfide bonds and bind antigen through antigen recognition domains within the F(ab) regions. The cytoplasmic tails of the mIg heavy chains are very short (3 amino acids for IgM) (4) and are presumably unable to bind to intracellular components that could initiate signal transduction. Therefore, the disulfide-linked heterodimer consisting of the Ig α /CD79a and Ig β /CD79b chains which interacts non-covalently with mIg, likely provides the necessary intracellular protein interaction domains for the activation of signal transduction pathways. This was demonstrated through the use of fusion proteins containing the extracellular and transmembrane domains of CD8 and the intracellular domains of Ig α or Ig β . Antibody-induced clustering of these fusion proteins expressed in B cell lines induced all of the major BCR signalling reactions including tyrosine phosphorylation and increased intracellular Ca²⁺ concentrations (10-12). The intracellular domains of the Ig α and Ig β invariant chains contains immunoreceptor tyrosine-based activation motifs (ITAMs) which have the characteristic YXXM (Tyr-X-X-Met) sequences

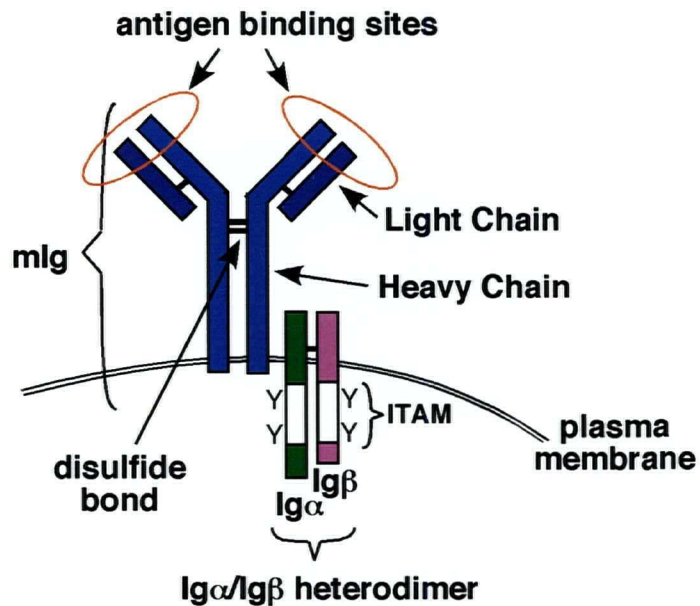


Figure 1.1 Structure of the B cell antigen receptor. The BCR is composed of a membrane bound immunoglobulin (mIg) and the non-covalently associated Ig α /Ig β heterodimer. The mIg is composed of a light chain and a heavy chain which are bound by disulfide bounds. Antigen is bound by both heavy and light chains at the variable region of the peptides. The Ig α /Ig β heterodimer is the signalling subunit of the BCR and contains the ITAM motifs (indicated by the white box) that upon phosphorylation recruit tyrosine kinases.

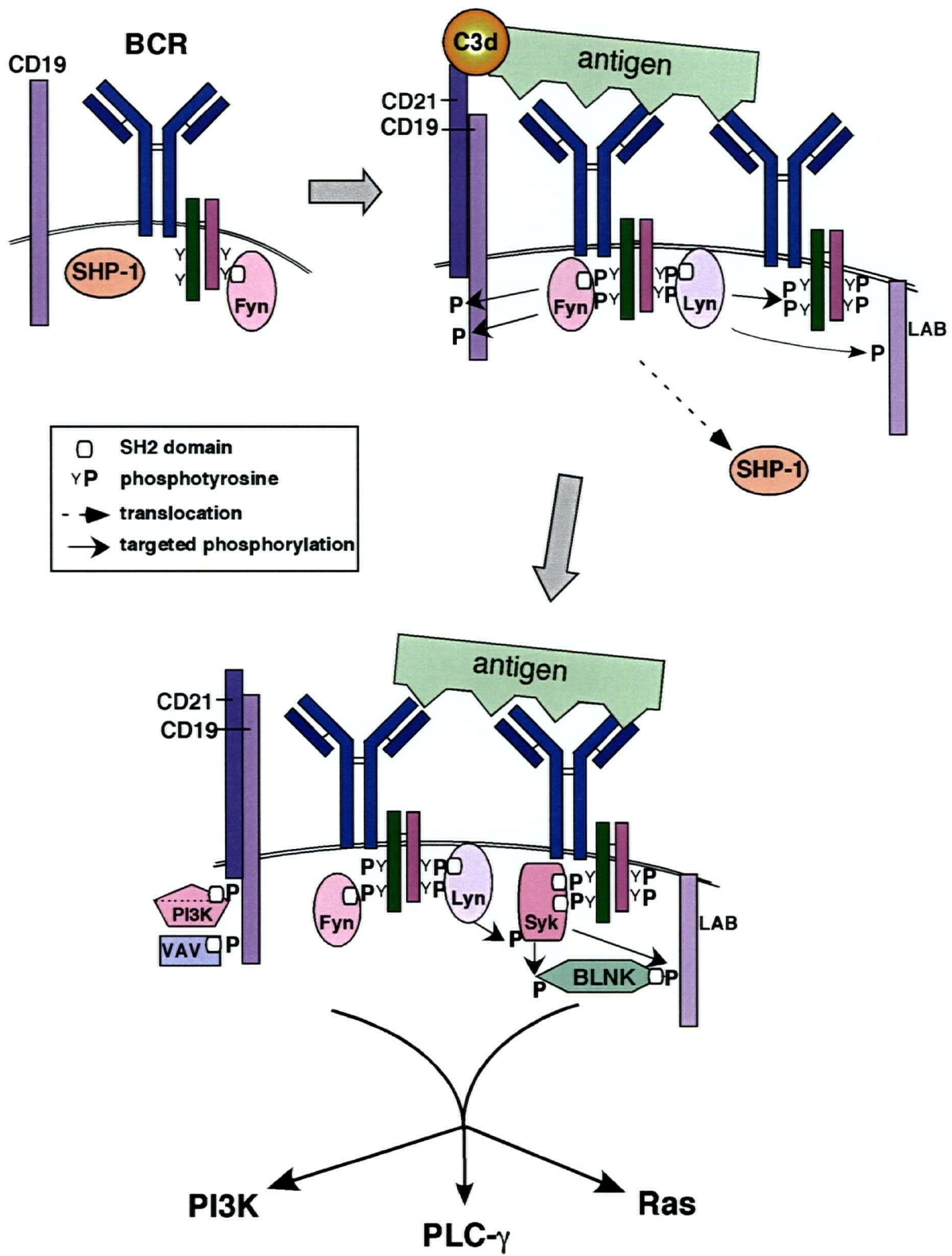
and are the site for intracellular protein interactions that initiate BCR signalling. It has been recently demonstrated that only one Ig α /Ig β heterodimer is associated with a single mIg (13).

Activation of BCR-dependent signalling pathways occurs through an ordered series of events (Fig.1.2), many of which have not been fully elucidated or identified. The classic view of the initiation of BCR signalling is that BCRs exist as monomers and the binding of a multivalent antigen or an antigen array on a surface by the BCR results in clustering of the BCR. Since only a few BCRs are associated with Src-family protein tyrosine kinases (PTK) such as Lyn, Fyn and Blk, this clustering allows the associated Src kinases to phosphorylate tyrosine residues on the now adjacent ITAM motifs of nearby BCR molecules.

Phosphorylated ITAMs in the Ig α /Ig β heterodimer then recruit the Syk tyrosine kinase via its Src homology 2 (SH2) domains. Activation of Src kinases by autophosphorylation and the Syk kinase by Src-mediated phosphorylation leads to the phosphorylation of other proteins which in turn leads to further activation of signal transduction pathways (3,8).

Recently, a new model for the initiation of BCR signalling has been proposed (14,15). Schamel and Reth have presented evidence that BCRs exist as oligomeric complexes prior to binding of antigen (13). According to this model the BCR exists as an ordered oligomeric structure where the ITAM motifs are not accessible for phosphorylation prior to binding of antigen (14). Upon binding of antigen, the structure of the oligomer becomes disordered which allows the phosphorylation of the ITAM motifs by associated Src kinases and subsequent recruitment and activation of the Syk PTK. Alternatively, the association of Src kinases with the unstimulated BCR oligomer may result in the continual phosphorylation of ITAM motifs of BCRs within the same oligomer allowing minimal recruitment and activation of Syk (15). Further activation of signalling pathways is prevented by the

Figure 1.2 Proximal signalling events induced upon BCR engagement. In the unstimulated cell, some BCRs associate with Src-family kinases (e.g. Lyn) independent of tyrosine phosphorylation. Association of the SHP-1 tyrosine phosphatase with the unstimulated receptor opposes the action of the Src-family kinases. Engagement of the BCR by antigen results in reorganization of BCR complexes and in the release of SHP-1 to allow associated Src-family tyrosine kinases (ex. Lyn and Fyn) to phosphorylate tyrosine residues on nearby BCR molecules and co-receptors such as CD19. The BCR-induced tyrosine phosphorylation of the transmembrane adapter proteins LAB/NTAL can be mediated by Src kinases but is more likely mediated by Syk. The CD19/CD21 complex binds the C3d complement fragment on complement coated antigens to induce co-clustering with the BCR which enhances BCR signalling. Tyrosine phosphorylated proteins recruit signalling proteins to the plasma membrane via interaction with their SH2 domains. Recruited proteins include the Syk tyrosine kinase which is activated by phosphorylation by a Src kinase and can in turn phosphorylate the LAB/NTAL and BLNK adapter proteins. The Rac GEF Vav and the lipid kinase PI3K are also recruited to the plasma membrane via their interaction with tyrosine phosphorylated CD19. Phosphorylation and/or activation of these proteins leads to the activation of three major signalling pathways: the PI3K pathway, the PLC- γ pathway and the Ras pathway.



association of the protein phosphatase SHP-1 (SH2 domain-containing protein phosphatase 1) with the unstimulated BCR which then dephosphorylates the ITAM motifs (16). This low level of constitutive PTK activity is sufficient to generate low level signals needed for B cell survival (17) but not sufficient to induce BCR activation (8,15). Binding of antigen to the BCR oligomer then causes the release or neutralization of the SHP-1 phosphatase allowing the efficient and rapid increase in ITAM phosphorylation and further activation of Src and Syk PTKs to promote further signalling events (8,15). Further experimental evidence in support of this model is still required.

Another level of regulation of BCR activation can be inferred from the observation that engagement of the BCR results in the translocation of the BCR into lipid rafts (18-20). Lipid rafts are low-density detergent resistant membrane microdomains enriched in glycosphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins, and acylated proteins (21). Translocation of the BCR into lipid rafts can facilitate BCR signalling by co-localizing the BCR with signalling components, such as Lyn which is constitutively localized in lipid rafts (21), and by excluding negative regulators, such as CD22 and CD45 (22,23). The CD45 phosphatase also plays a positive role in BCR signalling by removing the negative regulatory phosphate from Src-family kinases (23a). Lipid rafts may also provide a specialized microenvironment for recruiting signalling proteins including PI3K and PLC- γ (24) as well as to mediate the association of multiple BCR oligomers (8,23). Further evidence is needed to define the role of lipid rafts in the initiation of BCR signalling since there have been conflicting reports on the effect on disrupting lipid rafts on downstream BCR signalling events (18,19).

Activation of the Src-family kinases and Syk in response to BCR engagement results in the phosphorylation of a number of different signalling proteins including Btk (25,26), the adaptor protein B cell linker protein (BLNK) (27) and the CD19 co-receptor (28) (Fig. 1.2).

BCR engagement induces the tyrosine phosphorylation and activation of the Tec-PTK family member Btk (26,29). The activation of both Lyn and Syk is required for the maximal BCR-induced phosphorylation of Btk (30). It is thought that Lyn mediates the initial phosphorylation of Btk while Syk promotes sustained Btk phosphorylation (31). Btk is known to promote activation of the PLC- γ pathway (see Section 1.7) and mutations in Btk result in X-linked agammaglobulinaemia, a severe immunodeficiency due to a lack of mature B cells (32).

Typical adaptor proteins do not have any enzymatic activity but mediate the interaction of other proteins through protein interaction domains (33-35). Examples of these domains include SH2 domains which bind to phosphotyrosine residues, Src homology 3 (SH3) domains which bind to proline rich sequences and pleckstrin homology (PH) domains which bind to phosphoinositides. One of the main functions of adaptor proteins is to recruit signalling proteins to the plasma membrane or to protein complexes. The co-localization of a kinase and its substrate to the plasma membrane or to a protein complex increases the efficiency of substrate phosphorylation and activation of subsequent downstream signalling events (36). This type of complex formation occurs frequently in the activation of signalling pathways since the physical separation of signalling components until the time of receptor engagement is an effective way of maintaining a low level of signalling in unstimulated cells while achieving rapid activation upon receptor engagement.

BCR engagement results in the phosphorylation of a number of adaptor proteins by the BCR-associated PTKs and the subsequent recruitment of important SH2 domain-

containing signalling enzymes. Tyrosine phosphorylation of the transmembrane adaptor protein LAB/NTAL results in the membrane recruitment of the cytosolic adaptor protein BLNK which is in turn phosphorylated by Syk (31,37,38) (Fig. 1.2) . Phosphorylated BLNK then recruits PLC- γ which is in turn phosphorylated and activated by both Btk and Syk (27,31,39). Tyrosine phosphorylation of the CD19 transmembrane adapter protein in response to BCR engagement recruits both Vav and PI3K to the plasma membrane (40). Vav is an activator of the Rac GTPase which regulates the actin cytoskeleton while PI3K generates inositol phospholipids that recruit PH domain-containing proteins to the plasma membrane (see below).

1.5 The phosphatidylinositol-3 kinase/Akt pathway

PI3K enzymes phosphorylate inositol phospholipids at the 3'-position on the inositol ring. There are three classes of PI3K enzymes in mammalian cells, which are classified according to substrate specificity and structure (41).

Members of the class I group are heterodimers consisting of a 110 kDa catalytic subunit and a regulatory subunit. This group can be further subdivided into the class IA and IB PI3Ks. Class IA members include the p110 α , p110 β and p110 δ catalytic subunits that can associate with one of five regulatory subunits encoded by three different genes, p85 α , p85 β and p55 γ . The p50 α and p55 α subunits are generated by alternative splicing of the p85 α gene. The only member of class IB is the p110 γ catalytic subunit, which is encoded by a unique gene and associates with the p101 regulatory subunit. Class I PI3Ks can phosphorylate phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI(4)P), and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), with PI(4,5)P₂ as the preferred substrate *in*

vivo. Class IA PI3Ks are generally activated downstream of tyrosine kinases while the class IB PI3K is activated downstream of heterotrimeric G protein-coupled receptors.

Class II PI3Ks consist of a single subunit of 170 kDa. These PI3Ks can phosphorylate PI, PI(4)P and PI(4,5)P₂ with a strong preference for PI over PI(4)P over PI(4,5)P₂. The regulation of class II PI3Ks is less well characterized than that of class I PI3Ks. However they can be activated by various extracellular stimuli including epidermal growth factor, insulin and the chemokine MCP-1 (41).

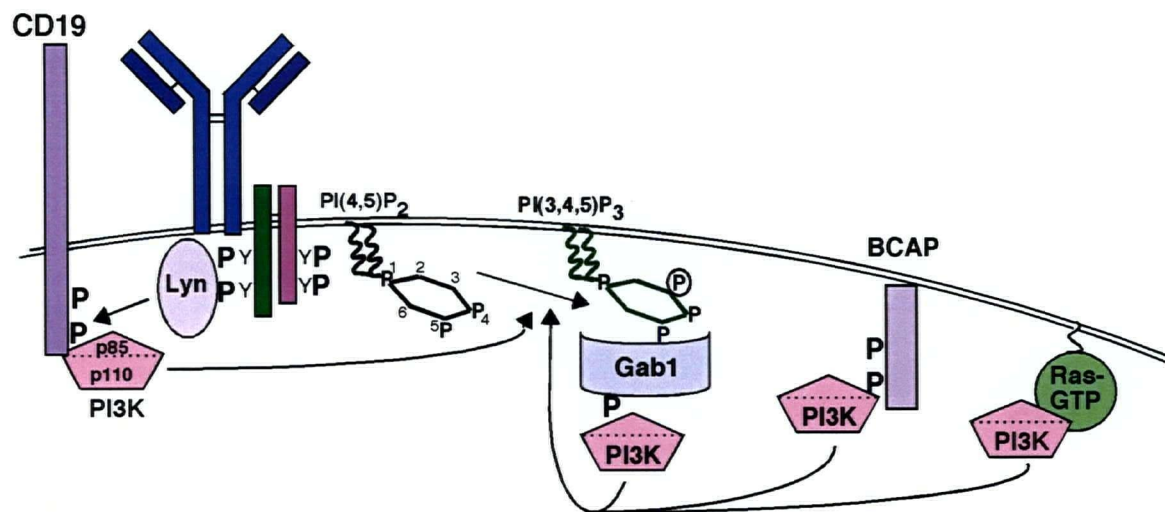
The class III PI3Ks are heterodimers comprised of a catalytic subunit and a 150 kDa regulatory subunit. Class III PI3Ks can only use PI as a substrate and are likely responsible for the generation of PI(3)P in the cell (41). The class III PI3K Vsp34p functions in regulating vesicle trafficking to the lysosome, an acidified organelle where protein degradation occurs, in both yeast and mammalian cells (42).

Class IA PI3Ks are the best characterized PI3Ks and play an important role in BCR signalling (43) (Fig. 1.3). The recruitment of PI3K to the plasma membrane brings it in close proximity to its inositol phospholipid substrates where it phosphorylates the 3' position of the inositol ring, thereby generating its primary product phosphatidylinositol (3,4,5) trisphosphate (PIP₃). The action of the 5'-inositol phosphatase, Src homology 2 domain containing inositol phosphatase-1 (SHIP-1), on PIP₃ generates another important lipid intermediate, PI(3,4)P₂ (44). The p85 subunit of the class IA PI3Ks contains two SH2 domains that allow the recruitment of the holoenzyme to the plasma membrane through the binding of phosphorylated tyrosine residues in a variety of adaptor/coreceptor molecules that are phosphorylated after BCR engagement including CD19, Gab1 and B cell adaptor for PI3K (BCAP) (45-49). Gene knockout studies have demonstrated that the phosphotyrosine-dependent recruitment of PI3K to CD19 (50) and BCAP (48) is essential for the maximal

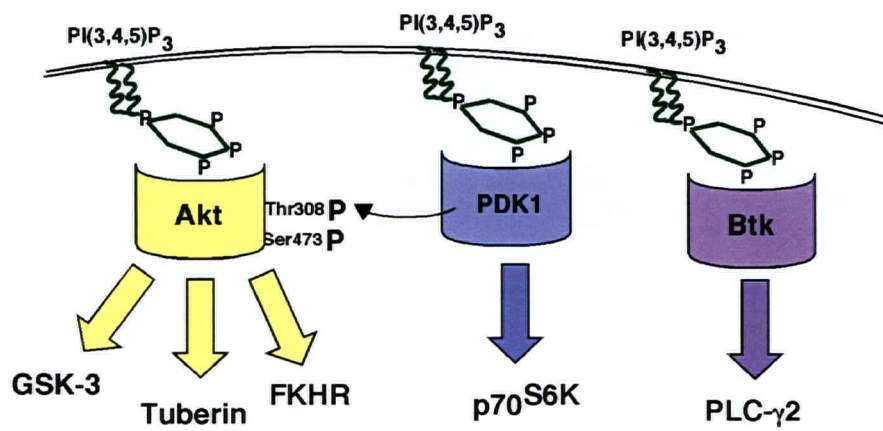
Figure 1.3 BCR-induced activation of the PI3K/Akt signalling pathway. *A*, Engagement of the BCR by antigen can lead to the activation of PI3K via multiple pathways.

Phosphorylation of CD19 by Src kinases recruits PI3K via binding of the SH2 domain of the p85 regulatory subunit. The recruitment of PI3K to the plasma membrane brings it in close proximity to its lipid substrate, phosphatidylinositol (4, 5) biphosphate (PI(4,5)P₂), which is phosphorylated by PI3K to generate phosphatidylinositol (3,4,5) trisphosphate (PIP₃). The positions on the inositol ring of PI(4,5)P₂ are indicated. Production of PIP₃ recruits the adapter protein Gab1, which upon phosphorylation by BCR associated protein kinases such as Syk recruits PI3K via its p85 domain. Association of PI3K with Gab1 promotes further activation of PI3K. Alternatively, PI3K can be recruited by association with the membrane associated adapter protein BCAP (B cell adapter for phosphoinositide-3 kinase) upon tyrosine phosphorylation of residues on BCAP by Syk. In addition, PI3K can be recruited to the plasma membrane by the active Ras-GTP through interaction with the p110 catalytic domain of PI3K. *B*, Production of PIP₃ recruits PH domain containing proteins to the plasma membrane where they are activated. These include the PDK1, Akt and Btk kinases. PDK1 phosphorylates Akt on Threonine 308 while phosphorylation on Serine 473 can be mediated by many different kinases. Major substrates of each kinase is indicated.

A



B



activation of BCR-induced PI3K-dependent signalling events. In addition, the activated form of the monomeric GTPase Ras has been shown to activate the class I PI3Ks by binding the p110 catalytic subunit (51,52), and thereby recruiting PI3K to the plasma membrane where Ras is localized. The PI3K products PIP_3 and $PI(3,4)P_2$ act as anchors that bind pleckstrin homology (PH) domain containing proteins and in this way recruit cytosolic signalling proteins to the membrane (53) (Fig. 1.3B). An important PH-domain containing protein that is involved in BCR signalling is the kinase Btk, which is required for the activation of PLC- γ (31). Also, the binding of Vav through its PH domain increases its ability to activate the Rac GTPase which activates the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) (54). The Gab1 adaptor protein also contains a PH domain. Once it is recruited to the plasma membrane, it is tyrosine phosphorylated by BCR associated PTKs, allowing it to recruit additional PI3K proteins to the plasma membrane and enhance PI3K-dependent signalling (47) (Fig. 1.3A). The 60 kDa serine/threonine protein kinase Akt, also known as Protein Kinase B, is also a PH-domain-containing protein and mediates the effects of PI3K on cell survival (55,56).

We and others have shown that BCR engagement activates Akt (57-62). Akt is the primary mediator of the anti-apoptotic signals generated by PI3K (56) and recent work has shown that Akt kinase activity is essential for the survival of the DT40 chicken B cell line (63). The activation of Akt is a multistep process that involves both localization and phosphorylation of Akt (64). Once recruited to the plasma membrane through interaction with PIP_3 and $PI(3,4)P_2$, Akt undergoes a conformational change that allows it to be phosphorylated and activated by upstream kinases. Upon BCR engagement, human Akt is phosphorylated on two sites, threonine 308 (Thr308) and serine 473 (Ser473).

Phosphorylation of both of these sites is required for maximal activation (65). These

modifications induce a structural reorganization of the kinase domain that is required for kinase activity (66).

The phosphorylation of Akt on Thr308 but not Ser473, is attributed to a kinase called PDK1 (PI3K-dependent kinase 1) (64,67). PDK1 also contains a PH domain that has very high affinity for PIP₃ (68). The kinase that phosphorylates Ser473 has not been characterized but is termed PDK2 (PI3K-dependent kinase 2). There is evidence that PDK1 can act as PDK2 in the presence of a PRK2 (Protein Kinase C-related kinase) derived peptide (69). Alternatively, other reports have shown that the integrin-linked kinase (ILK) is both necessary and sufficient for phosphorylation of Akt on Ser473 (70,71). Akt has also been demonstrated to undergo autophosphorylation on Ser473 following phosphorylation of Thr308 by PDK1 (72) and more recently, a novel PDK2 kinase that is distinct from ILK or PDK1 has been isolated by Hill et al. (73). Thus, there may be multiple kinases that can phosphorylate Akt on Ser473 and these kinases may be preferentially regulated in different cell types or by different receptors.

Akt-dependent phosphorylation regulates the activity of a number of proteins that are involved in cell survival, cell growth and cell cycle progression (74). Substrates of Akt include Bad (75,76), tuberlin (77), and p21^{Cip1/Waf} (78,79). The Akt-mediated phosphorylation of Bad inhibits Bad-induced apoptosis by promoting the association of Bad with the 14-3-3 protein (80-82). Unphosphorylated Bad promotes apoptosis by associating with and neutralizing the pro-survival Bcl-2 family members to induce the release of cytochrome C from the mitochondria thereby activating the pro-apoptotic caspase cascade (55,83). Phosphorylation of tuberlin by Akt promotes cellular growth likely by relieving the tuberlin-mediated inhibition of the mTOR/p70 S6-kinase module which promotes protein synthesis (84). Phosphorylation of the cell cycle inhibitor p21^{Cip1/Waf} by Akt causes p21^{Cip1/Waf} to

translocate from the nucleus to the cytoplasm. This prevents p21^{Cip1/Waf} from associating with cyclin dependent kinase/cyclin complexes promoting cell cycle progression (85).

Akt also promotes cell survival by regulating transcription factors that control genes involved in cell survival and apoptosis. Akt positively regulates the transcription factor NF- κ B which controls transcription of pro-survival factors such as Bcl-X_L and cyclin D2 (86-89). Akt-mediated phosphorylation inhibits the activity of Forkhead transcription factors that induce the expression of pro-apoptotic proteins including Fas ligand, a Bcl-2 family member called Bim and the p27^{KIP1} cell cycle inhibitor (90,91).

Akt can also phosphorylate the protein-serine/threonine kinases glycogen synthase kinase-3 α (GSK-3 α) and GSK-3 β (92). GSK-3 α and GSK-3 β are constitutively active in resting cells but receptor-stimulated phosphorylation of GSK-3 α at Ser21 or GSK-3 β at Ser9 inhibits their enzymatic activity (93). These negative regulatory sites on GSK-3 α and GSK-3 β can be phosphorylated not only by Akt but also by several other kinases including the p90^{RSK} kinase, integrin-linked kinase, and several protein kinase C (PKC) isoforms (92,94-100). BCR engagement induces the phosphorylation of GSK-3 α and GSK-3 β on these negative regulatory sites and inhibits the activity of GSK-3 α (57).

GSK-3 activity can regulate the activity of the transcriptional activators, NF-ATc (nuclear factor of activated T cells) and β -catenin (101,102). NF-ATc is a calcium sensitive transcription factor that translocates to the nucleus in response to BCR engagement (103). Phosphorylation of the transcription factor NF-ATc by GSK-3 promotes its nuclear export (104). Inhibition of GSK-3 would increase the ability of NF-ATc to translocate to the nucleus and initiate gene transcription from NF-ATc dependent promoters (93). Therefore, BCR mediated inhibition of GSK-3 activity could potentially result in the upregulation of NF-ATc

dependent transcription. Regulation of the transcriptional co-activator, β -catenin by GSK-3 has been most well studied in the Wnt signalling pathway and will be discussed in detail below (Section 1.6).

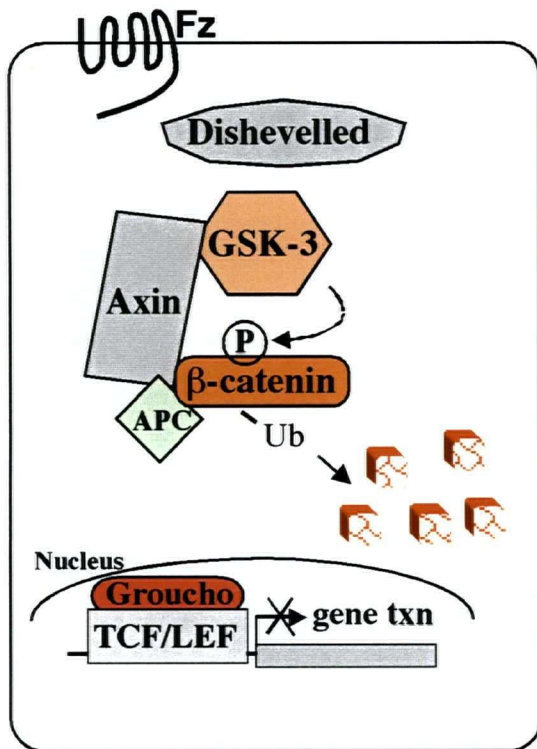
1.6 Regulation of the transcriptional activator β -catenin by the Wnt signal transduction pathway

One of the major targets of GSK-3 is β -catenin, a transcriptional co-activator that has important roles in early development (105,106). The regulation of β -catenin has been studied most extensively in the context of the Wnt signalling pathway (Fig. 1.4). In unstimulated cells, β -catenin is maintained at a low level by the action of GSK-3. The phosphorylation of β -catenin by GSK-3 on N-terminal serine and threonine residues promotes its ubiquitination by the FWD1 (F-box/WD40 repeat protein 1) protein, which in turn promotes its degradation by the proteasome degradation machinery (107-109). Activation of the Wnt signalling pathway, by binding of the Wnt hormone to its receptor, Frizzled, results in the inhibition of GSK-3 activity which prevents phosphorylation of β -catenin. This allows the accumulation of β -catenin in the cytosol and subsequent translocation to the nucleus where unphosphorylated β -catenin promotes gene transcription (106,110,111). Wnt signalling inhibits GSK-3 activity via the Disheveled protein in a manner that is not well understood (106,112).

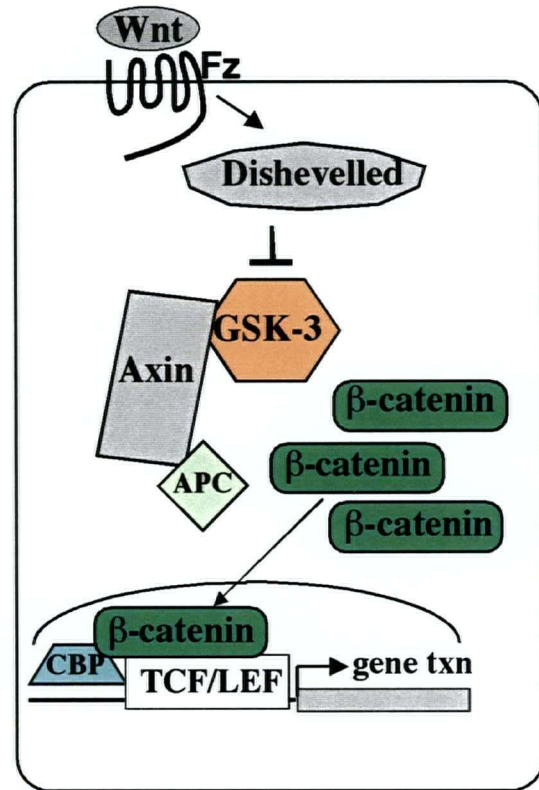
Dephosphorylated β -catenin moves into the nucleus where it promotes transcription (111,113). However, the mechanism for the nuclear translocation of β -catenin remains unclear. β -catenin does not contain a nuclear localization signal but translocates into the

Figure 1.4 Activation of β -catenin by Wnt hormone stimulation. In the resting cell (left panel) the Axin/APC/GSK-3 protein complex promotes the phosphorylation of β -catenin by GSK-3. Phosphorylation of β -catenin targets it for ubiquitination (Ub) and subsequent degradation by the proteasome. Wnt hormone binding to the Frizzled (Fz) receptor (right panel) results in the inhibition of GSK-3 via the protein Dishevelled. Inhibition of GSK-3 prevents phosphorylation of β -catenin and allows β -catenin to accumulate in the cytosol. Translocation of β -catenin into the nucleus occurs by an unknown mechanism. Once in the nucleus β -catenin relieves LEF/TCF gene repression by displacing the Groucho protein and promotes transcription in a complex with the LEF/TCF family of transcription factors and the CBP DNA binding protein.

Resting Cell



Wnt Stimulated Cell



nucleus in an ATP-dependent manner through the nuclear pore complex in a manner that is independent of both the Ran GTPase and the importin nuclear import pathway (114,115). Recent studies have shown that β -catenin can bind to the androgen receptor which upon ligand binding translocates into the nucleus (116-119). Therefore, androgen receptors provide a potential mechanism to shuttle β -catenin into the nucleus. However, there is no apparent cross-regulation of the androgen and Wnt pathways (116). Thus, the mechanism for the nuclear import of β -catenin in response to Wnt activation remains undefined.

Once in the nucleus, β -catenin relieves LEF-1/TCF mediated gene repression by displacing the Groucho/TLE family of repressors (120-123) or by sequestering histone deacetylase activity (124). β -catenin then acts as a transcriptional activator in association with the LEF-1/TCF transcription factors (120,123) and the CREB-binding protein (125-127) to promote gene expression from promoters with LEF-1/TCF binding sites. Recent work by Clevers and colleagues has shown that β -catenin only promotes transcription in its unphosphorylated form (111,113) indicating that the inhibition of GSK-3-mediated phosphorylation of β -catenin is essential for the accumulation of β -catenin in the nucleus and for β -catenin-dependent transcription.

The phosphorylation-dependent degradation of β -catenin is facilitated by a large protein complex that includes APC, which is a tumour suppressor encoded by the *adenomatous polyposis coli* gene and the Axin scaffolding protein (112). β -catenin is a poor substrate for GSK-3 *in vitro* and it is thought that Axin facilitates phosphorylation of β -catenin by GSK-3 by creating complexes in which β -catenin and GSK-3 are in close proximity (112,128,129). Mutations resulting in the inactivation of APC are associated with the 85% of sporadic and hereditary colorectal cancers and result in increases in β -catenin

protein levels (130,131). APC binds to both β -catenin and Axin which it is thought to stabilize the association of β -catenin with Axin since mutations in the interaction domains of APC promote the accumulation of β -catenin (112). APC may also play a role in inhibiting β -catenin activity in unstimulated cells by binding nuclear β -catenin, exporting it from the nucleus and shuttling it to the degradation complex (132).

Other proteins have been found in this β -catenin regulatory complex including casein kinase 1 and protein phosphatase-2A (PP2A) (112,133). Casein kinase 1 has recently been shown to be responsible for the “priming” phosphorylation of β -catenin on Ser 45 which is required for subsequent phosphorylation of β -catenin by GSK-3 at Thr 41, Ser 37 and Ser 33 (134-136). PP2A mediates the Wnt-induced dephosphorylation of Axin which prevents the association of GSK-3 with Axin. PP2A therefore promotes the stabilization of β -catenin by decreasing the association of GSK-3 with Axin. (129,137).

The upregulation of β -catenin in response to activation of the Wnt pathway regulates early developmental processes (106,112). β -catenin-mediated transcription results in the transcription of genes required for both embryonic development, cell cycle progression and cellular proliferation (112). In *Xenopus*, target genes of the LEF-1/TCF promoter family include *Siamese* and *gooseoid*, both of which play a role in body axis formation (138,139). In mammalian cells, β -catenin regulates the transcription of both *cyclin D1* and *c-myc*, genes that promote cell growth and proliferation (140-142).

Recent evidence has shown that the Wnt-mediated activation of β -catenin-dependent transcription is important for B cell development (143). Treatment of pro-B cells with the Wnt hormone promotes cell proliferation while LEF-1 knock-out mice have reduced proliferation and increased apoptosis of pro-B cells (143). β -catenin is a major mediator of

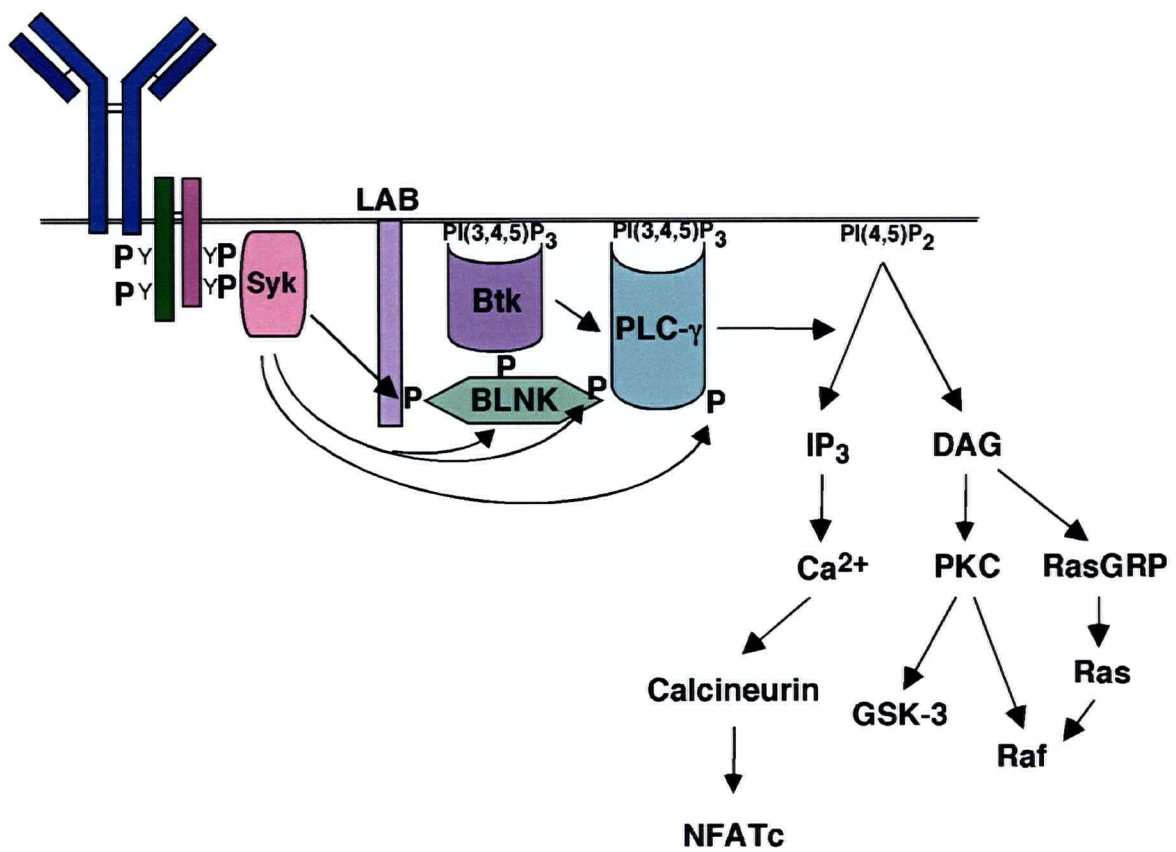
Wnt signalling and in cooperation with LEF-1 upregulates gene transcription. Therefore, β -catenin likely mediates the observed effect of Wnt and LEF-1 on pro-B cells. One of the major aims of this thesis was to determine if β -catenin protein levels and β -catenin-mediated transcription are regulated in mature B cells.

1.7 The phospholipase-C γ pathway

Engagement of the BCR results in tyrosine phosphorylation and activation of both PLC- γ 1 and PLC- γ 2 (144-147) (Fig. 1.5). Since PLC- γ 1 and PLC- γ 2 are cytoplasmic proteins they must be recruited to the plasma membrane in order to act on their membrane bound lipid substrate PI(4,5)P₂ (2). Activation of the BCR results in tyrosine phosphorylation of the membrane bound adaptor protein LAB/NTAL which is in turn phosphorylated by Syk allowing it to recruit the cytosolic adaptor protein BLNK (37,38). PLC- γ binds to BLNK via its SH2 domains. This facilitates the phosphorylation and activation of PLC- γ by the Btk tyrosine kinase and the Syk tyrosine kinase (31).

Activated PLC- γ generates the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP₃) via the cleavage of PI(4,5)P₂ (148). Increases in DAG levels lead to the activation of DAG-dependent enzymes including PKC and Ras guanosine nucleotide releasing protein (RasGRP), an activator of the Ras GTPase (149-152). The targets of PKC include the protein serine/threonine kinase GSK-3 (96,98-100) and Raf-1 kinase (153). IP₃ activates IP₃ receptors on the endoplasmic reticulum resulting in release of calcium from intracellular calcium stores (31,148). The increase in intracellular calcium results in activation of calmodulin-dependent kinase II and the calmodulin-activated phosphatase calcineurin (31). Calcineurin (PP2C) dephosphorylates the transcription factor

Figure 1.5 BCR-induced activation of the PLC- γ signalling pathway. Engagement of the BCR by antigen (not shown) results in the recruitment and activation of the protein tyrosine kinases Lyn and Syk. Tyrosine phosphorylation of the adapter protein LAB/NTAL promotes the recruitment of BLNK. Phosphorylation of BNLK by Syk recruits Btk and PLC- γ to the plasma membrane. Both Btk and PLC- γ have PH domains and can also be recruited to the plasma membrane by the activation of the PI3K pathway. The Btk and Syk kinases then phosphorylate and activate PLC- γ . Active PLC- γ cleaves phosphatidylinositol (4, 5) bisphosphate (PI(4,5)P₂) into the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP₃). Increased levels of DAG lead to the activation of DAG-dependent enzymes including PKC and RasGRP. RasGRP is an activator of Ras which in turn activates the kinase Raf-1 (see Section 1.8). Phosphorylation of GSK-3 by PKC is inhibitory while phosphorylation of Raf-1 by PKC contributes to the activation of Raf-1 by Ras. Increases in IP₃ result in the release of intracellular calcium (Ca²⁺) which leads to the activation of the calcineurin phosphatase (PP2C). Calcineurin-mediated dephosphorylation of NF-ATc promotes the translocation of NF-ATc to the nucleus where it promotes transcription.



NF-ATc. This reveals a nuclear localization signal and promotes its translocation into the nucleus where it can activate transcription (154).

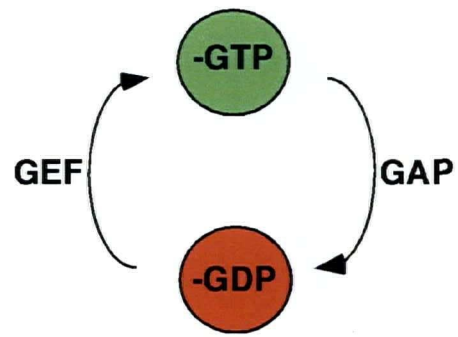
Activation of the PLC- γ pathway in response to BCR engagement is essential for proper B cell activation and development (155). B cells that are deficient in PLC- γ activation have impaired development at multiple stages of B cell development and defective response to BCR stimulation *in vitro* and *in vivo* (156,157). PLC- γ activation is also critical in the anti-IgM induced apoptosis of immature B cells (158). Therefore, PLC- γ can contribute to both negative and positive selection of B cells. The importance of PLC- γ in B cell development is supported by the observation that mice lacking proteins that are involved in PLC- γ activation such as BLNK and Btk have blocks in B cell development at the same stages as PLC- γ deficient mice (155).

1.8 The Ras and Rap monomeric GTPases

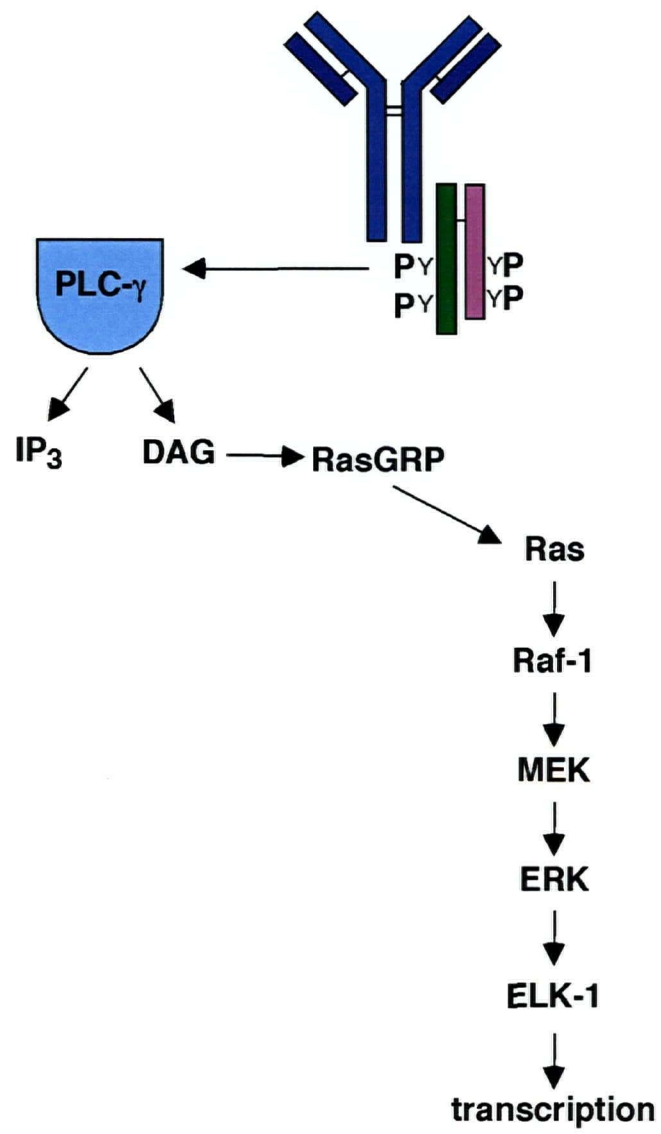
Monomeric GTPases act as molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form. Ras is the best characterized member of this family of proteins (159). The active form of GTPases can bind downstream effector proteins and thereby regulate their localization, conformation, and activity. The conversion between the GTP- and GDP-bound forms of a GTPase is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Fig. 1.6A). GEFs activate GTPases such as Ras by promoting the release of bound GDP which allows the more abundant intracellular GTP to bind to the GTPase and activate it by inducing a conformational change. Most monomeric GTPases hydrolyze GTP at a very slow rate in the absence of regulatory proteins. GAPs increase the rate of intrinsic GTPase activity thereby

Figure 1.6 BCR-induced activation of the Ras signalling pathway. *A*, Monomeric GTPases are molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form. Guanine nucleotide exchange factors (GEFs) regulate the exchange of GDP for GTP while GTPase-activating proteins (GAPs) induce the hydrolyzation of GTP to GDP. *B*, Engagement of the BCR by antigen (not shown) results in the activation of the PLC- γ pathway (see Figure 1.4) resulting in the production of DAG. Increases in DAG lead to the activation the Ras GEF, RasGRP. RasGRP can then activate the Ras monomeric GTPases which results in the activation of the kinase Raf-1. Raf-1 then phosphorylates and activates the MEK kinase which in turn phosphorylates and activates the ERK1/2 MAPK. ERK1/2 then phosphorylates and activates transcription factors, such as ELK-1, to promote transcription of pro-proliferative or pro-survival genes.

A



B



promoting the conversion of the GTPase to its inactive GDP-bound form.

The BCR activates at least three monomeric GTPases, namely Ras, Rac and Rap (160-163). Ras is a key regulator of cell growth and its effects are mediated primarily by the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) MAPK (164,165). Rac regulates the kinase cascades leading to the activation of the JNK and p38 MAPKs (166). The three major classes of MAPKs (ERK1/2, JNK, and p38) phosphorylate a variety of transcription factors, increasing their ability to promote transcription (167). Rap has been shown to regulate cell migration and integrin-mediated adhesion in B and T cells (168-170).

The Ras GTPase is activated upon engagement of the BCR via the PLC- γ dependent generation of DAG which results in the activation of RasGRP, a Ras-specific GEF (Fig.1.6B) (150-152,160,161,171). A major function of the Ras GTPase is to activate the Raf-1/MEK/ERK1/2 signalling pathway (Fig. 1.6B). Activated Ras-GTP leads to the activation of the protein kinase Raf-1. Activation of Raf-1 involves the interaction with Ras-GTP as well as the phosphorylation of Raf-1 (172,173) that can be mediated by PKC (153), the kinase PAK (174) and kinases of the Src-family (175). Active Raf then phosphorylates and activates the dual-specificity kinases MEK1/2 (171,176). Active MEK in turn phosphorylates and activates the MAPKs ERK1/21 and ERK1/22 (177). Upon activation the ERK1/2 kinases translocate to the nucleus where they phosphorylate and activate transcription factors including Ets-1, Ets-2 and ELK-1 and Sap1a which are members of the ternary complex factor family (2,167).

Previous work in our lab has shown that the BCR activates the Rap1 GTPase via the PLC- γ -dependent increase in DAG (163). The GEF responsible for activation of Rap in B cells remains unknown. The BCR also activates the Rap2 GTPase, as is shown in this thesis.

Recent work has demonstrated that the Rap GTPases play a key role in receptor-induced integrin activation and, hence cell adhesion, in many cell types (178,179), including B cells (S. J. McLeod and M.R. Gold, manuscript in preparation). However, the Rap GTPases were first described as potential antagonists of Ras-mediated signalling.

The idea that Rap is an antagonist of Ras-mediated signalling originated with experiments showing that overexpressing Rap1 caused Ras-transformed 3T3 cells to assume a more normal fibroblast-like morphology (180). Similarly, activated Rap1 can oppose Ras-dependent *Xenopus* oocyte differentiation (181). Since Ras and Rap1 have identical core effector binding domains (residues 32-40) (182) and activated Rap1 can bind *in vitro* to Ras effectors, including Raf-1 (183,184), it was proposed that activated Rap1 limits Ras-mediated signalling by sequestering Ras effectors in inactive complexes. Although Rap2 has a phenylalanine at position 39 instead of the serine present in Rap1(185), this substitution does not appear to have any functional consequences (182) indicating that activated Rap2 can also bind Ras effectors and act as a Ras antagonist. Indeed, several groups have shown that both activated Rap1 and activated Rap2 can inhibit Ras-dependent activation of ERK1/2 (185-188). Other studies however did not find that Rap activation impaired Ras-dependent ERK1/2 activation (189-191), indicating that this could be a cell type-specific effect.

In contrast to the ability of activated Rap to inhibit Ras-dependent ERK1/2 activation in some cell types, Rap-GTP can in fact act as a positive regulator of both ERK1/2 and p38 in some situations. In neuronal cells such as the PC12 cell line, Stork and colleagues have shown that Rap1-GTP activates B-Raf, leading to sustained activation of ERK1/2 (192,193). Brummer *et al.* (194) have recently shown that B-Raf plays a significant role in the activation of ERK1/2 by the BCR, suggesting that a Rap/B-Raf pathway could contribute to BCR-

induced ERK1/2 activation. Thus, depending on the cell type, the Rap GTPases may act as either positive or negative regulators of ERK1/2 activation.

Signalling pathways regulated by Ras play an essential role in BCR signalling. Loss-of-function studies have shown that activation of the Ras/Raf-1/MEK/ERK1/2 signalling pathway is essential for B cell development (195) and for BCR-induced proliferation of mature B cells (196). Transgenic mice expressing a dominant-negative form of Ras exhibit a block in maturation at early stages of B cell development (195). Expression of constitutively-active Raf-1 in these mice rescued the developmental block indicating that a Ras/Raf-1 pathway regulates early B cell development (195). Moreover, inhibition of the Ras/ERK1/2 pathway in mature B cells by treatment with MEK inhibitors blocks B cell proliferation induced by BCR and IL-4 stimulation (196). Thus, understanding the mechanisms by which the Ras pathway is regulated in B cells is important, since activation of the Ras pathway is crucial to B cell development and proliferation.

In addition to activating the Raf-1/MEK/ERK1/2 pathway, Ras can also promote signalling via PI3K. Activated Ras can bind directly to the p110 catalytic subunit of PI3K (52,197), thereby recruiting PI3K to the plasma membrane where it can produce the lipid second messengers PIP_3 and $\text{PI}(3,4)\text{P}_2$ which activate downstream targets such as Akt. Akt has been shown to play a critical role in regulating survival versus apoptosis in B cells (63). Consistent with the idea that Ras regulates the PI3K/Akt pathway in B cells, recent work has shown that expressing constitutively-active Ras leads to Akt activation in the A20 B cell line (198). The BCR can also initiate PI3K-dependent signalling by inducing the SH2 domain-dependent binding of PI3K to membrane-associated scaffolding/adaptor proteins discussed in Section 1.5. Thus there may be two distinct mechanisms for BCR-induced activation of PI3K. Nevertheless, if activated Rap were to act as an antagonist of Ras-mediated signalling

in B cells, it would have the potential to limit the activation of two critical BCR signalling pathways, the Ras/Raf-1/MEK/ERK1/2 pathway and the Ras/PI3K/Akt pathway. One of the goals of this thesis was to examine whether Rap activation regulated the ability of the BCR to activate these two signalling pathways.

1.9 Summary of thesis

This thesis is divided into three sections. In this thesis I have identified downstream targets of the PI3K/Akt signalling pathway and examined the regulation of the PI3K/Akt pathway in B cells (Fig. 1.7). In the first section I elucidated the mechanism by which the BCR regulates the serine/threonine kinase GSK-3 and the transcriptional activator β -catenin, two potential downstream targets of PI3K and Akt. In the second section I examined the effect of the Rap GTPase on BCR-regulated signalling pathways including the PI3K pathway and the ERK1/2 MAPK pathway. In the third section, I used cDNA microarray analysis to identify genes that are regulated by the serine/threonine kinase Akt in B cells.

My first hypothesis was that BCR signalling would lead to upregulation of β -catenin protein levels and an increase in β -catenin-dependent transcription. Activation of both the Wnt signalling pathway and the BCR signalling pathway results in the inhibition of GSK-3 (57,112), the kinase that maintains β -catenin at low levels in resting cells. It has been previously shown that activation of Akt results in the inactivation of GSK-3 via the Akt-mediated phosphorylation of GSK-3 (92,93). Since engagement of the BCR also results in the activation of Akt (57), I hypothesized that the BCR-induced inhibition of GSK-3 would lead to increases in β -catenin protein levels and β -catenin-dependent transcription and that these responses would be mediated by the activation of Akt (Fig. 1.8).

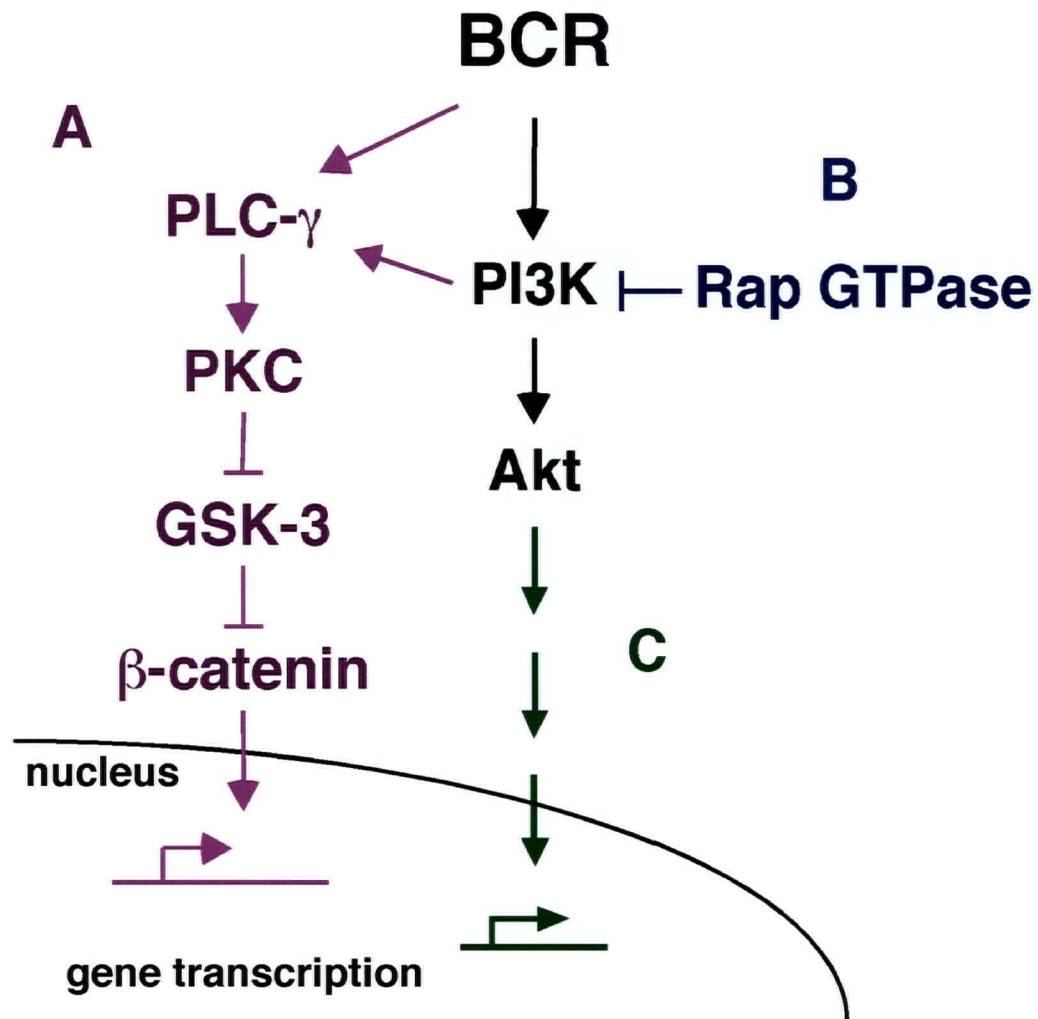


Figure 1.7 Summary of thesis. *A*, The BCR was found to regulate the transcriptional co-activator β -catenin by the PI3K-dependent and independent activation of the PLC- γ /PKC/GSK-3 pathway (purple). *B*, Rap GTPases were found to negatively regulate the BCR-induced activation of the PI3K/Akt pathway (blue). This opposes the Akt-mediated downregulation of the FKHR/p27^{Kip1} pro-apoptotic module (not shown). *C*, Novel gene targets of Akt in B cells were identified by cDNA microarray analysis (green).

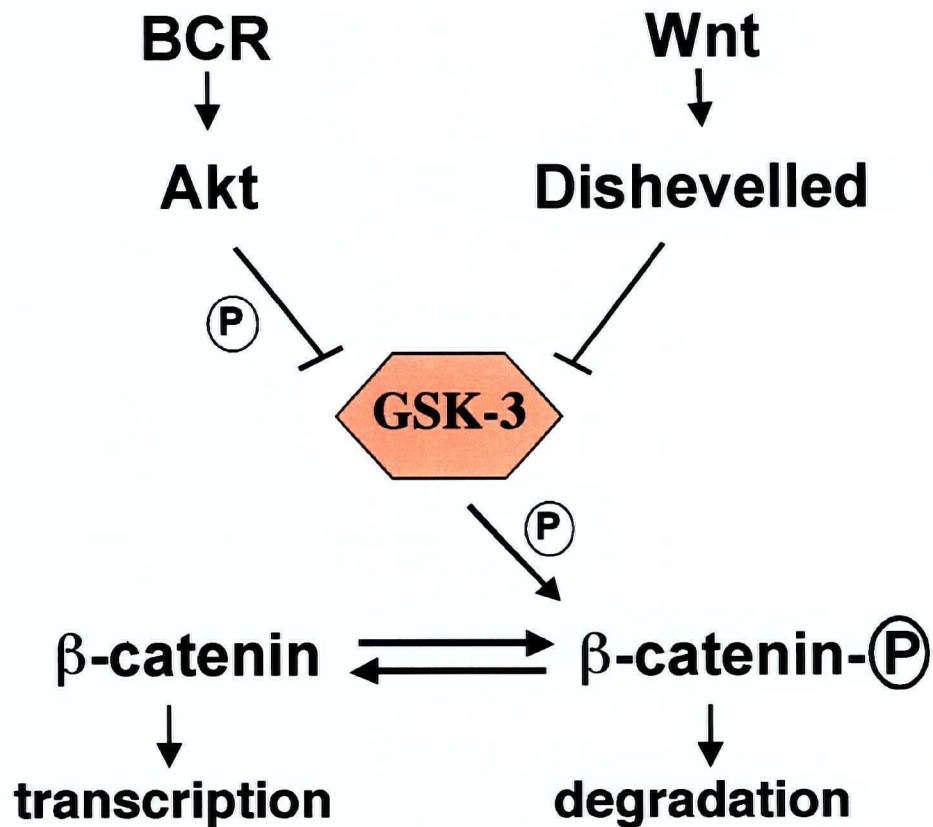


Figure 1.8 Proposed regulation of β -catenin by the BCR stimulation and the Wnt signalling pathway. Wnt and BCR induced activation of signalling pathways result in the inhibition of GSK-3. In other systems, inhibition of GSK-3 can be induced by Akt-mediated phosphorylation. Since the BCR activates Akt and inhibits GSK-3, the BCR-induced inhibition of GSK-3 may also be due to the Akt-mediated phosphorylation of GSK-3. In addition, the BCR-induced inhibition of GSK-3 would be predicted to prevent the degradation of β -catenin protein and promote β -catenin mediated transcription in B cells.

As hypothesized, I found that engagement of the BCR results in increased nuclear and cytosolic β -catenin protein levels and in increased β -catenin dependent transcription. However, even though Akt phosphorylates GSK-3 in other cell types (199,200), I found that both the BCR-induced phosphorylation of GSK-3 as well as BCR-induced upregulation of β -catenin were mediated primarily by PLC-dependent activation of PKC and not by Akt. Thus, the BCR regulates β -catenin via a PLC- γ 2/PKC/GSK-3 signalling pathway.

The monomeric GTPases Ras and Rap have nearly identical effector binding domains but Rap does not activate Ras effectors. Therefore, it has been hypothesized that Rap could inhibit Ras-mediated signalling by sequestering Ras effectors. In B cells, Ras activates the ERK1/2 signalling pathway as well as the PI3K/Akt signalling pathway (159,198). In the second Section of this thesis I have asked whether the Rap GTPases modulate BCR-induced Ras-dependent signalling pathways that lead to the activation of ERK1/2 or Akt (Fig. 1.9). I found that Rap is neither a positive or negative regulator of BCR-induced ERK1/2 activation. Rap activation also had no effect on the ability of the BCR to activate the JNK and p38 MAPKs. In contrast, I found that activation of the endogenous Rap GTPases limits the activation of Akt by the BCR. BCR-induced phosphorylation of Akt on critical activating sites was increased when Rap activation was blocked. Preventing Rap activation also increased the ability of the BCR to stimulate Akt-dependent phosphorylation of the FKHR transcription factor on negative regulatory sites and decreased the levels of p27^{Kip1}, a pro-apoptotic factor whose transcription is enhanced by FKHR. Moreover, preventing Rap activation reduced BCR-induced cell death in the WEHI-231 B cell line. Thus activation of endogenous Rap by the BCR limits BCR-induced activation of the PI3K/Akt pathway, opposes the subsequent inhibition of the FKHR/p27^{Kip1} pro-apoptotic module, and enhances

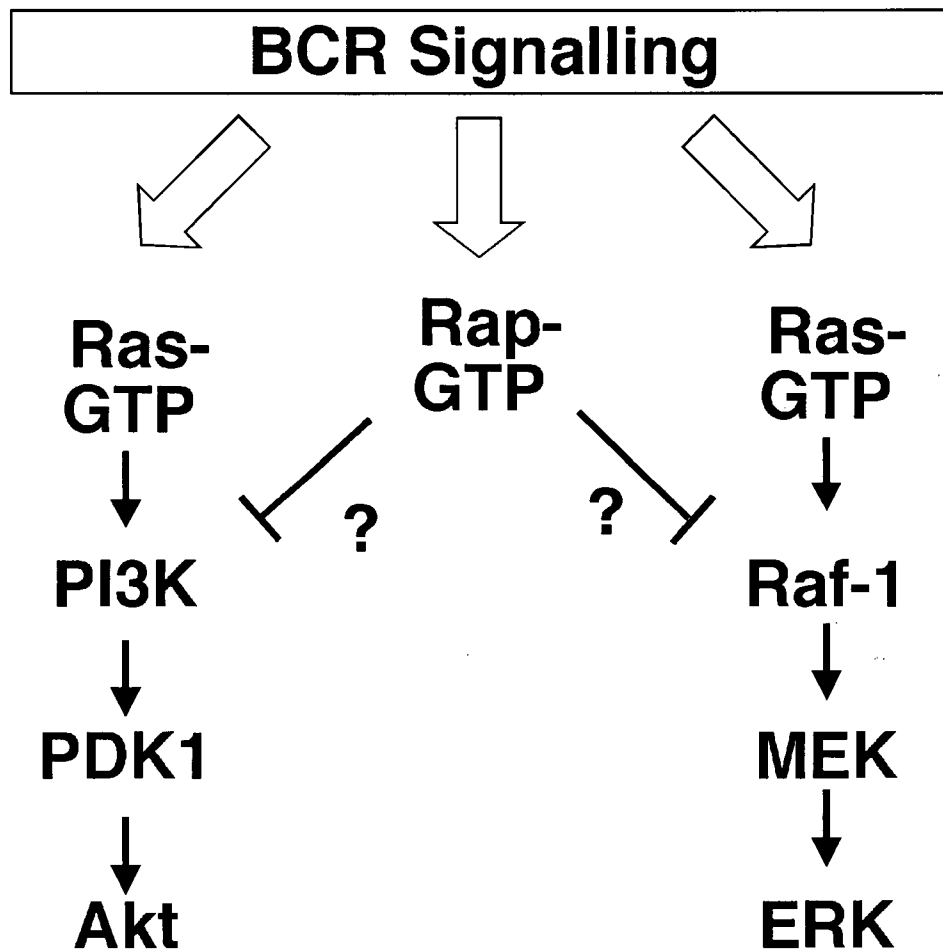


Figure 1.9 Potential regulation of Ras-dependent signalling pathways by the Rap GTPase. Engagement of the BCR leads to the activation of the Ras and Rap GTPases. Since Rap and Ras have identical effector binding domains, but Rap does not activate Ras effectors, it was proposed that Rap could inhibit Ras-dependent signalling. In B cells, activation of Ras can lead to activation of the PI3K/PDK1/Akt pathway and activation of the Raf/MEK/ERK1/2 pathway. Therefore, active Rap could inhibit the BCR-induced activation of the PI3K pathway or the Raf-1 pathway.

BCR-induced cell death. Consistent with the idea that Rap-GTP is a negative regulator of the PI3K/Akt pathway, expressing constitutively active Rap2 (Rap2V12) reduced BCR-induced phosphorylation of Akt and FKHR. Finally, I found that Rap2V12 can bind PI3K and inhibit its activity in a manner that depends upon BCR engagement. This provides a potential mechanism by which Rap-GTP limits activation of the PI3K/Akt pathway, a central regulator of B cell growth and survival.

In the third Section of this thesis I used cDNA microarray analysis to identify gene targets of Akt in B cells. I used a conditionally-active form of Akt that can be specifically activated upon addition of 4-hydroxytomoxifen (4-HT). I identified over 900 genes that are regulated by selectively increasing Akt kinase activity. I examined whether Akt activation resulted in changes in the mRNA levels for 16 of these genes using quantitative Real-Time PCR analysis. Approximately 50% of the expression changes identified by microarray analysis correlated with expression changes determined by Real Time-PCR analysis. These genes are involved in regulating a large variety of processes including regulation of cell cycle progression, cell adhesion, vesicle trafficking, apoptosis and nuclear transport. Therefore, activation of Akt results in changes in gene expression that can potentially influence B cell survival and activation.

1.10 Publications arising from this thesis

1. **S. L. Christian**, R. L. Lee, S. J. McLeod, A. E. Burgess, A. H.Y. Li, M. Dang-Lawson and M. R. Gold. 2003. Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAP kinase activation. **Journal of Biological Chemistry**. 278:41756-41767.

- 2 **S. L. Christian.**, P. V. Sims, and M. R. Gold. 2002. The B cell antigen receptor activates the transcriptional activator β -catenin via protein kinase C-mediated inhibition of glycogen synthase kinase-3. **Journal of Immunology.** 169:758-769

3. H. C.Ting, **S. L. Christian**, A. E. Burgess and M. R. Gold. 2002. Activation and phosphatidylinositol 3-kinase-dependent phosphorylation of protein kinase C- ϵ by the B cell antigen receptor. **Immunology Letters.** 82:205-215

Chapter 2

Materials and Methods

2.1 General Materials and Methods

2.1.1 Antibodies

Goat antibodies against mouse IgM (μ -chain specific), mouse IgG (γ -chain specific) and Armenian hamster Ig were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-mouse- κ light chain antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). The hybridoma producing the HM79-16 hamster anti-mouse Ig β monoclonal antibody was a gift from Dr. Tetsuya Nakamura (University of Tokyo) (201). The 4G10 monoclonal antibody was purchased from Upstate Biologicals Inc. (Lake Placid, NY). The hybridoma producing the anti-murine CD40 monoclonal antibody, 1C10, was a gift from Dr. M. Howard (DNAX Research Institute, Palo Alto, CA) (202). The HM79-16 and the 4G10 monoclonal antibodies were purified from the hybridoma supernatant using a protein G-Sepharose column and 1C10 monoclonal antibody was purified from hybridoma supernatant using a protein A-Sepharose column. Antibodies used for immunoblotting were purchased as indicated in Table 2.1.

Table 2.1 Antibodies used for immunoblotting

The dilution of the primary antibody, the secondary reagent and source for each antibody used for immunoblotting in this thesis is listed. (* anti-mouse-Ig-horseradish peroxidase (HRP) was used at 1:10000, anti-rabbit-Ig-HRP was used at 1:20000 and protein A-HRP or protein G-HRP were used at 1:3000 unless otherwise indicated (Bio-Rad, Hercules, CA); anti-goat-Ig-HRP (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at 1:3000.)

Antibody	1° antibody, diluted in Tris-buffered saline (TBS)	2° Reagent, diluted in Tris-buffered saline* (TBS)	Source
Actin	1:10 000	Anti-mouse-Ig-HRP	Sigma-Alrich (Oakville, ON, Canada).
Phospho-Akt Ser473	1:1000 1% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
Phospho-Akt Thr308	1:1000 1% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
Akt	1:1500 5% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
β -catenin	1:500 5% BSA	Anti-mouse-Ig-HRP 5% BSA (1:2000)	BD Transduction Laboratories (Lexington, KY)
Bim1	1:1000	Anti-rabbit-Ig-HRP	Affinity BioReagents (Golden, CO)
Pan-Cadherin	1:100 5% milk	Anti-goat-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
Phospho-ERK1/21/2 Thr202/Tyr204	1:2000 5% BSA	Anti-mouse-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
ERK1/21	1:2000 1% milk	Anti-rabbit-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
M2 anti-FLAG	1:50 (10 μ g/mL) TBS	Protein G-HRP (no milk)	Sigma-Alrich (Oakville, ON, Canada).
Phospho-FKHR Ser 256	1:500 5% BSA or milk	Protein A-HRP 5% BSA or milk	Cell Signalling Technologies (Beverly, MA)
FKHR	1:500 5% BSA or milk	Protein A-HRP 5% BSA or milk	Cell Signalling Technologies (Beverly, MA)
Phospho-GSK3 α/β Ser9	1:1000 5% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
GSK3	1:1000 5% BSA	Anti-mouse-Ig-HRP	Chemicon International (Temecula, CA)

phospho-JNK Thr183/Tyr185	1:1000 5% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
JNK	1:5000 5% BSA	Anti-rabbit-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
p27 ^{KIP1}	1:2000	Anti-mouse-Ig-HRP	BD Transduction Laboratories (Lexington, KY)
LEF-1	1:200	Anti-mouse-Ig-HRP	ExAlpha Biologicals (Watertown, MA)
phospho-p38 Thr180/Tyr182	1:1000 5% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
p38	1:5000 5% BSA	Anti-rabbit-Ig-HRP	Cell Signalling Technologies (Beverly, MA)
p85 α	1:200	Anti-mouse-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
PDK1	1:1000	Anti-mouse-Ig-HRP	BD Transduction Laboratories (Lexington, KY)
PKC ϵ	1:1000 (1 μ g/mL) 1% BSA	Anti-mouse-Ig-HRP	BD Transduction Laboratories (Lexington, KY)
PKD	1:200 5% milk	Anti-rabbit-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
Phospho-tyr (4G10 hybridoma)	2hr RT: 1:10 TBS 1% BSA	Anti-mouse-Ig-HRP	Upstate Biologicals Inc. (Lake Placid, NY).
Rap1	1:500 5% milk	Protein A-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)
Rap 2	1:2000 5% milk	Anti-mouse-Ig-HRP	BD Transduction Laboratories (Lexington, KY)
Sam68	1:500 5% milk	Anti-rabbit-Ig-HRP	Santa Cruz Biotechnologies (Santa Cruz, CA)

2.1.2 B cell lines and murine splenic B cells.

The B cell lines used in this thesis are described in Table 2.2. All cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 50 μ M 2-ME, 1 mM pyruvate, 2 mM glutamine, 15 U/ml penicillin and 50 μ g/ml streptomycin (complete medium). WEHI-231 cells expressing a myristoylated Akt-estrogen receptor chimeric protein (mER-Akt) or the non-myristoylated version (A2-ER-Akt) were maintained in complete medium supplemented with 0.25 μ g/ml puromycin (Calbiochem, La Jolla, CA), as were WEHI-231 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector (203). A20 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector were maintained in complete medium supplemented with 4 μ g/ml puromycin. Small resting B cells were isolated from the spleens of C57BL/6 mice by Percoll density centrifugation after antibody- and complement-mediated lysis of T cells (57).

Table 2.2 Summary of B cell lines

Cell Line	Description	Original source or person responsible for generation of cell line
K40-B1	Mouse Pro-B cell Expresses Ig α /Ig β subunits of BCR only	gift from Dr. Anthony DeFranco (University of California, San Francisco) (204)
WEHI-231	Immature/transitional mouse B cell Expresses surface IgM Undergoes anti-IgM-induced growth arrest	American Type Culture Collection (Manassas, VA)
WEHI-231 mER-Akt	WEHI-231 cells expressing mER-Akt protein (vector: pMX-PIE)	Sherri L. Christian
WEHI-231 A2-ER-Akt	WEHI-231 cells expressing control A2-ER-Akt protein (vector: pMX-PIE)	Sherri L. Christian
WEHI-231 MSCV	WEHI-231 cells transfected with pMSCV empty vector	Sarah J. McLeod
WEHI-231 RapGAPII	WEHI-231 cells expressing RapGAPII protein (vector: pMSCV)	Sarah J. McLeod
WEHI-231 Rap2V12	WEHI-231 cells expressing Rap2V12 protein (vector: pMSCV)	Sherri L. Christian
A20	Mature mouse B cell Expresses surface IgG	American Type Culture Collection (Manassas, VA)
A20 MSCV	A20 cells transfected with pMSCV empty vector	May Dang-Lawson
A20 RapGAPII	A20 cells expressing RapGAPII protein (vector: pMSCV)	May Dang-Lawson
A20 Rap2V12	A20 cells expressing Rap2V12 protein (vector: pMSCV)	Anita E. Burgess
Ramos	Immature human B cell Expresses surface IgM	American Type Culture Collection (Manassas, VA)

2.1.3 Retrovirus-mediated transfection of WEHI-231 cells

To generate WEHI-231 cell lines expressing the mER-Akt or A2-ER-Akt fusion protein, cDNA encoding these proteins (a gift from Dr. Richard Roth, Stanford University) (205) were subcloned into the pMX-puro-IRES-EGFP retroviral vector, a derivative of pMX-puro (DNAX, Palo Alto, CA) (206). The resulting plasmid (2 µg) was transfected into the BOSC 23 packaging cell line by calcium phosphate transfection and the viral particles produced were used to infect WEHI-231 cells as described (207). Expression of the mER-Akt or A2-ER-Akt protein was detected by immunoblotting with an anti-Akt antibody to detect the 90 kDa fusion protein and the 66 kDa endogenous protein. Stable bulk populations of WEHI-231 cells expressing these chimeric proteins were selected using 0.25 µg/ml puromycin. Bulk populations of WEHI-231 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector were also similarly generated by retroviral-mediated transfection. The cDNAs encoding FLAG-tagged RapGAPII or Rap2V12 in pMSCVpuro were gifts from Dr. M. Matsuda (Osaka University, Osaka, Japan). Expression of FLAG-RapGAPII or FLAG-Rap2V12 was detected by immunoblotting with the M2 anti-FLAG monoclonal antibody.

2.1.4 Transfection of A20 cells by electroporation

A20 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector were generated by electroporation-based transfection. The plasmid DNA was linearized by restriction enzyme digestion then resuspended with 10 million cells in 0.8 ml phosphate buffered saline (PBS) lacking calcium chloride and magnesium chloride (2.7 mM KCl, 1.5 mM KH₂PO₄, 1.4 M NaCl, 8 mM Na₂HPO₄, pH 7.2) in an electroporation cuvette. The cells

were incubated on ice for 10 min then subjected to an electroshock of 400 V and 975 μ F using a Bio-Rad Gene Pulser. The cells were incubated on ice for an additional 10 min, then resuspended in 10 ml complete medium for 36-48 hours. Stable clones were obtained by single cell cloning and selection in medium containing 4 μ g/ml puromycin (Calbiochem, La Jolla, CA).

2.1.5 B cell stimulation and preparation of cell lysates

To reduce basal signalling caused by serum components, WEHI-231 cells were cultured in complete medium with the FCS reduced to 1% for 12 to 18 h prior to stimulation while A20 cells were grown in complete medium with 0.5 mg/ml BSA instead of FCS. The cells were washed once with modified HEPES-buffered saline (25 mM HEPES [pH 7.2], 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1 mg/ml glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 nM 2-ME), resuspended in this buffer at 1×10^7 or 2.5×10^7 per ml, and warmed to 37°C for 10 to 30 min. Where indicated, the cells were incubated with wortmannin (BioMol, Plymouth Meeting, PA), Ly294002 (BioMol), acetyl-leucine-leucine-norleucinol (ALLN) (Sigma, St. Louis, MO), safinol (Calbiochem), U73122 (BioMol), U73343 (BioMol), PD-98059 (BioMol) or U0126 (BioMol) for 20 to 30 min prior to stimulation at the concentrations indicated in the figure legends. The cells were then stimulated with either anti-Ig antibodies, phorbol dibutyrate (PdBu), ionomycin, 4-hydroxytamoxifen (4-HT) (Sigma), LiCl or bisindolylmaleimide I (BIM I) (Calbiochem) at the concentrations indicated in the figure legends for the indicated times. For stimulations lasting more than 1 h, the cells were resuspended at 5×10^5 per ml in complete medium and treated with anti-Ig antibodies at 10 μ g/ml for the indicated times. The reactions were

terminated by adding ice-cold PBS containing 1 mM Na₃VO₄ and then centrifuging the cells for 1 min at 1100 x g in a cold microfuge. For cell lines, unless otherwise indicated, the cell pellets were solubilized in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 8], 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄, 25 mM β-glycerophosphate). Splenic B cells were solubilized in buffer B (20 mM HEPES [pH 7.4], 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1mM PMSF, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄, 25 mM β-glycerophosphate) containing 0.5% Igepal (ICN, Costa Mesa, CA). For Rap activation assays, anti-FLAG or 4G10 immunoprecipitation assays, PI3K enzyme assays and detection of p27^{Kip1} or Bim1 protein levels, the cell pellets were solubilized in Rap lysis buffer (1% Igepal, 50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1mM PMSF, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄, 25 mM β-glycerophosphate). For ERK1/2 *in vitro* kinase assays, the cell pellets were solubilized in ERK1/2 assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM MoO₄, 0.2 mM Na₃VO₄, 1 mM DTT, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 0.7 µg/ml pepstatin, 40 µg/ml PMSF, 10 µg/ml soybean trypsin inhibitor). In all cases, the samples were left on ice for 10 min and then insoluble material was removed by centrifugation. Protein concentrations determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

2.1.6 Immunoblotting

Total cell extracts, cytoplasmic and nuclear fractions (20 µg protein unless otherwise indicated) or immunoprecipitated proteins were separated on SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were blocked for 1 to 2 h with 5%

(w/v) skim milk powder in TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween-20) and then incubated overnight at 4°C with the primary antibody. Antibodies were diluted as specified in Table 2.1. The membranes were then washed with TBST and incubated with the appropriate HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature. Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). To reprobe the membranes, bound antibodies were eluted by incubating the membrane in 10 mM Tris-HCl [pH 2], 150 mM NaCl for 30 min. The membranes were then re-blocked and probed as described above. To quantitate results, scans of ECL exposures were saved as TIFF files and analyzed using ImageQuant 1.2 software (Molecular Dynamics, Sunnyvale, CA).

2.2 Materials and Methods specific for Chapter 3

2.2.1 Preparation of cytoplasmic and nuclear fractions

Nuclear and cytoplasmic fractions were prepared as described by Dignam *et al.* (208). After stimulation, 5×10^6 cells were resuspended in 200 μ l buffer A (10 mM HEPES [pH 7.4], 10 mM KCl, 1.5 mM MgCl₂, 20% glycerol, 0.5 mM DTT, 1mM PMSF, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Na₃VO₄, 25 mM β -glycerophosphate). After 15 min on ice, the non-ionic detergent Igepal was added to a final concentration of 0.5%. The samples were then centrifuged for 1 min at 1100 x g in a microfuge at 4°C. The supernatant was removed and used as the cytosolic fraction. The pellets were rinsed once with buffer A and then extracted with 100 μ l buffer B for 20 min on ice. The insoluble material was removed by centrifuging for 3 min at full speed in a microfuge at 4°C. The supernatant was collected

and used as the nuclear fraction. Protein concentrations for the cytosolic and nuclear fractions were determined using the bicinchoninic acid assay.

2.2.2 β -catenin pull-down assays using a GST-ECT fusion protein

A glutathione S-transferase (GST) fusion protein containing the C-terminal portion of the cytoplasmic domain of E-cadherin (GST-ECT) was used to precipitate unbound β -catenin. The pGEX-4t1-ECT plasmid encoding this fusion protein (a gift from Drs. H. Aberle and R. Kemler, Max Planck Institute for Immunobiology, Freiburg, Germany) (209) was transformed into the *E. coli* strain DH5 α . Fusion protein production was induced by growing the bacteria in the presence of 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) for 12 h at 37°C. The bacteria were then resuspended in sonication buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mg/ml lysozyme, 0.1 mg/ml DNase I, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml aprotinin, 1 mM PMSF) and lysed by sonication (setting 4, 3 x 30 sec). The lysate was centrifuged at 154,000 x g for 45 min at 4°C. The supernatant containing the fusion protein was collected and stored as aliquots at -70°C. To precipitate β -catenin, 30 μ l of bacterial lysate containing the GST-ECT fusion protein were incubated with 15 μ l packed glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. After washing, the beads were mixed with Triton X-100 cell lysates (0.5 mg protein) for 1 h at 4°C. The beads were then pelleted and washed three times with Triton X-100 lysis buffer. Bound proteins were eluted using SDS-PAGE sample buffer.

2.2.3 Luciferase reporter assays

The TOPtk and FOPtk plasmids were obtained from Dr. Marian Waterman (University of California, Irvine) (121). Transient transfection of WEHI-231 cells was performed using the DMRIE-C lipid reagent (Invitrogen, Burlington, Ontario, Canada). Briefly, lipid:DNA complexes were formed by adding the DMRIE-C lipid reagent (12 μ l) and the DNA (4 μ g) to 1 ml of OPTI-MEM medium and incubating for 45 min at 21°C. WEHI-231 cells (4×10^6) that had been resuspended in 0.2 ml complete medium lacking antibiotics were added to this mixture. After 4 hours at 37°C, 2 ml of complete medium lacking antibiotics were added and the cells were grown for an additional 20 h at 37°C. The transfected cells were then resuspended in fresh complete medium and divided into multiple wells of a 24-well plate. The cells were cultured with medium only, 10 μ g/ml goat anti-mouse IgM antibody or 20 mM LiCl for 4 h at 37°C. After washing with PBS, the cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was determined using the Promega luciferase assay system. Readings were made using a MicroLumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) set for a 10 sec acquisition window. Relative luciferase units were derived by normalizing the luciferase activity to the amount of protein in each sample.

2.3 Materials and Methods specific for Chapter 4

2.3.1 Rap activation assay

Cells were stimulated as described, solubilized in Rap lysis buffer (see Section 2.1.5), and then assayed for Rap activation as described previously (163). Briefly, a GST fusion protein containing the Rap1/2-binding domain of the RalGDS protein (GST-RalGDS(RBD)) was used to selectively precipitate the active GTP-bound forms of 1 and Rap2. The

precipitated GTP-bound Rap1 and Rap2 were detected by immunoblotting with antibodies to Rap1 or Rap2. In some experiments the filters were probed with anti-Rap2 antibodies, stripped, and then reprobed with anti-Rap1 antibodies.

2.3.2 PKC- ϵ bandshift assay

Cell lysates (20 μ g protein) were separated on 12% acrylamide gels with an acrylamide to acrylamide:bis ratio of 118:1 and then transferred to nitrocellulose as described by Ting *et al.* (210). Basally phosphorylated PKC- ϵ , as well as a slower migrating form corresponding to a hyper-phosphorylated form of PKC- ϵ that is induced by BCR signalling (210), was detected by immunoblotting with a monoclonal antibody specific for PKC- ϵ .

2.3.3 Immunoprecipitation

Cell lysates were pre-cleared by incubating with Sepharose-CL-4B beads (Sigma) for 30 min at 4°C. For anti-FLAG immunoprecipitation, cell lysates were transferred to tubes containing 10 μ l of agarose beads covalently coupled with the M2 anti-FLAG-monoclonal antibody (Sigma-Aldrich) plus 10 μ l of Sepharose-CL-4B beads as filler. For anti-phosphotyrosine immunoprecipitation, the cell lysates were transferred to tubes containing protein G-Sepharose beads (Sigma-Aldrich) that had been incubated with the 4G10 monoclonal antibody for 1 h at 4°C and then washed with Rap lysis buffer. The cell lysates and the antibody-coated beads were mixed for 1 h at 4°C. The beads were then pelleted and washed three times with Rap lysis buffer. Bound proteins were eluted using SDS-PAGE sample buffer.

2.3.4 ERK1/2 *in vitro* kinase assay

ERK1/22 was immunoprecipitated using monoclonal anti-ERK1/21/2 antibodies conjugated to agarose beads (Santa Cruz Biotechnologies). ERK1/2 *in vitro* kinase assays were performed as described previously using myelin basic protein (Sigma-Aldrich) as a substrate (177). In other experiments, 10 µg/ml GST-ELK-1 (Cell Signalling Technologies) was used as the substrate. Briefly, reactions were initiated by adding 30 µl of kinase assay buffer containing the substrate and 5 µCi ^{32}P - γ -ATP to the immunoprecipitated ERK1/22. After 15 min at room temperature, the reactions were stopped by adding 30 µl of 2X SDS-PAGE sample buffer. The resulting ^{32}P labelled substrate was separated from free ^{32}P - γ -ATP on a 15% polyacrylamide gel and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.3.5 PI3K *in vitro* kinase assay

PI3K enzyme assays were performed on anti-phosphotyrosine immunoprecipitates or on anti-FLAG immunoprecipitates as described by Gold *et al.* (211). The immunoprecipitates were washed twice with Rap lysis buffer and three times with 10 mM Tris-HCl, pH 7.5. The immunoprecipitates were then incubated with 10 µg phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and ^{32}P - γ -ATP for 15 min at room temperature. The resulting ^{32}P -labeled phosphatidylinositol 3-phosphate was separated from the ^{32}P - γ -ATP by thinlayer chromatography and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.3.6 Transient transfection of HEK293 cells

HEK293 cells were transiently transfected with FLAG-tagged Rap1 V12 in the MSCV vector or empty vector. Lipid:DNA complexes were formed by adding the LF2000 lipid reagent (15 μ l, Invitrogen) and the DNA (5 μ g) to 0.5 ml of OPTI-MEM medium and incubating for 20 min at 21°C. The lipid:DNA complexes were then added to a confluent layer of HEK293 cells grown in 6-well tissue culture dishes and incubated at 37°C for 36 h. The cells were solubilized in 0.5 ml Triton X-100 lysis buffer and protein concentrations determined using the bicinchoninic acid assay.

2.4 Materials and Methods specific for Chapter 5

2.4.1 RNA isolation and purification

WEHI-231 cells expressing either mER-Akt or A2-ER-Akt were generated by retroviral-mediated gene transfer as described above (Section 2.1.3). WEHI-231 cells expressing these chimeric proteins were resuspended at 5×10^5 cells/ml in complete medium and stimulated with 2 μ M 4-HT for 3 h, 20 h or left unstimulated. The cells were then pelleted by centrifugation and total RNA isolated using Trizol™ (Invitrogen, Burlington, ON) according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and the concentration determined by spectrophotometry after every manipulation. Contaminating genomic DNA was removed by DNase treatment with the DNA-free kit from Ambion (Austin, TX). Briefly, 10 μ g of total RNA were incubated with 5 Units DNase for 30 min at 37°C. DNase-inactivation reagent (10% v/v) was then added to remove the DNase enzyme as well as buffer salts. The RNA was concentrated by adding 0.5 M ammonium acetate and 2.5 volumes of 95% ethanol and incubating at -20°C for 20 min. The RNA was then pelleted by centrifugation for 15 min and redissolved in 10 μ l of RNase-

free water. The absence of genomic DNA contamination was confirmed by polymerase-chain reaction (PCR) analysis using primers for β -actin, which do not amplify across introns (see Table 2.3 for primer sequences). PCR reactions were carried out using puRe Taq Ready-To-Go PCR beads (Amersham Biosciences, NJ) using the following PCR protocol: 1 min at 95°C, followed by 35 cycles of 45 sec at 95°C, 45 sec at 54°C, and 45 sec at 72°C, with a final incubation of 5 min at 72°C. PCR products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gels.

Table 2.3 Sequences of primers used in this thesis

PCR¹

GenBank ID; description	Forward Primer ²	Reverse Primer	Amplicon length
6671508; b-actin	GATGTCACGCACGATT CC	GTCCCTGTATGCCTCTGG TC	200

Real-time quantitative PCR³

GenBank ID; description	Forward Primer	Reverse Primer	Gene Symbol	Amplicon length
<i>Housekeeping genes</i>				
6671508; b-actin	CGTGGGTGACATCAAG AGAA	AACCGCTCGTTGCCAAT AGT	b-act	141
NM009736; b-2-microglobulin	TGAATTCACCCCCACTG AGAC	TCATGCTTAACTCTGCA GGCG	b2M	51
XM132897; GAPDH	ATGTGTCCGTCGTGGAT CTGA	TGCCTGCTTCACCACCTT CTT	GAPDH	80
AF539799; NADPH oxidase	AGTGGCATCCCTTCACTC TGA	AGTCCCTGCTGCTCGA ATAT	NADPH	75
<i>Select genes from cDNA microarray analysis for gene expression validation by Real-Time PCR</i>				
C80807; Mouse mRNA for zeta-crystallin/quinone reductase_ partial cds ⁴	GACCGGCATGCAGTATT CTGA	TAAAGCCTCTGGAAAGCT TCGG	zcrq	104
AW557767; Mus musculus Lps/Ran GTPase (lps(d)) mRNA_ complete cds ⁴	TAAATAAGGTGGCACAC GCG	TCCCAGATGAGGATGAT GACC	ran	118

Table 2.3 cont'd

C79683; Mus musculus catenin alpha 1 (Catn1)_ mRNA	CCCCAGTACCCAATGCA GATA	TGACTGATGGTACCCCC TCATA	acat	107
AU045048; Bases 1-139 hom to SOS&Rab11a	CTCAGGAGCTGAAAGTT GGCAG	GGATGTGAACAAAGAGA ACCCA	sos	103
AW538600; Homo sapiens RAP1_ GTPase activating protein 1 (RAP1GAP1) mRNA	TGACCCCTATCCACAACC AAGG	TCTGCTTCACCAATCACC CA	rpgp	111
AW550755; Mus musculus H19 and muscle-specific Nctc1 genes_ complete sequence	ATTGAGCCACCAACTCC CTCT	TTCGCGTACGTTTGAAG GA	nctc	105
AW556230; Mus musculus cell division cycle 42 homolog (S. cerevisiae) (Cdc42)_	TGACTGGTCCCCCATGTT GGTT	GGCTGATGATTGCCATC TCCT	Cd42	110
C85275; Homo sapiens poly(A)-binding protein_ nuclear 1 (PABPN1)_ mRNA	GGCCTGCTGTAAAACC ACTG	ACCAGGCATCAGCACAA CAGA	pbnp	111
C85448; Mus musculus apoptosis inhibitor 1 (Api1)_ mRNA bases 2040-2676	CCAGCTCCCATTCGGAAC TTGA	TTTGCGGGTGTCTCGTG CTAT	api1	53
AW559047; Mus musculus RAB11B_ member RAS oncogene family (Rab11b)_ mRNA	CGGTCAGCGATTGTGCTT CTGT	CCGTGCCCTTTGCAGAAA AGA	rab	135
AU017072; Mouse mRNA for syntaxin 1B_ complete cds 285-656 ⁴	CTCCTGGCCCTTGCTCTGA TACT	AGCCGTGTCTGACACCA AGAA	syn	51
AU041108; Mus musculus mitogen activated protein kinase kinase 1 (Map2k1)_ mRNA	GACTGAAGCCCACGCTAT CATGG	TCAAAAACCCCTAAGCA GAGCC	mapk	52
AW545241; Mouse mRNA for nonmuscle tropomyosin 5	GATGGCACTTTAGAGAC CGCA	GAACATCATCTCATCGC CCTC	trop	51

Table 2.3 cont'd

AW546008; Mus musculus heterogeneous nuclear ribonucleoprotein A1 (Hnripal)	GGAGTTTTTGCCACAC ACAT	AACTCAGCCAAGCACAG TGGT	hm	88
AW546078; Rat mRNA for ribosomal phosphoprotein P2	AACCCCTGAGCGATGACA TCCT	TCGGCTCAACAAGGTCA TCA	P2	65
Aw557306; Mus musculus cyclin D2 (Ccd2) mRNA ⁴	GCTTCCAGTTGCAATCA TCGA	TGTGCGCTACCGACTTC AAGT	CyD2	59

¹The annealing temperature for primers was 54°C.

²All primers are written 5' to 3'.

³Primers for real-time PCR were designed to the following specifications: primer T_m: 58-60°C, %CG: 20-80, amplicon length: 50-150 bases, amplicon T_m: 75-80 °C. Primers were used at 300 nM with the exception of those indicated.

⁴Primers were used at 900 nM.

2.4.2 Microarray hybridization

Purified RNA was reverse transcribed using an oligod(T) primer and either a Cy3- or Cy5-dUTP label incorporated during the reverse transcription reaction using protocols provided by the Gene Array Facility at the Prostate Center (Jack Bell Research Centre, Vancouver, BC). Specifically, 20 µg of RNA were resuspended along with 100 µM oligo d(T) primer, 6.7 mM of dATP, dCTP and dGTP, 2mM dTTP, 0.1 mM DTT and 1 mM Cy-dUTP in a final volume of 25 µl. The reaction was incubated at 65°C for 5 min then 42°C for 5 min to resolve secondary RNA structure and allow stable binding of the primer. Superscript II (200 U, Invitrogen) and 10,000 Units of RNasin (Invitrogen) were then added and the mixture incubated at 42° C for 3 h. To stop the reaction, 0.2 M NaOH was added to the reaction mixture and the reaction mixture was heated to 65°C for 15 min. The pH was neutralized by addition of HCl (0.13 M final concentration) and Tris-Cl, pH 7.5 (0.6 M final concentration). The labeled cDNA preparations were combined as indicated in Table 2.4. Fluorescently-labeled cDNA encoding GFP (green-fluorescent protein) was added to the cDNA and purified using a Qiagen PCR purification kit (Mississauga, ON). The labeled cDNA was precipitated by adding 0.3 M sodium acetate and 2.5 volumes of 95% ethanol, then incubating overnight at -20°C. The solution was centrifuged for 10 min to pellet the cDNA and the pellet washed with 70% ethanol. After drying by air, the pellet was resuspended in 50 µl hybridization buffer (0.5X Formamide, 5X SSC [0.75 M NaCl, 75 mM sodium citrate, (pH 7.0)], 0.1% SDS, 25 µg polyA, 10 µg BSA, 20 µg salmon testes DNA). The NIA (National Institute of Aging) 15K cDNA microarray slides (printed on Array-It slides, TeleChem International Inc, Sunnyvale, CA) were obtained from the Gene Array Facility in the Prostate Center (Jack Bell Research Centre, Vancouver, BC). The NIA 15K array set

was created by PCR amplification of a random cDNA library generated from mouse re- and periimplantation embryos, E12.5 female gonad/mesonephros, and newborn ovary cells (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>). The slides were washed twice with 0.1% SDS for 5 min and then five times 1 min with distilled water. All wash steps were done with shaking on a vortex at low speed. The slides were heated for 3 min in a 95°C water bath to denature the bound cDNA probes just before use and dried by centrifugation. The labelled cDNA probe was denatured for 3 minutes at 95°C and then kept at 65°C until used. The probe was pipetted onto the denatured slide, as indicated in Table 2.4, and a cover slip placed on top. The slide was placed in a hybridization chamber and incubated overnight at 42°C in a humidified chamber. The cover slip was then removed by floating the slide on 0.2X SSC and then the slide was washed 3 times for 5 minutes each in Wash Solution I (0.1X SSC, 0.1% SDS) and 3 times for 5 minutes in Wash Solution II (0.1X SSC). The slides were then dried by centrifugation.

Table 2.4 Experimental layout of Microarray analysis of Akt target genes in the WEHI-231 B cell line by slide identification number and experiment.

Slide Barcode Number	Cy3-labelled (_1) Cell type; treatment (shorthand symbol)	Cy5-labelled (_2) Cell type; treatment (shorthand symbol)
Experiment #1		
PRMS02-S3-0001-CD011-031	mER-Akt; untreated (mER-0h)	mER-Akt; + 4-HT 3 h (mER -3h)
PRMS02-S3-0001-CD011-032	A2-ER-Akt; + 4-HT 3 h (A2-3h)	A2-ER-Akt; untreated (A2-0h)
PRMS02-S3-0001-CD011-032	mER-Akt; + 4-HT 20 h (mER -20h)	A2-ER-Akt; + 4-HT 20 h (A2-20h)
Experiment #2		
PRMS02-S3-0001-CF013-001	mER-Akt; untreated (mER -0h)	mER-Akt; + 4-HT 3 h (mER -3h)
PRMS02-S3-0001-CF013-002	A2-ER-Akt; + 4-HT 3 h (A2-3h)	A2-ER-Akt; untreated (A2-0h)
PRMS02-S3-0001-CF013-003	mER-Akt; + 4-HT 20 h (mER -20h)	A2-ER-Akt; + 4-HT 20 h (A2-20h)
Experiment #3		
PRMS02-S3-0001-CF013-004	mER-Akt; + 4-HT 3 h (M-3h)	mER-Akt; untreated (M-0h)
PRMS02-S3-0001-CF013-005	A2-ER-Akt; untreated (A2-0h)	A2-ER-Akt; + 4-HT 3 h (A2-3h)
PRMS02-S3-0001-CF013-006	A2-ER-Akt; + 4-HT 20 h (A2-20h)	mER-Akt; + 4-HT 20 h (mER -20h)

2.4.3 Real-time PCR

RNA was isolated and purified as described above. cDNA was generated by reverse transcription using an oligo d(T)₁₆ primer and the TaqMan Reverse transcription reagents (P/N N808-0234, Applied Biosystems Inc, Foster City, CA). Briefly, 2 µg purified RNA were incubated with 1X TaqMan RT buffer, 5.5 mM MgCl₂, 500 µM dNTP, 2.5 µM oligo d(T)₁₆, 4 Units RNase inhibitor and 12.5 Units MultiScribe reverse transcriptase for 10 min at 25°C, followed by a 30 min incubation at 48°C and a 5 min incubation at 95°C to inactivate the enzyme. Real-time PCR was performed using the SYBR Green PCR master mix (P/N 4309155, Invitrogen) following the manufacturer's protocol as follows. Briefly, the cDNA generated from 10 ng RNA were incubated with 1X SYBR Green PCR master mix and either 300 or 900 nM of each primer as indicated in Table 2.3. PCR reactions (25 µl final volume) each were added to the 96-well ABI Optical Reaction Plate (P/N 4306737). The thermal cycling conditions were as follows: 10 min at 95°C for AmpliTaq Gold activation followed by 43 cycles of 15 sec at 95°C and 1 min at 60°C. Double-stranded PCR amplicons fluorescently-labeled with SYBR Green were detected using the ABI sequence detection system.

The relative levels of starting template were calculated using the $\Delta\Delta C_t$ method as shown below. C_t is defined as the cycle number where the amplification product crosses a defined threshold. Since one PCR cycle is equivalent to a two-fold difference in PCR product and this is proportional to the amount of starting material the difference in C_t from the treated to the untreated sample is indicative of the relative levels of starting template in each sample. This was calculated as follows.

1. A correction for loading was calculated for each sample

$$Ct_{\text{normalization factor}} + Ct_{\text{gene of interest-n}} = \Delta Ct_n$$

The normalization factor for each treatment or sample was calculated as the difference between the geometric mean of 4 housekeeping genes in the unstimulated sample and the treated sample as outlined by Vandesompele *et al.* (212). The geometric mean is a measure of the arithmetic mean appropriate for numbers that are in a logarithmic scale such as is the case with the Ct measurement where the difference of one Ct is the equivalent of a 2-fold change. The geometric mean of the Ct of the housekeeping genes was calculated as the antilog of the following formula, where x is the Ct of each gene and i represents a specific gene.

$$\overline{\log x} = 1/n \sum_{i=1}^n \log x_i$$

2. The difference in ΔCt 's between reference and treated sample was then calculated.

$$\Delta Ct_r - \Delta Ct_n = \Delta \Delta Ct_n$$

3. To calculate the relative fold difference between the reference sample and the treated sample $\Delta \Delta Ct_n$ is transformed to the base 2.

$$2^{(\Delta \Delta Ct_n)} = \text{relative fold difference}$$

Chapter 3

The B cell antigen receptor regulates β -catenin levels via Protein Kinase C-mediated inhibition of Glycogen Synthase Kinase-3

3.1 Introduction

β -catenin is a transcriptional activator that has important roles in early development, cancer, and stem cell proliferation. During development, β -catenin is an essential component in dorsal-ventral axis formation in *Xenopus* embryos, cuticle patterning in the *Drosophila* embryo, and asymmetric cell division in early *Caenorhabditis elegans* embryos (106). β -catenin has also been shown to play an important role in mammalian development since it is necessary for correct embryonic development of the anterior-posterior axis (138) and for hair follicle morphogenesis (213) in mice. β -catenin is also an important factor in haematopoietic stem cell (HSC) proliferation (214). Ectopic expression of β -catenin in murine HSCs promotes cell cycle progression and proliferation, while maintaining the cells in an apparently undifferentiated state. In contrast, inhibition of β -catenin-dependent signalling inhibits HSC proliferation. In adult animals, β -catenin appears to be involved in cell survival and proliferation as mutations that result in the accumulation of β -catenin are found in approximately 85% of colorectal cancers. β -catenin performs many of its diverse roles by regulating gene transcription. For example, β -catenin regulates the expression of genes involved in *Xenopus* development such as *siamois* and *gooseoid* (138,139) as well as genes involved in promoting mammalian cell growth and proliferation including *c-Myc* and *cyclin D1* (140-142). Since β -catenin is expressed in many different types of cells including

colorectal cells, epidermal cells (215), and lymphocytes (143,214) it is possible that β -catenin may function as a general survival factor.

β -catenin promotes gene transcription by interacting with members of the LEF-1/TCF family of transcription factors (120,123). Nuclear accumulation of β -catenin, in response to signals from surface receptors, allows β -catenin to relieve LEF-1/TCF mediated gene repression by displacing the Groucho/TLE family of repressors (121-123) or by sequestering histone deacetylase activity (124). β -catenin then promotes gene transcription from promoters with LEF-1/TCF binding sites in association with a member of the LEF-1/TCF family of transcription factors (120,123) and the CREB-binding protein (125-127).

The regulation of β -catenin is best understood in the context of the Wnt signalling pathway. In resting cells, phosphorylation of β -catenin by GSK-3 promotes the ubiquitination and subsequent degradation of β -catenin by the proteasome degradation machinery. However, in the presence of Wnt hormones, GSK-3 activity is inhibited by a poorly understood mechanism that involves the Disheveled protein. Inhibition of GSK-3-dependent phosphorylation of β -catenin allows β -catenin to accumulate in the cytoplasm resulting in its translocation into the nucleus where it promotes transcriptional activation in cooperation with members of the LEF-1/TCF family of DNA-binding proteins (110,120,123).

In addition to being regulated by Wnt signalling, the GSK-3/ β -catenin pathway can also be regulated by Akt. Akt has been shown to phosphorylate GSK-3 α and β on their negative regulatory sites (92) and receptors such as the insulin receptor (93,200) and the epidermal growth factor receptor (216) can inhibit GSK-3 activity via Akt-mediated phosphorylation of GSK-3. In B cells, BCR stimulation results in GSK-3 phosphorylation

and concomitant inhibition of GSK-3 kinase activity (57). Since inhibition of GSK-3 kinase activity by Wnt signalling results in an increase in β -catenin levels, I hypothesized that the inhibition of GSK-3 that occurs after BCR engagement would also result in increased β -catenin levels (see Fig. 1.9). Although β -catenin-dependent signalling induced by Wnt hormones plays an important role in early B cell development (143), the possibility that β -catenin could be involved in BCR signalling in mature B cells had not been investigated.

In this chapter, I show that BCR signalling causes an increase in β -catenin protein levels as well as an increase in β -catenin-dependent transcription in B cells. I show that the low level of β -catenin protein present in unstimulated B cells is maintained by proteasome-mediated degradation. In addition, I found that signalling by CD40 potentiated the BCR-induced increase in β -catenin-mediated transcription.

I also examined the mechanism by which the BCR-induces phosphorylation of GSK-3. Although the BCR activates Akt (57), I found that Akt activation by itself was unable to cause significant phosphorylation of GSK-3 or upregulation of β -catenin in B cells. In vitro, the phosphorylation of GSK-3 on its negative regulatory sites (serine 9 of GSK-3 β , serine 21 of GSK-3 α) can also be carried out by a variety of kinases including PKC enzymes and p90^{RSK} (94,96,98-100,217). Since the BCR causes strong activation of a number of PKC isoforms, I investigated whether the BCR regulates GSK-3 and β -catenin via PKC. I found that both BCR-induced phosphorylation of GSK-3 and BCR-induced upregulation of β -catenin were mediated primarily by PLC-dependent activation of PKC and not by Akt or by the ERK1/2/p90^{RSK} pathway. Thus, the BCR regulates β -catenin protein levels and β -catenin-mediated transcription via a PLC- γ 2/PKC/GSK-3 signalling pathway.

3.2 BCR engagement causes an increase in nuclear and cytosolic β -catenin protein levels in B cell lines and in splenic B cells

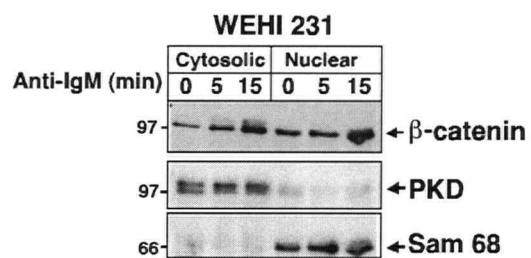
GSK-3 is a constitutively active kinase that normally keeps β -catenin levels low by phosphorylating β -catenin such that it becomes a target for ubiquitin-mediated degradation. Since BCR signalling inhibits GSK-3 activity (57), I hypothesized that BCR engagement would lead to an increase in β -catenin levels. To test this, I prepared cytosolic and nuclear fractions from WEHI-231 B lymphoma cells that had been stimulated with anti-IgM antibodies (Abs) for various times. Cytosolic and nuclear fractionation was performed by inducing hypotonic swelling of the cells followed by a low detergent extraction to obtain the cytosolic fraction and subsequent re-extraction with 0.42 M NaCl to obtain the nuclear fraction. I found that BCR engagement caused an increase in β -catenin levels in both cellular compartments within 5 to 15 min (Fig. 3.1A). Since β -catenin is a transcriptional activator, in all subsequent experiments I focused on the levels of β -catenin in the nuclear fraction of the cells. I found that BCR engagement consistently caused an increase in the levels of β -catenin in the nuclear fraction of WEHI-231 cells and that this increase persisted for at least 1 h (Fig. 3.1B,D).

To show that the BCR-induced increase in the amount of β -catenin in the nuclear fractions of the cells was not due to contaminating cytosolic β -catenin, I validated my cell fractionation technique by reprobing the blots with Abs to the cytosolic protein PKC μ /protein kinase D (PKD) (218) and the nuclear protein Sam 68 (Fig. 3.1A, middle and lower panels). PKD is a cytosolic protein kinase while Sam 68 is a 68-kDa nuclear RNA-binding protein that is phosphorylated by Src during mitosis (219,220). I found that the Sam 68 was entirely

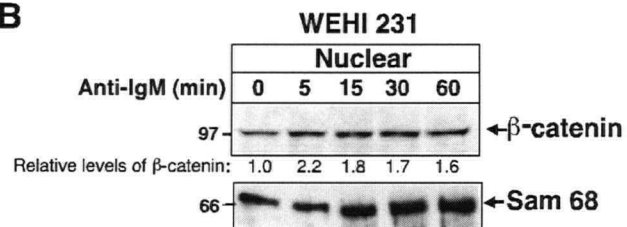
Figure 3.1. BCR engagement increases β -catenin protein levels in the WEHI-231 B cell

line. *A*, WEHI-231 cells were stimulated with 40 μ g/ml goat anti-mouse IgM antibodies for the indicated times. *Upper panel*, cytoplasmic and nuclear fractions (20 μ g protein) were analyzed for β -catenin levels by immunoblotting with an antibody specific for β -catenin. *Middle and lower panels*, the membrane was reprobed with antibodies to the cytosolic protein PKD and the nuclear protein Sam 68 to show that there was little cross-contamination of the nuclear and cytoplasmic fractions. *B*, WEHI-231 cells were stimulated with 40 μ g/ml goat anti-mouse IgM antibodies for the indicated times. Nuclear fractions (20 μ g protein) were analyzed for β -catenin levels by immunoblotting. As a loading control, the blot was reprobed with antibodies to the nuclear protein Sam 68. Molecular mass standards (in kDa) are indicated to the left of each panel. The normalized amount of β -catenin for each sample was calculated by dividing the intensity of the β -catenin band by the intensity of the corresponding Sam 68 band. The relative β -catenin levels were calculated by setting the value for unstimulated cells to 1. *C*, Sam 68 levels do not change significantly after BCR engagement ($p > 0.09$). The nuclear Sam 68 levels were determined by comparing the intensity of the Sam 68 band from the stimulated samples to the band from the unstimulated sample. For each point the mean \pm SEM of the relative level of Sam 68 (unstimulated = 1.0) is shown. For each time point, the data represent results from 10 or more independent experiments except for the 60 min time point which is from 5 experiments. *D*, WEHI-231 cells were stimulated with 40 μ g/ml goat anti-mouse IgM antibodies for the indicated times and the relative levels of nuclear β -catenin were determined for all experiments performed. The mean \pm SEM for each time point is shown. n is the number of independent experiments. Significance as determined by Student's t -test: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

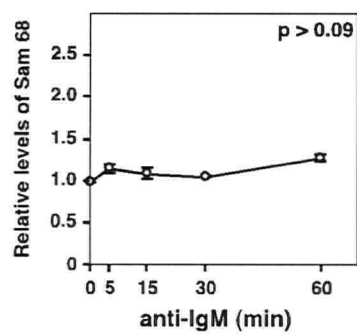
A



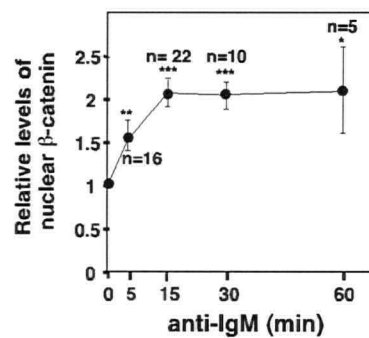
B



C



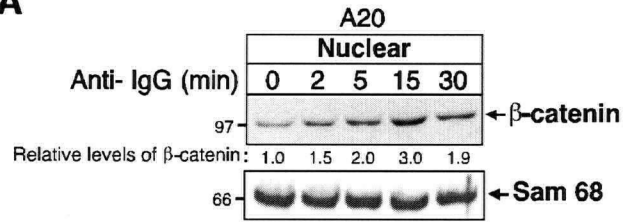
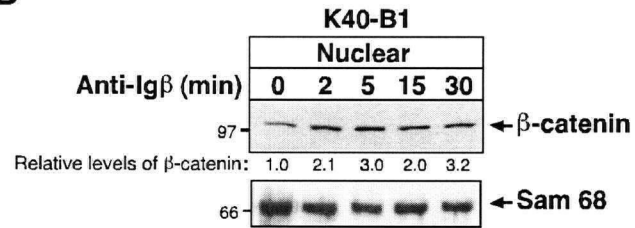
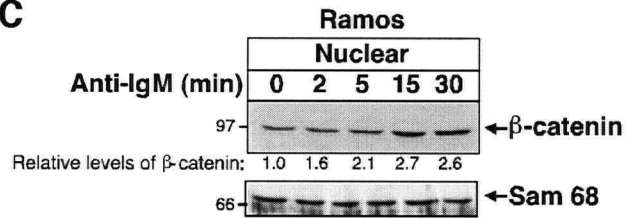
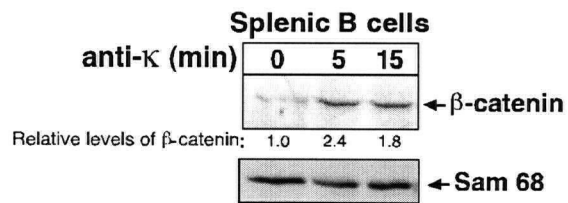
D



in the nuclear fractions while only a small amount of the total PKD was in the nuclear fractions. Thus, the nuclear fractions were only minimally contaminated with cytosolic proteins. Since the amount of β -catenin in the nuclear fractions was equal to or greater than that in the cytosol fractions, neither the basal nor the BCR-stimulated levels of β -catenin in the nuclear fractions can be accounted for by the small amount of cytosolic contamination, as determined by the presence of PKD in the nuclear fractions. Further analysis showed that the levels of Sam 68 in the nuclear fractions increased slightly after BCR engagement but that this was not statistically significant as assessed using Student's T-test ($p > 0.09$) (Fig. 3.1C). I therefore used the levels of Sam 68 as a loading control and calculated the relative levels of β -catenin in the nuclear fraction of each sample by dividing the intensity of the β -catenin band by the intensity of the corresponding Sam 68 band for that sample (Fig. 3.1B,D). Even if Sam 68 levels do increase slightly after BCR engagement, then I am underestimating the BCR-induced increases in the levels of β -catenin in the nucleus. In any case, figure 3.1D shows that engaging the BCR for 15 to 60 minutes caused a statistically significant 2- to 2.5-fold increase in the levels of β -catenin in the nuclear fractions of WEHI-231 B lymphoma cells.

To determine if upregulation of β -catenin is a consistent characteristic of BCR signalling I extended this analysis to include two other murine B cell lines as well as a human B cell line and murine splenic B cells (Fig. 3.2). The A20 and K40-B1 murine B cell lines represent different stages of B cell development than WEHI-231 cells. While WEHI-231 cells express membrane IgM and represent immature/transitional B cells that are susceptible to antigen-induced clonal deletion, A20 cells express surface IgG and represent mature B cells that have undergone Ig class switching. K40-B1 cells represent pro-B cells that express

Figure 3.2. BCR engagement increases β -catenin levels in B cell lines and in murine splenic B cells. *A*, A20 cells were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse IgG antibodies. *B*, K40-B1 pro-B cells were stimulated with 30 $\mu\text{g/ml}$ of the HM79-16 anti-Ig β monoclonal antibody plus 15 $\mu\text{g/ml}$ goat anti-hamster Ig antibodies. *C*, Ramos cells were stimulated with 40 $\mu\text{g/ml}$ goat anti-human IgM antibodies. Nuclear fractions of the cells (20 μg protein = 5×10^5 cell equivalents) were analyzed for β -catenin levels by immunoblotting. *D*, Splenic B cells were stimulated with 30 $\mu\text{g/ml}$ goat anti-mouse κ light chain antibodies. Total cellular extracts (1.6×10^7 cell equivalents) were analyzed for β -catenin levels by immunoblotting. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 1. Molecular mass standards (in kDa) are indicated to the left of each panel. Each experiment was performed at least three times with similar results.

A**B****C****D**

the Ig α /Ig β heterodimer on their surface in the absence of mIg (204). The Ig α /Ig β heterodimer on K40-B1 cells is associated with several proteins including calnexin and Src family kinases (221) and may therefore be part of a pro-B cell receptor. Moreover, crosslinking this putative pro-BCR with anti-Ig β antibodies induces a subset of the signalling reactions characteristic of the mIg-containing BCR (ref. 221 and Fig. 3.3). I found that engaging the BCR on A20 cells resulted in an increase in the levels of β -catenin in the nuclear fractions of these cells (Fig. 3.2A). Similarly, clustering the putative pro-B cell receptor on K40-B1 cells with anti-Ig β antibodies also caused an increase in nuclear β -catenin levels (Fig. 3.2B).

To extend this analysis beyond murine B cell lines, I showed that BCR engagement caused an increase in nuclear β -catenin levels in the Ramos human B cell line (Fig. 3.2C). Finally, to confirm that this response also occurs in normal B cells, I showed that engaging the BCR on murine splenic B cells caused an increase in β -catenin levels (Fig 3.2D). Both the time course and the magnitude of the BCR-induced increases in β -catenin levels were similar in all of the cells I examined (Figs. 3.1, 3.2). The upregulation of β -catenin occurred within 2 to 5 min and was sustained for at least 30 min, with the maximal increases in the range of 2- to 3-fold. This response is similar in magnitude to the increase in nuclear β -catenin levels caused by LPS in macrophages (222). β -catenin levels remain elevated in response to LPS stimulation of macrophages and monocytes for up to 5 h (222,223), in response to fibroblast growth factor stimulation of HUVEC cells for up to 6 h (224) and in response to epithelial growth factor stimulation of epithelial cells for up to 9 h (225). My preliminary data suggest that β -catenin levels may be sustained only up to 3 h after BCR

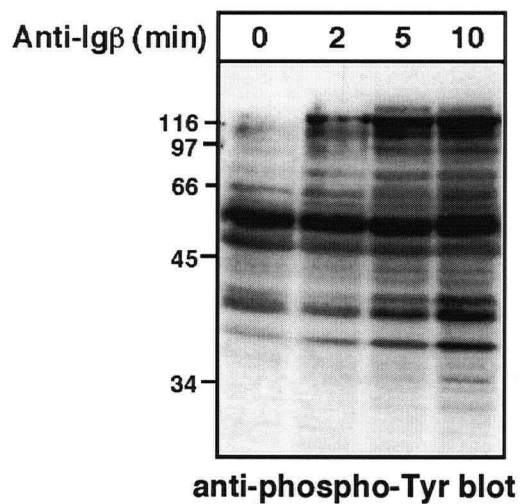


Figure 3.3. Stimulation of K40-B1 cells through the pro-BCR results in increased global tyrosine phosphorylation. K40-B1 pro-B cells were stimulated with 30 $\mu\text{g}/\text{ml}$ of the HM79-16 anti-Ig β monoclonal antibody plus 15 $\mu\text{g}/\text{ml}$ goat anti-hamster Ig antibodies for indicated times. Triton X-100 extracts (20 μg protein) were analyzed for tyrosine phosphorylation by immunoblotting with the 4G10 anti-phosphotyrosine (anti-P-Tyr) monoclonal antibody. Molecular mass standards (in kDa) are indicated to the left of each panel. This experiment was performed three times with similar results.

engagement (data not shown). However, the increase in β -catenin was not consistent past 1 h of BCR engagement. Nevertheless, upregulation of nuclear β -catenin levels is a consistent characteristic of BCR signalling that occurs in normal B cells and in B cell lines representing multiple stages of B cell development.

3.3 BCR engagement increases β -catenin-mediated transcription

Since β -catenin is a transcriptional co-activator, I examined whether the BCR-induced increase in β -catenin protein levels in the nuclear fraction of B cells correlated with an increase in β -catenin-mediated transcription. In other cell types, β -catenin promotes transcription by binding to LEF-1/TCF DNA-binding proteins and the p300/CBP co-activator. To assess β -catenin-dependent transcription, I performed reporter gene assays using the TOPtk and FOPtk plasmids. The TOPtk plasmid contains multiple LEF-1/TCF binding sites, as well as the minimal thymidine kinase promoter, upstream of the luciferase gene (121). The binding of β -catenin-LEF-1/TCF complexes to the TOPtk promoter has been shown to stimulate transcription of the luciferase reporter gene. The FOPtk plasmid is identical except that it contains mutated LEF-1/TCF binding sites and therefore does not bind LEF-1/TCF proteins that can recruit β -catenin. Figure 3.4 shows that crosslinking the BCR on WEHI-231 cells caused a 2.2-fold increase in luciferase activity in cells transfected with the TOPtk plasmid but did not cause a significant increase in luciferase activity in cells transfected with the FOPtk plasmid. This increase was significant ($p < 0.05$) and comparable to the receptor induced increases in β -catenin-mediated transcription of 2- to 2.5-fold observed by others (224). Thus, BCR signalling can stimulate β -catenin-dependent

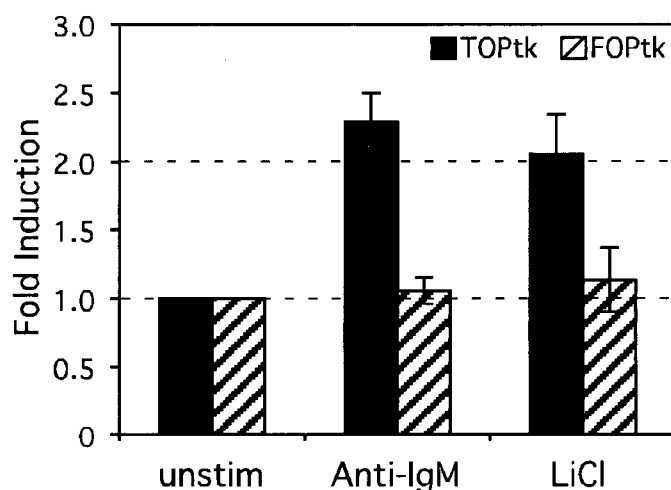


Figure 3.4. BCR engagement increases β -catenin-dependent transcription.

WEHI-231 cells were transiently transfected with the TOPtk or FOPtk plasmids and then cultured for 20 h at 37°C. Triplicate or duplicate samples were then cultured for 4 h at 37°C with 10 μ g/ml goat anti-mouse IgM, 20 mM LiCl, or no addition (unstim). Luciferase activity assays were performed on cell extracts. Luciferase units were normalized to the protein concentration for each sample. The values for unstimulated cells that had been transfected with either the TOPtk or FOPtk plasmid were set to 1 and the values for the anti-IgM- and LiCl-treated samples are reported as “fold induction” relative to the corresponding unstimulated sample. The data represent the mean \pm SEM from four independent experiments. Significance as determined by Student’s t-test: * $p < 0.05$.

transcription driven by the LEF-1/TCF-binding sites in the TOPtk promoter. This suggests that the BCR-induced increase in β -catenin protein levels in B cells could result in increased transcription of genes that contain LEF-1/TCF binding sites. I also found that treating WEHI-231 cells with a GSK-3 inhibitor, LiCl, resulted in a 2-fold increase in luciferase activity (Fig. 3.4). As will be discussed in Section 3.7, this indicates that inhibition of GSK-3 in the absence of other signals is sufficient to increase β -catenin-dependent transcription in WEHI-231 cells.

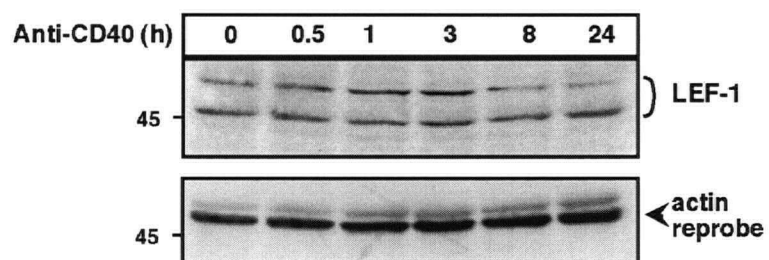
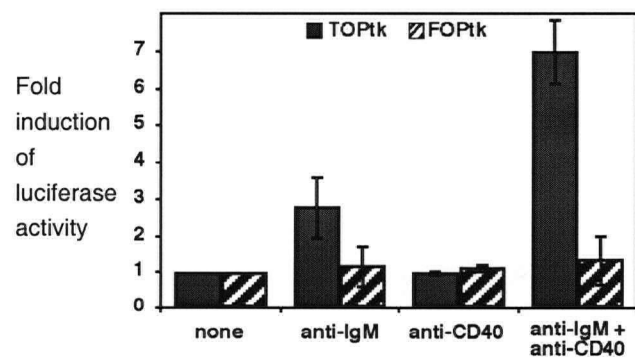
3.4 CD40 and BCR co-stimulation results in a synergistic increase in β -catenin-mediated transcription

Proliferation of immature B cells requires two signals, the first from the binding of antigen by the BCR and a second co-stimulatory signal that is generated by the binding of CD40 to CD154 (CD40 ligand) on an activated T helper cell (1). Engagement of the BCR in the absence of CD40 co-stimulation results in the apoptosis of immature/transitional B cells and renders mature B cells anergic (8). This two signal requirement prevents the activation B cells in the absence of T cell help. This is an important mechanism for preventing the activation of autoreactive B cells and preventing autoimmunity, particularly since the deletion of autoreactive T cells is very efficient. The WEHI-231 B cell line is an ideal model for studying this two signal model. This cell line resembles an immature/transitional B cell in that prolonged stimulation through the BCR results in growth arrest and cell death while co-stimulation through CD40 prevents these responses (226-228).

Since BCR signalling results in an increase in β -catenin mediated transcription of a reporter gene construct in B cells (Section 3.3), β -catenin could regulate gene expression in

B cells, presumably by interacting with its usual binding partners which are members of the LEF-1/TCF family of DNA-binding proteins. Dadgostar *et al.* (229) has recently shown by cDNA microarray analysis that LEF-1 mRNA is present in splenic B cells and that CD40 signalling results in an increase in LEF-1 mRNA levels. This CD40-induced increase in LEF-1 protein levels could synergize with the BCR-induced increase in β -catenin protein levels to further increase β -catenin-mediated transcription. To determine if CD40 signalling could potentiate β -catenin-mediated gene transcription, I first investigated whether CD40 engagement resulted in increased LEF-1 protein levels. Preliminary data suggested that CD40 stimulation might cause a small increase in LEF-1 protein levels (Fig. 3.5A). This is the first evidence that LEF-1 protein is present in immature B cells. Even though it was not clear whether this CD40-induced increase in LEF-1 levels was significant, I went on to ask whether CD40 signalling potentiated the ability of the BCR to stimulate β -catenin-dependent transcription. I found that CD40 stimulation alone did not result in an increase in β -catenin-mediated transcription of luciferase activity driven by the TOPtk promoter but that simultaneous engagement of both the BCR and CD40 resulted in a synergistic increase in β -catenin-mediated transcription (Fig. 3.5). The combination of BCR signalling and CD40 signalling for 3 hours resulted in a 7-fold increase in luciferase expression from the TOPtk promoter compared to the usual 2.5-fold increase caused by BCR engagement alone. This preliminary evidence suggests that CD40 signalling can synergize with the BCR-induced increase in β -catenin to increase β -catenin-mediated transcription. Although other mechanisms are possible (see page 112), this may reflect the ability of CD40 signalling to increase the expression of β -catenin binding partners that mediate the ability of β -catenin to promote transcription.

Figure 3.5. CD40 engagement enhances the ability of the BCR to stimulate β -catenin-mediated gene transcription. *A*, WEHI-231 cells were stimulated with 10 μ g/ml anti-CD40 antibody for the indicated times. Triton X-100 extracts (40 μ g protein) were analyzed by immunoblotting with an anti-LEF-1 antibody. Molecular mass standards (in kDa) are indicated to the left of each panel. This experiment was repeated twice with similar results. *B*, WEHI-231 cells were transiently transfected with the TOPtk or FOPtk plasmids and then cultured for 20 h at 37°C. Triplicate or duplicate samples were then cultured for 4 h at 37°C with 10 μ g/ml goat anti-mouse IgM, 10 μ g/ml anti-CD40, both anti-mouse IgM and anti-CD40 or no addition (unstim). Luciferase activity assays were performed on cell extracts. Luciferase units were normalized to the protein concentration for each sample. The values for unstimulated cells that had been transfected with either the TOPtk or FOPtk plasmid were set to 1 and the values for the anti-IgM- and LiCl-treated samples are reported as “fold induction” relative to the corresponding unstimulated sample. The data represent the mean \pm SEM from three independent experiments.

A**B**

3.5 β -catenin levels are regulated via proteasome-mediated degradation in B cells

In other cell types, receptor-induced increases in nuclear β -catenin levels are a consequence of decreased degradation of β -catenin in the cytosol. In unstimulated cells, the constitutive phosphorylation of β -catenin by GSK-3 keeps β -catenin levels low by targeting it for ubiquitination and proteasome-mediated degradation. Wnt signalling, for example, inhibits GSK-3 and allows β -catenin to accumulate in the cytosol and then rapidly translocate into the nucleus. Since BCR engagement caused a rapid increase in the levels of both cytosolic and nuclear β -catenin, I asked whether this was due to the inhibition of proteasome-mediated degradation of β -catenin. To test this, I treated WEHI-231 cells with the proteasome inhibitor N-acetyl-Leu-Leu-NorLeucinal (ALLN) in order to allow ubiquitinated proteins to accumulate. I then used a GST fusion protein containing the cytoplasmic domain of E-cadherin (GST-ECT) to selectively pull down β -catenin present in Triton X-100 cell extracts. This GST-ECT fusion protein has been widely used to selectively isolate free cytoplasmic β -catenin that can potentially translocate into the nucleus, as opposed to β -catenin that is bound to cadherins, transmembrane proteins that function as adhesion molecules (209).

I found that treating WEHI-231 cells with the proteasome inhibitor ALLN for 20 min was sufficient to cause a 4-fold increase in the levels of free β -catenin (Fig. 3.6, compare 0 min ethanol lane to 0 min ALLN lane). This argues that proteasome-mediated degradation normally prevents the accumulation of β -catenin in B cells. This is expected since previous studies have shown that inhibition of proteasomal degradation of β -catenin leads to increased cytosolic β -catenin levels (109). The ALLN-induced increase in β -catenin levels was similar in magnitude to that caused by anti-IgM in the ethanol-treated control cells (Fig. 3.6).

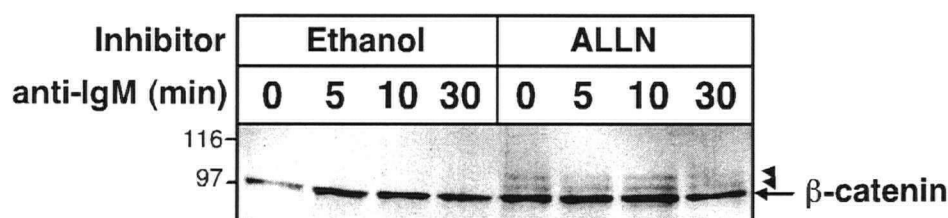


Figure 3.6. β -catenin levels in B cells are regulated by proteasome-mediated

degradation. WEHI-231 cells were pre-treated with the proteasome inhibitor ALLN (25 (M) or an equivalent volume of ethanol for 20 min at 37(C prior to stimulation with 40 (g/ml goat anti-mouse IgM for the indicated times. Triton X-100 cell extracts (1.5 x10⁷ cell equivalents) were then incubated with glutathione-Sepharose beads pre-loaded with the GST-ECT fusion protein. β -catenin levels were analyzed by immunoblotting. The slower migrating forms of β -catenin are indicated by the arrowheads. Note that in the absence of anti-IgM stimulation (0 min lanes), ALLN alone caused an increase in β -catenin levels, presumably by inhibiting proteasome-mediated degradation of β -catenin. Molecular mass standards (in kDa) are indicated to the left. Similar results were obtained in two independent experiments using the GST-ECT to pull down free β -catenin and in two additional experiments in which β -catenin was immunoprecipitated with an anti- β -catenin antibody.

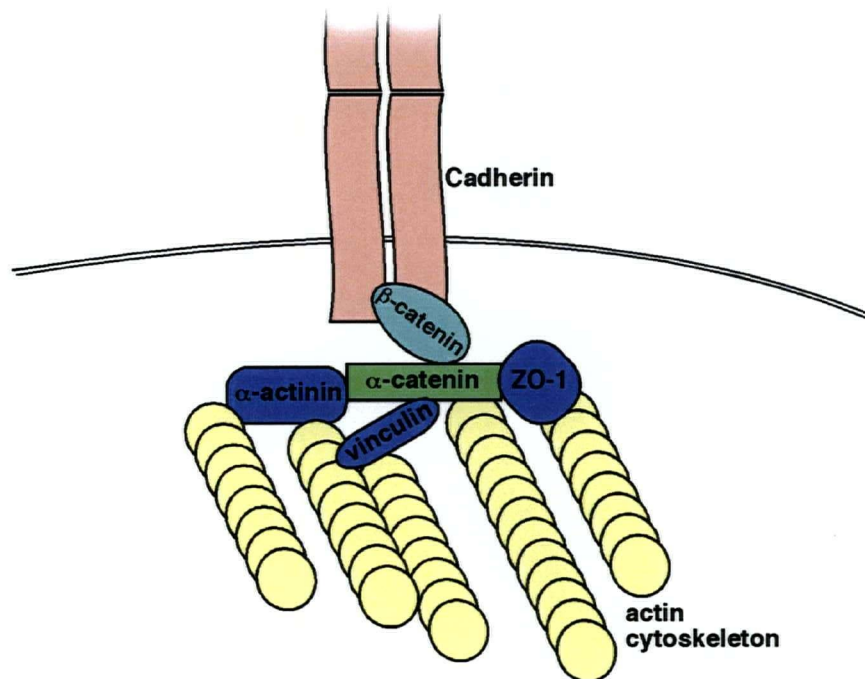
Moreover in the presence of ALLN, anti-IgM treatment did not cause a further increase in β -catenin levels (Fig. 3.6). These results are consistent with the idea that the BCR-induced increase in β -catenin levels is due to inhibition of proteasome-mediated degradation of β -catenin. When the cells were treated with ALLN, I also observed slower migrating forms of β -catenin. These may be phosphorylated or ubiquitinated forms of β -catenin that are normally degraded very rapidly by proteasomes.

3.6 Expression of a cadherin protein by some B cell lines

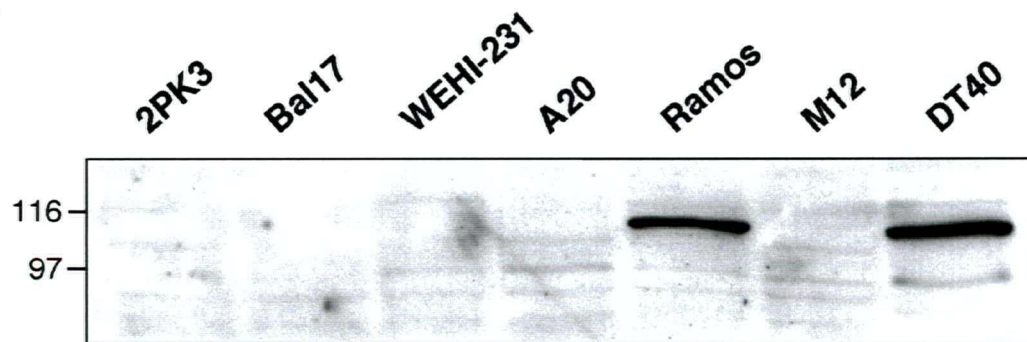
In addition to its role as a transcriptional co-activator, β -catenin is also an essential component of cadherin-mediated cell adhesion. Cadherins are calcium-dependent transmembrane proteins that mediate cell-cell adhesion contacts. The classical cadherins include the E-, N- and P-cadherins which interact in a homophilic manner (230) while protocadherins, in particular α -protocadherin, can interact either homophilically or heterophilically with the protein Reelin acting as a ligand (231). β -catenin provides a crucial link between the cadherins and the actin cytoskeleton. β -catenin binds to both the intracellular domain of the cadherin protein and to the cytoplasmic protein α -catenin. α -catenin binds to actin filaments directly or via linker molecules such as ZO-1, α -actinin or vinculin (27, Fig. 3.7A). Therefore, in cells containing cadherin proteins, much of the β -catenin in the cell is bound to cadherins at the plasma membrane. Cadherin proteins are expressed by fibroblast cells, epithelial cells and some leukemic cell lines (232,233). The consequence of the cadherin/ β -catenin interaction is that there are two different pools of β -catenin, a large pool that associates with the cytoplasmic domain of cadherin adhesion receptors and a smaller pool that is involved in signalling to the nucleus. Experimentally,

Figure 3.7. Analysis of cadherin expression in B cell lines. *A*, Schematic diagram of the cadherin-catenin complex and the protein interactions that mediate the association of α - and β -catenin with the actin cytoskeleton. α -catenin has been shown to bind to the actin cytoskeleton directly and via the linker proteins α -actinin, vinculin and ZO-1. β -catenin acts as an adapter protein that bridges α -catenin to the cytoplasmic domain of the cadherin. *B*, Triton X-100 extracts (30 μ g) from the indicated cell lines were analyzed by immunoblotting with an anti-pan cadherin antibody. Molecular mass standards (in kDa) are indicated on the left.

A



B



Anti-pan-cadherin blot

overexpression of cadherins sequesters free β -catenin at the membrane and downregulates Wnt-induced β -catenin-mediated transcription (234,235). Additionally, the accumulation of β -catenin in response to Wnt signalling is able to increase the formation of cadherin-catenin complexes at the membrane which leads to increased adhesion (232). Therefore, cross-talk can occur between the two pools of β -catenin. Alternatively, these two pools of β -catenin may function independently. *Caenorhabditis elegans* has two β -catenin homologues that perform the two different functions of β -catenin, BAR-1 and HMR-1. BAR-1 interacts with the *C. elegans* TCF homologue POP-1 and is involved in regulating transcription while HMP-2 interacts with the cadherin HMR-1 and plays a role in adhesion (236).

Since β -catenin is expressed in a number of B cell lines and cadherin proteins are expressed in some leukemic cell lines I examined a number of B cell lines for expression of cadherin proteins. To do this I made use of an anti-pan-cadherin antibody that recognizes murine, rat and human classical P-, N-, E-, K- M- and R-cadherin isoforms but not members from the related protocadherin family. I found that both the Ramos human B cell line and the DT40 chicken B cell line express a cadherin protein of approximately 100 kDa, a molecular mass that is consistent with that of classical cadherins (Fig. 3.7B, lanes 5 and 7). However, this antibody did not detect cadherin proteins in the 2PK3, BAL17, A20, WEHI-231 or M12 murine B cell lines (Fig. 3.7B, lanes 1-4 and 6). Thus classical cadherin proteins are not expressed by the majority of B cell lines.

Since the Ramos cell line expresses a classical cadherin protein (Fig 3.7) and BCR signalling upregulates β -catenin protein levels in these cells (Fig. 3.2), I investigated whether the cadherin and β -catenin proteins interact in Ramos cells. The cadherin/ β -catenin interaction is an essential component in cadherin-mediated adhesion. However, I did not

detect β -catenin protein in the anti-cadherin immunoprecipitates (data not shown). Thus it seems unlikely that this cadherin protein is involved in β -catenin-dependent adhesion and its function in the Ramos cell line remains to be determined.

3.7 GSK-3 regulates β -catenin levels and β -catenin-mediated transcription in B cells

In Wnt-responsive cells, GSK-3 normally phosphorylates β -catenin and targets it for degradation while Wnt signalling increases β -catenin levels by inhibiting GSK-3. Since BCR signalling inhibits GSK-3 (57), my hypothesis was that the BCR also regulates β -catenin via GSK-3. To determine whether GSK-3 normally targets β -catenin for degradation in B cells, I asked whether inhibiting GSK-3 activity with LiCl would be sufficient to cause an increase in β -catenin levels. Lithium ions have been shown to specifically inhibit GSK-3 kinase activity by displacing the Mg^{2+} cofactor (237). I found that treating WEHI-231 cells with 20 mM LiCl for 15 to 30 min resulted in an increase in nuclear β -catenin levels (Fig. 3.8A). As a specificity control, I showed that treating the cells with 20 mM KCl did not increase β -catenin levels. Another inhibitor of GSK-3, bisindolylmaleimide I (BIM I) (238), also increased the levels of β -catenin in the nuclear fraction of WEHI-231 cells (Fig. 3.8B). Thus, inhibition of GSK-3 is sufficient to allow the accumulation of β -catenin in the nuclear fraction of B cells. This indicates that GSK-3 normally prevents the accumulation of β -catenin in B cells and is consistent with the idea that BCR upregulates β -catenin by inhibiting GSK-3.

I also examined whether inhibition of GSK-3 activity is sufficient to increase β -catenin-mediated transcription in WEHI-231 cells. I found that LiCl treatment caused a 2-fold increase in β -catenin-dependent transcription from the TOPtk promoter while having

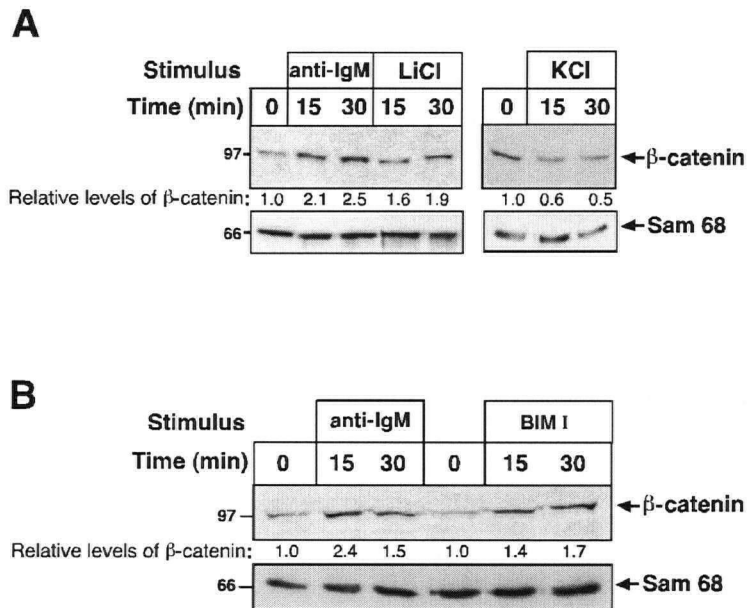


Figure 3.8. Inhibition of GSK-3 increases nuclear β-catenin levels in B cells.

A, WEHI-231 cells were treated with 40 μg/ml goat anti-mouse IgM, 20 mM LiCl, or 20 mM KCl for the indicated times. *B*, WEHI-231 cells were treated with 40 μg/ml goat anti-mouse IgM or 10 μM bisindolylmaleimide I (BIM I) for the indicated times. Nuclear fractions were analyzed by immunoblotting with a β-catenin-specific antibody. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β-catenin were determined as in figure 3.1. Molecular mass standards (kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments.

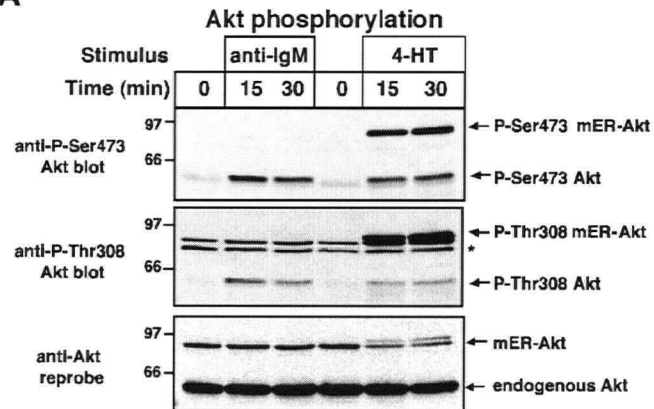
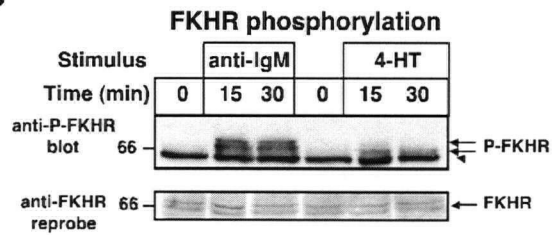
little or no effect on transcription driven by the control FOPTk promoter (see Fig. 3.4). Thus, inhibition of GSK-3 is sufficient to increase both β -catenin protein levels and β -catenin-dependent transcription in B cells.

3.8 Akt activation is not sufficient to induce phosphorylation of the negative regulatory sites on GSK-3 or increase β -catenin protein levels

My next goal was to determine the mechanism by which the BCR regulates GSK-3 and therefore β -catenin. Receptor-induced inhibition of GSK-3 kinase activity is due to phosphorylation of serine 21 of GSK-3 α or serine 9 of GSK-3 β (93). The serine/threonine kinase Akt was a good candidate for BCR-induced GSK-3 phosphorylation since the BCR activates Akt (57) and Akt has been shown to phosphorylate these negative regulatory sites on GSK-3 α/β in insulin-stimulated cells (92).

To assess the contribution of Akt to the regulation of GSK-3 and β -catenin in B cells, I expressed a conditionally active form of Akt (mER-Akt) in the WEHI-231 cell line. The mER-Akt protein lacks the PH domain of Akt but contains a myristoylation sequence at the N-terminus to localize it to the inner leaflet of the plasma membrane. This altered form of Akt is fused to a mutant form of the estrogen receptor that is responsive to the estrogen analogue 4-hydroxytamoxifen (4-HT). In response to 4-HT, the estrogen receptor portion of the mER-Akt protein undergoes a conformational change that exposes the Akt activation sites, allowing the mER-Akt protein to be phosphorylated and activated by PDK1 and PDK2 (205). I found that 4-HT treatment of WEHI-231 cells expressing the mER-Akt protein resulted in activation of this 90-kDa Akt fusion protein as indicated by its phosphorylation on the key Akt regulatory sites that correspond to threonine 308 and serine 473 of wild type Akt

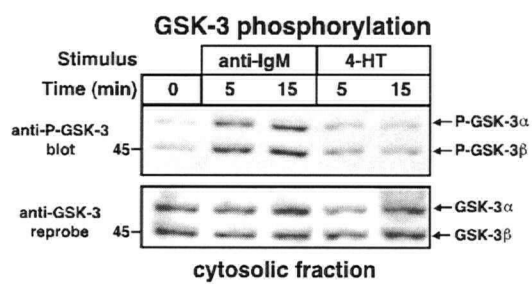
Figure 3.9. Activation of the mER-Akt fusion protein results in phosphorylation of the Forkhead-related transcription factor, FKHR. WEHI-231 cells expressing the mER-Akt fusion protein were stimulated for the indicated times with 40 μ g/ml goat anti-mouse IgM or with 2 μ M 4-HT to specifically activate the mER-Akt protein. *A*, Triton X-100 cell extracts were analyzed for the activation of mER-Akt and the endogenous Akt by immunoblotting with antibodies that recognize Akt phosphorylated on serine 473 (anti-P-Ser473 Akt) (*upper panel*) or threonine 308 (anti-P-Thr308 Akt) (*middle panel*). Equal loading was analyzed by reprobing the membranes with an anti-Akt antibody (*lower panel*). A non-specific band in the anti-P-Thr308 Akt blot (*middle panel*) is indicated by an asterisk. *B*, Triton X-100 cell extracts were analyzed by immunoblotting with an antibody to phosphorylated FKHR/FOXO1 (anti-P-FKHR). The arrowhead indicates a non-specific band while the arrows indicate more highly phosphorylated forms of FKHR that are induced upon treating the cells with anti-IgM or 4-HT. Equal loading was analyzed by reprobing the membranes with an antibody recognizing FKHR.

A**B**

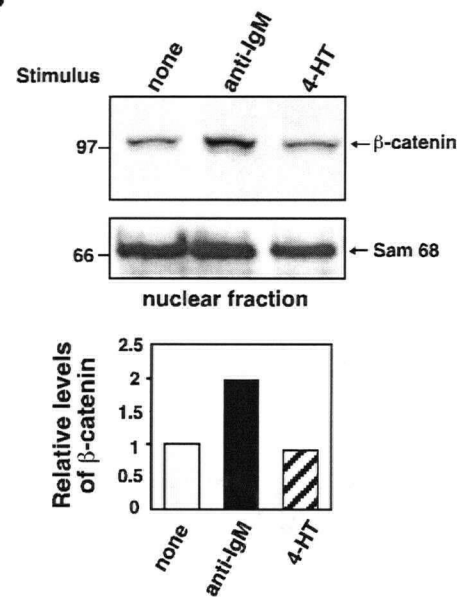
(Fig. 3.9A). Although mER-Akt is localized to the membrane, endogenous Akt can move off the membrane in order to phosphorylate endogenous substrates. In addition, the 60-kDa endogenous Akt was also activated when the mER-Akt-expressing cells were treated with 4-HT (Fig. 3.9A), possibly due to an interaction between the mER-Akt protein and the endogenous Akt. The 4-HT-induced activation of the endogenous Akt was similar in magnitude to that caused by BCR engagement (Fig. 3.9A). If the mER-Akt also phosphorylates cytoplasmic substrates such as GSK-3, then the total 4-HT-induced Akt activation could be greater than the BCR-induced activation of endogenous Akt. To test whether 4-HT treatment of mER-Akt-expressing cells could stimulate the phosphorylation of known Akt substrates, I examined the phosphorylation of the Forkhead-related transcription factor (FKHR/FOXO1), a protein that is found in both the cytoplasm and nucleus. Akt has been shown to phosphorylate FKHR on serine 256 and threonine 24 (90). To assess FKHR phosphorylation, I used an Ab that recognizes FKHR that is phosphorylated on serine 256. Treating WEHI-231 cells with 4-HT for 15 or 30 min caused the appearance of phosphorylated FKHR forms (Fig. 3.9B). A lower band was also detected (indicated by an arrowhead) which was not observed in later experiments (see figure 4.13). It is likely a non-specific band that was recognized by the earlier lots of the anti-phospho-FKHR antibody. Note that BCR engagement caused the appearance of additional forms of FKHR that migrated even more slowly than those induced by 4-HT treatment. This may reflect the phosphorylation of FKHR on additional sites that are targeted by other BCR signalling pathways. Indeed, Ras-dependent phosphorylation of FKHR has been reported (239). In any case, these data indicate that 4-HT treatment of mER-Akt-expressing cells can stimulate Akt-dependent phosphorylation events.

Figure 3.10 Activation of the mER-Akt fusion protein does not lead to GSK-3 phosphorylation or upregulation of β -catenin. *A*, WEHI-231 cells expressing mER-Akt were stimulated with 40 μ g/ml goat anti-mouse IgM or 2 μ M 4-HT for the indicated times. Cytoplasmic fractions (5 μ g protein) were analyzed for GSK-3 phosphorylation using an anti-P-GSK-3 antibody that recognizes GSK-3 α phosphorylated on serine 21 and GSK-3 β phosphorylated on serine 9. The membrane was then stripped and reprobed with an antibody recognizing all forms of GSK-3. *B*, WEHI-231 cells expressing mER-Akt were stimulated with 40 μ g/ml goat anti-mouse IgM or 2 μ M 4-HT for 15 min. Nuclear fractions were analyzed for β -catenin levels by immunoblotting (*upper panel*). The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 3.1. The results from this experiment are presented graphically in the *lower panel*. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in at least three independent experiments.

A



B



I then asked whether 4-HT-induced Akt activation could lead to phosphorylation of GSK-3 α/β on their negative regulatory sites. Phosphorylation of GSK-3 α and β at serine 21 and serine 9, respectively, was assessed using phosphorylation state-specific antibodies. Figure 3.10A shows that while BCR engagement caused a significant increase in the phosphorylation of both GSK-3 α and GSK-3 β , 4-HT treatment caused only a very small increase in GSK-3 phosphorylation even though it activated endogenous Akt to the same extent as BCR engagement. Thus while Akt activation is sufficient to inhibit GSK-3 activity in muscle cells (199), in B cells the amount of Akt activation stimulated by the BCR does not cause significant phosphorylation of GSK-3 on the negative regulatory sites. This may be a cell-type specific phenomenon or due to the localization of Akt since the dually acylated ER-Akt used in muscle cells (199) can be targeted to lipid rafts while the single myristoylation of the mER-Akt that I used does not target it to lipid rafts. This indicates that BCR-induced phosphorylation and inhibition of GSK-3 is mediated by a kinase other than Akt.

Since the inhibition of GSK-3 is sufficient to increase β -catenin levels in WEHI-231 cells (Fig. 3.8), the inability of Akt activation to cause significant phosphorylation of GSK-3 in these cells suggested that Akt activation would be unable to stimulate the upregulation of β -catenin. Indeed, I found that 4-HT treatment of mER-Akt-expressing WEHI-231 cells did not cause an increase in β -catenin levels (Fig. 3.10B). Thus, Akt either does not regulate the GSK-3/ β -catenin pathway in B cells or is not sufficient by itself to induce these responses.

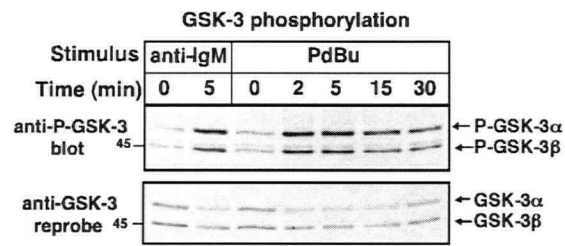
3.9 Protein kinase C activity is necessary for the BCR-induced increase in β -catenin levels

In addition to Akt, several PKC isoforms can phosphorylate GSK-3 *in vitro* (96,98-100). Moreover treating cells with phorbol esters, compounds that activate both conventional PKC isoforms (PKC- α , - β_I , - β_{II} and - γ) and novel PKC isoforms (PKC- δ , - ϵ , - η and - θ), can inhibit GSK-3 activity (240). Phorbol esters are analogues of diacylglycerol, one of the second messengers produced by the hydrolysis of PIP2 by PLC. IP₃, the other second messenger produced by PLC causes increases in intracellular calcium, which, in combination with DAG, contributes to the activation of the conventional PKC isoforms (149). Since the BCR activates PLC- γ (144-147) and induces both PKC activation and increases in intracellular calcium (31), I asked whether PKC enzymes might link the BCR to the GSK-3/ β -catenin pathway.

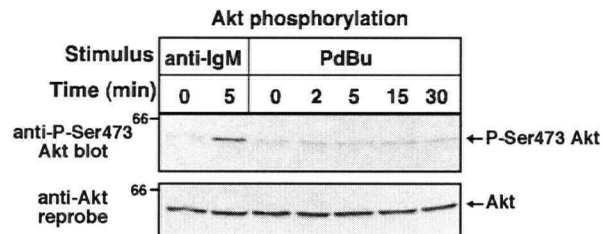
First, I asked whether treating B cells with phorbol esters could stimulate GSK-3 phosphorylation. Treating WEHI-231 cells with low concentrations of PdBu causes activation of PKC that is approximately equivalent to that induced by anti-IgM engagement and is therefore very similar to the physiological concentrations of DAG produced by PLC- γ activation (241). Figure 3.11A shows that treating WEHI-231 cells with 20 nM of the DAG mimic, phorbol dibutyrate (PdBu), caused significant phosphorylation of both GSK-3 α and GSK-3 β . This PdBu-induced GSK-3 phosphorylation was equal to or greater than that caused by anti-IgM. Moreover, the PdBu-induced GSK-3 phosphorylation was not dependent on Akt, since this concentration of PdBu did not activate Akt, as judged by phosphorylation of Akt on serine 473 (Fig. 3.11B), a very sensitive measurement of Akt activation.

Figure 3.11 PdBu, but not ionomycin, induces GSK-3 phosphorylation and upregulates β -catenin in WEHI-231 cells. *A and B*, WEHI-231 cells were stimulated with 40 μ g/ml anti-IgM or 20 nM PdBu for the indicated times. Triton X-100 cell extracts (5 μ g protein) were analyzed for GSK-3 phosphorylation using the anti-P-GSK-3 α/β antibody (*A*) or for Akt phosphorylation using the anti-P-Ser473 Akt antibody (*B*). To ensure equal loading, the membranes were stripped and reprobed with antibodies against GSK-3 or Akt. *C*, WEHI-231 cells were stimulated with 40 μ g/ml anti-IgM, 10 nM PdBu, or 30 nM PdBu for the indicated times. Nuclear fractions were analyzed by immunoblotting with a β -catenin-specific antibody. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 3.1. *D*, WEHI-231 cells were stimulated with 40 μ g/ml anti-IgM, 30 nM PdBu, or 1 μ M ionomycin for the indicated times. Nuclear fractions were analyzed by immunoblotting with a β -catenin-specific antibody. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 3.1. Molecular mass standards (in kDa) are to the left of each panel. For each panel, similar results were obtained in at least two independent experiments.

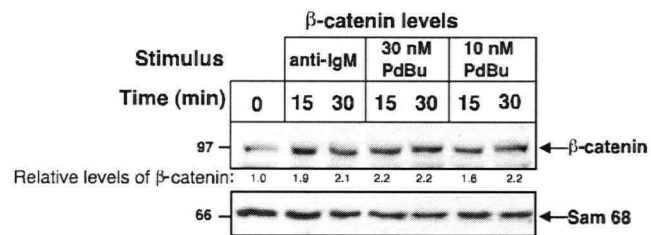
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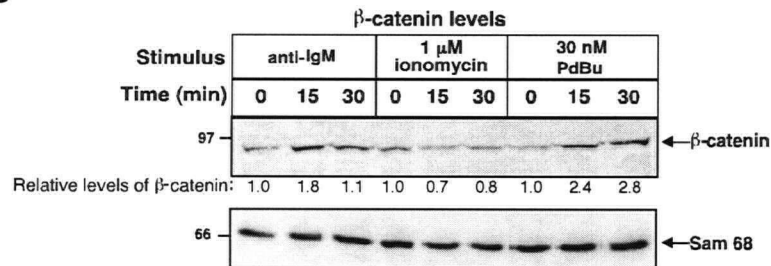
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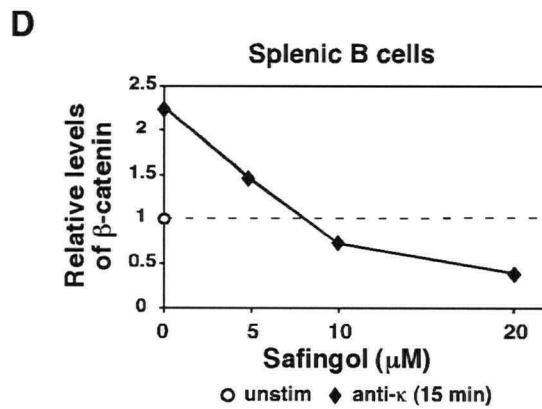
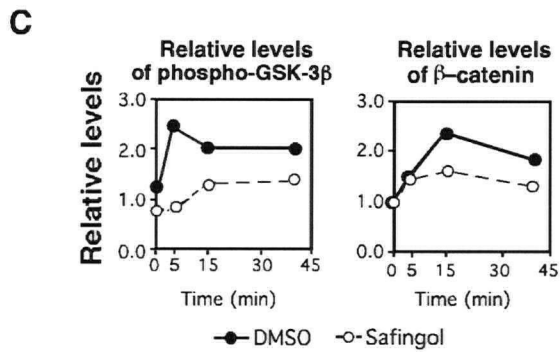
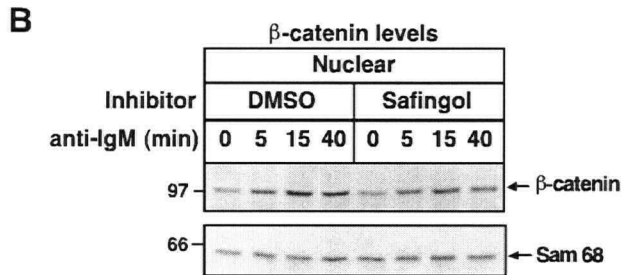
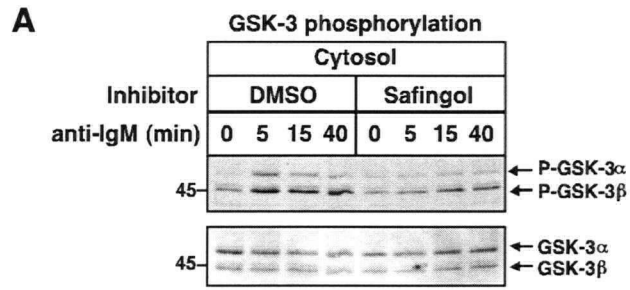


Since PdBu treatment of WEHI-231 cells could stimulate the phosphorylation of GSK-3 α/β on their negative regulatory sites, I asked whether it could also cause an increase in β -catenin levels. Figure 3.11C shows that low concentrations of PdBu (10-30 nM) could increase the level of β -catenin in the nuclear fraction of WEHI-231 cells to the same extent as anti-IgM treatment.

Lastly, I asked if increases in intracellular calcium, the other second messenger produced concurrently with DAG, could regulate β -catenin levels. Treating WEHI-231 cells with ionomycin, a calcium-selective ionophore that induces an influx of extracellular calcium into the cytosol, did not increase β -catenin levels (Fig. 3.11D). Thus, phorbol ester-induced activation of PKC is sufficient to induce GSK-3 phosphorylation and to increase nuclear β -catenin levels. Moreover, since the amount of PKC activation caused by anti-IgM treatment of WEHI-231 cells is similar to that caused by 10 nM PdBu (241), this suggests that PKC activation is sufficient to mediate the effects of BCR engagement on GSK-3 and β -catenin.

To test whether PKC activation is necessary for BCR-mediated regulation of GSK-3 and β -catenin, I used the PKC inhibitor safinol to inhibit PKC activity. Safinol inhibits PKC activity by binding to the DAG-binding C1 domain of PKC enzymes and preventing DAG from activating them. Treating WEHI-231 cells with 25 μ M safinol inhibited both the BCR-induced phosphorylation of GSK-3 α/β (Fig. 3.12A) and the BCR-induced increase in nuclear β -catenin levels (Fig. 3.12B) by 30 - 70% compared to the untreated cells (Fig. 3.12C). This is consistent with a model in which GSK-3 phosphorylation and inactivation is responsible for the increase in β -catenin levels. Moreover, these data argue that the BCR regulates GSK-3 and β -catenin via PKC-mediated inhibition of GSK-3. In support of this

Figure 3.12. The BCR regulates GSK-3 and β -catenin via PKC. *A-C*, WEHI-231 cells were pre-treated with 25 μ M safinol or the equivalent volume of DMSO for 20 min at 37°C. The cells were then stimulated with 40 μ g/ml goat anti-mouse IgM for the indicated times. *A*, Cytosolic fractions (5 μ g protein) were analyzed for GSK-3 phosphorylation by immunoblotting with the anti-P-GSK-3 α/β antibody. The blots were then reprobed with anti-GSK-3 antibodies. *B*, Nuclear fractions from the same samples were analyzed for β -catenin levels by immunoblotting. The blots were then reprobed with anti-Sam 68 antibodies as a loading control. *C*, The relative levels of β -catenin in this experiment were determined as in figure 3.1 (right panel). The relative levels of phosphorylated GSK-3 β were determined using ImageQuant Software and are shown in the left panel. Note that anti-IgM-induced phosphorylation of GSK-3 α was also inhibited by safinol treatment (see panel A). Similar results were obtained in three independent experiments. *D*, Splenic B cells were pretreated with the indicated amount of safinol or with DMSO for 20 min at 37°C. The cells were then stimulated with 30 μ g/ml goat anti-mouse κ light chain antibodies for 15 min. Total cellular extracts were analyzed for β -catenin levels by immunoblotting. The relative levels of β -catenin were determined as described in figure 3.1. The results from a representative experiment are shown. Similar results were obtained in three independent experiments. Molecular mass standards are indicated to the left of each panel.



idea, I found that inhibiting PKC activity with safingol completely blocked BCR-induced upregulation of β -catenin protein levels in murine splenic B cells (Fig. 3.12D).

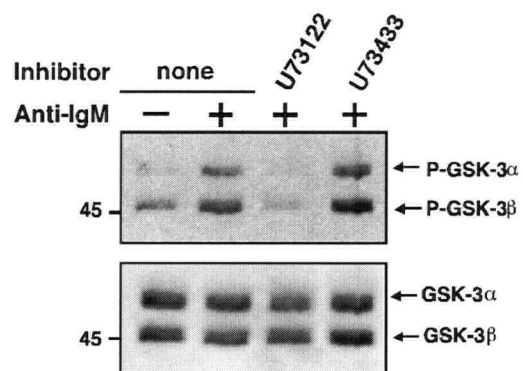
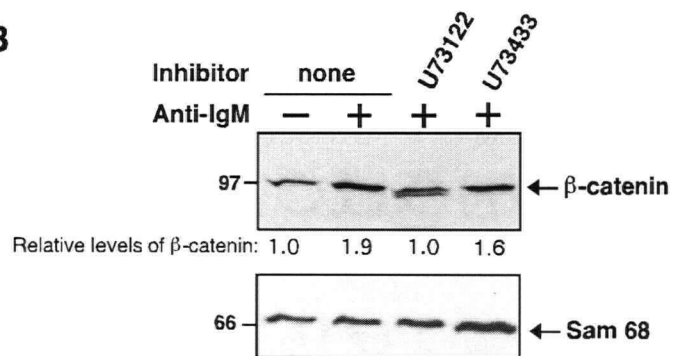
3.10 Phospholipase C activity is necessary for the BCR-induced increase in β -catenin levels.

My data suggest that the BCR regulates GSK-3 and β -catenin via conventional (PKC- α , - β , - γ) or novel (PKC- δ , - ϵ , - η , - θ) PKC isoforms that are dependent on DAG for their activation. These PKC isoforms are inhibited by safingol and are activated by phorbol esters such as PdBu which mimic the action of DAG. During BCR signalling, these PKC isoforms would be activated via the production of DAG by PLC- γ (31). Therefore, preventing the PLC- γ activation should inhibit the ability of the BCR to activate this putative PKC/GSK-3/ β -catenin pathway. Indeed, I found that treating WEHI-231 cells with U73122, an inhibitor of PLC activity, blocked both the BCR-induced increase in GSK-3 phosphorylation (Fig. 3.13A) and the BCR-induced increase in β -catenin levels (Fig. 3.13B). In contrast, an inactive structural analogue of U73122, U73343, had no significant effect on BCR-induced GSK-3 phosphorylation or β -catenin upregulation (Fig. 3.13). Inhibition of PLC- γ by U73122 completely blocked the ability of the BCR to induce GSK-3 phosphorylation and increase β -catenin protein. This is in contrast to the partial inhibition of these responses caused by the inhibition of PKC by safingol. One possible explanation is that U73122 treatment of WEHI-231 cells inhibits PLC to a greater extent than safingol inhibits PKC. Alternatively, a PLC- γ -dependent PKC-independent pathway may contribute to BCR-induced phosphorylation of GSK-3 and upregulation of β -catenin. A more comprehensive study using multiple PLC- γ and PKC inhibitors could differentiate between these two

Figure 3.13. PLC activity is required for the BCR to regulate GSK-3 and β -catenin.

WEHI-231 cells were incubated with or without the PLC inhibitor U73122 (10 μ M) for 20 min at 37°C. The cells were then stimulated for 15 min with 40 μ g/ml goat anti-mouse IgM.

A, Cytosolic fractions (5 μ g protein) were analyzed for GSK-3 phosphorylation by immunoblotting with the anti-P-GSK-3 α/β antibody. The blots were then reprobed with anti-GSK-3 antibodies. *B*, Nuclear fractions from the same samples were analyzed by immunoblotting for β -catenin. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 3.1. Similar results were obtained in two independent experiments. Molecular mass standards are indicated to the left of each panel.

A**B**

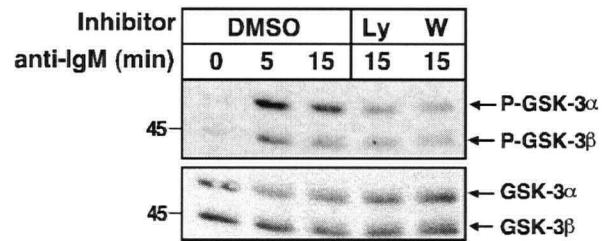
possibilities. However there is a limited number of appropriate and specific PLC- γ and PKC inhibitors that are available for this type of study (242). The finding that both PLC and PKC activities are required for the BCR to regulate GSK-3 and β -catenin suggests that the BCR regulates β -catenin via a PLC- γ /PKC/GSK-3 pathway.

3.11 The BCR-induced increase in β -catenin levels is partially dependent on PI3K activity

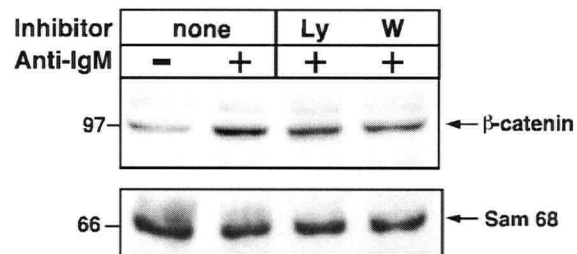
Previous work in our lab had shown that BCR-induced GSK-3 phosphorylation, as well as the subsequent inhibition of GSK-3 activity, is dependent on PI3K (57). Since Akt is an important downstream target of PI3K, I had initially assumed that the BCR would regulate GSK-3 via Akt. However, I found that the BCR regulates GSK-3 via a PLC- γ 2/PKC pathway. This does not preclude a role for PI3K since PI3K regulates the membrane recruitment and activation of the Btk tyrosine kinase. Phosphorylation of PLC- γ 2 by Btk is required for maximal activation of PLC- γ 2 (31) and previous work has shown that inhibition of PI3K reduces anti-IgM-induced production of PLC- γ 2-derived second messengers by approximately 60% (243). Therefore, I predicted that inhibition of PI3K would partially block the ability of the BCR to regulate GSK-3 and β -catenin. Indeed, I found that two structurally-distinct PI3K inhibitors, wortmannin and Ly294002, inhibited both the BCR-induced increase in GSK-3 phosphorylation and the BCR-induced increase in nuclear β -catenin levels by 60-75% (Fig. 3.14). Thus, PI3K does contribute to the ability of the BCR to regulate β -catenin but this may reflect the role of PI3K in the activation of PLC- γ 2 as opposed to Akt.

Figure 3.14 PI3K activity contributes to the ability of the BCR to regulate GSK-3 and β -catenin. WEHI-231 cells were pre-treated with 25 μ M Ly294002 (Ly), 30 nM wortmannin (W) or an equivalent volume of DMSO for 20 min at 37°C. The cells were then stimulated with 40 μ g/ml goat anti-mouse IgM for 5 min or 15 min. *A*, Cytosolic fractions (5 μ g protein) were analyzed for GSK-3 phosphorylation by immunoblotting with the anti-P-GSK-3 α/β antibody. The blots were then reprobed with anti-GSK-3 antibodies. *B*, Nuclear fractions from the 15 min samples were analyzed by immunoblotting for β -catenin. The blots were then reprobed with anti-Sam 68 antibodies as a loading control. Similar results were obtained in four independent experiments. *C*, The relative levels of β -catenin were determined for each experiment and the mean \pm SEM for each point is shown. Note that the PI3K inhibitors did not alter the level of β -catenin in cells that were not treated with anti-IgM (data not shown).

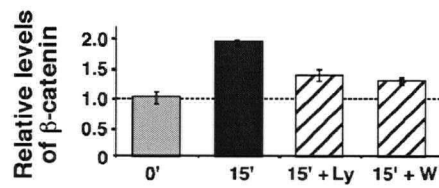
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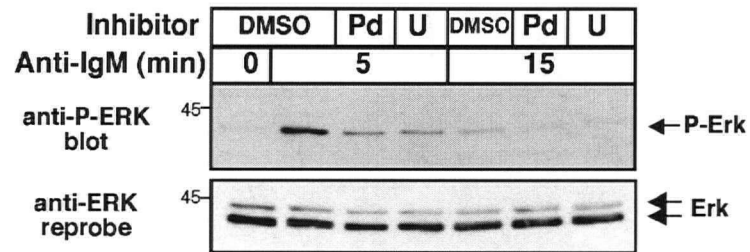
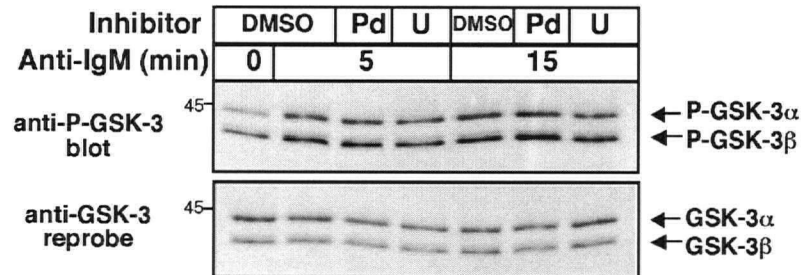
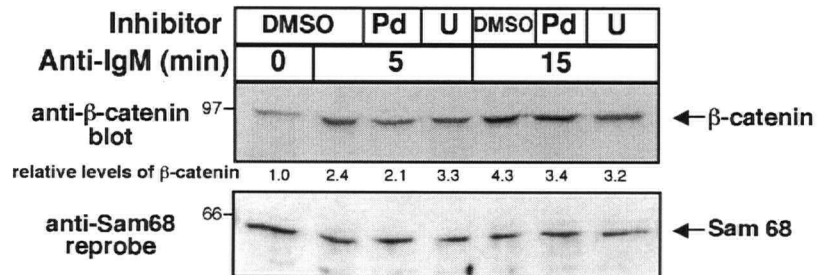
C



3.12 The BCR does not regulate GSK-3 phosphorylation via Protein kinase C-mediated activation of the MEK/ERK1/2/p90^{RSK} pathway

I have shown that PKC activity is required for BCR-induced phosphorylation of GSK-3. However, PKC is only one of many kinases that can phosphorylate GSK-3, at least in vitro (92,94-100). Thus, it was not clear whether PKC directly phosphorylates GSK-3 or whether it activates another kinase that phosphorylates GSK-3. In particular p90^{RSK}, a downstream target of ERK1/2, can phosphorylate GSK-3 (94,217,244,245) and DAG-responsive PKC isoforms can activate p90^{RSK} via the Raf/-1MEK/ERK1/2 pathway (153,246). Since GSK-3 is a target of p90^{RSK} (94,217), it is possible that the BCR may induce GSK-3 phosphorylation via PKC-induced activation of the Raf-1/MEK/ERK1/2/p90^{RSK} pathway. To determine whether GSK-3 phosphorylation was regulated via this pathway, I inhibited BCR-induced activation of ERK1/2 by pre-treating WEHI-231 cells with two different MEK inhibitors PD-98059 and U0126. Treating WEHI-231 cells with these compounds dramatically inhibited BCR-induced ERK1/2 phosphorylation (Fig. 3.15A), confirming that these compounds inhibited the activation of MEK. However, I found that inhibition of the MEK/ERK1/2 pathway had no effect on BCR-induced phosphorylation of GSK-3 (Fig. 3.15B) or the BCR-induced increase in nuclear β -catenin protein levels (Fig. 3.15C). Therefore, PKC does not regulate the GSK-3/ β -catenin pathway via the MEK/ERK1/2/p90^{RSK} pathway. Although the simplest hypothesis is that PKC phosphorylates GSK-3 directly, we cannot rule out that PKC regulates GSK-3 phosphorylation indirectly, via the activation of a kinase other than p90^{RSK}.

Figure 3.15 The MEK/ERK1/2/p90^{RSK} pathway is not involved in BCR-induced phosphorylation of GSK-3 or BCR-induced upregulation of β -catenin. WEHI-231 cells were incubated with 60 μ M PD-98059 (Pd), 2 nM U0126 (U) or an equivalent volume of DMSO for 20 min at 37°C. The cells were then stimulated for the indicated times with 40 μ g/ml goat anti-mouse IgM. *A*, Cytosolic fractions (5 μ g protein) were analyzed for ERK1/2 phosphorylation by immunoblotting with the anti-P-ERK1/2 antibody. The blots were then reprobed with anti-ERK1/2 antibodies. *B*, Cytosolic fractions (5 μ g protein) were analyzed for GSK-3 phosphorylation by immunoblotting with the anti-P-GSK-3 α/β antibody. The blots were then reprobed with anti-GSK-3 antibodies. *C*, Nuclear fractions (20 μ g) from the same samples were analyzed by immunoblotting for β -catenin. The blots were reprobed with antibodies to the nuclear protein Sam 68 and relative levels of β -catenin were determined as in figure 3.1. Similar results were obtained in two independent experiments.

A**B****C**

3.13 Discussion

In this chapter I have shown that the transcriptional activator β -catenin is a target of BCR signalling. I show that BCR engagement increases β -catenin protein levels as well as β -catenin-dependent transcription. I demonstrated that β -catenin levels are regulated by proteasomal degradation in B cells and that CD40 co-stimulation can synergize with BCR signalling to increase β -catenin-mediated transcription. I also provide evidence that the BCR-induced increases in β -catenin protein levels and β -catenin-mediated transcription are mediated by the BCR-induced inhibition of GSK-3 activity that had been described previously (57). I also showed that the BCR-induced phosphorylation of GSK-3 on its negative regulatory sites is mediated by a PI3K/PLC- γ /PKC signalling pathway (Fig. 3.16).

I found that BCR signalling increased the levels of β -catenin in WEHI-231 cells, A20 cells, Ramos cells, and murine splenic B cells. WEHI-231 and Ramos cells resemble immature/transitional B cells since they are susceptible to anti-IgM-induced apoptosis while A20 cells are IgG⁺ cells that are presumably derived from memory B cells. The function of β -catenin in immature/transitional B cells or in mature B cells is not known. In differentiated cells such as epithelial cells, upregulation of β -catenin promotes cell cycle entry and proliferation by driving the transcription of the genes encoding cyclin D1 and c-Myc genes (140-142). LiCl, which I showed upregulates β -catenin in WEHI-231 cells, has been shown to promote the proliferation of fetal liver B cell progenitors (143). Thus, it is possible that β -catenin could be involved in BCR-induced proliferation.

Although the role of β -catenin in mature B cells is not clear, work by Grosschedl and colleagues indicates that β -catenin is important for the proliferation and survival of pro-B

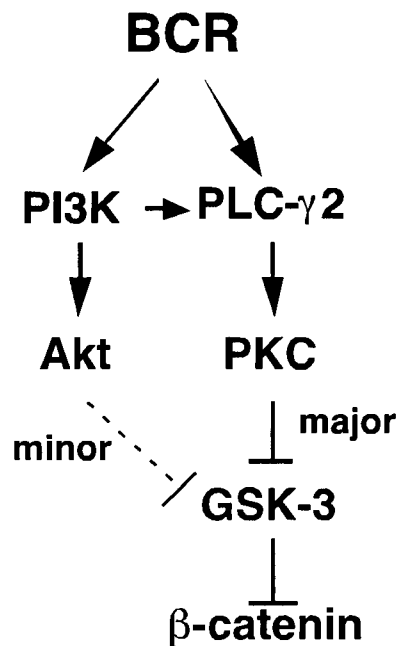


Figure 3.16. Model for the regulation of β -catenin by the BCR. The BCR regulates β -catenin levels primarily via a PLC- γ 2/PKC/GSK-3 pathway in which the activation of PLC- γ 2 is partially dependent on PI3K. PKC activation leads to the inhibition of GSK-3. Since GSK-3 normally targets β -catenin for degradation, inhibition of GSK-3 allows β -catenin to accumulate. In contrast to PKC, Akt makes only a minor contribution to the inhibition of GSK-3 and the upregulation of β -catenin by the BCR.

cells. In pro-B cells, Wnt signalling increases β -catenin levels and stimulates proliferation (143). Moreover, disrupting the genes encoding the β -catenin binding partner LEF-1 results in increased apoptosis of pro-B cells (143), suggesting that this pathway is essential for the survival of pro-B cells. I found that clustering the Ig β on the surface of the K40-B1 pro-B cell line resulted in the upregulation of β -catenin. This suggests that the putative pro-B cell receptor, which consists of the Ig α /Ig β subunit associated with calnexin and several unidentified proteins (221), could deliver survival and proliferative signals via β -catenin. If this is the case, then the pro-B cell receptor may have a role similar to that of the pre-B cell receptor which delivers Syk- and Btk-dependent signals that promote the survival and further differentiation of pre-B cells.

β -catenin is a transcriptional activator. During early development it regulates the expression of genes that determine cell fate while in differentiated cells it regulates the expression of genes that promote proliferation (106,112). My data indicate that β -catenin can also function as a transcriptional activator in B cells. I found that BCR engagement could stimulate β -catenin-dependent transcription, as judged by a luciferase reporter gene assay. However, the gene targets of β -catenin in B cells remains to be determined (see Section 6.5.1).

In all of the systems studied thus far, β -catenin promotes transcription by cooperating with LEF-1/TCF family proteins. The LEF-1/TCF proteins bind specific DNA sequences while β -catenin provides a transactivation domain that can recruit CBP/p300. Although Wnt-responsive bone marrow pro-B cells express LEF-1, previous reports have not detected this protein in mature B cells (143). However, more recent RT-PCR studies in our lab indicate that TCF-1 mRNA is present in murine splenic B cells as well as a number of murine B cell

lines (B. Biagioni and M.R. Gold, unpublished observations). Low amounts of LEF-1 mRNA are also present in WEHI-231 cells (B. Biagioni and M.R. Gold, unpublished observations). Dadgostar *et al.* (229) showed that LEF-1 mRNA is present in murine splenic B cells. By immunoblotting, I showed that LEF-1 protein is present in WEHI-231 cells (Fig. 3.5A). This is the first demonstration that LEF-1 protein is present in mature B cells. It remains to be determined whether TCF-1 protein is also present in WEHI-231 cells and whether normal B cells, as well as other B cell lines, express LEF-1 and TCF-1 proteins.

The survival, activation, and proliferation of B cells is regulated by many receptors. In particular, CD40 signalling prevents BCR-induced apoptosis and synergizes with BCR signals to promote B cell proliferation. I found CD40 engagement did not induce β -catenin-dependent transcription by itself but significantly enhanced the ability of the BCR to stimulate β -catenin-dependent transcription from the TOPtk promoter (Fig. 3.5). The ability of CD40 to synergize with the BCR to induce β -catenin-dependent transcription may reflect the ability of CD40 to upregulate the expression of LEF-1/TCF proteins. Dadgostar *et al.* (229) reported that CD40 signalling increased the levels of LEF-1 protein in murine splenic B cells. I found that CD40 signalling caused a very small increase in the levels of the LEF-1 protein in WEHI-231 cells. It is not clear whether this small increase in LEF-1 levels could account for the significant increase in β -catenin-dependent transcription. It is possible that CD40 signalling upregulates the expression of other β -catenin binding partners such as TCF-1 or that it contributes to the accumulation or activation of β -catenin in some other way. Preliminary evidence suggests that engagement of CD40 alone or in combination with engagement of the BCR does not affect the level of β -catenin in B cells (B. Biagioni and M.R. Gold, unpublished observations). Therefore it is likely that CD40 affects the regulation

of β -catenin-dependent transcription in some other manner. Staal *et al.* (111) have shown that the level of β -catenin dephosphorylated at serine 37 and threonine 41, but not the overall level of β -catenin, correlates with the transcriptional activity of β -catenin. Therefore an alternative possibility is that CD40 may act to enhance the dephosphorylation of β -catenin at these N-terminal sites. Nevertheless, CD40 co-stimulation can enhance β -catenin-dependent transcription in WEHI-231 cells, suggesting that it can enhance the ability of the BCR to cause β -catenin-mediated changes in gene expression. *In vivo*, co-stimulation by CD40 may result in increased expression of β -catenin-induced gene products compared to antigenic stimulation alone. Since β -catenin controls genes that can influence cell survival and proliferation, including genes encoding c-Myc and cyclin D1 (140-142), the synergistic increase in β -catenin-mediated gene transcription caused by the combination of BCR and CD40 signalling could be one mechanism by which CD40 signalling prevents BCR-induced apoptosis and enhances BCR-induced proliferation of mature B cells.

Since treating B cells with a proteasome inhibitor caused a substantial increase in β -catenin levels, it may be that β -catenin is rapidly degraded in B cells and that BCR signalling causes β -catenin to accumulate by decreasing the rate at which β -catenin is degraded. Studies in several cell types have shown that β -catenin is rapidly degraded in unstimulated cells via a GSK-3-dependent mechanism (107-109,247). Free β -catenin in the cytoplasm binds to a protein complex that contains GSK-3 as well as the APC and the Axin scaffolding protein. Axin and APC bind both GSK-3 and β -catenin, facilitating the phosphorylation of β -catenin by GSK-3. Phosphorylation of β -catenin by GSK-3 targets it for ubiquitination and proteasome-mediated degradation. My finding that the proteasome inhibitor ALLN increases β -catenin levels in B cells argues that proteasome-mediated degradation keeps β -

catenin levels low in unstimulated B cells. Wnt hormones, the best studied regulators of β -catenin, cause increases in β -catenin levels by inhibiting GSK-3. This allows β -catenin to accumulate since it is no longer efficiently targeted for degradation. My data suggest that the BCR also increases β -catenin levels by preventing the degradation of β -catenin. I found that BCR engagement did not cause a further increase in β -catenin levels in ALLN-treated cells in which β -catenin degradation was already inhibited, consistent with the idea that inhibition of β -catenin degradation is the major way in which the BCR regulates β -catenin levels. In addition, BCR signalling caused β -catenin levels to increase within 5 to 15 min, kinetics that are more consistent with inhibition of β -catenin degradation as opposed to an increase in transcription or translation.

In addition to regulating gene transcription, β -catenin is involved in cadherin-mediated adhesion. Therefore I also investigated whether B cell lines express classical cadherins. I found that classical cadherins were expressed in only two out of the seven B cell lines I examined. This suggests that classical cadherins either do not play an essential role in B cell physiology or function only in specific subsets of B cells. It is unclear what role classical cadherins may play in B cells since cadherins mediate homophilic interactions and homophilic B cell interactions do not appear to have an essential role in B cell development or activation. This is consistent with the observation that the classical cadherin, N-cadherin, is not expressed in normal leukocytes (233). However, integrin-mediated homophilic B cell interactions can be induced in B cell lines by stimulation with various extracellular stimuli (226,248), suggesting that homophilic interactions may occur in some situations. Interestingly, the heterophilic interaction between the classical E-cadherin and $\alpha_E\beta_7$ integrin have been reported to mediate the adhesion of epithelial cells to intraepithelial lymphocytes

(249). Thus classical cadherins can mediate heterophilic interactions with specific cell types in some situations. It remains to be determined if protocadherins, which are not recognized by the anti-pan-cadherin antibody I used, are expressed in these B cell lines since protocadherins are expressed in pro-B cells (250). Heterophilic interactions mediated by protocadherins may play a role in B cell development since heterotypic cell interactions with bone marrow stromal cells are essential interactions for proper B cell development (251). Although these interactions have been shown to be integrin-mediated (251-255), protocadherins might contribute to these cell-cell interactions.

In epithelial cells and other cell types in which Wnt signalling has been studied, the constitutive phosphorylation of β -catenin by GSK-3 keeps β -catenin levels low by targeting it for ubiquitination and proteasome-mediated degradation. Our data indicate that GSK-3 kinase activity is also responsible for keeping β -catenin levels low in unstimulated B cells. GSK-3 is constitutively active in B cells (57) and I found that two different inhibitors of GSK-3, LiCl and BIM I, caused β -catenin levels to increase in B cells. LiCl inhibits GSK-3 kinase activity by displacing the Mg^{2+} cofactor (237) while BIM I competitively inhibits the ATP binding site of GSK-3 (238). Lithium ions are highly specific inhibitors of GSK-3 while BIM I inhibits a greater variety of kinases including GSK-3, PKC α and others (242). Although BIM I inhibits the activity of several PKC isoforms (242,256), its ability to increase β -catenin levels in B cells most likely reflects its inhibitory effect on GSK-3 since inhibiting PKC activity blocks increases in β -catenin levels (Fig. 3.12). In any case, the use of these two different GSK-3 inhibitors indicates that GSK-3 is a central regulator of β -catenin levels in B cells. Two recently described, novel and specific GSK-3 inhibitors, SB-

216763 and SB-415286 (257), could now be used to support the conclusion that GSK-3 does indeed regulate β -catenin levels in B cells.

Inhibition of GSK-3 is thought to be the major mechanism by which receptor signalling increases β -catenin levels. My data suggest that the BCR also regulates β -catenin via the inhibition of GSK-3. Our lab had previously shown that BCR signalling leads to the phosphorylation of GSK-3 α/β on their negative regulatory sites and the concomitant inhibition of GSK-3 activity (57). As described above, I found that inhibition of GSK-3 is sufficient to cause an increase in β -catenin levels in B cells. Moreover, I found that agents that blocked the BCR-induced phosphorylation of GSK-3 on its negative regulatory sites also blocked the ability of the BCR to increase β -catenin levels. Thus, it appears that the inhibition of GSK-3 is both necessary and sufficient for the BCR to upregulate β -catenin.

I found that treating WEHI-231 cells with the GSK-3 inhibitor LiCl was sufficient to stimulate β -catenin-dependent transcription to a similar extent as anti-IgM treatment. Thus, at least in WEHI-231 cells, other BCR signalling pathways are not required to induce the expression or activation of proteins that cooperate with β -catenin to drive transcription. This is in contrast to Jurkat T cells where LiCl inhibits GSK-3 activity but is not sufficient to increase β -catenin-dependent transcription of the same TOPtk luciferase reporter gene construct (258). Although the BCR activates Akt (57), I found that inducing Akt activation using the mER-Akt fusion protein was not sufficient to induce significant GSK-3 phosphorylation or upregulation of β -catenin in WEHI-231 cells, even though the endogenous Akt was activated to a similar extent as that caused by anti-IgM. In contrast to the situation in B cells, overexpressing a membrane-bound form of constitutively-active Akt

does lead to the inhibition of GSK-3 in L6 skeletal muscle cells (199). This suggests that the degree of coupling between Akt and GSK-3 could be cell type-specific.

Although activation of Akt alone is not sufficient to regulate GSK-3 phosphorylation or β -catenin protein levels, the possibility that Akt activation may contribute to the regulation of GSK-3 and β -catenin has not been ruled out. To determine if the activation of Akt is necessary for the full inhibition of GSK-3 and upregulation of β -catenin it would be necessary to inhibit the BCR-induced activation of Akt activity via the expression of a dominant negative mutant form of Akt or through the use of RNAi to downregulate expression of the various Akt isoforms in B cells. Inhibition of GSK-3 phosphorylation or the upregulation of β -catenin in cells expressing a dominant negative Akt mutant would indicate that activation of Akt contributes to the regulation of GSK-3 and β -catenin. Unfortunately, we have been unable to express dominant negative forms of Akt in B cell lines, presumably because the expression of these mutants reduces basal Akt activity to levels below that needed for cell survival (63).

My results indicate that Akt does not play a major role in linking the BCR to GSK-3 and β -catenin and that the BCR regulates GSK-3 and β -catenin primarily via PKC. PKC activity is also required for Wnt-induced inhibition of GSK-3 (240). Treating B cells with the PKC activator PdBu was sufficient to induce GSK-3 phosphorylation and cause an increase in nuclear β -catenin levels. This was not dependent on Akt since PdBu did not stimulate the phosphorylation of Akt on sites that are required for its activation. In support of the idea that the BCR regulates GSK-3 and β -catenin via PKC, I showed that the PKC inhibitor safinol blocked BCR-induced GSK-3 phosphorylation and BCR-induced upregulation of β -catenin to the same extent. The inhibition of the BCR-induced GSK-3

phosphorylation and β -catenin upregulation by safingol was much more dramatic in splenic B cells than in the WEHI-231 cells line. One possibility is that there is a PKC-independent pathway that regulates GSK-3 phosphorylation and β -catenin levels in WEHI-231 cells but not in splenic B cells. Alternatively, since WEHI-231 is a lymphoma cell line these cells may express multi-drug resistance proteins that efficiently pump out drugs (259) and prevent the intracellular accumulation of safingol to the same levels as in splenic B cells. In any case, my results show that PKC makes a major contribution to the ability of the BCR to regulate GSK-3 and β -catenin. Safingol prevents the binding of DAG to the C1 domains of both conventional and novel PKC isoforms. Thus, activation of a DAG-dependent PKC isoform is both necessary and sufficient for the BCR to stimulate the phosphorylation of GSK-3 α/β and the accumulation of β -catenin.

Although other groups have shown that PKC can phosphorylate and inhibit GSK-3 (96,240,260,261), this is the first report showing that PKC-induced inhibition of GSK-3 is sufficient to cause the accumulation of β -catenin. In epithelial cells, PKC activity is necessary for Wnt-induced accumulation of β -catenin but phorbol ester-induced PKC activation is not sufficient to increase β -catenin levels (260). This suggests that other signals are required for the upregulation of β -catenin but that these signals are already present in WEHI-231 cells.

BCR engagement leads to the activation of PKC- α , - β , - δ , - ϵ , and - ζ (262-266). Each PKC isoform is likely to have a unique set of substrates and my data suggest that either a conventional PKC isoform (PKC- α , - β I, - β II, - γ) or a novel PKC isoform (PKC- δ , - ϵ , - η , - θ) mediates GSK-3 phosphorylation since these PKC isoforms are responsive to increases in DAG. In addition, my finding that U73122 blocked BCR-induced phosphorylation of GSK-3

and upregulation of β -catenin supports the idea that these responses are mediated via a PLC- γ 2/PKC pathway. I also found that PI3K contributes to the ability of the BCR to regulate GSK-3 and β -catenin. This presumably reflects the role of PI3K in the BCR-induced activation of PLC- γ 2 (31).

As discussed above, there are many different kinases which have been shown to phosphorylate GSK-3 *in vitro* and *in vivo* (92,94-100,217). I have shown that activation of PKC is both necessary and sufficient for BCR-induced GSK-3 phosphorylation. However, PKC may be mediating GSK-3 phosphorylation directly or indirectly via activation of another kinase. Therefore, I explored the possibility that PKC-dependent activation of the Raf-1/MEK/ERK1/2/p90^{RSK} pathway might be necessary for the BCR-induced phosphorylation of GSK-3. While this pathway is activated by BCR engagement (177) (Fig. 3.15) I found that inhibition of MEK and ERK1/2 did not affect BCR-induced GSK-3 phosphorylation or β -catenin upregulation. Therefore, BCR-induced PKC activation does not mediate GSK-3 phosphorylation via MEK/ERK1/2-dependent activation of p90^{RSK} in response to BCR stimulation. Since there is no evidence for a MEK-independent PKC-dependent pathway of p90^{RSK} activation, my data suggest that it is unlikely that PKC-mediated phosphorylation of GSK-3 is dependent on p90^{RSK} activity. PKC may induce GSK-3 phosphorylation via activation of another kinase but the simplest hypothesis is that PKC directly phosphorylates GSK-3 in B cells.

To determine if GSK-3 is a direct target of PKC in B cells and to determine which isoform of PKC regulates GSK-3 phosphorylation, I investigated whether GSK-3 and PKC- α , - δ , - β ₁ or - ϵ associate in B cells. One indication of the ability of a kinase to directly phosphorylate its substrate *in vivo* is the ability to bind its substrate *in vivo*. Unfortunately, I

was unable to conclusively demonstrate by immunoprecipitation studies that GSK-3 associates with any of the PKC isoforms examined in B cells (data not shown). Multiple lines of evidence are required to establish that a protein is likely a direct substrate of a kinase in the cellular milieu. First, it is essential to demonstrate that the purified kinase can phosphorylate its purified substrate *in vitro*, in the absence of other proteins, and that this phosphorylation is on the same sites as that observed *in vivo*. This has been demonstrated for the phosphorylation of GSK-3 by multiple PKC isoforms (96,99). Second, inhibition of the kinase through the use of chemical inhibitors or expression of a catalytically inactive dominant negative kinase should prevent phosphorylation of the substrate *in vivo*. In contrast, agonists that activate the kinase should lead to phosphorylation of the substrate. Both of these criteria have been demonstrated in B cells by myself as presented in this thesis and in response to Wnt stimulation of fibroblast cells (240). The association of a kinase with its putative substrate is also an indication that the kinase has the potential ability to phosphorylate this substrate. However, most kinase/substrate interactions are likely to be transient and may therefore be undetectable. Lastly, the kinase should be unable to phosphorylate a mutant version of the substrate containing substitutions at the sites of phosphorylation when expressed in the cell of interest. In the case of GSK-3 this would not be very informative since many different kinases, including PKC, have been shown to phosphorylate the same sites on GSK-3 *in vitro* (92,94-100). Thus although it is quite likely that GSK-3 is a direct substrate of PKC in B cells, the involvement of another kinase has not been ruled out.

The combinatorial use of gain-of-function experiments and loss-of-function experiments are important in proving a causal relationship between a kinase and its downstream target(s). A gain-of-function experiment (i.e. overexpression of a constitutively

active kinase) demonstrates that activation of a kinase is sufficient to elicit the observed response. In contrast, a loss-of-function experiment (i.e. the activity of a kinase is inhibited) demonstrates that the activity of a particular kinase activity is necessary for the observed response. The combination of these two types of experiments provides the most compelling evidence that a particular protein regulates a target protein, signalling pathway or other process.

Gain-of-function experiments can make use of the overexpression of constitutively active or wild type proteins to mimic the endogenous activation of the protein of interest. However, overexpression of an active protein can result in non-physiological effects due to the presence of excess protein that can result non-physiological interactions with endogenous proteins. This can be especially problematic if the proteins in question are normally sequestered in different cellular compartments. An alternative approach is to activate the protein of interest using specific chemical or small-molecule activators. This approach allows temporal control and potentially quantitative control of the activation of the protein of interest allowing one to more closely mimic physiological activation of the kinase. However, since related proteins can be activated by the same chemical or small-molecule agonists, the reliability of this approach depends highly on the specificity of the activator used.

Inhibition of a kinase (loss-of-function) can be accomplished through the use of chemical inhibitors, overexpression of a dominant negative form of the kinase or overexpression of a protein inhibitor of the kinase. Each of these approaches has its own drawbacks. Chemical inhibitors can inhibit other kinases in addition to the protein of interest resulting in non-specific effects (242). Dominant negative proteins function by binding and sequestering either upstream activators or downstream effectors thereby preventing activation of the downstream effectors of the endogenous kinase. Dominant negative proteins can exert

non-specific effects by blocking the activation of related proteins or pathways that the protein of interest does not normally interact with (267). Overexpression of a protein inhibitor can also result in non-specific effects by interacting with and inhibiting the activity of endogenous proteins with which the protein inhibitor does not normally interact. An alternative loss-of-function approach involves knocking-out expression of the protein of interest by inserting a non-functional gene into the protein coding region of the genome. While this is a very specific approach, potential drawbacks include the possibility that related proteins can functionally compensate for the loss of the protein thereby negating any phenotypic effect due to the knock-out. In addition, the loss of protein expression may be lethal in a particular organism or cell type thereby making functional analysis of the protein in this manner impossible.

Since every one of these approaches has its drawbacks and caveats a combination of approaches must be used in order to avoid making erroneous conclusions about the involvement of a kinase or protein in the regulation of a downstream pathway or process. This is because it is unlikely that the same non-specific reaction will occur with different approaches. Therefore, a combination of gain-of-function and loss-of-function experiments, using both chemical and biological approaches if possible, must be employed to determine a causal relationship between a kinase and its target(s).

In this chapter, I have shown that basal β -catenin protein levels are regulated by proteasome-mediated degradation in unstimulated B cells and that inhibition of the proteasome is sufficient to allow accumulation of β -catenin. In addition, BCR signalling increases the levels of β -catenin protein in the nuclei and cytosol of B cells and leads to an increase in β -catenin-mediate gene transcription. Since β -catenin can promote survival,

differentiation or proliferation, depending on the cell type, it may play an important role in the regulation of B cell development and activation by the BCR. Consistent with this idea, TCR-induced inhibition of GSK-3 is essential for T cell activation, including T cell proliferation and IL-2 production (268), although it is not known whether this involves β -catenin.

In summary, I have shown that the BCR regulates the levels of β -catenin in the nuclei of B cells via a PI3K/PLC- γ /GSK-3 pathway. Because β -catenin can promote survival, differentiation or proliferation, depending on the cell type, it may play an important role in the regulation of B cell development and activation by the BCR.

Chapter 4

Activation of the Rap GTPase in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAP kinase activation

4.1 Introduction

Monomeric GTPases are molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form. There are several families of monomeric GTPases, each of which has unique functions. In broad terms, the Arf, Rab and Ran family of GTPases are involved in the movement of vesicles and/or proteins within the cell (269-271). The Rho family of GTPases includes Rho, Rac and Cdc42. These GTPases links cell surface receptors to the dynamic organization of the actin cytoskeleton, which is essential for functional cellular migration and adhesion (166). In addition, Rac and Cdc42 link surface receptors, including the BCR, to the activation of the JNK and p38 MAPKs (8,166). The Ras family of GTPases is a diverse family containing at least 20 members including the classical Ras proteins as well as Rap1 and Rap2 (272). Activation of the H-Ras, N-Ras and K-Ras GTPases leads to activation of the canonical Ras pathway in which the ERK1/21 and ERK1/22 MAPKs are activated via a pathway that involves the Raf-1 and MEK kinases (164,165). Activation of the canonical Ras pathway in B cells is essential for B cell development (195) and for BCR-induced proliferation of mature B cells (196). Inhibition of Ras activity blocks early B cell development and prevents antigen-induced B cell proliferation (195,196). In support of a role for the Ras pathway in B cell activation, ERK1/2 kinase activity correlates with BCR-induced growth arrest and apoptosis in immature B cells (177,273). Therefore it is important to understand the mechanisms of the regulation of the Ras pathway in B cells.

In addition to activating the canonical Raf-1/MEK/ERK1/2 pathway, active Ras can also bind directly to the p110 catalytic subunit of PI3K and promote activation of the PI3K/Akt signalling pathway (52,197). Recent work showing that expression of constitutively active Ras leads to Akt activation in the A20 B cell line supports the idea that Ras promotes activation of the PI3K pathway in B cells (198).

The Rap monomeric GTPases are closely related to the classical Ras GTPase and were initially described as potential antagonists of Ras-mediated signalling. There are 4 different Rap proteins. Rap1A and Rap1B have over 97% identity at the protein level as do Rap2A and Rap2B. Rap1 and Rap2 have approximately 60% identity at the amino acid level over the entire protein but are nearly identical within the core effector binding domain (182,185). Rap1 is activated by the BCR via the PLC- γ -dependent increase in DAG (163). Rap1 and Rap2 share the same regulator GEF and GAP proteins (185) and preliminary evidence suggests that Rap2 is also regulated by the BCR in a PLC- γ -dependent manner (S.J. Mcleod, A.H.Y. Li and M.R. Gold, unpublished observations). CalDAG-GEF1, one of the Rap GEF proteins, can be activated by increases in DAG (188) and may be responsible for Rap activation downstream from the BCR.

Rap1A was originally identified by its ability to reverse the Ki-Ras-mediated transformation of NIH 3T3 cells (180) suggesting that Rap can inhibit Ras-mediated signalling. Since Rap1 and Ras have identical core effector binding domains (residues 32-40) (182), it was postulated that Rap1 might sequester Ras effectors in inactive complexes. Rap2 has a serine to phenylalanine substitution at position 39 in the effector binding region, compared to Ras and Rap1, but this substitution does not appear to have any functional consequences (182) and Rap2 is also able to act as a negative regulator of Ras signalling

(185). Activated Rap1 can bind Ras effectors *in vitro*, including Raf-1 (183,184). Several groups have shown that both activated Rap1 and activated Rap2 can inhibit Ras-dependent activation of ERK1/2 (185-188). Other studies however did not find that Rap activation impaired Ras-dependent ERK1/2 activation (189-191), suggesting that this could be a cell type-specific effect.

In contrast to the ability of Rap to negatively regulate ERK1/2 signalling in some cell types, Rap can also positively regulate ERK1/2 and p38 MAPK activation. In the neuronal PC12 cell line, active Rap1-GTP activates B-Raf which results in the sustained activation of ERK1/2 (192,193). Sawada et. al. have also provided evidence that Rap can positively regulate p38 activation in response to cell stretching (274).

Since the Rap GTPases can act as negative regulators of Ras-dependent signalling, I investigated whether Rap activation in B cells modulates the BCR-induced activation of the PI3K/Akt pathway or the MAPK pathways, critical mediators of BCR signalling. I found that Rap is neither a positive nor negative regulator of BCR-induced ERK1/2 activation and that Rap activation has no effect on the ability of the BCR to activate the JNK and p38 MAPKs. In contrast, I found that activation of the endogenous Rap GTPases limits the activation of Akt by the BCR. Preventing the activation of endogenous Rap by the BCR enhanced BCR-induced Akt phosphorylation while expressing Rap2V12, a constitutively active form of Rap2, inhibited BCR-induced Akt phosphorylation. Consistent with the idea that Rap-GTP opposes Akt activation, I found that Rap activation inhibits Akt-dependent signalling events, in particular phosphorylation of the FKHR transcription factor. I also found that the ability of Rap-GTP to limit BCR-induced signalling via Akt correlated with effects on B cell survival. Finally, I have shown that Rap2V12-GTP can bind to PI3K and inhibit its enzymatic activity. Thus, the Rap GTPases act as negative regulators of the

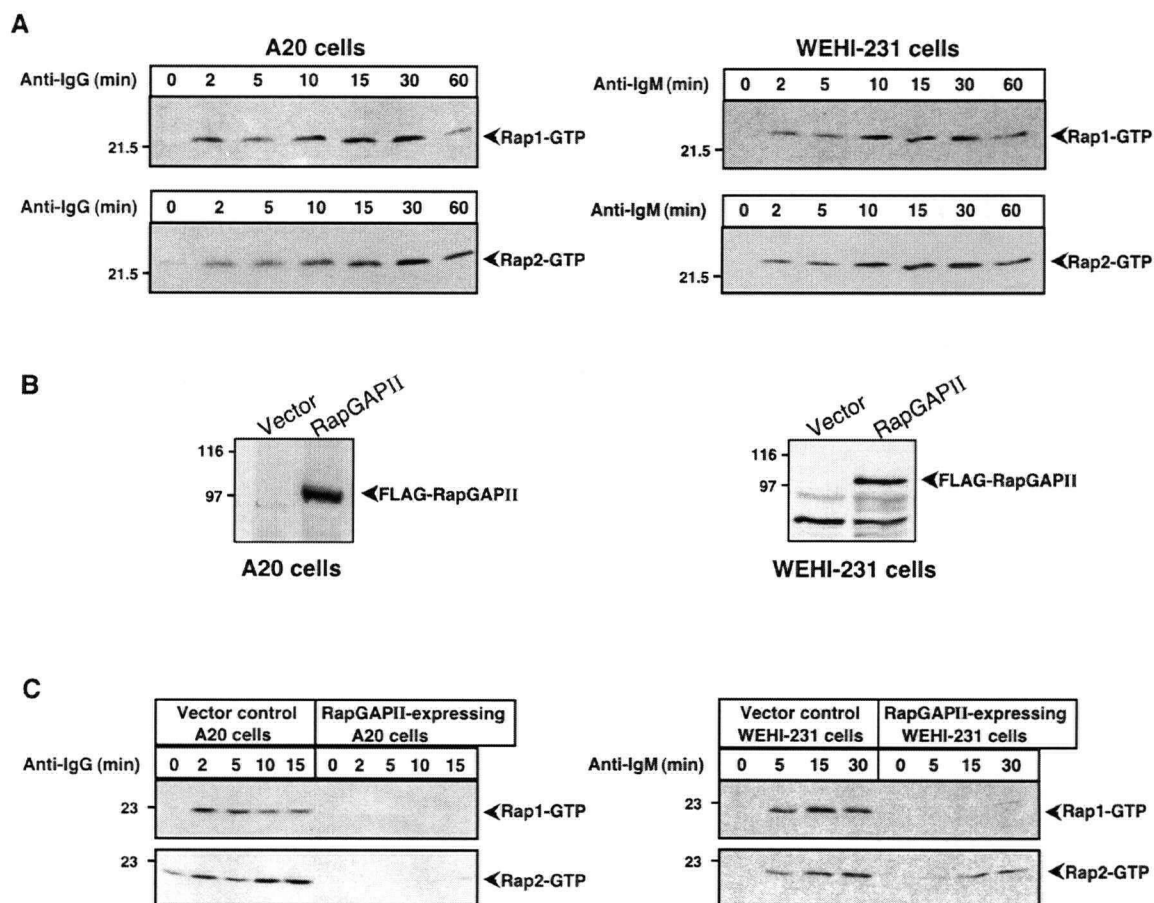
PI3K/Akt pro-survival signalling pathway in B cells and may do so via a mechanism that involves the binding of Rap-GTP to PI3K, the upstream activator of Akt.

4.2 Expressing RapGAPII inhibits BCR-induced activation of Rap1 and Rap2

BCR engagement results in an increase in the amount of active GTP-bound Rap1 (163). The activation of Rap2 by the BCR was also investigated since Rap2 has a nearly identical effector region to that of Rap1 (182), can bind Ras effectors, and can inhibit Ras-dependent ERK1/2 activation (185). Inducing BCR signalling with anti-Ig antibodies resulted in the activation of both Rap1 and Rap2 in the WEHI-231 and A20 murine B cell lines (Fig. 4.1A). WEHI-231 is an IgM⁺ B cell line that resembles an immature/transitional B cell that can undergo antigen-induced clonal deletion while A20 is an IgG⁺ B cell line that resembles a mature B cell that has undergone Ig class switching. The activation of Rap1 and Rap2 occurred with similar kinetics, consistent with the idea that they are regulated by the same GEFs and GAPs.

To investigate whether activated Rap GTPases modulate BCR signalling pathways, a loss-of function approach was employed in which the activation of endogenous Rap1 and Rap2 was blocked. To do this, a Rap-specific GAP called RapGAPII was stably expressed in both WEHI-231 and A20 cells (Fig. 4.1B). RapGAPII converts Rap1 and Rap2 to their inactive GDP-bound forms. A20 clones expressing RapGAPII were obtained by single-cell cloning and all experiments were performed using clone 16 and results confirmed using clone 3. Bulk populations of WEHI-231 cells expressing RapGAPII were generated by retrovirus-mediated gene transfer which has previously been shown to result in expression of the protein of interest in over 90 % of the cell population (207).

Figure. 4.1 BCR-induced activation of Rap1 and Rap2 is inhibited by expression of RapGAPII. *A*, A20 or WEHI-231 cells were stimulated with 40 µg/ml goat anti-mouse anti-Ig antibodies. Cell lysates from 2.5×10^6 cells were incubated with immobilized GST-RalGDS(RBD) in order to precipitate the active GTP-bound forms of Rap1 and Rap2 which were detected by immunoblotting with antibodies specific for Rap1 or Rap2. *B*, A20 and WEHI-231 cells were transfected with the empty pMSCVpuro vector or with pMSCVpuro containing the cDNA encoding RapGAPII. A20 clones and WEHI-231 bulk populations were analyzed for expression of the FLAG-tagged RapGAPII by immunoblotting with the anti-FLAG M2 antibody. *C*, A20 or WEHI-231 cells expressing either the empty vector or RapGAPII were stimulated with 40 µg/ml goat anti-mouse Ig antibodies. Rap activation assays were performed on the cell lysates as in *A*. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments.



All experiments were performed using cells which had been tested for expression of RapGAPII and for the efficient block in BCR-induced activation of Rap1 and Rap2. RapGAPII is normally expressed in brain (203) but not in B cells (275) and has been shown to specifically inhibit the activation of Rap1 and Rap2 (185,203) while having no effect on the activation of other closely related GTPases such as Ha-Ras, R-Ras (203), RhoA (276), and Rac1 (275). The expression of RapGAPII in B cell lines completely blocked BCR-induced activation of Rap1 and inhibited BCR-induced activation of Rap2 by at least 80% (Fig. 4.1C).

4.3 Inhibiting Rap activation has no effect on BCR-induced activation of the ERK1/2 MAPK

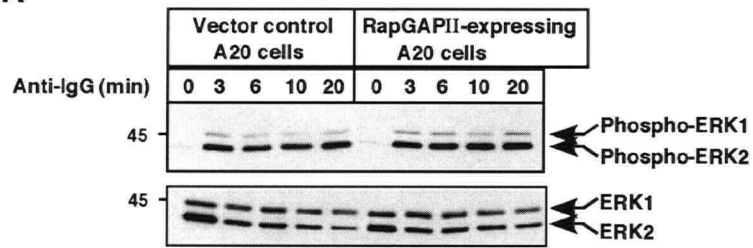
The BCR activates ERK1/2 (171,177) in a Ras-dependent manner (162,277) and this is important for both B cell development (195) and BCR-induced proliferation of mature B cells (196). Depending on the cell type, activated Rap may be either a positive or a negative regulator of ERK1/2 activation. In fibroblasts, expression of the constitutively active forms of Rap1 or Rap2 can inhibit ERK1/2 activation, perhaps by competing with Ras-GTP for Raf-1, the first kinase in the Raf-1/MEK/ERK1/2 cascade (185,186). In contrast, Rap-GTP can activate ERK1/2 in the PC-12 neuronal cell line by activating B-Raf (192,193). Thus I was interested in determining whether the Rap GTPases act as either positive or negative regulators of BCR-induced ERK1/2 activation.

To assess whether Rap activation modulated the ability of the BCR to activate ERK1/2, we compared BCR-induced ERK1/2 activation in vector control cells versus RapGAPII-expressing cells where activation of Rap1 and Rap2 was inhibited (see Fig. 4.1C).

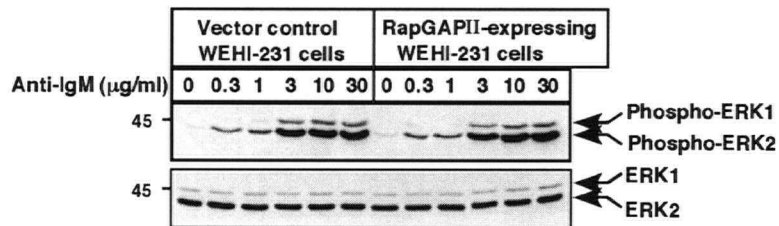
Figure 4.2 Preventing Rap activation does not affect BCR-induced ERK1/2 activation.

A and B, Cell extracts were analyzed by immunoblotting with antibodies that recognize the phosphorylated, active forms of ERK1/21 and ERK1/22. **A**, Vector control and RapGAPII-expressing A20 cells were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times. **B**, vector control and RapGAPII-expressing WEHI-231 cells were stimulated with the indicated concentrations of goat anti-mouse IgM antibodies for 5 min (experiment performed by R. L. Lee). For each panel, equal loading was analyzed by reprobing the membranes with an anti-ERK1/2 antibody. Molecular mass standards (in kDa) are indicated to the left of each panel. **C**, ERK1/22 *in vitro* kinase assays were performed on ERK1/22 immunoprecipitates. Vector control and RapGAPII-expressing A20 cells were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times and ERK1/22 activity was measured using GST-ELK1 as a substrate. For each panel, similar results were obtained in two to five independent experiments.

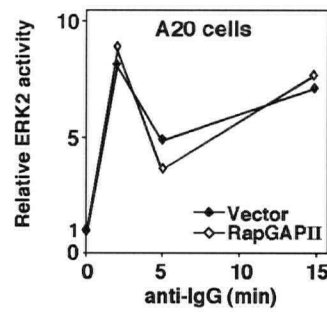
A



B



C

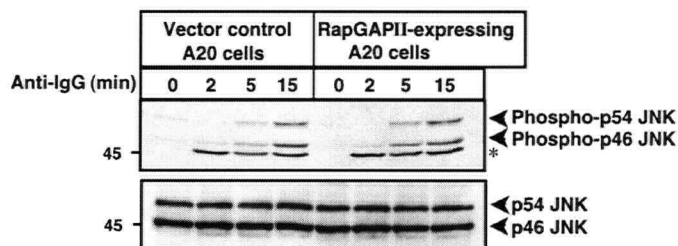
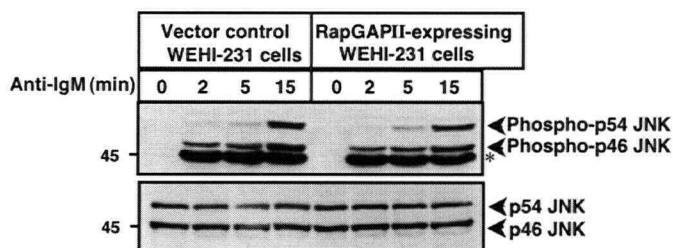
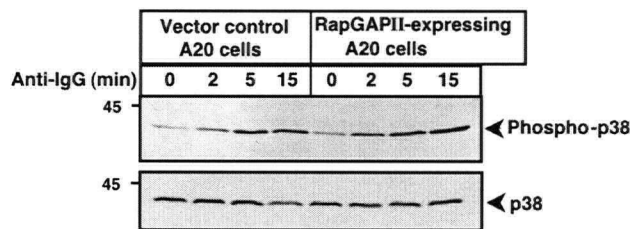
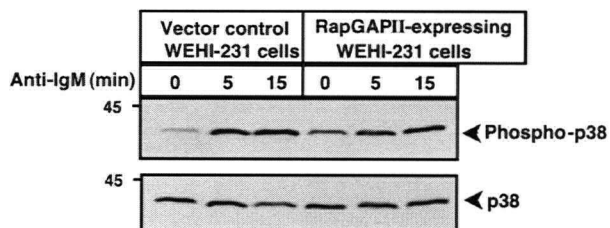


I first assessed ERK1/2 activation by using phosphorylation state-specific antibodies to detect the phosphorylation of ERK1/21 and ERK1/22 on sites that are required for their activation. The BCR causes strong activation of ERK1/22 and weaker activation of ERK1/21 (Fig. 4.2A), consistent with previous findings in the lab (177). When the activation of Rap1 and Rap2 was blocked by expressing RapGAPII, I found that this had no effect on either the magnitude or duration of anti-Ig-induced ERK1/2 phosphorylation in A20 cells (Fig. 4.2A) or WEHI-231 cells (S.J. McLeod and M.R. Gold, data not shown). To rule out the possibility that the effects of Rap-GTP might only be evident when there are low levels of ERK1/2 activation, the cells were stimulated with different concentrations of anti-Ig antibodies. Figure 4.2B shows that the dose response for anti-Ig-induced ERK1/2 phosphorylation was identical in vector control and RapGAPII-expressing WEHI-231 cells. We went on to perform ERK1/22 *in vitro* kinase assays to show that Rap does not modulate ERK1/2 activation directly. We found that the magnitude and duration of BCR-induced ERK1/22 activation was virtually identical in the vector control and RapGAPII-expressing A20 cells (Fig. 4.2C) and WEHI-231 cells (S.J. McLeod and M.R. Gold, data not shown). Thus, Rap-GTP is neither a positive nor a negative regulator of BCR-induced ERK1/2 activation.

4.4 Inhibition of Rap has no effect on BCR-induced phosphorylation of p38 or JNK MAPKs

I also investigated whether Rap activation modulated BCR-induced activation of the JNK and p38 MAPKs. Sawada *et al.* (274) showed that preventing Rap activation via the expression of RapGAPII inhibits cell stretching-induced activation of p38, but not JNK, in the L-929 fibroblast cell line. Using phospho-specific antibodies to detect the activated

Figure. 4.3 Preventing Rap activation does not affect BCR-induced activation of the JNK or p38 MAPKs. Vector control and RapGAPII-expressing A20 cells (*A and C*) or WEHI-231 cells (*B and D*) were stimulated with 40 μ g/ml goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize the phosphorylated, active forms of JNK (*A and B*) or p38 (*C and D*). Equal loading was analyzed by reprobing the membranes with antibodies to JNK or p38. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments. A non-specific band that is recognized by the anti-phospho-JNK antibody is indicated by an asterisk (*). Note that the decrease in JNK and p38 phosphorylation in RapGAPII-expressing WEHI-231 cells was not consistently observed.

A**A20 cells****B****WEHI-231 cells****C****A20 cells****D****WEHI-231 cells**

forms of JNK and p38, I found that preventing Rap activation by expressing RapGAPII (data not shown) did not have a significant effect on BCR-induced activation of JNK or p38 in either A20 cells or WEHI-231 cells (Fig. 4.3). Thus, activation of endogenous Rap1 and Rap2 does not modulate the ability of the BCR to activate any of the MAPKs.

4.5 Rap activation negatively regulates BCR-induced Akt phosphorylation

PI3K is also a downstream effector of Ras (52,197,278,279), and recent work has shown that expressing constitutively active Ras in the A20 B cell line leads to increased activation of the PI3K/Akt pathway (198). Since the effector-binding domains of Rap1 and Rap2 are nearly identical to that of Ras (182), Rap1 and Rap2 may also bind PI3K and modulate the activation of PI3K-dependent signalling pathways. An important downstream target of PI3K is the serine/threonine kinase Akt/protein kinase B. The BCR activates Akt in a PI3K-dependent manner (57) and Akt activity opposes BCR-induced apoptosis and promotes B cell survival (63). Therefore, I investigated whether Rap activation modulates BCR-induced activation of Akt.

The activation of Akt involves two steps, recruitment of Akt to the plasma membrane via the binding of the Akt PH domain to PIP₃ and PI(3,4)P₂ and subsequent phosphorylation of Akt on Thr308 by PDK1 (280) and on Ser473 by one of several candidate PDK2 enzymes (69-71,73). Phosphorylation of Akt on these two sites induces a structural reorganization of the kinase domain that is required for kinase activity (66). I therefore assessed Akt activation by using phospho-specific antibodies to analyze the phosphorylation of Akt on Thr308 and Ser473.

To determine whether activation of endogenous Rap1 and Rap2 by the BCR modulates its ability to activate Akt, I compared BCR-induced Akt phosphorylation in

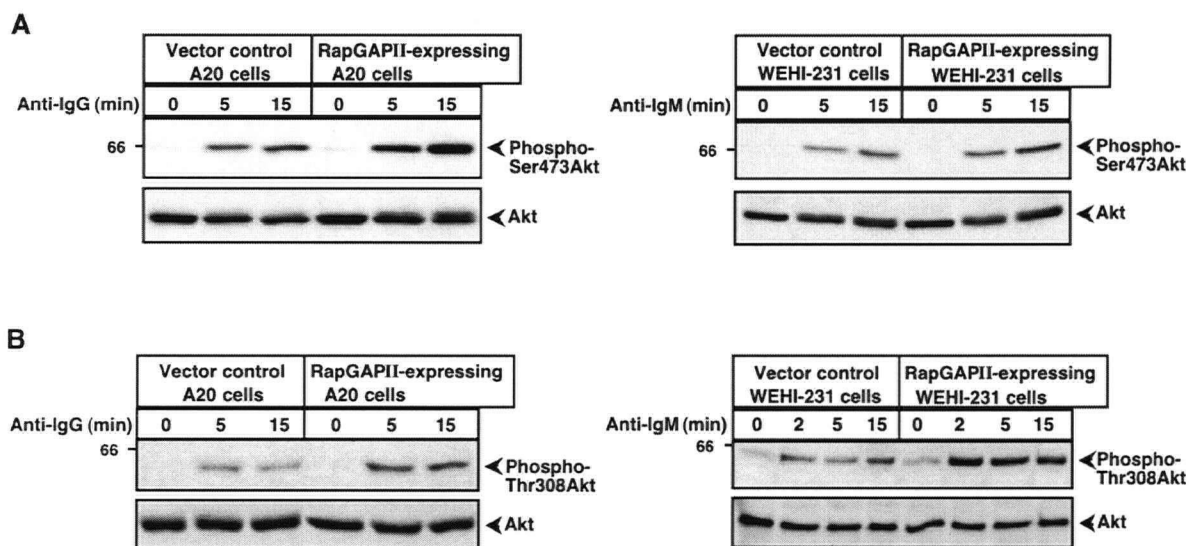


Figure. 4.4 Preventing Rap activation increases BCR-induced phosphorylation of Akt.

Vector control and RapGAPII-expressing A20 cells (left panels) or WEHI-231 cells (right panels) were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt that is phosphorylated on Ser473 (*A*) or on Thr308 (*B*). Equal loading was analyzed by reprobing the membranes with anti-Akt antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three to six independent experiments.

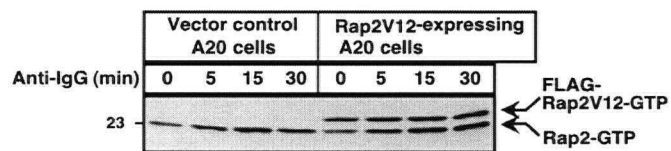
RapGAPII-expressing cells to that in vector control cells. I found that inhibiting Rap activation resulted in increased BCR-induced phosphorylation of Akt on both Ser473 (Fig. 4.4A) and Thr308 (Fig. 4.4B). Similar results were obtained in both the A20 and WEHI-231 B cell lines. BCR-induced phosphorylation of Akt on Ser473 and Thr308 was at least two-fold higher in the RapGAPII-expressing cells than in the vector control cells, and in some experiments was as much as 5-fold higher. Since inhibiting Rap activation resulted in increased phosphorylation of Akt, it suggests that BCR-induced activation of endogenous Rap1 or Rap2 normally limits BCR-induced phosphorylation of Akt. Thus, the Rap GTPases act as negative regulators of Akt activation in B cells.

To support the conclusion that Rap-GTP is a negative regulator of the PI3K/Akt pathway, I wished to perform the complementary gain-of-function experiment and show that increasing the amount of activated Rap results in a decrease in Akt activation. To do that, I expressed a constitutively active form of Rap2, Rap2V12, in both the A20 and WEHI 231 B cell lines. Figure 4.5A shows that the FLAG-tagged Rap2V12 is indeed constitutively active and that the total amount of activated Rap2 in these cells after BCR engagement was greater than in the vector control cells. Rap2V12 expression did not have a significant effect on BCR-induced ERK1/2 phosphorylation (Fig. 4.5B), indicating that Rap2V12 did not impair BCR signalling in general and supporting the idea that Rap does not regulate ERK1/2 activation in B cells. In contrast, Rap2V12 expression inhibited BCR-induced phosphorylation of Akt on Ser473 by approximately 50% (Fig 4.6A). BCR-induced phosphorylation of Akt on Thr308 phosphorylation was also decreased by approximately 50% in the Rap2V12-expressing cells (Fig. 4.6B). These data show that activated Rap can inhibit BCR-induced phosphorylation of Akt and is consistent with the idea that BCR-induced activation of endogenous Rap normally limits the ability of the BCR to activate Akt.

Figure. 4.5 Expression of the constitutively active Rap2V12 does not affect BCR-induced ERK1/2 phosphorylation. Vector control and Rap2V12-expressing A20 cells (*A and C*) or WEHI-231 cells (*B and D*) were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse Ig antibodies for the indicated times. *A and B*, Rap2 activation assays were performed on cell extracts. The active GTP-bound form of endogenous Rap2 or the transfected FLAG-tagged Rap2V12 was precipitated using a GST-RalGDS(RBD) fusion protein and detected by immunoblotting with an antibody specific for Rap2. *C and D*, Cell extracts were analyzed by immunoblotting with antibodies that recognize phosphorylated, active ERK1/21 and ERK1/22. Equal loading was analyzed by reprobing the membranes with anti-ERK1/2 antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three to four independent experiments. Note that the small decrease in ERK1/2 phosphorylation in Rap2V12-expressing A20 cells at 15 min was not a consistent finding.

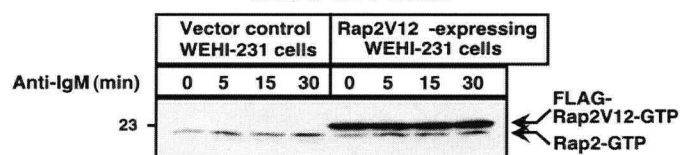
A

A20 cells



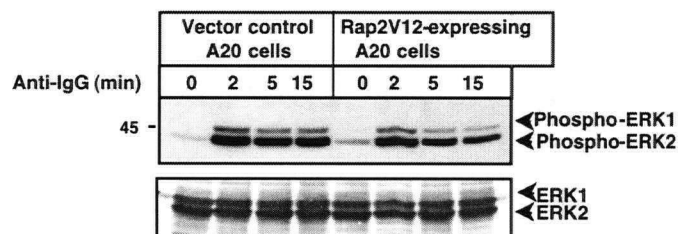
B

WEHI-231 cells



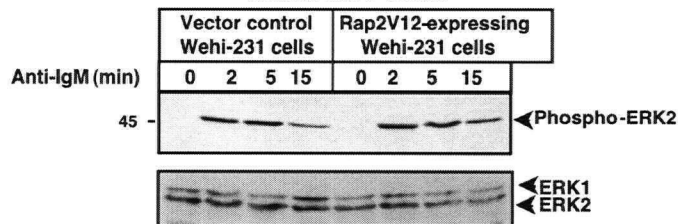
C

A20 cells



D

WEHI-231 cells



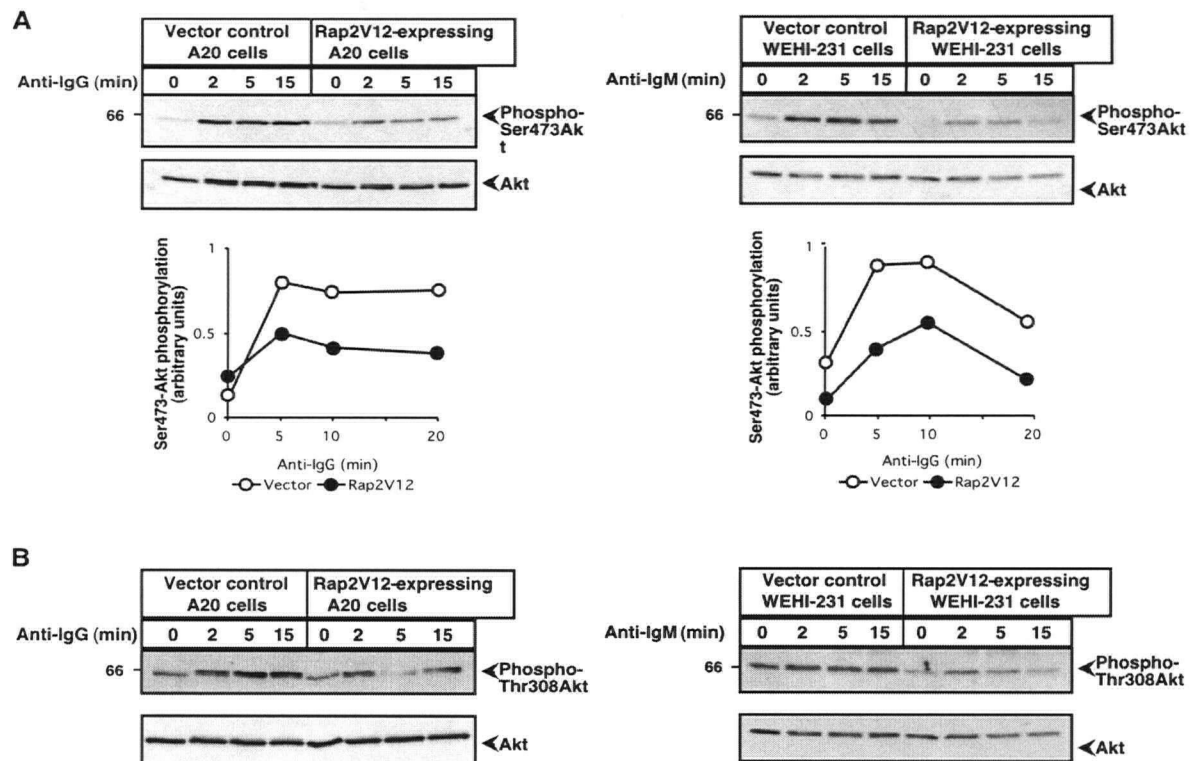


Figure. 4.6 Constitutively active Rap2V12 inhibits BCR-induced phosphorylation of Akt. Vector control and Rap2V12-expressing A20 cells (left panels) or WEHI-231 cells (right panels) were stimulated with 40 μ g/ml goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt that is phosphorylated on Ser473 (*A*) or on Thr308 (*B*). Equal loading was analyzed by reprobing the membranes with anti-Akt antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. To calculate the relative levels of Ser473 phosphorylation, the intensity of each band in the upper panel of (*A*) was determined by densitometry and divided by the intensity of the corresponding total Akt band in the lower panel. For each panel in this figure, similar results were obtained in three independent experiments.

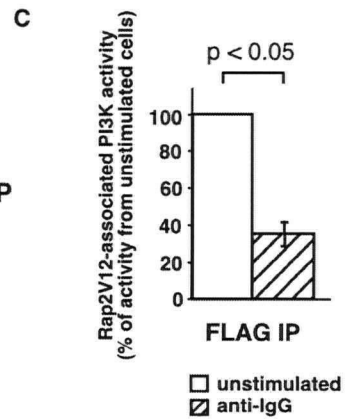
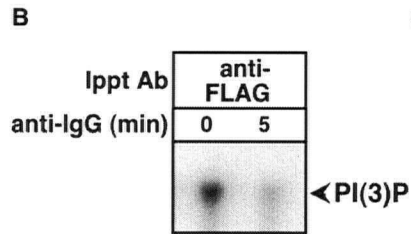
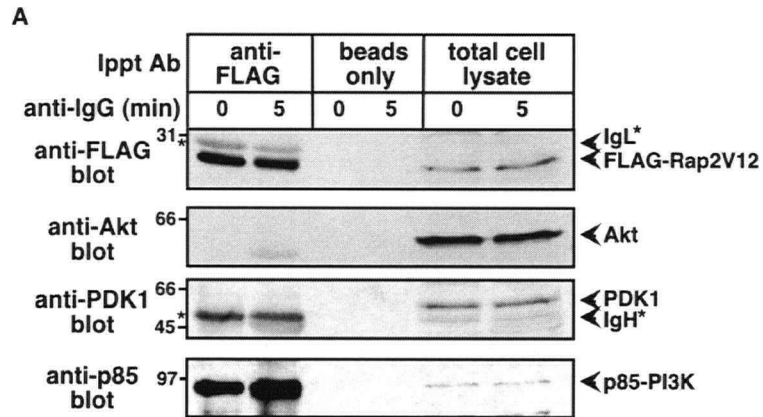
4.6 Rap2V12 and Rap1V12 can associate with PI3K

I was interested in determining the mechanism by which Rap-GTP inhibits BCR-induced activation of the PI3K/Akt pathway. I hypothesized that activated Rap may bind to one of the components in this pathway and inhibit its activity. Therefore, I immunoprecipitated the FLAG-tagged Rap2V12 and performed immunoblots to determine whether it associated with PI3K, PDK1, or Akt. Figure 4.7A shows that neither Akt nor PDK1 associated with Rap2V12 to a significant extent. The bands observed in the anti-PDK1 blots have a different mobility than the PDK1 in the cell lysates and are most likely the Ig heavy chains of the anti-FLAG antibody used for immunoprecipitation. In contrast, Rap2V12 bound very efficiently to PI3K, as judged by immunoblotting with antibodies to the p85 α subunit of PI3K (Fig 4.7A, bottom panel). Moreover, similar amounts of the PI3K p85 subunit co-precipitated with Rap2V12 from unstimulated cells and anti-Ig-treated cells, indicating that this was a constitutive association. These data suggest that activated Rap might exert its inhibitory effect on the PI3K/Akt pathway by binding to PI3K.

I went on to perform *in vitro* kinase assays to assess the amount of PI3K enzyme activity that was associated with Rap2V12 precipitated from unstimulated and anti-Ig-stimulated cells. A substantial amount of PI3K enzyme activity associated with the Rap2V12 precipitated from unstimulated cells (Fig. 4.7B). However, considerably less PI3K enzyme activity was associated with Rap2V12 precipitated from anti-Ig-stimulated cells (Fig. 4.7B). The amount of PI3K activity associated with Rap2V12 isolated from anti-Ig-treated cells was only $36 \pm 6\%$ ($n = 4$ independent experiments) of the PI3K activity associated with Rap2V12 isolated from unstimulated cells (Fig. 4.7C). Thus, even though similar amounts of the PI3K p85 subunit associated with Rap2V12 in unstimulated and anti-Ig-treated cells, considerably less PI3K enzyme activity was associated with the Rap2V12 isolated from anti-Ig-treated

Figure. 4.7 Rap2V12 associates with PI3K. *A*, A20 cells expressing FLAG-Rap2V12 were incubated with or without 40 µg/ml goat anti-mouse IgG antibodies for 5 min. Cell lysates from 1.25×10^7 cells were incubated with the anti-FLAG M2 antibody conjugated to agarose beads or with an equivalent volume of Sepharose-CL-4B beads. Precipitated proteins, as well as total cell lysates (1.25×10^6 cell equivalents) were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against the p85 subunit of PI3K. The upper portion of the membrane was subsequently reprobed with antibodies to PDK1 and Akt while the lower portion of the membrane was reprobed with the anti-FLAG antibody to show that equivalent amounts of FLAG-Rap2V12 had been precipitated from the unstimulated and anti-Ig-stimulated cells. Molecular mass standards (in kDa) are indicated to the left of each panel. The asterisks represent bands derived from the Ig heavy chains (IgH) or Ig light chains (IgL) of the anti-FLAG antibody used for immunoprecipitation. Similar results were obtained in two independent experiments.

B and C, A20 cells were stimulated as in *A*. Cell lysates were incubated with the anti-FLAG M2 antibody coupled to agarose beads and PI3K enzyme assays were performed on the immunoprecipitates using phosphatidylinositol as a substrate. The phosphatidylinositol 3-phosphate (PI(3)P) produced by PI3K was separated by thin-layer chromatography (*B*). The results are depicted graphically in (*C*). The amounts of ^{32}P -PI(3)P produced were quantitated using a phosphorimager and are expressed as a % of the ^{32}P -PI(3)P for the unstimulated (time 0) samples. The data are presented as the mean \pm SEM for four independent experiments. P-values were calculated using Student's T-test.



cells. The simplest interpretation of this result is that some BCR-induced signalling event allows Rap2V12 to inhibit the enzymatic activity of PI3K. An alternative explanation is that Rap2V12 binds to the p85 subunit of PI3K and that this interaction, only in the context of another BCR-induced signalling event, promotes the dissociation of the p110 catalytic subunit from this population of Rap2V12-bound PI3K molecules. I was not able to test this latter hypothesis since available antibodies to p110 α , p110 β and p110 δ were unable to detect any p110 protein associated with Rap2V12 isolated from either the unstimulated or anti-Ig-treated cells. In any case, these results suggest that activated Rap can inhibit the activation of the PI3K/Akt pathway by negatively regulating PI3K activity in some manner.

Rap1 and Rap2 have nearly identical effector domains and therefore Rap1 should also have the ability to bind to PI3K. However, our lab has not been able to express the constitutively active Rap1V12 protein in B cell lines, perhaps because Rap1V12 expression suppresses Akt activation to below the point required for cells to survive. Therefore, to test if Rap1 could also bind to endogenous PI3K, and potentially inhibit PI3K signalling in this way, I expressed Rap1V12 in the HEK293 human embryonic kidney cell line and performed PI3K *in vitro* kinase assays to assess whether PI3K could associate with FLAG-tagged Rap1V12. (Fig. 4.8). These kinase assays revealed that endogenous PI3K could associate with Rap1V12 in HEK293 cells. Preliminary experiments also suggested that the amount of PI3K activity that was associated with Rap1V12 was comparable to that associated with Rap2V12 (data not shown). However, I was unable to obtain consistent expression of Rap2V12 in HEK293 cells. Nevertheless, the constitutively-active Rap1V12 is able to bind to PI3K in HEK293 cells and can therefore potentially inhibit the activation of PI3K signalling by the same mechanism as active Rap2V12. Further work is necessary to determine whether activated endogenous Rap1-GTP binds to PI3K in B cells.

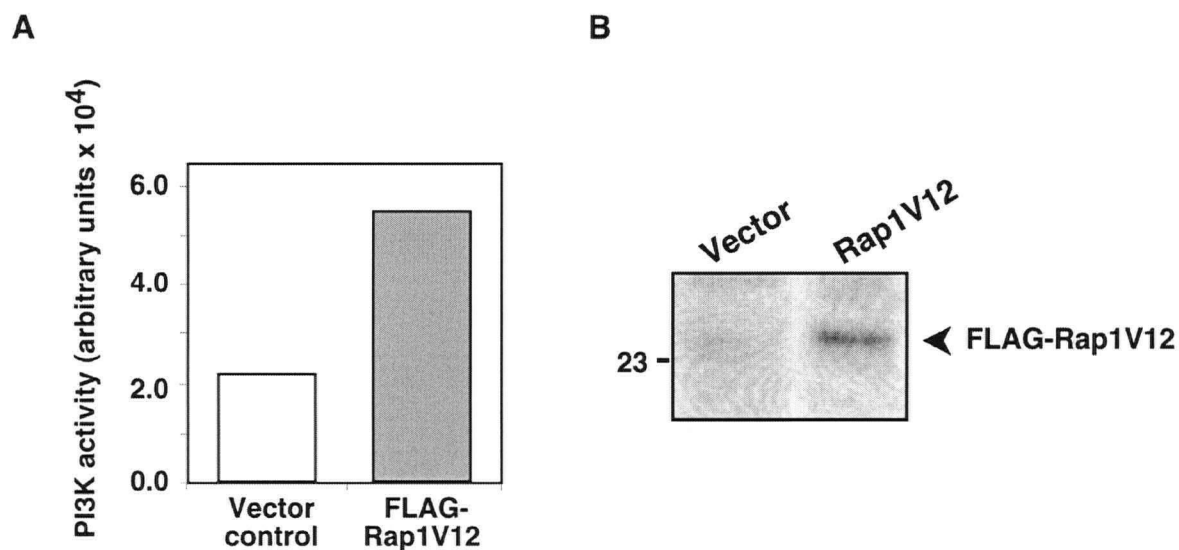


Figure. 4.8 Rap1V12 can associate with PI3K in HEK293 cells. HEK293 cells were transfected with the empty pMSCV vector or the same vector containing cDNA encoding FLAG-tagged Rap1V12. *A*, The cells were lysed 36 h after transfection and cell lysates (500 μ g protein) were incubated with the agarose-conjugated anti-FLAG M2 antibody and PI3K enzyme assays were performed on the immunoprecipitates using phosphatidylinositol as a substrate. The phosphatidylinositol 3-phosphate (PI(3)P) produced by PI3K was separated by thin-layer chromatography and the amount of 32 P-PI(3)P produced was quantitated using a phosphorimager. The results are depicted graphically. A representative experiment is presented, similar results were obtained in three different experiments. *B*, Cell lysates (20 μ g protein) were analyzed for Rap1V12 expression by immunoblotting with anti-FLAG antibodies. Molecular mass standards (in kDa) are indicated to the left of the panel.

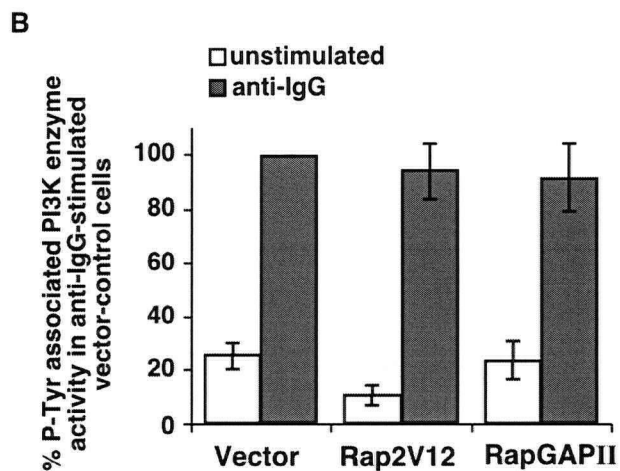
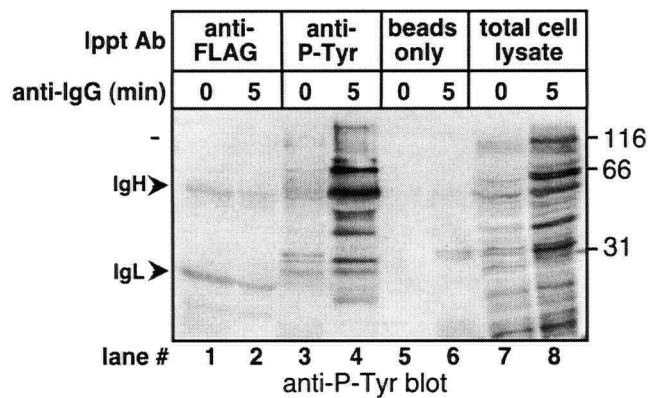
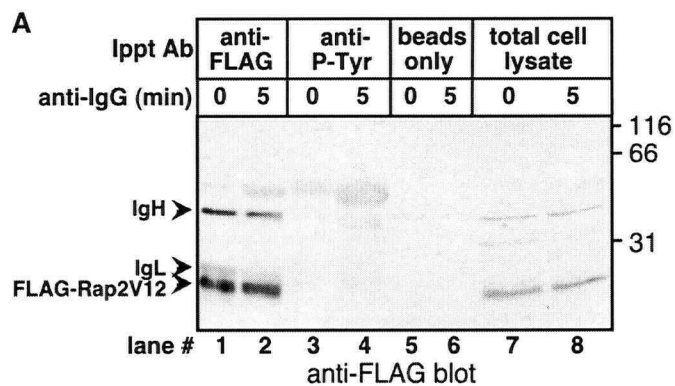
4.7 Rap does not associate with or modulate the activity of PI3K molecules that bind tyrosine-phosphorylated docking proteins

Upon BCR engagement PI3K is recruited to the plasma membrane where it can phosphorylate PI(4,5)P₂ and produce PIP₃. This BCR-induced membrane recruitment of PI3K can occur via binding of the SH2 domains of PI3K to membrane-associated docking proteins such as CD19, BCAP, Gab1 and Gab2 (45,46,48), all of which are phosphorylated on YxxM sequences by BCR-activated tyrosine kinases. It is also possible that PI3K can be recruited to the plasma membrane in a phosphotyrosine-independent manner, for example by binding to activated Ras. Indeed, recent work has suggested that Ras-dependent PI3K activation is important for BCR-induced Akt phosphorylation (198). Thus, the BCR may mobilize PI3K via both phosphotyrosine-dependent and -independent mechanisms.

Since gene knockout studies have shown that phosphotyrosine-dependent recruitment of PI3K to CD19 (50) and BCAP (48) is important for the BCR to fully stimulate PI3K-dependent signalling events, I asked whether activated Rap associates with and negatively regulates the PI3K that binds to tyrosine-phosphorylated proteins after BCR engagement. To test whether activated Rap associates with the pool of PI3K that binds to tyrosine-phosphorylated proteins, I used the 4G10 anti-phosphotyrosine (anti-P-Tyr) monoclonal antibody to immunoprecipitate tyrosine-phosphorylated proteins from Rap2V12-expressing A20 cells. While phosphotyrosine-containing proteins were precipitated from anti-Ig-treated cells (Fig. 4.9A, lower panel, lane 4) and PI3K enzyme activity was present in these anti-phosphotyrosine immunoprecipitates from Rap2V12-expressing cells (Fig. 4.9B), there was no detectable FLAG-Rap2V12 associated with these tyrosine-phosphorylated proteins (Fig. 4.9A, upper panel, lane 4). Similarly, when anti-FLAG immunoprecipitations were performed, FLAG-tagged Rap2V12 was efficiently immunoprecipitated from anti-Ig-treated

Figure. 4.9 Rap2V12 does not associate with tyrosine-phosphorylated proteins and does not inhibit the activity of PI3K that associates with tyrosine-phosphorylated proteins.

A, A20 cells expressing FLAG-Rap2V12 were incubated with or without 40 µg/ml goat anti-mouse IgG antibodies for 5 min. Cell lysates (1.25×10^5 cell equivalents) were immunoprecipitated with either the anti-FLAG M2 monoclonal antibody bound to agarose beads, the 4G10 anti-phosphotyrosine monoclonal antibody bound to protein G-sepharose beads, or Sepharose-CL-4B beads as a negative control. Cell lysates (1.25×10^6 cell equivalents) were included as a positive control. The presence of FLAG-Rap-2V12 was detected by immunoblotting with the anti-FLAG M2 antibody (upper panel) while tyrosine-phosphorylated proteins were detected by immunoblotting with the 4G10 anti-phosphotyrosine monoclonal antibody (lower panel). Molecular mass standards (in kDa) are indicated to the right of each panel. Similar results were obtained in two independent experiments. *B*, Rap2V12-expressing A20 cells, RapGAPII-expressing A20 cells, and vector control A20 cells were incubated with or without 40 µg/ml goat anti-mouse IgG antibodies for 5 min. Cell lysates were immunoprecipitated with the 4G10 anti-phosphotyrosine monoclonal antibody and PI3K enzyme assays performed on the immunoprecipitated proteins. The amounts of ^{32}P -PI(3)P produced were quantitated using a phosphorimager and are expressed as a % of the ^{32}P -PI(3)P for the anti-IgG-stimulated vector control cells. The data are presented as the mean \pm SEM for three independent experiments.



cells (Fig. 4.9A, upper panel, lane 2) but no phosphotyrosine-containing proteins were associated with the Rap2V12 (Fig. 4.9A, lower panel, lane 2). These findings indicate that Rap-GTP does not bind to tyrosine-phosphorylated proteins after BCR engagement and suggests that Rap-GTP does not modulate the enzymatic activity of PI3K molecules that associate with these phosphotyrosine-containing proteins. It is possible that Rap2V12 interacts with tyrosine phosphorylated proteins at a level below detection by immunoblotting. However, as discussed below, since Rap2V12 does not affect the phospho-tyrosine mediated activation of PI3K it is unlikely that Rap2V12 associates with this pool of PI3K.

To test whether the binding of Rap-GTP to PI3K negatively regulates the activity of PI3K associated with tyrosine phosphorylated proteins, I compared the amount of PI3K enzyme activity that could be immunoprecipitated with anti-phosphotyrosine antibodies from anti-Ig-stimulated Rap2V12-expressing A20 cells versus vector control A20 cells. I found that Rap2V12 expression did not decrease the amount of PI3K activity that could be immunoprecipitated with anti-phosphotyrosine antibodies from anti-Ig-treated cells (Fig. 4.9B). In both the vector control and Rap2V12-expressing cells, BCR engagement caused a substantial increase in the amount of PI3K activity associated with phosphotyrosine-containing proteins. Therefore, Rap2V12 apparently lacks the ability to prevent the association of PI3K with tyrosine phosphorylated proteins or to regulate this pool of PI3K. This suggests that endogenous Rap-GTP does not act as a negative regulator for this pool of PI3K. To directly test this idea, I examined the effect of preventing Rap activation via RapGAPII expression on the interaction of PI3K with tyrosine phosphorylated proteins. Figure 4.9B shows that preventing the activation of endogenous Rap did not increase the amount of PI3K activity associated with P-Tyr proteins. The amount of PI3K enzyme activity associated with phosphotyrosine-containing proteins was identical in control cells

and in RapGAPII-expressing cells in which Rap activation was suppressed (Fig. 4.9B).

Thus, endogenous Rap does not act as a negative regulator of the PI3K that associates with tyrosine-phosphorylated proteins. Taken together, these data suggest that Rap-GTP selectively regulates a pool of PI3K that the BCR mobilizes in a phosphotyrosine-independent manner. This phosphotyrosine-independent mode of PI3K mobilization by the BCR may involve Ras or other proteins that are not tyrosine phosphorylated.

4.8 BCR engagement induces the PI3K-dependent phosphorylation of PKC- ϵ

By preventing Rap activation via the expression of RapGAPII, I have shown that activation of the endogenous Rap proteins limits BCR-induced Akt phosphorylation, a process that is dependent on PI3K as shown previously (57). Since activated Rap can bind to a subset of PI3K in the cell, and apparently inhibit its activity in anti-Ig-stimulated cells, the question is raised of whether Rap-GTP negatively regulates other PI3K-dependent responses in B cells. In other work (210), I have investigated the regulation of PKC- ϵ by PI3K. I will first describe the regulation of PKC- ϵ by the BCR-induced activation of PI3K then go on to describe the effect of Rap activation on the PI3K-mediated phosphorylation of PKC- ϵ in Section 4.9.

Engagement of the BCR results in the activation of numerous PKC isoforms, including PKC- α (262), PKC- β (264), PKC- δ (266) and, as shown here, PKC- ϵ (210). There are 11 different PKC enzymes that are classified according to their mode of activation (149). Activation of the conventional PKC isoforms (PKC- α , - β_I , - β_{II} and γ) requires an increase in the products of PLC- γ activation, which are the second messengers DAG and Ca^{2+} . The novel PKCs (PKC- δ , - ϵ , - η and - θ) are activated by increases in DAG but do not require

Ca^{2+} . The full activation of PKC- δ and PKC- ϵ also require a phosphorylation event mediated by kinases such as PDK1 and PKC- ζ , which are regulated by the PI3K pathway (281-284). The atypical PKCs (PKC- ζ , - ι and - λ) do not require either DAG or Ca^{2+} for their activation and PKC- ζ appears to be regulated by a PI3K/PDK1 pathway (282,285).

Each PKC isoform is likely to have different substrates and therefore unique functions (286,287). An important step towards elucidating the function of each PKC isoform in B cells is to determine which PKC isoforms are activated upon BCR engagement. Together with H. C. Ting and A. E. Burgess, I examined the regulation of PKC- ϵ by the BCR (210).

The activation of novel PKCs, including PKC- ϵ , requires at least two phosphorylation events. Newly synthesized proteins undergo a priming autophosphorylation step that allows them to bind to DAG at the plasma membrane via their C1 domain. This binding induces a conformational change that allows the subsequent phosphorylation by PDK1 and PKC- ζ , kinases that are activated in a PI3K-dependent manner (283,284,288). These phosphorylation events significantly increase the kinase activity of novel PKCs and can therefore be used as a measure of their activation. Phosphorylation of PKC- ϵ results in the appearance of slower migrating forms as detected in anti-PKC- ϵ immunoblots. These bandshifts can be enhanced by the use of "low bis" SDS-PAGE gels where the ratio of acrylamide to bisacrylamide is 118:1 as opposed to the standard 37.5:1 ratio (289).

Engagement of the BCR resulted in the appearance of a higher molecular weight form of PKC- ϵ in murine splenic B cells, the murine A20 cell line, and the human Ramos cell line (Fig. 4.10A; ref. 210). This bandshift was eliminated when PKC- ϵ immunoprecipitates were treated with alkaline phosphatase indicating that the observed bandshift was due to a

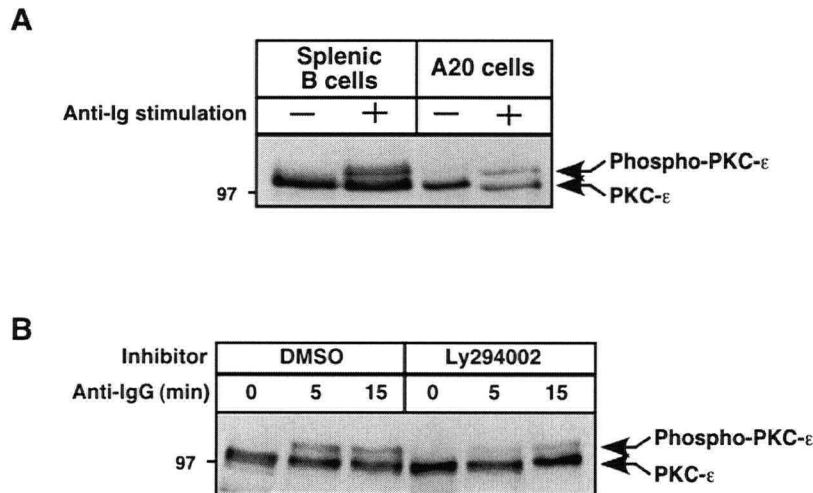


Figure. 4.10 BCR engagement induces PKC- ϵ phosphorylation in a PI3K-dependent manner. *A*, Splenic B cells were stimulated with 50 $\mu\text{g/ml}$ anti-mouse κ antibodies for 5 min. A20 cells were stimulated with 40 $\mu\text{g/ml}$ anti-mouse IgG antibodies for 5 min. *B*, A20 cells were pre-treated with 25 μM Ly294002 or an equivalent volume of DMSO for 20 min and then incubated with 40 $\mu\text{g/ml}$ goat anti-mouse IgG antibodies for the indicated times. Cell extracts (20 μg) were separated on 10% low bis gels and analyzed by immunoblotting with a monoclonal antibody to PKC- ϵ . Molecular mass standards are indicated to the left of each panel. Similar results were obtained in two independent experiments.

phosphorylation event (210). Thus, engagement of the BCR results in activation of PKC- ϵ , as determined by its phosphorylation state.

The PI3K-dependent kinases PDK1 and PKC- ζ have previously been shown to be responsible for the phosphorylation of PKC- ϵ (283,284,288). To determine whether the BCR-induced phosphorylation of PKC- ϵ was dependent on PI3K activity, A20 cells were pre-treated with the PI3K inhibitors, Ly294002 (Fig. 4.10B) or wortmannin (210) followed by stimulation through the BCR. Both of these inhibitors decreased but did not abolish the BCR-induced phosphorylation of PKC- ϵ . Therefore, phosphorylation of PKC- ϵ is dependent in part on PI3K activity, possibly due to the PI3K-dependent activation of PDK1 and/or PKC- ζ .

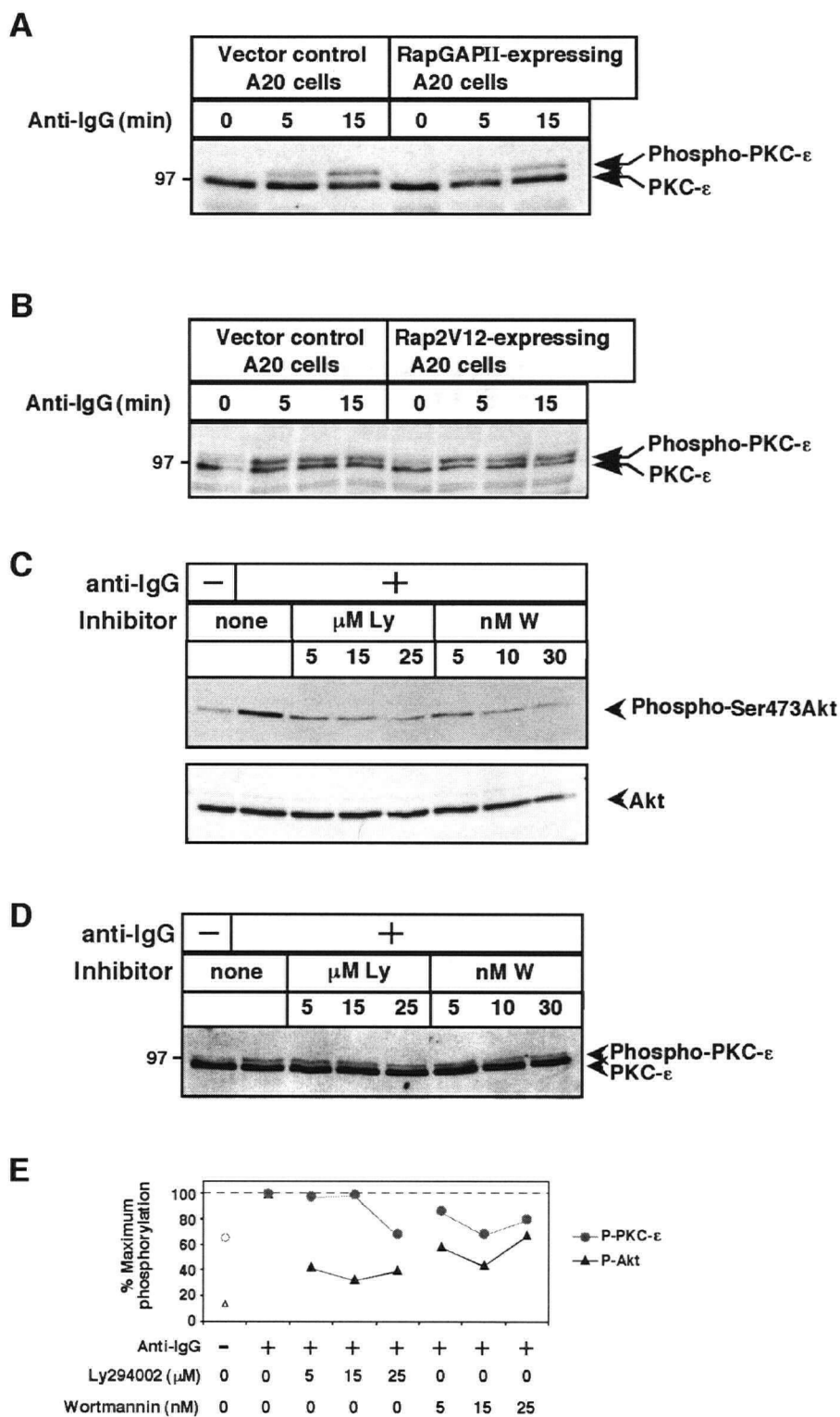
4.9 Activation of endogenous Rap limits PI3K-dependent phosphorylation of Akt but does not affect PI3K-dependent phosphorylation of PKC- ϵ

To address whether Rap-GTP negatively regulates all PI3K signalling or just specific PI3K-dependent pathways, I asked whether activation of the endogenous Rap proteins also limits the PI3K-dependent phosphorylation of PKC- ϵ that occurs in response to BCR engagement (210). In contrast to the 2- to 3-fold enhancement of BCR-induced Akt phosphorylation seen in RapGAPII expressing A20 cells, BCR-induced phosphorylation of PKC- ϵ was hardly affected by blocking Rap activation (Fig. 4.11A). Thus, it appears that activation of endogenous Rap selectively limits a subset of PI3K-dependent responses.

A possible explanation for this differential regulation of PI3K-dependent responses by Rap-GTP is that BCR-induced phosphorylation of Akt requires high levels of PI3K-

Figure 4.11 BCR-induced Akt phosphorylation is more sensitive than PKC- ϵ

phosphorylation to PI3K inhibition and Rap activation. *A*, A20 cells expressing empty vector or RapGAPII were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse IgG antibodies. *B*, A20 cells expressing empty vector or Rap2V12 were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse IgG antibodies. *A and B*, Triton X-100 cell extracts were separated on a 10% low bis gel and analyzed by immunoblotting with a monoclonal antibody to PKC- ϵ . *C-E*, A20 cells were incubated with the indicated concentrations of Ly294002 (Ly), Wortmannin (W) or an equivalent volume of DMSO for 20 min at 37°C. The cells were then stimulated for 5 min with 40 $\mu\text{g/ml}$ goat anti-mouse IgG. *C*, Triton X-100 cell extracts were analyzed by immunoblotting with antibodies to Akt that is phosphorylated on serine 473. Equal loading was analyzed by reprobing the membranes with an anti-Akt antibody. *D*, Triton X-100 extracts were separated on a 10% low bis gel and analyzed by immunoblotting with a monoclonal antibody to PKC- ϵ . Molecular mass standards (kDa) are indicated to the left of each panel. Similar results were obtained in three independent experiments. *E*, The intensity of the bands in *C* and *D* were determined by densitometry. The fraction of Akt or PKC- ϵ that was phosphorylated in each sample was determined by dividing the intensity of the phosphorylated protein band (phospho-Ser473-Akt or upper band of PKC- ϵ) by the total intensity of the Akt or PKC- ϵ bands. The data are expressed as the percent of maximum phosphorylation observed in anti-Ig-stimulated cells that were not treated with inhibitor. The results from one representative experiment are plotted as shown.



derived second messengers whereas BCR-induced phosphorylation of PKC- ϵ requires lower levels of PIP₃ and PI(3,4)P₂. Such differential thresholds for activation would make PKC- ϵ phosphorylation less sensitive than Akt phosphorylation to the inhibition of a subset of PI3K molecules by activated Rap. To test this idea that PKC- ϵ has a lower threshold for activation by PIP₃ and PI(3,4)P₂ than Akt, and is therefore less sensitive to alterations of PI3K activity, I asked whether PKC- ϵ phosphorylation is less sensitive to PI3K inhibitors than Akt phosphorylation. Figure 4.11B-D shows that this is indeed the case. While BCR-induced Akt phosphorylation was substantially inhibited by 5 μ M LY294002, BCR-induced phosphorylation of PKC- ϵ in the same experiment was unaffected by either 5 or 10 μ M LY294002 and only inhibited when the cells were pre-treated with a 25 μ M LY294002. Similar results were obtained using another PI3K inhibitor, wortmannin (Fig. 4.11D). This indicates that Akt is very sensitive to inhibition of PI3K activity and requires high levels of PI3K-derived lipids for its activation whereas maximal PKC- ϵ phosphorylation can still occur when PI3K is partially inhibited by low concentrations (5-10 μ M) of LY294002. Since endogenous Rap-GTP inhibits only a subset of PI3K molecules Rap-GTP is more likely to limit Akt phosphorylation than PKC- ϵ phosphorylation. This may explain why phosphorylation of Akt, but not PKC- ϵ , is enhanced when Rap activation is blocked. Thus, activation of endogenous Rap appears to selectively limit those PI3K-dependent responses that have the highest thresholds for activation by PI3K-derived second messengers.

4.10 Rap activation modulates phosphorylation of the FKHR transcription factor, a downstream target of Akt

Akt-dependent phosphorylation regulates the activity of a number of proteins that are involved in cell survival, cell growth and cell cycle progression (55,74). Substrates of Akt include Bad (75,76), GSK-3 (92), mTOR (290), tuberlin (77), and p21^{Cip1/Waf} (78,79). In addition, Akt-mediated regulation of the NF- κ B and Forkhead transcription factors leads to changes in gene expression that promote cell survival (86,87,90,239). Akt enhances the activation of NF- κ B, a transcription factor that regulates the expression of multiple pro-survival factors. In contrast, Akt inhibits the activity of Forkhead transcription factors which normally induce the expression of pro-apoptotic proteins. Akt-mediated phosphorylation of Forkhead transcription factors in the nucleus causes them to translocate from the nucleus to the cytoplasm and in this way inhibits their transcriptional activity (91). Gene targets of Forkhead include the cell cycle inhibitor p27^{Kip1} (291), a pro-apoptotic member of the Bcl-2 family called Bim1 (292) and the Fas ligand (90). Phosphorylation of Forkhead transcription factors by Akt results in decreased transcription of these pro-apoptotic genes and represents one mechanism by which Akt promotes cell survival. Since I found that Rap-GTP limits BCR-induced activation of Akt, an important question was whether Rap-GTP negatively regulates Akt-dependent anti-apoptotic/pro-survival signalling pathways in B cells.

To test whether Rap-GTP regulates Akt-dependent pro-survival signalling pathways, I analyzed the effect of RapGAPII expression and Rap2V12 expression on BCR-induced phosphorylation of the Forkhead-related transcription factor FKHR/FOXO1, a known target of Akt in B cells (Section 3.8) (293). Akt-mediated phosphorylation of FKHR on serine 256 and threonine 24 induces the cytoplasmic translocation of FKHR and inhibits FKHR-

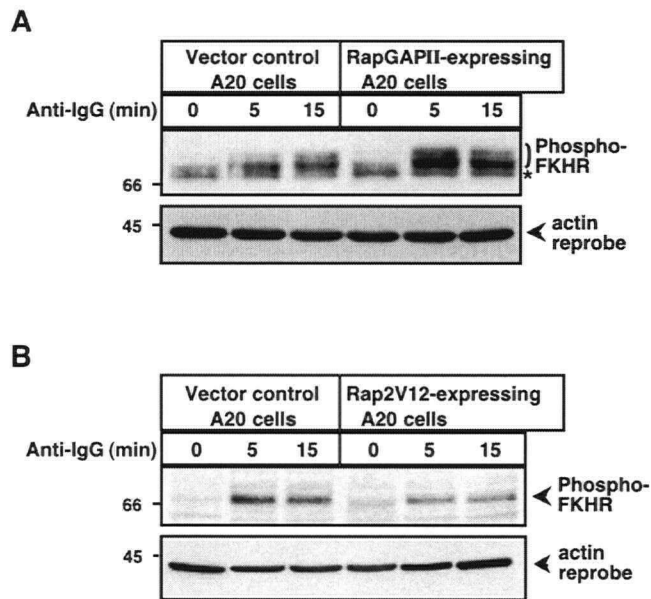


Figure. 4.12 Rap activation modulates BCR-induced phosphorylation of FKHR. *A*, Vector control and Rap2V12-expressing A20 cells were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse Ig antibodies for the indicated times. *B*, Vector control and RapGAPII-expressing A20 cells were stimulated with 40 $\mu\text{g/ml}$ goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize FKHR that is phosphorylated on serine 256. Equal loading was assessed by reprobing the membranes with antibodies to actin. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments. A non-specific band in *A* is indicated by an asterisk (*).

dependent transcription (91). Consistent with the finding that inhibition of endogenous Rap1 and Rap2 enhanced BCR-induced activation of Akt, I found that preventing the activation of endogenous Rap by expressing RapGAPII enhanced BCR-induced phosphorylation of FKHR on serine 256 by approximately 2-fold (Fig. 4.12A). This indicates that activation of endogenous Rap limits the ability of the BCR to induce FKHR phosphorylation, an Akt-dependent pro-survival pathway. Supporting the idea that Rap-GTP negatively regulates Akt-dependent phosphorylation of FKHR, I found that expressing the constitutively active Rap2V12 in A20 cells reduced BCR-induced phosphorylation of FKHR by approximately 60% (Fig. 4.12B).

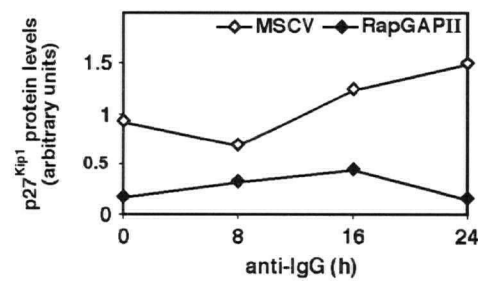
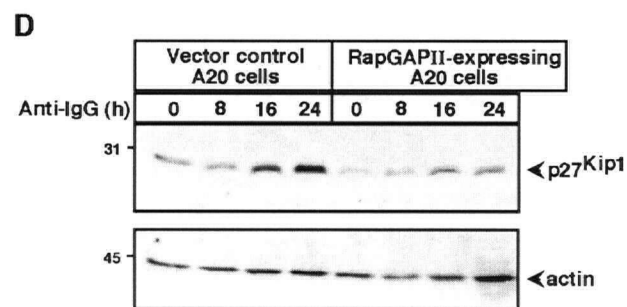
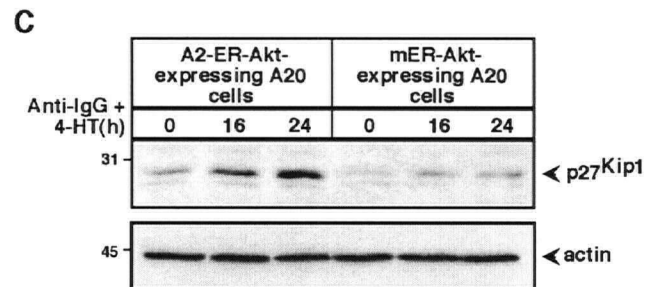
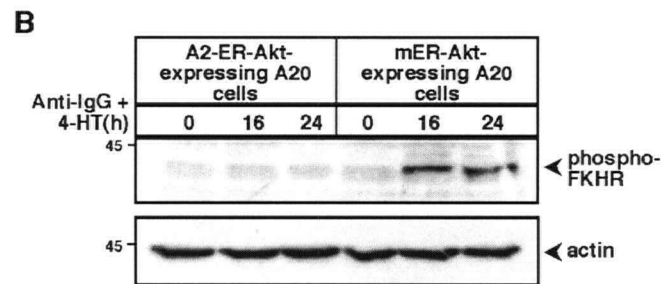
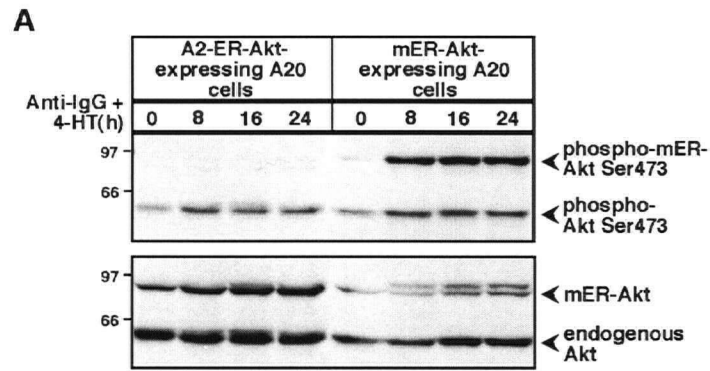
4.11 Endogenous Rap modulates expression of the FKHR target p27^{Kip1}

Since FKHR regulates the expression of proteins that promote cell cycle arrest and apoptosis, I wished to determine if modulation of the Akt/FKHR signalling module by activated Rap altered the expression of these proteins. In particular I focused on expression of the p27^{Kip1} cell cycle inhibitor, an FKHR target that can promote apoptosis when ectopically expressed (294-298). p27^{Kip1} plays a key role in BCR-induced growth arrest and apoptosis (295,298,299) in B cell lines such as WEHI-231 and CH31 which correspond to immature/transitional B cells that undergo clonal deletion in response to antigen engagement. Prolonged BCR signalling in these immature B cell lines leads to increases in the expression of p27^{Kip1} (298,299). Moreover, ectopic expression of p27^{Kip1} can induce apoptosis in these immature B cell lines (295,298) while antisense-induced decreases in p27^{Kip1} levels prevent apoptosis (295). This suggests that p27^{Kip1} expression is a key determinant of survival versus apoptosis in B cells.

Consistent with the idea that Akt is an important pro-survival factor for B cells, expression of constitutively active Akt in immature B cell lines delays BCR-induced upregulation of p27^{Kip1} and apoptosis (298). Suppressing the expression of p27^{Kip1} may therefore be an important mechanism by which Akt promotes B cell survival. My finding that activation of endogenous Rap by the BCR limits the activation of Akt, as well as the ability of Akt to phosphorylate FKHR on negative regulatory sites, suggested that Rap activation would enhance the expression of p27^{Kip1}. If this were the case, then preventing BCR-induced activation of endogenous Rap via the expression of RapGAPII should inhibit the expression of p27^{Kip1} by increasing BCR-induced Akt activation (as shown in Fig. 4.4) and increasing the phosphorylation of FKHR on negative regulatory sites (as shown in Fig. 4.12A) such that FKHR would have a decreased ability to promote the transcription of p27^{Kip1} (see pathway model in Fig. 4.14A)

To test this hypothesis, I first developed an approach to show that enhancing Akt activation leads to increased BCR-induced phosphorylation of FKHR and decreased expression of p27^{Kip1}. I expressed in A20 cells a conditionally active form of Akt (mER-Akt) that consists of the kinase domain of Akt fused to a mutant form of the estrogen receptor that is responsive to the estrogen analog 4-HT. Although the mER-Akt protein lacks the PH domain of Akt, it contains a myristoylation sequence at the N-terminus that localizes it to the inner leaflet of the plasma membrane. In response to 4-HT, the mER-Akt estrogen receptor undergoes a conformational change that exposes the Akt activation sites, allowing it to be phosphorylated and activated by PDK1 and PDK2 (205). As a negative control for any effects of 4-HT, I also expressed a variant of the mER-Akt protein called A2-ER-Akt that has an alanine to glycine mutation at position 2 and therefore cannot be myristoylated. This A2-ER-Akt fusion protein is restricted to the cytosol and cannot be activated by PDK1 and

Figure. 4.13 Both Akt activation and inhibition of endogenous Rap oppose BCR-induced accumulation of p27^{Kip1}. *A-C*, A20 cells expressing either A2-ER-Akt or mER-Akt were stimulated with 10 µg/ml goat anti-mouse IgG antibodies plus 2 µM 4-HT for the indicated times. *A*, Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt phosphorylated on serine 473. Equal loading was analyzed by re-probing the membranes with anti-Akt antibodies. *B*, Cell extracts were analyzed by immunoblotting with antibodies that recognize FKHR that is phosphorylated on serine 256. Equal loading was analyzed by re-probing the membranes with a monoclonal antibody to actin. *C*, Cell extracts were analyzed by immunoblotting with antibodies against p27^{Kip1}. Equal loading was analyzed by re-probing the membranes for actin. *D*, Vector control and RapGAPII-expressing A20 cells were stimulated with 10 µg/ml goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies against p27^{Kip1}. As a loading control, the membrane was re-probed with antibodies to actin. To calculate the relative levels of p27^{Kip1}, the intensity of each band in the p27^{Kip1} blot (upper panel) was determined by densitometry and divided by the intensity of the corresponding actin band in the lower panel. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in two to four independent experiments.



PDK2 (205).

Figure 4.13A shows that 4-HT stimulation of the mER-Akt protein could enhance Akt activation over a 24 h time course. Treating the control A2-ER-Akt-expressing A20 cells with the combination of anti-IgG plus 4-HT for 8-24 h resulted in sustained phosphorylation of only the endogenous Akt protein since the A2-ER-Akt protein cannot be activated by PDK1 and PDK2. In contrast, treating mER-Akt-expressing A20 cells with the combination of anti-IgG plus 4-HT induced sustained phosphorylation of the 90-kDa mER-Akt fusion protein and also enhanced the phosphorylation of the endogenous 60-kDa Akt protein compared to A2-ER-Akt-expressing cells (Fig. 4.13A). The phosphorylation of the endogenous Akt protein was about 30 % higher in the mER-Akt-expressing cells than in the A2-ER-Akt-expressing cells. The enhanced phosphorylation of endogenous Akt may be due to an interaction between the mER-Akt protein and the endogenous Akt protein. It has been shown that Akt can dimerize via a region adjacent to the PH domain and this association enhances the activity of Akt *in vitro* (300) potentially via autophosphorylation. This is significant since the endogenous Akt protein, unlike the mER-Akt fusion protein, is not restricted to the plasma membrane and can phosphorylate nuclear substrates such as FKHR.

I then examined the consequence of enhanced Akt activation on FKHR phosphorylation in A20 cells. Although BCR signalling induces FKHR phosphorylation on the 5 to 15 min time scale (Fig. 4.12), in A2-ER-Akt-expressing cells stimulated with anti-IgG plus 4-HT, signalling via the BCR alone caused very little sustained phosphorylation of FKHR at 8 to 24 h (Fig. 4.13B). In contrast, in mER-Akt-expressing cells stimulated with anti-IgG plus 4-HT, the increased activation of Akt resulted in significant and sustained phosphorylation of FKHR at 16 h and 24 h (Fig. 4.13B). I then examined the effect of enhanced Akt activation on the levels of the FKHR target p27^{Kip1}. BCR signalling alone in

the A2-ER-Akt-expressing cells resulted in a significant increase in p27^{Kip1} protein levels at 16 h and 24 h (Fig. 4.13C). In contrast, the enhanced Akt activation in the mER-Akt-expressing cells treated with anti-IgG plus 4-HT strongly suppressed the accumulation of p27^{Kip1} (Fig. 4.13C). This is consistent with previous findings showing that ectopic expression of constitutively active Akt suppresses BCR-induced accumulation of p27^{Kip1} in WEHI-231 cells (298).

Since inhibition of endogenous Rap via expression of RapGAPII increases the ability of the BCR to activate Akt and phosphorylate FKHR, I predicted that preventing Rap activation in this way would suppress the BCR-induced accumulation of p27^{Kip1}, similar to what I observed when I increased Akt activity using the mER-Akt fusion protein. Indeed, p27^{Kip1} levels were lower, and the BCR-induced accumulation of p27^{Kip1} was smaller, in RapGAPII-expressing A20 cells than in the vector control cells (Fig. 4.13D). These findings indicate that activation of endogenous Rap limits Akt activation and in doing so prevents Akt from suppressing the expression of p27^{Kip1}, a potent inducer of cell cycle arrest and apoptosis in B cells.

4.12 Activation of endogenous Rap modulates anti-Ig induced cell death

I have shown that preventing the activation of endogenous Rap, via RapGAPII expression, enhances the ability of the BCR to activate Akt. Since Akt is a potent pro-survival factor for B cells (63), I predicted that the enhanced Akt activation in RapGAPII-expressing cells would make the cells less sensitive to apoptotic stimuli. To test this hypothesis, anti-Ig-induced cell death was compared in RapGAPII-expressing or vector control WEHI-231 cells by S.J. McLeod and K.B.L. Lin in the lab and these data have been included in this thesis for the purpose of discussion. WEHI-231 cells resemble

immature/transitional B cells and undergo growth arrest followed by apoptosis upon prolonged treatment with anti-IgM antibodies (299). Moreover, expressing a constitutively active form of Akt in WEHI-231 cells has been shown to reduce BCR-induced cell death (298). Consistent with the enhanced BCR-induced Akt activation in RapGAPII-expressing WEHI-231 cells (see Fig. 4.4), RapGAPII-expressing WEHI-231 cells underwent less anti-IgM-induced cell death than the vector control cells (Table 4.1). At both the 48 h and 72 h time points, anti-IgM induced cell death was consistently 15% lower in the RapGAPII-expressing WEHI-231 cells than in the vector control cells. The finding that preventing Rap activation via RapGAPII expression reduces anti-Ig-induced cell death suggests that activation of endogenous Rap by the BCR favors anti-Ig-induced cell death, presumably by limiting the activation of Akt and Akt-regulated survival pathways such as the downregulation of the FKHR/p27^{Kip1} module.

Table 4.1. Preventing Rap activation via expression of RapGAPII reduces anti-IgM-induced cell death in WEHI-231 cells

Vector control and RapGAPII-expressing WEHI-231 cells were cultured with 10 µg/ml goat anti-mouse IgM antibodies for 48 h or 72 h. The cells were stained with 7-AAD, a probe for membrane integrity, and the percent of cells that were dead was determined by flow cytometry. For cells cultured in the absence of anti-IgM, cell death at 48 h and 72 h was less than 15%. P values were calculated using Student's T test.

% dead cells					
48 h			72 h		
Expt. I	MSCV:	70.2%	Expt I	MSCV:	87.2%
	RapGAPII:	55.0%		RapGAPII:	74.0%
Expt. II	MSCV:	70.0%	Expt IV	MSCV:	80.8%
	RapGAPII:	53.1%		RapGAPII:	65.4%
Expt. III	MSCV:	66.2%	Expt V	MSCV:	73.2%
	RapGAPII:	53.4%		RapGAPII:	58.8%
Difference in % dead cells (Mean \pm SEM)					
MSCV - RapGAPII: 15.0 \pm 2.1% (n=3)			MSCV - RapGAPII: 14.4 \pm 1.1% (n=3)		
P < 0.01			P < 0.005		

4.13 Discussion

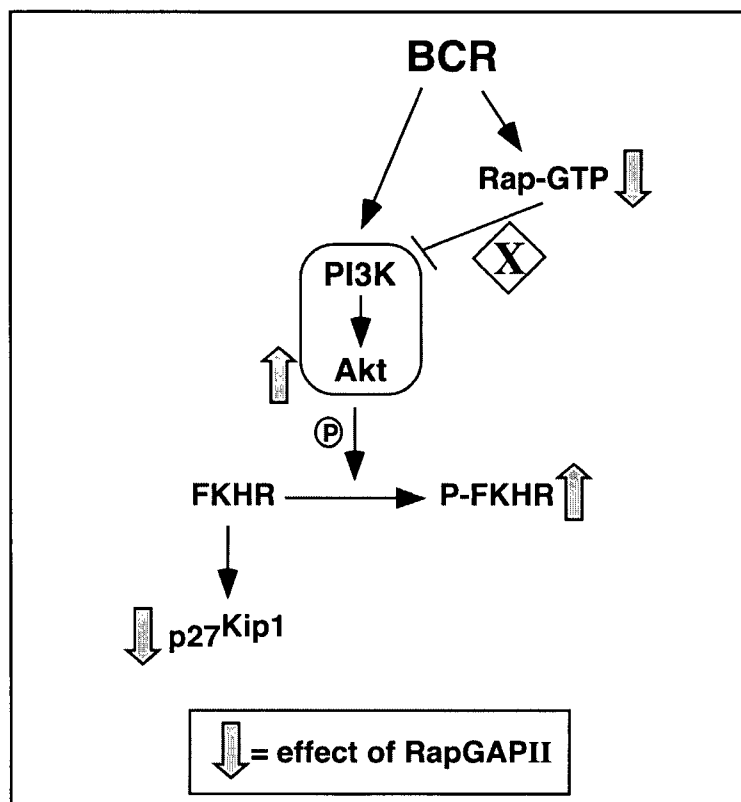
I have shown that activation of the Rap GTPases during BCR signalling selectively limits the ability of the BCR to activate the PI3K/Akt pathway while having no effect on other BCR-induced signalling events including phosphorylation of the ERK1/2, JNK, and p38 MAPKs. I found that preventing the activation of endogenous Rap by the BCR enhanced BCR-induced phosphorylation of Akt and potentiated Akt-dependent effects on the phosphorylation of the FKHR transcription factor and the expression of the FKHR target p27^{Kip1}. These findings indicate that BCR-induced Rap activation normally limits the ability of the BCR to activate Akt, inhibit FKHR, and suppress the expression of p27^{Kip1}, a cell cycle inhibitor that can promote apoptosis (Fig. 4.14A). Thus Rap activation functions as a negative regulator of a key Akt-regulated pro-survival pathway. Consistent with this idea, we found that blocking Rap activation enhanced the ability of WEHI-231 cells to survive prolonged treatment with anti-IgM antibodies. Finally, my finding that the constitutively active Rap2V12 can associate with PI3K and inhibit its activity in a manner that depends upon BCR engagement provides a potential mechanism by which Rap-GTP limits the activation of the PI3K/Akt pathway by the BCR.

The Rap GTPases were originally described as negative regulators of Ras signalling. *In vitro*, the active GTP-bound forms of Rap1 and Rap2 can bind Ras effectors such as Raf-1 and RalGDS (183-187) but apparently does not activate them. This suggested that activated Rap might limit Ras signalling by sequestering Ras effectors in inactive complexes. In support of this idea, expressing constitutively active forms of Rap1 or Rap2 can inhibit Ras-dependent ERK1/2 activation in a variety of cell types (185,186). Moreover, inhibiting the activation of endogenous Rap by expressing RapGAPII limits Ras-dependent ERK1/2 activation

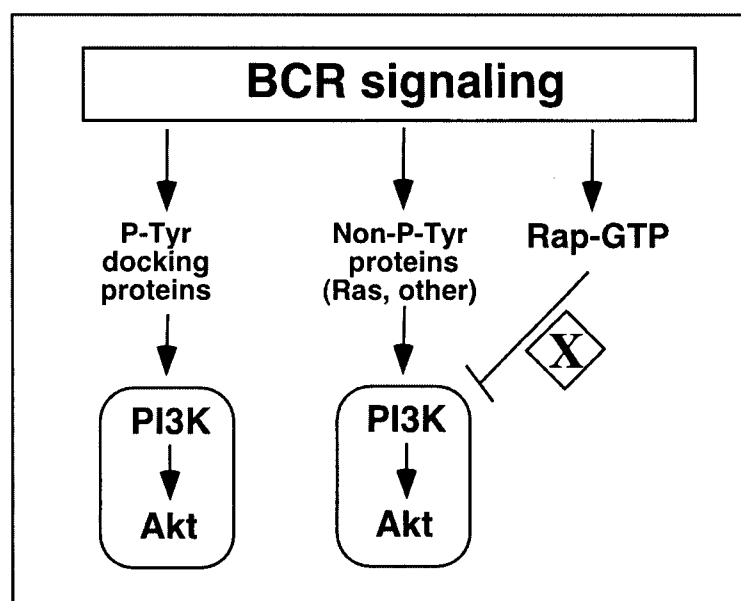
Figure. 4.14 Model depicting the effects of Rap activation and RapGAPII expression on the PI3K/Akt pathway. *A*, BCR-induced activation of endogenous Rap proteins limits the activation of the PI3K/Akt pathway by the BCR. In conjunction with other BCR signalling events (X), activated GTP-bound Rap can inhibit PI3K-dependent activation of Akt, apparently by binding to PI3K. Inhibition of Akt activation by activated Rap reduces BCR-induced phosphorylation of FKHR, resulting in increased levels of the FKHR target p27^{Kip1}. Thus, Rap activation limits the Akt-mediated downregulation of the FKHR/p27^{Kip1} pro-apoptotic module. The thick gray arrows represent the effect of preventing Rap activation via the expression of RapGAPII. Under these conditions, the negative effects of Rap-GTP on the PI3K/Akt pathway do not occur. The net result is that Akt activation is enhanced, FKHR phosphorylation is enhanced, and expression of the p27^{Kip1} cell cycle inhibitor is suppressed.

B, Model for regulation of the PI3K/Akt pathway by the Rap GTPases. The BCR may recruit PI3K to the plasma membrane via two distinct mechanisms. One involves the binding of the PI3K SH2 domains to tyrosine-phosphorylated (P-Tyr) docking/adaptor proteins such as CD19, BCAP, and Gab1. The other involves a phosphotyrosine-independent mobilization of PI3K that may involve the binding of PI3K to activated Ras or other proteins at the plasma membrane that are not tyrosine-phosphorylated. Activated Rap-GTP binds only to this latter pool of PI3K molecules. The ability of Rap-GTP to inhibit the enzymatic activity of this non-phosphotyrosine-associated pool of PI3K molecules appears to depend on other BCR signalling events, for example a phosphorylation event that might allow Rap-GTP to recruit an inhibitor of PI3K (X).

A



B



in 293T cells (203) and in a CD45R0⁺ T cell line (301). However other studies have found that ERK1/2 activation is not affected by activated Rap. ERK1/2 activation is not inhibited when constitutively active Rap1 is expressed in the T cells of transgenic mice (302) or when endogenous Rap1 and Rap2 are selectively activated in NIH 3T3 cells (191). One possible explanation for these divergent results is that the ability of Rap-GTP to effectively compete with Ras for binding to Raf-1 is cell type-specific. In B cells, we found that Rap-GTP did not act as a negative regulator of Ras-dependent ERK1/2 activation. Preventing the activation of endogenous Rap1 and Rap2 did not augment BCR-induced ERK1/2 activation, indicating that endogenous Rap does not limit the ability of the BCR to activate ERK1/2. Moreover, expressing the constitutively active Rap2V12 protein did not inhibit BCR-induced ERK1/2 activation. Thus Rap-GTP is unable to interfere with Ras-dependent ERK1/2 activation in B cells. This may reflect the fact that the amount of Raf-1 is not limiting in B cells or that Ras and Raf-1 are coupled very tightly by scaffolding proteins, such that Rap-GTP cannot effectively interfere with their interaction.

In some cell types, particularly neuronal cells, Rap-GTP acts a positive regulator of ERK1/2 by activating B-Raf (192,193). B-Raf is an upstream activator of the ERK1/2 pathway that functions similarly to Raf-1. Recent work by Brummer *et al.* showed that BCR-induced ERK1/2 activation involves both B-Raf and Raf-1, with B-Raf making the major contribution (194). This suggested that Rap-GTP could be a positive regulator of ERK1/2 activation in B cells. However, we found that blocking Rap activation via the expression of RapGAPII did not reduce the ability of the BCR to activate ERK1/2. This indicates that Rap-GTP does not contribute to ERK1/2 activation in B cells by activating the B-Raf/ERK1/2 pathway. Consistent with this idea, Brummer *et al.* (194) argue that B-Raf-dependent ERK1/2 activation in B cells is controlled by Ras as opposed to Rap. It is not clear why Rap-

GTP activates the B-Raf/ERK1/2 signalling module in neuronal cells such as the PC-12 cell line but not in B cells. There are multiple alternatively spliced isoforms of B-Raf, so it is possible that there are neural-specific B-Raf isoforms that are regulated by Rap-GTP whereas the B cell-specific isoforms are regulated by Ras. An alternate possibility is that an intermediary protein or scaffolding protein that couples Rap to B-Raf is expressed in neurons but not in B cells.

Although Rap-GTP did not inhibit the activation of the Ras/ERK1/2 pathway in B cells, it was possible that it selectively inhibited the activation of other Ras effectors. PI3K is a downstream effector of Ras in many cell types (197,278,279), including B cells (198) where it plays a key role in promoting B cell survival by activating Akt (63). Using a combination of loss-of-function and gain-of-function approaches, I found that Rap-GTP is a negative regulator of the PI3K/Akt pathway in B cells. Preventing Rap activation by expressing RapGAPII enhanced the ability of the BCR to stimulate Akt phosphorylation. This indicates that activation of endogenous Rap limits the ability of the BCR to activate Akt. This may be important since Akt regulates multiple pro-survival pathways and the excessive activation of these pathways could lead to oncogenic transformation. Consistent with the idea that Rap-GTP inhibits the activation of the PI3K/Akt pathway, expressing the constitutively active Rap2V12 in B cell lines reduced the ability of the BCR to induce Akt phosphorylation. Interestingly, our lab has not been able to express the constitutively active Rap1V12 protein in B cell lines, perhaps because it is a stronger inhibitor of Akt activation than Rap2V12 and reduces Akt activity to the point where the cells are unable to survive.

The ability of Rap-GTP to regulate Akt phosphorylation and activation has been reported previously in the context of cAMP-mediated cell signalling. Depending on the cell type, cAMP can either activate or inhibit Rap activation and Rap-GTP can act as either a

positive or a negative regulator of Akt activation. In 293 cells, cAMP analogues activate the Rap-specific nucleotide exchange factor EPAC, resulting in Rap activation and Rap-dependent Akt phosphorylation (303). Rap-GTP also appears to be a positive regulator of Akt in the C6 rat glioma cell line where cAMP inhibits Rap activation and this leads to decreased Akt phosphorylation (304). In contrast, Rap-GTP acts as a negative regulator of Akt in the PCCL3 thyroid cell line. In these cells, cAMP-induced activation and phosphorylation of Rap1b results in inhibition of Akt phosphorylation (305). The mechanisms by which Rap regulates the PI3K/Akt pathway in these systems are not known.

My findings suggest that Rap-GTP inhibits BCR-induced Akt phosphorylation by binding to PI3K. The constitutively active Rap2V12 associated with the p85 subunit of PI3K in A20 B lymphoma cells but did not associate with PDK1 or Akt. This indicates that Rap-GTP most likely inhibits the PI3K/Akt pathway in B cells by acting at the level of PI3K. This is the first report to demonstrate the association of Rap2 with PI3K. It had been proposed previously that Rap1 can bind PI3K (51,306) since Rap1 and Ras have identical effector-binding regions and Rap1 can bind most other Ras effectors. My preliminary data indicates that both Rap1V12 and Rap2V12 can bind endogenous PI3K when expressed in HEK293 cells. Thus, it is likely that both Rap1-GTP and Rap2-GTP can associate with PI3K in B cells and inhibit its ability to activate Akt. I have not been able to test this directly since I am unable to express the constitutively active Rap1-V12 in B cell lines as discussed above.

How the binding of Rap2-GTP to PI3K impairs the ability of PI3K to signal to Akt remains to be elucidated. I found that Rap2V12 associated with PI3K in both unstimulated and anti-Ig-stimulated A20 B lymphoma cells, as judged by immunoblotting with antibodies against the p85 subunit of PI3K. However, PI3K enzyme assays revealed that the amount of PI3K enzyme activity associated with these Rap2V12/PI3K complexes was significantly

lower when the complexes were isolated from anti-Ig-treated cells than when the complexes were isolated from unstimulated cells. Thus, Rap2V12 appears to inhibit the activity of PI3K in a manner that depends upon BCR engagement.

Although the nature of this BCR signalling contribution to Rap-mediated inhibition of PI3K is not known, a number of possibilities can be envisioned. One possibility is that Rap-GTP recruits an inhibitor of PI3K but can only do so in BCR-stimulated cells. The interaction between Rap-GTP and this putative inhibitor may depend on BCR signalling events, for example the phosphorylation of Rap-GTP or the putative inhibitor. The adaptor protein RUK (also known as CIN85, SETA and SH3KBP1 (307)) is a recently described inhibitor of PI3K (308) and it is possible that Rap2V12 recruits RUK upon BCR engagement. The adaptor protein Cbl can also negatively regulate PI3K and binds to PI3K in B cells in response to BCR engagement (309,310). Cbl targets proteins for ubiquitination in its role as an E3 ubiquitin ligase (311). In T cells, Cbl has been shown to target PI3K for ubiquitination (312) which inhibits plasma membrane recruitment of PI3K in a manner independent of PI3K degradation (313). Therefore, another possible mechanism for the Rap2V12-mediated inhibition of PI3K is through the recruitment of Cbl by Rap2V12.

Conversely, as opposed to recruiting an inhibitor of PI3K, the binding of Rap-GTP to PI3K in anti-Ig stimulated cells could cause an activator to dissociate from PI3K. Another possibility is that Rap-GTP recruits a lipid phosphatase such as PTEN that dephosphorylates the lipid second messengers produced by PI3K. The co-localization of PTEN with PI3K would prevent the accumulation of PIP_3 and $PI(3,4)P_2$ *in vivo* as well as the accumulation of phosphatidylinositol 3-phosphate in my *in vitro* PI3K assays in which phosphatidylinositol is used as the substrate. However, I was unable to detect an interaction between PTEN and Rap2V12 by immunoblotting.

Another possibility that should be considered is that Rap2V12, in conjunction with a BCR signalling event, induces the dissociation of the p110 catalytic subunit of PI3K from the p85 regulatory subunit. I have shown that Rap2V12 associates constitutively with the p85 subunit but the observed decrease in enzyme activity may be due a decrease in associated p110 subunit when these Rap2V12/p85 complexes are isolated from anti-Ig-stimulated cells than from unstimulated cells. Dissociation of the p110 subunit from the p85 subunit would also destabilize the p110 subunit as p85 functions to protect p110 from proteolytic degradation (314). I have been unable to test this hypothesis since the available antibodies against the p110 domain are not of sufficient sensitivity to detect the presence of specific p110 isoforms in the Rap2V12 complexes. Further work is required to test these various models for how Rap-GTP, in the context of BCR signalling, inhibits PI3K activity.

The initiation of PI3K signalling requires the recruitment of PI3K to the plasma membrane. Since PI3K is a central regulator of B cells survival and activation, it is not surprising that the BCR uses multiple mechanisms to recruit PI3K to the plasma membrane. The membrane-associated proteins that recruit PI3K to the plasma membrane after BCR engagement include a variety of tyrosine-phosphorylated docking proteins such as CD19, BCAP, Gab1 and Gab2 (45,46,48). Ras-GTP may also recruit PI3K to the plasma membrane after BCR engagement (198) and there may also be other non-phosphotyrosine-dependent mechanisms for initiating PI3K signalling. Interestingly, I found that Rap2V12 expression did not inhibit the activity of PI3K that associated with tyrosine-phosphorylated proteins and that Rap2V12 was not present in the same complexes with these tyrosine-phosphorylated proteins. This suggests that Rap-GTP inhibits the activity of a subset of PI3K molecules that the BCR mobilizes in a phosphotyrosine-independent manner that may involve Ras-GTP or a yet undescribed mechanism for PI3K recruitment to the plasma membrane (Fig. 4.14B).

Since Ras and Rap have nearly identical effector binding regions, the simplest model is that Rap-GTP prevents the binding of PI3K to Ras-GTP. However in preliminary experiments I found that expressing either the constitutively active Rap1V12 or Rap2V12 in COS cells or HEK293 cells did not impair the ability of Ha-Ras-V12 to bind endogenous PI3K. This argues against a model in which activated Ras and Rap compete for Ras effectors. An alternative model is that Rap-GTP binds only to Ras-GTP/PI3K complexes and inhibits these Ras-associated PI3K molecules. There is a precedent for trimeric complexes containing Ras-GTP, Rap-GTP and a Ras effector. Hu *et al.* (315) have shown *in vitro* that activated Ras and Rap can bind simultaneously to different sites on Raf-1 and that in this circumstance Rap-GTP impairs the ability of the Ras-GTP to activate Raf-1. A final possibility is that Rap-GTP prevents a non-tyrosine-phosphorylated protein other than Ras from binding PI3K and initiating PI3K-dependent signalling.

An important question is whether Rap activation limits the activation of other PI3K targets besides Akt in B cells. The Rap-dependent inhibition of PI3K may only impair processes that require high levels of PI3K-derived lipids. I found that BCR-induced phosphorylation of Akt, which is substantially inhibited at low concentrations (5 μ M) of the PI3K inhibitor LY294002, was inhibited by Rap activation whereas BCR-induced phosphorylation of PKC- ϵ , which is only inhibited by higher concentrations (25 μ M) of LY294002, was not affected by expression of either RapGAPII or Rap2V12. The finding that Rap activation preferentially inhibits PI3K-dependent signalling events that are most sensitive to PI3K inhibitors is consistent with the idea that Rap activation selectively limits only those PI3K-dependent signalling events that require high levels of PI3K-derived lipids for their activation.

Since Rap-GTP appears to selectively inhibit the PI3K/Akt pathway in B cells, I asked whether Rap activation limits the ability of the BCR to activate Akt-dependent pro-survival signalling pathways. An important Akt-dependent survival pathway involves the phosphorylation of FKHR by Akt, a modification that prevents FKHR from promoting the transcription of pro-apoptotic genes such as p27^{Kip1}. Consistent with my finding that activation of endogenous Rap by the BCR limits BCR-induced activation of Akt, I found that Rap activation also opposes the Akt-dependent inhibition of the FKHR/p27^{Kip1} module (Fig. 4.14A). Preventing the activation of endogenous Rap by the BCR mimicked the effects of enhancing Akt activation by increasing BCR-induced phosphorylation of FKHR and suppressing the expression of p27^{Kip1}. Thus activation of the Rap GTPases by the BCR limits the ability of the BCR to activate Akt and also limits the ability of Akt to suppress a pro-apoptotic pathway that involves FKHR-dependent accumulation of p27^{Kip1}. Rap-dependent accumulation of p27^{Kip1} is not limited to B cells. Although they did not implicate Akt as an intermediate, Katagiri *et al.* showed that expressing constitutively active Rap1 in T cells results in the accumulation of p27^{Kip1}, independent of T-cell receptor activation (316).

The ability of Rap-GTP to negatively regulate the activation of Akt and Akt-dependent survival pathways in B cells may be an important factor that limits B cell survival and activation. The BCR activates both pro-survival and pro-apoptotic pathways (3) and Rap activation may tip the balance in favor of apoptosis in certain situations where it is appropriate, such as the deletion or silencing of self-reactive B cells. Consistent with this idea, Rap activation has been associated with T cell anergy and activation-induced cell death (316-318). Our experiments show that preventing BCR-induced Rap activation reduces the ability of anti-IgM antibodies to cause cell death in WEHI-231 cells, a model for antigen-induced clonal deletion. This suggests that Rap activation makes immature/transitional B

cells more sensitive to antigen-induced clonal anergy or clonal deletion. Similarly, Rap activation may favor the induction of anergy in mature B cells. Further *in vivo* experiments are required to support this idea that Rap activation affects the signalling threshold for the elimination or silencing of self-reactive B cells. In particular, it would be of interest to test whether preventing Rap activation *in vivo* in murine B cells favors the development of autoimmunity.

In summary, I have shown for the first time that activated Rap2 can bind to PI3K and that in the context of BCR signalling, Rap2-GTP can inhibit the activity of PI3K molecules that are not involved in phosphotyrosine-dependent interactions. The net result is that Rap activation may modulate a subset of PI3K-dependent signalling reactions that require high levels of PI3K-derived lipids for their activation. I have also shown that activation of endogenous Rap limits the ability of the BCR to activate the PI3K/Akt pathway, limits the subsequent Akt-mediated inhibition of the FKHR/p27^{Kip1} pro-apoptotic module, and modulates the sensitivity of an immature B cell line to anti-Ig-induced cell death.

Chapter 5

Identification of Akt-regulated genes in B cells

5.1 Introduction

Akt was first described as a proto-oncogene where overexpression of the unregulated active kinase resulted in uncontrolled cell growth (319-321). Many reports have demonstrated the pro-survival function of Akt (reviewed in (55) and have attributed this function to the ability of Akt to inhibit, by phosphorylation, the activity of many pro-apoptotic factors, including Bad (75,76), GSK-3 (92) and caspase-9 (322). Akt also promotes cell survival by regulating the activity of the NF- κ B transcription factor (86,87) and members of the forkhead family of transcription factors (90,91). Akt-mediated phosphorylation and activation of the IKK kinase, the kinase that activates NF- κ B by phosphorylating the inhibitor of NF- κ B (I κ B), results in increased NF- κ B-dependent transcription and increased expression of NF- κ B-regulated pro-survival genes. In contrast, phosphorylation of forkhead transcription factors by Akt results in inhibition of forkhead-mediated transcription and reduced expression of forkhead-regulated pro-apoptotic genes. Thus, Akt acts by multiple mechanisms to prevent cell death and promote cell survival. Akt has been shown to be essential for cell survival of the DT40 B cell line (63) but the precise mechanism underlying the pro-survival role of Akt in B cells is unclear. The identification of genes that are regulated by Akt in B cells could reveal how Akt influences cell survival and other processes in B cells.

5.2 Induction of Akt-dependent changes in gene expression via activation of a conditionally-active Akt fusion protein

To detect changes in gene expression that were specifically attributable to the activation of Akt I made use of a conditionally active form of Akt (mER-Akt) that I expressed in the WEHI-231 B cell line. The mER-Akt protein lacks the PH domain of Akt but contains a myristoylation sequence at the N-terminus to localize it to the inner leaflet of the plasma membrane. This altered form of Akt is fused to a mutant form of the estrogen receptor that is responsive to the estrogen analogue 4-HT. In response to 4-HT, the estrogen receptor portion of the mER-Akt protein undergoes a conformational change that exposes the Akt activation sites, allowing the mER-Akt protein to be phosphorylated and activated by PDK1 and PDK2 (205). To distinguish changes in gene expression due to mER-Akt activation from changes in gene expression caused by the potential action of 4-HT on other signalling pathways, I expressed in WEHI-231 cells an analogous Akt fusion protein, A2-ER-Akt, which cannot be activated. A2-ER-Akt is identical to the mER-Akt protein except that it lacks the myristoylation sequence. Thus, the A2-ER-Akt fusion protein is not targeted to the membrane and cannot be activated by PDK1/PDK2-mediated phosphorylation. The A2-ER-Akt protein therefore acts as a negative control and any changes in gene expression elicited by 4-HT in both mER-Akt and A2-ER-Akt cells would be discounted as not being Akt-dependent.

Figure 5.1 shows that treating mER-Akt-expressing WEHI-231 cells with 4-HT for 3 h or 20 h resulted in sustained phosphorylation of the mER-Akt protein on the activation site corresponding to Ser473 of Akt. In addition, 4-HT treatment of the mER-Akt-expressing WEHI-231 cells resulted in phosphorylation of endogenous Akt at early time points (15-30 min; see figure 3.9) but did not affect phosphorylation of endogenous Akt at the 3 h and 20 h

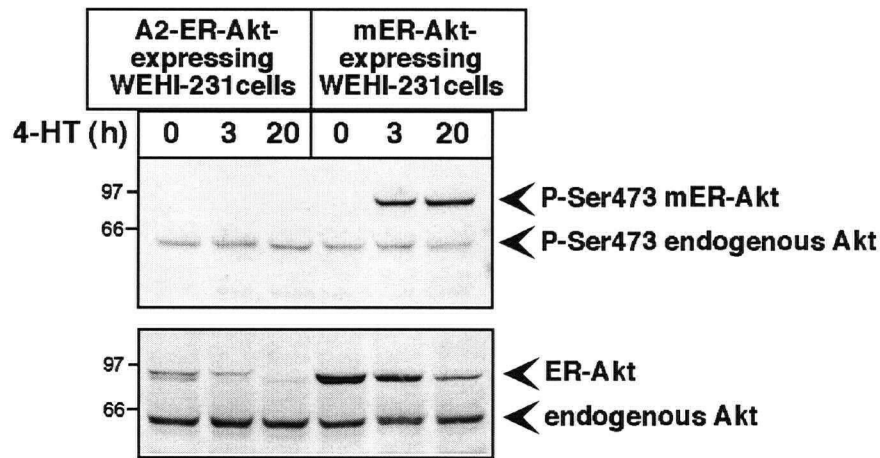


Figure 5.1 Treating WEHI-231 cells with 4-HT induces phosphorylation of the mER-Akt fusion protein but not the control A2-ER-Akt fusion protein. WEHI-231 cells expressing either the A2-ER-Akt or mER-Akt fusion proteins were stimulated with 2 μ M 4-HT for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt phosphorylated on serine 473. Equal loading was analyzed by re-probing the membranes with anti-Akt antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. Similar results were obtained in two independent experiments. Note that the disappearance of the ER-Akt protein upon 4-HT treatment was not a consistent finding.

time points shown in figure 5.1. The early 4-HT-induced phosphorylation of endogenous Akt may be due to interactions between the mER-Akt and endogenous Akt and is significant since the endogenous Akt is not constrained to the plasma membrane and can therefore phosphorylate cytoplasmic and nuclear proteins, including transcription factors. In contrast, 4-HT treatment of the A2-ER-Akt-expressing WEHI-231 cells did not result in phosphorylation of the A2-ER-Akt fusion protein or the endogenous Akt protein (Fig 5.1). Although the A2-ER-Akt protein was expressed at lower levels than the mER-Akt fusion protein, the A2-ER-Akt-expressing cells still serve as a control for the possible effects of Akt on other signalling pathways.

To detect changes in gene expression the mER-AKT- or A2-ER-Akt-expressing WEHI-231 cell lines were left untreated or treated with 4-HT for either 3 h or 20 h. These time points were chosen in order to attempt to identify genes that were regulated with short term activation of Akt and are more likely direct targets of Akt as well as genes that are regulated with long term Akt activation and may be indirect gene targets of Akt. Total RNA was then isolated from the cells and cDNA was reverse transcribed using an oligo-d(T) primer to target mRNA transcripts that possess 3'-poly A tails. This avoids the transcription of the more abundant ribosomal RNAs. The cDNA library that was used as the basis for the NIA (National Institute of Aging) 15K array set used in this thesis was also created by transcribing mRNA from an oligo-d(T) primer (<http://lgsun.grc.nia.nih.gov/cDNA/Library3.html#We>). Therefore, both the cDNA that I transcribed and the DNA on the array will contain the 3' ends of genes which is important since both will contain the same region of the genes represented on the array. The relative levels of gene expression were then determined via hybridization to a cDNA microarray (see Materials and Methods, Sections 2.4.1-2.4.2).

5.3 Discussion of the microarray technique

Microarray technology allows the simultaneous quantitation of the mRNA expression level of tens of thousands of genes. Thousands of different DNA sequences, each representing a unique gene, are spotted or printed on a “chip” or glass slide depending on the array printing technology that is employed. A set of probes, most often labeled with a fluorescent marker, is created from mRNA from the cell of interest. This set of labeled probes is then hybridized to the chip or glass slide and the relative intensity of the bound probe is quantified. The fluorescence intensity of the bound probe is indicative of the expression level of the gene in that cell at the point in time when the mRNA was isolated. Genes that are highly expressed and have many copies of the mRNA transcript from which the labeled cDNA was generated, will yield higher relative fluorescence intensities compared to genes that are expressed at lower levels.

The microarray technique is attractive because tens of thousands of genes can be examined simultaneously. It allows one to obtain a snapshot of the global condition of a cell at a certain point in time. Many different types of genes can be analyzed including genes that have not been characterized as well as genes that are thought to change or be expressed in the cell type of interest. The analysis of a large number of genes using microarrays avoids the bias inherent in other methods for analyzing gene expression (RT-PCR, Northern blotting) that focus on small numbers of genes that the researcher has hypothesized to be relevant. One of the most exciting uses of microarrays has been in disease diagnosis. For example, the predicted outcome of disease (i.e. metastasis and survival) of patients with stage I and stage II breast cancer can be vastly improved using microarray technology (323) and subsequent treatment can be adjusted to reflect the diagnosis. Microarray profiling has also been used to

differentiate diffuse large B-cell lymphoma into two forms with distinct gene array profiles that originate from different precursor B cell populations and have different prognoses (324).

In addition to obtaining a global view of the transcriptional state of the cell, microarray analysis can be a useful tool to identify genes that are differentially regulated in response to a treatment. Since so many genes can be examined simultaneously there is an increased chance of discovering novel gene targets including genes that have not yet been assigned a function. Cluster analysis of genes can identify genes that are regulated in a similar pattern. These genes may form a functional module of genes that work together to regulate a complex process (e.g. cell division or protein synthesis).

The complete set of genes on the slide or chip is called a genome. Each genome will contain different genes depending on the source cell type or organism and the method used to generate the genome. For example, in oligonucleotide-based arrays (e.g. Affymetrix array technology, Affymetrix Inc., Santa Clara, CA), 25- to 70-base oligonucleotides are synthesized, each specific for one of the thousands of genes of interest. These oligonucleotides are attached to the chip using one of several different technologies and then hybridized with the labeled cDNA probe. In PCR-based arrays, PCR amplicons are amplified from cDNA libraries of interest. The PCR amplicons are then attached to the chip and hybridized with the labeled cDNA probe. The cDNA libraries from which the PCR amplicons are generated can be designed to reflect all the genes expressed in an organism or the genes that are actively transcribed in a particular cell type (e.g. the "lymphochip" used for profiling B cell tumours (324)). The NIA 15K genome used in this thesis was created by PCR amplification of a random cDNA library generated from mouse embryonic cells (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>). This genome was selected because it was the most extensively used and least problematic array set available from the Gene Array Centre

at UBC representing a large number of genes. This genome presumably includes many genes expressed in B cells but some B cell-specific genes may not be represented in this gene set. In addition, many of the genes contained within the NIA 15K genome do not have an assigned function.

5.4 Normalization methods used for microarray analysis

Although obtaining data representing the expression levels of tens of thousands of genes can be very useful, interpretation of the data requires that a number of factors be taken into account. As with every experimental procedure variability can be introduced that is not due to the biological treatment. In the microarray technique variability can be introduced due to differences in RNA purification efficiency, reverse transcription efficiency, labeling efficiency of the cDNA, hybridization efficiency to the slide, all of which can be affected by the experience of the person performing the experiment. In addition, the different samples that are being compared to each other are often labeled with different fluorescent dyes in order to directly compare the hybridization of the samples to the same slide, thereby minimizing any variation due to differences between slides. When this is done, any differences in efficiency of the cDNA labeling must be taken into account and intrinsic differences in the fluorescence intensities of the two dyes must be factored in (325).

Various mathematical transformations are applied to data obtained from microarray analysis in order to correct for these potential sources of non-biological variability (325). The ideal normalization method would be to normalize to an internal control that is affected by experimental variability to the same degree and in the same manner as the experimental data but that is consistent between experimental conditions. One approach that is often used is to normalize to the expression level of a gene that is thought to be unaffected by the

experimental condition. This category of genes is often referred to as housekeeping genes. However, the expression of housekeeping genes is often variable (212,326) and the assumption that the expression of these genes does not change may not be correct for the specific cell type and comparison being examined. In addition, normalizing to one or two “housekeeping” genes may not be reliable when performing the analysis with the high number of variables present in the microarray.

Given the potential shortcomings of normalizing data to “housekeeping” genes, mathematical methods have been developed to normalize microarray data based on invariant features intrinsic to microarray data (327). For example, the invariant set normalization method ranks all fluorescence values according to intensity. Genes with similar ranks between conditions are then identified as unchanged and these genes are used to normalize the rest of the genes against (328-330). Another method involves fitting the data to a linear regression model and then scaling the fluorescence intensities of each array set so that the mean intensity of each array is identical (331). The normalization method chosen is important since the number of genes detected as differentially expressed can vary as much as 3-fold depending on the normalization method used (327). Unfortunately, there is no standard normalization technique that has proven to be superior and it is therefore necessary to choose a normalization technique with the appropriate balance of sensitivity to detect small differences in expression levels and specificity to detect changes that have a high degree of certainty for the particular experiment in question. The appropriate balance of sensitivity and specificity usually has to be determined by trial-and-error. Higher sensitivity will result in a larger number of genes detected as having a change in expression but will generate more false positives. Higher specificity will result in a smaller number of genes

detected as having a change in expression but with fewer false positives. However, this will increase the frequency of false negatives, true gene targets that are missed.

For this analysis, I chose to use a normalization technique with greater specificity but less sensitivity since I was interested in identifying genes that are the most likely to be regulated by Akt activation. This will decrease the amount of false positives identified but I may not identify genes with small but real changes or genes with higher variability of expression. To increase the likelihood of identifying genes that are true targets of Akt, I used more than one normalization technique to analyze the array data. Comparing the genes obtained by more than one technique decreases the possibility that a gene is identified by chance and increases the specificity of the analysis.

All of the data analysis was performed using GeneSpring Software (Silicon Genetics, Redwood City, CA). First, a background correction was applied where the mean intensity from the lowest 10% of the spots on the array was subtracted from the intensities of the rest of the spots. The array was divided into 60 separate "subgrids" based on the position of the spotted DNA and this background correction was performed independently for each subgrid. The underlying assumption is that the lowest 10% of hybridization intensities represent genes that are not expressed or expressed at very low levels and this was observed at the Gene Array Center at UBC. Then, any values in the array set that were below 0 were set to 0.01 since negative numbers adversely affect further calculations. To normalize the data, each value was then divided by the value representing the 50.0th percentile of all the values for that sample. This normalization scales the intensities of each array set so that the mean intensity of each array is identical and will be referred to as the scaled normalization. At this point two different ways of determining the relative expression level of each gene was used

(Figure 5.2). The first method applied one more level of normalization while the second method analyzed the data directly.

In the first method the measurements from the scaled normalization from each replicate for each gene in the treated samples were divided by the mean of that gene's measurements in the corresponding untreated control samples to give the normalized expression level. The mean normalized expression level of each gene was then determined from the replicate experiments. For these samples, genes with a mean normalized expression level below 0.5 or above 2 were identified as having a significant change in expression compared to the control samples. The mean fold change could then be determined by dividing the mean normalized expression level of the treated sample by the untreated control. The second method I used determined the mean of the normalized expression level directly from the scaled normalization. Then the fold change of each gene in the treated samples was calculated by comparing the mean normalized expression level of each gene from the treated samples to the mean normalized expression level the untreated control samples. Genes with a fold change of 2-fold greater or 2-fold less than the untreated sample were identified as having a significant change in expression. These normalization methods were recommended by the technicians at Silicon Genetics and were part of the software program available to me. In addition, these two methods resulted in different gene lists because the mean of the relative fold change was determined at different points in the analysis. In the first method, the mean of the normalized expression level was determined and then fold change was determined while in the second method the normalized expression level was determined independently for each replicate and then the mean of the fold change was determined. As discussed below, each normalization method identified different but overlapping sets of genes as being regulated by mER-Akt activation. Genes that were

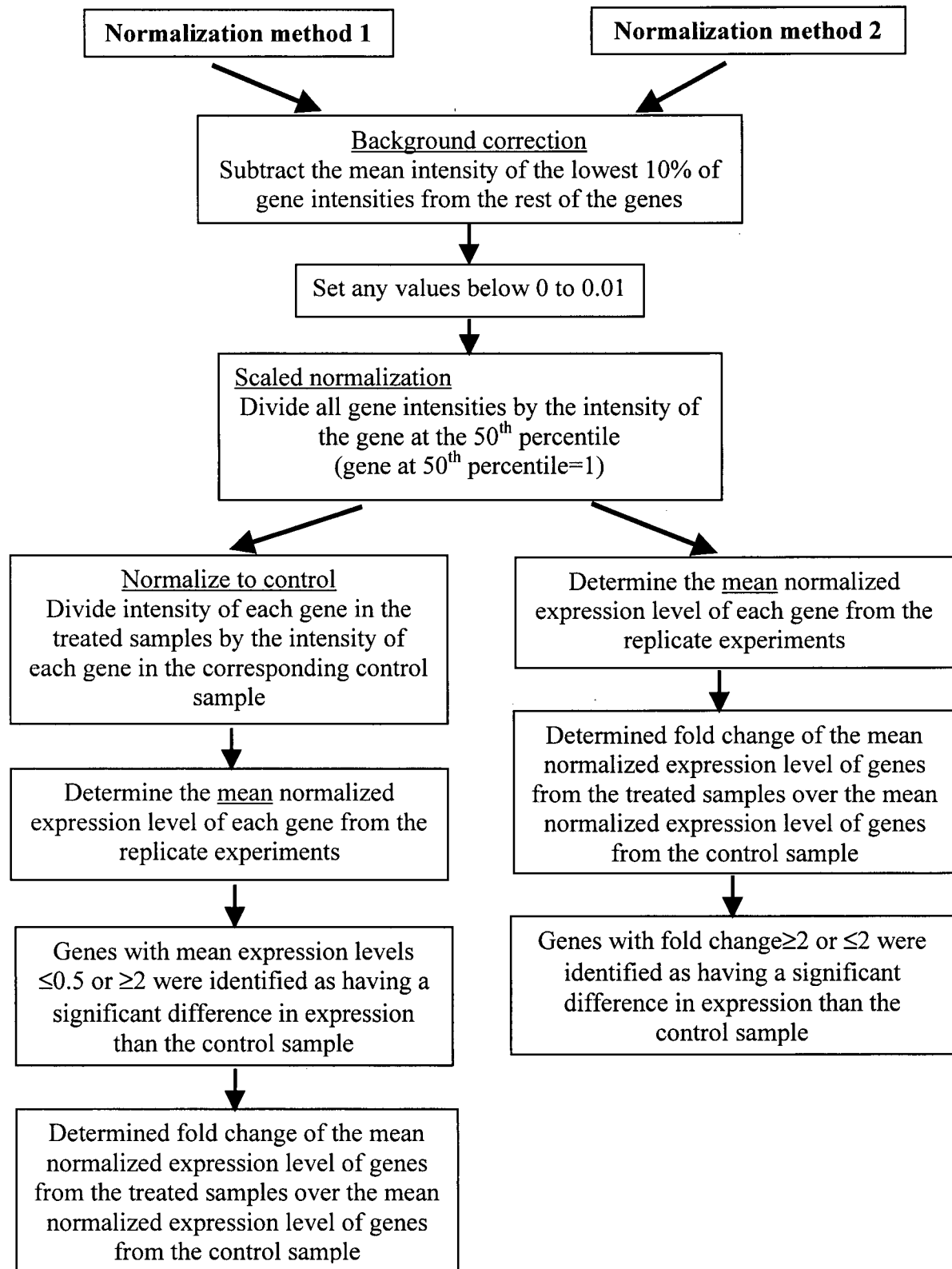


Figure 5.2 Flowchart of the two different normalization methods used for microarray analysis in this thesis.

identified as having a 2-fold change in both lists are more likely to be true gene targets of Akt since they were identified by two different methods.

5.5 Identification of Akt-regulated genes in WEHI-231 cells

Genes whose expression showed a significant increase or decrease after 4-HT treatment of the mER-Akt-expressing cells and the A2-ER-Akt-expressing cells were identified as described in Section 5.4. In both the mER-Akt- and A2-ER-Akt-expressing WEHI-231 cells, treating cells with 4-HT for 3 h or 20 h resulted in changes in gene expression (Table 5.1). Since the A2-ER-Akt protein is not activated by 4-HT, this indicates that 4-HT can affect gene expression by acting on endogenous proteins. Indeed, 4-HT is marketed as a PKC inhibitor by Calbiochem (La Jolla, CA) and part of its effect on gene transcription may be due to its effects on PKC or other endogenous kinases. Inhibition of basal PKC activity by 4-HT could cause changes in gene expression since PKC regulates transcription by activating ERK1/2, MAPKs that phosphorylate multiple transcription factors (3,162). I also cannot rule out the possibility that 4-HT regulates the activity of other cellular proteins that control gene expression. In any case, genes whose expression was regulated in the same manner by 4-HT in both the mER-Akt-expressing cells and the A2-ER-Akt-expressing cells were eliminated from the list of genes potentially regulated by Akt.

Since gene expression changes that were identified by both normalization techniques are more likely to be real changes, I focused only on those genes. This narrowed down the number of genes to those that are most likely to be true targets of Akt regulation.

Table 5.1 Summary of the number of genes whose expression changed upon treatment with 4-HT

The number of genes whose change in expression level (normalization method 1) or fold change (normalization method 2) was greater than 2-fold after 4-HT treatment of mER-Akt or A2-ER-Akt expressing WEHI-231 cells is indicated. The genes that showed a change in expression in either cell type were identified. Of those genes, the number whose expression changed in the 4-HT-treated mER-Akt-expressing cells but not in the A2-ER-Akt-expressing cells was determined and is presented in the rightmost column. These genes are most likely Akt-regulated genes. The number of those Akt-regulated genes whose calculated change was more than 2-fold using both normalization techniques is indicated in bold.

Normalization technique	Expression or Fold change level	# genes with changes in each cell type		# genes with changes in mER-Akt but not A2-ER-Akt expressing cells
		mER-Akt	A2-ER-Akt	
Normalized to untreated	>2 at 3 h	560	560	549
	<0.5 at 3 h	178	176	178
	>2 at 20 h	945	112	844
	<0.5 at 20 h	128	171	88
	TOTAL	1811	1019	1659
Fold Change over untreated	↑ 2-fold at 3 h	393	916	385
	↓ 2-fold at 3 h	33	117	33
	↑ 2-fold at 20 h	1328	391	983
	↓ 2-fold at 20 h	89	83	76
	TOTAL	1843	1507	1477
Present in both normalizations	↑ 2-fold at 3 h	--	--	213
	↓ 2-fold at 3 h	--	--	25
	↑ 2-fold at 20 h	--	--	666
	↓ 2-fold at 20 h	--	--	57
	TOTAL	--	--	961

The genes with the largest changes in expression in response to activation of mER-Akt at each time point are shown in table 5.2. The genes listed are divided into subcategories corresponding to the conditions described in table 5.1. The mean fold change is indicated and although a probability value is assigned to each gene by the GeneSpring analysis software, there was never a clear indication from the statisticians at Silicon Genetics as to what this probability value represents. Therefore this probability value was left out. The range of the normalized expression level is indicated when there was a range in fold change between replicate experiments.

The profile of the changes in gene expression for the complete set of genes is depicted graphically in figure 5.3. Genes with increased expression at 3 h or 20 h (Fig. 5.3A) as well as genes with decreased expression at 3 h or 20 h (Fig. 5.3B) are plotted on separated graphs and coloured accordingly. A small number of genes (14 genes) that showed increased expression at 3 h also had increased expression at 20 h. However, none of the genes analyzed had significantly decreased expression at both 3 h and 20 h. This observed exclusion of genes that changed at 3 h versus 20 h may be a result of only analyzing genes with a greater than 2-fold change. It is possible that a gene with a greater than 2-fold increase at one time point also increased at the other time point but did not change greater than 2-fold and so was not included in this analysis.

As shown in table 5.1, there are a total of 238 genes with changes in gene expression after 3 h of Akt activation and 723 genes with changes in gene expression after 20 h of Akt activation. Therefore, the longer that Akt is activated the more genes that are affected. Since 4-HT treatment of mER-Akt-expressing cells for long periods of time results in activation of Akt for the entire time, the increased number of genes with changes in gene expression could be a direct result of activation of Akt. Alternatively, 4-HT-induced changes in gene

Table 5.2 Select list of genes with increased or decreased gene expression upon 4-HT-induced mER-Akt activation.

A subset of the 961 genes identified with an expression change greater than 2-fold in response to activation of mER-Akt is shown here. The genes shown were chosen on the basis of a high magnitude of change with no bias for predicted gene function. Mean fold changes are calculated compared to the untreated sample (normalization method 2). The mean expression levels of 3 independent replicates is shown the range indicated when available. Decreases in gene expression are indicated by a negative (-) symbol preceding the magnitude of the fold change (e.g. a 10-fold downregulation is expressed as -10 which indicates that the stimulated sample was 10% of the untreated control). The normalized expression level was determined by setting the gene at the 50th percentile to 1 and determining the relative expression level of every other gene to the intensity of the gene at the 50th percentile. (*nd= no detectable expression)

Gen-Bank ID	Description	Normalized expression level in untreated sample	fold change at 3h	Normalized expression level at 3 h	fold change at 20h	Normalized expression level at 20 h
Decreased expression at 3 h						
AU019491	Drosophila melanogaster genomic scaffold 142000013386047	2.479	-33.50	0.074	nd	nd
AW555191	Human DNA sequence from clone RP3-324N14 on chromosome 6q23.1-24.3	2.308	-10.03	0.23	nd	nd
AW559036	Homo sapiens cDNA FLJ10386 fis_clone NT2RM2002142	2.276	-8.10	0.281	nd	nd
AU023481	Mus musculus CD4 antigen (Cd4 gene_partial sequence	1.582	-8.07	0.196	nd	nd

C76882	Homo sapiens clone RP11-478M12_ complete sequence	1.791	-7.92	0.226	nd	nd
C87101	Homo sapiens PAC clone RP4-676L20 from 7q35-q36 complete	2.953	-7.36	0.401	nd	nd
AW557482	Mus musculus neural precursor cell expressed_ developmentally	1.64	-6.10	0.269	nd	nd
AA407565	Mus musculus heparan sulfate (glucosamine 3-O-sulfotransferase 1	2.057	-4.89	0.421	nd	nd
C77490	Homo sapiens nuclear VCP-like (NVL mRNA	1.388	-3.70	0.375	nd	nd
C86468	Rattus norvegicus intermediate conductance calcium-activated	1.415 (1.143 to 1.762)	-3.39	0.418 (0.279 to 0.689)	-0.59	2.387 (1.346 to 4.231)
C87419	No Hits Found	0.797 (0.422 to 1.171)	-3.14	0.254 (0.213 to 0.295)	nd	nd
AU024689	Mus musculus chromosome 11_ BAC clone 111-181 (LBNL M01 complete	1.112 (0.813 to 1.406)	-3.06	0.363 (0.28 to 0.401)	-0.69	1.609
C76819	Homo sapiens_ clone RP11-29A1_ complete sequence	1.244	-2.89	0.431	nd	nd
C77648	Human DNA sequence from clone RP3-390M24 on chromosome 6_ complete	1.402	-2.85	0.492	nd	nd
Decreased expression at 20 h						
AA409134	Homo sapiens Chromosome 13 Cosmid Clone 97h8_ complete sequence	2.361 (2.196 to 2.525)	-0.56	4.231 (3.908 to 4.555)	-65.58	0.036
C85448	Mus musculus apoptosis inhibitor 1 (Api1 _ mRNA	1.623 (1.581 to 1.665)	-1.19	1.369 (1.199 to 1.539)	-40.58	0.04
AW549928	Mus musculus major histocompatibility locus class III region:	2.429 (2.053 to 2.804)	-0.66	3.69 (3.568 to 3.811)	-25.84	0.094

AW554 058	Homo sapiens eukaryotic translation initiation factor 3 subunit 9	1.994 (1.766 to 2.223)	-1.20	1.658 (1.643 to 1.674)	-24.62	0.081
AW538 298	Homo sapiens cDNA FLJ20826 fis_clone ADSE00129	2.14 (2.123 to 2.158)	-1.11	1.931 (1.862 to 2.000)	-17.69	0.121
AU043 193	Mus musculus frizzled-3 protein mRNA_complete cds	1.92 (1.374 to 2.466)	-0.72	2.667 (2.484 to 2.851)	-16.27	0.118
AU018 483	Homo sapiens genomic DNA_chromosome 21q21.1-q21.2_clone:B732H1	1.555 (1.380 to 1.729)	-0.46	3.375 (2.706 to 4.044)	-15.71	0.099
AA408 424	Homo sapiens mRNA for U2 snRNP-specific A' protein alternative	1.655 (1.496 to 1.814)	-0.87	1.913 (1.910 to 1.915)	-12.08	0.137
AW550 195	Homo sapiens crystallin_zeta (quinone reductase - like 1 (CRYZL1	2.148 (1.974 to 2.322)	-0.66	3.253 (3.184 to 3.323)	-11.87	0.181
AU042 966	Homo sapiens 12p13.3 PAC RPCI5-1154L15 (Roswell Park Cancer Institute	2.353 (2.196 to 2.509)	-1.52	1.544 (1.459 to 1.629)	-11.77	0.2
AW551 165	Mouse calpactin I heavy chain (p36 mRNA_complete cds.	1.527 (1.519 to 1.535)	-0.38	4.032 (3.789 to 4.274)	-11.15	0.137
C78257	Rattus norvegicus dithiolethione-inducible gene-1 (DIG-1 mRNA	1.783 (1.643 to 1.923)	-0.39	4.611 (3.630 to 5.591)	-10.94	0.163
AU045 202	H.sapiens CpG island DNA genomic MseI fragment_clone 70g11	1.232 (0.988 to 1.476)	-0.57	2.15 (1.817 to 2.484)	-10.90	0.113
C80408	Homo sapiens clone RG107G13_complete sequence	2.371 (2.326 to 2.416)	-1.27	1.87 (1.828 to 1.913)	-10.68	0.222 (0.152 to 0.292)
C76712	Human DNA sequence from cosmid L241B9_Huntington's Disease Region	1.573	-0.73	2.16	-10.35	0.152 (0.135 to 0.169)
Increased expression at 3h						
AU015 463	Homo sapiens mRNA; cDNA DKFZp586C1021 (from clone DKFZp586C1021 ;	0.338	25.92	8.762	nd	Nd
C87312	Caenorhabditis elegans cosmid C05D9	0.157	24.31	3.817	nd	nd

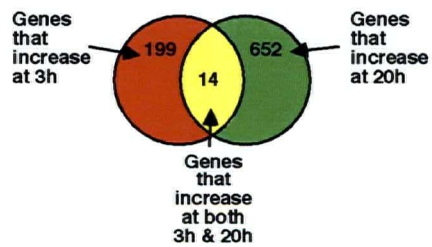
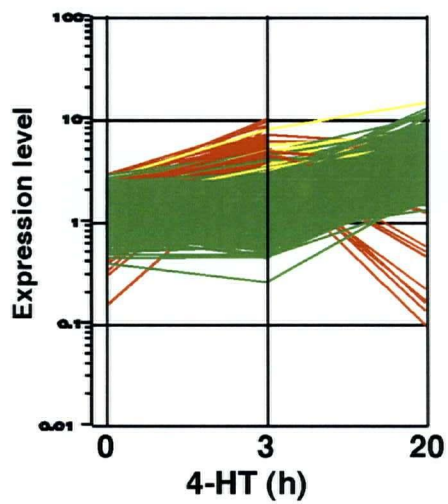
AU018 969	Homo sapiens cDNA FLJ10401 fis_clone NT2RM4000421_ highly similar to	0.304	18.78	5.709	nd	nd
AU016 251	Mus musculus decay accelerating factor 1 (Daf1 mRNA	0.397	10.86	4.311	nd	nd
AU040 128	Caenorhabditis elegans cosmid W01D2_ complete sequence	0.412 (0.346 to 0.479)	8.08	3.331 (3.083 to 3.58)	nd	nd
AU043 563	Human DNA sequence from PAC 274L7 on chromosome X contains ESTs	0.575 (0.485 to 0.664)	7.91	4.548 (4.537 to 4.558)	nd	nd
AU016 090	Human DNA sequence from clone 479J7 on chromosome Xq21.33-23 Contains	0.889	7.89	7.015	nd	nd
AU021 062	Mus musculus chromosome 16 clone ct7- 581i11 strain 129/Sv ES cell line	0.924	7.69	7.11	nd	nd
AU021 146	Mus musculus apoptosis activator Mtd (Mtd mRNA_ complete cds	0.494 (0.425 to 0.563)	7.29	3.601 (3.321 to 3.881)	nd	nd
AW538 653	Subacute sclerosing panencephalitis (SSPE virus mRNA for fusion	0.634	7.26	4.602	nd	nd
C78835	Mus musculus melanoma X-actin (Actx_ mRNA	0.543 (0.445 to 0.642)	7.01	3.804 (3.652 to 3.957)	12.72	6.907
AU022 620	Homo sapiens PAC clone RP4-672O11 from 7q22- 31.2_ complete sequence	0.688 (0.461 to 0.915)	6.44	4.428 (3.613 to 5.244)	nd	nd
C86598	Arabidopsis thaliana BAC F1K3 from chromosome IV near 21 cM_ complete	0.526	6.30	3.312	nd	Nd
C86246	Homo sapiens BAC clone GS1-98E2 from 7p11.2- p21_ complete sequence	1.383	6.27	8.677	nd	nd
AU020 827	Human DNA sequence from clone CTA-277P6 on chromosome 1q25.3-31.2	0.758	5.75	4.358	nd	nd

Increased expression at 20h						
C78835	Mus musculus melanoma X-actin (Actx _ mRNA	0.543 (0.445 to 0.642)	7.01	3.804 (3.652 to 3.957)	12.72	6.907
AU040 375	Mus musculus chromosome 17 clone BAC 205A4_ complete sequence	0.732	4.06	2.975	8.90	6.512 (6.147 to 6.877)
C80229	Human DNA sequence from clone RP5-860F19 on chromosome 20p12.3-13	0.986 (0.899 to 1.072)	1.31	1.289 (1.127 to 1.451)	7.30	7.199
AU023 118	Homo sapiens BAC clone RP11-187G20 from 2_ complete sequence	1.109 (0.802 to 1.417)	2.24	2.479 (1.521 to 3.437)	7.23	8.02 (6.351 to 9.689)
AU044 066	Homo sapiens mRNA; cDNA DKFZp564G1162 (from clone DKFZp564G1162	0.753 (0.731 to 0.775)	1.76	1.326 (1.161 to 1.490)	7.06	5.315
AU022 363	Human DNA sequence from clone RP4-652L8 on chromosome 1q24.1-25.2	1.151	1.59	1.157	6.88	8.069
AU016 461	Mus musculus major histocompatibility complex class I region	1.785 (1.3 to 2.270)	1.16	2.845 (2.255 to 3.435)	6.85	12.285 (10.662 to 13.907)
AA408 801	Mouse orotidine-5'-monophosphate decarboxylase mRNA_ 3' end.	1.005 (0.550 to 1.468)	1.21	1.167 (0.741 to 1.786)	6.71	6.885 (6.615 to 7.155)
AW537 155	Mus musculus Pax transcription activation domain interacting protein	1.006 (0.883 to 1.13)	1.57	1.215 (1.189 to 1.241)	6.64	6.754
AW549 646	Mus musculus heat-responsive protein 12 (Hrsp12 _ mRNA	0.972 (0.875 to 1.07)	1.17	1.526 (1.313 to 1.739)	6.57	6.454
AU016 154	Mus musculus calcium binding protein A6 (calyculin (S100a6 _ mRNA	1.672 (1.655 to 1.689)	2.55	1.948 (1.853 to 2.044)	6.52	10.979
AW554 373	Homo sapiens clone FLB1120 PRO0225 mRNA _ complete cds	1.044 (1.012 to 1.076)	1.64	2.66 (2.583 to 2.737)	6.52	6.807 (1.681 to 11.932)
AU045 030	Homo sapiens chromosome 16 clone RPCI-11 523L20	0.915 (0.717 to 1.113)	1.90	1.497 (1.237 to 1.756)	6.24	5.963 (5.503 to 6.422)

Figure 5.3 Profile of genes whose expression changes in response to 4-HT-induced activation of mER-Akt. *A*, Genes with a 2-fold or greater increase in transcription in response 4-HT stimulation were identified. The intensity of the fold change was plotted against the time after 4-HT treatment. Genes with increased transcription at 3 h are coloured in red, genes with increased transcription at 20 h are coloured in green and genes with increased transcription at both 3 h and 20 h are coloured in yellow. The numbers of genes in each colour category are depicted graphically in a Venn diagram below the gene profiles. *B*, Genes with a 2-fold or greater decrease in transcription in response 4-HT stimulation were identified. The intensity of the fold change was plotted against the time after 4-HT treatment. Genes with increased transcription at 3 h are coloured in red, genes with increased transcription at 20 h are coloured in green and genes with increased transcription at both 3 h and 20 h are coloured in yellow. The numbers of genes in each colour category are depicted graphically in a Venn diagram below the gene profiles.

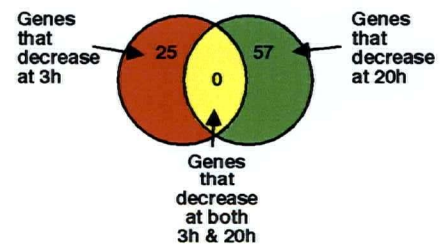
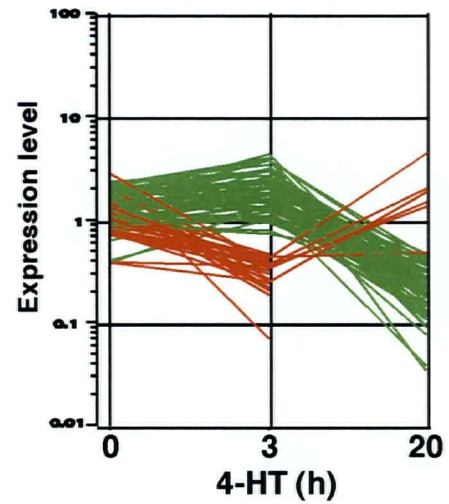
A

Profile of genes with increased expression



B

Profile of genes with decreased expression



expression at the later time point could be a consequence of indirect effects of Akt activation since Akt may induce the expression of genes whose products can themselves regulate gene expression. It is likely that both of these mechanisms contribute to the 4-HT-induced changes in gene expression observed at the 20 h time point. There is a total of 879 genes that were upregulated in response to mER-Akt activation and 82 genes that were downregulated in response to mER-Akt activation. The range of expression levels for genes that were either upregulated or downregulated by Akt activation was analyzed by sorting all the genes into categories as defined by the magnitude of the fold change and then determining the number of genes in each category (Fig. 5.4). The fold changes for genes that were upregulated by Akt activation ranged from 2-fold to 26-fold greater than the untreated sample (Fig 5.4A). In contrast, the range of fold changes for genes that were downregulated by Akt activation had a much larger range, of 2-fold to 66-fold less than the untreated sample (Fig 5.4B). The 4-HT-induced change in expression for most genes was in the range of 2- to 3-fold. Although some genes exhibited large changes in expression in response to mER-Akt activation, the expression of the majority of the mER-Akt regulated genes was affected to a much smaller degree. While the tendency is to focus on the genes with the largest changes in gene expression, microarray analysis by other groups has shown that physiologically relevant changes in gene expression that underlie cellular responses are often relatively small, in the range of 2- to 3-fold or less (332-334).

Since activation of mER-Akt resulted in the upregulation of more genes than were downregulated, this suggests that the major function of Akt with respect to transcriptional regulation may be to increase gene expression. Akt has been shown to increase the activity of the NF- κ B transcription factor and it is likely that it also affects the activity of other transcription factors either directly or indirectly. The significant number of genes that were

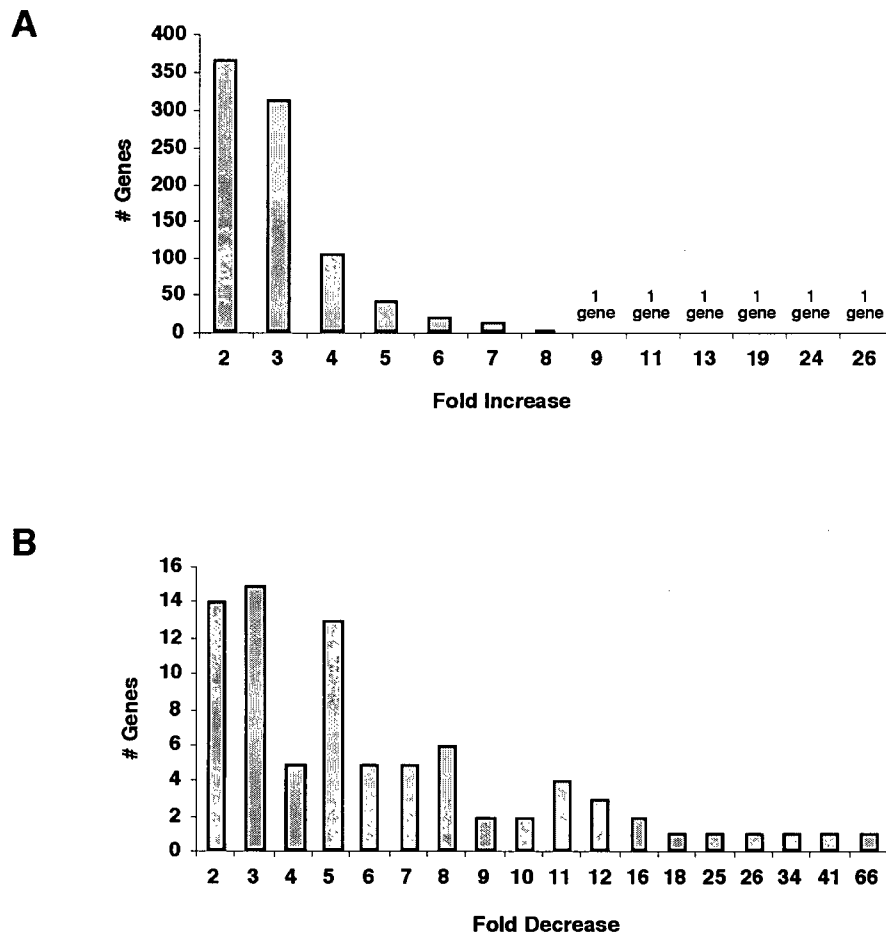


Figure 5.4 Magnitudes of changes in gene expression caused by 4-HT-induced activation of mER-Akt. *A*, 879 genes with a 2-fold or higher increase in transcription (calculated by normalization method 2) in response 4-HT treatment were identified. The value of each fold change was rounded down to the nearest integer and genes with the same value were grouped together. The number of genes in each fold change category is shown. *B*, 82 genes with a 2-fold or greater decrease in transcription in response 4-HT treatment were identified. The value of each fold change was rounded down to the nearest integer and genes with the same value were grouped together. The number of genes in each fold change category is shown.

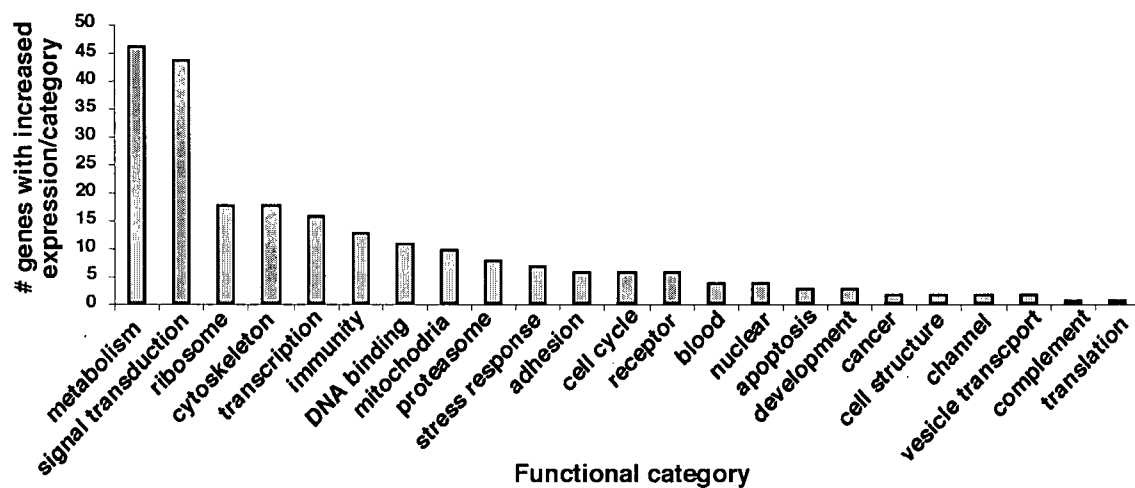
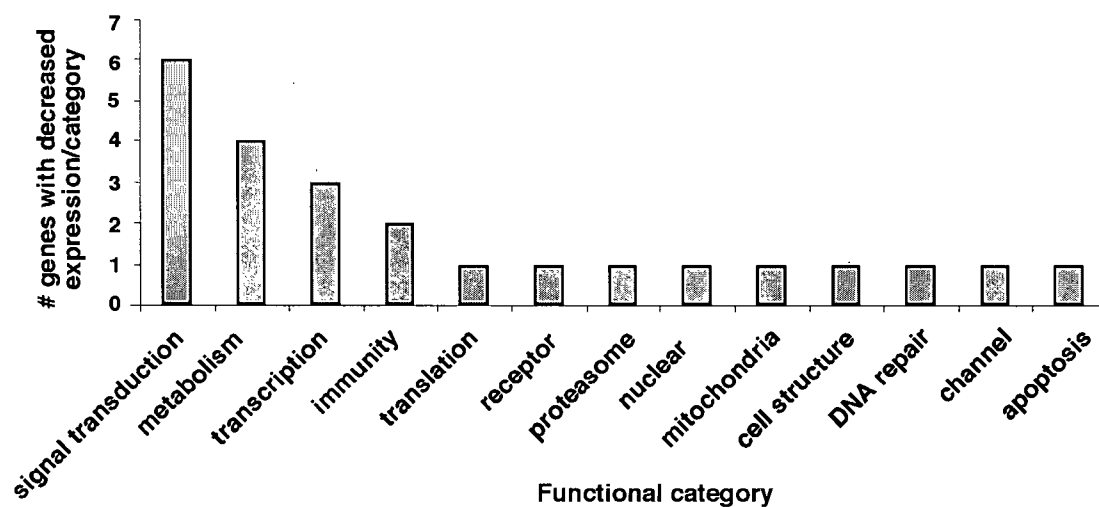
downregulated in response to Akt activation was also to be expected since Akt has an inhibitory effect on some transcription factors, in particular the forkhead-related transcription factors which control the expression of pro-apoptotic proteins.

5.6 Functional analysis of mER-Akt-regulated genes

As mentioned previously, Akt plays a pro-survival role in B cells (63) and other cell types (55,56). Therefore, the genes regulated by Akt would be predicted to function in regulating cell survival and apoptosis. To determine if the genes regulated by mER-Akt play a role in cell survival in some way, I assessed the potential function of the genes that had a 2-fold or greater increase or decrease in response to mER-Akt activation. The simplified gene ontology function of the GeneSpring was used to assist this analysis. A large number of genes that were identified as being regulated by mER-Akt activation do not have an assigned function as is the case for many of the genes in the NIA 15K genome. The remaining 256 genes were assigned to broad functional categories by the simplified gene ontology analysis in GeneSpring and by eye based on their known or predicted cellular functions (Fig. 5.5). These categories were loosely defined in order to keep the number of categories to a reasonable number. In addition a number of genes fell into more than one category. Therefore, when necessary and if appropriate, I assigned a gene to the broadest appropriate category (e.g. signal transduction) followed by narrower definition (e.g. proteasome if the gene functions in proteasomal degradation associated with signal transduction).

Over 200 mER-Akt regulated genes with a predicted or known function were categorized. For discussion purposes and brevity a representative sampling of these genes is shown in table 5.3. Genes that were upregulated by mER-Akt activation (Fig. 5.5A) fell into a wider range of categories than genes that were downregulated by mER-Akt activation

Figure 5.5 Functional clustering of 4-HT-induced changes in gene expression. *A*, 232 genes with a known or predicted function and a 2-fold or higher increase in transcription in response 4-HT treatment were identified. Each gene was assigned to a category by eye based on its known or predicted cellular function. The number of genes in each functional category is shown. *B*, 24 genes with a known or predicted function and a 2-fold or greater decrease in transcription in response 4-HT treatment were identified. Each gene was assigned to a category by eye based on its known or predicted cellular function. The number of genes in each functional category are shown.

A**B**

(Fig. 5.5B). This may simply reflect the fact that more genes have increased transcription than decreased transcription in response to mER-Akt activation and there is more room for variation with a greater number of genes. Alternatively, a greater variety of transcription factors may be activated by mER-Akt than are inhibited by mER-Akt resulting in a greater variety of genes that are upregulated in response to the activation of these transcription factors. The majority of genes that were up- or down-regulated by mER-Akt were genes involved in metabolism or signal transduction. These included genes encoding proteins that are activated in response to surface receptor engagement (e.g. *calmodulin* and *cdc42*) and proteins that are necessary to maintain cellular growth or proliferation (e.g. *RNA polymerase II* and *glucose phosphate isomerase I*). There was also large number of genes that encode proteins involved in transcriptional regulation. This category included genes encoding DNA binding proteins, transcription factors and components of the transcriptional machinery. A large number of genes involved in immune regulation were regulated by mER-Akt activation including a significant number of major histocompatibility complex (MHC) genes. This is not surprising since B cells can act as antigen-presenting cells that activate T cells. A significant number of Akt-regulated genes encoded proteins involved in proteasome-mediated degradation including proteases, ubiquitin and ubiquitin-linking enzymes.

Although Akt is a pro-survival protein relatively few of the Akt target genes identified in this analysis encoded proteins involved in regulating the apoptotic machinery. However, the majority of genes regulated by Akt can regulate cell survival indirectly, for example, by acting as a component of receptor signalling pathways that may regulate cell survival or apoptosis. The potential role of some of these genes in B cell survival and proliferation will be discussed in further detail in Section 5.8.

Table 5.3 Functional analysis of genes regulated by mER-Akt activation

Genes with a 2-fold or greater increase or decrease (256 genes) in expression in response to mER-Akt activation at 3 h or 20 h were sorted into categories based on their cellular function using the simplified gene ontology function in GeneSpring and by eye. A sample of these genes is presented here (82 genes). The mean fold change of the treated sample over the untreated sample is shown (normalization method 2). The mean expression levels of 3 independent replicates is shown the range indicated when available. Decreases in gene expression are indicated by a negative (-) symbol preceding the magnitude of the fold change. The normalized expression level was determined by setting the gene at the 50th percentile to 1 and determining the relative expression level of every other gene to the intensity of the gene at the 50th percentile. (*nd= no detectable expression)

Gen-Bank ID	Description	Normalized expression level in untreated sample	fold change at 3h	Normalized expression level at 3 h	fold change at 20h	Normalized expression level at 20 h
adhesion						
C80729	Mus musculus catenin alpha 1 (Catna1)_ mRNA	1.114 (0.419 to 1.836)	1.74	1.933 (1.536 to 2.354)	2.54	2.828
C77281	Mus musculus catenin src (Catns)_ mRNA	0.894 (0.417 to 1.736)	1.01	0.906 (0.478 to 1.399)	4.28	3.829
AU021 862	Rattus norvegicus protocadherin (Fat) mRNA complete cds	1.492 (1.068 to 1.915)	1.38	2.064 (1.869 to 2.260)	4.23	6.311 (5.570 to 7.051)
apoptosis						
AU021 146	Mus musculus apoptosis activator Mtd (Mtd) mRNA complete cds	0.494 (0.425 to 0.563)	7.29	3.601 (3.321 to 3.881)	nd	nd
C85448	Mus musculus apoptosis inhibitor 1 (Api1)_ mRNA	1.623 (1.581 to 1.665)	-1.19	1.369 (1.199 to 1.539)	-40.58	0.04

AW558 429	Mus musculus caspase 6 (Casp6)_ mRNA	1 (0.55 to 1.308)	1.26	1.263 (0.653 to 1.855)	2.08	2.08 (0.88 to 3.283)
AW539 228	Mus musculus Fas antigen ligand (Fasl)_ mRNA	1 (0.834 to 1.161)	1.09	1.094 (0.558 to 1.869)	2.31	2.314 (2.137 to 2.491)
blood						
AW546 817	Mus musculus hemoglobin alpha_ adult chain 1 (Hba- a1)_ mRNA	1.114 (0.736 to 1.492)	4.01	4.464 (4.345 to 4.583)	nd	nd
cancer						
C79593	Homo sapiens herstatin (HER-2) mRNA_ alternatively spliced_	1.414 (0.893 to 1.936)	1.57	2.217 (1.594 to 2.839)	4.65	6.576
AU018 365	Mus musculus myeloblastosis oncogene- like 2 (Mybl2) mRNA	1.88 (1.853 to 1.907)	-1.85	1.015 (0.986 to 1.044)	2.72	5.109 (4.55 to 5.668)
cell cycle						
AW557 878	M.musculus GAS 6 mRNA associated with growth-arrest	1.103	3.27	3.603	nd	nd
AU019 625	Mouse mRNA for nucleosome assembly protein-1_ complete cds	1 (0.301 to 2.163)	1.07	1.068 (0.514 to 1.633)	4.00	4.002 (3.55 to 4.454)
cell structure						
AW543 626	Homo sapiens caveolin 1 (CAV1) gene_ exon 3 and partial cds	0.813 (0.381 to 1.245)	1.58	1.281 (1.181 to 1.382)	-5.89	0.138
channel						
AW559 127	Mus musculus potassium voltage-gated channel_ subfamily Q_ member 1	1.221 (1.220 to 1.222)	2.29	2.794 (2.723 to 2.865)	2.14	2.613 (1.820 to 3.405)
C86468	Rattus norvegicus intermediate conductance calcium-activated	1.415 (1.143 to 1.762)	-3.39	0.418 (0.279 to 0.689)	1.69	2.387
complement						
AU016 251	Mus musculus decay accelerating factor 1 (Daf1)_ mRNA	0.397	10.86	4.311	nd	nd
cytoskeleton						
C86292	Cricetulus griseus mRNA for kinesin light chain isoform D	1.417 (1.286 to 1.548)	2.31	3.28 (3.099 to 3.461)	nd	nd

AU014 582	Homo sapiens myosin X (MYO10) mRNA_ complete cds	2.063 (2.013 to 2.113)	2.48	5.119 (4.221 to 6.018)	3.89	8.027 (7.766 to 8.288)
AW551 643	Mus musculus microtubule-actin crosslinking factor (Macf) mRNA	1.412 (1.095 to 1.73)	1.75	2.469 (2.099 to 2.838)	5.92	8.359 (7.653 to 9.064)
AW537 152	Mus musculus Serf1 protein (Serf1)_ survival of motor neuron protein	0.768 (0.709 to 0.827)	1.58	1.217 (1.111 to 1.323)	5.98	4.593 (4.181 to 5.005)
C78835	Mus musculus melanoma X-actin (Actx)_ mRNA	0.543(0.445 to 0.642)	7.01	3.804 (3.652 to 3.957)	12.72	6.907
AU021 475	smooth muscle myosin light chain kinase {3' region}	1.801 (1.723 to 1.880)	2.03	3.665 (3.613 to 3.716)	nd	nd
Development						
AW550 210	Mus musculus H19 and muscle-specific Nctc1 genes complete sequence	2.095 (2.03 to 2.16)	2.28	4.785 (4.477 to 5.094)	-3.51	0.597 (0.531 to 0.664)
DNA binding						
AW553 643	Mus musculus LZIP-1 and LZIP-2 proteins gene_ 5'end	1.733	3.82	6.613	nd	nd
AW558 264	Mus musculus zinc finger protein (Peg3) mRNA_ complete cds	2.034 (1.841 to 2.227)	2.40	4.89 (4.815 to 4.964)	nd	nd
AU016 534	R.norvegicus ZnBP gene for zinc binding protein	0.781 (0.439 to 1.122)	2.14	1.673 (1.364 to 1.982)	3.96	3.094
AW544 260	Mus musculus excision repair 1 (Ercc1)_ mRNA	0.43 (0.218 to 0.641)	2.53	1.086 (1.012 to 1.160)	-2.24	0.192
immunity						
C77559	Mouse DNA for t-haplotype-specific elements (located in H-2 complex)	0.97 (0.856 to 1.083)	1.22	1.188 (1.1 to 1.275)	2.66	2.576
AU023 481	Mus musculus CD4 antigen (Cd4) gene_ partial sequence	1.582	-8.07	0.196	nd	nd
AU016 461	Mus musculus major histocompatibility complex class I region	1.005 (0.550 to 1.468)	1.16	1.167 (0.741 to 1.786)	6.85	6.885 (6.615 to 7.155)

AW558 476	Mus musculus major histocompatibility complex class I region	0.947 (0.563 to 1.411)	1.28	1.148 (0.786 to 1.476)	3.15	2.745 (2.387 to 3.102)
AU042 150	Mus musculus major histocompatibility locus class II region: major	1.978 (1.669 to 2.288)	-1.05	1.889 (1.792 to 1.986)	2.45	4.855
AW549 928	Mus musculus major histocompatibility locus class III region:	2.429 (2.053 to 2.804)	1.52	3.69 (3.568 to 3.811)	-25.84	0.094
AW557 191	Mus musculus MHC class III protein RP1 (Rp1) mRNA partial cds	1.333 (1.197 to 1.470)	1.40	1.863 (1.785 to 1.941)	2.42	3.22 (2.746 to 3.693)
metabolism						
AW550 195	Homo sapiens crystallin_zeta (quinone reductase)-like 1 (CRYZL1)	2.148 (1.974 to 2.322)	1.51	3.253 (3.184 to 3.323)	-11.87	0.181
C80807	Mouse mRNA for zeta-crystallin/quinone reductase partial cds	0.619	5.32	3.294	nd	nd
AA408 801	Mouse orotidine-5'-monophosphate decarboxylase mRNA_3' end.	1.006 (0.883 to 1.13)	1.21	1.215 (1.189 to 1.241)	6.71	6.754
AU015 967	Mus musculus beta-galactosidase complex (Bgl) mRNA	2.264 (2.124 to 2.404)	1.39	3.152 (2.207 to 4.098)	2.52	5.716 (2.965 to 8.467)
AW550 689	Mus musculus glucose phosphate isomerase 1 complex (Gpi1) mRNA	0.889 (0.803 to 0.976)	1.37	1.22 (1.059 to 1.380)	3.98	3.535 (2.575 to 4.494)
AA407 565	Mus musculus heparan sulfate (glucosamine) 3-O-sulfotransferase 1	2.057	-4.89	0.421	nd	nd
C78257	Rattus norvegicus dithiolethione-inducible gene-1 (DIG-1) mRNA	1.783 (1.643 to 1.923)	2.59	4.611 (3.630 to 5.591)	-10.94	0.163
AU044 245	tmk=thymidylate kinase {alternatively spliced} [mice BALB/c 3T3	1.054 (0.94 to 1.168)	1.24	1.305(1.133 to 1.477)	2.13	2.241
nuclear						
AU020 818	Homo sapiens ubinuclein (UBN1) mRNA_ alternatively spliced_ complete	1.879 (1.406 to 2.353)	1.81	3.395 (2.735 to 4.055)	4.89	9.182 (7.963 to 10.400)
AU043 903	Mouse nuclear protein (mdm-1) mRNA_ complete cds	2.197 (1.854 to 2.54)	-1.43	1.538 (1.462 to 1.614)	2.91	6.394

AA408 077	Rattus norvegicus nucleolar phosphoprotein of 140kD_ Nopp140 mRNA	1.246 (0.644 to 2.178)	1.52	1.898 (1.335 to 2.415)	2.22	2.766 (1.966 to 3.566)
proteasome						
C77542	Homo sapiens ubiquitin specific protease 7 (herpes virus-associated)	1.183 (0.756 to 1.687)	1.42	1.681 (1.148 to 2.002)	2.33	2.756 (0.745 to 5.084)
AW546 384	Mus musculus proteasome (prosome_ macropain) subunit_ alpha type 3	2.207 (2.117 to 2.296)	0.68	1.494 (1.385 to 1.603)	2.36	5.208 (4.216 to 6.201)
AW543 412	Mus musculus proteasome (prosome_ macropain) subunit_ beta type 2	0.947 (0.736 to 1.159)	-1.14	0.831 (0.636 to 1.026)	-4.78	0.198
AA407 187	Mus musculus ubiquitin B (Ubb)_ mRNA	1 (0.33 to 1.651)	1.29	1.286 (0.617 to 2.749)	2.64	2.644 (1.751 to 3.788)
AW544 299	Mus musculus ubiquitin specific protease 9_ X chromosome (Usp9x)	0.912 (0.714 to 1.11)	1.19	1.085 (0.990 to 1.179)	2.96	2.7 (2.264 to 3.135)
AW550 624	Rat mRNA for polyubiquitin (ten completely repetitive ubiquitins in	1 (0.416 to 2.005)	1.18	1.177 (0.465 to 1.994)	2.38	2.375 (0.673 to 3.351)
receptor						
N/A	Mouse hepatitis virus receptor mRNA_ complete cds	1.654 (1.574 to 1.734)	1.64	2.707 (2.170 to 3.244)	2.81	4.642
AU015 632	Rattus norvegicus putative G protein-coupled receptor mRNA_ complete	1.141 (0.858 to 1.424)	2.04	2.33 (2.122 to 2.538)	2.32	2.652 (2.085 to 3.219)
ribosome						
AW536 186	Mus musculus acidic ribosomal phosphoprotein PO (Arbp)_ mRNA	1 (0.114 to 3.077)	-1.14	0.88 (0.353 to 2.025)	2.75	2.746 (1.511 to 4.466)
AW538 438	Mus musculus gene for ribosomal protein L27A_ complete cds	0.732 (0.471 to 0.995)	1.63	1.193 (0.733 to 1.584)	3.46	2.535
AW543 721	Mus musculus ribosomal protein S18 (Rps18)_ mRNA	1 (0.482 to 1.9)	-1.05	0.955 (0.508 to 1.256)	3.03	3.027 (2.599 to 3.454)
AW537 247	Rat mRNA for ribosomal protein L17	1 (0.438 to 2.596)	-1.03	0.974 (0.5 to 1.640)	3.45	3.449 (1.412 to 4.458)

signal transduction						
C87091	Homo sapiens retinoblastoma-binding protein 5 (RBBP5) mRNA	0.763	-2.24	0.34	nd	nd
C78125	Homo sapiens WNT1 inducible signalling pathway protein 1 (WISP1) gene	1.087 (0.757 to 1.416)	1.10	1.197 (0.729 to 1.665)	2.76	3.002 (1.77 to 4.234)
C75970	M.musculus mRNA for phosphatase 2A catalytic subunit isotype beta	1 (0.250 to 2.331)	-1.02	0.985 (0.118 to 1.866)	2.55	2.554 (2.456 to 2.652)
AU015 150	Mouse c-fos gene; cellular homolog to viral oncogene	1.247 (1.005 to 1.489)	2.86	3.565 (3.363 to 3.767)	nd	nd
AU044 498	Mus musculus B-cell receptor-associated protein 37 (Bcap37) mRNA	1 (0.562 to 1.482)	1.29	1.287 (0.958 to 1.963)	2.39	2.389 (1.810 to 2.967)
AU041 991	Mus musculus calmodulin (Calm) mRNA	2.579 (2.351 to 2.807)	-1.12	2.302 (2.132 to 2.472)	2.42	6.246 (5.344 to 7.147)
AU046 285	Mus musculus cell division cycle 42 homolog (S. cerevisiae)	1.941 (1.708 to 2.173)	-1.34	1.45 (1.368 to 1.531)	2.73	5.302 (4.328 to 6.275)
AU043 193	Mus musculus frizzled-3 protein mRNA_complete cds	1.92 (1.374 to 2.466)	1.39	2.667 (2.484 to 2.851)	-16.27	0.118
AU014 886	Mus musculus guanine nucleotide binding protein beta-2 related	1 (0.629 to 1.744)	1.16	1.158 (0.339 to 1.798)	2.35	2.353 (0.810 to 4.048)
AU041 108	Mus musculus mitogen activated protein kinase kinase 1 (Map2k1) mRNA	1.123 (0.217 to 1.861)	-2.42	0.465 (0.444 to 0.524)	-2.21	0.509
AU044 690	Mus musculus phospholipase A2_group 4 (Pla2g4) mRNA	1.809	1.23	2.23	2.77	5.013
AU021 496	Mus musculus serine protease inhibitor 1-5 (Spi1-5) mRNA	0.923	4.30	3.969	nd	nd
AU020 373	Mus musculus TGFb inducible early protein and hypothetical TGFb	0.904 (0.782 to 1.027)	4.22	3.818 (3.609 to 4.027)	nd	nd
AU023 245	Mus musculus transforming growth factor beta 1 induced transcript 4	1.087	3.66	3.978	4.86	5.279 (5.132 to 5.426)

stress response						
C81438	Mouse heat-shock protein hsp84 mRNA	1.987 (1.912 to 2.062)	-1.77	1.12 (1.058 to 1.181)	2.60	5.174 (4.404 to 5.944)
AW549 646	Mus musculus heat-responsive protein 12 (Hrsp12) mRNA	1.672 (1.655 to 1.689)	1.17	1.948 (1.853 to 2.044)	6.57	10.979
transcription						
AU021 882	Homo sapiens TATA box binding protein (TBP)-associated factor RNA	2.235 (1.989 to 2.481)	1.81	4.045 (3.964 to 4.125)	-5.37	0.416
AW553 445	Mus musculus Cbp/p300-interacting transactivator with Glu/Asp-rich	1.191 (0.979 to 1.403)	2.73	3.248 (2.951 to 3.544)	nd	nd
C86799	Mus musculus RNA polymerase II 3 (Rpo2-3) mRNA	1.32 (1.24 to 1.400)	1.44	1.902 (1.627 to 2.176)	3.29	4.348 (4.251 to 4.444)
AW554 173	Mus musculus RNA polymerase II transcriptional coactivator	0.996 (0.688 to 1.324)	1.14	1.131 (0.902 to 1.321)	-2.26	0.44
AW536 770	Mus musculus transcription factor EB (Tcfef) mRNA	1.226 (1.082 to 1.294)	1.04	1.277 (1.002 to 1.714)	5.21	6.386 (5.635 to 7.138)
translation						
AW554 058	Homo sapiens eukaryotic translation initiation factor 3 subunit 9	1.994 (1.766 to 2.223)	-1.20	1.658 (1.643 to 1.674)	-24.62	0.081
vesicle transport						
AU015 139	Homo sapiens GTP-binding protein RAB21 (RAB21) mRNA_ complete cds	1.876 (1.639 to 2.112)	1.45	2.714 (2.708 to 2.719)	3.16	5.932
AU017 072	Mouse mRNA for syntaxin 1B_ complete cds	1.399 (1.225 to 1.573)	2.87	4.015 (3.679 to 4.350)	nd	nd

5.7 Validation of changes in gene expression of selected genes induced by activation of mER-Akt using quantitative Real-time reverse transcriptase polymerase chain reaction

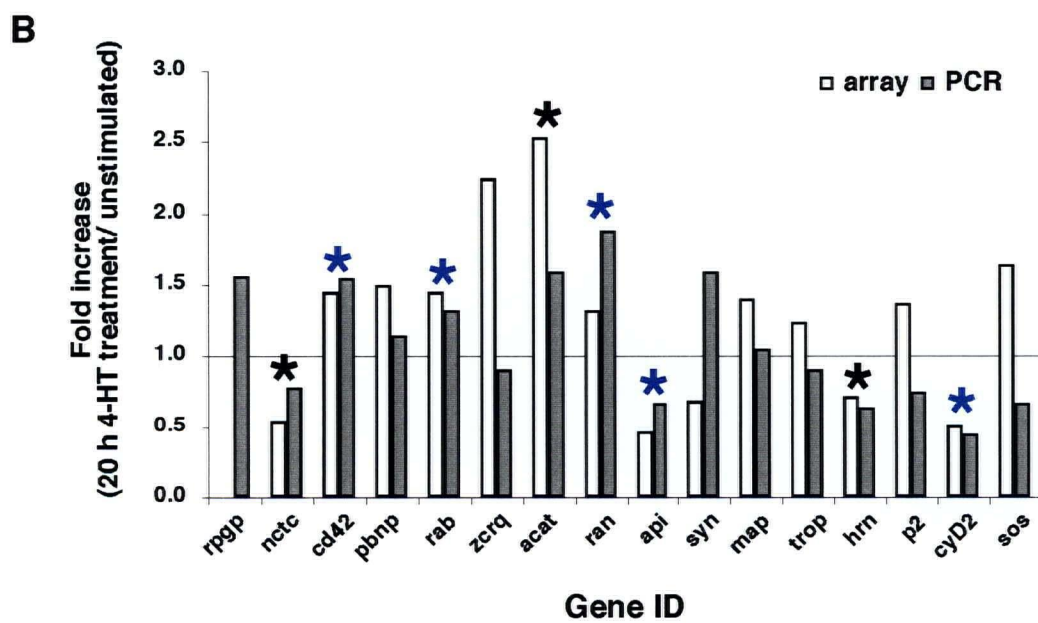
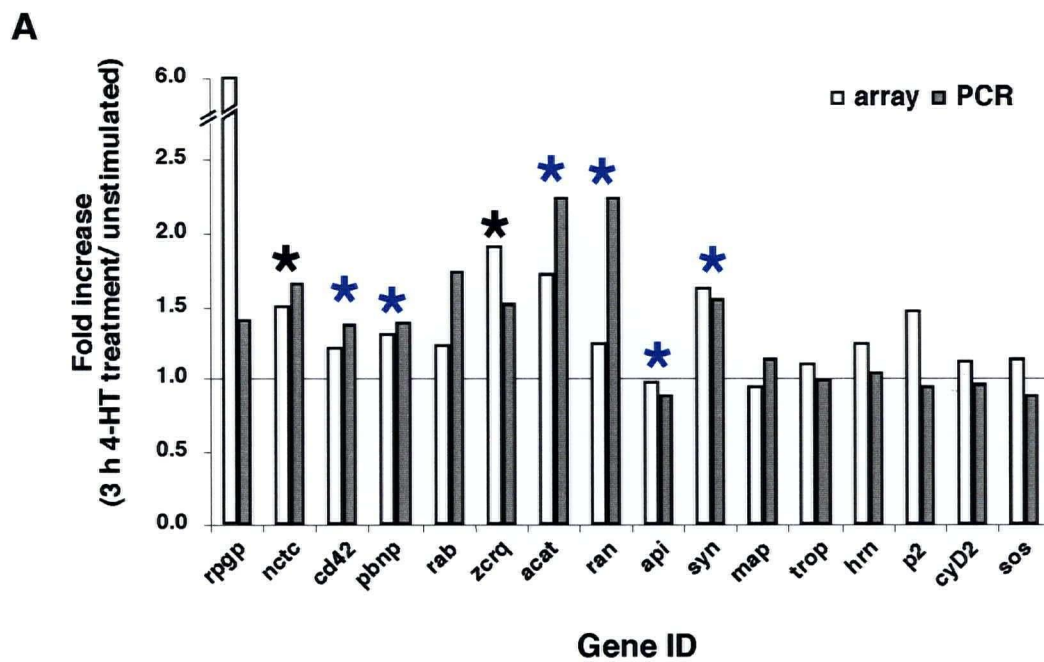
The microarray analysis I performed identified over 900 genes that were potentially regulated by mER-Akt activation in the WEHI-231 B cell line. Array analysis methods often identify a large number of genes by chance. This false discovery rate can be as high as 60-84% with normalization methods similar to the ones used here (335). Even with a very low false discovery rate of 1% an array containing 15,000 genes could have 150 genes identified by chance and a more typical false discovery rate of 50% could result in the identification of up to 7500 genes by chance. Therefore, it was necessary to validate the changes in gene expression predicted by my microarray analysis. I chose 16 of these genes and assessed the 4-HT-induced changes in the expression of these genes in mER-Akt-expressing WEHI-231 cells by quantitative Real-Time PCR analysis. These genes were selected based on their reproducible changes in response to 4-HT treatment in replicate experiments. These genes examined include *α -catenin*, *H19* and *muscle-specific Nctc1* gene, *cdc42*, *poly(A)-binding protein*, *apoptosis inhibitor-1*, *Rab11B*, *syntaxin 1B*, *MAP-2-kinase*, *non-muscle tropomyosin*, *heterogenous nuclear ribonucleoprotein A1*, *ribosomal phosphoprotein P2*, *cyclin D2* and a gene with homology to *Sos* and *Rab11a* (see Table 2.3). Genes with no known or predicted function were not chosen. In addition, 4 genes (*β -actin*, *GAPDH*, *NADPH oxidase* and *β_2 -microglobulin*) that have been previously identified by Vandesompele et. al. as appropriate “housekeeping” genes were chosen as internal controls for normalization of the Real-Time PCR data (212).

Real-time PCR takes advantage of the sensitivity of PCR and fluorescent detection of PCR-generated products to detect the amount of these products over a large concentration

range. The accumulation of PCR product is monitored by fluorescent detection in real time where the amount of starting template is proportional to the rate at which the PCR products accumulate. Since the final concentration of PCR products is often unrelated to the amount of starting material, a comparison of the rate at which the PCR products accumulate is a more accurate indication of the relative amounts of starting template. The rate is determined by identifying the cycle number at which the amount of PCR product crosses a defined fluorescence intensity threshold (Ct). Since the amount of PCR product doubles with every amplification cycle a difference of one Ct between two conditions is equivalent to a 2-fold difference in the amount of starting material.

I found that approximately 50% of the expression changes identified by microarray analysis correlated with expression changes detected by Real Time-PCR analysis (Fig. 5.6), which increased to 60% correlation if the genes that did not have observable changes with either method are included in this analysis. I observed a relatively good correlation between the magnitude of gene expression changes determined by the microarray analysis and those determined by Real-Time PCR analysis. Both Real-Time PCR analysis and microarray analysis showed that expression of *apoptosis protein inhibitor-1 (api-1*, also known as *c-IAP-2*) was downregulated at both 3 h and 20 h of mER-Akt activation while the expression of the Ran monomeric GTPase gene (*ran*) was upregulated at both time points. Other microarray-predicted changes in gene expression that were validated by Real-Time PCR included *α -catenin (acat)*, *syntaxin (syn)*, *cyclin D2 (cyD2)* and *cdc42 (cd42)*. *Syntaxin 1B* was upregulated after 3 h of mER-Akt activation as determined by both PCR and microarray analysis. At 20 h of mER-Akt activation, *syntaxin 1B* expression was downregulated as determined by microarray analysis but upregulated as determined by Real-Time PCR analysis. Therefore, the expression level of *syntaxin 1B* at 20

Figure 5.6 Comparison of changes in gene expression determined by Real-Time PCR versus cDNA microarray analysis. The fold change for each gene was determined by dividing its expression level in cells treated with 4-HT by its expression level in untreated cells. Fold changes were determined from both cDNA microarray analysis (array) and Real-Time PCR analysis (PCR). *A*, Changes in gene expression in response to 3 h of 4-HT treatment expressed as a fold change. *B*, Changes in gene expression in response to 20 h of 4-HT treatment expressed as a fold change. Genes that had similar fold changes by eye by both microarray and real-time PCR analysis are indicated with an asterisk while genes that are discussed in detail in Section 5.8 are indicated by a blue asterisk.



h was not validated and no conclusions can be drawn concerning the regulation of syntaxin 1B by mER-Akt activation at this time point. Both methods showed that *α-catenin* and *cdc42* exhibited increased expression at 3 h as well as 20 h of mER-Akt activation. Similarly, both methods showed that *cyclin D2* transcript levels decreased with 20 h of stimulation while activation of mER-Akt for 3 h had no effect on cyclin D2 levels.

Interestingly, expression of the housekeeping genes, *β-actin*, *β₂-microglobulin*, *GAPDH* and *NAPDH* oxidase, was also altered by mER-Akt activation (Table 5.5). Transcript levels for all 4 genes were upregulated with 3 h of 4-HT stimulation. At 20 h of 4-HT stimulation *β-actin* and *β₂-microglobulin* gene expression remained elevated while the level of *GAPDH* and *NAPDH* oxidase transcripts decreased to the same level as the unstimulated sample. In fact, *β-actin* had the greatest response to activation of mER-Akt of all genes examined by Real-Time PCR with a 4-fold increase in expression at 20 h. These findings are consistent with other reports showing that the expression level of “housekeeping” genes can change depending on the cell type or treatment (212,326). Since mER-Akt activation also leads to changes in the expression level of these “housekeeping” genes these genes cannot be used individually as internal normalization controls since they fail to meet the definition of a housekeeping gene whose expression level does not change. Therefore a combination of the level of all 4 genes were used as the internal normalization control as suggested by Vandesompele *et al.* (212). The geometric mean of the Ct from all 4 genes was calculated, which varied less than 15% on average between the treated and untreated samples, and was used as the normalization factor. If the geometric mean of the Ct was calculated without using the Ct of *β-actin* and used as the normalization factor, the resulting fold change of the genes of interest did not change more than 10%.

Table 5.4 Real-time PCR analysis of the effect of mER-Akt activation on the expression of potential “housekeeping” genes

The expression level of each housekeeping gene was normalized to the geometric mean of all 4 genes and then the relative expression level was determined as described in Section 2.4.3.

The fold changes from three replicate Real-Time PCR analyses were averaged and are presented as mean fold change \pm SEM.

Housekeeping gene	Fold change over unstimulated after 3 h 4-HT stimulation	Fold change over unstimulated after 20 h 4-HT stimulation
β -actin	1.93 ± 0.32	4.00 ± 1.49
β -2-microglobulin	1.18 ± 0.02	1.40 ± 0.03
GAPDH	1.48 ± 0.04	0.98 ± 0.07
NADPH oxidase	1.33 ± 0.38	0.87 ± 0.51

5.8 Discussion

In this chapter I used cDNA microarray analysis to identify Akt-regulated genes in B cells. To do this, I made use of the conditionally active mER-Akt fusion protein that can be specifically activated by treating cells with the estrogen analogue 4-HT. I compared 4-HT induced changes in gene expression in mER-Akt-expressing cells to the 4-HT-induced changes in gene expression in cells expressing the control A2-ER-Akt fusion protein. This microarray analysis indicated that activation of mER-Akt resulted in increased expression of 879 genes and decreased expression of 82 genes whose expression did not change in the 4-HT treated A2-ER-Akt-expressing cells. Although many of these genes have no known function, a number of the mER-Akt regulated genes were ones with known or predicted roles in signal transduction, cell metabolism, immune function and proteasomal degradation. I went on to use Real-Time PCR analysis to validate the changes in gene expression predicted by the changes predicted by the microarray analysis. Of the 16 genes analyzed in this manner, approximately 50% of the gene changes identified in the microarray analysis were confirmed by Real-Time PCR analysis. Thus I identified 9 genes whose expression changed when Akt was selectively activated in the WEHI-231 murine B cell line.

My observation that only 50% of the gene expression changes predicted by the microarrays were validated by Real-Time PCR analysis which is consistent with published false discovery rates of 60-84% (335). Therefore, it is not unexpected to find some gene changes that are not validated and the observation that not all gene changes identified by microarray analysis are validated by Real-Time PCR has been reported in other studies (332,336). The normalization methods that I employed were intended to increase the likelihood of identifying true gene targets of Akt while decreasing the change of detecting false positives. However, since the Real-Time PCR analysis revealed that only 8 out of 16

gene changes at each time point correlated with the microarray data a large percentage false positives was still obtained. Therefore, a more stringent normalization method might have yielded a lower rate of false discovery. Unfortunately, increasing the stringency of the analysis using the options available in GeneSpring did not result in the identification of any additional mER-Akt-regulated genes. One way to increase the reliability of microarray data is to increase the number of replicates or independent experiments that are analyzed. Therefore, I performed three independent experiments in order to increase the probability of identifying likely gene targets of Akt. However, the findings of Dow *et al.* have recently demonstrated that sample sizes of 12 to 16 generated more reliable results while sample sizes below 10 have a lower probability of detecting differentially expressed genes compared to a sample size of 12 (336). Therefore, in order to significantly improve the reliability of the microarray data, additional replicate experiments would need to be performed. The presence of additional replicates in the analysis would increase the likelihood of identifying true gene targets by reducing the total number of potential gene targets to only those with a probability of each gene of being a true Akt-regulated gene.

An analysis of a time course of 4-HT-induced activation of mER-Akt with more time points examined would also increase the reliability of the microarray analysis since a gene would likely show a pattern of regulation and have increased or decreased expression at more than one time point. However, as with all statistical analysis, a minimum of three replicates of each time point (with an ideal of 12 replicates) is needed. The high cost of microarray experiments often precludes this. Recently, more statistical analysis programs have become freely available (e.g. lcDNA; <http://receptor.seas.ucla.edu/lcDNA> or 'R'; <http://hajek.stat.ubc.ca/~jenny/teaching/rwkshp/rwkshp.htm>) that claim to increase the reliability of microarray data analysis with a lower number of 2 to 3 replicates. Since these

analysis programs often specify the experimental design to be used and the array type that can be analyzed (e.g. designed for arrays purchased from Affymetrix) it is essential to design the microarray experiment with the recommended parameters in mind. My recommendation for future microarray-based experiments would be to consult many different statistical analysis programs and a biostatistician with expertise in microarray analysis prior to designing the microarray experiment. The microarray experiment can then be designed to fit the appropriate statistical analysis method. Performing the microarray experiment with the recommended number of sample replicates (analyzing the same sample multiple times) and biological replicates (analyzing independently isolated samples with the same experimental design) and using the parameters suggested by the statistical analysis method will increase the reliability of the microarray data obtained.

For the genes whose changes in expression were validated by the Real-Time PCR analysis, I found that there was a good correlation between the magnitude of the expression changes determined by the microarray analysis and those determined by the Real-Time PCR analysis (Fig. 5.5). The magnitudes of these changes were in the range of 1.5 to 2.5-fold. An expression level of approximately 2-fold can be considered physiologically relevant since the expression level of genes from identical cultures rarely vary by as much as 2-fold (337). Moreover, small changes but physiologically relevant in gene expression are often observed in microarray analysis (332-334). Even though I observed some large gene changes in the microarray analysis, these changes did not exhibit good reproducibility across replicate experiments and so were not selected for validation by Real-Time PCR. The observed magnitudes of the validated gene changes induced by mER-Akt activation were not over 3-fold and this is likely to be a result of activating a single kinase, as opposed to the activation of multiple signalling pathways by the BCR (338). It is likely that Akt-dependent

signals act in concert with other BCR signalling pathways to regulate gene expression and other cellular functions. While my experiments identified changes in gene expression that can be induced by Akt activation alone, the identification of BCR-induced changes in gene expression that are inhibited by expression of a dominant negative form of Akt may better reveal the role of Akt in BCR signalling. Unfortunately, we have not been able to express dominant negative forms of Akt in B cell lines at this time.

Since only 50% of the genes identified by microarray analysis were validated by Real-Time PCR analysis care must be taken before drawing any specific conclusions from the overall microarray data set. I have chosen to focus on genes that were validated as targets of mER-Akt activation by Real-Time PCR analysis in order to avoid making erroneous conclusions. Below, I will discuss in detail a subset of the Akt-regulated genes that were identified by microarray analysis and validated by Real-Time PCR (see Fig. 5.5, genes identified by a blue asterisk).

Fruman and colleagues have previously examined the effect of BCR engagement on the transcriptome of splenic B cells using the Affymetrix array system (338). The contribution of the PI3K signalling pathway to BCR-induced changes in transcription was assessed by treating the cells with the PI3K inhibitor Ly294002 before stimulating them with anti-Ig antibodies. The complete set of microarray data for these experiments is published online at www.mbb.bio.uci.edu/fruman/Array_data_set.html. I have compared the PI3K-dependent and -independent changes in gene expression caused by BCR engagement that Fruman *et al.* (338) reported to the gene changes that I observed in response to activation of the mER-Akt protein. Since PI3K activates Akt in response to BCR engagement in B cells, the BCR-regulated genes that are dependent on PI3K activity may also be regulated by mER-Akt activation. As detailed in each Section below, many of the genes that I showed to be

regulated by mER-Akt activation were also regulated by BCR engagement. This includes *Ran*, *α -catenin*, *api-1*, *cyclin D2* and *Cdc42*. Surprisingly, except for *cyclin D2* and *api-1*, the BCR-induced changes in the expression of these genes was not blocked by the inhibition of PI3K activity. This suggests that although activation of Akt may be sufficient to regulate the expression of these genes, the pathways used by BCR to regulate these genes are largely independent of the PI3K/Akt pathway. This discrepancy may be due to intrinsic differences between splenic B cells and the WEHI-231 cell line, which is a tumour cell line, or may simply indicate that B cells use more than one signalling pathway to regulate expression of these genes. The signalling pathways used by the BCR to regulate expression of these genes remain to be determined. Another possibility is that other receptors in B cells regulate the expression of these genes by activating Akt.

In the next Sections, I describe six of the genes whose mER-Akt-induced regulation was confirmed by Real-Time PCR and discuss their possible roles in BCR signalling and B cell function.

5.8.1 The Ran GTPase

Expression of the gene encoding the Ran GTPase was increased at both 3 h and 20 h of mER-Akt activation. Ran is a Ras-related monomeric GTPase and functions in many different cellular processes, including nuclear transport, mitotic spindle assembly, post-mitotic nuclear assembly and cell cycle regulation (339-341).

Ran regulates nuclear import and export by regulating the loading and unloading of protein cargo on nuclear import and export receptors such as importins and exportins. In interphase, Ran activity is distributed asymmetrically with Ran-GTP predominantly in the nucleus and Ran-GDP predominantly in the cytosol (342) due to the asymmetric distribution

of its regulators. RCC1, the mammalian GEF specific for Ran, is localized in the cytosol (343) while RanGAP, the GTPase activating protein specific for Ran, is localized to the nucleus (344,345). The high concentration of Ran-GTP in the nucleus results in the release of cargo protein from import receptors in the nucleus while the hydrolysis of Ran-GTP to Ran-GDP in the cytosol causes export receptors to release their cargo in the cytosol (339).

In addition to its well defined role in nuclear transport, Ran has many other functions within the cell. Ran also regulates mitotic spindle assembly and post-mitotic nuclear envelope assembly independently of its role in nuclear transport (339). Ran has also been implicated in the regulation of the onset of mitosis (341). Inhibition of RCC1, resulting in a block in Ran-GTP formation, causes cells to prematurely enter mitosis in the presence of unreplicated DNA which would normally prevent the onset of mitosis (346). The early entry into mitosis occurs in conjunction with nuclear envelope breakdown, premature chromosome condensation and activation of cdc2/cyclin B (346). Most recently, Ran has been shown to regulate DNA replication by preventing the assembly of pre-replication complexes in intact nuclei to ensure that DNA is replicated only once per cell division (347).

The role of Ran in promoting spindle assembly suggests that it may be important for mitosis and may be upregulated prior to cell division. In addition, the Ran-mediated regulation of nuclear transport, spindle assembly, nuclear envelope assembly and regulation of entry into mitosis is essential to proper cell division. Therefore, the mER-Akt-mediated increase in Ran gene transcription could affect cell survival by increasing the amount of Ran protein available for these diverse functions.

BCR signalling in splenic B cells also results in increased expression of the Ran gene at 2 h and 12 h of stimulation (338). This increase is not blocked by treatment with the PI3K inhibitor, Ly294002 (338). Thus while I found that Akt activation is sufficient to increase

Ran gene expression in WEHI-231 cells, the BCR-induced upregulation of Ran transcription in splenic B cells is mediated by a PI3K/Akt-independent pathway.

5.8.2 α -catenin

I found that α -catenin mRNA levels were increased after 3 h and 20 h of mER-Akt activation. α -catenin is an essential component of cadherin-mediated adhesion and links the β -catenin/cadherin complex to the actin cytoskeleton (232,348). Although WEHI-231 cells do not express classical cadherin proteins (see Figure 3.7) that could interact with α -catenin, pro-B cells and pre-B cells express a non-classical or protocadherin, called BLL-cadherin (250). Protocadherins exhibit weak Ca^{2+} -dependent homophilic and heterophilic adhesive activity (231,349). The intracellular domain of the protocadherin Pcdh2 can interact with α -catenin and β -catenin (350) and protocadherins are present in endothelial and neuronal cellular junctions (231). This suggests that protocadherins could be anchored to the actin cytoskeleton in a similar manner as to the classical cadherins (231). It is not known if immature or mature B cells express protocadherins that could associate with α -catenin and mediate cellular adhesion. However, the Akt-mediated upregulation of α -catenin could potentially contribute to increased protocadherin-mediated adhesion of B cells in those cells which protocadherins are expressed.

Fruman and colleagues found that BCR stimulation resulted in an 2-fold increase in α -catenin transcript levels at 2 h and a slight increase in transcript levels at 12 h (338). This increase was not blocked when PI3K activity was inhibited. Although my data suggest that activation of Akt is sufficient to increase α -catenin gene transcription, the data from anti-IgM stimulated splenic B cells suggests that activation of the PI3K pathway is not necessary for

the BCR-induced increase in α -catenin gene expression in splenic B cells and that this increase through a PI3K/Akt-independent mechanism.

5.8.3 Api-1/IAP-2

The *api-1* gene encodes the apoptosis inhibitor protein-1, also known as IAP-2. I found that *api-1/IAP-2* gene expression was essentially unchanged at 3 h of mER-Akt activation but decreased by approximately 2-fold after 20 h of mER-Akt activation.

The IAP (inhibitor of apoptosis) proteins regulate activation of the caspase proteases (351). Caspases are synthesized in a pro-caspase form and proteolytic cleavage of the pro-caspase results in generation the active enzyme which itself is a protease. Death receptor induced activation of the initiator caspases, caspase-8 and caspase-9, leads to proteolytic cleavage and activation of the executioner caspases, caspase-3 and caspase-7 (83). Activated executioner caspases cleave essential intracellular proteins such as I^{CAD}/DFF45, which normally inhibits DNA fragmentation, and Bcl-2, which normally inhibits mitochondrial release of cytochrome C (83,352). The cleavage of these proteins results in apoptosis. The IAP proteins bind to and inhibit the activity of the executioner caspases (351). IAP proteins are in turn regulated by the Smac/DIABLO protein which promotes apoptosis by binding to IAP and preventing the IAP-dependent inhibition of caspase activity (351). BCR stimulation results in decreased expression of the *api-1/IAP-2* transcript which appears to be partially dependent on PI3K activation (338). This suggests that the PI3K/Akt pathway may play a role in the regulation of *api-1/IAP-2* by the BCR and may contribute to the BCR-induced activation of caspases by suppressing *api-1/IAP-2* expression (353).

However, since both Akt and *api-1/IAP-2* function to promote cell survival, the downregulation of the *api-1/IAP-2* gene by activation of mER-Akt is unexpected as the

downregulation of api-1/IAP-2 expression would result in increased cell death via increased caspase activation. Recent evidence has indicated that activation of caspases can in some instances promote cell survival, cell cycle progression and proliferation (354,355). The IAP proteins also have roles in processes other than apoptosis. In particular, IAP proteins can interact with the TRAF adapter proteins that link tumour necrosis factor receptor (TNFR) family members to the activation of the JNK MAPK and the NF- κ B transcription factor (356-359). Therefore, both caspases and the caspase regulatory IAP proteins have the potential to function independently of the regulation of apoptosis. The suppression of api-1/IAP-2 expression by mER-Akt activation may modulate these other processes, as opposed to cell survival. It has been demonstrated that caspase activation is necessary for CD40-induced B cell proliferation (360). It is not known if Akt plays a role in the induction of caspases in response to CD40 signalling.

5.8.4 Syntaxin 1B

I found that *syntaxin 1B* gene expression was increased 1.5-fold upon 3 h of Akt activation and that the magnitude of the change was similar by Real-Time PCR and microarray analysis. In contrast, the change in *syntaxin 1B* gene expression level at 20 h that was predicted by the gene array was not validated by Real-Time PCR analysis. Thus, there appears to be a transient increase in *syntaxin 1B* levels upon mER-Akt activation.

Syntaxin is an integral membrane protein that is essential for vesicular exocytosis of neurotransmitters at neuronal junctions (361). It is also involved in vesicular exocytosis and degranulation in various hematopoietic cells including macrophages and eosinophils (362). In addition, syntaxin has been shown to influence neurotransmitter reuptake through the GAT1

transmitter (363) and inhibit voltage-gated calcium channel function (364) in neuronal cells. A function for syntaxin in B cells has not yet been reported.

Exocytosis involves the fusion of cytosolic granules or vesicles with the plasma membrane followed by release of granule/vesicle contents to the extracellular environment. Syntaxin is involved in SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) complex assembly (365). The stable interaction of syntaxin with the other components of the SNARE complex, synaptobrevin and SNAP-25 (soluble N-ethylmaleimide sensitive factor attachment protein 25), drives fusion of the granule/vesicle with the plasma membrane and is necessary for subsequent release of vesicle contents (365). Syntaxin 1A overexpression inhibits voltage-gated calcium channel activity via a decrease in channel cell surface expression (364) and inhibits GAT1-mediated reuptake of the GABA neurotransmitter (363). The syntaxin isoforms 2, 3, 4 and 7 have been demonstrated to function in granular exocytosis in macrophages, eosinophils, neutrophils, mast cells and platelets (362).

Syntaxin 1A and 1B are 84 % identical at the protein level but only the *syntaxin 1B* gene has perfect identity with the PCR amplicon corresponding to the microarray probe. Whether or not there is a functional difference between syntaxin 1A and 1B is not known since very little work has been done on syntaxin 1B. However they are likely to have similar functions since they have such high sequence identity.

Although B cells do not undergo degranulation, fusion of cytosolic vesicles with the plasma membrane is essential for the expression of the BCR on the cell surface and for secretion of soluble antibody from plasma cells (3,366,367). Since the membrane-bound BCR is continually internalized into endosomal vesicles and new BCR is expressed on the cell surface (366,368-371), the increased rate of cycling following antigen engagement of the

BCR (372) may require further expression of the proteins required for vesicle fusion. Thus, the Akt-mediated increase in syntaxin 1B may increase the amount of syntaxin protein available for vesicle fusion. Alternatively, the Akt-mediated upregulation of syntaxin 1B may be an important step in the development of a B cell into an antibody-producing plasma cell since secretion of soluble antibody requires vesicle fusion.

The *syntaxin 1B* gene was represented on the array set used by Fruman *et al.* (338), however, there was no detectable expression in murine splenic B cells with or without anti-IgM stimulation. This suggests that splenic B cells and WEHI-231 B cells may have intrinsic differences in the expression pattern of some genes. An alternative possibility is that different genes were detected in the independent analyses because PCR-based arrays such as the NIH 15K array that I used and the oligonucleotide-based arrays manufactured by Affymetrix used by Fruman *et al.* (338) detect inherently different expression patterns even when RNA from the same sample is analyzed (373). Li *et al.* (373) found that the Affymetrix array detected more genes with changes in expression than the PCR-based array. It is proposed that the major reason for this discrepancy is because of the increased variation of hybridization efficiencies within the PCR-based array and the increased possibility of non-specific hybridization of the labelled DNA probes when longer cDNA sequences are used in the PCR-based array design. However, since the *syntaxin 1B* gene was detected on the PCR-based array but not on the oligonucleotide array, the different expression patterns are probably not due to the different array designs since the oligonucleotide array is reported to be more sensitive (373). Therefore, it is likely that intrinsic differences in the transcriptomes of splenic B cells and WEHI-231 B cells are responsible for the different expression patterns observed for the *syntaxin 1B* gene.

5.8.5 Cyclin D2

I found that expression of the cyclin D2 gene was downregulated when mER-Akt-expressing WEHI-231 cells were treated with 4-HT for 20 h, but not when they were treated with 4-HT for 3 h. Cell cycle entry and progression is regulated by the activity of cyclin-dependent kinases (CDKs), which in turn are positively regulated by association with cyclin proteins and negatively regulated by association with CDK inhibitors (374,375). The G1-type cyclins (D and E cyclins) regulate the G1 to S phase transition (376). Cyclin-D1, -D2 and -D3 (D-type cyclins), in association with CDK4 and CDK6, regulate progression through the restriction point in the G1 phase of the cell cycle (377,378). Cyclin E functions later in the G1 phase of the cell cycle, and in association with CDK2, regulates entry into S phase (377,379). Cyclin abundance varies throughout the cell cycle with the level of each cyclin being tightly controlled by ubiquitin-mediated degradation. The varying levels of cyclins regulates cell cycle progression via regulation of CDK activity (374,376).

Cyclin D2 levels are regulated by the BCR via activation of the PI3K pathway and overexpression of cyclin D2 is sufficient to prevent anti-IgM-induced growth arrest and apoptosis in WEHI-231 cells (298). Banerji *et al.* (298) have shown that in WEHI-231 cells, in response to BCR engagement, cyclin D2 mRNA levels decline significantly by 12 h, are undetectable at 24 h, but begin to increase again by 48 h. Inhibition of PI3K activity prevented these BCR-induced changes in cyclin D2 expression levels and maintained cyclin D2 at a low level over the entire 48 h time period. My data show that cyclin D2 mRNA levels are no different in untreated cells than in cells with 3 h of mER-Akt activation but decline by 20 h of mER-Akt activation. This is consistent with the findings of Banerji *et al.* (298) who observed that cyclin D2 mRNA levels did not change during the first 6 h of anti-IgM stimulation but were significantly lower at 24 h. I found that cyclin D2 mRNA levels

were decreased by 2-fold after 20 h of mER-Akt stimulation while Banerji *et al.* (298) observed that cyclin D2 protein levels were undetectable by 24 h. In addition my data suggest that the decrease in cyclin D2 expression levels is regulated by Akt activation while Banerji *et al.* (298) demonstrated that cyclin D2 upregulation is dependent on the PI3K pathway. Together, these data suggest that cyclin D2 levels in WEHI-231 cells are regulated by proteasome-mediated protein degradation and by suppression of cyclin D2 transcription.

BCR stimulation of splenic B cells also induces changes in cyclin D2 expression levels (338). Cyclin D2 mRNA levels were upregulated approximately 8-fold with 2 h of stimulation but only 2-fold with 12 h of stimulation. Although the quantitative changes in mRNA expression are not consistent between stimulation through the BCR and activation of mER-Akt there is a similar pattern of activation. Both mER-Akt activation in WEHI-231 cells and BCR stimulation of splenic B cells result in a decrease in cyclin D2 mRNA levels at later time points (12 and 20 h) compared to early time points (2 and 3 h). The increase in cyclin D2 expression was inhibited slightly at 2 h and completely at 12 h by inhibition of PI3K activity in splenic B cells. Therefore, the regulation of cyclin D2 expression at 12 h in response to anti-IgM treatment in splenic B cells is dependent on PI3K activity while I have shown that activation of Akt in WEHI-231 cells is sufficient for the downregulation of cyclin D2 expression at later times.

The PI3K/Akt pathway can therefore regulate both the upregulation and downregulation of cyclin D2 mRNA and protein levels over the course of the cell cycle.

5.8.6 Cdc42

I found that the expression of the gene encoding the monomeric GTPase Cdc42 was increased with both 3 h and 20 h of mER-Akt activation. Cdc42 is a member of the Rho-family of monomeric GTPases and is an important regulator of the actin cytoskeleton (166,380,381). Activation of Cdc42 in fibroblasts leads to the formation of filopodia, which are small ruffles of the plasma membranes at the leading edge of a migrating cell (381). Cdc42 modulates the actin cytoskeleton formation through association with the N-WASP scaffold protein which, in response to extracellular stimuli, induces activation of the Arp2/3 complex of actin-nucleating proteins (380). Activation of Cdc42 triggers actin filament assembly, promoting filopodia formation, cell polarization and contributes to cell-cell adhesion, all of which are necessary for cell motility and migration (381). Migration of B cells into peripheral lymphoid organs such as the spleen and lymph nodes is necessary for proper B cell development and activation (382). Therefore, the upregulation of Cdc42 expression in response to Akt activation may contribute to proper B cell development and activation by making the cell competent for actin remodeling that is required for cellular migration.

The array study performed by Fruman *et al.* (338) showed that anti-IgM stimulation of murine splenic B cells for 2 or 20 h results in upregulation of *Cdc42* gene expression by approximately 1.5-fold which is similar to the increase of 1.3- to 1.6-fold that I observed in response to mER-Akt activation. Inhibition of PI3K activity in these cells did not reduce the BCR-induced increase in Cdc42 expression. Therefore, PI3K activation is not necessary for the in the BCR-induced increase in Cdc42 expression and the BCR regulates Cdc42 expression through pathways other than the PI3K pathway. However, my data suggest that Akt activation is sufficient to induce Cdc42 expression in WEHI-231 cells. It is possible that

Cdc42 expression is regulated by different mechanisms in WEHI-231 cells and splenic B cells. This suggests that there may be more than one mechanism to induce Cdc42 expression in B cells.

5.8.7 Summary

A number of the mER-Akt-regulated genes that I identified by microarray analysis and verified by Real-Time PCR analysis play some role in cell survival either by maintaining proper cell homeostasis or by affecting the regulation of the cell cycle. As discussed above, Ran, cyclin D2 and api-1 can all affect cell survival and cell cycle progression. Conversely, syntaxin, α -catenin and cdc42 do not have a clear role in the regulation of cell survival but may be important for B cell activation and/or homeostasis. Of the mER-Akt-regulated genes that I validated by Real-Time PCR, there were no obvious direct connections to cell survival, as would have been predicted from the important role of Akt in B cell survival (63). Nevertheless, I found that the Akt-mediated regulation of transcription in WEHI-231 cells results in changes in the expression of many genes that could have direct and indirect effects on cell survival and function.

Chapter 6

Discussion

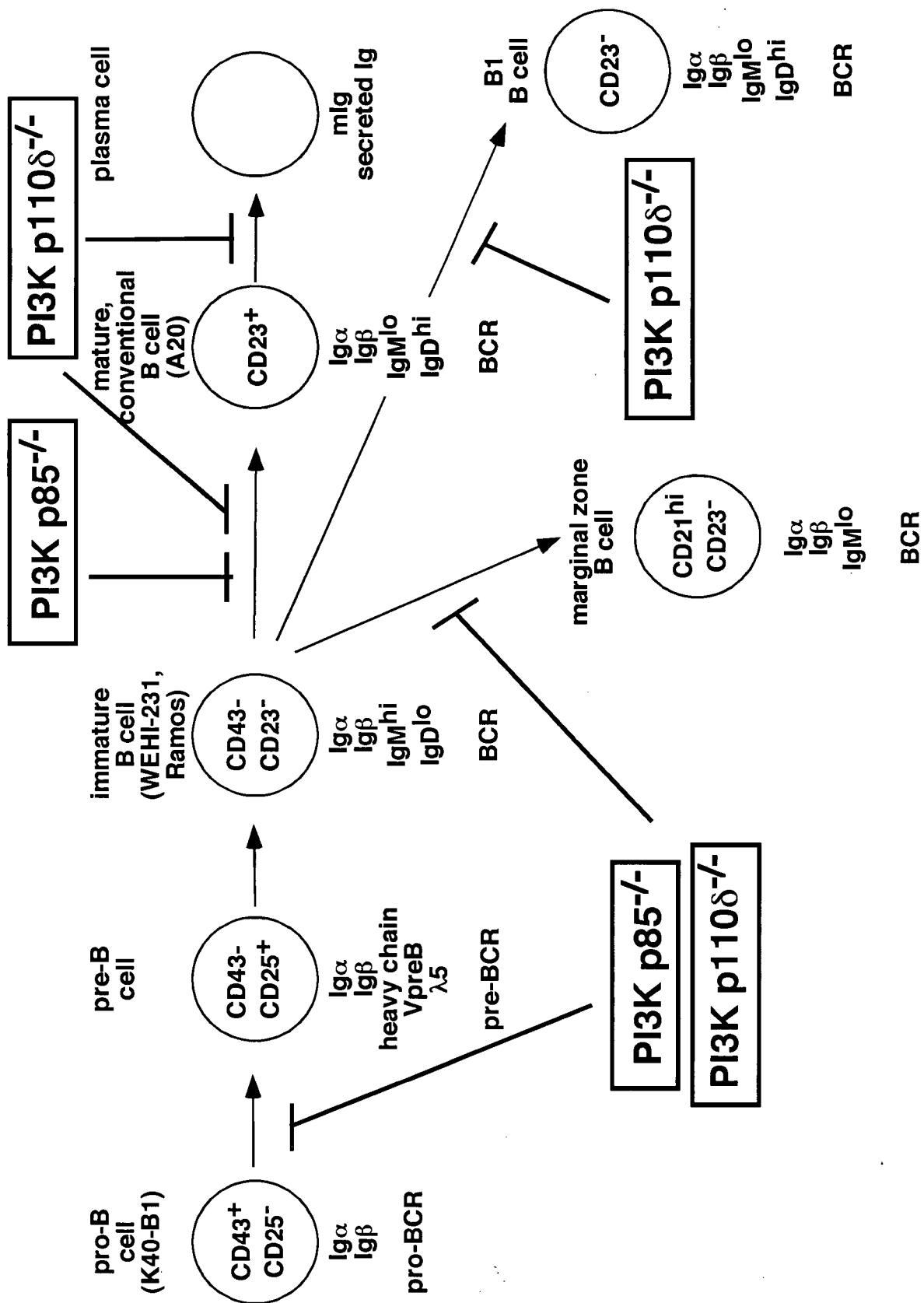
6.1 Summary of the thesis

In this thesis I investigated the regulation and function of PI3K and two of its downstream targets, the Akt and GSK-3 kinases, in the context of BCR signalling in B lymphocytes. I presented evidence showing that the BCR regulates the transcriptional activator β -catenin via a PLC- γ /PKC/GSK-3 pathway that is partially dependent on PI3K. I have also shown that the Rap GTPases negatively regulate the BCR-induced activation of the PI3K/Akt pathway as well as the ability of Akt to suppress the FKHR/p27^{KIP1} pro-apoptotic signalling module. Lastly, I used microarray technology to identify novel gene targets of Akt, an important mediator of cell survival in B cells. The findings that I presented in this thesis have contributed to the understanding of the regulation and targets of the PI3K pathway in B cells and will hopefully contribute to the overall understanding of the regulation of B cell development and activation, important processes for the proper functioning of the immune system.

6.2 B cell development and activation

The development of B cells from early progenitors to terminally differentiated antibody-producing plasma cells is defined by a series of stages that are regulated by signals through the BCR and other receptors (155,383) (Fig. 6.1). The first committed progenitor of the B cell lineage is the pro-B cell which expresses the Ig α /Ig β invariant chains of the BCR at the cell surface in the absence of mIg. The Ig α / β appears to be associated with other

Figure 6.1 The role of PI3K in B cell development. B cells go through multiple stages of development identified by the expression of various markers on their cell surface (indicated within the cell) and by the expression of different forms of the BCR (indicated beneath the cell). The stage that is represented by the cell lines used in this thesis is indicated in parentheses. The stage at which B cell development is blocked due to the absence of PI3K (p85 α regulatory subunit or p110 δ catalytic subunit) is indicated.



polypeptides of the surface of pro B cells (201,221). As shown in figure 3.3 and by Nagata *et al.* (221), engagement of the pro-BCR leads to activation of at least a subset of the signal transduction pathways activated by the mature BCR. Expression of a rearranged heavy chain in association with the surrogate light chains (VpreB and $\lambda 5$) and the Ig α /Ig β heterodimer is a marker of the transition to the pre-B cell stage. Expression of the pre-BCR at the cell surface, and presumably the signals transduced from this receptor, are essential for progression out of the pre-B cell stage (384-386). Both pro- and pre-B cells are found in the bone marrow where their survival and development is dependent on stimuli and cell-cell contact from their interaction with stromal cells and other cells in the bone marrow environment (383).

The transition from the pre-B cell to the immature B cell is accompanied by rearrangement of the light chain locus and expression of the light chain. Association of the light chain with the heavy chain and the Ig α /Ig β heterodimer generates the mature BCR. Immature B cells express BCRs containing membrane bound IgM (mIgM), one of the immunoglobulin isotypes expressed by B cells. Encounter of autoantigen by an immature B cell in the bone marrow results in receptor editing to prevent autoreactivity of the B cell (387). If receptor editing has failed to prevent autoreactivity, encounter of autoantigen by the immature B cell promotes the apoptosis of the autoreactive B cell and is one mechanism to prevent autoimmunity (388). Immature cells with successful rearrangements migrate to periphery where they undergo the transition to mature B cells. The transition from the immature to mature stage is defined by the appearance of the surface expression of receptors containing the mIgD immunoglobulin isotype and decrease in mIgM-expressing receptors (383).

Recognition of antigen by the BCR on the mature B cell stimulates proliferation and subsequent differentiation of B cells into antibody-producing plasma cells (4). B cells that have been activated via their interactions with antigen and with activated T cells can undergo further differentiation events. These include affinity maturation of variable regions of the Ig genes to increase the affinity of the BCR for antigen, isotype switching which results in the expression of other mIg isotypes such as mIgG, mIgA and mIgE and the induction of long-lived memory B cells (4).

6.3 Role of the PI3K signalling pathway in B cell activation and development

Genetic deletion studies of PI3K have revealed the involvement of this signalling pathway at multiple points in B cell development (Fig. 6.1) (155,314). Disruption of the genes encoding either the p110 α or the p110 β catalytic subunits of PI3K in mice results in embryonic lethality and precludes the analysis of the contribution of these subunits to B cell development or activation (314). However, the p110 δ PI3K subunit is expressed primarily in leukocytes (389,390) and mice deficient in p110 δ are viable and amenable to studies of B cell development and activation (391-393). Similarly, mice deficient in all three p85 α splice variants (p85 α , p55 α , p50 α) (394,395) or just the p85 α variant have been generated (396,397). The p85 α /p55 α /p50 α knock-out mice exhibit a more severe phenotype than the single knock-out mice with respect to liver necrosis but they have similar phenotypes with respect to B cell activation and development and will be not be differentiated from this point forward. None of the PI3K-deficient mice exhibited any deficiency in T cell development or activation.

Mice deficient for expression of the regulatory p85 α subunit of PI3K show a profound block in the pro-B to pre-B transition (394,396). Analysis of p110 δ -deficient mice also revealed a defect in the ability of bone marrow B cells to undergo the pro-B to pre-B transition (391,392). In addition, mice lacking the Btk tyrosine kinase a PI3K effector involved in the activation of PLC- γ , also show a block, albeit less severe, at the pro-B cell stage (398). Thus, functional PI3K signalling is required for progression through the pro-B to pre-B developmental checkpoint.

PI3K signalling also contributes to the immature to mature transition in B cell development (155,314). Both p85 α and p110 δ -deficient mice exhibit a block in the immature to mature B cell transition (391-394,397) indicating that PI3K activity is necessary for progression through developmental checkpoints in the periphery as well as the bone marrow. The Btk-deficient mice show a similar but less severe block in peripheral development (398) suggesting that PI3K promotes B cell maturation via the regulation of other downstream targets in addition to Btk.

Mature B cells can be classified into different subtypes. In addition to the conventional recirculating follicular B cells there are two other distinct subsets of B cells, B1 and marginal zone (MZ) B cells, whose development is also regulated by PI3K function (155,314). MZ B cells and follicular B cells make up the B2 cell population (399). The MZ B cells reside in the spleen at the boundary between the red and white pulp and are a much smaller population than the follicular B cells which make up the follicles and are capable of recirculating (399). B1 cells are found predominantly in the peritoneal cavity, exhibit a restricted V_H repertoire and express antibodies with low affinity and broad specificity that

primarily recognize bacterial polysaccharides (400). It has been suggested that B1 and B2 cells derive from a common progenitor although this remains controversial (400).

Analysis of PI3K-deficient mice has revealed that PI3K is necessary for the development of MZ and B1 B cells. Mice deficient for p85 α showed a considerable reduction in B1 cells (394,397) as did mice deficient for p110 δ (391-393). Lack of p110 δ also resulted in a dramatic reduction in MZ B cells (391-393). Recently PTEN-deficient mice have been generated which have sustained PI3K signalling since PTEN negatively regulates the production of the PI3K lipid products PIP₃ and PI(3,4)P₂ (401). These PTEN-deficient mice have increased numbers of MZ and B1 cells. This supports a role for PI3K signalling in the development of these cell populations. CD19-mediated activation of PI3K may be essential for MZ and B1 B cell development (402), which is not unexpected since CD19 deficient mice also exhibit a defect in marginal zone B cell and B1 cell development (403,404) and CD19 plays a key role in recruiting PI3K to the plasma membrane (45).

The PI3K pathway is also essential for B cell activation. Analysis of p85 α - and p110 δ -deficient mice revealed that the level of serum Ig is reduced in the absence of normal PI3K activity (391,393,394). The production of antibody in response to both T-dependent and T-independent antigens is also impaired in the absence of p110 δ (391-393). Importantly, these groups also found that functional PI3K activity is necessary for anti-Ig-induced B cell proliferation (391-394,397). Therefore, PI3K activity is essential for the proper response of B cells to antigenic stimulation. In support of this conclusion, Donahue and Fruman have recently reported that BCR-induced PI3K activity is necessary for the initial activation of B cells via BCR stimulation and is also required to sustain multiple rounds of proliferation *in vitro* (405).

The presence of functional PI3K is also important for B cell survival (394,405). B cells from p85 α -deficient mice showed increased apoptosis in culture (394). When these cells are stimulated with anti-Ig antibodies, they proliferate normally and do not show increased apoptosis compared to wild-type cells (394). However, inhibition of PI3K activity by Ly294002 treatment results in the death of anti-Ig-stimulated cells after one or two cell divisions (405). This suggests that PI3K activity, but not p85 α , is required for sustained anti-Ig-induced proliferation of splenic B cells. The p85 α -deficient mice still express the p85 β and p55 γ adapter proteins which could compensate for the loss of the p85 α subunit and its p55 α and p50 α splice variants (394) while Ly294002 treatment inhibits the activity of all PI3K enzymes (314).

These genetic studies have thus revealed an essential role for PI3K at multiple points in B cell development, activation and survival.

6.4 The role of PI3K in regulating B cell development and activation at the molecular level

It is clear from the above studies that PI3K activity is essential for B cell development and activation. The contribution of PI3K to these processes can be further examined at the molecular level. In various cell types, PI3K has been shown to regulate cell survival, cell cycle progression and cell growth (41,74). The regulation of these interconnected processes can be attributed to the regulation of various intracellular targets of PI3K signalling including Akt and GSK-3.

The protection of cells from apoptosis by PI3K can generally be attributed to the activation of its downstream target Akt (41). In support of this, activation of Akt has been

shown to be essential for survival of the DT40 B cell line (63). Proposed protein substrates of Akt which could directly influence cell survival include the Bcl-2 family member BAD and the pro-apoptotic protease caspase-9 (41). Phosphorylation of BAD prevents it from associating with and inhibiting the pro-survival functions of the Bcl-2 or Bcl-XL proteins which normally prevent cytochrome c-induced apoptosis (406). However, some studies have indicated that Akt-mediated phosphorylation of BAD does not correlate with cell survival and not all cell types express BAD (407,408). Therefore, the involvement of BAD in the regulation of apoptosis by the PI3K/Akt pathway may be cell type specific. Caspase-9 is a member of the pro-apoptotic caspase cascade and human caspase-9 has been reported to be phosphorylated and inhibited by Akt (322). However, the residue phosphorylated by Akt is not conserved in the mouse, rat or monkey caspase-9 homologues and mouse caspase-9 is not phosphorylated by Akt *in vitro* (409). Therefore, the involvement of Akt in regulating the pro-apoptotic caspase pathway via inactivation of caspase-9 is not likely to be a major anti-apoptotic mechanism. There have been many other recent reports of additional Akt substrates that can potentially influence cell survival. These include the apoptosis signal-regulating kinase-1 (ASK1) (410), the Yes-associated protein (YAP) (411) and tuberin (TSC2) (77,412). Recently, the Akt-mediated phosphorylation of tuberin has been shown to promote cell growth by inhibiting the ability of tuberin to act as a GAP for the Rheb GTPase which is a positive regulator of protein synthesis (413).

Another mechanism by which the PI3K/Akt pathway can influence cell survival is via the Akt-mediated regulation of the forkhead transcription factors and the NF- κ B transcription factor (86,87,90,239). Phosphorylation of forkhead transcription factors inhibits their ability to promote transcription of pro-apoptotic genes including Bim, Fas ligand and p27^{KIP1} (90,291). Akt-mediated phosphorylation of Forkhead transcription factors inhibits their

transcriptional activity by causing them to translocate from the nucleus to the cytoplasm where they are bound by 14-3-3 family proteins (91).

In contrast to its inhibitory effects on forkhead family transcription factors, Akt is a positive regulator of NF- κ B, a transcription factor that regulates the expression of many pro-survival genes. In unstimulated cells, NF- κ B is inhibited by its interaction with the I κ B protein. Phosphorylation of I κ B by the IKK kinase complex results in its proteasome-mediated degradation and exposes NF- κ B's nuclear localization signal, resulting in the translocation of NF- κ B into the nucleus (414). Phosphorylation of IKK by Akt promotes activation of this pathway (86,87,415) and promotes expression of NF- κ B regulated pro-survival genes including c-myc (415) and Bcl-X_L (416). Regulation of gene expression via the actions of Akt on NF- κ B and forkhead family transcription factors is likely to be a major mechanism by which the PI3K/Akt-pathway promotes cell survival.

The PI3K pathway is also an important regulator of cell cycle progression (41,74). Enhancement of PI3K activity results in accelerated cell cycle progression while inhibition of PI3K activity decreases the rate of this process (417,418). Cell cycle progression is coordinated by cyclins and CDKs. CDKs become activated upon association with specific cyclins and promote progression through the cell cycle (374,376). PI3K can positively regulate cyclin D1 levels by promoting its transcription (419) and inhibiting its phosphorylation-induced degradation (420). PI3K mediates its effects on cyclin D1 levels through the activation of Akt and p70 S6 kinase (p70 S6K) (41). Phosphorylation of cyclin D1 by GSK-3 promotes the degradation of cyclin D1 (420), which can be inhibited by the phosphorylation of GSK-3 by Akt on its negative regulatory sites (92). Conversely, the PI3K-mediated activation of p70 S6K can promote protein translation of cyclin D1. In

addition to regulating cyclin D1 levels directly, PI3K can also regulate cyclin-dependent kinase regulators including p21^{CIP1} and p27^{KIP1} via Akt-mediated phosphorylation and regulation of new protein synthesis (74).

Recently, a role for PI3K in controlling cell growth has been described (421). Alvarez et. al. (421) have shown that increased PI3K activity increases the protein synthesis rate in cycling cells via the increased activation of p70 S6K and mammalian target of rapamycin (mTOR) proteins. Both p70 S6K and mTOR regulate the synthesis of components of the ribosomal transcriptional machinery and activation of translational machinery to increase protein synthesis (422,423). Expression of constitutively active PI3K resulted in larger cells while transient increases in PI3K activation resulted in increased cell division times to compensate for the increased protein synthesis rate and to maintain proper cellular size (421). Therefore, PI3K regulates cell growth which is tightly regulated to cell division time in order to maintain the appropriate cell size.

The regulation of mTOR and p70 S6K by PI3K has recently been shown to be mediated through the Akt-mediated suppression of the tumour suppressor tuberin, encoded by the *Tsc2* gene (77,412,413). In normal, unstimulated cells the association of tuberin with the hamartin protein, encoded by the *Tsc1* gene, promotes the GAP activity of tuberin towards the Rheb monomeric GTPase. The action of the tuberin:hamartin complex prevents the Rheb-GTP-mediated activation of mTOR thereby inhibiting protein synthesis (413). Activation of the PI3K pathway promotes the Akt-mediated phosphorylation of tuberin causing the disassociation of tuberin from hamartin (412). This dissociation prevents the tuberin-mediated inhibition of the Rheb GTPase thereby promoting protein synthesis via activation of mTOR and p70 S6K (413). Therefore, PI3K promotes protein synthesis

through the Rheb-mediated activation of mTOR resulting in increased cell growth and proliferation.

Thus, the PI3K signalling pathway could potentially contribute to B cell development and activation in many different ways. Identification of the molecular mechanisms responsible for the effects of PI3K on the different stages of B cell development and activation remain to be fully elucidated.

6.5 Future directions

6.5.1 Identification of the gene targets and the function of β -catenin in B cells

Since β -catenin has been shown to influence cell development, cellular proliferation and oncogenic transformation in other cell types (112,130), β -catenin may also play a role in B cell development, proliferation or survival. Therefore, it is important to determine the function of β -catenin in B cells as β -catenin may be a crucial player in processes that are essential for proper immune system function.

β -catenin is a transcriptional co-activator and engagement of the BCR results in activation of β -catenin-mediated transcription (Section 3.3). However, the genes that are regulated by β -catenin in B cells have not been determined. Identification of the genes regulated by β -catenin in B cells would provide clues to the function of β -catenin in B cells.

A wide variety of β -catenin gene targets have been identified in mammalian cells including *c-myc* (140), *cyclin D1* (141,142), *Notch 1* and *HoxB4* (214), among others (424-430). Regulation of *c-myc* expression in response to BCR stimulation is an important mechanism for the control of apoptosis versus survival in immature B cells, including the WEHI-231 B cell line (431). Prolonged anti-IgM treatment of WEHI-231 cells results in an

initial rapid increase in *c-myc* expression followed by a drastic decline in *c-myc* levels that correlates with growth arrest followed by apoptosis. In contrast, co-stimulation of the BCR and CD40 results in the maintenance of elevated *c-myc* levels which correlates with CD40-mediated inhibition of anti-IgM growth arrest. Transcription of the *c-myc* gene in response to anti-BCR and CD40 signalling is regulated primarily by NF- κ B (432) but the additional β -catenin-mediated regulation of *c-myc* could also promote cell survival. Cyclin D1 is essential for cell proliferation (374) and increases in cyclin D1 expression can promote the proliferation of many types of cells (433-436). β -catenin-mediated upregulation of cyclin D1 expression (142) could therefore promote the proliferation of B cells. β -catenin mediated increases in *Notch* and *HoxB4* expression was observed in hematopoietic stem cells (HSC) (214). Notch is an important pro-proliferative signal while HoxB4 is involved in regulating the self-renewal of HSCs (214). The regulation of any one of these genes in B cells by β -catenin would be an indication that β -catenin functions in the regulation of B cell proliferation or survival.

DNA microarray analysis is an ideal technique by which to identify genes that are regulated by β -catenin in B cells. This method has been used previously to identify β -catenin regulated genes in many different types of cells, including colorectal carcinomas, melanoma and primary hepatocytes (425-430). Expression of a constitutively active and a transcriptionally deficient β -catenin protein has been used previously to identify β -catenin regulated genes in human hepatocytes (425).

β -catenin proteins with mutations in the GSK-3 target sites are not phosphorylated by GSK-3 and do not undergo degradation and can constitutively induce transcription (108). Therefore, a direct way to identify genes that are regulated by increased β -catenin protein

levels in B cells would be to express this type of constitutively active β -catenin protein in a B cell line, such as the WEHI-231 B cell line. Genes that are differentially expressed in cells expressing the mutant β -catenin versus vector control cells could be identified by microarray analysis.

While expressing a mutant form of β -catenin is a gain-of-function approach that would allow us to identify proteins for which elevated β -catenin levels are sufficient to regulate their transcription, a loss-of-function approach in which β -catenin-dependent transcription is prevented would reveal the function of β -catenin in BCR signalling. A truncated mutant form β -catenin has been generated by Levy *et al.* (425) which lacks the transactivation domain of β -catenin and therefore lacks any transactivation activity. This protein acts as a dominant negative by sequestering transcription co-activators of β -catenin such as LEF-1 and TCF, thereby preventing endogenous β -catenin from promoting transcription. Expression of this transactivation deficient β -catenin (TD β -catenin) protein in WEHI-231 cells would be a complementary loss-of-function approach that would identify gene targets that are regulated by β -catenin in response to BCR engagement. Genes that are not regulated in the TD β -catenin-expressing cell line as compared to a vector control cell line may be gene targets of β -catenin in B cells.

Overexpression of a protein of interest is not always achievable in B cell lines such as WEHI-231 since expression of the protein may itself inhibit survival of the cells. Therefore an alternative analysis that does not require the overexpression of mutant proteins is an important consideration. Two novel and specific inhibitors of GSK-3 have recently been described and are now available commercially from BioMol (Plymouth Meeting, PA) (257). Treatment of cells with these compounds would mimic the BCR-induced inhibition of

GSK-3. Microarray analysis could then identify genes that are regulated in response to GSK-3 inhibition. A subset of these genes would be regulated by the GSK-3 mediated increase in β -catenin-dependent transcription. Although direct identification of β -catenin regulated genes would be precluded in this analysis, potential β -catenin regulated targets would be identified in the context of GSK-3 inhibition. Since GSK-3 can also regulate proteins that influence cell cycle and proliferation independently of its regulation of β -catenin (437), identification of GSK-3 related genes in B cells would be useful as an independent analysis as well.

Although Wnt mediated regulation of β -catenin has been shown to promote the survival of pro-B cells (143) the function of β -catenin in more mature B cell types remains to be determined. Analysis of the effects of expressing mutant forms of β -catenin on B cell proliferation and survival would allow us to determine the function of β -catenin in mature B cells.

In addition to identifying β -catenin regulated gene targets, expression of the mutant β -catenin proteins in B cells would allow the direct analysis of the function of β -catenin on B cell proliferation and survival. If β -catenin promotes B cell proliferation then expression of the constitutive active mutant should increase the proliferation rate in the absence of other stimuli. Moreover, if β -catenin promotes cell survival in immature B cells, then expressing the constitutively active β -catenin might inhibit anti-IgM induced growth arrest in the WEHI-231 immature B cell line. Conversely, expression of the TD β -catenin mutant should decrease cell proliferation and may even promote apoptosis if β -catenin normally promotes survival in B cells.

6.5.2 Mechanism of the Rap-dependent inhibition of the PI3K/Akt pathway

In chapter 4, I showed that Rap activation impairs BCR-induced activation of the PI3K/Akt pathway. I also showed that the constitutively active Rap2V12 can associate with PI3K and that the Rap2V12-associated PI3K enzyme activity is reduced upon BCR engagement. I also found that the PI3K p85 regulatory subunit associates constitutively with Rap2V12. Therefore, my findings suggest that the Rap GTPases inhibit PI3K/Akt signalling by binding to PI3K.

How the association of Rap-GTP with PI3K leads to the inhibition of PI3K activity, only in the context of BCR signalling, is not known. One possibility is that BCR engagement induces the association or dissociation of another protein from the Rap-GTP:PI3K complex. For example, Rap-GTP induced association of the PI3K inhibitor RUK (308) could result in the inhibition of PI3K activity. Alternatively, Rap-GTP, in conjunction with other BCR signalling events, may promote the dissociation of a protein that activates PI3K such as Ras (52,197,278,279) or that enhances PI3K activity such as the GTPase PIKE (438). Identification of proteins that associate with the Rap-GTP:PI3K complex in untreated versus anti-Ig stimulated cells may distinguish between these possibilities and identify proteins that mediate Rap-dependent regulation of PI3K.

Since Ras and Rap have identical effector domains (182) and Ras can activate PI3K by interaction with the p110 subunit of PI3K (52,197,278,279), Rap may prevent the Ras-mediated activation of PI3K by binding to PI3K. To test this hypothesis, I expressed constitutively active Rap and Ras along with the p110 subunit of PI3K in HEK293 cells and investigated if the expression of Rap prevented the association of Ras with p110. However, my preliminary data suggest that interaction of Ras and Rap do not compete for association

with p110. Therefore, it is likely that Rap2V12 inhibits the BCR-induced PI3K activation in some other manner.

Identification of proteins that associate with Rap2V12 in B cells could be accomplished in multiple ways. Mass spectrometry could be used to identify proteins that co-precipitate with a FLAG tagged Rap2V12 that can be isolated using immobilized anti-FLAG antibodies. Comparing the FLAG-RAP2V12-associated proteins from anti-Ig stimulated versus unstimulated cells could lead to the identification of proteins that associate with Rap2V12. The BCR-induced association or dissociation of a protein from the Rap2V12 complexes would support the involvement of this protein in the regulation of PI3K activity. Expression of this putative PI3K regulator would then be necessary to test whether it regulates PI3K activity *in vitro*.

Another method to identify a potential PI3K regulatory protein that interacts with Rap2V12 would be to isolate the protein from cell extracts derived from anti-Ig treated cells. Addition of fractionated cell lysates to purified Rap2V12 and PI3K, followed by the analysis of PI3K activity would allow the identification of the fraction containing the Rap-dependent PI3K regulatory activity. Further purification through the use of column chromatography techniques could result in purification of the compound or protein regulating PI3K activity. Protein sequencing would then allow the identification of the PI3K regulatory protein.

I have shown that a similar level of the p85 subunit associated with Rap2V12 is isolated from anti-Ig-stimulated and unstimulated cells but that there is less associated PI3K enzyme activity. Thus, another possible mechanism by which Rap2V12, in combination with a BCR signalling event, could inhibit PI3K activity is by causing the dissociation of the p110 catalytic subunit from the p85 regulatory subunit. I have attempted to test this hypothesis by immunoblot analysis of the Rap2V12 immunoprecipitates. Unfortunately, the available

antibodies against p110 subunits were unable to detect the presence of any of the p110 isoforms tested (p110 α , p110 β and p110 δ) in immunoprecipitates from either anti-Ig-stimulated or unstimulated cells, even though PI3K catalytic activity was present in both. The enzyme assays are clearly more sensitive than immunoblotting with these antibodies. One way to determine if the p110 subunit dissociates from the p85 subunit would be to generate an anti-p110 antibody with increased sensitivity and repeat the p110 immunoblots on the Rap2V12 immunoprecipitates. Generation of an antibody would require purification of the p110 protein followed by injection of the purified protein into rabbits and isolation and characterization of the antibody specificity and reactivity. An alternative method to detect the presence of the p110 subunit in the Rap2V12 immunoprecipitates would be to perform mass spectrometry analysis of these immunoprecipitates as mentioned above. The dissociation of the p110 subunit from the p85 subunit would be detected since peptide sequences from the p110 subunit would be detectable from Rap2V12 immunoprecipitates isolated from unstimulated cell lysates but not isolated from BCR stimulated cell lysates.

A final possibility, that is not exclusive from the mechanisms described above, is that Rap2V12, in conjunction with a BCR signalling event, induces the phosphorylation of Rap2V12 or an associated protein. This phosphorylation event could be responsible for inducing the association of an inhibitor of PI3K or the dissociation of an activator of PI3K. Such a phosphorylation event could be identified by mass spectrometry analysis. The presence of a phosphate induces a shift in the molecular weight of the phosphorylated peptide by 80 kDa. Therefore, identification of this type of shift in response to engagement of the BCR would indicate that a phosphorylation event could regulate the Rap-GTP-mediated inhibition of PI3K activity. It would then be necessary to map the site of phosphorylation on the identified protein and then mutate the phosphorylation site. Overexpression of the PI3K

regulatory protein containing mutated residues at the sites of phosphorylation in B cells should prevent the Rap-GTP mediated decrease in PI3K activity observed upon BCR engagement.

Determining how Rap and PI3K interact is another important factor in elucidating the mechanism for the Rap-GTP-mediated inhibition of PI3K. One way this could be accomplished is to co-express Rap2V12 with tagged forms of the p85 and p110 subunits in COS or HEK293 cells. The interaction of Rap2V12 with either p85 or p110 could be then be determined by immunoprecipitating Rap2V12 and immunoblotting for the p85 or p110 subunit. To map the sites of interaction, truncated versions of the tagged p85 and p110 proteins could then be expressed and examined for interaction with Rap2V12. This analysis could reveal if Rap-GTP interacts with the p85 or p110 subunits or both. This will also allow further functional studies to be performed by selective mutational analysis of Rap binding sites on PI3K to potential distinguish between the effects of Rap-GTP binding and association of other proteins on PI3K signalling.

There may be additional ways in which Rap-GTP could regulate BCR-induced PI3K activity such as sequestering PI3K away from its substrate. This could potentially be accomplished via targetting PI3K to different membrane subdomains such as lipid rafts or to different membrane compartments such as the endoplasmic reticulum. Clearly, elucidating the mechanism of the Rap-GTP-mediated inhibition of PI3K will require a great deal more work.

6.5.3 Further identification and characterization of Akt-regulated gene targets in B cells

Akt is an important pro-survival factor in B cells (63) and, as discussed in Section 5.1, the Akt-mediated regulation of gene transcription can contribute to its pro-survival role. I have identified a number of potential Akt gene targets in B cells by microarray analysis (see chapter 5). Further analysis of a subset of these genes led to the identification of eight genes for which Akt-dependent changes in expression were validated by RT-PCR. Further characterization of the regulation and the function of these eight potential gene targets could reveal if they contribute to B cell survival or proliferation and will be discussed in detail below.

The verification of other potential Akt gene targets in B cells identified by the microarray analysis could lead to the identification of other important survival or proliferation factors. Other potential gene targets would be identified by their reproducible changes across replicate experiments. A short list of potential Akt gene targets identified in this way are shown in Table 6.2. The changes in expression of these genes must then be verified by Real-Time PCR analysis in order to have confidence that they are indeed likely targets of mER-Akt. These genes could then also be subject to further characterization of their function and regulation in B cells as discussed below.

An important first step in the characterization of the regulation of genes is to determine if they are regulated in response to the activation of surface receptors as well as in response to the activation of Akt. Determining if the BCR or other surface receptors, such as CD40 or chemokine receptors, regulate the expression of these genes in the same manner as activation of mER-Akt will reveal if the changes in gene expression also occur in response to physiological stimulation of B cells. Defining the time course and magnitude of the change

Table 6.1 Potential mER-Akt gene targets for future analysis

Ten genes with reproducible changes in expression across replicate experiments and with a 2-fold or greater increase or decrease in expression in response to mER-Akt activation at 3 h or 20 h were selected. These genes were sorted into categories based on their cellular function using the simplified gene ontology function in GeneSpring and by eye. The mean fold change of the treated sample over the untreated sample is shown (normalization method 2). The mean expression levels of 3 independent replicates is shown the range indicated when available. Decreases in gene expression are indicated by a negative (-) symbol preceding the magnitude of the fold change. The normalized expression level was determined by setting the gene at the 50th percentile to 1 and determining the relative expression level of every other gene. (*nd= no detectable expression)

Gen-Bank ID	Description	Functional Category	Normalized expression level in untreated sample	fold change at 3h	Normalized expression level at 3 h	fold change at 20h	Normalized expression level at 20 h
AU021146	Mus musculus apoptosis activator Mtd (Mtd) mRNA_complete cds	apoptosis	0.494 (0.425 to 0.563)	7.29	3.601 (3.321 to 3.881)	nd	nd
AW546817	Mus musculus hemoglobin alpha_adult chain 1 (Hba-a1) mRNA	blood	1.114 (0.736 to 1.492)	4.01	4.464 (4.345 to 4.583)	nd	nd
AU018652	Rattus norvegicus microtubule-associated protein 1A (MAP1A)	Cyto-skeleton	1.98 (1.925 to 2.035)	-1.44	1.377 (1.168 to 1.586)	2.39	4.741 (4.571 to 4.911)

AW55 6120	myosin regulatory light chain isoform C [rats_ Sprague- Dawley_	Cyto- skeleton	2.476 (2.156 to 2.797)	2.56	6.325 (6.063 to 6.587)	2.68	6.643
AW55 8264	Mus musculus zinc finger protein (Peg3) mRNA_ complete cds	DNA binding	2.034 (1.841 to 2.227)	2.40	4.89 (4.815 to 4.964)	nd	nd
AU022 716	Homo sapiens single-stranded- DNA-binding protein (SSBP2)_ mRNA	DNA binding	2.045 (1.874 to 2.216)	2.85	5.82 (5.590 to 6.05)	3.62	7.41 (0.696 to 14.123)
AW55 4748	Mus musculus MHC class I heavy chain precursor (H- 2D(b)) mRNA	immunity	2.801 (2.691 to 2.910)	-1.83	1.528 (1.446 to 1.610)	2.10	5.884 (5.734 to 6.035)
AW55 1816	Rattus norvegicus liver nuclear protein p47 mRNA	nuclear	1.213(1.063 to 1.363)	2.09	2.54 (2.381 to 2.699)	-3.13	0.387 (0.172 to 0.601)
N/A	Mus musculus DNA_ 3' flanking region of interleukin 12 receptor beta	receptor	8.5 (2.47 to 2.563)	1.27	3.193 (3.038 to 3.348)	-8.50	0.296 (0.061 to 0.530)
AW55 1149	Mus musculus protein kinase inhibitor gamma (PKIg) mRNA_ complete	signal trans- duction	1.281 (0.924 to 1.583)	1.10	1.408 (0.904 to 1.991)	2.57	3.295 (3.293 to 3.297)

in gene expression in response to Akt activation and receptor engagement is a major component of this characterization. These analyses should be performed on both the gene transcripts and the proteins themselves since protein level does not always correlate with the level of mRNA transcript. Determination of transcript level can be performed by Quantitative Real-Time PCR or Northern blot analysis while determination of the protein level can be performed by immunoblot analysis.

Analysis of the promoter region of the Akt-regulated gene could provide insight into the regulation of the gene by identifying potential binding sites for transcription factors. This can be accomplished by fusing the 5' untranslated region of the gene to a reporter gene such as luciferase or β -galactosidase and transfecting the resulting plasmid into a B cell line. Insertion of a region of DNA containing the promoter into such a plasmid will allow expression of the reporter protein upon activation of the signalling pathways responsible for the regulation of the endogenous gene. The minimal region required for regulation of gene expression can be determined by deletion analysis of the larger sequence of DNA containing the promoter region. Mutational and truncation analysis of the promoter region will allow the identification of known transcription factor binding sites as well as novel binding sites thus providing an indication of the upstream regulatory mechanisms.

Further analysis of the signalling pathways used by Akt or other surface receptors to regulate the level of gene expression should also be performed. A loss-of-function analysis of pathways that are activated by Akt will indicate which pathways are necessary for the change in gene expression in response to Akt. This can be accomplished by treating cells with inhibitor molecules that block activation of downstream effectors of Akt or BCR activation, such as the NF- κ B transcription factor, the MAPK pathways or the PI3K

pathway. In contrast, the gain-of-function analysis will indicate if the activation of a downstream effector of Akt is sufficient to induce the change in gene expression. For example, expression of constitutively active downstream effector could result in similar changes in gene expression if activation of that component by Akt normally regulates the observed changes in gene expression. Another important analysis is to determine whether regulation of a specific gene requires the transcription of new proteins. Treatment of cells with cycloheximide blocks *de novo* protein synthesis. Therefore, if treatment with cycloheximide prevents the Akt-induced change in gene expression the production of new proteins is required. This indicates that the regulation of gene expression is indirectly through the Akt-mediated regulation of other protein(s). In contrast, Akt regulates gene expression by regulating the activity of a pre-existing transcription factor such as NF- κ B or forkhead proteins if cycloheximide treatment has no effect on the Akt-induced changes in gene expression. The combination of these approaches will allow the elucidation of the mode of regulation in response to Akt or surface receptor activation.

Lastly, the function of the proteins encoded by the Akt-regulated genes in B cells should be determined. Analysis of cell death and proliferation in response to overexpression of these Akt-regulated proteins in B cells would indicate if they contribute to the pro-survival functions of Akt. Other important B cell processes that could also be examined include B cell adhesion or migration since the PI3K pathway is involved in the regulation of these processes in leukocytes (439).

6.6 Concluding statement

In this thesis I have investigated the signalling pathway used by the BCR to regulate the transcriptional activator β -catenin. I have shown that the Rap GTPases negatively regulate the BCR-induced activation of the PI3K/Akt pathway resulting in increased sensitivity of an immature B cell line to anti-Ig-induced cell death. I have also identified novel gene targets of Akt, an important mediator of cell survival in B cells. These findings have already contributed to the understanding of the regulation and targets of the PI3K pathway in B cells and will hopefully contribute to the overall understanding of the regulation of B cell development and activation. Moreover, the findings presented here have opened up new areas of investigation into the role of β -catenin in mature B cells, into a novel mechanism for the negative regulation of PI3K and into the roles of many different Akt regulated genes in B cells.

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