ABSTRACT

RasGRPI is a guanine nucleotide exchange factor (GEF) that activates Ras GTPases downstream of the B and T lymphocyte antigen receptors. RasGRPI is a critical regulator of T cell homeostasis, and contributes to the maintenance of T cell-mediated tolerance in the host. Although Ras signalling is important during B cell development, relatively little is known about a role for RasGRPI in B cells, or its contribution to B cell receptor (BCR) signalling. RasGRPI expression is detected in the bone marrow and some B cell lines including the murine immature B cell line WEHI 231. The WEHI 231 cell line, which mimics immature B cell responses to self-antigen by undergoing cell cycle arrest and apoptosis in response to antigen stimulation, was used to determine whether RasGRPI has the ability to modulate BCR-mediated responses in B cells.

WEHI 231 cell populations with increased RasGRPI expression (RasGRPI\textsuperscript{hi} cells) were generated by retroviral transduction. A two-fold increase in RasGRPI protein levels correlated with increased Ras activity. A three-fold increase in the fraction of cells undergoing apoptosis was detected in RasGRPI\textsuperscript{hi} cells following BCR ligation, compared with control cells. Mutation of the GEF domain of RasGRPI, which is required for Ras activation, prevented the protein from sensitizing WEHI 231 cells to BCR-induced apoptosis. Expression of constitutively active Ras GTPases was sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis. These results suggest that RasGRPI acts as a positive regulator of BCR signalling, and has the ability to sensitize WEHI 231 cells to BCR-induced apoptosis via activation of Ras GTPases.

Although increased RasGRPI expression caused sustained activation of the Ras effectors ERK1/2, this effect was not required for the ability of RasGRPI to sensitize WEHI 231 cells to BCR-induced apoptosis. Instead, increased RasGRPI expression was found to inhibit the NF-κB pathway, a critical regulator of life and death decisions in WEHI 231 cells. While stimuli that activate NF-κB prevented increased BCR-induced apoptosis of RasGRPI\textsuperscript{hi} cells, inhibition of NF-κB was sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis. These results suggest that RasGRPI sensitizes WEHI 231 cells to BCR-induced apoptosis by causing down-regulation of the NF-κB pathway.
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<table>
<thead>
<tr>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ARF</td>
<td>adenosine diphosphate ribosylation factors</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker protein</td>
</tr>
<tr>
<td>BSAP</td>
<td>B cell-specific activator protein</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>cDNA</td>
<td>complement deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CKIs</td>
<td>cyclin-dependent kinase inhibitors</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DN</td>
<td>double-negative</td>
</tr>
<tr>
<td>DP</td>
<td>double-positive</td>
</tr>
<tr>
<td>EBF</td>
<td>early B cell factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine biphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of IκB</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol (1,4,5) triphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosomal entry site</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB Essential MOdulator</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cell</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
</tbody>
</table>
PDK-1  PI (3,4,5) dependent kinase
PH    pleckstrin-homology
PI    propidium iodide
PI-3 kinase  phosphatidylinositol-3 kinase
PI (4,5)P₂  phosphatidylinositol 4,5 biphosphate
PLC   phospholipase C
PLD1  phospholipase D1
PKA   cAMP-dependent protein kinase
PKC   protein kinase C
PMA   phorbol myristate acetate
pRb   retinoblastoma protein
RAG   recombination activating genes
RalBP  Ral binding protein
RBD   Ras-binding domain
SLC   surrogate light chain
SP    single-positive
TCR   T cell receptor
TK    thymidine kinase
TNF   tumour necrosis factor
TRAF  TNF receptor-associated factor
VDJ   variable, diversity and joining immunoglobulin gene regions
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CHAPTER 1: INTRODUCTION

1.1 RASGRP1 IS A GUANINE NUCLEOTIDE EXCHANGE FACTOR THAT ACTIVATES RAS FAMILY GTPASES

1.1.1 Structure and biology of the classical Ras GTPases

1.1.1.1 Introduction

Ras family proteins are membrane-bound, low molecular weight GTPases that become transiently activated to propagate cell-surface receptor-derived signals mediated by growth factors, neurotransmitters and antigen binding, among others (Fig. 1-1) [1, 2]. The conformation state of Ras GTPases is regulated by two protein families: the GTPase activating proteins (GAP), which increase endogenous GTPase activity by 100-fold, thereby enabling GTP hydrolysis and the adoption of an inactive GDP-bound form [3, 4]; and guanine nucleotide exchange factors (GEF), which promote the release of GDP from the inactive Ras and enable the GTPase to adopt an active conformation by binding GTP [5].
The classical Ras family includes the highly conserved H-Ras, N-Ras, as well as K-RasA and K-RasB, the latter two being splice variants of a single K-Ras gene [6]. The classical Ras proteins are 21-kDa, 189 amino acid proteins (188 for K-RasB) that contain an N-terminal effector region that is highly conserved within the Ras family of small GTPases, while the C-terminus bears a sequence that directs their localization [7]. Despite differential expression of the three Ras genes in various tissues, as well as during gestation, the four classical Ras proteins seem to overlap considerably in terms of function [7]. For example, of the three Ras genes, only K-Ras seems to be required for proper murine embryo development, since deletion of the K-Ras gene is embryonic lethal, whereas deletion of H-Ras or N-Ras individually or in combination has very little effect on development [8, 9]. Nevertheless, signal transduction by the classical Ras GTPases is thought to contribute to several cellular responses that include developmental progression, differentiation, survival, proliferation and apoptosis, depending on cell type and context [10-13]. Although several putative effectors of Ras have been described, the best studied are the Raf family members, which include...
Raf-1, B-Raf and A-Raf, as well as the lipid kinase phosphatidylinositol-3 (PI-3) kinase and the Ral GTPase activator RalGDS [1]. These will be described in more detail below.

1.1.1.2 GTPase and effector binding domain structure and function

The N-terminal 165 amino acids of the classical Ras GTPases contain the critical domains required for GTP binding, GTPase function, and the effector binding domain [14]. The first 86 amino acids encode the switch I (residues 30-37) and switch II (residues 60-76) regions, which are thought to confer the ability of the Ras protein to change their conformation into the active or inactive form, depending on nucleotide-binding state (Fig. 1-2) [15]. Overlapping the switch I region is the Ras effector binding domain, spanning amino acids 32-40 [16]. A conformational change at the switch I region is critical for effector binding, although amino acids in the switch II region have been found to contribute as well [16]. Mutational analysis of the effector binding domain has revealed a role for specific residues in binding individual Ras effectors. For example, a point mutation at residue 38 prevents Ras from binding to PI-3 kinase or RalGDS but enables binding to Raf-1, while a Ras mutant with a substitution at residue 40 is only capable of interacting with PI-3 kinase [17]. Another critical residue in the N-terminal portion of the classical Ras family members is the serine at position 17. Substitution for an asparagine residue results in a dominant-negative form of Ras (N17Ras), which has the ability to bind guanine nucleotide exchange factors but is unable to adopt an active conformation and bind to effectors, even when GTP-bound [18, 19]. Activating mutations at residues 12, 13 and 61 have been detected in several human malignancies, and have been found to cause tumorigenic transformation of fibroblasts [20]. Mutation of these residues is thought to prevent interaction with GAP proteins, and therefore results in constitutively active Ras GTPases [20, 21].
<table>
<thead>
<tr>
<th>GTPase</th>
<th>% H-Ras identity (N-terminal)</th>
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</tr>
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<td>TC21</td>
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<tr>
<td>M-Ras</td>
<td>62</td>
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<td>No</td>
</tr>
</tbody>
</table>

Figure 1-2. Ras GTPase structure and divergence within the classical and R-Ras GTPase families. The classical and R-Ras GTPases have a high degree of homology within their N-terminus, and vary in their C-terminal modification pattern. Adapted from Reuther, 2000 [22].

1.1.1.3 C-terminal modifications affect Ras GTPase localization

The C-terminus of classical Ras proteins contains a 24 amino acids (23 for K-RasB) region referred to as the hypervariable region (due to very low homology between individual Ras members). Proper localization of Ras GTPases to membranes is mediated in large part via a C-terminal motif composed of a cysteine residue, followed by two aliphatic residues and a single serine or methionine residue, referred to as the CAAX motif, which is the target of posttranslational modifications [23-25]. The first modification occurs in the cytosol, where a prenyl group is added to the cysteine of the CAAX motif by a soluble enzyme termed prenyltransferase [26-28]. A prenylation signal is sufficient to target the Ras protein to the outer membrane of the ER and Golgi, where it undergoes further modification [26, 29, 30]. A polybasic domain comprised of six consecutive lysines present in the C terminus of K-RasB, or the addition of one or two palmitoyl lipid group(s) to cysteine residues in the C terminus of N-Ras, K-RasA, and H-Ras also affect specific localization within membrane microdomains (Fig. 1-2) [31, 32].
Variations in posttranslational modifications are thought to account, at least in part, for differences observed in trafficking and localization of different Ras members. Studies using microscopy and brefeldin A - an inhibitor of vesicular transport and secretion [33] - have shown that while H- and N-Ras employ the exocytic pathway to migrate to the plasma membrane, K-RasB migrates independently of this pathway, and at a much faster pace [30, 34]. Furthermore, whereas K-RasB is constitutively excluded from sphingolipid- and cholesterol-rich membrane microdomains (termed "lipid rafts"), H-Ras is located in lipid rafts in its GDP-bound, inactive state but becomes excluded from these following activation [35-37]. However, despite both being excluded from lipid rafts in their active state, H-Ras and K-Ras seem to be associated with distinct raft-excluded sites within plasma membranes [38, 39]. These differences in localization may constitute an additional regulatory mechanism, whereby microlocalization of individual Ras members could affect their ability to become activated by individual GEFs, as well as their potential to interact with and activate different effector pathways. Ras GTPase activity is generally thought to occur exclusively on plasma membranes. However, several recent studies have demonstrated that Ras activation and signalling has the potential to occur on endosomal membranes as well [40-42]. The extent to which this contributes to Ras signalling and the specific biological significance of these observations is currently under intense investigation [43].

1.1.1.4 The R-Ras GTPases: structure and homology to the classical Ras proteins

The R-Ras family of small GTPases, which include R-Ras, TC21 (R-Ras2) and M-Ras (R-Ras3), share about 55% amino acid identity with the classical Ras family members (Fig. 1-2). R-Rases bear a CAAX motif at their C-terminus and are also prenylated before they become associated with membranes (Fig. 1-2) [22]. Furthermore, R-Ras family members share some of the regulatory proteins associated with the classical Ras GTPases, including GEFs and GAPs [44]. Although similar to the classical Rases, relatively little is known about specific roles for these proteins.
R-Ras was initially cloned with a low-stringency H-Ras probe, and bears an amino-terminal 26-residue extension resulting in a 23 kDa protein [45]. The N-terminal effector-binding of R-Ras shares about 62% identity with H-Ras, although it is much less efficient at causing transformation of fibroblasts than are the classical Ras family members [46]. This is thought to be due to the relatively weak ability of R-Ras to interact with and activate the Raf pathway, although its PI-3 kinase pathway activation potential is equivalent to that observed in H-Ras [47].

TC21 is a 21 kDa protein whose effector-binding domain shares 69% identity with that of H-Ras, and is able to transform fibroblasts with a phenotype that is indistinguishable from that caused by the classical Ras proteins [48, 49]. Activating mutations of TC21 have also been found in a variety of tumour cell lines, indicating that this protein has the potential to contribute to human oncogenesis [50-52]. The effector mechanism used by TC21 involves PI-3 kinase activation, and although TC21 appears to bind to both Raf-1 and B-Raf, it is not clear whether it can cause their activation [49, 53-56].

M-Ras is a 29 kDa protein that bears a polybasic group in its C-terminus, similar to that seen in K-RasB [57]. M-Ras appears to share GEFs and GAPs with the classical Ras GTPases, although the effectors of M-Ras are largely uncharacterized [44]. These may include the RalGDS-related proteins Rif and RPM/RGL3, the putative tumour suppressor Nore-1 as well as the cell junction regulator AF6 [44, 58, 59]. M-Ras does possess the ability to weakly bind to and activate Raf family members, resulting in weak transformation of fibroblasts [58].

A high degree of homology, as well as overlap between the ability of classical and R-Ras GTPase family members to activate downstream effectors suggests some functional redundancy within this group [60]. However, with new effectors being constantly discovered, and implications for a role for Ras GTPases in cellular events ranging from cell growth to development and apoptosis, further studies will ultimately be required to enable the characterization of individual roles played by each mammalian Ras family member.
1.1.2 Activation of Ras proteins by GEFs

1.1.2.1 Mechanism of guanine nucleotide release

Guanine nucleotide exchange factors that activate Ras family members contain a highly conserved catalytic domain termed the GEF domain, which is homologous to that found in the yeast exchange factor CDC25 [61]. This domain is composed of several alpha-helical domains, and contains basic residues that are critically important in mediating interaction with acidic residues located in the switch II region of Ras proteins [61]. GEFs promote Ras activation by forming a complex with an inactive GDP-bound Ras protein, and facilitating the release of GDP by stabilizing the nucleotide-free form of the protein [5, 62, 63]. The crystal structure of Sos-1 bound to Ras has shown that the interaction between an alpha-helix of Sos causes a significant displacement of the switch 1 region of Ras [63]. This effect is thought to distort the switch 2 region and cause destabilization of the nucleotide phosphate-magnesium ion interaction, thereby enabling release of the nucleotide [63]. The nucleotide-free Ras protein is then able to bind GTP, which is 10 times more abundant than GDP in the cell, resulting in dissociation of Sos from Ras and adoption of an active conformation [63]. This reversible catalytic step thus regulates the maintenance of an equilibrium between GDP- and GTP-bound Ras proteins [62].

1.1.2.2 Ras family members are activated via three families of GEFs

Three families of GEFs with divergent regulatory mechanisms can promote GDP release from Ras GTPases: Sos (Sos-1 and Sos-2), RasGRF (RasGRF-1 and -2), and RasGRP (RasGRP1 through 4) (Fig. 1-3). Sos proteins are ubiquitously expressed and are regulated via a C-terminal proline-rich region that directs recruitment to cell surface receptors via interaction with the SH3 region of the adaptor protein Grb2 [1]. Sos-1 and -2 also bear an N-terminal lipid-binding pleckstrin-homology (PH) domain [1]. RasGRF-1 and -2 are found mostly in brain tissue and are
thought to become activated primarily in response to elevated calcium levels via a calmodulin-binding IQ domain, thereby linking calcium signalling to Ras activation in neurons [64, 65]. The RasGRP family of exchange factors are defined by the presence of a C1 domain, which enables their recruitment to diacylglycerol (DAG)-rich membranes as a result of phospholipase C (PLC) activation [60]. Mammalian RasGRP family members are predominantly expressed in the brain and haemapoietic tissues [60]. A RasGRP-like homolog has been detected in C. elegans using GenBank homology search, suggesting that RasGRPs were present prior to the evolution of the immune system (R. Kay, unpublished observation).

As exchange factors differ in their regulatory mechanisms, they also differ in their ability to bind and activate Ras family members. For example, whereas Sos-1 binds to and activates the classical Ras proteins as well as M-Ras, it cannot activate R-Ras or TC21 [60]. In contrast, RasGRP1 has been shown to activate all three classical Ras proteins as well as the three R-Ras family members, albeit with varying strength [60]. Furthermore, unlike the RasGRP family members, Sos and RasGRF family members are also able to activate Rho-family GTPases, via a Dbl homology (DH) domain (Fig. 1-3). As a result, both the cell type and the nature of the stimulus have the potential to determine which exchange factors become activated, thereby specifying the activation kinetics (duration and intensity) of individual Ras family members. Furthermore, studies performed in fission yeast have shown that exchange factors have the potential to direct the effector pathway utilised by an individual Ras GTPases [66], potentially adding yet another level of complexity to Ras regulation and the activation of its effector pathways.
Figure 1-3. Ras GTPases are activated via three families of guanine nucleotide exchange factors. DH: Dbl homology domain; PH: pleckstrin homology domain; GEF: guanine nucleotide exchange factor homology domain; PxxP: proline-rich region; IQ: calmodulin-binding motif; EFH: EF-hand motif; C1: DAG-binding C1 domain; α-Helix: alpha-helical domain.

1.1.2.3 RasGRP1 structure and regulation

RasGRP1 is a ~90-kDa protein whose major structural components include an N-terminal GEF domain and a pair of calcium-binding EF-hands, as well as a C-terminal DAG/phorbol ester binding C1 domain and alpha-helical motif (Fig. 1-3) [67-69]. RasGRP1 expression is predominantly found in the mammalian brain as well as lymphoid tissues and cell lines [67-69]. RasGRP1 has the ability to activate the classical Ras GTPases as well as the R-Ras family members, but not the Ras-like proteins Rap1, RhoA or RalA [44, 67-69]. The C1 domain of RasGRP1 binds DAG and phorbol esters with high affinity, which enables recruitment of the protein to DAG rich membranes where they can activate Ras family members, [41, 42, 69, 70]. RasGRP1 activates Ras GTPases both at the cell surface and on endomembrane structures, such as the Golgi apparatus [41, 42, 69, 70]. DAG is generated in membranes following receptor-mediated activation of PLC enzymes, which convert phosphatidylinositol 4,5 biphosphate (PI (4,5)P2) to DAG and inositol (1,4,5) trisphosphate (IP3) [71]. RasGRP1 therefore has the potential to link any cell surface receptor that activates PLC enzymes to Ras activation.

EF-hand motifs are composed of two α-helices bearing a calcium-binding site and typically act as calcium sensors that modulate protein activity [72]. RasGRP1 has two EF-hands, one of which has been shown to bind calcium [67]. Treatment of RasGRP1-transfected 293T cells with a
calcium ionophore (to mimic intracellular calcium mobilization) marginally augments Ras activation [68]. In contrast, the EF-hands are not required for the ability of RasGRP1 to cause transformation of fibroblasts [69]. Thus the nature and extent of the contribution provided by the EF-hands to RasGRP1 activity is currently unclear. RasGRP1 also bears an alpha-helical domain in its C-terminus that has the potential to mediate protein-protein interactions [69]. However, deletion of this C-terminal alpha-helical domain does not affect RasGRP1 localization to endomembranes in COS cells, nor does it prevent transformation of fibroblasts [42, 69]. Therefore whether the C-terminal alpha-helix of RasGRP1 mediates protein-protein interactions, and whether it contributes to localization and activation of RasGRP1 remains to be determined.

1.1.3 Characterized Ras effector pathways

1.1.3.1 Signalling via the Raf pathway

1.1.3.1.1 Raf family kinase structure and activation

Raf family kinases are serine/threonine kinases which, upon activation of Ras GTPases, translocate form the cytosol to the plasma membrane where they become activated [1]. The mammalian Raf kinase family is composed of three isoforms with overlapping functions that are termed A-Raf, B-Raf, and Raf-1, the latter being the most extensively studied [73]. Each Raf kinase bears an N-terminal regulatory domain composed of two conserved regions (CR-1 and CR-2). These regions contains two separate domains required for proper interaction with Ras GTPases: the Ras binding domain (RBD, residues 51-131); and a cysteine-rich domain (CRD, residues 139-184) (Fig. 1-4) [74]. The C-terminal portion of Raf family members (CR-3) contains a conserved kinase domain whose activity is regulated via phosphorylation [75, 76]. Although Ras GTPases are responsible for the initial recruitment of Raf kinases to membranes, activation of Raf proteins is governed by a complex set of regulatory steps that involve phosphorylation, de-phosphorylation
and interaction with several proteins. The regulation of Raf-1 will be summarized to illustrate the complexity of Raf family member regulation.

![Diagram of Raf-1 structure](image)

**Figure 1-4. The structure of Raf-1.**
CR: conserved region; RBD: Ras-binding domain; CRD: cysteine-rich domain; S259: serine at amino acid position 259.

In its inactive cytosolic form, Raf-1 is basally phosphorylated on serines at positions 259 and 621, which enables binding to the regulatory protein 14-3-3 (Fig. 1-4) [77-80]. Interaction of Raf-1 with 14-3-3 is essential both for the repression and the activation of Raf-1 [77-80]. Activation of Ras GTPases enables binding and recruitment of Raf-1 to plasma membranes, where it subsequently becomes phosphorylated on serine and tyrosine residues located on the N-terminal portion of the catalytic domain, by kinases that include PAK-3 and JAK-2 [76, 81-83]. However before Raf-1 can be phosphorylated and activated, an inhibitory phosphate group at serine residue 259 must be removed, a step catalyzed by protein phosphatase 2A [84, 85]. An active conformation is then stabilized by 14-3-3, and subsequent re-phosphorylation of serine 259 by cAMP-dependent protein kinase (PKA) results in transition back to its inactive state [80, 86]. Thus, an active form of Raf-1 can be generated by targeting it to the plasma membrane via fusion with a prenylation signal [87, 88], or alternatively, by deleting its N-terminal auto-inhibitory region [89-91].

1.1.3.1.2 *The Raf/MEK/ERK signalling cascade*

Raf family members serve as critical effectors of Ras GTPases by activating two dual-specificity MAPK kinases, MEK1 and MEK2, which recognize and phosphorylate the threonine and tyrosine residues in a TEY motif present in ERK1 and ERK2 [92]. ERK1/2 are proline directed kinases that
activate a multitude of downstream effectors, including protein kinases and transcription factors [93]. These include the ribosomal S6 kinases RSK1 and RSK2, which can phosphorylate the cAMP responsive element binding protein (CREB) involved in the activation of several genes that regulate immune function and the formation of long-term potentiation in the nervous system [93].

Transcription regulators activated through direct phosphorylation by ERK1/2 include the Ets-family transcription factors Ets-1, Ets-2 and Elk-1, which regulate genes involved in a variety of cellular responses including cytokine production, cell cycle progression and apoptosis [93]. ERK-1/2 play a critical role during cell cycle entry, a process regulated to a great extent by cyclin-dependent kinases (CDK). Activation of the MEK/ERK pathway promotes entry into the cell cycle via at least two independent mechanisms: by increasing expression of the cyclin-dependent kinase activator cyclin D1, via CREB- and Elk-1-mediated transcription of the transcription factor c-Fos; and by modulating the expression and degradation of CDK inhibitors p21\(^{Waf1}\) and p27\(^{Kip1}\) [94]. The RAF/MEK/ERK pathway has been shown to play a crucial role in several cellular processes, including thymocyte development [12], growth and differentiation of neurons [95], as well as the transformation of fibroblasts [1], which underscores the great potential of this pathway to modulate cellular responses.

1.1.3.1.3 MEK/ERK-independent Raf signalling pathways

The best characterized signaling cascade activated by Raf family members is by far the MEK/ERK pathway. However, several recent studies have addressed the possibility that Raf family members have the ability to signal independently of MEK. Initial studies found that activated Raf-1, but not activated MEK, is able to promote neuronal differentiation, and that an activated Raf mutant that lacks the ability to activate MEK is still able to activate several downstream effector pathways [96, 97]. One putative effector cascade of Raf signalling involves activation of the NF-κB family of transcription factors, which regulate cell survival [98]. However it is still unclear whether this occurs via an ERK-dependent or independent mechanism [98]. Other putative MEK/ERK-independent targets of Raf signalling include the cell cycle regulators pRb (retinoblastoma protein) and Cdc25 [99, 100], although further study would be required to establish that these proteins are bona fide
Raf effectors. In addition, Raf proteins have the ability to localize to mitochondrial membranes, where they can inhibit pro-apoptotic proteins such as ASK-1 and BAD, and activate pro-survival factors such as Bcl-2 [98]. Thus, although Raf proteins have been described primarily as MEK activators, several new targets are emerging as putative effectors of the Ras-Raf signalling cascade.

1.1.3.2 The phosphatidylinositol-3 (PI-3) kinase pathway

Ras family members have the ability to bind to and activate type I PI-3 kinases in a GTP-bound-dependent manner [101-103]. Class I PI-3 kinases are heterodimeric lipid kinases that are composed of a regulatory subunit (55, 85 or 101 kDa), which associates with adaptor proteins and tyrosine-phosphorylated receptors, and a catalytic subunit (termed p110-α, -β, -δ or -γ) [104]. Ras binds to a Ras-binding domain located within the catalytic subunit of PI-3 kinase [101]. This interaction is thought to promote PI-3 kinase activity by causing allosteric changes within the kinase domain of the molecule, as well as providing a means by which the enzyme may become localized in close proximity to phosphoinositide substrates located in membranes [105]. Type I PI-3 kinases contribute to cellular signalling by catalyzing the phosphorylation of position 3 within the inositol ring of phosphatidylinositol (4,5)-biphosphate (PI(4,5)P2) to produce PI(3,4,5)P3, a lipid messenger that promotes the recruitment of proteins that contain a PH domain [106].

PH domain-containing proteins that have been shown to act as downstream effectors of PI-3 kinases include the serine / threonine kinase Akt, as well as Tec kinases. Although Akt can localize to membranes solely via its PH domain, it needs to become phosphorylated via two kinases, PI (3,4,5) dependent kinase (PDK)-1 and possibly integrin-linked kinase (ILK), in order to become fully activated [107-109]. Akt has been shown to regulate metabolism, survival and proliferation in many cellular systems, via the regulation of enzymes such as glycogen synthase kinase (GSK)-3, and the inhibition of pro-apoptotic proteins such BAD and members of the FOXO family of Forkhead transcription factors [110-112]. Tec kinases are PH domain-containing tyrosine kinases expressed
in hematopoietic tissues that regulate several cellular events, including calcium mobilization and cytoskeletal rearrangements [113]. The best studied Tec kinase family member is Bruton’s tyrosine kinase (Btk), which is responsible for phosphorylation and activation of PLCγ, and whose loss of function results in X-linked immunodeficiency (xid) in mice and X-linked agammaglobulinemia (XLA) in humans [114, 115]. Thus initiation of signalling cascades via the PI-3 kinase pathway leads to complex cellular responses generally associated with survival and proliferation.

1.1.3.3 Ral GEFs and other Ras effector pathways

1.1.3.3.1 The Ras/RalGEF pathway

RalA and RalB are small Ras-related GTPases that have been shown to modulate vesicular transport as well as cytoskeletal rearrangements, and are thought to contribute to Ras-mediated tumorigenicity [116]. Targets of the Ral proteins (RalA and RalB) include the Ral binding protein (RalBP), which activates the Rho-like GTPases Cdc42 and Rac, both regulators of cytoskeletal rearrangements [117-119]. Ral GTPases have also been found to associate with ADP ribosylation factors (ARFs) and phospholipase D1 (PLD1), thereby causing PLD1 activation [120]. PLD1 hydrolyzes phosphatidylcholine to phosphatidic acid, a lipid messenger that contributes to vesicular transport by promoting membrane budding [121, 122]. Ral GTPases are activated by a family of Ral GEFs that are thought to be activated in a calcium dependent manner via binding to Ras GTPases [123]. Ras GTPases have been found to bind to and promote the activation of at least three Ral GEFs: RalGDS, Rlf and Rgl, all three of which possess a Ras binding domain [124]. A dominant-negative form of Ras inhibits Ral activity in response to various growth factor receptors, suggesting that Ral GEFs are direct effectors of Ras signalling [125]. Thus, by modulating Ral activity via Ral GEFs, Ras GTPases have the ability to regulate cytoskeletal changes as well as vesicular transport.
Other putative effectors of Ras

Several other proteins have been implicated as effectors of Ras signalling. These include the putative tumour suppressor Nore-1, which has been shown to promote K-Ras-induced apoptosis in various cell lines [126]. AF-6, a putative scaffolding protein that plays an important role in morphogenesis by regulating intercellular tight junctions, binds to activated Ras, which promotes its biological activity [127, 128]. Another putative Ras effector is Rin1, which interferes with Raf-1 activation and potentiates activation of the breakpoint cluster region gene (BCR)-Abl fusion that is responsible for the onset of chronic myeloid leukemias [1, 129]. Further studies will be required to establish specific roles for these putative effectors during Ras signalling. Nevertheless, the multitude of effectors pathways described in this section underscores the extent to which Ras GTPase activity has the potential to regulate a wide range of cellular events, through a complex web of effectors (Fig. 1-5).

Figure 1-5. Ras GTPases activate a wide variety of signalling cascades.
1.2 RASGRP1 AND THE CONTROL OF MURINE LYMPHOCYTE DEVELOPMENT

1.2.1 RasGRP1 contributes to positive selection during T cell development

1.2.1.1 T cell development and implications for Ras family members

Successful development of thymocytes is critically dependent upon the generation and cell-surface expression of a functional T cell receptor (TCR). Immature CD4⁻/CD8⁻ (double-negative, DN) T lymphocytes must express a functional TCRβ-chain as part of a pre-TCR complex that promotes proliferation and differentiation to the CD4⁺ CD8⁺ (double-positive, DP) stage, a process known as β-selection (Fig. 1-6) [130]. Several studies point to a central role for the Ras family of small GTPases during lymphocyte development and selection. Expression of an active form of H-Ras, or the Ras effector Raf-1, has been shown to enhance the DN to DP transition via activation of ERK1/2 [131-133]. Further thymocyte maturation is induced upon TCRα chain rearrangement and expression. This results in the formation of a TCR αβ complex that, in conjunction with the engagement of a MHC class-specific coreceptor CD4 or CD8, promotes differentiation to the CD4⁺/CD8⁻ or CD4⁻/CD8⁺ (single-positive, SP) lineage [134, 135]. The affinity of the TCRαβ for a peptide/MHC complex determines the signalling outcome: a weak signal leads to death by neglect, a strong signal leads to deletion (negative selection), and an intermediate signal results in differentiation to either the CD4 or CD8 SP lineage (positive selection) [135]. Several lines of evidence suggest that positive selection is regulated via the Ras/Raf-1/MEK/ERK pathway. Positive selection is impeded by the expression of a dominant-negative form of Ras, Raf-1 or MEK in DP thymocytes, whereas expressing an active form of Raf-1 or MEK can increase the efficiency of positive selection [136-140]. These results suggest that the activation of one or more Ras-specific guanine nucleotide factors downstream of the pre-TCR and the TCR could play a significant role in the regulation of thymocyte differentiation.
1.2.1.2 Evidence for a role for RasGRP1 during T cell development

RasGRP1 mRNA expression is low in DN thymocytes but increases 10-fold in DP and SP thymocytes, as a result of pre-TCR signalling [141]. RasGRP1 becomes activated following TCR-mediated activation of PLCγ1 and links the TCR to Ras-mediated ERK activation [142]. Transgenic expression of RasGRP1 in developing thymocytes augments DN to DP progression (β-selection) and promotes differentiation of DP thymocytes to the CD8 SP lineage [141]. RasGRP1 appears to play a critical role in the later stages of thymocyte development, as SP thymocyte populations become severely depleted in RasGRP1−/− mice [143]. However DN and DP populations do not appear significantly affected in these mice [143]. RasGRP1 is also critical for maintenance of peripheral T cells as RasGRP1−/− mature naïve T cells have reduced proliferative capabilities, and do not differentiate into effector T cells following antigen stimulation [144, 145]. Furthermore, the few mature T cells that are generated in these mice are defective in their responses to apoptosis-inducing stimuli, and deletion of RasGRP1 can cause a T cell-mediated, late-onset autoimmune phenotype with characteristics similar to that observed in systemic lupus erythematosus [145].
ERK activation is abolished in RasGRP1⁺ T cells, and this appears to account for the multiple effects caused by deletion of RasGRP1 in these cells [143-145]. Thus, RasGRP1 has the ability to modulate TCR-dependent activation of the Ras/Raf/ERK pathway, and is therefore critical to the development, positive selection and maintenance of T cells, as well as the maintenance of self-tolerance in the host.

1.2.2 RasGRP1 has the potential to regulate the outcome of signals generated from the B cell receptor

1.2.2.1 An overview of B cell development in the bone marrow

1.2.2.1.1 Introduction

From the moment they are committed to the B cell lineage, B cells undergo a series of differentiation and selection steps that ensure that only those clones that have a functional BCR with minimal self-reactivity are permitted to differentiate further and become mature resting B cell in the periphery. The formation of a functional BCR is a critical process in the generation of B cells that requires proper rearrangement of the immunoglobulin (Ig) heavy and light chain gene loci. These Ig loci are composed of multiple gene segments that are randomly assembled, which is responsible for creating a diverse repertoire of antigen-receptor specificities [146]. Ig gene rearrangement is mediated by a complex somatic recombination mechanism that requires the recombination activating genes (RAG)1 and RAG2, the lymphoid-specific enzyme terminal deoxynucleotidyl transferase (TdT), as well as various other DNA repair proteins [147].

The earliest committed B lineage stage, which arises from a common lymphoid progenitor, is referred to as the progenitor (pro)-B cell stage [148]. Pro-B cell clones that successfully rearrange their heavy chain gene and express a heavy chain, which in combination with a surrogate light
chain forms the pre-BCR at the cell surface, are positively selected to become pre-B cells. Further positive selection occurs at the pre-B cell stage, as only those cells that are able to receive pre-BCR-mediated signals survive, proliferate and rearrange their heavy chain gene to form a mature BCR and then enter the immature B cell stage. Immature B cells are the target of negative selection, where those cells bearing self-reactive antigen receptors are eliminated, either by receptor editing or clonal deletion. The following section reviews these differentiation steps that ensure the generation of a functional, non-self-reactive mature B cell population that is highly polyclonal. This which provides the immune system with the ability to recognize and protect against a multitude of potential pathogens. B cell development is summarized in Figure 1-7.

**Figure 1-7. An overview of B cell development.**

BCR: B cell receptor; HC: heavy chain; Ig: immunoglobulin; LC: light chain; SLC: surrogate light chain; VDJ: variable, diversity and joining immunoglobulin gene regions.

1.2.2.1.2 B cell lineage commitment is regulated by a complex transcriptional program

All murine lymphocytes are thought to be derived from a common lymphoid progenitor (CLP) characterized by a short life-span and the potential to reconstitute the T, B and natural killer (NK) cell compartments, but not the myeloid compartments [149-151]. Unlike the hematopoietic stem-
cell from which it is derived, the CLP lacks the ability to self-renew [150-152]. Commitment to the B cell lineage and further development and differentiation of B cells is mediated via a highly complex and coordinated transcriptional program. Transcriptional regulators critical for B lineage commitment include E2A, which encodes for two members of the basic helix-loop-helix family of transcription factors, E12 and E47, as well as the early B cell factor (EBF) [153, 154]. Together, these transcriptional regulators control the expression of B cell specific genes, including those that encode Ig α/β, RAG1/2, as well as the surrogate light chain (SLC) components λ5 and VpreB [153, 154]. Targeted disruption of the genes encoding either of these transcription factors prevents Ig gene rearrangements, resulting in a block in B cell differentiation at a very early phase of the pro-B cell stage [155-157].

Pax-5, also known as B cell-specific activator protein (BSAP), is another transcription factor that regulates the expression of several B cell-specific proteins, including CD19, Igα, as well as the B cell linker protein (BLNK), a B cell-specific adaptor protein [158]. Ablation of Pax-5 results in a severe block in B cell development at the pro-B cell stage [159]. Pax-5−/− pro-B cells are multipotent and possess self-renewal capacities, and treatment of Pax5−/− pro-B cells with appropriate cytokines results in the generation of functional macrophages, osteoclasts, NK cells, granulocytes and dendritic cells [160, 161]. Pax5−/− cells have the ability to reconstitute T cell development [161, 162]. These results suggest that Pax-5 expression is not only required for the generation of B cells, but is also critical for the maintenance of lineage commitment in developing B cells. Other transcription factors with important roles during the generation and maintenance of B cells include the Ikaros family members Ikaros and Aiolos, as well as the NF-κB family of transcription activators, although a specific role for these transcription factors in B lineage commitment has not been clearly established [153, 163].
1.2.2.1.3 Pro-B cells undergo heavy chain gene rearrangement before they transit to the pre-B cell stage

The first stage of B cell development is referred to as the progenitor B cell, or pro-B cell stage, and is highly dependent on interleukin (IL)-7 production by stromal cells in the bone marrow [164, 165]. Igα and Igβ are expressed at the surface of pro-B cells in association with calnexin, in a complex that must be signalling-competent to promote transition to the pre-B cell stage and further differentiation [166-170]. Commitment to the B cell lineage seems to occur at this stage, immediately prior to initiation of Ig gene rearrangement [171, 172]. The first step in Ig gene rearrangement is initiated when recombination of the diversity (D) and joining (J) regions of the HC gene occurs in pro-B cells [173]. This is followed by rearrangement of the variable (V) region to the rearranged D-J region of the HC gene [173]. Proper rearrangement and expression of the HC gene corresponds with the transition from the pro-B to the large pre-B cell stage, which is accompanied by the expression of a pre-BCR complex at the surface of the cell and a burst of proliferation [174-177].

Proper assembly of a signalling-competent pre-BCR, in the presence of low concentrations of stromal cell-derived IL-7, is required for the positive selection of large (early) pre-B cells and transition to the small (late) pre-B and later B cell stages [178]. The pre-BCR is composed of two disulfide-linked Ig heavy chains associated with surrogate light chains that are made of two peptides, VpreB and λ5 [179]. Signals that emerge from the pre-BCR are thought to be generated in a ligand-independent manner, via phosphorylation of a single transmembrane Ig α/β heterodimer following assembly and cell-surface expression of the pre-BCR [180-184]. However, recent studies suggest that pre-BCR signalling is dependent upon binding of a stromal cell surface ligand that remains to be clearly defined [185, 186]. Signalling through the pre-BCR results in the rapid down-regulation of the RAG1/2 and the SLC components VpreB and λ5 [187, 188]. This prevents rearrangement and expression of a second heavy chain allele, a process referred to as allelic exclusion [187-189]. Pre-BCR signalling mediated via Ig α/β results in the recruitment of Syk and BLNK, which together with PI-3 kinase signalling contribute to activation of the NF-κB pathway.
pathway, thereby promoting survival and proliferation of pre-B cells [190]. Recent studies point to a role for pre-BCR-mediated ERK activation in enabling pre-B cell proliferation and survival under conditions where IL-7 is limiting, thereby allowing further differentiation [191, 192].

1.2.2.1.4 Light chain gene rearrangement, transition to the immature B cell stage and receptor-mediated tolerance in immature B cells

Following a pre-BCR-induced proliferative burst, pre-B cells enter a resting stage and increase the expression of components required for the rearrangement of their LC loci [165, 188]. This stage is referred to as the late pre-B cell stage. Two distinct LC gene loci, κ and λ, can be rearranged to form functional light chain genes that give rise to LC polypeptides and permit trafficking of the BCR complex to the cell surface [173]. Proper LC expression is necessary for the generation of a B cell receptor and subsequent transition to the immature B cell stage [173]. Rearrangement of the κ and λ LC loci is thought to occur independently, although rearrangement of the κ locus seems to occur first and more frequently [193, 194]. Unlike heavy chains, light chains lack the D region, generally making them less diverse than heavy chains [146]. However, the V-J structure of the light chain facilitates recombination, and thus promotes secondary rearrangement events [146]. Pre-B cells can undergo several light chain gene rearrangement events at a single allele before they are selected to become immature B cells [195]. The rearrangement machinery is maintained active through to the immature B cell stage; this allows these cells to undergo secondary rearrangement of their LC loci in order to tailor the affinity of their BCR and to avoid self-reactivity [196-198]. This process is referred to as receptor editing.

Proper rearrangement of the HC and LC leads the expression of IgM molecules on the surface of newly generated immature B cells [199]. Subsequent alternative splicing of the HC transcript leads to surface expression of IgD and the ratio of surface IgD to IgM molecules increases as the immature B cells transit to the mature B cell stage in the periphery [199]. Immature B cell clones bearing a newly generated BCR (phenotypically IgMlo/IgDal-) encounter a variety of self-antigens within the bone marrow and later in the periphery as they become transitional immature B cells.
(IgM\textsuperscript{hi}/IgD\textsuperscript{+}) on their way to secondary lymphoid organs [199-201]. Only about 10% of the 2\times10^7 immature B cells generated daily in the murine bone marrow make it to the periphery and become mature circulating (IgM\textsuperscript{lo}/IgD\textsuperscript{+}) B cells, the result of tight regulation mediated in large part via tolerance induction [202].

Self-tolerance in B cells is achieved by selecting those clones with minimal reaction to self-antigen that have competent BCR signalling [202, 203]. The response of an immature B cell clone to self-antigen binding is determined at least in part by receptor affinity for a particular self-antigen (the strength with which the receptor binds antigen at a single site), as well as ligand avidity (the total strength of receptor-antigen binding at multiple sites) [201]. Immature B cell clones that react strongly to self antigen undergo receptor editing or deletion, while clones that react weakly to self-antigen are rendered anergic, and thus are unable to mount an immune response [203]. The precise factors that determine the outcome of BCR signalling in a self-reactive immature B cell clone is the subject of much study. Cross-linking the IgM molecules on the surface of isolated bone marrow immature B cells results in rapid apoptosis [204]. However, experiments have shown that there exists a latent period between self-antigen-mediated developmental arrest and apoptosis, during which receptor editing can occur to rescue self-reactive clones [205-207]. The level of IgM expression has been shown to affect the outcome of BCR signalling in self-reactive B cells. Whereas self-antigen causes receptor editing in bone marrow immature B cells that express low amounts of IgM on their surface, transitional immature B cells with high levels of surface IgM respond to self-antigen by undergoing apoptosis [208]. This difference in response might also be attributable to the environment in which the immature B cell encounter antigen. An ill-defined, Thy\textsuperscript{1}\textsuperscript{lo}/DX5\textsuperscript{+} cell type present in the bone marrow environment appears to protect immature B cells from self-antigen-mediated cell death [209, 210]. The affinity of self-antigen, as well as it's ability to cause robust signalling downstream of the BCR, are critical determining factors in the induction of receptor editing [211, 212]. Moreover, the absence of sufficient BCR levels on the surface of immature B cells has been shown to promote receptor editing instead maturation [213]. These findings suggest that several checkpoints exist during the generation and selection of immature B cell clones for further maturation and migration to the secondary lymphoid organs.
1.2.2.2 Ras signalling contributes to the development of B cells

Evidence for a role for Ras signalling during B cell development has emerged from studies where a dominant-negative form of Ras (N17Ras) was expressed from a transgene specifically designed to be active in the earliest stages of B cell development [214, 215]. Expression of N17Ras from the earliest B cell stage (early pro-B cells) significantly impairs progression to the late pro-B and pre-B cell stages, resulting in a transgene-dose dependent depletion of bone marrow B cells [214]. Despite severe depletion of bone marrow B cells in the later stages of development, N17Ras transgenic mice have relatively normal mature B cell populations in the spleen, albeit with lower numbers (3-fold reduction) and an increase in the immature to mature B cell ratio [214]. N17Ras expression in splenic B cells appears to impair B cell responses to mitogens, although the experiments were performed in whole B cell populations [214]. The authors note that a difference in the ratio of immature to mature B cell could at least partially reflect the differences seen in N17Ras transgenic vs control mice [214]. Using a slightly different promoter, Nagaoka et. al. were able to generate N17Ras transgenic mice with expression restricted to the later stages of B cell development (late pro-B and pre-B cell stages) [215]. Selective expression of N17Ras at the pre-B cell stage severely reduces pre-B cell viability, as well as progression to the late pre-B and immature B cell stages [215]. Taken together, these results suggest that B cell development is severely impaired when Ras signalling is inhibited.

A separate yet equally convincing set of data was generated using mice that had their recombination machinery impaired by deletion of RAG1 or RAG2, which causes a block in B cell differentiation at the pro-B cell stage [216, 217]. Expression of a constitutively active form of Ras (V12Ras) promotes the progression of pro-B cells from RAG1-deficient mice to later stages of B cell development [218]. Although V12Ras cannot promote Ig gene rearrangement in the absence of RAG1, B cells from the periphery of RAG1-deficient, V12Ras-expressing mice were found to express markers and gene transcripts consistent with later stages of B cell development [218]. V12Ras expression was also found to promote light chain gene rearrangement and developmental progression in B cells that lack a functional HC gene [219, 220]. HC deletion normally prevents
progression past the pro-B cell stage [219, 220]. Thus, taken together, the results from these experiments suggest that Ras signalling has the ability to promote developmental progression of B cells.

To date, the PI-3 kinase and Raf/MEK/ERK pathways are the only Ras effector pathways to have been studied extensively in the context of B cell development. Although there is data pointing to a critical role for PI-3 kinase in the survival and proliferation of B cells during development [104], the precise contribution of PI-3 kinase activation to Ras signalling in these cells is still unclear. A constitutively activated form of Raf that bears a membrane localization signal (Raf-CAAX) is sufficient to rescue the block in B cell development found in N17Ras transgenic mice [214]. Transgenic expression of Raf-CAAX was also found to augment B cell developmental progression from the pro-B to later stages, without affecting mature B cell compartments [133, 214]. Further evidence for a role for the Raf/MEK/ERK pathway during B cell development was obtained from studies on the transition from IL-7-dependent late pro-B cells to IL-7-independent pre-B cells [192]. These authors found that positive selection of early pre-B cells clones was highly dependent on their ability to activate the Raf/MEK/ERK pathway via a newly generated pre-BCR when IL-7 levels are limiting [192]. Thus, while the Ras/Raf/MEK/ERK pathway has the potential to play a major role in the outcome of Ras signalling during B cell development, further studies are needed to directly link PI-3 kinase signalling to Ras functions in this process.

1.2.2.3 Ras GTPases have the potential to become activated via multiple guanine exchange factors during BCR signalling

Ligation of the BCR causes activation of Ras GTPases, as well as the Ras/MEK/ERK pathway [221-225]. Ras activators that have been detected in B lymphoid tissues and/or cell lines include the Sos family members Sos-1 and -2, as well as the RasGRP family members RasGRP1 and RasGRP3 (reviewed in [60]), as well as RasGRP4 (S. Arishenkof, unpublished result). No reports are available that describe RasGRF1/2 expression or function in lymphocytes, although RasGRF2 expression has been detected in the spleen [65]. Thus, members from at least two guanine
nucleotide exchange factor families have the potential to contribute to Ras activation following BCR ligation: Sos and RasGRP. The BCR is a multiunit complex which bears an extracellular Ig receptor component composed of heavy and light chains, as well as a trans-membrane signalling component made of Igα and Igβ molecules [226]. BCR ligation initiates signalling events via the activation of several kinases, including the Src-family kinase Lyn, Blk and Fyn, as well as the tyrosine kinases Syk and Btk, which activate a wide variety of signalling effectors. Lyn, Blk and Fyn initially becomes recruited to the BCR complex following ligation and phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) on Igα/β molecules, which promotes recruitment and activation of Syk [226]. Btk becomes recruited to PIP3-rich membranes in proximity to the BCR signalling complex via a PH domain [227]. Btk then become phosphorylated and activated by Syk and Lyn [226].

RasGRP family members promote activation of Ras GTPases following BCR-mediated activation of PLC enzymes [228, 229]. BCR-mediated PLC activation occurs when Syk, once recruited to the BCR complex, phosphorylates BLNK and enables it to interact with and recruit PLCγ to BCR signalling complexes [226]. Btk directly phosphorylates PLCγ thereby causing its activation [226]. PLCγ converts membrane PI (4,5)P2 to diacylglycerol (DAG), which promotes the recruitment of C1 domain-containing proteins such as PKC enzymes and RasGRP family members [71]. The C1 domain-containing GEFs of the RasGRP family are thought to contribute significantly to BCR-mediated Ras activity [228, 229].

The GEFs Sos-1 and Sos-2 have also been shown to become recruited to the BCR complex, via the adapter Grb2 [230]. Grb2 becomes recruited to the BCR complex via binding to a phospho-tyrosine residue on the cytoplasmic tail of the BCR co-receptor CD22 [231-233]. CD22, a transmembrane glycoprotein associated with the BCR, becomes phosphorylated on several tyrosine residues following BCR ligation and contributes to the recruitment of several SH2-containing proteins, including PLCγ, PI-3 kinase, Lyn and Syk, in addition to Grb2 [231-233]. Despite this potential, deletion of Sos-1 and Sos-2 in DT40 cells does not affect the levels of active
Ras or ERK following BCR ligation, suggesting that Sos family members do not contribute to BCR-mediated Ras activation [229].

**Figure 1-8. BCR activates Ras GTPases via Sos and RasGRP family members.**
BLNK: B cell Linker protein; BCR: B cell receptor; DAG diacylglycerol; P: phosphate groups; PLC: phospholipase C.

### 1.2.2.4 RasGRPI has the potential to contribute to BCR-mediated signalling responses

Although expressed predominantly in T cells, RasGRPI mRNA is detected at low levels in bone marrow as well as in the immature B cell lines WEHI 231 and DT-40 [69]. Furthermore, RasGRPI protein levels are far higher in WEHI 231 cells than in either thymocytes or the T28 T cell hybridoma line (see Figure 3-1, below). However the presence of RasGRPI protein in bone marrow B cells has yet to be demonstrated. Ligation of both the pre-BCR and the BCR has been shown to cause PLCγ activation [184, 234, 235]. RasGRPI therefore has the potential to be recruited to membranes following pre-BCR and BCR ligation via its DAG-binding C1 domain. The avian B cell line DT-40 expresses both RasGRPI and RasGRP3, the latter appearing to be B cell-specific and expressed at moderately higher levels [229]. Recent studies performed in DT40 cells have shown that RasGRPI does in fact become recruited to the plasma membrane following BCR engagement [228]. However deletion of RasGRPI in DT40 cells does not affect activation of the
Ras-ERK pathway [229]. Instead, it appears that RasGRP3 accounts for most of the BCR-mediated Ras-ERK activation in this system. This therefore suggests that RasGRP3 provides compensatory signals in the absence of RasGRPI, or that RasGRPI is not important in this process. Deletion of RasGRPI in mice does not seem to affect B cell development significantly, as RasGRPI-/- mice have relatively normal numbers of mature B cells that respond normally to proliferation- and apoptosis-inducing signals [142, 145]. Thus if RasGRPI plays a role in B cell development, it is one that is relatively subtle and somewhat redundant with the role of RasGRP3.
1.2.3 The immature B cell line WEHI 231 as a model for BCR-induced deletion of developing B cells

1.2.3.1 The WEHI 231 B cell line

The B-lymphoma-derived WEHI 231 murine cell line mimics immature B cells by expressing high levels of cell surface IgM, and undergoing cell cycle arrest and apoptosis in response to BCR clustering [236-238]. Treatment of WEHI 231 cells with even low amounts (0.1 µg/ml) of anti-IgM antibodies results in cell cycle arrest, whereas commitment to apoptosis requires prolonged treatment with higher (1-10 µg/ml) concentrations of anti-IgM [239]. Molecular signals that block apoptosis in primary B cells, such as LPS and CD40L, also suppress anti-Ig-induced apoptosis in WEHI 231 cells, thus making the WEHI 231 cell a physiologically relevant model for antigen receptor-induced apoptosis induction in B cells [236, 240]. As a result, this cell line has been used extensively to study signalling events that occur downstream of the B cell receptor, as well as the molecular events leading to antigen-induced apoptosis [225, 241-246].

1.2.3.2 Ligation of the BCR activates a complex cascade of signalling events in WEHI 231 cells

Three major signalling cascades thought to become activated following BCR ligation are the PLCγ pathway, the Ras pathway and the PI-3 kinase pathway (Fig. 1-9) [230]. Following BCR ligation, Syk phosphorylates the BLNK which enables it to interact with and recruit PLCγ to BCR signalling complexes [226, 230]. Btk directly phosphorylates PLCγ thereby causing its activation, resulting in the generation of DAG and IP3 [226, 230]. An increase in membrane DAG promotes the recruitment of C1 domain-containing proteins such as PKC enzymes and RasGRP family members [226, 230]. IP3 binds to IP3 receptors located on the ER membranes to promote release of calcium from intracellular stores, as well as the consequent entry of extracellular calcium [226]. An increase
in cellular calcium is responsible for modulating the activation of several calcium-dependent proteins, such as calcineurin, a serine/threonine phosphatase that promotes activation of the transcription factor nuclear factor of activated T cell (NFAT) [226]. An increase in intracellular calcium is thought to be required for BCR-induced cell death of WEHI 231 cells [247, 248].

BCR-mediated Ras activation in WEHI 231 cells leads to activation of the Raf/MEK/ERK pathway, which regulates the transcription of several early response genes, including egr-1, involved in B cell activation, survival and proliferation [244, 249]. RasGRP family members, notably RasGRP3, contribute most of the BCR-induced Ras activation in DT40 B cells [229, 250]. BCR-mediated activation of PLCγ is thus likely to play a key role in the modulation of Ras activation in WEHI 231 cells [229]. However Sos family members also have the potential contribute to BCR-induced Ras activation in WEHI 231 cells, as WEHI 231 cells express CD22 [229, 251].

Another major signalling pathway activated via the BCR in WEHI 231 cells is the PI-3 kinase pathway. PI-3 kinase is thought to become activated via recruitment to membranes following BCR-induced phosphorylation of tyrosine residues on the cytoplasmic domain of BCR-associated CD19 molecules, which provide a docking site for the SH2 domain of PI-3 kinase, and binding to Ras GTPases [227, 252]. PIP3 produced by PI-3 kinase promotes the recruitment of PH domain-containing molecules [253]. PH domain-containing molecules found to become activated as a result of BCR-mediated PI-3 kinase signalling include the kinases Akt and Btk [253, 254]. PI-3 kinase mediated signalling are essential for the survival of WEHI 231 cells [243].

The signalling cascades described above represent a fraction of the multitude of proteins involved in the complex process of transmitting signals from the BCR to the nucleus of WEHI 231 cells. The specific signals that are responsible for the induction cell cycle arrest and apoptosis following BCR ligation of WEHI 231 cells and primary immature B cells are the subject of intense investigation.
Figure 1-9. BCR ligation activates multiple signalling cascades in WEHI 231 cells. BCR: B cell receptor; DAG diacylglycerol; P: phosphate groups; PLC: phospholipase C. Broken lines indicate specific signalling pathways that remain to be thoroughly tested in WEHI 231 cells.

1.2.3.3 Effectors of BCR-mediated arrest and apoptosis in WEHI 231 cells.

BCR ligation leads to an increase in the expression of the cyclin-dependent kinase inhibitors (CKIs) p27^KIP1, and p21[WAF1] [243, 255-257]. Up-regulation of CKIs has the ability to induce cell cycle arrest by inhibiting CDK activation, which is critically required during cell cycle entry [258]. A key regulator of cell growth and apoptosis in WEHI 231 cells is the transcription factor Myc, whose levels first become transiently increased, but then drop drastically following BCR ligation [241, 259]. Myc has been shown to repress the expression of p27^KIP1, suggesting that BCR-induced reduction in Myc may therefore account for the increase in p27^KIP1 activity observed following BCR ligation of WEHI 231 cells. Another potentially critical regulator of cell cycle arrest and apoptosis in WEHI 231 is the transcription factor CTCF, whose expression is upregulated following BCR ligation.
CTCF has been found to cause both the down-regulation of Myc, and the upregulation of CKIs [257]. Thus the transcription factors CTCF and Myc appear to act simultaneously to induce events leading to cell cycle arrest and apoptosis following BCR ligation of WEHI 231 cells.

Apoptosis occurs as the result of tightly regulated events that include shrinking of the cell, chromatin condensation and cleavage, and the orderly disintegration and clearing of the cell. This process is primarily regulated by a group of cysteine proteases referred to as caspases [260]. Initiator caspases (caspase-8, and -9) are present in the cytoplasm as inactive pro-caspases that, once cleaved and activated, activate effector caspases (3,6, and 7). Activation of effector caspases can occur via two independent mechanisms: activation of caspase-9 following mitochondrial damage and release of cytochrome c; or alternatively, direct recruitment and activation of caspase-8 following activation of tumour necrosis factor (TNF)-family receptors [261]. In WEHI 231 cells, antigen-induced cell death correlates with disruption of mitochondrial membranes [239, 262]. What is still unclear however is whether caspases are solely responsible for BCR-mediated apoptosis, and whether cytochrome c-mediated activation of caspase-9 actually occurs. The primary effector caspase activated following BCR ligation appears to be caspase-7 [246, 263]. Although no significant cytochrome c release is detected following BCR engagement, an increase in caspase-9 activity occurs [246]. Close association of caspase-9 with the mitochondrial membrane is thought to result in high sensitivity of the protease to low concentration of cytochrome c release [246]. Thus although caspases have the potential to regulate the onset of apoptosis in WEHI 231 cells, the specific caspase mechanism(s) involved remain unclear.

Mitochondrial integrity, the loss of which is a critical event in the induction of apoptosis, is regulated by the Bcl-2 family of pro-apoptotic (e.g. Bax, Bid, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-xl, A1) proteins, which is composed of 20 members [261]. Activated pro-apoptotic members appear to disrupt mitochondrial integrity by acting as membrane channels, by directly activating membrane channels. Anti-apoptotic Bcl2-family proteins are thought to act by directly binding and inhibiting the activation of the pro-apoptotic members, although some evidence suggests that the anti-apoptotic members can inhibit caspase activation directly [261]. Whereas BCR signalling leads
to down-regulation of both Bcl-xL and A1 in WEHI 231 cells, both are up-regulated following CD40 activation, and forced expression of either protein alone is sufficient to rescue cells from BCR-induced apoptosis [264-267]. Transcription of both A1 and Bcl-xL is at least partially regulated via NF-κB proteins (described below), which become activated following stimulation of the CD40 pathway in WEHI 231 cells [268, 269]. The mechanism through which BCR-mediated signals lead to down-regulation of A1 and Bcl-xL is unclear although it may occur via inhibition of the NF-κB pathway. The transcription factor Myc is also thought to be partially regulated via NF-κB [241, 259]. Therefore the control of pro- and anti-apoptotic proteins, mediated at least in part via NF-κB, significantly affects the outcome of signalling events that occur in WEHI 231 cells.

1.2.3 4 Activation of the NF-κB pathway promotes survival of WEHI 231 cells

In WEHI 231 cells, CD40 ligation as well as treatment with lipopolysaccharide (LPS) prevents BCR-induced apoptosis by activating the NF-κB pathway [236, 268, 270]. Members of the NF-κB / Rel family act as homo- and hetero-dimers that regulate the transcription of several survival genes by binding the κB region located in their promoters [271]. The NF-κB / Rel family is composed of five members, termed p50, p65, c-Rel, p52 and Rel B; each protein contains an N-terminal Rel homology region that is responsible for DNA binding, dimerization as well as a nuclear localization sequence [271]. NF-κB activity is regulated by inhibitor proteins termed inhibitor of κB (IκB), which bind NF-κB members and retain them in the cytoplasm by concealing their nuclear localization signal [272]. IκB binding of NF-κB members can occur inside the nucleus, resulting in the termination of DNA binding and export of NF-κB out of the nucleus [272]. This is mediated by an nuclear export sequence present in IκB proteins [272]. The major IκB proteins are termed IκBα, -β, -ε, and -γ. Activation of the NF-κB pathway occurs following activation of IκB kinase (IKK) complex, which phosphorylates IκBs on serines 32 and 36, and targets them for proteosome-mediated degradation [272]. The IKK complex is composed of several proteins, including the kinases IKKα and IKKβ, as well as a regulatory subunit termed IKKγ, or NEMO (NF-κB Essential
MOdulator) [273, 274]. CD40 (and LPS, using toll-like receptors) activates the IKK complex via the TNF receptor-associated factor (TRAF) family of signal transducers, which form a complex that binds the cytoplasmic tail of CD40 [275, 276]. In addition to promoting the transcription of survival factors such as A1 and Bcl-xL, major anti-apoptotic effectors of NF-κB activation include the inhibitor of apoptosis proteins (IAP), which act as caspase inhibitors [277]. However, further study is needed to confirm that IAP proteins contribute to the CD40 and LPS-mediated rescue of WEHI 231 cells from BCR-induced apoptosis.
1.3 RATIONALE AND SCIENTIFIC QUESTIONS ADDRESSED IN THIS THESIS

RasGRP1 is a Ras activator that is recruited to DAG-rich membranes following PLC activation by receptors such as the B and T cell antigen receptors. RasGRP1 is expressed in lymphoid tissues and cell lines, and has been shown to modulate T cell receptor-mediated responses that regulate positive selection of developing thymocytes, as well as survival, proliferation and maintenance of mature T cells. Several lines of evidence point to a role for Ras GTPases during B cell development, and BCR ligation has the potential to activate at least two individual families of GEFs, the RasGRP family and the Sos family. RasGRP1 has been shown to translocate to membranes following BCR ligation, and studies performed in a B cell line suggest that BCR-mediated activation of Ras GTPases is achieved in great part via activation of RasGRP family members. Combined, these observations suggest that RasGRP1 has the potential to modulate BCR-mediated responses in developing B cells.

The general objective of this thesis was to determine how RasGRP1 contributes to BCR signalling and BCR-mediated responses during B cell development. To specifically address this objective, the effect of increasing RasGRP1 levels on the outcome of BCR-mediated response in WEHI 231 cells, a murine immature B cell line that mimics BCR-mediated negative selection in immature B cells, was studied. The levels of RasGRP1 expression in WEHI 231 were increased using retroviral transduction of a murine RasGRP1 cDNA. WEHI 231 cells with increased RasGRP1 expression were used to address two specific scientific questions: Does an increase in RasGRP1 levels affect the outcome of BCR ligation in WEHI 231 cells?; and what are the molecular mechanisms involved in the ability of RasGRP1 to modulate BCR-mediated responses in WEHI 231 cells?
CHAPTER 2: MATERIALS AND METHODS

2.1 CELLS, REAGENTS AND ANTIBODIES

WEHI-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 \( \mu \text{M} \) 2-mercaptoethanol, 2 \( \text{mM} \) glutamine, 1 \( \text{mM} \) pyruvate, 15 units/ml penicillin, and 50 \( \mu \text{g/ml} \) streptomycin (complete medium). LPS and antibiotics (G418 and puromycin) were from Sigma-Aldrich (St-Louis, MS). Bay 11-7082, the PI3-K inhibitors Wortmannin and LY294002 as well as the MEK inhibitors PD98059 and U0126 were from Calbiochem (San Diego, CA). Anti-mouse CD40 and anti-human Thy-1 were from Pharmingen (San Diego, CA), goat anti-mouse IgM was from Jackson Immunoresearch Laboratories Inc. (West Grove, PA), and anti-HA was from BABCO/Covance (Denver, PA). Antibodies detecting extracellular signal-regulated kinase (ERK)-1/2 and phosphorylated ERK1/2, as well as Akt and phosphorylated Akt (Ser473) were from Cell Signaling Technology (Beverly, MA). The anti-RasGRP1 antibody (199), anti-IkB\( \alpha \) antibody (C-21), and the anti-Raf-1 antibody (C-12) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against Bcl-xL was from BD Transduction Laboratories (Lexington, KY). CFSE was from Molecular Probes (Eugene, OR). Trypsin and cell culture reagents were from StemCell Technologies (Vancouver, BC).
2.2 TISSUE CULTURE AND CELL BIOLOGY TECHNIQUES

2.2.1 cDNA constructs and retroviral transduction of WEHI-231 cells

2.2.1.1 The CTV 313 and 318 vectors

CTV vectors are retroviral vectors composed of the following elements: a 5' LTR and extended gag region derived from the Moloney murine leukemia virus (MMLV); a polylinker site for cDNA insertion; a supF gene, which provides a marker for bacterial transformation; a drug resistance marker for cDNA recovery driven by a herpes simplex virus thymidine kinase (TK) promoter; a 3' LTR derived from the myeloproliferative sarcoma virus (MPSV); as well as simian virus 40 (SV40) -derived origin of replication [89]. The CTV 313 vector bears a puromycin-resistance gene, and an internal ribosomal entry site (IRES)-driven green fluorescence protein (GFP) gene, whereas the CTV 318 vector bears a neomycin-resistance gene and an IRES-driven signalling-defective human Thy-1 gene. The Thy-1 protein is thought to be signalling-defective because its glycosylphosphatidylinositol (GPI)-linkage signal has been replaced by a trans-membrane domain. The individual resistance and label markers within these two vectors enables their use to co-transduce, select and sort for expression of two individual cDNAs into individual WEHI 231 populations.

2.2.1.2 cDNAs used in this thesis

The full-length HA-tagged RasGRP1 cDNA is identical to the XFL construct previously described [69]. The full-length, HA-tagged R271E, EF hand-deleted and C1 domain-deleted forms of RasGRP1 were derived from the GEFµ, ΔC1+ and EFA mutants [69]. HA-tagged, wild-type and G12V mutants of Ras GTPases were constructed in the Robert Kay Laboratory. The wild-type Ras GTPases had a GFP N-terminal fusion. M-Ras Q71L was a generous gift of L. Quilliam (Indiana
2.2.1.3 Retroviral transduction, selection and sorting of WEHI 231 cells

The human BOSC23 packaging cell line, which converts cDNAs to RNA molecules that are packaged into retroviruses, was transfected with cDNAs via calcium phosphate precipitates [279]. Supernatants containing retroviruses were harvested, filtered and used to infect $10^4$ WEHI 231 cells per ml, in the presence of fibronectin to enhance retrovirus-binding to the cells [280]. Transduced cells were drug-selected with either puromycin (0.25 μg/ml), or the neomycin analog G418 (2.5 mg/ml), and resistant clones were expanded and further selected for expression of human Thy-1 or GFP by sorting on a FacsVantage flow cytometer (BD Biosciences). The resulting WEHI 231 populations were generally over 80-100% positive for marker expression. Multiple vials were frozen for use in several experiments.

2.2.2 Stimulation of cells with anti-IgM and reversal of apoptosis induction by trypsin.

WEHI 231 cells in complete medium were stimulated through the BCR by the addition of anti-IgM at the concentrations indicated. To reverse the apoptosis induction signal provided by anti-IgM, cells were rinsed in phosphate buffered saline (PBS), treated with 0.025% trypsin in PBS for 10 minutes at 20°C, rinsed with PBS and returned to culture in complete medium. The mechanism by which trypsin treatment reverses the induction of apoptosis that is initiated by anti-IgM treatment is unknown. Trypsinization does not result in the immediate removal of anti-IgM from the cell surface, but could cause peptide cleavages that reduce the extent of cross-linking within surface IgM/anti-IgM complexes.
2.2.3 Apoptosis detection using propidium iodide (PI) staining of fixed nuclei

WEHI-231 cells cultured in complete medium at 5 to 10×10^5 cells/ml and stimulated as indicated were rinsed in PBS containing 0.1% glucose and fixed in 70% ethanol at 4°C for at least 24 hours. Cells were then rinsed in PBS and stained with PBS containing 5 μg/ml propidium iodide and 200 μg/ml RNaseA overnight in the dark at 20°C. Cells were then analysed for PI content by flow cytometry using a FacsCalibur cytometer and Cellquest software (BD Biosciences).

2.2.4 Cell division analysis using carboxyfluorescein succinimidyl ester (CFSE) labelling.

WEHI 231 cells at 10^6 cells/ml were cultured in complete medium with or without anti-IgM for 24 hours. Cells were then treated with PBS containing 0.025% trypsin as described above to terminate BCR signalling, and plated in fresh medium for 30 minutes. Up to 2×10^7 cells were incubated in 2 ml PBS containing 9 μM CFSE for 10 minutes at 37°C, rinsed twice in ice cold PBS and incubated overnight at 37°C. Cells were then sorted using a 20-channel gate within the FL1 parameter to select for a population of cells with highly similar CFSE brightness. Initial brightness was determined, and the cells were cultured for the indicated time and analysed by flow cytometry.

2.3 PROTEIN BIOCHEMISTRY AND TRANSCRIPTION ASSAYS

2.3.1 Preparation of cell lysates

WEHI 231 cells were cultured in complete medium at 5 to 10×10^5 cells/mL, treated as indicated, rinsed with PBS, and lysed in ice cold Mg+ lysis buffer (25 mM HEPES pH 7.2, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol; 25 mM NaF, 10 mM MgCl2, 1mM EDTA, 1 mM sodium vanadate, 1 mM sodium molybdate, plus protease inhibitors). Cytoplasmic fractions were
isolated by preparing lysates in 50 mM HEPES (pH 7.5), 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 4 mM EDTA, 0.2% NP-40, 10 mM MgCl₂ containing protease inhibitors [281] [281]. Total or cytoplasmic lysates were then centrifuged at 13,000 rpm for 5 minutes. Protein content was measured by colorimetric analysis using the bicinchoninic acid assay (Pierce, Rockford IL) prior to western blot analysis.

2.3.2 Ras activation assay

2.3.2.1 Short-term cell stimulations in the absence of serum

Cells were incubated at $1 \times 10^7$ cells/ml in activation buffer (25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 2 mM Na pyruvate, 0.1% glucose, 50 μM β-mercaptoethanol) for 15 min at 37°C. Anti-IgM was then added to 20 μg/ml for the indicated times. Stimulations were terminated by the addition of ice cold, Mg⁺ lysis buffer and lysate protein content was measured.

2.3.2.2 Affinity precipitation of active (GTP-bound) Ras GTPases

Ras activation assays were performed using the Ras-binding domain (RBD) of Raf-1 as described by Taylor and Shalloway [282]. Briefly, GST-RBD fusions were isolated from transduced E.Coli following induction with 200 μM IPTG by resuspending cells in cold PBS containing protease inhibitors, followed by sonication. E. Coli cell lysates were stirred for 30 minutes at 4°C after addition of 1% Triton X-100. E. Coli lysates were centrifuged at 13,000 RPM, 10% glycerol was added and were aliquoted stored for up to 4 weeks at -70°C.

RBD-GST was precipitated from prepared E.coli lysates using GST-agarose beads (Sigma), rinsed with lysis buffer, and used to precipitate active Ras proteins from WEHI 231 lysates.
(prepared simultaneously) by incubating 40 µl of a 50% bead:buffer suspension with 50 µg WEHI 231 lysate for 30 minutes at 4°C. Beads were then washed 1X with lysis buffer and protein was eluted by boiling in the presence of loading buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol) containing bromophenol blue.

2.3.3 Western blots

Cell lysates were combined with one volume of loading buffer, separated by electrophoresis on 12.5% SDS-PAGE gels, and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked overnight with 5% bovine serum albumin in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). Membranes were then rinsed twice in TBST, incubated overnight at 4°C, or 90 minutes at room temperature with primary antibody diluted in TBST + 3% bovine serum albumin. Membranes were then rinsed 4×10 minutes at room temperature and incubated with a horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch). The ECL chemiluminescence system (Santa Cruz) was used to visualize immunoreactive bands either by film or using the Versadoc 5000 imaging system (Bio-Rad Laboratories, Hercules, CA). Band volume analysis was performed using Quantity One software (Bio-Rad Laboratories).

2.3.4 NF-κB luciferase assay

WEHI 231 cells (5 × 10^6 cells/ml) were suspended in complete medium containing a reporter gene construct (10 µg/ml, pNF-κB-LUC; Stratagene, La Jolla, CA) in combination with 2 µg/ml of thymidine kinase promoter-dependent renilla luciferase construct to assess transfection efficiency (pRL-TK; Promega, Madison, WI), placed on ice for 20 minutes and electroporated at 270 V and 950 µF in 4 mm cuvettes (Gene Pulser; Bio-Rad Laboratories). Cells were then rinsed twice with medium, and 10^6 cells/ml were plated in fresh medium and incubated at 37°C for 4 hours. Cells were then rinsed with PBS and lysed for luciferase assays performed according to the manufacturer’s instructions (Dual Luciferase Reporter Assay; Promega).
CHAPTER 3: RESULTS

3.1 INCREASED RASGRP1 EXPRESSION AUGMENTS IGM-MEDIATED APOPTOSIS OF WEHI 231 CELLS

3.1.1 Generation and characterization of WEHI 231 cells with increased RasGRP1 expression

We used the WEHI 231 B cell line to determine whether differences in RasGRP1 expression could affect the outcome of BCR signalling. Although RasGRP1 mRNA levels are lower in WEHI 231 cells than in the T cell hybridoma T-28 or total thymocytes [69], RasGRP1 protein levels are 3-fold higher in WEHI 231 cell than in thymocytes, and 7-fold higher than T-28 cells (Fig. 3-1A). WEHI 231 populations were generated that had RasGRP1 protein levels double that of wild-type (Fig. 3-1A). This was achieved by retrovirally transducing WEHI 231 cells with a vector containing a cDNA encoding full-length, N-terminally hemagglutinin (HA) epitope-tagged murine RasGRP1 whose expression was monitored via IRES-mediated expression of GFP or of the extracellular domain of human Thy-1 (Fig. 3-1B). Transduced WEHI 231 cells were drug-selected. High-expressing clones within the selected population, as measured by GFP or Thy-1 expression, were then sorted by flow cytometry. WEHI 231 cells with increased RasGRP1 protein levels will from here on be referred to as RasGRP1_{high} cells. Unless indicated otherwise, at least two independently derived RasGRP1_{high} cell populations were used for each figure described in this thesis.

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Figure 3-1. Expression of endogenous and transduced RasGRP1 in WEHI-231 cells.

A. Lysates from WEHI 231 cells transduced with empty vector (control) or with vector encoding HA-tagged RasGRP1 (RasGRP1\textsuperscript{high}), the murine T cell hybridoma line T-28 and primary thymocytes were analyzed by western blot, using anti-RasGRP1 or anti-HA antibodies for detection (left panel). Numbers in the left panel indicate the total relative value of all detected bands, normalized to the endogenous RasGRP1 in control cells. Detailed analysis of individual transduced HA-RasGRP1 bands are indicated by arrowheads (right panel), with the numbers indicating relative quantities of each band. The main endogenous band (see control sample) has an estimated molecular mass of 89 kDa and the main transduced (HA\textsuperscript{+}) band has an estimated molecular mass of 91 kDa. B. Schematic diagram representing the structure of RasGRP1-bearing provirus used to infect WEHI 231 cells. LTR: long terminal repeat; IRES: internal ribosome entry site; GFP: green-fluorescence protein; TK: herpes simplex virus thymidine kinase promoter; G418\textsuperscript{r}: neomycin-resistance gene.

To determine whether increased RasGRP1 expression was able to augment activation of Ras family members, control and RasGRP1\textsuperscript{high} cells were transduced with HA-tagged H-Ras/GFP, K-
Ras/GFP or N-Ras/GFP fusions. An affinity precipitation method that utilizes the Ras-binding domain of Raf-1, which specifically binds GTP-Ras and not GDP-Ras, was used to measure the levels of activated Ras (Ras/GFP-GTP) in each cell population. Control and RasGRP1<sup>hi</sup> cells were treated with an anti-IgM antibody to initiate BCR ligation, which results in the activation of several downstream signalling events including the activation of PLC, Ras GTPases as well as phosphorylation of multiple protein tyrosine kinases in WEHI 231 cells [230]. BCR ligation of control cells caused an increase in levels of GTP-bound H-, K-, and N-Ras/GFP (Fig. 3-2A, B). An increase in H-, K- and N-Ras/GFP activation was detected in RasGRP1<sup>hi</sup> cells, compared with control cells (Fig. 3-2A, B). However statistical analysis of GTP-bound Ras values derived from three independent experiments reveal relatively high p-values (p > 0.05) at most time points evaluated, prompting caution in the interpretation of the results for individual time points. Furthermore, measurements of endogenous GTP-Ras levels would constitute a more physiologically relevant approach to determine whether increased RasGRP1 expression correlates with increased Ras activity in WEHI 231 cells. However, detection of endogenous Ras activity using Ras-specific antibodies proved to be difficult due to the inability to obtain reproducible results using currently available reagents (personal observation). Nevertheless, the results presented in Figure 3-2 suggest that increased RasGRP1 expression generally correlates with increased Ras activity.
Figure 3-2. Elevated RasGRP1 expression causes increased GTP loading of Ras GTPases.

A Control or RasGRP1<sup>high</sup> cells expressing HA-tagged K-Ras/GFP, N-Ras/GFP or H-Ras/GFP fusion proteins were stimulated for the indicated times with 20 μg/ml anti-IgM. GTP-bound Ras GTPases were purified by binding to GST-RafRBD and quantified by western blot using anti-HA for detection. The numbers indicate relative quantities of each band, normalized to control cells at 0 minutes. Control and RasGRP1<sup>high</sup> cell lysates had equivalent HA/GFP-tagged Ras content. Blots shown are from one of three experiments. B GTP-bound Ras proteins were quantified as in A. Bars are means of three values, except where indicated by *, with error bars indicating standard deviations. Bars indicated with a * are the means of two values, with error bars indicating standard deviations. Statistical significance of differences is indicated by p-values derived from one sample, two-tailed t-tests (IgM 0 min) or paired, two-tailed t-tests (IgM 1, 5 and 15 min) to compare GTP-bound Ras quantities from RasGRP1<sup>high</sup> cell lysates to those found in control at the indicated time points.

Doubling the levels of RasGRP1 had no significant effect on the basal rate of apoptosis of WEHI 231 populations grown in serum-containing medium, and the fraction of cells entering the S and G<sub>2</sub> phase of the cell cycle was unchanged compared with control populations, as measured by propidium iodide (PI) staining of fixed nuclei (Fig. 3-3A). RasGRP1<sup>high</sup> cells did divide marginally slower than control cells when cultured in serum-containing medium, as measured using dilution of
CFSE as well as mixed population studies (Fig. 3-3B,C). CFSE is a fluorescein-based dye that covalently binds proteins and is split equally between daughter cells. This enables determination of the number of divisions an individual cell has undergone based on relative brightness measured by flow cytometry. A small but reproducible reduction in cell division rate was measured in RasGRP1\textsuperscript{high} cells stained with CFSE, compared with control cells (Fig. 3-3B). The effect of increasing RasGRP1 expression on the proliferation of WEHI 231 cells in complete medium was also assessed by monitoring the ratio of control versus RasGRP1\textsuperscript{high} cells in co-cultured mixed populations. In these studies, GFP-labelled control cells were used as an internal control and were co-cultured with Thy-1 expressing control or RasGRP1\textsuperscript{high} cells. GFP and Thy-1 served as labels to monitor individual populations by flow cytometry (Fig. 3-3C). Proliferation capability relative to GFP control cells was indirectly measured as a change in the ratio of Thy-1-expressing cells over time. Whereas the ratio of Thy-1\textsuperscript{+} control cells to GFP\textsuperscript{+} control cells remained constant over 7 days of culture in complete medium, Thy-1\textsuperscript{+}RasGRP1\textsuperscript{high} cells became gradually depleted over time from cultures (Fig. 3-3C). These results suggest that increased RasGRP1 expression in WEHI 231 cells confers a modest proliferative disadvantage when cultured in complete medium, compared with control cells.
Figure 3-3. Increased RasGRP1 expression reduces the proliferative potential of WEHI 231 cells.

A. Cell cycle and apoptosis analysis using PI staining of permeabilized cells. Control or RasGRP1\textsuperscript{high} cells grown in complete medium were permeabilized, stained with PI, and analyzed by flow cytometry. The percentages of cells with sub-diploid (apoptotic) or greater than diploid (in S or G2 phases of cell cycle) quantities of DNA are indicated. Results are representative of three experiments. B. Cell division analysis. Control or RasGRP1\textsuperscript{high} cells grown in complete medium were stained with CFSE and cultured in complete medium for 48 hours. The progress of cell division was quantified by measuring dilution of CFSE fluorescence. Non-viable cells (PI\textsuperscript{+}) were gated out of the analysis. CFSE fluorescence intensity is displayed on a log scale, and peaks corresponding to two-fold reductions in fluorescence intensity represent sequential cell divisions. The fluorescence intensity of undivided cells was determined by analysis of cells after CFSE labelling but prior to culture. The percentages of cells in each peak are indicated. Results are representative of three experiments. C. RasGRP1\textsuperscript{high} become depleted from mixed cultures with control cells. Thy-1 expressing control or RasGRP1\textsuperscript{high} cells were mixed with control cells expressing GFP. The mixed populations were cultured in complete medium for 7 days, stained with an anti-human Thy-1 antibody at the indicated times and analyzed by flow cytometry. Non-viable cells (PI\textsuperscript{+}) were gated out of the analysis. The percentage of Thy-1\textsuperscript{+} cells within individual mixed populations is indicated at the bottom right corner of each dot plot. The data are representative of three experiments.

3.1.2 Increased RasGRP1 expression sensitzes WEHI 231 cells to BCR-induced apoptosis

Increased RasGRP1 expression promotes survival and proliferation of developing thymocytes following antigen receptor ligation [141]. One specific objective of this thesis was to determine whether RasGRP1 plays a similar pro-survival role during BCR signalling, a process that leads to
growth arrest and apoptosis in WEHI 231 cells [236, 237]. In order to study the effects of increased 
RasGRP1 expression on the outcome of BCR signalling in WEHI 231 cells, we used PI staining of 
fixed nuclei, a relatively simple method commonly used to monitor cell cycle arrest and levels of 
apoptosis that occur following BCR ligation of WEHI 231 populations [239]. As is the case for 
control cells, treatment of RasGRP1\textsuperscript{\textnormal{h}} cells with anti-IgM resulted in cell cycle arrest (Fig. 3-4A, 
upper panel). A greater proportion of RasGRP1\textsuperscript{\textnormal{h}} cells were undergoing apoptosis after 48 hours 
of treatment, compared with control cells (Fig. 3-4A, upper panel). An average 2.5-fold increase in 
apopoptosis was observed in RasGRP1\textsuperscript{\textnormal{h}} cells, compared with control cells after 48 hours of BCR 
ligation. To gain more insight into the ability of RasGRP1 to predispose cells to BCR-induced 
apoptosis, we monitored the ability of WEHI 231 populations to recover from BCR ligation following 
removal of the stimulus after 24 hours of treatment (see section 2.2.2 of the Materials and 
Methods). Control populations recovered promptly from short-term BCR stimulation, as measured 
by the relatively small proportion of cells undergoing apoptosis and a significant number of cells in 
the S/G\textsubscript{2} phase of the cell cycle 24 hours following removal of the stimulus (Fig. 3-4A, lower panel). 
In contrast, almost half the RasGRP1\textsuperscript{\textnormal{h}} cells were undergoing apoptosis, and very few cells 
entered the S/G\textsubscript{2} phase at that time (Fig. 3-4A, lower panel). This result was consistent with the 
observation that fewer cell division events occurred in RasGRP1\textsuperscript{\textnormal{h}} cells in the 24 hours following 
removal of the BCR ligand, compared with control cells (Fig. 3-4B).
Co-cultured mixed population assays were used to confirm the importance of RasGRP1-mediated increased apoptosis on WEHI 231 population responses to BCR ligation. In these experiments, the ratio of Thy-1-expressing control or RasGRP1<sup>high</sup> to GFP-expressing control cells was monitored following BCR ligation. The ratio within mixed cultures remained constant for control cells in the absence of anti-IgM, and the proportion of RasGRP1<sup>high</sup> cells was only slightly reduced after 48 hours of culture (Fig. 3-5). Treatment of mixed cultures with anti-IgM for 48 hours resulted in an increase in the proportion of cells permeable to PI (PI<sup>+</sup> cells), indicating that they were undergoing apoptosis and thus eventually becoming depleted from the cultures (Fig. 3-5). The fraction of PI<sup>+</sup> cells following 48 hours of BCR ligation was moderately higher in mixed cultures containing both control and RasGRP1<sup>high</sup> cells, compared with cultures containing control cells only (Fig. 3-5). Furthermore, while the proportions of control cells were constant in mixed cultures following treatment with IgM for 48 hours, the proportion of RasGRP1<sup>high</sup> cells was reduced.
indicating that RasGRPI$^{\text{high}}$ cells became depleted more rapidly than control cells from the mixed cultures (Fig. 3-5).

**Figure 3-5. Differential depletion of RasGRPI$^{\text{high}}$ cells following BCR ligation.**

Control cells or RasGRPI$^{\text{high}}$ cells co-expressing signaling-defective human Thy-1 were mixed with control cells expressing GFP. The mixed populations were cultured for 48 hours without or with 10 μg/ml anti-IgM, stained with an anti-human Thy-1 antibody and then analyzed by flow cytometry. The percentage of Thy-1$^+$ cells within individual mixed populations is indicated at the bottom right corner of each dot plot. Non-viable cells (Pl$^+$) were gated out of the analysis, and the percentage Pl$^+$ cells is indicated in bold font, at the top-right corner of each dot plot. The data are representative of three experiments.

### 3.1.3 Conclusions

Although it is possible to generate and maintain WEHI 231 populations with increased RasGRPI expression, these populations appear to possess slightly reduced proliferation capabilities, compared with control cells. In addition, a reduction in endogenous RasGRPI protein levels was detected in RasGRPI$^{\text{high}}$ populations. This could be the result of either selection for clones with lower RasGRPI expression, or negative feedback regulation of endogenous RasGRPI expression, or both. RasGRPI is detected as a doublet, with a lower main band and a fainter upper band.
Although expressed both in cell lines and thymocytes, the specific nature of the upper band is unknown, and could consist of a modified (e.g. phosphorylated) version of RasGRP1.

Increased RasGRP1 expression significantly increases the fraction of WEHI 231 cells undergoing apoptosis following 48 hours of BCR ligation. Studies performed using a short stimulation time (24 hours), followed by removal of the stimulus, provide evidence that RasGRP1 expression can affect the commitment of WEHI 231 cells to apoptosis following BCR ligation: unlike control cells, which recover promptly following removal of the stimulus, a significant proportion of the RasGRP1\textsuperscript{high} cells (almost half) undergo apoptosis. Furthermore, those RasGRP1\textsuperscript{high} cells that survive short-term BCR ligation treatment undergo fewer division events on average than control cells. Thus, increased RasGRP1 expression sensitizes cells to BCR-induced apoptosis and appears to delay re-entry of surviving cells into the cell cycle. The significance of these effects was confirmed using mixed population studies, where RasGRP1\textsuperscript{high} cells became depleted from cultures at a greater rate than control cells, following BCR ligation. These results suggest that RasGRP1 expression has the potential to affect the outcome of BCR signalling in immature B cells, where cells with increased RasGRP1 protein levels could be more susceptible to antigen-induced negative selection.
3.2 RASGRP1 SENSITIZES WEHI 231 CELLS TO BCR-INDUCED APOPTOSIS VIA ACTIVATION OF RAS FAMILY MEMBERS

3.2.1 RasGRP1 requires the ability to activate Ras family members in order to sensitize WEHI 231 cells to BCR-induced apoptosis

RasGRP1 is a Ras activator that has been shown to possess specific activity for both the classical Ras GTPases and the R-Ras family of GTPases [60]. Therefore, the next objective of this thesis was to determine whether activation of Ras GTPases by RasGRP1 was necessary and sufficient for its ability to sensitize WEHI 231 cells to BCR-induced apoptosis. An arginine-to-glutamate substitution at position 271 in the GEF domain of RasGRP1 prevents it from causing transformation of fibroblasts [69]. This point mutation reverses the charge of a residue that is critical for interaction with Ras GTPases, and is thought to prevent interaction with and activation of Ras proteins, without altering GEF domain structure [61]. To determine whether RasGRP1 requires the ability to interact with and activate Ras family members, the RasGRP1 GEF mutant (RasGRP1 R271E) was tested for its ability to cause increased BCR-induced apoptosis of WEHI 231 cells.

Two independent WEHI 231 populations were generated that expressed RasGRP1 R271E. One population (transduction 1, RasGRP1 R271E++) had RasGRP1 R271E protein levels that were nine times that of endogenous RasGRP1 in control cells (Fig. 3-6A). A second population (transduction 2, RasGRP1 R271E+) had RasGRP1 R271E protein levels that were three times that of endogenous RasGRP1 in control cells (Fig. 3-6A). No significant increase in BCR-induced apoptosis was detected in RasGRP1 R271E-expressing WEHI 231 cells, compared with control cells (Fig. 3-6B). This indicates that the ability of RasGRP1 to bind to and activate Ras GTPases is essential for its ability to sensitize WEHI 231 cells to BCR-induced apoptosis. A significant reduction in apoptosis was detected in the RasGRP1 R271E++ population, compared with control cells (Fig. 3-6B). The mechanism underlying this dominant-negative phenotype is unknown, but could reflect interference of RasGRP1 R271E with the function of endogenous RasGRP1.
3.2.2 **Ras signalling is sufficient to augment BCR-induced apoptosis of WEHI 231 cells**

In order to determine whether Ras activity alone was sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis, WEHI 231 populations expressing activated Ras family members were generated. A glycine to valine (G12V) substitution results in Ras proteins that are constitutively GTP-bound due to lack of GTPase activity, and thus signal independently of GEFs [20, 21]. WEHI 231 cells were transduced with vectors containing constitutively activated, HA-tagged H-, K-, or N-Ras GTPases (RasV12), whose expression was linked to an IRES-driven GFP. Transduced cells were drug-selected and sorted for GFP expression by flow cytometry. RasV12 proteins were not detectable by western blot analysis, suggesting that expression levels were very low in these cells,
despite GFP protein levels detectable by flow cytometry. Nevertheless, RasV12-transduced populations generally had a higher incidence of apoptosis compared with control cells following BCR ligation, albeit at levels lower than observed in RasGRP1 cells (Fig. 3-7A).

Figure 3-7. Ras signalling is sufficient to sensitize WEHI 231 cells to apoptosis.

WEHI-231 cells were transduced with retroviral vectors expressing the indicated proteins linked to an IRES GFP, drug-selected, sorted for GFP and treated with 10 μg/ml anti-IgM for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells, as shown in Fig. 3-4A. To control for variation between experiments in the extent to which anti-IgM treatment causes apoptosis, data is presented as % apoptotic cells in transduced cells divided by % apoptotic cells in control cells. Each circle indicates the mean of a single experiment with duplicate cultures, and lines indicate means of the five experiments. Statistical significance of differences is indicated by p-values derived from one sample, two-tailed t-tests to compare apoptosis in RasV12- or RasGRP1-transduced cells to control.

In addition to the classical Ras proteins, RasGRP1 has been shown to possess the ability to promote activation of the three Ras proteins, including M-Ras [44]. M-RasL71 is an activated Ras GTPase that, in comparison with H-RasV12, provides lower signaling intensity through Ras-activated signaling pathways [58]. To test the ability of M-RasL71 to sensitize WEHI 231 cells to BCR-induced apoptosis, WEHI 231 cells were transduced with vectors containing a cDNA that encodes HA-tagged M-RasL71 whose expression is linked to an IRES-driven GFP. Transduced cells were drug-selected and sorted for GFP expression by flow cytometry. Unlike the RasV12 proteins, M-RasL71 was detectable by western blot analysis. WEHI 231 cells transduced with M-RasL71 had greater levels of BCR-induced apoptosis, compared with control cells (Fig. 3-8A).
Figure 3-8. M-Ras signalling has the potential to sensitize WEHI 231 cells to BCR-induced apoptosis.

A. WEHI-231 cells were transduced with retroviral vectors expressing the indicated proteins linked to an IRES GFP, drug-selected, sorted for GFP and treated with 10 μg/ml anti-IgM for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells, as shown in Fig. 3-4A. To control for variation between experiments in the extent to which anti-IgM treatment causes apoptosis, data is presented as % apoptotic cells in transduced cells divided by % apoptotic cells in control cells. Each circle indicates the mean of a single experiment with duplicate cultures, and lines indicate means of the five experiments. Statistical significance of differences is indicated by p-values derived from one sample, two-tailed t-tests to compare apoptosis in M-RasL71-transduced cells to control. 

B Control or RasGRP1<sup>high</sup> cells expressing HA-tagged, wild-type M-Ras (HA-M-Ras) were stimulated for the indicated times with 20 μg/ml anti-IgM. GTP-bound HA-M-Ras was purified by binding to GST-RafRBD and quantified by Western blot using anti-HA for detection. The numbers indicate relative quantities of each band, normalized to control cells at 0 minutes. An increase in HA-M-Ras content within RasGRP1<sup>high</sup> cell lysate was factored into the relative quantity values. A single experiment was performed.

To confirm the previously published observation that RasGRP1 can activate M-Ras [44], levels of GTP-bound M-Ras were measured in control and RasGRP1<sup>high</sup> WEHI 231 populations expressing HA-tagged M-Ras, both prior to and following BCR ligation. GTP-bound M-Ras levels were moderately higher after 5 minutes of BCR ligation in RasGRP1<sup>high</sup>, compared with control cells (Fig. 3-8B). This preliminary result is consistent with previous reports demonstrating the ability of RasGRP1 to activate M-Ras [44], and is also consistent with the results presented in Figure 3-2, which suggest that increased RasGRP1 expression in WEHI 231 correlates with increased Ras activation.
Further experiments would have to be conducted to determine the extent to which increased RasGRP1 expression can augment BCR-induced M-Ras activation in WEHI 231 cells, or whether M-Ras acts as a RasGRP1 effector in these cells. Although M-Ras is capable of weakly activating Raf proteins, its effectors differ significantly from the classical Ras family members, and include the RalDGS-related protein Rif [58], and the RASSF1 homolog Nore-1 [126]. Therefore, in addition to using the Ras binding domain of Raf, experiments would have to be performed that use the Ras binding domain of Rif or Nore-1 to measure activity of M-Ras via affinity precipitation of active Ras proteins.

3.2.3 RasGRP1-mediated increased apoptosis occurs independently of its ability to cause sustained ERK2 phosphorylation

The best-studied effectors of Ras signalling are the MAP kinases ERK1/2, which become phosphorylated and activated following activation of the Ras-Raf-MEK kinase cascade [1]. BCR ligation of WEHI 231 cells leads to a robust and short-lived increase in ERK activity [224, 225]. The hypothesis that RasGRP1 mediates its effect by increasing basal or BCR-induced activation of ERK proteins was therefore tested by comparing ERK phosphorylation in RasGRP1\textsuperscript{high} to control cells, with a phospho-specific anti-ERK1/2 antibody commonly used to monitor ERK activation. Short-term ERK activation studies performed under serum-free conditions failed to demonstrate any effect of increased RasGRP1 expression on BCR-induced ERK activation in WEHI 231 cells (Fig. 3-9A). However, increased RasGRP1 expression was found to cause a marginal but reproducible increase in ERK activity after 15 minutes of BCR ligation when WEHI 231 cells were grown in the presence of serum (Fig. 3-9B). Whereas levels of active ERK were back to base levels in control cells following 60 minutes of BCR ligation, ERK activation was maintained well above base levels in RasGRP1\textsuperscript{high} cells (Fig. 3-9B). This result suggests that increased RasGRP1 expression promotes sustained BCR-mediated ERK activation.
Figure 3-9. Increased RasGRP1 expression causes sustained BCR-induced ERK activation in WEHI 231 cells.

A. Short-term anti-IgM stimulations under serum-free conditions. Control or RasGRP1^{high} cells were plated in activation buffer and treated for the indicated times with 20 μg/ml of anti-IgM, as described for the Ras activation assays (short-term stimulations under serum-free conditions, section 2.3.2.1). Cell lysates were quantified for T202/Y204-phosphorylated ERK1/ERK2 by western blot. The numbers indicate relative quantities of each ERK2 band, normalized to control cells at 0 minutes. The arrowheads indicate the positions of the pERK1 and pERK2 bands, which had estimated molecular masses of 44 kDa and 42 kDa respectively. Results are representative of three experiments.

B. Long-term anti-IgM stimulations. Control or RasGRP1^{high} cells were treated for the indicated times with 10 μg/ml of anti-IgM. Cell lysates were quantified for T202/Y204-phosphorylated ERK1/ERK2 by western blot. The numbers indicate relative quantities of each ERK2 band, normalized to control cells at 0 minutes. The arrowheads indicate the positions of the pERK1 and pERK2 bands, which had estimated molecular masses of 44 kDa and 42 kDa respectively. Results are representative of three experiments. Statistical significance of differences is indicated by p-values derived from one sample, two-tailed t-tests (IgM 0 min) or paired, two-tailed t-tests (IgM 15, 60 and 240 min) to compare relative quantities of ERK2 bands in RasGRP1^{high} versus control cells from three experiments.

ERK activation can be suppressed by treating WEHI 231 cells with the MEK1/2-specific inhibitors PD 98059 and U0126 [244]. In order to determine whether an increase in ERK activation was required for the ability of RasGRP1 to augment apoptosis, control and RasGRP1^{high} cells were treated with either PD 98059 or U0126 before and during BCR ligation (Fig. 3-10A). RasGRP1 retained its full ability to augment apoptosis when phosphorylated ERK levels in RasGRP1^{high} populations were reduced to that of control cells (Fig. 3-10B). These results suggest that activation...
of ERK proteins does not contribute to the ability of RasGRP1 to cause increased apoptosis of WEHI 231 cells following BCR ligation, as previously described by another group [244].

Figure 3-10. ERK1 and ERK2 activation is not required for enhancement of BCR-induced apoptosis by RasGRP1.

A. Control or RasGRP1\textsuperscript{high} cells were treated for the indicated time with 10 \mu g/ml of anti-IgM. As indicated, the MEK1/2 inhibitors PD98059 (20 \mu M) or U0126 (10 \mu M) were added 60 minutes prior to and during treatment with anti-IgM. Cell lysates were quantified for phosphorylated ERK1/2 by western blot. Results are representative of two experiments. B. Control or RasGRP1\textsuperscript{high} cells were left untreated or treated with 10 \mu g/ml anti-IgM for 48 hours in the presence or absence of MEK inhibitors as indicated. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of duplicate cultures from one experiment, with standard deviations indicated by error bars. Results are representative of two experiments.

### 3.2.4 An active form of Raf-1 promotes BCR-induced apoptosis of WEHI 231 cells

BCR ligation leads to the activation of both Raf-1 and B-Raf, and studies performed in the DT40 B cell line suggest that while B-Raf significantly contributes to BCR-mediated ERK activation, Raf-1 serves as an accessory molecule in the process [283]. Raf-1 must overcome auto-inhibition in order to become fully activated, a process that can be by-passed by removing a significant portion of its N-terminus, including the serine residue at position 259 [90, 91]. To investigate whether signalling downstream of Raf-1 could contribute to the ability of RasGRP1 to promote BCR-induced apoptosis, a constitutively active form of Raf-1 that lacks the N-terminal auto-inhibitory domain (Raf-1\textsuperscript{NT}) was transduced into WEHI 231 cells (Fig. 3-11A). Despite a strong ability to transform
fibroblasts [89], Raf-1NT failed to promote ERK phosphorylation in WEHI 231 cells, either prior to or following BCR ligation (Fig. 3-11B). Raf-1NT-transduced cells had a higher incidence of BCR-induced apoptosis cells after 72 hours of treatment with anti-IgM, albeit at levels lower than RasGRP1high cells, and with modest statistical significance (Fig. 3-11C). These preliminary results suggest that Raf-1 signalling has the potential to promote BCR-induced apoptosis in WEHI 231 cells. The use of additional constitutively active mutants of Raf-1, such as prenylated forms of wild-type or Raf-1NT that are constitutively localized to plasma membranes [87], in addition to solid data on Raf-1 activity, would be required to confirm that Raf-1 has the ability to promote BCR-induced apoptosis of WEHI 231 cells.
**Figure 3-11.** An active form of Raf-1 has the ability to sensitize WEHI 231 cells to BCR-induced apoptosis. 

**A.** WEHI-231 cells were transduced with retroviral vectors expressing the indicated proteins, drug-selected and sorted for GFP as a marker for protein expression. Cells were treated with anti-IgM for the indicated times and Western blot analysis was performed on cell lysates using an anti-Raf-1 antibody to measure relative Raf-1 expression. Estimated molecular mass for wild-type Raf-1 is 76 kDa, while Raf-1NT construct lead to the production of two separate bands, one at approximately 45 kDa (expected mass), and the lower band at approximately 28 kDa. The nature of the lower band is not known, but could be the product of cleavage or degradation of Raf-1NT. 

**B.** Western blot analysis performed on lysates generated as in A, using anti-ERK and anti-pERK antibodies. The numbers indicate relative quantities of each ERK2 band, normalized to control cells at 0 minutes. 

**C.** WEHI 231 cells expressing the indicated proteins were either untreated or treated with 10 μg/ml anti-IgM for 48 or 72 hours. Apoptosis was quantified by PI staining of permeabilized cells at 48 and 72 hours, as shown in Fig. 3-4A. Data is presented as the mean % apoptotic (sub-diploid) cells of duplicate cultures from two (72 h) or three (48 h) experiments, with error bars indicating standard deviation. Statistical significance of differences is indicated by p-values derived from paired, two-tailed t-tests performed on % apoptosis values in Raf-1NT versus control cells for the indicated times.

### 3.2.5 PI-3 kinase signalling is not required for the ability of RasGRP1 to augment BCR-induced apoptosis in WEHI 231 cells

PI-3 kinase is a Ras effector that becomes activated in a Ras-dependent manner following BCR ligation [252]. In WEHI 231 cells, BCR-induced PI-3 kinase activation leads to activation of the kinase Akt [243, 254]. Inhibition of PI-3 kinase activity in WEHI 231 cells results in cell cycle arrest and apoptosis, while transfection of an activated form of Akt strongly reduces BCR-induced cell
cycle arrest and apoptosis in these cells [243]. The PI-3 kinase pathway is therefore a contradictory candidate for mediating the apoptosis-enhancing effects of RasGRP1. Nevertheless, the hypothesis that RasGRP1 sensitizes WEHI 231 cells to BCR-induced apoptosis via activation of the PI-3 kinase pathway was tested.

The ability of RasGRP1 to activate PI-3 kinase was measured indirectly by monitoring the levels of phosphorylated Akt in WEHI 231 cells. The levels of phosphorylated Akt in RasGRP1\textsuperscript{high} cells were compared to those in control cells before and after BCR ligation. Increased levels of phosphorylated Akt were detected in RasGRP1\textsuperscript{high} cells, compared to control cells (Fig. 3-12A). This preliminary result suggests that RasGRP1 promotes rather than inhibits PI-3 kinase activity, although more experiments would be required to establish the extent to which increased RasGRP1 expression can affect Akt phosphorylation in WEHI 231 cells.

The PI-3 kinase inhibitor LY294002 was used to determine whether inhibition of the PI-3 kinase pathway prevents the ability of RasGRP1 to promote BCR-induced apoptosis. LY294002 has previously been shown to reduce signalling downstream of PI-3 kinase in WEHI 231 cells [243, 254]. Microscopic examination revealed that treatment of WEHI 231 cells with high levels of LY294002 (25 μM) resulted in spontaneous cell death in the absence of BCR ligation, as previously observed by at least one other group [243]. Addition of 5 μM LY294002 to complete medium had no effect on the rate of apoptosis of WEHI 231 cells, as detected by PI staining of fixed nuclei (Fig 3-12B). However, an increase in apoptosis was observed following BCR ligation in the presence of 5μM LY294002 in both control and RasGRP1\textsuperscript{high} cells (Fig. 3-12B). This preliminary result suggests that blocking PI-3 kinase signalling promotes, rather than inhibits BCR-induced apoptosis of WEHI 231 cells.

Combined, these preliminary data suggest that RasGRP1 could increase PI-3 kinase activity, but does not cause increased apoptosis by increasing PI-3 kinase signalling. However, more extensive studies would be required to establish whether RasGRP1 plays any significant role in the modulation of Ras-mediated PI-3 kinase activation, including more thorough studies using PI-3
kinase inhibitors, PI-3 kinase assays, and studies performed to assess the effect of increased RasGRP1 expression on PI-3 kinase effector pathways.

Figure 3-12. RasGRP1-mediated PI-3 kinase activation does not appear to contribute to BCR-mediated apoptosis.

A Short-term IgM stimulations of control or RasGRP1\textsuperscript{high} cells were performed as described in Figure 3-9A. Cell lysates were quantified for protein content, and western blot analysis was performed using anti-Akt and phospho-specific anti-Akt (Ser473) antibodies. The estimated molecular masses of Akt and phospho-Akt are 65 kDa. The numbers indicate relative quantities of each pAkt band, normalized to control cells at 0 minutes. A single experiment was performed. B Control or RasGRP1\textsuperscript{high} cells were left untreated (nil) or treated with 10 μg/ml anti-IgM for 48 hours in the presence or absence of 5μM LY294002. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of duplicate cultures from one experiment, with standard deviations indicated by error bars.

3.2.6 Conclusions

RasGRP1 likely requires the ability to activate Ras family members in order to sensitize WEHI 231 cells to BCR-induced apoptosis since expression of RasGRP1 R271E failed to increase BCR-induced apoptosis of WEHI 231 cells. Furthermore, WEHI 231 cells that expressed levels of RasGRP1 R271E nine times higher than endogenous RasGRP1 displayed a dominant-negative phenotype, and were significantly less sensitive to BCR-induced apoptosis than control cells. This phenotype could be the result of a reduction in BCR-induced Ras activation caused by to the ability
of the RasGRP1 R271E to compete for positive regulators of RasGRP1 (e.g. DAG) and its inability to cause Ras activation. This would prevent wild-type GEFs from binding to and activating GTPases, resulting in an overall reduction of GTPase activity as RasGRP1 R271E maintains these GTPases in an inactive, GDP-bound state. This effect has previously been observed in Jurkat T cells following TCR ligation, as RasGRP1 R271E caused a significant reduction in Ras activation [41]. However the ability of RasGRP1 R271E to reduce Ras activation was not tested during the course of these studies, and these experiments would be required to establish that RasGRP1 R271E does indeed act as a dominant-negative in WEHI 231 cells. Alternatively, RasGRP1 R271E has the potential to inhibit signalling via other proteins by limiting access to DAG-rich membrane regions. Signalling by proteins such as PKC family members, which require DAG binding in order to become activated, could therefore be impeded. This effect has the potential to contribute to the ability of increased RasGRP1 expression to sensitize WEHI 231 cells to BCR-induced apoptosis. The need for a relatively high ratio of RasGRP1 R271E to wild-type GEF to reduce GTPase activity could explain why a significant dominant-negative effect was not observed in cells expressing moderate amounts of RasGRP1 R271E.

The generation of WEHI 231 populations with stable and detectable expression of activated classical Ras (V12) GTPases was not achieved, suggesting the possibility that negative selection for clones with high expression occurred due to deleterious effects caused by RasV12 in the absence of BCR ligation. Nevertheless, transduced populations selected for IRES-driven GFP expression but with levels of activated Ras below detection were generally more sensitive to BCR-induced apoptosis than control cells. The degree of variation observed in the experiments could reflect the instability and fluctuation of RasV12-expressing WEHI 231 populations. However, WEHI 231 populations expressing detectable levels of activated M-Ras (L71), which signals via classical Ras effectors albeit with lower efficiency, were generated and were consistently more susceptible to BCR-induced apoptosis than control cells. These results suggest that when the amount of Ras signalling gets too high, as appears to be the case in RasV12-transduced cells, WEHI 231 cells are deleted even in the absence of BCR ligation. Thus, the results described above suggest that
RasGRP1 sensitizes WEHI 231 cells to BCR-induced apoptosis via the activation of Ras GTPases, and that Ras signalling alone can augment BCR-induced apoptosis in WEHI 231 cells.

Increased activity of two downstream effectors of Ras signalling, PI-3 kinase and Raf, as measured by Akt phosphorylation and ERK phosphorylation respectively, was detected in RasGRP1<sup>high</sup> cells. This suggests that increased RasGRP1 expression augments Ras signalling in WEHI 231 cells. However treatment with PI3-kinase inhibitors, or MEK inhibitors to reduce ERK activation failed to prevent the ability of RasGRP1 to sensitize WEHI 231 cells to BCR-induced apoptosis, suggesting that activation of the PI-3 kinase and Raf-ERK pathways were not required for the ability of RasGRP1 to sensitize WEHI 231 cells to BCR-induced apoptosis. Expression of an activated form of Raf-1 in WEHI 231 did cause a modest increase in BCR-induced apoptosis without affecting the magnitude of ERK activation. These results point to the possibility that RasGRP1 sensitizes WEHI 231 cells via an ERK-independent signalling cascade that could include Raf-1 activation. Raf-1 has been shown to possess signalling capabilities that are not dependent of ERK activation [98]. However, experiments that test Raf-1 activation directly would be required to determine whether RasGRP1 can actually cause an increase in Raf-1 activity. Alternatively, RasGRP1 could promote BCR-induced apoptosis via a combination of signals that do not include Raf-1, thus making Raf-1 signalling by itself insufficient to mimic signals generated downstream of RasGRP1.
3.3 RASGRP1-MEDIATED INHIBITION OF NF-κB AS A PUTATIVE MECHANISM FOR AUGMENTING SENSITIVITY OF WEHI 231 CELLS TO BCR-INDUCED APOPTOSIS

3.3.1 Increased RasGRP1 expression represses NF-κB activity by causing an increase in cytoplasmic IκBα levels

NF-κB activity is critical in the life and death decisions that occur in WEHI 231 cells. Signalling events that rescue WEHI 231 cells from BCR-induced apoptosis, such as LPS and CD40 signalling, are thought to operate by causing upregulation of NF-κB activity [268, 270, 284, 285]. NF-κB regulation is mediated by IκB proteins that maintain NF-κB family members in an inactive state in the cytoplasm. Receptor signalling results in IκB degradation that is mediated by activation of IKKs [272]. As opposed to CD40 signalling or LPS treatment, which trigger degradation of IκB proteins thereby increasing NF-κB activity, BCR ligation of WEHI 231 cells leads to an increase in IκB protein levels [268, 286, 287]. Since RasGRP1 promotes BCR-induced apoptosis, the effect of increased RasGRP1 expression on the NF-κB pathway was studied. A luciferase–based transcription assay was used to compare basal levels of NF-κB activity in RasGRP1\textsuperscript{high} versus control cells. A two-to three fold decrease in NF-κB activity was detected in RasGRP1\textsuperscript{high} cells grown in complete medium, compared with control cells (Fig. 3-13).
To determine whether RasGRP1 is able to modulate NF-κB regulation via the IκB proteins, the effect of increased RasGRP1 expression on cytoplasmic IκB levels in control versus RasGRP1\textsuperscript{high} cells prior to and during BCR ligation was investigated. BCR ligation in WEHI 231 cells for up to 60 minutes had little effect on cytoplasmic IκB\textsubscript{α} levels, but caused a sharp increase at 4 hours (Fig. 3-14). Increased RasGRP1 expression caused a 2- to 3-fold increase in the cytoplasmic IκB\textsubscript{α} levels both before and following 15 minutes of BCR ligation, and a more modest increase following 60 minutes and 4 hours of stimulation, compared to control cells (Fig. 3-14). BCR ligation in WEHI 231 cells lead to an initial decrease in IκB\textsubscript{β} levels, followed by an increase back to control levels (Fig. 3-14). Cytoplasmic IκB\textsubscript{β} levels were generally unaffected by increased RasGRP1 expression (figure 3-14). These results suggest that increased RasGRP1 expression lowers NF-κB activity by causing an increase in cytoplasmic IκB\textsubscript{α} levels.
To determine whether inhibition of NF-κB activity is required for the ability of RasGRP1 to promote BCR-induced apoptosis, signals that promote the activation of NF-κB, LPS and CD40 signalling, were tested for their ability to inhibit BCR-induced apoptosis in control and RasGRP1^{high} cells. CD40 and LPS have previously been shown to cause degradation of IkB proteins in WEHI 231 cells [268, 286]. Both CD40 and LPS were able to suppress BCR-induced apoptosis in control and RasGRP1^{high} cells, suggesting the ability of RasGRP1 to sensitize WEHI 231 cells to BCR-induced apoptosis could be dependent on inhibition of NF-κB activity (Fig. 3-15A).

BAY 11-7082, is an inhibitor of IkBα phosphorylation and degradation that blocks nuclear translocation of NF-κB in WEHI-231 cells [270]. This inhibitor was therefore used to test whether the ability of increased RasGRP1 expression to inhibit NF-κB activity is sufficient to increase BCR-induced apoptosis of WEHI 231 cells. At 5 μM, BAY 11-7082 caused a significant increase in levels of IkBα and induced spontaneous apoptosis of WEHI-231 cells, even in the absence of
BCR ligation (Fig. 3-15B, C). BAY 11-7082 at 1 μM had no effect on apoptosis in the absence of BCR ligation but increased the incidence of apoptosis following BCR ligation, despite a very subtle effect on IkBα levels in whole cell lysates (Fig. 3-15B, C). Thus inhibition of IkBα degradation is sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis.

Figure 3-15 RasGRP1-mediated enhancement of BCR-induced apoptosis is blocked by NF-κB activators and mimicked by stabilization of cytoplasmic IkBα.

A Control or RasGRP1high cells were left untreated (nil) or treated with 10 μg/ml anti-IgM with or without 0.1 μg/ml anti-CD40 or 5 μg/ml LPS for 48 hours. After permeabilization and staining with PI, cells were analysed by flow cytometry to determine the percentages of apoptotic cells. Bars indicate mean values from three experiment each with duplicate cultures, with standard deviations indicated by error bars. B Control and RasGRP1high cells were untreated, or treated with Bay 11-7082 as indicated. Western blot analysis was performed on whole cell lysates using an anti-IkBα antibody. Estimated molecular weight for IkBα is 35 kDa. The numbers indicate relative quantifies of each band, normalized to control cells at 0 minutes. A single experiment was performed. C WEHI-231 cells were treated with for 48 hours Bay 11-7082 and/or 10 μg/ml anti-IgM, as indicated. After permeabilization and staining with PI, cells were analysed by flow cytometry. The percentages of apoptotic cells are indicated. Results are representative of three experiments.
3.3.2 Increased RasGRP1 expression causes a reduction of Bcl-xL protein levels

Bcl-xL is an anti-apoptotic member of the Bcl-2 family that becomes down-regulated following BCR ligation in WEHI 231 cells, and whose regulation is at least partially mediated via NF-κB family members [265, 269]. To determine whether RasGRP1 expression could affect Bcl-xL protein levels, Bcl-xL content in RasGRP1^{high} cells was compared to that of control cells. RasGRP1^{high} cells had Bcl-xL levels that were significantly lower than control cells prior to BCR ligation (Figure 3-16A). As expected, BCR ligation in control cells resulted in a strong reduction in Bcl-xL levels, an effect that was modestly amplified in RasGRP1^{high} cells (Figure 3-16A).

Constitutive expression of Bcl-xL has previously been shown to block BCR-induced apoptosis of WEHI 231 cells [264, 265]. To determine whether RasGRP1-mediated sensitization of WEHI 231 cells to BCR-induced apoptosis could blocked by constitutive Bcl-xL expression, control and RasGRP1^{high} cells were transduced with either control vectors or vectors bearing cDNAs encoding for Bcl-xL, drug-selected and monitored for BCR-induced apoptosis. Transduction with Bcl-xL reduced BCR-induced apoptosis of control cells by about 50%, and was sufficient to reduce BCR-induced apoptosis levels in RasGRP1^{high} cells well below control levels (Fig. 3-16B). This result suggests that RasGRP1-mediated sensitization of WEHI 231 cells can be blocked by constitutive expression of Bcl-xL. This raises the possibility that the modest decrease in Bcl-xL levels observed in RasGRP1^{high} cells has the potential to contribute to the hyper-sensitivity of RasGRP1^{high} cells to BCR-induced apoptosis.
Elevated RasGRP1 expression causes a decrease in Bcl-xL levels, and enforced expression of BC1-XL suppresses RasGRP1-induced apoptosis.

A. Control or RasGRP1<sup>high</sup> cells were treated with 10 µg/ml anti-IgM for the indicated time. Cell extracts were quantified for Bcl-xL by western blot. The arrowhead indicates the position of the Bcl-xL band, which had an estimated molecular mass of 26 kDa. The numbers indicate relative quantities of each band, normalized to control cells at 0 minutes. Results are representative of three experiments. Statistical significance of differences is indicated by p-values derived from either one sample, two-tailed t-tests (IgM 0 h) or paired, two-tailed t-tests (IgM 1, 4, and 24 h) performed to compare relative Bcl-xL quantities in RasGRP1<sup>high</sup> cells to control in three experiments. B. Control or RasGRP1<sup>high</sup> cells were transduced with empty vector or retroviral vector containing a cDNA encoding for human BC1-XL, drug-selected, and treated with 10 µg/ml anti-IgM for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of duplicate cultures from two experiments, with standard deviations indicated by error bars. Results are representative of three experiments.

3.3.3 Conclusions

NF-κB regulates proteins such as Bcl-xL, A1 and c-Myc, whose levels influence the survival of WEHI 231 cells [268, 269]. Increased RasGRP1 expression caused accumulation of IκBα proteins.
in the cytoplasm of WEHI 231 cells, thereby inhibiting NF-κB activity. This effect proved to be sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis, as using low concentrations of the IκBα degradation inhibitor Bay 11-7082 to inhibit NF-κB also sensitized WEHI 231 cells to BCR-induced apoptosis. Increased RasGRP1 expression also caused a reduction of at least one NF-κB-regulated pro-survival protein, Bcl-xl. Treatment of WEHI 231 cells with agents that cause the active degradation of IκBα and are known to induce NF-κB activity, CD40 signalling and LPS, were sufficient to prevent RasGRP1-mediated sensitization of WEHI 231 cells to BCR-induced apoptosis. Together, these results suggest that RasGRP1 sensitizes WEHI 231 cells by inhibiting NF-κB activity.

A clear link between Ras activation and accumulation of IκBα has yet to be established and requires further investigation. Furthermore, accumulation of IκBα could be the result of a Ras-independent function of RasGRP1, or the result of the ability of RasGRP1 to block signalling via other proteins that promote the degradation of IκBα. Nevertheless, the ability to suppress RasGRP1-mediated increased apoptosis by CD40 signalling and LPS suggest that RasGRP1-mediated sensitization simply enhanced physiological mechanisms normally resulting in the apoptosis of WEHI 231 cells. Further investigation into the ability of RasGRP1 to suppress the activity of survival factors other than Bcl-xl, such as A1 or Myc, might provide further insight into the role of RasGRP1 during BCR signalling in WEHI 231 cells.
3.4 ASSESSMENT OF THE CONTRIBUTION OF THE REGULATORY DOMAINS OF RASGRP1 DURING BCR SIGNALLING IN WEHI 231 CELLS

3.4.1 RasGRP1 requires an intact C1 domain and EF-Hand motif to sensitize WEHI 231 cells to BCR-induced apoptosis

RasGRP1 activation is highly dependent on the protein’s ability to become recruited to membranes via a DAG binding C1 domain, where it comes into contact with Ras GTPases [41, 42, 67, 69]. Deletion of the C1 domain prevents RasGRP1 from translocating to membranes, and abolishes its ability to activate Ras family members and therefore cause transformation of fibroblasts [42, 69]. Since ligation of the BCR leads to activation of PLC enzymes and the generation of DAG in membranes, it is highly likely that the primary mode of RasGRP1 activation in WEHI 231 cells consists of recruitment to DAG-rich membranes via the C1 domain. Ligation of the BCR has indeed been observed to promote the recruitment of RasGRP1 to plasma membranes in DT-40 cells [228]. Therefore, to test the hypothesis that RasGRP1 requires a C1 domain to promote BCR-induced apoptosis of WEHI 231 cells, a mutant of RasGRP1 that lacks the C1 domain (RasGRP1 C1Δ, Fig. 3-17A) was transduced in WEHI 231 cells and tested for its ability to augment BCR-induced apoptosis. Expression levels of RasGRP1 C1Δ in transduced WEHI 231 populations exceeded those of exogenous RasGRP1 found in simultaneously transduced RasGRP1high cells (Fig. 3-17B). Expression of RasGRP1 C1Δ had no effect on the sensitivity of WEHI 231 cells to BCR-induced apoptosis, indicating that RasGRP1 requires a C1 domain to sensitize WEHI 231 to BCR-induced apoptosis (Fig. 3-17C).
Figure 3-17. Deletion of either the C1 domain or EF-hand motifs prevent RasGRP1 from sensitizing WEHI 231 cells to BCR-induced apoptosis.

A. Schematic diagram illustrating the location of the deletions within the mutant forms of RasGRP1 used in these experiments. GEF: guanine nucleotide exchange factor motif; EFH: EF-hands; C1: diacylglycerol-binding C1 domain.

B. WEHI 231 cells were transduced with retroviral vectors expressing the indicated proteins, selected and sorted. Western blot analysis was performed using an anti-HA antibody to confirm expression of exogenous RasGRP1 and mutant RasGRP1 proteins. C. Control or RasGRP1* cells, or WEHI-231 cells transduced with EF hand-deleted or C1 domain-deleted forms of RasGRP1, were left untreated (nil) or treated with 10 μg/ml anti-IgM for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of two experiments each with duplicate cultures, with standard deviations indicated by error bars. Statistical signiﬁcance of differences is indicated by p-values derived from paired, two-tailed t-tests performed to compare % apoptosis in RasGRP1-expressing versus control cells in two experiments.

RasGRP1 has a two calcium-binding EF-hand motifs, which together form a regulatory domain that has the ability to modulate the activity of several proteins, such as calmodulin [67, 72]. EF-hands typically serve as calcium sensors that enable proteins to alter their structural conformation following an increase in intracellular calcium, usually resulting in the adoption of an active
conformation [72]. The EF-hands of RasGRP1 therefore have the potential to act as a calcium-sensing motif that promotes activation of the protein. However, a role for the calcium-binding EF-hands of RasGRP1 has yet to be described, since mutant forms of RasGRP1 that either lack the EF-hands completely, or are unable to bind calcium, retain the ability to cause transformation of fibroblasts [69]. Furthermore, an increase in cellular calcium alone is not sufficient for RasGRP1 to cause significant Ras activation [68]. To determine whether RasGRP1 requires the ability to bind calcium via its EF-hands in order to sensitize WEHI 231 cells to BCR-induced apoptosis, a mutant form of RasGRP1 that has its EF-hand motifs deleted (RasGRP1 EFDelta, Fig. 3-17A) was transduced in WEHI 231 cells and tested for its ability to augment BCR-induced apoptosis. Expression levels of RasGRP1 EFDelta in transduced WEHI 231 populations were similar to exogenous RasGRP1 levels found in simultaneously transduced RasGRP1^{high} cells (Fig. 3-17B). WEHI 231 cells expressing RasGRP1 EFDelta had levels of BCR-induced apoptosis equivalent to control cells, suggesting that RasGRP1 requires intact EF-hands and the ability to bind calcium in order to increase sensitivity of WEHI 231 cells to BCR-induced apoptosis (Fig. 3-17C).

3.4.2 Chemical reagents that mimic accumulation of membrane diacylglycerol and increased intracellular calcium are sufficient for RasGRP1 to promote apoptosis of WEHI 231 cells

Ligation of the BCR on the surface of WEHI 231 cells activates a multitude of signalling effectors, including tyrosine kinases, phospholipases and Ras GTPases [230]. RasGRP1 activation is thought to be primarily the result of PLC activity, which promotes the generation of DAG in membranes as well as an increase in intracellular calcium in WEHI 231 cells [230]. Since RasGRP1 has the potential to be regulated via accumulation of membrane DAG as well as an increase in intracellular calcium, it would be useful to study these signals in isolation to test for their ability to modulate RasGRP1 activity. One method used to study individual signals in isolation consists in mimicking the signals using chemical reagents. To this effect, phorbol myristate acetate (PMA), a lipophilic phorbol ester that binds the C1 domain of RasGRP1 and thus confers
membrane localization [69], and the calcium ionophore ionomycin, are commonly used in WEHI 231 cells to mimic DAG accumulation in membranes and increased intracellular calcium, respectively [288-290]. Treatment of WEHI 231 cells with PMA and ionomycin results in cell cycle arrest and apoptosis [290]. Therefore, to determine whether generation of DAG in membranes combined with an increase in intracellular calcium are sufficient to promote RasGRP1 activation, the ability of increased RasGRP1 expression to sensitize WEHI 231 cells to treatment with PMA and ionomycin was tested. Stimulation of WEHI 231 cells with a combination of PMA and ionomycin caused a strong apoptosis response, and RasGRP1\textsuperscript{high} cells were more susceptible to PMA and ionomycin-induced apoptosis than control cells (Fig. 3-18). These results suggest that signalling events that occur downstream of PLC activation, DAG accumulation in membranes and increased intracellular calcium, are sufficient to activate RasGRP1.

![Figure 3-18. RasGRP1 sensitizes WEHI 231 cells to apoptosis induced by treatment with PMA and ionomycin.](image)

Control and RasGRP1\textsuperscript{high} were either left untreated (nil) or treated with a combination of 1.6 μM PMA and 1μg/ml ionomycin for 48 hours. Cells were then permeabilized and stained with PI prior to analysis by flow cytometry. The percentages of cells with sub-diploid (apoptotic) or greater than diploid (in S or G2 phases of cell cycle) quantities of DNA are indicated. Results are representative of three experiments.

To characterize the minimal signals required for RasGRP1 activation, the fraction of apoptotic cells in control and RasGRP1\textsuperscript{high} populations were measured following treatment with PMA or ionomycin, used in isolation. PMA alone was sufficient to promote apoptosis in WEHI 231 cells,
albeit at levels lower than when used in combination with ionomycin (Fig 3-19A). RasGRP1\textsuperscript{high} cells were twice more susceptible to PMA-induced apoptosis than control cells (Fig. 3-19A). These results suggest that an increase in membrane DAG, as mimicked by PMA treatment, is sufficient to activate RasGRP1. In order to address the relationship between accumulation of membrane DAG and the activation of RasGRP1 as a result of C1-mediated recruitment to membranes, the ability of RasGRP1 C1Δ to sensitize WEHI 231 cells to PMA-induced apoptosis in the presence or absence of ionomycin was tested. RasGRP1 C1Δ failed to sensitize WEHI 231 cells to apoptosis induced by PMA alone or in combination with ionomycin, confirming that RasGRP1 requires a C1 domain for recruitment to membranes and activation in WEHI 231 cells (Fig. 3-19B). Treatment of WEHI 231 cells with ionomycin caused a marginal apoptosis response, and RasGRP1\textsuperscript{high} cells treated with ionomycin had marginally higher apoptosis levels than control cells in response to ionomycin (Fig. 3-19A). A requirement for the EF-hand motif during RasGRP1 activation was tested by comparing the ability of RasGRP1 EFΔ to promote apoptosis of WEHI 231 cells in response to PMA alone or in combination with ionomycin. RasGRP1 EFΔ was unable to promote PMA-induced apoptosis of WEHI 231 cells, whether in the presence or absence of ionomycin, suggesting a strict requirement for calcium binding during RasGRP1 activation (Fig. 3-19B).
Figure 3-19. Enhancement of PMA- or PMA and ionomycin-induced apoptosis requires the C1 domain and EF hands of RasGRPI.

A. Control or RasGRP1\textsuperscript{hi} cells were left untreated (nil), or treated with either 1.6 \( \mu \text{M} \) PMA, or 1 \( \mu \text{g/ml} \) ionomycin, or both, for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of two experiments each with duplicate cultures, with standard deviations indicated by lines. B. Control or RasGRP1\textsuperscript{hi} cells, or WEHI-231 cells transduced with EF hand-deleted or C1 domain-deleted forms of RasGRPI, were untreated (nil) or treated with 1.6 \( \mu \text{M} \) PMA alone or in combination with 1 \( \mu \text{g/ml} \) ionomycin for 48 hours. Apoptosis was quantified by PI staining of permeabilized cells. Bars indicate means of two experiments each with duplicate cultures, with standard deviations indicated by error bars.

3.4.3 Conclusions

A C1 domain-deleted mutant form of RasGRPI was unable to sensitize WEHI 231 cells to BCR-induced apoptosis, suggesting that the C1 domain is critical in the ability of RasGRPI to promote BCR-induced apoptosis of WEHI 231 cells. These results are consistent with previous data pointing to a critical role for C1-mediated translocation of RasGRPI to membranes in order to become activated [42, 69]. Increased RasGRPI expression sensitized WEHI 231 cells to treatment with PMA, either alone or in combination with ionomycin, in a C1-dependent manner. One possible interpretation of these results is that signals that are sufficient to cause translocation of RasGRPI enable its activation, measured in this case as the ability to sensitize WEHI 231 cells to apoptosis. This is consistent with previous results showing that PMA is indeed sufficient to cause translocation and activation of RasGRPI in various cell types, including primary T cells and fibroblasts [42, 67, 69, 144].
BCR ligation in WEHI 231 cells results in a significant increase in intracellular calcium [230], which has the potential to modulate RasGRP1 activity via a pair of EF-hands [67]. Deletion of the EF-hand motif prevented RasGRP1 from sensitizing WEHI 231 cells to BCR-induced apoptosis, suggesting that RasGRP1 requires the ability to bind calcium in order to become activated. Alternatively, deletion of the EF-hand could affect the conformation of the protein, and render it non-functional. However, the mutant protein used in the experiments described above was fully capable of causing transformation in fibroblasts [69], suggesting that RasGRP1 EFA retains at least some degree of functionality. The ability of RasGRP1 to sensitise WEHI 231 cells to PMA-induced apoptosis indicates that an increase in intracellular calcium, as mimicked by ionomycin, is not required for its activation. Alternatively, PMA by itself could trigger calcium signalling that is sufficient to enable full activation of RasGRP1. Furthermore, deletion of the EF-hands prevented RasGRP1 from sensitizing WEHI 231 to PMA treatment, suggesting that calcium binding is required even in the absence of a robust increase in intracellular calcium, as caused by ionomycin. Together, these results point to an essential role for the EF-hands in the activation of RasGRP1. Although it appears that calcium binding by RasGRP1 is required for the adoption of an active conformation, it remains to be determined whether the EF-hands serve as a calcium sensor that triggers activation of RasGRP1 upon an increase in intracellular calcium.
CHAPTER 4: DISCUSSION

4.1 RASGRP1 PROMOTES BCR-INDUCED APOPTOSIS OF WEHI 231 CELLS

RasGRP1 is a Ras-specific GEF that becomes activated following recruitment to DAG-rich membranes via a C1 domain, and thus has the potential to activate Ras GTPases downstream of receptors that promote activity of PLC enzymes [67, 69]. RasGRP1 is activated following pre-TCR and TCR ligation as a result of PLCγ activation, and acts as a critical modulator of the Ras-ERK pathway during T cell responses to TCR ligation [142, 143]. As a result, RasGRP1 plays a significant role in the positive selection as well as the survival and maintenance of T lymphocytes [141, 143, 144]. Primarily expressed in brain and lymphoid tissues, RasGRP1 expression is detectable in bone marrow, as well as some B lymphoid lines [69]. Ligation of the pre-BCR and the BCR results both in PLCγ activation and activation of the Ras-ERK pathway [230]. Furthermore, BCR ligation has previously been shown to cause translocation of RasGRP1 to plasma membranes [228]. Thus our hypothesis was that RasGRP1 contributes to the modulation of Ras activation during B cell responses to BCR ligation.

To determine whether RasGRP1 expression affects the outcome of BCR signalling, RasGRP1 levels were increased in the immature B cell line WEHI 231 cells, a model for negative selection in developing B cells [237, 238, 240]. A two-fold Increase in RasGRP1 expression significantly increased the sensitivity of WEHI 231 cells to 48-hour α-IgM antibody treatment, as measured using propidium iodide staining of fixed nuclei. Experiments performed using shorter α-IgM stimulation times suggested that increased RasGRP1 expression accelerates commitment of WEHI 231 cells to BCR-induced apoptosis, and significantly inhibits re-entry into the cell cycle, as measured by dilution of CFSE following re-culture. The significance of these effects was confirmed
by using mixed population studies, which suggest that RasGRP1<sup>hi</sup> cells become rapidly depleted for cultures, compared with control cells. Taken together, these results suggest that increased RasGRP1 expression sensitizes WEHI 231 cells to BCR-induced apoptosis. Although this is the first time a Ras GEF has been implicated in promoting BCR-induced apoptosis in immature B cells, these findings are consistent with previous results that suggest PLCγ is required for BCR-derived signals to promote apoptosis in the avian immature B cell line DT40 [291].

A major objective of this thesis was to determine whether the ability of RasGRP1 to sensitize WEHI 231 cells to BCR-induced deletion was the result of its ability to promote activation of Ras GTPases. Increased RasGRP1 expression correlated with an increase in Ras activity, both prior to and following BCR ligation, as measured using an affinity precipitation assay that uses the Ras-binding domain of Raf-1 to purify and measure active (GTP-bound) Ras molecules (see Figure 3-2). These results suggest that the exogenous RasGRP1 proteins transduced in RasGRP1<sup>hi</sup> cells were capable of activating Ras GTPases. Increased Ras activity observed in RasGRP1<sup>hi</sup> cells prior to BCR ligation also suggested that RasGRP1 activation was occurring at low levels in the absence of BCR ligation, perhaps as a result of basal PLC activity. In addition to being activated following BCR ligation, PLC enzymes have the potential to become activated downstream of several membrane receptors, such as G-coupled receptors and integrins, in a BCR-independent manner [292]. Thus basal PLC activity resulting in mild RasGRP1 activation could explain why a modest growth disadvantage was observed in RasGRP1<sup>hi</sup> cells compared with control cells, in the absence of BCR ligation.

A RasGRP1 mutant that lacks the ability to activate Ras GTPases (RasGRP1 R271E) was completely unable to promote BCR-induced apoptosis. Instead, high levels of expression of the RasGRP1 R271E mutant actually reduced the incidence of BCR-induced apoptosis, suggesting this mutant had a dominant-negative effect. RasGRP1 R271E has previously been shown possess dominant-negative properties, as it was found to inhibit Ras activation following TCR ligation in a human T cell line [41]. However, Ras activity in RasGRP1 R271E-expressing WEHI 231 cells was not tested, and would have to be in order to establish a dominant-negative effect of this mutant in
WEHI 231 cells. A dominant-negative effect of RasGRP1 R271E could indicate that inhibition of Ras activity can actually decrease BCR-induced apoptosis. This specific observation is the only loss-of-function evidence provided in this thesis to suggest that RasGRP1 may normally be involved in the promotion of BCR-induced apoptosis in WEHI 231 cells. Furthermore, the specificity of RasGRP1 R271E has yet to be characterized. This mutant could potentially be non-specific and inhibit Ras activation mediated by other Ras exchange factors, including other RasGRP family members.

Thus a major caveat to the interpretation of the results presented in this thesis is the lack of evidence to support that the effects seen in RasGRP1\textsuperscript{high} cells simply reflect an exaggeration of the normal function of RasGRP1 during BCR signalling in WEHI 231 cells. This is due in part to the nature of the experiments performed in the course of these studies, which constituted in deriving data from gain-of-function (over-expression) studies, as opposed to deriving data from WEHI 231 cells where RasGRP1 expression has been reduced or eliminated. One potential approach that could be used to reduce RasGRP1 levels in WEHI 231 cells consists in treating cells with small interfering RNA molecules, which inhibit expression of the targeted gene transcript by causing its degradation [293]. However, studies performed in the Robert Kay lab suggest that the WEHI 231 cell line is not readily amenable to such an experimental approach. Another approach that could have been used to derive data from loss-of-function experiments consists in ablating RasGRP1 expression in DT-40 cells, an immature B cell line commonly used to perform genetic deletions for the study of gene function during BCR signalling [226]. Members of the Kay lab and another group have generated RasGRP1\textsuperscript{-/-} DT-40 cells and found that deletion of RasGRP1 does not reduce BCR-mediated Ras activation [229]. Furthermore, members of the Kay lab were unable to induce BCR-mediated apoptosis with the DT-40 cells currently available in the laboratory, making assessment of the contribution of RasGRP1 to BCR-induced-apoptosis impossible using this system. Alternatively, dominant-negative forms of the classical Ras GTPases (RasN17) could have been expressed in WEHI 231 cells, but these have been shown to lack the specificity required to serve as a powerful tool, which strongly limits their usefulness [19].
Despite these limitations, an augmentation in Ras signalling achieved via expression of constitutively active (V12 or L71) Ras GTPase mutants, was sufficient to sensitize WEHI 231 cells to BCR-induced apoptosis. Furthermore, the inability to maintain WEHI 231 clones expressing high RasV12 levels suggest that Ras signalling can promote apoptosis independently of the BCR. Thus despite being unable to prove that RasGRP1 is entirely, or even partially, required to transmit pro-apoptosis signals from the BCR, the results presented in this thesis suggest that increased Ras signalling alone is sufficient to promote apoptosis of WEHI 231 cells.
4.2 A PUTATIVE MECHANISM FOR BCR MEDIATED SIGNALS LEADING TO APOPTOSIS VIA RASGRP1

4.2.1 Activation of Ras family members by RasGRP1

The next objective of this thesis was to determine the mechanism via which increased RasGRP1 expression could sensitize WEHI 231 cells to BCR-induced ligation. RasGRP1 has been shown to activate both the classical and R-Ras GTPase family members [60]. Although the classical Rases share many of the same effectors (Raf kinases, PI-3 kinase, RalGDS), divergence among them is observed at the C-terminus. H- and N-Ras have C-terminal palmytoyl groups, whereas K-RasB bears a C-terminal basic region [36]. Divergence at the C-terminus is thought to account for differential membrane localization, whereby H- and N- Ras tend to associate with membrane lipid rafts, while K-Ras tends to be excluded from such membrane micro-compartments [36]. In WEHI 231 cells, BCR ligation has been shown to initiate activation of several signalling effectors outside lipid rafts [242]. Thus raft-excluded Ras family members, such as K-RasB, could be expected to become more readily activated than H- and N-Ras. However, it has to be noted that Ras localization has been shown to be highly dynamic, especially in the case of H-Ras, which is released from lipids rafts following activation [37].

Although RasGRP1\textsuperscript{high} cells were found to have increased activation levels of all three classical Ras GTPases, no significant differences were observed in one versus another classical Ras family member. Furthermore, signals derived from constitutively active H-, K-, and N- Ras were found to promote apoptosis of WEHI 231 cells indistinguishably. Therefore, although it would be tempting to speculate that RasGRP1 preferentially activates K-Ras over N- and H-Ras, no evidence was provided to that effect. Another putative effector of RasGRP1 in WEHI 231 is the 29 kDa M-Ras, which bears a basic region similar to that seen in K-RasB [44, 57]. Although M-Ras was quite
capable to sensitize WEHI 231 cells to BCR-induced apoptosis, further studies would be required establish that RasGRP1 activates M-Ras in WEHI 231 cells.

Thus, although RasGRP1 probably sensitizes WEHI 231 to BCR-induced apoptosis via the activation of Ras GTPases, whether RasGRP1 preferentially activates one or another Ras family member in WEHI 231 cells is still unclear. Experiments that would need to be performed to address these questions include the use of highly specific and sensitive antibodies that detect individual endogenous Ras family members in affinity precipitation assays that measure Ras activation. Also, the use of an inducible system for the expression of constitutively active Ras family members could circumvent the issue of selective depletion of high expressing clones, and be used to compare the ability of various Ras family members to sensitize WEHI 231 cells to BCR-induced apoptosis. Furthermore, investigations on co-localization of RasGRP1 with individual Ras family members in WEHI 231 membranes would be required to confirm the ability of RasGRP1 to activate individual Ras GTPases.

4.2.2 Well-characterized Ras effectors are not involved in sensitizing WEHI 231 cells to BCR-induced apoptosis

RasGRP1 has been shown to regulate Ras-mediated activation of ERK-1/2 in T cells, seemingly the most critical effectors of Ras signalling in T cell responses to TCR ligation during development and activation [12]. However, Ras GTPases have been shown to activate a complex network of signalling cascades, initiated as a result of the activation of a variety of effectors that include Raf kinases, PI-3 kinase, RalGDS as well as many others [1]. Considerable evidence suggests that the Ras/MEK/ERK pathway is required for the positive selection of thymocytes, although this signalling cascade does not appear to play a significant role during negative selection events in these cells [12]. Deletion of RasGRP1 results in a severe block in positive selection of thymocytes, but does not appear to affect negative selection events [142, 144]. Similarly, recent evidence suggests that the Ras/Raf/MEK/ERK pathway plays a significant role in pre-BCR selection, but does not appear to be required for negative selection of immature B cells [192, 244]. Thus, although Ras signalling
has the potential to contribute to negative selection of lymphocytes, it would appear that this contribution is independent of the Raf/MEK/ERK pathway. The apparent contrast between the lack of a role for RasGRP1 during negative selection of DP thymocytes and the putative role of RasGRP1 during negative selection of immature B cells could simply reflect differential expression and involvement of RasGRP family members in these cells, or could highlight differences in antigen receptor signalling events between the two cell types. For example, whereas the Src-family kinases Syk and Lyn, the Tec-family kinase Btk, as well the adapter protein BLNK are critical for BCR signalling, TCR signalling events are highly dependent upon the Src-family kinases Lck and Zap-70, as well as the adaptor SLP-76 [12, 227].

The hypothesis that negative selection of WEHI 231 cells occurs independently of Raf/MEK/ERK signalling was confirmed in experiments using WEHI 231 cells with increased RasGRP1 expression. In WEHI 231 cells, BCR ligation leads to both Ras activation as well as a robust, but transient increase in ERK phosphorylation [230]. Although increased RasGRP1 expression was able to cause sustained ERK phosphorylation, treatment with inhibitors of ERK phosphorylation failed to prevent RasGRP1 from sensitizing WEHI 231 cells to BCR-induced apoptosis. This result suggests that ERK activation is not involved in BCR-induced apoptosis. This is consistent with observations previously described by another group, although there is still controversy in the literature with regards to a role for ERK during BCR-induced apoptosis [244, 294, 295]. Furthermore, these results also suggest that the primary effectors of RasGRP1-mediated Ras activation following BCR ligation differ from those observed during TCR signalling. Transduction of an active form of Raf-1 was able to modestly sensitize WEHI 231 cells to BCR-induced apoptosis. This preliminary result could point to a potential role for Raf effectors other than ERK1/2 in the ability of RasGRP1 to cause increased BCR-induced apoptosis. However, further studies are required to establish a direct link between increased RasGRP1 expression in WEHI 231 cells and increased Raf-1 activation following BCR ligation.

Another putative effector of Ras signalling during B cell receptor signalling is the lipid kinase PI-3 kinase [254]. Culture of WEHI 231 cells with a high dose of the PI-3 kinase inhibitor LY294002 led
to spontaneous cell death in WEHI 231 cells, as previously observed [243], and a sub-lethal dose was sufficient to promote BCR-induced apoptosis. These results, along with a large body of evidence suggesting that the PI-3 kinase pathway generally promotes survival [104, 110], indicates that Ras-mediated activation of the PI-3 kinase pathway is not involved in BCR-mediated apoptosis. However, the studies performed in this thesis did not address PI-3 kinase activation directly, and relied on previous observations that PI-3 kinase inhibitors are indeed specific, thus commanding caution in the interpretation of these results. Effects of PI-3 kinase inhibitors on control and RasGRP1\textsuperscript{high} cells would need to be monitored via measurements of Akt phosphorylation. Nevertheless, these results suggest that RasGRP1-mediated sensitization of WEHI 231 cells to BCR-induced apoptosis is not the result of alterations in the PI-3 kinase pathway.

The possibility that other effectors of Ras signalling contribute to the ability of RasGRP1 to sensitize WEHI 231 cells to BCR-induced deletion has not been addressed during the course of this thesis. Although conclusions can tentatively be drawn with respect to the lack of involvement of the RAS/Raf/ERK pathway, as well as the Ras/PI3-K pathway, by no means have the studies performed in this thesis thoroughly addressed the exact nature of the effectors of Ras signalling responsible for the ability of RasGRP1 to promote BCR-induced deletion of WEHI 231 cells.

4.2.3 RasGRP1-mediated downregulation of NF\textsubscript{K}B activity sensitizes WEHI 231 cells to BCR-induced apoptosis

NF-\textkappa B activation has been clearly linked to survival in WEHI 231 cells, most notably during CD40-mediated rescue of cells from BCR-induced apoptosis [268, 284, 285]. However, although BCR ligation has been associated with an increase in \textkappa B levels [268, 286, 287], the exact molecular mechanism that links the BCR to a reduction in NF-\textkappa B activity leading to apoptosis needs further investigation. Three lines of evidence are presented to suggest that increased RasGRP1 expression augmented BCR-induced apoptosis by repressing NF-\textkappa B activity. First,
RasGRP1<sup>hi</sup> cells had significantly increased IκBα levels in their cytoplasm, which was accompanied by a significant and reproducible repression of NF-κB activity as measured using a luciferase reporter construct. Second, CD40 and LPS signalling, both of which promote IκB degradation and NF-κB activation [268, 270], were able to reduced BCR-induced apoptosis levels in RasGRP1<sup>hi</sup> cells to levels found in control cells. Third, a specific inhibitor of IκBα degradation was able to mimic the effects on BCR-induced apoptosis seen in RasGRP1<sup>hi</sup> cells. In addition, Bcl-X<sub>L</sub> levels, thought to be at least partially regulated via NF-κB, were found to be repressed in RasGRP1<sup>hi</sup> cells. Although this observation could be incidental and not a direct result of RasGRP1-mediated effects, forced expression of this anti-apoptotic effector was sufficient to repress BCR-induced apoptosis in RasGRP1<sup>hi</sup> cells. Thus it is highly probable that increased RasGRP1 sensitizes WEHI 231 cells to BCR-induced apoptosis by repressing NF-κB activity.

What is less obvious, not to say enigmatic, is the nature of the mechanism through which RasGRP1 mediates this effect. Ras activation has generally been associated with an increase in NF-κB activity [296-298], and only a single report has linked the Ras effector Raf-1 to inhibition of TNFα-mediated IκB degradation [299]. Members of a new family of Ras-family GTPases that serve as IκB degradation inhibitors, kappaB-Ras1 and kappaB-Ras2, have recently been characterized [300]. These have been found to bear an amino-acid signature that is analogous to that found in oncogenic Ras, notably at positions 12 and 61 [300]. This suggests that these Ras GTPases are constitutively active and therefore unlikely to be under the control of GEFs [300].

IκBα levels in WEHI 231 cells are regulated by a rapid, calpain-mediated degradation mechanism that is independent of IKKs, combined with constant protein synthesis [301, 302]. One study provides evidence that constitutive degradation of IκBα is mediated by the protein kinase CK2 (also known as casein kinase 2), which phosphorylates IκBα and promotes its degradation via calpain [302, 303]. Thus it is conceivable that Ras activation either inhibits CK2 activity or prevents interaction between CK2 and IκBα, thereby repressing phosphorylation and degradation of IκBα. However, very little is known about a link between Ras and CK2, if only that the two cooperate in
causing transformation of fibroblasts [304]. The involvement of CK2 in RasGRP1-mediated apoptosis could be addressed by testing the ability of increased CK2 expression to repress RasGRP1-mediated sensitization of WEHI 231 cells. Alternatively, the effect of increased RasGRP1 expression on CK2 activation could be assessed using a CK2 kinase assay [302]. Whether increased RasGRP1 expression affects the calpain-mediated IκBα degradation pathway in WEHI 231 cells could be tested directly by monitoring the effects of calpain treatment on BCR-induced apoptosis of control and RasGRP1<sup>high</sup> cells. Thus, further studies are required to provide a satisfactory mechanistic model that could directly link Ras activation to inhibition of NF-κB activity in WEHI 231 cells.
4.3 A POTENTIAL ROLE FOR RASGRP1 DURING NEGATIVE SELECTION OF DEVELOPING B CELLS?

4.3.1 Little is known about the role of Ras signalling during negative selection of B cells

Several lines of evidence point to role for Ras signalling during B cell development: expression of an activated Ras mutant can partially overcome the developmental block at the pro-B cell stage seen in RAG-deleted mice and induce light-chain gene rearrangement [218, 220]; and expression of a dominant-negative form of Ras has the ability to block B cell development at the pro-B cell stage, and inhibits pre-B cell proliferation and transition to the immature B cell stage [214, 215]. Severe depletion of immature B cells caused by transgenic N17Ras expression does not prevent the formation of mature B cells in the spleen, although these are in relatively low numbers, and respond weakly to mitogenic stimuli [214]. This result suggest that although Ras signalling is required for efficient transition from the early pro-B to the pre-B and immature B cell stages, it is not critical for the transition from the immature to the mature B cell stage.

Although studies performed on cultured immature B cells using MEK inhibitors suggest that ERK signalling is not involved in negative selection of B cells [244], the nature of the contribution of Ras signalling during this process has not been thoroughly addressed. Expression of an active form of Raf-1 causes a reduction of immature B cell numbers, although no studies were performed to determine whether this was a result of increased deletion, or accelerated maturation to the mature B cell stage [133, 214]. Thus, the results described in this thesis are the first direct evidence for a contribution by Ras GTPases during negative selection of developing B cells. Although increasing the expression of RasGRP1 resulted in sensitization of the immature B cell line WEHI 231 to BCR-induced apoptosis, caution has to be taken when attempting to extend this observation to a role for RasGRP1 during negative selection of primary immature B cells. Small quantities of RasGRP1 mRNA have been detected in bone marrow [69], but the detection of RasGRP1 protein in bone
marrow B cells has yet to be reported, and initial analysis of splenic B cell populations has failed to
detect any effect caused by deletion of RasGRP1 in mice [143, 145]. However, it should be noted
that although splenic B cells from RasGRP1-deficient mice appear to respond normally to
apoptosis-inducing signals such as Fas ligand [145], no studies have been performed to determine
the effect of deleting RasGRP1 on BCR-induced apoptosis in immature B cells, a death-inducing
pathway that significantly differs from Fas-induced cell death at the molecular level [305]. For
instance, whereas Fas-induced apoptosis is induced via activation of caspase-8, BCR-induced
apoptosis appears to be induced via caspase-9 activation, and unlike Fas-induced apoptosis, is
dependent upon new gene transcription [305]. As RasGRP1 and RasGRP3 have been found to
become recruited to plasma membranes and account almost entirely for BCR-induced Ras
activation in the immature B cell line DT40 [228, 229], studies performed in primary immature B
cells with increased or reduced levels of RasGRP1 or RasGRP3, or both, should be used to further
investigate a role for Ras GTPases during BCR-induced negative selection of immature B cells.

4.3.2 Differential RasGRP1 expression has the potential to affect the outcome of antigen
receptor signalling in B cells

Ligation of the BCR induces different cellular responses based on the developmental stage of the
B cell. For instance, BCR ligation leads to growth arrest and apoptosis in transitional immature B
cells; mature B cells become activated and proliferate in response to the same stimulus; and
signalling through the pre-BCR results in survival and proliferation and is required for the transition
from early to late pre-B cell stage [178, 199]. An active area of research in B cell biology consists in
elucidating the molecular differences between pre-B, immature B and mature B cells that could
account for such divergent responses to antigen stimulation. Both the pre-BCR and the BCR
appear to provide survival signals thought to be ligand-independent [183, 306]. Signalling through
the pre-BCR as well as ligation of the BCR in both immature and mature B cells results in ERK
activation and PLCγ activation [190]. One major difference in BCR signalling events between
immature and mature B cells is that, whereas BCR ligation leads to translocation of BCR molecules
into lipid rafts in mature B cells, signalling occurs outside lipid rafts following BCR ligation in
immature B cells [242, 307, 308]. It is therefore possible to speculate that this difference in signalling events significantly affects the nature of the signalling molecules that are recruited to the BCR following ligation.

As is the case for WEHI 231 cells, the NF-κB pathway contributes to the regulation of pro-survival factors such as Bcl-2, Bcl-xL and A1 in primary B cells, and is critical during B cell development and for the survival of mature B cells [309]. Whereas BCR signalling is associated with activation of the NF-κB pathway in mature B cells [310], previous studies using the WEHI 231 immature B cell line have found that BCR ligation leads to an initial increase in NF-κB activity that is followed by a reduction below control levels during prolonged stimulation [259, 268, 286, 287]. This phenomenon is supported by data generated in this thesis that describes BCR-induced increases in IκBα protein after 4 hours of BCR ligation. In mature B cells, activation of the NF-κB pathway occurs as a result of activation of PI-3 kinase and PKCβ, which become recruited to lipid rafts to activate the IKK complex following BCR-ligation [311-313]. Recent data suggest that BCR-induced activation of PKCβ, a calcium- and DAG-regulated protein kinase, initiates molecular events leading to the recruitment of a signalling complex that is critical for BCR-mediated NF-κB activation in mature B cells [314]. PKCβ is thought to phosphorylate the lymphoid-specific adaptor CARMA1, thereby altering its conformation to promote the assembly of multi-unit signalling complexes to membranes [314]. CARMA1 then recruits Bcl10, which associates with paracaspase/MALT1 and the ubiquitin-conjugating enzyme 13 at the plasma membrane to form a ubiquitin ligase complex that targets NEMO for ubiquitination [315]. It is believed that NEMO ubiquitination alters protein function to promote activation of the NF-κB pathway [315].

In immature B cells, prolonged BCR signalling has been associated with inhibition of NF-κB activity, although this remains to be thoroughly tested in primary immature B cells [268, 286, 287]. Deletion of the molecular components promote BCR-induced NF-κB activation in mature B cells, such as PKCβ, CARMA1 or Bcl10 has very little effect on the development of B cells in the bone marrow [316-318]. This suggest that although PKCβ, Bcl10 and CARMA1 are critical for the
maintenance and differentiation of mature B cells, additional mechanisms must contribute to the regulation of the NF-κB pathway during B cell development. The lack of prolonged BCR-induced NF-κB activation, combined with BCR signals that repress NF-κB-mediated expression of several pro-survival factors, could therefore at least partially account for the induction of apoptosis that occurs following BCR ligation in immature B cells. The data presented in this thesis suggest that RasGRP1 can act as a PLCγ-dependent BCR signalling effector with the potential to inhibit NF-κB activity via the activation of Ras proteins, thereby promoting apoptosis of immature B cells.

Combined, these observations point to a tentative (and oversimplified) model that could in part explain the differences in the outcome of BCR ligation in immature versus mature B cells (Fig. 4-1). Whereas BCR-induced PLCγ activation leads to NF-κB activation via PKCβ-mediated recruitment of the IKK complex to lipid rafts in mature B cells, BCR-induced PLCγ activation leads to NF-κB repression via RasGRP1 in immature B cells. BCR signalling in mature B cells is associated with NF-κB activation, thereby resulting in BCR-induced survival. On the other hand, the absence of BCR transition to lipid rafts following ligation, as well as the presence of RasGRP1 in immature B cells (but not mature B cells, based on limited expression studies performed in mature B cell lines [69]), results in inhibition of NF-κB activity and BCR-induced cell death. Further studies on expression patterns of RasGRP family members during B cell development, as well as expression and membrane localization studies on PKCβ and various components of the IKK complex in immature B cells would be required to validate this model.
Figure 4-1. RasGRP1 could contribute to differential outcome of BCR signalling in immature versus mature B cells.
REFERENCES


