BIOCHEMICAL CHARACTERIZATION OF SIGNALING PATHWAYS
REGULATING CELL SURVIVAL

By

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Date April 6, 2001
ABSTRACT

The granulocyte/macrophage colony stimulating factor (GM-CSF) exerts its anti-apoptotic effects on hemopoietic cells by activating multiple cellular signaling pathways including the phosphatidylinositol 3'-OH kinase (PI3K) cell survival pathway and the Ras-Raf-MEK-ERK kinase cascade. I hypothesized that cytokines activate each of these pathways to regulate components of the apoptotic machinery, including members of the Bcl-2 family. The mechanism by which cytokines such as GM-CSF regulate expression of Bcl-2 family members was examined in several models, including primary human eosinophils and basophils, and the hemopoietic cell lines TF-1 and MC/9. Protein expression of one pro-survival Bcl-2 family member, Mcl-1, was found to be dependent upon phosphatidylinositol (PI) 3-kinase. The cytokine-induced increase in Mcl-1 mRNA transcription was not dependent upon PI3K, thus dissociating the immediate-early transcription factors responsible for Mcl-1 transcription from the PI3K signaling pathway. In contrast, Mcl-1 mRNA levels were dependent upon MEK activation, suggesting a role for the Ras-MEK-ERK pathway in Mcl-1 transcription. However, activation of PI3K was shown to be necessary for GM-CSF to stimulate Mcl-1 protein translation. This was not due to any effect on prolonging the half-life of the protein.

I was also interested in other pathways that may regulate PI3K and Mcl-1 to affect cell survival. The second messenger ceramide has been implicated in a host of cellular processes including growth arrest and apoptosis. The relationship between ceramide signaling and the activation of PI3K and its downstream target, protein kinase B (PKB) was examined in detail. PKB activation was observed following stimulation of cells with GM-CSF. Addition of cell-permeable analogs, C2- or C6-ceramide, caused a partial loss
(50-60%) of PKB activation and resulted in reduced Mcl-1 translation. These reductions were not a result of a decrease in PI(3,4,5)P₃ or PI(3,4)P₂ generation by PI3K. Two residues of PKB (threonine 308 and serine 473) require phosphorylation for maximal PKB activation. Ser^{473} phosphorylation was consistently reduced by treatment with ceramide, whereas Thr^{308} phosphorylation remained unaffected. In further experiments, ceramide appeared to accelerate Ser^{473} dephosphorylation, suggesting the activation of a phosphatase. Consistent with this, the reduction in Ser^{473} phosphorylation was inhibited by the phosphatase inhibitors okadaic acid and calyculin A. Surprisingly, Thr^{308} phosphorylation was abolished in cells treated with these inhibitors, revealing a novel mechanism of regulation of Thr^{308} phosphorylation. These results demonstrate that PDK2-catalyzed phosphorylation of Ser^{473} is the principal target of a ceramide-activated phosphatase, which may regulate cell survival by reducing the level of pro-survival proteins such as Mcl-1.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inhibiting factor</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>APAF-1</td>
<td>A protease activating factor-1</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;-associated death inducer</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated factor X</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow derived mast cell</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CAPP</td>
<td>Ceramide-activated protein phosphatase</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-aspartic acid specific protease</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>jCAD</td>
<td>Inhibitor of caspase-activated deoxyribonuclease</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<tr>
<td>JAK</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LY</td>
<td>LY-294002</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>p110*;ER&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>p110-estrogen receptor molecule (tamoxifen mutant)</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>PD</td>
<td>PD98059</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PDK-1</td>
<td>3-Phosphoinositide dependent kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3'-OH kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase with homology to Tensin</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology-2</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-domain containing 5'-inositol phosphatase</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>WM</td>
<td>Wortmannin</td>
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</tbody>
</table>
Courage is the price that Life exacts for granting peace,
The soul that knows it not, knows no release
From little things.

- Amelia Earhart
1. INTRODUCTION

1.1. HEMOPOIETIC CYTOKINE RECEPTOR SIGNALING

1.1.1. Receptor Structure and Function

A plethora of extracellular molecules control cell growth, differentiation and survival. In the hemopoietic system, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) bind to cell surface receptors resulting in the activation of several intracellular signal transduction cascades. IL-3 acts as a panspecific hemopoietin stimulating the growth, survival and differentiation of erythroid and myeloid cells, eosinophils, basophils, mature macrophages, megakaryocytes and mast cells (Miyajima et al., 1993). GM-CSF is more restricted in its action, promoting the growth of macrophages, neutrophils and eosinophils (Miyajima et al., 1993).

The IL-3 receptor (IL-3R) and the GM-CSF receptor (GM-CSFR) are comprised of a ligand-specific α chain and a common β chain (βc; Nishinakamura et al., 1995). The murine IL-3R has two types of β chains, an IL-3-specific β-subunit and a common β-subunit (Nishinakamura et al., 1995). Studies of cytokine receptors in knockout mice reveal functional redundancy in the biological roles of GM-CSF and IL-3. For example, double-knockout mice lacking both the βc chain and the IL-3 gene display defects that are similar to those observed in the βc chain knockout mice, suggesting that in hemopoietic cells there are other cytokines that can compensate for the loss of IL-3 and GM-CSF signaling (Nishinakamura et al., 1996). Relatively little is known about cytokine-induced signaling via the α-chain. However, studies suggest an intact βc chain is required to mediate α-chain-dependent cell signaling (Nishinakamura et al., 1995).
GM-CSFR and IL-3R belong to the cytokine receptor superfamily, members of which have a conserved extracellular domain homologous to the fibronectin type-III domain. This 200 amino acid region contains four conserved cysteine residues and a conserved WSXWS sequence motif that may be involved in protein folding or ligand interaction (Bazan, 1990; Hilton et al., 1995; Yoshimura et al., 1992). The intracellular portion of the \( \beta_c \) has no intrinsic enzymatic activity and is divided into membrane-distal and membrane-proximal domains. Cytokine-induced signaling is initiated by ligand-induced heterodimerization of \( \alpha \) and \( \beta_c \) receptor subunits followed by receptor oligomerization and activation of associated tyrosine kinases (Brown et al., 1997; Lia et al., 1996). Members of the Janus tyrosine kinase family (JAK) are constitutively associated with conserved domains in the juxtamembrane region of the \( \beta_c \) chain called Box1 and Box2 (Mirua et al., 1993; Da Silva et al., 1994). JAK activity is required for the phosphorylation of specific tyrosine residues in the receptor subunits that then act as binding sites for proteins containing phosphotyrosine binding (PTB) domains (van der Geer and Pawson, 1995) or Src-homology (SH)-2 domains (review by Pawson, 1994), such as STATs (signal transducers and activators of transcription). Once bound to phosphotyrosine residues, STAT molecules are phosphorylated by JAKs resulting in the formation of STAT homo- and heterodimers, which then translocate to the nucleus where they function as transcription factors (Ihle, 1995; Yoshimura, 1998). For example, work by Ihle (1995) revealed that IL-3 and GM-CSF stimulation results in JAK2 activation and the recruitment and subsequent tyrosine phosphorylation of STAT5. Additional tyrosine kinases involved in IL-3 and GM-CSF signaling include Lyn, Fes, Tec, Yes, Fyn and Btk (Parganas et al, 1998). Further, the receptor-associated proteins Shc, SHIP and PI3K, are
also involved in IL-3 and GM-CSF mediated signal transduction (Sato et al., 1993; Yi et al., 1993; Gold et al., 1994). SHIP and PI3K will be discussed in greater detail below.

1.2. PI3K Signal Transduction Pathway

1.2.1. PI3K Family of Enzymes

The PI3K family of lipid kinases exists as heterodimers consisting of a regulatory/adapter subunit tightly associated with a catalytic subunit. PI3K can be divided into 3 categories on the basis of sequence homologies, lipid substrate specificity and structure (review by Vanhaesebroeck et al., 1997a). The class 1a PI3K consist of several isoforms of both the adapter/regulatory and the catalytic subunits. There are three highly homologous, class 1a catalytic subunit isoforms, p110α, p110β and p110δ (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997b). The p110 α and β isoforms are thought to mediate receptor tyrosine kinase signaling and are widely expressed. Expression of the δ isoform is restricted to hemopoietic cells. p110 isoforms contain a C-terminal kinase domain and a N-terminal regulatory domain. In addition, the amino terminus of p110 contains a Ras-binding site, which may play a positive role in PI3K activation.
There are two isoforms of the class 1a PI3K adapter/regulatory subunits designated p85α and p85β. Both isoforms contain two src-homology 2 (SH2) domains and an inter-SH2 domain (ISH2; Dhand et al., 1994; Otsu et al., 1991; Escobedo et al., 1991; Skolnik et al., 1991; Klippel et al., 1994). The ISH2 region mediates interaction between subunits and is required for the catalytic activity of p110 (Cohen et al., 1995). The role of the SH2 domains is two-fold: (1) to activate the p110 catalytic subunit by coupling p85 isoforms to tyrosine phosphorylated receptors, receptor associated proteins and cytosolic proteins containing the consensus sequences pYXXM or pYMXM (Songyang et al., 1993), and (2) to bring the catalytic subunit in proximity to its major substrate, PI(4,5)P₂, at the plasma membrane (Kelly et al., 1993; Ricort et al., 1996; Nave...
N-terminal to the SH2 domains, p85 isoforms contain a SH3 domain, a Bcr/Rac GTPase-activating protein (GAP) homology domain and two proline-rich domains (Gout et al., 1992). The proline-rich domains and the SH3 domain may play important roles in the association of PI3K with other proteins, such as dynamin (Gout et al., 1993), p125Fak (Guinebault et al., 1995), Grb2 (Wang et al., 1995), α-actin (Shibaski et al., 1994) and the Src family of tyrosine kinases (Pleiman et al., 1994). Further, intramolecular interactions between the SH3 and the proline-rich regions of p85 may provide an autoregulatory mechanism analogous to that used by the Src family kinases (Andreotti et al., 1997).

**Figure 1.2.** Isoforms and domain organization of the PI3K catalytic subunits. (2000). Vanhaesebroeck, B., and Alessi, D.R. *Biochem. J.*, 346, 561-76.
The class 1b PI3K consist of a distinct family of adapter proteins that are activated by binding of βγ subunits of heterotrimeric G-proteins to specific sites in both the p101 adapter subunit and the catalytic subunit (Stephens et al., 1997; Stoyanov et al., 1995). Much less is known about the class 2 and class 3 PI3K and these will not be discussed further here.

Once recruited to the inner surface of the plasma membrane, PI3K catalyze the transfer of the γ-phosphate from ATP to the D-3 position of the inositol ring of phosphoinositides yielding 3'-phosphorylated phosphoinositolides (Carpenter and Cantley, 1996). The principal phospholipids generated by class 1a PI3K activation are phosphatidylinositol 3,4,5 triphosphate PI(3,4,5)P₃ and phosphatidylinositol 3,4 biphosphate PI(3,4)P₂ (Vanhaesebroeck and Alessi, 2000).

The levels of PI(3,4,5)P$_3$ is tightly controlled in cells by the lipid phosphatases, PTEN (phosphatase with homology to tensin) and SHIP (SH2 domain-containing inositol phosphatase). PTEN is a D-3 specific inositol phosphatase that dephosphorylates PI(3,4,5)P$_3$ yielding PI(4,5)P$_2$ (Stambolic et al., 1998). SHIP, on the other hand, dephosphorylates PI(3,4,5)P$_3$ at the 5' position generating PI(3,4)P$_2$ and is the major 5’-phosphatase in hemopoietic cells (Damen et al., 1996; Lioubin et al., 1996; Kavanaugh et al., 1996). Through the dephosphorylation of PI(3,4,5)P$_3$, PTEN and SHIP constitute an important class of negative regulators. Moreover, by increasing the levels of surrogate lipid species, lipid phosphatases may function to activate alternate signaling pathways.

PI3K-generated phospholipids can regulate downstream signal transduction cascades through direct binding to the pleckstrin homology (PH) domains of target proteins. First recognized in a protein called pleckstrin, PH domains have been identified in over 100 proteins and are thought to mediate protein-lipid and protein-protein interactions (Haslam et al., 1993; Mayer et al., 1993; Shaw, 1996). Proteins that contain PI(3,4,5,)$\_3$-binding PH domains include: $\beta$-spectrin (Macias et al., 1994), dynamin (Ferguson et al., 1994; Salim et al., 1996), Bruton’s tyrosine kinase (Btk; Salim et al., 1996), son of sevenless (SOS; Wang et al., 1995), phospholipase C (Cifuentes et al., 1993; Lomasney et al., 1996), protein kinase B (PKB) and 3-phosphoinositide-dependent kinase-1 (PDK1) (Alessi et al., 1997a; Stokoe et al., 1997).

Several mechanisms have been identified by which phospholipid-PH domain interactions can alter the activity of downstream effector molecules: (1) membrane targeting: recruitment of downstream effectors to the inner surface of the plasma
membrane may bring target molecules into proximity of regulatory kinases and various signaling complexes; (2) allosteric regulation: phospholipids may function in an allosteric manner to alter the catalytic activity of substrates; and (3) conformational changes: binding of phosphoinositides to the PH domains may induce conformational changes required for full activation of the PH domain containing protein (Alessi and Cohen, 1998a; review by Blomberg et al., 1999). A good example of this is PKB, which will be discussed in more detail below.

To assess the role of PI3K in a variety of cellular processes, two specific and cell-permeable pharmacological inhibitors have been employed: (1) wortmannin (Okada et al., 1994) and (2) LY-294002 (Vlahos et al., 1994). The fungal metabolite wortmannin is a potent (IC$_{50}$ ~3nM) and irreversible inhibitor of mammalian class 1a PI3K. Wortmannin forms covalent bonds with Lys$^{802}$ in the ATP-binding site of PI3K, resulting in alkylation of the nucleophilic residue and inhibition of PI3K catalytic activity. The bioflavonoid 2-(4-morpholinyl)-8-phenylchromone (LY-294002) is also a specific and efficient inhibitor of PI3K (IC$_{50}$ ~1.40µM). LY-294002 functions as a competitive antagonist for the ATP-binding site of PI3K. The main disadvantage in using these biochemical reagents is that both LY-294002 and wortmannin can affect the activity of other enzymes. For example, both LY-294002 and wortmannin can inhibit the mammalian target of rapamycin (mTOR) (Brunn et al., 1996:Abraham, 1998). Additionally, at high concentrations (>100nM), wortmannin can inhibit phospholipase D (Bonser et al., 1991), myosin light chain kinase (Nakanishi et al., 1992), phospholipase A$_2$ (Cross et al., 1995b) and PI4kinase (Nakanishi et al., 1995).
A number of molecular tools have been developed to define the role of class Ia PI3K in various intracellular signaling cascades. For example, dominant negative forms of the p85 subunit that are unable to bind p110 inhibit endogenous PI3K activity by competing for phosphotyrosine docking sites (Hara et al., 1994). Likewise, overexpression of a catalytic-inactive p110 subunit blocks signaling by class Ia PI3K (Takayanai et al., 1996). Addition of a N-terminal myristoylation motif and an N-terminal fragment of the p85iSH2 domain to the p110 catalytic subunit (p110\**) results in constitutive PI3K activity independent of p85 regulation (Hu et al., 1995). Work by Klippel and coworkers (1998) describe an inducible system for studying the role of PI3K. In this system PI3K activity is induced by the addition of a cell permeable estrogen analog, 4-hydroxytamoxifen (4-OHT), to cells overexpressing a chimeric p110:estrogen receptor molecule (p110\*:*ER\textsuperscript{TM}). Finally, biologically active membrane-permeable forms of PI(3,4,5)P\textsubscript{3} and PI(3,4)P\textsubscript{2} have recently been developed to further define the role of PI3K in growth factor signaling (Jiang et al., 1998).

### 1.2.2. PI3K and Cell Survival

PI3K was first identified as an important regulator of growth-factor survival in studies by Yao and Cooper (1995) which demonstrated that treatment of PC12 cells with PI3K inhibitors blocks nerve growth factor (NGF) mediated cell survival. Further, platelet derived growth factor (PDGF) stimulation is unable to promote cell survival in PC12 cells overexpressing mutant PDGF receptors incapable of recruiting PI3K. Analogous studies revealed that PI3K is required for growth factor-dependent cell survival in a variety of cell types including fibroblasts, hemopoietic cells and neurons.
(Scheid et al., 1995; Yao and Cooper 1995; Takashima et al., 1996; Vemuri and McMorris 1996; Dudek et al., 1997; Philpott et al., 1997; Berra et al., 1998; Crowder and Freeman 1998; Stambolic et al., 1998). Additionally, overexpression of constitutively active PI3K is sufficient to abrogate cytokine withdrawal-induced apoptosis (Yao and Cooper 1995; Chen et al., 1998; Crowder and Freeman 1998). Further support for PI3K as a key player in cell survival is provided from studies involving PTEN and SHIP. As discussed above, PTEN and SHIP are phosphatidylinositol phosphatases that act upon PI(3,4,5)P3 to generate PI(4,5)P2 and PI(3,4)P2 respectively. PTEN knock out mice have elevated levels of PI(3,4,5)P3 and die during embryogenesis as a result of a failure in developmental apoptosis (Stambolic et al., 1998; Suzuki et al., 1998). Additionally, PTEN-deficient immortalized mouse embryonic fibroblasts are resistant to various apoptotic stimuli and have an increased propensity to form tumors. These data strongly suggests that PTEN decreases the apoptotic threshold for a cell by limiting the amount of PIP3 in the cell. Similarly, SHIP loss-of-function transgenic mice display impaired apoptosis and have excessive myeloid cell survival. This suggests that PI(3,4,5)P3 is a key mediator of PI3K-dependent cell survival (Helgason et al., 1998; Liu et al., 1998). Consistent with this theory, overexpression of SHIP in hemopoietic cells is sufficient to induce apoptosis (Liu et al., 1997). Finally the transforming efficiencies of various oncogenes, including activated Ras and Bcr/Abl, depends in part on their ability to increase intracellular PI3K activity (Skorski et al., 1997).
1.2.3. **Downstream Targets of PI3K**

Numerous enzymes have been shown to be regulated by PI3K, one of which is the p70 ribosomal protein S6 kinase (p70\textsuperscript{S6k}). p70\textsuperscript{S6k} is a cytoplasmic serine/threonine kinase that phosphorylates key residues in the S6 protein of the 40S ribosomal subunit. These phosphorylation events are necessary for translational initiation, protein synthesis and entry into S phase. There is strong evidence to indicate that activation of PI3K is both necessary and sufficient for activation of p70\textsuperscript{S6k}. For example, inhibition of PI3K activity by use of dominant negative p110 subunits (Burgering et al., 1995), mutated receptors that can no longer bind p85 (Chung et al., 1994; Ming et al., 1994), or pharmacological inhibitors of PI3K all abolish p70\textsuperscript{S6k} activity (Cheatham et al., 1994; Moule et al., 1995; Weng, et al., 1995). Further, addition of 4-OHT to cells overexpressing the conditionally active p110*:ER\textsuperscript{TM} chimera results in an increase in PI3K activity and a concomitant increase in p70\textsuperscript{S6k} phosphorylation (Klippel et al., 1998).

p70\textsuperscript{S6k} has 8 phosphorylation sites; Thr\textsuperscript{229}, Ser\textsuperscript{371}, Thr\textsuperscript{389}, Ser\textsuperscript{404}, Ser\textsuperscript{411}, Ser\textsuperscript{418}, Thr\textsuperscript{421} and Ser\textsuperscript{424} (Pullen and Thomas, 1997). Phosphorylation of Thr\textsuperscript{389} is wortmannin sensitive and may be an *in vivo* target of PKB activity (Dennis et al., 1996). Conversely, phosphorylation of Thr\textsuperscript{229} is wortmannin-insensitive and may be a target of PDK1 activity (Dennis et al., 1996). The immunosuppressant rapamycin inhibits mTOR resulting in the activation of phosphatases that can dephosphorylate Thr\textsuperscript{389} and inactivate p70\textsuperscript{S6k} (Pullen and Thomas, 1997; Dennis et al., 1996). Work by several groups revealed that indirect inhibition of p70\textsuperscript{S6k} with rapamycin has no effect on growth factor mediated survival indicating that PI3K promotes survival independently of p70\textsuperscript{S6k} activity (Scheid et al., 1996; Yao and Cooper, 1996; Kaufmann-Zeh et al., 1997).
1.2.3.1. Ras-ERK Signaling Pathway

The extracellular regulated kinases 1 and 2 (ERK 1 and ERK 2) are members of the mitogen-activated family of protein kinases and were originally described as proteins that undergo growth factor-mediated tyrosine phosphorylation (Ray and Sturgill, 1988; Rossomando et al., 1998). ERK 1 and ERK 2 are proline-directed serine/threonine kinases (Boulton et al., 1991; Charest et al., 1993; Clark-Lewis et al., 1991) that serve to activate a broad spectrum of downstream kinases including p90<sup>rsk</sup> (Blenis, 1993), Mnk1 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), Mnk2 (Waskiewicz et al., 1997), Rsk2 (Xing et al., 1996) and mitogen-activated protein kinase-activated protein (MAPKAP) kinase-5 (Ni et al., 1998). Additionally, ERK regulates the transcriptional activity of ELK1, Sap1 and c-Myc, which in turn regulate the expression of many genes critical to mitogenesis, such as, cyclin D1 (Greulich and Erikson, 1998; Lavoie et al., 1996).

Several research findings support the idea of PI3K as an upstream regulator of MEK and ERK in some model systems. Wortmannin can partially inhibit cytokine-stimulated ERK activation in a MEK-dependent manner (Karnitz et al., 1995; Cross et al., 1994; Sakanaka et al., 1994). As well, overexpression of constitutively active, membrane-bound p110 results in Ras and ERK activation (Hu et al., 1995). Finally, PI3K has been shown to be required for Raf-induced ERK activation (Ferby et al., 1994).

The mechanism by which PI3K impacts on ERK is at present not clear. ERK 1 and ERK 2 are targets of MAP kinases termed MEK1 and MEK2 (ERK kinase). MEK1 and MEK2 are dual-specificity kinases that activate ERK1 and ERK2 by phosphorylating threonine and tyrosine residues of the TEY motif (Brott et al., 1993; Crews et al., 1992;
Zheng and Guan, 1993). The amino terminus of MEK1 and MEK2 contains a nuclear export signal (NES) and an ERK-binding site which functions to anchor ERK in the cytoplasm (Fukuda et al., 1997). Following stimulation, ERK and MEK translocate to the nucleus and MEK subsequently relocates back into the cytosol.

Numerous research findings have placed MEK and ERK activation downstream of the small monomeric G-protein Ras (de Vries-Smits et al., 1992; Leevers and Marshall, 1992). Ras proteins are localized at the inner surface of the plasma membrane in an inactive GDP-bound form. Cytokine-stimulated receptor activation triggers the exchange of GDP for GTP on Ras and is catalyzed by the nucleotide exchange factor SOS. Thus Ras proteins act as molecular switches that are inactive in the GDP-bound form and active in the GTP-bound form. A constitutively active Ras (Val\(^{12}\)) mutant can rapidly activate ERK, whereas, a dominant interfering Ras (Asn\(^{17}\)) mutant blocks growth factor mediated ERK activation. Together these data suggest that MEK and ERK are downstream targets of active Ras (de Vries-Smits et al., 1992).

The best-described effectors of GTP-bound Ras are the serine/threonine protein kinases Raf-1 and A-Raf. Raf proteins contain an N-terminal Ras-binding domain and exhibit a greater affinity for GTP-bound Ras compared to GDP-bound Ras (Vojtek et al., 1993). In its GTP-bound form, Ras recruits Raf to the plasma membrane where it becomes phosphorylated within the RSXSXP motif and interacts with members of the 14-3-3 family of proteins (Thorson et al., 1998). The precise mechanism by which Raf becomes activated is poorly defined (Vojtek et al., 1993; review by McCubrey et al., 2000).
Research by several groups revealed that Raf is an upstream component of ERK activation, for example, over-expression of activated Raf leads to Ras-independent activation of ERK (Kyriakis et al., 1992; Dent et al., 1992; Howe et al., 1992). In addition, MEK1 and MEK2 are activated by phosphorylation on two serine residues by Raf. Taken together, these results reveal a linear phosphorylation-signaling cascade of Ras→Raf→MEK→ERK.

Some studies have suggested that PI3K-activated pathways may act upon specific isoforms of Raf (Sutor et al., 1999), thus providing a route for PI3K to ERK. This effect appears to be cell type specific, since studies by Scheid and Duronio (1996) revealed that PI3K activity is neither sufficient nor necessary for ERK activation in the mast cell line MC/9. Therefore, when examining the effects of PI3K inhibition on a cell response, attention should be made as to whether ERK activation is similarly affected.

1.3. PKB: A MULTIFUNCTIONAL MEDIATOR OF PI3K ACTIVATION

1.3.1. PKB structure

PKB is 57 kDa serine/threonine protein kinase originally cloned in screens to identify proteins homologous to the catalytic domains of PKA and PKC (Coffer and Woodgett, 1991; Jones et al., 1991). PKB (c-AKT) is the cellular homologue of the transforming oncogene product v-AKT, isolated from the AKT8 retrovirus (Bellacosa et al., 1991, 1993). Sequence analysis of viral- and cellular-PKB cDNAs revealed that v-PKB is a gag-c-PKB fusion protein resulting from a recombination event between viral gag sequences and the N-terminal region of the c-PKB gene (Staal et al., 1987; Staal 1997). Proteins encoded by v-PKB and c-PKB differ in their post-translational
modification, subcellular localization, and oncogenic potential. The viral gag-PKB protein is myristoylated at its N-terminus and is targeted to membrane surfaces where it becomes phosphorylated and active. Cellular PKB is primarily localized in the cytosol (Ahmed et al., 1993).

There are three known mammalian isoforms of PKB encoded by three different genes sharing 90% sequence homology (Coffer and Woodgett, 1991; Jones et al., 1991; Bellacosa et al., 1993). PKBα and PKBβ are widely expressed while PKBγ expression is restricted to the brain, testes, heart, spleen, lung and skeletal muscle. All PKB isoforms contain an N-terminal PH domain (Mayer et al., 1993; Musacchio et al., 1993; Datta et al., 1995), a proline rich C-terminal tail and a central kinase domain with serine/threonine specificity (Bellacosa et al., 1991; Coffer and Woodgett 1991).

The PH domain is responsible for lipid-protein interactions between PI3K-generated phospholipids and the amino-terminus of PKB. Direct binding of phosphoinositides to the PH domain results in membrane translocation and is critical for PKB activation (Franke et al., 1995, 1997; Bellacosa et al., 1998; Sable et al., 1998). Phospholipid-mediated membrane targeting brings PKB in close proximity to activating kinases (Andjelkovic et al., 1996; Bellacosa et al., 1991; Coffer and Woodgett 1991; Burgering and Coffer 1995; Kohn et al., 1996b; Soskic et al., 1999). Research by the Alessi group (1996a) revealed that phosphorylation of PKB is mediated by kinases that are themselves a target of PI3K activity. Four sites of PKB phosphorylation exist in vivo: Ser\textsuperscript{124}, Thr\textsuperscript{308}, Thr\textsuperscript{450} and Ser\textsuperscript{473}. Mutational analysis revealed that phosphorylation of Ser\textsuperscript{473} and Thr\textsuperscript{308} is both sufficient and necessary for full enzymatic activity (Alessi et al., 1996a). Thr\textsuperscript{308} is located in the activation loop of the central kinase domain of PKB and
is phosphorylated by PDK1 (Alessi et al., 1997b). PDK1 is a 63 kDa serine/threonine kinase with a C-terminal PH domain and a N-terminal kinase domain (Alessi et al., 1997a; Stephens et al., 1998; Hanks et al., 1998). PDK1 is constitutively active under basal conditions and its activity does not increase upon cytokine stimulation (Alessi et al., 1997a). Following stimulation, phosphoinositides bind to the PH domain of PDK1 and anchor it to the plasma membrane where it phosphorylates members of the AGC subfamily, such as PKB (Alessi et al., 1997b; Stokoe et al., 1997; Walker et al., 1998), PKC (Le Good et al., 1998), PKA (Cheng et al., 1998) and p70s6k (Alessi et al., 1998b; Pullen et al., 1998).

Figure 1.4. A. Structure and organization of the PKB isoforms. B. Structure of PDK1 and potential interaction with PRK2. C. v-Akt is a fusion protein consisting of PKB and an N-terminal viral gag protein. Myristoylation of the gag protein results in membrane targeting. Vanhaesebroeck, B., and Alessi, D.R. (2000). Biochem. J., 346, 561-76.
Some studies suggest that binding of phospholipids to the PH domain of PKB induces a critical conformational change permitting phosphorylation of Thr\textsuperscript{308} by PDK1. For example, \textit{in vivo} phosphorylation of Thr\textsuperscript{308} by PDK1 is abolished by mutations affecting PKB PH domain function. Further, PKB mutants lacking a PH domain undergo \textit{in vitro} PDK1 phosphorylation in the absence of lipid (Stokoe et al., 1997; Alessi et al., 1997b).

The identity of the Ser\textsuperscript{473} kinase is currently unknown, however, it is thought to function in a phospholipid-dependent manner and is therefore operationally termed PDK2. Ser\textsuperscript{473} is located in the carboxy-terminal regulatory domain of PKB. MAPKAP kinase-2, a member of the p38 MAPK stress activated signaling pathway, can phosphorylate Ser\textsuperscript{473} \textit{in vitro}, however, stress stimuli that enhance \textit{in vivo} MAPKAP kinase-2 activity fail to induce PKB activation. Moreover, PKB activation still occurs in the presence of the potent and specific p38 inhibitor, SB203580 (Alessi et al., 1996b). Some groups postulate that the integrin-linked kinase, ILK, is the elusive PDK2 (Delcommenne et al., 1998). ILK can phosphorylate Ser\textsuperscript{473} \textit{in vitro} and its activity is directly regulated by phosphoinositides, presumably through binding of the lipids to a cryptic PH domain within ILK. Moreover, a kinase-dead mutant of ILK inhibits \textit{in vivo} phosphorylation of Ser\textsuperscript{473}. Recent studies by Balendran and co-workers (1999) suggest that PDK1 may complex with the C-terminus of PRK-2 to phosphorylate Ser\textsuperscript{473}. On the other hand, Toker and Newton (2000) suggest that PKB undergoes autophosphorylation at Ser\textsuperscript{473}. Some of these issues have been resolved using PDK1-deficient embryonic stem cells. In this model, PKB activity is abolished, consistent with a lack of Thr\textsuperscript{308} phosphorylation. Interestingly, PDK1 knockout cells still undergo Ser\textsuperscript{473} phosphorylation.
in response to insulin-like growth factor 1 (IGF1) suggesting that PDK1 is not required for in vivo PKB Ser$^{473}$ phosphorylation. Moreover, PKB is inactive in PDK1 deficient cells revealing that PKB does not undergo autophosphorylation at Ser$^{473}$ (Williams et al., 2000).

Taken together, these results suggest four mechanisms by which PI3K-generated phospholipids act to regulate PKB activation: (1) phospholipids anchor PKB to the plasma membrane (Andjelkovic et al., 1997; Meier et al., 1997; Wijkander et al., 1997; Sable et al., 1998); (2) phospholipids elicit an “activating” conformational change in PKB, permitting phosphorylation in the activation loop (Datta et al., 1996; James et al., 1996; Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997); (3) phospholipids regulate upstream activating kinases of PKB (Currie et al., 1991); and (4) some studies suggest that phospholipid binding is responsible for the formation of PKB homomultimers (Datta et al., 1995; Franke et al., 1997; Alberti 1998).

1.3.2. PKB and Survival

PKB is emerging as a key player in cytokine-regulated signaling. For example, stimulation of 32D cells with IL-3 results in a rapid increase in PKB activity, while dominant-negative mutants of PKB block IL-3-stimulated proliferation (Songyang et al., 1997). Further, overexpression of wild-type PKB protects 32D cells from cytokine-withdrawal-induced apoptosis (Songyang et al., 1997). Similar studies in a variety of cell types reveal that constitutively active PKB can inhibit TNF-α-, drug- and ionizing-radiation-induced apoptosis (Chen et al., 1998; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja and Downward, 1997; Khwaja et al., 1997; Kulik et
al., 1997; Philpott et al., 1997; Xiong and Parsons 1997; Crowder and Freeman 1998; Eves et al., 1998; Gerber et al., 1998; Hausler et al., 1998; Kulik and Weber, 1998; Rohn et al., 1998; Blair et al., 1999).

Several dominant-negative forms of PKB have been generated to examine the role of PKB in cytokine-mediated survival. However these constructs often provide contradictory results. For example, overexpression of wild-type PKB containing a RasCAAX membrane targeting sequence (CAAX-PKB) inhibits growth factor-induced PKB-mediated cell survival (van Weeren et al., 1998), whereas, expression of N-terminally myristoylated PKB enhances the survival promoting ability of platelet derived growth factor (PDGF) (Kohn et al., 1996a; Andjelkovic et al., 1997). Dominant-negative PKB mutants may function by altering endogenous PKB activity or by directly interacting with specific downstream targets of PKB. On the other hand, dominant-negative PKB mutants may act non-specifically by binding to other PH domain-containing proteins or by sequestering PI3K-generated phospholipids away from other PH domain-containing proteins.

Genetic analysis of the *Drosophila* PKB homologue, dAKT1, provides additional support for PKB-dependent cell survival. *Drosophila* with loss of function dAKT1 alleles show significant ectopic apoptosis and die during embryogenesis (Staveley et al., 1998). This lethality is suppressed by transgenic expression of wild type PKB, supporting the existence of a PKB-mediated anti-apoptotic signaling pathway.

One mechanism by which PKB may mediate cell survival signaling is by inactivating members of the death-promoting machinery. This theory has led to
systematic examination of putative PKB phosphorylation sites in members of the apoptotic machinery, which is discussed below.

1.3.3. **Downstream Targets of PKB**

Several components of the apoptotic machinery contain the PKB consensus phosphorylation sequence, RXRXXS/T and have proven to be downstream targets of PKB activity (Alessi et al., 1996b; Walker et al., 1998). The first Bcl-2 family member found to be a substrate for PKB was the pro-apoptotic protein Bad. *In vitro* analysis revealed that PKB preferentially phosphorylates Bad at Ser\textsuperscript{136} (Datta et al., 1997a). Additional support for PKB as a potent Bad Ser\textsuperscript{136} kinase is provided by overexpression studies. Constitutively active PKB is sufficient to induce Ser\textsuperscript{136} phosphorylation of both endogenous and transfected Bad, whereas kinase-dead PKB inhibits Bad Ser\textsuperscript{136} phosphorylation (Datta et al., 1997a; Blume-Jensen et al., 1998; Wang et al., 1999a,b). Further, constitutively active PKB alleles are unable to rescue cells coexpressing Bad mutants in which Ser\textsuperscript{136} has been converted to alanine (Datta et al., 1997a; Blume-Jensen et al., 1998; Wang et al., 1999a).

Work by Scheid and Duronio (1998) suggested the existence of PKB-independent pathways leading to Bad phosphorylation. For example, GM-CSF stimulates Bad phosphorylation in the absence of PKB activation. Additionally, IL-4 promotes PKB activation in a PI3K-dependent manner but does not induce phosphorylation of Bad. Taken together, these results suggest that PKB-mediated cell survival occurs by Bad phosphorylation-dependent and -independent pathways.
The mammalian Ced-3 homologue, caspase-9, may also function as a downstream target of PKB activation. Human caspase-9 has two AKT motifs, Ser^{183} (RTRTGS) and Ser^{196} (RRRFSS). Work by Cardone and coworkers (1998) revealed that Ser^{196} is the primary PKB phosphorylation site and that phosphorylation of this site inhibits caspase-9 protease activity. This phosphorylation event is thought to be biologically significant as overexpression of constitutively active PKB alleles can suppress caspase-9-induced cell death. Further, ectopic apoptosis observed in fruit flies with loss of function mutations in the dAKT1 gene, can be rescued by expression of P35, an inhibitor of caspase-9 activation (Morris et al., 1996; Staveley et al., 1998). A recent study by Fujita and coworkers (1999) revealed that the PKB phosphorylation site found in human caspase-9 is absent in mouse, dog and rat caspase-9, suggesting that inhibition of caspase-9 activation by PKB-dependent phosphorylation is not evolutionarily conserved, raising concerns over the significance of this phosphorylation for preventing apoptosis.

In addition to regulating cytoplasmic apoptotic events, there are several nuclear targets of PKB activation. The mechanism by which PKB translocates to the nucleus is poorly defined, however, neither the PH domain nor kinase activity is required for this process (Andjelkovic et al., 1997). Once in the nucleus, PKB targets members of the Forkhead family of transcription factors. The Forkhead family member, DAF 16, was originally identified in genetic screens for suppressors of daf-2 mutations in Caenorhabditis elegans (Lin et al., 1997). Three DAF-16 orthologs have been identified in mammalian cells, FKHR, FKHRL, AFX (Davis et al., 1995; Sublett et al., 1995; Borkhardt et al., 1997; Hillion et al., 1997).
All Forkhead family members have a C-terminal transcriptional activation domain and a central DNA-binding domain termed the 'Forkhead domain' (Kops et al., 1999). Sequence analysis of daf-16 and the three human Forkhead isoforms revealed three consensus PKB phosphorylation motifs. Expression of constitutively active PKB is sufficient to induce phosphorylation at all three sites; however, the major in vitro target of PKB phosphorylation is located within the DNA-binding domain (Brunet et al., 1999; Datta, S.R., Brunet, A., and Greenberg, M.E. (2000). Genes Dev., 13, 2905-2927.)
Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). Consequent to phosphorylation, Forkhead family members are sequestered in the cytosol, away from their nuclear targets, through interaction with 14-3-3 proteins (Brunet et al., 1999).

Forkhead family members bind to insulin-response sequences within the insulin-like-growth-factor-binding-protein-1 promoter (Brunet et al., 1999; Kops et al., 1999; Tang et al., 1999) and to Forkhead binding sites within the Fas ligand promoter (Brunet et al. 1999). Once bound to the promoters, Forkhead isoforms may exert their pro-apoptotic effects through the upregulation of death promoting genes. For example, Forkhead family members may induce expression of endogenous FasL, which in turn can trigger a death-signaling cascade (Le-Niculescu et al., 1999; Brunet et al., 1999).

In contrast to PKB effects on restricting expression of pro-apoptotic proteins through Forkhead phosphorylation, recent studies suggest that PKB can also mediate the upregulation of survival promoting genes. For example, PKB can enhance the degradation of the NF-κB inhibitor IκB, permitting NF-κB nuclear translocation where it can initiate transcription of various pro-survival genes, such as Bfl-1/A1 and the caspase inhibitors c-IAP1 and c-IAP2 (Ozes et al., 1999; Hu et al., 1998).

Through its control of GSK-3, PKB has been found to regulate a number of metabolic processes including membrane translocation of the glucose transporter 4 (GLUT4), induction of GLUT 1 synthase and the activation of 6-phosphofructose-2-kinase (PFK2) (Kohn et al., 1996a, 1998; Deprez et al., 1997; Tanti et al., 1997; Hajduch et al., 1998; Summers et al., 1998; Wang et al., 1998). Glycogen synthase kinase (GSK-3) inhibits the synthesis of glycogen from glucose by phosphorylating and inactivating glycogen synthase. Phosphorylation of GSK-3 leads to its inactivation and promotes
glycogen. Sequence analysis of GSK-3 isoforms has identified Ser$^{21}$ in GSK3α and Ser$^{9}$ in GSK3β as potential targets of PKB-mediated phosphorylation (Cross et al., 1995a).

1.4. APOPTOSIS

1.4.1. Bcl-2 Family Members

Programmed cell death or apoptosis is a cell suicide mechanism that plays a critical role in development, tissue homeostasis and protection against pathogens. Intranucleosomal DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage are all characteristics of the apoptotic phenotype. The process of apoptosis allows the efficient removal of tissues without the peripheral damage caused by inflammation during necrosis. Apoptotic morphology can be induced by DNA damage, ultraviolet- and x-irradiation, chemotherapeutic drugs, cytokine deprivation and coupling of death receptors. Dysregulation of apoptosis can result in pathologies including cancer, autoimmunity and neurodegenerative disorders. Genetic analysis has identified a family of genes that are essential for programmed cell death in *Caenorhabditis elegans*, including *ced-9, ced-4, and ced-3* (Hengartner 1996). Cloning of the *C. elegans ced-9* gene revealed that it is structurally and functionally homologous to the mammalian cell survival mediator Bcl-2. Bcl-2 was originally isolated from the t(14;18) chromosomal translocation found in human follicular lymphomas and was subsequently shown to inhibit cytokine withdrawal-induced apoptosis in hemopoietic cells. Analogous studies in other cell lines confirmed Bcl-2's ability to promote survival in the presence of various apoptotic stimuli (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1995).
Following the initial identification and description of Bcl-2, other apoptosis regulators have been identified by sequence homology and in cell survival genetic screens. This growing family consists of pro- (Bad, Bak, Bax, Bcl-xS Bid, Bik, Bim, Blk, Bok (Mtd), Mcl-1s) and anti-apoptotic (A1, Bcl-2, Bcl-w, Bcl-xL, Boo, Mcl-1L) proteins that can be divided into three subfamilies on the basis of shared conserved regions termed Bcl-2 homology domains-1 through 4 (BH1 to BH4; review by Chao and Korsmeyer, 1998). All members of the Bcl-2 subfamily contain the BH1 and BH2 conserved sequence motifs and promote survival. Bax, Bak, Bcl-xS, and Bok are members of the...
Bax subfamily and contain BH1, BH2 and BH3 domains. The BH3 group consists of Bad, Bid, Bik, Bim, Blk, HRK, Mcl-1s, NIP3, NIX and the nematode homologue EGL-1. Overexpression studies reveal that the BH3 domain of pro-apoptotic family members is both necessary and sufficient to induce apoptosis (Conradt and Hovitz, 1998; Chittenden et al., 1995a).

One way in which Bcl-2 family members are thought to function is via heterodimerization, in which the BH3 domain of a pro-apoptotic protein inserts into a hydrophobic binding cleft created by the BH1, BH2 and BH3 regions of a pro-survival protein (Sattler et al., 1997). The Bcl-2 homologous antagonist/killer, Bak, was originally described as a cellular host protein that could interact with the adenovirus E1B 19K protein to promote apoptosis of virally infected cells (Farrow et al., 1995; Chittenden et al., 1995b; Kiefer et al., 1995). In uninfected cells Bak may promote apoptosis by binding to and inhibiting the pro-survival proteins Bcl-2 and Bcl-xL (Chittenden et al., 1995b). Similarly, the BH3 cohort, Bax, was first identified by coimmunoprecipitation with Bcl-2 (Oltvai et al., 1993). Work by several groups revealed that Bax homodimerization is both sufficient and essential for Bax-dependent apoptosis (Gross et al., 1998; Ink et al., 1997). However, recent work by Hsu and Youle (1998) suggests that Bax homodimerization may be a detergent-induced phenomenon and may not be required for Bax-mediated cell death.

Three-dimensional structural analysis of Bcl-xL revealed seven α helices and a nonconserved, flexible loop located between the α helices 1 and 2 (Muchmore et al., 1996). Helices α5 and α6 contain the BH1 and BH2 region of Bcl-xL and are similar to the pore-forming domains of some bacterial toxins, suggesting that Bcl-2 family
homologues having BH1 and BH2 conserved sequence motifs may regulate cell survival by forming pores in organelles, such as the mitochondria (Muchmore et al., 1996; Sattler et al., 1997). In keeping with this hypothesis, Bcl-2, Bcl-x\textsubscript{L}, and Bax can form ion channels in lipid bilayers \textit{in vitro} and this channel formation requires intact BH1 and BH2 domains (Minn et al., 1997; Antonsson et al., 1997; review by Schendel et al., 1998).

In addition to Bcl-2 homology domains, many pro- and anti-apoptotic molecules are targeted to various intracellular membranes via a carboxy-terminal transmembrane domain (Nyguyen et al. 1993). Work by Reed and coworkers showed that Bcl-2 and Bcl-x\textsubscript{L} are localized at the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope (1993). While anchored at the mitochondria, Bcl-2 and Bcl-x\textsubscript{L} are thought to inhibit mitochondrial membrane permeability transition (PT) (Vander Heiden et al., 1997) and the subsequent release of apoptogenic factors, such as cytochrome \textit{c} and apoptosis-inducing factor (AIF; Yang et al., 1997).

Mitochondrial PT is characterized by a loss in membrane potential (\(\Delta \psi\)) and requires an increase in cytoplasmic \(\text{Ca}^{2+}\). Work by several groups suggest that Bcl-2 and Bcl-x\textsubscript{L} may promote cell survival by maintaining \(\Delta \psi\) through enhanced proton efflux and by inhibiting the release of \(\text{Ca}^{2+}\) from the mitochondrial matrix (Susin et al., 1996; Shimizu et al., 1998). Alternatively, Bcl-2 and Bcl-x\textsubscript{L} may suppress apoptosis by inhibiting the release of mitochondrial caspases such as caspase-2 and -9 (Susin et al., 1999).

The Bcl-2 interacting mediator of cell death, Bim, also contains a C-terminal hydrophobic region. However unlike Bcl-2 and Bcl-x\textsubscript{L}, pro-apoptotic Bim is localized to the microtubule-associated dynein motor complex in healthy cells (Puthalakath et al. 1999). Following a death signal, Bim dissociates from the microtubules and translocates...
to the mitochondria where it antagonizes Bcl-2 and Bcl-xL-mediated cell survival. Likewise, Bax translocation from the cytosol to the mitochondria requires an intact C-terminal transmembrane domain (Wolter et al., 1997; Xiang et al., 1996).

Once localized at the mitochondria, pro-apoptotic Bcl-2 homologues mediate mitochondrial dysregulation and antagonize Bcl-2 and Bcl-xL survival promoting activities. For example, recombinant Bax, Bak, and Bid can induce mitochondrial membrane permeabilization in vitro (Minn et al., 1997; Antonsson et al., 1997; Schendel et al., 1999).

Mitochondrial PT involves the opening of an oligo-protein channel called the PT pore. The PT pore consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) and cyclophilin D (Kroemer et al., 1998). Although the exact mechanisms by which Bak and Bax function to induce apoptosis are not known, some studies suggest that mitochondrial-based Bak and Bax may interact with the VDAC to promote ΔΨ-loss (Narita et al., 1998; Shimizu et al., 1999) and with the ANT to promote cytochrome c release (Marzo et al., 1998; Saito et al., 2000). Conversely, research by Eskes and colleagues (2000) suggests that oligomeric Bax functions independently of the PT pore complex to form a nonspecific membrane channel thereby promoting ΔΨ-loss and cytochrome c release.

In addition to subcellular localization and dimerization, some Bcl-2 homologues are regulated by phosphorylation. Bcl-2 and Bcl-xL are regulated by phosphotransfer at evolutionarily conserved sites in the flexible loop region located between the BH3 and the BH4 domain (Chang et al., 1997; Ito et al., 1997; Maundrell et al., 1997). For example, IL-3 stimulates Bcl-2 Ser70 phosphorylation by PKCα leading to enhanced cell survival.
(May et al., 1994; Ruvolo et al., 1998). Recent work by Deng and coworkers (2000) revealed direct phosphorylation of Bcl-2 Ser$^{70}$ by ERK1/2 and suggests the existence of PKC-dependent and -independent pathways leading to Bcl-2 mediated cell survival. Similarly, a current study by the Craig group (2000) demonstrated phosphorylation of the pro-survival protein Mcl-1L in a PKC-and ERK-dependent manner; however, the biological significance of this phosphorylation event has yet to be determined. Microtubule-damaging drugs such as paclitaxel and vincristine induce PKA-mediated Bcl-2 hyperphosphorylation and promote apoptosis, presumably by altering the stability of Bcl-2:Bax heterodimers (Srivastava et al., 1998; Ito et al., 1997; Haldar et al., 1998; Yamamoto et al., 1999). Chemotherapeutic drugs, such as taxol, stimulate Bcl-2, Bcl-xL and Mcl-1L phosphorylation and are associated with decreased cell viability (Domina et al., 2000). Taxol-induced Bcl-2 phosphorylation may be mediated by the mitogen-activated protein kinase pathway member Raf or by the stress activated protein kinase JNK (Haldar et al., 1998). Taken as a whole these findings revealed that multiple signaling pathways converge on Bcl-2 family members and suggest that phosphorylation may be a general mechanism employed by various signaling pathways to regulate the function of pro-survival Bcl-2 family members. Moreover these studies revealed that phosphorylation of anti-apoptotic molecules can provide both growth inhibitory and growth promoting effects depending on stimulus and co-stimulatory signals.

The death agonist Bad (Bcl-xL/Bcl-2-associated death promoter) was first described as a protein that heterodimerizes with Bcl-2 and Bcl-xL to promote apoptosis (Yang et al., 1995). Following cytokine stimulation mitochondrial-based Bad is phosphorylated on Ser$^{112}$ and Ser$^{136}$ and dissociates from Bcl- xL to form an inactive
complex with the cytoplasmic sequestering factor 14-3-3 (Zha et al., 1996; Datta et al., 1997a; del Peso et al., 1997). Bad Ser\textsuperscript{112} is directly phosphorylated by p90\textsuperscript{rsk} via a PKC/MEK-dependent pathway (Scheid et al., 1999; Tan et al., 1999), whereas phosphorylation of Bad Ser\textsuperscript{136} is mediated by PKB-dependent and independent pathways depending on cell type (Scheid and Duronio, 1998; Datta et al., 1997a; Blume-jennson et al., 1998). Recent work by several groups have identified Ser\textsuperscript{155} as a novel site of Bad phosphorylation and demonstrated the direct phosphorylation of Bad Ser\textsuperscript{155} by PKA (Datta et al., 2000). In the presence of a death signal, cytosolic Bad is dephosphorylated by the Ca\textsuperscript{2+}-activated protein phosphatase calcineurin and relocalizes to the mitochondria where it antagonizes the pro-survival functions of Bcl-2 and Bcl-x\textsubscript{L} (Wang et al., 1999).

Bcl-2 family members may also be regulated by proteolytic cleavage. In healthy cells, the BH3 family member Bid is localized in the cytosol (Gross et al., 1999). Upon exposure to a death signal, p22 Bid is cleaved by a cysteine-aspartic acid specific protease, caspase-8, generating a p15 carboxy-terminal cleavage fragment (tBid). Cytosolic p21 Bax also undergoes amino-terminal cleavage by the calcium-dependent cysteine protease, calpain, resulting in a p18 carboxy-terminal fragment (Wood and Newcomb, 2000). Following caspase cleavage, tBid and p18 Bax translocate to the mitochondria where they regulate apoptotic mitochondrial changes. Interestingly, the truncated forms of both Bax and Bid possess greater intrinsic cytotoxicity compared to their full-length counterparts (Wood and Newcomb, 2000; Wood and Newcomb, 1999). Bcl-2 and Bcl-x\textsubscript{L} are also substrates for caspase activity. Surprisingly, proteolytic cleavage of Bcl-2 and Bcl-x\textsubscript{L} by caspase-3 and caspase-1, respectively, converts these pro-survival factors into potent death effectors (Clem et al., 1998; Kirsch et al., 1999).
In addition to post-translational modifications, alternative splicing of pre-mRNAs regulates the function and sub-cellular location of some Bcl-2-family proteins. For example, the Mcl-1s splice variant of Mcl-1 lacks the membrane-anchoring domain present on Mcl-1L and antagonizes the pro-survival function of Mcl-1L (Bingle et al., 2000; Bae et al., 2000). Similarly, the Bcl-2 β isoform of Bcl-2 lacks the c-terminal transmembrane region found in Bcl-2α and exhibits diminished pro-survival activity (Hockenbery et al., 1990; Tanaka et al., 1993). Functionally distinct isoforms have also been described for Bax (Bax-α, Bax-β, Bax-γ), Bcl-x (Bcl-xL and Bcl-xS) and Bim (BimEL, BimL, BimS; review by Jiang and Wu, 1999).

Some pro-survival Bcl-2 homologues undergo transcriptional regulation, for example, de novo gene expression of Bfl-1 is induced by inflammatory cytokines, tumor necrosis factor and interleukin-1 (Karsan et al., 1996). Moreover, recent studies identified Bfl-1 as a direct target of NF-κβ transactivation (Hu et al., 1998; Zong et al., 1999; Lee et al., 1999; Wang et al., 1999). Mcl-1 is a moderate viability-promoting member of the Bcl-2 family first identified as an early gene induced during differentiation of ML-1 myeloid leukemia cells (Kozopas et al., 1993). Work by Chao and coworkers (1998) revealed that Mcl-1 is regulated at the transcriptional level in response to cytokine stimulation, further, this group suggests that induction of Mcl-1 transcription occurs via a PI3K-PKB-dependent pathway (1999; discussed further in Chapter 4).

1.4.2. Caspases

The ced-3 gene product is homologous to the mammalian family of cysteine-aspartic acid specific proteases termed caspases (Yuan et al., 1993; Thornberry et al.,
1992). Caspases are expressed as proenzymes containing an N-terminal polypeptide (prodomain) and a large (~20 kDa) and small (~10 kDa) subunit (Nicholson and Thornberry, 1997). Zymogen activation involves autocatalysis or caspase-mediated proteolysis at an interdomain linker segment allowing the large and small subunit to associate to form a heterodimeric enzyme. The prodomain is highly variable in sequence and length and is involved in the regulation of enzymatic activity. Caspases can be divided into two groups: the initiators (caspases-8, -9, and -10) and the effectors or the executioners (caspases-2, -3, -6, and -7). Initiator caspases are activated in response to proapoptotic stimuli and require interaction with specific cofactors (Boldin et al., 1996; Li et al., 1997). For example, association of the cofactor FADD (Fas-associated protein with death domain) with the DED (death effector domain) of pro-caspase-8 results in caspase-8 activation. Similarly, APAF-1 (a protease activating factor), the mammalian homolog of the ced-4 gene product, interacts with the CARD (caspase recruitment domain) of pro-caspase-9, contributing to its proteolysis (Boldin et al., 1996; Muzio et al., 1996). The additional cofactors, cytochrome c and deoxyadenosine triphosphate are also required for caspase-9 activation (Li et al., 1997). Together, cytochrome c, APAF-1 and pro-caspase-9 form a signaling complex called the apoptosome.

Initiator caspases integrate extracellular and cell-autonomous cues to coordinate the activation of a specific set of executioners. For example, death receptor induced apoptosis triggers caspase-8 activation (Nguyen et al., 1994; Borner et al., 1994) and cytotoxic agents stimulate caspase-9 activation (Ng and Shore, 1998; Xu and Reed, 1998). Further, caspase-9 deficient embryonic stem cells and embryonic fibroblasts are resistant to UV- and irradiation-induced apoptosis. Effector caspases employ three
different strategies to mediate a select program of cell disassembly: (1) caspases inactivate inhibitors of apoptosis, such as ICAD, an inhibitor of the caspase-activated deoxyribonuclease (CAD) (Enari et al., 1998; Liu et al., 1997); (2) caspases destroy cell structures, such as the nuclear lamina, resulting in chromatin condensation (Takahashi et al., 1996; Orth et al., 1996); and (3) caspases reorganize cell structures by deregulating the activity of proteins involved in cytoskeleton regulation, including gelsolin (Kothakota et al., 1997), focal adhesion kinase (FAK), (Wen et al., 1997) and p21-activated kinase (PAK; Rudel and Bokoch, 1997). Caspase activation also leads to the loss of phosphatidylserine asymmetry in the plasma membrane, a process that may be critical in the recognition and clearance of apoptotic bodies by phagocytic cells such as macrophages (Blankenberg et al., 1999).

Emerging mechanistic evidence suggests that pro-survival Bcl-2-family proteins may function by suppressing caspase-dependent apoptosis. For example, binding of Bcl-xL to Apaf-1 may inhibit Apaf-1-mediated caspase-9 activation. In contrast, members of the Bax and BH3 Bcl-2 subfamilies may promote apoptosis by inhibiting Bcl-xL–Apaf1 association (Pan et al., 1998; Hu et al., 1998). A prerequisite of caspase-9 activation by Apaf-1 is the presence of sufficient quantities of the cofactors ATP and cytochrome c. It has also been proposed that loss of cytochrome c and other oxidative species from the mitochondria, controlled by Bcl-2 family proteins, ensures the demise of a cell even if the downstream executor caspases fail or are inactivated (Thornberry and Lazebnik, 1998).

1.5. Sphingomyelin Signal Transduction Pathway

1.5.1. Ceramide Generation and Metabolism
A wide variety of physiological signals, such as those generated by cytokines and growth factors, and various apoptotic stimuli including TNF-α and FasL, can activate the sphingomyelin pathway resulting in the production of the lipid second messenger ceramide (N-alkysphingosine) (Mathias et al., 1998). Generation of ceramide can occur by de novo synthesis involving ceramide synthase or by sphingomyelin hydrolysis involving various sphingomyelinases (Spiegel et al., 1996; Kolesnick, 1991; Spiegel and Merrill, 1996). De novo synthesis of ceramide occurs when serine and palmitoly-CoA undergo condensation to form 3-ketosphinganine. 3-ketosphinganine is quickly reduced to dihydrosphingosine by NADPH-dependent reductase and acylated by ceramide synthase (sphinganine N-acyltransferase) yielding


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Introduction of a trans-4, 5 double bond into dihydroceramide generates ceramide.

Ceramide generation can also occur by hydrolysis of sphingomyelin. Various sphingomyelinases can hydrolyze the phosphodiester bond of sphingomyelin yielding ceramide and phosphocholine. Sphingomyelinase isoforms can be grouped into five categories: (1) an acid sphingomyelinase first identified as a lysosomal enzyme defective in persons with types A and B Niemann-Pick disease (Levade, et al., 1986); (2) a ubiquitous, Zn\(^{2+}\)-stimulated, acid sphingomyelinase (Schissel et al., 1996); (3) a membrane bound, Mg\(^{2+}\)-dependent, neutral sphingomyelinase (Spence, 1993); (4) a cytosolic, cation-independent neutral sphingomyelinase (Okazaki et al., 1994); and (5) an alkaline sphingomyelinase found in the intestinal mucosa and bile (Nyberg et al., 1996; Duan et al., 1996).

Newly synthesized ceramide may accumulate transiently in the cell before being converted into various metabolites. Hydrolysis of ceramide generates sphingosine and phosphorylation of sphingosine by sphingosine-kinase generates sphingosine-1-phosphate (Spiegel et al., 1996). Phosphorylation of ceramide by ceramide kinase generates ceramide 1-phosphate (Bajjalieh et al., 1989; Kolesnick, 1990) and addition of phosphocholine to ceramide by sphingomyelin-synthase regenerates sphingomyelin (Kolesnick 1990). In addition, glycosylation of ceramide in the Golgi apparatus by glucosylceramide transferase produces various complex glycosphingolipids (Koch et al., 1996) that can function as second messengers for such diverse processes as proliferation (Spiegel et al., 1996; Gomez-Munoz et al., 1997), differentiation (Kolesnick and Golde, 1994; Mathias et al., 1993; Ballou et al., 1990; Kim et al., 1991), growth arrest (Jayadev
et al., 1995; Dbaido et al., 1995) and apoptosis (Brenner et al., 1998; Schwandner et al., 1998).

1.5.2. Ceramide and Apoptosis

Early evidence supporting a role for ceramide in apoptosis emerged from biochemical studies involving cell-permeable ceramide analogs; for example, Hannun and coworkers (1993) observed that C2-ceramide could mimic the toxic effects of TNFα in U937 cells. Additional studies involving cell-free systems revealed that TNF-α or ionizing radiation could act on cellular membranes to generate ceramide and initiate apoptosis (Haimovitz-Friedman et al., 1994; Dressler et al., 1992). Furthermore, ceramide-induced cell death is accompanied by DNA cleavage, chromatin condensation and organelle dismantling, all hallmarks of apoptotic morphology. Production of the pro-apoptotic lipid mediator, is initiated in cells in response to numerous stresses, including ionizing radiation (Haimovitz-Friedman et al., 1994; Michael et al., 1997; Chmura et al., 1997; Bruno et al., 1998), chemotherapeutic drugs (Bose et al., 1995; Jaffrezou et al., 1996; Zhang et al., 1996; Bradshaw et al., 1996; Whitman et al., 1997), UV light (Datta et al., 1997b), hyperosmolarity (Kyriakis et al., 1994, 1995) and activation of the TNF receptor (Obeid et al., 1993).

One mechanism of ceramide-mediated apoptotic signaling is thought to involve activation of the stress-activated protein kinase cascade (SAPK/JNK) (Verheij et al., 1996; Coroneos et al., 1996; Cuvillier et al., 1996; Westwick et al., 1995). The JNK pathway is activated in response to various stress stimuli, such as UV irradiation, hyperosmolarity, cytokines, and chemotherapeutic drugs leading to the trans-activation of
various transcription factors, such as c-jun and jun-D, (Fanger et al., 1997) and the subsequent transcription of death promoting genes, such as Fas ligand and TNF-α (Zhang et al., 2000; Le Niculescu et al., 1999). Moreover, in response to physical, chemical and environmental stresses, JNK translocates to the mitochondria where it can phosphorylate Bcl-2 and Bcl-x<sub>L</sub>, presumably leading to their inactivation (Fan et al., 2000).

Several studies link ceramide-induced apoptosis to activation of the JNK signaling pathway. For example, exposure of U937 monoblastic leukemia cells and bovine aortic endothelial cells to ionizing radiation, UV light, or TNF-α results in rapid ceramide generation and JNK-dependent apoptosis (Verheij et al. 1996). Additionally, treatment of cells with exogenous sphingomyelinase or cell-permeable ceramide derivatives activates the JNK signaling cascade (Westwick et al. 1995) and disruption of JNK signaling by overexpression of dominant-negative c-jun abrogates ceramide-induced apoptosis. Finally, B cells from patients with NPD are resistant to UV-induced JNK activation yet retain responsiveness to ceramide analogs (Huang et al., 1997). Ceramide may activate the JNK signaling pathway either via transforming-growth-factorβ-activated kinase (TAK1) (Shirakabe et al., 1997) or via the small G-protein Rac-1 (Brenner et al., 1997). Taken together, these data suggest an essential role for the JNK signaling pathway in ceramide-mediated apoptosis.

In addition to activation of the JNK pathway, ceramide may regulate apoptosis by altering mitochondrial function. The role of ceramide in mitochondria-dependent cell death is two-fold: (1) to disrupt the mitochondrial membrane potential (ΔΨ) (Castedo et al., 1996; Pastorino et al., 1996; Susin et al., 1997; Szalai et al., 1999) and (2) to generate reactive oxygen species (ROS) (Gudz et al., 1997; Garcia-Ruiz et al., 1997). Work by
Szalai and colleagues (1999) suggest that ceramide may promote $\Delta\Psi$-loss by reducing the threshold level of calcium required for PT. Additionally, ceramide-induced $\Delta\Psi$-loss may be mediated by a ceramide-activated protein phosphatase (CAPP; discussed below).

Consequent to ceramide-induced mitochondrial dysregulation, several apoptogenic factors are released from the mitochondria including AIF. AIF is a ubiquitous flavoprotein that undergoes mitochondri-o-nuclear translocation upon exposure to various death inducing agents, including ceramide (Susin et al., 1999). Moreover, nuclear translocation of AIF occurs in the presence of caspase inhibitors, suggesting the existence of caspase-independent death mechanisms. Once in the nucleus AIF induces chromatin condensation and DNA fragmentation (Susin et al., 1999).

1.5.3. Direct Targets of Ceramide Action

Three putative targets of ceramide action have been described in mammals: ceramide-activated protein phosphatase (CAPP); ceramide-activated protein kinase (CAPK); and protein kinase C$\zeta$. CAPP is a member of the protein phosphatase 2A (PP2A) family of serine/threonine phosphatase (Doborowsky et al., 1993; Law and Rossie, 1995). During ceramide-mediated apoptosis, CAPP may promote mitochondrial PT by dephosphorylating Bcl-2 Ser$^{70}$ (Ruvolo et al., 1999) directly or through the dephosphorylation and inactivation of PKCa (Lee et al., 1996). Further, studies described in Chapter 5 suggest that PKB Ser$^{473}$ may be a target of CAPP activity resulting in a decrease in PKB activity.

CAPK was first identified as a 97 kDa membrane-bound, proline-directed, serine/threonine-specific protein kinase (Mathias et al., 1991; Joseph et al., 1993; Liu et
al., 1994). Studies by Yao and coworkers (1995) suggested that CAPK mediates proliferative and pro-inflammatory responses to TNF-α by phosphorylation of c-Raf-1, leading to ERK activation. In contrast, work by Yu and colleagues (1997) demonstrated CAPK-mediated ERK inactivation via a MEK1 dependent pathway. Moreover, work by the Kharbanda group (2000) suggested that CAPK functions to couple the TNF-α receptor to the JNK cascade. Together these studies showed that ceramide might promote apoptosis through activation of the stress-activated pathways (JNK), and possibly through inhibition of the ERK pathway. As discussed above, ERK may couple pro-survival signals to Bad inactivation. This highlights how ceramide may use several levels of cross-talk to initiate cell death, which may be both cell type and stimulus specific.

Several research findings have identified the atypical PKC isoform, PKCζ, as a ceramide activated kinase, for example, treatment of NIH-3T3 fibroblasts cells with ceramide results in a threefold increase in PKCζ activity both in vivo and in vitro (Lozano et al, 1994). Moreover, work by Wang and colleagues (1999) and Muller and coworkers, (1995) suggests that ceramide is a bifunctional lipid second messenger; stimulating PKCζ activity at low concentrations and inhibiting PKCζ function at high concentrations. During ceramide-mediated apoptosis, PKCζ translocates to the nucleus, where it is thought to regulate components of the transcriptional machinery (Bertolaso et al., 1998).

1.6. Summary

A plethora of extracellular molecules regulate cell cycle progression, proliferation, differentiation and apoptosis. In the hemopoietic system, granulocyte-macrophage colony-stimulating factor (GM-CSF) binds to cell surface receptors resulting in the
activation of several intracellular signal transduction cascades. Among these are the PI3K-PKB and the Ras-ERK signal transduction pathways. In addition, the lipid second messenger ceramide may also function as a modulator of PKB- and ERK-dependent signaling.

Several research findings suggest that these signaling pathways may converge on members of the Bcl-2 family (review by Adams and Cory, 1998). While these studies have been useful in delineating the intracellular function of Bcl-2 family members, the primary molecular mechanisms by which these signaling pathways function to regulate Bcl-2 family members has not been completely defined. There are three outstanding questions that I addressed in this thesis:

1) Is there a role for PI3K-PKB in regulating Bcl-2 family member expression? In addition, are Bcl-2 family members regulated at the post-translational level by the PI3K-PKB signaling pathway?

2) The pro-survival Bcl-2 family member Mcl-1 is controlled by an immediate-early transcription factor (Wang et al., 1998). Do cytokines utilize the PI3K or ERK pathways in this process? Additionally, do the PI3K or ERK pathways play a role in Mcl-1 regulation at the translational level?

3) Ceramide may inhibit PI3K, leading to a decrease in PKB activation (Zundel and Garcia, 1998). Do the effects of ceramide on PI3K-PKB signaling result in modulation of Bcl-2 family members, such as Mcl-1?
1.6.1. Hypotheses

My working hypothesis is two-fold:

1) Various pathways mediate changes in Bcl-2 family member expression in response to cytokine stimulation.

2) Cross-talk between PI3K-PKB, Ras-ERK, and ceramide-signaling pathways plays a critical role in Bcl-2 family member regulation.

1.6.2. Objectives

These hypotheses led to three objectives that I wanted to pursue:

1) To determine whether PI3K, MEK or ceramide plays a role in the regulation of specific Bcl-2 family members.

2) To examine the mechanism of regulation of Mcl-1 in hematopoietic cells.

3) To further define the role of ceramide in the regulation of PI3K and PKB.

2. MATERIALS AND METHODS

2.1. Antibodies and reagents. TRIZOL reagent was purchased from Canadian Life Technologies (Burlington, ON). The RiboQuant Multi-Probe RNase Protection Assay System, including the multi-probe template sets mAPO-2 (45354P), hAPO-2 (45121P) and hAPO-2c (45609P) were purchased from Pharmingen (San Diego, CA). The anti-Mcl-1 antibody (sc-819) was purchased from Santa Cruz. The anti-Bcl-xL (B22630) antibody was from Transduction Laboratories (Lexington, KY). Bak specific antibodies
were purchased from Upstate Biotechnologies (Lake Placid, NY; 06-536) and from Transduction Laboratories (Lexington, KY). Anti-Bax specific antibodies were from Upstate Biotechnologies (Lake Placid, NY; 06-499) and from Santa Cruz (Santa Cruz, CA; sc-493) and anti-Bad antibodies were purchased from Santa Cruz (Santa Cruz, CA; sc-943) and from Transduction Laboratories (Lexington, KY; B36420). C2-, C2-dihydro-, and C6-ceramides were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). LY-294002 was from Calbiochem (La Jolla, CA). U0126 was from Promega (Madison, WI). [35S]-methionine and [α-32P]-UTP were purchased from ICN Biomedicals (Costa Mesa, CA). Recombinant human GM-CSF was from R&D Systems (Minneapolis, MN). The anti-phospho-Thr308 PKB antibody (9271), the anti-phospho-Ser473 PKB antibody (9270), and the anti-phospho-MAPK (9106) were purchased from New England Biolabs (Beverly, MA). The anti-PKB antibody (06-558) and Crosstide were from Upstate Biotechnology Inc. (Lake Placid, NY). Okadaic acid and calyculin A were from Sigma Chemical Co. (Oakville, ON). U0126 was from Promega (Madison, WI). RPMI 1640, fetal bovine serum, and Protein-G Sepharose beads were purchased from Canadian Life Technologies (Burlington, ON).

2.2. **Cell Culture Conditions and Stimulations.** RPMI 1640 was supplemented with 10% FBS, 10 μM 2-mercaptoethanol (2-Me), 100 units of penicillin G/ml, and 100 μg of streptomycin/ml prior to using for cell culture. The factor-dependent hematopoietic cell lines MC/9 and TF-1 (American Type Culture Collection) were passaged in RPMI 1640 supplemented with 1% WEHI-3B (mouse IL-3) conditioned medium or 1% CGMI (human GM-CSF) conditioned medium, respectively. Prior to stimulation, cells were
starved of cytokine and cultured for 8 h in low-serum medium (0.5% FBS). Cells were then washed 1 time with phosphate-buffered saline (PBS) and incubated at 10^6 cells/ml in low-serum medium with and without GM-CSF (50 ng/ml).

2.3. RNase Protection Assays. Total RNA was isolated from cultured cells using TRIZOL Reagent (Canadian Life Technologies) and subjected to RiboQuant Multi-Probe RNase Protection Assay System (Pharmingen, San Diego, CA) according to the manufacturer's specifications. Briefly, a 2 µg portion of total RNA was incubated overnight at 56°C with a high-specific-activity, [32P]-labeled anti-sense RNA probe set generated by T7 polymerase-directed in vitro transcription. Irrelevant mouse, human and yeast RNA were also incubated overnight with probe sets to control for non-specific hybridization. Following hybridization, excess probe and other single-stranded RNA were subjected to RNase digestion and “RNase-protected” probes were purified and separated on denaturing polyacrylamide gels. Protected mRNA species in the original RNA sample were quantified based on the intensity of the corresponding protected probe fragment as determined by direct quantitation with a PhosphorImager (Bio Rad). To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping gene transcript, L32.

2.4. Stimulation and Immunoblotting. Cells were deprived of cytokine and plated in low-serum (0.5%) medium for 8 h. Cells were then restimulated with 10 ng/ml GM-CSF for the indicated times in the presence or absence of a PI3K inhibitor (LY-294002; 50 µM), a MEK inhibitor (U0126; 25 µM) or ceramide analogs (25 µM). Cells were lysed
with ice-cold solubilization buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% deoxycholic acid, 0.25 mM PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor) and nuclei were pelleted by centrifugation (20,000 x g, 1 min). Supernatants were transferred to new tubes and normalized for total protein using the Pierce BCA protein assay kit (BioLynx, Brockville, ON). An equal volume of 2X reducing sample buffer was added to each sample followed by boiling for 5 minutes. Immunoblotting procedures were performed as described previously (Duronio et al., 1992; Welham et al., 1994; Welham and Schrader, 1992). Cell extracts (50 μg) were resolved on SDS-PAGE (12%) and proteins were transferred to nitrocellulose by semi-dry blotting. Membranes were blocked with 5% (w/v) skim milk containing 0.05% sodium azide overnight at room temperature and probed with 1:500 dilution of antibodies specific for Mcl-1, Bak, Bax or Bcl-xL at room temperature. Primary antibody was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

2.5. Determination of Mcl-1 half-life. To determine the effect of PI3K- and MEK-inhibition on the half-life of Mcl-1 in TF-1 cells, exponentially growing cells were precultured for 30 min in methionine-free medium supplemented with 0.5% dialyzed FBS and 10 ng/ml of GM-CSF. Cells were pulse-labeled with 1 mCi/ml [35S]-methionine (ICN; 10 mCi/ml, >1,000 Ci/mmol) for 15 min at 37°C and chased in growth medium containing 0.5% FBS, 10 ng/ml GM-CSF and 10 mM methionine in the presence and absence of LY-294002, in the presence and absence of U0126 or in the presence and absence of ceramide. At various times after the chase, 1 x 10^7 cells per sample (1 mg
solubilized protein) were lysed in 500 μl of solubilization buffer (10mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% deoxycholic acid, 0.25 mM PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor) immediately followed by removal of nuclei by centrifugation (20,000 x g, 1 min). Whole cell lysates were normalized for total protein using the Pierce assay. The supernatant was transferred to new chilled tubes and pre-cleared 2 x 45 min with 20 μl packed volume of protein-G Sepharose. After pre-clearing, the beads were pelleted and lysates transferred to new chilled tubes followed by the addition of 4 μg of anti-Mcl-1 antibody. Immunoprecipitations were performed overnight at 4°C with continuous mixing. The samples then received 20 μl of a 1:1 suspension of protein-G Sepharose beads in solubilization buffer and mixed at 4°C for an additional 1 h. The beads were pelleted and washed five times with fresh solubilization buffer. Between each wash, the beads and bound Mcl-1 protein were transferred to new chilled tubes to minimize non-specific binding of [35S]-methionine. The beads were then resuspended in 20 μl of 2X SDS sample buffer followed by boiling for 5 min. Mcl-1 immune complexes were resolved by SDS-PAGE (12%) and visualized by autoradiography. The Mcl-1 bands were quantified with a phosphorimager.

2.6. Translation studies. TF-1 cells were starved of cytokine and cultured for 8 h in low-serum medium (0.5% FBS). Cells were then washed 3 times with phosphate-buffered saline (PBS) and seeded in methionine-free medium supplemented with 0.5% dialyzed FBS. After 30 min at 37°C, cells were pretreated with LY-294002 (50 μM),
U0126 (25 µM), C₆- or C₂- ceramide (25 µM), or vehicle alone for an additional 30 min. Pulse-labeling was initiated by adding 1 mCi/ml of ³⁵[S]-methionine in growth medium containing 0.5% dialyzed FBS and 10 ng/ml GM-CSF. At various times after pulse-labeling, 1 x 10⁷ cells were lysed in 500 µl solubilization buffer (10mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% deoxycholic acid, 0.25 mM PMSF, 1 µM pepstatin, 0.5 µg/ml leupeptin and 10 µg/ml soybean trypsin inhibitor) immediately followed by removal of nuclei by centrifugation (20,000 x g, 1 min). Whole cell lysates were normalized for total protein using the Pierce assay. Cell extracts were pre-cleared and subjected to immunoprecipitation with 4 µg anti-Mcl-1 antibody as described above. Immunoprecipitated complexes were resolved on SDS-PAGE gel and visualized by fluorography. Mcl-1 bands were quantified with a phosphorimager.

2.7. Stimulation and Immunoprecipitation Conditions. Prior to stimulation, cells were starved of cytokine and cultured for 8 h in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cells were washed once with PBS and cultured at 10⁷ cells/ml in serum-free RPMI 1640 buffered with 20 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH 7.2 immediately prior to assay. Cells were incubated at 37°C for 15 min and treated with ceramides or vehicle for 30 minutes prior to the addition of 10 ng/ml GM-CSF for various times, which was previously found to induce maximal tyrosine phosphorylation. In experiments involving okadaic acid or calyculin A, these compounds were added 30 min prior to the addition of ceramide. To stop reactions, cells were pelleted and solubilized in ice-cold lysis buffer (50 mM Tris (pH 7.4), 0.5% (v/v) NP-40, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na₃VO₄, 1 µg/ml microcystin-LR,
0.25 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor) and removal of nuclei by centrifugation at 20,000 rpm for 1 min. Supernatants were incubated with 2 μg anti-PKB-α antibody at 4°C for 1 hour, with continuous mixing. Immune complexes were captured with 20 μl of Protein-G Sepharose beads at 4°C for 1 hour. The beads were washed 3 times with fresh solubilization buffer and resuspended in 1x reducing sample buffer followed by boiling for 5 minutes. Samples were fractionated on 9% SDS-PAGE gels and transferred to nitrocellulose. Blots were blocked in 5% skim milk and incubated with 1:1000 dilution of the appropriate antibody diluted in TBS containing 1% bovine serum albumin. Primary antibody was detected using the appropriate Horseradish peroxidase (POD)-coupled secondary antibody (DAKO), followed by enhanced chemiluminescence Western blot system as instructed by the manufacturer (Amersham Pharmacia Biotech).

In one set of studies, experiments were designed to investigate the effect of adding ceramide to cells that were previously stimulated with GM-CSF. TF-1 cells were starved of cytokine as described above, washed once in PBS and resuspended at 1 x 10^7 cells/ml in serum-free RPMI 1640 buffered with 20 mM HEPES. Cells were preincubated at 37°C for 15 min followed by GM-CSF (10 ng/ml) stimulation for 30 sec. Stimulation was terminated by washing cells 2 times with HEPES buffered medium followed by resuspension in 1 ml of the same solution. Immediately after washing, the cells were treated with C6-ceramide (25 μM) or vehicle for the indicated times. To stop reactions, cells were pelleted and solubilized in ice-cold lysis buffer (50 mM Tris (pH 7.4), 0.5% (v/v) NP-40, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na3VO4, 1 μg/ml microcystin-LR, 0.25 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.5 μg/ml leupeptin, 10 μg/ml
soybean trypsin inhibitor). Nuclei-free cell extracts were incubated with 2 μg anti-PKB-α antibody and immunoprecipitations were performed as described previously.

2.8. PKB Kinase Assays. Beads with immunoprecipitated kinases were washed three times with fresh solubilization buffer containing 500 mM NaCl and once with kinase buffer (20 mM HEPES (pH 7.2), 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.25 mM PMSF, 1 mM Na₃VO₄, 0.5 μg/ml leupeptin, 1 μg/ml microcystin-LR). Beads were resuspended in 25 μl kinase buffer containing 60 μM Crosstide. Five μl of ATP solution (200 μM ATP, 10 μCi of [γ-³²P]ATP in kinase buffer) was added, followed by incubation for 15 min at 30°C. Reactions were stopped by spotting 20 μl of the reaction volume onto 2 cm² squares of P81 filter paper, followed by extensive washing with 1% (v/v) phosphoric acid and measurement of associated radioactivity by liquid scintillation counting.

2.9. HPLC Analysis of PI(3,4,5)P₃ and PI(3,4)P₂. To measure the in vivo labeled lipids directly by HPLC, cells were labeled with 0.5 mCi/ml ³²P-orthophosphate for two hours at 37°C in phosphate free-RPMI-1640, stimulated and extracted as described above. The dried lipids were deacylated as described previously (Gold et al., 1994), and applied to a Partisil 10 SAX ion exchange column. Following a 10 min washing of the column with water, a 60 min 0-0.25 M ammonium phosphate gradient was performed, followed by a 50 min 0.25-1.0 M ammonium phosphate gradient. One ml fractions were collected and monitored for radioactivity by scintillation counting. ATP, ³H-inositol(1,4,5)P₃ (ICN)
and $^3$H-inositol(1,3,4,5)P$_4$ (ICN) were used to calibrate the column and eluted at 75 min, 84 min, and 106 min, respectively.

3.0. Metabolic Labelling of Bad, Bel-x$_L$, Bak and Bax. Cells were starved of cytokine, washed in phosphate-free medium, and then placed in phosphate-free RPMI medium buffered with 10 mM HEPES pH 7.4 with 1 mCi/ml $^{32}$P-orthophosphate at 37°C for 2 hours. Bad, Bel-x$_L$, Bak or Bax was immunoprecipitated from detergent-solubilized lysates as described above. Immunoprecipitates were fractionated on a 10% gel with an acrylamide-bisacrylamide ratio of 118:1. The gel was dried under heat and vacuum. $^{32}$P-labelled proteins were detected by autoradiography.
3. ANALYSIS OF BCL-2 FAMILY MEMBER REGULATION IN MULTIPLE HEMOPOIETIC CELL TYPES

3.1 RATIONALE AND HYPOTHESIS

Cytokine stimulation activates signaling pathways that can directly affect components of the apoptotic machinery to promote cell survival and/or growth. Through binding to their cognate receptors, granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) can suppress apoptosis. The exact mechanisms by which GM-CSF and IL-3 mediate cell survival in hemopoietic cells are not fully understood. However, recent reports suggest that they are likely to involve regulation of the Bcl-2 family members (Review by McCubrey et al., 2000). This may involve regulation at the mRNA level, the protein level, or post-translational stage.

3.2 RESULTS

I began my studies by asking whether various Bcl-2 family members were targets of protein phosphorylation cascades. To start, I examined each of the sequences of mouse Bad, Bax, Bak and Bcl-xL with an algorithm that predicts potential phosphorylation sites (Blom et al., 1999). The algorithm provided a score from 0.0 to 1.0 for each serine, threonine and tyrosine residue in each protein, with 1.0 representing a high probability for in vivo phosphorylation. The known phosphorylation sites for Bad - Ser\textsuperscript{112}, Ser\textsuperscript{136} and Ser\textsuperscript{155} - all scored above 0.9 (high probability). Table I lists each of the serine, threonine and tyrosine residues for Bax, Bcl-xL and Bak that received a score higher than 0.9. Because I found that all of these Bcl-2 family members contain residues
Table I - Prediction of potential phosphorylation for Bax, Bcl-xL, Bad and Bak

<table>
<thead>
<tr>
<th>Protein</th>
<th>Pos</th>
<th>Context</th>
<th>Score</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bak</td>
<td>91</td>
<td>RRYDSEFQT</td>
<td>0.950</td>
<td><em>S</em></td>
</tr>
<tr>
<td>Bak</td>
<td>117</td>
<td>KIATSLFES</td>
<td>0.988</td>
<td><em>S</em></td>
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<tr>
<td>Tyrosine predictions</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Protein</td>
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<td>Context</td>
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<td>AENAYEYFT</td>
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<td><em>Y</em></td>
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<td>Serine predictions</td>
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<tr>
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<td>Context</td>
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<tr>
<td>Bad</td>
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<td>QEDASATDR</td>
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<td>RSRHSSYPAP</td>
<td>0.997</td>
<td><em>S</em></td>
</tr>
<tr>
<td>Bad</td>
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<td>SRHSSYPAG</td>
<td>0.986</td>
<td><em>S</em></td>
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<td>Pos</td>
<td>Context</td>
<td>Score</td>
<td>Prediction</td>
</tr>
<tr>
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<td>FVDLYGNNA</td>
<td>0.954</td>
<td><em>Y</em></td>
</tr>
<tr>
<td>Serine predictions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Protein</td>
<td>Pos</td>
<td>Context</td>
<td>Score</td>
<td>Prediction</td>
</tr>
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<td><em>S</em></td>
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<tr>
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<td>0.919</td>
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<td>Bax</td>
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<td>VDTDSREV</td>
<td>0.996</td>
<td><em>S</em></td>
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with probability scores higher than 0.9, I hypothesized that some of these sites were phosphorylated in whole cells.

To determine if these Bcl-2 family members were regulated post-translationally by phosphorylation in response to cytokine stimulation, it was necessary to first confirm the identity of pro- and anti-apoptotic specific bands present on a SDS-PAGE gel. To address this matter, Bcl-x<sub>L</sub>, Bak and Bax proteins were immunoprecipitated from unstimulated murine MC/9 cells using anti-Bcl-x<sub>L</sub> (Transduction Laboratories), anti-Bak (Transduction Laboratories) and anti-Bax (Upstate Biotechnology) antibodies respectively. Following immunoprecipitation, the samples were fractionated on SDS-PAGE gels, transferred to nitrocellulose membrane, and subsequently immunoblotted with anti-Bcl-x<sub>L</sub> (Santa Cruz), anti-Bak (Upstate Biotechnologies) and anti-Bax (Santa Cruz) polyclonal antibodies. In the reverse experiment, the various Bcl-2 family members were immunoprecipitated using the Santa Cruz antibodies and immunoblotted with anti-Bcl-x<sub>L</sub> (Transduction Laboratories), anti-Bak (Transduction Laboratories) and anti-Bax (Upstate Biotechnology) antibodies. Figures 3.1 and 3.2 verified the presence and apparent molecular mass of Bax and Bak in MC/9 cells.

Next, the Bcl-2 homologues were immunoprecipitated from <sup>32</sup>P-orthophosphate-labeled MC/9 cells lysates stimulated with GM-CSF. Results from these studies (shown in Figure 3.3, 3.4 and 3.5) revealed that Bcl-x<sub>L</sub>, Bak, and Bax were not phosphorylated in response to GM-CSF stimulation or withdrawal. Our lab has previously demonstrated in vivo phosphorylation of the pro-apoptotic protein Bad (Scheid and Duronio, 1998); therefore, to serve as a positive control, Bad was immunoprecipitated and its
Figure 3.1. The pro-apoptotic protein Bax is expressed in MC/9 cells. A. MC/9 cells (5 x 10^6) were lysed with solubilization buffer and Bax was immunoprecipitated and blotted with anti-Bax antibodies from Upstate Biotechnologies (UBI) or Santa Cruz (SC). B. Same as in A with varying amounts of cell lysate and additional controls shown. Cell lysates and immunocomplexes were resolved on an 12% polyacrylamide gel, transferred to nitrocellulose and probed with anti-Bax antibodies.
Figure 3.2. The pro-apoptotic protein Bak is present in MC/9 cells. MC/9 cells (1 x 10^7) were lysed with solubilization buffer and Bak was immunoprecipitated with anti-Bak antibody (Transduction Laboratories). Cell lysates were resolved on an 10% polyacrylamide gel, transferred to nitrocellulose and probed with anti-Bak antibody (Transduction Laboratories). Proteins were detected with ECL (Amersham).
Figure 3.3. Bak is not phosphorylated in response to GM-CSF stimulation in MC/9 cells. MC/9 cells were starved of cytokine for 8 h and then metabolically labeled with $^{32}$P-orthophosphate for 2 h, followed by stimulation with GM-CSF (50 ng/ml) for the indicated times. Bak was immunoprecipitated and fractionated by SDS-PAGE. $^{32}$P-labelled proteins were detected by autoradiography. This experiment was performed in duplicate with one set of samples shown. B. The membrane was probed with anti-Bak antibody (Transduction Laboratories).
Figure 3.4. **Bcl-x\textsubscript{L} is not phosphorylated in MC/9 cells in response to GM-CSF stimulation.** MC/9 cells were starved of cytokine for 8 h and then metabolically labelled with \textsuperscript{32}P-orthophosphate for 2 h, followed by stimulation with GM-CSF for the indicated times. Bcl-x\textsubscript{L} was immunoprecipitated and fractionated by SDS-PAGE. \textsuperscript{32}P-labelled proteins were detected by autoradiography. The membrane was probed with anti-Bcl-x\textsubscript{L} antibody (Transduction Laboratories). Arrow indicates position of Bcl-x\textsubscript{L}. * indicates position of putative Bad protein.
Figure 3.5. Bcl-xL coimmunoprecipitates with Bad.  

**A.** MC/9 cells were starved of cytokine for 8 h and then metabolically labelled with $^{32}$P-orthophosphate for 2 h, followed by stimulation with GM-CSF for the indicated times. Bcl-xL, Bak, Bax, and Bad were immunoprecipitated and fractionated by SDS-PAGE. $^{32}$P-labelled proteins were detected by autoradiography. This experiment was performed in duplicate with one set of samples shown.  

**B.** The membrane was probed with anti-Bad antibody sc-943 (Santa Cruz Biotechnology).
phosphorylation state monitored along side Bcl-x<sub>L</sub>, Bak, and Bax. GM-CSF could promote a significant increase in Bad phosphorylation, consistent with this earlier work. Serendipitously, these studies led to the finding that Bcl-x<sub>L</sub> coimmunoprecipitates with Bad protein in unstimulated and GM-CSF-stimulated MC/9 cells (Figure 3.5). Cytokines have previously been shown to regulate Bcl-x<sub>L</sub>-Bad association in Bcl-x<sub>L</sub>-Bad transfected cells (Zha et al., 1996). In addition to confirming this earlier work, this study was the first to demonstrate Bcl-x<sub>L</sub>-Bad association in non-transfected cells.

In addition to phosphorylation, cytokine-stimulated signaling pathways may regulate the apoptosis threshold by increasing or decreasing Bcl-2 family member protein levels, and this became a focus for my work. Initially, the mRNA levels of various Bcl-2 family members were examined in the same factor-dependent cell line used for the phosphorylation experiments, MC/9. MC/9 cells were starved of cytokine for 8 h and then resuspended in medium containing 0.5% FBS and GM-CSF for 16 h. Total RNA was isolated from the cells using standard protocols, and is shown in Figure 3.6. Multi-probe RNase protection assays were performed using the Riboquant (Pharmingen) mAPO-2 template set as described in Material and Methods section 2.1.4. As shown in Figure 3.7, mRNAs corresponding to the pro-survival proteins Bcl-2, Bcl-w, and Bcl-x<sub>L</sub> and mRNAs corresponding to the pro-apoptotic proteins Bax and Bak were present in MC/9 cells. GM-CSF stimulation caused an increase in Bcl-x<sub>L</sub> mRNA in a time-dependent fashion, while it had no effect on the expression of the other Bcl-2 family members tested. I next wanted to see if protein expression levels matched the changes seen in mRNA in response to GM-CSF. For this experiment, MC/9 cells were starved of
Figure 3.6. Total RNA isolated from MC/9 cells. Total RNA was isolated from MC/9 cells using TRIZOL Reagent (Candian Life Technologies) according to the manufacture’s specifications. A small portion of each sample was resolved on a 1% agarose gel to determine the integrity of isolated RNA.
Figure 3.7. In MC/9 cells Bcl-xL mRNA levels increase in response to GM-CSF stimulation. MC/9 cells were plated in low-serum (0.5%) medium with and without GM-CSF. At 16 h, total RNA was isolated and analyzed by RNase protection assays using the mAPO-2 multiprobe template set (Pharmingen). Changes in the mRNA levels induced by 16 h with GM-CSF for Bad, Bax, Bak, Bcl-xL and Bcl-w were plotted relative to unstimulated mRNA levels (set at 1). Radioactivity was quantitated by a Molecular Imager (Bio-Rad). To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping gene transcript, L32. The data represent the average of triplicate determinations with error bars representing standard deviation.
cytokine and restimulated with GM-CSF or IL-3 for 16 h, and the lysates probed with antibodies specific to mouse Bak, Bax, and Bcl-x\textsubscript{L} proteins. Interestingly, Figure 3.8 revealed that Bcl-x\textsubscript{L} protein levels remained unchanged in response to cytokine stimulation, even though GM-CSF caused an increase in Bcl-x\textsubscript{L} mRNA over the same time course.

Studies performed in a human model system may provide a greater understanding of the molecular events underlying the regulation of Bcl-2 family members and may grant insight into the role of these proteins in human cellular responses to growth and survival signals. Therefore, the regulation of Bcl-2 family members in response to cytokine stimulation was examined in the human erythroleukemia cell line TF-1. Similar to murine MC/9 cells, TF-1 cells are dependent upon exogenous cytokine for growth and survival. TF-1 cells were suspended in low-serum medium containing human GM-CSF for various times. Total RNA was isolated from the cells and multi-probe RNase protection assays were performed using the Riboquant hAPO-2 template set. The results in Figure 3.9 revealed that GM-CSF caused an increase in Mcl-1 and Bcl-x\textsubscript{L} mRNA levels in a time-dependent fashion. In contrast, mRNA species encoding other Bcl-2-related proteins did not change significantly, nor did the expression of the housekeeping gene L32. This experiment was repeated numerous times, and the relative increases in Bcl-x\textsubscript{L} and Mcl-1 mRNA are shown in Figure 3.9.

The regulation of Mcl-1 and Bcl-x\textsubscript{L} protein levels in response to GM-CSF stimulation in TF-1 cells was also examined. Similar to the effects on mRNA levels, GM-CSF caused a dramatic increase in Mcl-1 protein levels, suggesting that GM-CSF does indeed regulate Mcl-1 at the transcriptional level. In accordance with my findings
Figure 3.8. Protein levels of Bak, Bax, and Bcl-xL do not change in response to GM-CSF or IL-3 stimulation. MC/9 cells were plated in low-serum (0.5%) medium containing IL-3 or GM-CSF. At 16 h, MC/9 cells (2 x 10⁶) were lysed in solubilization buffer (see Material and Methods) and equal amounts (50 μg) of their protein lysates were analyzed by immunoblotting for the expression of various Bcl-2 family members.
Figure 3.9. GM-CSF treatment increases Mcl-1 and Bcl-xL mRNA levels. TF-1 cells were deprived of cytokine and plated in low-serum (0.5% v/v) medium for 8 h followed by restimulation with GM-CSF for 0, 2, and 4 h. Total RNA was isolated and analyzed by RNase protection assays using the hAPO-2 multiprobe template set (Pharmingen). Radioactivity was quantitated by a Molecular Imager (Bio-Rad). To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping gene transcript, L32. The data represent the average of triplicate determinations with error bars representing standard deviation.
Figure 3.10. Analysis of Bcl-xL, Bax and Mcl-1 protein levels following stimulation with GM-CSF. TF-1 cells were starved of cytokine for 16 h and then stimulated with GM-CSF for the indicated times. Equal amounts of detergent-solubilized lysates were fractionated by SDS-PAGE and immunoblotted with anti-Mcl-1, anti-Bcl-xL and anti-Bax antibodies.
in MC/9 cells, there was no significant change in the level of Bcl-x\textsubscript{L} protein or in the level of other Bcl-2 family members tested (Figure 3.10).

I also wanted to examine the expression profile of Bcl-2 family mRNA in primary human cells, to compare with the data generated from TF-1 cells. Eosinophils and basophils play a major role in the pathogenesis of allergic diseases such as asthma (Review by Walsh, 1997; Holgate et al., 2000). Enhanced survival of basophils and eosinophils in airway tissues during allergic responses is one of the hallmarks of these disease processes. The severity of inflammation may be a result of defects in pathways controlling eosinophil and basophil apoptosis.

To gain a better understanding of the mechanisms underlying primary human eosinophil and basophil apoptosis, I began a collaborative study with Dr. R. Schellenberg of St. Paul's Hospital. Expression of Bcl-2 family members in both primary human eosinophils and basophils was examined. RNase protection analysis revealed that mRNAs corresponding to the pro-survival proteins, Bcl-2, Bcl-x\textsubscript{L}, Bfl-1 and Mcl-1 and mRNAs corresponding to the anti-survival proteins Bak and Bax were present in eosinophils (Figure 3.11). Basophils expressed Mcl-1 and Bcl-x\textsubscript{L/S} mRNAs. Interestingly, Mcl-1 mRNA level was five-fold higher in eosinophils compared to basophils and Bcl-x\textsubscript{L} mRNA level was six-fold higher in basophils compared to eosinophils. These findings revealed that human eosinophils have a specific profile of Bcl-2 family mRNA expression distinct from that of basophils. These observations, along with a greater understanding of the molecular mechanisms that contribute
Figure 3.11. Mcl-1 message level is higher in eosinophils whereas Bcl-xL message level is higher in basophils. A. Total RNA was isolated from primary human eosinophils and basophils and analyzed by RNase protection assays using the hAPO-2 multiprobe template set (Pharmingen). Eosinophils also expressed Bfl-1 mRNA.
to eosinophil and basophil apoptosis may provide an opportunity for therapeutic intervention during allergic reactions. Future studies will compare the responsiveness of various Bcl-2 family members at both the mRNA and protein level to cytokines and chemokines, as well as the potential role of the PI3K pathway in both chemotaxis and survival.

3.3 DISCUSSION

The mechanisms by which cytokines function to promote cell survival are not fully understood. Several research studies suggest that cytokines may inhibit components of the apoptotic machinery, such as the pro-apoptotic protein Bad (Datta et al., 1997a; Blume-Jensen et al., 1998) or caspase-9 (Cardone et al., 1996) to protect cells from apoptosis. Other studies suggest that cytokines can activate pro-survival factors, like the Bcl-2 family members Bcl-2 and Bcl-x\textsubscript{L}, to suppress apoptosis. The results in this chapter have compared the expression of Bcl-2 family members in a number of hemopoietic model systems and demonstrate the selective regulation of the pro-survival factors Bcl-x\textsubscript{L} and Mcl-1 by GM-CSF-stimulated signal transduction pathways.

Work by Sevilla and coworkers (1999) identified Bcl-x\textsubscript{L} as a potential target of the Ets2 transcription factor. The Sevilla group demonstrated cytokine-induced Ets2 transactivation of the Bcl-x\textsubscript{L} promoter in colony-stimulating factor-1 dependent macrophages. Moreover, constitutive expression of Ets2 protects these cells from cytokine-withdrawal-induced cell death, suggesting a putative role for Ets2 in Bcl-x\textsubscript{L}-dependent cell survival. Finally, work by McCarthy (1997) demonstrated ERK-
dependent activation of Ets2. A potential role for the Ras-Raf-MEK-ERK signaling pathway in GM-CSF-induced Bcl-x{sub}L upregulation will be discussed further in Chapter 4.

The data in this chapter shows GM-CSF-induced increase in Mcl-1 mRNA. Mcl-1 protein levels were also upregulated in response to GM-CSF stimulation, providing a possible mechanism whereby GM-CSF may promote survival in hemopoietic cells. GM-CSF stimulates both the PI3K-PKB and the Ras-ERK pathways. In Chapter 4 the question of whether either of these pathways controls GM-CSF-induced upregulation of Mcl-1 in hemopoietic cells will be addressed in greater detail.

Our lab (Scheid and Duronio, 1999) and others (Datta et al., 1997a; Blume-Jensen et al., 1998) have previously demonstrated cytokine-induced phosphorylation of the pro-apoptotic protein Bad. In my work, I demonstrated that the Bcl-2 family members Bak, Bax, and Bcl-x{sub}L were not phosphorylated in response to cytokine stimulation, suggesting that the activities of these proteins may be regulated by expression levels, subcellular localization (Puthalakath et al. 1999; Wolter et al., 1997; Xiang et al., 1996) and/or proteolytic cleavage (Gross et al., 1999; Wood and Newcomb, 2000; Clem et al., 1998; Kirsch et al., 1999).

The results in this Chapter also show that Bcl-x{sub}L interacts with both phosphorylated and unphosphorylated forms of Bad in GM-CSF-stimulated MC/9 cells. This finding led to other work in our laboratory showing that Bad is phosphorylated on Ser{sup 112} by a MEK-dependent pathway, whereas phosphorylation of two other residues occurs in a MEK-independent manner (Scheid et al., 1999). Ser{sup 112} phosphorylation was found to be required for dissociation of Bad from Bcl-x{sub}L. These results demonstrated for the first time the involvement of a Ras-controlled signaling pathway leading to the
phosphorylation and inactivation of a pro-apoptotic Bcl-2 family member in mammalian cells (Scheid et al., 1999).

Finally, I showed differences in the mRNA expression of Bcl-2 family members between primary human eosinophils and basophils. This is a significant finding as eosinophils and basophils have been shown to play a role in the pathogenesis of asthma (Holgate et al., 2000). Further examination of the potential differences in the protein levels of Bcl-2 family members in eosinophils versus basophils may lead to the identification of critical survival pathways operating in these cells and may provide novel therapeutic targets for the treatment of asthma.
4. DISTINCT ROLES FOR CERAMIDE, ERK AND PI3K IN MCL-1 REGULATION

4.1 RATIONALE AND HYPOTHESIS

In hemopoietic cells, GM-CSF exerts its anti-apoptotic effects by activating multiple cellular signaling pathways including the PI3K cell survival pathway and the Ras-Raf-MEK-ERK kinase cascade. In contrast, the apoptotic lipid second messenger ceramide may promote apoptosis by inhibiting components of these pathways. One pro-survival factor known to be a target of GM-CSF stimulation (Chapter 3) is the Bcl-2 pro-survival factor Mcl-1. Thus, my working hypothesis is that PI3K- and ERK-dependent pathways may mediate GM-CSF-induced Mcl-1 upregulation.

4.2 RESULTS

To test this hypothesis, I began by examining the mRNA levels of Mcl-1 in cells stimulated with GM-CSF in the presence of the potent and specific MEK inhibitor U0126. As shown in Figure 4.1, blocking the ERK pathway significantly diminished GM-CSF-induced upregulation of Mcl-1 mRNA. Another inhibitor of MEK, PD98059 gave similar results (data not shown). These data revealed that MEK activation was an essential element of GM-CSF-induced Mcl-1 gene expression.

Bcl-xL message also increased in response to GM-CSF stimulation, so I asked whether MEK was involved in this upregulation. Similar to the effects seen with Mcl-1 message, Bcl-xL message was downregulated by U0126, suggesting the involvement of MEK (Figure 4.1). Together, these results suggested that a MEK-controlled pathway regulated Bcl-xL transcription.
Figure 4.1. MEK activity is required for GM-CSF induced upregulation of Mcl-mRNA. TF-1 cells were starved of cytokine and cultured overnight in low serum medium (0.5% v/v). Cells were then restimulated with GM-CSF (lane 1, GM-CSF alone) for 2 h in the presence or absence of LY-294002 (50 μM; lane 2), C6-ceramide (50 μM; lane 3) or the MEK inhibitor U0126 (25 μM; lane 4). Total RNA was isolated and analyzed by RNase protection assays using hAPO-2 multiprobe template set (Pharmingen). Radioactivity was quantitated by a Molecular Imager (Bio-Rad). To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping gene transcript, L32. Data for the unstimulated, GM-CSF alone and the LY-294002-treated samples are the average of triplicate determinations and error bars indicate standard deviation. The C6-ceramide and U0126-treated samples are the average of duplicate determinations and the error bars indicate the range.
Our lab (Scheid and Duronio, 1995) and others (Yao and Cooper 1995; Alessi et al., 1997; Songyang et al., 1997) showed that PI3K and its downstream effectors PDK1 and PKB constitute a major element of cytokine-mediated cell survival in various cell types. Using RNase protection assays, I asked whether there was a dependence upon PI3K for the expression or stability of Mcl-1 mRNA. In contrast to the effects of inhibiting MEK, inhibition of PI3K with two potent and specific pharmacological inhibitors, LY-294002 and wortmannin (not shown), had no effect on GM-CSF-induced upregulation of Mcl-1 mRNA levels (Figure 4.1). These observations suggested that either the expression or the stability of Mcl-1 mRNA was controlled by a MEK-dependent pathway and not by a PI3K-controlled pathway.

I next examined the regulation of Mcl-1 protein by PI3K- and ERK-dependent pathways. TF-1 cells were starved of cytokine and incubated for 8 h in low-serum media. Cells were then restimulated with GM-CSF for 0, 2, and 4 h in the presence or absence of U0126 or PD-98059. The level of Mcl-1 protein was then determined by immunoblot analysis. As anticipated, MEK inhibition completely abolished GM-CSF-induced increase of Mcl-1 protein, which is consistent with reduced levels of Mcl-1 mRNA under these conditions (Figure 4.2). Interestingly, this effect was mirrored in cells treated with LY-294002 (Figure 4.2) or wortmannin (not shown). This suggested that pathways activated by GM-CSF that increase Mcl-1 protein levels consist of elements controlled by both PI3K and MEK. Since inhibition of PI3K had no effect on the level of Mcl-1 mRNA, these results suggested that PI3K was controlling an aspect of Mcl-1 protein translation or stability. In contrast to Mcl-1, there was no significant change in the level
Figure 4.2. PI3K activity and MEK activity are required for GM-CSF-induced Mcl-1 upregulation. TF-1 cells were deprived of cytokine, plated in low-serum medium for 8 h and restimulated with GM-CSF in the presence or absence of LY-294002 (50 μM) or the MEK inhibitor U0126 (25 μM). Cells were harvested after 0, 2 and 4 h and equal amounts of protein lysates were analyzed by immunoblotting for the expression of Mcl-1. The same membrane was subsequently reprobed with antibodies specific to Bax, Bak, and Bcl-xL.
of Bcl-x_L protein or in the level of other Bcl-2 family members tested (Figure 4.2), suggesting that PI3K and MEK were acting specifically on Mcl-1 regulation.

To elucidate the role of PI3K in the regulation of Mcl-1 protein levels, I performed pulse-chase experiments. In these experiments, cells grown in the presence of GM-CSF were metabolically labeled with [35S]-methionine, followed by addition of cold methionine for various lengths of time. The half-life of Mcl-1 was determined to be approximately 40 minutes (Figure 4.3), consistent with the half-life previously reported by Chao and coworkers (1998). In two independent experiments, I consistently observed that PI3K-inhibition had no effect on the half-life of Mcl-1. This suggested that the PI3K-dependent upregulation of Mcl-1 was not at the level of protein stability or protein degradation. To further investigate the mechanism by which PI3K was regulating Mcl-1 protein levels, translation studies were performed in which cells were metabolically labeled with [35S]-methionine for 1, 3, and 4 h in the presence or absence of LY-294002 (Figure 4.4). Taken together, these results suggested that GM-CSF-induced Mcl-1 upregulation was controlled at the transcriptional level by a MEK-dependent pathway and at the translational level by a PI3K-controlled pathway.

Research by several groups suggests that PI3K may activate p70^s6k in response to cytokine stimulation resulting in the initiation of protein synthesis (Chung et al., 1994; Valius and Kazlauskas, 1993). To determine if PI3K was functioning via p70^s6k-dependent pathway to regulate Mcl-1 protein synthesis in response to GM-CSF stimulation, TF-1 cells were stimulated with GM-CSF in the presence and absence of the potent and specific p70^s6k inhibitor, rapamycin. As shown in Figure 4.5, treatment with
Figure 4.3. Inhibition of PI3K activity has no effect on the half-life of Mcl-1. A. TF-1 cells were pulse-labelled with \[^{35}\text{S}]\text{Met}\) for 15 min and chased in low-serum growth medium containing methionine (10 mM) and GM-CSF in the presence or absence of LY-294002 (50 \(\mu\text{M}\)). At the indicated times after chase (min), whole cell lysates were prepared and Mcl-1 was immunoprecipitated. Mcl-1 was resolved on SDS-PAGE gel and visualized and quantitated by a Molecular Imager (Bio-Rad). The level of Mcl-1 at each time point was converted to the percentage of Mcl-1 level at the zero time point and plotted to determine the half-lives. This experiment was performed in duplicate with similar results. One set of samples is shown.
Figure 4.4. Regulation of Mcl-1 protein synthesis by PI3K- and ceramide-dependent pathways. TF-1 cells were placed in medium containing $[^{35}\text{S}]$-methionine and then stimulated with GM-CSF alone or GM-CSF with LY-294002, C6-ceramide or U0126. After 1, 3, or 4 h Mcl-1 was immunoprecipitated, fractionated by SDS-PAGE and quantitated by a Molecular Imager (Bio-Rad).
rapamycin only partially inhibited Mcl-1 upregulation, thus pointing to other effectors of PI3K besides just p70^S6k.

To rule out the question of cross-talk between the Ras-ERK pathway and the PI3K pathway regulating Mcl-1 protein synthesis, TF-1 cells were stimulated with GM-CSF in the presence or absence of LY-294002 or U0126. Cells were then harvested and protein lysates were analyzed by immunoblotting for phospho-ERK. As expected, the MEK inhibitor U0126 completely blocked the appearance of phospho-ERK, while PI3K inhibition has no effect on ERK phosphorylation (Figure 4.6). Taken together these studies show that signals emerging from PI3K- and Ras-dependent pathways were functioning interdependently to regulate Mcl-1 synthesis in response to GM-CSF stimulation.

The generation of ceramide from the breakdown product of sphingomyelin is potentially an important second messenger in apoptosis signaling in a variety of cell types and may serve as a negative regulator of pro-survival pathways (Kolesnick, 1998). For example, ceramide has been shown to downregulate both the Ras-ERK pathway (Yu et al., 1997) and components of the PI3K-PKB pathway (Zundel and Garcia, 1998; Schubert et al., 2000). This led me to test whether ceramide could have an impact on Mcl-1 transcription or translation. Once again total RNA was isolated from cells stimulated with GM-CSF in the presence or absence of C_2- or C_6-ceramide and subjected to RNase protection assays. The results in Figure 4.1 showed that treatment with C_6-ceramide did not effect GM-CSF-induced upregulation of Mcl-1 mRNA, which is consistent with a lack of effect of C_6-ceramide on ERK phosphorylation (Figure 4.6). Identical results were obtained when cells were treated with C_2-ceramide (data not shown).
Figure 4.5.  p70S6K activity is partially required for Mcl-1 upregulation. TF-1 cells were deprived of cytokine (lane C), plated in low-serum medium for 8 h and restimulated with GM-CSF in the presence or absence of the PI3K inhibitor LY-294002 (50 μM, 25 μM or 10 μM) or in the presence or absence of the p70S6K inhibitor rapamycin (100 ng/ml, 50 ng/ml, or 25 ng/ml). Cells were harvested 6 h after restimulation and equal amounts of protein lysates were analyzed by immunoblotting for the expression of Mcl-1.
Figure 4.6. Inhibition of PKB and MAPK by various inhibitors. TF-1 cells were starved of cytokine for 8 h followed by a 15 min pre-incubation with LY-294002 (50 μM), C₆-ceramide (25 μM) or U0126 (25 μM) where indicated, followed by a 10 min stimulation with GM-CSF. Cells were harvested and equal amounts of protein lysates were analyzed by immunoblotting for phospho-ERK (top panel) or phospho-PKB (middle and lower panels).
To explore the possibility that ceramide may promote apoptosis via down-regulation of PI3K-dependent Mcl-1 protein synthesis, cells were stimulated with GM-CSF in the presence or absence of C₂- or C₆-ceramide. As shown in Figure 4.7 the addition of C₆-ceramide to TF-1 cells blocked GM-CSF induced Mcl-1 protein upregulation, which paralleled the effects of inhibiting PI3K activity. Further, pulse-chase analysis (Figure 4.8) and translation studies confirmed that ceramide treatment inhibited GM-CSF-induced upregulation of Mcl-1 at the level of translation (Figure 4.4). Together the data suggests that reduced Mcl-1 protein levels caused by ceramide-inhibition of a PI3K-dependent signaling pathway may be a contributing factor in ceramide-mediated cell death.

Use of SHIP-/- Mast Cells in Regulation of Bcl-2 Family Proteins

Our lab has been collaborating with Dr. G. Krystal’s group in investigating the regulation of phosphoinositides and PKB by SHIP (manuscript submitted). SHIP is an SH2-containing phosphatase that dephosphorylates PI(3,4,5)P₃ at the 5’ position to produce PI(3,4)P₂ and is the major 5’ phosphatase in hemopoietic cells. SHIP loss-of-function transgenic mice display impaired apoptosis and have excessive myeloid cell survival (Helgason et al., 1998; Liu et al., 1998). One reason for this may be that SHIP negatively regulates PI3K function, by reducing the amount of PI(3,4,5)P₃ generated by PI3K. In the previous sections I showed that inhibition of PI3K downregulated Bcl-xₐ mRNA, but without any effect on Bcl-xₐ protein. I wanted to use the SHIP-/- system to test if upregulation of PI3K-derived lipids would lead to increased Bcl-2 family member expression. To address the question of whether SHIP is an important component of Bcl-2
Figure 4.7. C₆-ceramide inhibits GM-CSF induced increase in Mcl-1 protein levels. A. TF-1 cells were deprived of GM-CSF, placed in low-serum (0.5%) medium for 16 hours and restimulated with GM-CSF in the presence or absence of C₆-ceramide (25 μM) or LY-294002 (50 μM). 4 hours after restimulation, cells were solubilized and equal amounts of protein lysates were analyzed by immunoblotting for the expression of Mcl-1 and Bak. B. Similar to A, except that LY-294002 and C₆-ceramide were used at the indicated concentrations and Mcl-1, Bax and Bcl-xₐ were immunoblotted.
Figure 4.8. The message levels of various Bcl-2 family members tested are present in equal amounts in SHIP+/+ and SHIP -/- BMMCs. A. Total mRNA from SHIP+/+ and SHIP -/- BMMCs was isolated and analyzed by RNase protection assays using the mAPO-2 multiprobe template set (Parmingen). B. Radioactivity was quantitated by a Molecular Imager (Bio-Rad), and Bcl-xl mRNA was normalized against the level of the housekeeping gene transcript, L32. Error bars represent the range of duplicate experiments.
family member regulation in bone marrow mast cells (BMMC), the mRNA and protein levels of Bcl-2 family members from SHIP$^{-/-}$ and SHIP$^{+/+}$ BMMC were examined. The data in Figure 4.8 revealed that mRNAs corresponding to Bad, Bak, Bax, Bcl-2, Bcl-xL, and Bcl-w were present in approximately equal amounts in wild type and SHIP knock out BMMC. Surprisingly, Western blot analysis (Figure 4.9) showed that Bcl-xL protein levels were greatly elevated in SHIP$^{-/-}$ compared to SHIP$^{+/+}$, suggesting that SHIP may negatively regulate the expression of Bcl-xL protein.

4.3 DISCUSSION

These studies revealed that a SHIP dependent pathway negatively regulates Bcl-xL levels in hemopoietic cells. It will be interesting to determine if SHIP is involved in a feedback regulation via a PI3K pathway to reduce Bcl-xL protein levels in BMMC. Other work in our laboratory (manuscript submitted) has shown that SHIP$^{-/-}$ BMMC have elevated PKB activity, possibly due to an increase in basal levels of PI(3,4,5)P$_3$, suggesting that an increase in PKB activation may lead to the observed increase in Bcl-xL expression. These results are consistent with recent studies showing that transgenic mice expressing gag-PKB (a constitutively active form) have greatly elevated Bcl-xL protein levels in the T-cell compartment (Jones et al., 2000).

Future studies will involve pulse-chase experiments to determine if the increased levels of Bcl-xL in SHIP$^{-/-}$ cells are due to an increase in the half-life of Bcl-xL or due to an increase in protein translation. In addition, SHIP$^{-/-}$ BMMC will be treated with PI3K inhibitors to look at the role of PI3K-PKB signaling pathway in the regulation of Bcl-xL protein. Finally, experiments will be performed to look at the level of Bcl-xL protein in
Figure 4.9. Bcl-x<sub>L</sub> protein levels are elevated in SHIP knockout cells. SHIP <br/>++/+ and SHIP −/- BMMCs were harvested and equal amounts of their protein lysates were <br/>analyzed by immunoblotting for the expression of Bcl-x<sub>L</sub> and other proteins as indicated <br/>in the figure. This experiment was performed in triplicate with one set of samples <br/>shown.
MC/9 cells overexpressing the conditionally active version of PKB, p110*:ER\(^\text{Tm}\). Work by Kohn and coworkers (1998) revealed that activation of p110*:ER\(^\text{Tm}\) is sufficient to induce the phosphorylation of PHAS-1 (phosphorylated heat- and acid-stable protein), a key step in the initiation of protein synthesis. It is anticipated that MC/9 p110*:ER\(^\text{Tm}\) transformants will express higher levels of Bcl-x\(_L\) protein in the presence of 4-OHT than in its absence, providing a possible mechanism by which Bcl-x\(_L\) levels may be regulated in hemopoietic cells.

GM-CSF induced signaling events trigger activation of both the Ras-ERK pathway and the PI3K cell survival pathway. The Ras-MEK-ERK pathway is involved in the transduction of anti-apoptotic signals in a variety of hemopoietic cells and couples survival stimulus initiating at the cell surface to transcription factors controlling gene expression. Our group has recently demonstrated that phosphorylation of the pro-apoptotic protein Bad at Ser\(^{112}\) is mediated by the MEK-ERK pathway and this MEK-dependent phosphorylation of Bad may be a contributing factor in the ability of cytokines to promote cell survival (Scheid et al. 1999). This present study suggests that the Ras-MEK-ERK pathway also serves a role in the transcriptional activation of the pro-survival factor Mcl-1, suggesting that in hemopoietic cells cytokines may suppress apoptosis by inducing de novo gene expression of death antagonists as well as the post-translational modification and inactivation of pro-apoptotic proteins. The Mcl-1 promoter contains a cAMP-response element (CRE-2) site and may be a target of a transcription factor complex containing the cAMP-response element-binding protein (CREB; Wang et al., 1999). Several studies have shown the ERK pathway leading to CREB transactivation activity (Review by McCubrey et al., 2000). Therefore, in TF-1 cells, Ras signaling to
CREB may take place through the Ras-Raf-MEK-ERK pathway resulting in an increase in *de novo* Mcl-1 gene expression.

Unlike inhibiting MEK, blocking PI3K did not alter the upregulation of Mcl-1 mRNA following stimulation with GM-CSF. However, inactivating PI3K did block the increase in Mcl-1 protein synthesis induced by GM-CSF and this effect was not due to an increase in Mcl-1 degradation or alterations in Mcl-1 protein stability. Thus, GM-CSF-induced Mcl-1 upregulation was controlled at the translational level by a PI3K-controlled pathway. My findings are contradictory to the work of Wang and colleagues (1999) who showed that PI3K regulates Mcl-1 gene expression through the activation of an immediate early transcription factor. In their study Wang and coworkers demonstrated that PI3K inhibitors or dominant negative PI3K mutants block the transcriptional upregulation of Mcl-1 mRNA. One emerging factor that may contribute to the discrepancies observed between the two studies involves cross talk between the PI3K and ERK pathways. For example, the Wang group performed most of their work in the IL-3 dependent pro-B cell line BAF/3. In these cells, blocking with a dominant negative p85 inhibits activation of ERK (Craddock, 1999). Furthermore, overexpression of dominant negative p85 was shown to block IL3-induced ERK activation in FDC-P1 cells (Sutor, 1999), but we have shown that inhibition of PI3K has no effect on IL-3-induced ERK activation in MC/9 cells (Scheid, 1996) as well as in FDC-P1 cells (D. Chiu and V.D., in preparation). Since the studies by Wang and coworkers did not address the effects of blocking PI3K on ERK activation, it is possible that the effects they see on Mcl-1 mRNA transcription are manifested through a reduction in ERK signaling. In the TF-1 cell line, blocking PI3K had no effect on ERK activation (Figure 4.6). Inhibiting PI3K had no
effect on Mcl-1 mRNA levels, however I could abolish Mcl-1 mRNA by inhibiting MEK. Thus, the method of inhibiting PI3K, and the corresponding effects on ERK activity must be considered prior to drawing conclusions about the specific role of PI3K.

A wide variety of physiological signals, such as those generated by cytokines and growth factors, can activate the sphingomyelin pathway resulting in the production of the lipid second messenger ceramide. My studies suggest that ceramide may decrease anti-apoptotic signaling in cells by inhibiting PI3K-mediated Mcl-1 upregulation. Reports by several groups have identified PKB as a downstream target of ceramide-mediated apoptosis (Zhou, 1998; Schubert, 2000). Recent work from our laboratory showed that PKB phosphorylation at an activating site, Ser$^{473}$, may be a target of ceramide-activated protein phosphatases (Schubert et al., 2000). Thus my current study suggests that the mechanism by which ceramide may function to inhibit GM-CSF-induced PI3K-dependent increase in Mcl-1 protein is through the inactivation of PKB. An involvement of PKB in the PI3K-dependent regulation of Mcl-1 protein appears probable, since PKB was shown recently to phosphorylate components of the translational machinery. For example, 4E-BP1 phosphorylation by PKB inactivates the function of 4E-BP1 to repress mRNA translation (Takata et al., 1999; Gingras et al., 1998; Dufner et al., 1999). In addition, PI3K, PDK1 and PKB may have multiple inputs on p70$^{66k}$. I have shown here that the effects of rapamycin (and thus complete p70$^{66k}$ inhibition) on Mcl-1 protein upregulation were not as dramatic as blocking PI3K (see Figure 4.4), thus pointing to other effectors of PI3K besides just p70$^{66k}$.

In summary, through binding to its cognate receptor, GM-CSF can induce anti-apoptotic signaling via stimulation of the ERK and PI3K signal transduction cascades.
Several research findings suggest that the cytoprotective actions of these signaling pathways may converge on members of the Bcl-2 family, supporting the view that cytokines promote cell survival by increasing signaling of anti-apoptotic pathways as well as by decreasing signaling of pro-apoptotic pathways. My studies here demonstrated that ERK- and PI3K-dependent pathways were required for GM-CSF-induced upregulation of the Mcl-1 pro-survival factor. In addition, I provided evidence that ceramide-induced apoptosis could occur by inhibiting the translation of Mcl-1 protein. A further understanding of the molecular events underlying regulation of Bcl-2 family members may grant insight into the pathogenesis of disease and provide novel targets for therapeutic intervention.
5. EXAMINATION OF PROTEIN KINASE B (PKB) AS A POTENTIAL TARGET OF CERAMIDE ACTION

5.1 RATIONALE AND HYPOTHESIS

The second messenger ceramide (N-alkysphingosine) has been implicated in a host of cellular processes including growth arrest and apoptosis. Ceramide may augment the execution of apoptosis through the inactivation of pro-survival pathways. Recently, several reports supported this notion by demonstrating negative regulation of the PI3K-PKB signaling pathway by ceramide (Basu et al., 1998; Summers et al., 1998; Zundel et al., 1998; Zhou et al., 1998). Thus, ceramide may specifically inhibit PI3K, resulting in a loss of PKB activity or ceramide may negatively regulate PKB activation independently of PI3K.

5.2 RESULTS

To investigate the effects of ceramide on PKB activation by cytokines, I began by measuring PKB activity in vitro following exposure of cells to GM-CSF, ceramide, or the PI3K inhibitor LY-294002. In human erythroleukemic TF-1 cells, stimulation with GM-CSF resulted in a large increase in PKB activity after 1 or 3-minute treatment (Figure 5.1). Inhibition of PI3K by treatment of cells with LY-294002 almost entirely blocked the activation of PKB. Phosphorylation of ERK was unaffected by ceramide treatment (Figure 5.2) indicating that C6-ceramide was not reducing the signaling capacity of the GM-CSF receptor non-specifically.
Figure 5.1. C₆-ceramide attenuates PKB activation by GM-CSF. A. TF-1 cells were pretreated with C₆-ceramide (25 μM) or LY-294002 (50 μM) and then stimulated with GM-CSF for 1 min or 3 min. PKB activity was measured in vitro using a peptide substrate (Crosstide). A blank containing protein G-Sepharose was carried through the assay, and its activity (~5,000 cpm) was subtracted from each of the other assay points. B. The reaction products were resolved by SDS-PAGE and transferred onto nitrocellulose. The membrane was immunoblotted with α-PKB antibody to assess loading and to confirm the absence of PKB in the blank sample. Error bars represent the range of duplicate samples.
Figure 5.2. C₆-ceramide and LY-294002 do not affect MAPK phosphorylation. TF-1 cells were pretreated for 20 min with 25 μM C₆-ceramide (lane 1), 50 μM LY-294002 (lane 2), or 25 μM U0126 (lane 3) and the stimulated with 50 ng/ml GM-CSF for 5 min (lanes 1-4). Cells in lane 5 received no treatment. Cell lysates were normalized for equal protein concentration and probed with anti-phospho-ERK antibody.
In addition, a potent MEK inhibitor, U0126, completely abolished the phosphorylation of ERK in this experiment, whereas LY-294002 had no effect. Thus, C₆-ceramide specifically reduces the maximal activity of PKB achieved by stimulation of cells with GM-CSF, without affecting the parallel MEK-ERK pathway.

I next examined the effects of ceramide on PI3K activity in whole cells. TF-1 cells were metabolically labeled with ³²P-orthophosphate and then stimulated with GM-CSF in the presence or absence of C₆-ceramide. Lipids were extracted, deacylated and separated by anion exchange HPLC (Scheid and Duronio, 1998). Figure 5.3 showed the elution profiles corresponding to both glycerol-PI(3,4)P₂ and glycerol-PI(3,4,5)P₃. Stimulation with GM-CSF caused a significant increase in the amounts of both of these lipids above unstimulated cells. Pre-treatment of cells with C₆-ceramide did not affect the increase of either lipid species caused by GM-CSF. However, pre-treatment of cells with LY-294002 abolished the generation of both lipid species. The total radioactivity loaded onto the column was very similar between samples, indicating that the differences in radioactivity caused by GM-CSF treatment were due to changes in mass for each lipid. Thus, C₆-ceramide did not affect the de novo generation of PI(3,4,5)P₃ or PI(3,4)P₂ caused by GM-CSF stimulation. Therefore, the effects of C₆-ceramide on PKB were not because of reduced levels of PI3K-generated lipids.

PKB activation occurs by multiple phosphorylations on several residues (Alessi et al., 1996a). Thr³⁰⁸ is located in the activation loop of the kinase domain and phosphorylation by PDK1 is required for activation. Ser⁴⁷³ phosphorylation near the carboxy-terminal region of PKB further potentiates activity. I monitored these
Figure 5.3. C₆-ceramide does not affect PI3K-generated PI(3,4)P₂ or PI(3,4,5)P₃. TF-1 cells were metabolically labeled with ³²P for 2 h. Cells were either left unstimulated (closed circles) or stimulated with GM-CSF in the presence of ceramide (open circles), LY-294002 (closed triangles), or vehicle alone (closed squares) for 3 min, and the lipids were extracted as described under “Materials and Methods”. Deacylated lipids were fractionated by anion exchange chromatography and detected by liquid scintillation counting. Results are representative of two independent experiments.
phosphorylation events separately using phospho-specific antibodies. In the first experiment, cells were pretreated with C_{6}-ceramide or vehicle alone and then stimulated for 1, 3 or 5 minutes with GM-CSF. Phosphorylation of PKB at Ser^{473} was observed by 1 minute, maximal by 3 minutes, and began to decline by 5 minutes (Figure 5.4). In the presence of ceramide, the extent of Ser^{473} phosphorylation was greatly reduced. Thr^{308} phosphorylation was also examined. In contrast to Ser^{473}, Thr^{308} phosphorylation was not altered by ceramide treatment. Treatment of cells with dihydroceramide, an inactive ceramide analog, had no effect (Figure 5.4), consistent with the findings of others (Zhou et al., 1998; Summers et al., 1998). In a separate experiment, I wanted to confirm that Thr^{308} phosphorylation was occurring by a PI3K-controlled pathway. Cells were treated with either C_{6}-ceramide or LY-294002, and the lysates were probed with anti-phospho-Thr^{308} (Figure 5.5). Consistent with Figure 5.4 ceramide treatment had no effect. In contrast, LY-294002 completely abolished Thr^{308} phosphorylation. This confirmed that Thr^{308} phosphorylation was occurring by a PI3K-activated pathway. Finally, I asked whether ceramide could accelerate PKB Ser^{473} dephosphorylation, if added after GM-CSF stimulation (Figure 5.6). In this experiment cells were stimulated with GM-CSF for 30 seconds, washed, and then ceramide or vehicle was added. In this way I could distinguish whether ceramide was blocking Ser^{473} phosphorylation or if it was accelerating its dephosphorylation. Ceramide significantly reduced Ser^{473} phosphorylation in this set of experiments, most consistent with an acceleration in Ser^{473} dephosphorylation (Figure 5.6). This result suggested that the mechanism of Ser^{473} dephosphorylation by ceramide most likely involved a ceramide-activated protein phosphatase.
Figure 5.4. Ceramide specifically reduces PKB Ser\textsuperscript{473} phosphorylation. A. TF-1 cells were pretreated with C\textsubscript{6}-ceramide for 20 min and then stimulated with GM-CSF for 1, 3, or 5 min. PKB was immunoprecipitated and fractionated by SDS-PAGE. Ser\textsuperscript{473} or Thr\textsuperscript{308} phosphorylation was monitored by immunoblotting with anti-phospho-Ser\textsuperscript{473} or anti-phospho-Thr\textsuperscript{308} antibody. Total PKB was immunoblotted to confirm equal loading.

B. TF-1 cells were pretreated with C\textsubscript{2}-ceramide or C\textsubscript{2}-dihydroceramide for 20 min and then stimulated with GM-CSF for 5 min. Ser\textsuperscript{473} phosphorylation was monitored by immunoblotting with anti-phospho-Ser\textsuperscript{473} antibody.
Figure 5.5. PI3K inhibition, but not ceramide, block PKB Thr$^{308}$ phosphorylation. Cells were treated with C$_6$-ceramide, LY-294002 (both at 25 μM), or vehicle and stimulated with GM-CSF for the indicated times. Cell lysates were normalized based on protein concentrations, fractionated by SDS-PAGE and
Figure 5.6. Ceramide accelerates PKB Ser\textsuperscript{473} phosphorylation. Cells were stimulated with GM-CSF for 30 s, washed twice with RPMI 1640 and treated for 5 min with vehicle or C\textsubscript{6}-ceramide. Cell lysates were fractionated by SDS-PAGE and immunoblotted with anti-phospho-Ser\textsuperscript{473}. Total PKB was immunoblotted to confirm
The results of Figure 5.6 suggested a mechanism involving ceramide-activated protein phosphatase in the regulation of PKB dephosphorylation. To examine this possibility further, cells were co-treated with ceramide and okadaic acid, a PP2A phosphatase inhibitor previously reported to inhibit ceramide-activated phosphatases (Dobrowsky et al., 1993), followed by stimulation with GM-CSF. Figure 5.7 showed the activity of PKB in each of these conditions. Okadaic acid was ineffective in reversing the decrease in activity caused by ceramide. This was consistent with previous reports (Summers et al., 1998; Zhou et al., 1998), and at first suggested that ceramide was not affecting PKB through dephosphorylation. However, I noticed that cells treated only with okadaic acid and GM-CSF also had reduced PKB activity. This suggested that okadaic acid might interfere with the normal activation of PKB, which may be independent of the inhibitory actions of ceramide. Immunoblotting for Ser$^{473}$ phosphorylation demonstrated that okadaic acid fully restored the decreased phosphorylation of this residue caused by ceramide (Figure 5.8). Because okadaic acid was restoring Ser$^{473}$ phosphorylation, but could not restore kinase activity, I monitored the phosphorylation state of the other regulatory site, Thr$^{308}$. Surprisingly, okadaic acid, either in the presence or absence of ceramide, blocked Thr$^{308}$ phosphorylation (Figure 5.8). I then performed additional experiments to examine the effects of okadaic acid on PKB phosphorylation. As shown in Figure 5.9, okadaic acid did not affect the kinetics or extent of Ser$^{473}$ phosphorylation induced by GM-CSF. However, at all times examined Thr$^{308}$ phosphorylation was significantly reduced.

To confirm the involvement of a serine phosphatase in these processes, additional experiments were performed with a functionally distinct phosphatase inhibitor, calyculin
Figure 5.7. **PKB is a substrate for a ceramide-activated phosphatase.** Cells were pretreated with 1 μM okadaic acid (O.A.) for 20 min followed by C₆-ceramide or vehicle for 20 min. Cells were then stimulated with GM-CSF for 3 min. PKB was immunoprecipitated and activity was measured *in vitro* using Crosstide as a substrate. *Error bars* represent the range of duplicate samples.
**Figure 5.8.** PKB Ser$^{473}$ is a substrate for a ceramide-activated phosphatase. Immunoprecipitates from two separate kinase assays were fractionated by SDS-PAGE and probed for Ser$^{473}$ phosphorylation (designated Exp. 1 and 2). A portion of the immunoprecipitate was also probed for Thr$^{308}$ phosphorylation and for total PKB.
Figure 5.9. Okadaic acid inhibits Thr\textsuperscript{308} phosphorylation. Cells were treated with 1 μM okadaic acid (O.A.) or vehicle (Control) for 20 min followed by GM-CSF for the indicated times. PKB was immunoprecipitated, fractionated by SDS-PAGE and probed for Ser\textsuperscript{473} phosphorylation, Thr\textsuperscript{308} phosphorylation or total PKB.

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<th>Control</th>
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<td>1 3 5 7</td>
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A. Figure 5.10 showed that cells pretreated with ceramide and calyculin A restored Ser\(^{473}\) phosphorylation. In another experiment, calyculin A was compared with okadaic acid. Both inhibitors restored Ser\(^{473}\) phosphorylation equally during ceramide treatment (Figure 5.10). Also both are shown here to inhibit Thr\(^{308}\) phosphorylation, demonstrating that this effect was not unique to okadaic acid. As a control to show that these phosphatase inhibitors were not disrupting the generation of PI(3,4,5)P\(_3\) following GM-CSF stimulation, lipid analysis was also performed. GM-CSF-stimulated generation of PI(3,4,5)P\(_3\) remained unaffected. Together these results indicated that (1) the effects of ceramide on Ser\(^{473}\) phosphorylation were through the activation of a phosphatase, and (2) okadaic acid and calyculin A could inhibit an activity necessary for Thr\(^{308}\) phosphorylation.

5.3 DISCUSSION

Recently, three groups have studied the effects of ceramide on PI3K and PKB activation. Zundel and Giaccia (1998) provided evidence that PI3K is a direct target of ceramide, with its inhibition leading to decreased PKB activation. In contrast, Zhou and coworkers (1998) and Summers and coworkers (1998) provided evidence that ceramide does not affect PI3K activation, but rather inhibits PKB directly. In these three reports, PI3K activity was determined by immunoprecipitating PI3K (either directly or associated with tyrosine phosphorylated proteins) followed by \textit{in vitro} kinase assays using PI as a substrate. Since this method does not examine the \textit{de novo} accumulation of the PI3K reaction products, I felt this issue required further examination, given the importance of PI3K in many different signaling pathways and the potential importance of ceramide as inducer of apoptosis. Therefore, I measured the \textit{de novo} 3'-phosphoinositide production
Figure 5.10. Calyculin A restores ceramide inhibition of Ser\textsuperscript{473}. A. Cells were treated with 50 nM calyculin A or vehicle (ethanol) for 20 min followed by GM-CSF for 5 min. Lysates were fractionated by SDS-PAGE, probed for Ser\textsuperscript{473} phosphorylation and reprobed for total PKB. B. Cells were treated with either 50 nM calyculin A, 1 μM okadaic acid, or vehicle (ethanol) for 20 min and stimulated with GM-CSF for 5 min. Lysates were fractionated by SDS-PAGE and probed for Ser\textsuperscript{473} phosphorylation, reprobed for Thr\textsuperscript{308} phosphorylation and then reprobed for total PKB.
in the presence of ceramide following stimulation with GM-CSF. In contrast to LY-294002, which completely abolished PI(3,4,5)P₃ and PI(3,4)P₂ generation, ceramide had no effect on the generation of these lipid species. Thus, I conclude that ceramide does not regulate the activation of PI3K.

Under the same conditions, I measured PKB activity and found it to be lowered by 50-60% by ceramide. This suggested to me that ceramide was altering the activation of PKB independently of any effect on PI3K. Ceramide could be acting on upstream kinases that regulate PKB, or it may block to some extent its translocation to the plasma membrane. Closer examination of the regulatory sites of PKB demonstrated that only Ser⁴⁷³ phosphorylation was largely attenuated by ceramide treatment. For Thr³⁰⁸ to become phosphorylated, both PDK1 and PKB must bind PI(3,4,5)P₃ to induce a conformational change providing access for PDK1. The observation that Thr³⁰⁸ phosphorylation was not affected by ceramide confirms that PKB retained access to PI(3,4,5)P₃, most likely through translocation to the plasma membrane, consistent with a lack of affect of ceramide on PI3K. Thus, my results demonstrated that the reduction in PKB activity was due to reduced overall Ser⁴⁷³ phosphorylation.

In order to understand the mechanism by which ceramide was reducing Ser⁴⁷³ phosphorylation, I asked if this was due to a ceramide-activated phosphatase. Little information is known about the critical enzymes responsible for returning PKB to an inactive state following receptor activation. However, ceramide has been demonstrated to activate a PP2A-like phosphatase activity (CAPP) through unknown mechanisms. This activity is sensitive to the phosphatase inhibitor okadaic acid (Dobrowsky et al., 1993), and okadaic acid has been used to implicate CAPP in the dephosphorylation of Bcl-2, Rb,
and PKC isoforms (Lee et al., 1996; Dbaibo et al., 1995; Ruvolo et al., 1999). In my experiments, okadaic acid fully restored the reduced phosphorylation of Ser\textsuperscript{473} caused by ceramide, consistent with the hypothesis that the effects of ceramide on Ser\textsuperscript{473} phosphorylation were due to phosphatase activation. While these results cannot support a definitive conclusion, they are consistent with CAPP being the mediator of PKB dephosphorylation at Ser\textsuperscript{473}.

I also examined the effects of okadaic acid and calyculin A on Thr\textsuperscript{308} phosphorylation. Interestingly, I found that both compounds greatly reduced Thr\textsuperscript{308} phosphorylation, suggesting that a dephosphorylation event is necessary for PDK-1 catalyzed Thr\textsuperscript{308} phosphorylation. The reduction in Thr\textsuperscript{308} phosphorylation by okadaic acid coincided with reduced activity. Thus, my experiments have revealed an unexpected mechanism of regulation of Thr\textsuperscript{308} phosphorylation. This was surprising, given that PDK1 is constitutively active. One possible mechanism is that an inhibitor protein of PDK1 exists which is activated by phosphorylation. Yet another possible mechanism of regulation is by phosphorylation of PDK1 itself, which has recently been reported (Casamayor et al., 1999). In addition, my findings that PDK1-catalyzed Thr\textsuperscript{308}, but not PDK2-catalyzed Ser\textsuperscript{473} phosphorylation, is reduced by okadaic acid would argue that the two enzymes are distinct. Some researchers have suggested that PDK2 is the integrin-linked kinase (Delcommenne et al., 1998), distinct from PDK1, and it will be interesting to see if ceramide similarly reduces ILK-phosphorylated substrates through phosphatase activation.

In conclusion, I have demonstrated the selective targeting of Ser\textsuperscript{473} of PKB by a ceramide-activated phosphatase and have conclusively shown that ceramide had no effect
on the lipids generated following activation of PI3K. This ceramide-mediated dephosphorylation may have profound effects on the activities of other downstream targets of PKB, such as caspase-9, Forkhead transcription factors, and GSK-3. However, preliminary studies revealed that treatment of cells with C2- or C6-ceramide analogs had no effect on GSK-3 phosphorylation. In addition, work by Fujita and coworkers revealed that the PKB phosphorylation site found in human caspase-9 is absent in mouse caspase-9 suggesting that caspase-9 phosphorylation and inactivation by PKB may not be physiologically relevant (Fujita et al., 1999). Thus, the most plausible candidate for PKB-dependent survival appears to be the Forkhead family of transcription factors. Future studies should focus on the role of Forkhead mediated transcription during ceramide-induced apoptosis.

Of equal significance, ceramide-activated phosphatases may target other phosphorylated proteins containing the conserved FXXF(S/T)(Y/F) motif corresponding to Ser\textsuperscript{473} of PKB. This carboxy-terminal phosphorylation motif is present in p70\textsuperscript{56k} (Thr\textsuperscript{389}) and in the atypical PKC's. PRK1/2 contain PDK-2 pseudosubstrate domains that do not require phosphorylation for activation, and the activity of these kinases may be immune to ceramide treatment. Thus, the possible effect of ceramide on other protein kinases that may be regulated by modulation of the PDK2-substrate residue will have to be investigated.
6. OVERALL DISCUSSION

6.1 Phosphorylation of Bcl-2 Family Members in Hemopoietic Cells

Cytokines such as GM-CSF and IL-3 probably mediate survival by inhibiting components of the apoptotic machinery. Direct phosphorylation of Bcl-2 family members by kinases activated by GM-CSF or IL-3, as well as dephosphorylation by phosphatases, could represent an important part of this control. Several precedents exist in this regard. For example, the pro-apoptotic protein Bad is a downstream target of multiple kinases, possibly including PKB (Datta et al., 1997a; del Peso et al., 1997), ERK-controlled Rsk (Scheid et al., 1999; Bonni et al., 1999) and cAMP-activated PKA (Harada et al., 1999; Datta et al., 2000). Besides hemopoietic-specific cytokines, other growth factors, such as such as nerve growth factor and insulin-like growth factor-1 modulate Bad. Phosphorylation of Bad neutralizes its ability to mediate apoptosis, through a poorly understood mechanism (reviewed by Datta et al., 2000). The multiplicity of different survival pathways leading to Bad phosphorylation may represent a redundancy to ensure inactivation during signaling through PI3K, Ras-ERK or cAMP generation.

Initially, I was interested in whether other Bcl-2 family members may undergo cytokine-stimulated phosphorylation, similar to Bad. Analysis of the primary sequence of Bax, Bak, and Bcl-x\textsubscript{L} identified several potential serine/threonine phosphorylation sites (Table 1). In addition, Bcl-x\textsubscript{L} contains a flexible loop domain, previously shown to be a site of phosphorylation in Bcl-2 (Chang et al., 1997). To test this possibility, I first established immunoprecipitation conditions for Bax, Bak and Bcl-x\textsubscript{L}, as well as Bad, and
then monitored their phosphorylation state using $^{32}$P-orthophosphate-labelling. Although Bad was clearly a target of GM-CSF-activated kinases, I did not detect changes in the phosphorylation level of the other family members examined. Thus, GM-CSF-mediated cell survival does not appear to involve changes in the phosphorylation state of Bax, Bak or Bcl-x$_L$.

These experiments revealed that endogenous Bad associates with endogenous Bcl-x$_L$ under both starvation and stimulation conditions. This was the first study to observe Bcl-x$_L$-Bad association in vivo in untransfected cells, and contributed to a study we published identifying MEK as an upstream regulator of Bad phosphorylation (Scheid et al., 1999). I also attempted to identify which sites of Bad were phosphorylated when bound to Bcl-x$_L$, as this might give some insight into the mechanism that Bad inactivates Bcl-x$_L$. These efforts are ongoing.

Although the phosphorylation of Bcl-x$_L$, Bax and Bak did not change with stimulation, an unexplored possibility was that cytokines might alter the relative expression levels of Bcl-2 homologous proteins. Therefore, the next focus of my research was to study cytokine-dependent, transcriptional and translational regulation of various Bcl-2 family members.

### 6.2 Analysis of Bcl-2 family Expression in Murine and Human Hemopoietic Cells

RNA-protection assays were initially performed with RNA isolated from the murine factor-dependent cell line MC/9. I began my studies by isolating RNA from cells that were deprived of cytokine for 16 hours, conditions that have previously been shown to induce apoptosis in MC/9 cells (Scheid and Duronio, 1998). Using a multi-probe
template set, mRNAs corresponding Bcl-2, Bcl-w, Bcl-xL, Bax, Bak and Bad were detected. During restimulation with cytokine, only Bcl-xL mRNA levels increased substantially. Other family members, such as Bak, Bax and Bad did not change significantly over time. This suggests that survival factors such as IL-3 and GM-CSF may activate signaling pathways that function to increase or sustain Bcl-xL mRNA levels; for example, Dumon and colleagues (1999) have shown that cytokines can stimulate Bcl-xL transcription via a Jak2-dependent mechanism. In addition, some studies have demonstrated a rapid increase in Bcl-xL mRNA in response to other survival hormones and growth factors (Sui et al., 2000; Puthier et al., 1999). Interestingly, when protein levels were subsequently determined by immunoblot analysis, little or no change in Bcl-xL protein was observed over the same time course. It may be that a small amount of mRNA is sufficient to sustain Bcl-xL protein levels; conversely Bcl-xL may have a long half-life. Previous studies from our lab (Scheid et al., 1995) have shown that MC/9 cells begin to undergo apoptosis during the time points examined therefore it is unlikely that changes in Bcl-xL protein are responsible for the cytoprotective effects of GM-CSF or IL-3.

6.3. GM-CSF selectively regulates Mcl-1 expression

The expression of Bcl-2 family mRNA in human cell lines was also examined. In the factor-dependent cell line TF-1, mRNAs corresponding to Bcl-xS, Bcl-xL, Bik, Bak, Bax and Mcl-1 were present. Similar to my findings in MC/9 cells, Bcl-xL mRNA was upregulated by cytokine stimulation or decreased during cytokine withdrawal. Moreover,
like the MC/9 cells, Bcl-xL protein levels did not change significantly under these conditions.

Expression of another Bcl-2 family member, Mcl-1, was also examined in TF-1 cells. Mcl-1 is a pro-survival factor originally identified based on its upregulation in a human myeloblastic leukemia cell line that was induced to differentiate along the monocyte/macrophage pathway (Kozopas et al., 1993). While the exact role of Mcl-1 in differentiation is unknown, Mcl-1 can act as a survival factor in multiple hemopoietic cell types both in tissue culture (Zhou et al., 1997) and when expressed in transgenic mice (Zhou et al., 1998). Recent work by Korsmeyer and coworkers showed that Mcl-1 knockout is embryonic lethal suggesting that in addition to its pro-survival function, Mcl-1 plays a critical role in embryonic development. TF-1 cells were incubated in the presence of GM-CSF and the mRNA and protein level of Mcl-1 were examined. GM-CSF stimulation causes an increase in Mcl-1 mRNA and protein levels, suggesting that GM-CSF may mediate survival through the up-regulation of pro-survival factors such as Mcl-1. Thus of all the Bcl-2 family members tested, Mcl-1 is unique in that its mRNA and protein levels are markedly affected by GM-CSF stimulation.

The mechanism by which Mcl-1 exerts its antiapoptotic influence is at present unclear. Several reports suggest that under apoptosis-inducing conditions, the pro-apoptotic protein Bax undergoes homodimerization to promote apoptosis, whereas the pro-survival factor Bcl-2 forms Bcl-2:Bax heterodimers to suppress apoptosis and promote cell survival. Work by Wang and colleagues (1998) suggest that Mcl-1 can also heterodimerize with Bax to enhance cell viability in hematopoietic FDC-P1 cells. Thus, GM-CSF-induced upregulation of Mcl-1 protein may promote cell survival through an
increase in Mcl-1:Bax heterodimers. Through the use of cross-linking reagents, future studies can attempt to address this issue further. It should also be noted that several studies have reported that dimerization among the Bcl-2 family members is a detergent-induced phenomenon and therefore may not be physiologically relevant (Hsu and Youle 1998).

6.4. Role of MEK in Mcl-1 Transcription

I next wanted to understand the signaling pathways that impact on Mcl-1 transcription and translation. Ligand-conjugated GM-CSF receptor activates a number of well-studied signaling pathways including Ras and its downstream targets. Research studies over the past several years have implicated Ras, and some of its downstream effectors, including Raf, in anti-apoptotic signaling. For example, Kinoshita and coworkers (1995) have demonstrated regulation of Bcl-2 expression by a Ras/Raf pathway in hematopoietic cells. In addition, work from our lab has recently revealed that phosphorylation of Bad on Ser-112 occurs by a MEK-dependent mechanism, and this phosphorylation regulates its binding with Bcl-xL.

Activation of Ras, leading to ERK1 and ERK2 activation, is associated with increased immediate-early gene expression. Research by several groups has demonstrated ERK-dependent regulation of Elk1 and camp response element (CRE) binding protein (CREB) transcription factors (Vanhouutte et al., 1999; Davis et al., 2000). For example, following activation, ERK undergoes translocation to the nucleus where it can directly phosphorylate and activate Elk-1. Once activated, Elk-1 can bind to serum response elements (SRE) located in the promoter regions of immediate early genes, such
as the c-fos transcription factor and cyclin D1 (Lavoie et al., 1996). In addition, activated ERK can directly phosphorylate and activate p90\textsuperscript{rsk}. ERK-dependent p90\textsuperscript{rsk} activity has been shown to phosphorylate CREB resulting in the binding of CREB to CRE elements in the promoter of c-fos and other immediate-early genes (Xing et al., 1996). p90\textsuperscript{rsk} can directly phosphorylate c-fos resulting in an increase in c-fos transcriptional activity (Bruning et al., 2000). Recent work by Wang and colleagues has shown the presence of a CRE-2 binding motif in the Mcl-1 promoter region and has demonstrated that IL-3 dependent stimulation of the Mcl-1 gene requires a CREB-like protein (Wang et al., 1999). In addition to a CRE-2 site, the Mcl-1 promoter also contains a sis-inducible element (SIE) very similar to the SIE binding motif found in the c-fos gene promoter (Wagner et al., 1990; Rajotte et al., 1996). Thus in TF-1 cells, cytokine-stimulated activation of the ERK pathway can lead to the activation of various immediate-early transcription factors necessary for Mcl-1 gene transcription.

To determine if ERK was required for GM-CSF-stimulated increase in Mcl-1 protein, two specific MEK inhibitors were employed. U0126 and PD-98059 block the activation of the ERKs by GM-CSF. I found that inhibition of MEK with U0126 or PD-98059 significantly inhibited GM-CSF-induced increase in Mcl-1 mRNA. To assess the role of ERK in Mcl-1 protein translation, cells were again treated with the MEK inhibitors U0126 or PD98059 in the presence of GM-CSF. As expected, the MEK inhibitors blocked the GM-CSF-induced elevation of Mcl-1 protein. This was consistent with the effects of MEK inhibition on reduced transcriptional upregulation of Mcl-1 mRNA in response to GM-CSF. Thus GM-CSF-mediated cell survival may involve the
upregulation of death antagonists, such as Mcl-1, as well as the post-translational modification and inhibition of death effectors, like Bad.

6.5. Role of PI3K in Mcl-1 Translation

In keeping with the finding that ERK regulates GM-CSF-induced Mcl-1 transcription, I wanted to know if this pathway and/or a PI3K-controlled pathway regulates Mcl-1 protein translation. Although I had determined that inhibition of the parallel, PI3K-PKB pathway had no effect on Mcl-1 mRNA, I wanted to know if this pathway might still be important for Mcl-1 protein expression in response to GM-CSF stimulation. To determine the role of PI3K in cytokine-stimulated Mcl-1 regulation, the PI3K pathway was selectively blocked with wortmannin or LY-294002, which had no effect on the ERK pathway. Addition of the PI3K inhibitors wortmannin or LY-294002 inhibited GM-CSF-induced upregulation of Mcl-1 protein. Thus, these experiments define separate pathways that impact on Mcl-1 expression; (1) a MEK-dependent pathway involved in transcriptional regulation, and (2) a PI3K-controlled pathway involved in translational regulation or enhanced protein stability. Pulse-chase experiments would argue against an effect of PI3K on increasing Mcl-1 stability, and thus I conclude that PI3K regulates the translation of Mcl-1.

There are indications that PI3K and its downstream target PKB may be centrally involved in translational control. Recent work by several groups suggest that PKB may be involved in regulating protein synthesis by phosphorylation of the mRNA cap protein, eIF4B, as well as its inhibitory binding protein, 4EBP1 (Khaleghpour et al., 1999; Takata et al., 1999). Thus, GM-CSF-induced PI3K activity may lead to PKB-dependent
phosphorylation events required for the initiation of Mcl-1 protein synthesis. There are no pharmacological inhibitors of PKB, however, experiments are currently underway to introduce a conditionally active form of PKB into TF-1 cells in order to test the hypothesis that a PI3K-PKB-dependent pathway leads to GM-CSF-induced Mcl-1 upregulation.

Additional PI3K-dependent pathways that may be involved in Mcl-1 translation include serum- and glucocorticoid-regulated kinase (SGK; Park et al., 1999), PKC isoforms, Bruton’s tyrosine kinase (BTK), PDK1, and Vav (review by Toker, 2000). For example, PDK1 has been shown to phosphorylate and activate p70S6k (Pullen et al., 1998; Alessi et al., 1997) in a PI3K-dependent manner (Balendran et al., 1999) and PKC has been shown to regulate the translation of the upstream stimulating factor-2 (USF2). USF2 is a transcription factor thought to have an important role in the proliferation of hematopoietic cells through its regulation of various cyclins and p53 (Zhang et al., 1998).

The results presented in Chapter 4 conflict with published accounts of PI3K-PKB dependent transcriptional regulation of Mcl-1. Through the use of dominant negative PI3K mutants and PI3K inhibitors, Wang and coworkers (1999) demonstrate transcriptional upregulation of Mcl-1 mRNA in the IL-3 dependent cell line, BAF/3. One obvious difference between the Wang study and my results is the difference in cell lines studied. Recent reports by Welham and coworkers (1999) have demonstrated PI3K-independent wortmannin-sensitive ERK activity in BAF/3 cells. Further, the Karnitz group (1995, 1999) has shown that overexpression of dominant negative p85 can block IL-3 induced ERK activation in FDC-P1 cells. The Wang group did not address the effect of blocking PI3K on ERK activation nor did they examine the role of ERK activity
in regulating Mcl-1 transcription. Thus the PI3K-PKB-mediated effects on Mcl-1 transcription observed by Wang and colleagues may be due to an inhibition of ERK activity and not necessarily a decrease in PI3K-PKB signaling. In the TF-1 cell line that I used, inhibiting PI3K activity had no effect on ERK activation. Together my results support a role for PI3K in translational regulation of Mcl-1 and a role for ERK in the transcriptional regulation of Mcl-1.

The Wang study suggests that *Mcl-1* transcription requires a CREB-like binding protein and indicate that a PI3K-PKB dependent pathway may be leading to phosphorylation of CREB Ser\(^{133}\). MEK-dependent p90\(^rsk\) activity has also been shown to result in phosphorylation of CREB Ser\(^{133}\) in response to cytokine stimulation (Xing et al., 1996), thus I would argue that in the TF-1 hematopoietic cell line, CREB transcriptional regulation of Mcl-1 gene may be occurring via a ERK-dependent and not a PI3K-controlled pathway. Alternatively, Mcl-1 transcription could be regulated by other ERK-dependent transcription factors as discussed in section 6.3.

### 6.6. SHIP as a regulator of Bcl-x\(_L\)

The effects of PI3K inhibition on Bcl-x\(_L\) mRNA transcription were interesting, and suggested that PI3K and its downstream target PKB may play a role in Bcl-x\(_L\) protein expression. Although I could not detect changes in Bcl-x\(_L\) in MC/9 cells during treatment with PI3K inhibitors, it may be that chronic suppression of PI3K-derived signals was necessary to modulate Bcl-x\(_L\) protein. The SH2-containing inositol 5' phosphatase SHIP, functions as a key regulator of PI(3,4,5)P\(_3\) and PI(3,4)P\(_2\) levels in hematopoietic cells (Liu et al., 1999). Liu and coworkers (1999) have shown that bone marrow-derived mast cells
from SHIP<sup>−/−</sup> are resistant to cell death induced by a variety of apoptotic-stimuli including growth factor-withdrawal. To determine the role of SHIP in the regulation of apoptosis, the mRNA and protein levels of various Bcl-2 family members were analyzed in SHIP knockout cells. The mRNA levels of several Bcl-2 family members were found to be present in equal amounts in both SHIP<sup>+/+</sup> and SHIP<sup>−/−</sup> cells. Surprisingly, Bcl-x<sub>L</sub> protein levels were greatly elevated in SHIP<sup>−/−</sup> cells, suggesting that in BMMC SHIP may negatively regulate Bcl-x<sub>L</sub> expression.

The PI3K-generated phospholipids PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> function as molecular switches to regulate the activity of downstream serine/threonine phosphorylation cascades in response to growth factor stimulation. For example, both PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> have been shown to regulate the activities and localization of PDK1, PKB and Btk in response to cytokine stimulation (review by Toker, 2000). Work by Liu and colleagues (1999) has revealed that SHIP<sup>−/−</sup> mice have elevated levels of PI(3,4,5)P<sub>3</sub> and PKB activity. Thus one way that SHIP may regulate Bcl-x<sub>L</sub> is through downregulation of PI(3,4,5)P<sub>3</sub> signaling pathways, possibly involving enzymes such as PDK1 and PKB.

Recent work by Jones and coworkers (2000) supports the idea of a PI3K-PKB-controlled pathway regulating Bcl-x<sub>L</sub> protein levels. The Jones group used T lymphocytes overexpressing v-PKB to show that a PKB-dependent pathway regulates Bcl-x<sub>L</sub> expression at the translational level. In contrast, research by Packham and coworkers (1998) has demonstrated the selective regulation of Bcl-x<sub>L</sub> protein levels by a PI3K-independent, Jak2-dependent pathway. The contradictory findings may be
explained by the fact that the Packham group utilized 32D.3-derived transfectants whereas my studies and those of Jones and coworkers were performed with primary cells.

Continuing studies will involve treating SHIP−/− bone marrow derived mast cells with the PI3K inhibitors LY-294002 or wortmannin to see if PI3K inhibition reduces the amount of Bcl-xL protein back to wild type levels. Another approach would be to retrovirally introduce kinase-dead mutants of PDK1 or PKB and see if these also interfere with Bcl-xL upregulation in SHIP−/− mice. The generation of PI3K knockout mice would help to define a role for PI3K in Bcl-xL regulation. Finally, one might predict that cells derived from PTEN-deficient mice might also display elevated Bcl-xL protein since PTEN acts similarly to SHIP in elevating PI(3,4,5)P3 and PKB activity (Stambolic et al., 1998).

6.7. Ceramide negatively regulates Mcl-1 protein translation

Apoptotic signaling mediated by ceramide can occur by several different mechanisms. Recent work suggests that ceramide may induce apoptosis by activating pro-death pathways such as the stress-activated protein kinase c-Jun N-terminal kinase signaling cascade (Verheij et al. 1996; Wang et al., 1999; Bourbon et al., 2000) and by promoting dephosphorylation of the pro-survival protein Bcl-2 (Ruvolo et al., 1999) and the pro-apoptotic protein Bad (Basu et al., 1998). Ceramide may also augment the execution of apoptosis by the inactivation of pro-survival pathways. Recently, several reports have supported this notion by demonstrating negative regulation of ceramide on the PI3K signaling pathway (Zundel and Giaccia et al., 1998).
To investigate the possibility that ceramide may decrease anti-apoptotic signaling by inhibiting GM-CSF-induced PI3K-dependent Mcl-1 upregulation, cells were treated with cell permeable ceramide analogs, and stimulated with GM-CSF. It was found that ceramide could inhibit GM-CSF-induced increase in Mcl-1 at the translational level, while having no effect on Mcl-1 transcription. Other Bcl-2 family members were also examined (Bcl-xL, Bak and Bax) and were unaffected by ceramide treatment. These results suggest that ceramide-mediated apoptosis may involve the downregulation of Mcl-1 via a PI3K-dependent pathway in hematopoietic cells.

6.8. Ceramide inhibits PKB by promoting dephosphorylation of Ser^{473}

Relatively little is known about the way in which ceramide acts upon signaling pathways. Zundel and Giaccia (1998) reported that ceramide specifically inhibits PI3K, which in turn results in the loss of PKB activation, a regulatory target of PI3K activity. Two other reports also demonstrated the negative effects of ceramide on PKB activation (Basu et al., 1998; Zhou et al., 1998; Summers et al., 1998); however, they suggest that ceramide-mediated PKB inhibition occurs via a PI3K-independent pathway. Here I provide biochemical evidence that ceramide regulates PKB activity not through PI3K modulation, but rather by selectively accelerating PKBSer^{473} dephosphorylation, a residue critical for maximal PKB activation. This reduction in phosphorylation could be restored by pretreatment of cells with okadaic acid or calyculin A, two potent phosphatase inhibitors. This study suggests that 3-phosphoinositide-dependent kinase (PDK)-2-targeted phosphorylation of PKB may be a target of a ceramide-activated phosphatase.
The reduction of PKB phosphorylation at Ser⁴⁷³ and the parallel decrease in activity caused by ceramide may be significant contributors to ceramide-mediated apoptosis (Schubert et al., 2000). At present, the mechanism by which PKB protects against apoptosis is not fully understood. Some groups have identified Bad as a target of PKB phosphorylation (Datta et al., 1997a; Blume-Jensen et al., 1998; del Paso et al., 1997) supporting the theory that ceramide induces apoptosis by inhibiting PKB-mediated Bad phosphorylation. However, we (1998) and others (Hinton and Welham, 1999) have provided examples in which one can dissociate endogenous Bad phosphorylation from endogenous PKB activation and PI3K-dependent survival.

With respect to PKB inactivation, ceramide may disrupt the ability of PKB to prevent apoptosis by phosphorylating transcription factors. In Caenorhabditis elegans, genetic experiments have ordered signaling through the insulin-like receptor Daf-2 to Age-1 (PI3K homologue), Pdk-1 and AKT activation, with Daf-16 phosphorylation, which promotes longevity and prevents developmental arrest at the dauer stage (Paradis et al., 1998). Null mutations in the pdk-1 gene cause constitutive arrest in the dauer stage, whereas activating mutations rescues null mutations of daf-2 or age-1. Daf-16 is the C. elegans homologue of the mammalian Forkhead transcription factor. Several groups have shown PKB phosphorylation of Forkhead members in mammalian cells (Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). Phosphorylation of Forkhead by PKB stimulates its export from the nucleus. This may prevent the transcription of pro-apoptotic proteins, including the Fas ligand. Thus, ceramide may prevent nuclear export of Forkhead transcription factors by reducing PKB activation. Further, as discussed above, PKB inactivation may prevent the initiation of protein
synthesis of pro-survival factors, such as Mcl-1. It will be interesting to see if Mcl-1 is regulated via a PKB controlled signaling pathway in hematopoietic cells.

6.9. Summary

My studies of the regulation of Bcl-2 family members have revealed distinct roles for the PI3K and ERK pathways in regulating the pro-survival factor, Mcl-1. I have found that while ERK activity is required for regulation of Mcl-1 gene expression, there is a need for PI3K activity in regulation of protein expression. I provide evidence that ceramide may promote apoptosis by inhibiting cytokine-stimulated Mcl-1 protein synthesis. In addition, these studies reveal that ceramide may inhibit cell survival through the down-regulation of PKB activity via a ceramide-activated protein phosphatase. Finally, the experiments presented here indicate that Bcl-xl protein levels may be negatively regulated by the src homology 2 (SH2)-containing inositol 5’ phosphatase, SHIP.
7. BIBLIOGRAPHY


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