PHOSPHATIDYLINOSITOL 3-OH KINASE: AN IMPORTANT ELEMENT IN SURVIVAL SIGNALLING

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B.Sc. (Honours), University of Guelph, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in THE FACULTY OF GRADUATE STUDIES

Department of Medicine

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February 1999

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Date April 19, 1999

DE-6 (2/88)

ABSTRACT

Homeostasis of blood cells is maintained by a tightly controlled system of cell division and cell death. Disruption of this homeostasis may have profound implications in the development of cancer. This disruption can be a result of uncontrolled cell division, and/or resistance of the cells to programmed cell death, termed apoptosis. Hemopoietic cytokines are integral in controlling both of these processes, and while the signalling pathways that control cell division are quickly being elucidated, less is known about how these cytokines promote survival.

One family of enzymes regulated by cytokine signalling are the phosphatidylinositol 3-kinases. By using pharmacological inhibitors of these enzymes, it was determined that some, but not all cytokines require PI 3-kinase to prevent apoptosis. A well known downstream target of PI 3-kinase is the p70 S6 kinase. However, we showed that p70 S6 kinase could be dissociated from this survival function.

The extracellular regulated kinases (termed $p42^{erk2}$ and $p44^{erk1}$) are MAPKs that may require a component of PI 3-kinase activity for full activation. Therefore the Erks could also be a component of the PI 3-kinase mediated survival pathway. We showed that inhibition of PI 3-kinase by wortmannin resulted in a 50% reduction in the activity of $p44^{erk1}$ following cytokine stimulation, consistent with published reports. However, full inhibition of PI 3-kinase by a structurally unrelated inhibitor, LY-294002, did not reduce the activation of $p44^{erk1}$, implicating targets other than PI 3-kinase in the action of wortmannin. These results agree with the observation that IL-4 can activate PI 3-kinase, and maintain survival dependent upon this activity, but IL-4 does not activate $p42^{erk2}$, $p44^{erk1}$, or other MAPK family members such as p38 or SAPK. Furthermore, inhibition of MEK, the upstream activator of the Erks, prevented the ability of cytokines to activate $p44^{erk1}$, but it did not have any effect on cell survival. Thus, PI 3-kinase probably does not mediate survival via Erks or other MAPKs.

Recently, a Bcl-2 family member, Bad, has been shown to undergo phosphorylation in response to cytokine stimulation. Phosphorylation of Bad may be crucial for the ability of cytokines to prevent Bad-induced apoptosis. Since PI 3-kinase activates PKB, a kinase that phosphorylates sequences similar to one found in Bad, we examined whether cytokines induce Bad phosphorylation and whether this was dependent on PI 3-kinase. Cytokine-induced phosphorylation of Bad was partially blocked by PI 3-kinase inhibitors. However phosphorylation of Bad induced by GM-CSF was unaffected by PI 3-kinase inhibitors. Conversely, IL-4 was found to stimulate PI 3-kinase, PKB, and promote cell survival, but was unable to induce Bad phosphorylation. These results suggest that other pathways besides PI 3-kinase lead to Bad phosphorylation and that phosphorylation of Bad is not required for cytokines to prevent apoptosis. Thus, the PI 3-kinase/PKB pathway may promote survival by as yet uncharacterized pathways that do not involve Bad phosphorylation.

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ABBREVIATIONS

Bad	Bcl-X_L-associated death inducer
BH	Bcl-2 homology
BMMC	Bone marrow derived mast cell
Btk	Bruton's tyrosine kinase
CAMPT	Camptothecin
Erk	Extracellular regulated kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK-3	Glycogen synthase kinase-3
HPLC	High pressure liquid chromatography
IL	Interleukin
kDa	Kilodalton
LY	LY-294002
MAPK	Mitogen activated protein kinase
MEK	MAPK/Erk kinase
mTOR	Mammalian target of rapamycin
MW	Molecular weight
NGF	Nerve growth factor
PAGE	Polyacrylamide gel electrophoresis
PD	PD98059
PDK	Phosphoinositide dependent kinase
PH	Pleckstrin homology
PI	Phosphatidylinositol
PKB	Protein kinase B
PKC	Protein kinase C
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SH2	Src-homology-2
SHIP	SH2-domain containing 5'-inositol phosphatase
SOS	Son of sevenless
TLC	Thin layer chromatography
WM	Wortmannin

ACKNOWLEDGMENTS

I would like to begin by thanking Vincent Duronio for providing me with an ideal environment to begin my scientific career. The direction and enthusiasm he provided was essential for the development of the work presented in this thesis. I would also like to thank the following individuals for their assistance during the course of this work. First, members the Duronio laboratory, including Ron Lauener, David Fong and Christtian Stevens provided technical advice and assistance. I would also like to thank Ian Foltz and James Wieler for their countless suggestions which helped the direction of this thesis. Jacky Damen deserves special thanks for her technical help and expertise. I am indebted to the members of my supervisory committee, Michael Gold, Gerry Krystal and Roger Brownsey, for taking the time to so carefully read and correct earlier versions of the thesis. Finally, I would like to extend my warmest thank you to Kathryn Schubert, whose love, understanding, and patience was tremendously appreciated.

1. INTRODUCTION

1.1 GENERAL

Blood cells begin as stem cells, the self-renewing progenitors from which all other major blood cells originate (Morrison et al., 1995). With the recognition that these progenitors formed colony forming units (CFU's) of specific cell types *in vitro*, the identification of the soluble factors that promoted the expansion, differentiation and survival of many types of hemopoietic cells was soon realized (Metcalf, 1984; Nicola, 1989). The subsequent isolation and cDNA cloning of the receptors for these hemopoietic factors, or cytokines, revealed a family of related receptors composed of multiple subunits, some of which were found to be shared between cytokines (Cosman et al., 1990; Cosman, 1993; Miyajima et al., 1993). Hemopoetic cytokines and their receptors, as well as the pathways through which they transmit intracellular signals, will be discussed below. For this initial discussion, the important physiological response to remember is that hemopoetic cytokines have a wide range of roles, one aspect of which includes survival, or prevention of apoptosis (Williams et al., 1990). Precise control of blood cell survival allows the levels of blood cells to be tightly regulated. This process is critical for the survival of the organism as a whole. Alteration of apoptosis may have disastrous effects. Gain- or loss-of-function mutations that allow cells to survive in the absence of exogenous survival factor may contribute to the development of cancers, as well as providing resistance against cancer treatment therapies (Thompson, 1995). Additionally, tissue damage associated with the normal roles of granulocytes during inflammation may be more profound as the lifespan of the cells is extended (Osbashi et al., 1992).

The mechanisms by which cytokine receptors prevent apoptosis were not well understood when this work was started. The principle aim of these studies was to better understand the cytokine-activated signalling pathways that mediate hemopoietic cell

1.2. APOPTOSIS

Apoptosis is a genetically conserved program of cell death whereby a damaged cell, a cell receiving an exogenous death signal, or a cell that is no longer receiving an exogenous survival signal, is eliminated from the organism (reviewed by Steller, 1995; Jacobson et al., 1997). Our understanding of apoptosis has increased dramatically in the past five years, and with it the appreciation of how important this process is. During development, the sculpturing of the animal is achieved by a combination of cell growth, differentiation and cell death (Jacobson et al., 1997). Apoptosis also plays an important role in many normal functions of adult animals, particularly in the immune system and organs such as the skin. Not surprisingly, dysregulation of apoptosis is manifest in many human disease states, including cancer, neurological, developmental and immunological disorders (Thompson, 1995).

2.1. THE BCL-2 FAMILY AND CASPASES - THE APOPTOSIS MACHINERY

The concept of apoptosis being an active, "programmed" event has its roots in the study of the nematode *Caenorhabditis elegans*, where a significant effort has been made to genetically identify effectors of the apoptosis program. Two mammalian gene families related to the *C. elegans* apoptosis-regulating genes have been identified: the first encodes a conserved family of CED-9-related proteins containing the inaugural Bcl-2 protein. To date 8 bona-fide mammalian members have been identified (Adams and Cory, 1998). This family appears to play a crucial role in determining the threshold of an apoptotic stimulus and initiating a final phase of irreversible cell death (described in more detail below).

The second family is related to the nematode *ced-3* gene which encodes cystein-aspartic acid specific proteases, hence the term "caspases" (Alnemri et al., 1997). These are thought to constitute the effector stage of apoptosis (Thornberry et al., 1992; Yaun et al., 1993). This mammalian family numbers 13 members to date, and they appear to function

as the central executioners of apoptosis by cleaving numerous protein targets necessary for DNA repair, membrane phospholipid distribution, and metabolic function (Cohen, 1997; Villa et al., 1997; Thornberry and Lazebnik, 1998). In addition, caspases may participate in the amplification of a death signal, by cleaving and activating other caspases (Hofmann et al., 1997; Li et al., 1997; Thornberry and Lazebnik, 1998). Experiments using caspase inhibitors that resemble the cleavage site of caspase substrates have shown that cell death can be prevented (or, in some circumstances delayed) by inhibiting caspases (Nicholson et al., 1995).

Caspases normally lie dormant in the cell, expressed as low activity zymogens which need an input stimulus to be processed into their active forms (Thornberry and Lazebnik, 1998). The activation process which has been best studied is cleavage of a prodomain at specific residues which are substrates for either distinct caspases or by auto-cleavage. Much like the recruitment of transmembrane receptors by extracellular ligands, caspases may be clustered together upon a death stimulus and, with sufficient localized activity, can become fully active as a result of autocatalytic cleavage (Thornberry and Lazebnik, 1998). This process probably requires the presence of cofactors, such as other proteins which contain a conserved protein domain present in several caspase family members, termed the death effector domain (DED). Fas, which is a membrane spanning receptor that can directly initiate caspase activation, recruits several cytoplasmic proteins, including the adapter protein FADD (Fas-associated protein with death domain; Ashkenazi and Dixit, 1998). FADD is instrumental in the activation of procaspase-8, following death receptor activation, by associating with both the cytosolic death domain of Fas and with the death domain of procaspase-8 (Boldin et al., 1996; Nagata, 1997; Muzio et al., 1998).

Caspase activation can also occur by localized cytosolic clustering. For example, Apaf1, the mammalian homologue of *C. elegans* CED-4 (Zou et al., 1997), functions in conjunction with the cofactors dATP and cytochrome c to bind to and activate procaspase-9 through another protein domain distinct from the DED, termed CARD (caspase recruitment

domain; Li et al., 1997). Colocalization of procaspase-9 with Apaf1 and dATP results in auto-cleavage and assembly into a fully active protease complex which can subsequently cleave and activate effector caspases such as caspase-3. Caspase-9 activation has been demonstrated in knockout mice to be essential for some stimuli to induce caspase 3-mediated apoptosis (Zou et al., 1997; Hakem et al,. 1998; Kuida et al., 1998). Caspase-3 can then cleave nuclear and cytosolic targets resulting in irreversible cell death (Thornberry and Lazebnik, 1998).

Genetic evidence in *C. elegans* has placed the *ced-9* gene family upstream of *ced-3* function. Overexpression of CED-9 or Bcl-2 in mammalian cells can prevent the activation of certain caspases. This would be consistent with experimental observations that pro-survival Bcl-2 proteins can protect well against cytotoxic agents which lead to caspase activation (reviewed by Adams and Cory, 1998). Indeed, Bcl-2 family members such as Bcl-2 itself and Bcl-X_L can protect cells from apoptosis caused by radiation, cytokine withdrawal, kinase inhibitors such as staurosporine, and chemotherapeutic drugs (Cory, 1995; Chao and Korsmeyer, 1998). Bcl-2 or Bcl-X_L may physically interact with Apaf1 and prevent its association and/or activation of procaspase-9 (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997; James et al., 1997).

Not surprisingly, a more complex scenario has developed in that caspase activation can be placed both upstream and downstream of Bcl-2 family regulation. For instance, as mentioned above, Fas directly activates caspases upon ligation by FasL or clustering antibodies (Ashkenazi and Dixit, 1998). Overexpression of Bcl-2 poorly inhibits Fas-induced apoptosis (Strasser et al., 1995; Newton et al., 1998) and does not affect early caspase activation. Activation of caspases upstream of Bcl-2 may induce a signalling cascade that eventually converges on Bcl-2 family proteins that amplify the death signal. One potential mechanism by which this pathway is regulated may involve caspase-8 mediated cleavage of the Bcl-2 family member Bid. This produces a truncated form of Bid which is a potent activator of cytochrome c release (Luo et al., 1998; Li et al., 1998). As

mentioned above, cytochrome c may play a role as a cofactor in regulating caspase activity (Yang et al., 1997; Kluck et al., 1997; Bossy-Wetzel et al., 1998), by altering the conformation of Apaf1, thereby allowing recruitment and activation of caspase-9 (Li et al., 1997; Kuida et al., 1998; Green and Reed, 1998). The cleavage of Bid by Caspase-8 was demonstrated following Fas activation (Li et al., 1998) but it remains unclear if this mechanism is important for the apoptosis of hemopoietic cells that occurs following removal of survival factors.

How does the Bcl-2 family of proteins regulate apoptosis? Sequence analysis has revealed conserved regions (termed "Bcl-2 homology" or "BH" domains) that are present in all Bcl-2 family members and which are necessary for both the pro- and anti-apoptotic effects of these proteins (reviewed by Adams and Cory, 1998). The most apparent role for these domains is to facilitate homo- and hetero-oligomerization (Yin et al., 1994; Chittenden et al., 1995), which is essential for the pro-apoptotic functions of BH3-domain containing proteins (Chittenden et al., 1995). Heterodimerization apparently is not required for the pro-survival functions of Bcl-2 proteins (Cheng et al., 1996; Kelekar et al., 1997), which was an earlier theory (Yin et al., 1994). X-ray crystallography and NMR analysis of Bcl-X_L demonstrated that the BH1, 2 and 3 domains are in close proximity in the folded monomer and form a hydrophobic pocket. This may account for the dimerization potential with other family members. For example, NMR analysis of a complex between the Bcl-X_L and Bak BH3 domains revealed that the hydrophobic pocket of Bcl-X_L interacts with the amphipathic α-helical peptide contained in Bak (Muchmore et al., 1996; Sattler et al., 1997). Future structural studies should reveal essential information about the role of Bcl-2 and Bcl-X_L in survival.

Another well studied Bcl-2 protein is the pro-apoptotic member Bax. Bax was originally described as a Bcl-2 binding partner which acts to prevent the survival function of Bcl-2 (Oltvai et al., 1993). More information about Bax has recently revealed that it may act in a unique way to induce death, perhaps through a non-apoptotic, caspase-independent

mechanism (Xiang et al., 1996; McCarthy et al., 1997). This form of death may be mediated through organelle damage, such as disruption of the mitochondrial membrane. Loss of mitochondrial function would lead to cell death, since the cell could no longer reduce reactive oxidative species or manufacture sufficient ATP. Finally, the evidence that Bcl-2 actually binds to Bax may be an artifact of detergent solubilization conditions (Hsu and Youle, 1998), further dissociating Bax from the other Bcl-2 family members.

Recent evidence using a chimeric FKBP-Bax expression system suggests that Bax homodimerization is critical for mitochondrial dysfunction and death (Gross et al., 1998). Cells expressing chimeric FKBP-Bax remain healthy in the presence of IL-3, but addition of the cell-permeable compound FK1012 (which enforces dimerization of the FKBP-Bax chimeras) results in translocation of FKBP-Bax to mitochondrial membranes and loss of mitochondrial membrane potential. In cells expressing normal Bax, translocation (of normal Bax) following IL-3-withdrawal was blocked by co-expression of the protective Bcl-X_L protein (Gross et al., 1998). In contrast, cells expressing FKBP-Bax were completely susceptible to FK1012-induced death even in the presence of overexpressed Bcl-X_L, arguing that Bcl-X_L or Bcl-2 play a role in preventing Bax translocation and/or homodimerization. However, if homodimerization is enforced, Bcl-X_L is unable to prevent death. These results suggest a model in which Bax is either held in the cytoplasm by a chaperone protein or is normally in an inert conformation in the cytosol and only translocates and homodimerizes in response to death signals.

2.2. CYTOKINE SURVIVAL VERSUS DEATH LIGANDS

Fas and other receptors in its family, including TNFR and TRAIL, provide a clear model for how an organism regulates immune function by influencing the survival of these receptor-bearing cells (reviewed by Nagata, 1997; Ashkenazi and Dixit, 1998). Ligation of one of these receptors under specific circumstances results in a rapid demise and clearance of the cell. This is probably due to the direct activation of caspases following receptor

activation, which can occur within seconds. This "active" induction of cell death is in contrast to the many cells of the hemopoietic system which require a survival factor bound to their receptors to prevent apoptosis, and it is the lack of such survival signals that results in apoptosis (Williams et al., 1990). In this respect, apoptosis is a default pathway which is held in check by the survival factors.

Given that the absence of these factors results in apoptosis, there are several possible ways that cytokines can protect against apoptosis. First, cytokines may induce changes in the *expression* of either Bcl-2 family proteins. For example, it has been noted that the protein and mRNA levels of Bcl-2 family proteins may rise or fall following cytokines stimulation or removal, respectively. Also, expression of the pro-survival or pro-apoptotic proteins may delay or enhance apoptosis following cytokine removal (Nunez, et al., 1990; Rinaudo et al., 1995; Silva et al., 1996; Gregoli et al., 1997; Sakai et al., 1997; Chao et al., 1998).

Additionally, cytokine signalling pathways may *directly* alter the function of these proteins or other components directly involved in the apoptotic machinery, such as caspases. This second possibility could for example involve direct phosphorylation of Bcl-2 family proteins as a way to alter their function. For instance, Bad phosphorylation has been reported to occur following IL-3, GM-CSF, or SCF stimulation (Zha et al., 1996; Scheid and Duronio, 1998). Phosphorylated Bad can bind the cytosolic 14-3-3 protein, which sequesters Bad from Bcl-X_L, presumably allowing Bcl-X_L to suppress apoptosis. Bad will be discussed in more detail below. Another protein involved in apoptosis which undergoes phosphorylation is Bcl-2 itself. Both anti-apoptotic and pro-apoptotic modifications of Bcl-2 appear to occur as a result of various cellular treatments. For instance, chemotherapeutic drugs such as taxol induce an increase in Bcl-2 phosphorylation which is correlated with diminished protective effects. Taxol-induced phosphorylation of Bcl-2 may be mediated by Raf, a target of Ras (Haldar et al., 1995) and/or c-Jun N-terminal/Stress activated protein kinases (Maundrell et al., 1997). Survival factors such as

IL-3 may also induce Bcl-2 phosphorylation through activation of the classical isoforms of PKC. PKC activation by bryostatin correlates with PKC-α colocalization with Bcl-2 and increased phosphorylation of Bcl-2 on Ser70. A functional role for Ser70 phosphorylation was supported by mutagenesis studies which showed that mutation of this residue to alanine abolished the protective effects of bryostatin (Ito et al., 1997; Ruvolo et al., 1998).

Like Bad, the pro-apoptotic *Drosophila* protein Hid appears to be a target of phosphorylation induced by survival factors. Activation of the Ras-Erk pathway antagonizes the ability of Hid to promote apoptosis (Bergmann et al., 1998). Yet another example comes from examination of the phosphorylation of caspase themselves. Recent evidence suggests that growth factor stimulation of certain kinases may lead to phosphorylation of caspase-9, which suppresses its activity (Cardone et al., 1998).

A likely scenario for the viability response to cytokines such as IL-3 or GM-CSF would probably involve protein translation, such as increased Bcl-X_L expression, as well as phosphorylation of Bcl-2 family proteins, such as Bad. The next section of this review will focus on the signalling pathways regulated by several receptors of the hemopoietic family.

1.3. HEMOPOIETIC CYTOKINE RECEPTOR SIGNALLING

1.3.1. Receptor Structure and Function

Cytokines control the growth, differentiation and survival of both progenitor and terminally differentiated hemopoietic cells. The individual cytokines studied here include IL-3, IL-4, GM-CSF and SCF. IL-3, GM-CSF and IL-5 have similar tertiary structure in spite of relatively low sequence homology (Miyajima et al., 1993). These three cytokines assemble into a four-α-helix bundle and bind to their cognate receptors through interactions between the N-terminal and C-terminal helixes. Studies of cytokine receptors in knockout mice have demonstrated redundancy in the biological role for these cytokines. For example, IL-3, GM-CSF and IL-5 all share similar biological actions and activate very similar signalling pathways (Duronio et al., 1992). An explanation for this redundancy and the observations that each can cross-compete for high affinity binding was made clearer when it was found that each signals through a common β-subunit in association with a ligand specific α-subunit (Tavernier et al., 1991; Miyajima et al., 1993).

A striking example of this redundancy comes as the result of targeted gene disruption. GM-CSF knockout mice exhibit normal hemopoiesis, suggesting that other factors can compensate for the loss of GM-CSF (Dranoff et al., 1994; Stanley et al., 1994). Erythropoietin (Epo) knockout mice, in contrast, display severe anemia and die as embryos, indicating that no other factor can substitute for Epo in the normal role for this hemopoietin in development and homeostasis (Wu et al., 1995). However, while the physiological roles for IL-3, GM-CSF and IL-5 all appear to be redundant, there may be subtle differences in the way in which each activates gene expression under various conditions. Additionally, very little is known regarding the signalling role played by the α-subunit for these receptors.

As described above, IL-3 and GM-CSF signal through a heterodimeric receptor consisting of a cytokine-specific α-subunit and a common, signal transducing β-subunit (AIC2A, βc). In mice, the IL-3 receptor can also consist of an IL-3-specific β-subunit

(AIC2B), although gene ablation studies have shown that the biological activities of these two receptor subunits overlap (Nishinakamura et al., 1995). Receptors of the hemopoietic superfamily retain certain structural criteria. In the extracellular regions of the receptors are either one or two hemopoietin domains containing four conserved cysteine residues, which form intramolecular disulfide linkages, and the sequence motif WSXWS (Cosman et al., 1990). Another important subfamily consists of receptors for IL-4, which are composed of α, β and γ subunits.

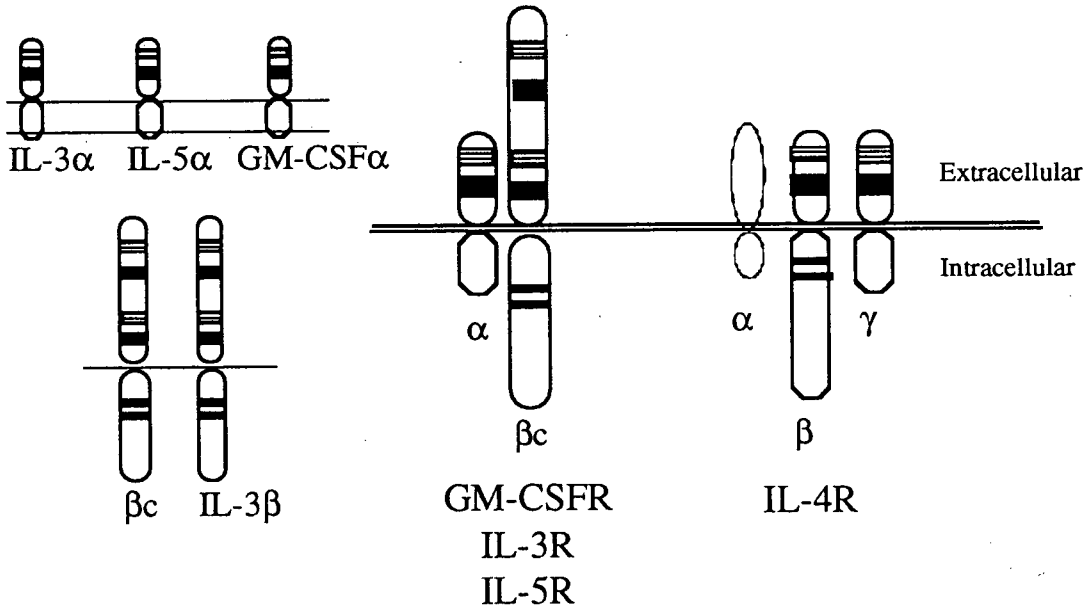


Figure 1.1. Hemopoietic cytokine receptor families for IL-3, IL-4, GM-CSF and IL-5. The striped boxes represent disulfide bonds between conserved cysteine residues. The black boxes represent the WSXWS region, and the intracellular lines of the β-subunits are the box1 and 2 motifs. The α-subunits are specific for IL-3, IL-5 and GM-CSF, which dimerizes with a common β-subunit to form the high affinity receptor complex. In mice, IL-3α can also associate with an IL-3-specific β-subunit. IL-4 binds a receptor complex composed of an IL-4 specific α and β subunit, as well as a γ subunits which is shared with IL-2R, IL-7R, IL-9R, IL-13R, and IL-15R.

The receptors of the hemopoietin cytokine family contain no enzymatic activity but function by recruiting and activating membrane-localized and cytosolic effectors. Of great importance in cytokine receptor signalling is tyrosine phosphorylation. All type I cytokines

(for example, IL-2, IL-3, IL-4, IL-5, GM-CSF and erythropoietin) activate some forms of the Janus kinase (Jak) family of tyrosine kinases. For example, Jak3 becomes activated by IL-2 and IL-4 signalling, while Jak2 is associated with the βc subunit of the receptor for IL-3, IL-5 and GM-CSF and becomes activated following ligand binding. Heterodimerization of receptor subunits results in trans-phosphorylation and activation of the Jak kinases, ultimately leading to the phosphorylation of tyrosine residues in the receptor subunits. This leads to the recruitment and tyrosine phosphorylation of receptor-associated proteins, including Shc, SHP-2, Grb2, and Vav. Recruitment of STAT (signal transducers and activators of transcription) proteins to phosphotyrosine residues targets C-terminal tyrosine residues in these molecules for phosphorylation by Jaks. This leads to bivalent homo- and heterodimerization of STAT's and translocation to the nucleus where they mediate transciptional events.

Considerable evidence points towards an essential role for Jak signalling in cytokine function (Argetsinger, et al., 1993; Witthuhn, et al., 1993, 1994; Miura, et al., 1993). Jaks bind to cytokine receptors via the Box1 and Box2 regions of the receptors (Miura, et al., 1993; Witthuhn, et al., 1993; DaSilva, et al., 1994). Genetic knockout experiments of Jak3 in mice results in severe immunodeficiencies, attributable to a loss of lymphoid cell production (Nosaka et al., 1995; Thomis, et al., 1995). Other protein tyrosine kinases have also been shown to associate with and become activated upon cytokine receptor binding. For the IL-3, IL-5 and GM-CSF family these include Lyn, Fes, Tec, Yes, Btk and Fyn (Torigoe et al., 1992; Hanazono et al., 1993; Corey et al., 1993; Sato et al., 1994).

Following tyrosine phosphorylation initiated by receptor activation, signalling molecules that contain phosphotyrosine binding structures, such as Src-homology (SH)-2 or phosphotyrosine binding (PTB) domains are recruited and assemble the receptor-proximal "signalsome" (reviewed by Pawson, 1995). Besides STATs, which are directly phosphorylated by Jaks, many other signalling proteins require localization to the plasma

membrane for activation. One such pathway is the Ras pathway. The cytosolic guanine-nucleotide exchange factor SOS must be brought close to membrane-tethered Ras, in order to promote the exchange of GDP for GTP on Ras and thereby enable Ras to bind to and activate downstream effectors. The best known target of GTP-Ras is the proto-oncogene Raf1, a serine-threonine kinase at the apex of a mitogen-activated protein kinase (MAPK) cascade, consisting of MEK, p44/42 Erk1/2 and $p90^{Rsk}$. Targets of this pathway include a number of nuclear transcription factors (reviewed by Ferrell, 1996; Denhardt, 1996). The Ras/Erk pathway and its mechanism of activation will be discussed in greater detail below.

Finally, another important class of signalling molecules activated by hemopoietic receptors is the family of lipid kinases that phosphorylate phosphatidylinositol on various hydroxyl residues of the inositol ring. The best known and most studied of these lipid kinases is phosphatidylinositol 3-kinase, which has been the major focus of my studies.

1.3.2. PI 3-kinase signalling

The phosphoinositide group of lipid second messengers has gathered much attention in recent years, as have the enzymes that regulate their synthesis. Of particular interest is phosphatidylinositol 3-kinase, a family of lipid kinases that have restricted substrate specificity to the D-3 position of phosphatidylinositol. Historically, these activities, along with a phosphoprotein of 85 kD, were isolated via their association with activated tyrosine kinase receptors, such as the PDGF receptor (Courtneidge and Heber, 1987). It was soon recognized that this PI kinase activity was responsible for producing a unique set of phosphoinositides (Whitman et al, 1988; Traynor-Kaplan et al., 1988), including PIP_3 and $PI(3,4)P_2$. $PI(3,4)P_2$ and PIP_3 have been implicated as major second messengers involved in signalling by virtually all growth factors (reviewed by Toker and Cantley, 1997; Duronio et al., 1998).

1.3.3. PI3K Isoforms, Structure and Tissue Distribution

Classical PI 3-kinase is a heterodimeric protein, comprised of an 85 kDa regulatory subunit, and a 110 kDa catalytic subunit (Hiles et al., 1992; Hu et al., 1993; Gout et al., 1992; Dhand et al., 1994). Each subunit has several possible isoforms. For the catalytic subunit, there are four classes; 1a, 1b, 2 and 3. In the class 1a there are three known isoforms: p110 α (Hiles et al., 1992), β (Hu et al., 1993), and δ (Vanhaesebroeck et al., 1997). The p110 α and β subunits are ubiquitous, whereas the δ isoform appears to be restricted to hemopoietic cells (Vanhaesebroeck et al., 1997). All three class 1a isoforms are closely related, and share very similar structural organization: the kinase domain is C-terminal while the Ras binding and p85 binding domains are N-terminal. Class 1b catalytic subunits are similar in sequence to class 1a subunits except for the absence of a p85 binding domain. Instead, these enzymes are regulated through interactions with the βγ subunits of heterotrimeric G-proteins (Stephens et al., 1997; Stoyanov et al., 1997). Class 2 and 3 catalytic subunits will not be discussed in detail here.

Regulation of the α, β, and δ isoforms of class 1a PI 3-kinase is modulated by a constitutively bound p85 subunit. The p85 subunit is expressed as two isoforms, α and β, which have a high degree of homology. The p85 subunit has no enzymatic activity but rather act as adapter proteins that contains two src-homology 2 (SH2) domains. SH2 domains function to couple the PI 3-kinase enzyme to tyrosine phosphorylated receptors, receptor associated proteins and cytosolic proteins containing the specific consensus sequences pYXXM or pYMXM (Songyang et al., 1993). The activity of p110 appears to be regulated by two factors, i) localization to the plasma membrane where its substrate is located (Kelly et al., 1993; Ricort et al., 1996; Nave et al., 1996), and ii) allosteric modifications by the p85 subunit once p85 is bound to tyrosine phosphorylated proteins (Herbst et al, 1994; Giorgetti et al., 1993). Association between the p85 and the p110 occurs between an N-terminal domain in the p110 subunit and a region of the p85 subunit localized between the two SH2 domains (termed the iSH2 region). How the p85 subunit

regulates p110 through this interaction is still poorly understood (Cohen et al., 1995). Other structures of note within the p85 subunit include two proline-rich regions, which may facilitate SH3 domain binding (Gout et al., 1992), a Bcr/Rac GTPase-activating protein (GAP) homology (BH) domain, and an N-terminal SH3 domain (Gout et al., 1992). The proline-rich regions and the SH3 domain may play important roles in the association of PI 3-kinase with other proteins, including dynamin (Gout et al., 1993), cbl (Hunter et al., 1997), p125Fak (Guinebault et al., 1995), Grb2 (Wang et al., 1995), α-actin (Shibaski et al., 1994) and Src family tyrosine kinases (Pleiman et al., 1994).

Once drawn to the plasma membrane in association with the p85 subunit, p110 can phosphorylate $PI(4,5)P_2$ to generate PIP_3. The accumulation of this lipid is tightly controlled by specific D3 and D5 phosphatases, including PTEN (Stambolic et al., 1998) and the SH2-domain containing 5'-phosphatase SHIP (Damen et al., 1996; Lioubin et al., 1996; Kavanaugh et al., 1996; Scheid et al., submitted), which dephosphorylates PIP_3, forming $PI(4,5)P_2$ and $PI(3,4)P_2$, respectively. SHIP and PTEN constitute an important class of negative regulators, by reducing the signal generated by PI 3-kinase. In general, both PIP_3 and $PI(3,4)P_2$ are believed to regulate downstream effectors of PI 3-kinase, although the relative importance of each may depend on the downstream enzyme. Clearly, the tight regulation of these lipid species, in contrast to PI(3)P which does not change during agonist stimulation (Gold et al., 1994), implies an important and specific role in downstream effector regulation.

PI 3-kinase derived phosphoinositides may activate downstream effectors by recruiting them to the plasma membrane. Several groups in 1993 characterized a novel protein motif (Haslam et al., 1993; Mayer et al., 1993), termed the pleckstrin homology (PH) domain. First recognized in a protein called pleckstrin, these domains have minimal primary sequence homology, but are similar in tertiary structure. By using computer programs that determine the predicted folding of known protein sequences, over 100 proteins have been suggested to have a PH domain (Musacchio et al., 1993; Gibson et al.,

1994; Saraste and Hyvonen, 1995). Some of the proteins shown to contain PH domains that can bind phosphoinositides include: β-spectrin (Macias et al., 1994), dynamin (Ferguson et al., 1994; Salim et al., 1996), son of sevenless (SOS; Wang et al., 1995), Bruton's tyrosine kinase (Btk; Salim et al., 1996), phospholipase C (PLC)-δ (Cifuentes et al., 1993; Lomasney et al., 1996), PKB, and 3-phosphoinositide-dependent kinase-1 (PDK1; Alessi et al., 1997; Stokoe et al,. 1997). The PH domains of these proteins differ in their affinity for the different phosphorylated phosphoinositides. For example, SOS has the greatest *in vitro* affinity for $PI(4,5)P_2$ (Kubiseski et al., 1997), while PDK1 and Btk appear to bind most strongly with PIP_3 (Alessi et al., 1997; Salim et al., 1996). This form of regulation clearly adds a great deal of complexity to cytokine signal transduction. Some enzymes may be continuously tethered to the plasma membrane by $PI(4,5)P_2$, while others may be recruited only upon the generation of PIP_3 or $PI(3,4)P_2$. Additional studies are required to demonstrate the *in vivo* specificity of PH domain binding with lipids. For example, a dominant negative mSos containing a point mutation that abolishes the *in vitro* interaction with $PI(4,5)P_2$ is still able to suppress Ras activation, implying that it can still function at the plasma membrane (Chen et al., 1997). Replacing the SOS PH domain with the PH domain of other $PI(4,5)P_2$ binding proteins is not sufficient for Ras activation, suggesting some specificity of PH domain function (de Mora et al., 1996). Similarly, point mutations in the PH domain of Btk, an important tyrosine kinase in B-cell development, are responsible for X-linked immunodeficiency in mice (Xid) and X-linked agammaglobulinaemia (XLA) in humans, again suggesting a very important role for the PH domain in this kinase (Salim et al., 1996). These point mutations reduce the affinity of the interaction between Btk and PIP_3, and, significantly, a genetic link between PI 3-kinase and Btk has recently been established in p85α gene knockout mice, which have an Xid-like phenotype (Suzuki et al., 1998; Fruman et al., 1998).

The binding of PIP_3 and $PI(3,4)P_2$ to PH domains may also induce conformational changes that lead to activation of the PH domain containing protein. For example, PKB

phosphorylation may require a conformational change induced by these lipids to make activating sites accessible to upstream kinases (Alessi and Cohen, 1998). This subject will be discussed in more detail below.

The role of PI 3-kinase activity in a variety of cell models has been examined using a number of pharmacological and molecular strategies. With the discovery of the fungal metabolite wortmannin as a potent, specific and irreversible inhibitor of PI3K (Arcaro and Wymann, 1993; Wymann et al., 1996), there was a bloom in the literature describing functional roles for PI3K. In order to verify an effect caused by wortmannin to be a result of PI3K inhibition, other tools are generally employed. For example, another unrelated inhibitor of PI 3-kinase was developed by a group at Eli Lilly who were screening compounds related to quercetin, itself a weak PI 3-kinase inhibitor. Their discovery of LY-294002 (Vlahos et al., 1994) proved to be most useful when used in conjunction with wortmannin. The corroborative effects of two unrelated compounds used at concentrations consistent with inhibition of PI 3-kinase provides confidence in the specificity of these drugs. Nevertheless, other targets of both of these drugs have been described. Wortmannin inhibits phospholipase D action (Bonser et al., 1991) and myosin light chain kinase (Nakanishi et al., 1992), although at concentrations several orders of magnitude higher than those required to inhibit PI 3-kinase. The activation of phospholipase A_2 also appears to be susceptible to wortmannin at low nanomolar concentrations (Cross et al., 1996). Although the specific target upstream of PLA_2 has not been identified, it may be the same enzyme that regulates wortmannin-sensitive Erk activation (Scheid and Duronio, 1996). Additionally, both wortmannin and LY294002 have been shown to block the activity of the mammalian target of rapamycin, mTOR, at concentrations similar to the IC_{50} of PI 3-kinase, although the biological significance of this, besides p70 S6 kinase inhibition, remains unexplored (Brunn et al., 1996; Abraham, 1998).

One molecular approach for modulating PI3K activity is to overexpress in cells a dominant negative form of the p85 subunit, which lacks the p110 binding domain, thereby

inhibiting endogenous PI 3-kinase by competing for phosphotyrosine docking sites (Hara et al., 1994). Another dominant negative approach involves expression of a mutated, catalytically-inactive p110 subunit which can bind p85 (Takayanai et al., 1996). In contrast, expression of a mutant p110 subunit engineered with a C-terminal myristolation signal and an N-terminal fragment of the p85 iSH2 region (p110*) results in constitutive PI 3-kinase activity independent of p85 regulation (Hu et al., 1995). Use of this reagent has allowed the identification of potential downstream targets of PI 3-kinase. However, caution should be used when interpreting these results since pathways activated secondarily, as a result of cytokine or prostaglandin secretion due to uncontrolled PI 3-kinase activity, can never be discounted. A more specific approach has recently been described (Klippel et al., 1998). Expression of a chimeric protein comprised of the p110 subunit and the estrogen receptor allows induction of PI 3-kinase activity by adding a cell permeable estrogen analog, 4-hydroxytamoxifen (4-OHT). Addition of 4-OHT leads to increased PI 3-kinase activity that is relatively rapid (within minutes) compared with the p110* construct described above (many hours).

1.3.4. PI 3-kinase and Apoptosis

1.3.4.1 Growth factor stimulated survival pathways

Signalling by members of the hemopoietin family provide an anti-apoptotic signal. Following removal of cytokine, activation of an apoptotic program is initiated in the cells. The proteins of the signalling pathway that mediate survival were not well understood when this work was initiated. Work by Yao and Cooper (1995) showed that NGF-mediated survival of PC12 cells (a neuronal cell line) required PI 3-kinase activity. This survival signal was dissociated from activation of the Ras/Erk pathway because expression of a dominant negative Ras did not prevent survival. Our group demonstrated that hemopoietic growth factors require PI 3-kinase activity for survival, also independently of $p21^{ras}$ (Scheid et al., 1995), although there are exceptions to this rule depending upon the

stimuli. For example, GM-CSF promotes survival but inhibiting PI 3-kinase does not block this effect (Scheid et al., 1995). All of these results are presented in this thesis, along with subsequent studies evaluating several potential mediators downstream of PI 3-kinase. Additional support for a role of PI 3-kinase in inhibition of apoptosis in hemopoietic cells came from Minshall et al., (1996) and Parrizas et al., (1997), who both showed that IGF-1 activation of PI 3-kinase provides a protective signal.

There was some logical justification prior to these studies to suggest that PI 3-kinase might be critical in signalling for survival. As described in the preceding section, PI 3-kinase is a conserved enzyme expressed ubiquitously in multicellular organisms. Phosphatidylinositol signalling has also been demonstrated in such lower and ancient eukaryotes as yeast and dictyostelium. More importantly, PI 3-kinase is activated by all mitogenic and survival-promoting agonists, via tyrosine phosphorylation of growth factor receptors or through the actions of G-protein-coupled receptors (Duronio et al., 1998). Furthermore, activation of PI 3-kinase activity, and the metabolism of the PI lipids, is tightly regulated - inhibition of the enzyme or decoupling from the receptor causes a rapid decrease in 3'-phosphorylated PI lipids to basal levels, presumably through the action of specific PI phosphatases. Thus, when the need arises to eliminate unwanted or immunologically dangerous cells, removal of growth factor from the extracellular environment will quickly turn off the pro-survival, PI 3-kinase-generated signal. Additionally, as discussed above, many cytosolic signalling proteins (perhaps hundreds) contain PH domains, which function in the recruitment of these enzymes and adapters to PIP_3 and $PI(3,4)P_2$ generated by PI 3-kinase, as well as the other phosphoinositides, such as $PI(4,5)P_2$ (Rebecchi and Scarlata, 1998). Finally, new evidence suggests that the transforming efficiency of various oncogenes requires PI 3-kinase activation, perhaps to allow survival during the establishment of a transformed phenotype (Skorski et al., 1997). Although it will be discussed below in more detail, a particular downstream target of PI 3-

kinase signals, PKB, has been demonstrated to be itself an important mediator of survival signals, further establishing an important role for PI 3-kinase activity.

Recently, there have been several examples where PI 3-kinase has been identified as a proto-oncogene. The retrovirus ASV-16 induces sarcomas in chickens and within its genome is an oncogene which has PI 3-kinase activity, called v-p3k (Chang et al., 1997). Expression of v-p3k in chicken embryo fibroblasts induced elevated levels of both PIP_3 and $PI(3,4)P_2$ lipids as well as imparting a transformed phenotype. Recently, a mutant regulatory domain of a PI 3-kinase has been identified in human cells which contains the first 571 residues of the p85α subunit linked with a region conserved in the eph tyrosine kinase receptor family (Jimenez et al., 1998). This mutant appears to induce constitutive PI 3-kinase activity, providing mitogenic signalling in the absence of ligand activation. An incidence of a human cancer which contains similar activating mutations has yet to be found, and the role for PI 3-kinase as a bona-fide oncogene has yet to be confirmed. Interestingly, loss of the tumor suppressor PTEN, which has been reported to have PIP_3 3'-phosphatase activity, results in increased levels of PIP_3 and increased tumor incidence in mice, which can be reversed with retroviral transfer of the functional gene (Stambolic et al, 1998).

1.3.4.2. Anoikis (*Homelessness*)

There are also other situations in which PI 3-kinase mediated survival may be important. Many cells (for example epithelial, endothelial and fibroblast) adhere to an extracellular matrix (ECM), such as vitronectin, fibronectin and collagen, and this matrix may act as a survival factor (Meredith et al., 1993; Frisch and Francis, 1994). The cell surface molecule that mediate the attachment to the ECM include heterodimeric receptor complexes called integrins. Binding of integrins to the ECM induces signalling pathways very similar to those of growth factor receptors, including induction of tyrosine phosphorylation, principally by c-Src and pp125Fak (reviewed by Frisch and Ruoslahti,

1997). These tyrosine phosphorylation events recruit SH2 domain containing proteins, among others, leading to the activation of a plethora of pathways, including those mediated by p21ras and PI 3-kinase. Various oncogenes can bypass the requirement for ECM:integrin binding and confer anchorage-independent growth and survival, which is a critical step in tumorogenesis and metastasis (Stoker et al., 1968). Significantly, loss of attachment to the ECM results in a default pathway leading to both growth arrest and apoptosis, and in this way, dangerous cells which detach from the ECM are eliminated. This form of apoptosis has been termed "anoikis", the Greek word for homelessness (Frisch and Francis, 1994).

Currently, there is intense investigation into the roles of various signalling pathways that prevent anoikis. Overexpression of constitutively activated forms of p125Fak are able to rescue epithelial cell lines from anoikis. An active Fak kinase domain is required, demonstrating that tyrosine phosphorylation is important in mediating the pro-survival signal (Frisch et al., 1996a). An important role for the c-Jun N-terminal kinase, JNK, has been suggested based on the finding that JNK activity increases during anoikis. Consistent with this idea, expression of dominant-negative mutants of the kinase partially attenuated anoikis (Frisch et al., 1996b). Paradoxically, JNK activation appears to be downstream of Bcl-2-regulated caspase activity, such that caspase inhibitors or the viral protein crmA block anoikis and JNK activation (Frisch et al., 1996b). This was later suggested to be a result of MEKK-1 (an upstream activator of the JNK kinase SEK1) cleavage and activation by caspases (Cardone et al., 1997). A lack of correlation between JNK activation and anoikis was reported by Khwaja and Downward (1997), who provided evidence for a protective role for PI 3-kinase (Khwaja et al., 1997). Loss of attachment with the ECM results in a decrease in PI 3-kinase activity. Expression of a constitutively active p110 catalytic domain, membrane localized v-Akt (a viral form of PKB), or expression of Ras mutants that activate PI 3-kinase but not Raf/MEK/MAPK all suppress anoikis (Khwaja et al., 1997).

MEKK-1 cleavage and JNK activation may fit into a model which also involves PI 3-kinase/PKB. If PKB activity normally prevents caspase activity when bound to the matrix, then loss of this signal may result in MEKK-1 cleavage, thus acting as a positive feedback loop to increase JNK activity and further potentiate apoptosis. Expression of dominant negative JNK's or mutated MEKK-1's were found to only delay or partially induce anoikis under various conditions (Cardone et al., 1997), suggesting that loss of PKB/PI 3-kinase may be more crucial in the induction of anoikis.

In light of our findings, as well as those of Yao and Cooper, a role for PI 3-kinase in growth factor-suppressed apoptosis appeared likely in the autumn of 1995. Over the next four years, a large body of work by many groups established in various model systems a requirement for PI 3-kinase in anti-apoptotic signalling, including the ECM:integrin example described above. Moreover, at least one downstream target of PI 3-kinase, PKB, has been implicated as a necessary component of this survival pathway. The next few sections of this review will examine PKB and other downstream targets of PI 3-kinase as well as the evidence placing PI 3-kinase at the head of a widely conserved survival pathway.

1.3.5. Downstream targets of PI 3-kinase

1.3.5.1. PKB/Akt

When Protein kinase B (PKB) was cloned, it was recognized to be similar to members of both the cAMP-dependent kinase and protein-kinase-C families (Coffer and Woodgett, 1991; Jones et al., 1991). It was also recognized to be the cellular homologue of v-Akt, a protein expressed in rodent T-cell lymphoma caused by the AKT-8 acute transforming retrovirus (Bellacosa et al., 1991). There are three isoforms of PKB, α, β and γ. All are closely related in sequence, and contain a PH domain N-terminal to the catalytic domain. PKBα and β are expressed widely, while PKB γ is restricted to brain,

testes, heart, spleen, lung and skeletal muscle (Coffer and Woodgett, 1991; Jones et al., 1991; Bellacosa et al., 1993).

Generation of 3'-phosphorylated phosphatidylinositols produces a wide range of cellular responses, yet relatively few downstream targets have been identified that are directly regulated by these lipids. As mentioned above, recent studies have demonstrated the importance of pleckstrin-homology (PH) domains in binding with PtdIns. PH domains resemble SH2 domains structurally and may bind electrostatically with the negatively charged inositol head groups of PIP_3 and $PI(3,4)P_2$ at the plasma membrane. PKB has been shown to have affinity for both PIP_3 and $PI(3,4)P_2$ *in vitro*. Several observations have led to the widely held idea that PKB is an *in vivo* target of PI 3-kinase. First, mutation of several tyrosine residues in the PDGF receptor (Y740 and Y751) to alanine abrogated both PI 3-kinase binding and PKB activation, although the Ras/Erk pathway remained functional (Franke et al., 1995). Secondly, blocking PI 3-kinase with inhibitors or dominant negative constructs prevent PKB activation (Burgering and Coffer, 1995). Finally, addition of $PI(3,4)P_2$ directly to cells induces activation of PKB (Franke et al., 1997).

Initially it was thought that PKB was the direct target of PI 3-kinase activity via activation by 3'-phosphorylated lipids (Frech et al., 1996; Klippel et al., 1997; Franke et al., 1997). The model of activation became more complex with the finding that several residues within the PKBα enzyme (Thr308 and Ser473) required phosphorylation for full enzymatic activity, and dephosphorylation of PKB rendered the kinase inactive (Alessi et al., 1996). As well, mutation of Thr308 or Ser473 to alanine abolished the activation of PKB by several ligands, while mutation of these residues to aspartic acid, to introduce a negative charge and mimic the effect of phosphorylation, produced a partially active kinase (Alessi et al., 1996). Thus it seemed unlikely that phosphoinositide binding alone could account for full activation. It remained a possibility that PKB binding to phosphoinositide

lipids induced autophosphorylation, but an equally possible scenario involved transphosphorylation by another protein kinase.

It was soon recognized that the phosphorylation on both Thr308 and Ser473 were mediated by kinases that are themselves targets of PI 3-kinase activity. Termed phosphoinositide dependent kinases, PDKs, these protein kinases also contain PH-domains and are targeted to the plasma membrane by PI 3-kinase-generated lipids (Alessi et al., 1997a and b; Stokoe et al., 1997; Stephens et al., 1998). *In vitro*, PIP_3 or $PI(3,4)P_2$ greatly enhances the phosphorylation of PKB by PDK1 (Alessi et al., 1997a), suggesting that PDK1 requires these lipids for *in vivo* activation. Later PDK1 was shown to be constitutively active, and it was the regulation of PKB by PIP_3 which was important: the PH domain of PKB must bind with PIP_3 or $PI(3,4)P_2$ to confer a structural change to allow access of PDK1 to Thr308 (Alessi et al., 1997b; Stephens et al., 1998). Thus, mutant forms of PKB which cannot bind with PIP_3 cannot be activated by PDK1 (Stokoe et al., 1997). Conversely, PKB mutants lacking a PH domain are phosphorylated by PDK1 in the absence of lipid (Stokoe et al., 1997; Alessi et al, 1997b). The identity of the Ser473 kinase remains unknown, although ILK (integrin-linked kinase) has been demonstrated to phosphorylate S473 *in vitro* and in transfected systems (Delcommenne et al., 1998). This model of activation is depicted in Figure 1.2.

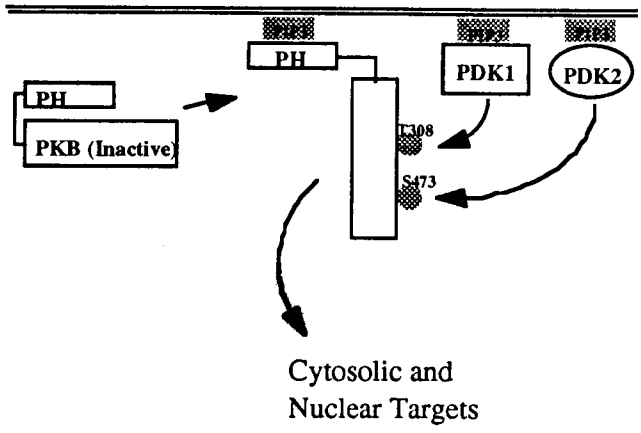


Figure 1.2. Activation of PKB by PDK1 and PDK2. PKB is normally cytosolic, and held in an inactive form requiring PIP_3 or $PI(3,4)P_2$ binding with the PH domain, which

renders the kinase susceptible to phosphorylation by PDK1 and PDK2. PDK1 is co-localized with PKB through interaction with PIP_3 via its PH domain, but is otherwise constitutively active. PDK2 remains to be cloned.

Subsequent to the first studies in 1995 showing that PI 3-kinase may be important in the prevention of apoptosis, several reports investigated the role of PKB in mediating the pro-survival activity of PI 3-kinase. Previous evidence had suggested that PKB may be an oncogene, which when overexpressed may contribute to tumorogenicity in ovarian and pancreatic carcinomas (Cheng et al., 1992; Cheng et al., 1996). In a report by Dudek and coworkers (1997), transfected mutant forms of PKB which blocked the activation of endogenous PKB promoted apoptosis. Conversely, expression of a membrane targeted PKB enhanced the ability of PDGF to promote survival. A role for PKB in survival by other cytokines has been demonstrated, including IGF-1 (Kulik et al., 1997), IL-2 (Ahmed et al., 1997), NGF (Ulrich et al., 1998), and IL-3 (Songyang et al., 1997). Furthermore it has been shown that PI 3-kinase/PKB signals provide protection from various pro-apoptotic stimuli, including c-Myc overexpression (Kauffmann-Zeh et al., 1997), Fas-induced cell killing (Hausler et al., 1998), UV irradiation (Kulik et al,. 1997), and matrix detachment ("anoikis", Khwaja et al., 1997). Viruses such at the polyomavirus may also utilize the PI 3-kinase/PKB pathway to support survival of infected cells (Dahl et al., 1998).

Targeted disruption studies also support a role for PKB in suppression of apoptosis. *Drosophila* with a single point mutation in a portion of the PKB gene encoding the catalytic domain which renders the kinase inactive, are embryonically lethal, and exhibit ectopic apoptosis (Staveley et al., 1998). Expression of an inhibitor of apoptosis protein (p35), protected these cells, demonstrating the specific activation of an apoptosis cascade during the loss of PKB signalling. Moreover, transgenic expression of wild type PKB can rescue the phenotype. In another model, transgene experiments where the pro-apoptotic protein Hid was targeted to the *Drosophila* eye, PI 3-kinase/PKB activation by Ras mutants

unable to signal to MAPK resulted in partial rescue of the eye ablation phenotype (Bergmann et al, 1998).

1.3.5.2. Mitogen Activated Protein Kinases ($p42^{erk1}$ and $p44^{erk2}$)

The mitogen activated protein kinases are a group of ubiquitously expressed serine/threonine kinases which belong to a linear cascade of signalling molecules activated by a wide array of stimuli. In my work, I have focused on two of the best characterized members of the mitogen activate protein (MAP) kinase family, $p44^{erk1}$ and $p42^{erk2}$. These kinases where originally described as proteins that undergo tyrosine phosphorylation in response to growth factors (Ray and Sturgill, 1987; Rossomando et al., 1989). Purification attempts from growth factor stimulated cell lysates and other models revealed that two phosphoproteins, p42 and p44, had protein kinase activity towards microtubule associated protein 2 (MAP2; Ray and Sturgill, 1987) and myelin basic protein (MBP) and were activated by a wide assortment of mitogenic stimuli (Cicirelli et al., 1988; Pelech et al., 1988; Sanghera et al., 1990; Boulton et al., 1991; Northwood et al., 1991). These two related kinases were later named extracellular regulated kinase (Erk) 1 and 2.

Erk MAP kinases require phosphorylation on threonine and tyrosine residues to become fully active (Ray and Sturgill, 1988; Sturgill et al., 1988; Hanks et al., 1988; Anderson et al., 1990; Pollack et al., 1991). Murine $p42^{erk2}$ phosphorylation was mapped to Thr-183 and Tyr-185 (Thr-202 and Tyr-204 in Erk1; Payne et al., 1991). These phospho-acceptor sites lie on either side of a conserved Glu, and this structural motif (TXY) is characteristic of all MAP kinase family members (reviewed by Ferrell, 1996). Conformational changes revealed using X-ray crystallography demonstrated that phosphorylation is required to increase enzymatic activity (Johnson et al., 1996; Canagarajah et al., 1997).

In purification experiments measuring Erk activation, a set of tyrosine/threonine activator kinases were isolated (Crews and Erikson, 1992; Nakielny et al., 1992; Matsuda

et al., 1992; Zheng and Guan, 1993). These MAP kinase kinases, termed MEK1 and MEK2 (for MAPK/Erk kinase) share 80% identity at the amino acid level. $p44^{erk1}$ and $p42^{erk2}$ are the only known *in vivo* substrates for MEK1 and MEK2, which appear to have a very specific substrate specificity. MEK proteins also contain a short amino-terminal region (residues 32-44) which facilitates export of MEK from the nucleus into the cytoplasm and has been termed the NES (nuclear export signal; Fukuda et al., 1997). Interestingly, following stimulation, both Erk and MEK translocate to the nucleus, shortly after which MEK is redistributed back into the cytosol. Mutation of the MEK NES sequence allowed Erk to diffuse passively into the nucleus, suggesting MEK also functions to anchor Erk in the cytoplasm. This is consistent with the observation that Erk and MEK specifically associate through a short 32 amino acid Erk-binding site peptide located at the amino-terminal of MEK (Fukada et al., 1997).

Several lines of evidence 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 normally resides at the plasma membrane in an inactive GDP-bound form. The cellular concentration of GTP is higher than GDP, but the dissociation of GDP from Ras is rate-limiting. Receptor activation leads to the exchange of GDP for GTP on Ras catalyzed by the nucleotide exchange protein Sos. Mutations of Ras which render it constitutively active (Val12) can rapidly activate Erk. Conversely, a dominant interfering Ras (Asn17) blocks growth factor activation of Erk2 (de Vries-Smits et al., 1992). Thus, the activation of Erk and its immediate kinase MEK, appear to be under the control of Ras.

It has also been demonstrated that Raf is an upstream component of Erk activation. Overexpressing activated forms of Raf1 in 3T3 cells leads to the activation of MAP kinases in a Ras-independent manner (Kyriakis et al., 1992; Dent et al., 1992; Howe et al., 1992). In addition, Raf1 can phosphorylate MEK on two residues, Ser218 and Ser222, leading to activation of this kinase. Ras GTP loading at the plasma membrane stimulates association with Raf and stimulates an increase in Raf kinase activity through a poorly defined

mechanism (Warne et al., 1993; Moodie et al., 1993; Vojtek et al., 1993). These signalling events collectively form the linear activation pathway of Ras -> Raf -> MEK -> Erk.

There is considerable evidence that cytokine receptors signal through this pathway to activate Erk. IL-3, GM-CSF and IL-5 rapidly induce Ras and Raf activation (Satoh et al., 1991; Duronio et al., 1991; Carrol et al., 1990; Kanakura et al., 1991). Deletion mapping of the βc receptor subunit revealed that a region between amino acids 544 and 763 is required for Ras/MAPK activation (Sato et al., 1993; Itoh et al., 1996). The critical tyrosine necessary for this function is probably Tyr-577, which is essential for Shc phosphorylation and association with the βc (Pratt et al., 1996; Sato et al., 1994; Inhorn et al., 1995). Mutation of Tyr-577 does not completely abolish MAPK activation (Durstin et al., 1996), which may be attributable to Grb2/Sos binding with SHP2 (Pazdrak et al., 1997). SHP2 associates with the βc through Tyr-612 (Pazdrak et al., 1997; Bone et al., 1997).

PI 3-kinase has been proposed to be an upstream regulator of MEK and Erk. Work by Karnitz et al. (1995) showed that wortmannin could partially inhibit IL-2-stimulated Erk activation, by reducing the activation of MEK, while having no effect on Ras or Raf activation. Similar findings using wortmannin have also been reported in other systems (Welsh et al., 1994; Cross et al., 1994; Sakanaka et al., 1994). Ferby et al. (1994) also reported a dependence on PI 3-kinase for PAF-induced Erk activation. Another inhibitor of PI 3-kinase, LY-294002, was also successful at reducing Erk activation, although doses higher than those required to inhibit PI 3-kinase were required. Hu et al. (1995) demonstrated that membrane-targeted, constitutively active p110 led to the activation of Ras and Erk, although the caveat of autocrine activation was not ruled out. Together these studies suggest that Erk lies downstream of PI 3-kinase.

This raises the question of whether PI 3-kinase promotes survival by activating Erk. Efforts to demonstrate a clear link between Erk activation and survival have been controversial. Some studies with mutant receptors deficient in Ras activation have shown

that the Ras pathway supports survival (Kinoshita et al., 1995). Introduction of active Ras mutants restores survival promoted by GM-CSF or IL-3. Complicating these observations are findings that Ras also leads to PI 3-kinase activation, (Rodriguez-Viciana et al., 1994), and survival was later attributed partially to the activation of this kinase rather than activation of other downstream effectors of Ras (Kinoshita et al., 1997; Kauffmann-Zeh et al., 1997).

As well, overexpression of constitutively active regulators of Erk have been used to assess the role of Erk in survival. Raf overexpression provides protection from growth factor withdrawal (Cleveland et al., 1994; Kinoshita et al., 1997). However, this mechanism may not involve Erk. Rather, Raf may undergo translocation to the mitochondria by binding to Bcl-2, and then phosphorylating Bad (Wang et al., 1994; 1996). The significance of Bad phosphorylation will be discussed below. Alternately, transient expression of a dominant negative Raf mutant suppresses IL-3-mediated survival in BaF_3 cells (Perkins et al., 1996), perhaps through a similar mechanism. In contrast, overexpression of Ras mutants deficient in PI 3-kinase activation, as well as overexpression of membrane targeted Raf, activates MEK and Erk but are ineffective in suppressing anoikis in detached MDCK cells (Khwaja et al., 1997). The MEK inhibitor PD98059 has also provided conflicting evidence in assessing the role of Erk activation in survival. In some studies, PD98059 had no effect on survival induced by cytokines (Scheid and Duronio, 1998) while in other systems it induced apoptosis in PC-12 cells incubated with NGF or IGF-1 (Xia et al., 1995; Parrizas et al., 1996). The requirement for Erk signalling may be more prevalent in neuronal cells, in which these later studies were performed.

Results from our laboratory (Scheid and Duronio, 1996), that are also presented in this thesis, provide some advances in these areas. We found that PI 3-kinase activity was neither sufficient nor necessary for the full activation of Erk. Furthermore, activation of Erk

was found not to be involved in cytokine-mediated survival. These results are outline in Chapter 4.

1.3.5.3. p70 S6 Kinase

Another protein that has been described as an *in vivo* target of PI 3-kinase activity is the S6 ribosomal protein kinase p70. This serine/threonine kinase has been shown to phosphorylate key residues in the S6 subunit of the 40S ribosome, which is necessary for translational initiation, protein synthesis and S cycle entry (Lane et al., 1993; Reinhard et al., 1994). p70 S6 kinase is required in part for the translational control of mRNA transcripts which contain start site-polypyrimidine tracts (Jefferies et al., 1997). Many of these transcripts encode proteins involved in the translation machinery, and thus increase overall translation.

There is strong evidence that p70 S6 kinase is downstream of PI 3-kinase. Inhibition of PI 3-kinase by either i) dominant negative p110 subunits, ii) mutated receptors which can no longer bind p85, or iii) pharmacological inhibitors of PI 3-kinase all abrogate p70 S6 kinase activity (Cheatham et al., 1994; Chung et al., 1994; Ming et al., 1994; Weng et al., 1995). Furthermore, the p110*-ER mutant described earlier results in immediate p70 S6 kinase phosphorylation with addition of 4-hydroxytamoxifen (Klippel et al., 1998).

Eight serine/threonine residues in the catalytic domain and linker regions of p70 S6 kinase undergo phosphorylation and this is associated with an increase in p70 S6 kinase activity (Pullen and Thomas, 1997). These sites are Thr229, Ser371, Thr389, Ser404, Ser411, Ser418, Thr421 and Ser424. Phosphorylation of Thr389 is wortmannin sensitive and PKB may be the physiological kinase (Dennis et al., 1996). Phosphorylation of Thr389 is absolutely necessary for Thr229 phosphorylation (Pullen et al., 1998), which relieves intramolecular constraints to allow access for a Thr229 kinase to its target. Phosphorylation of Thr229 is catalyzed by a constitutively active, wortmannin-insensitive kinase (Dennis et al., 1996). Recent work by the Thomas group has identified PDK1 as the

potential Thr229 kinase (Pullen et al., 1998). Thr229 is analogous to Thr308 in PKB, which is also phosphorylated by PDK1 (Alessi et al., 1997a; Stokoe et al., 1997). Phosphorylation of Thr389 of p70 S6 kinase is sensitive to the immunosuppressant rapamycin (Chung et al., 1992), which functions by binding and inhibiting the mammalian target of rapamycin, mTOR (Pullen and Thomas, 1997). This renders p70 S6 kinase susceptible to inactivating phosphatases (Pullen and Thomas, 1997; Dennis et al., 1996). A schematic of this three step activation model is given in Figure 1.3.

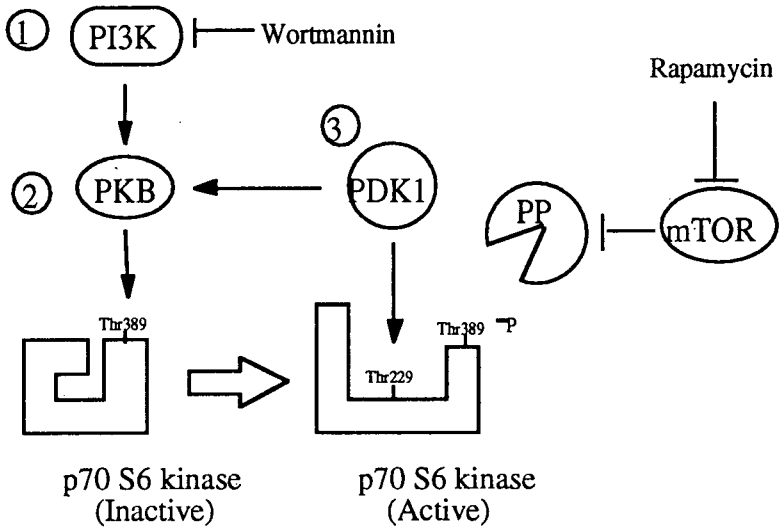


Figure 1.3. The proposed involvement of PKB and PDK1 in p70 S6 kinase activation. PP represents protein phosphatases.

Since p70 S6 kinase may be a critical regulator of the translation of many different proteins, and given that PI 3-kinase appears to be an upstream activator of p70 S6 kinase, we and others investigated the role of p70 S6 kinase in inhibition of apoptosis. Our group (Scheid et al., 1996), Yao and Cooper (1996) and Kauffmann-Zeh et al. (1997) all demonstrated that inhibition of mTOR and p70 S6 kinase (indirectly) with rapamycin had no effect on growth factor mediated survival or PKB-mediated protection from c-Myc overexpression. The details of these experiments are presented in Chapter 5.

1.3.5.4. Glycogen synthase kinase-3

Phosphorylation of glycogen synthase by glycogen synthase kinase-3 (GSK-3) renders the enzyme inactive and inhibits the net synthesis of glycogen from glucose, a critical step in glycogenesis (He et al., 1995). When insulin and other growth factors stimulate GSK-3 phosphorylation and inactivate GSK-3, glycogen synthase activity increases and glycogen is synthesized more rapidly. GSK-3 and its *Drosophila* and *Xenopus* homologues SHAGGY and Xgsk-3 are also important in polarity determination, as a component of the Wnt signalling pathway. In this context, GSK-3 phosphorylates and destabilizes β -catenin, reducing its transcriptional activity (Ikeda et al., 1998).

GSK-3 has been demonstrated to phosphorylate a diverse collection of other cellular targets (Welsh et al., 1996), including translation initiation factor eIF-2B (Welsh et al., 1998) and several transcription factors, including c-Jun, the $p90^{rsk}$ protein kinase, and the nuclear transcription factors NF-AT and CREB (Wang et al., 1996; Beals et al., 1997; Foil et al., 1994). With such a diverse array of cellular targets, it is not surprising that GSK-3 has an important role in cell fate and development in organisms such as *Dictyostelium*, *Drosophila* and *Xenopus* (Harwood et al., 1995; Siegfied et al., 1992; He et al., 1995; Pierce and Kimelman, 1995; Dominguez et al., 1995). This predicts that GSK-3 will have similar importance in mammalian development.

A connection between GSK-3 and PI 3-kinase activity has been suggested, since inhibitors of PI 3-kinase block GSK-3 phosphorylation (Welsh et al., 1994; Cross et al., 1994; Hurel et al., 1996). The upstream kinase that phosphorylates GSK-3 could be PKB, since PKB can phosphorylate serine-21 in GSK-3α or the equivalent site of serine-9 in GSK-3β (Cross et al., 1995; van Weeren et al., 1998). Additional regulatory phosphorylations occur on GSK-3, both activating and inactivating, at tyrosine and other serine/threonine residues. Besides input from a PI 3-kinase/PKB signalling branch, some PKC isoforms and $p90^{rsk}$ activities may also lead to GSK-3 phosphorylation (Welsh et al.,

1994; Eldar-Finkelman et al., 1995). Clearly, GSK-3 is a node for a multitude of signalling pathways.

Recent work has suggested that the pro-survival function of PI 3-kinase may be mediated through the inactivation of GSK-3 (Pap and Cooper, 1998). In this study, the authors demonstrate that expression of a constitutively active form of GSK-3 leads to apoptosis in the expressed cells. Conversely, over-expression of a dominant-negative form of GSK-3 rescues cells from apoptosis following PI 3-kinase inhibition by either pharmacological inhibition (LY-294002) or expression of a dominant-negative PI 3-kinase. Additionally, over-expression of wildtype p53 induces apoptosis that can be blocked by a dominant negative GSK-3. In contrast, a mutant p53, which acts as a repressor of endogenous p53, prevents GSK-3 mediated cell death.

Clearly, these data implicate GSK-3 as an important, *positive* inducer of apoptosis. Phosphorylation of downstream targets of GSK-3 may be responsible for initiating apoptosis. For example, GSK-3 may catalyze the phosphorylation of CREB on an activating site distinct from Ser133, the target of PKA and MAPKAP kinase-2 (Fiol et al., 1994). CREB phosphorylation and transcriptional activity are modulated by a diverse array of both positive and negative survival stimuli, and may have a role in apoptosis under specific circumstances (Scheid et al., 1999).

1.3.5.5. Bcl-X_L-associated death inducer (Bad)

Downstream targets of PKB which may be involved in prevention of apoptosis are currently the subject of intense investigation. As discussed above, GSK-3 may be a very important target. Also, another possible target was identified in 1995 (Yang et al., 1995). Using yeast two-hybrid analysis to search for Bcl-X_L associating proteins, researchers identified and cloned Bad (Bcl-X_L-associated death inducer), which when overexpressed blocked IL-3-induced survival. Further analysis revealed that Bad is phosphorylated during stimulation by unidentified kinases. Moreover, experiments using trasfected Bad showed

that this phosphorylation occurs at two sites - serine 112 and 136 (Zha et al., 1996). Sequences surrounding these sites match with the consensus binding sites for 14-3-3 proteins. Phosphorylation of Bad on these residues promotes association with cytosolic 14-3-3 and dissociation from membrane bound Bcl-X_L. Furthermore, mutation of Bad at these serine residues abolishes its ability to bind with 14-3-3 and restricts Bad localization to Bcl-X_L-localized compartments (Zha et al., 1996). The theory therefore developed that growth factor-stimulated phosphorylation of Bad provides a mechanism for protection against apoptosis. By sequestering Bad to the cytosol, Bcl-X_L can perform its pro-survival function. When Bcl-X_L is bound to Bad, the anti-apoptotic functions of Bcl-X_L is inhibited, and apoptosis proceeds.

Efforts to establish a link between the pro-survival role of PI 3-kinase and the control of Bad in 1997 and in early 1998 by three groups provided evidence that PKB was potentially the kinase that phosphorylates Bad on serine 136 following growth factor stimulation. del Peso et al. (1997) demonstrated that over-expressed AU1-tagged Bad could be phosphorylated by IL-3 via a PI 3-kinase dependent mechanism. Overexpression in the same cells of activated or dominant negative PKB mutants induced or inhibited AU1-Bad phosphorylation, respectively. Interestingly, Bad phosphorylation in their model was increased more than 10 fold by IL-3 stimulation and this effect was completely blocked in the absence of PI 3-kinase signalling. This would suggest that the vast majority of Bad phosphorylation was occurring on PI 3-kinase targeted sites. Datta et al. (1997), performed similar experiments, again using overexpressed, membrane targeted PKB mutants and epitope tagged Bad. This group showed that under these conditions PKB was targeting serine 136 and not serine 112. Blume-Jensen (1998) used human embryonic kidney 293 cells overexpressing murine wild-type or mutant c-Kit to demonstrate that overexpressed murine Bad was phosphorylated by overexpressed PKB at serine 136. The phosphorylation site surrounding Ser136 resembles in primary sequence the motif required for efficient phosphorylation of peptide substrates by PKB (Alessi et al., 1996a).

Comparisons of these regions in Bad, GSK-3, and 6-phosphofructo 2-kinase are made in Figure 1.4.

GSK3a	Arg-Ala-Arg-Thr-Ser-**Ser**-Phe	(Ser 21)
GSK3b	Arg-Pro-Arg-Thr-Ser-**Ser**-Phe	(Ser 9)
PFK-2	Arg-Pro-Arg-Asn-Tyr-**Ser**-Val	(Ser 483)
BAD	Arg-Gly-Arg-Ser-Arg-**Ser**-Ala	(Ser 136)

Figure 1.4. Sequence comparisons for potential *in vivo* targets of PKB. The serine residue which undergoes phosphorylation is shown in boldface, with its corresponding position in the protein shown in parentheses.

However, the case for a role for PKB in Bad phosphorylation has not been fully established. As described above, all of the work attempting to establish a role for PKB in Bad phosphorylation has utilized transfected mutants of PKB and transfected, epitope-tagged Bad. In none of these studies has endogenous Bad phosphorylation by endogenous PKB activity been demonstrated. Overexpression of Bad may alter its susceptibility to PKB, an observation that is supported by the results of the three papers themselves. In each case, Bad phosphorylation was stimulated by agonists, while the extent of phosphorylation of Ser136 (the residue phosphorylated by PKB) varied significantly. Therefore, a closer examination of the upstream kinases that phosphorylate endogenous Bad is required before any definitive statement can be made regarding the role of PKB in Bad phosphorylation. In studies from our laboratory, PKB activity was dissociated from Bad phosphorylation, since IL-4 could not stimulate Bad phosphorylation, even though it could activate PI 3-kinase and PKB (Scheid and Duronio, 1998). Additionally, GM-CSF stimulation led to Bad phosphorylation in the absence of PI 3-kinase or PKB activation. These experiments will be presented and discussed in Chapter 5.

1.4. OBJECTIVES

The studies that will be described in this thesis were undertaken with the following objectives:

1. To determine whether PI 3-kinase plays a role in cytokine-mediated cell survival.

2. If PI 3-kinase plays a role in survival, it would be necessary to determine the relevant downstream targets which relay the survival signal. At the time these studies were initiated, few targets downstream of PI 3-kinase signalling were known, but one was p70 S6 kinase. Determining the importance of p70 S6 kinase in survival became important as that PI 3-kinase plays a role in survival became clear.

3. There was also little known about the crosstalk between the Ras/Erk and PI 3-kinase pathways. One of the goals of my research was to investigate the relative importance of Erk activation in survival and the role of PI 3-kinase in this event.

4. A potential target of PI 3-kinase signals was the Bcl-2 family member Bad, which was shown to be an important element of the survival pathway. Bad phosphorylation negatively regulates its death-promoting activity. One objective therefore was to characterize the role PI 3-kinase plays in Bad phosphorylation. Other signalling pathways to Bad may also occur and these are also important events to characterize.

5. The PI 3-kinase generated lipid products may be important regulators of apoptosis, so an understanding of the metabolic pathways which directly regulate the levels of these lipids is important. One element of this regulation is the 5' inositol phosphatase SHIP, and an objective of this work was to assess the relative importance for SHIP for controlling the levels of PI 3-kinase generated PIP_3 and $PI(3,4)P_2$.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals and their sources

Acetic Acid	Fisher Scientific
Acrylamide	BioRad
Adenosine 5'-triphosphate salt	Sigma
Agarose	Gibco BRL
Ammonium bicarbonate	Fisher Scientific
Ammonium hydroxide	Fisher Scientific
Ammonium persulphate	Fisher Scientific
Ampicillin	Sigma
Bovine serum albumin	Boehringer Mannheim
1-Butanol	Fisher Scientific
b-glycerophosphate	Sigma
Chloroform	Fisher Scientific
Coomasie brilliant blue R	BioRad
Dimethyl sulfoxide	Fisher Scientific
Dithiothreitol (DTT)	Boehringer Mannheim
Ethidium bromide	Fisher Scientific
Glutathione	Sigma
Glycerol	Fisher Scientific
Glycine	Fisher Scientific
HEPES	Sigma
Hydrochloric acid	Fisher Scientific
Isopropyl β-D-thiogalactopyranoside (IPTG)	Gibco BRL
Lithium chloride	Sigma
Magnesium chloride	Sigma
2-Mercaptoethanol	Sigma
Methanol	Fisher Scientific
Myelin basic protein	Kinetek
Ninhydrin	Fisher Scientific
Nonidet P-40 (10% solution)	Calbiochem
Petroleum ether	Fisher Scientific
Phosphoric acid	Fisher Scientific
Phosphoric acid, ^{32}P	NEN radiochemicals
Ponceau S concentrate	Sigma
Sodium azide	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Fisher Scientific
Sodium fluoride	Fisher Scientific
Sodium orthovanadate (see preparation protocol)	Sigma
TEMED (N,N,N',N'-tetramethylelthylenediamine)	BioRad
Tris hydroxylmethyl aminomethane hydrochloride (Tris-HCl)	Fisher Scientific
Triton X-100	Sigma
Tween-20	Fisher Scientific
Urea	Fisher Scientific

2.1.2. Tissue culture reagents

Fetal Bovine Serum	Gibco BRL
L-glutamine	Sigma
Penicillin/Streptomycin	Gibco BRL
Phenol Red	Sigma
RPMI-1640	Gibco BRL
Sodium Bicarbonate	Sigma
Trypsin	Gibco BRL

2.1.3. Consumables

Chromatography paper (3MM)	Whatman
Conical tubes (14 and 50 ml)	Falcon
Membrane filter units (0.22 um)	Nalgene
Microcentrifuge tubes	ESBI
Nitrocellulose membrane	Schleicher and Schuell
P81 phosphocellulose chromatography paper	Whatman
Phosphocellulose TLC plates	Sigma
Pipet tips (p20, p200 and p1000)	ESBI
Silica gel TLC plates	Sigma
Tissue culture flasks (175 cm2)	Corning
Tissue culture plates (60 and 100 mm)	Falcon

2.1.4. Protease Inhibitors

Aprotinin - Serine protease inhibitor	Sigma
Ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) - Metalloprotease inhibitor	Sigma
Ethylene diamine tetraacetic disodium salt (EDTA) - Metalloprotease inhibitor	Sigma
Leupeptin - Serine protease inhibitor	Sigma
Phenylmethylsulphonylfluoride (PMSF) - Serine protease inhibitor	Sigma
Soybean trypsin factor - Trypsin and Factor Xa	Sigma

2.1.5. Antibodies

anti-Bad mouse monoclonal (B36420)	Transduction labs
anti-Bad mouse monoclonal (B32140)	Transductoin labs
anti-Bad rabbit polyclonal (SC-943)	Santa Cruz
anti-phosphoS112 Bad rabbit polyclonal	New England Biolabs
anti-phosphoS136 Bad rabbit polyclonal	New England Biolabs
anti-erk1/2 rabbit polyclonal	Santa Cruz
anti-phospho-erk1/2 rabbit polyclonal	New England Biolabs
anti-p70 S6 kinase rabbit polyclonal	Kinetek
anti-PKBalpha sheep polyclonal	Upstate Biotechnology
anti-GSK-3 sheep polyclonal	Upstate Biotechnology
anti-phosphoS21 GSK-3 rabbit polyclonal	Upstate Biotechnology

2.2. METHODS

2.2.1. Cell Culture

The following cell lines were obtained from the American Type Culture Collection: MC/9, a murine mast cell line, CTLL-2, a murine cytotoxic T-cell cell, and HL-60, a human promyeloid cell line. Each line was grown in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. The factor dependent cell line MC/9 also received 10% (v/v) supernatant from WEHI-3 cells as a source of IL-3. CTLL-2 cells were expanded with recombinant murine IL-2 (5 ng/ml). All cell lines were grown on non-tissue culture treated 100 mm plates at 37°C and 5% CO_2 with humidity. Generally, all cell lines were maintained at densities between 0.1 and 1 x 10^6/ml.

2.2.2. DNA fragmentation and Annexin-V apoptosis assays

After the indicated times of treatment with cytokines and/or inhibitors, 2 x 10^5 cells were pelleted and solubilized in 400 µl lysis buffer (0.6% SDS and 10 mM EDTA, pH 8.0). Following addition of 100 µl of 5M NaCl, the samples were mixed by gentle inversion of the tubes. After overnight incubation at 4°C, samples were pelleted by centrifugation for 20 min in a micro-centrifuge tube. Supernatants containing DNA fragments were transferred to clean tubes and the pellets were discarded. One µl of 1 mg/ml RNase A was added and samples were incubated 20 min at 37°C. 500 µl of Tris-buffered phenol (pH 8.0): chloroform (1:1) was added, the samples were briefly vortexed and the aqueous layer retained. To this layer were added 55 µl of 3M sodium acetate and 1.0 ml of ice-cold absolute ethanol. Samples were mixed and incubated 10 min at -20°C. DNA was pelleted by centrifugation for 10 min at 14,000 rpm and then separated by electrophoresis on a 2% agarose:TBE gel. Visualization of DNA bands was performed by staining with ethidium bromide, destaining in water and viewing on a UV transilluminator. Video images of the stained gels were obtained using a GelPrint 2000 system.

Cells undergoing apoptosis were also distinguished from healthy cells by staining with Annexin-V-FITC or PE, according to the manufacturers protocol (Pharmingen, California) and measured by flow cytometry (Coulter EPICS V). Late apoptotic cells were also distinguished by their ability to take up propidium iodide. The number of cells staining with annexin-V alone and with both annexin-V and propidium iodide were added together, giving the total number of cells at both early and late stages of apoptosis.

2.2.3. Measurement of Intracellular PI(3,4,5)P_3

Cells were deprived of cytokines overnight, then washed three times with phosphate-free RPMI and incubated in the same medium with 0.5 mCi/ml ^{32}P-orthophosphate (ICN) for two hours at 37°C. Cells were stimulated with cytokine in the presence or absence of inhibitors, then stopped by the addition of 3.5 ml of $CHCl_3$: methanol (1:2, v:v). Lipids were extracted as described previously (Gold et al., 1994), spotted onto oxalate-treated silica gel plates, and chromatographed using $CHCl_3$:acetone:methanol:acetic acid:water as the solvent (46:17:15:14:8, v:v:v:v:v). Lipids were visualized by autoradiography and PIP_3 was identified by co-migration with PIP_3 standard generated in an *in vitro* reaction in which immunoprecipitated PI 3-kinase phosphorylated PI(4,5)P_2 in the presence of γ-^{32}P-ATP. The PIP_3 spot was removed from the plate and quantitated by liquid scintillation counting. Values were normalized based on the total radioactivity in each lane. To confirm the identity of the above TLC spot as PIP_3, the lipids scraped from the TLC plate were deacylated, followed by HPLC separation of the water-soluble products using procedures and conditions similar to those described previously (Gold et al., 1994). Following 10 min washing of the Partisil 10 SAX ion exchange column with water, a 20 min 0-0.25M ammonium phosphate gradient was followed by a 50 min 0.25 - 1.0 M ammonium phosphate gradient. The peak of ^{32}P radioactivity eluted between ^{3}H-inositol-P_3 (53 min) and ^{3}H-inositol-P_4 (70 min), as expected.

2.2.4. HPLC Analysis of PIP_3 and $PI(3,4)P_2$

To measure the *in vivo* labelled lipids directly by HPLC, cells were labelled with 0.5 mCi/ml ^{32}P-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, ^{3}H-inositol(1,4,5)P_3 (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.

2.2.5. XTT Mitochondrial activity assay.

Mitochondrial reduction potential was measured using a colorimetric assay in which the amount of 2,3-bis(2-methoxt-4-nitro-5-sulphenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium (XTT, Sigma) reduction is measured. Following various treatments for 18 - 48 hrs, 25 µl of a mixture of 1 mg/ml XTT and 25 µM phenazine methosulfate (Sigma) in RPMI was added and incubations continued for 4 hours at 37°C. The absorbance of the coloured reaction product was measured at 450 nM.

2.2.6. p70 S6 Kinase Phosphorylation

For immunoblot analysis, a 10 µl aliquot of the solubilized cell extract was mixed with 10 µl of 2X SDS sample buffer and boiled for 5 minutes. Samples were separated by SDS-PAGE on an 8% gel (acrylamide:bis ratio of 118:1) followed by transfer by semi-dry blotting to nitrocellulose. Membranes were blocked with 5% (w/v) skim milk containing 0.05% sodium azide for 2 h at room temperature and probed with a 1/1000 dilution of anti-p70 S6 kinase antibody (Kinetek) in Tris-buffered saline, pH 7.4, (TBS) containing 2 %

(w/v) BSA and 0.05% (w/v) sodium azide for 2 h at room temperature. Membranes were washed repeatedly with TBS and TBS plus 0.05% (v/v) Tween-20 and incubated in peroxidase-conjugated goat anti-rabbit antibody for one hour, followed by further washing. Bound antibody was detected with enhanced chemiluminescence (ECL; Amersham).

2.2.7. p70 S6 kinase immunocomplex kinase assay

Cells were washed three times with Hanks balanced salt solution buffered with 20 mM HEPES-NaOH, pH 7.4, and resuspended in RPMI 1640 buffered with 20 mM HEPES-NaOH, pH 7.4, to a concentration of 1 x 10^7 cells/ml. Cells were incubated at 37°C for 20 min followed by the addition of rapamycin (100 ng/ml), wortmannin (100 nM) or LY-294002 (25 μM) for 10 min. Cells were then stimulated with the following cytokines: MC/9 cells received recombinant GM-CSF (60 U/ml), synthetic IL-3 (10 μg/ml) or synthetic IL-4 (10 μg/ml). CTLL-2 cells were stimulated with X63-OMIL-2-conditioned medium (10%) or synthetic IL-4 (10 μg/ml). All stimulations were for 10 min, and were terminated by rapid centrifugation of cells and lysis in ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.7, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 20 mM β-glycerophosphate, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MO_4, 0.25 mM phenylmethylsulphonyl fluoride (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). An aliquot (25 μl) was removed for Western blotting analysis. For immunoprecipitations, the remaining supernatant was incubated with 5 μg/ml anti-p70 S6 kinase antibody with continuous mixing at 4°C for 2 hours. The samples then received 20 μl of a 1:1 suspension of protein-A-Sepharose beads (Pharmacia) in kinase buffer with mixing at 4°C for an additional 1 h. Beads were washed 3 times with fresh solubilization buffer and once with kinase buffer (20 mM HEPES, pH 7.2, 25 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulphonyl fluoride, 2 mM Na_3VO_4 and 0.5 μg/ml leupeptin). Beads were

resuspended in 50 µl of kinase buffer containing 1 mg/ml p70 S6 kinase synthetic peptide substrate (AKRRRLSSLRASTSKSESSQK) which is based on the S6 protein of the 40S ribosome. Ten µl of ATP solution (final concentration of 100 µM ATP, with 1 µCi ^{32}P-ATP in kinase buffer) were added followed by incubation for 15 minutes at 30°C. Twenty five µl of the reaction mixture were spotted onto a 2 cm^2 sheet of P81 filter paper (Whatman), followed by washing with numerous (>5) volumes (~200 ml) of 1% (v/v) phosphoric acid and quantitation of associated radioactivity by liquid scintillation counting.

2.2.8. MAP kinase immunocomplex kinase assay

For use in assays, cells were washed free of cytokine with Hanks balanced salt solution and incubated in complete RPMI medium without cytokine for 3 - 5 hours prior to assay. Alternatively, cells were incubated overnight with 1% WEHI-3-conditioned medium prior to assay. At time of assay, cells were again washed and resuspended to 1.0 x 10^7 cells/ml in RPMI medium buffered with 20 mM HEPES, pH 7.4. Cells were incubated at 37°C for 15 min followed by addition of the PI 3-kinase inhibitors at various concentrations for 10 min. Cells were stimulated with either recombinant GM-CSF (60 U/ml), synthetic IL-3 (10 µg/ml) or phorbol dibutyrate (200 nM) for either 5 min (GM-CSF) or 10 min (IL-3, phorbol dibutyrate). Reactions were stopped by rapid pelleting of the cells followed by lysis in ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.7, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MO_4, 0.25 mM PMSF, 1 µM pepstatin, 0.5 µg/ml leupeptin and 10 µg/ml soybean trypsin inhibitor), and removal of nuclei by centrifugation (20,000 x g, 1 min). Supernatants were incubated with 40 µg/ml anti-p44*erk-1* antibody coupled to agarose beads (Santa Cruz Biotechnology, CA) at 4°C for 4 hours, with continuous mixing. The beads were washed 3 times with fresh solubilization buffer and once with kinase buffer (20 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.25 mM PMSF, 2 mM Na_3VO_4, 0.5 µg/ml leupeptin). The beads were

then resuspended to 25 µl in kinase buffer containing 1 mg/ml MBP. Five µl of ATP solution (100 µM ATP, 1 µCi ^{32}P-ATP in kinase buffer) was added followed by incubation for 15 min at 30°C. Reactions were stopped by addition of 30 µl of 2X SDS sample buffer, followed by boiling for 2 min. Samples were separated by SDS-PAGE (15%), and proteins were transferred to nitrocellulose by semi-dry blotting. Phosphorylation of MBP was analyzed by autoradiography, and measured by liquid scintillation counting of the excised bands. Immunoblotting was performed using anti-p44$^{erk\text{-}1}$ (Santa Cruz Biotechnology, CA) followed by detection with enhanced chemiluminescence. Alternatively, Erk activity was measured by spotting 25 µl of the reaction volume onto 2 cm^2 squares of P81 filter paper (Whatman), followed by repeated washing with numerous (>5) volumes (~200 ml) of 1% (v/v) phosphoric acid. Radioactivity bound to the filter was quantitated by liquid scintillation counting.

2.2.9. Immunoprecipitation and blotting of Bad

For analyzing the electrophoretic mobility shift of Bad, MC/9 cells stimulated under various conditions were lysed with ice-cold solubilization buffer (20 mM Tris-HCl pH 7.4, 137 mM NaCl, 0.25% Nonidet P40, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MoO_4, 1 µg/ml microcystin-LR, 0.25 mM PMSF, 1 µM pepstatin, 0.5 µg/ml leupeptin and 10 µg/ml soybean trypsin inhibitor) and incubated on ice for 10 minutes. Samples were centrifuged (20,000 x g, 1 min) and supernatants were transferred to clean tubes. Five µg of anti-Bad monoclonal antibody (B36420; Transduction Labs) was added and the samples were rotated overnight at 4°C. Bad 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 in a 12.5% polyacrylamide gel with a 118:1 acrylamide:bisacrylamide ratio and transferred to nitrocellulose. The blots were blocked with 3% skim milk solution for 1 hour and then

incubated with 1 μg/ml anti-Bad antibody (either SC-943 from Santa Cruz or B36420 from Transduction Labs) overnight at room temperature. Primary antibody was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

2.2.10. Metabolic Labelling of Bad

MC/9 cells were starved of cytokine as described in section 2.2.8, 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 was immunoprecipitated from detergent-solubilized lysates as described above. Immunoprecipitates were fractionated on a 12.5% gel with an acrylamide:bisacrylamide ratio of 118:1. The gel was dried under heat and vacuum. ^{32}P-labelled Bad was detected by autoradiography, and quantified by either excision from the gel followed by liquid scintillation counting or by using a phosphorimager (Molecular Dynamics or BioRad).

2.2.11. Two-dimensional phosphopeptide mapping

Labelling and SDS-PAGE fractionation of Bad was performed as described above. Gel fragments containing ^{32}P-labelled Bad were sliced into 1 mm^3 pieces, dried under vacuum, and rehydrated in 1 ml of 50 mM ammonium bicarbonate, pH 7.8, containing 100 μg TPCK-treated trypsin (Sigma). The protein was digested overnight at 37°C and an additional 100 μg of TPCK-treated trypsin was added for a further 4 hours. Gel fragments were pelleted by centrifugation, the supernatant was transferred to a clean tube and dried under vacuum. The digested protein was washed with diminishing volumes of water, resuspended in 5 μl of pH 8.9 buffer (1% ammonium carbonate), and applied to a cellulose TLC plate (microcrystalline cellulose, 200 μm thickness; Kodak). The plate was wetted with pH 8.9 buffer, and electrophoresis was performed at 600 V for 45 min at 8°C. The plate was rotated 90° ascending chromatography in n-butanol:pyridine:H_2O:acetic acid (37.5:25:20:5) was performed for 3 hours, followed by autoradiography. Non-radioactive

synthetic peptides were run concurrently, and stained with ninhydrin (0.5% v/v in ethanol). For *in vitro* phosphorylation of GST-BAD, 1μg of GST-BAD (a gift from Dr. A. Karson) was incubated in a reaction mixture containing various amounts of nuclear-free MC/9 cell lysate, 10 μCi γ-^{32}P-ATP, 300 μM ATP and 50 mM $MgCl_2$ for 10 min at 30°C. Reactions were stopped by addition of an equal volume of 2X sample buffer and boiling at 95°C for 5 min. GST-BAD was fractionated by SDS-PAGE, and its position determined by coomassie blue staining and autoradiography. Bands were excised from the gel and digested by trypsin as described above.

2.2.12. Phosphoamino acid analysis

^{32}P-labelled peptides isolated from two dimensional mapping were scraped from the plate and extracted from the cellulose in 200 μl of water. The samples were dried under vacuum and resuspended in 200 μl 6N HCl at 50°C for 40 min. The samples were cooled, and again dried under vacuum. The samples were then resuspended in 5 μl of water containing 1 μg each of phospho-serine, phospho-threonine and phospho-tyrosine. Samples were applied to a cellulose plate, and separated by electrophoresis in H_20:Acetic acid:pyridine (950:45:5) at 600 V for 30 min at 8°C, followed by autoradiography. Non-radioactive standards were visualized by ninhydrin staining.

2.2.13. PKB immunocomplex kinase assay

Cells were washed free of cytokine with Hanks balanced salt solution and incubated in the above medium without cytokine for 3 - 5 hours prior to assay. Alternatively, cells were incubated overnight with 1% WEHI-3-conditioned medium prior to assay. At time of assay, cells were again washed and resuspended to 1.0 x 10^7 cells/ml in RPMI medium buffered with 20 mM HEPES, pH 7.4. Cells were incubated at 37°C for 15 min followed by addition of the PI3K inhibitors LY-294002 or wortmannin for 10 min. Cells were stimulated with either recombinant GM-CSF (100 ng/ml), synthetic IL-3 (10 μg/ml), synthetic IL-4 (10 μg/ml) or recombinant SCF (100 ng/ml) for various times. These

concentrations of cytokines were previously shown to induce maximal increases in tyrosine phosphorylation. Reactions were stopped by rapidly pelleting the cells followed by lysis in ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.7, 0.5% Nonidet-P40, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MoO_4, 1 μg/ml microcystin-LR, 0.25 mM PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor) and removal of nuclei by centrifugation (20,000 x g, 1 min). Supernatants were incubated with 2 μg anti-PKB-α antibody (Upstate Biotechnology Incorporated) 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 containing 500 mM NaCl and once with kinase buffer (20 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.25 mM PMSF, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin). Beads were resuspended in 25 μl kinase buffer containing 60 μM Crosstide (Upstate Biotechnology Inc.). Five μl of ATP solution (200 μM ATP, 10 μCi ^{32}P-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 cm^2 squares of P81 filter paper (Whatman), followed by extensive washing with 1% (v/v) phosphoric acid and measurement of associated radioactivity by liquid scintillation counting.

3. REQUIREMENT FOR PI 3-KINASE IN THE PREVENTION OF APOPTOSIS

3.1. RATIONALE AND HYPOTHESIS

Hemopoietic cells usually depend upon cytokines to maintain survival (Williams, et al., 1990). Cytokines stimulation activates signal transduction pathways which prevent apoptosis, but the elements of these signalling pathways were largely unknown when these studies were initiated. IL-4 can protect cells from apoptosis, but this cytokine is unable to promote growth, suggesting that these two processes are separable. The lack of cell growth in the presence of IL-4 has been attributed to a lack of activation of the Ras/Erk cascade. Less is known regarding IL-4's ability to promote survival. All survival and growth-promoting cytokines activate PI 3-kinase, including IL-4. This observation led to the hypothesis that PI 3-kinase activity is responsible for the ability of IL-4 and other cytokines to inhibit apoptosis.

3.2. RESULTS

Cells of the murine mast cell line MC/9 are factor dependent, requiring the addition of specific cytokines to the growth medium to support continued survival and/or growth. These cytokines include any one of IL-3, IL-4, GM-CSF, SCF and IL-5. In the first set of experiments, cells were washed free of cytokine and placed in medium containing only 10% fetal bovine serum. Under these conditions, MC/9 cultures displayed a gradual increase in DNA fragmentation (Figure 3.1), even as early as 2 h after cytokine withdrawal. DNA fragmentation is a unique hallmark of apoptosis, and is the result of a specific, caspase-activated DNase (Enari et al., 1998). To test if PI 3-kinase was providing anti-apoptotic signals, wortmannin was added to parallel dishes. Wortmannin had the effect

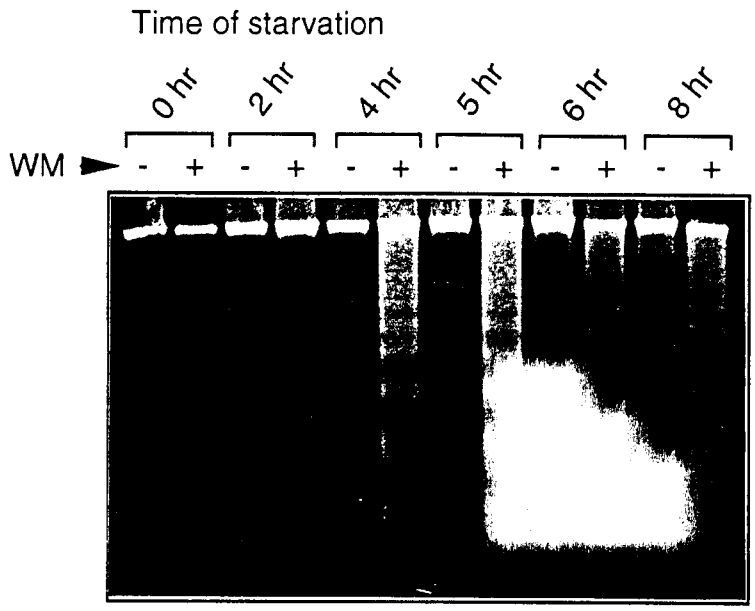


Figure 3.1. Apoptosis in MC/9 cells following starvation. Cells were washed three times and incubated in RPMI plus 10% fetal bovine serum at 37°C. At the indicated times of starvation, samples were taken and prepared as described. In samples indicated, 200 nM WM was added at 0 time.

of accelerating apoptosis (compare 4 h and 5 h time points), which may be attributable to inhibition of basal or serum-stimulated PI 3-kinase activity.

The preceding experiment was performed with wortmannin at a concentration of 200 nM, which is higher than the reported concentration of the drug needed to completely inhibit PI 3-kinase. To test the effects of wortmannin at lower concentrations, to lessen the possibility that wortmannin could be affecting other cellular enzymes, it is important to recognize that the drug is sensitive to hydrolysis at physiological temperature and pH, and has a half-life of approximately 1 h under these conditions (R. Lauener and M. Scheid, unpublished observations). To overcome this problem, wortmannin was used in subsequent experiments at a lower initial concentration and added hourly at half the initial concentration during the course of the experiment. Following this protocol, MC/9 cells were incubated in the presence of IL-4, and various concentrations of wortmannin for 8 h (Figure 3.2). Fragmented DNA was isolated from cells treated with as low as 1 nM wortmannin, which suggested that wortmannin was having an effect on apoptosis due to its selective inhibition of PI 3-kinase. In addition, HL-60 cells which do not require the addition of cytokine to the growth media for survival were resistant to wortmannin (Figure 3.2), demonstrating that wortmannin was not activating a component directly involved in the apoptotic machinery. Camptothecin, a topoisomerase inhibitor, potently induced apoptosis in these cells confirming that HL-60 have a functional apoptosis cascade.

These results demonstrated that the ability of IL-4 to promote survival was dependent upon PI 3-kinase, so we then asked whether survival mediated by other cytokines, such as IL-3, was also PI 3-kinase dependent. The effect of wortmannin at various doses was tested on MC/9 cells stimulated with either IL-3, IL-4 or GM-CSF

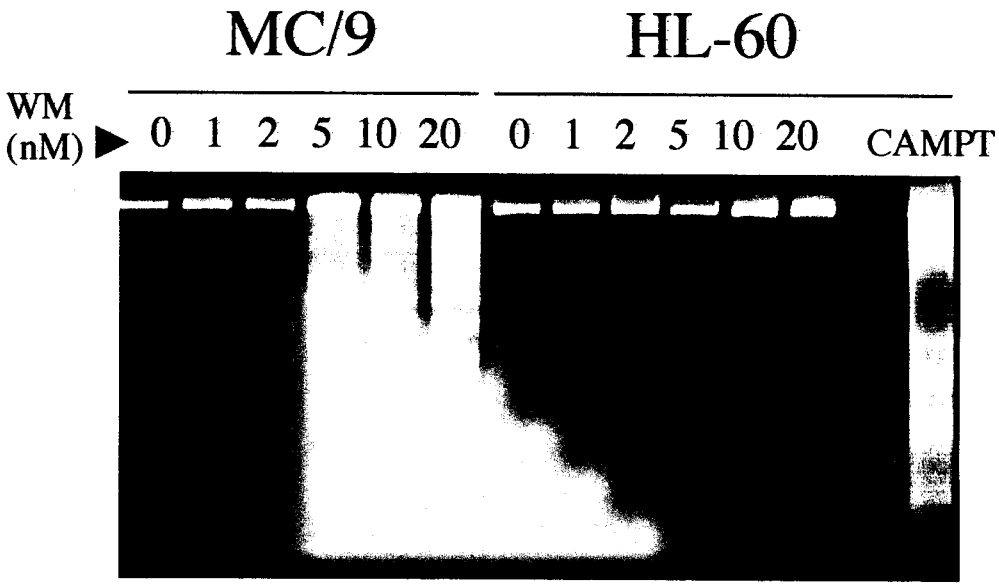


Figure 3.2. PI 3-kinase inhibition induces apoptosis in IL-4-stimulated MC/9 cells, but not HL-60 cells. MC/9 cells or HL-60 were incubated with WM with twice the indicated concentration added at 0 time, followed by hourly additions of the indicated concentration. Incubations were for 8 hours. CAMPT indicates HL-60 cells incubated in parallel with 1 μM camptothecin for 6 hours to induce DNA fragmentation.

(Figure 3.3). As expected, wortmannin caused DNA fragmentation in cells incubated in IL-4, and as well as cell incubated with IL-3. On the other hand, cells incubated with GM-CSF were completely resistant to wortmannin, even at 25-fold higher concentrations, indicating that GM-CSF was able to bypass the inhibition of PI 3-kinase by wortmannin, presumably via an alternate signalling pathway. This was an unexpected finding, since IL-3 and GM-CSF receptors share a common, signal transducing β-subunit, and have been shown to activate very similar signalling pathways (Duronio et al., 1992; Miyajima et al.,1993). To further establish the role of PI 3-kinase in the inhibition of apoptosis, another inhibitor of the enzyme, LY-294002, which is unrelated to wortmannin and functions by a different mechanism, was used. LY-294002 used at concentrations of 10 and 25 μM induced apoptosis in cells stimulated with IL-3 or IL-4, but not GM-CSF (Figure 3.4).

So far, these experiments have measured DNA fragmentation, and while being a hallmark of apoptosis, it is not very quantifiable. To extend these findings, annexin-V binding to surface phosphatidylserine in conjunction with propidium iodine staining was undertaken (Figure 3.5). When used together, both early and late apoptosis can be quantitated using flow cytometry. LY-294002 accelerated apoptosis in cells starved of cytokine, and induced apoptosis in >50% of a population of IL-3-grown cells by 12 hrs. In contrast, there was no increase in either annexin-V binding or propidium iodine uptake into cells incubated with GM-CSF.

To further characterize the effects of wortmannin and LY-294002 on cytokine-dependent cells, viability assays which measure metabolic activity were conducted. In the first set of experiments, cells were plated with various cytokines in the presence of wortmannin at several initial concentrations. After 48 hours, mitochondrial activity was measured using XTT conversion (Figure 3.6). Cells starved of cytokine displayed no mitochondrial activity, while cells grown in the presence of GM-CSF, SCF, IL-4, or IL-3 were fully active. Increasing initial concentrations of wortmannin potently inhibited the

ability of SCF, IL-4 and IL-3 to maintain metabolic activity in a dose-dependent manner, whereas GM-CSF-stimulated cells were unaffected. The high concentrations of wortmannin required for this effect were probably due to the susceptibility of wortmannin to hydrolysis, as described above, as no further additions of this compound were made during the 48 hour time course. These results demonstrated that PI 3-kinase inhibition following GM-CSF stimulation did not result in an alternate form of cell death such as necrosis, which would lead to mitochondrial death without the appearance of apoptosis.

To test whether GM-CSF was stimulating a wortmannin- or LY-294002-resistant PI 3-kinase activity, phospholipid analysis was conducted to measure PIP_3 levels. ^{32}P-labelled MC/9 cells were treated with various concentrations of wortmannin or LY-294002 and stimulated with GM-CSF. Lipids were extracted and dried, followed by thin layer chromatography to isolate the labelled PIP_3 species (Figure 3.7). Table 1 lists the fold induction of each lipid species following stimulation with GM-CSF. Both inhibitors efficiently blocked the ability of GM-CSF to induce any increase in PIP_3 at 50-100 nM wortmannin or 25 μM LY-294002. To confirm the identity of the spot attributed to PIP_3, the spot was scraped from the TLC plate and deacylated, followed by HPLC analysis. This glycerol-inositol-3,4,5-P_3 spot co-migrated with deacylated PIP_3 generated from an *in vitro* kinase assay using immunoprecipitated PI 3-kinase and $PI(4,5)P_2$ as substrate (Figure 3.8).

3.3. DISCUSSION

At the time of these studies, these results demonstrated for the first time the requirement for PI 3-kinase in the inhibition of apoptosis by some, but not all cytokines. The identical results obtained with the use of two structurally unrelated inhibitors of PI 3-kinase argues against the possibility that nonspecific inhibition of unrelated enzymes may have caused the apoptosis. Additionally, the concentrations of wortmannin or

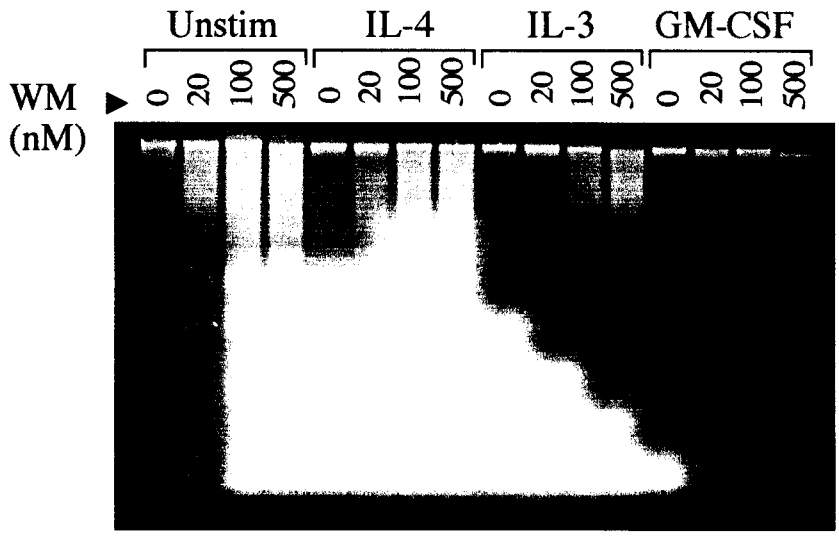


Figure 3.3. Dose-response for wortmannin. MC/9 cells were incubated in the presence of twice the indicated concentrations of WM, added at 0 time, followed by the indicated concentration (nM) added hourly. Cells were incubated either in RPMI plus 10% fetal bovine serum (starved), or in the same medium with 500 ng/ml synthetic IL-3, 500 ng/ml synthetic IL-4, or 60 units/ml of recombinant GM-CSF. After 8 hours, cells were prepared as described.

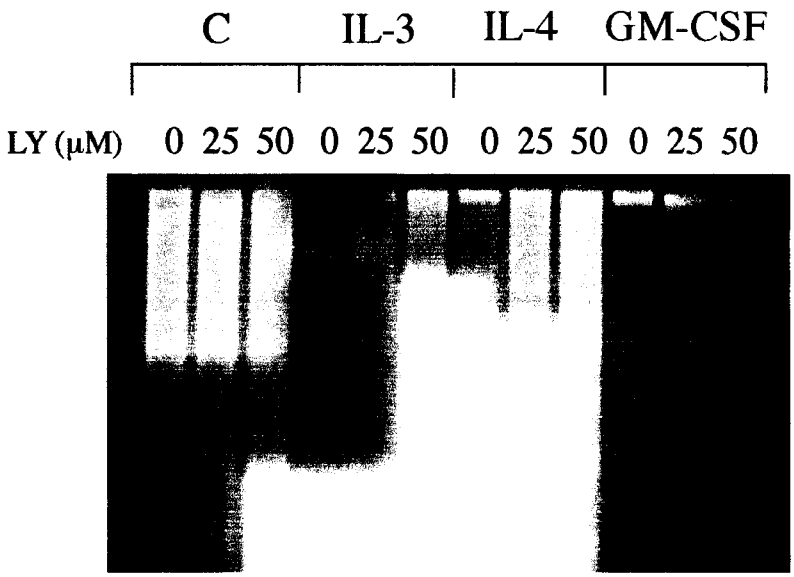


Figure 3.4. Apoptosis in MC/9 cells treated with LY294002. Cells were washed and incubated in the presence of optimal concentrations of the indicated cytokines along with a single addition of the indicated concentrations of LY294002. Cells were incubated for 15 hours, then harvested and treated as described in Methods and Materials.

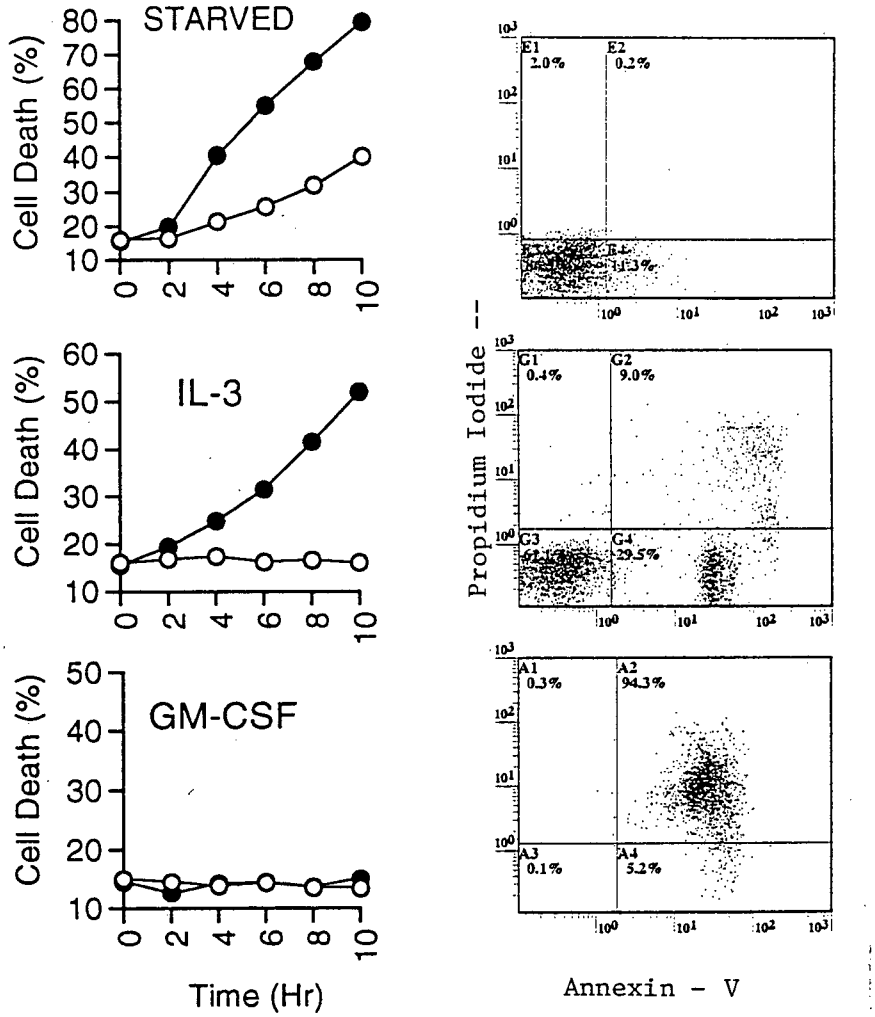


Figure 3.5. Cell death resulting from PI3K Inhibition. **Left panels.** MC/9 cells were washed and resuspended in complete medium containing the indicated cytokine, or without cytokine (starved). LY-294002 (25 μM; closed circles) or vehicle (open circles) were added at time 0 and at the indicated hours duplicate aliquots of cells were removed, washed, stained with annexin-V-FITC and propidium iodide, and analyzed using flow cytometry. Cells undergoing early apoptosis showed an increase in annexin-V binding, but excluded propidium iodide. At later time points the percentage of propidium iodide staining cells gradually increased. Reported is the total amount of annexin-V-FITC and propidium iodide staining, which is representative of populations containing cells at both stages of apoptosis. **Right panels.** Representative analysis of cells staining with propidium iodide and annexin-V in control (top), earlier apoptosis (middle) and late apoptosis (bottom) samples. Results are representative of 4 independent experiments. The standard deviation for each time point for each condition was less than 2%.

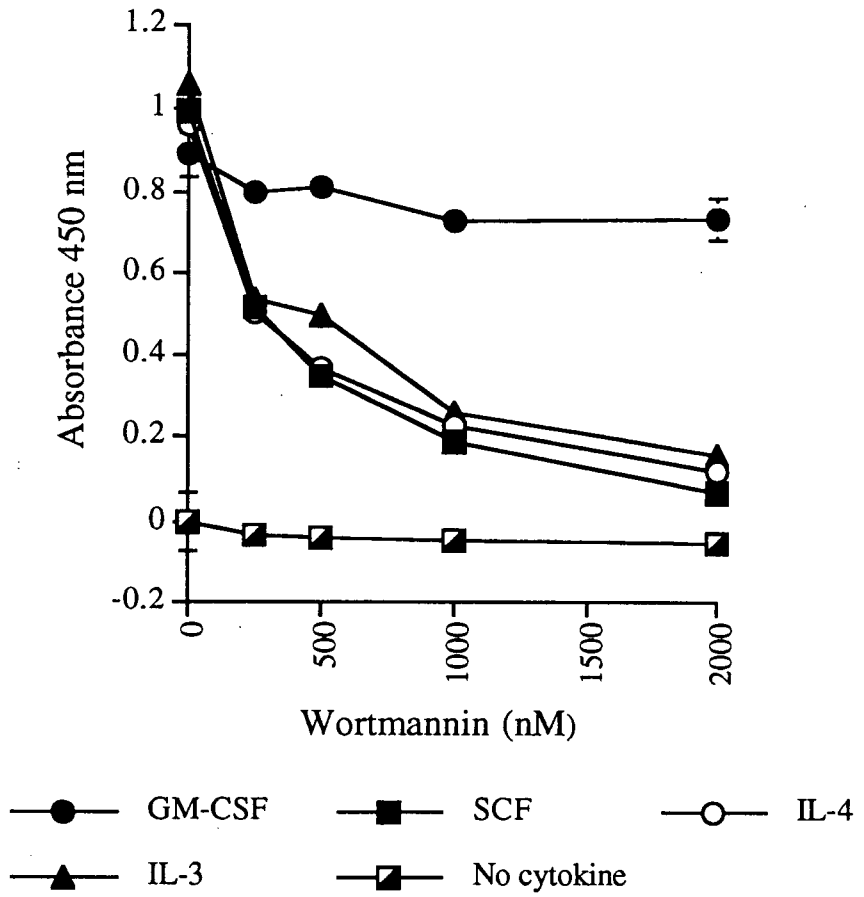


Figure 3.6. Metabolic activity in GM-CSF stimulated cells but not IL-3, IL-4 or SCF following treatment with wortmannin or LY-294002. MC/9 cells were incubated with the indicated cytokines along with the indicated concentrations of wortmannin. After 48 hours, an XTT assay to measure mitochondrial activity was performed as described in Materials and Methods. Each point represents three independent determinations, +/- standard deviation. Where no error bars are shown, they were smaller than the symbol.

Table 1

Inhibition of GM-CSF-stimulated PI-3,4,5-P_3 by wortmannin and LY-294002

Values represent triplicate determinations of cpm in PI-3,4,5-P_3 spots, with unstimulated control samples normalized to a value of 1.00.

Inhibitor	Condtion	Fold Stimulation[a]
[LY-294002] (μM)		
0	Unstimulated	1.00 +/- 0.11
0	GM-CSF	1.77 +/- 0.27
10	GM-CSF	1.72 +/- 0.15
25	GM-CSF	1.09 +/- 0.23
50	GM-CSF	0.49 +/- 0.06
[Wortmannin] (nM)		
0	Unstimulated	1.00 +/- 0.01
0	GM-CSF	2.92 +/- 0.38
10	GM-CSF	1.83 +/- 0.40
25	GM-CSF	1.42 +/- 0.56
50	GM-CSF	1.36 +/- 0.09
100	GM-CSF	1.16 +/- 0.07

[a]Compared to cpm in unstimulated cells in the absence of inhibitor.

LY-294002 used were carefully selected to achieve full PI 3-kinase inhibition without approaching concentrations known to inhibit less-sensitive enzymes. Nevertheless, the use of pharmacological inhibitors of an enzyme can never completely ensure the lack of non-specific effects. An example of wortmannin and LY-294002 inhibiting other enzymes became apparent following the completion of these studies: both can also inhibit the PI 3-kinase relative, mTOR (Brunn et al., 1996). Inhibition of mTOR is also mediated by the immunosuppressant rapamycin, which is examined in the next chapter.

To this end, another approach that could be used to demonstrate the requirement for PI 3-kinase would be to express in these MC/9 cells dominant-negative forms of PI 3-kinase. Others have demonstrated that expression of a catalytically dead p110 subunit abolishes ligand-induced PIP_3 generation, probably by displacing active p110 from the p85 regulatory subunit (Takayanagi et al., 1996). Alternatively, expression of a mutant p85 subunit that cannot bind p110 also suppresses PI 3-kinase activity, by acting in a dominant negative role (Hara et al., 1994). Either of these methods should confirm our pharmacological results, although expression of mutant signalling proteins may introduce other non-specific effects that may also be difficult to predict.

The effects of PI 3-kinase inhibition on survival are not limited to MC/9 cells. Other cell lines, such as the human TF-1 cell line and the murine cell line U937, also undergo apoptosis in the absence of PI 3-kinase signalling (M. Scheid and K. Schubert, unpublished observations). As well, primary human eosinophil survival was attenuated when cultured with various cytokines and inhibitors of PI 3-kinase (M. Rebbetoy, M. Scheid and V. Duronio, unpublished observations). The identification of critical survival pathways operating in eosinophils may lead to therapies which induce apoptosis. One such disease where this could have some clinical benefits is asthma, since eosinophils have been shown to play a role in the pathogenesis of the disease.

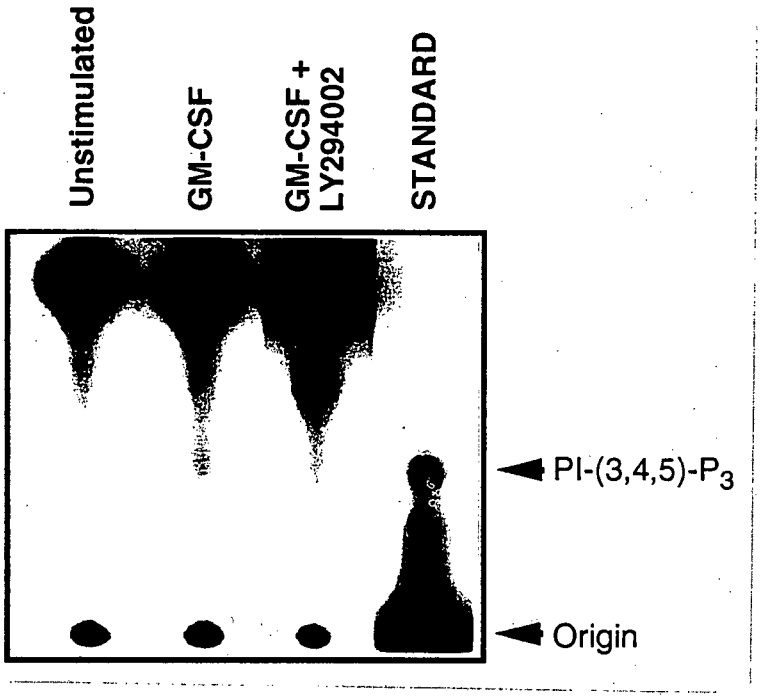


Figure 3.7. A representative example of PIP_3 separated by thin layer chromatography. Lipids were extracted as described in Methods and Materials from cells that were either unstimulated, or stimulated with GM-CSF for 5 minutes following a 10 min preincubation in the absence or presence of 50 μM LY-294002. The radioactivity in the PIP_3 spots were quantitated and normalized based on the relative radioactivity per lane, and used to generate the results in Table 1. The PIP_3 standard was prepared by using immunoprecipitated PI 3-kinase in an *in vitro* reaction with ^{32}P-ATP, using $PI(4,5)P_2$ as substrate.

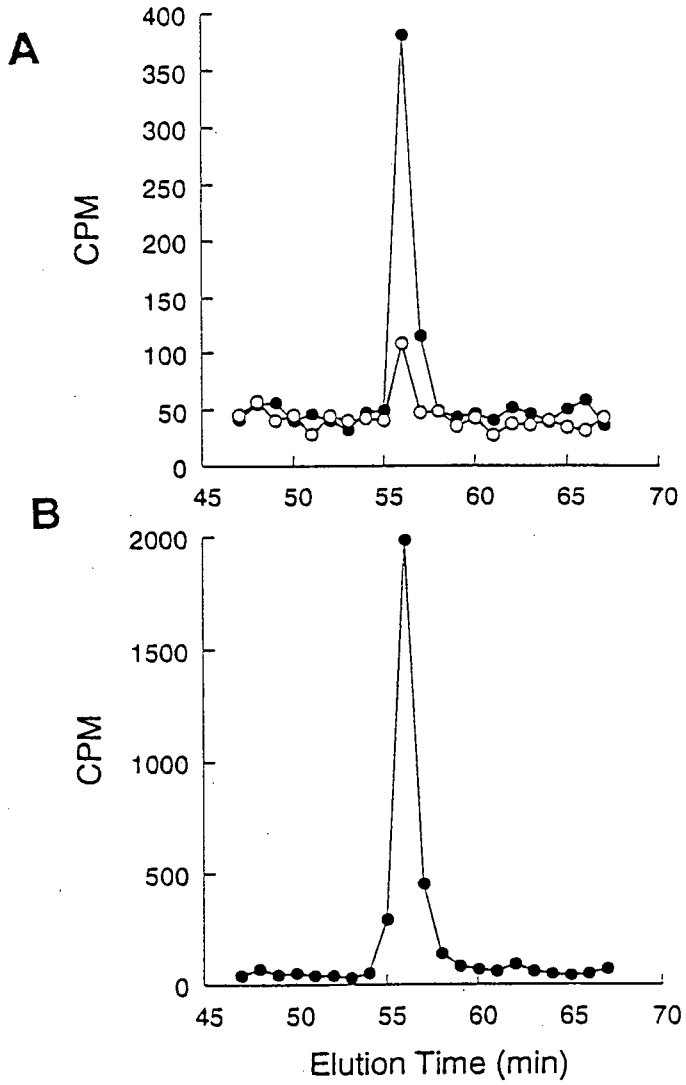


Figure 3.8. Identification of the separated spot by HPLC analysis. **A**. The spots separated by TLC of whole cell extracts from unstimulated (O) or GM-CSF-stimulated (•) cells were removed, deacylated, and chromatographed as described in Experimental Procedures. **B**. The ^{32}P-labeled products from the *in vitro* reaction described above also eluted at the identical time. The peaks from HPLC corresponded to the expected elution time for glycerol-P-IP_3.

Our results with GM-CSF are intriguing, since GM-CSF and IL-3 receptors share a common β-subunit, thought to be the signalling element in the dimeric receptor complex. Since GM-CSF can clearly bypass the requirement for PI 3-kinase in the prevention of apoptosis, it must either be activating a completely separate signalling pathway or activating an enzyme downstream of PI 3-kinase by an independent upstream event. Another possibility is that both IL-3 and GM-CSF activate other survival signalling pathways, independent of PI 3-kinase, which are sufficient to maintain survival when activated in response to GM-CSF but not IL-3. This possibility is supported by the observation that IL-3-treated cells were protected to a greater extent compared to unstimulated cells when PI 3-kinase was blocked (for example, compare the kinetics of cell death between these two conditions in Figure 3.5).

In light of these initial results, it became clear that a detailed evaluation of several downstream targets of PI 3-kinase was required to further define the survival pathway(s) activated by PI 3-kinase. At about the same time as these studies were published, several punitive downstream targets of PI 3-kinase were becoming recognized, including PKB/Akt, p70 S6 kinase, and Erk. In addition, several novel and atypical PKC isoforms appeared to be under the control of PI 3-kinase generated signals. Gradually it became clear that PI 3-kinase was controlling a large set of downstream kinases, and that a systematic approach would be necessary to evaluate which were critical for survival.

4. DISSOCIATION OF MAPK AND P70 S6 KINASE AS EFFECTORS OF PI 3-KINASE REGULATED SURVIVAL SIGNALS

4.1. RATIONALE AND HYPOTHESIS

The p70 S6 kinase is believed to be a physiological target downstream of PI 3-kinase. The extracellular regulated kinases (Erk's) may also be targets of PI 3-kinase. Since PI 3-kinase is a key regulator of survival, p70 S6 kinase and/or Erks may be effectors of this survival pathway.

4.2. RESULTS

4.2.1. p70 S6 Kinase

p70 S6 kinase is required for the phosphorylation of the S6 subunit of the 40S ribosome, which appears to be necessary for assembling the 40S subunit into translating polysomes (Jefferies et al., 1996). S6 phosphorylation preferentially enhances the translation of mRNA with 5'-terminal polypyrimidine tracts. Mitogen activated p70 S6 kinase occurs in conjuncture with cell division, when new protein synthesis is required for G1 - S phase progression. Activation of p70 S6 kinase occurs rapidly (minutes) following cytokine receptor activation, indicating the presence of direct signalling pathways to this enzyme. Moreover, it is coupled closely with the action of the PI 3-kinase-related protein kinase mTOR.

Phosphorylation of p70 S6 kinase retards it mobility through SDS-PAGE, resulting in several bands with slower migration, which is a good indication of protein phosphorylation (Klippel et al., 1998). In the MC/9 cell line, IL-3 and GM-CSF stimulated phosphorylation of p70 S6 kinase as indicated by a mobility shift (Figure 4.1). IL-4 was also able to induce the same characteristic shift. (Figure 4.2). Induction of phosphorylation was blocked in cells pre-incubated with either LY-294002, wortmannin or rapamycin,

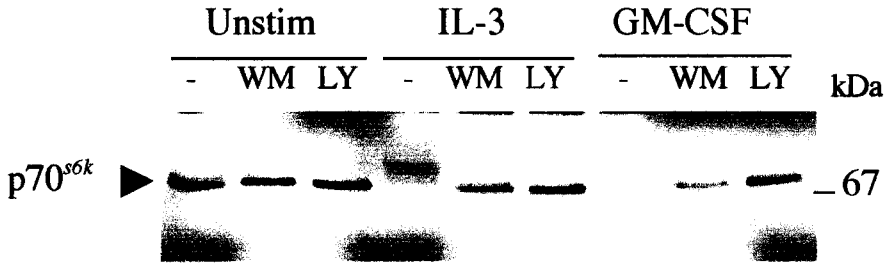


Figure 4.1. PI 3-kinase inhibitors block the phosphorylation of p70 S6 kinase in cytokine-stimulated MC/9 cells. MC/9 cells were treated with wortmannin (WM; 100 nM) or LY-294002 (LY; 25 μM) as indicated and either remained unstimulated, or stimulated for 5 min with IL-3 or GM-CSF. Cells were lysed with solubilization buffer and lysates were separated on an 8% polyacrylamide gel (acrylamide:bisacrylamide ratio 118:1). Proteins were transferred to nitrocellulose, probed with anti-p70 S6 kinase, and detected with ECL (Amersham). WM or LY was also able to inhibit IL-4-stimulated p70 S6 kinase band-shift in MC/9 cells (Figure 4.2).

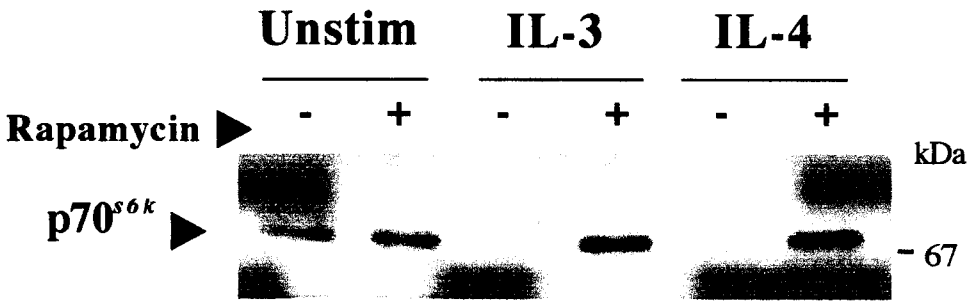


Figure 4.2. Rapamycin inhibits p70 S6 kinase phosphorylation. MC/9 cells (2 x 10^6) were treated with rapamycin or vehicle followed by stimulation with medium alone, IL-3 or IL-4. p70 S6 kinase was detected as described in Figure 4.1.

demonstrating the upstream requirement for PI 3-kinase and mTOR activities on p70 S6 kinase activation (Figures 4.1 and 4.2).

The T cell line CTLL-2 was also used to investigate the ability of two cytokines, IL-2 and IL-4, to regulate p70 S6 kinase activity. IL-2 is well known to activate both the Ras/MAPK pathways and the PI 3-kinase pathway (Karnitz et al., 1995). IL-2 effectively activated p70 S6 kinase, measured by both a decrease in mobility and measured *in vitro* using a peptide substrate (Figure 4.3). In contrast to MC/9 cells, IL-4 was an ineffective agent at stimulating p70 S6 kinase phosphorylation or activity in CTLL-2 cells. Treatment of these cells with rapamycin completely abolished all p70 S6 kinase activity (Figure 4.3), confirming a role for mTOR in IL-2-mediated p70 S6 kinase activation. Since IL-4 could not induce p70 S6 kinase activity, it was important to determine if IL-4 receptors were functional on this cell line. Whole cell tyrosine phosphorylation was measured by immunoblotting cell lysates with an anti-phosphotyrosine antibody (Figure 4.4). IL-2 treatment resulted in dramatic increases in tyrosine phosphorylation compared with unstimulated cells. Although IL-4 was less dramatic, it did clearly increase tyrosine phosphorylation of several proteins, including an approximately 170 kD protein, that was likely IRS-2, a previously reported target of IL-4 stimulation. Therefore, further experiments will be needed to fully characterize the effect of IL-4 on p70 S6 kinase, but clearly this enzyme is not activated by targets of the IL-4 receptor in CTLL-2 cells.

Having established that the cytokines tested on MC/9 could induce phosphorylation of p70 S6 kinase, and that IL-2 but not IL-4 could increase p70 S6 kinase activity in CTLL-2 cells, we next determined the effects of blocking p70 S6 kinase on survival. First, rapamycin at concentrations which blocked the mobility shift of p70 S6 kinase induced by IL-3 or GM-CSF had no effect on the survival of MC/9 cells incubated in the presence of these cytokines, nor was rapamycin able to prevent apoptosis resulting from cytokine withdrawal (Figure 4.5). Secondly, CTLL-2 cells deprived of cytokine for 12 hours accumulated DNA fragments, while cells incubated in IL-2, and to a slightly

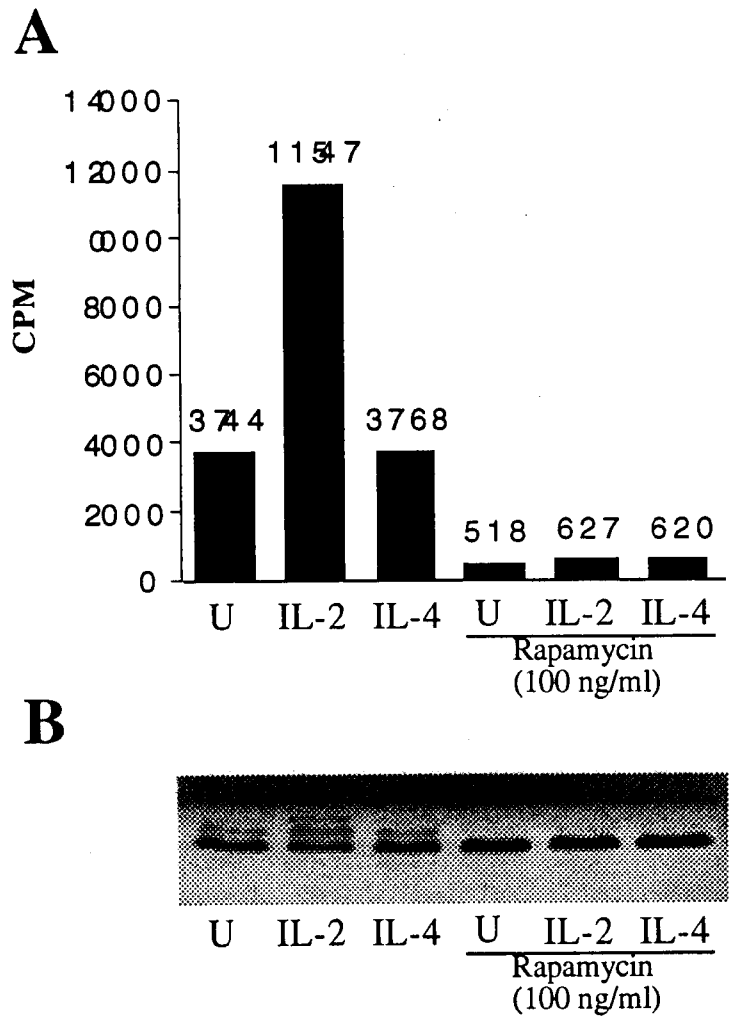


Figure 4.3. IL-2, but not IL-4, stimulates p70 S6 kinase activity in the CTLL-2 cell line. CTLL-2 cells (1 x 10^7) treated with rapamycin (100 ng/ml) or vehicle were stimulated with the indicated cytokines and lysed with solubilization buffer. **A**. p70 S6 kinase was immunoprecipitated and an *in vitro* kinase assay using a peptide substrate was performed as described in Materials and Methods. The average cpm activity of duplicate determinations is shown above each bar. **B**. Cell lysates were resolved on an 8% polyacrylamide gel (acrylamide:bisacrylamide ratio 118:1), transferred to nitrocellulose and probed with anti-p70 S6 kinase antibody. Proteins were detected with ECL (Amersham).

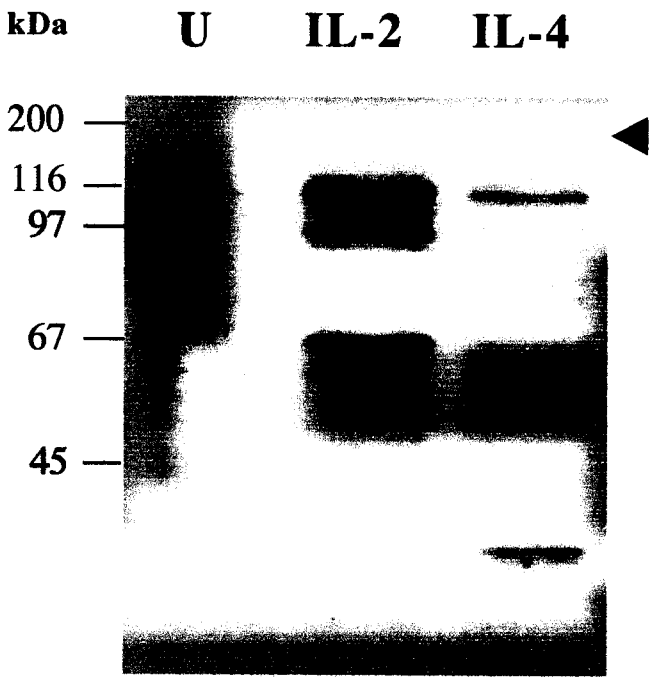


Figure 4.4. IL-4 is able to induce tyrosine phosphorylation in CTLL-2 cells. Cells (2 x 10^6) were stimulated with cytokine and lysed with solubilization buffer. Cell lysates were resolved on a 12% polyacrylamide gel, transferred to nitrocellulose and probed with anti-phosphotyrosine 4G10 antibody. Proteins were detected with ECL (Amersham). Arrow indicates the p170 phosphopeptide with increased tyrosine phosphorylation in response to IL-4.

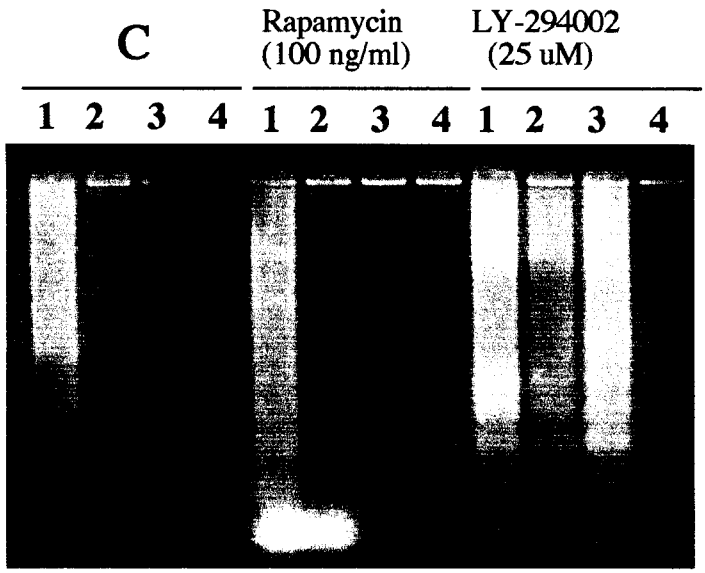


Figure 4.5. LY-294002, but not rapamycin, induces apoptosis in MC/9 cells stimulated with various cytokines. MC/9 cells (0.25 x 10^6) washed free of cytokine were cultured in the presence of IL-3 (500 ng/ml. #2), IL-4 (500 ng/ml. #3) or GM-CSF (60 U/ml, #4) or medium alone (#1) and treated with rapamycin (100 ng/ml) or LY-294002 (25 μM) for 8 h. Cells were harvested and DNA fragments were isolated as described in Methods and Materials. Similar results were also obtained when low concentrations (25 nM) of wortmannin were substituted for LY-294002 (data not shown).

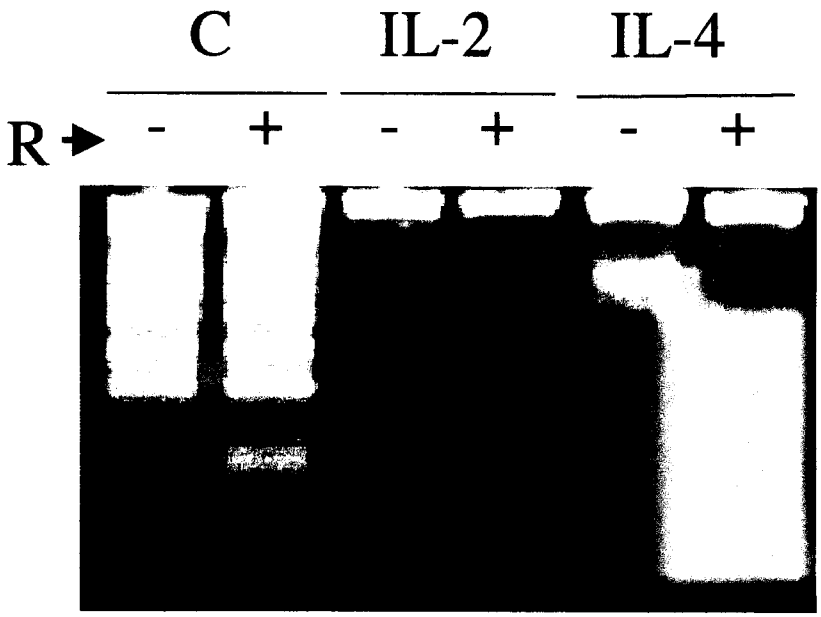


Figure 4.6. Rapamycin treatment does not induce DNA fragmentation in IL-2 or IL-4-stimulated CTLL-2 cells. Cells (0.25×10^6) washed free of cytokine were cultured for 16 h in the presence or absence of rapamycin (100 ng/ml; R) as well as the indicated cytokine. Cells were harvested and DNA fragments were isolated as described in Materials and Methods.

lesser extent IL-4, were protected from apoptosis (Figure 4.6). Co-treatment with rapamycin in each of these conditions did not effect the ability of either cytokine to prevent apoptosis. Since it was clear that rapamycin was able to block p70 S6 kinase at the concentrations used, while it had no effect on apoptosis, these findings support the conclusion that p70 S6 kinase is not a critical enzyme activity in the protective effects of the tested cytokines.

4.2.2. Mitogen activated protein kinase

MAPK signalling "cassettes" are structured cascades that begin generally with activation of a monomeric G-protein at the plasma membrane. In the case of $p42^{erk2}$ and $p44^{erk1}$, the classical MAPK enzymes, GTP loading of $p21^{ras}$ results in the activation of Raf1, a serine/threonine kinase which phosphorylates and activates MEK1 (MAPK/Erk kinase) and MEK2, two dual specificity kinases which are the direct upstream activators of both $p44^{erk1}$ and $p42^{erk2}$. Phosphorylation of $p44^{erk1}$ and $p42^{erk2}$ at a tyrosine and threonine residue in the conserved TEY motif results in full activation. The MAPK enzymes have both cytosolic and nuclear targets, including transcription factors such as c-Fos, Elk-1 and c-Myc that are involved in the expression of immediate early gene products and are important for the initiation of cell division (Denhardt, 1996). Downstream targets of Erk1 and 2 include the $p90^{Rsk}$ and p70 S6 kinases.

Numerous publications have suggested a requirement for PI 3-kinase activity in the activation of Erk (Welsh et al., 1994; Cross et al., 1994; Karntiz et al., 1995; Hu et al., 1995). These reports have generally relied on the effects of wortmannin to assess the importance of PI 3-kinase in Erk activation by growth factors. Also, transient expression of catalytically active p110 enzyme results in an increase in Erk activation over a lengthy time period (Hu et al., 1995). However, it may be difficult to exclude an autocrine mechanism in these experiments. It is possible that the differential ability of GM-CSF compared with IL-3 to protect cells from apoptosis in the presence of PI 3-kinse inhibitors could be

accounted for by a difference in MAPK activation. Therefore, this was addressed in the next set of studies. To begin, Erk activation was measured following stimulation with several cytokines (Figure 4.7). Both IL-3 and GM-CSF were strong inducers of kinase activity, to similar degrees, while IL-4 could not activate the enzyme. Immunoblotting of a fraction of the IL-3 and GM-CSF-stimulated cell lysates demonstrated a characteristic bandshift of both p44erk1 and p42erk2 (Figure 4.7), indicative of phosphorylation, during conditions of IL-3 and GM-CSF treatment. Generally, these results are consistent with previous reports (Welham et al., 1993).

Since IL-3 and GM-CSF could both activate Erk, we then tested the effects of PI 3-kinase inhibition on this activation. Wortmannin treatment of cells prior to stimulation dramatically reduced the activation of Erk, as measured by immunocomplex kinase assays using myelin basic protein (MBP) as a substrate (Figure 4.8). The maximal reduction of Erk activation was approximately 50%, occurring with 100 nM wortmannin. Higher doses of wortmannin had no greater affect. Additionally, wortmannin up to 2 μM had no effect on Erk activity when added directly to the kinase reaction (data not shown), demonstrating that it was the activation of Erk which was sensitive to wortmannin as opposed to Erk enzyme activity. The maximal activation of Erk by GM-CSF was similar to that of IL-3 and the inhibition by wortmannin was also similar for both. LY-294002 was also tested and was found to be effective in blocking MAPK activation at concentrations of about 100 μM. Autoradiographs of MBP from these experiments and corresponding immunoblots for $p44^{erk1}$ are shown in Figure 4.9. Additional experiments testing the effects of wortmannin and LY-294002 on GM-CSF-stimulated Erk activation are presented in Table 2. Wortmannin was consistently able to inhibit $p44^{erk1}$ activation by about 50% at concentrations between 100 and 200 nM, while LY-294002 had no effect up to the highest concentration tested, 50 μM.

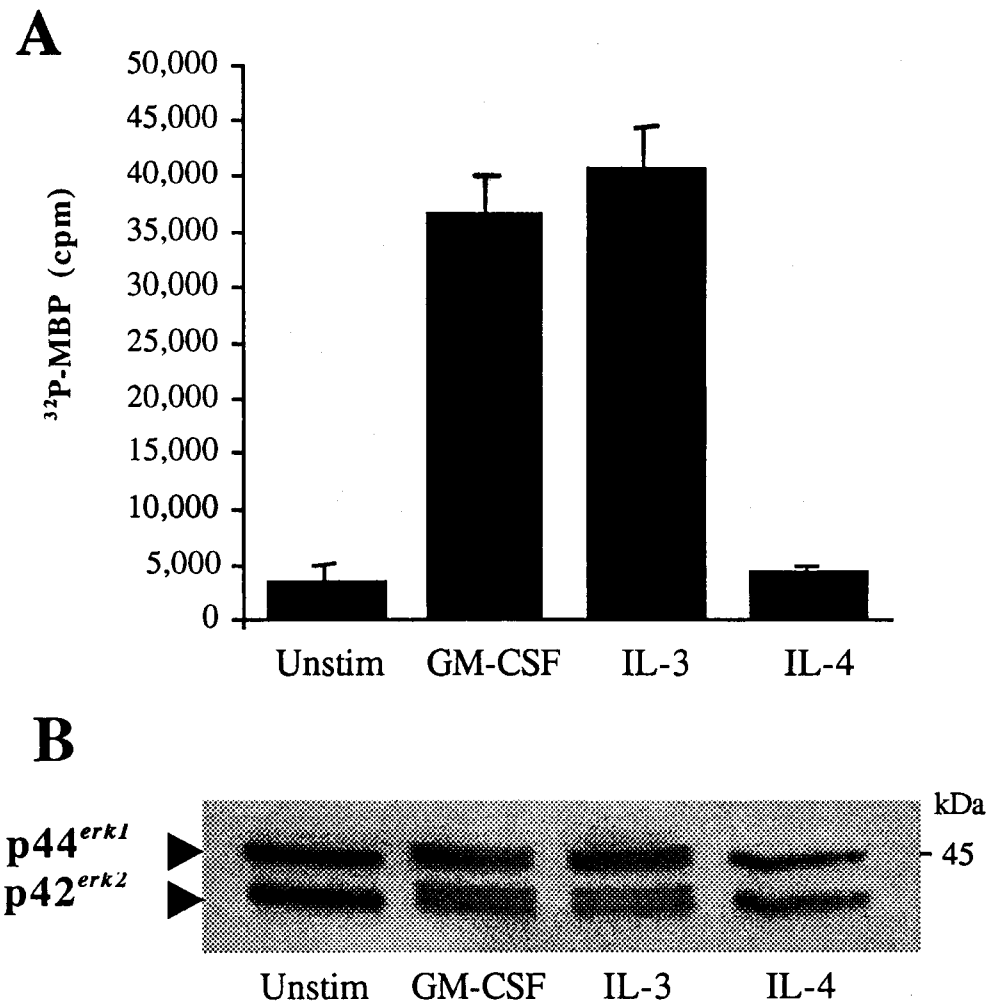


Figure 4.7. Erk activation by IL-3, GM-CSF, but not IL-4. MC/9 cells were starved overnight in complete medium containing 1/10 the normal amount of IL-3, washed, and prepared as described in Materials and Methods. Cells were then stimulated with synthetic IL-3 (10 μg/ml), 60 U/ml recombinant murine GM-CSF, or 10 μg/ml synthetic IL-4 for 5 min and then isolated and detergent-solubilized as described in Materials and Methods. Lysates were immunoprecipitated with anti-p44^{erk-1} coupled to agarose beads. **A.** Activity of washed immunoprecipitates were measured by the incorporation of ^{32}P into myelin basic protein as determined by scintillation counting following fractionation by SDS-PAGE (15% polyacrylamide gel) and transfer to nitrocellulose. Results are the average +/- standard deviation from three independent experiments. **B.** Some of the detergent-solubilized lysates were separated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose, and probed with an anti-Erk antibody (lower panel).

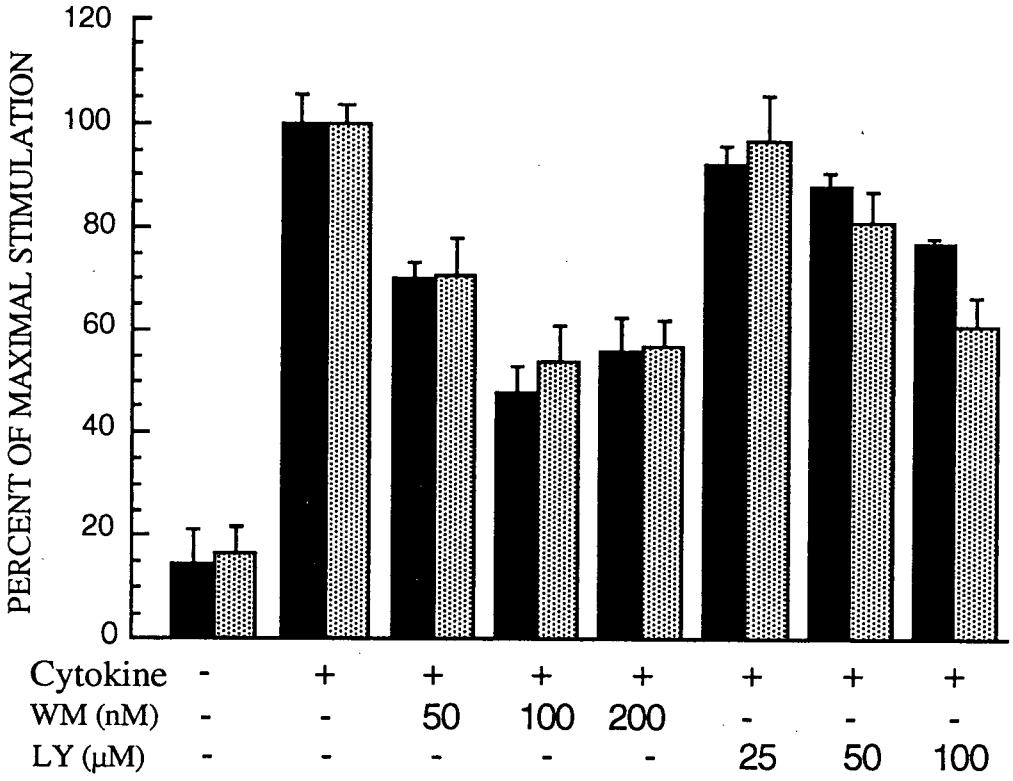
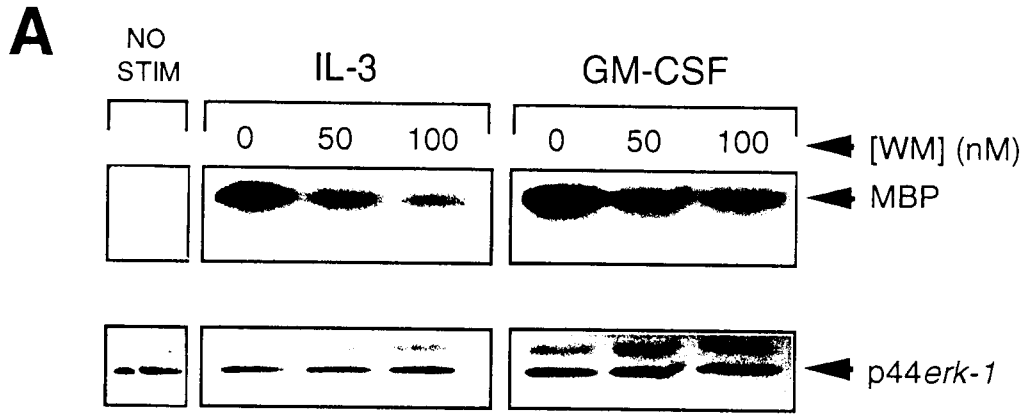
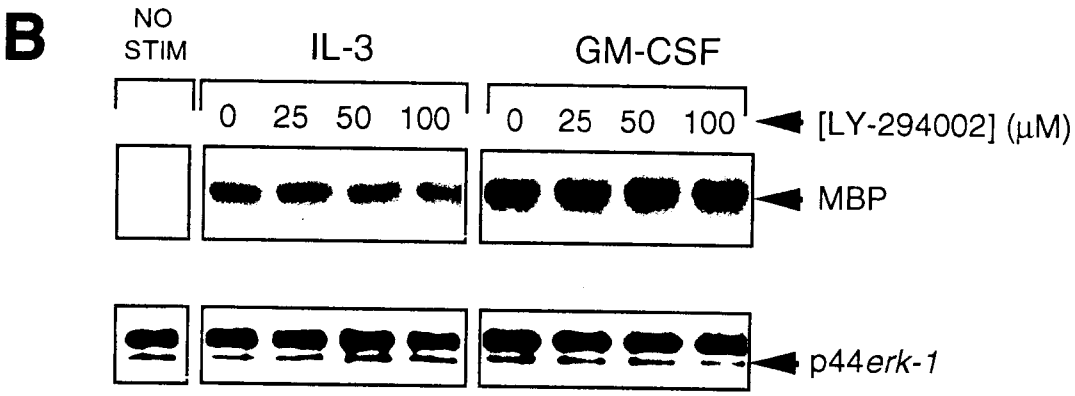


Figure 4.8. Inhibition of Erk activity by wortmannin and LY-294002. MC-9 cells were prepared as described in Materials and Methods and treated with medium alone or medium containing the indicated concentrations of WM or LY-294002 for 10 min. Following stimulation by either 60 U/ml recombinant murine GM-CSF (shaded bars) for 5 min or 10 μg/ml synthetic IL-3 (solid bars) for 10 min cells were isolated and detergent-solubilized as described in Materials and Methods. Lysates were immunoprecipitated with anti-p44$^{erk\text{-}1}$ coupled to agarose beads. Activity of washed immunoprecipitates were measured by the incorporation of ^{32}P into myelin basic protein as determined by scintillation counting following fractionation by SDS-PAGE (15% polyacrylamide gel) and transfer to nitrocellulose. Results are the average +/- standard deviation from four independent experiments. Values for unstimulated samples were typically in the range of 2000 to 4000 cpm.

Figure 4.9. Representative experiment of MBP phosphorylation by anti-p44$^{erk\text{-}1}$ immunoprecipitates and corresponding anti-p44$^{erk\text{-}1}$ blot. **A.** Kinase reactions of MBP phosphorylation by immunoprecipitated p44$^{erk\text{-}1}$ cell lysates from indicated conditions were separated on a 15% polyacrylamide gel, transferred to nitrocellulose and exposed to film (upper panel). The membrane was then immunoblotted with anti-p44$^{erk\text{-}1}$ (lower panel). **B.** Experiment was similar to that shown in A, but with the indicated concentrations of LY-294002. **C.** To confirm the identity of the p44$^{erk\text{-}1}$ in the immunoprecipitates, whole cell extract (lane 1), antibody beads alone (lane 2), or immunoprecipitated extracts (lane 3) were immunoblotted using the anti-p44$^{erk\text{-}1}$ antibody. This antibody detects both p44$^{erk\text{-}1}$ and p42$^{erk\text{-}2}$, but only p44$^{erk\text{-}1}$ was immunoprecipitated by the anti-p44$^{erk\text{-}1}$ beads. The IgG heavy chain present in samples containing anti-p44$^{erk\text{-}1}$ beads is indicated. Identical results were obtained when a different p44$^{erk\text{-}1}$ antibody was used to immunoblot (results not shown).


A
NO STIM
IL-3
GM-CSF
0 50 100
0 50 100
[WM] (nM)
MBP
p44erk-1


B
NO STIM
IL-3
GM-CSF
0 25 50 100
0 25 50 100
[LY-294002] (μM)
MBP
p44erk-1

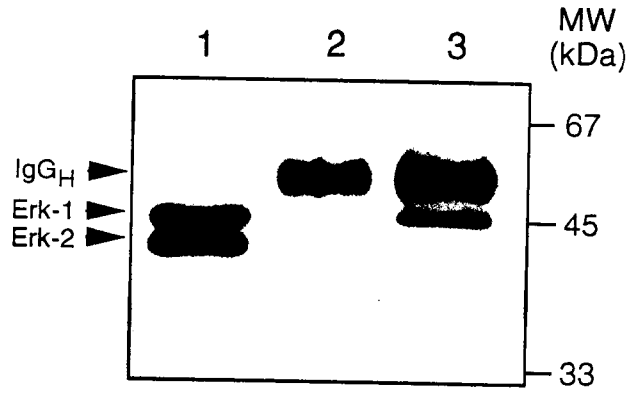

C
1
2
3
MW (kDa)
67
45
33
IgG$_H$
Erk-1
Erk-2

Table 2

Inhibition of basal and GM-CSF-stimulated MAP kinase activity by wortmannin and LY-294002

Values represent triplicate determinations of MAP kinase activity from filter paper binding assays as described under "Methods and Materials." Cell treatments were carried out as described in Fig 4.8

Inhibitor	cpm +/- S.D.		Fold Stimulation [a]
	Unstimulated	GM-CSF	
[LY-294002] (μM)			
0	2085 +/- 196	11,052 +/- 488	5.3
10	2109 +/- 154	12,051 +/- 1683	6.0
25	2009 +/- 168	9,655 +/- 242	4.6
50	2403 +/- 150	10,228 +/- 583	4.9
[Wortmannin] (nM)			
0	3655 +/- 132	15,685 +/- 800	4.3
10	4779 +/- 744	12,751 +/- 582	3.5
25	5348 +/- 481	12,007 +/- 953	3.3
50	4674 +/- 90	9972 +/- 90	2.7
100	5065 +/- 141	7193 +/- 345	2.0

[a]Compared to cpm in unstimulated cells in the absence of inhibitor.

It became clear at this point that wortmannin may be acting non-specifically in the inhibition of Erk, suggesting that PI 3-kinase was not upstream of Erk. The results of Table 1 become important to revisit: LY-294002 pretreatment of 25 μM abolished all PIP_3 generated in these cells, which is completely consistent with the reported potency of this inhibitor (Vlahos et al., 1994), yet when used at this concentration, LY-294002 had no effect on Erk activation. These results clearly dissociate Erk activation from the activation of PI 3-kinase, and points to the existence of non-specific targets for wortmannin.

It appeared likely that LY-294002 at high (100 μM) concentrations was acting on targets besides PI 3-kinase to reduce Erk activation. To further test this, wortmannin- or LY-294002-treated cells were stimulated with phorbol ester, which activates Erk through a PKC-dependent mechanism, and is unable to activate PI 3-kinase. Erk activation by phorbol esters does not appear to involve any wortmannin-sensitive enzymes, since wortmannin had no affect at a concentration of 200 nM (Figure 4.10). LY-294002 pretreatment of 25 μM did not attenuate Erk activation by phorbol esters, as expected, although higher concentration such as 100 μM caused a similar decrease as see following GM-CSF stimulation. Since PI 3-kinase is not activated by phorbol esters, this result clearly demonstrates that LY-294002 at high concentrations must be inhibiting other enzymes besides PI 3-kinase necessary for the activation of Erk.

Finally, the effect of blocking Erk activation on survival was investigated. PD98059 is a cell permeable, stable, potent and selective inhibitor of MEK (Alessi et al., 1995). Treatment of cells with PD98059 (50 μM) over an 11 hr time course did not induce apoptosis in cytokine-stimulated cells nor did it potentiate apoptosis in cytokine-starved cells (Figure 4.11). MEK inhibition was confirmed by stimulating cells with IL-3 or GM-CSF and probing cell lysates with an anti-phospho-Erk antibody, which only detects the phosphorylated, activated forms of Erk 1 and 2 (Figure 4.11.B).

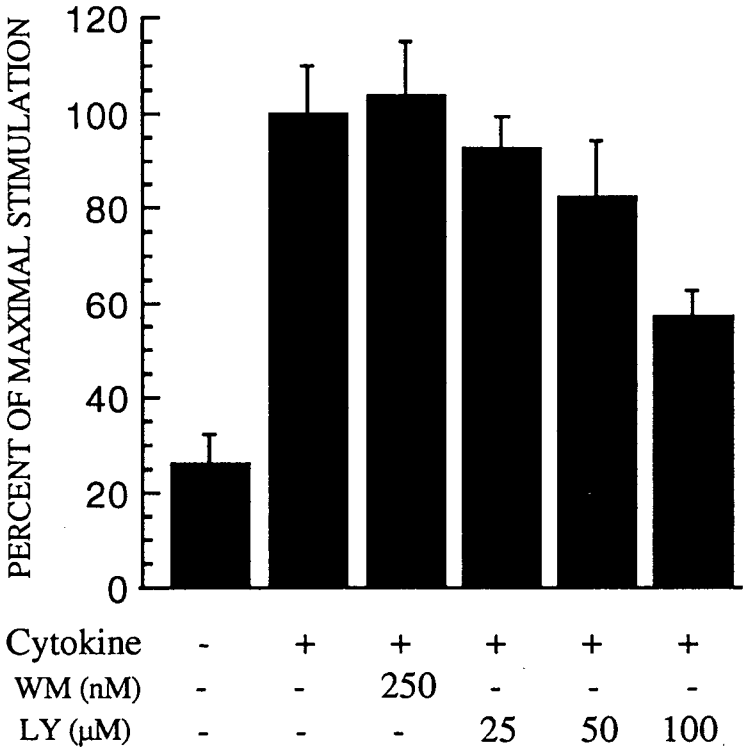


Figure 4.10. Erk activity is attenuated by LY-294002 but not WM following stimulation of cells with phorbol ester. MC/9 cells were treated with medium or medium containing indicated concentrations of WM or LY-294002 for 10 min and then with 100 nM phorbol dibutyrate or vehicle alone. Erk assay was performed as in Figure 4.7. Results are the average +/- standard deviation of four independent experiments. Unstimulated samples were generally in the 2000 - 4000 cpm range.

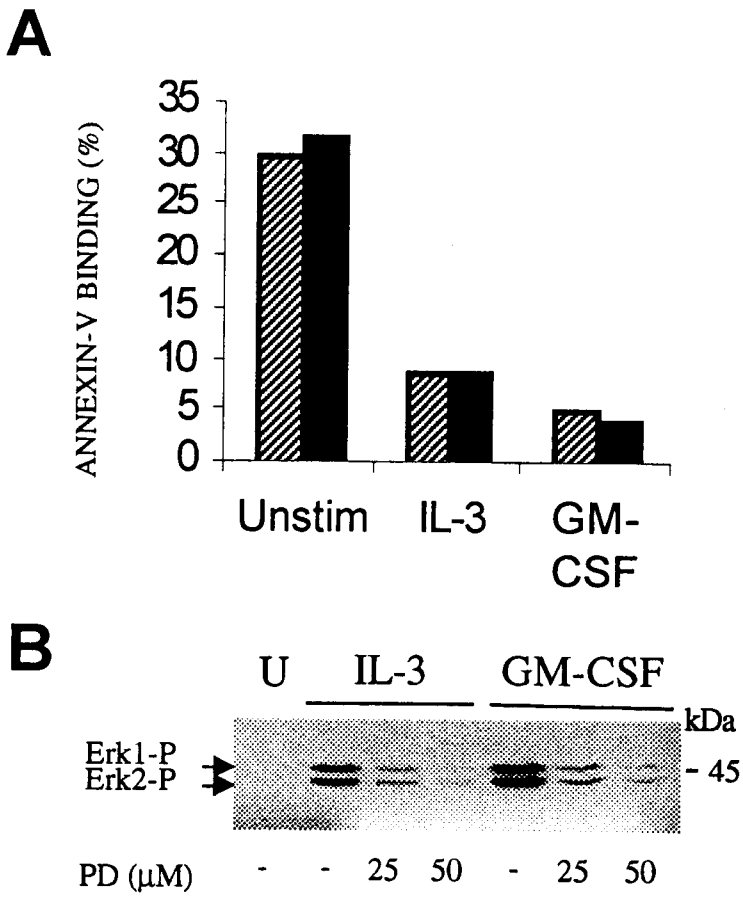


Figure 4.11. Effect of MEK inhibition on survival. MC/9 cells were incubated with IL-3, GM-CSF or medium alone and with 50 μM PD98059 (shaded bars) or vehicle alone (solid bars) for 11 hours. **A.** Apoptosis was determined by annexin V binding by using flow cytometry. Results shown are averages of duplicate samples and are representative of two independent experiments. The range for each duplicate was less than 2%. **B.** Cells were incubated with PD98059 (PD) at the indicated concentrations for 30 min followed by stimulation with IL-3 or GM-CSF for 5 min. Detergent solubilized cell lysates were fractionated by SDS-PAGE and immunoblotted with anti-phospho-Erk (New England Biolabs).

4.3. DISCUSSION

These studies have demonstrated that PI 3-kinase-mediated survival is not dependent on p70 S6 kinase or Erk. First, with respect to p70 S6 kinase, inhibition of this enzyme with rapamycin did not attenuate the ability of several cytokines to promote PI 3-kinase-dependent survival. Additionally, IL-4 was able to promote survival of CTLL-2 cells without activating p70 S6 kinase, adding further evidence for a separation between survival and p70 S6 kinase activation.

Rapamycin binding with its cellular receptor protein FKBP12 forms a complex which inhibits the protein kinase activity of mTOR (Brown et al., 1994; Sabatini et al., 1994; Chiu et al., 1994; Sabers et al., 1995). Introduction of mutant mTOR which is no longer sensitive to rapamycin restores p70 S6 kinase activity (Brown et al., 1995), establishing that mTOR is upstream of p70 S6 kinase. The studies presented here have established that mTOR inactivation by rapamycin does not abrogate the ability of cytokines to promote survival. This is important, since both wortmannin and LY-294002 appear to inhibit mTOR directly (Brunn et al. 1996). Without the availability of rapamycin as an inhibitor of mTOR, the results of Chapter 4 could not have distinguished between PI 3-kinase and mTOR as the critical element for survival signalling.

With respect to Erk, IL-4 promoted PI 3-kinase dependent survival in the absence of Erk activity. As well, PI 3-kinase inhibition by LY-294002 resulted in cell death in the presence of IL-3, while not affecting receptor signalling to Erk. Finally, treatment of cells with the MEK inhibitor PD98059 had no effect on cell survival while blocking Erk phosphorylation following IL-3 or GM-CSF stimulation.

A noteworthy report which appeared after the publication of our findings suggested that PI 3-kinase signalling by IGF was synergizing with Erk activation to promote survival (Parrizas et at, 1997). It was demonstrated that Erk inhibition alone was able to induce a fraction of IGF-1 stimulated cells into apoptosis. Using the MEK inhibitor PD98059 in combination with wortmannin produced a greater degree of apoptosis than PI 3-kinase

inhibition alone. These results may reflect a degree of cooperation between the PI 3-kinase pathway and the Erk pathway in promoting survival. However, it should be noted that LY-294002 was not also tested, raising doubts as to whether this synergy was entirely a result of PI 3-kinase inhibition. In this respect, Erk signalling may constitute a minor or major role in survival depending upon the cell system and environmental context. Furthermore, this effect may involve the activation state of other MAPK family members, such as JNK or p38 MAPK. The balance between Erk activity and those of the JNK family has been proposed as a regulator of cell death (Xia et al., 1995). In the studies presented here, it appears that Erk activity is not necessary for the survival effects of cytokines. In the developing eye of Drosophila, on the other hand, apoptosis-suppressing phosphorylation of Hid by a Ras-dependent Erk pathway appears to play a dominant role over a minor survival effect provided by PI 3-kinase and PKB (Bergmann et al., 1998). A caveat of these experiments is that the role for a Ras/MAPK pathway was assessed during Hid overexpression. From this work, it was clear that Ras/MAPK leads to Hid inactivation, but since Hid was overexpressed, any pathway which inactivates it becomes the dominant survival pathway to prevent death and the eye-ablation phenotype.

While our results here would suggest that Erk activation is not essential for protection from apoptosis, there may be a MEK-dependent pathway which leads to the Bcl-2 family member Bad. These results will be discussed in detail in later, and highlight the possibility for redundant pathways operating under specific situations depending upon the cellular environment. For instance, IL-3 survival signals may predominantly use the PI 3-kinase pathway to prevent apoptosis while other cell types in other environments may preferentially use a Erk component to achieve the same goal. This possibility should not be overlooked when examining the survival signals generated by a survival factor receptor on any particular cell type.

The cross-talk between different signalling pathways is an important aspect of our understanding of signal transduction since it will likely have great effect on the final

biological endpoint. Prior to this work, accumulating evidence in the literature suggested that a degree of cross-talk existed between PI 3-kinase and Erk. The work demonstrated here points strongly to a lack of cross-talk of PI 3-kinase and the pathways which lead to Erk activation. This study, however, does not rule out the possibility that wortmannin, but not LY-294002, inhibits a normal function of PI 3-kinase separate from PIP_3 generation. This may be related to the role of PI 3-kinase as a protein kinase (Hunter, 1995).

Finally, a noteworthy result of this work comes from the use of "specific" inhibitors, such as wortmannin. This work was one of the first to recognize a potential PI 3-kinase-independent target of wortmannin that is affected by low concentrations similar to those used to inhibit PI 3-kinase (Scheid and Duronio, 1996), and suggests that the earlier papers which utilized wortmannin to place Erk downstream of PI 3-kinase should be re-evaluated. Furthermore, the results with phorbol ester-stimulated Erk suggests that LY-294002 can also attenuate Erk independently of PI 3-kinase, if used at high enough concentrations. After our work was accepted for publication, Cross et al. (1996) published a report demonstrating that phospholipase A_2, itself a downstream target of Erk, was inhibited by wortmannin via a PI 3-kinase independent mechanism. Additionally, Ferby followed up on previous work by demonstrating that PAF-stimulated Erk activation was insensitive to expression of a dominant negative p85, which blocked PIP_3 formation, but remained sensitive to the effects of wortmannin (Ferby et al., 1996). Together, these three reports strongly suggest that the effect of wortmannin on Erk are due to targets distinct from PI 3-kinase. Furthermore, they stress the need to carefully evaluate wortmannin-sensitive effects at concentrations consistent with PI 3-kinase inhibition. Supportive evidence using alternate methods of inhibition, such as the use of LY-294002 or dominant negative strategies are also essential.

5. EXAMINATION OF BCL-X_L-ASSOCIATED DEATH INDUCER (BAD) AS A POTENTIAL TARGET OF PI 3-KINASE ACTIVITY

5.1. RATIONALE AND HYPOTHESIS

PKB has recently been identified as a potential downstream target of PI 3-kinase responsible for a survival signal. One target of PKB could potentially be Bad, based on the consensus PKB phosphorylation site surrounding Ser136. Phosphorylation of Bad inactivates the pro-apoptotic function of the protein. Thus, PI 3-kinase dependent phosphorylation of Bad via PKB could be one means by which PI 3-kinase prevents apoptosis.

5.2. RESULTS

To test this hypothesis, we began by measuring Bad phosphorylation by electrophoretic mobility shift. Bad phosphorylation on two or more sites causes it to migrate slower than monophosphorylated or unphosphorylated Bad during SDS/PAGE (Zha et al., 1996), a characteristic shared by many phosphoproteins, including p70 S6 kinase and Erk shown earlier. To establish immunoprecipitation and immunoblotting methods, several commercially available antibodies were evaluated. Immunoprecipitation of endogenous Bad from 500 μg of MC/9 lysates with 2.5 μg B36420 (Transduction Labs) completely immuno-depleted all Bad protein (Figure 5.1). Blotting with this same antibody detected the Bad doublet (indicated by arrow), as well as a very strong immunoreactive band at approximately 23 kDa that was observed in whole cell lysates. This protein was not co-immunoprecipitated with Bad (since it remained in the sample following immuno-depletion), nor was it recognized by any other anti-Bad antibody used for immunoblotting. Another anti-Bad antibody was tested (Pharmingen 2G11) and was found not to be effective in immunoprecipitating Bad. An equivalent amount of an unrelated mouse IgG antibody served as a control, and was also not able to bring down the

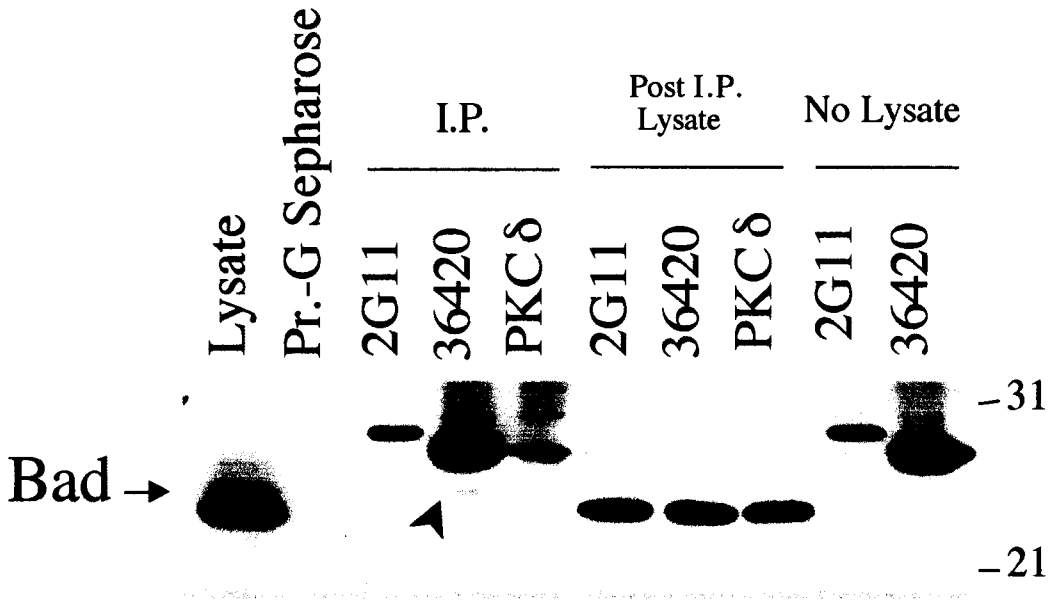


Figure 5.1. Immunoprecipitation and immunoblotting for murine Bad. MC/9 cells (15 x 10^7) were lysed in Bad solubilization buffer (see Materials and Methods) and divided into three tubes. Anti-Bad antibodies were added (2 μg of 2G11 (Pharmingen) or 2 μg of B36420 (Transduction Labs)) or 2 μg of an unrelated murine monoclonal (PKC δ (Transduction Labs)), followed by rotation at 4°C overnight. Antibodies were captured with 10 μl of Protein-G sepharose and boiled in sample buffer. 10 μl of the whole cell lysate, Protein-G sepharose beads, immunoprecipitations, post immunoprecipitation lysates, or antibodies alone were fractionated by SDS/PAGE (12% polyacrylamide gel) and transferred to nitrocellulose. The blot was probed with a 1 μg/ml solution of anti-Bad (B36420) and detected with 1:10000 dilution of goat anti-mouse secondary conjugated to HRP using ECL (Amersham). The Bad doublet is indicated by arrows.

doublet at 24/25 kD. Several other antibodies could immunoprecipitate this doublet, including B31240 (Transduction labs) and SC-943 (Santa Cruz), and both could also detect Bad on fractionated whole cell lysates (SC-943 is shown in Figure 5.2). In most of the experiments shown here, Bas was immunoprecipitated and blotted with B36420, which produced the most consistent results.

Next, experiments were performed to test the effect of cytokine stimulation on Bad phosphorylation. Stimulation with IL-3 rapidly induced the appearance of the slower migrating, 25 kDa form of Bad, demonstrating that Bad undergoes phosphorylation following this stimulus (Figure 5.2a). GM-CSF also induced phosphorylation of Bad (Figure 5.2b), while IL-4 on the other hand was ineffective in producing a change in the mobility of Bad. The specificity of the immunoprecipitating antibody was confirmed by immunoblotting whole cell lysates with a different antibody (SC-943), which produced identical results (Figure 5.2c).

The finding that IL-4 did not induce a bandshift suggested that Bad was not a target of signalling pathways activated by IL-4. However, if Bad was only partially phosphorylated following treatment with IL-4, the shift in mobility may not have been evident by immunoblotting. To test directly whether Bad was being phosphorylated in response to IL-4, *in vivo* phosphate labelling was performed. Bad was immunoprecipitated from ^{32}P-labelled cells following stimulation with IL-3, GM-CSF or SCF. A 70 - 100% increase in radioactivity was caused by IL-3, GM-CSF and SCF (Figure 5.3a and b). IL-4, on the other hand, did not increase radioactivity incorporated into Bad above the levels observed in unstimulated cells (Figure 5.3a and b).

Three recent reports have suggested that Ser136 of Bad is a target for the serine/threonine kinase PKB (Datta et al., 1997; del Peso et al., 1997; Blume-Jenson et al., 1998). These studies have been based on the use of epitope-tagged Bad in transfected systems and the expression of dominant negative forms of PKB. To address whether endogenous Bad was the target of PI 3-kinase-activated PKB, it was first determined

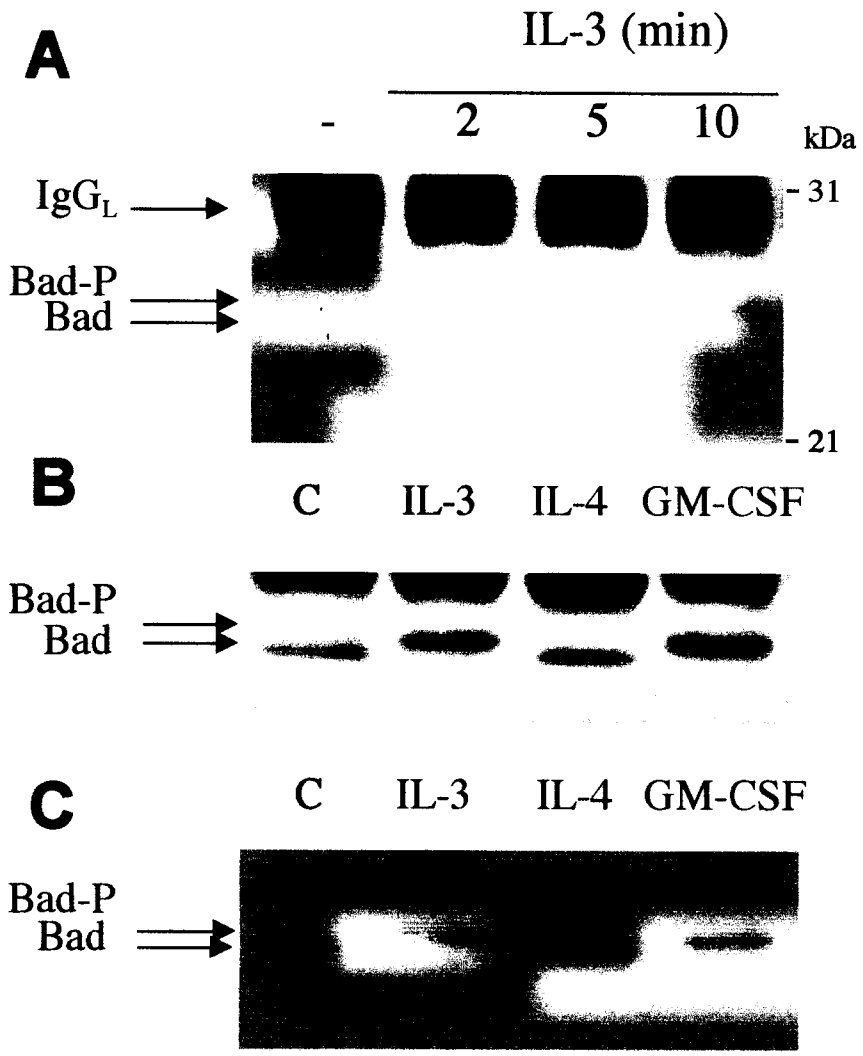


Figure 5.2. Bad mobility shift is induced by treatment with IL-3, GM-CSF or SCF but not IL-4. **A.** Cells were starved of cytokine for 3 - 5 hours and stimulated with IL-3 for the indicated times, or were left unstimulated. Cells were isolated and solubilized as described in Materials and Methods. Bad was immunoprecipitated (5 μg of B36420, Transduction Labs) and separated by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting for Bad. **B.** Immunoprecipitated Bad from cells stimulated with IL-3, IL-4 or GM-CSF for 10 min and immunoblotted with B36420. **C.** Whole cell lysates from a similar experiment immunoblotted with anti-Bad antibody SC-943 (Santa Cruz Biotechnology). Results shown are representative of 4 independent experiments.

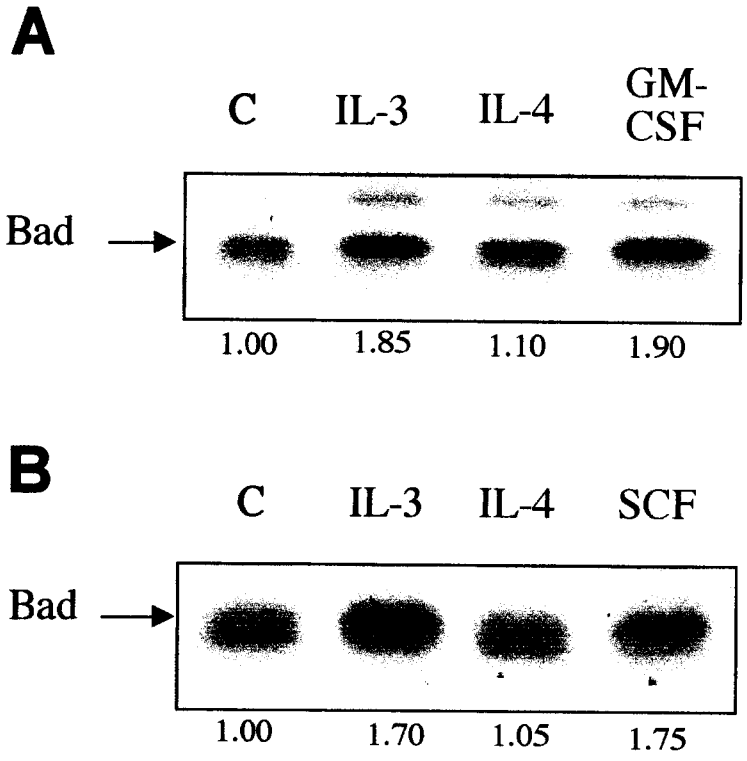


Figure 5.3. IL-4 does not stimulate Bad phosphorylation. **A.** Cells were starved of cytokine for 3 to 5 hours and then metabolically labelled with ^{32}P-orthophosphate for 2 hours, followed by stimulation with the indicated cytokine for 10 min. Bad was immunoprecipitated and fractionated by SDS-PAGE. ^{32}P-labelled Bad was detected by autoradiography and quantitated by either a phosphorimager or by excising the bands and counting by liquid scintillation. This experiment was performed in duplicate, with one set of samples shown. **B.** Identical experiment as A, but SCF was tested instead of GM-CSF. The numbers beneath each lane corresponds to the average fold stimulation above untreated for each set of duplicates.

which cytokines could activate PKB. PKB immunoprecipitated from unstimulated cells was completely inactive, and did not incorporate ^{32}P into a peptide substrate any better than a Protein-G sepharose control. Stimulation with IL-3, IL-4, GM-CSF or SCF all induced an 8 - 10 fold increase in PKB activity (Figure 5.4a), with SCF being the most potent activator (40 fold maximal induction). Pretreatment with LY-294002 completely abolished PKB activation (Figure 5.4a). Next, a time course of activation was performed with GM-CSF in the presence of LY-294002. This compound inhibited all PKB activation following GM-CSF stimulation (Figure 5.4b). These results demonstrated that GM-CSF was not activating PKB independently of PI 3-kinase, which could have accounted for the lack of requirement for PI 3-kinase in GM-CSF-mediated survival. Additionally, since IL-4 activated PKB to levels similar to IL-3 or GM-CSF, our results argue that Bad is not a target of PKB since IL-4 did not increase Bad phosphorylation significantly above unstimulated levels.

We next tested the effects of PI 3-kinase inhibitors on Bad phosphorylation. Cells were pretreated with LY-294002, stimulated with either IL-3 or GM-CSF, and Bad phosphorylation assessed by mobility shift (Figure 5.5a). LY-294002 had a partial effect on IL-3 stimulated Bad phosphorylation, reducing the slower migrating form to a doublet, indicating the presence of both hypo- and hyperphosphorylated forms. The GM-CSF-induced bandshift of Bad on the other hand was only reduced to a slight degree by LY-294002. This effect was generally not consistent between experiments, and is exemplified below. These results suggested a partial requirement for PI 3-kinase in IL-3 stimulated Bad phosphorylation and a complete absence in the case of GM-CSF stimulation. A dose response study was also performed using LY-294002 (Figure 5.5b). At low concentrations (5 μM) the reduction in bandshift was observed following IL-3 treatment and did not change with higher concentrations. Again, Bad isolated from cells stimulated with GM-CSF appeared not to change with any concentration of LY-294002. *In vivo* ^{32}P-labelling

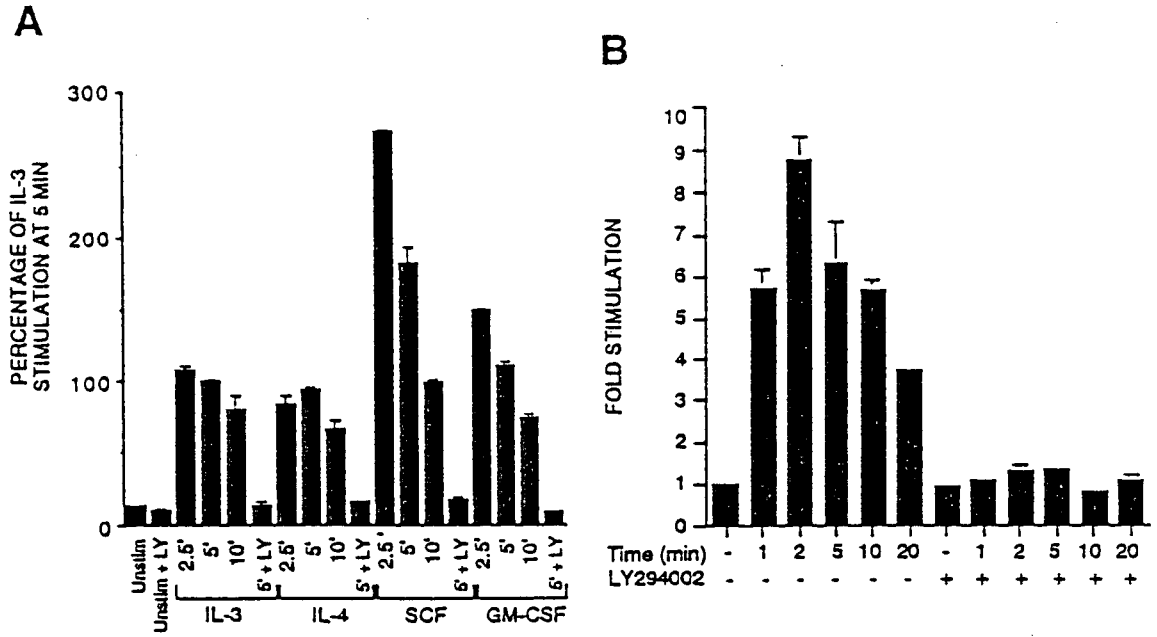


Figure 5.4. Cytokine activation of PKB and Requirement for PI3K. **A.** MC/9 cells starved of cytokine for 3 - 5 hours were stimulated with the indicated cytokines at concentrations which have previously been shown to induce maximal tyrosine phosphorylation and PI3K activity, for the indicated times. Also, some cells were pretreated with LY-294002 (25 μM) for 10 min and stimulated for 5 min with cytokine. Cells were lysed in ice-cold solubilization buffer. PKB was immunoprecipitated and its activity was measured in an *in vitro* kinase assay. Individual experiments have been normalized to the percentage of stimulation induced by 5 min treatment with IL-3, which was performed concurrently. Typical maximum stimulations for IL-3, IL-4 and GM-CSF ranged between 8 - 12-fold above unstimulated samples, which generally were in the 2000 - 3000 CPM range. **B.** Cells were prepared as above and pretreated with LY-294002 (25 μM) or vehicle alone for 10 min followed by stimulation with GM-CSF for the indicated times. PKB immunoprecipitation and kinase assay was performed as above. The results presented are from 3 experiments using duplicate samples, with error bars representing standard error.

with IL-3 or GM-CSF was again performed: PI 3-kinase inhibition was ineffective in attenuating the increase in Bad phosphorylation following GM-CSF stimulation (Figure 5.5c).

While our earlier results suggested that Erk activation was not responsible for the ability of IL-3 or GM-CSF to inhibit apoptosis, it remained a possibility that this pathway could be responsible for some component of Bad phosphorylation. To test this possibility, cells were treated with PD98059, a potent and specific inhibitor of MEK, and Bad phosphorylation was examined following stimulation. MEK inhibition resulted in a reduction in the bandshift of Bad induced by IL-3 or GM-CSF (Figure 5.6a). This correlated with the degree of inhibition of $p44^{erk1}$ or $p42^{erk2}$ phosphorylation (Figure 5.6b). ^{32}P-labelling and immunoprecipitation of Bad was once again performed. MEK inhibition resulted in a significant reduction in IL-3 or GM-CSF-stimulated Bad phosphorylation (Figure 5.6c). This finding suggested that there was a selective inhibition of one or more sites on a multiply-phosphorylated Bad protein, or that the phosphorylation at all sites were decreased.

In order to discern which residues of Bad where undergoing phosphorylation in our model, two dimensional tryptic mapping was performed. First, 1 μg of GST-BAD were phosphorylated *in vitro* with detergent solubilized lysate from cells stimulated with GM-CSF. Following boiling in sample buffer, the phosphorylated products were separated by SDS-PAGE, dried, and exposed to film (Figure 5.7). The GST-Bad was digested with trypsin overnight and the resulting digest was subjected to 2D-peptide mapping. As can be seen in Figure 5.8a, GST-Bad phosphorylated *in vitro* produced an array of phosphorylated peptides. Two peptides from each comigrate exactly with synthetic peptides corresponding to the peptides containing S112 and S136 (Figure 5.8b).

Having established a two-dimensional tryptic mapping protocol, endogenous Bad was isolated from ^{32}P-labelled cells following stimulation with various agonists and

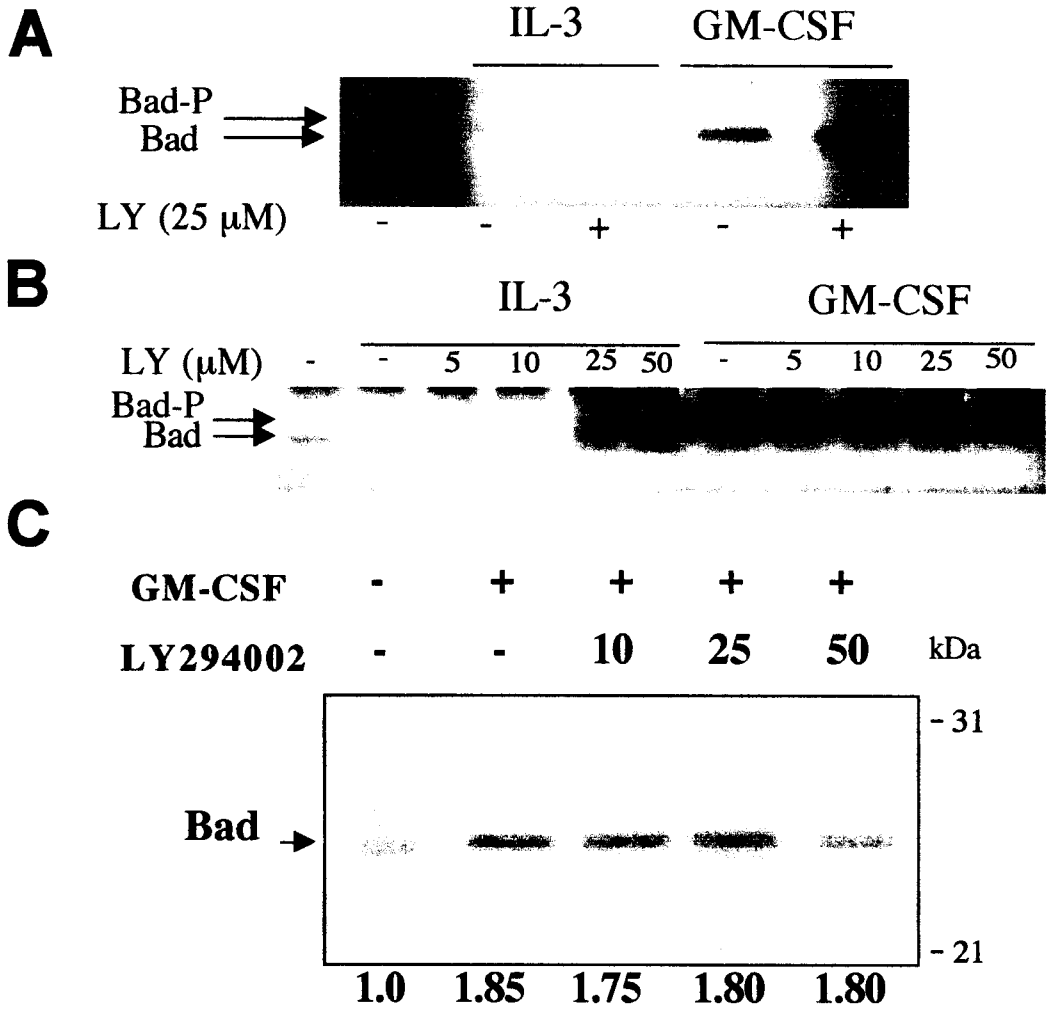


Figure 5.5. PI3K inhibition partially blocks IL-3, but not GM-CSF induced Bad Phosphorylation. **A.** Cells were preincubated with LY-294002 (25 μM), or vehicle alone for 10 min, followed by stimulation with IL-3 or GM-CSF for 10 min. Bad was immunoprecipitated and the mobility shift was examined by immunoblotting with a polyclonal anti-Bad antibody (SC-943). **B.** Cells were pretreated with the concentrations of LY-294002 indicated above the lanes for 10 min and stimulated with IL-3 or GM-CSF for 10 min. Bad was immunoprecipitated and immunoblotted with B36420 (Transduction Labs) to determine mobility shift. **C.** MC/9 cells were ^{32}P-labelled and treated with the indicated concentrations of LY-294002 (μM), followed by stimulation with GM-CSF (60 U/ml). Bad was immunoprecipitated and fractionated by SDS-PAGE. ^{32}P-labelled Bad was quantitated by phosphorimager (BioRad). The fold stimulations indicated below each lane are the average of duplicate determinations.

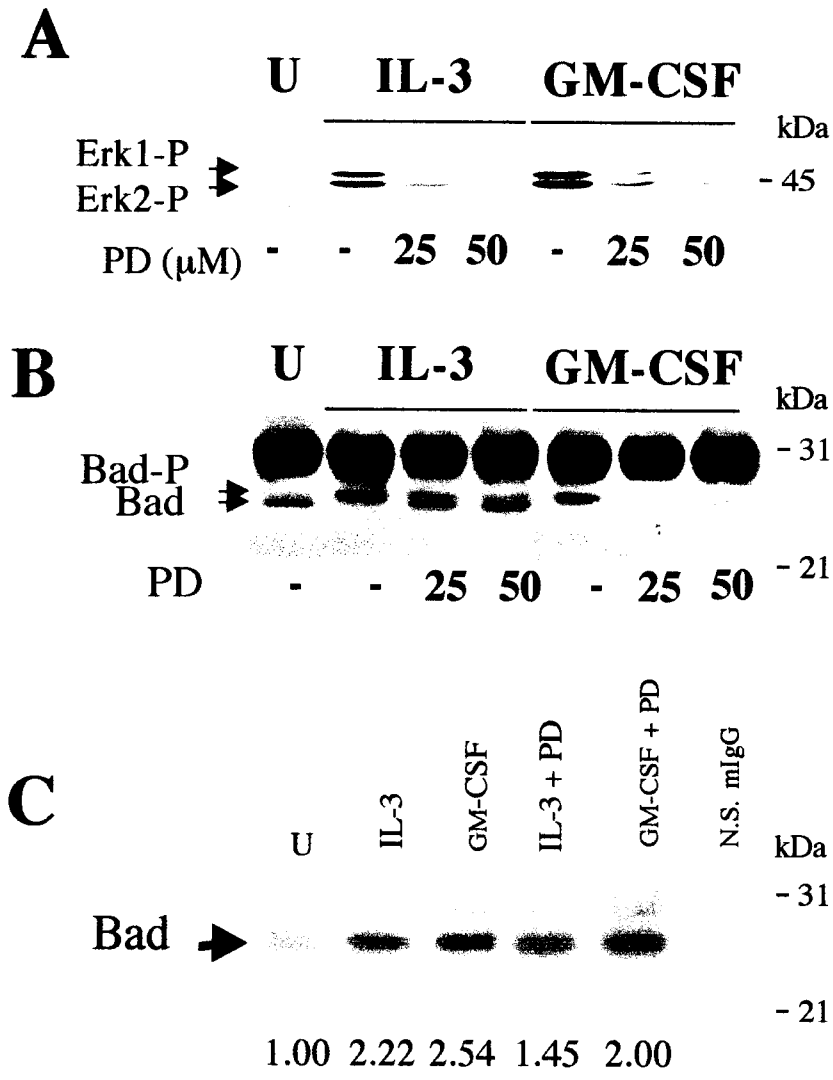


Figure 5.6. MEK inhibition blocks Bad phosphorylation. **A.** Cells were incubated with PD98059 (PD) at the indicated concentrations for 30 min followed by stimulation with IL-3 or GM-CSF for 5 min. Detergent solubilized cell lysates were fractionated by SDS-PAGE and immunoblotted with anti-phospho-MAPK. **B.** Bad was immunoprecipitated from the same cells lysates and immunoblotted with B36420 to determine mobility shift. **C.** MC/9 cells were labelled with ^{32}P and treated with PD98059 (50 μM) or vehicle alone (DMSO) for 10 min followed by stimulation with the indicated cytokine. Bad was immunoprecipitated, fractionated by SDS/PAGE, and detected by autoradiography. Bad radioactivity was quantitated by a Molecular Imager (BioRad). N.S. mIgG represents a control immunoprecipitation using 5 μg of an unrelated murine monoclonal antibody.

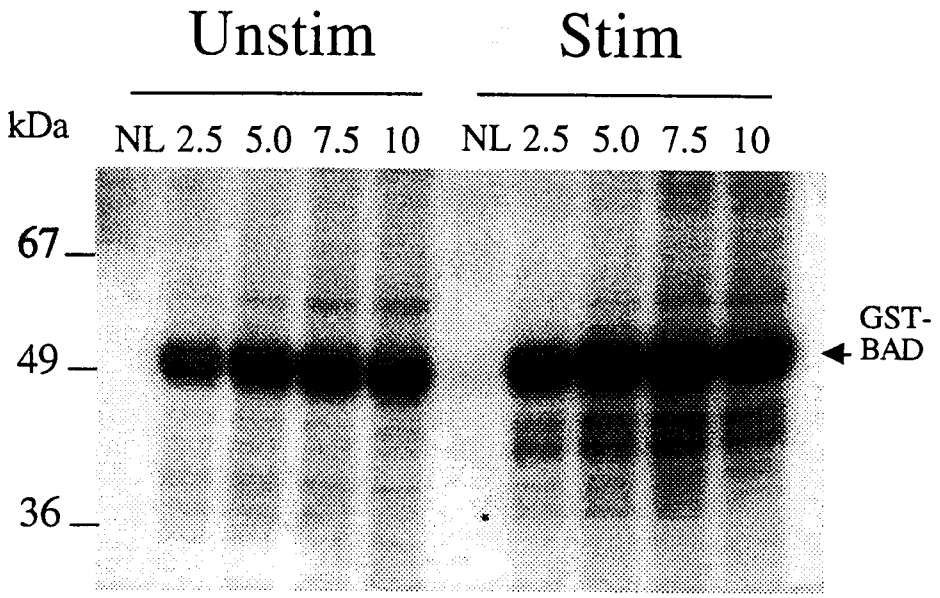


Figure 5.7. *In vitro* phosphorylation of GST-BAD. One μg of GST-BAD was incubated with the indicated volumes (in μl) of a 500 μg/ml nuclear free cell extract from stimulated (Stim) or unstimulated (Unstim) MC/9 cells in kinase buffer containing 10 μCi ^{32}P-γ-ATP for 10 min at 30°C. Reactions were stopped with an equal volume of 2X sample buffer followed by boiling for 5 min at 95°C. GST-BAD was fractionated by SDS-PAGE, the gel dried under heat and vacuum, and exposed to film. For the 7.5 μl reactions, the cpm incorporated were 5,302 (unstim) and 11,046 (stim). NL; no lysate was added.

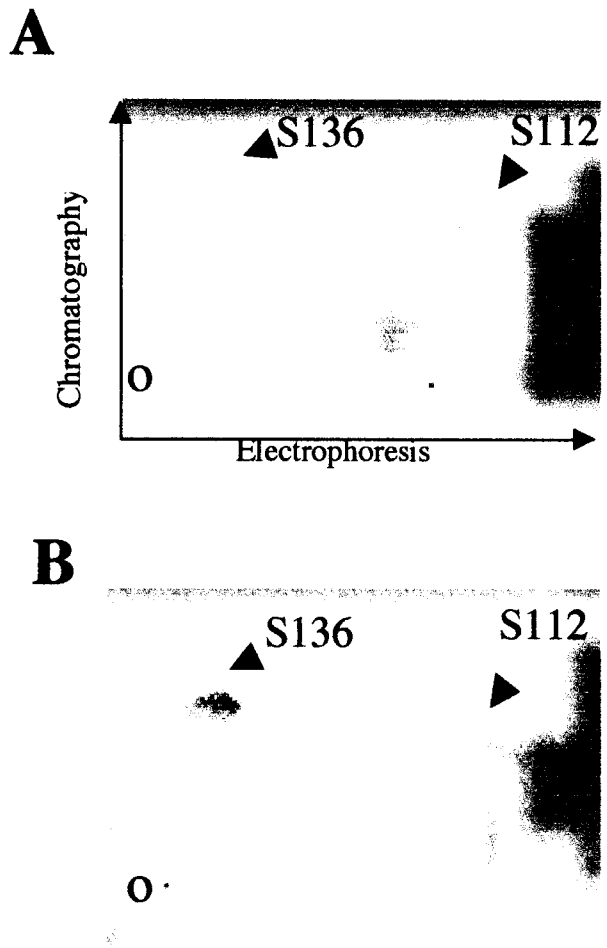


Figure 5.8. Two dimensional tryptic mapping of *in vitro* and *in vivo* phosphorylated Bad. **A**. GST-BAD from Figure 5.7 was cut from the gel and digested by trypsin as described in Materials and Methods. Tryptic fragments were separated by charge on cellulose sheets at 600 V for 45 min in pH 8.9 buffer at 8°C, followed by thin layer chromatography in methanol:pyridine:H_2O:acetic acid (37:25:20:5) for 2 hours at room temperature. The plates were dried and exposed to film. **B**. In some experiments non-radioactive synthetic phosphopeptides containing phospho-Ser136 or phospho-Ser112 were applied to the cellulose sheet and co-chromatographed, followed by visualization by ninhydrin staining.

inhibitors. After digestion with trypsin, Bad from unstimulated cells produced only two spots - neither of which co-migrated with peptides containing either Ser112 or Ser136 (Figure 5.9a, left panel). Stimulation with GM-CSF resulted in an increase in the activity of both spots as well as the appearance of a third spot (Figure 5.9a, middle panel). This third peptide co-migrated with the synthetic peptide containing phosphorylated Ser112. Phosphorylation of Ser136 was not apparent following stimulation. Next, Bad from PD98059-treated cells was examined (Figure 5.9a, right panel). The spot that comigrates with the synthetic peptide containing Ser112 was reduced to unstimulated levels, whereas the other two spots were relatively unaffected. These results would suggest that stimulation with GM-CSF results in phosphorylation of three residues - one appears to be Ser112 and the other two are unidentified. The Ser112-containing peptide contains more than one possible phospho-acceptor site (ie. three serines and a threonine), so to add further proof that PD98059 was blocking Ser112 phosphorylation, Bad that was precipitated from cell lysates was probed with an antibody specific for phosphorylated Ser112 (Figure 5.9b). IL-3 stimulated phosphorylation at Ser112 and MEK inhibition resulted in a complete inhibition of this phosphorylation. Thus, Ser112 of Bad may be phosphorylated by MEK (or a downstream kinase) and not by PKB.

The unidentified phosphopeptides are of considerable interest, as they may reveal novel information about the functional role Bad plays in apoptosis. Each of the three spots were scraped from the TLC plate and phosphoamino acid analysis was performed. Each peptide was phosphorylated exclusively on serine residues (Figure 5.10).

5.3. DISCUSSION

The Bcl-2 family member Bad is a BH3-containing protein whose role in cell death remains relatively obscure. Overexpression of Bad in FL-5.12 cells, NIH 3T3 cells or 293 cells results in cell death. Mutation of two serine residues (Ser136 and Ser112) to alanine potentiates this killing effect, suggesting that phosphorylation negatively regulates its ability

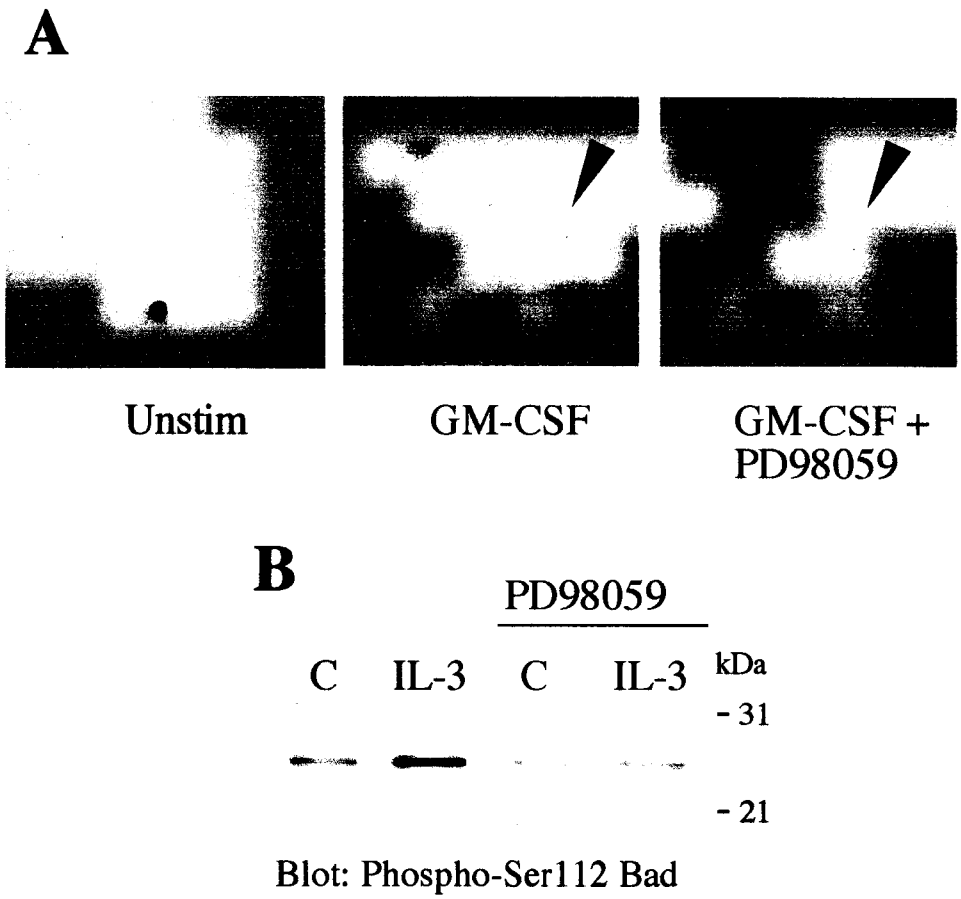


Figure 5.9. MEK inhibition selectively blocks Ser112 phosphorylation. **A.** ^{32}P-labelled MC/9 cells were pretreated with PD98059 for 10 min and stimulated with GM-CSF for 5 min. Tryptic analysis was performed as described in Figure 5.8 and in the Materials and Methods. The arrow indicates the position of the radioactive spot which co-migrates with the cold synthetic tryptic peptide containing Ser112. The origin is not shown on these autoradiographs. See figure 5.10.A. **B.** MC/9 cells treated with PD98059 or vehicle alone for 10 min were stimulated with medium or IL-3 for 5 min and solubilized in sample buffer. 25 μg of protein was fractionated by SDS-PAGE, transferred to nitrocellulose and blotted with an antibody specific for phospho-Ser112 (New England Biolabs).

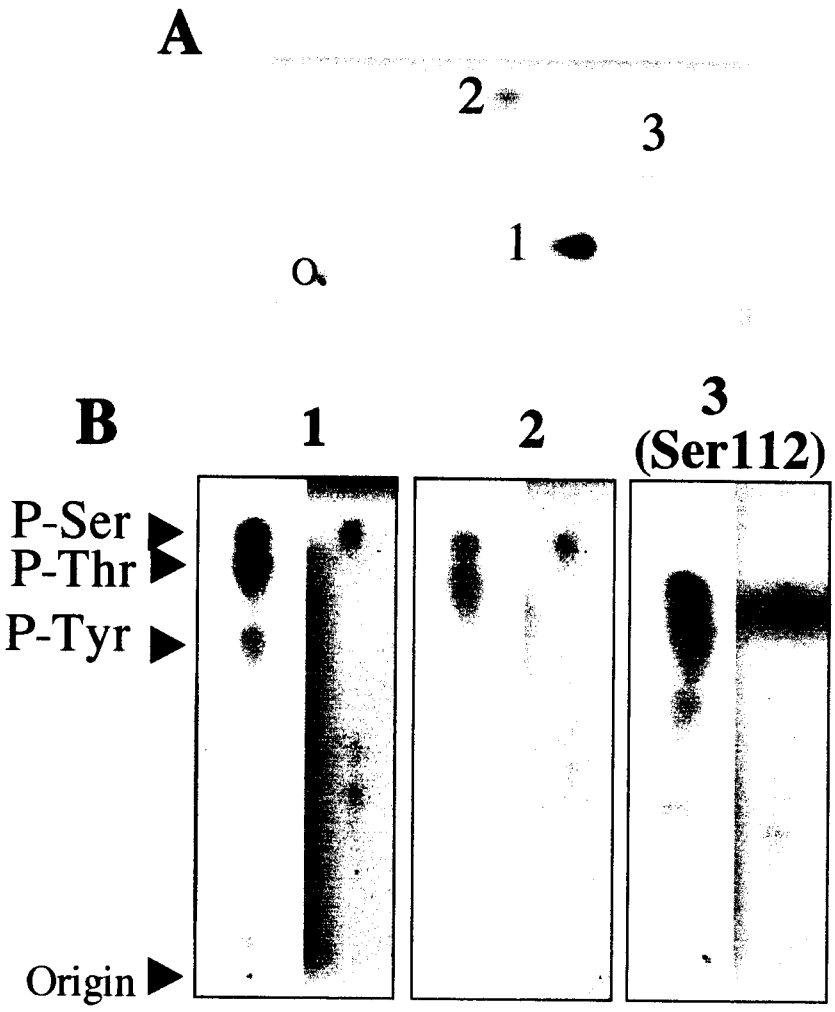


Figure 5.10. Phosphoamino acid analysis of *in vivo* phosphorylated Bad. Bad was immunoprecipitated from ^{32}P-labelled, GM-CSF-stimulated MC/9 cells and tryptically digested as described in Materials and Methods. Tryptic fragments were separated by electrophoresis and ascending chromatography as described in Figure 5.8. Each of the three spots were removed from the cellulose plate, hydrolyzed in 6 N HCl for 50 min at 110°C, dried and washed with H_20 extensively, and applied to cellulose plates along with 1 μg of each cold phospho-Ser, phospho-Thr and phospho-Try. Electrophoresis was performed in H_20:acetic acid:pyridine (85:10:5) for 30 min at 1000 V and 8°C. In each of the three panels, the left lane represents the ninhydrin stained cold phosphoamino acid, with the autoradiograph in the right lane.

to induce apoptosis. The phosphorylation of Bad on one or both of these residues in response to survival factor stimulation has been associated with decreased apoptosis (Zha et al., 1996). Furthermore, it has been observed in unstimulated cells that Bad associates with Bcl-X_L. This association is prevented by IL-3 stimulation, which stimulates phosphorylation of Bad (Zha et al., 1996). In vitro binding analysis with phospho-GST-Bad and Bcl-X_L supports this theory (Zha et al., 1996). A potential cytosolic binding partner of phosphorylated Bad may be the 14-3-3 proteins (Zha et al., 1996). Therefore the model that has emerged suggests that survival agonists stimulate Bad phosphorylation, resulting in the binding of Bad to 14-3-3 proteins, resulting in the sequestration of Bad away from Bcl-X_L. This translocation correlates with a diminished capacity for Bad to induce apoptosis, possibly by relieving some negative influence on Bcl-X_L.

Phosphopeptide mapping of Bad has been described previously (Zha et al., 1996). This characterization was done with overexpressed HA-tagged Bad, and while Ser136 and Ser112 clearly undergo phosphorylation under these conditions, it remains possible that endogenous Bad is phosphorylated differently. In MC/9 cells, endogenous Bad appears to be phosphorylated on Ser112 in response to IL-3, GM-CSF or SCF. In addition, two other peptides show an increase in specific activity compared to resting cells. Ser136 is not a major target of signalling pathways activated by these cytokines since very low amounts of radioactivity were incorporated into this peptide following stimulation. Activation of PKC by phorbol esters led to both Ser136 and Ser112 phosphorylation, as well as other sites of phosphorylation, indicating that Ser136 of Bad can be phosphorylated in MC/9 cells.

Since Bad was undergoing phosphorylation in response to cytokines independently of PI 3-kinase or PKB, we asked what other pathways may be involved. Inhibition of MEK with PD98059 had the effect of reducing the phosphorylation of Ser112 exclusively - the other two phosphopeptides remained unaffected by treatment with this drug. A caveat of these findings is the specificity of PD98059 as an inhibitor of MEK 1 and 2. An alternate approach could be through the expression of a constitutively active or inducible Ras, Raf or

MEK construct, which is currently being tested. Since a PD98059-sensitive pathway leads to Ser112 phosphorylation, it brings into question whether Ser112 phosphorylation is important for survival, since inhibition of MEK does not block survival promoted by IL-3 or GM-CSF. Additionally, the observation that IL-4 can maintain survival without stimulating Bad phosphorylation on this residue suggests that Ser112 phosphorylation does not play a critical role in IL-4 mediated survival. An important set of experiments that are underway will examine the subcellular location of Bad following stimulation with cytokines in the presence or absence of the MEK inhibitors.

The identity of the other residues that undergo phosphorylation following cytokine stimulation are of considerable interest. The unknown peptides were scraped from the TLC plate and subjected to phosphoamino acid analysis, which demonstrated exclusive serine phosphorylation (Figure 5.9). Currently, our laboratory is utilizing the phosphorylation of GST-Bad to generate sufficient quantities of these peptides for mass-spectroscopy analysis.

6. SHIP IS A NEGATIVE REGULATOR OF PIP_3 AND PKB

6.1. RATIONALE AND HYPOTHESIS

The lipids produced by PI 3-kinase activate pro-survival signalling pathways. However, besides PI 3-kinase, little is known about the enzymes that regulate the levels of PI 3-kinase-derived lipids. Considering that PI3-kinase-derived lipids are an important regulator of cell survival, these enzymes may be extremely relevant therapeutic targets. SHIP is a 5' phosphoinositide phosphatase which may have a direct role in PI lipid turnover. SHIP-mediated conversion of PIP_3 to PI $(3,4)P_2$ may then have an overall positive or negative effect on PKB, depending upon which lipid is primarily responsible for kinase activation.

6.2. RESULTS

In order to discern the importance of SHIP as a regulator of PI 3-kinase generated signals, bone marrow mast cells from SHIP knockout mice were used to measure PIP_3 and $PI(3,4)P_2$ levels following SCF. These cells were derived from knockout mice generated previously (Helgason et al., 1998) and have been demonstrated to contain no functional SHIP-1. However, the importance of other 5' phosphatases, such as SHIP-2, in the hydrolysis of PIP_3 remains unknown. To address the question of whether SHIP-1 is an important component of PIP_3 turnover, ^{32}P-orthophosphate labelling was performed to directly label the ATP pool used by PI 3-kinase to phosphorylate its substrates. Following solvent extraction and deacylation, the water soluble glycero-phosphoinositides were separated by HPLC as described in the Materials and Methods. Figure 6.1 represents a typical elution profile of PIP_3 and $PI(3,4)P_2$ generated in response to SCF stimulation of $SHIP^{-/-}$ and $SHIP^{+/+}$ bone marrow mast cells (BMMC). As can be seen, the accumulation of PIP_3 in the $SHIP^{-/-}$ cells greatly exceeds that in $SHIP^{+/+}$ cells (by approximately 25 fold) following stimulation with SCF. In contrast, the SCF-generated $PI(3,4)P_2$ levels are reduced by about 30% in the $SHIP^{-/-}$ cells compared with their

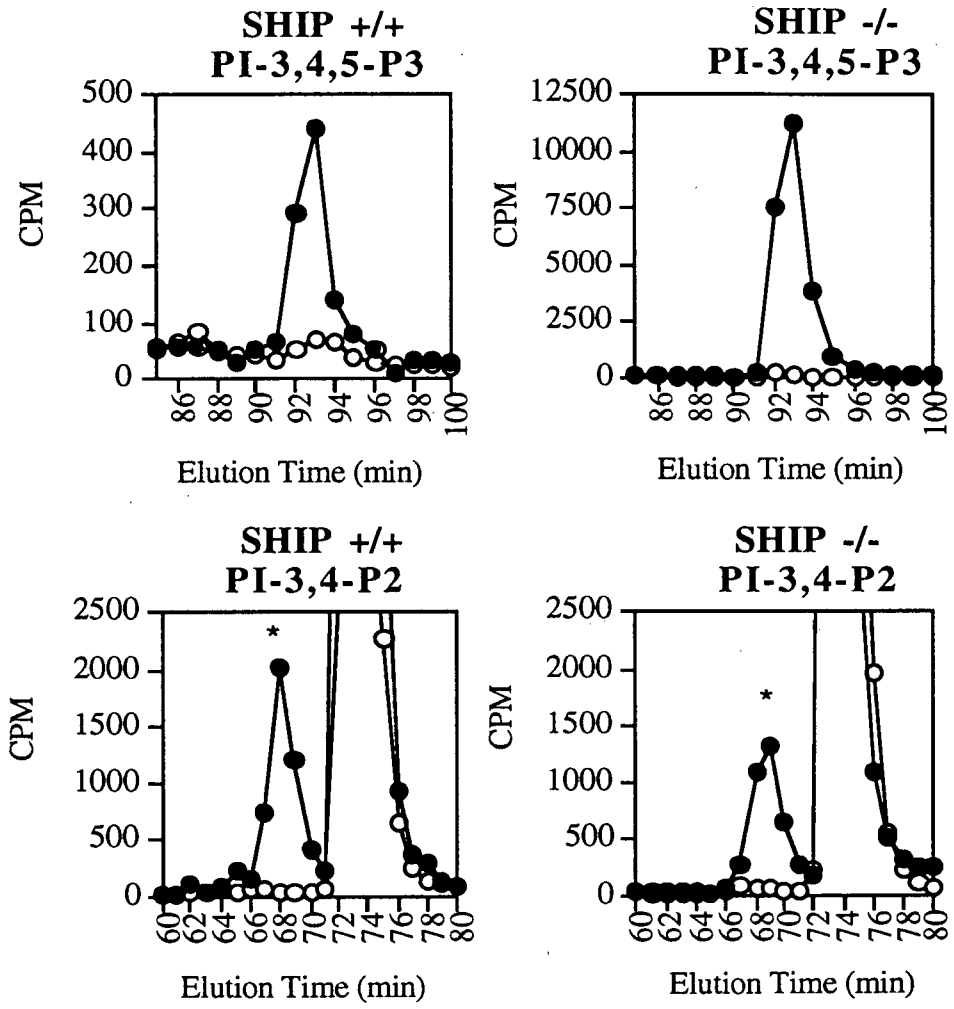


Figure 6.1. SHIP decreases PIP_3 and elevates $PI(3,4)P_2$. Partisil 10 SAX HPLC elution profiles of deacylated PIP_3 and $PI(3,4)P_2$ from $SHIP^{+/+}$ and -/- BMMCs following 2 min with (•) and without (O) 100 ng/ml SCF. The asterisk indicates the elution position of $PI(3,4)P_2$. The profiles are representative of 4 separate experiments.

$SHIP^{+/+}$ counterparts. The differences in radioactivity most likely reflects increases in mass, as opposed to differences in specific activity of the labelled products between the knockout and wildtype cells. The specific activity of other lipid species, which did not change with stimulation, were similar between knockout and wildtype cells. Furthermore, total recovered soluble radioactivity between knockout and wildtype differed by less than 30% between samples. Thus, the 25-fold increase in PIP_3 radioactivity can only be explained by increase in mass.

We next determined the extent of PIP_3 and $PI(3,4)P_2$ production at various concentration of SCF. This allowed an assessment of SHIP's role as a regulator of PIP_3 at more physiological concentrations of SCF. Figure 6.2 represents the radioactivity corresponding to PIP_3 and $PI(3,4)P_2$ at 2 min stimulation with 100, 30, 5 and 1 ng/ml SCF. At all concentrations tested, PIP_3 isolated from $SHIP^{-/-}$ cells greatly exceeded that from $SHIP^{+/+}$ cells. In fact, the fold increase for PIP_3 at 100 ng/ml SCF in $SHIP^{+/+}$ BMMC was approximately 5 fold, consistent with increases in other systems, while PIP_3 generation in $SHIP^{-/-}$ BMMC was more than 50 fold. In other experiments higher concentrations of SCF (>200 ng/ml) induced even higher (>90 fold) increases in PIP_3 (data not shown). In contrast, $PI(3,4)P_2$ was markedly reduced under each condition in the $SHIP^{-/-}$ BMMCs, which strongly suggests that a large component of this lipid was generated as a result of PIP_3 breakdown at the 5' phosphate by SHIP. The fact that there was generation of $PI(3,4)P_2$ suggested that either other, less efficient 5' phosphatases are functioning in the $SHIP^{-/-}$ BMMCs, or that a PI 3- or 4-kinase may have been phosphorylating PI(4)P or PI(3)P to generate $PI(3,4)P_2$ directly. These possibilities cannot be distinguished at this time.

We next examined the kinetics of PIP_3 and $PI(3,4)P_2$ turnover in SHIP knockout and wildtype BMMC. This was important, since the data so far could not determine whether the loss of SHIP would maintain the high levels of PIP_3 produced after cytokine stimulation. As can be seen in Figure 6.3, generation of PIP_3 in the $SHIP^{-/-}$ BMMC

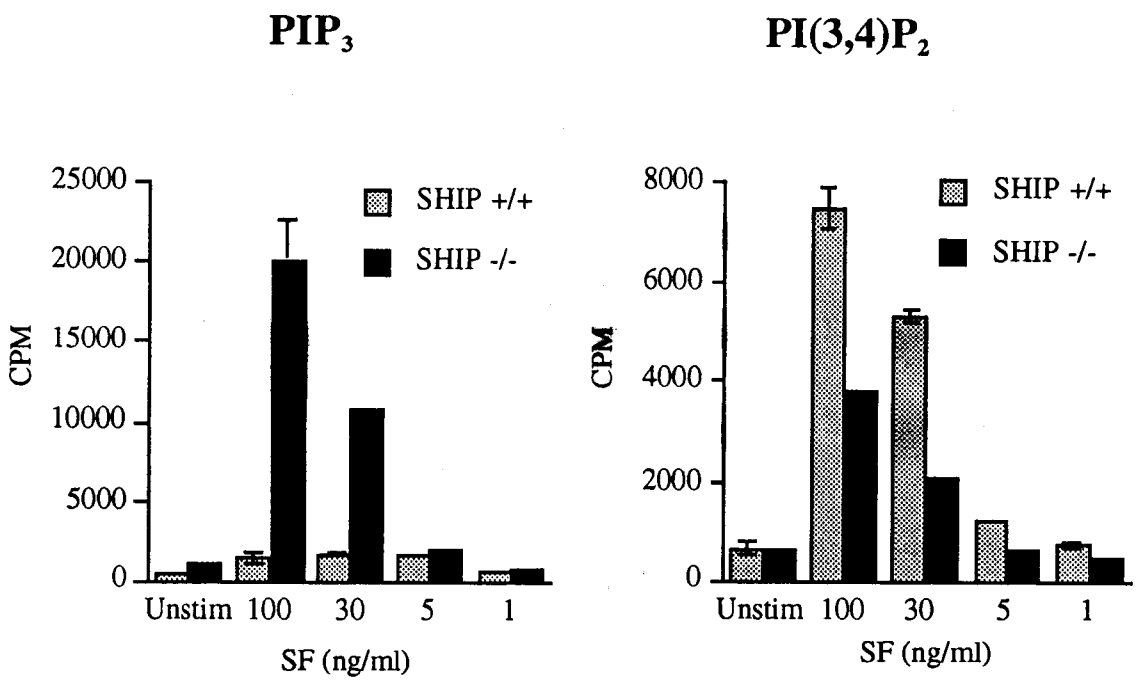


Figure 6.2. Dose-response to SCF. PIP_3 (left panel) and $PI(3,4)P_2$ (right panel) levels were measured in $SHIP^{+/+}$ and -/- BMMCS following 2 min of stimulation with the indicated concentrations of SCF. Results are the mean +/- SD of duplicate determinations and are representative of 3 separate experiments.

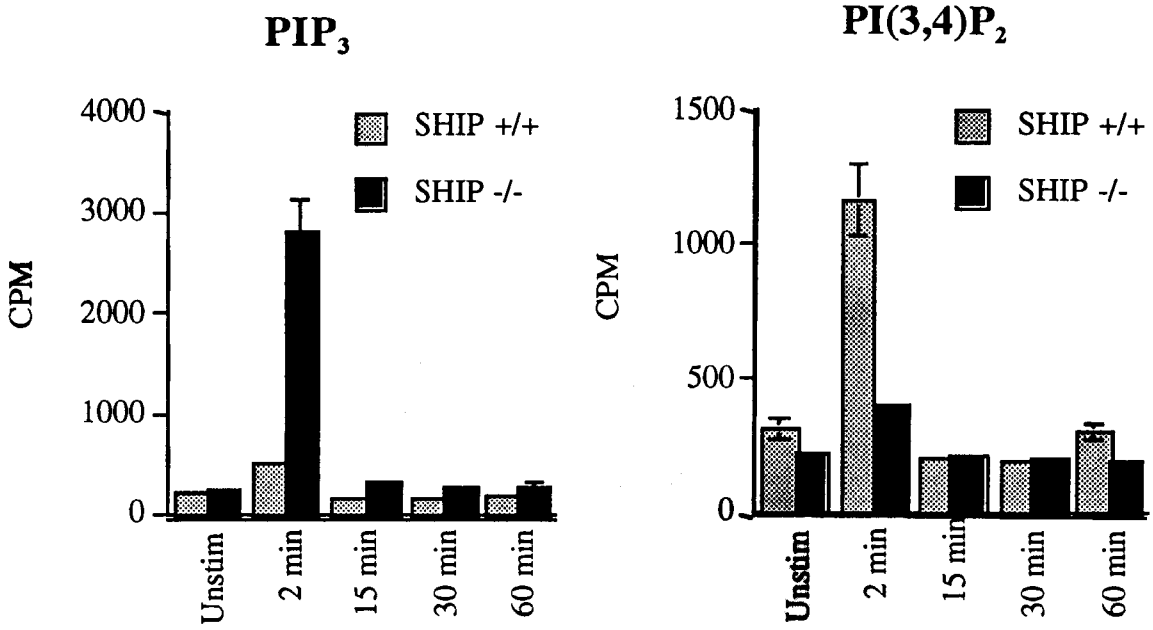


Figure 6.3. Time course of PIP_3 and $PI(3,4)P_2$ generation. PIP_3 (left panel) and $PI(3,4)P_2$ (right panel) levels were measured in $SHIP^{+/+}$ and -/- BMMCs following stimulation with 30 ng/ml of SCF for the indicated times. Results are the mean +/- SD of duplicate determinations and are representative of 3 separate experiments.

returned to basal levels by 15 - 30 min following stimulation. This suggested that PIP_3 was the target of other phosphatases, either 3' or 5' phosphatases. The quick reduction in PIP_3 could also be a function of negative regulation by PIP_3 itself. The large increases may compete for and dissociate PI3K from tyrosine phosphorylated proteins, as suggested by Rameh et al. (1995), thus reducing the amount of lipid kinase activity stimulated by c-kit ligation.

Having established that SHIP was required for the breakdown of PIP_3, the next set of studies asked how the activation of PKB would be affected in the $SHIP^{-/-}$ cells. In the first set of experiments, PKB activation was measured using immunocomplex kinase assays from knockout or wildtype cells stimulated with a maximal dose of SCF over a time course (Figure 6.4a). SCF produced maximal stimulation in both the $SHIP^{-/-}$ and $SHIP^{+/+}$ cells measured at 2 minutes. This would argue that a saturation of PDK1, PDK2 and PKB activation has been reached and no further elevation in lipid species could lead to greater activation. At later time points the PKB activity remained elevated in the $SHIP^{-/-}$ cells but dropped significantly in wildtype cells. This suggests that the sustained activation of PKB may be the result of increased recruitment and phosphorylation of PKB by PDK1 and PDK2 due to the sustained levels of PIP_3 at the plasma membrane.

To further examine the ability for SCF to activate PKB in wildtype or knockout cells, a dose-response study was performed (Figure 6.4b). PKB was measured following 2 minutes stimulation with the indicated concentrations of SCF. Maximal PKB activity was observed at 100 ng/ml SCF, while 5 and 1 ng/ml SCF showed an increase in the ability of SCF to induce PKB activation in $SHIP^{-/-}$ cells compared with $SHIP^{+/+}$ BMMC. Immunoblotting with an anti-phospho-S473-PKB antibody (which only recognizes the Ser473-phosphorylated PKB) demonstrated an increase in the phosphorylation of this residue at the lower concentrations of SCF in the $SHIP^{-/-}$ cell, consistent with activity (Figure 6.4c). Also, PKB was observed to undergo an electrophoretic mobility shift upon stimulation, which is indicative of dual (Ser473 and Thr308) phosphorylation (Alessi et al.,

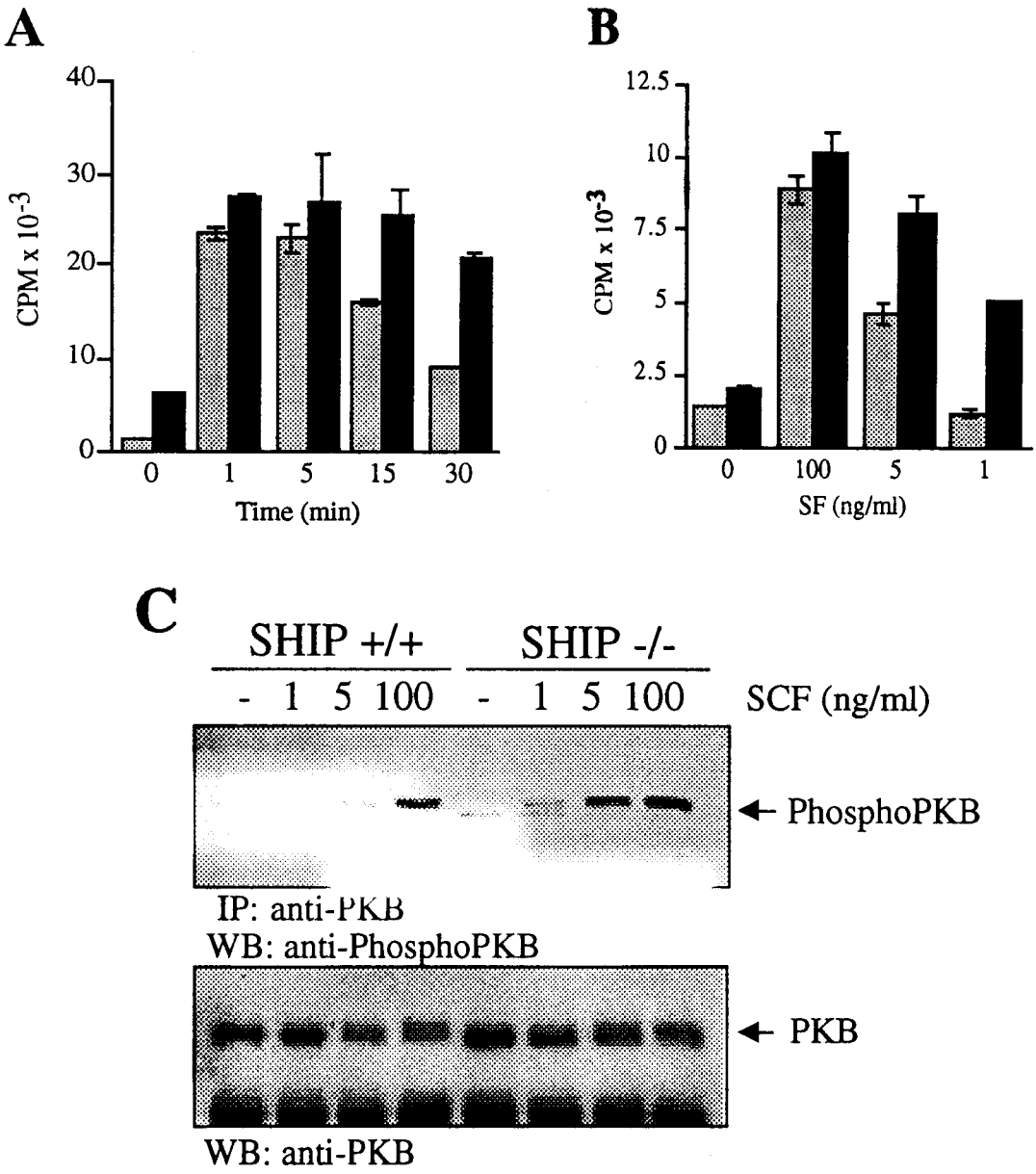
1997). Importantly, this mobility shift occurred at the lower concentrations of SCF only in SHIP-/- BMMC, indicating that SHIP restricts the extent of phosphorylation of PKB by both PDK1 and PDK2. Immunoblotting for total PKB revealed equal amounts in each immunoprecipitation (Figure 6.4c). Thus, these experiments indicate that SHIP normally restricts the ability of cytokines to activate PKB, and this is most likely through a reduction in the amount of PIP_3 following its generation by PI 3-kinase.

6.3. DISCUSSION

The results presented in this chapter expand upon the biological role of an inositol 5' phosphatase, SHIP. The significance of SHIP in mice development has been previously characterized (Helgason et al., 1998). The mice are viable and fertile, but suffer from splenomegaly, myeloid infiltration of the lungs, wasting and shortened lifespan. Granulocyte/macrophage progenitors are more responsive to a variety of cytokines than wild type littermates. Expression of dominant negative or constitutively active forms of SHIP have proven unsuccessful in determining whether SHIP is a major regulator of PI 3-kinase generated lipid products (Lioubin et al., 1996; Liu et al., 1997). While SHIP can clearly hydrolyze PIP_3 into $PI(3,4)P_2$ *in vitro*, prior to the studies present here the question of whether it actually performs this role *in vivo* remained unclear.

The role and regulation of SHIP became an important subject of this thesis, since it may directly influence the downstream targets activated by PI 3-kinase, which are important for survival signalling. Therefore, the objectives of the studies presented here were an effort to establish a role for SHIP in PIP_3 regulation, and whether this regulation influences a primary downstream target of PIP_3, the protein kinase PKB. Clearly, SHIP is a major regulator of PI 3-kinase generated lipid products, since BMMC lacking SHIP produced significantly more PIP_3 and less $PI(3,4)P_2$ in response to SCF stimulation. The second major finding was that loss of SHIP led to increased and prolonged stimulation of PKB activity, consistent with PIP_3 being an activator of this kinase. This was not due to a

Figure 6.4. PKB activation is elevated and prolonged in SHIP knockout BMMC. **A.** SHIP-/- (solid bars) or +/+ (shaded bars) BMMC were stimulate for the indicated times with 100 ng/ml SCF and PKB activity was measured as described in Materials and Methods. **B.** Cells were stimulated with the indicated concentrations of SCF for 2 min and PKB activity was measured as described in Materials and Methods. **C.** Cells were stimulated with the indicated concentrations of SCF for 2 min and PKB was immunoprecipitated, followed by fractionation by SDS-PAGE and immunoblotting with an anti-phospho-S473-PKB Ab (upper panel). The blot was then reprobed with an anti-PKB Ab (lower panel). The results in A and B represent the average of duplicate determinations +/- range, and are consistent among 4 independent experiments. The results of C are representative of two independent experiments.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IP: anti-PKB
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PKB
WB: anti-PKB

greater expression of PKB, since immunoblot analysis revealed that equal amounts of PKB were immunoprecipitated from $SHIP^{-/-}$ and $SHIP^{+/+}$ cells. Further, additional experiments demonstrated that equivalent PI 3-kinase activities measured *in vitro* were associated with the c-kit receptor following ligation (M.S., Michael Huber, Vincent Duronio and Gerald Krystal, manuscript submitted). Therefore, the simplest explanation for the increased PKB activity associated with SCF stimulation is the massive elevation of PIP_3 compared with $SHIP^{+/+}$ BMMC.

Continuing studies will also examine the effect the loss of SHIP has on the survival of hemopoietic cells. Given the results of this thesis, one may predict that elevated PIP_3 and PKB activity would result in a diminished requirement for cytokines to maintain survival. The $SHIP^{-/-}$ mice apparently do not develop lymphomas (Helgason et al., 1998), an important observation which suggests that additional regulatory mechanisms are in place to ensure against the development of neoplasia. One pathological condition which may not require the additional genetic mutations necessary for lymphomas is asthma. It may be predicted that a prolonged lifespan of invading granulocytes, due to a loss of SHIP, may contribute to the severity of the disease. This would be consistent with the observation that $SHIP^{-/-}$ mice have increased granulocytes in the lung. These predications await formal investigation, although it may prove difficult to develop a mouse asthma model.

7. OVERALL DISCUSSION

7.1. PI 3-kinase in cytokine-mediated survival

The role of phosphatidylinositol 3-kinase in cytokine-mediated survival was examined in a hemopoietic model. MC/9 survival was maintained when cells were incubated in the presence of IL-3, IL-4 or SCF . This survival which was blocked by the addition of the PI 3-kinase inhibitors wortmannin or LY-294002. Wortmannin and LY-294002 act by different mechanisms. Wortmannin is a fungal metabolite which covalently binds with and destroys the catalytic activity of PI 3-kinase, while LY-294002 is an ATP competitor. GM-CSF, in contrast to the other cytokines, promoted survival in a manner that was completely independent of PI 3-kinase activity. The cytosolic release of mitochondria-localized proteins may be important for the execution of apoptosis, which also results in the death of the mitochondria. While the results presented here do not directly address this aspect of apoptosis, it was determined that GM-CSF could maintain mitochondrial activity measured using XTT conversion. Furthermore, GM-CSF but not IL-3, IL-4 or SCF-stimulated cells maintained functional mitochondria following extended treatments with PI 3-kinase inhibitors. These results would suggest that GM-CSF signalling may be influencing the cells' decision to undergo apoptosis prior to mitochondrial death. It will be important to directly verify this hypothesis. Importantly, PI 3-kinase remains critical for cell cycle progression because although the cells do not die, they do stop dividing. This re-inforces the concept that pro-survival pathways and mitogenic pathways are often separate. It would be interesting to see whether GM-CSF-stimulated cells resume cell division upon removal of PI 3-kinase inhibitors from the culture medium.

This difference in signalling between IL-3, IL-4 and SCF, compared with GM-CSF, cannot be explained based on the potency of each cytokine to activate PI 3-kinase, because the concentrations of drugs used to inhibit the enzyme completely blocked all stimulated PI 3-kinase activity. Furthermore, SCF has been shown previously to be the

most potent among these cytokines in its activation of PI 3-kinase (Gold et al, 1994), as well as PKB (Scheid and Duronio, 1998; see Chapter 5). However, in these cells, SCF was not able to overcome inhibition of PI 3-kinase and protect against apoptosis.

The ability of other growth and survival factors to prevent apoptosis, independently of PI 3-kinase has been shown in several other recent studies. A similar conclusion regarding IGF-1 signalling was reached by Kulik and Weber (1998). In their model, PI 3-kinase/PKB signalling was not required for IGF-1 mediated survival of Rat-1 fibroblasts if the receptor was overexpressed. The authors concluded that IGF-1 can signal through novel pathways to survival, independently of PI 3-kinase, depending on the extent of receptor activation. Likewise, Philpott and co-workers (1997) showed that primary sympathetic neurons require PI 3-kinase signalling through NGF stimulation only in the absence of serum. In the presence of serum, NGF could overcome the pro-apoptotic actions of PI 3-kinase inhibitors or dominant negative forms of PKB.

7.2. Role of p70 S6 kinase in PI 3-kinase-mediated survival

Several downstream targets of PI 3-kinase were evaluated as potential mediators of a pro-survival signal. The serine kinase p70 S6 kinase is activated by a mechanism involving multiple phosphorylations on serine and threonine residues. One residue, Thr389, is a target of PI 3-kinase mediated signals. This may not be mediated by PKB, since PKB cannot phosphorylate this site *in vitro* (Alessi et al., 1998). This phosphorylation, along with other phosphorylations in the auto-inhibitory region of the kinase, leads to the opening of the kinase domain and its phosphorylation on Ser229, probably by PDK1. The latter enzyme catalyzes the phosphorylation of Ser229 in a PI 3-kinase-independent manner, since this action is wortmannin-insensitive (Pullen et al., 1998; Alessi et al., 1998). Ultimately, this combination of phosphorylations leads to full activation.

The activation of p70 S6 kinase is important for the phosphorylation of the S6 subunit of the 40S ribosome, and the translation of start sites in mRNA containing polypyrimidine tracks is contingent upon this modification. Inhibition of p70 S6 kinase can be achieved by treatment of cells with rapamycin, a low molecular weight drug that binds to the mammalian homologue of TOR (target of rapamycin). This appears to increase the susceptibility of p70 S6 kinase to serine/threonine phosphatases, which render the kinase inactive.

In our models, cytokine receptor stimulation by IL-3, GM-CSF and IL-4 (in MC/9 cells) and IL-2 (in CTLL-2 cells) all led to p70 S6 kinase phosphorylation. Activation of p70 S6 kinase was also measured in CTLL-2 cells, demonstrating that IL-2 was a potent activator of p70 S6 kinase. Prior treatment of cells with PI 3-kinase inhibitors, wortmannin or LY-294002, blocked the phosphorylation of p70 S6 kinase, consistent with a role for PI 3-kinase upstream of this kinase. Likewise, rapamycin was also effective in promoting dephosphorylation and inactivation. However, rapamycin treatment did not effect the ability of any of these cytokines to promote survival. These results indicate that the death-promoting actions of the PI 3-kinase inhibitors do not involve inhibition of p70 S6 kinase.

7.3. Erk as a survival mediator

Other targets reported to be downstream of PI 3-kinase activation are the MAPK family members $p44^{erk1}$ and $p42^{erk2}$. Other studies have relied significantly on the use of wortmannin as an inhibitor of PI 3-kinase in defining this pathway. To determine a requirement for Erk in cytokine-mediated survival, the effects of wortmannin on the ability of several cytokines to activate Erk were initially investigated. It was found that although wortmannin significantly inhibited Erk activation by IL-3 or GM-CSF, the extent of inhibition was the same for each. These results suggested that the pro-survival ability of GM-CSF compared with IL-3 during PI 3-kinase inhibition was not mediated through Erk. Furthermore, complete inhibition of Erk with PD98059 had no effect on the ability of GM-

CSF or IL-3 to promote survival, nor did it even accelerate apoptosis in cytokine-starved cells. These findings, coupled with the observation that IL-4 can promote survival in the absence of signalling to Erk, argues against a role for Erk in survival model.

Another important observation that became apparent during these studies was the crosstalk between the PI 3-kinase and Erk pathways. Following stimuli that do not activate Erk, it was clear that PI 3-kinase cannot, independently of other events, induce the activation of this molecule. For example, in hemopoietic cells, IL-4 stimulation leads to PI 3-kinase activation without any effect on Ras, Raf1, MEK, or Erk. More recently, expression of a mutant TrkA receptor, which was an effective activator of PI 3-kinase, was shown to be incapable of activating Ras/Erk (Hallberg et al., 1998). These observations do not themselves rule out the possibility that PI 3-kinase regulates Erk following stimuli which normally leads to its activation. For example, in order for PI 3-kinase to exert any effect on Erk, Grb2/SOS translocation leading to Ras/Raf1/MEK activation may also need to occur besides just PI 3-kinase activation – in other words the proper molecules must be assembled into place. While our results using wortmannin are in agreement with what others have reported, there was a surprising discrepancy in the ability of LY-294002 to inhibit Erk to the same extent. Detailed dose-response analysis revealed that LY-294002 could abolish all PIP_3 generation in cells following cytokine stimulation without having any effect on Erk activation. Much higher (>100 uM) concentrations of LY-294002 were needed to inhibit Erk activation, but under these conditions LY-294002 could also block phorbol ester-stimulated Erk activation. Erk activation by phorbol esters is independent of PI 3-kinase, since it was not affected by wortmannin. Therefore, the conclusion that PI 3-kinase activity is required for the activation of Erk appears to be based on observations using an inhibitor (wortmannin) which may be acting through targets other than PI 3-kinase.

In agreement with these results, Ferby et al. (1996) recently dissociated PI 3-kinase activity from Erk activation. By using a dominant-negative p85 subunit of PI 3-kinase, they

showed in transient expression experiments that inhibition of PI 3-kinase did not lead to diminished Erk activation, however wortmannin remained effective in partially inhibiting Erk, but probably due to non-specific effects.

The target of wortmannin upstream of MAPK remains unknown. Since MEK is the only known physiological activator of Erk, it is likely to involve the activation of this protein kinase. Wortmannin does not appear to have any effect on Raf activation (Karintz et al., 1995), so it may be that other signalling pathways converge on MEK besides Raf. Elucidation of these potential wortmannin sensitive targets may provide some insight into roles for these molecules in signalling which had originally been attributed to the actions of PI 3-kinase.

7.4. The role of PI 3-kinase in Bad phosphorylation

The Bcl-2 family member Bad was also examined for its role in PI 3-kinase signalling pathways. The biological role for Bad was unknown at the start of these studies, but several lines of evidence suggested that it plays a role in growth factor stimulated survival. First, it contains a BH3 domain that is conserved in other pro-apoptotic Bcl-2 family members, such as Bak, Bik and Bid (Chittenden et al., 1995; Hunter and Parslow, 1996; Zha et al., 1996b; Han et al., 1996; Wang et al., 1996) . The BH3 domain of Bad alone is a potent death inducer when transfected into various cell types (Zha et al., 1997). It presumably promotes apoptosis by heterodimerizing with pro-survival proteins such as Bcl-2 and Bcl-X_L. Consistent with this, Bad heterodimerization with Bcl-X_L may prevent the pro-survival function of Bcl-X_L. Phosphorylation of Bad on two serine residues, Ser136 and Ser112, promotes dissociation from Bcl-X_L and association of Bad with 14-3-3. The Ser112 site resembled consensus sites targeted by the PKA class of protein kinases (Zha et al., 1996). The Ser136 phosphoreceptor site is contained in a Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd sequence, where Xaa is any amino acid and Hyd is a hydrophobic residue, which has been shown to be a good target region for PKB/Akt (Alessi and Cohen, 1998).

This observation led to the hypothesis that PI 3-kinase activated pathways results in Bad phosphorylation on Ser136, possibly through the activation of PKB.

Interestingly, peptide mapping from ^{32}P-labelled Bad revealed that very little of the Ser136 residue was undergoing phosphorylation in MC/9 cells, either from starved or stimulated cells. This would be consistent with a lack of Bad phosphorylation in response to IL-4, even though IL-4 treatment results in activation of PKB. In addition, GM-CSF-stimulated Bad phosphorylation is not affected following PI 3-kinase inhibition, which prevents PKB activation. One concern however is the susceptibility of Bad to phosphatases during immunoprecipitation. To address this concern, several steps were taken to minimize the possibility of phosphatase activity. First, cells were lysed and diluted 20 - 40 fold in immunoprecipitation buffer. Secondly, all steps following cell lysis were performed at 4°C. Thirdly, NaF, β-glycerophosphate, sodium vanadate and microcystin-LR were added to the immunoprecipitation buffer to inhibit any serine, threonine and tyrosine phosphatase activity. Finally, to serve as a positive control, Ser136 phosphorylation could be observed following PMA stimulation.

Ser112 was a target of a kinase or kinases activated in response to IL-3, GM-CSF or SCF, but not IL-4. The pathway leading to Ser112 phosphorylation appears to involve MEK1 and or MEK2, since pretreatment of cells with PD98059 selectively inhibited Ser112 phosphorylation. Experiments are currently underway to introduce a selectively inducible Raf-1 into MC/9 cells in order to further test the hypothesis that a MEK-dependent pathway leads to Bad phosphorylation at Ser112. Since MEK has a very narrow substrate specificity, limited to Erk1 and Erk2, according to the available data, the Erk's are likely to be involved. The Ser112 site does not lie within the consensus Pro-Xaa-Ser/Thr-Pro Erk phosphorylation motif (Clark-Lewis et al., 1993), indicating that other kinases downstream of Erk (or MEK) most likely catalyze the phosphorylation of this site. One potential kinase is p90^{rsk}. Of particular interest, several other phosphorylated peptides were generated from endogenous Bad. The identity of these peptides and the residues which are

phosphorylated in them remain unknown, although phosphoamino acid analysis indicates that they are also phosphorylated on serine residues.

7.5. SHIP as a regulator of PI 3-kinase generated lipids

The role of SHIP in the turnover of PI 3-kinase generated PIP_3 and $PI(3,4)P_2$ was assessed using bone marrow mast cells derived from SHIP-deficient mice. The levels of PIP_3 in $SHIP^{-/-}$ cells were much greater following stimulation with SCF compared with wildtype cells. This contrasts with a decrease in the levels of $PI(3,4)P_2$ in $SHIP^{-/-}$ cells, indicating that one of SHIP's function is to generate this lipid. Since the levels of PIP_3 returned to base line shortly after stimulation, $SHIP^{-/-}$ cells must have other enzymes which function to breakdown PIP_3. Nevertheless, these studies have clearly shown that SHIP is an important regulator of PI 3-kinase generated lipids, and points to an important role in regulating the signalling by PI 3-kinase. In this respect, PKB activation was examined and found to be activated to a greater degree in $SHIP^{-/-}$ at low SCF concentrations, consistent with a role for PIP_3 in the activation of PKB.

7.6. Summary

In conclusion, the work presented here in has provided insight into the relative importance of several signalling molecules in survival signalling by hemopoietic cytokines. The importance of PI 3-kinase-generated signals in prevention of apoptosis by some, but not all cytokines, was demonstrated. Several putative downstream targets of PI 3-kinase, including Erk and p70 S6 kinase, were dissociated from PI 3-kinase-mediated survival. These studies also revealed that PI 3-kinase activity was not required for the activation of Erk, and points to nonspecific actions of the PI 3-kinase inhibitor wortmannin. The phosphorylation of Bad was also dissociated from PI 3-kinase and PKB activity. These results conflict with published accounts of PI 3-kinase/PKB dependent phosphorylation of Ser136 of Bad. The discrepancy between the work presented here and these reports may be

due to cell type, environmental context, or caveats in expression studies. Finally, the experiments presented here indicate that SHIP$^{-/-}$ BMMC will prove to be an important and useful tool for evaluating the ability of PI 3-kinase to activate downstream targets and promote survival.

7.7. Future Directions

There are a number of questions that must still be addressed in future work. The pro-survival pathway activated by GM-CSF in MC/9 cells, which can mediate survival independently of PI 3-kinase, remains unknown. In preliminary experiments not presented here, inhibition of GSK-3 by GM-CSF appears to be independent of PI 3-kinase, whereas GSK-3 inhibition by IL-3-dependent inhibition of GSK-3 is dependent on PI 3-kinase (Scheid and Duronio, unpublished observations). This may be particularly relevant in light of the recent finding that GSK-3 inactivation may be necessary for growth factor survival signals (Pap and Cooper, 1998). Therefore, the upstream pathways leading to GSK-3 phosphorylation on activating and inactivating sites should be closely examined for GM-CSF and IL-3 generated signals.

Another question regarding GM-CSF survival is at what point is this pathway negatively interfering with apoptosis. PI 3-kinase/PKB signals have been shown to lead to caspase-9 phosphorylation and inactivation (Cardone et al., 1998). The question arises as to whether GM-CSF can still mediate caspase-9 phosphorylation independently of PI 3-kinase and PKB activity. Additionally, this raises another question: does PI 3-kinase signalling block cytochrome c release from mitochondria, or does caspase-9 phosphorylation prevent apoptosis exclusively? If this were the case, Bcl-2 may be predicted to not play a role in cytokine mediated survival, but clearly it does (Williams et al., 1990; Vaux et al., 1988). Hypothetically, this combination of both upstream and downstream mitochondrial targets would suggest that cytokine signalling utilizes multiple pathways to prevent caspase activation. PI 3-kinase would not have to participate in all of

these pathways. It is possible that PKB phosphorylation of caspase-9 prevents or delays apoptosis regardless of cytochrome c release, which may be under the control of other, PI 3-kinase-independent pathways. For example, the prevention of cytochrome c release could be mediated through the Ras/Erk pathway. Thus, while IL-4 cannot stimulate Ras, Erk or Bad phosphorylation, cells stimulated by IL-4 remain protected from apoptosis by PI 3-kinase-mediated inactivation of caspase-9. Likewise, inhibition of MEK with PD98059 does not have any effect on IL-3 or GM-CSF mediated survival perhaps for the same reason. This hypothesis is consistent with observations by others (Parrizas et al., 1997), who showed that MEK inhibition acts synergistically with PI 3-kinase inhibition to induce apoptosis. A model that would be supported by these findings might be that cytochrome c release in conjunction with caspase-9 dephosphorylation activates caspase-9 to a greater degree than dephosphorylation alone. It may also explain why in some cellular contexts MEK inhibition leads to apoptosis, since in these models the extent of cytochrome c release, and the degree of inhibition provided by PKB-mediated caspase-9 phosphorylation, could be critical. Recent findings in the *Drosophila* model system also support this model. Erk signalling by Ras rescues cell death from Hid expression, presumably by direct phosphorylation on several residues (Bergmann et al., 1998). PI 3-kinase/PKB activation partially rescues against this background by inactivating a downstream component of Hid-mediated cell death. A schematic of this is provided in Figure 7.1.

In closing, the work presented here should help in our understanding the ways that survival pathways activated by cytokine receptors prevent apoptosis. This may contribute to the development of treatments for diseases such as cancer or asthma by blocking these survival pathways.

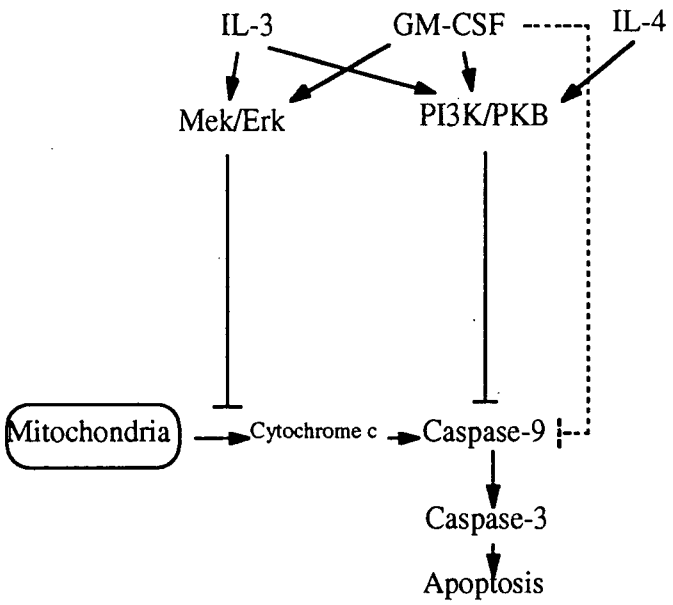

Figure 7.1. Proposed model for Ras/MAPK and PI 3-kinase/PKB signalling pathways. The activation of Ras -> Raf -> MEK -> Erk provides protection from apoptosis by preventing cytochrome c release and caspase-9 activation, possibly through modulation of Bcl-2 family proteins such as Bad. This requirement is bypassed by direct phosphorylation of Caspase-9 by PKB, which inactivates it and protects against amplification of caspase activity leading to apoptosis. GM-CSF-mediated inactivation of caspase-9 independent of PKB may provide an explanation for PI 3-kinase-independent survival by this cytokine.

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