INVESTIGATION OF PHOSPHATIDYLINOSITOL-3-KINASE (PI3K) AND EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 (ERK1/2) ACTIVATION

by

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

2001

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Experimental Medicine Program

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ABSTRACT

There have been conflicting studies in the past several years regarding the relationship between PI3K activation and its potential role in ERK1/2 activation. Some studies indicate that PI3K activation is required for ERK1/2 activation, yet other studies suggest that there is no relationship between the two. In this thesis we hypothesize that PI3K activation is not required for ERK1/2 activation. To investigate this hypothesis we used several cell systems, including a human, hemopoietic cell line, TF-1, and murine, hemopoietic cell lines FDC-P1 and BAF-3 cells. Also, we used a human embryonic kidney cell line, HEK 293. With the use of pharmacological inhibitors of PI3K, LY294002 and wortmannin it was concluded that ERK1/2 activation does not require PI3K activation.

We also addressed the question of what role PI3K plays in the regulation of protein synthesis. However, in order to test this, we first investigated whether our cell systems contained the important components of the translational protein machinery including, eIF-4E and 4E-BPs. Afterwards, we tested the effects of using various inhibitors, including the PI3K inhibitors LY294002, wortmannin, MEK1/2 inhibitors, U0126 and the FRAP/mTOR inhibitor rapamycin. From our preliminary experiments we determined that 4E-BP1 is present in TF-1 cells and 4E-BP2 is present in FDC-P1, MC-9 and TF-1 cells. Furthermore, eIF-4E is present in all three cell systems. With the use of the inhibitors, we observed that LY294002 inhibits the phosphorylation of 4E-BP1, U0126 did not block the phosphorylation of 4E-BP1 and rapamycin partially blocked the phosphorylation of 4E-BP1. Surprisingly, wortmannin did not block the phosphorylation of 4E-BP1. Due to the preliminary nature of the results, more experiments are needed.
to fully understand the role that PI3K may play in the regulation of protein translation.
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<th>Description</th>
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<tr>
<td>4E-BP</td>
<td>eIF-4E binding protein</td>
</tr>
<tr>
<td>5'UTR</td>
<td>5'-untranslated leader sequence</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase</td>
</tr>
<tr>
<td>BMK1</td>
<td>big MAP kinase 1</td>
</tr>
<tr>
<td>BCR</td>
<td>breakpoint cluster region</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>eIF-3</td>
<td>eukaryotic initiation factor-3</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>eukaryotic initiation factor-4E</td>
</tr>
<tr>
<td>ERF</td>
<td>Ets2 repressor factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FRAP</td>
<td>FKBP-rapamycin associated protein</td>
</tr>
<tr>
<td>Grp1</td>
<td>general receptor for 3-phosphoinositides</td>
</tr>
<tr>
<td>HSP27</td>
<td>heat shock protein 27</td>
</tr>
<tr>
<td>IL-3</td>
<td>interleukin-3</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>iSH2</td>
<td>inter SH2</td>
</tr>
<tr>
<td>JAK</td>
<td>janus family of tyrosine kinases</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP</td>
<td>mitogen-activated protein kinase-activated protein</td>
</tr>
<tr>
<td>MKK</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>MAP kinase phosphatase</td>
</tr>
<tr>
<td>MLK</td>
<td>mixed lineage kinase</td>
</tr>
<tr>
<td>Mnk1</td>
<td>MAPK activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MUK</td>
<td>MAPK upstream kinase</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDGF-R</td>
<td>platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIF</td>
<td>PDK1-interacting fragment</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
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PP2  protein phosphatase 2
PRD  proline rich domain
PtdIns phosphatidylinositol
rhGM-CSF recombinant human granulocyte macrophage-colony stimulating factor
RNP  ribonucleoprotein
RRM  RNA-recognition motif
rRNA  ribosomal RNA
SAPK  stress activated protein kinase
SH2  src-homology-2
SH3  src-homology-3
SOS  son-of-sevenless
SRE  serum response element
SRF  serum response factor
TAO  thousand and one amino acid protein kinase
TPA  tetradecanoylphorbol 13-acetate
TPL-1  tumor progression locus-2
tRNA  transfer RNA
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Chapter 1: Introduction

1.1 Hemopoietic Cytokine Receptor Signaling

Extracellular molecules are important in initiating the various responses required for cell functions, such as cell growth, differentiation and survival. In the hemopoietic system the extracellular molecules include, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-3 stimulates the growth and differentiation of erythroid and myeloid cells, eosinophils, basophils, megakaryocytes, mature macrophages and mast cells. GM-CSF promotes the growth of macrophages, neutrophils and eosinophils (Miyajima et al., 1993).

Both the IL-3 receptor and GM-CSF receptor consist of a ligand specific \( \alpha \) chain and a common \( \beta \) chain. In the murine IL-3 receptor there are two types of \( \beta \) chains, an IL-3 specific \( \beta \)-subunit and a common \( \beta \)-subunit (Nishinakamura et al., 1995). These receptors are activated via the binding of the cytokine to the receptor inducing the heterodimerization of the \( \alpha \) and \( \beta_c \) receptor subunits which causes receptor oligomerization and activation of the associated tyrosine kinases (Brown et al., 1997). For instance, members of the Janus tyrosine kinase family (JAK) are constitutively associated with the \( \beta_c \) subunit. Thus, upon receptor activation JAK phosphorylates specific tyrosine residues in the receptor subunits which function as binding sites for proteins containing phosphotyrosine binding (PTB) domain or Src-homology2 (SH2) domains (Pawson, 1995). For instance, signal transducers and activators of transcription (STATs) possess SH2 domains. STATs bind to phosphotyrosine residues and are phosphorylated by JAKs which causes the STAT homo and heterodimerization. These STAT complexes
translocate to the nucleus and function as transcription factors (Ihle, 1995). There are also other receptor-associated proteins, such as Shc, SHIP and PI3K which are important in IL-3 and GM-CSF mediated signal transduction (Sato et al., 1993; Yi et al., 1993; Gold et al., 1994).

1.2 Phosphatidylinositol-3 kinase (PI3K)

1.2.1 Background

Cell membranes play an important role in cell recognition and cell signaling, and allow regulation of responses to certain cell stimuli. The cell membrane consists of phospholipids, such as phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (PtdIns) (Stryer et al., 1995). PtdIns and its phosphorylated derivatives, phosphatidylinositol-3-phosphate (PtdIns(3)P), phosphatidylinositol-3,4-diphosphate (PtdIns(3,4)P$_2$) and phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P$_3$), are known to participate in signal transduction upon activation via mitogens. Therefore, the discovery of phosphatidylinositol-3 kinase (PI3K), which is an enzyme which phosphorylates the 3'OH of the inositol ring, contributed significantly to elucidating the mechanisms of cellular signaling.

Early studies of PI3K showed that it was able to form complexes with some viral oncoproteins, such as v-src and v-ras (Sugimoto et al., 1984). Furthermore, PI3K was shown to be involved in viral transformation (Cantley et al., 1991) and cells were able to undergo malignant transformation when transfected with DNA containing the fragment of viral or cellular PI3K gene (Chang et al., 1997). Thus, these experiments were critical in demonstrating the
importance of PI3K in cancer development. Ultimately, a more complete understanding of its mechanisms of action could be important for cancer therapy.

1.2.2 Structure

There are multiple isoforms of mammalian PI3Ks that are divided into three classes based on their structure and substrate specificity (Wymann et al., 1998; Vanhaesebroeck et al., 1997; Fruman et al., 1998). The three classes are designated as class I, class II and class III.

Class I PI3Ks are heterodimers with ~110 kDa catalytic subunit and a regulatory subunit (Kapeller, 1994). The substrates of these PI3Ks are PtdIns, PtdIns(4)P, PtdIns(4,5)P$_2$. However, the preferred substrate is PtdIns(4,5)P$_2$. Class I PI3Ks are further subdivided into class IA and class IB enzymes, which signal downstream of protein-tyrosine kinases and heterotrimeric G protein-coupled receptors, respectively (Vanhaesebroeck and Waterfield, 1999). The class IA p110 subunit has various isoforms (p110α, β, δ). The p110α and β isoforms are found in most mammalian tissues; however, p110δ is mainly found in leukocytes. The adaptor proteins are p85α, p85β and p55γ. The class IB PI3K only consists of a p110γ catalytic subunit and a 101 kDa regulatory subunit, p101. Class IB PI3Ks are mainly found in white blood cells (Vanhaesebroeck and Waterfield, 1999).

Class II PI3Ks are large molecules, greater than 170 kDa, which have a C-terminal C2 domain. This C2 domain binds to phospholipids; however, this binding requires the presence of Ca$^{2+}$. Also, no adapter proteins are known for
class II PI3Ks (Arcaro et al., 1998). There are three class II isoforms, PI3K-C2α, β, and γ. PI3K-C2α, β are found in most mammalian tissue types; however, PI3K-C2γ are found mainly in the liver. Class II PI3Ks have the same substrates as class I PI3Ks, but prefer PtdIns(4)P (Vanhaesebroeck and Waterfield, 1999).

Class III PI3Ks are homologues of the yeast vesicular sorting protein, Vps34p (Stack et al., 1992). The catalytic subunit of PI3Ks are Vps34p analogues and the regulatory subunit is a 150 kDa protein, p150. Class III PI3Ks, unlike the other classes, can only use PtdIns as a substrate. Thus, the generation of PtdIns(3)P in cells are due mainly to class III PI3Ks (Vanhaesebroeck and Waterfield, 1999).

<table>
<thead>
<tr>
<th>Class</th>
<th>Structural features of catalytic subunits</th>
<th>Subunits</th>
<th>Regulation</th>
<th>Lipid substrates in vitro</th>
<th>in vivo</th>
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<tr>
<td>I</td>
<td>p110α, β, δ</td>
<td>p110α, p85α, p50γ</td>
<td>Tyr kinases &amp; Ras</td>
<td>PtdIns, PtdIns(4)P, PtdIns(4,5)P_2</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>p110γ, p101</td>
<td>p110γ, p101</td>
<td>heterotrimeric G proteins &amp; Ras</td>
<td>PtdIns(4)P, PtdIns(4,5)P_2</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Vps34p analogues</td>
<td>p150</td>
<td>Constitutive?</td>
<td>PtdIns</td>
<td></td>
</tr>
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Figure 1.1 Schematic representation of the different classes of mammalian PI3Ks and structural features of the catalytic subunits (Vanhaesebroeck and Waterfield, 1999).
Generally, the regulatory p85 subunit consists of one Src-homology-3 (SH3) domain, which binds to polyproline domains and two proline rich fragments. Also, the p85 subunit consists of two Src-homology-2 (SH2) domains, which bind to phosphorylated tyrosine residues. The two SH2 domains are separated by an inter SH2 (iSH2) region. The iSH2 domain is the site of interaction between the p85 and p110 subunits and the two SH2 domains allow the p85/p110 heterodimer to bind to receptor protein-tyrosine kinases or other tyrosine phosphorylated proteins via phosphotyrosines in a YXXM motif (Kapeller et al., 1994; Yu et al., 1998).

**Figure 1.2** Schematic representation of a general PI3K heterodimer. The p85 subunit consists of two SH2 domains, separated by an iSH2 domain, two proline rich domains (PRDs), separated by breakpoint cluster region-homologous domain (BCR) and one SH3 domain. The catalytic p110 subunit binds to iSH2 domain. The arrows indicate the binding sites for PI3K activation. The SH2 domains bind to phosphotyrosine proteins (P) and small G-proteins (G), such as ras, bind to p110 subunit. (Krasilnikov, 2000).
1.2.3 PI3K activation

For class I PI3K to be activated the two subunits, p85 and p110 must assemble to form a heterodimer. Furthermore, the assembled PI3K requires interaction with specific activator proteins prior to activation. For class IA PI3K the main activator proteins have phosphorylated tyrosine residues on receptors or non-receptors. For instance, any growth factor or cytokine that stimulate tyrosine phosphorylation results in phosphorylated tyrosine sites (Ruderman et al., 1990). These phosphorylated tyrosine residues bind to the SH2 domains of the PI3K regulatory subunit (p85) and induce a conformational change leading to enzyme activation via the recruitment of the p110 catalytic subunit (Yu et al., 1998). Class IB PI3K activation requires interaction with Gβγ subunits of heterotrimeric Gproteins via the p101 regulatory subunit. For example, a seven-transmembrane receptor, such as angiotensin receptor binding to its ligand activates the heterotrimeric Gprotein and subsequently GDP is replaced with GTP on the Gα subunit and the Gβγ dissociates and activates PI3K (Krugmann et al., 1999).

However, there are other mechanisms of class I PI3K activation. For instance, p21-Ras, a cellular protein, is known to complex with p110 between amino acid residues 133 and 314 and induce PI3K activation. Only the Ras-GTP form, but not the Ras-GDP form is able to activate PI3K. (Rodriguez-Viciana et al., 1996).

Some examples of stimuli which activate class II PI3Ks are insulin, epidermal growth factor, platelet-derived growth factor and integrins. The
mechanism of PI3K activation for class II is unclear and currently under investigation (Brown et al., 1999).

Class III PI3Ks seem to be constitutively activated; thus, there does not seem to be any external stimuli which specifically activates class III PI3Ks (Panaretou et al., 1997).

1.2.4 Function

The catalytic p110 subunit of PI3K contains both protein-serine-threonine kinase and phosphoinositide kinase activities. The main role of the serine-threonine kinase activity of p110 appears to be playing a regulatory role via the phosphorylation of the p85 subunit at Ser-608. A study by Dhand et al. (1994) suggested that the phosphorylation of the p85 subunit causes an 80% reduction in the PI3K activity. The function of the lipid kinase activity of PI3K is the phosphorylation of the D3 position on the inositol ring of PtdIns, and PtdIns(4)P and PtdIns(4,5)P2, yielding PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, respectively (Carpenter et al., 1990; Carpenter et al., 1993; Dhand et al., 1994). These phosphorylated derivatives are second messengers in signal transduction.

Figure 1.3 Simplified diagram of PtdIns and point of action of PI3K (Vanhaesebroeck and Alessi, 2000).
1.2.5 Downstream Targets of PI3K

1.2.5.1 Protein Localization

The phosphoinositide, lipid products of PI3K action function as docking sites to which a variety of proteins may bind. These recruited proteins contain pleckstrin homology (PH) domains, which are protein modules of ~120 amino acids (Ferguson et al., 2000). PH domains all share a conserved core fold, consisting of a seven stranded β-barrel capped on one end by a C-terminal α-helix (Lietzke et al., 2000). Proteins with PH domains bind preferentially to PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. For instance, PKB and PDK1 all possess PH domains which bind to PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> and resulting in PKB and PDK1 activation. Also, exchange factors for GTPases also contain PH domains. More specifically, the general receptor for 3-phosphoinositides (Grp1), a PI3K-activated exchange factor for Arf GTPases, binds to PtdIns(3,4,5)P<sub>3</sub> with high affinity and thus increasing its activity (Lietzke et al., 2000).

The lipid product of PI3K, PtdIns(3)P, functions as a recruiter of proteins containing a FYVE domain. Proteins involved in membrane trafficking all posses a FYVE domain on the carboxy-terminal. The FYVE domain was named after four proteins in which they were first indentified: Fab1p, YOTB, Vac1p and Early endosome antigen 1 (Leevers et al., 1999). Via its interaction with proteins containing FYVE domains, PtdIns(3)P, functions to control many processes at the plasma membrane. Such processes include phagocytosis, pinocytosis, exocytosis and cytoskeletal organization (Czech 2000; Greenwood et al., 2000). Furthermore, PtdIns(3)P is found in endosomes where it is postulated to regulate
endosome fusion and mobility (Lawe et al., 2000). PI3K products are also required to activate focal adhesion kinase (FAK) to promote cell migration (Reiske et al., 2000).

The p85/p110, PI3K heterodimer, itself may also form complexes with certain cellular molecules. For example, the interaction of Ras with PI3K has been clearly documented. Ras binds to the p110 subunit of the heterodimer and has been suggested to function as an regulator of PI3K activity perhaps by increasing PI3K activation, allosterically or by contributing to the recruitment of the p85/p110 heterodimer to the plasma membrane (Rodriguez-Viciana et al., 1996)

1.2.5.2 PKB

Protein kinase B (PKB), a protein-serine-threonine kinase, also known as Akt is an important signaling molecule activated by PI3K. PKB plays an important role in protecting cells from apoptosis, which is programmed cell death. The PI3K lipid product, PtdIns(3,4)P$_2$ binds to PKB thereby, recruiting it to the membrane and allowing its activation (Franke et al., 1997). For PKB to be fully active it requires phosphorylation of Thr-308 and Ser-473 residues. The phosphorylation of these residues is also dependent on PI3K. The Thr-308 kinase, phosphoinositide-dependent kinase (PDK1), binds to PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ prior to Thr-308 phosphorylation (Stephens et al., 1998). Then Ser-473 is phosphorylated. The method of Ser-473 phosphorylation is still unclear (Wick et al., 2000). One study indicated that it is phosphorylated by another kinase, PDK2 (Alessi et al., 1997) and it has been suggested that PDK2
is actually integrin-linked kinase (ILK) (Persad et al., 2000). Persad et al. (2000) demonstrated that ILK phosphorylates Ser-473 by using a dominant-negative form of ILK or an inhibitor of ILK to show a reduction in the phosphorylation of Ser-473. Yet another study indicated that PDK1, after interacting with a molecule, PDK1-interacting fragment (PIF), is able to change substrate specificity and phosphorylate Ser-473 (Balendron et al., 1999). However, a recent study suggested that Ser-473 is phosphorylated by undergoing autophosphorylation (Toker et al., 2000). The activation of PKB regulates the transduction of cell survival signals.

1.2.5.3 **PLCγ and PKC**

Some isoforms of phospholipase C and protein kinase C are known to function downstream of PI3K. PLCγ activity is enhanced by binding to PtdIns(3,4,5)P3 via its PH domain (Bae et al., 1998; Falasca et al., 1998). During cell division, phosphoinositides serve as substrates for phospholipase Cγ (PLCγ), which is activated by receptor tyrosine kinases. The products of this reaction are diacylglycerol and inositol phosphates, which in turn activate classical and novel protein kinase C (PKC) which is a serine/threonine-specific protein kinase (Orr et al., 1994; Carpenter et al., 1996).

Currently, there are 11 isoenzymes of PKC identified and they are subdivided into three groups based on cofactors required for maximal activation. The classical PKCs require diacylglycerol and calcium, the novel PKCs require only diacylglycerol and the atypical PKCs do not require calcium nor diacylglycerol. The role that PtdIns(3,4,5)P3 and PtdIns(4,5)P2 may play in the
activation of the classical PKCβ1, and the novel PKCe, PKCη and PKCμ is quite complex. Palmer et al. (1995) suggested that PtdIns(3,4,5)P$_3$ and PtdIns(4,5)P$_2$ activation of PKCs may be due to charge affects on the lipid vesicles or liposomes and not due to the phosphoinositide lipid itself. The reason being that there is a lack of an identifiable conserved binding site between the PKC and phosphoinositol lipid and the fact that the activation of the PKCs by both phosphoinositides behaves in a non-specific manner (Palmer et al., 1995). Briefly, PKCs are involved in diverse cellular functions which include cell proliferation, tumor promotion, differentiation and apoptotic cell death (Nishizuka, 1992; Clemens et al., 1992; Hug and Sarre, 1993; Nishizuka, 1995; Goodnight et al., 1994; Hofmann, 1997; Casabona, 1997)

1.3 Mitogen-Activated Protein Kinases

1.3.1 Background

Mitogen-activated protein kinases (MAPKs) are key players of cellular signal transduction systems and they are highly conserved. MAPKs are protein-serine/threonine kinases. These proteins all contain a characteristic activation motif, TXY (threonine-glutamate/glycine/proline-tyrosine). This motif is located between subdomains VII and VIII of the conserved kinase core sequence (Lewis et al., 1998). MAPKs are activated when both the threonine and tyrosine residues are phosphorylated. There are three main types of mammalian MAPKs. These are extracellular signal-regulated kinase 1/2 (ERK1/2) which will be discussed later in detail, p38 MAPK and c-Jun N-terminal kinase (JNK). Both
p38 MAPK and c-Jun N-terminal kinase are also known as stress-activated protein kinases.

1.3.2 JNK and p38 MAPK

Cells continuously respond to the environment and the stress activated protein kinases (SAPKs) play an important role in regulating the cells responses. The two major groups of SAPKs are p38 MAPK and JNK.

JNK was named based on its activity as the c-jun N-terminal kinase, which regulates the activity of the transcription factor c-jun (Hibi et al., 1993). There are ten JNK isoforms, encoded by three genes, JNK1, JNK2, JNK3. These JNK isoforms are 46 kDa or 55 kDa (Gupta et al., 1996). All JNKs have the dual phosphorylation site, TPY (threonine-proline-tyrosine), which are phosphorylated on the threonine and tyrosine residues for activation (Martin-Blanco, 2000). JNK1 and JNK2 genes are expressed ubiquitously; whereas, JNK3 gene is predominantly expressed in the brain (Gupta et al., 1996).

Some of the variety of extracellular stimuli which activate JNKs include: irradiation, ultraviolet light, heat shock, high osmotic stress, and proinflammatory cytokines, such as interleukin 1. The activation of JNK causes changes to cell transcription, protein synthesis, cell surface receptor expression and cytoskeletal structure, ultimately affecting cell survival. (Han et al., 1994; Rouse et al., 1994; Wang et al., 1997; Derijard et al., 1994; Yuasa et al., 1998).

There are four, identified mammalian isoforms of p38 MAPKs. They are p38α, p38β, p38γ and p38δ. These isoforms are all similar in size, are in the range of 360-372 amino acids, show 60-75% sequence homology and all
possess a TGY (threonine-glycine-tyrosine) dual phosphorylation site between kinase domains VII and VIII. All p38 MAPKs are activated by TNF, IL-1, UV irradiation and high osmotic stress (Raingeaud et al., 1995; Obata et al., 2000). However, there are differences in the location of expression between these isoforms. p38α is ubiquitously expressed in most tissues. p38β are predominantly expressed in brain and heart tissues. p38γ is primarily expressed in skeletal muscle. p38δ is mainly expressed in lung, kidney, intestines, salivary gland epithelium, testis, ovary, adrenal and pituitary gland (Wang et al., 1997; Hu et al., 1999).

1.3.3 Extracellular Signal-Regulated Kinases

The important discovery of kinases mediating growth factor and hormone action on the cell during the 1970s stimulated research into signaling events, which eventually led to the identification of the MAPK cascades. Tyrosine phosphorylation and receptor kinases were discovered in the early 1980s which further credited MAPK cascades as the major pathway involved in growth regulation. ERK1 and ERK2 were the first MAPKs to be characterized (Boulton et al., 1990).

ERK1 is a 44 kDa protein, while ERK2 is a 42 kDa protein. In mammals, they have 90% sequence homology (Boulton et al., 1991). ERK1/2 both contain a conserved catalytic core, C-terminal extensions and 25 amino acid insert between subdomains IX and X. Both ERK1/2 contain the activation motif TEY (threonine-glutamate-tyrosine). They both have similar 3-D architecture, having a
two-lobed structure, where the active site is found at the domain interface (Zhang et al., 1994).

ERK1/2 are activated in the presence of extracellular stimuli, such as growth factors, cytokines and mitogens. The receptors for these stimuli include receptors that stimulate protein-tyrosine phosphorylation, for instance, tyrosine kinase receptors and single transmembrane, hemopoietic receptors. The activated receptor can recruit Grb-Sos (son-of-sevenless) complex, which in turn catalyzes the conversion of Ras-GDP to Ras-GTP. The activated Ras-GTP has an effector domain that binds to Raf, thus activating it. Then the activated raf phosphorylates MAP kinase kinase 1/2 (MKK1/2) also known as MEK1/2 which is the direct activator of ERK1/2 by phosphorylating the threonine and tyrosine residues of the TEY activation motif (Lewis et al., 1998).

An increase in intracellular calcium levels may play a role in stimulating MKKs and thus activating ERKs. Intracellular calcium levels can increase due to a response to any stimuli that causes an opening of calcium channels. Pyk2, a member of the focal adhesion kinase (FAK) family of nonreceptor tyrosine kinases, which is expressed in neuronal cells, is tyrosine phosphorylated in response to an increase in calcium levels and membrane depolarization (Lev et al., 1995) and activates Ras.

PKC involvement in the activation of ERK1/2 is still unclear. PKC activating agonists, such as phorbol 12-myristate 13-acetate (PMA) which has shown an increase in activation of ERK1/2. However, a study showed that Pyk2, a protein tyrosine kinase, is also activated by PMA and is known to activate Ras.
(Lev S. et al., 1995) indicating that perhaps PKCs are not directly involved in ERK activation. However, conflicting studies using experiments in cells have evidence that Raf is phosphorylated and activated by PKC (Carroll and May, 1994).

ERK1/2 can also be activated via heteromeric G proteins (G$_{ai}$, G$_{ao}$ or G$_{aq}$) which are usually associated with serpentine receptors. These receptors are activated in response to agonists such as thrombin, endothelin and lysophosphatidic acid. The G$_{by}$ subunits of the heterotrimeric G$_{ai}$ protein activates Ras through Shc-Grb-Sos interactions which activates Ras/Raf/MKK and ERK1/2 (Van Biesen et al., 1995; Hawes et al., 1995). However, signaling through G$_{ao}$ or G$_{aq}$-coupled receptors is independent of G$_{by}$ or Ras and activates Raf-MKK1/2-ERK1/2 in a PKC-dependent manner (Van Biesen et al., 1996; Hawes et al., 1996). Yet, not all heterotrimeric proteins activate ERK1/2. The G$_{as}$-mediated signaling through cAMP-dependent protein kinase suppresses MKK1/2 and consequently suppresses ERK1/2 activation in some cell systems. Also, the cAMP-dependent protein kinase inhibits Raf activation (Lewis et al., 1998).

Some cytokine signalling pathways lead to interactions between receptors and members of the Janus family of protein-tyrosine kinases (JAKs) which has been demonstrated to activate ERK1/2. It has been suggested that JAK causes the activation of Ras through a Shc/Grb-coupled pathway (Chauhan et al., 1995; He et al., 1995).
There is great controversy over the role that PI3K may play in the activation of ERK1/2. During the past years there have been conflicting studies regarding ERK1/2 dependency on PI3K activation. Some studies indicated that PI3K activation is required for ERK1/2 activation (Standaert et al., 1995; von Willebrand et al., 1996; Grammer and Blenis, 1997; Bondeva et al., 1998; Sutor et al., 1999). However, others have suggested that PI3K activation is not required for ERK1/2 activation (Cheatham et al., 1994; Scheid and Duriono, 1996; Frevert and Kahn, 1997).

1.3.3.1 Function

There are many and various downstream targets of ERK1/2. Some of these targets are protein kinases (including Rsk1), transcription factors (including Elk), signaling components (including Sos) and cytoskeletal proteins (including synapsin1) (Lewis et al., 1998).

Protein kinases that are substrates of ERKs include Rsk1, Rsk2, Rsk3 and MAPKAP kinase 2, Mnk1 and Mnk2. Generally, the main role that these protein kinases play is to control various processes such as gene transcription and protein synthesis. For example, the downstream target of Rsk is p70S6 kinase (Chung et al., 1992) and p70S6 kinases is required to phosphorylate ribosomal protein S6 and thus regulates protein translation (Pullen and Thomas, 1997). Mnk are involved in activating eukaryotic initiation factor 4E (eIF4E) (Waskiewicz et al., 1997); thus, controlling translation. Some substrates of MAPKAP kinases are involved in transcription, such as cAMP response element-
binding protein (CREB), CREB binding protein (CBP) and c-fos (Xing et al., 1996; Nakajima et al., 1996).

ERKs can also directly phosphorylate many transcription factors. One of the transcription factors which is phosphorylated by ERKs is Elk2. Elk2 belongs to the Ets family of helix-turn-helix transcription factors. This factor regulates serum response element (SRE) by activating serum response factor (SRF) (Price et al., 1996). Furthermore, Ets2 repressor factor (ERF) is phosphorylated by ERKs and inhibited. In some cases c-Jun and c-Myc are also phosphorylated and activated by ERKs (Perverali. et al., 1996; Seth et al., 1992).

ERKs also phosphorylate cytosolic substrates which include signaling components that may be involved in feedback regulation or cross-regulation of other pathways. For instance, ERKs phosphorylate the Ras guanine nucleotide exchange factor, Sos1 at several residues. These phosphorylations of Sos1 cause disruption in the interactions of Sos1-Shc, Sos1-EGFR, and Sos1-Grb2; thus, interfering with Ras/Raf activation (Rozakis-Adcock et al., 1995). Consequently, ERK serves as a negative regulator of its upstream activators.

Some cytoskeletal proteins are phosphorylated by ERKs. For instance, ERK1/2 was originally identified to phosphorylate microtubule-associated proteins 1 and 2. However, the physiological consequence of this phosphorylation has not been determined (Hoshi et al., 1988). Tau, a microtubule-binding protein, is also highly phosphorylated by ERKs. The effects of Tau phosphorylation has not yet been clearly determined (Drewes et al., 1992).
The ultimate function of the ERK1/2 pathway is to regulate cell growth. It is involved in cell cycle progression through G1. It has been shown that cells with constitutively active mutants of MKK1, and thus overactivation of ERKs, have elevated levels of DNA synthesis and increased cell growth (Seger et al., 1994). Furthermore, when constitutively active mutants of MKK1 and MKK2 were transfected into 3T3 cells, the cells underwent transformation and exhibited characteristics of cell transformation. Some of these characteristics included high saturation density, anchorage independent growth, cell rounding and solid tumor formation in mice (Okazaki and Sagata, 1995).

ERK pathways also play a role in regulating embryonic development and cell differentiation. For instance, the activation of MKK and ERK is essential for vulval development in C. elegans. Also, photoreceptor cell specification and anterior-posterior body patterning in Drosophila. Furthermore, ERK1/2 pathways have been shown to regulate the differentiation of mammalian cell lines that undergo processes of commitment along neuronal, blood cell, or fat cell lineages. (reviewed in Lewis et al., 1998)

1.3.4 Inactivation of MAPKs

MAP kinase phosphatases (MKPs) are dual-specific protein phosphatases and inactivate MAPKs by dephosphorylating both the threonine and tyrosine residues of the TXY activation motif. Nine members of these phosphatases have been identified. These phosphatases are MKP1, MKP2, MKP3, MKP4, MKP5, PAC1, hVH2, hVH5 and Pyst1 (Rohan et al., 1993; Misra-Press et al., 1995; Muda et al., 1996; Muda et al., 1997; Dowd et al., 1998; Tanoue et al., 1999).
The substrate specificity of MKPs is still unclear. Also two other groups of phosphatases, protein serine/threonine phosphatases and tyrosine phosphatases also inactivate MAPKs (Keyse, 2000).

### 1.3.5 Novel MAPK Isoforms

The importance of MAPKs in cellular responses to external stimuli is evident by the vastness of the MAPK superfamily. Thus, novel MAPK isoforms, with the aid of the advancement in technology, are continuously being discovered. For instance, two new ERK isoforms, 110 kDa ERK5/Big MAP kinase 1 (BMP1) and 61 kDa ERK7, which possess the characteristic TEY motifs, were identified. ERK5 was initially found to be activated by oxidant and osmotic stresses. However, a recent study by Kamakura et al. (1999) demonstrated that receptor tyrosine kinase, growth factors, such as epidermal growth factor and nerve growth factor can also activate ERK5. ERK7 activation is very different from ERK1/2/5. ERK7 is constitutively activated in serum-starved cells and this activation is dependent on the presence of its C-terminal domain (Abe et al., 1999). Also, a recent study identified ERK1b, a novel 46 kDa ERK isoform. ERK1b, unlike ERK1/2 is not regulated by MEK1 (Yung et al., 2000).

### 1.4 Eukaryotic Protein Translation

Protein translation is the synthesis of new proteins which are crucial for cells to function properly. Protein translation involves converting the genetic message from the messenger RNA (mRNA) into the protein. Briefly, there are three stages in protein translation: initiation, the important regulatory step in eukaryotic cells which involves the recruitment of the ribosomal complex,
elongation, which is the synthesis of the polypeptide chain, and termination, which is when the polypeptide chain is released from the ribosomal complex.

The role of PI3K in the regulation of protein translation has been under investigation for the past several years. It has been carefully documented in the case of insulin receptor signaling. A study conducted by Mendez et al. (1997), indicated that PI3K regulates protein synthesis via two separate pathways. One pathway is the activation of PKC zeta to promote general protein synthesis. The other pathway is the via Akt and leads to the activation of p70S6K which is involved in growth related protein synthesis and cell cycle progression, such as via c-myc (Mendez et al., 1997).

1.4.1 Ribosomal complex

The ribosomal complex, which consists of ribosomal RNA (rRNA) and numerous small proteins, reads the genetic code and is the site of peptide bond formation. It controls the recognition between the triplet, nucleotide codon of the mRNA and the anticodon of the transfer RNA (tRNA). The ribosome moves along the mRNA one codon at a time. The eukaryotic ribosome complex, which is 80S, consists of two subunits, the large ribosomal subunit (~60S) and the small ribosomal subunit (~40S). The ribosome attaches to the mRNA at or near the 5' end of a coding region and it moves along the mRNA toward the 3' end. It translates each triplet codon into an amino acid. As the ribosome moves along the message it associates with a specific aminoacyl-transfer RNA (tRNA) which adds amino acids to the growing polypeptide chain. The ribosome can accommodate two tRNAa, thus allowing the peptide bond to form (Lewin, 1995).
1.4.2 Eukaryotic Translation Initiation

The initiation step is the most important in eukaryotic protein translation because many factors are required to recruit the ribosomal complex to mRNA to allow translation to occur. It is the slowest step of protein translation, thus, it is the rate-limiting step. More specifically, the very first step in the initiation process is the recruitment of the small ribosomal subunit (40S) to the mRNA.

The mRNA is brought to the ribosome by eukaryotic initiation factor-4F (eIF-4F). eIF-4F is a multiprotein complex which consists of the subunits: eIF-4E, eIF-4A and eIF-4G. eIF-4E binds to the 5' terminal 7-methyl-GTP (m\(^7\)GTP) cap. eIF-4A is a subunit of an ATP-dependent RNA helicase that cooperates with eIF-4B, and unwinds the secondary structure of mRNA. eIF-4G is a scaffolding protein. eIF-4G has a binding site for eIF3 and eIF3 is complexed with the 40S ribosomal subunit. This interaction allows the association of the eIF-4F-mRNA complex to the 40S ribosomal subunit (Sachs and Varani, 2000). Also, eIF-4G serves as a regulator of initiation by enhancing eIF-4E's ability to bind to the 5' cap via phosphorylation (Waskiewicz et al., 1999). The eIF-4F complex scans the mRNA until the translation initiation codon (usually AUG) is located (Zimmer et al., 2000). eIF-4G, does not bind directly to mRNA, but it associates with a 5' cap structure, m\(^7\)GTP and the poly (A) tail at the 3' end. The 5' m\(^7\)GTP cap, formed by guanine 7-methyltransferase, and the 3' poly (A) tail, formed by poly(A) polymerase, are found in all eukaryotic mRNA (Quiocho et al., 2000). eIF-4G also binds to the cap and poly (A) tail indirectly through its interaction with the cap binding protein eIF-4E and the poly (A) binding protein, Pab1p,
respectively. In yeast, this association causes the mRNA to circularize (Sachs et al., 1997; Wells et al., 1998).

**Figure 1.4** Organization of the eukaryotic translation initiation apparatus. The yeast mRNA circularizes via eIF-4E's interaction with the 5' m'GTP cap and the 3' poly (A) tail (Sachs and Varani, 2000).

1.4.2.1 **Structure of Pab1p**

Pab1p binds with high affinity to the poly (A) tail and is a member of the RNA binding protein family. These proteins contain the ribonucleoprotein (RNP) or RNA recognition motif (RRM), a universal RNA recognition module (Varani and Nagai, 1998). All Pab1p proteins contain four RRM domains each separated by highly conserved linkers. The two N-terminal RRM domains, which are located in the first 200 amino acids of the protein, contain the poly (A) binding activity (Deardoff and Sachs, 1997). The RRM1 and RRM2 domains bind to eIF-
4G. However, the exact mechanism of how eIF-4G binds specifically to Pab1p still remains to be determined (Kessler and Sachs, 1998).

1.4.2.2 Structure of eIF-4G

There are two functional homologues of eIF-4G, denoted as eIF-4G (171 kDa) and eIF-4G (176 kDa). These homologues are 46% identical and possess similar biochemical activities. Both these isoforms behave as scaffolding bridges between eIF-4E and eIF-4A to form the multisubunit complex eIF-4F. They also both bind directly to Pab1 (Marcotrigiano et al., 1999). The N-terminal region of eIF-4G interacts with eIF-4E, while the C-terminal region contains separate binding sites for eIF-4A and eIF3. Also, the C-terminal region contains docking sites for Mnk1, also known as MAPKAPK (Pyronnet et al., 1999). The region that binds to eIF-4E which is identified to be the conserved Tyr-X-X-X-X-Leu-Φ eIF-4E recognition motif where X is variable and Φ is Leu, Met or Phe. This region is common among other eIF-4E-binding proteins, such as 4E-BPs (Marcotrigiano et al., 1999).

Figure 1.5 Schematic representation of the characterized eIF-4G binding sites (Waskiewicz et al., 1999).
1.4.2.3 eIF-4G Interactions with eIF-4E and Pab1p

The association of eIF-4G with eIF-4E increases eIF-4E affinity for the cap by greater than 10-fold (Haghighat and Sonenberg, 1997). Furthermore, the C-terminal region of eIF-4G provides a docking site for Mnk1 which has been identified as the kinase which phosphorylates eIF-4E at Ser-209. This phosphorylation of eIF-4E enhances its affinity to bind to the 5' cap and eIF-4G. Also, Mnk1 interacts with p97, an eIF-4G-related protein that does not bind to eIF-4E, indicating that p97 can block the phosphorylation of eIF-4E by sequestering Mnk1 (Pyronnet et al., 1999).

eIF-4G associates with the mRNA poly (A) tail through Pab1p. This association of both eIF-4E and Pab1p with eIF-4G allows for synergistic activation of translation initiation (Tarun et al., 1997). However, the mechanism of the synergy is still unknown. mRNAs that are both capped and polyadenylated are translated more efficiently than mRNA that is either capped or polyadenylated.

1.4.3 eIF-4E

eIF-4E is an important part of the eIF-4F complex. eIF-4E is found in the smallest amounts compared to all the other initiation factors. eIF-4E is present at 0.01-0.2 molecules/ribosome as compared to 0.5-3 molecules/ribosome for other initiation factors (Duncan et al., 1987) and thus, it is the rate-limiting subunit. Furthermore, the highly conserved sequences of eIF-4E from higher to lower eukaryotes demonstrate the universal importance of eIF-4E (McKendrick et al., 1999). Since eIF-4E serves as one of the rate-limiting components of the
translation initiation machinery, its regulation by signal transduction events is a crucial part of the control of protein translation.

eIF-4E is a 25 kDa protein. It has an αβ structure and its shape resembles a baseball glove (Marcotrigiano et al., 1997). The eIF-4E protein contains a curved eight-stranded anti-parallel β-sheet that contains the cap-binding site, while the convex surface contains the three long α-helices. Two short helices are found within the loops bridging the β1-β2 and β3-β4 strands of the β-sheet. It contains conserved tryptophan residues involved in cap-binding (Sachs and Varani, 2000).

1.4.3.1 Regulation of eIF-4E at Multiple Levels

The mechanisms regulating transcription of the eIF-4E gene are not completely understood. However, the eIF-4E promoter was demonstrated to possess two myc binding sites (Jones et al., 1996). A study has also demonstrated that in cells over-expressing c-myc, there is an increase in expression of eIF-4E mRNA (Rosenwald et al., 1993). While Myc protein is an important regulator of cell proliferation very few myc-regulated genes have been characterized (Grandori and Eisenman, 1997). eIF-4E expression has also been shown to increase seven-fold in fibroblasts in response to serum and growth factors and in the activation of human T cells (Rosenwald et al., 1993; Mao et al., 1992).

eIF-4E requires phosphorylation in order to increase its affinity for the 5' cap on the mRNA. eIF-4E is phosphorylated on Ser-209 and Thr-210. However, Ser-209 is the main residue to be phosphorylated (Raught and Gingras, 1999).
PKC has been demonstrated to phosphorylate Ser-209 \textit{in vitro}, but the protein kinase responsible for its phosphorylation \textit{in vivo} is less clear. It is suggested that the phosphorylation of Ser-209 is via Mnk1. In turn, Mnk1 is phosphorylated and activated by p38 MAP kinase and ERK1/2 (Wang \textit{et al.}, 1998; Fukunaga and Hunter, 1997).

The phosphorylation state of elf-4E correlates with the translation rate and growth status of the cell. For example, elf-4E is underphosphorylated in mitosis, where translation rates are low (Bonneau and Sonenberg, 1987). There is an increase in elf-4E phosphorylation when cells are exposed to hormones, growth factors or cytokines. Also, there is an increase in elf-4E phosphorylation when there is an increase in cardiac load (Wada \textit{et al.}, 1996). To complicate matters, some types of cellular stresses, such as arsenite and TNF\(\alpha\) and IL-1\(\beta\) have shown to increase the phosphorylation of elf-4E, but translation rates actually decrease (Wang \textit{et al.}, 1998; Raught and Gingras, 1999). However, other types of cellular stresses, such as heat-shock or infection with adenovirus are accompanied by a decrease in elf-4E phosphorylation (Duncan \textit{et al.}, 1987; Feigenblum \textit{et al.}, 1993). Thus the effects and regulation of elf-4E phosphorylation are not completely understood and more studies are still required for a complete explanation.

There appears to be a role of Ras/Raf/MAPK pathway in the phosphorylation of elf-4E. Some studies have demonstrated an increase in phosphorylation of elf-4E in ras or src-transformed cells (Frederickson \textit{et al.}, 1991; Rinker-Schaeffer \textit{et al.}, 1992). Furthermore, the MEK inhibitor, PD98059,
blocks the increase in eIF-4E phosphorylation caused by growth factors (Dudley 
et al., 1995). Activation of the p38 MAPK pathway also shows an increase in
eIF-4E phosphorylation as shown by its inhibition in the presence of the p38
MAPK inhibitor, SB20358 (Young et al., 1997). Both the ERK pathway and p38
MAPK pathway converge at a common protein, Mnk1. Mnk1 phosphorylates
Ser-209 in vitro and is the most likely candidate to phosphorylate eIF-4E in vivo.
(Pyronnet et al., 1999; Raught and Gingras, 1999).

![Diagram of signaling pathways](image)

**Figure 1.6** Two pathways leading to the phosphorylation and activation of eIF-4E (Raught and Gingras, 1999).

eIF-4E is further regulated through its interaction with a family of inhibitory
binding proteins, known as 4E-BPs or phosphorylated, heat-and-acid-stable
(PHAS), which describes the properties of the protein (Gingras et al., 1999b).
This family consists of three small acidic proteins that compete with eIF-4G for
the common binding site on eIF-4E. The 4E-BPs are very small proteins. 4E-
BP1 is ~10 kDa, 4E-BP2 is ~12 kDa and 4E-BP3 is ~15 kDa. 4E-BP1 shares a 56% sequence identity with 4E-BP2 and 4E-BP3 shares a 57% sequence identity with 4E-BP1 and a 59% sequence identity with 4E-BP2 (Fletcher et al., 1998; Poulin et al., 1998). All these 4E-BPs share a small amino acid motif, Tyr-X-X-X-X-Leu-Φ, with elF-4G. Thus, these two proteins, 4E-BPs and elF-4G compete with each other for the elF-4E binding site. Over expression of 4E-BPs have been shown to inhibit cap-dependent, but not cap-independent translation (Gingras et al., 1999a).

Binding of 4E-BPs to elF-4E is reversible and is dependent on the phosphorylation status of 4E-BP. Hypophosphorylated 4E-BPs interact strongly with elF-4E, but hyperphosphorylated 4E-BPs have decreased binding efficiency to elF-4E (Gingras et al., 1999a).

Treatment of cells with various stimuli, such as hormones, growth factors, cytokines and G-protein-coupled receptor agonists cause an increase in 4E-BP phosphorylation and in most cases, an increase in translation rates. However, in conditions of nutrient or growth factor deprivation this deprivation results in 4E-BP dephosphorylation, an increase in elF-4E binding and ultimately results in the decrease of cap-dependent translation (Raught and Gingras, 1999).
Figure 1.7  Simplified diagram of the regulation of the formation of the eIF-4F complex. Phosphorylated 4E-BPs does not bind to eIF-4E, thus allowing the formation of the eIF-4F complex; whereas, dephosphorylated 4E-BPs does not allow the formation of the eIF-4F by binding and blocking eIF-4E's activity (Raught and Gingras, 1999).

A recent study, demonstrated that 4E-BPs bind and function without folded structure. This unfolded structure of 4E-BPs is interesting, because proteins usually exhibit their function via a folded 3-D conformational shape (Fletcher et al., 1998). Furthermore, the eIF-4E binding region of the 4E-BPs are flanked with PEST sequences. These PEST sequences may regulate 4E-BP turnover by making them targets for degradation by the Ca^{2+} activated protease, calpain (Fletcher et al., 1998).

The three isoforms of 4E-BPs do not have any functional differences. However, their tissue distribution varies. On the one hand, 4E-BP1 is found in most tissues, but is predominantly expressed in adipose tissue, pancreas and skeletal muscle. On the other hand 4E-BP2 is ubiquitously expressed (Tsukiyama-Kohara et al., 1998). 4E-BP3, like 4E-BP1, is expressed in most
tissues, but is predominantly expressed in skeletal muscle, heart, kidney, pancreas and very little in brain and thymus (Poulin et al., 1998).

4E-BPs are phosphorylated at specific serine/threonine residues. Some of these sites have been identified and include, Thr-37, Thr-46, Ser-65, Thr-70, Ser-85 and Ser-112. It is important to mention that there may be other sites, not yet identified and that there are possibly different phosphorylation sites in different cell types (Gingras et al., 1999b). The phosphorylation of Thr-37 and Thr-46 are considered to be early phosphorylation events, because the 4E-BPs are still complexed to eIF-4E. However, these phosphorylation events are required for subsequent phosphorylation. The phosphorylation of Ser-65 and Thr-70 are the sites which cause the release of 4E-BP from eIF-4E (Gingras et al., 1999b).

The phosphorylation of 4E-BPs does not appear to be regulated through the Ras/Raf/MAPK pathway, but appears to be regulated by PI3K pathway. It has been demonstrated that overexpression of PI3K catalytic subunit, p110α induces the phosphorylation of 4E-BP1. Furthermore, wortmannin, an inhibitor of PI3K, was shown to block the phosphorylation of 4E-BP1 (Gingras et al., 1999c). The ultimate protein kinase which phosphorylates 4E-BP1 appears to be FKBP-rapamycin associated protein/mammalian target of rapamycin (FRAP/mTOR) (Raught and Gingras, 1999). The inhibitor, rapamycin, which blocks the activity of FRAP/mTOR has been shown to prevent the phosphorylation of 4E-BP1 (Gingras et al., 1999c). It has been shown that in vitro FRAP/mTOR phosphorylates Thr37 and Thr46 (Gingras et al., 1999b).
The inactivation of 4E-BPs occurs via dephosphorylation. This dephosphorylation occurs via the increased activity of protein phosphatase 1 and protein phosphatase 2A (PP1, PP2A). It has been demonstrated in cardiac myocytes that in the presence of oxidative stress, 4E-BPs undergo dephosphorylation and subsequently, protein synthesis is inhibited (Pham et al., 2000).

An interesting study conducted by Khaleghpour et al. (1999) demonstrated that overexpressed eIF-4E up to five fold over endogenous levels may activate a negative feedback loop that targets a component of the PI3K signaling pathway which lies downstream of PI3K. In this study the overexpressed eIF-4E caused the dephosphorylation of both 4E-BP1 and p70 S6K (Khaleghpour et al., 1999). However, there is still work to be done to provide a clearer picture of the regulation of 4E-BPs.
Figure 1.8 A model showing the role of PI3K in the phosphorylation and inactivation of 4E-BP and ultimately leading to an increase in protein synthesis (Gingras et al., 1999c).
1.4.3.2 **Function of eIF-4E**

eIF-4E can control cell growth and proliferation. eIF-4E activity plays an important role in the regulation of cell cycle, particularly in the G1/S progression. Protein synthesis is needed for entry into and throughout the cell cycle. (Polunovsky *et al.*, 1996).

Overexpression of eIF-4E can transform cells. Some tumor cells have been identified to have an increase in eIF-4E levels (Zimmer *et al.*, 2000). Furthermore, eIF-4E has been shown to cooperate with v-myc in the transformation of primary rodent fibroblasts (Lazaris-Karatzas and Sonenberg 1992).

eIF-4E can inhibit apoptosis. Under normal conditions, serum deprivation will induce apoptosis in cells. However, in cells with high levels of eIF-4E apoptosis is inhibited. For instance, overexpression of eIF-4E in NIH3T3 cells prevents apoptosis induced by serum deprivation (Polunovsky *et al.*, 1996).

1.4.4 **Competition Among mRNA for Translation**

In normal cellular conditions, the amount of eIF-4E is very low ~one-tenth the molar concentration of mRNA. Thus, in order for the mRNA to be translated, it must compete for available eIF-4E. The competitiveness of the mRNA depends on its rate-constant of initiation. This rate-constant of initiation is determined by the sequence context around the translation initiation codon, position of the translation initiation codon relative to the cap structure, the secondary structure of the mRNA, which is primarily in the 5' untranslated leader sequence (5'UTR) (Sonenberg and Gingras, 1998). Thus, mRNAs that contain
5'UTRs which are short, unstructured and GC poor are translated more efficiently due to the cap-structure accessibility to binding of eIF-4E. For example, housekeeping proteins, such as globins and histones are translated more efficiently. However, 5'UTRs which are lengthy, GC-rich, highly structured or have one or more upstream initiation codon are translated less efficiently due to cap inaccessibility and lack of identification of the true translation initiation codon. Examples of these mRNAs are mRNAs that code for growth regulatory molecules, and c-myc (Sonenberg and Gingras, 1998; Zimmer et al., 2000).

1.4.5 Translation of Some mRNAs That Are Not Dependent on eIF-4E

Some viral mRNA are uncapped, thus, translation occurs through a process termed internal initiation. Internal initiation involves the direct binding of an initiation factor/ribosome complex to structural elements in the RNA 5'UTR called internal ribosome entry sites (IRES). This process still requires components of cellular translation, such as eIF4G, eIF4A and eIF3. However, it does not require eIF-4E (Raught and Gringras, 1999).

1.5 Summary

The work in this thesis consists of two main sets of studies as summarized here.

1.5.1 The Relationship Between PI3K and ERK1/2 Activation

Determining the relationship between PI3K activation and ERK1/2 activation is an important step to unraveling the mysteries of various signaling pathways within the cell system. In the past several years there have been conflicting studies over the relationship between PI3K activation and ERK1/2
activation. Thus, it is important that this controversial issue be clarified. This thesis addressed the question of whether or not PI3K activation is required for ERK1/2 activation in various cell systems.

We hypothesized that PI3K is not required for ERK1/2 activation. To test this hypothesis we investigated the following objectives.

1. To observe the effects of PI3K inhibition (using PI3K inhibitors: LY294002 and wortmannin) on ERK1/2 activation using immunoblots and apoptotic studies.

2. To determine if the concentration of the cytokine in the presence of PI3K inhibition (using effective, constant concentration of LY294002) will affect the activation of ERK1/2.

1.5.2 PI3K Regulation of Protein Synthesis

One of the main questions in protein translation which remains to be answered is what mechanisms are involved which directs the cell to translate one mRNA and not another mRNA? To attempt to address this issue, one must first investigate which protein plays a predominant role in regulation of protein translation. Thus, it has been suggested, but not clearly established, that PI3K may play a role in regulation of protein translation. For instance, Jefferies et al. (1994) has suggested that the PI3K pathway, via PDK1 phosphorylation activates p70S6 kinase which enhances protein translation. Furthermore, Gingras et al. (1999c) suggested that PI3K-dependent pathway is required for the phosphorylation of 4E-BP1 and its deactivation and causing its release from eIF-4E and allowing mRNA translation to occur.
We hypothesized that PI3K is involved in protein translation and were interested in establishing protocols for future studies regarding protein translation. To test this hypothesis, we undertook the following studies.

1. To observe if our cell systems have the important components of the translational protein machinery, eIF-4E and 4E-BPs and, if so, do we observe a difference between stimulated and unstimulated cell?

2. What are the effects of the inhibitors LY294002, wortmannin, U0126 and rapamycin on the phosphorylation of 4E-BP1 and 4E-BP2?
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Materials

- Autoradiography Film (MR)
- Chromatography Paper (3MM)
- Conical Tubes (15 and 50 ml)
- Membrane Filter Units (0.22 µm)
- Microfuge Tubes
- Nitrocellulose Membrane (pore size 0.45 µm)
- Pipette Tips
- Tissue Culture Plates/Flasks (Untreated and Treated) Falcon
- Tubes for Flow Cytometer

2.1.2 General Chemicals

- 30% Acrylamide/Bis Solution, 37.5:1
- Ammonium Persulphate
- BCA Protein Assay Reagent
- Bovine Serum Albumin
- Bromophenol Blue
- Cap Column (mGDP coupled to Sepharose)
- Dextrose
- Dimethyl Sulfoxide
- Enhanced Chemiluminescence (ECL)
- Ethanol
- Ethylene Diamine Tetraacetic Disodium Salt (EDTA)
- Glycerol
- Glycine
- HEPES
- Hydrochloric Acid
- 2-Mercaptoethanol
- Methanol
- Ponceau S concentrate
- Potassium Chloride
- Potassium Dihydrogen Phosphate
- Propidium Iodide
- Protein A-Sepharose
- RNaseA
- SDS-PAGE Molecular Weight Standards
  (Broad Range and Low Range)
- Sodium Azide
<table>
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<th>Fisher Scientific</th>
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<tbody>
<tr>
<td>Sodium Dihydrogen Phosphate</td>
<td>Fisher Scientific</td>
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<tr>
<td>Sodium Dodecyl Sulphate (SDS)</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Skim Milk</td>
<td>Safeway Brand</td>
</tr>
<tr>
<td>TEMED (N,N,N',N'-tetramethylethylenediamine)</td>
<td>BioRad</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane base</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Fisher Scientific</td>
</tr>
</tbody>
</table>

### 2.1.3 Tissue Culture Reagents

- Dulbecco’s Modified Eagle Medium (DMEM) | Gibco BRL Products |
- Fetal Bovine Serum (FBS) | Gibco BRL Products |
- L-Glutamine | Gibco BRL Products |
- Penicillin/Streptomycin | Gibco BRL Products |
- RPMI 1640 Medium | Gibco BRL Products |
- Sodium Pyruvate | Gibco BRL Products |
- Trypsin | Gibco BRL Products |

### 2.1.4 Pharmacological Inhibitors

- LY294002 | Calbiochem |
- Rapamycin | Calbiochem |
- U0126 | Calbiochem |
- Wortmannin | Calbiochem |

### 2.1.5 Protease Inhibitors

- Aprotinin | Sigma |
- Leupeptin | Sigma |
- Pepstatin A | Sigma |
- Phenylmethylsulphonylfluoride (PMSF) | Sigma |
- Soybean Trypsin Inhibitor | Sigma |

### 2.1.6 Tyrosine Phosphatase Inhibitors

- β-Glycerophosphate | Sigma |
- Sodium Molybdate | Sigma |
- Sodium Orthovanadate | Sigma |

### 2.1.7 Serine Phosphatase Inhibitor

- Microcystin-LR | Calbiochem |
2.1.8 Cytokines

cGMI
Recombinant Human GM-CSF
Synthetic GM-CSF, IL-3, IL-4
WEHI-3

Biosource International
Gift from Dr. Ian Clark-Lewis (University of BC)

2.1.9 Antibodies

Anti-elf-4E
Anti-4E-BP1 (11208 for Western blots)
Anti-4E-BP1 (11209 for IP)
Anti-4E-BP2 (11211)

Gifts from Dr. Sonenburg (McGill, Montreal)

Anti-p85

Upstate Biotech. Inc.

Anti-p70 S6Kinase
Anti-phospho-4E-BP1 (Ser-65)
Anti-phospho-elf-4E (Ser-209)
Anti-phospho-p44/42 MAPK (Thr-202/Tyr-204)
Anti-phospho-Akt (Ser-473)

Cell Signaling Technologies

Goat Anti-Rabbit Immunoglobulin
Goat Anti-Mouse Immunoglobulin

DAKO

2.2 Methods

2.2.1 Tissue Culture

FDC-P1 cells are cytokine-dependent and derived from murine, bone
marrow tissue (Dexter et al., 1980). These cells were cultured in RPMI 1640
medium, supplemented with 10% FBS, 20mM 2-mercaptomethanol, 1mM sodium
pyruvate, 2 mM L-glutamine and 5% WEHI-3-conditioned medium was added as
a source of interleukin-3 (IL-3). BAF-3 cells are also derived from a hemopoietic,
murine tissue that was initially identified as a pro-B-cell type (Palacios and
Steinmetz, 1985). MC-9 cells are derived from a murine, mastocytoma (Galli et
al.,1982). Both MC-9 and BAF-3 cells are cultured in the same growth medium
as FDC-P1. TF-1 cells were previously derived from an eurythroleukemic cancer
patient (Kitamura et al., 1989). The TF-1 cells are grown in similar growth medium as the previous cells, except 1% conditioned medium containing recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) denoted as cGMI was used instead of the WEHI-3 conditioned medium. HEK 293 cells are a human embryonic kidney cell line (Graham et al., 1997), grown in DMEM medium supplemented with 10% FBS, 1X L-glutamine and 50 units/ml penicillin and 50 μg/ml streptomycin. FDC-P1, BAF-3, TF-1 and MC-9 cell lines are all suspension cells and grown in non-treated, tissue culture plates. However, the HEK 293 cells are adherent cells and grown in treated, tissue culture plates and detached using 0.25% trypsin. All these cells were grown at 37°C and 5% CO₂ with humidity.

2.2.2 Cell Stimulations and Lysis Conditions

Cells were starved of cytokine by overnight incubation in medium containing 1% WEHI-3-conditioned medium for FDC-P1 cells and BAF-3 cells, or no GM-CSF for TF-1 cells, or no FBS for HEK 293 cells. Following the overnight incubation, the cells were washed 3 times with sterile, 1X PBS. The HEK 293 cells were harvested using 2X Versene prior to the PBS washes. The cells were then counted using the Beckman Coulter Counter. The cells were incubated at 37°C for 0.5 hour in 20 mM HEPES (pH 7.4)-buffered RPMI or DMEM prior to the assay. Cells were treated with PI3K inhibitors, LY294002 or wortmannin or MEK-1 and MEK-2 inhibitor, U0126 at varying concentrations. Cells were incubated with the above inhibitors for 10 minutes prior to stimulation with the appropriate cytokines at concentrations shown to cause maximal stimulation of tyrosine
phosphorylation. The FDC-P1s were stimulated with either synthetic IL-3 or synthetic GM-CSF, BAF-3 cells were stimulated with synthetic IL-3 and TF-1 cells were stimulated with GM-CSF. The cells were stimulated with the indicated cytokines for 5 minutes. However, HEK 293 cells were stimulated with 10% FBS for 10 minutes. To stop the treatments, the cells were pelleted and then solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM Na$_3$MoO$_4$, 10 mM β-glycerophosphate, 1 μg/ml microcystin-LR, 1 μg/ml aprotinin, 40 μg/ml PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor). Nuclei were pelleted by centrifuging at 13,000 rpm for 1 minute at 4°C and the supernatants were transferred to new tubes for immunoblotting analysis.

For studies examining phosphorylation of 4E-BPs the cells were treated similarly as in the previous section except for the following modifications. In addition to treatments with LY294002, wortmannin and U0126 inhibitors they were also treated with the FRAP/mTOR inhibitor, rapamycin at varying concentrations. FDC-P1 cells were stimulated with synthetic IL-3, MC-9 cells were stimulated with either synthetic IL-4 or PMA and the TF-1 cells were stimulated with either GM-CSF or human IL-3 for 5 minutes. To stop the treatments the cells were pelleted and resuspended in ice-cold buffer not containing detergent (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 50 mM NaF, 1 mM EGTA, 1 mM DTT, 1 mM Na$_3$VO$_4$, 1 mM Na$_3$MoO$_4$, 10 mM β-glycerophosphate, 1 μg/ml microcystin-LR, 1 μg/ml aprotinin, 40 μg/ml PMSF, 1
μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor). The cells were then lysed by using three, freeze-thaw cycles (freeze in liquid N₂ and thawed in 37°C water bath). The nuclei were pelleted by centrifuging at 13,000 rpm for 5 minutes at 4°C and the supernatants were transferred to new tubes. The samples were heat enriched to remove unwanted proteins by boiling at 100°C, and cooled on ice for 5 minutes. Then the supernatant was collected by centrifuging at 13,000 rpm for 5 minutes at 4°C.

A different lysis buffer was used for eIF-4E. The procedure was similar to that of 4E-BPs except the lysis buffer consisted of 20 mM Hepes-KOH, pH 7.5, 75 mM KCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM Na₃MoO₄, 10 mM β-glycerophosphate, 1 μg/ml microcystin-LR, 1 μg/ml aprotinin, 40 μg/ml PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. Three freeze-thaw cycles were used to lyse the cells and the nuclei were removed by pelleting. Then the supernatant was incubated with m⁷-GDP Sepharose resin, which binds to the eIF-4E, for one hour while rotating at 4°C. Then the samples were centrifuged at 13,000 rpm for 1 minute at 4°C. The supernatant was discarded and the Sepharose beads were washed three times with lysis buffer. eIF-4E was eluted by boiling the Sepharose beads for 5 minutes, containing lysis buffer and a final concentration of 1X SDS sample buffer. The Sepharose beads were removed by centrifuging the samples. The supernatant was transferred to new tubes for immunoblotting.
2.2.3 Immunoblotting Assays

Concentrated sample buffer to give a final concentration of 1X (2% SDS, 10% glycerol and 1% β-mercaptoethanol) was added to the total protein lysate. The samples were boiled for 5 minutes prior to immunoblotting analysis.

Before the addition of the sample buffer, the amount of protein in sample was approximated by counting cells using the approximation that 1 X 10^6 cells is equivalent to 100 μg of protein. In some experiments, the cell count approximation was verified using BCA protein assay reagent (Pierce), according to the manufacturer's directions.

Nine % SDS-PAGE gels, 1.5 mm thick, were casted and 50-100 μg of total protein were loaded onto the gels and broad-range, prestained molecular weight standards were run alongside the lysates. The gels were electrophoresed at 200 volts for 30-45 minutes. The gels were then transferred to nitrocellulose blots using semi-dry transfer apparatus. The blots were stained with Ponceau S to ensure that there was equal loading of the lysates. Then the blots were blocked in 5% skim milk for 1 hour while gently rocking and incubated in the appropriate primary antibody, anti-phospho-Akt (Ser-473), 1:1000 dilution or anti-phospho-p44/42 MAPK (Thr-202/Tyr-204), 1:1000 dilution, in 1X TBS, 1 % BSA and 0.03% sodium azide for overnight. Following the incubation, the blots were washed twice with 1X TBS and once with 1X TBS with 0.05% Tween for 5 minutes for each wash. The primary antibody was detected by incubating in the appropriate secondary antibody (goat anti-rabbit or goat anti-mouse, diluted in 1X TBS, 0.05% Tween) coupled to horseradish peroxidase. After the one hour
secondary antibody incubation, the blots were washed twice with TBS and twice
with TBS with 0.05% Tween for 10 minutes for each wash. The bands were
visualized using enhanced chemiluminescence. The blots were exposed to MR
Kodak film for autoradiography.

The protein levels in the blots were also normalized by probing for p85
levels. The blots were stripped using stripping buffer, consisting of 60 mM Tris-
HCl, pH 6.7 and 2% SDS. The blots were rocked at 50°C for 0.5 hour. Then the
blots were blocked in 5% skim milk for 1 hour and then incubated in anti-p85
1:2000 dilution for 1 hour. The same wash procedure was followed as above and
then the blot was incubated in the secondary antibody, goat anti-rabbit, for one
hour. Then the blots were visualized using enhanced chemiluminescence and
exposed to MR Kodak film for autoradiography.

The immunoblotting assay for 4E-BPs and eIF-4E was similar to the
procedure mentioned previously except for the following modifications. 15%
SDS-PAGE (37.5:1) gels or 15% low bisacrylamide gels (made up using
acrylamide:bisacrylamide ratio of 118:1), 1.5 mm thick were casted and 100 μg-1
mg of total protein were loaded onto the gels alongside, low molecular weight
standards. Following transfer to nitrocellulose, the primary antibodies used were
anti-eIF-4E, 1:1000 dilution or anti-4E-BP1, 1:1000 dilution or anti-4E-BP2,
1:2500 dilution or anti-phospho-4E-BP1 (Ser-65), 1:1000 dilution or anti-
phospho-eIF-4E (Ser-209), 1:1000 dilution in 1X TBS, 1% BSA and 0.03%
sodium azide for overnight. Washes and detection with the appropriate
secondary antibody were described as above.
2.2.4 Apoptosis Analysis by Staining for Subdiploid DNA

The FDC-P1 cells, BAF-3 cells and TF-1 cells were washed three times in sterile, 1X PBS prior to treatments. The cells were counted using Beckman-Coulter Counter and resuspended in growth medium to a concentration of 1 X $10^6$ cells/ml, without any cytokine and treated as follows. The cells were either treated with or without cytokines (5% WEHI-3 and 1% GM-CSF) and with or without inhibitors (LY 294002 and U0126). The cells were incubated at 37°C, 5% CO$_2$ in humidified air for either 16 hours or 24 hours.

The following method for the fixation of the cells, is a modified version from Current Protocols of Immunology. One ml aliquots of the cells, following various treatments, were collected and pelleted. Then the supernatant was poured off and 1 ml of ice-cold, 70% ethanol was added to the cells drop-wise, while vortexing. Then the cells were incubated at 4°C for at least 18 hours.

The cells were pelleted by spinning at 3000 g for 5 minutes. Then the ethanol was poured off and the cells resuspended in staining buffer containing 1X PBS, 0.1% dextrose, 100 μg/ml RNaseA, 50 μg/ml propidium iodide. The samples were kept at room temperature, in the dark, for 45 minutes prior to flow cytometry analysis.

2.2.5 Flow Cytometry

The propidium iodide stained cells were analyzed using the Beckman Coulter EPICS XL-MCL, flow cytometer. Fluorescence detector FL3 was used because propidium iodide emits in the 605-635 nm range. EXPO 32 Data Acquisition Software was used to collect the data which was displayed in four
histograms. SS vs. FS, FL3 vs. FS, number of events vs. FL3 log and number of events vs. FL3. EXPO 32 Analysis Software was used to analyze the data. The background noise and autofluorescence of the cells were gated out using the negative control sample in the FL3 vs FS histogram. The same gate was applied to all subsequent treatment samples. The amount of subdiploid DNA content was quantified using the number of events vs. FL3 histogram by marking the area starting from the y-axis to the first largest peak, which corresponds to the cells in the G1 cycle.

2.2.6 Laser Densitometry

Some of the autoradiography films (FDC-P1 films) were quantified using laser densitometry. The films were scanned using Personal Densitometer SI Molecular Dynamics. The files were saved and analyzed using BioRad Quantity One software. The data was imported into Excel and normalized against the p85 control.
Chapter 3: Relationship Between PI3K and ERK1/2 Pathways

3.1 Rationale

A complex network of signaling pathways is activated in cells in response to various cytokines. This complex network is further complicated by the fact that different cell types appear to trigger different signaling cascades. Furthermore, one of the predominant controversial issues in signal transduction is the relationship between phosphatidylinositol 3-kinase (PI3K) activation and mitogen activated protein kinase (MAPK) activation, or more specifically, activation of ERK1/2. Some studies indicate that PI3K activation is required for ERK1/2 activation (Grammer and Blenis, 1997; Standaert et al., 1995; Bondeva et al., 1998; von Willebrand et al., 1996; Sutor et al., 1999) while others suggest that PI3K activation is not required for ERK1/2 activation (Cheatham et al., 1994; Scheid and Duriono, 1996; Frevert and Kahn, 1997). The intent of this study was to clarify this issue by determining whether PI3K activation is required for ERK1/2 activation in several different cell lines.

Our lab had previously demonstrated that PI3K activation is not required for activation of ERK1/2 in MC-9 cells (Scheid and Duriono, 1996). Thus, we decided to readdress this issue due to a more recently published study which suggested that PI3K activation is required for the activation of ERK1/2 in a related murine, hemopoietic cell line, FDC-P1 (Sutor et al., 1999). Sutor et al., suggested that this difference in response was due to their use of a different cell type.
3.2 Hypothesis and Objectives

We hypothesize that PI3K activation is not required for ERK1/2 activation. To test this hypothesis we undertook the following studies:

1. To observe the effects of PI3K inhibition (using PI3K inhibitors: LY294002 and wortmannin) on ERK1/2 activation using immunoblots and apoptotic studies.

2. Does the concentration of the cytokine in the presence of PI3K inhibition (using effective, constant concentration of LY294002) affect the activation of ERK1/2?

3.3 Results

3.3.1 Does ERK1/2 Activation Require the Activation of PI3K? Studies in the FDC-P1 Cell Line

To investigate the relationship between PI3K and ERK1/2, we decided to start by using FDC-P1 cells, which are a murine, hemopoietic cell line. The factor-starved FDC-P1 cells were treated with PI3K inhibitors, LY294002 and wortmannin at varying concentrations and the MEK-1/2 inhibitor, U0126 at varying concentrations for 10 minutes. The cells were then stimulated with either synthetic GM-CSF (10 µg/ml) or synthetic IL-3 (10 µg/ml) for 5 minutes. The cells were subsequently lysed and proteins were isolated for immunoblotting. This experiment was repeated several times and the results are similar to those as seen in the following immunoblots. It is important to note that the phosphorylation of Ser-473 in the PKB blot was used as an indirect measure of...
the level of PI3K activity. Accordingly, when PI3K activation was blocked by PI3K inhibitors there was no phosphorylation of Ser-473.

From the immunoblots (Fig. 3.1 and 3.2a) we observed that in both GM-CSF and IL-3 treated cells the activation of ERK1/2 was not blocked by LY294002 to any significant extent, except perhaps at the highest concentration (50 μM) which was much higher than that required for PI3K inhibition. Even the lowest concentration of LY294002 (10 μM) completely inhibited the phosphorylation of Ser-473 of PKB. Wortmannin partially blocked the activation of ERK1/2, even at 50 nM, and to a greater extent at higher concentrations. This result of wortmannin was also seen in a previous study by Sutor et al. (1999) and similar results to these were obtained in the previous study from our laboratory using MC-9 cells (Scheid and Duronio, 1996). The MEK inhibitor, U0126 completely inhibited the activation of ERK1/2, while not having any effect on PI3K activation, as determined by the phosphorylation of PKB. Interestingly, from our phosphorylated ERK1/2 blots we saw four bands which could have included degradation products, or novel isoforms of MAPKs that are yet to be identified. When the blots were stripped and reprobed with anti-ERK1/2 antibodies it was the upper two bands, that are indicated in the figures, which correspond to ERK1 and ERK2 (Figure 3.2b).

These results indicated that in FDC-P1 cells, wortmannin was behaving in a non-specific manner to block PI3K activation as well as ERK1/2 as seen in the previous results using MC-9 cells (Scheid and Duronio, 1996). However, since complete inhibition of PI3K activation with LY294002 had no affect on ERK1/2
activation, we can conclude that cytokine-dependent activation of ERK1/2 in FDC-P1 cells can occur completely independently of PI3K activation.

**Figure 3.1** FDC-P1 cells pretreated with LY294002 and wortmannin followed by stimulation with GM-CSF. FDC-P1 cells were starved of cytokine overnight. Cells were pretreated with PI3K inhibitors, LY294002 and wortmannin at the indicated concentrations for 10 minutes. Then the cells were stimulated with GM-CSF (10 μg/ml) for 5 minutes. The cell lysate proteins were resolved using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB, anti-phospho-ERK1/2. The levels of p85 were used for normalization.
Figure 3.2  FDC-P1 cells pretreated with LY294002 and wortmannin followed by stimulation with IL-3. FDC-P1 cells were starved of cytokine overnight. Cells were pretreated with PI3K inhibitors, LY294002 and wortmannin. Also MEK inhibitor, U0126 was used at the indicated concentrations for 10 minutes. Then the cells were stimulated with IL-3 (10 µg/ml) for 5 minutes. A. The cell lysate proteins were resolved using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB, anti-phospho-ERK1/2. The levels of p85 were used for normalization. B. The blot in panel A was stripped and reprobed with anti-ERK1/2.
The immunoblots from several independent experiments were quantified using laser densitometry. The data from the laser densitometry were analyzed in the following manner: the density of the background was subtracted from each band. This data was then normalized by dividing by the density from the p85 bands which also had the background subtracted. Then the positive control, which is the sample treated with only cytokine was set to 100% and the protein levels of the other samples were compared to the positive control. The standard deviation was calculated by using three independent repetitions of the experiment.

A

![Bar chart showing Phospho-ERK1/2 Protein Levels (%)](chart.png)
Figure 3.3 Quantification of FDC-P1 immunoblots by laser densitometry. A. Laser densitometry results of the FDC-P1 cells stimulated with GM-CSF in the presence of various inhibitors and probed for anti-phospho-ERK1/2. B. Laser densitometry results of the same blot in A, except probed with anti-phospho-PKB. C. Laser densitometry results of FDC-P1 cells stimulated with IL-3 in the presence of various inhibitors and probed for anti-phospho-ERK1/2. D. Laser densitometry of the same blot in C, expect probed with anti-phospho-PKB. Results are the average ± the standard deviation of three independent experiments. Note standard deviation bars were depicted on all columns of the graph; however, in some cases, there is little standard deviation and can not be seen on bars.

The bar graphs seen in Figure 3.3 clearly show that 10 μM LY294002 and 50 nM wortmannin completely block the phosphorylation of PKB (Ser 473) and thus, the activation of PI3K. Generally from these laser densitometry results it appears that when stimulating with GM-CSF and using LY294002 at 50 μM there was a reduction in phosphorylation of ERK1/2. However, since maximal inhibition of PI3K was achieved using 10 μM LY294002 and there was no affect on ERK1/2 at this concentration, thus, using 50 μM LY294002 may be eliciting non-specific inhibition. Furthermore, when stimulating these cells with IL-3 there did not seem to be a significant reduction in ERK1/2 phosphorylation when using LY294002 at any concentration. However, it is quite clear that the use of wortmannin reduces the phosphorylation of ERK1/2.
The results from the immunoblots suggested that ERK1/2 activation was not dependent on PI3K activation. In addition to analysis of the kinases, we compared the functional affects of blocking PI3K or ERK1/2 selectively. The classical function of PI3K is to inhibit apoptosis, while many studies have indicated that ERK1/2 activation is not required for inhibition of apoptosis; thus, we decided to treat cells with the inhibitors and determine their effects on the FDC-P1 cells in terms of apoptosis. Analysis was carried out by propidium iodide staining.

Propidium iodide stains DNA and the cells staining for subdiploid DNA indicates the number of cells that have undergone apoptosis. The FDC-P1 cells were treated with the inhibitors, LY294002 (50 μm) and U0126 (25 μm) in the presence of 5% WEHI-3 for 24 hours and then stained with propidium iodide. The experiment was repeated numerous times, each time with similar results. A representative sample is seen in Figure 3.4.
Figure 3.4  

FDC-P1 apoptosis studies. FDC-P1 cells were washed 3 times with 1X PBS and resuspended in RPMI prior to addition of LY294002 (50 μM), U0126 (25 μM) and 5% WEHI-3. DMSO served as a control for the inhibitor solvent. The cells were incubated for 24 hours at 37°C in humidified conditions in the presence of 5% CO₂. Cells were fixed in 70% ethanol overnight, stained with propidium iodide in the presence of RNAseA, and then analyzed on the flow cytometer (Beckman Coulter EPICS XL-MCL).
In the presence of LY294002 there was an increase in cell death, as expected. However, in the presence of U0126, there was not much significant death. These functional results indicate that while loss of PI3K activity has a major role in causing apoptosis, cells can survive in the absence of ERK1/2 activation. Furthermore, these functional results indicate that the promotion of cell survival signals by the PI3K pathway does not involve the ERK1/2 pathway.

3.3.2 Studies in the TF-1 Cell Line

We wanted to further investigate the relationship of PI3K activation and ERK1/2 activation in a human hemopoietic cell line, TF-1, which were derived from an erythroleukemic patient. The factor-starved TF-1 cells were treated with PI3K inhibitors, LY294002 and wortmannin at varying concentrations and the MEK inhibitor, U0126 at varying concentrations for 10 minutes. The cells were then stimulated with GM-CSF (10% of conditioned medium containing human GM-CSF, cGMI) for 5 minutes. The cells were then lysed and proteins were isolated for immunoblotting. This experiment was repeated several times. The immunoblots (Fig. 3.5 and 3.6) show that neither LY294002 nor wortmannin significantly block the phosphorylation of ERK1/2. Furthermore, the increasing concentrations of the inhibitors showed little or no difference in the phosphorylation of ERK1/2. For instance, LY294002 used at a concentration of 2.5 μm and 50 μm showed similar levels of ERK1/2 phosphorylation. Also, as seen previously in the FDC-P1 results, both LY294002 and wortmannin blocked the activation of PI3K and U0126 completely blocks the activation of ERK1/2, without affecting the phosphorylation of Ser 473 of PKB.
**Figure 3.5** TF-1 cells pretreated with various concentrations of LY294002 followed by stimulation with cGMI. TF-1 cells were starved of cytokine overnight. Cells were pretreated with LY294002 and also MEK inhibitor, U0126 was used at the indicated concentrations for 10 minutes. Then the cells were stimulated with cGMI (10%) for 5 minutes. The cell lysate proteins were resolved using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB, anti-phospho-ERK1/2. The levels of p85 were used for normalization.
Figure 3.6 TF-1 cells pretreated with various concentrations of wortmannin followed by stimulation with cGMI. TF-1 cells were starved of cytokine overnight. Cells were pretreated with wortmannin at the indicated concentrations for 10 minutes. Then the cells were stimulated with cGMI (10%) for 5 minutes. The cell lysate proteins were resolved using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB and anti-phospho-ERK1/2. The levels of p85 were used for normalization.
For TF-1 stimulations, we have been using conditioned GM-CSF media which may also contain other factors which may not be a true representation of the effects of GM-CSF alone. Thus, we repeated the experiments by stimulating with 50 ng/ml of recombinant human GM-CSF (rhGM-CSF). As seen in Figure 3.7, the results were similar to those seen previously. However, the only difference is that a higher concentration of LY294002 was required to fully inhibit PI3K. In this case, LY294002 had to be 25 μM or greater to completely block PI3K. There was no significant reduction in the phosphorylation of ERK1/2 in the presence of complete PI3K blockage. Again, the ERK1/2 pathway does not appear to be dependent on the PI3K pathway.

The functional responses of the cells, in the presence of inhibitors, were also investigated using propidium iodide staining for apoptosis studies. The TF-1 cells were treated with the inhibitors, LY294002 (50 μM) and U0126 (25 μM) in the presence of 1% GM-CSF for 24 hours and then stained with propidium iodide. The experiment was repeated numerous times each time with similar results. A representative sample is seen in Figure 3.8.
Figure 3.7 TF-1 cells pretreated with various concentrations of LY294002 followed by stimulation with rhGM-CSF. TF-1 cells were starved of cytokine overnight. Cells were pretreated with LY294002 and also MEK inhibitor, U0126 was used at the indicated concentrations for 10 minutes. Then the cells were stimulated with rhGM-CSF (50 ng/ml) for 5 minutes. The cell lysates were subjected to electrophoresis using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB and anti-phospho-ERK1/2. The levels of p85 were used for normalization.
Figure 3.8  TF-1 apoptosis analysis. TF-1 cells were washed 3 times with 1X PBS and resuspended in RPMI prior to addition of LY294002 (50 μm), U0126 (25 μm) and 1% GM-CSF. DMSO served as a control for the inhibitor solvent. The cells were incubated for 24 hours at 37°C in humidified conditions in the presence of 5% CO₂. Cells were fixed in 70% ethanol overnight, stained with propidium iodide in the presence of RNAseA and then analyzed on the flow cytometer (Beckman Coulter EPICS XL-MCL).
The results were similar to those seen in FDC-P1s. More specifically, in the presence of LY294002 there was an increase in apoptosis as seen by the increase in subdiploid DNA; yet, in the presence of U0126, there was no significant increase in apoptosis. When both LY294002 and U0126 were used together we observed a synergistic action.

### 3.3.3 Studies In The BAF-3 Cell Line

We decided to use another cell line, BAF-3 cells, which is also a murine, cytokine-dependent, hemopoietic cell line, because previous studies using these cells indicated that ERK1/2 activation was dependent on PI3K when dominant negative mutants of PI3K were used (Craddock et al., 2001). Factor-starved BAF-3 cells were treated with PI3K inhibitors, LY294002 and wortmannin at various concentrations and the MEK inhibitor, U0126 at various concentrations for 10 minutes. The cells were then stimulated with synthetic IL-3 (10 μg/ml) for 5 minutes. The cells were then lysed and proteins were isolated for immunoblotting. This experiment was repeated several times and the results are similar to those as seen in the Figure 3.9.
IL-3

Figure 3.9  BAF-3 cells pretreated with LY294002 and wortmannin followed by stimulation with IL-3. BAF-3 cells were starved of cytokine overnight. Cells were pretreated with PI3K inhibitors, LY 294002 and wortmannin. Also MEK inhibitor, U0126 was used at the indicated concentrations for 10 minutes. Then the cells were stimulated with IL-3 (10 μg/ml) for 5 minutes. The cell lysates were subjected to electrophoresis using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB and anti-phospho-ERK1/2. Ponceau S staining was used to ensure equal loading between lanes.

Again, the results were similar as in the other cell lines; however, lower concentrations of inhibitors were used, because in this cell line lower concentrations of LY294002 and wortmannin were needed to inhibit PI3K-
dependent phosphorylation of Ser 473 in PKB. Both PI3K and wortmannin did not block the phosphorylation of ERK1/2. This demonstrates once again that ERK1/2 activation does not require PI3K activation.

Apoptosis studies were also done using BAF-3 cells. The BAF-3 cells were treated with the inhibitors, LY294002 (50 µm) and U0126 (25 µm) in the presence of 5% WEHI-3 for 16 hours and then stained with propidium iodide. The experiment was repeated numerous times each time with similar results. A representative sample is seen in Figure 3.10.
Figure 3.1  BAF-3 apoptosis analysis. BAF-3 cells were washed 3 times with 1X PBS and resuspended in RPMI prior to addition of LY294002 (50 μm), U0126 (25 μm) and 5% WEHI-3. DMSO served as a control for the inhibitor solvent. The cells were incubated for 16 hours at 37°C in humidified conditions in the presence of 5% CO₂. Cells were fixed in 70% ethanol overnight, stained with propidium iodide in the presence of RNAseA, and then analyzed on the flow cytometer (Beckman Coulter EPICS XL-MCL).
The results are similar to those seen in TF-1s. In the presence of LY294002 there was an increase in death, but this was not observed in the presence of U0126. Furthermore, when both LY294002 and U0126 were used together we observed a synergistic action.

Figure 3.11 is a bar-graph depicting a summary of all the cell types that were tested for apoptosis. In the cases of FDC-P1 and BAF-3 cells, complete starvation led to the most number of apoptotic cells (55% and 80% apoptotic cells, respectively), while TF-1 have more resistant to starvation (10%). In all three cell types, the use of LY294002 definitely increased the number of apoptotic cells as compared to the use of U0126, which seemed to be quite similar to the negative-control sample (stimulated only). The use of both inhibitors LY294002 and U0126 seemed to behave synergistically in TF-1 and BAF-3 cells.

Figure 3.11  Summary of apoptosis studies of the three cell types. Series 1 refers to experiments using FDC-P1 cells, series 2 refers to experiments using TF-1 cells and series 3 refers to experiments using BAF-3 cells.
3.3.4 Studies in the HEK 293 Cell Line

We wanted to examine a non-hemopoietic cell type to observe the relationship between PI3K and ERK1/2. We decided to use a human, epithelial, kidney cell line and test its response to growth factors in serum. The serum-starved HEK 293 cells were treated with PI3K inhibitors, LY294002 and wortmannin at various concentrations and the MEK inhibitor, U0126 at various concentrations for 10 minutes. The cells were then stimulated with FBS (10%) for 10 minutes. The cells were then lysed and proteins were isolated for immunoblotting. HEK 293 cells differed from the previous cell lines used as they had low levels of endogenous activated ERK1/2 in the unstimulated lane (Figure 3.12).

Surprisingly, levels of LY294002 which completely blocked the phosphorylation of PKB (25μM) partially blocked the activation of ERK1/2; whereas, wortmannin had no effect on the activation of ERK1/2 even at concentrations greater than those required to block the phosphorylation of PKB. However, as seen in the previous cell lines; U0126 completely blocked the activation of ERK1/2, without having any affect on the phosphorylation of PKB. These results indicate that LY294002 may have some non-specific effects in this particular cell line that are distinct from the action of wortmannin.
Figure 3.12  HEK 293 cells pretreated with LY294002 and wortmannin followed by stimulation with FBS. HEK 293 cells were starved of FBS overnight. Cells were pretreated with PI3K inhibitors, LY294002 and wortmannin. Also the MEK inhibitor, U0126 was used at the indicated concentrations for 10 minutes. Then the cells were stimulated with 10% FBS for 10 minutes. The cell lysates were subjected to electrophoresis using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB and anti-phospho-ERK1/2. The levels of p85 were used for normalization.
3.3.5 Does the Concentration of Cytokine Affect the Activation of ERK1/2 in the Presence of LY294002?

We determined from our previous results, that in the presence of a specific, constant amount of cytokine the activation of ERK1/2 is not affected when PI3K is inhibited... Thus, we wanted to observe the effects of using various concentrations of the cytokinein the presence of a constant, effective concentration of LY294002 and determine whether the phosphorylation of ERK1/2 is affected. We decided to use the human TF-1 cells for this experiment. The factor-starved TF-1 cells were treated with the PI3K inhibitor, LY294002 for 10 minutes (10 µM of LY294002 was used, because this concentration has been shown to be sufficient to completely inhibit the activation of PI3K). The cells were then stimulated with varying concentrations of GM-CSF (0.5%-10%) for 5 minutes. The cells were lysed and proteins isolated for immunoblotting. This experiment was repeated several times. From the representative immunoblot seen in figure 3.13, we observed that complete inhibition of PI3K had no effect on ERK1/2 activation, regardless of the concentration of cytokines used to stimulate the cells. Even at low amounts of cytokine, such as 0.5% GM-CSF, we saw that ERK1/2 was almost maximally phosphorylated, even when PI3K was completely inhibited. In all the lanes there appeared to be similar levels of ERK1/2 phosphorylation, with perhaps a slight increase in ERK1/2 phosphorylation when using concentrations of GM-CSF at greater than 8%. In the phospho-PKB blot, we observed that maximal phosphorylation required 4% of GM-CSF. However, in the presence of 10 µm LY294002, activation of PI3K and phosphorylation of
PKB was completely blocked. These observations indicated that the varying concentrations of cytokine, in the presence of complete PI3K inhibition, did not effect ERK1/2 activation, and provide further supporting for the hypothesis that ERK1/2 activation can be independent of PI3K activation.

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<th>cGMI</th>
<th>0.5%</th>
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Figure 3.13  TF-1 cells pretreated with a constant concentration of LY294002 followed by stimulation with various concentrations of cGMI. TF-1 cells were starved of cytokine overnight. Cells were pretreated with 10 μM LY294002 for 10 minutes. Then the cells were stimulated with various concentrations of cGMI for 5 minutes. The cell lysates were subjected to electrophoresis using 9% SDS-PAGE and immunoblotted for anti-phospho-PKB and anti-phospho-ERK1/2. The levels of p85 were used for normalization.
3.4 Discussion

The postulation that PI3K activation is required for ERK1/2 activation has been quite controversial over the past several years. As mentioned previously, studies were conducted that indicated that ERK1/2 activation required PI3K activation (Standaert et al., 1995; von Willebrand et al., 1996; Grammer and Blenis, 1997; Bondeva et al., 1998; Sutor et al., 1999) while others suggested that PI3K activation is not required for ERK1/2 activation (Cheatham et al., 1994; Scheid and Duronio, 1996; Frevert and Kahn, 1997). Of particular interest to our lab, was the study conducted by Sutor et al. (1999). In this study, they used PI3K dominant-negative mutants and wortmannin. They suggested that in FDC-P1 cells, PI3K differentially, activates Raf isoforms to activate the ERK1/2 pathway. However, these results differ from the results seen previously in a study conducted by Scheid and Duronio (1996). In this study using MC-9 cells, it was shown that LY294002 completely blocked the activation of PI3K, yet had no effect on ERK1/2 activation. Sutor et al. (1999) suggested that these differences may be due to the use of different cell types. However, Sutor et al., (1999) failed to use LY294002, the more selective PI3K inhibitor to confirm their results. Hence, in this study we used four different cell lines, including FDC-P1s, to compare the effects of wortmannin and LY294002 on the activation of the ERK1/2. We probed for the phosphorylation of PKB (Ser-473) which indicates the activity of PI3K. It is well accepted that the phosphorylation of PKB is indicative of PI3K activation (Downward 1998; Alessi and Cohen 1998).
Furthermore, we probed for the phosphorylation of ERK1/2 (Thr-202/Tyr-294) which is indicative of its activation state (Haystead et al., 1992).

Two important pharmacological inhibitors of PI3K have been widely used to study the relationship of activated PI3K to that of ERK1/2 activation. These inhibitors are LY294002 and wortmannin. Wortmannin is a fungal metabolite and an irreversible inhibitor of PI3K. It has been shown in previous studies not to be a specific inhibitor of PI3K (Cross et al., 1995; Standaert et al., 1995) unless used at low nM concentrations. However, LY294002, which is not structurally related to wortmannin, blocks the ATP-binding site of PI3K, and is shown to be much more specific than wortmannin (Vlahos et al., 1994).

Furthermore, dominant-negative mutants of the PI3K's p85 regulatory subunit have also been used to help define the relationship between PI3K and ERK1/2 activation (Sutor et al., 1999; Craddock et al., 1999). The dominant-negative mutants have been constructed via the deletion of the inter-SH2 of the p85 regulatory subunit which is necessary for binding to the catalytic p110 subunit of PI3K (Hara et al., 1994). Thus, this deletion renders the p85 subunit inactive, although the SH2 domains of p85 can still compete for phosphotyrosine binding sites and block endogenous enzymes.

Our experiments clearly show that in FDC-P1 cells, LY294002 completely blocks the activation of PI3K, yet does not block the activation of ERK1/2, indicating the lack of dependency. This result differs from Sutor et al. (1999) who used dominant-negative mutants of PI3K, and wortmannin. When we used wortmannin, we also observed some decrease in the phosphorylation of ERK1/2.
However, this is likely due to non-specific effects of wortmannin, such as blocking kinases that phosphorylate ERK1/2. It is important to mention that in studies where dominant-negative mutants of PI3K are used to show that ERK1/2 activation is dependent on PI3K activation, the results could also be due to non-selective effects. As mentioned above, the PI3K mutant is manufactured via the deletion of the inter-SH2 domains on the p85 subunit. Thus, there are more functional SH2 domains present in the cells than normally. These SH2 domains may still function as docking sites for phosphorylated tyrosine molecules such as those used by other signalling molecules in the Ras/Raf/MAPK pathway. This increasing amount of functional SH2 domains in the cells may elicit unwanted responses via triggering or inhibiting other signalling pathways which may explain why in studies using dominant-negative mutants there seems to be a reduction in ERK1/2 activation. In accordance with our speculation, a study conducted by Craddock et al. (1999) demonstrated that when dominant-negative PI3K mutants were used (same as used by Sutor et al., 1999) they observed an increase in tyrosine phosphorylation of additional proteins in both total cell lysates and complexed with the mutant p85 subunit (Craddock et al., 1999).

A study by Duckworth and Cantley (1997) indicated that differential effects on ERK1/2 activation may be observed with the PI3K inhibitor, wortmannin depending on the concentration of the cytokine used. In this study, they demonstrated that wortmannin blocks the activation of ERK proteins if Swiss 3T3 cells were stimulated with lower levels of platelet-derived growth factor (Duckworth and Cantley, 1997). However, again in this study, they failed to
compare the affects of LY294002. We investigated this issue by stimulating TF-1 cells with various concentrations of GM-CSF in the presence of complete PI3K inhibition, using LY294002. At all concentrations of GM-CSF used and complete PI3K blockage, there was no inhibition of ERK1/2 activation.

It is important to mention that in our study there appeared to be a difference in the specificity of the PI3K inhibitors in the types of cells used. In the FDC-P1 cells wortmannin blocked the phosphorylation of ERK1/2, but LY294002 did not. However, in HEK 293 cells, the reverse was true. In other words, LY294002 had some affect in blocking the activation of ERK1/2 while wortmannin did not. These results indicate that both wortmannin and LY294002 may be behaving in a non-specific manner in different cell types and effect upstream components leading to ERK1/2 activation. However, the main conclusion that can be drawn in all these cases is that when PI3K activation can be completely blocked by any one of the inhibitors, the lack of affect on the activation of ERK1/2 proves that PI3K is not required.

The HEK 293 results are also similar to some of the results observed by Scheid et al. (1996). In this study MC-9 cells were also treated with phorbol 12-myristate 13-acetate (PMA) for stimulation of ERK1/2. When the cells were treated with either LY294002 or wortmannin it was shown that LY294002 partially blocked the phosphorylation of ERK1/2, while wortmannin had no effect (Scheid et al., 1996). Thus, it is possible to suggest that the target of LY294002’s effect on ERK1/2 activation may be a common element in the pathway activated by PMA and by serum stimulation.
One of the main functions of PI3K is the prevention of apoptosis. Apoptosis is programmed cell death and differs from necrosis which is a nonspecific mode of cell death and is characterized by swelling of the cell and mitochondria, which disrupts the cell membrane and ultimately causing cell lysis (Bertho et al., 2000). Apoptosis is characterized by chromatin condensation, cell shrinkage, membrane blebbing and formation of apoptotic bodies (Darzynkiewicz et al., 1992). Furthermore, the gold standard for determining apoptosis is DNA fragmentation (Darzynkiewicz et al., 1997) which is not present in necrotic cells.

From the previous experiments using immunoblot analyses, it can be clearly concluded that PI3K activation is not required for ERK1/2 activation. However, we wanted to further investigate the potential functional responses of PI3K and ERK1/2. The well-known functional response of PI3K is its regulation of cell survival (Kuwahara et al., 2000; Krasilnikov, 2000; Hutchinson et al., 2001). Thus, in our study we conducted DNA fragmentation analysis using propidium iodide. This dye binds to DNA, and using the flow cytometer we are able to deduce the number of cells having subdiploid DNA, which corresponds to the number of apoptotic cells. We observed an increase in apoptosis when PI3K inhibitor, LY294002 was used. However, the complete inhibition of ERK1/2 with the use of U0126 had little effect on apoptosis. The results clearly showed that the inhibition of PI3K causes apoptosis, while the inhibition of ERK1/2 still allowed the cell to survive. Therefore, the results provided evidence that the functional role of the PI3K pathway (cell survival) is distinct from the functional role of ERK1/2 pathway. In other words, these functional results prove that
promotion of cell survival signals by the PI3K pathway does not have to involve the ERK1/2 pathway.

When both inhibitors LY294002 and U0126 were used, there was a significant increase in apoptotic cells in TF-1 and BAF-3 cells, as compared to addition of LY294002 alone. This synergistic effect indicates that perhaps the U0126, which inhibits ERK1/2 activation, may enhance apoptosis in the presence of PI3K inhibition. It is generally accepted that ERK1/2 is involved in cell growth. As mentioned in the introduction, ERK1/2 is involved in the cell progression through the G1 phase; thus, the inhibition of growth may condition the cells to be more susceptible to apoptosis when PI3K is inhibited. However, since this synergistic effect is only observed in two cell types, TF-1 and BAF-3 and not FDC-P1; this indicates that inhibitors may elicit slightly different responses in different cell types. Furthermore, there is a strong possibility that different cell types may possess different signaling proteins or slightly modified pathways.

3.5 Conclusions

The conclusions that can be drawn from the results in this chapter are the following. Due to lack of selectivity, wortmannin blocks the activation of ERK1/2 in FDC-P1 cells, while LY294002 does not. In TF-1 and BAF-3 cells both wortmannin and LY294002 do not block the activation of ERK1/2. In HEK 293 cells, wortmannin does not block the activation of ERK1/2. However, LY294002 seems to slightly block the activation of ERK1/2. Also, the inhibitors LY294002 and wortmannin may elicit different responses in different cells and these responses may be due to nonspecific mechanisms. The overall conclusion is
that when either inhibitor LY294002 or wortmannin completely blocked the activation of PI3K there was no significant blockage of ERK1/2 activation which proves that ERK1/2 activation does not require PI3K activation.
Chapter 4: PI3K Regulation of Protein Synthesis

4.1 Rationale

A role for PI3K in protein translation has been suggested, but has not been clearly established. There have been previous studies which alluded to the idea that PI3K may play a significant role in the regulation of protein synthesis. It was demonstrated that the PI3K pathway via PDK1 phosphorylates and activates p70 S6 kinase. This kinase was shown to phosphorylate the S6 subunit of the 40S ribosome and thereby enhancing protein translation (Jefferies et al., 1994). It has been suggested that the PI3K pathway may be involved in the phosphorylation of 4E-BPs and thus, their deactivation and release from eIF-4E and allowing translation of the mRNA to occur (Gingras et al., 1999c). Furthermore, our laboratory recently demonstrated that Mcl-1, the pro-survival Bcl-2 family member is regulated by both PI3K and MAPK pathways. PI3K inhibitors, LY294002 and wortmannin inhibited the translation of the Mcl-1 mRNA, while the MEK-1/2 inhibitor U0126, inhibited the transcription of the Mcl-1 mRNA (Schubert and Duronio, 2001). Therefore, we were interested in assessing the role of PI3K in regulating proteins involved in protein translation in our cell systems.

4.2 Hypothesis and Objectives

Our preliminary hypothesis was that PI3K is involved in protein translation. It is important to mention that these types of studies, investigating the components of the protein translation machinery, have not been previously
done in our laboratory. We were interested in establishing protocols for future studies regarding protein translation.

To test the hypothesis we undertook the following studies:

1. To observe if our cell systems have the important components of the translational protein machinery, eIF-4E and 4E-BPs and, if so, do we observe a difference between stimulated and unstimulated cells?

2. What are the effects of the inhibitors LY294002, wortmannin, U0126 and rapamycin on the phosphorylation of 4E-BP1 and 4E-BP2?

4.3 Results

4.3.1 Presence of 4E-BPs and eIF-4E in the FDC-P1 Cell Line

We wanted to identify which 4E-BPs were present in our cell systems and to determine if it was necessary to heat-enrich our protein lysate for cleaner blots as has been done previously (Gingras et al., 1999c). The first experiment was done using FDC-P1 cells. The cells were unstarved and were washed three times with 1X PBS. Some samples were stimulated with 10 μg/ml of synthetic IL-3 for 5 minutes while others were not stimulated. Some samples involved heat-treatments while others did not. The lysates were subjected to electrophoresis on a 15% SDS PAGE and the blots were probed with anti-4E-BP1 and anti-4E-BP2 antibodies (gifts from Dr. N. Sonenberg; Figure 4.1).
Figure 4.1 In FDC-P1 cells 4E-BP2 is detected, but 4E-BP1 is not. FDC-P1 cells were either stimulated with 10 μg/ml of IL-3 for 5 minutes or left unstimulated. The cell lysates (both heat-enriched and non heat-enriched) were fractionated using 15% SDS-PAGE and immunoblotted for anti-4E-BP2 and anti-4E-BP1. Only the immunoblot probed with anti-4E-BP2 is shown in the above figure. The anti-4E-BP1 immunoblot was blank.
From this experiment we observed that the heat-treated protein lysates produced cleaner blots as compared to lysates that were not heat-treated. We were unable to visualize any 4E-BP1, but we were able to detect 4E-BP2. It is important to note that this 4E-BP2 antibody is known to cross-react with both 4E-BP1 and 4E-BP3, thus, causing the diffuse band. According to Dr. Gingras (personal communication), 4E-BP2 is ~18 kDa, whereas, 4E-BP1 is ~20-21 kDa. Furthermore, it appeared that in stimulated samples, the bands were slightly darker and had slightly slower molecules. This upward band shift could indicate phosphorylation of the 4E-BP2 protein after stimulation.

We then wanted to determine whether eIF-4E was present in FDC-P1 cells. We used nonstarved cells and washed three times with 1X PBS. Some samples were stimulated with 10 μg/ml of synthetic IL-3 for 5 minutes while others were not stimulated. A pull down assay was performed using 750 μg of total protein lysate. The lysate was incubated in the m7GDP Sepharose column, which should affinity purify eIF-4E. This pull down assay was performed in order to concentrate the amount of eIF-4E, because this protein is in such low abundance in the cells. eIF-4E normally binds to the m7GTP cap of mRNA, thus, this procedure allowed us to mimic this interaction and isolate eIF-4E. The eluant from the m7GDP Sepharose column was electrophoresed on a 15% SDS PAGE and probed with anti-eIF-4E antibody (Figure 4.2).
Figure 4.2  FDC-P1 cells contain eIF-4E. FDC-P1 cells were either stimulated with 10 μg/ml of IL-3 for 5 minutes or left unstimulated. The whole cell lysates were purified by running through a mGDP Sepharose column. The eluants from the column were subjected to electrophoresis using 15% SDS-PAGE and immunoblotted for anti-eIF-4E.
Our results showed that eIF-4E (25 kDa) is present in FDC-P1. However, a difference in mobility between unstimulated and stimulated cells was not observed.

4.3.2 Presence of 4E-BPs and eIF-4E in the MC-9 Cell Line

We next wanted to determine if 4E-BPs and eIF-4E are present in the murine, mast cell line, MC-9. In this experiment we starved the cells overnight in 1% WEHI-conditioned medium because we wanted to observe if there is a more significant difference between the stimulated and unstimulated samples. The cells were washed 3 times with 1X PBS prior to stimulation. The samples were stimulated with 10 μg/ml of synthetic IL-4 or 10 μg/ml of synthetic IL-3 or 20 nM PMA for 5 minutes. IL-4 was used because it is known to only activate the PI3K pathway and not the ERK1/2 pathway. We wanted to compare this to IL-3 which activates both the PI3K and ERK1/2 pathways (Welham et al., 1994; Scheid and Duronio, 1998). PMA is known to activate PKCs, thus, we were interested in the role that PKCs may play in regulating 4E-BPs and eIF-4E. The cells were lysed and heat-enriched. The lysates were electrophoresed on a 15% low-bisacrylamide gel (ratio of acrylamide:bis was 118:1) and transferred to nitrocellulose blots. Low-bis acrylamide gels allows one to obtain a greater separation between phosphorylated and non-phosphorylated proteins. The blots were probed with either anti-4E-BP1 or anti-4E-BP2 antibody (Figure 4.3).
Figure 4.3 In MC-9 cells 4E-BP2 is detected, but 4E-BP1 is not. The MC-9 cells were starved of cytokine overnight. **A.** The cells were either stimulated with 10 μg/mL of IL-4 or 20 nM of PMA for 5 minutes. The cell lysates were heat-enriched and electrophoresed on a 15% low-bis gel and blotted for anti-4E-BP2 and anti-4E-BP1. The anti-4E-BP1 blot is not shown in the above figure. **B.** The cells were stimulated with 10 μg/mL of IL-4, 10 μg/mL of IL-3 or 20 nM of PMA for 5 minutes. The cell lysates were heat-enriched and electrophoresed on a 15% low-bis gel and blotted for anti-4E-BP2.
The results showed that again, 4E-BP1 was not detected and 4E-BP2 was present. The results indicated that upon stimulation with IL-4 or IL-3 we observed an increase in phosphorylation of 4E-BP2 as seen by the band shift. This band shift was also observed in the sample treated with PMA; however, the band shift was not as prevalent as seen in the samples treated with IL-4 and IL-3.

For determining if MC-9 cells contain eIF-4E, a similar experiment was performed as stated previously using FDC-P1 cells. A 15% low-bisacrylamide gel was used to resolve the lysate proteins after purification using the m\(^7\)GDP Sepharose column. The blot was probed with anti-eIF-4E antibody (Figure 4.4). The results clearly indicate that MC-9 cells contain eIF-4E; however, there was no band shift observed in the treated samples.

**Figure 4.4** **MC-9 cells contain eIF-4E.** MC-9 cells were starved of cytokine overnight. The cells were then either stimulated with 10 μg/ml of IL-4 for 5 minutes or 20 nM PMA. The whole cell lysates were purified by following a m\(^7\)GDP Sepharose column chromatography. The eluants from the column were electrophoresed using 15% low-bis gel and immunoblotted for anti-eIF-4E.
4.3.3 Presence of 4E-BPs and eIF-4E in the TF-1 Cell Line

We next wanted to determine if 4E-BPs and eIF-4E are present in the human cell line, TF-1. In this experiment we starved the cells overnight and washed the cells three times with 1X PBS prior to the stimulations. The procedure is similar to that used in the MC-9 cells, as stated previously, except the cells were stimulated with 10% GM-CSF-containing medium (cGMI) for 5 minutes (Figure 4.5).

Interestingly, unlike the other two cell lines used previously, we were able to detect 4E-BP1. Furthermore, a clear upward bandshift was seen in 4E-BP1 when comparing the stimulated and unstimulated samples. These results indicate that upon stimulation, the 4E-BP1 protein is phosphorylated. Also, there appeared to be three bands present. These bands corresponded to the α (fastest-migrating and darkest band), β (middle band) and γ (slowest-migrating and lightest band) forms of 4E-BP1. Also, 4E-BP2 was present in this cell line. However, no bandshifts were easily observed between the unstimulated and stimulated cells.

To determine the presence of eIF-4E in TF-1 cells we conducted an experiment similar to that used previously, for FDC-P1 cells. A 15% low-bisacrylamide gel was used to resolve the lysate proteins after purification using the m⁷GDP Sepharose column. The blot was probed with anti-eIF-4E antibody (Figure 4.6). The results clearly indicate that TF-1 cells contain eIF-4E. However, bandshifting was not observed between the stimulated and unstimulated samples.
Figure 4.5  TF-1 cells contain both 4E-BP1 and 4E-BP2. The TF-1 cells were starved of cytokine overnight. The cells were either stimulated with 10% GM-CSF for 5 minutes or not stimulated. The cell lysates were heat-enriched and electrophoresed on a 15% low-bis gel and blotted for A. anti-4E-BP1 and B. anti-4E-BP2.
TF-1 cells contain eIF-4E. TF-1 cells were starved of cytokine overnight. The cells were either stimulated with 10% GM-CSF for 5 minutes or not stimulated. The whole cell lysates were purified by chromatography through a mGDP sepharose column. The eluants from the column were electrophoresed using 15% low-bis gel and immunoblotted for anti-eIF-4E.
4.3.4 What Are the Effects of the Inhibitors (LY294002, wortmannin, U0126 and rapamycin) On the Phosphorylation of 4E-BP1 and 4E-BP2?

To determine the effects of the inhibitors on 4E-BPs we chose to use TF-1 cells, because we previously determined that both 4E-BPs are present in this cell system. The cells were cytokine-starved overnight and prior to treatment the cells were washed three times with 1X PBS. The cells were then treated with PI3K inhibitors, LY294002, wortmannin at various concentrations, MEK inhibitor, U0126 at various concentrations and the FRAP/mTOR inhibitor, rapamycin at various concentrations for 10 minutes. The cells were then stimulated with either 10% GM-CSF or 100 ng/ml of hIL-3 for 5 minutes. The cell lysates were heat-enriched and electrophoresed on 15% low-bisacrylamide gels and probed for either 4E-BP1 or 4E-BP2 (Figure 4.7).

In the samples stimulated with GM-CSF, rapamycin inhibited the phosphorylation of 4E-BP1. Also, LY294002 inhibited the phosphorylation of 4E-BP1, yet interestingly, wortmannin did not. Surprisingly, in the unstimulated sample there was an upward bandshift indicating the phosphorylation of 4E-BP1. Similar results were observed for the 4E-BP2 blot, however, these results were inconclusive due to the unspecificity of the 4E-BP2 antibody. The bandshift we saw may be due to the bandshifting of the 4E-BP1 protein. In Figure 4.8, where the samples are treated with hIL-3, the results were similar to the results as seen in Figure 4.7. It is important to note that LY294002 seemed to block the phosphorylation of 4E-BP1 more efficiently than rapamycin.
Figure 4.7 TF-1 cells pretreated with LY294002, wortmannin, U0126 and rapamycin followed by stimulation with 10% GM-CSF. The TF-1 cells were starved of cytokine overnight. The cells were pretreated with the indicated inhibitors at various concentrations for 10 minutes. Then the cells were stimulated with 10% GM-CSF for 5 minutes. The cell lysates were heat-enriched and electrophoresed on a 15% low-bis gel and blotted for A. anti-4E-BP1 and B. anti-4E-BP2.
Figure 4.8 TF-1 cells pretreated with LY294002, wortmannin, U0126 and rapamycin followed by stimulation with 100 ng/ml human IL-3. The TF-1 cells were starved of cytokine overnight. The cells were pretreated with the indicated inhibitors at various concentrations for 10 minutes. Then the cells were stimulated with 100 ng/ml of human IL-3 for 5 minutes. The cell lysates were heat-enriched and electrophoresed on a 15% low-bis gel and blotted for anti-4E-BP1.
We stripped the previous blot (Figure 4.8) and probed for phospho-4E-BP1 (Ser65). 4E-BP1 is phosphorylated at multiple residues. However, Ser 65 is considered to be a more important residue since it is involved in eIF-4E release (Figure 4.9). The superposition of the two blots showed that the top bands corresponded to the phosphorylated forms of 4E-BP1. These results confirmed the above results seen when using the non-phosphorylated form of the 4E-BP1 antibody. Furthermore, this blot clearly showed that LY294002 blocked the phosphorylation of 4E-BP1 more efficiently than rapamycin. And that in unstimulated cells there was a basal level of phosphorylation of 4E-BP1.

![Human IL-3](image)

Figure 4.9 Immunoblot shown in Figure 4.8 was stripped and reprobed with anti-phospho-4E-BP1(Ser 65).
To address the issue of endogenous basal level of phosphorylation of 4E-BP1 in TF-1 cells, we speculated that perhaps the 10% FBS in the starvation media may elicit protein translation responses. Therefore, we reduced the FBS to 0.5% in the cytokine-starvation medium. We stimulated the samples with 10% GM-CSF and we subjected the cell lysates to 15% SDS PAGE and probed for phospho-4E-BP1 (Ser 65) (Figure 4.10). Again, we observed that the unstimulated samples contained phosphorylated 4E-BP1. However, there seemed to be a slight reduction in phosphorylation as compared to the stimulated samples where there appears to be more phosphorylation. Furthermore, two different lysis procedures were used: detergent-method and freeze-thaw cycles. From the results it seemed that both methods worked effectively. However, the detergent method seemed to be slightly better.

From our previous low-bisacrylamide eIF-4E blots, we were unable to observe any differences in bandshifts between stimulated and unstimulated samples. Therefore, we tested an antibody raised against phosphorylated Ser 209 of eIF-4E, which is considered to be the main residue phosphorylated on eIF-4E. We stripped the blot used in Figure 10 and reprobed with the phospho-eIF-4E antibody (Figure 4.11). There were faint bands present in the stimulated samples that were not present in the unstimulated samples. These faint bands corresponded to ~25 kDa, which is the molecular mass of eIF-4E. Thus, it appeared that eIF-4E is phosphorylated upon stimulation of the cell.
Figure 4.10  TF-1 cells were cytokine starved and serum starved (0.5% FBS instead of the normal 10%) overnight. The cells were either stimulated with 10% GM-CSF for 5 minutes or not stimulated. The cells were lysed using either detergent containing lysis buffer or non-detergent containing lysis buffer, thus cells were lysed using three freeze-thaw cycles. The cell lysates were subjected to on 15% SDS-PAGE and immunoblotted for anti-phospho-4E-BP1(Ser 65).

Figure 4.11  Immunoblot shown in Figure 4.10 was stripped and reprobed with anti-phospho-eIF-4E (Ser 209).
4.4 Discussion

Proteins play an integral part in cell function from structural support, such as cytoskeletal proteins, to enzymes which are required for cell metabolism and regulation. If any of these proteins are either under-expressed or over-expressed the normal functioning of the cell would be greatly compromised. Therefore, the regulation of protein synthesis has always been of immense interest, particularly the translation of proteins which may play a role in cancer development. Thus, identifying the key players involved in protein synthesis regulation is very important.

As mentioned previously, PI3K is involved in the regulation of protein synthesis. It has been established that PI3K via PDK-1, phosphorylates p70 S6 kinase which in turn phosphorylates the S6 protein of the 40 ribosomal subunit. This phosphorylated ribosomal complex specifically, promotes the translation of a subset of mRNA (Vinals et al., 1999). This subset of mRNA are characterized by an oligopyrimidine tract at their 5'-untranslated region. These mRNAs encode for components of the translational apparatus, such as ribosomal proteins and translational elongation factors which are important for cell growth and proliferation (Dufner and Thomas, 1999). Furthermore, PI3K has been implicated in the phosphorylation of 4E-BPs, and their inactivation by phosphorylation permits the initiation of translation (Gingras et al., 1999c). The direct upstream kinase that phosphorylates both p70 S6 kinase and 4E-BPs appear to be FRAP/mTOR (Peterson et al., 2000).
From our results we observe that 4E-BPs and eIF-4E are present in our cell systems. However, it is not clear whether 4E-BP1 is present in our murine cells, MC-9s and FDC-P1s. We tried to change the parameters in order to detect 4E-BP1 in our murine cells. We tried to use more cell lysate protein (up to 1 mg) or more antibody, yet we were still unable to observe any 4E-BP1. The possible reasons for this are that perhaps the amount of 4E-BP1 present in murine cells is so low that we are unable to detect it. However, loading even more total protein lysate (>1 mg) may result in unspecific bands being detected. Therefore, the next step was to try to immunoprecipitate using large quantities of total protein lysate using the 4E-BP1 antibody and probe for phosphorylated 4E-BP1. Another possibility may be that perhaps these murine cell types do not have any 4E-BP1. Even still, perhaps this antibody may only work well for human 4E-BP1 and may not work as efficiently for murine 4E-BP1, because the antibody was raised in rabbit using human 4E-BP1.

It is important to mention that previous studies determined that there are a total of three 4E-BP1 forms that can be detected. There is the hyperphosphorylated slow migrating form, identified as 4E-BP1γ, which does not interact with eIF-4E. Also, there is the middle form, identified as 4E-BP1β, which is phosphorylated and binds to eIF-4E with low affinity and there is the unphosphorylated form, which migrates the fastest and is identified as 4E-BP1α. This α isoform interacts very strongly with eIF-4E (Gingras et al., 1999c). As we observed from our TF-1 samples, we definitely could see three bands present in our 4E-BP1 blots. In the unstimulated samples there appeared to be both the α
form and β form present. However, it appear that in stimulated samples, there was a decrease in the α form and an increase in γ forms. These differences in phosphorylation states coincide with the idea that the hyperphosphorylated form, γ, is unable to bind and inhibit eIF-4E, consequently, allowing protein translation to occur.

In the TF-1 cells we observe that in the unstimulated samples, 4E-BP1 is also phosphorylated. This result is similar to that seen in cardiac myocytes. In this study, Pham. et al. (2000) also observed that in unstimulated cardiac myocytes there are phosphorylated forms of 4E-BP1. When the TF-1 cells were treated with LY294002 there was no phosphorylation of 4E-BP1. Thus, these results indicate that PI3K is required for the basal phosphorylation of 4E-BP1 to allow a basal level of protein synthesis to occur, for example proteins that are required for survival. However, when the TF-1 cells are stimulated we notice an increase in the amount of phosphorylated 4E-BP1.

Surprisingly, wortmannin, another PI3K inhibitor did not inhibit the phosphorylation of 4E-BP1. This result is in conflict with Pham et al. (2000), who demonstrated that wortmannin, like LY294002, also blocks the phosphorylation of 4E-BP1. More experiments must be conducted in order to determine the reason for this discrepancy.

Rapamycin is an inhibitor which directly inhibits the phosphorylation of FRAP/mTOR. However, from our results it appears that LY294002 is more effective at blocking the phosphorylation of 4E-BP1 than rapamycin. Previously, a study conducted by Peterson et al. (2000) indicated that FRAP/mTOR
underwent autophosphorylation at Ser 2481 located in the carboxy-terminal tail. This phosphorylation is required for FRAP/mTOR kinase activity. Furthermore, this study indicated that when PI3K was inhibited, this resulted in the complete blockage of FRAP/mTOR autophosphorylation. However, rapamycin and serum withdrawal did not block FRAP/mTOR autophosphorylation (Peterson et al., 2000). Consequently, it can be speculated that LY294002 completely blocks FRAP/mTOR activation and significantly reduces 4E-BP1 phosphorylation, while rapamycin does not completely block FRAP/mTOR activation and resulting in some 4E-BP1 phosphorylation.

4.5 Future Experiments

These results are only preliminary and further experiments must be done in order to clearly identify the role of PI3K in protein translation. The experiment with wortmannin must be resolved, perhaps a longer incubation time with this inhibitor is needed to inhibit the activity of FRAP/mTOR. Also, earlier studies have indicated that PI3K is not involved in the phosphorylation of eIF-4E. Thus, an interesting experiment would be to observe what kind of effect that PI3K inhibitors may have on the phosphorylation of eIF-4E to confirm or disprove the results of PI3K in our cell systems.

An important issue that still must be addressed is the mechanism of 4E-BP1 dephosphorylation. It has been suggested that the dephosphorylation occurs via the increase in PP1 and PP2A activity (Pham et al., 2000). Thus, it will be interesting to observe the effects of using PP1 and PP2A inhibitors, okadaic acid and calyculin A in the presence of PI3K inhibitors.
Chapter 5: Overall Summary

5.1 PI3K Activation Is Not Required for ERK1/2 Activation

During the past several years there have been great controversy over the role that PI3K activation may play in regulating the activation of ERK1/2. Some studies have indicated that PI3K activation is required for ERK1/2 activation (Sutor et al., 1999; Craddock et al., 1999). However, other studies have revealed that PI3K activation is not required for ERK1/2 activation (Scheid and Duronio, 1996; Frevert and Khan, 1997). Some studies that claimed that PI3K activation is required for ERK1/2 activation used dominant-negative PI3K mutants, which are constructed via the deletion of the inter-SH2 region of the p85 regulatory subunit (Hara et al., 1994; Sutor et al., 1999; Craddock et al., 1999). Consequently, there are more functional SH2 domains present in the cells than normally, which may still function as docking sites for phosphorylated tyrosine proteins and eliciting misleading responses, such as the reduction in ERK1/2 activation. Our speculation is supported by Craddock et al. (1999) who demonstrated that there was an increase in tyrosine phosphorylated proteins which complexed with the mutant p85 subunit.

The development and use of pharmacological inhibitors that specifically inhibited PI3K have made dramatic progress in determining the role of PI3K in the activation of ERK1/2. Wortmannin has been shown to not be a very specific inhibitor of PI3K (Cross et al., 1995), as we have also seen in our study using FDC-P1s. However, another inhibitor LY294002, has been shown to be a much more specific inhibitor that wortmannin (Vlahos et al., 1994). Even though
wortmannin is considered to be not as specific as LY294002, it should still be used alongside LY294002 for a comparison of responses.

This study used both pharmacological inhibitors, LY294002 and wortmannin to determine if PI3K is required for ERK1/2 activation in various cell systems. In all the cell systems used (FDC-P1, TF-1, BAF-3 and HEK 293), it can be observed that when PI3K activation is completely blocked by either inhibitors, there is no effect on the activation of ERK1/2. Consequently, proving that PI3K is not required for ERK1/2 activation.

The question of differential effects on ERK1/2 activation in the presence of PI3K inhibition using various concentrations of cytokines was also addressed in TF-1 cells. This study demonstrated that at all concentrations of cytokine used in the presence of PI3K blockage there was no inhibition on ERK1/2 activation.

Functional studies using FDC-P1 cells, TF-1 cells and BAF-3 cells suggested that the inhibition of ERK1/2 activation had no effect on cell survival (as seen by lack of apoptosis during blockage of ERK1/2), but the blocking of PI3K activation had an effect on cell survival (as seen by the presence of apoptosis during blockage of PI3K). Thus, it can be concluded that these functional results prove that the promotion of cell survival signals by PI3K pathway does not necessarily involve the ERK1/2 pathway.

In conclusion, the results clearly demonstrate that PI3K activation is not required for ERK1/2 activation. Resolving this PI3K and ERK1/2 controversy is beneficial for scientific advances by helping to determine the placement of the jigsaw pieces of the signal transduction puzzle. Ultimately, understanding the
complexities of signal transduction pathways within a cell system would provide a valuable tool for development of drug therapies.

5.2 PI3K Regulation of Protein Synthesis

What role does PI3K play in the regulation of protein synthesis? Before one can answer this question the presence of regulatory proteins of protein translation must first be determined in our cell systems. We determined that 4E-BP1 is present in TF-1 cells, and 4E-BP2 is present in FDC-P1, MC-9 and TF-1 cells. 4E-BPs are proteins that bind to eIF-4E and inhibit it from binding to eIF-4G, which makes up the translational machinery. Furthermore, eIF-4E is present in all three cell systems.

Preliminary experiments were also performed using inhibitors, such as LY294002, wortmannin, U0126 and rapamycin in the presence of cell stimulations. Surprisingly, wortmannin did not block the phosphorylation of 4E-BP1, while LY294002 did. U0126, as expected, did not block the phosphorylation of 4E-BP1. Rapamycin only partially blocked the phosphorylation of 4E-BP1.

These are only preliminary results; thus, more experiments are needed to fully understand the role that PI3K may play in the regulation of protein translation. However, these initial experiments importantly, established necessary protocols for future experiments to clearly elucidate the role of PI3K.
Chapter 6: References


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