CYTOKINE REGULATION OF GLYCOGEN SYNTHASE KINASE-3 (GSK-3)

by

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ABSTRACT

Cytokines are soluble growth factors that are essential for the continued survival of numerous hematopoietic cell lines. For example, in cells of the erythromyeloid lineage, interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) are powerful anti-apoptotic agents. Glycogen synthase kinase-3 (GSK-3) is an ubiquitous and yet enigmatic protein kinase implicated in various cellular functions, including glycogen metabolism, embryonic development, cell survival, proliferation, and protein translation. Numerous extracellular stimuli can promote the inactivation of GSK-3 through serine phosphorylation of the amino-terminal region. Growth factors are among the agents capable of inactivating GSK-3, presumably to promote cell survival and proliferation. Interestingly, an extensive literature search has found no studies examining GSK-3 in the context of cytokine-mediated signaling. This study is the first to demonstrate the elevation of GSK-3α and GSK-3β serine phosphorylation by IL-3, IL-4, and GM-CSF in several hematopoietic cell lines. IL-4 required PI3-K activity, and presumably PKB activity, for full GSK-3 modification, while IL-3 and GM-CSF did not. Although MAPK and p70S6K did not regulate the control of GSK-3 by IL-3, IL-4 or GM-CSF, PKC did demonstrate a strong effect. Inhibition of PKC activity, through the addition of pharmacological compounds, led to a dramatic abrogation of GSK-3 serine phosphorylation induced by all three cytokines. However, the lack of specificity of these inhibitors made it difficult to identify the class of PKC or the individual isoforms responsible for GSK-3 regulation. Furthermore, the inhibition of diacylglycerol production also impacted on the cytokines' ability to phosphorylate GSK-3. Diacylglycerol is a necessary component for the activation of numerous PKC isoforms. However, like the PKC inhibitors, some evidence suggests that these PLC inhibitors may be acting on non-specific targets. Interestingly, increased
serine phosphorylation of GSK-3 did not appear to correlate with a decrease in catalytic activity, although the quality of the assay system is questionable. Regardless, this study is the first to examine GSK-3 in cytokine-mediated signaling, and to implicate PI3-K and PKC as mediators of this event. Further studies will help develop a clear signaling model for the regulation of GSK-3 and determine GSK-3's role in the cellular effects induced by cytokines.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>viii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>x</td>
</tr>
<tr>
<td>Dedication</td>
<td>xi</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td></td>
</tr>
<tr>
<td>1.1. Hematopoiesis</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Hematopoietic Growth Factors</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1. Interleukin-3</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2. Granulocyte-Macrophage Colony Stimulating Factor</td>
<td>5</td>
</tr>
<tr>
<td>1.2.3. Interleukin-4</td>
<td>6</td>
</tr>
<tr>
<td>1.3. Cytokine Receptors</td>
<td>7</td>
</tr>
<tr>
<td>1.4. Cytokine Signaling Pathways</td>
<td>10</td>
</tr>
<tr>
<td>1.4.1. JAK</td>
<td>10</td>
</tr>
<tr>
<td>1.4.2. MAPK</td>
<td>14</td>
</tr>
<tr>
<td>1.4.3. p90(^{sk})</td>
<td>19</td>
</tr>
<tr>
<td>1.4.4. Phosphatidylinositol 3' - kinase (PI3-K)</td>
<td>20</td>
</tr>
<tr>
<td>1.4.5. Protein kinase B</td>
<td>21</td>
</tr>
<tr>
<td>1.4.6. Protein kinase C</td>
<td>26</td>
</tr>
<tr>
<td>1.4.7. Protein kinase A</td>
<td>31</td>
</tr>
<tr>
<td>1.4.8. p70(^{S6K})</td>
<td>34</td>
</tr>
<tr>
<td>1.5. Glycogen synthase kinase-3 (GSK-3)</td>
<td>35</td>
</tr>
<tr>
<td>1.5.1. Isoforms</td>
<td>35</td>
</tr>
<tr>
<td>1.5.2. Regulation</td>
<td>36</td>
</tr>
<tr>
<td>1.5.3. Embryonic development</td>
<td>39</td>
</tr>
<tr>
<td>1.5.4. Substrates</td>
<td>40</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

## CHAPTER 1: INTRODUCTION

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Hematopoiesis</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Cytokine receptors</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>JAK/STAT pathway</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>MAPK pathways</td>
<td>15</td>
</tr>
<tr>
<td>1.5</td>
<td>PI3-K/PKB pathway</td>
<td>22</td>
</tr>
<tr>
<td>1.6</td>
<td>Diacylglycerol formation</td>
<td>27</td>
</tr>
<tr>
<td>1.7</td>
<td>PKC isoforms</td>
<td>28</td>
</tr>
<tr>
<td>1.8</td>
<td>PKA activation</td>
<td>32</td>
</tr>
</tbody>
</table>

## CHAPTER 3: RESULTS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Effect of cytokines on serine phosphorylation of GSK-3α/β</td>
<td>56</td>
</tr>
<tr>
<td>3.2</td>
<td>Effect of cytokines on GSK-3α/β is post-translational</td>
<td>59</td>
</tr>
<tr>
<td>3.3</td>
<td>Role of PI3-K in GSK-3 regulation by cytokines</td>
<td>61</td>
</tr>
<tr>
<td>3.4</td>
<td>PKB activity is sufficient for GSK-3 serine phosphorylation</td>
<td>64</td>
</tr>
<tr>
<td>3.5</td>
<td>The role of MAPK in GSK-3 regulation</td>
<td>67</td>
</tr>
<tr>
<td>3.6</td>
<td>The role of p70S6K in GSK-3 regulation</td>
<td>69</td>
</tr>
<tr>
<td>3.7</td>
<td>Cell specific regulation of GSK-3 by forskolin</td>
<td>71</td>
</tr>
<tr>
<td>3.8</td>
<td>Effect of phorbol esters on GSK-3α/β</td>
<td>73</td>
</tr>
<tr>
<td>3.9</td>
<td>GSK-3 phosphorylation via PKC in MC/9</td>
<td>76</td>
</tr>
<tr>
<td>3.10</td>
<td>GSK-3 phosphorylation via PKC in TF-1</td>
<td>77</td>
</tr>
<tr>
<td>3.11</td>
<td>GSK-3 phosphorylation via PKC in FDC-P1</td>
<td>78</td>
</tr>
</tbody>
</table>
Figure 3.12. PKCδ does not regulate GSK-3 phosphorylation by cytokines .......... 80
Figure 3.13. Diacylglycerol formation upon cytokine treatment .................. 83
Figure 3.14. Role of PLC in GSK-3 regulation by cytokines ....................... 85
Figure 3.15. Regulation of GSK-3α activity ........................................... 88
Figure 3.16. Regulation of PKB activity .................................................... 90
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF-1</td>
<td>activating transcription factor-1</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCGF</td>
<td>B-cell growth factor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer core binding protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>eIF4E</td>
<td>elongation initiation factor 4E</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>GADD153</td>
<td>growth arrest and DNA-damage-inducible 153</td>
</tr>
<tr>
<td>GAS</td>
<td>γ-IFN activated sequences</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>IL-3</td>
<td>interleukin-3</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin-4</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>JAK homology</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal kinases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF2C</td>
<td>myocyte enhancer factor 2C</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>N-linked</td>
<td>amino-terminus</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxide</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated protein kinase</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylyserine</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine binding</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal S6 kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinases</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>She</td>
<td>SH2-containing</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
</tbody>
</table>
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To Mom
CHAPTER 1: INTRODUCTION

1.1. Hematopoiesis

The numerous types of cells that comprise mammalian blood perform a variety of crucial physiological functions. The formation and development of these cells is termed hematopoiesis. The process originates with a population of pluripotent stem cells from which every cell type in the blood derives.\(^1\) To ensure continual and extensive cell production, stem cells have an unlimited capability for self-renewal and can differentiate into any one of the types of cells found in the peripheral blood.\(^2,3\) Although stem cells reside primarily in the bone marrow, these cells are also able to circulate through the bloodstream. Unfortunately, research on stem cells has been complex due to their low numbers in the body and the difficulty of culturing them \textit{in vitro}. However, recent advances are facilitating studies using stem cells.\(^4,5\)

Pluripotent stem cells can exist in several states. A large portion of the population of stem cells are quiescent, halted at an intermitotic phase of the cell cycle which can be maintained for a long period of time.\(^6\) This reserve pool of resting cells ensures a continual capacity for hematopoiesis. Meanwhile, a smaller population of stem cells can continue to divide without differentiating, allowing for a self-renewal capacity.\(^7\) Finally, a very small group of stem cells can divide and differentiate into progeny cells of a specific type.\(^8\)

Hematopoiesis is an on-going process beginning in the embryonic stage. Early on, hematopoiesis takes place in the embryonic yolk sac.\(^9\) In humans, between the third and seventh gestation months, stem cells migrate to the fetal liver and then later to the spleen.\(^9,10\) After seven months, and from then on, hematopoiesis occurs primarily in the bone marrow.\(^11\) Most large bones participate in blood cell production, including the limb bones, pelvis, sternum, vertebra,
and ribs. In times of severe hematopoietic stress however, the liver and the spleen can once again lend themselves to blood cell production.\textsuperscript{12}

The developmental path a stem cell will follow is dependent on the microenvironment, particularly the presence of soluble growth factors and matrix components.\textsuperscript{13} Blood cells can be categorized into two major lineage groups (Figure 1.1).\textsuperscript{3} The erythromyeloid lineage gives rise to neutrophils, macrophages, eosinophils, basophils, mast cells, erythrocytes, megakaryocytes and platelets. Neutrophils and macrophages provide protection against bacteria by engulfing foreign pathogens through phagocytosis. Eosinophils defend against parasitic infections and downregulate allergic responses, while basophils participate in inflammatory and allergic responses through IgE binding and granular release. Meanwhile, mast cells, which are morphologically and functionally similar to basophils, are found mainly in the tissues. Erythrocytes carry oxygen to the tissues through a hemoglobin carrier, while megakaryocytes produce platelets, which in turn promote healing of wounds to the circulatory system. The other major group is the lymphoid lineage, which includes B and T lymphocytes and natural killer cells. B cells secrete immunoglobulins against specific antigens, while T cells provide a cellular-based immunity. Natural killer cells, which are less specific in their targets, are also cytotoxic cells that act on microbes and tumour cells.\textsuperscript{14}

Hematopoiesis is an essential function for survival. Humans are estimated to produce some $3.7 \times 10^{11}$ blood cells a day. As such, there exists a need for delicate balance between blood cell production/survival and cell death via apoptosis. This is often achieved through the presence or absence of necessary growth factors. If this process becomes unbalanced however, the physiological results can range from immune deficiencies to leukemia.
Figure 1.1 - Hematopoiesis. The development of all blood cells begins with a pluripotent stem cell that is capable of differentiating into members of both the myeloid and lymphoid lineages. This process typically takes place in the bone marrow, with the effector cells later moving into the blood and surrounding tissues to perform their critical functions.3
1.2. Hematopoietic Growth Factors

An efficient means of communication between cells is necessary in any large multicellular organism. Neighbouring cells are able to communicate through direct cell-to-cell contact, while cells of the nervous system can propagate signals via neurotransmitters at synaptic connections. In contrast, cells of the blood are mostly regulated by the release of proteins within the circulatory system. These secreted peptides can travel through the bloodstream to target cells that are far away from the original source.

Soluble hematopoietic growth factors called cytokines are glycosylated proteins that can regulate cell differentiation, survival, and proliferation of pluripotent and mature blood cell lines. Cytokines can also mediate direct functional responses in many blood cells. To date, a wide range of cytokines have been characterized, however, many of these exhibit redundant responses as well as functional pleiotropy. In fact, there is ample evidence that various cytokines are evolutionarily related. For example, the genes for IL-3, IL-4, and GM-CSF are all clustered on chromosome 5 of the human genome.

1.2.1. Interleukin-3 (IL-3)

Interleukin-3 (IL-3) is a hematopoietic growth factor responsible for the growth, survival and proliferation of pluripotent and committed cell types, particularly those of the erythromyeloid lineage. The cDNA encoding the murine IL-3 was first identified in 1984 and the human form two years later. The human protein is comprised of 152 amino acids (murine 166 amino acids) and contains two putative N-linked glycosylation sites and a single disulfide bond. While the disulfide linkage is necessary for cytokine activity, the glycosylation process only promotes...
an increase in IL-3’s half life in vivo. Remarkably, the homology between the human and murine forms is only 29%, which is consistent with the high species specificity of IL-3. The cytokine is produced primarily by CD4+ T helper cells, but other sources, such as thymic epithelial cells, and stimulated mast cells and eosinophils can also contribute small amounts.

IL-3’s effects are generally directed at cells of the hematopoietic system. As Figure 1.1 illustrates, IL-3 can promote differentiation of hematopoietic cells in the bone marrow, particularly those of the myeloid lineage. As well, IL-3 promotion of cell survival and proliferation in progenitor and terminally differentiated blood cell types is well documented. In fact, several myeloid and lymphoid cell lines depend on IL-3 to protect against apoptosis. For example, IL-3 is sufficient for the survival and proliferation of the MC/9 mast cell line, the BaF/3 pro B-cell line, the TF-1 myelomonocytic cell line and the hematopoietic myeloid progenitor FDC-P1 cell line. IL-3 is also a potent regulator of the functional activity of mast cells, basophils, eosinophils, and macrophages. For example in mast cells, IL-3 induces the synthesis of vasoactive histamines.

1.2.2. Granulocyte-macrophage colony stimulating factor (GM-CSF)

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a versatile cytokine that regulates specific sets of hematopoietic cells. Murine GM-CSF was first cloned in 1984, and the human gene a year later. The mature human GM-CSF, derived from a precursor with a secretory signal sequence, is a 127 amino acid protein sharing only 54% homology with the 124 amino acid murine form. GM-CSF contains two sites for N-linked glycosylation that enhance biological activity, and two disulfide bonds of which one is essential for cytokine activity.
There exists a wide variety of cells capable of producing GM-CSF, including T cells, endothelial cells, macrophages, mast cells, and stromal cells in the bone marrow.\textsuperscript{36-40}

Similar to IL-3, GM-CSF regulates differentiation of myeloid blood cells. As its name implies, it is also an essential factor for the growth and differentiation of macrophages and granulocytes such as neutrophils and eosinophils. The factor-dependent cell lines MC/9, TF-1, BaF/3, and FDC-P1 all exhibit long-term proliferation in the presence of GM-CSF.\textsuperscript{41-44} This cytokine is also a mediator of certain cell functions in neutrophils, macrophages, and eosinophils.\textsuperscript{45-47} For example, GM-CSF can enhance the phagocytic capabilities of neutrophils and the cytotoxicity of eosinophils.\textsuperscript{45,47}

1.2.3. Interleukin-4 (IL-4)

Interleukin-4 (IL-4) is a pleiotropic cytokine responsible for various cell functions. First identified as a B-cell growth factor (BCGF), both the murine and the human form of IL-4 were cloned in 1986.\textsuperscript{48,49} Mature human and murine IL-4 are derived from precursor proteins containing hydrophobic secretory signal sequences, which are cleaved to produce the 129 amino acid and 120 amino acid species respectively. IL-4 has three N-linked glycosylation sites and six cysteine residues involved in disulfide linkages, the latter being essential for cytokine activity.\textsuperscript{50,51} This cytokine is produced primarily by CD4\textsuperscript{+} T cells, but also in smaller amounts by mast cells and basophils.\textsuperscript{52-54}

IL-4 can elicit responses in a diverse subset of cells from many hematopoietic lineages. B cells and basophils differentiate in response to this cytokine, and IL-4 can direct differentiation of naive T cells into cells of the T\textsubscript{H}2 subclass of T cells.\textsuperscript{55-57} Unlike IL-3 and GM-CSF, interleukin-
4 does not typically act as a growth factor on its own. However, IL-4 can synergize with co-stimulatory agents to support proliferation of various cell lines. For example, IL-4 in conjunction with IL-10 can support proliferation of MC/9 mast cells, however, neither cytokine alone can accomplish the same task.\textsuperscript{58} FDC-P1 and MC/9 cells grown in IL-4 supplemented media display survival for only a few days with minimal proliferation.\textsuperscript{59,60} In fact, IL-4 can inhibit proliferation of various cell types, including FDC-P1 cells treated with IL-3 and GM-CSF.\textsuperscript{61} On the other hand, TF-1 cells can proliferate in response to IL-4 without co-stimulatory agents.\textsuperscript{62} Therefore IL-4’s ability to upregulate proliferation is dependent on cell type. As well, IL-4 can also trigger functional responses in B cells, T cells, macrophages, mast cells, and endothelial cells.\textsuperscript{63-67} For example, IL-4 induces the expression of MHC II on the cell surface of B cells.\textsuperscript{63} As well, this cytokine’s role in the allergic response is of particular interest due to its unique ability to induce immunoglobulin class switching to IgE.\textsuperscript{68}

1.3. Cytokine receptors

Soluble cytokines exert their effects through their respective receptor molecules found on the surface of various cell types. These receptors are biochemically quite similar, but are unique in comparison to other receptor families. The type I cytokine receptor family includes the receptors for IL-3, GM-CSF, IL-4 and many more.\textsuperscript{69} They are single-spanning transmembrane glycoproteins lacking intrinsic kinase activity. Many cytokines utilize a dimeric receptor system comprised of a unique receptor for each ligand (\(\alpha\) chain), and a common chain to assist in transduction of an intracellular signal (Figure 1.2).\textsuperscript{70} Both chains contain a 200 amino acid stretch in the extracellular domain with four conserved cysteine residues and a conserved region
Figure 1.2 - Cytokine receptors. Schematic display of the type I cytokine receptors for IL-3, GM-CSF, and IL-4. All three cytokines bind to a unique receptor (α chain), which associates with a shared receptor component (β or γ chain). The intracellular portions of these receptor complexes contain domains essential for protein recruitment and propagation of an intracellular signal."
containing a WSXWS sequence. As well, some receptor subunits possess Box 1/Box 2 domains in the cytoplasmic region of the receptor that are important for the binding of associated JAK proteins. Box 1 is a membrane proximal region rich in proline residues while Box 2 is less conserved, and typically contains several hydrophobic residues followed by negatively charged amino acids and ending in one or two positively charged residues. Cytokine receptors may also contain other motifs to mediate protein-protein interactions, such as the putative SH2 domain found in the IL-4 receptor.

The binding of a cytokine by the α receptor chain is a low affinity interaction. Association of the α subunit with the non-ligand-binding common receptor subunit creates a high-affinity heterodimeric receptor complex that can better respond to the physiological concentrations of cytokines. A signal is then propagated into the cell, a process that is primarily driven by the common chain, but which requires the intracellular portions of both receptor subunits in IL-3, GM-CSF, and IL-4 signalling.

The 70 kDa IL-3 receptor α chain (IL-3Rα) and the 45 kDa GM-CSF receptor α chain (GM-CSFRα) are both glycoproteins that conform to the standards of type I cytokine receptors. Both bind their respective cytokines with low affinity. As such, the receptor complexes for IL-3, GM-CSF, and IL-5 all utilize the 130 kDa β common (βc) chain for increased binding affinity and the transmission of an intracellular signal. This subunit houses the Box 1 and Box 2 motifs which are essential for receptor function. Interestingly, the murine system has a βIL-3 chain as well as a βc chain, with the former being found only in IL-3 receptors. In contrast, human cells do not possess this restricted β chain.
The IL-4Rα is a 140 kDa transmembrane receptor that binds IL-4 with low affinity and houses the Box 1 and Box 2 motifs that mediate some of the receptor's protein interactions within the cell. To enhance physiological sensitivity, the IL-4 receptor, along with the IL-2, IL-7, IL-9, IL-13 and IL-15 receptors, also contain the 65 kDa γc receptor subunit. The importance of the γc protein is highlighted in the human disease X-linked severe combined immunodeficiency (X-SCID), where a mutated γc chain results in dysfunctional B cells and diminished production of T cells and natural killer (NK) cells.

1.4. Cytokine signaling pathways

Cytokine association with its respective receptor initiates a cascade of various signaling pathways within the target cell. A diverse array of protein kinases can mediate the plethora of cellular activity that cytokines promote.

1.4.1. JAK

Type I cytokine receptors contain no intrinsic kinase activity, and as such require the recruitment of other protein kinases. The Janus kinases (JAK's) are key components in the early stages of cytokine-induced intracellular signalling. Members of the JAK tyrosine kinase family, JAK1, JAK2, JAK3, and TYK2, contain seven conserved JAK homology (JH) regions (JH1-7) and two tyrosine kinase domains. The latter are localized at the carboxyl-terminus with a catalytic tyrosine kinase domain (JH1) and a pseudo-kinase domain (JH2). The pseudo-kinase domain regulates JAK activity by interacting with the JH1 domain and inhibiting JAK activity in
the absence of ligand stimulation. Meanwhile, the amino terminus houses the region responsible for binding to the cytokine receptor.

JAK-receptor interactions promote tyrosine phosphorylation of the receptor subunits and association of other adapter molecules at the receptor. In resting cells, JAK's are constitutively associated with the proline-rich receptor motifs Box 1 and Box 2. JAK2 is the predominant isoform found at the βc receptor, with minor JAK1 and TYK2 presence, while the γc chain of the IL-4 receptor favors JAK3 and JAK1. Cytokine-receptor binding promotes heterodimerization of the α and common receptor chains into a single receptor complex, followed by oligomerization of these dimeric units. As such, two JAK molecules, one at each receptor complex, come into close proximity. This permits transphosphorylation of each JAK by the other, resulting in the upregulation of JAK tyrosine kinase activity (Figure 1.3). The activated JAK's then target key tyrosine residues within the receptor chains themselves. These phosphorylations allow for binding of various SH2-containing proteins at the receptor. Extensive mapping of receptor docking sites has been accomplished through truncation and amino acid substitution studies.

Recruitment of the STAT family of transcription factors to the receptors is a direct result of receptor phosphorylation by JAK's. There are seven known STAT isoforms, all of which contain an amino-terminal oligomerization domain, a DNA binding domain, an SH2 domain, and a carboxyl-terminal transactivation domain. IL-4 potently activates STAT6 and to a lesser extent STAT5a/b, while STAT5a/b plays a key role in IL-3/GM-CSF signalling with some STAT1, STAT3, and STAT6 activation. STAT's are normally latent in the cytoplasm, but cytokine stimulation promotes their nuclear translocation, DNA binding, and transactivation
Figure 1.3 - JAK/STAT pathway. Cytokine association with its respective receptor activates the JAK family of tyrosine kinases and the STAT family of transcription factors. Upon cytokine binding, the receptor-associated JAK’s phosphorylate each other and the receptor chains. This promotes STAT recruitment to the receptor complex where it is phosphorylated by JAK, and becomes an active transcription factor.
abilities. Upon receptor phosphorylation, the STAT proteins bind to these sites via their SH2 domains. For example, tyrosine phosphorylation of the IL-4Rα at Tyr578 and Tyr606 permits recruitment of STAT6 to the receptor. Once associated with the receptor, the JAK's then phosphorylate the STAT's directly at a conserved tyrosine residue which initiates SH2-mediated homo- or heterodimerization of various STAT molecules (Figure 1.3). STAT can then pass across the nuclear membrane and target the palindromic GAS (γ-IFN activated sequences) element TTNACNNNA found in various gene promoter regions. Other kinases can further modulate the transactivational and DNA binding abilities of the STAT's via phosphorylation of key serine residues. Once bound to a gene's promoter sequence, STAT's can either act alone, or in concert with other weak DNA-binding proteins, such as CBP/p300, to initiate gene transcription.

JAK's can play an important role in regulating both cell survival and cell proliferation in factor-dependent hematopoietic cells. Several transcription factors implicated in proliferative responses, including c-fos and c-myc, are themselves regulated by STAT proteins. For example, promotion of c-fos transcription in GM-CSF-treated TF-1 cells is partially controlled by STAT family members. In BaF/3 cells, expression of a dominant negative JAK2 blocks c-fos and c-myc promoter activation, and IL-3/GM-CSF-mediated cellular proliferation. As well, STAT5 repression can block IL-3-induced cell growth in BaF/3 cells and 32D myeloid cells, while STAT5a-deficient bone marrow-derived macrophages show impaired proliferative responses to GM-CSF. However, there is mounting evidence that JAK can also mediate these cellular effects without STAT. For example, constitutive activation of JAK2 in BaF/3 cells demonstrates no STAT activity but can confer IL-3-independent growth nevertheless. This apparent paradox
of STAT involvement in IL-3-mediated proliferation is still under scrutiny. It is plausible that JAK’s utilize alternative pathways that bypass the need for STAT-mediated transcription. For example, IL-3-induced JAK activity can upregulate the expression of the anti-apoptotic Bcl-XL protein independent of STAT in FDC-P1 cells. As well, JAK’s can activate the Ras/MAPK cascade without STAT.

1.4.2. MAPK

The mitogen-activated protein kinase (MAPK) family is a group of evolutionarily conserved kinases that are one of the most commonly used kinase pathways involved in eukaryotic cell regulation. Various MAPK isoforms have been isolated, with the three most predominant being the extracellular signal-related kinases (ERK1 and ERK2), the p38 family (p38 α, β, γ, δ), and the Jun amino-terminal kinases/stress-activated protein kinases (JNK1, 2, 3/SAPK γ, α, β). The ERK’s are stimulated by a variety of growth factors, while p38 and JNK isoforms are often activated by stress stimuli and have been implicated in the apoptotic process.

The mechanism of MAPK isoform activation is highly conserved and utilizes protein kinase modules and sequential phosphorylations. As Figure 1.4 illustrates, all three major MAPK modules utilize a GTP-binding protein, followed by activation of a MAPK kinase kinase (MEKK), a MAPK kinase (MEK), and finally the MAPK itself.

ERK is largely activated by the monomeric GTPase Ras. Ras is a plasma membrane-localized protein, the result of post-translational isoprenylation. It acts as an adapter, targeting the Raf family of proteins (A-Raf, B-Raf, Raf-1) and recruiting these to the plasma membrane as
Figure 1.4 - MAPK pathways. A cascade of protein kinase phosphorylations regulates all three families of MAPK isoforms, including ERK, JNK, and p38. Small GTP-binding proteins transduce a signal initiated by extracellular stimuli, promoting activation of MEKK, MEK, and finally the MAPK isoforms.
All Raf isoforms are serine/threonine kinases activated by association with Ras. Raf may also require prior phosphorylation at key residues by p21-activated protein kinase (PAK) and/or PKC to elicit full activation. All three Raf isoforms are regulated by similar means, although B-raf does not require phosphorylation to achieve activation. In turn, the Raf proteins can phosphorylate MEK1 and MEK2 within their respective activation loops, resulting in full kinase activity. MEK1 and MEK2 are dual specificity MAPKK that phosphorylate both the threonine and the tyrosine found in a Thr-Glu-Tyr motif in the activation loop at kinase subdomain VIII within ERK1 and ERK2 (Thr185 and Tyr187 of ERK2). These modifications promote full ERK activity.

Meanwhile, the p38 and JNK family of MAPK isoforms are also regulated by similar modules of protein kinases induced by a variety of pro-inflammatory and stress stimuli. The Rho family of GTP-binding proteins, including Rac and Cdc42, initiate a MAPK cascade similar to Ras in the ERK pathway. These GTPases can then activate a wide range of MEKK proteins including TAK1, ASK1 (MEKK5), MEKK1, Tpl-2, MEKK2, MEKK3, MUK and TAO. Each of these can then lead to propagation of the JNK pathway, the p38 pathway, or both. The numerous players at this stage create a complex protein kinase network. As in ERK regulation, these MEKK’s phosphorylate MEK’s, which in turn promote p38 and JNK activation. p38 activity is upregulated by MEK3 and MEK6 through the dual phosphorylation of a Thr-Gly-Tyr sequence analogous to the crucial ERK motif. Likewise, the JNK pathway utilizes MEK4 and MEK7 to target a Thr-Pro-Tyr motif within the JNK activation loop.

All MAPK’s phosphorylate substrates at a serine or threonine residue that precedes a proline site. As such, MAPK’s are commonly referred to as proline-directed protein kinases. A
common target of MAPK's are transcription factors. ERK can target ELK-1, c-fos, and c-jun transcription factors resulting in enhanced expression or transactivational ability.\textsuperscript{114-116} As well, ERK can regulate cell proliferation by enhancing the activity of carbamoyl phosphate synthetase, a rate-limiting enzyme involved in nucleotide synthesis.\textsuperscript{117} JNK's can phosphorylate the transcription factors c-jun and ATF-2, while p38 members can target MEF2C, ATF-1, C/EBP\beta, GADD153, and SRF transcription factors.\textsuperscript{118-124}

The MAPK cascades are common intracellular pathways utilized by a variety of cytokines. With IL-3 or GM-CSF stimulation, phosphorylation of the $\beta_c$ receptor at Tyr\textsuperscript{577} by JAK2 allows for the binding of the adapter protein Shc via its phosphotyrosine binding (PTB) domain.\textsuperscript{89} Shc is then itself phosphorylated, whereupon another adapter protein, Grb2, binds to Shc. The guanine nucleotide exchange factor, Sos, can associate with Grb2 and catalyze the exchange of GDP with GTP, converting Ras to an active form. From there, the Ras-Raf-MEK-ERK pathway can proceed. Meanwhile, IL-4 is less consistent in the activation of MAPK compared to IL-3 and GM-CSF, with IL-4-mediated upregulation of MAPK being a cell type-specific response.\textsuperscript{125} In some cases, the IL-4 receptor complex recruits the insulin receptor substrate molecules (IRS) to Tyr\textsuperscript{497} of the $\gamma_c$ chain, where IRS is phosphorylated by Fes and/or JAK2.\textsuperscript{126,127} From there, Grb2 can bind to IRS and exert similar effects on Ras as seen in IL-3 signalling. However, these general mechanisms do not account for alternative means that exist to promote ERK activity.\textsuperscript{128} Meanwhile, IL-3 and GM-CSF can also activate the JNK/SAPK and p38 isoforms.\textsuperscript{129-130} In a pattern similar to IL-4's ability to activate ERK in a cell specific manner, enhanced p38 activity is also restricted to certain cell lines.\textsuperscript{131,132} However, how p38 and JNK isoforms are activated by cytokine treatment is less well understood.
MAPK isoforms have been shown to be important in cell proliferation and cell survival in factor-dependent cell lines. Expression of a Raf or a MEK1 construct fused to the estrogen receptor permitted survival and proliferation of FDC-P1 and TF-1 cells in the presence of estrogen, despite a lack of IL-3 or GM-CSF.\textsuperscript{133} Meanwhile, the expression of an active Ras blocked cell death in BaF/3 cells possessing a truncated βc receptor chain. Furthermore, transfection of an oncogenic form of Raf suppressed apoptosis in IL-3-deprived BaF/3 and 32D cells.\textsuperscript{134} As well, expression of a dominant negative MEK in BaF/3 blocked IL-3-induced survival. However, Ras activity is not essential for BaF/3 proliferation in response to IL-3.\textsuperscript{135} This suggests an alternative mechanism for ERK activity is available, or that ERK is not essential for cell proliferation in all cases.

JNK and p38 isoforms are primarily activated by stress and inflammatory cytokines and often mediate pro-apoptotic processes. For example, the removal of IL-3 leads to apoptosis in TF-1 cells, a process blocked by the co-presence of a p38 inhibitor.\textsuperscript{129} In contrast, p38 inhibition cannot deter ceramide-induced apoptosis in the MC/9 cell line.\textsuperscript{136} As such, there exists a complex role for these MAPK isoforms. The activation of the JNK/SAPK and p38 families have also been reported in the IL-3/GM-CSF-stimulation of various factor-dependent cell lines.\textsuperscript{132, 137} Interestingly, JNK may in fact promote IL-3-induced proliferation.\textsuperscript{137} Some have suggested that the pro-apoptotic effects of JNK and p38 may be offset by the activation of ERK during mitogen stimulation.
1.4.3. \textit{p90^{rsk}}

Another downstream target of the ERK isotypes is the family of serine/threonine kinases termed p90 ribosomal S6 kinase (p90\textsuperscript{rsk} or MAPKAP-K1). This group is comprised of three isoforms, RSK1, RSK2, and RSK3, all encoded by distinct genes.\textsuperscript{138} RSK, first isolated from the \textit{Xenopus laevis} oocyte, is peculiar in that it has two catalytic domains. The amino-terminus domain is responsible for RSK's ability to phosphorylate a variety of cellular substrates involved in cell proliferation, protein synthesis, and gene transcription. Meanwhile, the carboxyl-terminus kinase domain plays a role in RSK activation.\textsuperscript{139} RSK is usually activated when ERK is stimulated, and mitogens are typically strong activators of both ERK and RSK. The activation of RSK by ERK is a multi-step process involving not only ERK, but also other protein kinases as well.\textsuperscript{140}

Cytokines such as IL-3 and GM-CSF are mitogens, and as such can activate RSK in factor-dependent cell lines. For example, the TF-1 response to GM-CSF leads to RSK activation and FDC-P1 cells demonstrate IL-3-dependent RSK activity.\textsuperscript{141,142} Numerous proteins are targeted by RSK, including the transcription factors cAMP response element binding (CREB) protein, c-fos, and IxB\textalpha/NFkB.\textsuperscript{143-145} CREB is phosphorylated by RSK2 at Ser\textsuperscript{133}, a site whose modification is essential for CREB transactivational ability and possibly cell survival. Meanwhile, the expression of the \textit{c-fos} gene can be upregulated by RSK proteins. As well, NFkB activation may also be upregulated by RSK's phosphorylation of IxB\textalpha, allowing for the degradation of IxB\textalpha, NFkB nuclear translocation, and transcription of important genes. Finally, the translation of a specific group of mRNA important for cell growth is upregulated by RSK via its phosphorylation of the 40S ribosomal subunit protein S6.\textsuperscript{146}
1.4.4. Phosphatidylinositol 3'-kinase (PI3-K)

Activation of the PI3-K/PKB pathway is another common response to cytokine signaling. Three classes of PI3-K have been defined with respect to substrate specificity and structural motifs. The Class I PI3-K isoforms, which are best understood, are all heterodimeric proteins comprised of a catalytic and a regulatory subunit. Members of this class preferentially phosphorylate phosphatidylinositol-4,5-biphosphate [PI(4,5)P$_2$] at the 3'-OH position within the inositol ring to form PI(3,4,5)P$_3$, but can also target PI and PI(4)P to a lesser extent to generate PI(3)P and PI(3,4)P$_2$ respectively. This class is further subdivided into two groups based on the type of adapter subunit associated with the catalytic unit. Class IA PI3-K’s are comprised of a 110 kDa catalytic subunit and a regulatory protein. There are three Class IA catalytic isoforms termed p110$\alpha$, $\beta$, and $\delta$. p110$\delta$ expression appears restricted to the hematopoietic lineage. The common regulatory subunit is an 85 kDa protein housing SH2 and SH3 domains for protein interactions. The Class IB PI3-K isoform is p110$\gamma$ associated with a 101 kDa regulatory protein. This isoform is sensitive to the $\beta\gamma$ subunits of the trimeric G-proteins and does not associate with the p85 regulatory protein.

The proposed regulation of Class IA PI3-K during IL-3/GM-CSF signaling involves multiple steps. Cytokine ligation to its respective receptor results in the phosphorylation of the $\beta_c$ chain at Tyr$^{612}$, which acts as a docking site for the tyrosine phosphatase SHP2. A novel adapter protein p100 may act as an intermediary, linking to both SHP2 and p85 via their SH2 domains. In IL-4 signalling, IL4-R$\alpha$-associated IRS molecules bind the p85 subunit of PI3-K. In both cases, the association of the p85 regulatory protein with the cytokine receptor
complex results in the activation of the p110 catalytic protein and membrane translocation. This permits proximity of the enzyme to the necessary lipid substrates for PI3-K.

1.4.5. Protein kinase B (PKB)

PKB was so named for its homology to the catalytic domain of PKA and PKC, and was identified as the cellular homolog of the viral oncogene Akt (v-Akt) from a transforming retrovirus (AKT8) in spontaneous thymoma of the AKR mouse. It is a serine-threonine protein kinase whose full activity requires membrane localization and phosphorylation at two sites conserved in the AGC kinase family. Three mammalian isoforms have been identified and cloned, PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3, with PKBα being the more abundant species in most tissues. All three isoforms are ubiquitously expressed, and share a high degree of homology and similar structural features. This includes an amino-terminus pleckstrin homology (PH) domain that binds to phospholipids and two conserved phosphorylation sites (Ser\textsuperscript{473} and Thr\textsuperscript{308} in PKBα).

The activation of PKB is a complex multi-step process (Figure 1.5). PI3-K activation generates elevated levels of PI(3,4,5)P\textsubscript{3} and PI(3,4)P\textsubscript{2} lipids, which can promote recruitment of the constitutively active phosphoinositide-dependent kinase 1 (PDK1) to the membrane surface via the latter's pleckstrin homology (PH) domain. PDK1 acts as a link between PI3-K and a variety of protein kinases implicated in mitogenic activation, including PKB. PDK1 targets a conserved threonine-serine found in the T-loop between subdomains VII and VIII of a
Figure 1.5 - PI3K-PKB pathway. Cytokine signaling often initiate PI3-K activation. The heterodimeric PI3-K produces a series of lipids, which promote PDK1 and PKB membrane translocation. PDK1 can then phosphorylated PKB at Thr\(^{308}\), while an elusive PDK2 can target Ser\(^{473}\). These phosphorylation promote activation of PKB.\(^{156}\)
variety of AGC protein kinases leading to catalytic activation, including PKB\(\alpha\) (Thr\(^{308}\)), PKC\(\zeta\) (Thr\(^{410}\)), p70\(^{S6K}\) (Thr\(^{252}\)), Rsk2 (Ser\(^{227}\)), and SGK2 (Thr\(^{193}\)).\(^{157-162}\)

Meanwhile, PI3-K-generated lipids promote cell membrane localization of protein kinase B (PKB). Initial studies on PKB regulation focused on the constitutive activity of its viral counterpart, v-Akt. A myristoylation signal in the gag domain targets v-Akt to the plasma membrane, suggesting that membrane localization was essential for PKB activation.\(^{156}\) Subsequent studies confirmed the translocation of a pool of PKB to the plasma membrane upon mitogen stimulation, a process mediated by PKB’s PH domain. In fact, point mutations in the PH domain that reduce lipid binding affinity impairs PKB activation, while enhanced lipid binding hyper-activated the enzyme.\(^{163}\) Both PI(3,4,5)P\(_3\) and PI(3,4)P\(_2\) bind to the PH domain of PKB, but the relative contributions of each in vivo is still a mystery.\(^{164}\) However, studies on SHIP, a 5'-phosphatase that converts PI(3,4,5)P\(_3\) to PI(3,4)P\(_2\), showed that SHIP is an inhibitor of PKB activity in vivo.\(^{165}\) This data suggests PI(3,4,5)P\(_3\) is primarily responsible for PKB translocation.

Upon membrane localization, PKB is phosphorylated at two residues. Thr\(^{308}\) is found within the T-loop of the kinase domain, and Ser\(^{473}\) on a hydrophobic region of the carboxyl-terminus. Both sites are necessary for PKB activation as demonstrated through mutation of each to a non-phosphorylatable alanine.\(^{166}\) The inhibition of PI3-K blocked phosphorylation of both these residues, demonstrating that the kinases responsible for these modifications are themselves dependent on PI3-K-generated lipids. The kinase responsible for Thr\(^{308}\) phosphorylation is in fact PDK1. The phosphorylation of Ser\(^{473}\) is more enigmatic. The integrin-linked kinase (ILK) has recently been suggested to regulate PKB activity through direct phosphorylation of Ser\(^{473}\) in a
PI3-K-dependent manner. Interestingly, PDK1 can phosphorylate both Thr\textsuperscript{308} and Ser\textsuperscript{473} when PDK1 associates with the carboxyl-terminal region of the PKC-related protein kinase PRK2. Another theory on the table is that Thr\textsuperscript{308} phosphorylation allows PKB to autophosphorylate at Ser\textsuperscript{473}, possibly via close proximity to other PKB molecules in a multimeric complex. Regardless, once Ser\textsuperscript{473} is phosphorylated, PKB detaches from the membrane region, re-enters the cytosol and translocates to the nucleus. The evidence to date suggests that this is the means of activation for PKB\textalpha, PKB\textbeta, and PKB\gamma. There have been, however, reports of PKB activation independent of PI3-K. In particular, activation of the PKA pathway can activate PKB without the need for its PH domain or Ser\textsuperscript{473} phosphorylation, although Thr\textsuperscript{308} is still required.

Numerous potential PKB targets have been implicated in the possible control of cell survival and proliferation. Recently, PKB was identified as a regulator of the pro-apoptotic Bcl-2 family member, BAD, via phosphorylation at Ser\textsuperscript{136}. This leads to sequestration of the protein and aids in the inhibition of apoptosis. Unsequestered, BAD can heterodimerize with the anti-apoptotic Bcl-2 and Bcl-x\textsubscript{L} and neutralize their pro-survival abilities. However, the regulation of BAD is still under scrutiny and its role in cytokine-mediated survival has been called into doubt. Furthermore, the expression level of BAD is low and not ubiquitous, suggesting that any role PKB has in cell survival may be accomplished independently of BAD. Meanwhile, PKB may also phosphorylate and inactivate caspase-9, blocking execution of the apoptotic program. However, the phosphorylation site targeted by PKB in caspase 9 is not conserved among the species. As well, PKB can inhibit the forkhead family of transcription factors that primarily target pro-apoptotic genes, including the Fas ligand. Phosphorylation of the forkhead family of transcription factors results in their sequestration in the cytoplasm, thus
preventing transcription of these destructive genes. It has been reported that the transcription factor CREB may also be targeted by PKB, inducing expression of the pro-survival Bcl-2 family member Mcl-1. Protein synthesis, a necessary component for cell proliferation, might be under PKB control as well. During basal conditions, a regulator of mRNA translation, eIF4E, is found to be inactive and in complex with a repressor of translation termed 4E-BP. 4E-BP phosphorylation by PKB might allow 4E-BP to dissociate from eIF4E and subsequently promote mRNA translation.

The PI3-K/PKB pathway is a potential regulator of cell survival, proliferation, and a variety of hematopoietic cell functions. Numerous factor-dependent hematopoietic cell lines have demonstrated enhanced PI3-K and PKB activity upon IL-3, IL-4, and GM-CSF stimulation. This activation of PKB may be essential for cell survival and/or proliferation. A dominant-negative form of PKB has been noted to abrogate IL-3-induced proliferation in the 32D factor-dependent cell line, while expression of the constitutively active v-Akt protects these cells from apoptosis induced by IL-3 withdrawal. However, the role of PI3-K and PKB in cytokine-mediated survival and proliferation has been a contentious issue. While constitutive PKB activity in BaF/3 is sufficient to protect against apoptosis induced by IL-3 withdrawal, PKB activity is not necessary for the IL-3-induced inhibition of apoptosis due to DNA damage. It has also been shown that there is little correlation between PKB activity and cell survival in MC/9 and other cell lines. BaF/3 proliferation in the presence of IL-3 is reduced with the expression of a dominant negative form of the p85 adapter protein (Δp85), but there is no effect on survival. However, recent evidence points to significant communication between the PI3-K and ERK pathways. Some data suggests that the ERK
pathway is regulated by PI3-K, and that the abrogation of PI3-K also blocks IL-3 induced ERK activity and myeloid cell proliferation.\textsuperscript{134,188} It is plausible that the MAPK and PI3-K pathways synergize to mediate cell survival and proliferation in many factor-dependent cells.

1.4.6. Protein kinase C (PKC)

Diacylglycerol (DAG) is commonly produced by the cell receptor-mediated hydrolysis of phosphatidylinositol and phosphatidylcholine by phospholipase C (PI-PLC and PC-PLC) and phospholipase D (PLD) isoforms (Figure 1.6).\textsuperscript{189} A potent second messenger, DAG targets the PKC family of serine/threonine kinases that are ubiquitously expressed in a variety of species.\textsuperscript{190} PKC is also activated through its role as an intracellular receptor for phorbol esters.\textsuperscript{191}

Among the eleven known mammalian isoforms of PKC, there are various reoccurring structural features. Four domains termed C1-C4 are found in some or all of the PKC isoforms (Figure 1.7).\textsuperscript{192} The amino-terminus C1 domain found in some PKC's contains two sets of zinc-finger motifs. DAG binds at this region, and is competitive with phorbol esters for the same binding domain. Beside the C1 domain is a pseudosubstrate region that binds to the PKC catalytic region and suppresses PKC activity prior to effector binding. The C2 domain permits calcium and phosphatidylserine association. Some PKC's have a pseudo-C2 domain that lacks key aspartate residues needed for calcium binding. Finally, the C-terminus C3 and C4 domains comprise the catalytic region. As well, a hinge region between the regulatory and catalytic portions becomes proteolytically labile upon plasma membrane association of PKC.\textsuperscript{193}
Figure 1.6 - Diacylglycerol formation. Cellular agonists often induce diacylglycerol formation through one of several mechanisms. The phospholipase C (PLC) enzymes, which can hydrolyze either phosphatidylcholine (PC-PLC) or phosphatidylinositol (PI-PLC) to produce DAG, are commonly activated after agonist stimulation. Meanwhile, phospholipase D (PLD) also acts on phosphatidylcholine to produce phosphatidic acid, which can be further dephosphorylated to yield diacylglycerol.
Figure 1.7 - PKC isoforms. The three classes of PKC, while sharing certain structural features, have unique cofactor requirements.
The classical or conventional PKC's (cPKC) include the α, βI, βII (an alternative splicing variant of βI) and γ subtypes and were the first to be cloned. This subset of PKC's is activated by a combination of calcium, phosphatidylserine (PS), and diacylglycerol, and is responsive to phorbol esters. DAG binding via the C1 region increases PKC’s affinity for PS and calcium into the physiological range. The novel isoforms of PKC (nPKC), δ, ε, θ, and η, are calcium-independent, but still require diacylglycerol and phosphatidylserine for activation. The absence of calcium regulation is due to the lack of key residues in the C2 domain. Like the classical PKC’s, these proteins act as receptors for phorbol esters as well. The atypical subgroup, with ζ and λ (mouse t) isoforms, are activated in a unique manner from the other two families. Without a C2 domain and only one cysteine-rich fold in the C1 region, neither diacylglycerol nor calcium regulate the function of the atypical members of PKC. Meanwhile, the enigmatic PKCμ/PKD is a unique family member, possessing a PH domain and an N-terminal hydrophobic region. Furthermore, it lacks the canonical pseudosubstrate region familiar in other PKC's. However, PKCμ is responsive to diacylglycerol and phorbol esters thanks to a C1-like domain. Meanwhile, a group of PKC-related protein kinases or PRK's, have also been identified. There are at least three members, including PRK1 (PKN), PRK2, and PRK3, which share similar properties with the atypical PKC's in that they are insensitive to calcium, DAG, and phorbol esters. Recently, a role for both the RhoA GTPase and PDK1 in PRK activation has been suggested.

The activation of PKC isoforms is a complex, multi-step process involving phosphorylation, membrane localization, and pseudosubstrate release. PKC is phosphorylated at three key residues soon after protein translation. The activation loop of classical, novel, and
atypical PKC’s are phosphorylated within a conserved residue (Thr\textsuperscript{500} of PKCβ\textsubscript{II}) by PDK1. Autophosphorylation within a turn motif in all PKC isoforms (Thr\textsuperscript{641} of PKCβ\textsubscript{II}) and within a hydrophobic motif in classical and novel types (Ser\textsuperscript{660} of PKCβ\textsubscript{II}), primes the kinase for activation. Atypical PKC’s possess a glutamic acid residue at the site corresponding to Ser\textsuperscript{660}, which mimics a phosphorylated residue. Later, agonist-induced production of DAG promotes translocation of PKC to the cell membrane. The actions of calcium, DAG, and PS promote membrane translocation, pseudosubstrate release and creation of a fully competent PKC enzyme.

Remarkably, the role of phospholipases and PKC isoforms in the cytokine regulation of cell survival and proliferation has undergone minimal investigation. However, cytokine-mediated elevation of intracellular DAG has been observed. Phosphatidylcholine-specific phospholipase C (PC-PLC) activity has been detected in cells upon IL-3, GM-CSF, and IL-4 stimulation, with concurrent elevation of diacylglycerol levels.\textsuperscript{199-201} Furthermore, phospholipase D activity has also been detected upon cell treatment with GM-CSF, but not with IL-4 treatment.\textsuperscript{202-204} Since cytokines do not initiate a calcium spike, and since a byproduct of phosphatidylinositol-directed phospholipase C (PI-PLC) activity is IP\textsubscript{3}, an activator of transient calcium fluxes, PI-PLC is not considered to be involved in the intracellular cytokine response.

Numerous investigators have reported cytokine-induced activation of PKC isoforms. MC/9, TF-1, and FDC-P1 cells all display heightened PKC function upon IL-3 and GM-CSF stimulation.\textsuperscript{204-208} Interestingly however, no information exists on IL-4-mediated PKC activity in these factor-dependent cells. Nevertheless, in IL-3 and GM-CSF signaling, PKC isoforms may play a critical role in cell survival. Avoiding apoptosis may require PKC for its ability to induce expression of the pro-survival \textit{bcl-2} gene and to phosphorylate the Bcl-2 protein. For example,
overexpression of PKCe in the TF-1 cell line exhibits elevated bcl-2 transcription and permits cell survival in the absence of IL-3. Chronic phorbol ester exposure results in reduced PKC activity and bcl-2 expression, as well as increased apoptosis even in the presence of IL-3. As well, pharmacological intervention of IL-3-stimulated PKC activity decreases bcl-2 transcripts. Furthermore, inhibition of PC-PLC in TF-1 cells also abrogates IL-3-enhanced bcl-2 expression and promotes apoptosis even in the presence of IL-3. However, some isoforms, including PKCδ and θ, may in fact promote apoptosis in certain cell types. They are targeted and activated by caspase cleavage during apoptosis, and over-expression of these in hematopoietic cells can further drive cell death.

1.4.7. Protein kinase A

The elevation of adenosine 3',5' cyclic monophosphate (cAMP) by extracellular signals is a powerful and ubiquitous intracellular second messenger. The classical mechanism of cAMP production is mediated by receptor activation of GTP-binding proteins (G-proteins). The G-protein subunit, Gαs, activates adenylyl cyclase and enhances conversion of ATP to cAMP. Increased levels of cAMP can then promote protein kinase A (PKA) activation.

The inactive PKA holoenzyme tetramer is comprised of two regulatory (R) and two catalytic (C) subunits (Figure 1.8). The R mammalian isoforms (RIα, RIβ, RIIα, RIIβ) derive from distinct genes and vary in tissue expression, although the physiological importance of these varieties is unresolved. Meanwhile, Cα, Cβ, and Cγ are the three known mammalian genes encoding the catalytic subunits, with three additional splice variants of Cβ (Cβ1, Cβ2, Cβ3). Each C isoform also differs in tissue expression profile and catalytic properties.
Figure 1.8 - PKA activation. Agonist stimulation of cells can in some cases stimulate PKA activation. The canonical pathway involves activation of G proteins, followed by elevated levels of cAMP induced by adenylyl cyclase activation. cAMP is then able to free PKA from the inhibition brought on by its regulatory subunits.
stages, the C subunits are inactive due to the inhibition conferred by the inhibitory pseudosubstrate domain of the R subunits. With increased intracellular cAMP, each R subunit is capable of binding two molecules of cAMP. With this event, their affinity for the C subunit is decreased, permitting dissociation of the R and C complex and activation of PKA catalytic activity. Negative feedback control is achieved through PKA-mediated phosphorylation and activation of cyclic nucleotide phosphodiesterases, which degrade intracellular cAMP and thus reduce PKA activity. PKA is also involved in negative feedback of cAMP production via phosphorylation of membrane receptors, leading to heterologous desensitization whereby receptors exhibit decreased affinity for their respective ligands.

PKA was first noted for its role in the hormonal regulation of glycogen metabolism. It is a serine/threonine protein kinase that targets a consensus sequence containing two basic adjacent residues (RRXS/TX). PKA can regulate gene expression through a variety of transcription factors, including ATF-1 and the cAMP-responsive element binding protein (CREB). The latter is phosphorylated by PKA at Ser^{133}, which enhances CREB’s transactivational ability. In this manner, PKA and cAMP can regulate various gene transcription events via promoter regions containing a cAMP-responsive element (CRE).

Although G-protein activity has not been routinely detected during cytokine signalling, several tyrosine kinase-based receptors have been shown to mediate marked cAMP accumulation, possibly via inhibition of phosphodiesterase isoforms. In B cells, IL-4 can induce sustained elevated cAMP levels and increased PKA activity. Meanwhile, IL-3-induced phosphorylation and inactivation of BAD at Ser^{112} exhibits PKA dependency. However, GM-CSF stimulation of TF-1 cells does not induce a cAMP spike nor PKA activity. Equally
confusing is PKA’s role in cell survival and cell proliferation. In B cells, forskolin-induced
elevation of cAMP results in enhanced apoptosis, while microinjection of cAMP into the
myeloid cell line IPC-81 displays a similar effect.\textsuperscript{222,223} In contrast, cAMP can abrogate
apoptotic events upstream of caspase 3 in human neutrophils.\textsuperscript{224} As such, it appears that the
effect of cAMP and PKA in cell survival is potentially cell type- and stimulus-specific.

1.4.8. p70\textsuperscript{S6K}

Targeting the S6 protein of 40S (small) ribosomal subunit, p70/p85\textsuperscript{S6K} are splice variants
of a serine/threonine kinase implicated in various mitogenic signaling pathways.\textsuperscript{225} The p85\textsuperscript{S6K}
isoform has an amino-terminal 23-amino acid nuclear localization signal that p70\textsuperscript{S6K} lacks. The
functional significance of these two isoforms is still being unraveled, and the term p70\textsuperscript{S6K} is still
often used to describe both species.

The regulation of p70\textsuperscript{S6K} is a complex process requiring multiple upstream inputs.
Activation of p70\textsuperscript{S6K} involves serial phosphorylations within the regulatory domain,
transactivation loop, and autoinhibitory domain. Activation via these critical phosphorylations is
controlled by the mammalian target of rapamycin (mTOR/FRAP) and PDK1, both of which are
essential for protein activation.\textsuperscript{226,227} The latter targets a conserved residue (Thr\textsuperscript{252}) that is
homologous to Thr\textsuperscript{308} in PKB and other residues throughout the AGC family of protein
kinases.\textsuperscript{160}

The cytokine activation of p70\textsuperscript{S6K} has been documented in a variety of cases, including
treatments with IL-3, GM-CSF, and IL-4.\textsuperscript{127,228-231} The kinase may mediate cell proliferation
through the phosphorylation of S6 ribosomal proteins. This enhances protein translation of a
class of mRNA containing tract of oligopyrimidines (TOP) at the 5' terminus. Many of these mRNA encode for proteins involved in cell cycle control, and p70^{S6K} activity is in most cases essential for progression past the G1 phase and thus cell proliferation. However, p70^{S6K} may not be essential for cell proliferation in all cases.\textsuperscript{230, 232}

1.5. Glycogen synthase kinase-3 (GSK-3)

In the 1980's, biochemists were keen to characterize the enzymes responsible for metabolic reactions. During this search, GSK-3, a serine/threonine protein kinase, was identified as an enzyme that phosphorylates and inactivates glycogen synthase, a key checkpoint in the regulation of glycogen synthesis.\textsuperscript{233, 234} In particular, insulin upregulation of glycogen production was found to be a result of GSK-3 inhibition and subsequent glycogen synthase activation. Since that discovery, the manner in which GSK-3 isoforms are regulated has been under scrutiny. Interestingly, since its initial characterization, the level of GSK-3 activity has been implicated in regulating a variety of cell functions including embryonic development, cell proliferation and survival, protein translation, cell cycle progression, and protein degradation.\textsuperscript{235-240} However, there has been no published data into GSK-3 and its role in cytokine stimulation of factor-dependent hematopoietic cells.

1.5.1. Isoforms

GSK-3, although originally purified from skeletal muscle, is ubiquitously expressed among cell types and evolutionarily conserved among diverse species such as yeast, plants, and mammals.\textsuperscript{239, 241, 242} In lower organisms, a large family of GSK-3 homologs have been
characterized. In mammals, genetic screening has identified two distinct isoforms of GSK-3. The 51 kDa GSK-3α and the 47 kDa GSK-3β share substantial sequence homology throughout the key regulatory and catalytic regions. Although most studies have focused on either GSK-3α or GSK-3β, little has been published with respect to the differences between the two. However, there is some evidence of dissimilarity in the regulation, function, and substrate specificity of each mammalian isoform.

1.5.2. Regulation of GSK-3

Extracellular stimuli typically lead to the inactivation of the constitutively active GSK-3 isoforms. The best-studied model of GSK-3 inactivation involves insulin stimulation of glycogen production. Further studies has found that a variety of stimulants can inhibit GSK-3 activity, including growth factors, immunological events, cardiac hypertrophy, and hypoxia.

These external signals can promote GSK-3 inactivation through the phosphorylation of an amino-terminus serine residue found in all GSK-3 isoforms studied. The modification of the Ser and Ser residues of GSK-3α and GSK-3β respectively, promote inactivation of GSK-3 protein kinase activity. Conversion of this critical serine residue to a non-phosphorylatable alanine results in a constitutively active GSK-3 enzyme that is non-responsive to extracellular signals.

A variety of protein kinases have been shown to phosphorylate these serine residues in vitro including PKB, p90RSK, PKA, ILK, and some PKC isoforms. If and how each of these kinases modulate GSK-3 activity is still under investigation in various model systems. The
classical model of insulin-mediated GSK-3 inactivation was found to involve the PI3-K and PKB pathways. The presence of a dominant negative PKB can abrogate insulin's effect on GSK-3, thus demonstrating the essential need for PKB activity in the serine phosphorylation of GSK-3. In fact, various other stimuli utilize the PI3-K/PKB pathway to inactivate GSK-3. In cultured neurons, insulin growth factor-1 (IGF-1) blocks GSK-3 activity, a process that is sensitive to PI3-K inhibitors. Furthermore, hypoxic and hypertrophic stimuli require the PI3-K/PKB pathway to mediate their inhibitory effects on GSK-3. However, there are several studies that have demonstrated PI3-K/PKB-independent regulation of both isoforms of GSK-3.

Several other growth factors mediate inhibition of GSK-3 via the MAPK/RSK pathway. In PC12 cells, epidermal growth factor (EGF) and nerve growth factor (NGF) inhibition of GSK-3 is blocked by the MEK inhibitor, PD98059. In vitro, GSK-3α was phosphorylated at Ser\(^{21}\) and inactivated by p90\(^{\text{rsk}}\). Furthermore, RSK has been implicated in mediation of GSK-3 activity in Xenopus.

As well, a role for some protein kinase C (PKC) isoforms in GSK-3 inhibition has also been demonstrated. In embryonic development, PKC mediates GSK-3 inactivation, despite an insulin/PKB/GSK-3 pathway in the same cell line. Over-expression of PKC\(_{\beta}\) in intestinal epithelium cells leads to deceased GSK-3 activity. As well, phorbol esters have been demonstrated to lead to phosphorylation and inactivation of GSK-3 in a variety of cellular models. However, there is evidence that each isoform of GSK-3 shows varying susceptibility to PKC's actions. In particular, GSK-3β is more responsive to many PKC isoforms than is GSK-3α.
Even the cAMP-dependent protein kinase (PKA) can associate and phosphorylate GSK-3α and β at the inhibitory serine residues both in vitro and in vivo. In cerebellar granule neurons, elevated cAMP promotes Ser⁹ phosphorylation of GSK-3 and subsequent enzyme inhibition.

Meanwhile, in EGF-treated human myoblasts, there is a decrease in GSK-3 activity and upregulation of glycogen synthesis. The increase in glycogen production is blocked by the mTOR inhibitor, rapamycin, which suggests a possible link between GSK-3 regulation and p70S6K. In fact, GSKα can be inactivated through phosphorylation of Ser²¹ by p70S6K.

Finally, another protein kinase implicated in GSK-3 control is the integrin-linked kinase (ILK). ILK is a serine/threonine protein kinase that associates with integrin subunits and whose activity is regulated by cell interaction with the extracellular matrix. ILK can directly phosphorylate and inactivate GSK-3 at the key serine residue. Transfected ILK promotes GSK-3 inactivation while those transfected with a kinase-dead version of ILK demonstrate enhanced GSK-3 activity. Furthermore, ILK has been described as the elusive PDK2, a PI3-K-dependent kinase that phosphorylates Ser⁴⁷³ of PKB, thus regulating PKB activation. As such, it remains to be determined whether or not ILK's primary effect on GSK-3 in vivo is direct or via the enhanced PKB activity.

In addition to the inhibitory serine phosphorylation sites, GSK-3 can also be phosphorylated on key tyrosine residues. These conserved residues (Tyr²⁷⁹ in GSK-3α and Tyr²¹⁶ in GSK-3β) are phosphorylated during the resting state, and are necessary for full GSK-3 activity. In fact, mutation of this site to phenylalanine results in a highly inactive GSK-3 possessing only a small level of residual catalytic activity. The importance of tyrosine
phosphorylation in regulating GSK-3 activity is demonstrated with GSK-3 constructs lacking the amino-terminus region and thus the critical serine residue. In these mutants, the enzyme is still inactivated through dephosphorylation of the tyrosine.\textsuperscript{267} The kinase responsible for tyrosine phosphorylation of GSK-3 is a novel study, although recent evidence suggests that PYK2 may serve that role.\textsuperscript{269} Although it appears these tyrosine residues are crucial for GSK-3 regulation, they might not be essential for GSK-3 activity in all systems.\textsuperscript{252} For example, in HEK-293 cells, neither IGF-1 nor insulin treatment, which lead to inactivation of GSK-3, induced tyrosine dephosphorylation of GSK-3\(\beta\). As such, the relationship between tyrosine and serine phosphorylation of GSK-3 in growth factor-mediated inhibition remains unsolved. Furthermore, there is evidence that GSK-3 can autophosphorylate itself at other amino acid residues.\textsuperscript{270} Therefore, this complex regulation of GSK-3 by phosphorylation is a continuing study.

\textbf{1.5.3. GSK-3 in embryonic development}

The study of GSK-3 has been conducted extensively in various embryonic development models. GSK-3 first garnered attention in \textit{Drosophila melanogaster} where it was implicated in the Wnt/Wg (wingless) signaling pathway. The Wnt ligand binds to serpentine receptors of the Frizzled family, leading to activation of the protein Dishevelled (Dsh).\textsuperscript{271} Dsh, through an unknown mechanism, promotes inactivation of the shaggy/zeste-white 3 protein, a homolog of the mammalian GSK-3.\textsuperscript{272,273} This inactivation leads to increased stability and nuclear accumulation of the \(\beta\)-catenin protein, a promoter of the lymphoid enhancer factor/T cell factor (Lef/Tcf) family of transcription factors. The resulting gene products are involved in the
formation of the dorsal axis during *Drosophila* development. In contrast, GSK-3 activity permits phosphorylation of β-catenin, ubiquitination, and subsequent proteasome-mediated degradation.

Meanwhile, in *Xenopus*, GSK-3 also modulates embryo polarity and development. Rotation of the outer cytoplasm relative to the inner cytoplasm upon fertilization results in rotation of the dorsalizing activity to the side opposite to the site of sperm contact.\(^{273,274}\) This promotes GSK-3 inactivation and nuclear accumulation of β-catenin. Meanwhile, the ventral side of the embryo exhibits active GSK-3 and limited β-catenin levels. As such, a polarity is established within the embryo.

Finally, in *Dictyostelium*, cell polarity is also mediated by GSK-3 activity. During starvation conditions, a buildup in cAMP levels results in the activation of the tyrosine kinase, ZAK1. This kinase is able to phosphorylate GSK-3 at the key tyrosine residues and promote GSK-3 activation. In turn, this regulates genes that direct cell determination.\(^{275,276}\)

Although much of the GSK-3 developmental studies have emphasized primitive organisms, embryo development and tumorigenesis in higher organisms appears to also utilize the canonical Wnt/Wg pathway involving GSK-3.\(^{277,278}\)

### 1.5.4. Substrates of GSK-3

Since the initial discovery of GSK-3 in glycogen metabolism, a number of other GSK-3 targets and effects have been noted. For the most part, active GSK-3 appears to be an antagonist of events leading to cell proliferation. In fact, GSK-3 has the potential to mediate a variety of cell functions through a number of cellular targets, including cellular metabolism, cell
proliferation, cell survival, and protein translation. Furthermore, GSK-3's role in the pathogenesis of Alzheimer's disease is of recent interest.

Many substrates of GSK-3 require pre-phosphorylation at a serine residue in the +4 position relative to the GSK-3 target site. In skeletal muscle, GSK-3 will phosphorylate and activate eIF-2B at Ser\(^{535}\) of its epsilon subunit, but only with a priming phosphorylation at Ser\(^{539}\). Mutation of Ser\(^{539}\) to a non-phosphorylatable alanine results in abrogation of GSK-3 phosphorylation at Ser\(^{535}\). However, GSK-3 can also target peptide regions containing no prior phosphorylation, as in the case of β-catenin. Interestingly, it is this variation in substrate recognition that allows GSK-3 to mediate certain downstream cellular effects without eliciting others. For example, insulin stimulation abrogated GSK-3 phosphorylation of the pre-phosphorylated glycogen synthase, but had no effect on GSK-3-mediated stability of β-catenin, a protein requiring no prior phosphorylation. Therefore, in a cellular context, GSK-3 can sort out its multiple effector pathways through differences in substrate modification.

GSK-3's founding role was in regulating cell metabolism, particularly the regulation of glycogen synthesis. Glycogen synthase is the rate-limiting enzymatic process in glycogen biosynthesis. At the carboxyl-terminus, GSK-3 can phosphorylate four serine residues (Ser\(^{640}\), Ser\(^{644}\), Ser\(^{648}\), Ser\(^{652}\)), which are known as sites 3a, 3b, 3c, and 4 respectively. Phosphorylation of each site is dependent on prior phosphorylation at the +4 residue, beginning with casein kinase 2 (CK2) phosphorylation of Ser\(^{656}\). This sequential phosphorylation leads to inactivation of glycogen synthase activity.

Cell proliferation and cell survival may also be regulated by GSK-3. For example, specific inhibitors of GSK-3 protect peripheral and central nervous system neurons from
apoptosis caused by growth factor withdrawal and PI3-K inhibition.\textsuperscript{261,282,283} As well, lithium inhibition of GSK-3 can prolong cell proliferation in various cell lines.\textsuperscript{236,284} Furthermore, a dominant negative GSK-3 transfectant can also prevent apoptosis.\textsuperscript{285} In contrast, constitutively active GSK-3 reduces cell proliferation, while overexpression of active GSK-3 can cause significant increase in cell death.\textsuperscript{236,283,261,285} However, active GSK-3 may not be essential for all forms of apoptosis.\textsuperscript{283,286} In fact, murine fibroblasts lacking GSK-3\textsubscript{β} demonstrated a greater impairment in cell survival.\textsuperscript{237}

GSK-3's pro-survival effects may be mediated in part by its interaction with various transcription factors. For example, phosphorylation of c-jun, a component of the AP-1 transcription factor, by GSK-3 \textit{in vitro} can inhibit AP-1's DNA binding capacity.\textsuperscript{287} Likewise, the degradation of c-myc is mediated by phosphorylation at Thr\textsuperscript{58}, a GSK-3 target site.\textsuperscript{288} GSK-3 can also regulate the nuclear accumulation of the calcium-regulated transcription factor NF-AT. Calcineurin, a calcium-dependent phosphatase, dephosphorylates key serine sites on NF-AT, thus promoting nuclear entry. However, GSK-3 can oppose this action through phosphorylation of these same serine residues, thereby supporting nuclear export of NF-AT.\textsuperscript{236,289} GSK-3 has also been implicated in modulating various other transcription factors including CREB, heat shock factor-1 (HSF-1) and NF-κB.\textsuperscript{290-292}

Cell cycle progression and protein translation may also be altered by GSK-3's actions. GSK-3 may promote cyclin D1 expression indirectly through β-catenin since the Lef/Tcf group of transcription factors regulates cyclin D1 transcription.\textsuperscript{293} Cyclin D1 phosphorylates and inactivates the retinoblastoma (RB) tumour suppressor protein allowing the cell to pass the G1/S phase restriction point. Furthermore, GSK-3 can also directly influence cyclin D1 through
phosphorylation of Thr$^{286}$. This modification promotes increased proteasomal degradation of the cyclin.\textsuperscript{294} Fumonisin B, a well-known rat hepatocarcinogen, leads to inhibition of GSK-3\(\beta\) and overexpression of cyclin D1.\textsuperscript{295} As such, GSK-3 may be a crucial factor in tumorigenesis. Meanwhile, the debate continues on GSK-3's role in regulating eIF-2B. Protein translation is mediated by this multimeric eukaryotic initiation factor, which can be downregulated through phosphorylation of the \(\epsilon\) subunit at Ser$^{535}$ by GSK-3.\textsuperscript{238} Indirect data shows a correlation between IGF-1-induced GSK-3 inactivation in cultured neurons, and activation of eIF-2B.\textsuperscript{247} As well, insulin treatment of skeletal muscle results in GSK-3-mediated phosphorylation and activation of eIF-2B via the PI3-K/PKB pathway.\textsuperscript{296} In contrast, when EGF-induced inactivation of GSK-3 is blocked, there is no change in eIF-2Be phosphorylation.\textsuperscript{255}

Finally, GSK-3's role in the pathology of neurodegenerative diseases is also a hot topic. In Alzheimer's disease, there is an accumulation of neurofibrillary fibers, which many believe to be a causative factor of this devastating disease. These fibers contain large amounts of hyperphosphorylated tau, and recent work has suggested that GSK-3 can directly phosphorylate tau.\textsuperscript{297,298} Furthermore, inhibition of GSK-3 can block hyperphosphorylation of tau both \textit{in vitro} and \textit{in vivo}.\textsuperscript{299} In fact, transgenic mice expressing the GSK-3\(\beta\) gene within the brain demonstrate advanced tau phosphorylation and high rates of neurodegeneration.\textsuperscript{300} As such, GSK-3 represents an interesting focus in the on-going study of Alzheimer's disease.
CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

- H$_3^{[32]P}$O$_4$
- 4-hydroxytamoxifen
- Acetic acid
- Acrylamide
- Acrylamide:bis
- Ammonium persulphate
- Adenosine 5'-triphosphate
- β-mercaptoethanol
- Bis
- Bovine serum albumin
- Bromophenol blue
- Butanol
- Calphostin C, Cladosporium cladosporioides
- Chloroform
- D609
- Diethylether
- DMEM
- DMSO
- Dithiothreitol
- Enhanced chemiluminescence reagents (ECL)
- Ethanol
- EDTA
- EGTA
- Ficoll-Paque
- Forskolin, Coleus forskohlii
- γ-$^{[32]P}$ATP
- Glycine
- Gö 6976
- Gö 6983
- Glycerol
- HEPES
- Hydrochloric Acid
- L-glutamine
- Leupeptin
- LY294002
- Magnesium chloride
- Methanol
- Microcystin

ICN
Sigma
Fisher Scientific
BioRad
BioRad
Sigma
Fisher Scientific
BioRad
Fisher Scientific
Fisher Scientific
BDH
Calbiochem
Fisher Scientific
Calbiochem
Fisher Scientific
Gibco
Fisher Scientific
Sigma
Amersham
Commercial Alcohols
BDH
BDH
Amersham
Sigma
ICN
BioRad
Calbiochem
Calbiochem
Fisher Scientific
Sigma
Fisher Scientific
Gibco
Sigma
Calbiochem
Fisher Scientific
Fisher Scientific
Calbiochem
Penicillin/streptomycin
Pepstatin A
Petroleum ether
Phorbol myristic acetate (PMA)
Phosphatidic acid
Phosphoric acid
PMSF
Ponceau S concentrate
Potassium oxalate
Rapamycin
Ro-31-8220/bisindolylmaleimide IX
Ro-31-8425/bisindolylmaleimide X
Rottlerin
Skim milk
Sodium azide
Sodium chloride
Sodium dodecyl sulphate (SDS)
Sodium fluoride
Sodium molybdate
Sodium pyruvate
Sodium orthovanadate
Soybean trypsin inhibitor
TEMED
Tris
Triton X-100
Tween-20
U0126
U73122
Urea
Wortmannin/KY 12420

2.1.2. Disposables

Aluminum-backed silica plates
Autoradiography film
Conical tubes
Disposable pipettes
Eppendorfs
Filter paper
Gel loading tips
Glass pipettes
Kimwipes
Latex gloves

Gibco
Sigma
Sigma
Sigma
Sigma
Sigma
Fisher Scientific
Sigma
BDH
VWR
Calbiochem
Calbiochem
ICN
Safeway Canada
BDH
Fisher Scientific
Fisher Scientific
Fisher Scientific
Sigma
Sigma
Sigma
Boehringer Mann.
BioRad
BioRad
Boehringer Mann.
BDH
Promega
Calbiochem
Fisher Scientific
Calbiochem
Liquid nitrogen
Membrane Filters
Nitrocellulose
P81 phosphocellulose
Petri plates
Pipette tips
Scintillation fluid
Scintillation vials

**2.1.3. Proteins/peptides**

CGMI
Crosstide
Fetal bovine serum
Human recombinant GM-CSF
Murine recombinant GM-CSF
Murine recombinant interleukin-3
Murine synthetic interleukin-4
Phospho-glycogen synthase peptide-2
Protein G-Agarose
RPMI-1640
WEHI-3 (from WEHI-3 cell line)

**2.1.4. Antibodies**

Akt1/PKBα polyclonal 1 μg/ml
GSK-3α polyclonal
GSK-3α polyclonal 1:1000
GSK-3β polyclonal 1:1000
Phospho-Akt (Ser^{473}) polyclonal 1:1000
Phospho-CREB (Ser^{133}) polyclonal 1:1000
Phospho-GSK-3α/β (Ser^{21/9}) polyclonal 1:1000
Phospho-GSK-3α/β (Ser^{9}) polyclonal 1:1000
Phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}) E10 monoclonal 1:1000
Phospho-p70^{56K} (Thr^{421}/Ser^{424}) polyclonal 1:1000
PI3-K (p85) polyclonal 1:2000
p70^{56K} polyclonal 1:1000
Rsk/MAPKAP-K1 polyclonal 1:1000

Praxair
Nalgene
BioRad
Whatman
VWR
Pharmacia
VWR

Chris Brown, Calgary
Upstate Biotech
Gibco
R&D
R&D
R&D
James Wieler, UBC
Upstate Biotech
Gibco
Gibco
ATCC

Upstate Biotech
Upstate Biotech
Santa Cruz
Stressgen
Cell Signaling
Cell Signaling
Cell Signaling
Cell Signaling
Cell Signaling
Cell Signaling
Upstate Biotech
Cell Signaling
Upstate Biotech
Cell Signaling
Upstate Biotech
2.2. Methods

2.2.1. Cell culture

2.2.1.1. FDC-P1, MC/9 and MC/9 (PKB-ER)

The murine mast cell line MC/9 and the murine myeloid progenitor cell line FDC-P1 (American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 growth medium, supplemented with 10% heat-inactivated FBS, 5-10% WEHI-3 conditioned medium containing murine recombinant IL-3, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 nM β-mercaptoethanol, and penicillin/streptomycin. Starvations were performed for 4 to 6 hours in the absence of any WEHI-3. All incubations were carried out in a humidified incubator at 5% CO₂. MC/9 (PKB-ER) cells are MC/9 clones expressing PKB fused with the estrogen receptor. This line was cultured and treated in the same manner as MC/9.

2.2.1.2. TF-1

The human erythroblast cell line, TF-1 (American Type Culture Collection, Manassas, VA), was grown in RPMI-1640 growth medium, supplemented with 10% FBS, 1% CGMI conditioned media containing human recombinant GM-CSF, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 nM β-mercaptoethanol, and penicillin/streptomycin. Starvations were performed for 12 to 16 hours in the absence of any CGMI. All incubations were carried out in a humidified incubator at 5% CO₂.
2.2.2. Cytokine stimulation

Starved cells were collected, spun down (1500 rpm, 5 minutes), and resuspended in warm RPMI-1640 with 20 mM HEPES to \(5 \times 10^6\) cells/ml. Immediately before and during stimulations, the cells were kept in a 37°C water bath and routinely vortexed to keep treatments homogenous. Upon completion of a stimulation time point, cells were spun down at 20,000 x g for 1 minute. The supernatant was aspirated, and the cell pellet resuspended in 16 μl of ice-cold lysis buffer (50 mM Tris-Cl, pH 7.7, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na3VO4, 1 mM NaMoO4, 40 μg/ml PMSF, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μM pepstatin). Cell lysates were left on ice for 10 minutes after which they were again spun at 20,000 x g for 1 minute. The supernatant was extracted and either used for immunoprecipitation or immunoblotting.

2.2.3. Protein Biochemistry

2.2.3.1. Immunoprecipitation

The lysates of approximately 8 million cells were reconstituted in 500 μl lysis buffer. Either 2 μg of anti-PKBα or 2 μg of anti-GSK-3α were added, and the samples were then placed at 4°C on a rotator for 1 hour. Protein G-Agarose was added (20 μl) and samples were again rotated for 1 hour. Afterwards, the agarose beads were isolated through centrifugation at 10,000 rpm for 1 minute and then washed as needed.
2.2.3.2. Immunoblotting

The lysates of approximately 1 million cells (16 μl) were added to 4 μl 5X SDS-PAGE loading buffer (14.81 ml 20% SDS, 9.26 ml 1M Tris, pH 6.8, 14.81 ml glycerol, 3.70 ml 0.1% bromophenol blue, 7.41 ml β-mercaptoethanol) and boiled for 10 minutes. Samples were used immediately or frozen down at −20°C. For immunoblotting, a 12.5% low-bis (120:1 acrylamide:bis) polyacrylamide gel (6.64 ml 30% acrylamide, 3.52 ml water, 1.68 ml 1% bis, 4 ml 1.5 M Tris, pH 8.8, 160 μl 10% SDS, 112 μl 10% APS, 11.2 μl TEMED) and a 5% stacking gel (453 μl 1.5 M Tris, pH 6.8, 50 μl 10% SDS, 835 μl 37.5:1 acrylamide:bis, 3.66 ml water, 16.7 μl 10% APS, 5 μl TEMED) were used for protein separation. Gels were run at 200 V in reservoir buffer (3 g. Tris, 14.4 g. glycine, 1 g. SDS; final volume of 1 L) for approximately 45 minutes, or until the dye front had bled off. The gel contents were then transferred to a nitrocellulose membrane at 40 mA for 1 hour 15 minutes in a semi-dry transfer apparatus. Transfer buffer consisted of 5.81 g. Tris, 2.93 g. glycine, 0.0375 g. SDS, and 200 ml methanol, in a final volume of 1 L. The nitrocellulose was soaked briefly in Ponceau S stain to visualize successful protein transfer, and then blocked in 3% skim milk in TBS for 1 hour at room temperature. Afterwards, blots were washed 3 times in TBS with 0.05% Tween-20 added (TBST) for 10 minutes each wash. Overnight incubation of the nitrocellulose in primary antibody followed. Antibody dilutions were in TBST as noted in section 2.1.4. The blots were again washed 3 times with TBST for 10 minutes each. Afterwards, the appropriate secondary antibody conjugated to the horseradish peroxidase enzyme was added at 1:5000 dilution for 1 hour in TBST. Three TBST washes followed, with a final wash in ECL for 1 minute. Proteins
were visualized through film exposure of the blot. Pre-stained molecular weight markers were used and their positions were noted on the developed film.

2.2.3.3. Stripping blots

Nitrocellulose blots were re-probed by stripping off the previous antibodies. Blots were kept moist in TBS, and then soaked in stripping buffer (60 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) for 30 minutes at 50°C with frequent agitation. The nitrocellulose was then washed with copious amounts of TBST, and then re-blocked in 3% skim milk. The immunoblotting procedure was continued from this point.

2.2.4. Kinase activity assays

2.2.4.1. GSK-3α

Anti-GSK-3α (2 μg) was added to cell lysates for one hour, followed by an hour with 20 μl of Protein G-Agarose, all undergoing rotation at 4°C. The beads were then washed 3 times with lysis buffer and once with kinase buffer (5 mM HEPES, pH 7.4, 0.5 mM EDTA, 0.2 mM Na₃VO₄). They were resuspended in 30 μl kinase buffer with 75 μM phospho-glycogen synthase peptide-2 substrate added. The assay proceeded with the addition of 10 μl containing 5 μCi γ-[³²P]ATP, 500 μM ATP, and 75 mM MgCl₂ in kinase buffer, with the reaction continuing for 15 minutes at 30°C. Immediately after, the contents were briefly spun down, and the phosphorylated products were isolated by spotting 25 μl of the reaction supernatant onto P81 phosphocellulose paper. The paper was allowed to dry for a few minutes, and was then washed six times in 1% phosphoric acid for 4 minutes each. Quantification was achieved through scintillation counting
of each P81 square. Background controls were done simultaneously in samples containing no cell lysate.

2.2.4.2. PKB

Cell lysates were immunoprecipitated with 2 μg of anti-PKB-α antibody for one hour under rotation at 4°C, followed by an addition of 20 μl of Protein G-Agarose for one hour. Upon completion, the beads were washed 3 times with lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 5 mM EGTA, 0.2 mM Na3VO4, 1 mM DTT, 1 μM microcystin, 1 mM MgCl2, 40 μg/ml PMSF, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). The beads were then resuspended in 10 μl kinase buffer and 10 μl of 100 μM Crosstide substrate. The assay was initiated with the addition of 10 μl containing 10 μCi γ-[32P]ATP, 500 μM ATP, and 75 mM MgCl2 in kinase buffer. The reaction continued for 18 minutes at 30°C after which the contents were briefly spun down. The phosphorylated products were isolated by spotting 20 μl of the reaction supernatant onto P81 phosphocellulose paper. The paper was allowed to dry for a few minutes, and was then washed six times in 1% phosphoric acid for 4 minutes each. Quantification was achieved through scintillation counting of each P81 square. Background controls were done simultaneously in samples lacking cell lysate.

2.2.5. Lipid analysis

2.2.5.1. Thin layer chromatography (TLC)

Cells were washed three times and starved for the final 2 hours in phosphate-free RPMI-1640 with 20 mM HEPES, and labeled with 0.25 mCi/ml [32P]PO4 at approximately 5X10^6
cells/ml. After 2 hours, the cells were spun down and resuspended in the same volume of RPMI-1640 with 20 mM HEPES in polypropylene tubes. Stimulation proceeded as in section 2.2.2. To terminate the reaction, 1.6 ml of 2:1 chloroform:methanol was added, and the contents were shaken vigorously. Another 1 ml of chloroform was then added to aid in separation of the organic and aqueous phases. The samples were spun at 3000 rpm for 3 minutes and the lower organic phase was removed and kept. Lipids were dried down using a speed-vac centrifuge, and resuspended in 70 μl chloroform. Meanwhile, aluminum-backed silica plates were soaked in 1% potassium oxalate for 1 minute and dried overnight. The plate was then baked at 80-100°C for 60 minutes. To the plate, 7 μl of each lipid sample was spotted and 5 μg of unlabelled phosphatidic acid standard was included on each side. The plate was placed in a solvent system containing 9:1:1 (v/v) chloroform:methanol:acetic acid, and the solvent front was allowed to climb to 10.5 centimetres from the origin. After a 10-15 minute drying period, the plate was soaked in a second solvent system (60:40:1 petroleum ether:diethylether:acetic acid) with this solvent front reaching the top of the plate. The silica plate was finally dried for 4 hours, and the unlabelled standards were visualized through iodine staining. The labeled lipids were detected through overnight exposure of a phosphoimaging plate.

2.2.5.2. Densitometry analysis

Quantification of lipid levels was achieved through phosphoimaging development and analysis using BioRad's Quantity One densitometry program.
CHAPTER 3: CYTOKINE REGULATION OF GSK-3

3.1. Rationale and Hypothesis

Regulation of cell survival and proliferation is a vital factor within the hematopoietic system. Since these cellular events are modulated by cytokines in many cases, the signaling cascades propagated by these growth factors deserve scrutiny. With mounting evidence of GSK-3’s role in cell proliferation and survival, an investigation into the effects of cytokines on GSK-3 seems warranted.236,237

Since a diverse array of extracellular signals can target GSK-3, it would be of no surprise if cytokines also show the same effect.236,247-249 In particular, serine phosphorylation of GSK-3 isoforms upon cytokine stimulation would be anticipated. Whether or not the PI3-K/PKB pathway is sufficient and/or essential to any GSK-3 phosphorylation event is difficult to postulate considering the diverse upstream kinases that have demonstrated an effect on GSK-3.245,251,255,260,263 As well, it would be of interest to determine whether or not the GSK-3α and GSK-3β isoforms share similar regulatory features in these model systems.

3.2. Results

Effects of cytokine stimulation on serine phosphorylation of GSK-3 – The murine MC/9 mast cell line is able to proliferate in IL-3- or GM-CSF-supplemented media. In contrast, media containing IL-4 only permits prolonged cell survival of MC/9 cells, but no proliferative response. An approximate dosage range and an appropriate incubation time for each cytokine was established from previous work.136,173 Using these values as a reference point, MC/9 cells were treated with a dose response of each cytokine. The phosphorylation status of Ser9 and Ser21 of
GSK-3β and GSK-3α respectively was analyzed using an equal mix of the phospho-GSK-3α/β antibody and the phospho-GSK-3β antibody (Figure 3.1 A). The two antibodies are required since the phospho-GSK-3α/β antibody alone displays a weak affinity for phospho-GSK-3β (data not shown). With IL-3 stimulation, both GSK-3 isoforms exhibited increased serine phosphorylation after 10 minutes, correlating with the increase in cytokine dosage. Even with the presence of both antibodies, the signal was strongest with the GSK-3α isoform. While the level of GSK-3α phosphorylation was saturated across all dosages of IL-3 used, phosphorylation of GSK-3β reached a maximal level with 1 μg/ml IL-3 treatment. As such, all further experiments performed with IL-3 were conducted at that dosage. Likewise, both IL-4 and GM-CSF induce elevated levels of serine phosphorylation of GSK-3α and β. Maximum levels of GSK-3 phosphorylation were reached at 10 μg/ml IL-4 and 25 ng/ml GM-CSF at 10 minutes and 5 minutes respectively. These doses and time points were also used in all subsequent experiments.

The human TF-1 cell line also proliferates in the presence of GM-CSF. Appropriate doses of GM-CSF and an ideal incubation time have also been previously established. A dose response ranging from 0.1 ng/ml to 10 ng/ml demonstrated increased serine phosphorylation of GSK-3 at a maximum induced by 10 ng/ml GM-CSF after 5 minutes (Figure 3.1 B).

Another factor-dependent cell line of murine origin is FDC-P1. Like MC/9, this cell line will proliferate in response to IL-3 and GM-CSF, but not IL-4. The cytokine doses and treatment times used were equivalent to those established for maximal serine phosphorylation of GSK-3 in MC/9. Despite a high background level of phospho-GSK-3α in the unstimulated sample, all three cytokines promoted a recognizable increase in the phosphorylation of GSK-3α (Figure 3.1
C). Likewise, GSK-3β also showed an increased phosphorylation signal with each cytokine. Once again, these treatment dosages were maintained in all further experiments utilizing FDC-P1 cells. Interestingly, there is a third unidentified band of about 40 kDa in size that was consistently observed in all phosphoserine-GSK-3 immunoblots utilizing FDC-P1 cell lysates. Like GSK-3α and GSK-3β, this band was found to increase in intensity with the presence of each cytokine. Whether this is an example of non-specific binding to a completely different protein, or proof of proteolytic cleavage of GSK-3, remains to be determined. However, there is no previous mention of a physiological GSK-3 cleavage event in the literature.
Figure 3.1. Effect of cytokines on serine phosphorylation of GSK-3α/β. A. MC/9 cells were cytokine-starved and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved and stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). C. FDC-P1 cells were cytokine-starved and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3. These results are typical of at least 4 experiments.
C

FDC-P1

IL-3

DMSO 1μg/ml

- P - GSK-3α
- P - GSK-3β
- ?

IL-4

DMSO 10μg/ml

- P - GSK-3α
- P - GSK-3β
- ?

GM-CSF

DMSO 25ng/ml

- P - GSK-3α
- P - GSK-3β
- ?
Cytokines do not alter expression of the GSK-3 proteins – Although the short cytokine incubation times utilized in Figure 3.1 make it unlikely that the effects seen on GSK-3 are the result of increased protein levels, it was prudent to establish that the apparent increase in GSK-3 phosphorylation was indeed due to post-translational modification of the protein.

In Figure 3.2 A, MC/9 cells were stimulated with each of the three cytokines at the established doses and incubation times. As before, IL-3, IL-4, and GM-CSF all induced increased serine phosphorylation of both GSK-3α and GSK-3β. After stripping the blot of this antibody mix, they were re-probed with antibodies specific for GSK-3α and GSK-3β, regardless of phosphorylation state. As the figure illustrates, there was no change in the protein levels of either isoform of GSK-3. As such, the changes demonstrated with the phospho-GSK-3 antibodies were solely due to increases in phosphate content at the amino-terminal serine residue of GSK-3. The use of the p85 antibody was to simply insure equal total protein content throughout the experimental samples. Likewise, Figure 3.2 B established the consistency of GSK-3 protein content between GM-CSF-stimulated TF-1 cells and unstimulated ones.
Figure 3.2. Effect of cytokines on GSK-3α/β is post-translational. A. MC/9 cells were cytokine-starved and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved and stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, GSK-3α, GSK-3β, and p85. These results are typical of 2 experiments.
Role of PI3-K in cytokine-induced GSK-3 inactivation – The first reports focusing on the regulation of GSK-3 involved the insulin-mediated inactivation of GSK-3. This inhibition was shown to require the phosphatidylinositol-3 kinase (PI3-K)/Protein kinase B (PKB) pathway. In previous studies, pharmacological abrogation of PI3-K activity eliminated phosphorylation of GSK-3 at the crucial amino-terminus serine residues. To assess the role of this pathway in the phosphorylation of GSK-3 in factor-dependent hematopoietic cell lines, two structurally distinct inhibitors of PI3-K were used. Both LY294002 (IC\textsubscript{50} = 1.4 \mu M) and wortmannin (IC\textsubscript{50} = 3 nM) are potent catalytic site inhibitors of PI3-K. Cells were pretreated with either inhibitor for 15 minutes prior to stimulation, at dose ranges well-established for \textit{in vivo} PI3-K inhibition. IL-3- and GM-CSF-stimulated phosphorylation of both GSK-3 isoforms was unchanged with either 100 \mu M LY294002 or 100 nM wortmannin present (Figure 3.3 A). Assessment of PKB phosphorylation at Ser\textsuperscript{473}, which is known to be phosphorylated downstream of PI3-K activation, allowed for assurances of total PI3-K inhibition. However, despite a total loss of Ser\textsuperscript{473} phosphorylation at 10 \mu M of either inhibitor, GSK-3 phosphorylation was unchanged with IL-3 and GM-CSF treatment. In contrast, IL-4 regulation of GSK-3 was completely impaired by the presence of either wortmannin or LY294002.

Meanwhile, TF-1 cells pretreated with either PI3-K inhibitor showed some reduction in GM-CSF-stimulated GSK-3 phosphorylation (Figure 3.3 B). However, despite total abrogation of PKB Ser\textsuperscript{473} phosphorylation at 100 \mu M LY294002 and 100 nM wortmannin, the GSK-3 phosphorylation signal was still greater than that found in the unstimulated samples.
Figure 3.3. Role of PI3-K in GSK-3 regulation by cytokines. A. MC/9 cells were cytokine-starved, pre-treated with LY294002 or wortmannin for 15 minutes, and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved, pre-treated with LY294002 or wortmannin for 15 minutes, and stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phosphoserine^473^ PKB, and p85. These results are typical of 3 experiments.
<table>
<thead>
<tr>
<th>LY294002 (µM)</th>
<th>Wortmannin (nM)</th>
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<tr>
<td>1</td>
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<tr>
<td>10</td>
<td>10</td>
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<tr>
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GM-CSF

- P - GSK-3α
- P - GSK-3β
- P - PKB (Ser473)
- p85
PKB activity is sufficient for induction of GSK-3 serine phosphorylation – Since PI3-K inhibition did have dramatic effects with IL-4 signaling for GSK-3 phosphorylation, and lesser effects with other cytokines, it would be of interest to see if PKB can in fact mediate phosphorylation of GSK-3. Unfortunately, there are numerous downstream targets of PI3-K, making it difficult to differentiate between the effects of PKB on GSK-3 versus other kinases. To examine PKB’s ability to mediate GSK-3 phosphorylation required a system whereby PKB could be stimulated without changes in PI3-K activity. As such, we utilized an MC/9 cell line stably transfected with a PKB protein fused to a receptor for estrogen (PKB-ER). The addition of an estrogen derivative, 4-hydroxytamoxifen (4-HT), provides a steroid group for the PKB-ER protein, promoting activation of the PKB construct. In fact, treatment with 4-HT alone led to increased GSK-3 α/β phosphorylation in a dose-responsive manner with maximal stimulation at 10 μM (Figure 3.4 A). As well, this dosage of 4-HT produced levels of GSK-3 phosphorylation comparable to those found with the standard panel of cytokines (Figure 3.4 B). While cytokine treatments promote PKB\textsuperscript{473} phosphorylation, they have no effect on the PKB-ER construct. In contrast, the 4-HT has no effect on endogenous PKB, but does promote phosphorylation of the transfected PKB mutant. Regardless, this system forcefully activates PKB and is not a fair representation of PKB activation in vivo during cytokine stimulation. However, in this experiment, PKB can promote phosphorylation of GSK-3 independent of PI3-K.
Figure 3.4. PKB activity is sufficient for GSK-3 serine phosphorylation. A. MC/9 (PKB-ER) cells were cytokine-starved and stimulated with varying dosages of 4-hydroxytamoxifen (10'). B. MC/9 (PKB-ER) cells were cytokine-starved and stimulated with either 10 μM 4-HT (10'), 1 μg/ml IL-3 (10'), 10 μg/ml IL-4 (10'), 25 ng/ml GM-CSF (5'), or the diluent alone (DMSO - 10'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phosphoserine PKB, and p85. These results are typical of at least 3 experiments.
ERK/RSK do not regulate GSK-3 phosphorylation - Several studies have suggested a role for the ERK/RSK pathway in the phosphorylation of the GSK-3 isoforms. To examine this, we used the MEK1 (IC$_{50} = 72$ nM) and MEK 2 (IC$_{50} = 58$ nM)-specific inhibitor U0126 to examine the role of these MEK isoforms, and their downstream target, ERK, on GSK-3 regulation during cytokine treatment. As is shown in Figure 3.5 A, both IL-3 and GM-CSF stimulated phosphorylation of the ERK1 and ERK2 proteins in the MC/9 cell line. This was assessed with an antibody specific for the dual phosphorylation of the threonine and tyrosine found in the TEY motif in p42/p44 MAPK. These MEK-mediated phosphorylations are essential for ERK activation. Unlike IL-3 and GM-CSF however, IL-4 did not demonstrate any upregulation of ERK phosphorylation above the control sample. Concomitantly, there was little to no change in GSK-3 serine phosphorylation by IL-3, IL-4, or GM-CSF with 25µM U0126 pretreatment in comparison to samples with no inhibitor. Likewise in the TF-1 cell line, GM-CSF-stimulated phosphorylation of GSK-3 was unhindered by the presence of U0126 in the media (Figure 3.5 B).

Since ERK is upstream of RSK, we examined whether U0126 pretreatment also blocked this kinase. RSK is activated by numerous phosphorylation events via ERK, as well as through autophosphorylation upon activation by ERK. In many cases, the change in phosphorylation state can be detected through the resulting change in molecular weight. For example, an antibody specific for p90rsk demonstrates a clear bandshift to a higher molecular weight with cytokine treatment, indicating enhanced phosphorylation and thus reduced electrophoretic mobility (Figure 3.5). This same bandshift can be abrogated, along with ERK activity, with U0126 pretreatment, demonstrating the inhibition of p90rsk phosphorylation and activity. With the
abrogation of RSK activity and no change in cytokine-induced serine phosphorylation of GSK-3, RSK's role in GSK-3 regulation must be minimal.
**Figure 3.5. The role of MAPK in GSK-3 regulation.** A. MC/9 cells were cytokine-starved, pretreated with either 25 μM U0126 or diluent for 15 minutes, and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved, pretreated with either 25 μM U0126 or diluent for 15 minutes, and stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). SDS-PAGE was performed on whole cell lysates and the resulting blots probed for serine phosphorylation of GSK-3, phospho-p44/p42 MAPK, p90\(^{Rsk}\) and p85. These results are typical of 3 experiments.
p70^{S6K} does not modulate GSK-3 phosphorylation upon cytokine stimulation – Another kinase that potentially phosphorylates GSK-3 isoforms is p70^{S6K}.\textsuperscript{263} To determine if p70^{S6K} does in fact regulate GSK-3 phosphorylation, MC/9 cells were pretreated with rapamycin, an inhibitor of p70^{S6K} activity (IC\textsubscript{50} = 0.05 nM). After 15 minutes of inhibitor treatment, cell samples were stimulated with the various cytokines to observe the effects on GSK-3 phosphoserine content. In MC/9 cells, IL-3 and GM-CSF induced elevated phosphorylation of p70^{S6K}, with IL-4 producing less of an effect. The antibody for p70^{S6K} phosphorylation recognizes the modification of both Thr\textsuperscript{421} and Ser\textsuperscript{424}. These residues must be phosphorylated to release pseudosubstrate inhibition of p70^{S6K} and to thereby promote full catalytic activity. As well, analysis using an antibody specific for the general p70^{S6K} protein also noted a corresponding upward bandshift with cytokine treatment. With all three cytokines, 100 ng/ml of rapamycin was able to reduce the phosphorylation of p70^{S6K} and the resulting bandshift. However despite the inhibition of p70^{S6K} activity, there was no change in GSK-3\alpha or GSK-3\beta phosphorylation in rapamycin-treated versus untreated cells (Figure 3.6 A). IL-3, IL-4 and GM-CSF upregulation of GSK-3 serine phosphorylation was not blocked by rapamycin. Furthermore, GM-CSF induction of GSK-3 phosphorylation in TF-1 cells was also uninterrupted by the cancellation of p70^{S6K} activity (Figure 3.6 B).
Figure 3.6. The role of p70<sup>65k</sup> in GSK-3 regulation. A. MC/9 cells were cytokine-starved, pretreated with either 100 ng/ml rapamycin or diluent for 15 minutes, and stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved, pretreated with either 100 ng/ml rapamycin or diluent for 15 minutes, and stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-p70<sup>65k</sup>, p70<sup>65k</sup> and p85. These results are typical of 3 experiments.
PKA activity may upregulate GSK-3 phosphorylation – Yet another protein kinase implicated in the regulation of GSK-3 serine phosphorylation is the cAMP-dependent protein kinase (PKA). To further examine whether or not PKA can in fact phosphorylate GSK-3 in vivo, forskolin, an activator of adenylyl cyclase, was used to induce PKA activation. The direct effect of this pharmacological treatment is an elevation in the levels of cAMP, which in turn can promote PKA activity. With previously established doses and incubation times for forskolin therapy, the effects of PKA activation were assessed in MC/9, TF-1, and FDC-P1 cell lines. While increased forskolin dosages elevated GSK-3 serine phosphorylation in TF-1, there was little to no change in MC/9 and FDC-P1 cells (Figure 3.7). The effect of forskolin on GSK-3 in TF-1 cells was primarily directed at the GSK-3α isoform, although a slight increase in phosphorylation was also detected in GSK-3β. The level of phosphorylation signal was highest at 40 µM of forskolin, but since this was the largest dosage tested, higher concentrations of forskolin may further promote GSK-3 phosphorylation. To assess whether PKA was in fact activated through forskolin treatment, the phosphorylation of CREB, a direct substrate of PKA, was also studied using antibodies specific for the phosphorylated residues targeted by PKA. While both TF-1 and FDC-P1 cells demonstrate elevated increases in CREB phosphorylation correlating with increasing forskolin dosage, MC/9 cells did not appear to respond to forskolin therapy since modification of the CREB protein was unchanged. Whether this is due to the cell line’s inability to respond to forskolin through PKA activation, or PKA’s inability to target CREB, remains undetermined. However, other work has clearly demonstrated CREB phosphorylation upon forskolin treatment in MC/9 cells.
Figure 3.7. **Cell specific regulation of GSK-3 by forskolin.** MC/9, TF-1 and FDC-P1 cells were cytokine-starved and stimulated with either forskolin (4, 10, 40 μM), or the diluent alone (DMSO) for 10 minutes. SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-CREB (Ser^{133}), and p85. These results are typical of 2 experiments.
Phorbol ester stimulation of GSK-3 serine phosphorylation – Of further interest is the possibility that protein kinase C (PKC) may also play a role in the regulation of GSK-3 isoforms. With that in mind, members of the classical and novel subsets of the PKC family were activated through the use of the phorbol ester, phorbol myristate acetate (PMA). PMA has a structure that resembles diacylglycerol, and is thus able to substitute for DAG in the activation of PKC. Phorbol esters increase the binding ability of PKC isoforms for Ca\(^{2+}\), resulting in the enzyme’s full activation without the need for calcium mobilization. In both the MC/9 (Figure 3.8 A) and the TF-1 (Figure 3.8 B) cell lines, 100 nM of PMA did promote GSK-3\(\alpha\) and GSK-3\(\beta\) serine phosphorylation to levels well above those found in samples treated with the delivery vehicle alone (DMSO).
Figure 3.8. Effect of phorbol esters on GSK-3α/β. A. MC/9 cells were cytokine-starved and stimulated with either PMA (100 nM) or the diluent alone (DMSO) for 10 minutes. B. TF-1 cells were cytokine-starved and stimulated with either PMA (100 nM) or diluent alone (DMSO) for 10 minutes. SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-PKB (Ser^{473}), p85. These results are typical of >5 experiments.
PKC regulates GSK-3 phosphorylation – Unfortunately, it is well established that phorbol esters can activate a variety of protein kinases in addition to PKC.\textsuperscript{307} As such, it became essential to utilize other methods to evaluate PKC’s effects on GSK-3 during cytokine stimulation. To that end, a panel of PKC inhibitors was selected to pre-treat each cell line, and observe the capacity to which each cytokine could serine phosphorylate GSK-3 under these conditions. Two of these inhibitors included the staurosporine analogs, Rö-31-8220 and Rö-31-8425, which target PKC at the C3 domain of the catalytic region as a competitive inhibitor of ATP.\textsuperscript{308-310} Both of these compounds are strong inhibitors of the classical PKC’s (IC\textsubscript{50} = 5-24 nM) and less so for PKCε (IC\textsubscript{50} = 24-39 nM). However, there is evidence that these two drugs are capable of inhibiting a wide range of members from all three PKC families.\textsuperscript{311,312} Two other staurosporine-based analogs also used were the Gö 6976 and Gö 6983 compounds. The classical PKC’s are strongly inhibited by both of these drugs (IC\textsubscript{50} = 2-10 nM), although their effects on the novel and atypical isoforms at higher concentrations remain unsolved.\textsuperscript{313-316} As well, these two inhibitors can be helpful in discerning between the effects of various PKC isoforms. For example, Gö 6976 also blocks PKCλ but not PKCζ (IC\textsubscript{50} > 20 μM), while Gö 6983 blocks PKCζ (IC\textsubscript{50} = 60 nM) but not PKCμ.\textsuperscript{317-318}

With this arsenal of PKC inhibitors, studies were performed to determine the ability of each to block cytokine-induced GSK-3 serine phosphorylation. Concentration and pre-incubation times were identified from dose response studies performed earlier (data not shown). In MC/9 cells, we can see the minor effect of PI3-K inhibitors on IL-3 and GM-CSF-responses again (Figure 3.9). Likewise, PI3-K inhibition totally blocked IL-4-mediated GSK-3 phosphorylation. As for the PKC inhibitors, Rö-31-8220, Rö-31-8425, and Gö 6976 all blocked
phosphorylation of GSK-3, irrespective of the cytokine used. This was true for both GSK-3α and GSK-3β. Interestingly, Gö 6983 consistently had a lesser effect on blocking GSK-3 phosphorylation. Meanwhile in TF-1 cells, the same three PKC inhibitors also blocked GSK-3 phosphorylation, although Rö-31-8220 was less effective in this case (Figure 3.10). Once again, Gö 6983 had little to no effect. Finally, a similar pattern was observed in FDC-P1 cells, although many of the inhibitors showed a weaker effect (Figure 3.11).

Ideally, these PKC inhibitors would be most useful to help distinguish between the role of PKC and PKB in GSK-3 regulation. Unfortunately, some of these PKC inhibitors produce an effect on PKB phosphorylation at Ser$^{473}$. Rö-31-8220 did not block cytokine-mediated PKB phosphorylation in MC/9 but it did have some effect in TF-1 and FDC-P1 cells. Rö-31-8425 also blocked PKB phosphorylation in MC/9 and TF-1, but had a variably effect in FDC-P1. Meanwhile, Gö 6976 consistently blocked PKB phosphorylation in all cases. Finally, Gö 6983 had no effect on PKB phosphorylation in any of these systems.
Figure 3.9. **GSK-3 phosphorylation via PKC.** MC/9 cells were cytokine-starved and pretreated with either LY294002 (100 μM), wortmannin (100 nM), Ro-31-8220 (10 μM), Ro-31-8425 (10 μM), Gö-6976 (5 μM), or Gö-6983 (5 μM) for 15 minutes. Samples were then stimulated with either IL-3 (10^5), IL-4 (10^5), GM-CSF (5') or the diluent alone (DMSO - 10'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-PKB (Ser^473), and p85. These results are typical of 3 experiments.
Figure 3.10. GSK-3 phosphorylation via PKC. TF-1 cells were cytokine-starved, pretreated with either Ro-31-8220 (10 μM), Ro-31-8425 (10 μM), Go-6976 (5 μM), or Go-6983 (5 μM) for 15 minutes. Samples were then stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-PKB (Ser^{473}), and p85. These results are typical of 3 experiments.
Figure 3.11. GSK-3 phosphorylation via PKC. FDC-P1 cells were cytokine-starved and pretreated with either LY294002 (100 μM), wortmannin (100 nM), Ro-31-8220 (10 μM), Ro-31-8425 (10 μM), Go-6976 (5 μM), or Go-6983 (5 μM) for 15 minutes. Samples were then stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or the diluent alone (DMSO - 10'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phospho-PKB (Ser^{73}), and p85. These results are typical of 2 experiments.
GSK-3 is not a target of PKCδ - The unconfirmed specificity of many PKC inhibitors, with respect to each PKC isoform, makes any study of PKC difficult. One drug that showed promising isoform specificity is rottlerin with strong effects on PKCδ (IC₅₀ = 3-6 μM). However, publications have also noted rottlerin’s effects on some classical PKC’s (IC₅₀ = 30-42 μM) and some novel and atypical PKC isoforms (IC₅₀ = 80-100 μM). However, the effect of rottlerin pre-incubation in MC/9 and TF-1 cells with respect to cytokine-mediated GSK-3 phosphorylation was still examined (Figure 3.12). As illustrated, 5 μM of rottlerin had no effect on GSK-3 phosphorylation in any case. As such, it appears that PKCδ is not an important regulator of GSK-3 serine phosphorylation.
Figure 3.12. PKCδ does not regulate GSK-3 phosphorylation by cytokines. A. MC/9 cells were cytokine-starved and pretreated with either 5 uM rottlerin or diluent only (DMSO) for 15 minutes. Samples were then stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5') or diluent alone (DMSO - 10'). B. TF-1 cells were cytokine-starved and pretreated with either 5 uM rottlerin or diluent only (DMSO) for 15 minutes. Samples were then stimulated with either GM-CSF (5') or diluent alone (DMSO - 10'). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3 and p85. These results are typical of 3 experiments.
Cytokines increase diacylglycerol levels – Considering the prominent role of PKC in the regulation of GSK-3 serine phosphorylation by cytokines, the regulation of PKC itself is an interesting focus. Presumably the inhibition of factors mediating PKC activation should also inhibit GSK-3 phosphorylation. Both classical and novel PKC isoforms, as well as PKCµ, require diacylglycerol production for the upregulation of PKC catalytic activity. Cytokines have been noted to increase diacylglycerol levels in other cell systems. To examine whether these cytokines promote DAG elevation in MC/9 cells, phosphate-free media was supplemented with H3[32P]O4, and the cells pre-incubated and treated with cytokines in this solution. Thin layer chromatography permitted the separation of various lipid products, and those radioactively labeled were visualized by exposure to X-ray film. However, diacylglycerol does not contain phosphate. As such, a metabolic byproduct of DAG was examined instead. Phosphatidic acid is formed from the actions of diacylglycerol kinase and changes in its intracellular level are intimately linked to diacylglycerol amounts. Quantification of radioactivity within each sample’s phosphatidic acid pool was measured through densitometry analysis. Both IL-3 and GM-CSF stimulated at least a two-fold increase in phosphatidic acid levels (Figure 3.13). However, IL-4’s effects were minimal in this study. To determine the effectiveness of certain diacylglycerol inhibitors, three candidate drugs were also selected for pretreatment in the cell samples prior to cytokine treatment. The DAG kinase inhibitor II (R59949) can block the transformation of diacylglycerol to phosphatidic acid, while the phospholipase inhibitors U73122 and D609 were also examined. U73122 is a broad phospholipase C inhibitor with a higher affinity for phosphatidylinositol-specific PLC (PI-PLC) while D609 is more selective for PC-PLC.
Effective dosages for these drugs were established through literature sources and previous dose response studies (Figure 3.14).

Pretreatment of MC/9 cells with DAG kinase inhibitor II, D609, or U73122 reduced IL-3- and GM-CSF-induced phosphatidic acid accumulation (Figure 3.13). Meanwhile, these inhibitors produced little difference in the already minimal phosphatidic acid change induced by IL-4. Interestingly, PMA also produced elevated phosphatidic acid levels but these perturbations were not significantly affected by any of these three inhibitors.
Figure 3.13. Diacylglycerol formation upon cytokine treatment. MC/9 cells were cytokine-starved and labelled with $^{32}$P-PO$_4$$. The cells were then pretreated with either DAG kinase inhibitor II (10 μM), U73122 (50 μM), D609 (100 μM) or diluent alone for 15 minutes. Samples were then stimulated with either IL-3 (10'), IL-4 (10'), GM-CSF (5'), PMA (10') or the diluent alone (DMSO - 5'). Samples were then stimulated with either GM-CSF (5') or diluent alone (DMSO - 5'). Lipids were extracted using the Bligh-Dyer method and run on silica plates. Results of autoradiography were quantified and graphed.
Diacylglycerol production and GSK-3/PKB phosphorylation – To further examine the effects of the phospholipase C inhibitors on GSK-3 phosphorylation during cytokine signaling, cells were pretreated with either U73122 or D609 in the dosages indicated (Figure 3.14A). In the MC/9 cell line, fifteen minute pretreatment with U73122 at dosages as low as 10 μM led to abrogation of both cytokine-induced PKB Ser^{473} phosphorylation and GSK-3α/GSK-3β Ser^{21}/Ser^{9} phosphorylation. Treatment with 50 μM U73122 further enhanced this effect in all three cases. Meanwhile, the D609 compound demonstrated no change in cytokine-mediated GSK-3 or PKB phosphorylation. A similar response was noted in the TF-1 cell line, although the effect on GSK-3 and PKB phosphorylation was first noted at 50 μM of the U73122 compound. While inhibition with U73122 implicates PI-PLC-mediated DAG production as having some function in the signalling pathway to GSK-3, this is unlikely since cytokines are not known to activate PI-PLC. Thus it is possible that the inhibitor acts non-specifically on some kinases such as PI3-K or PDK1. These possibilities will have to be addressed by testing the effect of the inhibitors on the various possible kinases.
Figure 3.14. Role of PLC in GSK-3 regulation by cytokines. A. MC/9 cells were cytokine-starved, pre-treated with U73122, D609, or diluent (DMSO) for 15 minutes, and stimulated with either IL-3 (10′), IL-4 (10′), GM-CSF (5′) or the diluent alone (DMSO - 10′). B. TF-1 cells were cytokine-starved, pre-treated with U73122, D609, or diluent (DMSO) for 15 minutes, and stimulated with either GM-CSF (5′) or the diluent alone (DMSO - 10′). SDS-PAGE was performed on whole cell lysates and the resulting blot probed for serine phosphorylation of GSK-3, phosphoserine \textsuperscript{473} PKB, and p85. These results are typical of 3 experiments.
B

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<th>U73122 (μM)</th>
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- P - GSK-3α
- P - GSK-3β
- P - PKB (Ser473)
- p85
Correlation of GSK-3 activity with serine phosphorylation – Although the above results demonstrate a pattern in the regulation of GSK-3 phosphorylation in cytokine-treated cells, the catalytic activity of GSK-3 needs to be directly examined to verify that the phosphorylation events are having their expected effects. Although the serine phosphorylation of GSK-3 has been intimately linked to the inactivation of this kinase, there is also evidence that other modifications of GSK-3 also play a crucial and maybe even a more important role.236,250,252,267 Using a pre-phosphorylated peptide substrate derived from the glycogen synthase protein, GSK-3α activity was examined in vitro. Interestingly, the pattern of GSK-3 activity did not correlate with the expected results established in the phospho-serine studies (Figure 3.15). In TF-1 cells, treatment with GM-CSF resulted in no significant change in GSK-3 activity, although Western blot analyses suggest that the serine residue of GSK-3α in this treatment shows elevated phosphorylation. Likewise, treatment of cells with LY294002 or U73122 also showed no conclusive change with respect to cytokine-stimulated TF-1’s and those treated with the vehicle alone (DMSO). However, the one pattern that did emerge was the lowered catalytic activity induced by the four PKC inhibitors. Rö-31-8220, Rö-31-8425, Gö 6976 and even Gö 6983 pretreatment reduced GSK-3 catalytic activity to levels between 50-70% of the control samples.
Figure 3.15. Regulation of GSK-3α activity. TF-1 cells were cytokine-starved and stimulated with either 10 ng/ml GM-CSF (5 min.) or diluent alone (DMSO - 5 min.), in some cases after pre-incubation (15 min.) with either LY294002 (100 μM), Rö-31-8220 (10 μM), Rö-31-8425 (10 μM), Gö 6976 (5 μM), Gö 6983 (5 μM), or U73122 (100 μM). GSK-3α was immunoprecipitated and its phosphotransferase activity assayed with the phosphoglycogen synthase substrate. The substrate was isolated on P81 phosphocellulose paper and scintillation analysis performed. The results are shown as a mean of three experiments ± standard error.
PKC inhibitors and PKB activity – Likewise, the direct analysis of PKB activity can also support, or refute, the ability of some of these PKC inhibitors to inhibit cytokine-induced PKB activity. To that effect, PKBα was immunoprecipitated and its catalytic activity assessed in vitro with a peptide substrate. Figure 3.16 clearly demonstrates a 6 to 10 times increase in the activity of PKB as compared to the control samples. Not surprisingly, the well-established PI3-K inhibitor, LY294002, reduced GM-CSF-stimulated PKB activity substantially. Of the four PKC inhibitors and the PLC inhibitor U73122, only Gö 6976 and U73122 reduced PKB activity by statistically significant amounts. In fact, these two downregulated PKB activity to levels found in cell pretreated with LY294002.
**Figure 3.16. Regulation of PKB activity.** TF-1 cells were cytokine-starved and stimulated with either 10 ng/ml GM-CSF (5 min.) or diluent alone (DMSO - 5 min.), in some cases after pre-incubation (15 min.) with either LY294002 (100 μM), Rö-31-8220 (10 μM), Rö-31-8425 (10 μM), Gö 6976 (5 μM), Gö 6983 (5 μM), or U73122 (100 μM). PKBα was immunoprecipitated and its phosphotransferase activity assayed with the phosphoglycogen synthase substrate. The substrate was isolated on P81 phosphocellulose paper and scintillation analysis performed. The results are shown as a mean of three experiments ± standard error.
CHAPTER 4: CONCLUSIONS

A variety of studies have closely examined the enigmatic enzyme, glycogen synthase kinase-3 (GSK-3). Many of these publications have noted GSK-3 to be an anti-proliferative and an anti-survival kinase. Several growth factors and other extracellular stimuli promote inactivation of GSK-3, presumably to allow for cell survival. This is supported through experiments where inactivation of GSK-3 protected cells from entering an apoptotic phase. Likewise, cells over expressing GSK-3 or expressing constitutively active GSK-3 demonstrated impaired cell survival and proliferation. However, no clear and consistent pattern has emerged as to whether GSK-3 is sufficient or even essential for cell survival in certain cell types. Surprisingly, there are no published reports on the effects of cytokines on GSK-3. Cytokines are soluble growth factors that target primarily hematopoietic cells, and promote enhanced cell survival and/or increased proliferation. In fact, a variety of cells require these cytokines for survival in culture and are thus called factor-dependent cell lines, such as MC/9, TF-1 and FDC-P1. IL-3 and GM-CSF are examples of cytokines that strongly promote cell growth and differentiation of the myeloid lineage of hematopoietic cells. Others, such as IL-4 are less potent in their proliferative effects, but still demonstrate potential as a growth factor, particularly with other co-stimulatory agents. Despite these powerful anti-apoptotic effects, a thorough search of the literature reveals that there is no data available on the role of cytokines in GSK-3 regulation.

In this study, IL-3, IL-4 and GM-CSF induced a dramatic increase in the serine phosphorylation of both GSK-3α and GSK-3β within five to ten minutes of initial stimulation in MC/9, TF-1 and the FDC-P1 cell lines. Although the results obtained from the kinase assays are
preliminary, and will have to be verified, it appears that the lack of inhibition of GSK-3 activity, despite its serine phosphorylation, contradicts numerous studies where the serine phosphorylation of GSK-3 was shown to be essential and necessary for GSK-3 inactivation.\textsuperscript{236, 250} In considering possible explanations for this result, it is known that numerous other residues within GSK-3 are also targets for post-translational modification. In particular, much focus has been directed on the conserved tyrosine residues found in both GSK-3 mammalian isoforms (Tyr\textsuperscript{279} in GSK-3\textsubscript{α}). Some research has demonstrated that this tyrosine is also an active player in the regulation of GSK-3 activity.\textsuperscript{267, 268} In particular, phosphorylation at this site promotes GSK-3 activity in cells during basal stages. It is plausible that, despite GSK-3 serine phosphorylation, the phosphorylation of the tyrosine residue continues to promote GSK-3 catalytic activity to levels comparable to that of untreated resting cells. As well, if GSK-3 does indeed mediate cytokine-induced growth and survival, the effects of GSK-3 inactivation would need to be observed over a longer time frame. In these studies, GSK-3 was examined only five to ten minutes after the initial addition of cytokines. Perhaps a longer cytokine exposure does promote a decline in GSK-3 catalytic activity to mediate long-term cellular effects, although insulin-mediated abrogation of GSK-3 occurs as quickly as five minutes.\textsuperscript{251} Furthermore, the possibility that the \textit{in vitro} kinase assay itself may have been flawed or misleading cannot be ruled out. The examination of GSK-3 activity is notoriously difficult due to the kinase’s high level of basal activity.\textsuperscript{323} As well, no clear positive control was established to confirm the accuracy of this assay system. In particular, comparison of GSK-3 activity from the lysates of untreated and IgM-treated A31 B-cells demonstrated no observable differences (data not shown). In other studies, IgM treatment of this particular cell line has been noted to reduce GSK-3\textsubscript{α} activity in comparison to untreated cells.\textsuperscript{324}
Numerous attempts to replicate this data using the same assay system and protocols have failed. It is possible that some of the reagents used in these assays, or the techniques employed by the examiner, were flawed. Alternatively, the substrate peptide utilized in these in vitro assays permits analysis of only one facet of GSK-3 catalytic activity. The substrate was based on a sequence from glycogen synthase, a pre-phosphorylated protein targeted by GSK-3. The peptide substrate is also pre-phosphorylated at the +4 residue to the site of GSK-3's target, as is the case with glycogen synthase in vivo. As such, this assay examined GSK-3's ability to phosphorylate this particular substrate, and is presumably indicative of GSK-3's affinity for pre-phosphorylated targets. However, GSK-3 can also target various non-phosphorylated substrates. One example is the transcription factor β-catenin, which can mediate the enhanced transcription of genes implicated in cell survival and cell proliferation. Interestingly, GSK-3 can be modulated to target one class of substrates but not the other. Thus, this assay only focused on the catalytic activity of GSK-3α towards pre-phosphorylated substrates, as opposed to those containing no prior modification.

Despite the apparent setback in correlating GSK-3 serine phosphorylation with GSK-3 activity, the pathways regulating GSK-3 serine phosphorylation were still crucial targets for examination. Since IL-3, IL-4 and GM-CSF all promote cell survival in MC/9 and TF-1 cells to some extent, the concomitant serine phosphorylation of GSK-3 could suggest a correlation between GSK-3 regulation and cell survival.

The PI3-K/PDK/PKB pathway has long been of interest in the study of how cytokines manifest their anti-apoptotic effects in hematopoietic cells. All three cytokines used in this study are well-known to activate both PI3-K and PKB. The question remains as to the need
for this pathway in mediating cell survival. In some cell systems, PKB presence is essential for cell survival and cell proliferation, while in other cell lines, PKB activity does not correlate with cell survival.\(^{50,184-187}\) Regardless, the PI3-K pathway has been demonstrated to be a key regulator of GSK-3 serine phosphorylation in other model systems. As well, PKB can phosphorylate GSK-3 at these critical serine residues \textit{in vitro}.

Examination of the PI3-K pathway \textit{in vivo} often involves use of the two structurally distinct inhibitors, wortmannin and LY294002. Wortmannin is a fungal metabolite that covalently associates with the p110 subunit of PI3-K with high affinity (IC\(_{50}\) = 3 nM), resulting in the abrogation of both the phosphatidylinositol kinase and the serine kinase activities of PI3-K.\(^{325}\) This drug is a potent inhibitor of PI3K, and at lower concentrations it is also selective for PI3-K. LY294002, an analog of the bioflavinoid quercetin, is also a potent inhibitor of PI3-K (IC\(_{50}\) = 1.4 \(\mu\)M).\(^{302}\) It inhibits PI3-K through a different mechanism from wortmannin by acting as a competitive inhibitor of ATP binding. In fact, LY294002 is considered to be a more reliable inhibitor of PI3-K than wortmannin.

In MC/9 cells, the PI3-K pathway is clearly an important player in the regulation of GSK-3 serine phosphorylation during IL-4 stimulation. However, the function of PI3-K in the inhibition of GSK-3 is less certain with IL-3 and GM-CSF treatment of MC/9 and TF-1 cells. This pattern between IL-4 and IL-3/GM-CSF regulation of GSK-3 is probably due to the differences in the cytokine receptor complexes and the signaling pathways promoted by each. The IL-4 receptor is comprised of the \(\alpha\) chain specific for the cytokine itself, but also includes the \(\gamma\) common chain found in receptors for IL-4, IL-2, IL-7, IL-9, IL-13, and IL-15.\(^{74}\) Meanwhile, the IL-3 and GM-CSF receptors contain the \(\beta\) common subunit. The primary role of the common
subunit chain is as an intracellular signal transducer of cytokine stimulation. In fact, the β and γ chains can activate different pathways from each other. As such, the similar cellular effects induced by IL-3 and GM-CSF are most probably due to the shared βc receptor subunit. Both IL-3 and GM-CSF activate ERK, promote cell survival and proliferation in many hematopoietic cells, and exhibit continued GSK-3 phosphorylation in the absence of PI3-K activity. Meanwhile, IL-4, with a γc receptor chain instead, does not activate ERK, does not induce cell proliferation in many cell lines, and demonstrates PI3-K-dependent GSK-3 regulation. As such, while PI3-K plays a predominant role in IL-4-mediated GSK-3 phosphorylation, IL-3 and GM-CSF may use other pathways to mediate GSK-3 phosphorylation, or utilize redundant pathways to circumvent the blockage of PI3-K activity. However, even in IL-3 and GM-CSF signaling, PI3-K activity still plays a minor role in GSK-3 phosphorylation, suggesting the existence of backup kinases to target GSK-3 in the event of PI3-K shutdown.

Numerous studies have suggested that PI3-K and PKB are required for cytokine-mediated survival. If this is true, the inability of PI3-K inhibitors to block GSK-3 serine phosphorylation in some cases would suggest that GSK-3 is not a key player in cytokine-mediated survival. However, studies have noted that GM-CSF and IL-3 can promote cell survival independent of PI3-K in MC/9 cells, although this is not true in TF-1 and FDC-P1 cells. As GSK-3 is not greatly affected by PI3-K inhibitors with these two cytokines in MC/9, this lends credence to the theory that GSK-3 may play a role in MC/9 cell survival. On the other hand, the minor role played by PI3-K during GSK-3 regulation by GM-CSF in TF-1 cells suggests that GSK-3 might not be an important factor in cell survival in all cytokine-responsive cell types. Clearly, further investigation of GSK-3 in cytokine-mediated cell survival is warranted.
The use of LY294002 and wortmannin establishes an important role for PI3-K in IL-4-induced GSK-3 phosphorylation, and a minor function in the IL-3 and GM-CSF stimulation of GSK-3 serine phosphorylation. It is, however, difficult to correlate this data with PKB as a component in GSK-3 signaling. PI3-K promotes elevated levels of 3'-phosphorylated phosphoinositides, which in turn can mediate the activities of many protein kinases. For example, PDK1 becomes membrane localized and promotes phosphorylation of various substrates upon agonist-mediated increase of PI3-K lipid products. These substrates include PKBα, PKCζ, p70S6K, RSK2, and serum glucocorticoid kinase 2 (SGK2).\textsuperscript{157-162} As well, PI3-K displays some protein serine kinase activity and can phosphorylate its p85 subunit and the IRS-1 protein. As such, inhibition of PI3-K activity could conceivably alter the activity of numerous other kinases other than PKB. Although several studies have targeted PKB activity directly to demonstrate changes in the serine phosphorylation of GSK-3, other studies have also pointed to a possible link between GSK-3 and kinases such as PKC, p70\textsuperscript{S6K}, and RSK2. As such, pharmacological inhibition of PI3-K in the study of GSK-3 phosphorylation is only an initial step in elucidating the upstream kinases responsible. Since no effective and/or specific inhibitor for PKB is commercially available, it is difficult to focus exclusively on PKB's effects on GSK-3 through chemical intervention. However, it is clear that PKB can promote the serine phosphorylation of GSK-3 in some instances. The MC/9 cell line expressing PKB fused to a portion of the estrogen receptor is one example of a system demonstrating the role of PKB in GSK-3 modification independent of PI3-K. Treatment of this altered MC/9 cell line with the estrogen derivative 4-hydroxytamoxifen (4-HT), clearly demonstrates that PKB can mediate phosphorylation of GSK-3 independent of PI3-K. However, the question remains as to whether
PKB is an *in vivo* regulator of GSK-3 phosphorylation during cytokine signaling, particularly upon IL-4 treatment.

Another putative regulator of GSK-3 serine phosphorylation was the MAPK/RSK pathway. In particular, RSK has been demonstrated to phosphorylate the crucial serine residues in GSK-3 *in vitro*. It is also plausible that some of the effects noted in the PI3-K inhibition studies could result from the inability of PDK1 to target RSK. However, RSK also requires phosphorylation by MAPK at key residues in order to be fully activated. IL-3 and GM-CSF can stimulate MAPK phosphorylation and activity, while IL-4 demonstrates no such response. With this in mind, RSK was inhibited through the abrogation of MEK activity. MEK is the dual-specific kinase that phosphorylates the threonine and tyrosine residues within the catalytic loop of MAPK, thus promoting full activity of the latter. As such, inhibition of MEK should abrogate RSK activity as well. The U0126 compound was utilized as a potent inhibitor of both MEK1 (IC$_{50}$ = 72 nM) and MEK2 (IC$_{50}$ = 58 nM). U0126 is a specific non-competitive inhibitor of MEK with respect to both ATP and ERK, and does not effect the activities of PKC, Abl, Raf, MEKK, ERK, JNK, MKK3, MKK6, Cdk2, or Cdk4. Pretreatment of MC/9 and TF-1 cells with this cell permeable inhibitor led to reduced IL-3- and GM-CSF-mediated MAPK phosphorylation. As well, abrogation of RSK phosphorylation was confirmed through bandshift analysis. However, there was no effect on the phosphorylation of either GSK-3α or GSK-3β by any of the cytokines in these cell models. As such, it is clear that neither MEK, ERK, nor RSK are responsible for the regulation of GSK-3 by IL-3, IL-4 or GM-CSF. Although MAPK is important for IL-3- and GM-CSF-mediated cell survival of some factor-dependent cell lines, many of these effects are cell-type specific. As well, there is on-going debate over the
extent to which the MAPK and PI3-K pathways crosstalk.\textsuperscript{134,188} As such, it is difficult to ascertain whether the lack of MAPK input into GSK-3 serine phosphorylation indicates a minimal role for GSK-3 in cell survival.

Closer scrutiny of the p70\textsuperscript{S6K} pathway also demonstrated its insignificance in the regulation of GSK-3. The mitogenic activation of p70\textsuperscript{S6K} is regulated by phosphorylation events that release the protein from pseudosubstrate inhibition and promote full catalytic activity. To examine this kinase more thoroughly in its effects of GSK-3, the macrolide antibiotic, rapamycin, derived from the filamentous bacterium \textit{Streptomyces hygroscopicus}, was utilized. This immunosuppressive drug associates with the FKBP protein, creating a complex which in turn binds to mTOR.\textsuperscript{329} Although the exact biochemical nature of mTOR remains elusive, mTOR is a putative protein kinase that lies upstream of the p70\textsuperscript{S6K} regulatory pathway. Treatment of cells with rapamycin can block p70\textsuperscript{S6K} activity in various cell systems. However, rapamycin is highly specific and has demonstrated no effects on PI3-K, Raf, MAPK, or RSK.\textsuperscript{330,331} Clearly, IL-3 and GM-CSF can induce phosphorylation of p70\textsuperscript{S6K} in MC/9 and TF-1 cells, while IL-4 cannot. Rapamycin can totally abrogate this effect, thus blocking p70\textsuperscript{S6K} activity. IL-4's inability to activate p70\textsuperscript{S6K}, combined with the absence of any inhibitory effect on GSK-3 serine phosphorylation with rapamycin treatment, suggests that p70\textsuperscript{S6K} is not a player in the mediation of either GSK-3\(\alpha\) or GSK-3\(\beta\) serine phosphorylation. The role of p70\textsuperscript{S6K} in cellular proliferation is well-established, although these effects appear to be cell-type specific.\textsuperscript{228-232} There is little information on p70\textsuperscript{S6K} and cell survival. As such, the fact that GSK-3 is not a target for p70\textsuperscript{S6K} still supports a putative role for GSK-3 in cell survival.
Recent evidence also illustrates enhanced GSK-3 phosphorylation in response to induced cAMP production. In fact, the cAMP-responsive PKA can phosphorylate GSK-3 at the crucial serine residues in vitro. The production of cAMP can be accelerated in vivo via treatment with various pharmacological compounds, including forskolin.\textsuperscript{260,261} This compound is a naturally occurring diterpene that directly activates adenylyl cyclase to promote elevated intracellular cAMP concentration.\textsuperscript{332} The EC\textsubscript{50} range for forskolin has been noted to be between 1 \(\mu\text{M}\) to 20 \(\mu\text{M}\) for cells and tissues. This compound generally induces cAMP-dependent effects, although the drug has also been noted to target various membrane transport proteins and receptors.\textsuperscript{333,334}

The elevation of cAMP induced by forskolin typically promotes PKA activation. One common substrate target for PKA is the transcription factor CREB. Forskolin clearly promoted CREB phosphorylation in TF-1 and FDC-P1, although MC/9 did not appear to respond to forskolin. Other groups have reported models in which forskolin is unable to promote increased cAMP production.\textsuperscript{335,336} However, previous work from this laboratory has established the role of forskolin in MC/9 cells as an activator of adenylyl cyclase, and further demonstrated increases in CREB phosphorylation.\textsuperscript{136} As such, the lack of forskolin-based responses in MC/9 remains a mystery. However, even with an expected forskolin response, only TF-1 cells exhibited increased GSK-3 serine phosphorylation. In particular, GSK-3\(\alpha\) phosphorylation appeared much more targeted by forskolin treatment then GSK-3\(\beta\). This would support the previous notion that PKA can target GSK-3 for phosphorylation in some cases. However, whether or not PKA repeats this role in cytokine signaling cannot be discerned from these results. While IL-4 can stimulate PKA activation in B cells, GM-CSF does not produce a cAMP spike in TF-1.
As such, it is improbable that cytokines regulate GSK-3 phosphorylation through PKA.

The PKC inhibitors dramatically affect cytokine-induced GSK-3 phosphorylation. The most efficient and consistent inhibitor for GSK-3 serine phosphorylation was the staurosporine analog, Gö 6976. Both PKC α and PKC β1 have been shown to be inhibited by this compound (IC$_{50}$ = 2-6 nM), while PKC δ, ε, η and ζ are not (IC$_{50}$ = 700-20,000 nM).$^{316}$ Indirect evidence also suggests that this inhibitor blocks PKC γ activation.$^{313}$ Furthermore, the unique PKC μ is also a target for Gö 6976 inhibition (IC$_{50}$ = 20 nM).$^{317,337}$ A search of the literature found no evidence of PKC θ, or λ/τ inhibition by Gö 6976. However, despite the actions of Gö 6976, it is unlikely that the classical PKC isoforms are involved in GSK-3 phosphorylation during cytokine signaling, since classical PKC's are typically activated in conjunction with an increase in intracellular calcium. Cytokines do not promote such calcium mobilization. On the other hand, in vitro activation of classical PKC requires very small amounts of calcium to be present. As such, it is plausible that the residual levels of intracellular calcium found in these hematopoietic cells may still be sufficient for activation of classical PKC's. However, it is more likely that the effects of Gö 6976 are due either to the inhibition of PKC μ or to a non-specific target of the inhibitor. Some of the non-specific targets of Gö 6976 include MAPKAP-K1b, MSK1, and PDK1.$^{338}$ Interestingly, this inhibitor consistently blocked phosphorylation of PKB at Ser$^{473}$, although other studies have suggested that this inhibitor has minimal effects on PKBα activity in vitro.$^{338}$ Regardless, the abrogation of both PKB phosphorylation and activity by Gö 6976 in this study makes it difficult to determine if PKC or PKB are indeed involved in the regulation of GSK-3 phosphorylation. However, since IL-3- and GM-CSF-induced GSK-3 phosphorylation is
not heavily dependent on PI3-K activity, it is more likely that Gö 6976’s effects on GSK-3 during IL-3 and GM-CSF stimulation is via inhibition of PKC μ. The inability of Gö 6983 to dramatically inhibit GSK-3 phosphorylation also suggests a role for PKC μ since Gö 6983 targets the classical PKC’s (IC$_{50}$ = 6-7 nM), but not PKC μ (IC$_{50}$ = 20,000 nM). However, Gö 6983 does not inhibit cytokine-induced PKB phosphorylation or activation either. Furthermore, phorbol ester treatment of MC/9 and TF-1 cells demonstrated elevated levels of GSK-3 serine phosphorylation. Phorbol esters are capable of regulating the classical and novel PKC family members, as well as PKC μ, further suggesting that PKC μ might be involved in GSK-3 regulation by some cytokines.

With concomitant loss of PKB activity during inhibition of GSK-3 phosphorylation by Gö 6976, it is difficult to determine if the inhibitor’s effect is due to PKB or PKC inhibition. However, Rö-31-8220, another PKC inhibitor, was also able to dramatically inhibit GSK-3 phosphorylation in MC/9 cells without affecting serine phosphorylation of PKB in some cell types. This would suggest that the actions of this PKC inhibitor on GSK-3 are independent of PKB activity. Rö-31-8220 has been noted to inhibit the activities of all classical PKC’s (IC$_{50}$ = 5-27 nM), as well as PKC ε (IC$_{50}$ = 24 nM). However, this same inhibitor can target a plethora of other protein kinases, including MAPKAP-K1b, MSK1, SGK, p70$^{S6K}$, and even GSK-3β itself. This makes any conclusions drawn from studies with Rö-31-8220, or any other PKC inhibitor, tenuous at best. While it is clear that PKC plays a predominant role in GSK-3 signaling by cytokines, the isoform(s) responsible remain unknown.

Although the inhibition of GSK-3 serine phosphorylation should promote elevated GSK-3 catalytic activity, the in vitro kinase assays does not appear to corroborate this theory. Once
again, this may be due to procedural flaws in the assay itself (see above). In previous studies, acute stimulation of cells with phorbol esters have been noted to decrease tyrosine phosphorylation and concurrent GSK-3 activity. However in the current study, inhibition of PKC led to further reduction in GSK-3 activity, and is thus the opposite effect of what previous groups have reported. Therefore, the data produced from these in vitro assays do not corroborate with the existing literature or signaling pathway mechanics. As such, the results of these assays must be viewed cautiously.

In an effort to further support the theory that PKC regulates cytokine-mediated GSK-3 serine phosphorylation, this study examined the production of diacylglycerol. DAG is an essential component for the activation of classical and novel PKC's. Furthermore, these DAG-dependent PKC isoforms have also been implicated as promoters of PKC μ activity, while PKC μ can also bind directly to diacylglycerol as well. IL-3 and GM-CSF both induce the production of phosphatidic acid, a byproduct of diacylglycerol metabolism. This process is susceptible to both the PI-PLC inhibitor U73122 and the PC-PLC inhibitor D609. However, PI-PLC activity has not been implicated in cytokine stimulation, primarily due to a lack of calcium mobilization that should result from the phosphoinositide hydrolysis product, IP₃. Meanwhile, PC-PLC activity has been detected with IL-3, GM-CSF, and IL-4 stimulation. Interestingly, PLD hydrolysis of phosphatidylcholine to produce DAG via phosphatidic acid has also been detected with GM-CSF stimulation, but not IL-4. However, neither U73122 nor D609 is known to inhibit PLD activity.

The specificities of the two phospholipase inhibitors used in this study are also suspect. Both U73122 and D609 inhibit phosphatidic acid production to some extent. However, the role
of PI-PLC in this production should be minimal, which cannot be corroborated with the apparent actions of U73122. Likewise, U73122 also blocks GSK-3 and PKB serine phosphorylation. Although U73122 has been shown to inhibit agonist-stimulated PKB serine phosphorylation with thrombin, thrombin is also known to promote PI-PLC activity and calcium mobilization. This is unlike cytokines where no PI-PLC activity and no calcium signal are recorded. It is therefore more likely that the non-specific nature of U73122 is the underlying cause for many of the results obtained.

The current study is the first to demonstrate that cytokines do promote GSK-3 serine phosphorylation in various factor-dependent hematopoietic cell lines. The pathways regulating this phosphorylation event are primarily the PI3-K and PKC pathways. IL-4 requires PI3-K activity in order to manifest its effects on GSK-3. In contrast, neither IL-3 nor GM-CSF stimulation of GSK-3 phosphorylation is dramatically altered with the presence of PI3-K inhibitors. While the MAPK/RSK and p70\textsuperscript{S6K} pathways show no evidence of playing a role in GSK-3 regulation, the use of PKC inhibitors suggests a strong function for this family of protein kinases. Unfortunately, the nature of the PKC pharmacological inhibitors makes it impossible to identify the specific class of PKC’s, or the specific PKC isoform responsible for the effects seen on GSK-3. As well, the effects of these PKC inhibitors on PKB make it difficult to distinguish the role of the PI3-K/PKB pathway from that of the PKC pathway. Likewise, studies of diacylglycerol production are also hampered by the questionable specificity of the inhibitors that are in common laboratory use. As such, it is difficult to establish which mitogenic pathways are essential for GSK-3 serine phosphorylation and cell survival by only using pharmacological methods. Genetic manipulation of GSK-3 and the various PKC isoforms within these cell
models would be a more productive exercise to determine the true effects GSK-3 and PKC on cell survival.
CHAPTER 5: REFERENCES


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