
Receptor tyrosine kinases, upon binding to their respective ligands, undergo dimerization, activation of intrinsic kinase domains and phosphorylation of intrinsic tyrosine residues. This results in the recruitment and activation of a number of intracellular signaling molecules. In the case of the receptor tyrosine kinase c-Kit, activation by its ligand Steel Factor (SLF) is important for a number of developmental systems including hematopoiesis, gametogenesis and germ cell formation. SLF stimulation of c-Kit induces the recruitment of a number of SH2-containing proteins including Phosphatidylinositol-3-kinase (PI3-kinase) and Phospholipase C gamma (PLC-γ). PI3-kinase generates D3 phosphoinositol second messengers. In the case of PLC-γ activation, hydrolysis of phosphatidylinositol bisphosphate into inositol trisphosphate and diacylglycerol leads to Ca2+ mobilization and activation of Protein Kinase C.

In this thesis, using c-Kit positive mast cells and myelomonocytic cell lines I have examined the involvement of these two signaling enzymes in SLF-stimulated survival and mitogenic signals as well as SLF-stimulated receptor internalization. The mechanism of SLF-stimulated c-Kit internalization is examined and a requirement for both Ca2+ influx and PI3-kinase activity in this process is identified. In addition, I have determined that mitogenic stimulation of c-Kit positive cells by membrane-bound SLF requires the activity of PLC-γ. Finally, I have identified an important role for PLC-γ-mediated Ca2+ influx in c-Kit-mediated survival signals and characterized a SLF-mediated apoptotic mechanism involving Ca2+ influx blockade. Therefore, the use of both c-Kit receptor mutants as well as specific inhibitors has allowed the identification of important roles for these signaling proteins and their second messengers in SLF-stimulated cellular processes. The relevance of these c-Kit associated signaling molecules in vivo will be discussed.
Acknowledgements

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<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor Protein</td>
</tr>
<tr>
<td>BFU</td>
<td>Burst Forming Unit</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone Marrow Derived Mast Cell</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CHK</td>
<td>Csk homologous kinase</td>
</tr>
<tr>
<td>CLP</td>
<td>Caecal Ligation and Puncture</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective Tissue Mast Cell</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas Associated Death Domain</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GRAP</td>
<td>Grb-2 related adaptor protein</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>Immune Response Element</td>
</tr>
<tr>
<td>KI</td>
<td>Kinase Insert</td>
</tr>
<tr>
<td>mIgM</td>
<td>membrane-associated Immunoglobulin M</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>PDK1&amp;2</td>
<td>Phosphatidylinositol-dependent protein kinase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B (Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PMA</td>
<td>Phorbol-12-myristate 13-acetate</td>
</tr>
<tr>
<td>SH2&amp;3</td>
<td>Src-homology domain</td>
</tr>
<tr>
<td>SLF</td>
<td>Steel Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>b-SLF</td>
<td>biotinylated-SLF</td>
</tr>
<tr>
<td>mSLF</td>
<td>membrane-bound SLF</td>
</tr>
<tr>
<td>sSLF</td>
<td>soluble SLF</td>
</tr>
<tr>
<td>TAM</td>
<td>Tyrosine-based Activation Motif</td>
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| TNF          | Tumour Necrosis Factor
"Children, be curious. Nothing is worse (I know it) than when curiosity stops. Nothing is more repressive than the repression of curiosity. Curiosity begets love. It weds us to the world. It's part of our perverse, madcap love for this impossible planet we inhabit. People die when curiosity goes. People have to find out, people have to know. How can there be any true revolution till we know what we're made of?"

_Graham Swift, Waterland. 1983_
CHAPTER I

Introduction to mast cells, c-Kit signaling and c-Kit biology.
The past 10 years have yielded advances in molecular genetics as well as biochemical techniques which have greatly facilitated our understanding of receptor biology. The ability to generate mice which are deficient in both cell-surface receptors and intracellular signaling proteins has revealed the role of some of these gene products in biological processes such as stem cell development. In addition, the use of mutagenesis techniques and the ability to overexpress mutant proteins in cell lines has permitted the identification of critical receptor motifs and signaling proteins which are required for the transduction of signals from the plasma membrane to the nucleus.

Understanding the biology of the c-Kit receptor (CD117), has been facilitated by the existence of naturally occurring mutant mice which are deficient in either the c-Kit receptor or its ligand Steel Factor (SLF). The phenotypes of these mice reveal that c-Kit signals are critical in a number of biological processes and that c-Kit deficient mice have very few mast cells. Adoptive transfer of c-Kit positive cells, and in particular c-Kit positive mast cells, into deficient mice allows one to evaluate the role of c-Kit-dependent processes in vivo.

The first chapter of this thesis deals with concepts that are pertinent to c-Kit signal transduction. The expression of c-Kit on mast cells is critical for mast cell development, function and maintenance. Therefore, mast cell biology will be reviewed in this chapter in addition to the function of c-Kit signaling in other cell types. The cloning, characterization and structure of both c-Kit and SLF will be detailed as well as some of the signaling molecules which are activated upon stimulation of c-Kit. Finally, the mechanism of c-Kit trafficking as well as some of the consequences of c-Kit activation such as mitogenesis and the maintenance of cell survival will be discussed.
(i) Overview of Mast Cells:

It has been postulated that the main function of mast cells is the clearance of parasitic worms. Indeed, mast cell receptors which are involved in the allergic or anaphylaxis response, a response which is deleterious to the host, evolved later than the mast cell itself. One of the key receptors on the surface of mast cells is c-Kit. Therefore, an account of mast cell development, function and maintenance is an important context for understanding the biology of the c-Kit receptor. In addition, analysis of c-Kit biology often involves the study of wild type mast cells or introduction of mutant c-Kit receptors into in vitro-generated mast cells from c-Kit deficient mice. This first section will deal with mast cell development, expression of key mast cell receptors, how mast cells are modulated by cytokines and growth factors and the role of the mast cell in protective immunity and immune dysregulation.

Mast cell development:

In 1981, a candidate precursor cell for the mast cell was identified. This cell type, termed the P-cell for "persisting cell" was derived from murine bone marrow, possessed histamine containing granules and was found to be Thy-1 negative and H-2K, H-2D positive. This putative mast cell precursor required a T cell derived growth factor. It was postulated that the P-cell gave rise to a subset of mast cells (1, 2). Further evidence of a mast cell progenitor was obtained in 1996 with the identification of a mastocyte precursor which had the capacity to give rise to all mast cells in vitro at high frequency. This precursor, which was isolated from murine fetal blood by Rodewald, et al., did not possess developmental potential for other hematopoietically derived lineages. The mastocyte progenitor was Thy-1<sup>lo</sup>-c-Kit<sup>hi</sup>, expressed RNAs for mast cell-associated proteases, contained cytoplasmic granules which are a hallmark of mast cell morphology, but did not express the mature
mast cell receptor FcεRI. Importantly, these progenitors were able to reconstitute mast cell deficient W/W^v mice to wild type levels when transferred intraperitoneally (3).

It is presumed that mast cells circulate briefly and mature in peripheral tissues by tissue migration and homing mechanisms mediated by adhesion molecules and soluble mediators. This homing mechanism, however, is not completely understood (4). Some of the tissues in which mast cells are distributed include connective tissues, respiratory system-associated tissues, tissues in the gastrointestinal and genitourinary tracts, at the blood or lymphatic vessel/tissue interface and within peripheral nerves (5-7). A key mediator in mast cell development is SLF as mouse strains that exhibit defects in either the mast cell-associated receptor tyrosine kinase, c-Kit (W/W^v) or the ligand for c-Kit, SLF, (S/S^v) are deficient in mast cells (8,9). It is possible that SLF expressed on fibroblasts and stromal cells in these tissues may play a role in the homing mechanism whereby Thy-1^hi, c-Kit^hi precursor cells migrate to these tissues and differentiate into FcεRI positive mast cells by virtue of a cognate c-Kit/SLF interaction (10).

Two major subsets of mast cells, connective tissue type mast cells (CTMC) and mucosal mast cells (MMC), have been described. The CTMCs reside in the skin and the peritoneal cavity and are considered to possess a more mature mast cell phenotype based on their granule content. Their phenotype is described as an ability to react with safranin dye and with the heparin-binding dye berberine sulfate. In addition, they possess high histamine content and incorporate [35S]sulfate into [35S]heparin. The MMC is considered to be a more immature mast cell type and is found in the mucosa of the gastrointestinal tract. IL-3 dependent bone marrow-derived cultured mast cells (BMMC), can be generated in vitro and are thought to represent MMCs in that they have an immature phenotype. Specifically, BMMCs do not exhibit cytoplasmic reactivity with safranin or berberine sulfate and have a low histamine content. Interleukin-3 (IL-3) is the major growth factor for MMCs.
BMMCs can mature and acquire the multiple phenotypic characteristics of CTMCs \textit{in vivo} or when co-cultured with SLF \textit{in vitro} but not with IL-3 alone (5, 11, 12).

\textit{Morphology, expression of FcεRI and secretion of mediators:}

More than 100 years ago Paul Ehrlich initially coined the term "mast zellen" or well-fed cells for mast cells. This term was given because of the prominence of large granules throughout the cytoplasm of these cells. As will be discussed, these granules are not only markers of the maturation state of the mast cells, but also play distinct and important roles in inflammatory and allergic responses. The two mast cell phenotypes described, the CTMC and MMC, do not necessarily represent a rigid phenotypic distinction as in fact, there exists a spectrum of intermediate phenotypes between the CTMC and MMC. This range of phenotypes is based on differences in granule mediators and sensitivity to a variety of agents (7, 13). These phenotypes can in fact be reversible, and are regulated by cytokines (5). In spite of the phenotypic variety exhibited by mast cells, one of the characteristics shared by all mast cell types is the expression of the high affinity receptor for the Fc portion of Immunoglobulin E (IgE) termed the FcεRI.

\textit{FcεRI regulation, activation and functions:}

The expression of FcεRI on the surface of mast cells is critical for the allergic functions of the mast cell. This was proven using FcεRI deficient mice which were unable to mount cutaneous and systemic anaphylactic responses (14). The mechanism for the involvement of FcεRI in allergic reactions has been determined. Allergic and anaphylactic reactions are stimulated by either circulating or pre-bound IgE which binds to multivalent antigen (Ag). FcεRI receptors on the surface of mast cells are then cross-linked by binding to the Fc portion of IgE/Ag complexes. This cross-linking event activates a signaling cascade
initiated by the FcεRI which ultimately results in the release of mediators from cytoplasmic granules (13). This sequence of events can be amplified by the presence of IgE molecules which have the ability to substantially upregulate FcεRI expression on murine mast cells both in vitro and in vivo. This upregulation allows mast cells to increase their ability to release proinflammatory and immunoregulatory mediators in response to IgE-dependent activation thus creating a strong amplification mechanism for the allergic response (15).

FcεRI is a multi-chain receptor composed of α, β and γ chains. The α chain is critical for binding of the Fc region of IgE. Aggregation of FcεRI by IgE and multivalent Ag results in the phosphorylation of Tyrosine based Activation Motifs (TAMs) which are found in the cytoplasmic domains of both the β and γ chains (16). An intracellular kinase which is a strong candidate for phosphorylation of these β and γ chain TAMs is the src family kinase, p56lyn (17, 18). These TAMs, when phosphorylated, provide binding sites for a number of cytosolic signaling proteins. In addition, these TAMs are rapidly de-phosphorylated by FcεRI-associated phosphatases. Some of the other kinases implicated in FcεRI-mediated signal transduction are the Syk and sphingosine kinases. Syk is likely involved in the phosphorylation of other proteins such as phospholipase C gamma (PLC-γ) which stimulates Ca²⁺ mobilization and the activation of protein kinase C (PKC). Sphingosine kinase may also be involved in FcεRI signaling by mediating Ca²⁺ mobilization.

Phosphatidylinositol 3-kinase (PI3-kinase) is involved in FcεRI signaling, and inhibition of this enzyme with wortmannin prevents mast cell degranulation (19). In addition to tyrosine residues, threonine residues on the γ chain and serine residues on the β chain are also phosphorylated (16, 20, 21). Thus, the intracellular signaling molecules listed here are activated in response to FcεRI aggregation by IgE and multivalent Ag. Furthermore, the activation of these signaling molecules ultimately leads to the release of mediators from the mast cell granules.
In addition to intracellular signaling molecules, however, the CD45 phosphatase surface receptor has also been implicated in mast cell degranulation. Specifically, CD45 deficient mast cells fail to degranulate upon FceRI aggregation (22). Therefore, CD45 is an essential transmembrane receptor which is required for mast cell degranulation, although the CD45-mediated signals which contribute to FceRI-mediated degranulation are not known.

Other substances can also induce mast cell degranulation. Ionomycin, which is an antibiotic produced by Streptomyces conglomeratus extracts Ca$^{2+}$ from the extracellular milieu and transports the cation across the cellular membrane and into the cytosol. Ionomycin has the ability to stimulate mast cell mediator secretion via this Ca$^{2+}$ transport function, thus implicating Ca$^{2+}$ signals in the degranulation process. (23). In addition, Substance P, a vasodilatory neuropeptide, induces degranulation (24) and this may also be due to its ability to mobilize Ca$^{2+}$ by G protein activation of PLC-β (25, 26). Aggregation of FcγRIII on the surface of mast cells also leads to the release of some mediators, but only in mast cells of the serosal or connective tissue (SMC) phenotype (27). FcγRI aggregation, however, fails to induce mast cell degranulation (28).

**Released mediators:**

Mast cell mediators perform functions such as regulation of vascular permeability, control of cell trafficking into tissues and extracellular matrix turnover and these functions are involved in both the pathogenesis associated with autoimmune disease and anaphylaxis as well as the clearance of bacteria and parasites. Mediators can be divided into those which are pre-formed and readily released from intracellular granules, those which are synthesized as a result of FctRI activation and various cytokines.
Pre-formed:

Histamine is one of the principle mediators in allergy and is fairly abundant with 1-5 µg of histamine per $10^6$ mast cells comprising 5-10% of the total granule weight. Histamine release exerts its effect on neighboring cells which express histamine receptors. A number of histamine-mediated biological effects have been observed both *in vitro* and *in vivo*. For instance, release of histamine from activated mast cells upregulates P-selectin on endothelial cells. P-selectin is an adhesion molecule which mediates the adhesion of lymphocytes to endothelial cells and induces leukocyte rolling in post-capillary venules, thus allowing leukocyte infiltration into the inflammatory micro-environment (29). Histamine also exerts its inflammatory effects by augmenting the production of IL-11 from human lung fibroblast cells (30) and by causing vasodilation (31).

Platelet Activating Factor is also released from mast cell granules and its role in inflammation is to aggregate platelets, neutrophils and monocytes. In addition, injection of Platelet Activating Factor causes an infiltration of eosinophils into the inflammatory micro-environment (32). Proteoglycans which exist as chondroitin sulfate in murine BMMCs and heparin in human mast cells are also released as pre-formed mediators. These agents stimulate fibroblast proliferation and potentiate the release of fibroblast growth factors. They also potentiate the binding of fibronectin to collagen (33).

Mast cells release a number of neutral proteases, the most prominent being tryptase, comprising 25% of the dry weight of mast cells. Mast cells are one of the only cell types which are capable of producing tryptase. Tryptase is found in a tetrameric conformation by forming a tight complex with heparin. Tryptases are also an important clinical marker in mast cell-mediated inflammation as they can be detected in the serum and plasma of affected individuals (34-36). Chymases are also neutral chymotrypsin-like proteases. Along with cathepsin G, these are both produced by mast cells (37). Recently it was determined that a
mast cell chymase is responsible for the cleavage of SLF from its membrane-bound form to a soluble form (38).

Newly synthesized mediators:
Upon aggregation of FcεRI receptors, some mediators are newly synthesized and released. Prostaglandin D2 (PGD2) and leukotriene C4 are the two major newly synthesized mast cell mediators. PGD2 causes bronchoconstriction, chemotraction of a variety of cell types, and vasodilation. Leukotrienes cause smooth muscle contraction and vasodilation as well as endothelial cell activation. These slow-reacting anaphylactic substances prolong the allergic reaction [reviewed in (7)].

Cytokines:
One of the most important inflammatory molecules released by activated mast cell is TNF-α. Mammalian mast cells are the only cell type which store TNF-α in its granules as a preformed mediator (39, 40). As will be further discussed, this cytokine is a critical molecule in mast cell-mediated bacterial clearance. In addition, mRNA for each IL3,4,5,6,8, GM-CSF, TGF-β, and chemokines (RANTES, MIP-1-α and IFN-α) are detected following IgE crosslinking in murine mast cells. These cytokines and chemokines contribute to the mast cell-mediated inflammatory response (41, 42).

Role of Mast cells in the immune system.

The distribution of mast cells throughout connective tissues, in the respiratory system, in the gastrointestinal and genitourinary tracts, at the blood or lymphatic vessel/tissue interface and within peripheral nerves (5-7) suggests that these cells are well positioned to respond to environmental antigens at skin or mucosal surfaces, blood-borne toxins or venoms, neuropeptides such as Substance P as well as parasites or bacteria (43). The release of
mediators such as histamine, heparin, proteases, leukotrienes and prostaglandins as well as TNF-α upon aggregation of FcεRI cause bronchoconstriction, increased vascular permeability and general inflammation. Under some circumstances this could potentiate IgE-mediated anaphylaxis (44, 45). Alternatively, in other circumstances, this inflammation could enable IgE-mediated responses to a parasite (46). Furthermore, mast cells have the ability to interact directly with bacteria and bacterial cell wall components such as lipopolysaccharides. This interaction promotes mediator release from mast cells (47, 48). Thus, depending on the circumstances, mast cells play diverse roles in the immune system. As will be discussed later in this section, these roles are dependent on the expression and function of c-Kit.

**Mast cell-assisted clearance of bacteria:**

There is a growing body of evidence that mast cells not only play a deleterious role in the organism (anaphylaxis) but may also be a key cell type for the clearance of pathogens such as bacteria and parasites. For example, Echtenacher and coworkers have demonstrated that reconstitution of mast-cell deficient W/W<sup>+</sup> mice with <i>in vitro</i> cultured BMMCs prevents death from surgically induced caecal ligation and puncture (CLP). The critical mast cell mediator which provided protection against CLP was determined to be TNF-α by blocking mast cell released TNF-α with specific antibodies (49). It has been demonstrated previously that mast cells are activated by complement components C3a and C5a (50). The CLP procedure was found to induce acute septic peritonitis in C4 and C3 deficient mice. It was then determined that in the C4 and C3 deficient mice, mast cells fail to produce TNF-α, a potent neutrophil recruiting factor. Thus mast cells represent an important link between the innate immune system and the subsequent clearance of peritoneal bacteria (51). Mast cells have also been shown to promote clearance of <i>Klebsiella pneumoniae</i> from the lungs and/or peritoneal cavity in mice and there again, TNF-α was the critical mediator.
Interestingly, TNF-α secretion from mast cells can be induced by direct contact between the mast cells and the bacterial type I fimbriae (52).

**Mast cell-mediated pathology:**

In addition to its role in the clearance of bacteria and parasites, mast cells have often been implicated in a number of pathological states. One mechanism which contributes to mast cell-mediated pathogenesis is the ability of mast cells to present Ag to T cells. Specifically, Ag/IgE complexes which are bound to FceRI become internalized. These internalized complexes are then degraded and antigenic peptides may be loaded onto MHC class I molecules and presented to T cells (53, 54). In addition, Interferon-gamma has been demonstrated to upregulate MHC class II on CTMCs. Therefore, FceRI-mediated internalization of Ag/IgE complexes may result in Class II presentation to CD4+ T cells (53). These mechanisms represent a potential amplification loop in mast cell-mediated autoimmunity. Indeed, in atopic individuals, langerhan cells and dermal dendritic cells both have the ability to trap Ag on FceRI. Whether this mechanism is directly involved in a number of auto-immune diseases and syndromes is not clear, however, there is evidence that mast cells do play a role in these disease states.

In the case of rheumatoid arthritis, it has been demonstrated using both W/W° mast cell deficient mice compared with wild-type littermates that mast cells may enhance the connective tissue damage which occurs in the chronic stages of arthritis. Co-culture of mast cells with human rheumatoid synovial cells was found to induce the release of collagenase and prostaglandins. It is postulated that mast cell secretion of TNF-α may also be a critical mediator in this pathogenic activity (54, 55).

In the case of the skin disease Scleroderma, large numbers of mast cells were found to be present in the affected tissues. It was therefore postulated that mast cells may contribute to
the dermal fibrosis which is associated with this disease (56). In Tsk mice, an animal model for Scleroderma, numerous degranulating mast cells have been observed in the thickened dermis of these animals, thus confirming an involvement of mast cells in the pathology of this disease (57).

The Arthus Reaction is a model for the pathogenesis observed in Ab/Ag complex formation which plays a role in Systemic Lupus Erythematosus, Rheumatoid Arthritis as well as Immune Glomerulonephritis and Vasculitis. Immune complex-mediated injury in mice by cutaneous Arthus reaction requires the expression of FcyRIII on mast cells (58). This was demonstrated by reconstitution of W/Wv mice with mast cells from wild-type or FcyR deficient mice. The involvement of mast cells in the pathology of the Arthus Reaction is similar to that which is observed in anaphylaxis, in that mast cells are stimulated to release mediators. However, the Fc receptor implicated in the Arthus Reaction is the FcγR rather than FceRI.

In the case of Asthma, there is a strong correlation with serum concentrations of IgE and pathogenesis. IgE-dependent acute reversible airway obstruction occurs by a mechanism that is mast cell dependent. Although other cell types such as T cells have been implicated in Asthma, one can experimentally induce a mast cell-mediated airway hyper-reactivity which can occur in the absence of leukocyte recruitment. In contrast, CD4+ T cell-mediated asthma likely requires eosinophil infiltration (reviewed in (61)).

Mast cells interact with the immune system in a variety of ways, and this interaction can either be helpful or deleterious to the organism, depending on the circumstances. The mediators released by mast cells may be critical for setting these immune responses. In Chapter 5 of this thesis, a model outlining different signals mediated by either membrane-
bound or soluble SLF will describe a role for c-Kit signaling in mast cell functions such as the clearance of peritoneal bacteria.

Effect of cytokines on mast cells:

As will be discussed in greater detail in the section on c-Kit receptor biology, c-Kit is highly expressed on mast cells and the ligand for c-Kit, SLF, stimulates a variety of biological outcomes in mast cells (59). The following are a number of effects that SLF as well as other cytokines exert on mast cells:

BMMCs are growth-factor dependent cells and can be grown in vitro with either Interleukin-3 (IL-3) or SLF. Withdrawal of either IL-3 or SLF from these cultures leads to apoptosis, and either of these factors can rescue BMMCs from apoptotic death. Thus, both IL-3 and SLF are survival factors for mast cells (60-62). In addition to being a survival factor, SLF under some circumstances can induce mast cells to proliferate. Injection of recombinant rat SLF into mice was found to induce the development of large numbers of dermal mast cells of the CTMC phenotype. Furthermore, addition of SLF was demonstrated to cause the in vitro proliferation of IL-3-dependent BMMCs (immature mast cell type) and purified peritoneal mast cells (mature type CTMC) in the absence of any other added factors. However, not only did SLF induce proliferation of BMMCs but it also induced the maturation of BMMCs of an alcian blue positive, safranin negative and low histamine content phenotype to the more mature CTMC phenotype which are safranin positive, have high histamine content and have the ability to synthesize heparin (63, 64).

SLF has been demonstrated to have a direct effect on the degranulation of mast cells. Recombinant SLF injected subcutaneously in patients with breast carcinoma induced a wheal and flare reaction which developed at each SLF injection site. At these locations,
most dermal mast cells exhibited extensive, anaphylactic-type degranulation. A fourteen
day time course significantly increased dermal mast cell density at sites distant from
injection. Therefore, a development of both mast cell hyperplasia and mast cell functional
activation was observed in vivo with SLF injections (65). Stimulation of mast cells in vitro
with SLF can also induce the release of IL-6 at a level 100 times higher than observed for
unstimulated cells, but less than the amount of IL-6 released in response to IgE plus Ag
(66). The effect of SLF on IgE-mediated mast cell degranulation, however, is variable.
Pre-incubation of mast cells with SLF was found to enhance the subsequent FcεRI-
stimulated mediator release (67). Conversely, mice which were pre-treated with
recombinant SLF for 21 days followed by sensitization with anti-DNP IgE and DNP Ag
exhibited no increase in IgE-dependent anaphylactic reactions. In fact, a slight decrease in
IgE-dependent anaphylaxis was observed (68). This could be due to the fact that the
chronic SLF treatment may also cause chronic degranulation. Therefore, sensitization with
IgE and Ag would not induce a strong degranulation response given that the intrinsic
mediators have yet to be re-generated. Thus, it appears as though SLF has a direct effect
on mast cell activation and mediator release, and it can also enhance the mediator release
cauised by aggregation of FcεRI by IgE and multivalent Ag if mast cells are not chronically
treated with SLF.

Other survival factors for mast cells include IL-3, IL-4, IL-5 and IL-6. Preincubation of
mast cells with IL-4, IL-5 or IL-6 for 24 hours during sensitization with IgE enhanced IgE
crosslinking-mediated histamine release (67). Notably, these cytokines are mostly Th2
cytokines, thus the participation of T cells in an allergic response may provide cytokine-
mediated amplification of the response. IL-4 has also been shown to promote human mast
cell maturation by inducing expression of the chymase protease as well as the tryptase
protease (69).
Therefore, mast cells play diverse roles in allergic and anaphylactic responses as well as autoimmunity and immunity against foreign pathogens. c-Kit has the ability to govern many mast cell functions by maintaining the survival of mast cells, inducing the maturation and proliferation of mast cells and enhancing the activation of mast cells. An understanding of c-Kit receptor biology and signal transduction will further illuminate the nature of the pleotropic functions of these cells.

(ii) Overview of the c-Kit receptor tyrosine kinase and its ligand SLF:

The *Dominant Spotting* (*W*) and *Steel* (*Sl*) loci in mice were described nearly 50 years ago (70, 71). These mice exhibited profound defects in three separate stem cell populations: neural crest-derived melanocytes, germ cells and hematopoietic precursor cells. Indeed, mice which are homozygous for mutations in either the *W* or *Sl* loci lack coat pigment, are sterile and anemic. In addition, these mice exhibited a profound deficiency in mast cells (72). More than 30 years ago, it was determined by E.A. MucCulloch that the defect in *W* mice could be rescued by transplantation of wild type bone marrow, whereas the defect due to the *Sl* mutation could not, suggesting that the *W* loci encoded a cell autonomous protein, whereas the *Sl* loci encoded an environmental factor (73).

Presently it is known that the *W* locus on mouse chromosome 5, encodes for the c-Kit proto-oncogene, a type-III receptor tyrosine kinase of the same family as the PDGFR and CSF-1 receptor (74, 75). The *W* locus spans approximately 30 kilobase-pairs of genomic DNA with 21 exons ranging from 100-200 base-pairs (76). The *Sl* locus on mouse chromosome 10 encodes the ligand for c-Kit termed in alphabetical order: kit ligand, mast cell growth factor, Steel Factor and stem cell factor (77-81). This section will address the structure of the c-Kit receptor and its ligand SLF, the regulation of the expression of these
two proteins and their tissue distribution, the various W and Sf mutant mice and their phenotypes and the biological function of this receptor/ligand interaction.

c-Kit structure:

c-Kit was first characterized as the viral oncogene (v-kit) of a feline sarcoma virus (HZ4-FeSV) (82). The cDNA cloning of the corresponding mouse and human cellular sequences confirmed that v-kit arose from the truncation of a cellular gene: the c-Kit proto-oncogene (83). Yarden and colleagues as well as Qiu et al. and Chabot et al. noted that the c-Kit 5.5 kilobase-pair mRNAs detected from mouse brain predicted an amino acid sequence which displayed significant homology to other type III transmembrane receptors such as the CSF-1 receptor encoded by c-fms and PDGFR-α and -β (75, 84).

The extracellular domain of c-Kit includes a signal peptide and a 500 amino acid ligand binding-domain with five immunoglobulin-like regions containing several intramolecular disulfide bonds and N-glycosylation sites (83, 84). A 23 amino acid hydrophobic transmembrane domain for insertion of the receptor in the plasma membrane lies downstream of the extracellular domain. A domain of 433 amino acids forms the intracellular segment with a kinase region (amino acids 575-915) containing an ATP-binding site (amino acid 622), intrinsic autophosphorylation sites and a noncatalytic, hydrophilic stretch of 77 amino acids known as the kinase insert domain (KI) which divides the kinase in half. This KI is poorly conserved and contains binding sites for signaling proteins which will be described. The structure of c-Kit is also illustrated in Figure 1.

Two alternatively spliced forms of the c-Kit receptor have been described (85-87).
The proto-oncogene c-Kit consists of a highly glycosylated extracellular domain which contains 5 immunoglobulin-like domains. The extracellular domain is followed by a membrane-spanning juxtamembrane domain. The intracellular portion of the receptor contains an intrinsic kinase domain divided in half by a kinase insert.
Type III receptor tyrosine kinase (c-Kit)

Glycosylated Extracellular Domain

Required for Dimerization

Juxtamembrane Domain

Kinase Domain (I)

Kinase Insert

Kinase Domain (II)
Alternate splicing generates two c-Kit transcripts with or without an in-frame 4 amino acid insertion Gly-Asn-Asn-Lys designated Kit⁺ in this thesis (83, 85). This four amino acid insertion is located in the extracellular domain, slightly upstream of the transmembrane segment. Hayashi et al. have correspondingly identified two potential 5’ splice donor sequences in the intron upstream of the transmembrane exon (88). The two receptors have similar affinities for soluble SLF by Scatchard analysis and they both respond to SLF by becoming tyrosine phosphorylated and recruiting signaling molecules. It has been demonstrated, however, that the Kit isoform is much more highly expressed than the Kit⁺ isoform on the surface of c-Kit positive cells (89).

**SLF structure:**

Although SLF was not cloned until 1990, prior to this there was strong biological evidence that mast cells and mast cell precursors required a factor produced by fibroblasts in either a soluble (90, 91) or membrane-bound form (92-95). This factor was thought to be the ligand for c-Kit as mast cells derived from culturing fetal liver W/W² cells in IL-3 for several weeks were unable to respond to this fibroblast-associated activity. It was then demonstrated that a purified and characterized mouse fibroblast-derived soluble factor (designated as Kit Ligand) stimulated proliferation of mast cells derived from wild-type but not W/W² littermates. This factor also promoted the development of erythroid burst-forming units (BFU-E) in synergy with IL-3 (96). This ligand, SLF, was then cloned by a number of groups (77-81). The genomic structure of SLF was found to have at least 8 exons ranging in size from 51-183 base-pairs in length with exons 2-7 encoding portions of the extracellular domain of the ligand, exon 7 encoding the transmembrane region and exon 8 encoding the cytoplasmic tail (97).
The protein structure of SLF was also examined. SLF is heavily glycosylated with O-linked and N-linked sugars and exists as a noncovalently associated dimer (98-100). The soluble form of SLF is comprised of the first 164-165 amino acids of the extracellular domain and is generated by a proteolytic cleavage event (38, 77, 81, 97, 98, 101). Most soluble SLF in serum, however, likely exists as a monomer, given that its concentration is less than 3 ng/ml (102). Three alternatively spliced murine forms of SLF mRNA have been reported: a full length form, a species lacking exon 6 thus producing a 28 amino acid deletion and another splice variant with a smaller 16 amino acid deletion of exon 6 (101, 103).

Tissue distribution of both c-Kit and SLF proteins.

During mouse embryogenesis c-Kit and SLF are expressed in the locations where proliferation and maturation of each hematopoietic, melanoblast and germ cell lineages occurs (104, 105). In the adult, ovarian c-Kit expression is confined to the oocyte and expression of SLF is observed in the ovarian follicle during postnatal life (104, 105). There is evidence for a chemotactic role for SLF in melanocyte migration which results in the development of pigmentation as SLF expression can be detected in the subdermal mesenchymal cells of the limb buds prior to melanocyte invasion (105). c-Kit is concordantly expressed on melanocytes and melanocyte precursors. SLF and c-Kit are expressed during early brain development where SLF is expressed at day 12 and 15 of gestation in the floor plate of the neural tube and c-Kit expression has been noted in migrating neural crest cells and in differentiating neurons in the dorsal neural tube and dorsal ganglia (104). In the adult mouse, c-Kit and SLF are expressed at high levels in the central nervous system (84).
In terms of hematopoietic cells, c-Kit is highly expressed at day 11.5 in the fetal liver (104, 105). In the adult hematopoietic system c-Kit mRNA is expressed in mast cells and in early erythroid and myeloid cell lines and some lymphocytes (106). Thus, it appears that c-Kit and SLF expression are at highest levels during the early stages in development and that one notable exception is the mature mast cell which continues to respond to SLF.

Regulation of expression of the c-Kit receptor.

Protein kinase C (PKC) has been reported to play an important role in modulation of the expression of the c-Kit receptor by inducing the release of the extracellular domain of c-Kit in M-07e cells by a poorly understood proteolytic mechanism (107). c-Kit mRNA levels have also been observed to be modulated in response to a number of agents. Both the PKC stimulator Phorbol-12-myristate 13-acetate (PMA) as well as IgE plus Ag reduces the level of c-Kit mRNA by more than 50% (108). In addition, cytokines such as IL-3, GM-CSF and Erythropoietin (Epo) but not IL-4 are able to down-regulate c-Kit mRNA expressed by mast cells and stem cell progenitors (109).

Mutations in both W and Sf loci.

There are several different mutations of both the W and Sf locus which result in various phenotypes. Comparison of the W locus with other loci which display observable phenotypes reveals that the spontaneous mutation rate for the W locus is exceptionally high (110). Generally, a double dose of mutant W or Sf alleles results in either embryonic lethality or yields a macrocytic anemia, lack of coat pigment, sterility and a profound mast cell deficiency. Embryonic lethality is likely due to absence of early hematopoietic cells, thus causing death in utero or in the prenatal period due to severe anemia (8, 9, 111, 112). The W mutation is a 78 amino acid deletion resulting in the absence of both cell surface c-
Kit, and c-Kit kinase activity (96). The $W^{42}$ mutation is a mutation resulting in loss of c-Kit intrinsic kinase activity. Mice homozygous for either the $W$ and $W^{42}$ mutations are embryonically lethal (113, 114). $W^{v}$ is a homozygous viable mutation which also maps to the kinase domain, but produces a less severe reduction in the level of intrinsic kinase activity of the c-Kit receptor (96, 115). $W$ mutations behave in a co-dominant fashion with other $W$ alleles. This behaviour is likely due to the requirement for c-Kit receptor dimerization in order to activate the intrinsic c-Kit kinase domains. Receptor dimerization and signaling will be discussed in greater detail in section (iii).

Mutations at the $Sl$ locus result in similar phenotypes as the $W$ mutations. $Sl^{d}$ is a deletion mutation which yields a truncated SLF protein, thus only generating the soluble form of the ligand (103). Interestingly, the $Sl^{d}$ mutation has the same phenotype as the $Sl$ mutation, specifically, sterility, lack of coat pigment and sever anemia. This suggests that the membrane-bound form of the SLF ligand is more important biologically than its soluble counterpart (116). There are several other examples of $W$ and $Sl$ mutations (74, 75, 115, 117), and, it is important to note that these mutations may be taken advantage of experimentally. Specifically, mast cell-positive mice can be generated by adoptive transfer of congenic wild-type mast cells into mast cell deficient $W^{42}$ mice. Several investigators have performed these adoptive transfer experiments in order to study the role of either mast cells or c-Kit mediated signals in the context of a variety of cellular processes (118).

**Biological functions of both c-Kit and SLF proteins.**

Signals mediated by the c-Kit receptor upon SLF binding lead to a number of different outcomes in a variety of cell types (reviewed in (108)). This thesis deals primarily with survival and proliferation signals in the context of mast cells. However, the following is a
brief description of some of the biological consequences mediated by this receptor/ligand system.

Adhesion:
Adhesion is an important component in the process of homing and migration of cells to their target tissues. There is strong biological evidence that SLF contributes to the migration of c-Kit positive cells to their target tissues as discussed in the section on tissue distribution. In vitro evidence of c-Kit/SLF mediated adhesion was demonstrated by Flanagan et al. where in vitro-derived mouse mast cells were shown to adhere to COS cells transfected with mSLF but not sSLF (103). Conversely, mast cells from W/W mice which express no c-Kit protein were unable to adhere to fibroblasts, however, mast cells from W/W mice which have diminished kinase activity but express the c-Kit extracellular domain exhibited no such impairment (119, 120). In addition, c-Kit can mediate adhesion to matrix proteins such as fibronectin (121) and a dependence on the c-Kit extracellular domain for this adhesion has been demonstrated (122).

Chemotaxis:
The distribution of SLF during development strongly suggests that germ cells of the melanocyte, oocyte and mast cell lineages migrate to their sites of development, likely by virtue of the c-Kit receptor (92). Evidence that SLF-stimulated chemotaxis occurs in vivo is derived from experiments where subcutaneous injections of recombinant SLF led to a profound recruitment of mast cells at dermal injection sites (64, 65). A direct demonstration of SLF-stimulated chemotaxis in vitro was observed by Blume-Jensen et al. using c-Kit transfected porcine aortic cells. Upon SLF stimulation, these cells underwent circular actin reorganization and SLF-directed chemotaxis (123). Other investigators have confirmed, using W/W mice mast cells which express kinase inactive c-Kit positive receptors, that SLF-directed chemotaxis requires kinase activity (124). SLF also has
indirect effects on chemotaxis induced by other factors. Incubation of human cultured mast
cells with SLF induced an enhancement of mRNA levels for MCP-1 (monocyte
chemoattractant protein) but not other chemoattractants such as interleukin-8 (IL-8),
macrophage inflammatory protein-1alpha (MIP-1alpha), MIP-1beta, or RANTES (125).
Thus, either directly or indirectly, SLF stimulates the chemotaxis of c-Kit positive cells.

**Survival and hematopoiesis:**

SLF is an essential hematopoietic cytokine which often functions in concert with other
cytokines in order to maintain viability of progenitor cells so that they may go on and
differentiate into a variety of cell types. SLF is incapable of driving noncycling
hematopoietic progenitor cells into the cell cycle but it does prevent their apoptotic death
(126). For example, c-Kit is expressed on 76% of CD34+ and all CD34+HLA-DR-
marrow cells. In combination with other cytokines, SLF was found to increase the cloning
efficacy of these immature precursor cells due to its anti-apoptotic properties (127). SLF
was also found to enhance the development of the most early progenitors (CD34+HLA-
DR‘c-Kit+) into pre-colony-forming cells which give rise to BFU-E (erythroid), CFU-G
(granulocyte), CFU-GEMM (granulocyte, erythroid, monocyte, megakaryocyte) and CFU-
GM (granulocyte,monocyte). The SLF-mediated enhancement in precursor differentiation
observed was determined to be due to protection from apoptosis (128). With respect to
progenitor cells of a variety of lineages, SLF is required for the survival of germ cells
(129), melanocytes (130), myeloid progenitors (131), erythroid progenitors (132), Natural
Killer cell progenitors (133) as well as hematopoietic stem cells (134-137). In addition,
SLF is a survival factor for terminally differentiated human mast cells (138, 139) as well as
murine mast cells as demonstrated in vitro (61) and in vivo (63, 64). The anti-apoptotic
properties of SLF in maintaining both immature progenitors and mature mast cells was
found to be inhibited by TGF-β (140). However, this may be due to a down-modulation
of c-Kit mRNA caused by this cytokine (109, 141).
Interestingly, the anti-apoptotic properties of SLF are only effective \textit{in vivo} if SLF is in its membrane-bound form. This is illustrated by the fact that normal mast cells injected subcutaneously into \textit{Slf} mice where the only form of SLF is the soluble form, failed to survive, whereas these mast cells could survive in the skin of \textit{W/W} mice (142). In addition, transfection of \textit{Slf} stromal cells with either the membrane-bound form of SLF (mSLF) or the soluble form of SLF (sSLF) revealed that mSLF maintains hematopoiesis for longer periods \textit{in vitro} (143). In addition, mSLF was found to be much more effective than sSLF in promoting mouse primordial germ cell survival \textit{in vitro} (144, 145).

\textit{Proliferation:}

In general, SLF is a survival factor, and proliferation due to SLF is often due to synergy with other cytokines (145, 146). For example, SLF induces human melanocytes to proliferate \textit{in vitro} if it is provided with PMA (147). SLF can act as a mitogen on its own, however this is usually only in the case of terminally differentiated mast cells or in malignant cell growth. Examples of SLF-mediated malignant growth are observed in small-cell lung cancers (148) and in \textit{CD34}+ acute myeloid leukemic blast cells (149, 150). SLF may also induce the proliferation of c-Kit expressing myelomonocytic cell lines \textit{in vivo}. Specifically, injection of c-Kit negative 32D cells into syngeneic mice was found to result in no apparent pathology. In contrast, expression of c-Kit in 32D cells greatly increased the leukemogenic potential of these cells \textit{in vivo}, as evidenced by a profound proliferation of c-Kit positive 32D cells \textit{in vivo} as well as \textit{in vitro} (151).

As mentioned, mast cells are one of the only non-malignant cell types which will also proliferate in response to SLF. Immature mouse mast cells or freshly isolated mature mouse peritoneal mast cells both proliferate in response to SLF when it is added as the only cytokine (63, 96). The proliferative effect of SLF on mast cells may also reflect the ability
of mast cells to secrete other cytokines such as IL-3 and IL-4 which also induce mast cell proliferation [reviewed in (108)].

**Differentiation and maturation:**

Differentiation has been defined as the development of precursor cells into a cell type which is distinct from its precursor. Maturation is defined as the attainment of full development and/or growth. An example of maturation for mast cells is the storage of increasing amounts of histamine in mast cell granules. In terms of differentiation, SLF is usually the base "survival" cytokine and additional cytokines are needed to drive differentiation down a particular pathway. It is therefore difficult to tell whether under these circumstances, SLF is contributing to a differentiation program or not. The following are examples where SLF plays a role in differentiation of a number of precursors:

SLF in combination with GM-CSF, G-CSF, IL-3, Epo or IL-6 was found to stimulate erythropoiesis and myelopoiesis in both short term (134, 152) and long term bone marrow cultures (137, 153). SLF also enhances IL-5, IL-3 and GM-CSF-stimulated eosinopoiesis from normal human bone marrow (154). Injection of SLF in vivo along with G-CSF results in neutrophil development (155) and SLF significantly enhances the development of megakaryocytes in combination with GM-CSF, IL-3 or IL-6 (156, 157).

In terms of lymphocyte hematopoiesis, SLF enhances IL-7 stimulated B lymphopoiesis (158). SLF is also a potent synergistic factor with IL-2 or IL-7 in promoting proliferation of CD4+/CD8+ adult mouse thymocytes (159). Incubation of human thymocytes with SLF and IL-4 results in the development of TCRγδ cells. However, if SLF is added with IL-2, one observes a ten-fold increase in CD56+ large granular cells, indicative of a Natural Killer cell phenotype (160).
In addition to the cooperative effect of SLF with other cytokines on progenitor differentiation, SLF has been shown to enhance the G-CSF mobilization of bone marrow stem and progenitor cells into the periphery, an important step in the differentiation program of a number of cell types (155, 161).

SLF also induces the maturation of mast cells. Mature CTMCs can be generated in SLF-deficient mice by administration of recombinant SLF (64). It is difficult to determine if this outcome is due to a maturation event or simply due to enhanced survival and/or proliferation. However, the maturational properties of SLF were confirmed by an in vitro co-culture experiment with immature mast cells and NIH3T3 fibroblasts. These co-culture conditions were found to induce a mature mast cell phenotype where mast cells increased the content of histamine in their granules among other phenotypic markings (162). This was also confirmed in vitro by incubating mast cells with soluble recombinant SLF as the only cytokine which was found to induce a mature mast cell phenotype (63). Thus, these experiments demonstrate that SLF is a maturation factor for mast cells.

**Activation:**

W/W^v^ mast cells can be generated in vivo by repeated treatment with PMA (142). When these c-kit negative mast cells are compared with wild-type mast cells, it was found that SLF induced mast cell activation and mast cell-mediated inflammation in vivo only in those mice with c-Kit positive mast cells (163). SLF was also found to induce mediator release from human skin mast cells in vitro in the absence of any other added cytokines (164). Thus, SLF is capable of activating mast cells to release their granule-stored mediators.

Therefore, these aforementioned SLF-mediated effects govern many of the activities of mast cells. The biochemical mechanisms for these outcomes will be discussed in the next section on c-Kit signal transduction.
(iii) c-Kit signal transduction

This section deals with how SLF stimulation of the c-Kit receptor at the plasma membrane generates the various signals which ultimately result in changes in gene transcription. This sequence of events begins with receptor dimerization, kinase activation, autophosphorylation of the receptor, recruitment of SH2-containing proteins and the enzymatic generation of second messengers, all of which will be discussed.

Ligand binding and receptor dimerization:

Receptor dimerization is an event which is thought to be critical for the initiation of signal transduction by growth factor receptor tyrosine kinases (165). The fact that most receptor tyrosine kinases span the plasma membrane only once raises the possibility that signal transduction may involve intermolecular rather than intramolecular interactions. This has been shown for the EGF<sup>R</sup>, CSF-1 receptor and PDGF<sup>R</sup> using covalent cross-linking reagents (166-169). In terms of the c-Kit receptor, the observed co-dominance of mutant <i>W</i> alleles also suggests that intermolecular events between c-Kit receptors are required for efficient kinase activation and signaling (170). The structure of SLF reveals that it has the capacity to exist as a non-covalent dimer (98-100). It was therefore predicted that c-Kit receptor dimerization, an event which occurs at the initiation of the signaling process in numerous receptors, would occur by virtue of the structure of its ligand SLF. Blechman <i>et al</i>. have demonstrated that dimerization of the c-Kit receptor is not only due to ligand bivalence, but that there is a putative dimerization site on the fourth immunoglobulin-like domain of the extracellular portion of c-Kit. Deletion of this domain abolished ligand-induced dimerization. In addition, deletion of this domain inhibited SLF-stimulated c-Kit signal transduction as measured by tyrosine phosphorylation of the receptor and [<sup>3</sup>H]-
thymidine incorporation of proliferating cells (171, 172). Other investigators later confirmed by Fluorescence Energy Transfer that c-Kit dimerization does indeed occur (173).

**Activation of the intrinsic tyrosine kinase domain:**

Dimerization presumably leads to the trans-phosphorylation of neighboring c-Kit kinase domains. This trans-phosphorylation causes activation of the kinase domains and the subsequent phosphorylation of intrinsic tyrosine residues in the intracellular portion of the receptor. In agreement with a process which leads to the activation of c-Kit tyrosine kinase domains, c-Kit positive M07E cells were found to be stimulated by SLF dimers leading to dose-dependent proliferation which was inhibitable by the tyrosine kinase inhibitor genistein. In addition, immunoblotting revealed tyrosine phosphorylation of the c-Kit receptor in response to SLP (174). Similarly, in normal human melanocytes, it was found that SLF stimulation led to the proliferation and tyrosine phosphorylation of the c-Kit receptor. These investigators also noted a number of other tyrosine phosphorylated proteins which may have been phosphorylated by the c-Kit kinase, one of which was identified as MAP-kinase (175). Stimulation of c-Kit transfected Cos cells with SLF was found to induce the recruitment and phosphorylation of phosphatidylinositol 3' kinase (PI3-kinase) and phospholipase C gamma 1 (PLC-γ). The introduction of point mutations associated with the \( W^{D2} \) mutant allele into c-Kit expression constructs reduced both SLF-induced autophosphorylation and association of the c-Kit receptor with these signaling proteins. This verified that it was c-Kit auto-kinase activity which was important for recruitment and activation of signaling proteins (176). In addition, autophosphorylation of the c-Kit receptor was found to be required for SLF-mediated activation of p21ras (177).
There are also examples where the c-Kit kinase domain has become dysregulated leading to cellular transformation, thus highlighting the biological importance of the c-Kit kinase domain in c-Kit signal transduction. The murine mastocytoma P815 was found to exhibit ligand-independent activation of the c-Kit receptor. Specifically, the P815 cells exhibited constitutive kinase activity, receptor auto-phosphorylation as well as factor-independent proliferation. This mutation was found to be an aspartic acid to tyrosine mutation at residue 814 in the kinase domain (178). Similarly, in a human mast cell leukemia, a mutation at residue 816 from valine to aspartic acid was noted (179). Moriyama et al. have studied the effect of this mutation in greater detail and found that it causes constitutive tyrosine phosphorylation and enhanced degradation of the receptor (180). Data generated in this laboratory using purified c-Kit kinase domain constructs with the PSI5 aspartic acid to tyrosine mutation suggests that these kinase domains likely pre-exist as dimers in solution. This is in contrast to non-mutated kinase domains which do not pre-exist as dimers. The pre-existence of kinase dimers may possibly explain the observed ligand-independent activation observed in the P815 mastocytoma (181). Thus, the kinase domain is critical for the initiation of SLF-mediated signal transduction, and dysregulation of this kinase domain has the potential to transform a variety of cell types.

**SLF-stimulated recruitment of SH2 domain-containing proteins.**

As mentioned, activation of the c-Kit receptor induces the recruitment and phosphorylation of a number of cellular proteins. These proteins are recruited to the activated c-Kit receptor by virtue of their SH2 domains. SH2 domains are protein modules of 100 amino acids and are found in diverse groups of cytoplasmic signaling proteins. Autophosphorylation of specific tyrosine residues on the c-Kit receptor following ligand-mediated dimerization reveals binding sites for SH2 domain containing molecules. High affinity binding of an SH2 domain requires a phosphorylated tyrosine residue imbedded within a specific amino
acid sequence. The sequence of phosphotyrosine and the three amino acids downstream of this phosphotyrosine confer the specificity for the different SH2 domains of molecules such as PI3-kinase and PLC-γ which bind to different sites on the same receptor. SH2-containing proteins often also have 50 amino acid protein modules called SH3 domains and these SH3 domains bind to proline-rich sequences (182). A diagram of the c-Kit receptor with its associated signaling proteins is illustrated in Figure 2.

The SH2-containing proteins which are recruited to the c-Kit receptor become phosphorylated and enzymatically active in order that they may generate various second messengers (165, 176, 183). c-Kit activation leads to the recruitment and phosphorylation of PI3-kinase, PLC-γ and GTPase activating protein (184). The specific site for PI3-kinase binding to the c-Kit receptor was found to be in the kinase insert at phosphorylated tyrosine residue 719 which is part of a four amino acid sequence, YMDM (tyrosine, methionine, aspartic acid, methionine) (185). In addition to these proteins, other molecules are also either directly or indirectly recruited to the c-Kit receptor. For example, SLF-stimulated recruitment of the p85 regulatory domain of PI3-kinase to the c-kit receptor results in the recruitment of other proteins such as the proto-oncogene CRKL which is also bound to p85. This p85-mediated recruitment of CRKL leads to the phosphorylation of CRKL and the association of CRKL with the c-Kit receptor via its SH3 domains. Therefore, PI3-kinase has the capacity to bring other proteins such as CRKL to the c-Kit receptor. In the case of CRKL, CRKL associates with p120(CBL). Thus a complex interaction between c-Kit, p85 and CRKL allows the p120(CBL) protein to associate with c-Kit (186).

Stimulation of c-Kit with SLF also induces SH2-mediated binding of non-enzymatic
Figure 2. Schematic representation of c-Kit associated signaling molecules.

Binding of SLF to c-Kit receptors results in receptor dimerization and activation of the intrinsic tyrosine kinase domain. This process results in the phosphorylation of tyrosine residues which serve as docking sites for SH2-containing proteins such as PI3-kinase, PLC-γ and Grb2.
Figure 2.

c-kit

Src 567 YVPL 569
Grb-2 702 YKNL
PI3K 719 YMDM
PLCy 728 YVVP
adaptor proteins. The adapter protein Grb2 has been demonstrated to bind to c-Kit via its SH2 domain. Grb2 then interacts with other proteins such as the guanine nucleotide exchange factor mSos1 which exchanges GTP for GDP on the Ras protein. Ras activation triggers a signaling cascade where its substrate Raf is recruited to the plasma membrane where it can then activate other kinases which ultimately activate MAP-kinase (187). GRAP, a Grb2 related adapter protein which contains the same SH3-SH2-SH3 domains as Grb2 also associates with c-Kit upon SLF induction. GRAP has been demonstrated to co-immunoprecipitate with mSos1, thus GRAP may also activate the Ras pathway (183).

Non-receptor tyrosine kinases have also been demonstrated to co-associate with the c-Kit receptor upon stimulation with SLF. Using the megakaryocyte line CMK, Jhun et al. demonstrated that SLF stimulation caused the co-immunoprecipitation of c-Kit with a fusion protein of GST and the SH2 domain of MAT-Kinase (188). More recently, the c-Kit binding site for this kinase which was renamed CHK for Csk homologous kinase, was mapped to both the tyrosine 561/567 diphosphorylated sequence as well as the monophosphorylated tyrosine 721 sequence.

Tyrosine phosphatases, as their name suggests, are enzymes which de-phosphorylate tyrosine residues and in many cases, down-regulate growth factor induced responses. SLF-stimulated c-Kit binds to either the amino or carboxyl-terminal SH2 domains of the phosphatase SHP-1. SHP-1 also becomes tyrosine phosphorylated as a result of recruitment to the c-Kit receptor and can dephosphorylate c-Kit in vitro (189). c-Kit associated tyrosine phosphatases will be discussed in greater detail in the section on negative regulation of c-Kit.
In addition to the SH2-containing proteins which inducibly associate with c-Kit upon SLF stimulation, there are a number of proteins which constitutively associate with c-Kit, and these proteins become phosphorylated upon SLF binding. Tec is a cytoplasmic src family kinase and is constitutively associated with c-Kit. Following SLF binding, Tec becomes phosphorylated and demonstrates kinase activity. Given that other hematopoietic cytokines such as IL-3, CSF-1 or GM-CSF do not activate Tec, the unique activation of Tec by SLF may induce synergy when combined with those signaling proteins activated by other receptors (190).

JAK-2 kinase has also been reported to constitutively associate with c-Kit. Upon SLF binding, greater quantities of JAK-2 kinase associate with the receptor and JAK-2 becomes transiently phosphorylated. Inhibition of JAK-2 expression with anti-sense oligonucleotides resulted in a marked reduction in SLF-mediated mitogenesis thus implicating this kinase in c-Kit mediated proliferation signals (191, 192). The Lyn kinase is also constitutively associated with c-Kit and it binds to the juxtamembrane region of the receptor. Stimulation with SLF leads to phosphorylation of lyn over a period of 60 minutes and SLF-stimulated in vitro lyn kinase activity has also been observed. Similar to JAK-2 kinase, inhibition of lyn kinase with antisense oligonucleotides leads to a dramatic reduction in SLF-mediated mitogenesis.

Among the panel of proteins which become tyrosine phosphorylated upon SLF stimulation, one of the most strongly phosphorylated proteins is the proto-oncogene p95vav. However, unlike the proteins listed above, although p95vav is strongly phosphorylated, it was not found to co-associate with the c-Kit receptor following SLF stimulation. The mechanism for SLF activation of p95vav therefore remains to be elucidated (193).
SLF-mediated generation of second messengers.

The activation of the various aforementioned signaling proteins as a result of stimulation with SLF leads to the enzymatic generation of second messengers (194). This section will deal primarily with the messengers generated by both PI3-kinase and PLC-γ. PLC-γ becomes phosphorylated upon SLF stimulation of the c-Kit receptor (184). PLC-γ, when activated, hydrolyses phosphatidylinositol-bis-phosphate (PIP2) into inositol-tris-phosphate (IP3) and diacylglycerol (DAG) (195). These lipid second messengers stimulate two main cellular outcomes: the mobilization of Ca2+ and the activation of PKC.

Specifically, IP3 binds to IP3 receptors on the surface of the endoplasmic reticulum. This interaction induces the opening of the IP3 receptor channel in the endoplasmic reticulum which releases stored internal Ca2+ into the cytosol. Ca2+ mobilization from internal stores leads to the influx of Ca2+ from the extracellular milieu via the ICRAC-channel in the plasma membrane. This channel is a "store operated Ca2+ channel" which is induced to open upon emptying of internal Ca2+ stores (196, 197). Ca2+ mobilization has been demonstrated in SLF-stimulated human adult skin mast cells (25).

The DAG second messenger activates the enzyme protein kinase C (PKC) (198). PKC is a serine/threonine kinase family of 12 known members where PKC-α, -β and -γ are Ca2+ and phospholipid-dependent. c-Kit is phosphorylated by PKC-α on 2 serine residues, Ser-741 and Ser-746 in the kinase insert and Ser-821 and Ser-959 in the C-terminal portion of the receptor. These last two, however, are not phosphorylated directly by PKC-α but depend on PKC activation (199). The function of PLC-γ second messengers will be described in more detail in section (γ) of this Chapter.
PI3-kinase has been shown to inducibly co-immunoprecipitate with the c-Kit receptor and the p85 regulatory subunit of PI3-kinase binds specifically at tyrosine residue 719 (85, 176, 184, 185). Upon recruitment of p85/PI3-kinase to the c-Kit receptor PI3-kinase becomes activated (184). The enzymatically active PI3-kinase molecule phosphorylates phosphatidylinositols (PI) substrates on the D3 position of the inositol ring thus generating PI(3)P, PI(3,4)P2 and PI(3,4,5)P3 (200). The functional role of these second messengers will be discussed in more detail in section (iv) of this Chapter.

Negative signaling mechanisms for c-Kit signaling down-modulation.

Negative regulation of receptor signaling is accomplished by a variety of mechanisms. One universal method of down-modulating signals at the cell membrane is by regulating the surface expression of receptors such as c-Kit by a mechanism termed ligand-stimulated receptor internalization, a process common to all receptor tyrosine kinases (201, 202). This process delivers receptors into lysozomal compartments where they may be degraded. In addition to receptor internalization, c-Kit can be removed from the cell surface by incubation with the PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA). This results in a release of the extracellular domain of the receptor, likely by proteolytic cleavage near the transmembrane domain. This receptor shedding event also limits the availability of SLF to the remaining trans-membrane c-Kit receptors as released free extracellular domains can competitively bind to SLF in the microenvironment (206).

In addition to these down-modulatory mechanisms, c-Kit receptors may also be ubiquitinated following receptor internalization. Protein ubiquitination is a mechanism for tagging short lived proteins so that they are directed for degradation. In MO7e cells, SLF-stimulation of c-Kit leads to rapid ATP and kinase-dependent polyubiquitination, as evidenced by its inhibition by both sodium azide and genistein. However, c-Kit also
c-Kit mRNA levels can also be decreased in response to a number of cytokines. IL-3, GM-CSF and Epo were found to down-regulate the level of c-Kit mRNA expressed in mast cells and stem cell progenitors and this down-regulation was paralleled by a decrease in receptor expression (109). In addition, it was found that proliferation of c-Kit positive, SLF-responsive blast cells from AML patients was almost completely abolished upon the addition TGF-β in vitro. This inhibition was found to be due to a decrease in c-Kit receptor expression on the surface of the blasts, as a result of an acceleration of c-Kit mRNA decay (141).

c-Kit signals themselves can be modulated by PKC. PKC has been demonstrated to participate in a negative feedback loop regulating c-Kit by phosphorylation of intrinsic c-Kit serine residues. Serine phosphorylation of c-Kit was found to correlate with a concomitant decrease in c-Kit autophosphorylation and kinase activity (204). It was also observed that inhibition of PKC enhanced SLF-mediated PI3-kinase activation by enhancing p85 binding to the receptor (205). Another negative feedback mechanism which is SLF-signaling dependent is the activation of a c-Kit associated tyrosine phosphatase termed syp or PTP1C or SHP-1 (this thesis will use the nomenclature SHP-1) (189, 206). SHP-1 is the product of the murine motheaten locus (me) and binds inducibly to the juxtamembrane region of the c-Kit receptor (207). In agreement with a negative regulatory function for SHP-1, a factor-independent mastocytoma was found to express SHP-1 which was chronically ubiquitinated and therefore effectively down-regulated. It was postulated that this chronic SHP-1 ubiquitination contributed to the transformed phenotype of these cells (208). In addition, in vivo evidence for the negative role of SHP-1 in c-Kit signaling has been...
demonstrated. $W/W^{+}$ mice and $W/+^{+}$ mice were bred with $m/m$ and $m/+^{+}$ mice in various combinations. It was found that adding a single dose of $m$ to $W/+^{+}$ mice increased the cloning efficiency of hematopoietic progenitor cells. This suggests that there is a potentiation of c-Kit signaling when $m$ mutations are introduced into the $W$ genetic background (209). Thus, there are a variety of mechanisms which negatively regulate c-Kit signaling, and dysregulation of these mechanisms may contribute to c-Kit mediated cellular transformation.

**Mechanisms of synergy with other growth factors.**

Synergy between different cytokines and growth factors likely occurs due to the stimulation of two or more signaling pathways unique to each factor in addition to shared overlapping signaling pathways. Convergence on one signaling molecule as a result of stimulation by several growth factors does occur. For example, JAK2 is activated by IL-3, GM-CSF and SLF in spite of the obvious structural differences of each respective receptor (IL-3 and GM-CSF share a common beta subunit devoid of kinase activity, while c-Kit has intrinsic kinase activity) (210). However, receptors such as the ones mentioned stimulate their own unique signaling pathways as well, which when combined, induce synergy.

The effect of SLF on IL-3 and GM-CSF induced proliferation of MO7e cells was examined. Although on their own each factor was capable of stimulating MO7e cells, addition of SLF to either IL-3 or GM-CSF enhanced proliferation three to six-fold. The pattern of cellular tyrosine phosphorylation obtained with IL-3 and GM-CSF was identical, however, SLF induced a different pattern of phosphorylation. It was postulated that this was due to the fact that SLF could induce the phosphorylation of PLC-γ1 yet both IL-3 and GM-CSF failed to activate this molecule (211). In addition to this example, a megakaryoblastic cell line, HML-2 was found to be modestly stimulated by both SLF and
GM-CSF. When these two factors were added together, however, GM-CSF was found to promote an upregulation of c-Kit expression thus enhancing phosphorylation of c-Kit and increasing the mitogenic responsiveness of these cells (212). Therefore, combinations of growth factors with SLF not only induce efficient cellular differentiation as described in a previous section, but also enhance mitogenesis by mobilizing distinct signaling pathways.

*Transcription factors which are activated upon SLF stimulation.*

Like other growth factor receptors, c-Kit signaling leads to the activation of transcription factors fos and jun (165, 213). Activation of PI3-kinase, which occurs upon stimulation with SLF, also leads to the activation of transcription factors NFE2 and AP-1 (214). As mentioned, in M07e cells, it has been demonstrated that JAK2 associates with c-Kit and is transiently phosphorylated. SLF-stimulated phosphorylation of JAK2 induces the subsequent activation and phosphorylation of the transcription factor Stat 1. Activated Stat 1 has been demonstrated to bind to an oligonucleotide representing the Sis Inducing Element (SIE) promoter sequence for fos, thus implicating Stat 1 in the c-Kit signal transduction pathway (215). In addition to the activation of fos, and jun, c-Kit likely activates other transcription factors. SLF-induced activation of the Ras pathway, thus leading to the phosphorylation of MAP-kinase results in the activation of various transcription factors including Elk-1 and c-myc (216). In addition to these transcription factors, however, MAP-kinase activates a transcription factor which is specific to c-Kit signaling. The phenotypes observed in W and Sl mutant mice in terms of mast cell and melanocyte development are strikingly similar to the phenotype observed in mice with mutations in the germline transcription factor Microphthalmia (Mi). It was found that SLF-stimulated MAP-kinase activation led to the phosphorylation of Mi at a consensus target serine. In melanocytes, this activation led to Mi transactivation of the tyrosinase pigmentation promoter (217). Therefore, the activation of signaling proteins described in
this section and the enzymatic generation of their second messengers ultimately result in changes in gene transcription which drive a variety of cellular processes.

As outlined in this section, a number of signaling molecules become activated and/or recruited to the c-Kit receptor upon exposure to SLF. Two of these recruited enzymes, PI3-kinase and PLC-γ will be described in greater detail in this chapter. These two enzymes generate very different second messengers and both have been shown to induce distinct cellular outcomes.

(iv) PI3-kinase mediated signals:

PI3-kinase is recruited to the c-Kit receptor upon stimulation with SLF. The second messengers generated by PI3-kinase and the biological role of these messengers in c-Kit mediated cellular outcomes will be addressed in this thesis and will be particularly relevant in Chapter 2. As illustrated in Figure 3, the recruitment of the PI3-kinase subunits to the c-Kit receptor results in the enzymatic generation of its products. These products have pleiotropic cellular effects and these effects will be discussed in this section.

**PI3-kinase structure:**

PI3-kinase activity was first observed in 1985 in association with the PDGF as well as the polyoma middle T/ pp60c-src complex. A lipid kinase activity was found to be associated with these complexes with the unique capacity to phosphorylate phosphatidylinositol (PI) and other phosphorylated PIs [PI(4)-P and PI(4,5)-P2] on the D3 position of the inositol ring. Thus, this lipid kinase was termed PI3-kinase (218-220).
Figure 3. Activation of PI3-kinase.

The recruitment of the p85 subunit to the activated c-Kit receptor results in the activation of the PI3-kinase enzymatic domain p110. Activated PI3-kinase phosphorylates phosphatidylinositol (PI) lipid substrates on the D3 position of the inositol ring thus generating three possible products. These lipid products stimulate a variety of cellular processes and activate, either directly or indirectly, a number of substrates.
Figure 3.

PI3 Kinase Signaling

- Membrane Ruffling
- Actin Reorganization
- Mitogenesis?
- Maintenance of cell viability
- Receptor Internalization?

Phosphorylation of Thr 252

PDK1 and other kinases

p70 S6 kinase
Purification of PI3-kinase from rodent liver, mouse fibroblasts and bovine brain revealed that it consists of an 85 kilodalton regulatory subunit and a 110 kilodalton catalytic subunit. (221-224). Both an α and β isoform of the p85 subunit have been described. These two isoforms possess extensive amino acid homology and were shown to have the ability to bind to activated PDGFR in vitro. P85α is ubiquitously expressed and p85β is expressed in bovine brain and lymphoid tissues (225).

p85 contains one SH3 and two SH2 domains as well as a BCR-like (breakpoint cluster region) domain which in other proteins confers GTPase activity for small GTP-binding proteins (226). Both p85 isoforms have amino-terminal SH3 domains, followed by a proline rich sequence, the BCR domain, another proline rich sequence and two SH2 domains which are separated by an inter-SH2 region [reviewed in (200)]. A 35 amino acid region in the inter-SH2 region is the binding site for an 88 amino acid region in the amino-terminal portion of p110 (227). The proline-rich region of the p85 subunit has been shown to associate with a number of SH3 containing molecules. Examples include the SH3 domains of c-abl, pp56src, pp59fyn, pp56lyn and pp60v-src (228-230).

p110 is a 1068 amino acid protein and was found to be catalytically active only when co-expressed with p85 (231). There exists two p110 isoforms, α and β (222). Resolution of p110 by SDS-PAGE reveals a 110 kilodalton doublet, with p110β corresponding to the lower molecular weight band. The p110 catalytic subunit is homologous to the VPS34 protein which regulates protein sorting in budding yeast (227). PI3-kinase activity in Saccharomyces cerevisiae is restricted to the conversion of PI to P(3)P (232). In the case of both VPS34 and p110, the lipid kinase activity of the catalytic domain is located in the carboxyl-terminal region whereas the amino-terminal region binds to p85.
Positive regulation:
PI3-kinase activity increases in response to a number of growth factors such as PDGF, IGF-1, CSF-1, NGF, hepatocyte growth factor (HGF), SLF and EGF (233-235). In addition, a variety of cytokines such as IL-1,2,3,4,5,7,8,10 and IL-13 activate PI3-kinase (214, 236-241). This activation is completely dependent on the associated kinase activity of the respective receptors or the autophosphorylation of these receptors at tyrosine containing motifs necessary for recruitment and activation of PI3-kinase (242). With respect to the PDGFR, a receptor which is often used to study growth factor induced activation of PI3-kinase, the binding site for p85 was located at a phosphorylated-tyrosine residue in the kinase insert of the receptor (243). Analysis using truncation mutants of p85 SH2 domains revealed that the carboxyl-terminal SH2 domain (SH2-C) was sufficient for directing high affinity interaction with the PDGFR. Furthermore, excess SH2-C was found to competitively block binding of the full length p85 to the PDGFR (244).

Stimulation of the c-Kit receptor with SLF was found to induce a complex containing c-Kit and p85 (245) and the specific residues required for p85 binding were located in the c-Kit kinase insert at tyrosine residue 719 (185).

It is postulated that recruitment of p85 to receptors such as c-Kit may facilitate the activation of PI3-kinase by the intrinsic receptor tyrosine kinase domain (reviewed in (200)). The p85 subunit of PI3-kinase becomes tyrosine phosphorylated in response to PDGF, insulin and NGF, however no change in PI3-kinase enzymatic activity was attributed to this tyrosine phosphorylation event (246, 247). Others have shown that it is only when both p85 and the PDGFR are transiently overexpressed does p85 become phosphorylated. However, when these molecules were expressed at physiological levels, p85, which is recovered in anti-phosphotyrosine immunoprecipitates, was found to not be
phosphorylated (248). Thus, p85 phosphorylation may not be an appropriate measure of the enzymatic activation of the holoenzyme.

In agreement with the concept that recruitment of p85 to activated receptors causes p85 activation, p85 was demonstrated to undergo a conformational change upon binding to tyrosine phosphorylated recognition sites. In order to demonstrate this, the structure of p85α was examined. Using a 17 amino acid peptide containing tyrosine 751 of the p85 binding site from the kinase insert of the PDGFR, p85 was observed to undergo a conformational change upon binding to the phosphorylated peptide. This was measured as a significant spectral change using circular dichroism and fluorescence spectroscopy. These conformational changes suggested that there are flexible linker regions between the various p85 domains. In addition, these results confirm that recruitment of p85 to binding motifs in the PDGFR induces a PI3-kinase activation step as evidenced by this conformational change (249).

Direct activation of PI3-kinase by the binding of p85 SH2 domains to phosphotyrosine containing sequence motifs on the PDGFR or polyoma middle T has also been observed in vitro with purified protein preparations. Doubly phosphorylated binding motifs with two tyrosines improved PI3-kinase enzymatic activation, suggesting that occupation of both SH2 domains optimizes activation (250, 251).

In spite of the evidence that recruitment and binding of p85 to phosphorylated sequences induces activation, the purpose for this recruitment process may be in order to translocate the enzyme to the plasma membrane where it can access substrate. In agreement with this hypothesis, p110 constructs which are targeted to the plasma membrane by either amino-terminal myristoylation or carboxyl-terminal farnesylation were expressed in cells. Membrane-localized p110 in the absence of activated receptors or p85, is able to activate
downstream molecules such as pp70 S6 kinase and Akt/PKB via kinases such as PDK1 and PDK2 (252). Membrane-targeting of a constitutively activated form of p110 (p110*) resulted in maximal activation of these downstream responses. Thus, these investigators postulated that p85 recruitment allows for PI3-kinase activation by virtue of the fact that PI3-kinase becomes translocated to the plasma membrane so that it can access its lipid substrates.

Negative regulation:
PI3-kinase has been demonstrated to exhibit auto-regulation. Mutagenesis of the p110 subunit in a sequence motif which is common to both protein and lipid kinases, revealed an intrinsic protein serine kinase activity. This activity is only obvious when the p110 and p85 subunit exist in a high affinity binding complex. The p110 serine kinase activity phosphorylates serine residue 608 of p85 both in vivo in cultured cells as well as in vitro with the recombinant enzyme. Phosphorylation of p85 at Ser608 was found to result in an 80% decrease in PI3-kinase activity and this reduction could be reversed upon treatment with phosphatase 2A (253). In agreement with this mechanism, several groups have observed that p85α subunits of PI3-kinase immunoprecipitated from quiescent cells are phosphorylated on serine and threonine residues (251, 254).

Interaction of PI3-kinase with other proteins.

Given the variety of protein binding domains in the p85 regulatory subunit such as SH2, SH3 and proline rich domains, it is not surprising that PI3-kinase interacts with other signaling molecules. In addition, PI3-kinase may influence other signaling pathways. The following are examples of molecules which interact with PI3-kinase:
The MAP kinase (MAP-K) pathway is a mitogen stimulated pathway which leads to growth and/or transformation. The pathway is a linear cascade where the serine/threonine kinase Raf is first activated and translocated to the plasma membrane via GTP-Ras. Raf translocation results in the activation of MEK and subsequently MAP kinase/Erk. Interestingly, growth factor-stimulated activation of Ras is necessary but not sufficient for Raf activity. Several candidates have been implicated in further activation of Raf including Src, PKC and PI3-kinase. In some cases activated forms of p110 lead to MAP-K activation whereas in others it does not (252, 255-258). Similarly, inhibition of PI3-kinase with wortmannin or dominant negative PI3-kinase blocks activation of MAP kinase in certain cell types, however there are exceptions (259-263). In order to resolve this confusion, Duckworth et al. have characterized two kinds of MAP-K pathway activation: a wortmannin sensitive and insensitive pathway (264). Specifically, wortmannin blocks the PDGF-dependent activation of Raf and the MAP-K cascade in CHO cells which have physiological levels of PDGFR. However, wortmannin has no effect on MAP-K activation in PDGFR over-expressing Swiss 3T3 cells when stimulated with PDGF. Interestingly, if Swiss 3T3 cells are stimulated with lower, physiological levels of PDGF, wortmannin is then able to inhibit the MAP-K pathway. It is postulated that the wortmannin-insensitive MAP-K pathway observed at high receptor levels may be stimulated by a PLC-γ dependent PKC. The mechanism for the wortmannin-sensitive stimulation of MAP-K is not clear, but may be as a result of activation of D3-phosphoinositide-dependent PKC isoforms.

A relationship between Ras activation and PI3-kinase activation has been observed. Specifically, PI3-kinase was found to directly interact with Ras-GTP but not with Ras-GDP and is activated in vitro and in vivo as a result of this interaction (265-267). In addition, Rodriguez-Viciana et al. have used a number of partial loss of function Ras mutants in order to demonstrate a correlation between Ras activation and cellular acin
rearrangement and membrane ruffling (267). Inhibition of PI3-kinase was found to block Ras induction of these cellular activities. The use of partial loss of function Ras mutants along with activated versions of both Raf and PI3-kinase also revealed that these two pathways need to be activated for PDGF-mediated transformation of PDGFR expressing fibroblasts. Furthermore, inhibition of PI3-kinase inhibits Ras-induced membrane ruffling and PI3-kinase activity alone is sufficient to induce membrane ruffling through the small GTPase Rac. Thus, some Ras-stimulated events require PI3-kinase.

Small GTPases such as Rac and Rho have been associated with PI3-kinase activity. Rac induces membrane ruffling and focal complex formation and Rho induces actin stress fibres and focal adhesion assembly (268, 269). A constitutively active PI3-kinase which is targeted to the plasma membrane was used to assess the contribution of PI3-kinase in Rac and Rho mediated cellular responses. Expression of the active PI3-kinase led to actin reorganization resulting in Rac-mediated lamellipodia and focal complexes as well as Rho-mediated stress fibres and focal adhesions. The expression of active PI3-kinase did not induce Ras/Rac/Rho pathways that regulate gene transcription events such as the c-fos serum response element. The identity of the direct targets for D-3 phosphoinositides that regulate the activation of Rac and Rho have not been identified. This target is likely a guanine nucleotide exchange factor which exchanges GDP for GTP on Rac and Rho (270).

In addition to the aforementioned interactions, a GST fusion protein including the SH3 domain of p85 was shown to bind to the microtubule-associated dynamin protein (271). This large GTP-binding protein possesses intrinsic GTPase activity and is implicated in clathrin-mediated internalization (272). The co-association between p85 SH3 and proline-rich regions of the dynamin molecule has only been demonstrated in vitro, but may have important consequences for ligand-stimulated internalization.
Pleckstrin homology (PH) domains are found on various proteins and facilitate some of the downstream signaling events mediated by PI3-kinase activation. PH domains mediate either protein-protein or lipid-protein interactions or both. Some of the proteins which contain PH domains include pleckstin, PLC-81, dynamin, Akt and SOS [reviewed in (273)]. Some PH domains have the ability to bind to D3 phosphoinositides. One of the prominent downstream elements of the PI3-kinase pathway is the PH domain-containing protein Akt, also known as protein kinase B (PKB). Akt is a serine/threonine kinase which was described by two groups in 1995 as a target for PI3-kinase signaling (274,275). In these cases Akt was demonstrated to be activated by PDGF, EGF and basic fibroblast growth factor. Activation of Akt/PKB was found to be inhibited by the PI3-kinase inhibitor wortmannin and by co-expression of a dominant negative mutant of PI3-kinase.

Deletion of the amino-terminal Akt PH domain was found to inhibit the ability of some agonists such as PDGF to activate Akt (274). In addition, P(3,4)P2 but not P(4,5)P2 or P(3,4,5)P3 can directly stimulate Akt activity and the ability to stimulate correlates with the affinity of these lipids for the Akt PH domain. P(3,4)P2 also induces Akt dimerization (276-278). However, it is likely that additional kinases, are required to activate Akt in addition to P(3,4)P2. Specifically, PI-dependent protein kinase-1 (PDK1) has been demonstrated to phosphorylate and activate Akt in vitro and is itself directly activated by both P(3,4)P2 and P(3,4,5)P3 (279). Importantly, PDK1 which contains an amino-terminal catalytic domain and a carboxyl-terminal PH domain phosphorylates threonine residue 308 of Akt (280). PDK1 is not the only kinase which phosphorylates Akt, as activated Akt contains multiple phosphorylated residues and other kinase candidates such as PKD2 are rapidly being identified. A model has emerged where P(3,4)P2 functions to induce Akt dimerization as well as to recruit Akt to the plasma membrane. This step results
in a partial activation of Akt which induces a conformational change so that Akt is available for phosphorylation by other lipid-dependent kinases such as PDK1. A role for PI3-kinase activated Akt in cell survival will be discussed in greater detail in section (vi).

It has been demonstrated that PDGF or insulin stimulated activation of PI3-kinase induces the serine phosphorylation of p70 S6 kinase. This serine phosphorylation step results in p70 S6 kinase-dependent phosphorylation of the 40S ribosomal protein S6. p70 S6 kinase is an important mediator for G1 cell-cycle transition in some cells (281). Two inhibitors of PI3-kinase, wortmannin and LY294002, were found to inhibit p70 S6 kinase activation by both PDGF and insulin thus further confirming that it is PI3-kinase which activates p70 S6 kinase. Rapamycin, an inhibitor of p70 S6 kinase (282) was also found to block p70 S6 kinase activation by each PDGF and insulin without affecting PI3-kinase activation, suggesting that p70 S6 kinase lies downstream of PI3-kinase.

The interaction between PI3-kinase and p70 S6 kinase has been clarified. PDK1 which has been shown to phosphorylate and activate Akt, strongly activates S6 kinase in vivo and in vitro by phosphorylation of threonine residue 252 in the activation loop of the p70 catalytic domain. The phosphorylation of threonine residue 252 was dependent on the phosphorylation of the p70 carboxyl-terminal tail and threonine residue 412 most importantly. There is a strong positive cooperativity between threonine residues 252 and 412 phosphorylation in that both sites must be phosphorylated to induce p70 activation. Phosphorylation of threonine residue 412 is inhibitable by wortmannin and thus is likely dependent on another D3-lipid dependent kinase. (279).

Three PKC isoforms have been demonstrated to be activated by PI(3,4)P2 and PI(3,4,5)P3 in vitro: PKC-γ, η and ζ (283, 284). However, only PKC-ε and PKC-λ have been demonstrated to be activated by PI3-kinase lipid products in stimulated cells (285, 286).
These D3-phosphoinositide-dependent PKC isoforms phosphorylate a number of substrates. For example, pleckstrin is phosphorylated \textit{in vitro} by PKC in the presence of PI(3,4)P2 and PI(3,4,5)P3 and this is inhibitable by the PI3-kinase inhibitor wortmannin (263). Activation of these PKCs has also been implicated in the Ras/Raf/MEK/MAP kinase pathway. PKCs can phosphorylate Raf-1 \textit{in vitro} and phorbol esters which activate PKC have been shown to activate Raf-1 \textit{in vivo}. In addition, wortmannin has been shown to block activation of Raf-1 in certain cell types. Thus taken together, PI3-kinase lipid products may activate certain PKCs which in turn activate Raf-1 (259). This model of D3-phosphoinositide-dependent PKC activation of Raf-1 remains speculative.

\textit{Role of PI3-kinase and second messengers in cellular processes:}

Over the past five years, the role of PI3-kinase in a variety of cellular processes has been the focus of a great deal of research. There are a number of advances which have facilitated these studies such as the discovery of wortmannin and other PI3-kinase inhibitors which are fairly specific in their inhibitory activity, as well as the construction of constitutively active PI3-kinase (p100*) and dominant negative PI3-kinase proteins. In addition, analysis of receptor tyrosine kinases where binding sites of various signaling molecules such as PI3-kinase are selectively mutated has provided another means for answering questions regarding the biological role of this enzyme.

There is increasing evidence that PI3-kinase plays an important role in cellular processes such as mitogenesis and transformation, however, assigning a key function to PI3-kinase which does not overlap with other signaling molecules has been difficult and can vary in different cell types. Nonetheless, there are examples of mutants of the PDGFR which fail to bind and activate PI3-kinase which are also mitogenically defective. For instance, downstream PI3-kinase substrates have been implicated in Insulin-stimulated mitogenesis.
Valius and Kazlauskas have shown that a PDGF mutant which lacks all SH2 binding sites fails to respond to PDGF. Re-addition of the PI3-kinase binding site to this mutant was found to restore the mitogenic response to PDGF (298). Using the same PDGFR mutants, the PI3-kinase binding site was also found to restore cellular transformation of Ph cells (288). However, in both of these studies, it was found that add-back of the PLC-γ binding site also restored mitogenesis or cellular transformation.

In addition to these studies, the constitutively active p110* was demonstrated to mediate Xenopus laevis oocyte maturation and p110* was found to act coordinately with Ras in order to drive these processes (257). As our understanding of how PI3-kinase interacts with other signaling proteins evolves, the cellular functions that are driven by PI3-kinase activity will likely become more clear.

One cellular process which has been studied in greater detail is PI3-kinase mediated cell survival. In 1995, it was first noted that Nerve Growth Factor (NGF)-mediated protection from apoptosis required PI3-kinase activity. NGF, which binds to the Trk receptor protein tyrosine kinase, mediates both differentiation and survival of neurons. The ability of NGF to prevent apoptosis of PC-12 cells was inhibited by both wortmannin and LY294002. In addition, PDGFR co-expressed with Trk receptors was able to prevent PC-12 apoptosis when stimulated with PDGF. This was not the case, however, if the PDGFR was a mutant receptor which failed to activate PI3-kinase (289). Other examples of PI3-kinase-mediated protection from apoptosis have also followed this study (290, 291).

It was later determined that PI3-kinase-dependent cell survival was likely mediated by activation of Akt. As mentioned, Akt is activated by coordinate binding of D3 phosphoinositides via its PH domain as well as activation and phosphorylation by kinases such as PDK1. It was found that overexpression of Akt prevented apoptosis of cerebellar
neurons which were induced to undergo apoptosis by factor withdrawal or PI3-kinase inhibition (292). In addition, activated Akt was demonstrated to prevent apoptosis induced by extracellular matrix detachment (anoikis) of MDCK cells (293). The mechanism of Akt-mediated prevention of apoptosis is becoming increasingly clear. Accumulating evidence indicates that Akt disrupts Bad/Bcl-2 or Bad/Bcl-xL heterodimers by phosphorylating the pro-apoptotic Bad protein. This phosphorylation event allows for the association of Bad with the cytosolic protein 14-3-3, thus sequestering pro-apoptotic Bad. The anti-apoptotic proteins Bcl-2 and Bcl-xL are then able to form homo and heterodimers, thus promoting cell survival (294-296).

Other PI3-kinase-dependent cellular processes have been described. As mentioned, the interaction of PI3-kinase with small GTP-binding proteins such as Rac results in cellular events such as membrane ruffling. Using PDGFRI mutants which lack the PI3-kinase binding site as well as PI3-kinase dominant negative molecules and PI3-kinase inhibitors such as wortmannin, Wennstrom et al. demonstrated that rearrangement of actin filaments resulting in membrane ruffling requires PI3-kinase activity (297). In addition, PI3-kinase activity is required for Rac-induced integrin-mediated mammary epithelial cell motility and invasiveness in a collagenous matrix (298). PI3-kinase activity is also required for a subset of mast cell responses such as histamine secretion and fluid pinocytosis which are stimulated by cross-linking of the FceRI by Ag and IgE (19).

The pleotropic effects of PI3-kinase activation likely reflect the multiple D3-phosphoinositide substrates. Each of the three major D3 phosphorylated lipid products generated by PI3-kinase activation likely bind to distinct PH domain-containing proteins, thus eliciting a variety of responses. As an understanding of the activities of these lipid second messengers evolves, the role of PI3-kinase in cellular outcomes will become increasingly clear.
PLC-γ mediated signals:

Upon stimulation of the c-Kit receptor with SLF, it has been demonstrated that the PLC-γ signaling enzyme becomes phosphorylated. The ability for SLF-stimulated c-Kit to activate this enzyme may account for some of the synergy observed with SLF and other growth factors and cytokines such as IL-3 as these are receptors which do not activate PLC-γ. In this section, PLC-γ structure, PLC-γ second messengers and the role of this enzyme in cellular functions will be discussed. These details are also illustrated in Figure 4.

PLC-γ protein structure:

The enzymatic activity of PLC is induced by a variety of stimuli such as neurotransmitters and their receptors, mediators of inflammation such as Substance P, hypothalamic and pituitary hormones, gastrointestinal hormones such as bombesin, growth factors such as PDGF, EGF, nerve growth factor (NGF) and SLF, some cytokines and a variety of Ag receptors such as the T cell receptor (TCR/CD3), membrane-IgM, FcεRI and FcγRIII (299). Ten mammalian isozymes of PLC have been identified and characterized. Initially, three distinct isoforms of PLC were purified from bovine brain: PLC-β1, PLC-γ1 and PLC-δ1 (300). Subsequently, seven additional mammalian, two drosophila and two fungal PLC enzymes were identified. The original three PLC isoforms form the three major subtypes (β, γ, and δ) which are 150kD, 145kD and 85kD in size respectively (301). These three subtypes have sequence similarity in two regions, region X which is 170 amino acids long and Y which is 260 amino acids long. There is a 300 amino acid amino-terminal region which precedes X. The region that separates X and Y is quite variable between the three subtypes in terms of length where PLC-γ has a long spacer and
Figure 4. Activation of PLC-γ.

The recruitment of PLC-γ to the activated c-Kit receptor results in the activation of PLC-γ enzymatic activity. Activated PLC-γ hydrolyses PI(4,5)P₂ into both IP₃ and DAG. Generation of IP₃ results in the binding of IP₃ to its cognate receptor on structures such as the endoplasmic reticulum. This interaction induces a mobilization of internally stored Ca²⁺ followed by an influx of extracellular Ca²⁺ through the ICRAC channel. Generation of DAG activates PKC.
Figure 4.

[Diagram showing Endoplasmic Reticulum with various labeled components and reactions such as Ca^2+, I_{CRAC}, PLC, PIP_2, DAG, PKC, InsP_3, and InsP_3^R.]
PLC-β and PLC-δ have short spacers. Mutational analysis of PLC-γ was performed where either constructs of PLC-γ1 or PLC-γ2 were expressed in COS-1 cells or Escherichia coli respectively (302, 303). Enzymatic activity of PLC-γ was only reduced 10-30% in PLC-γ molecules where the SH2 and SH3 domains were deleted. Deletion of either X or Y region, however, resulted in a complete loss of enzymatic activity. Two histidine residues in the X domain were found to be critical for enzymatic activity. Importantly, the long 400 amino acid PLC-γ spacer contains two SH2 domains and one SH3 domain. In addition, the two PLC-γ isoforms, PLC-γ1 and γ2 possess PH domains. The 100 amino acid PH domains found in a number of signaling proteins are normally intact, however in the case of PLC-γ, the PH domain is split by the SH2 and SH3 domains (304). The 3-dimensional structure of the molecule predicts that the PH domains are brought physically close together so that they may function. A second PH domain is located in the amino-terminal region of PLC-γ and it is intact.

Activation of PLC-γ isoforms via growth factor receptor tyrosine kinases:

Some growth factor receptor tyrosine kinases such as the PDGFR, EGFR, fibroblast growth factor receptor (FGFR), and NGFR stimulate PLC-γ-mediated phosphoinositide turnover upon stimulation (305-307). Stimulation of c-Kit with SLF also activates PLC-γ (25, 166, 176, 184). Other growth factors receptors such as CSF-1 receptor and insulin receptor do not have this effect (308).

Unlike PLC-β, PLC-γ activation is independent of G protein activation but rather requires the intrinsic protein tyrosine kinase activity of the aforementioned receptors. PDGF and EGF receptors which lack protein tyrosine kinase activity fail to stimulate hydrolysis of PIP2 upon ligand binding (301). Growth factor stimulation of tyrosine kinase competent receptors with EGF, PDGF, NGF or SLF mitogens results in an increase in

I-58
phosphorylation of PLC-γ1 on serine and tyrosine residues (166, 309-314). This phosphorylation is rapid and correlates with PIP2 hydrolysis. There is also evidence that receptor tyrosine kinases such as c-Kit become physically associated with PLC-γ1 SH2 domains (176, 305, 315).

Once PLC-γ is activated by phosphorylation of serine and tyrosine residues, a conformational change is thought to occur. Theoretically, this proposed conformational change exposes the PLC-γ SH3 domain which then interacts with the cytoskeleton thus allowing the X and Y domains to gain accessibility to membrane-associated lipid substrates. However, what is known is that exposure of cells to EGF or PDGF induces the translocation of PLC-γ from cytosolic to particulate fractions (316). In addition, truncated PLC-γ1 enzymes which contain the SH3 domain were found to localize to the actin cytoskeleton when microinjected into cells (317). Other evidence that activated PLC-γ associates with the cytoskeleton is provided by the recent discovery of the 110 kilodalton protein Dynamin. Dynamin was found to bind to fusion proteins of GST and the PLC-γ1 SH3 domain (271). Therefore, accumulating evidence indicates that PLC-γ associates with the cellular cytoskeleton upon activation with a variety of mitogens, and this association may be required for the accessibility of lipid substrates to the PLC-γ enzymatic domains.

Activation of PLC by non-receptor protein tyrosine kinases.

There are two major PLC-γ isoforms (1 and 2) which may be expressed in hematopoietically derived cells. The ratio of PLC-γ2 to PLC-γ1 in cells decreases in the order of human B cell lines (Daudi) > rat basophilic cell line RBL-2H3 > human monocytic cell line U937 > human T cell line Jurkat. In human T cells, PLC-γ1 is preferentially phosphorylated whereas in B cells PLC-γ2 was found to be preferentially phosphorylated. Non-receptor protein tyrosine kinases can phosphorylate and activate PLC-γ isozymes and
these tyrosine kinases are activated as a result of ligation of surface receptors such as the T cell receptor, FcεRI and membrane-IgM (mIgM) as well as FcγRIII. ZAP-70, a 70 kilodalton protein tyrosine kinase which binds phosphorylated TAMs (tyrosine-based activation motifs) on CD3 ζ and ε chains via its tandem SH2 domains, rapidly phosphorylates PLC-γ on tyrosine and serine (318-321). Kinases implicated in mIgM-mediated tyrosine phosphorylation of PLC-γ are Fyn, Lyn and Blk Src-family kinases as well as the Syk protein tyrosine kinase (322). In the case of FcεRI, tyrosine phosphorylation of PLC-γ occurs following aggregation with Ag plus IgE (21), and this is likely mediated by the protein tyrosine kinase lyn (18).

Generation of Ca²⁺ influx and activation of PKC via PLC-γ enzymatic activity.

All three PLC subtypes γ, β, and δ, are dependent on Ca²⁺ for their catalytic activity. The carboxyl-terminal portion of the Y region is thought to be the Ca²⁺ binding site. As mentioned, the enzymatic activity of PLC-γ is activated by phosphorylation of intrinsic tyrosine and serine residues. Receptor tyrosine kinases, either cellular or receptor associated are critical for this process. Once phosphorylated and activated, PLC-γ hydrolyses PIP₂ into IP₃ and DAG and access to this lipid substrate may be facilitated by the SH3 domain of the PLC-γ enzyme [reviewed in (299, 323)].

IP₃ generation and Ca²⁺ mobilization:

Intracellular Ca²⁺ stores are membrane stores where Ca²⁺ is inducibly released. These stores express IP₃ receptors that span their membranes as well as ATP-dependent pumps which sequester Ca²⁺ into the stores. IP₃-sensitive stores are typically located in the endoplasmic reticulum (324), and a family of IP₃ receptors has been identified (325, 326). The IP₃ receptor is a tetrameric complex which contains membrane spanning domains in the carboxyl-terminal region, thus anchoring it into intracellular endoplasmic reticulum
membranes, and the four subunits combine to form the IP3-sensitive Ca\(^{2+}\) channel. A large amino-terminal domain is located in the cytoplasm with an IP3 binding site at its terminus. Upon IP3 binding to the amino-terminal region, the IP3 receptor undergoes a large conformational change perhaps related to the opening of this channel for release of Ca\(^{2+}\) from these internal stores (327).

Ca\(^{2+}\) in intracellular stores which is released into the cytosol upon IP3 binding to the IP3 receptor induces capacitative entry of extracellular Ca\(^{2+}\) (328). The term “capacitative entry” is used to describe the external Ca\(^{2+}\) influx which is regulated by the Ca\(^{2+}\) content in the endoplasmic reticulum. When internal Ca\(^{2+}\) stores are emptied, influx of Ca\(^{2+}\) from the extracellular milieu is induced. This has been demonstrated with mast cells following injection of IP3 (197). The mechanism for how the IP3 receptor on the endoplasmic reticulum communicates with the Ca\(^{2+}\) channel (ICRAC) on the plasma membrane remains speculative.

**Generation of DAG and activation of PKC:**

PKC is a cellular enzyme which plays a role in a number of biological responses stimulated by various growth factor and Ag receptors. PKC, as mentioned, is activated by an increase in amounts of DAG in cellular membranes as a result of PLC-mediated hydrolysis of PIP\(_{2}\) [reviewed in (198)]. In stimulated cells, Ca\(^{2+}\) responses are transient whereas PKC activation is prolonged. Ca\(^{2+}\) mobilization and PKC activation act synergistically in driving cellular responses and PKC may also regulate ion channels themselves (329). In the presence of high concentrations of Ca\(^{2+}\), activation of PKC requires less PLC-γ-stimulated production of DAG, whereas, when large amounts of DAG are produced, less Ca\(^{2+}\) is required to activate PKC (some isoforms of PKC are Ca\(^{2+}\)-dependent enzymes). In addition, PKC may play a negative feedback role in agonist-induced IP3 production, thus “resetting” the cell for future responses. This is evidenced by the observation that
Phorbol esters inhibit Ca\(^{2+}\) mobilization and this inhibition is overcome with PKC inhibitors (330). Ten subspecies of PKC have been identified. PKC-\(\alpha\) is the target for PLC-\(\gamma\) and this PKC isoform is activated by DAG and Ca\(^{2+}\) as well as by phosphatidylserine, cis-unsaturated fatty acids and lysophosphatidylcholine [reviewed in (198)].

**Role of PLC-\(\gamma\) and second messengers in cellular processes:**

It first became clear that PLC-\(\gamma\) hydrolysis of cellular PI(4,5)P2 was biologically significant when mitogenesis induced by bombesin or PDGF was observed to be blocked by injection of a monoclonal antibody to PIP2 (331). A role for PLC-\(\gamma\) in different cellular processes is emerging (195), and in some cases, the particular second messenger involved has been identified:

**PLC-\(\gamma\)-associated responses:**

There are a number of examples where PLC-\(\gamma\) has been demonstrated to drive a particular cellular fate. However, this section will focus on those occasions where PLC-\(\gamma\) performs a unique and non-overlapping function independent of other enzymes such as PI3-kinase. For example, a truncated form of c-Kit was found to be critical for oocyte activation following fertilization and the addition of PLC-\(\gamma\)-inhibitors revealed that PLC-\(\gamma\) was essential for this function (332). Using site-directed mutants of the PDGF\(R\), Alimandi et al. demonstrated that PDGF-stimulated monocytic differentiation of FDC-P2 cells and PDGF-stimulated mitogenesis of these cells was found to require both PLC-\(\gamma\) activation and PKC activation (333). In addition, microinjection of the SH2-SH2-SH3 PLC-\(\gamma\) domains was found to inhibit the association of PLC-\(\gamma\) with either the PDGF\(R\) or EGFR. This inhibition prevented PDGF or EGF induced S phase entry (334).
PKC associated responses:

PKC activity has been implicated in a number of negative regulatory events for a large variety of growth factor and cytokine receptors. PKC causes the shedding of extracellular domains of the IL-6R (335). Similarly, PKC has been shown to induce the shedding of the extracellular domain of the homing receptor gp90MEL-14 which is used for lymphocyte entry into lymph nodes and Peyer's Patches by adhesion to high endothelial venules. Stimulation of lymphocytes with PMA resulted in loss of surface expression of gp90 within 1 hour (336). CSF-1 down-modulation was similarly induced by TPA activation of PKC (337). As mentioned in a previous section, c-Kit extracellular domains are also cleaved by PKC (201).

In addition, to these negative regulatory functions, PKC has been observed to play an important role in actin reorganization and motility of c-Kit positive cells in response to SLF (204). Activators of PKC such as PMA also induce the development of c-Kit negative mast cells in W/W^v mice, however the mechanism for this mast cell development are unknown (142). Activation of PKC by other receptors, such as the insulin receptor, induces DNA synthesis and mitogenesis (285) and PMA has pleotropic effects on a number of cell types.

Ca^2+ associated responses:

A role for Ca^2+ in cell survival and apoptosis will be discussed in more detail in section (vi). However, there have been a number of other biological functions which are dependent on intracellular increases in Ca^2+ levels. For example, Ca^2+ influx likely plays an important role in mast cell degranulation. Specifically, incubation of mast cells with ionomycin leads to rapid degranulation (23). In addition, integrin recycling in neutrophils, which is critical for their migration, requires an intracellular increase of Ca^2+ ions (338). There are several Ca^2+-dependent proteins which become activate by PLC-γ-mediated...
Ca\textsuperscript{2+} influx. The cytosolic Ca\textsuperscript{2+}-binding protein Calmodulin mediates multiple cellular effects and indirectly activates the phosphatase Calcineurin. Calcineurin is required for the nuclear translocation of the transcription factor NFAT (339). Therefore, the activation of PLC-γ and the generation of its enzymatic products are critical for a number of cellular responses for a number of receptor systems including c-Kit.

(vi) Mechanisms of apoptosis and the role of Ca\textsuperscript{2+}.

Both SLF and IL-3 have been demonstrated to protect hematopoietic stem cells, myeloid progenitor cells and mast cells from apoptosis (60, 126, 128, 340). Some of the biochemical signals mediated by SLF are, however, different than IL-3-generated signals, thus these factors likely utilize different mechanisms for protecting cells from apoptosis (184). In agreement with these differences, while stimulation of the IL-3 receptor induces the upregulation of the survival factor Bcl-2, c-Kit stimulation does not (61, 341, 342). In Chapter 4, a role for PLC-γ-stimulated Ca\textsuperscript{2+} influx in SLF-mediated protection from apoptosis is described. This section will detail the principal molecules, both pro-apoptotic and anti-apoptotic, as well as the role of Ca\textsuperscript{2+} ions involved in programmed cell death.

Apoptosis which is also termed programmed cell death is defined by morphological changes which result in nonpathologic cell loss. Some of the morphological hallmarks associated with apoptosis are DNA fragmentation, cell shrinkage and membrane blebbing (343). Elimination of specific cell populations by apoptotic mechanisms occurs during the normal development of multicellular organisms. In addition, apoptosis occurs in the mature organism such as in B and T cell selection, and dysregulation of apoptosis can lead to various pathological states [reviewed in (344)]. Apoptotic mechanisms can be divided into two major categories: apoptosis induced by withdrawal of growth and survival factors and apoptosis induced by the aggregation of specific receptors. In the case of factor-
withdrawal induced apoptosis, a family of pro- and anti-apoptotic play a role in this process.

An example of an apoptosis-inducing receptor is the Fas antigen which displays homology with the B cell antigen CD40, with nerve growth factor receptor and with tumor necrosis factor (TNF) receptor. Aggregation of the Fas antigen with anti-Fas antibody induces apoptosis (345). In addition, aggregation of TNFRI induces apoptosis as well as the activation of the transcription factor NF-κB (346). Other related receptors which induce apoptosis upon cross-linking continue to be characterized, such as TRAMP, a novel member of the TNF receptor family (347).

The mechanisms of either receptor-stimulated apoptosis or factor-withdrawal induced apoptosis involve the use of distinct as well as overlapping families of effector proteins. The function and structure of these gene products will be described in the following section.

Role of the Bcl-2 family of proteins in apoptosis.

The Bcl-2 anti-apoptotic gene was first identified at the t(14;18) translocation in human follicular lymphomas and was later shown to be expressed in non-transformed cells [reviewed in (348)]. Dysregulation of Bcl-2 has been associated with resistance to apoptosis induced by a number of drugs (349). The concept that Bcl-2 is a negative regulator of programmed cell death arose from a number of observations. Transfer of a Bcl-2 vector into IL-3 dependent pre-B-cells was shown to induce prolonged cell survival upon withdrawal of IL-3 (350). Following this observation as well as other examples similar to it, it was formally demonstrated by Hockenberg et al. that Bcl-2, which was described as an inner mitochondrial membrane protein, blocked programmed cell death...
This was further confirmed by Reed et al., where antisense-mediated reduction in Bcl-2 expression was demonstrated to accelerate the rate of cell death upon growth factor withdrawal (352).

\textit{Bcl-2 sensitive and insensitive apoptotic death.}

As mentioned, Bcl-2 protects many cell types from both drug-induced apoptosis as well as growth factor withdrawal-induced apoptosis. However, there are examples where Bcl-2 fails to protect cells in response to certain apoptotic stimuli. For example, in B lymphoma cell lines, Bcl-2 overexpression fails to protect against mIgM-induced apoptotic death of WEHI-231 B cells, yet Bcl-2 can protect against death induced by heat shock in the same cells (353). Bcl-2 has been shown to block glucocorticoid-induced apoptosis in the mouse T hybridoma 2B4.11. However, "activation-induced apoptosis", a model for elimination of extra-thymic self-reactive cells via Fas/Fas Ligand interactions, was not inhibited by expression of Bcl-2 in 2B4 cells (354). A similar Bcl-2 insensitive apoptotic program was observed in Fas/Fas Ligand transduced apoptosis in B lymphoid, cell lines and thymocytes (366). Therefore, in general, receptor-mediated apoptosis is not inhibited by the expression of Bcl-2.

In addition, the ability of different growth factors to rescue starved cells from apoptosis is not universally dependent on the up-regulation of Bcl-2. Some of the survival cytokines which induce Bcl-2 expression include IL-4 and IGF-1 (355) whereas the Flt3 ligand and SLF fail to induce Bcl-2 expression in both primary acute myeloid leukemia cells (356) and mast cells (61) in spite of the fact that these two factors can protect cells from apoptosis. Therefore, the anti-apoptotic properties of Bcl-2 may not always be sufficient to protect cells from programmed cell death. In the case of protection from apoptosis by factors such as SLF, there are likely cellular mediators other than Bcl-2 which confer this protection.
**Bcl-2 family members and protein structure:**

The Bcl-2 family of proteins contain an increasing number of members ranging in size between 20-26 kilodaltons, some of which are positive regulators of apoptosis and some of which are negative regulators. Among the pro-apoptotic Bcl-2 family members are Bax, Bak and Bad whereas the anti-apoptotic members include Bcl-2 and Bcl-XL [reviewed in (357)]. There are two notable features of many of these proteins which are related to their function. Firstly, these proteins have the ability to dimerize. Dimerization of Bcl-2 with other family members can result in either a survival-promoting effect or alternatively in the induction of apoptosis (348). For example, Bcl-2 homodimers promote cell survival, whereas conversion of Bcl-2/Bax heterodimers to Bax homodimers leads to apoptosis (358). Interestingly, Bax expression is upregulated by the pro-apoptotic tumour suppressor gene p53, whereas Bcl-2 expression is conversely down-regulated by p53 (359, 360). Concordant upregulation of Bax and down-regulation of Bcl-2 would therefore favour the formation of apoptosis-inducing Bax/Bax homodimers. In a previous section, the role of Akt in cell survival was described. When activated by D3 phosphoinositide products of PI3-kinase as well as other kinases, Akt is able to phosphorylate Bad so that it interacts with the cytosolic protein 14-3-3. This Akt-mediated event effectively uncouples Bcl-XL/Bad heterodimers so that anti-apoptotic Bcl-XL homodimers may form (294-296). Thus, the cell survival properties of various growth factors may be due to the activation of PI3-kinase and subsequently, Akt.

In addition to the ability to form dimers, Bcl-2 proteins exhibit a striking similarity to the pore-forming domains of bacterial toxins such as diphtheria toxin which act as channels for ions or proteins [reviewed in (361)]. This observation is particularly intriguing given that Bcl-2 is located on multiple intracellular membranes. The protective capacity of Bcl-2 may be dependent on its membrane location. In some cell types such as in Rat-1/myc cells but not MDCK cells, an endoplasmic-reticulum associated Bcl-2 protein is protective. In
contrast, a mitochondrial associated Bcl-2 protein is protective against serum deprivation-induced apoptosis in MDCK cells but not Rat-1/myc cells (362). There is now direct evidence for ion-channel activity from experiments with recombinant Bel-2 or Bcl-XL in synthetic lipid membranes (363, 364). With respect to channel formation, overexpression of Bel-2 was found to inhibit mitochondrial permeability transition whereas Bax overexpression induces permeability (365, 366). Bax has also been shown to form channels in lipid membranes in vitro, which, interestingly, could be antagonized by the co-expression of Bel-2. Thus, although Bel-2 has channel forming properties, its insertion in inner membranes may serve as a gate-keeper for the ion-channels formed by related proteins such as Bax.

The pore-forming abilities of Bel-2 family members have gained particular interest in light of recent findings that secretion of cytochrome c from the mitochondria induces an irreversible apoptotic cascade of events (367). It has been postulated that mitochondrial permeability transition which may be caused by Bax can be inhibited by expression of mitochondrial localized Bel-2 and that this in fact inhibits apoptosis (368). It was later demonstrated that this Bel-2-mediated prevention of apoptosis was attributable to a block in the release of cytochrome c from the mitochondria (369). Kharbanda et al. confirmed that cells overexpressing the Bel-2 related protein Bcl-XL also failed to accumulate cytosolic cytochrome c or undergo apoptosis. In this case it was demonstrated that Bcl-XL was found to bind directly and specifically to cytochrome c (370). The functional significance of the Bel-2 family members as pore-forming structures has yet to be completely understood. However, what is known is that Bel-2 family members function in concert with other cytosolic proteins to either protect or induce apoptosis. Furthermore, these family members play an important role in growth factor withdrawal-induced apoptosis.
ICE-family proteases (caspases) and apoptosis:

Recent advances in the understanding of apoptosis have revealed that Bcl-2 may interact with a series of pro-apoptotic proteases called caspases. The molecular basis of programmed cell death was first elucidated by genetic studies of the nematode Caenorhabditis elegans. It was discovered that two genes, ced-3 and ced-4, are required for all apoptotic events in the development of the nematode, and another gene, ced-9, inhibited the actions of ced-3 and ced-4, thus promoting cell survival. The mammalian homologue for ced-9 was found to be Bcl-2 and the vertebrate homologue for ced-3 was found to be one of several cysteine proteases with aspartic acid specificity termed caspases. These caspases exist as inactive polypeptides that are activated upon removal of a regulatory prodomain thus resulting in conversion to an active protease. Caspases are the main effectors of apoptosis in mammalian cells.

The mammalian ced-3-related protease which is necessary for apoptosis was identified as CPP32, and a peptide aldehyde inhibitor was developed which potently inhibits CPP-32 activity (Ac-DEVD-CHO). CPP-32 is also designated as caspase-3. The human homologue of the ced-4 gene has recently been cloned. This molecule, termed Apaf-1, was found to participate in cytochrome c-dependent activation of caspase-3. In fact, cytochrome c binds directly to Apaf-1, and it is believed that this may trigger the apoptotic caspase cascade.

An understanding of how the three major C. elegans effectors CED-3, CED-4 and CED-9 interact has given some insight into the interdependence of caspase-3, Apaf-1 and Bcl-2. Chinnaiyan et al. devised a framework for the interaction of the C. elegans proteins where CED-9 was able to interact with and inhibit the function of CED-4 and that this interaction was upstream of CED-3. This was based in part on the finding that CED-4 could...
physically interact with CED-3 (376). The same pattern of interactions was confirmed in vitro for the mammalian homologues (377). Specifically, Apaf-1 was shown to participate in caspase-3 activation. Interestingly, however, there is no identified intrinsic aspartic acid-directed proteolytic activity for the Apaf-1 protein. Therefore, cytochrome c-dependent caspase-3 cleavage requires an initiating caspase. Recently, caspase-9 has been identified as the very first initiating caspase in cytochrome-c-dependent apoptosis (367). A model has emerged where cytochrome c released from the mitochondria is bound by Apaf-1 (369). Apaf-1 and caspase-9 interact with each other via their respective N-terminal domains. In the presence of cytochrome c and dATP, this interaction leads to the activation of caspase-9. Caspase-9 then cleaves and activates caspase-3 which results in the initiation of a caspase cascade resulting in apoptosis (378).

In the cases of apoptosis induced by aggregation of surface receptors such as the Fas antigen, cytochrome-c-activated caspase-9 is not the initiating effector. In contrast, a distinct caspase termed caspase-8 (FLICE) is recruited to the aggregated Fas receptor. It is postulated that caspase-8 is activated by its own intrinsic proteolytic activity and this occurs upon dimerization or aggregation of the caspase-8 protease (379). Auto-activation of caspase-8 then induces a caspase cascade which results in apoptosis. Therefore, although factor-withdrawal and receptor-mediated apoptosis are initiated by distinct caspases, in each case a caspase cascade is triggered. In the case of factor-withdrawal apoptosis, there appears to be an involvement of mitochondrial factors.

**Role of Ca^{2+} ions in apoptosis:**

Two reagents have proven to be very useful in elucidating the role of Ca^{2+} in apoptosis. Thapsigargin is a high affinity inhibitor of the intracellular Ca^{2+} transport ATPases. This agent induces elevations of intracellular Ca^{2+} and depletes the IP_{3}-sensitive Ca^{2+} stores.
while bypassing a requirement for PLC-γ activation. By inhibiting the intracellular Ca\textsuperscript{2+} ATPases associated with cellular structures such as the endoplasmic reticulum, thapsigargin prevents re-filling of internal stores (380). The second experimental tool often used in experiments determining the role of Ca\textsuperscript{2+} in apoptosis is the substance ionomycin. Ionomycin is a Ca\textsuperscript{2+} ionophore which has been shown to selectively bind Ca\textsuperscript{2+} at a one to one ratio and transport the Ca\textsuperscript{2+} ion across lipid membranes (381). Thus, incubation of cells with ionomycin results in increases of intracellular Ca\textsuperscript{2+} independent of the emptying of internal stores.

Increases in intracellular Ca\textsuperscript{2+} concentrations have been shown to induce apoptosis in a variety of cell types. Thapsigargin has been shown to activate apoptosis in thymocytes and apoptosis was inhibited in cells which were incubated in Ca\textsuperscript{2+}-free medium or pretreated with the intracellular Ca\textsuperscript{2+} chelator bis-(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid/acetoxymethyl ester (BAPTA) (382). Thus, the intracellular Ca\textsuperscript{2+} increases caused by thapsigargin treatment induced apoptosis in these cells. In addition, CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes undergo apoptosis upon incubation with Ca\textsuperscript{2+} ionophore (Ca\textsuperscript{2+}-mediated cell death) (383, 384). Interestingly, peripheral T lymphocytes, unlike thymocytes, are resistant to apoptosis caused by Ca\textsuperscript{2+} elevation, however, the immunosuppressant cyclosporin A (CsA) primes splenic lymphocytes to undergo Ca\textsuperscript{2+}-mediated cell death induced by ionomycin. It was shown that CsA and ionomycin synergize to induce TGF-β secretion and that this in turn caused apoptosis (385). The mechanism for Ca\textsuperscript{2+}-mediated cell death is not clearly understood. A correlation between sustained increases in cytosolic Ca\textsuperscript{2+} concentrations and oxidative stress has been observed (386). In addition, Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent endonucleases are likely responsible for the DNA digestion observed in lymphocyte apoptosis and increases of intracellular Ca\textsuperscript{2+} may activate these endonucleases (387).
In spite of these examples where increases in intracellular Ca\(^{2+}\) concentrations result in apoptosis, there are several examples where these Ca\(^{2+}\) increases actually protect cells from apoptosis. 32D cells are an IL-3-dependent hematopoietic cell line. Baffy et al. transfected these cells with the Bcl-2 protein. Withdrawal of IL-3 from 32D cells induced apoptosis in only those cells not expressing Bcl-2. IL-3 withdrawal was also observed to result in a decrease in cytosolic free Ca\(^{2+}\) concentration as measured by Indo-1 fluorescence, however, Bcl-2 expressing 32D cells did not exhibit this same decrease. Importantly, 32D cells could not only be rescued by addition of IL-3 but also by Ca\(^{2+}\) ionophores, and both treatments blocked DNA fragmentation. Thus, in the case of this IL-3 dependent cell line, decreases in intracellular Ca\(^{2+}\) concentrations lead to apoptosis and cells could be rescued by increasing these Ca\(^{2+}\) concentrations (342). Other IL-3 dependent cell lines such as BAF3 and FDCP-MIX, were found to be protected from growth factor withdrawal-induced apoptosis by Ca\(^{2+}\) ionophores (348). In the case of rat sympathetic neurons, deprivation of NGF induced programmed cell death and prevention of apoptosis was achieved by prolonged neuronal depolarization which mediates a rise in intracellular Ca\(^{2+}\) concentration caused by Ca\(^{2+}\) influx (389, 390).

Some of the apparent confusion as to the role of Ca\(^{2+}\) in apoptosis is highlighted in a study by Kelley et al. This study demonstrated that Epo-dependent erythroid progenitor cells underwent apoptosis upon removal of Epo. Decreasing the intracellular Ca\(^{2+}\) levels by extracellular Ca\(^{2+}\) chelation with EGTA facilitated DNA breakdown. However, increasing Ca\(^{2+}\) concentrations with ionophores also resulted in DNA cleavage, although the DNA fragments generated by high intracellular Ca\(^{2+}\) concentration were much larger than those observed in the absence of Epo or in the presence of EGTA (391). Thus, high and low Ca\(^{2+}\) levels may have distinct effects on the apoptotic program, and this will be discussed in further detail in Chapter 4.
Effect of Calcineurin on apoptosis:

Cyclosporin A or otherwise known as FK506, is an immunosuppressive drug which binds to the immunophilin protein FKBP12. The immunosuppressive effect of this drug, however, is mediated by FK506-FKBP12 inhibition of the Ca\(^{2+}\)-activated phosphatase calcineurin. Calcineurin, which is activated by calmodulin, dephosphorylates substrates such as the transcription factor NFAT so that it may translocate to the nucleus and regulate genes which are important for cellular activation. Recently, the actions of FK506 have become more clear. FKBP12 is found to be associated with the IP3 receptor / Ca\(^{2+}\) channel on internal Ca\(^{2+}\) stores located in organelles such as the endoplasmic reticulum. Calcineurin itself is anchored to the IP3 receptor via FKBP12. Because calcineurin regulates the phosphorylation status of the IP3 receptor, disruption of the calcineurin-FKBP12-IP3 receptor tri-molecular complex by FK506 also results in disruption of Ca\(^{2+}\) mobilization (339). These observations have important implications given that FK506 has been shown to inhibit activation-induced cell death in T cell hybridomas and thymocytes (392). In agreement with this observation, other groups have confirmed that calcineurin is involved in Ca\(^{2+}\)-mediated cell death and growth factor withdrawal induced death in a variety of cell types including neuronal cells (393, 394).

The role of intracellular Ca\(^{2+}\) levels in apoptosis remains unresolved, and the discovery that calcineurin may play a regulatory role in programmed cell death may provide some further clarification. These issues will be addressed in the context of c-Kit mediated survival signals in Chapter 4.
Ligand-stimulated receptor internalization and endocytosis.

Chemotaxis, adhesion and receptor internalization are mechanisms whereby growth factor receptors may respond to their environments. Ligand-stimulated receptor internalization can result in lysosomal degradation of the receptor/ligand complex, thus serving as a mechanism for attenuating signals mediated by surface receptors. In the context of a developmental program such as hematopoiesis, down-modulation of surface receptors may signal discrete stages in development. In addition, internalization of receptors such as c-Kit which adhere to and are stimulated by mSLF expressed by stromal cells, may serve as a means for mobilizing c-Kit positive precursors from the bone marrow environment to the periphery.

Following ligand binding to growth factor receptor tyrosine kinases on the cell surface, receptor/ligand complexes enter the cell by a process termed ligand-stimulated internalization. The term internalization refers to the initial steps involved in receptor removal from the cell surface whereas the term endocytosis refers to later routing steps which follow internalization. The process of receptor internalization involves plasma membrane structures called clathrin coated pits as well as adaptor proteins, dynamin and the cooperation of the receptor-mediated signals which have been described in earlier sections.

With the advent of the electron microscope in the mid-1950s, internalization and endocytosis began to be recognized as processes which were not only reserved to phagocytic cells but in fact occurred in all cells in order to internalize proteins that have become bound to surface receptors. The first receptors which were studied for their internalization and endocytic properties were the transport proteins low density lipoprotein (LDL) as well as the growth factor receptor for EGF (395-397). From these studies,
important properties regarding ligand-stimulated internalization were elucidated. Firstly, internalization of a ligand is coupled to the process of ligand binding to the receptor, and once the protein is bound to the receptor, the surface half-life of the receptor is typically less than ten minutes. Following ligand binding it was observed that receptors cluster in coated pits and the internalized proteins are usually delivered to lysosomes where they are degraded. These steps will be outlined in detail in this section.

**Clathrin coated pits and receptor internalization:**

Internalization of receptor tyrosine kinases requires the assembly of a protein "coat" from the soluble, cytosolic compartment of the cell. Clathrin coats are assembled from clathrin triskelions which consist of three 190 kDa heavy and three 35 kDa light chains. Heterotetrameric adaptor protein complexes (AP) often associate with these triskelions [reviewed in (398)].

These clathrin triskelions are remarkably flexible and together, they form polyhedral lattices. The clathrin light chain is more diverse than the heavy chain with tissue specific alternatively spliced forms. There are two forms of clathrin light chain, LCa and LCb which are distributed in a random fashion within the lattice. A domain in the central third of the clathrin light chains was found to bind to the clathrin heavy chain. Clathrin light chains bind Ca\(^{2+}\) with 1-5% of clathrin light chains bound to Ca\(^{2+}\) at steady-state intracellular Ca\(^{2+}\) concentrations. High concentrations of Ca\(^{2+}\) have been shown to rapidly induce assembly of purified clathrin *in vitro* (399).

Ligand-bound receptors are recruited into clathrin-coated pits by first associating with adaptor proteins by a process which does not appear to require ATP or GTP. The interaction between AP-2 and transmembrane receptors is via a tyrosine-based sorting
signal YXXø where X is any amino acid and ø is an amino acid with a bulky hydrophobic side chain (400). The adaptor protein distinguishes clathrin-coated vesicles that bud from the plasma membrane versus those which bud from the trans-Golgi network. The plasma membrane-associated adaptors are the AP2 complexes which consist of 100 kDa adaptin subunits designated α, β2 μ2 and σ2 (401). The α chain of the AP-2 adaptor is the clathrin binding subunit (402). The μ2 subunit mediates the recruitment of AP-2 to the tyrosine based motif on the transmembrane receptor (400) and this interaction between μ2 and the tyrosine-based signal is greatly strengthened if AP-2 is present in clathrin coats. This indicates that there exists an interactive cooperativity between the AP-2 μ2 subunit with tyrosine motifs and the AP-2 α subunit with clathrin cages (403). The exact mechanism for AP-2 recruitment to activated receptors, however, is not entirely clear. Santini et al. have demonstrated that activation of FcεRI receptors on RBL-2H3 cells is not sufficient to induce recruitment of clathrin complexes by using immobilized rather than soluble antigen (404). Specifically, immobilized antigen was found to induce RBL-2H3 activation as exhibited by cytoskeletal changes, however, this form of Ag fails to induce internalization of FcεRI receptors. Thus, there are likely other unidentified components involved in the recruitment of AP-2 and clathrin to the plasma membrane.

AP-2 complexes, which are recruited to activated receptors, along with clathrin mediate the assembly of clathrin triskelions into a planar lattice on the inner surface of the plasma membrane. The clathrin planar lattices subsequently gain curvature by a process that is not well understood (398). The constriction of the clathrin lattice is facilitated by the GTPase dynamin (discussed below). Clathrin assembly selectively traps receptors via their interaction with adaptor proteins and this leads to the formation of a spherical coat around a naked budded membrane vesicle. Receptors are concentrated into clathrin-coated pits so that the number of receptors in these structures is 100-fold greater than the concentration of receptors on the plasma membrane.
Some receptors do not undergo clathrin-coated pit-mediated internalization. Less than 1 in 100 Thy-1 molecules are found in coated pits. Thy-1 is a GPI-anchored protein and it is found to be concentrated and internalized by structures called caveolae. These structures are non-coated vesicles containing the caveolin 21 kilodalton integral membrane phosphoprotein as a major structural component (405). In addition, fluid-phase endocytosis represents another clathrin-independent mechanism for removing proteins from the cell surface. This process has been described as clathrin-independent pinocytosis (406).

Following clathrin coated pit invagination and constriction, vesicles bud from the plasma membrane in an ATP and GTP-dependent fashion. This step likely requires other cytosolic factors and may be facilitated by dynamin. Subsequent to the clathrin vesicle budding step, clathrin molecules are released and this process requires an ATP-dependent reaction mediated by cytosolic factors such as the uncoating ATPase hsc70 (407). This process allows for fusion of the uncoated vesicle with early and late endosomes. Concomitant with this fusion event, the stripped vesicles may also become homo-aggregated. Homo-aggregation is dependent on the α subunit of AP-2 as demonstrated in vitro (408).

Early endosomes are tubular-vesicular membrane structures which are located at the cell periphery. Early endosomes gradually mature into late endosomes with late endosomes containing the major site for entry of newly synthesized lysosomal hydrolases via the mannose 6-phosphate receptor (409). The accumulation of EGF and EGF receptors can be detected in the early endosomal compartment within 5 minutes of exposure to EGF at the cell surface. In contrast to transferrin receptors which ultimately recycle back to the cell membrane, EGF and EGF receptors move from early endosomes and accumulate in late endosomes within the perinuclear area 10-20 minutes after exposure to EGF at 37°C.
These late endosomes are thought to be a transient compartment which fuses with lysosomes where the receptor/ligand complexes are proteolytically degraded. Movement of receptor/ligand complexes through endosomal compartments is accompanied by a lowering of endosome pH where early endosomes have a pH of 6.0-6.5 and late/mature endosomes have a pH of 4.5-5.5. It is likely that the majority of internalized ligand remains bound to the receptor at several steps of the endocytic process. The multiple steps described above are illustrated in Figure 5.

**Dynamin:**

Although the mammalian dynamin protein has been characterized only recently, the *Drosophila* homologue of the dynamin gene, *shibire*, was discovered approximately 20 years ago. Cells from *shibire* flies which have mutations in the dynamin protein were found to have a profound defect in the ability to internalize surface receptors. The *shibire* flies also exhibited a temperature sensitive paralysis. Using electron microscopy, the *shibire* defect in internalization was revealed as an inability to pinch off clathrin coated pits which remained deeply invaginated.

Mammalian dynamin was originally defined as a microtubule-binding protein. Upon further characterization, dynamin was described as a high molecular weight intracellular protein (94-96 kDa) with intrinsic GTPase activity *in vitro*. The protein was found to contain three intrinsic GTP-binding consensus sequence elements [reviewed in (272)]. Dynamin self-assembles into rings and stacks of interconnected rings thus forming a collar-like structure around the neck of the invaginated clathrin coated pit. It was postulated that a conformational change would close the rings to pinch off the budding coated vesicle. This step requires that dynamin be in a GTP-bound form and that the GTPase domain be
Figure 5. The process of ligand-stimulated receptor internalization.

Following ligand binding to receptor tyrosine kinases, receptors dimerize, become activated and recruit signaling molecules. Coincident with this process, adaptor molecules such as AP-2 are recruited to the plasma membrane where they mediate the assembly of a clathrin planar lattice. This lattice then gains curvature, and the membrane invaginates thus creating a clathrin coated pit. Constriction and budding of the clathrin coated pit is facilitated by dynamin. Following budding of the clathrin coated pit, clathrin coats are disassembled and the naked vesicle then fuses with early endosomes, thus delivering the contents of the vesicle down the endocytic pathway for lysosomal degradation.
Hinshaw et al. have demonstrated that invaginated coated pits accumulate in mammalian cells which overexpress a dominant negative dynamin molecule. In nerve terminals, GTP-bound dynamin permits tubule formation and these dynamin molecules were visualized by electron microscopy as ring structures around the invaginated pit which facilitated constriction. These experiments established that dynamin was required for the constriction of coated pits and for clathrin coated pit budding.

The regulation of dynamin is not well understood. Associations with known signaling molecules have, however, been noted. Phosphorylation of dynamin by PKC was found to stimulate the intrinsic GTPase activity of dynamin. Dynamin has been observed to bind to SH3-containing proteins via its proline-rich domain in the carboxyl-terminal tail in vitro. In addition, dynamin contains a PH domain and it was found that cooperative binding of both phosphatidylinositol (4,5) bisphosphate (PIP2) to the dynamin PH domain as well as Grb-2-SH3 binding to the dynamin proline-rich domain resulted in a four-fold increase in dynamin GTPase activity. It was postulated that Grb-2 interaction with dynamin induced the recruitment of dynamin to the plasma membrane where its activity was further stimulated by phosphorylated lipids.

**Internalization of receptor tyrosine kinases:**

The majority of the early experiments which were performed to understand the process of receptor tyrosine kinase internalization studied the EGF receptor (EGFR). Using fluorescent tagging, two studies in 1978 visualized binding, aggregation and internalization of the EGFR as well as the insulin receptor in human carcinoma A-431 cells and fibroblasts respectively. Haigler et al. observed that the EGFR was rapidly internalized by using fluorescently-tagged EGF. Following incubation with tagged EGF at 37°C, receptors were observed to leave the cell surface and become randomly distributed in endocytic vesicles in
the cytoplasm. By twenty minutes, fluorescent vesicles were observed to form a perinuclear ring (416). Similarly, Schlessinger et al. found that both fluorescently tagged insulin and EGF bound diffusely to the cell surface at 4°C and within minutes at 37°C the receptor/ligand complexes became aggregated and internalized (417). Almost ten years later, some of the requirements for EGFR internalization were characterized. Honegger et al. characterized a mutant EGFR which contained a point mutation at lysine residue 721, a critical residue in the ATP binding site. This mutant was expressed in NIH3T3 cells, but unlike wild-type receptors, the mutant did not possess intrinsic protein-tyrosine kinase activity. Although mutant EGF receptors were internalized upon incubation with EGF, the receptors were not degraded and were found to be recycled back to the cell surface in contrast to wild-type receptors which were properly degraded (418). In contrast to this study, EGF-stimulated receptor internalization was found to be completely blocked by microinjection of anti-phosphotyrosine antibodies. In addition, a kinase-defective EGFR mutant failed to undergo ligand-induced internalization (419).

This issue was more recently resolved with an examination of EGFR recruitment into clathrin-coated pits. Consistent with a requirement for intrinsic kinase activity for ligand-stimulated receptor internalization, Lamaze et al. found, using a cell-free assay to selectively measure recruitment of EGFR into coated pits, that EGF bound to wild-type receptors is efficiently sequestered into clathrin coated pits. In contrast, sequestration of kinase-deficient receptors into coated pits occurred very inefficiently and at the same rate as what was observed for unoccupied receptors. Addition of a fully functional kinase domain to this assay system completely restored clustering into clathrin-coated pits (406). In addition to the requirement for kinase activity, a carboxyl-terminal sequence containing an 18 amino acid highly negatively charged region in the EGFR has been implicated in EGF-dependent receptor internalization. This region has also been implicated in increases in
cytosolic Ca\textsuperscript{2+} following ligand stimulation (420). This "internalization domain" was further narrowed down to three regions containing endocytic motifs (421).

Attempts have been made to characterize other internalization motifs in receptor tyrosine kinases. The internalization of the CSF-1 receptor, or c-fms, has been evaluated. Carlberg et al. have shown that a mutant c-fms protein lacking tyrosine kinase activity was rapidly internalized but not degraded following binding of CSF-1. A similar result was observed for a CSF-1 receptor where tyrosine kinase activity was found to be intact but two autophosphorylation sites in the kinase insert were removed (422). In addition, tyrosine residue 569 in the c-fms juxtamembrane domain was found to be essential for kinase activity and CSF-1-stimulated receptor internalization (422). In the case of the PDGFR\textsuperscript{R}, hydrophobic regions in the carboxyl-terminal domain of the receptor were demonstrated to be required for receptor internalization. Deletion of the carboxyl-terminal 155 amino acids led to both loss of kinase activity and an inhibition of PDGF-stimulated receptor internalization. A hydrophobic 14 amino acid sequence downstream of the kinase domain was later implicated in PDGFR\textsuperscript{R} internalization (424). Thus, mutational analysis of receptor tyrosine kinases in an attempt to identify intrinsic sequences which are required for internalization has not revealed a universal consensus sequence. For this reason, it is important to consider the signaling molecules which are being activated by these receptors and the second messengers produced by these signaling molecules in order to determine their effect in the process of ligand-stimulated receptor internalization.

**Role of Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent molecules in receptor internalization.**

One of the major signaling molecules recruited by a number of receptor tyrosine kinases is PLC-γ which, when activated, induces increases in intracellular Ca\textsuperscript{2+} concentration which have been implicated in cellular protein trafficking. Ca\textsuperscript{2+} may influence some of the early
stages of receptor internalization as it was found that clathrin light chains are in fact Ca\(^{2+}\) binding proteins and that elevated Ca\(^{2+}\) levels stabilize these light chains (425, 426). In agreement with a role for Ca\(^{2+}\) in the first clathrin-mediated internalization steps, Lin et al. demonstrated that clathrin coated pit budding requires the Ca\(^{2+}\)-dependent annexin VI molecule. Using annexin VI depleted cytosol, these investigators demonstrated that clathrin pit budding only occurs when purified annexin VI is added back to the cytosol, and that this activity required both Ca\(^{2+}\) and ATP (427). However, Smythe et al. have argued that internalization of the transferrin receptor occurs independently of annexin VI (428).

Other Ca\(^{2+}\) binding proteins have been implicated in receptor internalization. Calpain has been characterized as a Ca\(^{2+}\)-dependent cysteine protease. Calpain was found to interact with the membranes of coated vesicles and has been implicated in the fusion between clathrin-coated pits and early endosomes. It has been postulated that calpain may assist in the digestion and shedding of the clathrin coat which is required for this fusion event (429). In addition, the Ca\(^{2+}\) binding molecule calmodulin has been implicated in receptor internalization. Calmodulin, a cytoplasmic protein which mediates Ca\(^{2+}\)-regulated processes, was found to bind to clathrin light chains in a Ca\(^{2+}\)-dependent manner. It was further determined that the carboxyl-terminus of the clathrin light chain was the calmodulin binding site (430). Using specific calmodulin antagonists such as N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) it was demonstrated that calmodulin plays a role in lysosomal transport (431). In addition, Columbo et al. demonstrated that increased Ca\(^{2+}\) levels were found to stimulate fusion among endosomes and that addition of recombinant calmodulin stimulates fusion beyond that produced by Ca\(^{2+}\) ions alone (432). Thus, a role for Ca\(^{2+}\) and Ca\(^{2+}\) binding proteins in various steps of the endocytic process is emerging, however, other unrelated signals are likely required at various points in the internalization pathway.
Role of PI3-kinase and D3 phosphoinositides in receptor internalization.

A role for PI3-kinase in protein trafficking was first suggested by S. Emr and colleagues who noted that the yeast homologue of the p110 catalytic subunit of PI3-kinase, Vps34, was essential for protein sorting to the yeast lysosome-like vacuole (433). These observations led to the concept that PI3-kinase may play a role in receptor endocytic events. Consistent with this hypothesis, Joly et al. analyzed a PDGFR mutant which lacked high affinity binding sites for PDGFR. They found that HepG2 cells expressing the mutant PDGFR, upon stimulation with PDGF, failed to concentrate in juxtanuclear vesicular structures. PDGFR mutants displayed no defect in the early internalization steps at the plasma membrane, however, endocytic degradation of the mutant PDGFR was severely impaired. Incubation of HepG2 cells expressing wild-type PDGFR with the PI3-kinase inhibitor wortmannin also resulted in a decrease in degradation of the receptor, suggesting that not only recruitment but also PI3-kinase activity were required for PDGFR endocytosis (434).

Endosome-endosome fusion (homotypic fusion) was also found to be blocked by the addition of either PI3-kinase inhibitors wortmannin or LY294002 in baby-hamster kidney cells. An inhibition of homotypic endosome fusion was further confirmed using an assay system where only membranous components were incubated with PI3-kinase inhibitors in vitro (435). In addition to receptor internalization, another related process, fluid phase endocytosis, was found to require PI3-kinase. Using the fluid phase marker horse radish peroxidase, wortmannin was found to inhibit the endocytosis of this marker into baby hamster kidney cells (436).

A direct role for the enzymatic activity of PI3-kinase in the process of receptor internalization and endocytosis is rapidly emerging, however, PI3-kinase association with
small GTPases has also been implicated in cellular protein movement. GTP-bound Rac was found to bind directly to PI3-kinase and this interaction could be stimulated by incubation of Swiss 3T3 cells with PDGF. On its own, Rac mediates growth factor-induced membrane ruffling as a result of actin reorganization at the plasma membrane. This activity may therefore have an effect on the cytoskeletal events associated with receptor internalization (437).

Although other signaling proteins such as Grb-2 have also been associated with the ligand-stimulated internalization of certain receptors, this thesis will concentrate on PI3-kinase and PLC-γ-mediated signals. The data presented in Chapter 2 will implicate both of these enzymes in SLF-stimulated c-Kit internalization. An important question which is related to ligand-stimulated internalization is the biological function of this process which will be addressed in greater detail in Chapter 3. Given that internalization of receptor/ligand complexes ultimately results in the endocytic delivery of these complexes into lysosomes for degradation, receptor internalization serves an obvious down-modulatory role for signals transmitted at the cell membrane. In agreement with this concept, a non-internalizing EGF receptor was found to induce cellular transformation (438). However, it is intriguing to note that in the case of the high affinity IL-2 receptor, antibody-mediated inhibition of IL-2 receptor internalization did not affect IL-2 binding but prevented the growth of IL-2-dependent T cell lines (439, 440). In addition, examination of non-internalizing EL4 cell variants revealed that activation of these cells with IL-1 was found to require the internalization of the IL-1 receptor (441). Therefore, in some cases there appears to be a correlation between receptor internalization and mitogenic signaling. This correlation is supported by findings that signaling molecules such as p85 are associated with early endosomes (200, 442), suggesting that the endocytic compartment may be an important structure for signal transduction. Thus, a role for receptor internalization in the propagation of growth factor mediated signals and the theory that these signals may in fact
be localized to an endocytic structure are emerging concepts in the field of signal transduction.

(viii) Summary and Thesis Preview

The concepts which have been discussed in this Chapter have highlighted a role for mast cells in the clearance of pathogens as well as in autoimmunity. One of the key receptors expressed by mast cells and other hematopoietic, germ cell and melanocyte precursors is c-Kit. The characterization of c-Kit and its ligand SLF has revealed that signals mediated by c-Kit have pleotropic functions such as adhesion, chemotaxis, differentiation, mitogenesis, and the maintenance of cell survival. In many cases, these outcomes are not mediated by SLF alone, but in combination with other growth factors such as IL-3, IL-7, GM-CSF or Epo. The basis for c-Kit mediated cellular functions as well as for the synergy observed between SLF and other cytokines is revealed in the array of signaling molecules activated by the binding of SLF to c-Kit. Two of the major signaling enzymes activated by this receptor/ligand complex are PI3-kinase and PLC-γ. These two SH2-containing proteins, when activated, generate very distinct second messengers which contribute to mitogenesis, the maintenance of cell viability and ligand-stimulated receptor trafficking, all of which have been described in detail in this Chapter.

Given the role of these two signaling enzymes in receptor-mediated processes, we have taken the approach of analyzing mutant c-Kit receptors for their internalization properties and mitogenic potential. In Chapter 2 of this thesis, the mechanism of c-Kit internalization is therefore addressed. In this Chapter, both PI3-kinase and PLC-γ-mediated signals will be implicated in SLF-stimulated c-Kit internalization. This process of internalization may serve as a mechanism for degrading receptors, thus down-modulating signal transduction. Paradoxically, the internalization and endocytosis processes may also potentiate receptor-
mediated signals. This concept is addressed in Chapter 3 where a correlation between receptor internalization and mitogenic signaling is established. In addition, an essential role for PLC-γ is revealed when c-Kit is stimulated by membrane-bound but not soluble SLF. This form of ligand, as mentioned in this Introduction, appears to be more biologically relevant than soluble SLF given that soluble SLF is unable to support many c-Kit mediated processes \textit{in vivo}. It is postulated that the requirement for PLC-γ-mediated signals in the context of membrane-bound SLF may be as a result of an impairment in the PI3-kinase pathway. This would potentially explain why SLF often acts in synergy \textit{in vivo} with other growth factors which readily activate PI3-kinase.

A number of examples have been described in this Introduction where SLF exerts an important survival function in the context of differentiation. In addition, this SLF-stimulated survival mechanism is different than IL-3-mediated survival signals in terms of up-regulation of the Bcl-2 anti-apoptotic protein. In Chapter 4, mechanisms of SLF-mediated cell survival are addressed. Specifically, a role for PLC-γ stimulated Ca\(^{2+}\) influx in cell survival is identified, and this Chapter characterizes an apoptotic mechanism where combinations of SLF and Ca\(^{2+}\) influx blockers leads to apoptosis. This apoptotic mechanism is demonstrated as caspase-mediated and Bcl-2-insensitive. Interestingly, this form of apoptosis is also shown to require the activation of PLC-γ.

SLF is a key modulator of mast cell function and mast cells express high levels of c-Kit. Determining the role of c-Kit associated signaling proteins in mast cell processes is therefore an important component to understanding the mechanisms required for mast cell functions. In Chapter 5, the results described in Chapters 2-4 are placed into the context of mast cell activation \textit{in vivo}. The question of how the signaling differences mediated by membrane versus soluble SLF apply in the organism is discussed. In addition, the question of the relevance of receptor internalization in terms of full activation of the PI3-
kinase enzyme will be raised. These questions will lead to new directions in the study of c-
KIT signal transduction.
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CHAPTER II

Phosphatidylinositol 3-Kinase and Ca^{2+} Influx Dependence for Ligand-stimulated Internalization of the c-Kit Receptor.

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**Introduction:**

The pleotropic effects of SLF on c-Kit positive cells such as mitogenesis and chemotaxis have been detailed in the previous Chapter. One important controlling mechanism required for the proper homeostasis of these cellular functions is the mechanism of ligand-stimulated receptor internalization, a process common to all receptor tyrosine kinases (1). Given the importance of this process in regulating c-Kit-mediated signals, the mechanistic requirements for SLF-stimulated c-kit internalization will therefore be addressed in this chapter.

As mentioned in the first chapter, stimulation of the c-kit receptor with SLF results in the recruitment and tyrosine phosphorylation of SH2-containing second messenger-generating enzymes (2) such as PLC-γ and PI3-kinase (3, 4). The phosphorylated lipid products of these enzymes stimulate a variety of intracellular processes including Ca^{2+} mobilization and actin reorganization (5-7). Coincident with second messenger generation, the process of receptor internalization is also initiated. Within minutes following ligand binding, receptors cluster in dimers or oligomers and internalize by endocytosis, likely through clathrin-coated pits (8-10). Eventually, clathrin coats are removed and the remaining vesicles fuse with endosomes, late endosomes, and ultimately lysosomes, resulting in receptor degradation.

Numerous deletion and mutagenesis studies have been carried out to map regions of receptor tyrosine kinases required for ligand-stimulated internalization (11-20). Tyrosine kinase activity (21, 22), autophosphorylation sites (12, 13) and interactions with second messenger-generating enzymes have been implicated. In particular, recruitment and activation of PI3-kinase has been associated with ligand-stimulated internalization of the PDGF receptor (17). Receptors carrying mutations within the cytoplasmic domain that...
disrupt the PI3-kinase binding site are impaired in the later stages of endocytosis (23). However, conflicting results with a deletion mutant encompassing this binding site demonstrate no impairment in any stage of endocytosis (14).

Ligand-stimulated endocytosis of c-kit was investigated by Yee et al. (24). They reported that a c-kit mutant with no kinase activity was impaired for ligand-stimulated internalization. Individual mutations converting tyrosines 719 and 821 to phenylalanines, however, failed to affect internalization, although Phe 821 was impaired for mitogenesis (25). Tyr 719 is located in the kinase insert region of the c-kit catalytic domain. In its phosphorylated form, it forms part of a consensus binding site for the p85 subunit of PI3-kinase, and has been shown to mediate this interaction in vitro and in vivo (4).

This chapter also examines the role of PI3-kinase activation on ligand-stimulated internalization of c-kit. It was found that in the absence of PI3-kinase activation, the c-kit receptor internalizes but remains localized near the inner aspect of the plasma membrane. However, when both PI3-kinase and Ca²⁺ influx are inhibited, clathrin fails to co-immunoprecipitate with c-kit and receptor internalization is completely prevented. These results show that concurrent inhibition of PI3-kinase activity and Ca²⁺ influx disrupts the earliest stages of c-kit internalization.
Materials and Methods

Cell culture and transfection
gp+e NIH 3T3 packaging cells were grown in Dulbecco's modified Eagle's medium
(DMEM - GIBCO) supplemented with 10% fetal bovine serum (FBS) and antibiotics. DA-
1 cells are an IL-3-dependent, c-kit negative murine lymphoma cell line (26). They were
routinely grown in RPMI supplemented with 10% FBS, 10-5M β-mercaptoethanol and 2% 
supernatant from WEHI-3 cells as a source of IL-3. BMMCs were isolated and cultured as
described (27).

The cDNAs for wild type (wt) murine c-kit or mutants in which Tyr 719 was replaced with
Phe, were cloned into the LXSN retroviral expression vector. These vectors were
transferred into the gp+e NIH 3T3 packaging cells by electroporation, and transfectants
were selected by growth in 400 μg/ml G418 (GIBCO). DA-1s were co-cultured with a
confluent layer of pooled gp + e transfectants for 24 hours. The non-adherent DA-1s were
removed and cultured for an additional 5 to 10 days in IL-3. c-Kit positive DA-1 cells were
enriched by two rounds of cell sorting (FacsStar Plus - Becton Dickinson). Surface
expression of c-kit was detected using biotinylated SLF and strepavidin-phycoerythrin
(Jackson). Stable populations of c-kit expressing DA-1s were then cloned by limiting
dilution (Figure 1.).

As reported by others (4), we confirmed that the p85 subunit of PI3-kinase co-
immunoprecipitates with c-kit following stimulation of the wt receptor with SLF but fails to
co-immunoprecipitate with YF719 (Figure 2.).
Figure 1. Expression of c-kit in the DA-1 cell line.

Expression of wt c-kit on the surface of DA-1 cells was detected with b-SLF and Strep-PE by FACS. (A) Uninfected DA-1 cells. (B) Expression of c-kit on DA-1 before sorting. (C) Expression of c-kit on DA-1 cells after one sort. (D) Expression of c-kit on DA-1 cells after two sorts, cloning by limiting dilution and several months in culture.
Figure 1.
**Figure 2.** Association of p85 with wt and mutant forms of c-kit.

$1 \times 10^7$ cells were either stimulated (+) or not stimulated (-) with 250ng/ml SLF for 5 minutes at 37°C. Cells were lysed with 1% NP-40 lysis buffer and precipitated with rabbit polyclonal antibodies. Upper gel shows precipitation with pre-immune serum in lane 1 or anti-kit antibodies in lanes 2-5 followed by probing with anti-kit antibodies. Lower gel shows precipitation with anti-p85 antibodies in lane 1 and anti-kit antibodies in lanes 2-5 followed by probing with anti-p85 antibodies.
Figure 2.

- 145 kD Kit blot
- 85 kD Kit blot

Legend:
- total p85
- wt
- YF719 -
- YF719 +

Kit IP
p85 blot
Production and labeling of recombinant SLF

Recombinant murine SLF was produced in soluble form in *E. coli* using the pFLAG.ATS IPTG-inducible secretion expression vector (Invitrogen). This vector includes an eight amino acid N-terminal FLAG epitope (InterScience). *E. coli* containing the pFLAG.ATS plasmid were incubated overnight at 37°C in LB with 100 μg/ml ampicillin. This culture was then diluted 20-fold and grown to an OD₆₀₀ of 0.4-0.5 before being induced with 0.033 g/L IPTG. The cultures were then incubated overnight at 37°C before they were centrifuged at 10,000 rpm for 20 minutes. The bacterial supernatant was passed through a 0.22 micron filter and stored at -80°C with 1mM CaCl₂ and 100 μM PMSF. FLAG-SLF was purified by passing supernatants over a column of Anti-FLAG M1 mouse monoclonal antibodies covalently attached to agarose gel. The column was first equilibrated with 30 ml PBS + 1mM CaCl₂. Bacterial supernatants were then passed over the M1 column 3 times. The FLAG-SLF fusion protein binding to the affinity column is Ca²⁺ dependent therefore elution of FLAG-SLF can be achieved by adding EDTA. 6 elutions with 1 ml of PBS + 2mM EDTA were performed. These were collected, concentrated and checked for purity by silver stain and western blot. The silver stain in Figure 3.A reveals a major band of 23kD as well as a minor band of lower molecular weight. The western blot in Figure 3.B confirms that the major band is SLF and the minor lower molecular weight bands are likely degradative products. SLF was also checked for activity by bioassay. In Figure 3.C the concentration of SLF for half-maximal proliferation of c-Kit positive mast cells was found to be approximately 100ng/ml. This concentration was similar to that observed by others (28). SLF was also conjugated with biotin (Sigma) as described (29).

Internalization assay - Flow cytometry

1 x 10⁶ cells of wt and YF719 DA-1 clones were resuspended in Phosphate Buffered Saline + 0.5% FBS (PBS/FBS) and washed twice with the same solution. MgCl₂ and
Figure 3. Analysis of SLF purity and bioactivity.

Starting material, flow through, wash and eluate fractions of purified SLF were resolved by SDS-PAGE (12%) and either subjected to silver stain (A) or transferred to nitrocellulose and probed with anti-Flag monoclonal antibody (B). Bioactivity of SLF was confirmed by incubating BMMCs with different concentrations of SLF for 18 hours followed by a 6 hour pulse with ^3HdT (C).
Figure 3.

A

B

C

Starting material
Flow Thru
Wash
Purified eluate

SLF

SLF

[SLF] ng/ml

Cpm

0 1 10 100 1000 10000

0 2000 4000 6000 8000 10000 12000

[SLF] ng/ml
CaCl₂, when added, were at 0.5 mM and 1 mM respectively. Biotin-conjugated SLF (b-SLF) was then added to the cells in PBS/FBS at a concentration which allowed for maximal FACS staining - typically 1:100 to 1:50 dilution. Cells were incubated on ice with b-SLF for 60 minutes. Cells were incubated for 0, 6, 9, 12 and 15 minutes at 37°C and washed three times with ice cold PBS/FBS. Cells were then fixed with 3% paraformaldehyde for 10 minutes on ice. This fixation procedure stabilized the staining pattern for several hours with little disruption of the cellular architecture. Following fixation, cells were washed three times with PBS/FBS and then incubated with streptavidin-conjugated phycoerythrin (Strep-PE: Jackson) for 30 minutes on ice. Cells were then washed twice with PBS/FBS and analyzed by FACS for expression and internalization of c-kit.

The internalization of c-kit was confirmed to be a ligand-dependent process using a FITC-conjugated anti-kit antibody (2B8) (Pharmingen) directed at the extracellular domain of c-kit. This antibody does not compete with SLF binding to the c-kit receptor. Cells were either first incubated with (or without) SLF for 1 hour at 4°C, followed by a 10 minute incubation at 37°C, washed, and then labeled with FITC-conjugated anti-kit for 20 minutes at 4°C. Cells were then analyzed by flow cytometry. In the absence of ligand, receptor levels did not decrease after incubation at 37°C. However, addition of ligand caused a loss of staining with the anti-kit antibody (Figure 4.) indicating that internalization of c-kit is stimulated by ligand binding.

**Internalization assay - microscopy**

1 x 10⁶ cells of wt and YF719 DA-1 clones were resuspended in PBS/FBS and washed twice with the same solution. FITC-conjugated SLF was then added to the cells in PBS/FBS at a dilution of 1:100 to 1:50. Cells were incubated on ice with FITC-SLF for
Figure 4. Flow cytometric analysis of ligand dependence for c-kit internalization.
DA-I cells expressing wt c-kit were either maintained at 37°C (solid line), heated at 37°C (hatched line) or heated at 37°C with SLF for 10 minutes (dashed line). wt DA-I cells were then stained with 2B8, a FITC-conjugated anti-kit antibody directed at the extracellular domain of c-kit. Loss of staining is indicative of internalized receptors.
40 to 60 minutes. Cells were then transferred to a 37°C water bath for a short incubation time and then subsequently placed back on ice. Cells were washed three times with ice-cold PBS/FBS then fixed with 3% paraformaldehyde for 10 minutes on ice. Following fixation, cells were washed three times with PBS/FBS. Cells were then labeled with an anti-FITC antibody tagged with Texas Red (TxR) (Molecular Probes) for 45 minutes on ice. Cells were washed three times with PBS/FBS then resuspended in 5 μl of 90% glycerol containing 1,4 Diazobicyclo-[2.2.2]-octane (DABCO purchased from Aldrich) which significantly reduces photobleaching (30). Cells were allowed to settle for 10 minutes on the microscope slide before applying a coverslip and nail polish. Incubation with glycerol was sufficient to immobilize the cells, while maintaining their three-dimensional structure relatively undistorted. Cells were observed by fluorescence microscope (Leitz DMR/BE) using a 100X oil-immersion objective with filters for both FITC and TxR. Photographs were always exposed for 90 seconds. When cells were photographed using both filters, the Texas-Red image was exposed first since it experienced greater photobleaching.

In order to test the co-localization of both the c-kit receptor and its ligand, SLF, wt and YF719 DA1 cells were incubated with b-SLF and a FITC conjugated anti-c-kit antibody (2B8, Pharmingen) for 60 min. at 4°C either in the presence or absence of extracellular Ca²⁺. As controls, cells were either incubated with b-SLF and a FITC labeled isotype control antibody (Pharmingen) followed by staining with streptavidin-conjugated TxR or cells were incubated with 2B8 alone followed by streptavidin-conjugated TxR. Cells were then warmed to 37°C for 0, 7.5 and 15 minutes. Cells were washed with ice cold PBS/FBS and fixed as described above. Cells were then permeabilized with 0.1% saponin for 15 min. at room temperature, washed and then resuspended in glycerol/DABCO and plated on microscope slides as described above.
**Wortmannin**

Wortmannin (purchased from Sigma) was dissolved at a concentration of 20mM in DMSO and stored in aliquots at -80°C. Cells were prepared for immunofluorescence microscopy and flow cytometric analysis as described above with the exception that 100 nM wortmannin was added to the binding buffer minutes before the 37°C incubation.

**Immunoprecipitation and Western Blotting**

For c-kit immunoprecipitations, 2.5 x 10^6 BMMCs per sample were starved overnight in RPMI + 0.5%FBS and then washed twice in PBS + 0.5% FBS. Cells were then incubated with 500 ng/ml SLF at 37°C for various time points using the indicated conditions and then immediately washed twice in ice cold PBS/FBS. Cells were then lysed in lysis buffer containing 1% Triton-X 100 (Caledon), 50mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 1.5mM MgCl₂ and 1mM EGTA with inhibitors: 500 μM Na-orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μM NaF and 1mM Na-molybdate (all Sigma). Lysates were then spun at 10,000 rpm for 20 minutes and supernatants were incubated with 50 μl of a 20% Protein A slurry (Pharmacia) and 5 μl of a polyclonal rabbit antibody raised against a GST-kit cytoplasmic tail fusion protein or 5 μl of pre-immune serum and 50 μl of a 20% Protein A slurry (R. Rottapel; unpublished). A whole cell lysate prepared from 1/10th the number of cells was used as a positive control on the Western blot. All the other lysates were incubated for 2 hours at 4°C and the beads were washed three times in HNTG wash buffer containing 0.1% Triton-X, 20 mM Heps (pH 7.0), 10% glycerol, 150 mM NaCl and 1mM Na-orthovanadate. Beads were resuspended in leading buffer with β2-mercaptoethanol, boiled for 5 minutes and released proteins were resolved on a 7.5% acrylamide gel, transferred to nitrocellulose and blocked in PBS plus 5% skim milk powder and 0.5% Tween-20. Blots were incubated with an anti-clathrin monoclonal antibodies (Transduction Laboratories) at a dilution of 1:5000 overnight followed by Goat-anti-mouse conjugated horseradish.
peroxidase (HRP) secondary antibodies (Jackson) at a dilution of 1:2,000 and visualized with chemiluminescence (Dupont). Blots were then stripped by acid treatment and re-probed with rabbit polyclonal anti-c-kit antisera at a dilution of 1:500 followed by incubation with protein A-HRP (Amersham) at a dilution of 1:30,000 and visualized again by chemiluminescence.
Results

FACS analysis of internalization

Labeled ligand was used in conjunction with flow cytometry to follow the average rate of internalization over a population of cells. Labeled ligand offers the advantage of following only those receptors that are activated, unlike the use of anti-receptor antibodies which cannot distinguish between activated and unactivated receptors. Cells were incubated with b-SLF at 4°C and then further incubated for varying amounts of time at 37°C. Internalization was arrested by adding ice cold PBS/FBS and shifting the cells back to 4°C. Following washing and fixing, the cells were then incubated with Strep-PE, and analyzed for surface expression. Figure 5. demonstrates typical FACS profiles with b-SLF, showing a decline in the intensity of the fluorescence signal as the bound ligand is internalized with the c-kit receptor.

Internalization kinetics of wt and YF719 c-kit receptors

Recruitment and activation of PI3-kinase has been implicated in both endocytic and exocytic processes in a number of different experimental systems. The ligand-stimulated internalization kinetics of both wt and YF719, a c-kit receptor mutant that fails to recruit and activate PI3-kinase was therefore examined. Figure 6. depicts the average of three flow cytometry experiments presented in graphical form. The mean fluorescence intensity of cells which were maintained at 4°C is denoted as 100% surface receptor expression. As shown in Figure 6.A, wt c-kit and the YF719 mutant expressed in DA-1 cells internalize with similar kinetics. The half life for loss of surface label is approximately 6 minutes.

Receptor internalization dependence on Ca^{2+}

Another early signaling event following ligand-stimulated activation of c-kit is the
Figure 5. Flow cytometric analysis of wt c-kit internalization.

DA-1 cells expressing wt c-kit were incubated with b-SLF at 4°C for 60 min. Cells were warmed at 37°C for 0, 7.5 and 15 min. Cells were then fixed, incubated with Strep-PE for 30 min. and analyzed by Flow Cytometry. Live cells were analyzed by gating out cells which were positive for 7AAD stain. The loss of PE signal is indicative of internalized receptor.
Figure 5.
Figure 6. The role of Ca^{2+} in wt and mutant c-kit internalization.

DA-1 cells expressing wt and YF719 c-kit were assessed for their ability to internalize c-kit. Loss of surface c-kit was measured as a function of the loss of mean fluorescence. Time zero was taken to represent 100% mean fluorescence. (A) c-kit internalization with Ca^{2+} and (B) c-kit internalization without Ca^{2+}. (C) c-kit internalization with Ca^{2+} and 2.5 mM Ni^{2+}. Circles and squares represent wt and YF719 receptors respectively. The data shown represent duplicates (± SD) and experiments were repeated three times with similar results.
Figure 6.
mobilization of Ca\textsuperscript{2+} from intracellular stores, followed by Ca\textsuperscript{2+} influx from the extracellular milieu (31). A number of Ca\textsuperscript{2+} binding proteins are associated with coated pits and endosomes, suggesting that high levels of intracellular Ca\textsuperscript{2+} may influence receptor endocytosis (32-34). We therefore investigated the effect on receptor internalization of removing extracellular Ca\textsuperscript{2+}. As shown in Figure 6B, when Ca\textsuperscript{2+} is excluded from binding and wash buffers, wt c-kit internalizes with kinetics similar to that observed in the presence of Ca\textsuperscript{2+}, however the YF719 receptor fails to internalize.

In order to confirm that Ca\textsuperscript{2+} influx is required for YF719 internalization, Ni\textsuperscript{2+} a competitive blocker of Ca\textsuperscript{2+} influx channels (33, was added to the incubation medium containing Ca\textsuperscript{2+} and the internalization kinetics were determined. As shown in Figure 6C, the inclusion of 2.5 mM Ni\textsuperscript{2+} in the binding and wash buffers blocks internalization of the YF719 mutant, but has little effect on the kinetics or the extent of internalization of wt c-kit. Taken together, these results demonstrate that internalization of YF719 is dependent on Ca\textsuperscript{2+} influx.

**Fluorescence microscopic analysis of internalization**

Receptor endocytosis is a multi-step process involving transfer of receptor from clathrin-coated pits, to early and late endosomes (9). PI3-kinase activity has been particularly associated with movement of receptor down later steps in the endocytic pathway (23). The distribution pattern of internalized wt and mutant c-kit receptors after activation with SLF using fluorescently labeled ligand and fluorescence microscopy was therefore examined. Cells were washed and incubated with FITC-conjugated SLF at 4°C to allow ligand binding. Cells were then incubated for a further period at 37°C to allow for internalization. Following washing and fixing, the cells were further stained with anti-FITC antibody coupled with Texas Red. Because cells were not permeabilized, the anti-FITC antibody binds only those occupied receptors remaining on the surface of the cell. As shown in
Figure 7A, when wt cells are incubated with FITC-SLF at 4°C, or for only a short time at 
37°C, the FITC and Texas Red stains are superimposable indicating that the ligand-receptor 
complex is on the cell surface. With longer incubations at 37°C, the fluorescence becomes 
increasingly punctate and there is an accompanying loss of Texas-Red staining. Finally, 
after 10-15 minutes of incubation at 37°C, little Texas-Red fluorescence is observed while 
the majority of the FITC staining is in large aggregates and appears to be internal to the 
cells. This pattern of staining is consistent with a process involving ligand-driven 
aggregation of the receptor, followed by internalization of the ligand-receptor complex and 
subsequent movement down the endocytic pathway.

The receptor distribution pattern following ligand binding was also investigated for the 
YF719 mutant. As shown in Figure 7B, in the presence of Ca²⁺, the YF719 receptor was 
also observed to aggregate and internalize. However, unlike the wt receptor, the 
distribution pattern of the YF719 receptor remained punctate and was primarily located near 
the interior of the plasma membrane, even after a 15 minute incubation. This suggests that 
although the YF719 receptor is internalizing, further movement down the endocytic 
pathway may be impaired.

When Ca²⁺ was omitted from the binding and wash buffers, the wt receptor internalized 
and displayed a pattern of staining identical to that observed in the presence of Ca²⁺ and 
with similar kinetics (Figure 8A). In contrast, although the YF719 receptor was still 
observed to aggregate, the Texas Red and FITC stains remained co-localized on the surface 
of the cell (Figure 8B). This observation indicates that the receptor-ligand complexes are 
not internalizing, in agreement with the flow cytometric data which also demonstrated an 
ability of the YF719 receptor to internalize in the absence of Ca²⁺. These observations 
have been extended to time points as long as 30 minutes. At these times, the YF719 mutant
Figure 7. Fluorescence microscopic analysis of wt and YF719 c-kit internalization.
DA-1 cells expressing (A) wt or (B) YF719 c-kit were incubated with FITC-SLF for 60 min. at 4°C. Cells were then warmed to 37°C for 0, 7.5 or 15 min. Cells were then fixed and stained with Texas Red conjugated anti-FITC antibody before plating on microscope slides and examining under the fluorescence microscope.
Figure 7A.
Figure 7B.
Figure 8. Fluorescence microscopic analysis of wt and YF719 c-kit internalization in the absence of extracellular Ca$^{2+}$.

DA-1 cells expressing (A) wt or (B) YF719 c-kit were incubated with FITC-SLF for 60 min. at 4°C in the absence of extracellular Ca$^{2+}$. Cells were then warmed to 37°C for 0, 7.5 or 15 min. Cells were then fixed and stained with Texas Red conjugated anti-FITC antibody before plating on microscope slides and examining under the fluorescence microscope.
Figure 8A.
Figure 8B.
still fails to internalize in the absence of Ca\textsuperscript{2+}.

As receptors undergo ligand-stimulated internalization, receptor/ligand complexes accumulate in endocytic compartments which are acidic. This acidic environment may lead to dissociation of the ligand from its receptor and therefore result in different endocytic routing patterns for each. In order to test whether c-kit and its ligand SLF follow the same endocytic route, wt and YF719 DA-1 cells were incubated with both b-SLF and a FITC conjugated anti-c-kit antibody, 2B8 for 60 minutes at 4°C followed by a 37°C incubation for either 0, 7.5 or 15 minutes. Cells were then fixed, permeabilized and incubated with streptavidin-conjugated TxR. As shown in Figure 9, after a 7.5 minute incubation at 37°C the TxR and FITC labels co-localize indicating that the receptor/ligand complex remains intact throughout the internalization process. The co-localization of SLF and c-kit is observed for both the wt and mutant YF719 receptor either in the presence or absence of extra-cellular Ca\textsuperscript{2+}.

\textit{Wortmannin inhibits wt internalization in the absence of Ca\textsuperscript{2+}}

The observation of impaired receptor internalization in the absence of Ca\textsuperscript{2+} for the YF719 mutant cannot distinguish between a dependence on PI3-kinase binding to the c-kit receptor or a further requirement for PI3-kinase reaction products generated by enzymatic activation. In order to address this question, internalization experiments were performed following pre-treatment of the cells with wortmannin, a specific inhibitor of the PI3-kinase enzyme (36). wt DA-1 cells were prepared for either flow cytometry or fluorescence microscopy. Minutes before incubation of the cells at 37°C, wortmannin was added to the binding buffer. In the presence of Ca\textsuperscript{2+}, and following wortmannin pre-treatment, the wt c-kit receptor was observed to internalize (Figure 10.B). However, when analyzed by fluorescence microscopy, the receptor appeared to localize predominantly at the cell
Figure 9. Fluorescence microscopic analysis of the co-association of c-kit and SLF.
DA-1 cells expressing either wt or YF719 receptors were incubated with b-SLF and FITC-
conjugated anti-Kit antibody for 60 min. at 4°C in the presence or absence of extracellular
Ca\(^{2+}\). Cells were then warmed to 37°C for 7.5 minutes, fixed with paraformaldehyde and
permeabilized with 0.1% saponin. Cells were then incubated with streptavidin-conjugated
Texas Red for 45 min. before plating on microscope slides and examining under the
fluorescence microscope.
Figure 9.
wt DA-1 cells were assessed for their ability to internalize in the presence of the PI3-kinase inhibitor wortmannin. Loss of surface c-kit was measured as a function of the loss of mean fluorescence. Time zero was taken to represent 100% mean fluorescence. (A) wt internalization with Ca\textsuperscript{2+}. (B) wt internalization with Ca\textsuperscript{2+} and wortmannin. (C) wt internalization without Ca\textsuperscript{2+}. (D) wt internalization without Ca\textsuperscript{2+}, with wortmannin. (E) Wortmannin titration with or without Ca\textsuperscript{2+}. Inhibition of internalization was assessed 15 min. following incubation with β-SLF at 37°C. Empty and hatched boxes represent conditions with Ca\textsuperscript{2+} and without Ca\textsuperscript{2+} respectively. The data shown represent triplicates (± SEM) and experiments were repeated three times with similar results.
Figure 10.

A

B

C

D

E

Mean Fluorescence Intensity

Relative Cell Number

[wortmannin] nMolar

% left on surface

0 0.125 0.25 0.5 1 10 100 1000 10000

neg.

0 min.

15 min.
membrane, with a punctate staining pattern similar to that observed for the YF719 receptor (Figure 11). This altered subcellular distribution suggests that PI3-kinase may be particularly important in later endocytic steps. As shown in Figure 10.D, wt c-kit receptors on the surface of cells incubated with wortmannin are not internalized in the absence of Ca^{2+}. A failure of the wt receptor to internalize in the absence of Ca^{2+} and following wortmannin pre-treatment is also observed by fluorescence microscopy (Figure 11). Titration of the concentration of wortmannin both in the presence and absence of Ca^{2+} revealed that the amount of wortmannin required to achieve 50% inhibition of c-kit internalization on BMMCs after a 15 minute incubation with SLF at 37°C was approximately 1nM (Figure 10.E). This level of wortmannin has been shown to be specifically inhibitory for PI3 kinase enzymatic activity in other systems (36, 37). In contrast, there was no inhibitory effect on internalization in the presence of Ca^{2+} at any concentration of wortmannin. These results therefore indicate that there is a dependence on PI3-kinase enzymatic activity and Ca^{2+} in c-kit receptor internalization.

c-kit receptor internalization is clathrin-associated

These results indicate that ligand-stimulated receptor internalization is modulated by PI3-kinase activation and Ca^{2+} influx. One of the earliest steps in endocytosis is association with clathrin-coated pits. However clathrin-independent endocytic mechanisms have also been reported (22). In order to determine if c-kit internalization is clathrin-associated, c-kit immunoprecipitates were analyzed for the co-immunoprecipitation of clathrin.

Cells were starved overnight, washed and then stimulated at 37°C with SLF with or without Ca^{2+} and with or without wortmannin. The cells were washed, lysed and immunoprecipitated with either pre-immune serum (lane 1), or anti-kit antibodies (lanes 3-14). Lane 2 contains whole cell lysates using 1/10th the amount of cellular material.
Figure 11. Fluorescence microscopic analysis of c-kit internalization in the presence of wortmannin.

DA-1 cells expressing wt c-kit receptor were treated with wortmannin prior to a 15 min. incubation with SLF at 37°C. wt DA-1 cells did not internalize with wortmannin in the absence of Ca²⁺. Top panel is FITC stain and bottom panel is Texas Red.
Figure 11.
Samples were separated by SDS-PAGE and analyzed by Western blot. The blot in the upper panel in Figure 12. was probed with an anti-clathrin heavy chain monoclonal antibody. This blot was stripped and re-probed with anti-kit antibodies (bottom panel of Figure 12.). As shown in lane 2, an immunoreactive band is observed in whole cell lysates which migrates at 180 kD, the expected molecular weight for the clathrin heavy chain. In the absence of stimulation, only very low levels of clathrin co-immunoprecipitated with anti-c-kit antibodies (Figure 12., lane 3). No clathrin was observed following immunoprecipitation with c-kit pre-immune serum (Fig 12., lane 1). As shown in Figure 12. lane 5, stimulation with SLF at 37°C for 5 minutes increased the level of clathrin that co-immunoprecipitates with c-kit. Increased co-immunoprecipitation of clathrin was also observed following stimulation of cells with SLF for 2.5 or 5 minutes in the absence of Cu²⁺ only, or in the presence of wortmannin only (lanes 6 to 11). However, in the absence of Cu²⁺ and in the presence of wortmannin, negligible levels of clathrin were found to co-immunoprecipitate with c-kit. These results indicate that in the absence of PI3-kinase activity, Cu²⁺ influx is required for the earliest stages of clathrin-associated receptor internalization.
Figure 12. c-Kit internalization is clathrin associated.

BMMCs were lysed and immunoprecipitated with either pre-immune (lane 1), or anti-c-kit antibodies (lane 3-14). Lane 2 contains a whole cell lysate from 1/10th the cell number used in the immunoprecipitations. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-clathrin monoclonal antibody (top panel), stripped, and then re-probed with anti-c-kit polyclonal antibody (bottom panel).
Figure 12.

Clathrin
Heavy chain

c-kit
Ligand-stimulated receptor internalization is a multi-step process involving assembly of an endocytic machinery composed of clathrin heavy and light chains, adaptors, dynamin, and other cytosolic factors. In addition to these elements, recruitment and activation of enzymes such as PI3-kinase have also been implicated in the endocytic process. This chapter demonstrates that ligand-stimulated internalization of the c-kit receptor is blocked when both PI3-kinase activation and Ca\(^{2+}\) influx are inhibited. While inhibition of either of these signals alone did not prevent the early stages of internalization, loss of PI3-kinase activity resulted in internalized receptors that appeared to accumulate in vesicles close to the membrane. This observation is consistent with the results of Joly et al. who demonstrated that a mutant PDGF receptor which cannot associate with PI3-kinase is internalized but not degraded (23).

PI3-kinase is a second-messenger-generating enzyme which has been linked to mitogenesis, receptor trafficking and maintenance of cell viability in other receptor systems (17, 25, 38). Indirect evidence suggests a role for PI3-kinase in vesicular sorting or movement of proteins in the cell. Vps34, a yeast protein with homology to the p110 catalytic subunit of PI3-kinase is involved in the transport of soluble hydrolases from the trans Golgi network to yeast vacuoles (39). Cantley and colleagues have shown that activated PDGF receptor and PI3-kinase remained complexed in endosomes and associate with microtubules in 3T3-L1 cells (40). Inhibition of PI3-kinase has also been reported to inhibit transferrin receptor endocytosis, non-specific fluid phase uptake and early endosome fusion, possibly via the small GTPase Rab5 (41). In addition, PI3-kinase activation is required for the reorganization of actin filaments and the induction of membrane ruffling by PDGF (7). Besmer and colleagues (24) found little to no effect of a
c-Kit activates PLC-γ (3, 6) resulting in the hydrolysis of PIP2 into DAG and IP3 (5). IP3 causes the mobilization of intracellular Ca^{2+} from stores in the endoplasmic reticulum, resulting in the stimulation of Ca^{2+} influx from the extracellular milieu through the Ca^{2+} Release Activated Channel (CRAC) (31). This Ca^{2+} influx step seems to be critical for c-kit internalization, since intracellular mobilization should still occur in the absence of extracellular Ca^{2+} or in the presence of Ni^{2+}.

These results do not identify the Ca^{2+}-dependent step in the early stages of receptor endocytosis. However a number of Ca^{2+}-dependent elements implicated in endocytosis have been identified. Clathrin light chains are Ca^{2+}-binding proteins and high levels of Ca^{2+} are thought to stabilize the clathrin cage (42, 43). The adaptor protein annexin VI, a Ca^{2+}-dependent phospholipid-binding protein, is required for budding of clathrin coated pits in a cell free system (32). However, annexin VI was not found to have a role in coated
pit formation and constriction, nor did it enhance transferrin receptor endocytosis in A431 cells (44). Another Ca\textsuperscript{2+}-dependent protein is calmodulin, a cytoplasmic mediator of many calcium-regulated processes (45). Calmodulin has been demonstrated to bind to clathrin light chains in a Ca\textsuperscript{2+}-dependent manner (46), and has been shown to regulate endosome fusion \textit{in vitro} (34) and is implicated in other membrane trafficking events (47). Future experiments will be required to more fully determine the role of these elements in the Ca\textsuperscript{2+} sensitive phase of c-kit internalization.
References


CHAPTER III

Phospholipase C-gamma Activation is Required for Cellular Stimulation by Membrane-Bound but not Soluble Steel Factor.
Introduction:

The results described in Chapter 2 highlight an important role for both PI3-kinase and Ca\(^{2+}\) influx in SLF-stimulated c-kit internalization. These results prompted an examination of the role of PI3-kinase and PLC-\(\gamma\) in SLF-stimulated mitogenic signals. In addition, the studies in Chapter 2 pose the interesting question of what is the functional relevance of SLF-stimulated c-Kit internalization. These questions will be addressed in this Chapter using different forms of the SLF ligand.

SLF is expressed on stromal cells or fibroblasts as either a membrane bound- or soluble form which is generated by chymase cleavage of the extracellular portion of the growth factor (1-3). Increasing evidence indicates that the two forms of the ligand stimulate qualitatively different responses. For example, the Steel-Dickie allele (St\(^{D}\)) expresses soluble SLF (sSLF) but not membrane-bound SLF (mSLF). Mice with this mutation exhibit hematopoietic, coat colour and germ cell defects suggesting that mSLF plays a unique biological role in these tissues (4). In vitro, mSLF but not sSLF supports long-term hematopoietic precursor growth by stromal cells (5-7) and erythropoietic development (8). In vivo, the concentration of sSLF in serum is such (3 ng/ml) that it is likely in a monomeric form, and thus poorly mitogenic (9). Therefore, mSLF is likely the dominant form of the ligand in vivo.

Although the biochemical basis for the differences between sSLF and mSLF are not completely understood, some signaling differences between the two ligand isoforms have been observed. Following ligand binding, the signal transduction cascade is initiated by the stimulation of receptor autophosphorylation on key tyrosine residues (10). In the case of sSLF, tyrosine phosphorylation of c-Kit is rapid (within minutes) followed by a decline in phosphorylation. This decline in phosphorylation coincides with receptor internalization.
and endocytosis, leading ultimately to receptor degradation. In contrast, phosphorylation of c-Kit by mSLF persists over much longer periods of time (11). This persistence in tyrosine phosphorylation was attributed to the enhanced stability of the c-Kit receptor on the cell surface following mSLF stimulation, likely due to the prevention of receptor internalization in this configuration.

Receptor autophosphorylation generates binding sites that recruit SH2-containing proteins to the receptor. For c-Kit, stimulation with sSLF results in recruitment of PI3-kinase, PLC-γ, and other signal transducing molecules (12). Activated PI3-kinase phosphorylates phosphatidylinositol lipids on the D3 position of the inositol ring (13). This enzyme and its phosphorylated lipid products have been implicated in a number of cellular responses including membrane ruffling (14, 15), chemotaxis (12, 16-18), adhesion to fibronectin (19) and receptor internalization (20-22) as well maintenance of cell survival and mitogenesis (23-25). Activation of PLC-γ stimulates the hydrolysis of PIP₂ to inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ promotes the mobilization of Ca²⁺ from internal stores followed by influx from the extracellular milieu while DAG activates protein kinase C (PKC) (26). A functional role for PLC-γ in a number of different systems has recently emerged [Reviewed in (27)]. PLC-γ activation was shown to be required for PDGF-stimulated mitogenesis and monocytic differentiation of myeloid progenitor FDC-P2 cells (28). In 3T3 fibroblasts, microinjection of a polypeptide corresponding to the SH2-SH2-SH3 domains of PLC-γ1 prevents endogenous PLC-γ1 binding to receptors and inhibits PDGF and EGF-mediated S-phase entry (29). In addition, a truncated form of c-Kit has been implicated in oocyte activation following fertilization, and this activity was inhibited by PLC-γ inhibitors or Ca²⁺ chelators (30). Furthermore, we have previously demonstrated that blockade of PLC-γ-stimulated Ca²⁺ influx reverses SLF-mediated survival signals in bone marrow-derived mast cells (31). Therefore, these two signaling
enzymes and their second messengers link receptor activation with a variety of distal responses.

Valius and Kazlauskas tested the functional relevance of PI3-kinase and PLC-γ activation using PDGF receptors where all the intrinsic tyrosine phosphorylation sites were mutated and then added back individually (32). They demonstrated that adding back either the PLC-γ binding site or the PI3-kinase binding site in the PDGFR is sufficient to mediate PDGF-stimulated mitogenesis (32). In addition, other studies have shown that cellular responses such as chemotaxis and adhesion require either PI3-kinase or PLC-γ (33-35). Therefore, despite their different biochemical activities, PLC-γ and PI3-kinase appear to stimulate overlapping cellular responses.

Given the apparent redundancy between PI3-kinase and PLC-γ, the objective of this study was to evaluate the requirement for activation of either of these enzymes following stimulation by soluble or membrane-bound SLF. Cells expressing receptors with either the PI3-kinase SH2-binding site or the PLC-γ SH2-binding site mutated in order to prevent recruitment of these signaling proteins (KitYF719 and KitYF728 receptors respectively) were tested for their ability to respond to sSLF, mSLF or plate-bound anti-c-Kit antibodies. I found that while both mutants responded equally to sSLF, KitYF728 expressing cells failed to respond to either mSLF or plate-bound anti-Kit antibodies. Additionally, I observed that a c-Kit isoform impaired for endocytosis also requires PLC-γ activation for mitogenic stimulation. These results therefore demonstrate that, while not required for stimulation by sSLF, PLC-γ activation is essential for mitogenic stimulation by mSLF, by an immobilized ligand such as plate-bound anti-c-Kit antibodies, or by receptors impaired for endocytosis.
Materials and Methods

Cell culture and Transfection

gp + a amphotrophic NIH 3T3 packaging cells (gift from Dr. R. Rottapel, Toronto, Canada) were grown in Dulbecco's modified Eagle's medium (Life Technologies Inc., Burlington, ON) supplemented with 10% fetal bovine serum (FBS) and antibiotics. 32D cells (gift from Dr. Joel Greenberger, Pittsburg, Pennsylvania) are an interleukin-3-dependent, c-kit negative myelomonocytic cell line (36). They were routinely grown in RPMI (Life Technologies) supplemented with 10% FBS and 2% supernatant from WEHI-3 cells as a source of interleukin-3 (IL-3). All cell cultures contained 55 μmol/L β-mercaptoethanol and antibiotics (both Sigma, Oakville, ON).

The cDNAs (obtained from Dr. R. Rottapel) for wild type (wt) murine c-kit or mutants in which either tyrosine 719 was replaced with phenylalanine (YF719) or tyrosine 728 was replaced with phenylalanine (YF728) as well as the Kit+ isoform sequence were cloned into LXSN retroviral expression vectors. These DNAs were digested with Nde I generating a 5.5 kb fragment containing the Kit+ vector and a 4.0 kb fragment containing each of the YF719 or YF728 mutations. The Kit+ vector was ligated with either YF719 or YF728 versions of the 4.0 kb fragment. The resulting plasmids were analyzed for proper orientation and correct sequence. The LXSN YF728 construct was used in order to generate a YF719/YF728 double mutant using a directed mutagenesis by PCR method. Briefly, synthetic primers containing both the YF719 mutation and an EcoRI site were combined with flanking primers and amplified to produce two fragments, overlapping in the region of the mutating primer. The two fragments were isolated and re-amplified with the flanking primers. The resulting 1 kb fragment was then TA cloned and verified by restriction digest followed by digestion with Apal and Sal I to produce a 630 bp fragment.
for subsequent ligation and subcloning into the LXSN Kit YF728 vector. Both junctions and mutations were verified by sequencing.

These vectors were transfected into gp + a NIH 3T3 packaging cells by lipofectamine treatment (Gibco, Grand Island, NY) and selected in 1 mg/ml G418 (also Gibco). 32D cells were co-cultured with a confluent layer of pooled, irradiated (20 Gy) gp + a transfectants for 24 hours. The non-adherent 32Ds were removed and cultured for an additional 2-5 days in IL-3 followed by selection for c-Kit positive cells in 1 mg/ml G418 and IL-3. c-Kit-positive 32D cells were further enriched by cell sorting (FacStar Plus, Becton Dickinson). Surface expression of c-Kit was detected using biotinylated SLF and streptavidin-conjugated phycoerythrin (Jackson, Westgrove PA) as described previously. Stable populations of c-Kit expressing 32D cells were then cloned by limiting dilution.

**Reagents.**

Recombinant murine soluble Steel Factor (SLF) was produced as described (22) and assessed for purity by silver staining and western blot. Bioactivity was always assayed on c-kit positive cells by 3H-thymidine incorporation. Anti-SLF antibodies used for flow cytometry were purchased from Genzyme (Cambridge, Mass). SLF used in internalization assays was conjugated with biotin as described (36). SLF used in the Ca2+ mobilization assay was obtained from Biosource (Camarillo, CA) and used at a concentration of 200 ng/ml.

**Western Blotting.**

5 x 10⁶ 32D cells of each non-infected, KitWT, KitYF719, KitYF728, Kit*WT, Kit*YF719 and Kit*YF728 constructs were starved overnight in RPMI + 0.5% FBS. Cells were then washed in FBS containing phosphate buffered saline (PBS-FBS) three times followed by stimulation with 500 ng/ml SLF for 2.5 minutes at 37°C. Cells were
immediately washed in ice-cold PBS-FBS then lysed in lysis buffer containing 1% NP-40, 50 mM Tris, 20 mM EDTA (pH 7.0) with the following inhibitors: 200 μM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin (all Sigma). Lysates were then spun at 10,000 rpm for 20 minutes and supernatants were incubated with 50 μl of a 20% Protein A slurry (Pharmacia Biotech Inc., Baie D'Urfe, Quebec) and 5 μl of Rb125, a polyclonal rabbit antibody raised against the intracellular portion of the c-Kit protein (gift from Dr. Herman Ziltener, Vancouver BC). Lysates were incubated for 2 hours at 4°C and the beads were washed three times in lysis buffer with inhibitors. Beads were resuspended in loading buffer with β2-mercaptoethanol and boiled for 5 minutes. Released proteins were resolved on a 7.5% acrylamide gel, transferred to nitrocellulose, and blocked in TBS plus 3% gelatin and 0.5% Tween 20. Blots were incubated with 4G10 anti-phosphotyrosine antibody (UBI, Lake Placid, NY) in TBS plus 1% gelatin and 0.5% Tween 20 at a dilution of 1:1000 for 1 hour followed by goat anti-mouse-conjugated horseradish peroxidase secondary antibodies (Jackson) at a dilution of 1:5,000 and visualized with chemiluminescence (NEN Life Science Products). Blots were stripped by acid treatment and re-probed with rabbit polyclonal anti-c-Kit antisera at a dilution of 1:500 or with rabbit polyclonal anti-p85 antisera followed by incubation with protein-A-horseradish peroxidase (Amersham Corp., Mississauga, ON) at a dilution of 1:30,000. Visualization was again by chemiluminescence.

**Calcium Mobilization Measurements:**

32D infectants were starved in RPMI with no FBS for 2 hours at 37°C, washed three times in Ca²⁺ mobilization buffer, (140 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂·2H₂O, 0.8 mM MgSO₄·7H₂O, 1 mM KH₂PO₄ and 10 mM glucose, pH 8) and then incubated with 10 μM Indo-1 AM ester dye (Molecular Probes, Eugene, OR) plus 0.03% pluronic acid (Molecular Probes) for 1 hour at 37°C. Cells were then washed in the same buffer at pH 7.4 and resuspended at a volume of 1 x 10⁷ cells/ml. Cells were kept at room temperature and for
Each Ca\(^{2+}\) measurement, were briefly warmed to 37°C and kept at this temperature for the duration of the 400 second run. SLF was added at a concentration of 200 ng/ml after 30 seconds and Ca\(^{2+}\) mobilization was monitored using flow cytometry by following the ratio of bound to unbound fluorophore over time.

\( ^{3}H\)-Thymidine incorporation assays:

32D infectants were washed three times in RPMI + 0.5% FBS and then plated at a density of 2.5 x 10⁴ cells per well in a 96 well flat bottom plate. Different concentrations of SLF were added to the wells. In the case of NIH3T3 co-culture assays, varying concentrations of NIH3T3 cells were added to a 96 well plate and incubated overnight in DMEM + 10% FBS. Cells were then irradiated at 20 Gy and 2.5 x 10⁴ 32D infectants were plated on the NIH3T3 monolayer. For the immobilized anti-c-kit antibody assays, 96 well plates were coated with 10 μg/ml of a mouse anti-rat monoclonal antibody (Sigma) for 1 hour at 37°C. Excess antibody was then washed from the plate followed by addition of varying concentrations of ACK-2 (gift from Dr. S. Nishikawa) in PBS/FBS (37). Plates were incubated for 1 hour at 37°C, washed several times and 2.5 x 10⁴ 32D infectants were added to each well. In all cases, after 18 hours, 1μCi of \(^{3}H\)-thymidine was added to each well for 6 hours. Cells were then harvested and incorporated radioactivity was determined by scintillation counting. Co-culture assays were plotted as a measure of stimulation with feeder cells over stimulation with media alone. Plate coated ACK-2 results were plotted as a measure of stimulation with both secondary and ACK-2 over stimulation with secondary alone. Similar results were obtained if results were plotted as stimulation with both secondary and ACK-2 over stimulation with ACK-2 alone.

Internalization Assay, Microscopy:

1 x 10⁵ cells of e-Kit and c-Kit\(^{+}\) 32D clones were resuspended in PBS/FBS and washed twice with the same solution. Biotinylated-SLF at a dilution of 1:800 and FITC-labeled
2B8 anti-kit antibody (Pharmingen) at a dilution of 1:200 were then added to the cells in PBS/FBS. Cells were incubated on ice for 40 to 60 minutes. Cells were then transferred to a 37°C water bath for a short incubation time and then subsequently placed back on ice. Cells were washed three times with ice-cold PBS-FBS then fixed with 3% paraformaldehyde for 10 minutes on ice. Following fixation, cells were washed three times with PBS/FBS. In some cases, cells were then labeled with an anti-FITC antibody tagged with Texas Red (TxR) (Molecular Probes) for 45 minutes on ice. For the determination of ligand/receptor co-localization, cells were permeabilized in 0.1% saponin for 10 minutes followed by three washes with PBS/FBS and incubation with streptavidin-Texas Red (Calbiochem, La Jolla, CA) for 45 minutes on ice. All cells were washed three times with PBS-FBS then resuspended in 5 μl of 90% glycerol containing 1,4 Diazo bicyclo-[2.2.2]-octane (DABCO purchased from Aldrich) which significantly reduces photobleaching (38). Cells were allowed to settle for 10 minutes on the microscope slide before applying a coverslip and nail polish. Incubation with glycerol was sufficient to immobilize the cells, while maintaining their three-dimensional structure relatively undistorted. Cells were observed by fluorescence microscope (Leitz DMR/BE) using a 100X oil-immersion objective with filters for both FITC and TxR. Photographs were always exposed for 90 seconds. When cells were photographed using both filters, the Texas-Red image was exposed first since it experienced greater photobleaching.

For the examination of internalization of the c-kit receptor when stimulated with plate-coated ACK-2 anti-c-kit antibodies, coverslips were coated with both mouse-anti-rat antibodies and ACK-2 as described above. In some cases, coverslips were incubated with only mouse-anti-rat antibodies. KitWT 32D cells were then added to coverslips for 30 minutes. In one case where coverslips were coated with mouse-anti-rat antibodies alone, KitWT 32D cells were incubated with sSLF. All samples were then fixed, saponized, and stained with 2B8 for 30 minutes.
Internalization Assay, Flow Cytometry:

1 x 10^6 cells of all 32D clones were resuspended in PBS/FBS and washed twice with the same solution. Biotinylated-SLF was then added to the cells in PBS/FBS at a concentration which allowed for maximal FACS staining - typically 1:100 to 1:50 dilution. Cells were incubated on ice with biotinylated-SLF for 60 minutes. Cells were incubated for 0, 5, 10, 15 and 20 minutes at 37°C and washed three times with ice cold PBS/FBS. Cells were then fixed with 3% paraformaldehyde for 10 minutes on ice. This fixation procedure stabilized the staining pattern for several hours with little disruption of the cellular architecture. Following fixation, cells were washed three times with PBS/FBS and then incubated with Streptavidin-PE for 30 minutes on ice. Cells were then washed twice with PBS/FBS and analyzed by flow cytometry for expression and internalization of c-kit.
Results

Expression of c-Kit on 32D cells.
To evaluate the role of PI3-kinase and PLC-γ activation by c-Kit following stimulation by sSLF or mSLF, WT and c-Kit receptors mutated in recruitment sites for these enzymes were transferred into 32D cells. 32D cells are a c-Kit negative, IL-3 dependent myelomonocytic cell line which have previously been shown to express high levels of c-Kit upon retroviral infection (36). 32D cells were infected with KitWT, KitYF719 and KitYF728 retroviral constructs, selected and sorted for c-Kit positive cells by FACS. Single clones were generated and tested for c-Kit expression by flow cytometry. As shown in Figure 1.A-D, 32D cells infected with either KitWT, KitYF719, KitYF728 or a double mutant, KitYF719/728 express detectable levels of c-Kit on their surface. The mean fluorescence intensities are similar within a factor of two indicating comparable levels of receptor on the cell surface of all infectants.

Tyrosine phosphorylation of 32D c-Kit and recruitment of p85.
The ability of the WT and mutant c-Kit receptors to undergo autophosphorylation upon ligand binding by exposing the infectants to sSLF, followed by immunoprecipitation with anti-Kit antisera and immunoblotting with anti-phosphotyrosine antibodies was evaluated. As shown in Figure 1.E, KitWT, KitYF719, KitYF728 were tyrosine phosphorylated upon stimulation with sSLF (middle panel). When this blot was stripped and re-probed with antibodies to the p85 regulatory domain of PI3-kinase, it was found that p85 co-immunoprecipitated with KitWT and KitYF728 receptors but not KitYF719 receptors. This is in accordance with previous results which demonstrated that Y719 is part of the consensus binding site for PI3-kinase (39). The amount of precipitated c-Kit (bottom panel) was found to be similar for all three infectants as determined by stripping and re-
Figure 1. Expression and tyrosine phosphorylation of 32D infectants.

32D cells were infected with the indicated c-kit constructs, selected, sorted and cloned. These clones were then measured for receptor levels by flow cytometry using biotinylated-SLF followed by Streptavidin-PE. Levels of c-kit receptor on Kitwt (A), KitYF719 (B), KitYF728 (C) and KitYF719/YF728 (D) cells was approximately equivalent as measured by the mean fluorescence intensity (MFI). Uninfected 32D cells are represented by the shaded histogram. (E): c-Kit receptors from Kitwt, KitYF719, KitYF728 and 32D cells were either not stimulated (-) or stimulated (+) with SLF for 2.5 minutes at 37°C. Receptors were then precipitated and resolved by 7.5% SDS-PAGE followed by transfer to nitrocellulose and blotting with anti-phosphotyrosine antibodies (middle panel). Blots were then stripped and re-probed with anti-p85 antibodies (upper panel). Equal levels of Kit were precipitated in each sample as evidenced by stripping and re-probing with anti-c-kit antibodies (bottom panel).
Figure 1.
Figure 1E.

**IP: Kit Blot: p85**

<table>
<thead>
<tr>
<th>KitWT</th>
<th>KIT Y719</th>
<th>KIT Y728</th>
<th>32D</th>
<th>p85</th>
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<tr>
<td>SLF</td>
<td>-</td>
<td>+</td>
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**IP: Kit Blot: pTyr**

**IP: Kit Blot: Kit**
probing the blot with anti-c-Kit antibodies (Rb 125). I failed to observe
immunoprecipitated c-Kit protein or tyrosine phosphorylation in uninfected 32D cells.
Therefore, all three of these infectants demonstrate kinase activity as exhibited by tyrosine
phosphorylation upon stimulation with sSLF, and only KitWT and KitYF728 receptors co-
immunoprecipitate p85.

KitYF728 receptors do not induce SLF-stimulated Ca2+ mobilization.
It has already been demonstrated that c-Kit activates PLC-γ (40). Residue Y728 in c-Kit is
part of a short sequence of amino acids which closely matches the consensus sequence
identified for the PLC-γ SH2 binding domain (41-43). To confirm the requirement for
Y728 in activating PLC-γ, Ca2+ mobilization, a downstream consequence of PLC-γ
activation, was followed after stimulation of cells expressing WT and mutant receptors with
sSLF (26). KitWT, KitYF719, KitYF728 and uninfected 32D cells were loaded with
INDO-1, a Ca2+-sensitive fluorophore, at 37°C. Cells were then stimulated with SLF and
analysed for Ca2+ mobilization by flow cytometry. In the case of either KitWT or
KitYF719 (Figure 2.A & B), a modest increase in intracellular Ca2+ is observed following
stimulation with sSLF. This increase is comparable to that observed by other groups (44).
Neither uninfected 32D cells nor KitYF728 32D cells responded to sSLF by mobilizing
Ca2+. These results are consistent with an inability of the YF728 mutant to activate the
PLC-γ enzyme.

KitWT, KitYF719 and KitYF728 but not KitYF719/YF728 32D cells respond to sSLF.
Both PI3-kinase and PLC-γ have been implicated in growth factor receptor-mediated
mitogenesis (23, 28, 24, 27, 25, 29) and we have previously demonstrated that PLC-γ
second messengers such as Ca2+ influx are critical for SLF-dependent cell survival (31).
The effect of the KitYF719 and KitYF728 mutations on SLF-stimulated mitogenesis by
Figure 2. SLF-stimulated mobilization of Ca$^{2+}$ in 32D infectants.

32D cells infected with Kitwt (A), KitYF719 (B) or KitYF728 (C) or uninfected (D) were loaded with 10μM INDO-1 for 1 hour at 37°C followed by analysis by FACStar plus for Ca$^{2+}$ mobilization. 200ng/ml SLF was added after 30 seconds to INDO-1 loaded cells. Ca$^{2+}$ mobilization was followed over time as a ratio of bound Ca$^{2+}$ signal / unbound Ca$^{2+}$ signal.
Figure 2.
incubating KitWT, KitYF719 and KitYF728 expressing 32D cells with varying concentrations of sSLF was determined. In addition to these three cell types, 32D cells expressing the KitYF719/YF728 double mutant were also tested. As shown in Figure 3, the three cell lines KitWT, KitYF719 and KitYF728 32D responded equally well to sSLF (empty circles, closed circles and empty squares respectively). In contrast, the KitYF719/YF728 double mutant 32D cell line (closed squares) failed to respond to sSLF exhibiting counts that were not significantly different than those obtained with the uninfected 32D c-kit negative cells (crosses). These data are therefore consistent with the requirement for either PI3-kinase or PLC-γ activation for a mitogenic signal. These results are also in agreement with those of Valius et. al. who demonstrated that either the PI3-kinase or the PLC-γ binding sites were sufficient to restore PDGF-mediated mitogenesis, but that receptors bearing mutations at both of these sites were mitogenically inert (32).

CytWt and KitYF719 receptors but not KitYF728 receptors respond to mSLF.

The observation that either PI3-kinase or PLC-γ activation is sufficient for sSLF-stimulated mitogenesis of c-Kit positive cells prompted an examination of whether this was also the case for mSLF. NIH3T3 fibroblasts were used as a source of mSLF (45). As shown in Figure 4.A, these cells express mSLF on their surface. NIH3T3 cells were irradiated, plated at varying concentrations and then co-cultured with KitWT, KitYF719, KitYF728 or uninfected 32D cells. As shown in Figure 4.B, both KitWT and KitYF719 32D infectants are stimulated by co-culture with NIH3T3 cells, although the density of feeder cells required for peak stimulation of the two cell lines differs, perhaps due to variations in c-Kit expression between the two cell lines. This same pattern of stimulation has been observed in a number of experiments using other mSLF expressing cell lines as well (not shown). In contrast, the KitYF728 32D infectants failed to be stimulated by NIH3T3 cells above that seen for c-Kit negative 32D founder cells. At the highest concentration of NIH3T3
Figure 3. $^3$H-Thymidine incorporation of SLF stimulated 32D infectants.

32D cells infected with Kitwt (open circles), KitYF719 (closed circles), KitYF728 (open squares), KitYF719/YF728 (closed squares) or uninfected (crosses) were incubated with various concentrations of sSLF for 18 hours at 37°C followed by a 6 hour $^3$H-thymidine pulse. Cells were then harvested and incorporated counts were determined by scintillation counting. Error bars represent the standard error determined from triplicate measurements.
Figure 3.
Figure 4. Stimulation of 32D infectants with mSLF on NIH3T3 cells. (A): NIH3T3 cells were measured for mSLF levels by incubation with a rabbit anti-SLF polyclonal antibody followed by a FITC-conjugated anti-rabbit secondary antibody. The shaded histogram represents NIH3T3 cells which were incubated with an isotype control. (B): 32D infectants were then co-cultured with various concentrations of NIH3T3 feeder cells. Kitwt (filled), KitYF719 (empty), KitYF728 (shaded) and 32D uninfected cells (hatched) were incubated with NIH3T3 cells for 18 hours followed by a 6 hour ³H-thymidine pulse. Cells were then harvested and incorporated counts were determined by scintillation counting. Error bars represent the standard error determined from triplicate measurements.
Figure 4.

A

B

Relative Cell Number

Fold Stimulation

# NIH3T3 feeder cells
cells some background stimulation of both KitYF728 and uninfected 32D cells was observed. This may be due to the secretion of soluble factors by this higher density of feeder cells. This experiment revealed that while KitYF728 is capable of being stimulated by sSLF as measured by tyrosine phosphorylation or \(^{3}H\)-thymidine incorporation, 32D KitYF728 cells are unable to be stimulated by mSLF on NIH3T3 cells. This indicates that PLC-γ activation may be critical for responding to the membrane-bound isoform of SLF.

**KitWT and KitYF719 receptors but not KitYF728 receptors respond to plate-bound anti-c-Kit antibodies.**

An alternate method of stimulation that has been reported to reproduce the stimulus by mSLF is through the use of plate-bound c-Kit-specific antibodies (42, 46). I therefore used this form of stimulation, which is not complicated by the presence of soluble SLF in the supernatant, to confirm the results obtained with fibroblast-associated SLF. I found that c-Kit positive 32D cells failed to be stimulated by rat anti-mouse c-Kit-specific monoclonal antibody ACK-2 alone or mouse-anti-rat IgG alone (data not shown).

However, when both plate-bound anti-rat antibodies, followed by ACK-2 were used as the stimulus, both KitWT and KitYF719 32D cells responded to the plate-bound antibodies in a dose-dependent fashion (Figure 5.). In contrast, neither the KitYF728 nor the uninfected 32D founder cell line responded to this stimulus. Therefore, although KitYF728 32D cells respond to sSLF, they fail to respond to plate bound anti-kit antibodies. Given the fact that plate-bound anti-c-kit antibodies have been shown to mimic mSLF (46), this experiment is consistent with a requirement for PLC-γ activation following stimulation with this form of ligand.

**Immobilized anti-Kit antibodies fail to stimulate c-Kit receptor internalization.**

It has previously been reported that stimulation with either mSLF or plate-bound anti-c-Kit
Figure 5. Stimulation of 32D infectants with plate-bound anti-c-kit antibodies.

96 well plates were coated with 10 μg/ml mouse-anti-Rat antibodies for 1 hour at 37°C followed by coating with various concentrations of anti-c-kit antibodies (ACK-2). Kitwt (filled), KitYF719 (empty), KitYF728 (shaded) and 32D uninfected cells (hatched) were then added to coated plates for 18 hours followed by a 6 hour ³H-thymidine pulse. Cells were then harvested and incorporated counts were determined by scintillation counting. Stimulation of 32D cells with both ACK-2 and secondary antibodies was measured as fold stimulation over counts obtained from stimulation with secondary antibodies alone. Similar results were obtained if fold stimulation was measured over counts obtained from ACK-2 alone. Error bars represent the standard error determined from triplicate measurements.
Figure 5.
antibodies enhances the stability of the c-Kit receptor (19, 54), possibly because these stimuli interfere with receptor internalization. Therefore established by fluorescence microscopy that the plate-bound anti-c-kit antibody stimulus prevented receptor internalization. KitWT 32D cells were incubated on coverslips coated first with anti-rat antibodies and then ACK-2 antibodies for 30 minutes at 37°C. Control cultures with anti-rat antibodies alone plus sSLF were also initiated. In each case, cells were fixed with paraformaldehyde followed by permeabilization with saponin and labeling with FITC-conjugated anti-c-kit antibodies (2B8). As shown in Figure 6., cells stimulated with sSLF for 30 minutes at 37°C on anti-rat antibody-coated coverslips display a punctate distribution of the receptor which is internal to the cell. In contrast, cells stimulated with coverslip-bound ACK-2 display a more uniform distribution of c-Kit with some surface aggregation. In the case of 32D KitWT cells incubated with coverslip-coated anti-rat antibodies alone, a uniform surface anti-Kit staining is observed with minimal internal aggregates (perhaps newly synthesized Kit protein) indicating that the c-Kit receptors are not internalized. Given that the staining patterns observed with anti-rat antibodies and ACK-2 versus anti-rat alone are nearly identical, it can be concluded that this form of stimulus fails to induce receptor internalization.

Expression of Kit*, Kit*YF719 and Kit*YF728 on 32D cells.

These results demonstrate that Kit*YF728 receptors fail to stimulate cells in response to either mSLF or plate-bound anti-c-Kit antibodies. Since these two stimuli likely do not induce effective receptor internalization, these data suggest that interference with receptor internalization may inhibit some mitogenic signals. Further investigated this hypothesis by making use of an endocytosis-defective c-Kit variant. A splice variant isoform of the c-Kit receptor, designated Kit+ has been described. The Kit+ isoform carries a 4 amino acid insertion in the extracellular domain of the receptor just N-terminal to the transmembrane region (12). We and others have observed that unlike the KitWT isoform, Kit+WT
Figure 6. Evaluation of internalization stimulated by either plate-bound ACK-2 or sSLF.

Kitwt 32D cells were plated on coverslips with either secondary alone plus sSLF (left panel), ACK-2 plus secondary (middle panel) or secondary alone (right panel). Kitwt 32D cells were stimulated for 30 minutes at 37°C followed by fixing with paraformaldehyde and permeabilization with saponin. Cells were then incubated with FITC-conjugated anti-c-kit antibodies (2B8) for 30 minutes and visualized by fluorescence microscopy.
Figure 6.
receptors appear to be retained in an early endocytic fraction of the cell. We introduced the YF719 and YF728 mutations onto the Kit+ isoform, and expressed the Kit+WT, Kit+YF719 and Kit+YF728 constructs in 32D cells. Figures 7.A-C show flow cytometric histograms of Kit+WT, Kit+YF719 and Kit+YF728 32D cells respectively which were stained with biotinylated-SLF and Streptavidin-PE demonstrating similar levels of c-Kit expression on their surfaces.

Tyrosine phosphorylation of 32D c-kit+ and recruitment of p85.

The three Kit+ variants were tested for their ability to become tyrosine phosphorylated and to recruit the p85 subunit of PI3-kinase upon stimulation with SLF. As illustrated in the middle panel of Figure 7.D, I observed that all three Kit+ variants become tyrosine phosphorylated upon incubation with sSLF (this experiment was performed on the same day as the blots in Figure 1E). The phosphotyrosine blot was then stripped and re-probed with anti-p85 antibodies. As expected, Kit+WT 32D cells were able to recruit p85, but Kit+YF719 32D cells failed to recruit p85 upon SLF induction (top panel). This result is consistent with that described by Reith et. al. (12). In the case of Kit+YF728, I observed modest induction of p85 association, but never to the same degree as Kit+WT, KitWT or KitYF728 32D cells. The amount of precipitated c-kit (bottom panel) was found to be equal for all three infectants by stripping and re-probing the blot with anti-kit antibodies. Therefore, all three of these infectants demonstrate kinase activity as exhibited by tyrosine phosphorylation upon stimulation with sSLF.

32D Kit+ cells exhibit an altered endocytic pattern and a limited capacity for internalizing c-kit receptors from the cell surface.

As mentioned above and illustrated in Figure 8., we have observed that the Kit+ isoform exhibits an altered endocytic routing compared to Kit in response to SLF. KitWT and
Figure 7. Expression and tyrosine phosphorylation of 32D infectants on the Kit+ isoform.

32D cells were infected with c-Kit+ c-kit constructs, selected, sorted and cloned. These clones were then measured for receptor levels by flow cytometry using biotinylated-SLF and Streptavidin-PE. Levels of c-kit receptor on Kit+wt (A), Kit+YF719 (B) and Kit+YF728 (C) cells was approximately equivalent as measured by the mean fluorescence intensity (MFI). (D): c-Kit receptors from Kit+wt, Kit+YF719, Kit+YF728 and 32D cells were either not stimulated (-) or stimulated (+) with SLF for 2.5 minutes at 37°C.

Receptors were then precipitated and resolved by 7.5% SDS-PAGE followed by transfer to nitrocellulose and blotting with anti-phosphotyrosine antibodies (middle panel). Blots were then stripped and re-probed with anti-p85 antibodies (upper panel). Equal levels of kit were precipitated in each sample as evidenced by stripping and re-probing with anti-c-kit antibodies (bottom panel).
Figure 7.

A

MFI = 136

B

MFI = 74

C

MFI = 119

Relative Cell Number

b-SLF
Figure 7D.

IP: anti-kit  Blot: anti-p85
Kit+WT  Kit+YF719  Kit+YF728  32D  p85

SLF  -  +  +  -  +  -  +  IP

p85

IP: anti-kit  Blot: anti-pTyr

Kit

IP: anti-kit  Blot: anti-kit

Kit
Figure 8. SLF-stimulated c-kit internalization of Kitwt and Kitwt 32D variants. Kitwt and Kitwt 32D infected cells were incubated with biotinylated-SLF and FITC-conjugated anti-c-kit antibodies (2B8) for 1 hour at 4°C followed by a 15 minute incubation at 37°C. Cells were then fixed with paraformaldehyde. In the case of the left and middle panels for Kitwt (A) and Kitwt (B) infectants, cells were then incubated with TxR-conjugated anti-FITC antibodies in order to detect receptors still remaining on the cell surface. In the case of the right panels for both Kitwt (A) and Kitwt (B) variants, cells were permeabilized with saponin followed by incubation with Streptavidin-TxR. All samples were resuspended in glycerol solution, plated on microscope slides and sealed with coverslips before visualizing by fluorescence microscopy. (C): 32D infectants were measured by flow cytometry for receptor internalization. Kitwt (closed squares), Kitwt (open squares), Kit*YF719 (closed circles) and Kit*YF728 (open circles) were incubated with biotinylated-SLF for 1 hour at 4°C followed by incubations at 37°C for 0, 5, 10, 15 and 20 minutes. Cells were then incubated with Streptavidin-PE and measured by flow cytometry. Receptor internalization was measured as loss of fluorescence signal over time. Error bars represent the standard error determined from triplicate measurements.
Figure 8A.

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Figure 8B.
Figure 8. C

![Graph](image_url)
Kit+WT 32D infectants were incubated with sSLF and FITC-conjugated anti-c-Kit antibodies (2B8) at 4°C. This was followed by a 15 minute incubation at 37°C to allow for receptor internalization. Cells (not permeabilized) were then washed, fixed, and stained with a Texas Red labeled anti-FITC antibody for 30 minutes in order to differentiate internalized receptors from receptors remaining on the surface of the cell. As shown in Figure 8.A, KitWT 32D cells exhibit a uniform pattern of staining with both FITC and TxR stains indicating that c-Kit receptors are distributed evenly on the surface of the cell. After a 15 minute incubation with biotinylated-SLF at 37°C, the FITC stain is found inside the cell in large aggregates and the TxR stain, which stains only receptor left on the cell surface, disappears, consistent with receptor internalization and endocytosis.

In order to demonstrate that the receptor and its ligand are being internalized together, cells were incubated with biotinylated-SLF and FITC-conjugated anti-c-Kit antibodies, incubated for 15 minutes at 37°C, washed fixed, permeabilized and stained with Streptavidin-TxR. In this case, the FITC and TxR stains are co-localized, indicating that both c-Kit and SLF are co-internalizing.

Kit+WT 32D cells were subjected to the same analysis and the results are shown in Figure 8.B. As with KitWT, Kit+WT receptors are internalized following a 15 minute incubation with sSLF at 37°C. However, we observed that the internalized Kit+WT receptors are localized close to the plasma membrane. This indicates that Kit+WT receptors may be impaired in their ability to undergo endocytosis compared to KitWT receptors.

The kinetics of internalization for the various receptors were then assessed. KitWT, Kit+WT, Kit+YF719 and Kit+YF728 cells were labeled with biotinylated-SLF and incubated at 37°C for 0, 5, 10, 15 and 20 minutes. Cells were then labeled with Streptavidin-PE and the remaining fluorescence on the surface of cells was measured by
flow cytometry. As shown in Figure 8.C, KitWT 32D cells rapidly internalize c-Kit receptors after incubation with biotinylated-SLF (closed squares). Kit*WT and Kit*YF719 32D also internalize their c-Kit receptors (empty squares and closed circles respectively), however the total amount of receptor left on the surface remain significantly elevated, possibly due to saturation of an early endocytic compartment. In contrast, the Kit*YF728 32D cells fail to internalize their c-Kit receptors at all (empty circles). Therefore the unidentified impairment in Kit*WT endocytosis cooperates with the PLC-γ binding mutation to completely abrogate receptor internalization.

Kit+ and Kit+ YF719 receptors respond sub-mitogenically to sSLF whereas Kit+ YF728 receptors completely fail to respond to sSLF.

In order to determine if the altered intracellular distribution of the Kit+WT isoform observed after sSLF-stimulated internalization affects mitogenesis, Kit+WT, Kit+YF719 and Kit+YF728 32D cell lines were tested for their ability to respond to sSLF. As shown in Figure 9., the Kit+WT and Kit+YF719 expressing 32D cells (open squares and closed circles respectively) respond sub-mitogenically to sSLF as compared to KitWT 32D cells (Closed squares. This experiment was performed on the same day as the experiment shown in Figure 3). Strikingly, Kit+YF728 32D cells completely fail to respond to sSLF. This result therefore provides an additional correlation indicating a requirement for PLC-γ activation following stimulation in circumstances where c-Kit receptor internalization or endocytosis is impaired.
Figure 9. 3H-Thymidine incorporation of SLF stimulated 32D Kit+ infectants.

32D cells infected with Kitwt (closed squares), Kit+wt (open squares), Kit+YF719 (closed circles) or Kit+YF728 (open circles), were incubated with various concentrations of sSLF for 18 hours at 37°C followed by a 6 hour 3H-thymidine pulse. Cells were then harvested and scintillation counted. Error bars represent the standard error determined from triplicate measurements.
Figure 9.
Discussion

In this chapter, I have investigated the role of two c-Kit associated signaling molecules, PI3-kinase and PLC-γ, in mitogenic stimulation by soluble and membrane-bound SLF.
We found that KitWT, and the mutants KitYF719 and KitYF728 transmitted equivalent mitogenic signals in response to sSLF. In contrast, KitYF719/YPF728, a receptor with both PI3-kinase and PLC-γ binding sites mutated, failed to respond to sSLF. This observation indicates that either PI3-kinase or PLC-γ activation (but not both) is required for a mitogenic response to sSLF. Since Valius et al. observed a similar dependence for stimulation through the PDGF receptor, a requirement for either PI3-kinase or PLC-γ activation may be a common characteristic of mitogenic stimulation by other growth factor receptors as well.

The ability of these c-Kit mutants to transmit mitogenic signals in response to membrane-bound SLF was also examined. I observed that while both KitWT and KitYF719 transmitted mitogenic signals in response to mSLF, KitYF728, the mutant that does not activate PLC-γ, failed to transmit a mitogenic signal. A similar response by these receptors was also observed when immobilized anti-c-Kit antibodies were used as the stimulus.
These data therefore indicate that while PLC-γ activation is not absolutely required in response to sSLF, it is essential when the stimulus is mSLF or an immobilized ligand.
Since mSLF is likely the dominant physiological form of the c-Kit ligand (4, 47, 8), these results indicate that PLC-γ activation may play a unique and indispensable role in the stimulation of mitogenesis by SLF in vivo. In light of these results, it is of interest that the Wv allele produces a c-Kit receptor that is unable to activate PLC-γ but retains the ability to activate PI3-kinase (48). Thus, some of the phenotypic defects observed in the Wv mouse may reflect the importance of PLC-γ activation in vivo.
It has been observed that stimulation with either mSLF or plate-bound anti-c-Kit antibodies leads to enhanced receptor stability compared with stimulation by sSLF (11, 46). This increased stability is likely due to interference with c-Kit receptor internalization and endocytosis by these stimuli. Indeed, I confirmed that stimulation of KitWT 32D cells with plate-bound anti-c-Kit antibodies prevents internalization of the c-Kit receptors (Figure 6). Our results therefore raise the possibility that in the absence of PLC-γ activation, receptor internalization and endocytosis may be required in order to stimulate mitogenesis. This hypothesis was investigated further by evaluating the ability of sSLF to stimulate mitogenesis through endocytosis-defective Kit+WT and mutant receptors. I found that while both Kit+WT and Kit+YF719 transmitted mitogenic signals in response to sSLF, Kit+YF728 failed to respond to sSLF. Since Kit+YF728 receptors do not internalize in response to sSLF, this result provides a further correlation indicating that in the absence of PLC-γ activation, receptor internalization may be required for mitogenesis. The fact that the Kit+WT and Kit+YF719 receptors are partially impaired for endocytosis and transmit sub-mitogenic signals compared to KitWT in response to sSLF further supports this inverse correlation between internalization and mitogenesis.

The biochemical basis for the impairment in Kit+ endocytosis remains unknown. However, we have noted that following sSLF stimulation, the intracellular distribution of Kit+WT receptors bears a strong resemblance to the distribution of sSLF-stimulated c-Kit receptors in circumstances where PI3-kinase is not activated (22) (and Chapter 2). It is therefore possible that the Kit+ isoform fails to fully activate PI3-kinase-dependent signaling. Nonetheless, we and others have observed that Kit+WT receptors recruit PI3-kinase following sSLF stimulation (12). Therefore signaling elements downstream of PI3-kinase may be impaired. An inability of Kit+ receptors to fully activate PI3-kinase signaling would also be consistent with the the non-mitogenic phenotype of Kit+YF728 32D cells, given the requirement for either PLC-γ or PI3-kinase activation in response to
sSLF (Figure 3). Future work will address whether full activation of PI3-kinase-dependent signaling requires either receptor internalization or further endocytic steps.

Ligand-stimulated receptor internalization is thought to be a negative regulatory mechanism whereby activated receptors are cleared from the cell surface and ultimately delivered into lysozomal compartments for degradation (49). In agreement with this concept, ligand-induced transformation has been observed by a non-internalizing EGF receptor (50). It has also been found that cells which express a dominant negative form of dynamin, a protein which is essential for receptor internalization, fail to stimulate EGF receptor internalization and exhibit enhanced EGF-stimulated mitogenesis (51). There are other examples however, where inhibition of receptor internalization results in loss of mitogenesis. For example, anti-IL-2 receptor antibodies that prevent receptor internalization have been found to be inhibitory for IL-2 stimulated mitogenesis (52, 53). Furthermore, Falk et. al. characterized two EL4 cell variants and found that one variant which failed to respond to IL-1 correspondingly failed to internalize its IL-1 receptors upon binding this cytokine (54). The observations on the importance of PLC-γ activation in response to immobilized ligand and the possible modulation of PI3-kinase signaling by receptor endocytosis may therefore clarify the relationship between receptor trafficking and mitogenesis.
References


CHAPTER IV

Protection from Apoptosis by SLF but not IL-3 is Reversed through Blockade of Calcium Influx

Portions of this chapter have been published in


(American Society of Hematology)
Introduction:

The results described in Chapter 3 identify a role for PLC-γ in c-Kit responses to mSLF. These results, in addition to the role identified for Ca^{2+} influx in c-kit internalization as described in Chapter 2 prompted an examination of the role of both PLC-γ and PLC-γ-mediated Ca^{2+} influx in SLF-mediated survival signals. Survival signals such as those provided by SLF as well as a variety of cytokines and growth factors, protect hematopoietic cells and mast cells from cell death. In the absence of these protective signals, cells undergo a series of morphological and biochemical changes including membrane blebbing, nuclear condensation, cell shrinkage, and DNA fragmentation (1), culminating within 12 to 48 hours in apoptotic or programmed cell death.

One documented mechanism for the protection of cells from apoptosis is through the up-regulation of bcl-2. The bcl-2 family of proteins are key regulators of apoptotic death (2) consisting of both anti-apoptotic and pro-apoptotic members. Although the precise mechanism by which bcl-2 family members influence apoptosis is unknown, recent structural and biochemical evidence indicates that bcl-2 proteins perform multiple functions which may influence cell death pathways. These include physical interactions with numerous cytoplasmic proteins, formation of ion channels, and regulation of the permeability transition in mitochondria (3).

For hematopoietic stem cells, myeloid progenitor cells and mast cells, two factors that protect cells from apoptosis are Steel Factor (SLF), the ligand for the c-kit receptor tyrosine kinase and interleukin-3 (IL-3) (4-7). Up-regulation of bcl-2 by IL-3 in myeloid cells has been well documented (8, 9, 1). In contrast, SLF has only been observed to up-regulate bcl-2 in natural killer cells (10) indicating that the mechanism by which SLF protects myeloid cells from apoptosis may differ from that of IL-3.
One biochemical change that has been associated with the induction of apoptosis in a number of cell types is the deregulation of intracellular Ca^{2+} concentrations (11, 12). A general model explaining the role of Ca^{2+} in apoptosis however, remains elusive.

Excessive intracellular Ca^{2+} levels such as those induced by Ca^{2+} ionophore, have been shown to induce apoptosis in a number of experimental systems (13, 14). Apoptosis in splenocytes appears to involve a Ca^{2+}-dependent endonuclease (15) and intracellular Ca^{2+} increases have been linked to apoptosis of both activated T cell hybridomas (16) and immature thymocytes (17). In contrast to these observations, some cells seem to be protected from apoptosis by Ca^{2+} influx. For instance, IL-3 dependent mast cells and cell lines are protected from growth factor withdrawal-mediated apoptosis by addition of Ca^{2+} ionophore (18), and programmed neuronal death is also suppressed by increases in intracellular Ca^{2+} (19).

Given the importance of intracellular Ca^{2+} in the apoptotic process, the effect of inhibitors of Ca^{2+} influx on cell survival promoted by SLF and IL-3 has been investigated. This study demonstrates that blockade of Ca^{2+} influx reverses the ability of SLF to protect cells from apoptosis, but does not affect cell viability promoted by IL-3. Notably, in the presence of Ca^{2+} influx blockers, higher concentrations of SLF induce greater levels of cell death, indicating that this form of apoptosis is dependent on cellular stimulation. SLF, unlike IL-3, stimulates the mobilization of Ca^{2+} from internal stores followed by influx of Ca^{2+} from the extracellular milieu (20) and this Ca^{2+} mobilization is accomplished by activation of the PLC-γ enzyme. Stimulation of mast cells with other PLC activators such as IgE plus Ag or Substance P in the presence of Ca^{2+} channel blockers, also induces apoptosis. Furthermore, incubation of KitYF728 32D cells (which do not activate PLC-γ) with SLF and Ca^{2+} channel blockers or incubation of 32D-kit cells in the presence of PLC-γ inhibitors with SLF and Ca^{2+} channel blockers fails to induce apoptosis. Taken
together, this suggests that apoptosis induced by SLF plus Ca\textsuperscript{2+} channel blockers is dependent on PLC-\(\gamma\) mediated signals. I also show that overexpression of bel-2 does not protect cells from the combination of SLF plus Ca\textsuperscript{2+} influx blockers, but caspase inhibitors provide significant protection. These results therefore demonstrate a role for Ca\textsuperscript{2+} influx in SLF-mediated protection from cell death and identify a new mechanism for inducing caspase-mediated apoptosis through the combination of a PLC-activating growth signals with blockade of Ca\textsuperscript{2+} influx.
Materials and Methods:

Cells culture and transfection.
32D-kit cells (from Dr. Mark Minden, Toronto) are an IL-3-dependent myelomonocytic cell line expressing c-kit (21). 32D-kit cells were grown in RPMI supplemented with 10% heat-inactivated FBS, 2% WEHI-3 supernatant, and 1 mg/ml G418 (Gibco). Bone marrow derived mast cells (BMMC) were generated as described previously (22). They were cultured in OPTI-MEM (Gibco) supplemented with 10% heat-inactivated FBS and 2% WEHI-3 supernatant as a source of IL-3. The P815 cell line is a murine mastocytoma (23). P815 cells were grown in RPMI supplemented with 10% heat-inactivated FBS. Bcl-2 gp+e retroviral producing NIH 3T3 packaging cells (gift from Dr. Y. Ben-David, Toronto) contain an LXSN-based retroviral vector expressing genes for both puromycin resistance and murine bcl-2. These were grown in DMEM and supplemented with 10% FBS and 2 μg/ml puromycin (Sigma). 32D-kit-bcl-2 cells were generated by co-culturing 32D-kit cells with packaging cells for 24 hours. Non-adherent 32D-kit cells were then removed, cultured for 48 hours and then selected for bcl-2 over-expressing cells in the presence of 2 μg/ml puromycin. YF728 32D cells were generated as described in Chapter 3. All cell cultures also contained 55 μM β-mercaptoethanol and antibiotics (both Sigma).

Production of recombinant SLF.
As described in Chapter 2.

Other reagents.
Ca²⁺ channel blockers, ionomycin, oleic and elaidic acid and Substance P were obtained from Sigma. Oleic and elaidic acids were stored in de-gassed ethanolic solutions at concentrations of 1M and 100mM respectively and stored at -20°C. Monoclonal murine dinitrophenyl (DNP) - specific IgE, clone SPE-7 and albumin - human DNP-HSA antigen
were also obtained from Sigma. YVAD-CHO ICE protease inhibitor peptide was obtained from Amersham. DEVD-FMK and Boc-Asp-FMK were both obtained from Enzyme Systems.

Cell death assays.
2.5 $\times$ 10⁴ BMCCs or 32D-kit cells were placed in 96-well flat bottom plates in a volume of 0.1 ml RPMI containing 0.5% FBS. Cells were supplemented with either SLF, IL-3 or Substance P plus $\text{Ca}^{2+}$ channel blocker. In the case of adding IgE, cells were incubated with 10 $\mu$g/ml SPE-7 for 45 minutes at 4°C followed by 3 washes with RPMI, 0.5% FBS before plating in 96 well plates and adding 100 ng/ml DNP-OVA. The proportion of dead cells was determined after 18 or 24 hours in culture by counting cells that could or could not exclude Trypan blue.

Semi-solid agar assays.
Cell death assays were plated as described above. Following an 18 hour incubation, cells were washed once in RPMI containing 10% FBS and plated in 35mm petri dishes along with 1mg/ml G418, 25% WEHI-3 supernatant and 0.3% agar (Gibco). Colonies of 40 or more cells were counted after 7 days at 37°C.

Analysis of DNA content.
1.25 $\times$ 10⁶ cells were incubated for 18 or 24 hours as described above. Cells were spun down and resuspended in Vindelov's reagent: 3.4mM Tris (pH 8), 75 $\mu$M Propidium Iodide (from Sigma), 0.1% NP-40, 700 u/l RNAse (Sigma) and 10 mM NaCl. Cells were then analyzed by flow cytometry.
**Western Blotting.**

For the Bcl-2 blot, 1 X 10^6 32D-kit and 32D-kit-bcl-2 cells were washed in PBS, resuspended in TBS lysis buffer containing 1% NP-40, 10% glycerol, protease inhibitors (500 μM sodium-orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF (all Sigma)), and incubated at 4°C for 20 minutes. Lysates were centrifuged at 12,000 rpm for 10 minutes, the supernatant was separated by 12% SDS-PAGE and transferred to nitrocellulose. The blot was blocked with 5% skim milk powder, 0.1% TWEEN-20 in PBS, probed with anti-bcl-2 antibodies (UBI) followed by a horseradish peroxidase labeled Goat anti-Mouse secondary antibody (Jackson) and developed with chemiluminescence reagents (Amersham). In order to confirm equal protein loading, the blot was stripped by acid treatment and re-probed with an anti-actin monoclonal antibody (Sigma) followed by the same horseradish peroxidase labeled Goat anti-Mouse secondary antibody (Jackson) and developed with chemiluminescence reagents.

For the PARP cleavage blot, 2 X 10^6 32D-kit cells were pre-treated with combinations of 10μM econazole, SLF, IL-3 and Boc-Asp-FMK. Cells were washed in PBS, resuspended in a hypotonic lysis buffer (20mM Hepes, 10mM KCl, 1mM EDTA and 1mM DTT) with protease inhibitors 1μg/ml both aprotinin and leupeptin and 1mM PMSF (all Sigma) and left at 4°C for 5 minutes before adding 0.1% NP40. Lysates were centrifuged at 6,000 rpm for 5 minutes at 4°C. The supernatant was removed and centrifuged again at 10,000 rpm for 10 minutes at 4°C. The nuclear pellets were re-suspended in sample buffer containing 10% β-mercaptoethanol and 8M Urea, boiled and sonicated for 10 seconds at a setting of 50%, resolved by 7.5% SDS-PAGE and transferred to nitrocellulose. The blot was probed and developed as described above using a rabbit polyclonal anti-PARP antibody (UBI) which was raised against a synthetic peptide corresponding to the region C-terminal to the cleavage site of human PARP. The blot was then probed with a Goat-anti-
Rabbit horse radish peroxidase labeled secondary antibody (Jackson) and then developed using chemiluminescence reagents.
Results:

Protection from apoptosis by SLF is reversed through blockade of Ca\textsuperscript{2+} influx.

Ca\textsuperscript{2+} influx has been observed to both induce and protect cells from apoptosis in different cell systems (13, 14, 18). In order to investigate the role of Ca\textsuperscript{2+} influx in protection from apoptosis the effect of Ca\textsuperscript{2+} influx blockers on cell viability supported by SLF, a growth factor that mobilizes Ca\textsuperscript{2+} or IL-3, a mitogenic cytokine that does not mobilize Ca\textsuperscript{2+} was determined (20, 24). For these experiments 32D-kit cells, an IL-3-dependent murine myelomonocytic cell line that has been transfected with the c-kit receptor tyrosine kinase gene were used. Expression of c-kit in these cells makes them mitogenically responsive to SLF in vitro and renders them tumorigenic in vivo (21). These cells die apoptotically within 12-24 hours upon removal of factor (9).

Although 32D-kit cells are normally protected from apoptosis by SLF, I observed, using Trypan blue exclusion analysis, that this protective signal is converted to a death signal if the cells are co-incubated with the Ca\textsuperscript{2+} influx blockers cco. o.o or ketotifen (Figs 1A & C). In contrast, death is not observed when 32D-kit cells are incubated with Ca\textsuperscript{2+} influx blocker plus the mitogen IL-3 (Figure 1.A and C, squares), nor do blockers accelerate cell death due to withdrawal of protective factors (not shown). Furthermore, simultaneous incubation of 32D-kit cells with SLF, Ca\textsuperscript{2+} influx blockers, and IL-3 still results in cell death (Figure 1.A, triangles), indicating that IL-3 is not protective for this particular death signal. Induction of cell death by SLF plus Ca\textsuperscript{2+} influx blocker increases with increasing concentrations of SLF (Figure 1.E). Therefore, taken together, the effect of the Ca\textsuperscript{2+} influx blockers is not simply to counteract or neutralize the protective effect of SLF. Rather, they combine with high levels of SLF to induce cell death.
Figure 1. Effect of SLF or IL-3 with Ca^{2+} influx blockers on 32D-Kit cells.

(A-D) 32D-Kit cells were incubated with either SLF (500 ng/ml in all cases) (circles) or IL-3 (25% WEHI-3 conditioned medium in all cases) (squares) or both SLF and IL-3 (triangles) with varying amounts of econazole or ketotifen. In the case of (A) and (C), the proportion of dead cells was determined after 18 hours in culture by counting cells that could or could not exclude Trypan blue. For (B) and (D), colony formation was determined by incubating cells in liquid culture with either SLF or IL-3 with econazole or ketotifen followed by a 7 day incubation of cells in semi-solid medium. (E) 32D-Kit cells were incubated with varying amounts of SLF. Circles: plus 7.5 μM econazole. Squares: No econazole. Error bars represent the standard error determined from triplicate measurements.
Figure 1.
Failure to exclude Trypan blue is a useful but late cell death endpoint that may not always correlate with other measures of cell viability. I therefore also determined the effect of combining Ca\(^{2+}\) influx blockers with SLF or IL-3 on the clonogenic capacity of cells. 32D-kit cells were exposed to blockers with either SLF or IL-3 for 18 hours. The cells were then collected, plated in semi-solid agar in the presence of IL-3, and colonies were counted 7 days later. As shown in Figs. 1B & D, overnight exposure to econazole or ketotifen in the presence of IL-3 has little effect on the ability of cells to form colonies. In contrast, exposure to SLF plus ketotifen or econazole reduces clonogenicity by 10 or 1,000-fold respectively. Therefore, the specific combination of SLF with Ca\(^{2+}\) influx blockers also results in clonogenic cell death.

Although 32D-kit cells are factor-dependent, they grow continuously in culture and are tumorigenic in vivo. We wished to determine if the ability to induce cell death by SLF plus Ca\(^{2+}\) influx blockers could also be observed in non-transformed cells. Therefore determined the effect of combining Ca\(^{2+}\) influx blockers with SLF or IL-3 on murine bone marrow-derived mast cells (BMMCs), which are also protected from apoptosis and mitogenically stimulated by IL-3 and SLF was determined. As shown in Figs. 2A, B & C, exposure of BMMCs to the combination of Ca\(^{2+}\) influx blockers ketotifen, econazole or Ni\(^{2+}\) with SLF but not IL-3 results in cell death. As with 32D-kit cells, greater stimulation by SLF leads to greater levels of cell death in the population (Figure 2.D). Thus, cell death induced by the combination of SLF plus Ca\(^{2+}\) influx blockers is not limited to transformed cells such as 32D-kit.

Ca\(^{2+}\) influx blockers alone induce cell death in P815 mastocytoma cells.

These data show that stimulation of factor-dependent cells with SLF in the presence of Ca\(^{2+}\) influx blockers leads to cell death. There are many examples of cells that exhibit
Figure 2. Effect of SLF or IL-3 with Ca^{2+} influx blockers on BMMCs.

(A-C) BMMCs were incubated with either SLF (500 ng/ml in all cases) (circles) or IL-3 (25% WEHI-3 conditioned medium in all cases) (squares) or both SLF and IL-3 (triangles) with varying amounts of econazole, ketotifen or Ni^{2+}. (D) BMMCs were incubated with IL-3 and varying amounts of SLF. Circles: plus 2 mM Ni^{2+}. Squares: no Ni^{2+}. In all cases, the proportion of dead cells was determined after 18 hours in culture by counting cells that could or could not exclude Trypan blue. Error bars represent the standard error determined from triplicate measurements.
Figure 2.
factor-independent growth due to expression of receptor tyrosine kinases with activating mutations. One example is the P815 mastocytoma, which exhibits constitutive tyrosine phosphorylation of the c-kit protein and factor-independent growth (23). These properties have been attributed to an activating point mutation in the c-kit cytoplasmic domain (25). My observations predict that these cells should be susceptible to death induced by Ca\textsuperscript{2+} influx blockers in the absence of SLF stimulus. As shown in Figure 3, treatment of P815 cells with Ca\textsuperscript{2+} channel blockers econazole, ketotifen or Ni\textsuperscript{2+} leads to cell death in the presence or absence of added SLF. Thus, the constitutive signals in P815 cells are sufficient to combine with Ca\textsuperscript{2+} channel blockers to induce death.

**BMMC and 32D-kit induced death is apoptotic.**

Visual inspection of 32D-kit cells following treatment with SLF plus Ca\textsuperscript{2+} influx blockers revealed some morphological characteristics of apoptosis including nuclear condensation and membrane blebbing (Figure 4.D). However, in order to further substantiate that 32D-kit and mast cells were undergoing apoptosis, DNA content, which characteristically fragments and decreases during apoptosis, was measured. As shown in Figure 5. A, C & D and summarized in Table I, treatment of 32D-kit cells (and BMMCs) with SLF or IL-3 alone or IL-3 with econazole for 18 hours did not generate a population of cells with sub diploid DNA content, whereas SLF plus econazole generated a large population of cells with sub diploid DNA (Figure 5.B). 32D-kit cells will undergo apoptosis when they are deprived of growth factor. After 18 hours of growth factor withdrawal, 32D-kit cells exhibited a modest proportion of cells with sub-diploid DNA content. This population is substantially increased after 24 hours of factor withdrawal. Thus the apoptotic process induced by Ca\textsuperscript{2+} influx blocker plus SLF is more rapid than the apoptosis observed from factor withdrawal.
Figure 3. Effect of SLF or IL-3 with Ca\(^{2+}\) influx blockers on P815 mastocytoma cells. P815 cells were incubated with either SLF (500 ng/ml, solid bars) or IL-3 (25% WEHI-3 conditioned medium, stippled bars) with Ca\(^{2+}\) channel blockers econazole, ketotifen or Ni\(^{2+}\). In all cases, the proportion of dead cells was determined after 18 hours in culture by counting cells that could or could not exclude Trypan blue. Error bars represent the standard error determined from triplicate measurements.
Figure 3.

Bar chart showing the percentage of death at different concentrations of inhibitors: no blocker, 4μM Ec, 80μM Ket, and 4mM Ni.
Figure 4. Morphology of 32D-Kit cells incubated with factor and Ca^{2+} influx blockers.

32D-Kit cells were incubated for 18 hours with (A) SLF alone, (B) IL3 + 8 μM econazole, (C) IL-3 alone, (D) SLF + 8 μM econazole or (E) Cells were incubated with no added factor.
Figure 4.
Figure 5. DNA content of 32D-Kit cells incubated with factor and Ca^{2+} influx blockers.

32D-Kit cells were incubated for 18 hours with (A) SLF alone, (B) SLF + 8 μM econazole, (C) IL-3 alone, (D) IL-3 + 8 μM econazole or (E) Cells were incubated with no added factor. Cell were then resuspended in Vindelöv's solution containing propidium iodide and analyzed by flow cytometry.
Figure 5.
Table 1. Sub G1 DNA Content of BMMCs or 32D-Kit cells treated with econazole plus factor.

<table>
<thead>
<tr>
<th>Sub G1 Content (%)</th>
<th>no econazole</th>
<th>8μM econazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>25.4</td>
<td>20.4</td>
</tr>
<tr>
<td>SLF</td>
<td>6.8</td>
<td>66.8</td>
</tr>
<tr>
<td>Factor Withdrawal</td>
<td>61.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>32D-Kit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>1.6</td>
<td>4.8</td>
</tr>
<tr>
<td>SLF</td>
<td>2.3</td>
<td>35.0</td>
</tr>
<tr>
<td>Factor Withdrawal</td>
<td>9.2</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

BMMCs were incubated with 8μM econazole plus IL-3 (25% WEHI conditioned media), SLF (500ng/ml), or no factor for 24 hours, stained with propidium iodide, and analyzed for DNA content. 32D-Kit cells were similarly incubated for 18 hours before analysis. n.d.: not determined.
Specificity for non-voltage gated Ca^{2+} influx blockers.

Ca^{2+} influx following receptor activation is mediated by the opening of Store-Operated Ca^{2+} Channels (26). It is likely that this channel is the target for inhibition since the efficacy with which the three compounds, ketotifen, econazole and Ni^{2+}, induce cell death in combination with SLF correlates with their ability to inhibit the SOC (27). We have observed that the voltage gated Ca^{2+} channel blockers verapamil and nifedipine are ineffective in inducing cell death when combined with SLF (not shown). This result suggests that the induction of cell death in combination with SLF is specific for non-voltage gated Ca^{2+} influx blockers. Other ion-blocking effects have been reported for both econazole and ketotifen (27). In order to determine if specific blockade of Ca^{2+} influx is critical for induction of cell death, the effect of the calcium ionophore ionomycin, on induction of apoptosis was examined. As shown in Figure 6., ionomycin protects 32D-kit cells from SLF plus Ca^{2+} influx blocker-induced death in a concentration-dependent manner, with maximal protection at 10 nM. Given the specificity of ionomycin for Ca^{2+} (28), these results indicate that it is the specific blockade of Ca^{2+} influx which is required for induction of apoptosis in combination with SLF. Importantly, higher concentrations of ionomycin, which generate excessive levels of intracellular Ca^{2+}, resulted in the restoration of cell death. This demonstrates that in the context of cell activation by SLF, both extremes of Ca^{2+} influx can induce cell death.

Other PLC-activating signals which result in apoptosis.

Although both SLF and IL-3 are mitogenic for mast cells, only SLF induces a modest Ca^{2+} influx (20, 24). This indicates that perhaps PLC-γ stimulated Ca^{2+} mobilization might be the signaling event required to induce cell death in combination with Ca^{2+} influx blockers. I therefore investigated the effect on cell viability of two other signals known to activate PLC-γ and mobilize Ca^{2+} in mast cells. Cross-linking of the high affinity receptor...
Figure 6. Effect of ionomycin on SLF plus blocker-induced cell death.

32D-Kit (closed squares and circles), or 32D-Kit-bcl-2 (open squares and circles) cells were incubated for 18 hours with 5 μM econazole plus varying amounts of ionomycin in the presence of either SLF (circles) or IL-3 (squares). The proportion of dead cells was determined by Trypan Blue exclusion. Error bars represent the standard error determined from triplicate measurements.
Figure 6.
for IgE with antigen initiates a series of signaling events including PLC-γ activation and 
Ca\textsuperscript{2+} mobilization, which result in mast cell degranulation (29). The effect on cell viability 
of stimulating mast cells with Ag plus IgE in the presence of Ca\textsuperscript{2+} influx blockers was 
therefore tested. As shown in Figure 7.A, cell death is also induced when mast cells are 
stimulated with IgE plus antigen in the presence of econazole, whereas antigenic 
stimulation alone or econazole alone have no effect on cell viability.

Mast cells also respond to amphiphilic cationic peptides such as Substance P (30, 31). These molecules directly stimulate heterotrimeric G-proteins resulting in PLC-β activation, 
Ca\textsuperscript{2+} mobilization and, in the presence of suboptimal levels of antigen plus IgE, mast cell 
degranulation. As shown in Figure 7.B, cell death is also induced when mast cells are 
stimulated with Substance P in the presence of econazole. As with SLF, the degree of cell 
death in the presence of Ca\textsuperscript{2+} influx blocker increases with increasing concentrations of Ag 
or Substance P. Since the three signals SLF, IgE and Substance P (but not IL-3) all 
mobilize Ca\textsuperscript{2+} leading to Ca\textsuperscript{2+} influx, it is likely that Ca\textsuperscript{2+} mobilization is the required 
signal for the induction of cell death in combination with Ca\textsuperscript{2+} influx blockers.

Oleic acid protects cells from SLF plus Ca\textsuperscript{2+} influx blocker induced apoptosis.

Our observation that Ca\textsuperscript{2+} mobilizing signals can combine with Ca\textsuperscript{2+} influx blockers to 
duce apoptosis suggests that this effect is mediated by activation of PLC. In order to 
etermine if PLC activation is important for cell death, the effect of oleic acid, which has 
been shown to inhibit PLC activation in response to epidermal growth factor (EGF) 
stimulation (32), on apoptosis induction was examined. This inhibitory effect, which does 
not alter EGF binding or EGF receptor tyrosine kinase activation, is only observed with 
cis-9-octadecenoic acid (oleic acid) but not trans-9-octadecenoic acid (elaidic acid) (33). 
We incubated 32D-kit cells with econazole and SLF or IL3 in the presence of either oleic or
Figure 7. Effect of IgE plus Ag or Substance P with Ca^{2+} channel blockers on BMMCs. (A) BMMCs were coated with IgE anti-DNP monoclonal SPE-7, then incubated with IL-3 plus varying amounts of DNP-HSA (Ag), with or without econazole. Circles: plus 5μM econazole, Squares: no econazole. (B) BMMCs were incubated with IL-3 plus varying amounts of Substance P. Circles: plus 5μM econazole, Squares: no econazole. In all cases, the proportion of dead cells was determined after 24 hours in culture by counting cells that could or could not exclude Trypan blue. Error bars represent the standard error determined from triplicate measurements.
Figure 7.
Figure 8. Oleic acid protects 32D-kit cells from SLF plus Ca^{2+} influx blocker induced apoptosis.

32D-kit cells were incubated for 18 hours with SLF plus 5μM econazole plus oleic acid (solid bars) or elaidic acid (hatched bars) or IL3 plus 5 μM econazole plus oleic acid (stippled bars). The proportion of dead cells was determined by Trypan Blue exclusion. Error bars represent the standard error determined from triplicate measurements.
Figure 8.
elaidic acid. As shown in Figure 8, 32D-kit cells treated with SLF plus Ca\(^{2+}\) channel blocker are rescued from cell death by oleic but not elaidic acid. The range of effectiveness of oleic acid is between 1-100\(\mu\)M which corresponds to concentrations required for PLC inhibition (32). This observation is therefore consistent with a requirement for activation of PLC in the induction of apoptosis in combination with Ca\(^{2+}\) influx blockers.

**PLC-\(\gamma\)activation is required for induction of apoptosis by SLF and Ca\(^{2+}\) channel blockers.**

In order to confirm that PLC is indeed required for Ca\(^{2+}\) channel blocker induced apoptosis, YF728, a c-kit receptor which possesses a mutation at the putative PLC-\(\gamma\) binding site, was expressed in 32D cells (see Chapter 3 for details). 32D cells expressing wt c-kit were able to mobilize Ca\(^{2+}\) upon SLF stimulation however SLF-stimulated YF728 cells were not able to mobilize Ca\(^{2+}\) (Chapter 3, Figure 2.). When KitWT and KitYF728 cells were incubated with SLF and econazole, KitWT cells underwent apoptosis, however the KitYF728 cells were resistant to the induction of apoptosis. Both KitWT and KitYF728 are resistant to apoptosis with IL-3 and Ca\(^{2+}\) channel blocker. This indicates that activation of the PLC-\(\gamma\)enzyme was required for induction of apoptosis by SLF plus Ca\(^{2+}\) channel blockers (Figure 9.).

**Bcl-2 fails to protect 32D-kit cells from SLF plus Ca\(^{2+}\) influx blocker induced apoptosis.**

The bcl-2 family of proteins have been strongly linked to protection of cells from apoptosis induced by a wide variety of agents. Expression of bcl-2 is correlated with proliferating cells (34, 35), is negatively regulated by the tumor suppressor p53 (36), and overexpression of bcl-2 protects 32D cells from apoptotic death following factor withdrawal (9). Given the importance of bcl-2 in regulating susceptibility to apoptosis, we were interested in the effect that overexpression of this protein might have on the induction of apoptosis by SLF plus Ca\(^{2+}\) influx blocker. 32D-kit cells were therefore infected with a
Figure 9. Incubation of KitYF728 32D cells with SLF and Ca²⁺ influx blocker does not result in apoptosis.

KitWT 32D (squares) and KitYF728 32D (circles) cells were incubated with either SLF (closed squares and circles) or IL-3 (open squares and circles) along with varying concentrations of econazole. The proportion of dead cells was determined after 18 hours in culture by counting cells that could or could not exclude Trypan blue. Error bars represent the standard error determined from triplicate measurements.
Figure 9.
Figure 10. Bcl-2 overexpression fails to protect 32D-Kit cells from SLF plus Ca\textsuperscript{2+} influx blocker.

(A) Western blot of cell lysates from 32D-Kit (lane 1) or 32D-Kit-bcl-2 (lane 2) cells. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-bcl-2 antibodies (top panel). The blot was then stripped and re-probed with an anti-actin monoclonal antibody to demonstrate equal protein loading (bottom panel). (B) 32D-Kit (solid bars) or 32D-Kit-bcl-2 (striped bars) cells were incubated for 18 hours with SLF or IL-3 plus 2.5, 5 or 10 μM econazole and the proportion of dead cells was determined by Trypan Blue exclusion. In additional cultures, IL-3 alone or no factor was added and cell viability was similarly determined. Error bars represent the standard error determined from triplicate measurements.
Figure 10.
retrovirus vector (37) containing the bcl-2 gene in order to generate a bcl-2 overexpressing line (Figure 10.A) and these cells were tested for susceptibility to apoptosis. I confirmed that bcl-2 overexpression protects 32D-kit cells from apoptosis induced by factor withdrawal (Figure 10.B). However, as also shown in Figure 6.B, overexpression of bcl-2 in 32D-kit cells fails to protect these cells from induction of apoptosis by SLF plus econazole. These observations demonstrate that induction of cell death by SLF plus blocker occurs in a bcl-2 independent manner. 32D-kit-bcl-2 cells were similarly protected from apoptosis by SLF plus blocker with low levels of ionomycin (Figure 6.), however, unlike the 32D-kit cells, they did not demonstrate significant cell death at higher levels of ionomycin. Taken together, these results therefore confirm that bcl-2 can protect cells from both factor withdrawal and high levels of intracellular Ca^{2+} but is unable to protect cells from the combination of SLF plus Ca^{2+} influx blockers.

Caspase inhibitors protect cells from Ca^{2+} influx blocker plus SLF-induced death

Members of the caspase family of intracellular proteases are common effectors of apoptotic death induced by a wide variety of agents. Current models of caspase involvement in apoptosis suggest that pro-apoptotic stimuli activate a cascade of proteases, with ICE-like caspases (caspase-1) acting upstream of CPP32-like caspases (caspase-3) (38, 39). ICE-like caspases demonstrate cleavage specificity for substrates with aspartate in the P1 position and hydrophobic amino acids in the P4 position. These enzymes are preferentially inhibited by tetrapeptides such as the aldehyde YVAD-CHO (40). In contrast, CPP32-like caspases preferentially cleave substrates with acidic amino acids in the P4 position and are inhibited by tetrapeptides such as DEVD-FMK and Boc-Asp-FMK (41, 42). In order to determine if caspases were involved in the induction of apoptosis by SLF plus Ca^{2+} influx blocker, these caspase inhibitors were tested for their protective ability. All three inhibitors protected 32D-kit cells from apoptosis induced by the topoisomerase inhibitor etoposide (not shown). These inhibitors also provided resistance to apoptosis induced by SLF plus
Figure 11. YVAD-CHO, DEVD-FMK and Boc-Asp-FMK protect 32D-Kit cells from SLF plus econazole.

32D-Kit cells were incubated with either SLF (solid bars) or SLF plus 10μM econazole (stippled bars). The proportion of dead cells was evaluated by Trypan Blue exclusion after 18 hours in culture. Error bars represent the standard error determined from triplicate measurements.
Figure 11.

[Diagram showing % Death for different treatments: no inhibitor, 100μM YVAD-CHO, 100μM Boc-Asp-FMK, 100μM DEVD-FMK]
econazole (Figure 1I), indicating that apoptosis induced through the combination of SLF stimulation with a Ca\textsuperscript{2+} influx blocker is likely mediated by a caspase cascade involving both ICE-like and CPP-32 enzymes.

Cleavage of PARP protein is observed with SLF treatment plus Ca\textsuperscript{2+} influx blockade. Poly(ADP-ribose) polymerase (PARP) is a nuclear repair enzyme which has also been implicated in transcription enhancement during preinitiation complex formation (43). A common endpoint in the caspase cascade is the cleavage of this 116 kD nuclear protein into an ~85 kD fragment (44-46). Since this cleavage event is likely mediated by the CPP-32 subfamily of caspases and my results indicate that CPP32-like caspases are involved in the induction of apoptosis through the combination of SLF and Ca\textsuperscript{2+} influx blockers, the effect of this treatment on PARP was evaluated. As shown in Figure 12., an ~85kD PARP cleavage product is observed in nuclear extracts when 32D-kit cells are treated with SLF plus econazole but is not detected in nuclear extracts from cells incubated with SLF alone, IL-3 alone, or IL-3 plus econazole. Co-incubation of cells with Boc-Asp-FMK results in partial inhibition of PARP cleavage, in agreement with my data showing partial protection from apoptosis by this inhibitor. Therefore it can be concluded that the combination of SLF plus Ca\textsuperscript{2+} influx blocker initiates a cascade involving ICE-like and CPP32-like caspases that ultimately results in cleavage of PARP.
Figure 12. Western blot analysis of nuclear PARP.

32D-Kit cells were incubated for 12 hours with either SLF (lane 1), IL-3 (lane 2), SLF plus 10 μM econazole (lane 3), IL-3 plus 10 μM econazole (lane 4) or SLF plus 10 μM econazole plus 100 μM Boc-Asp-FMK. Nuclear lysates from these cultures were separated by 7.5% SDS-PAGE, transferred to nitrocellulose and probed with anti-PARP antibodies.
Figure 12.
Discussion:

An important property of many hematopoietic growth factors and cytokines is the protection of cells from apoptosis. Here we have shown that blockade of Ca^{2+} influx reverses the ability of SLF but not IL-3, to protect cells from apoptotic death, highlighting the importance of Ca^{2+} influx in SLF-mediated cell survival. These data indicate however, that the effect of Ca^{2+} influx blockade extends beyond simple neutralization of the protective properties of Ca^{2+} influx. Since higher levels of SLF stimuli lead to greater levels of cell death, and induction of cell death by SLF plus blockers occurs even in the presence of IL-3, this suggests that activation by SLF is an essential component of this form of apoptosis. Thus concurrent blockade of Ca^{2+} influx effectively converts the SLF-mediated protective signal into a death signal.

Several endpoints indicate that the cell death induced by the combination of SLF and Ca^{2+} influx blockers is apoptotic. These include specific morphological characteristics such as membrane blebbing and nuclear condensation, loss of membrane integrity as indicated by failure to exclude Trypan Blue, DNA fragmentation, protection by caspase inhibitors and PARP cleavage. Although many of these endpoints are also characteristic of apoptotic death caused by factor withdrawal (4), an important difference is that while IL-3 or bcl-2 overexpression protects cells from factor withdrawal, they do not protect these cells from apoptosis induced by SLF plus Ca^{2+} influx blockade.

Other apoptotic signals have also been found to be bcl-2 insensitive (3). These include cell death induced by TNF, Fas activation, Activation-Induced Cell Death, and superantigen-mediated clonal deletion (47-51). It is therefore possible that disruption of Ca^{2+} influx in the context of activation may be an important component of such signals as well. In support of this possibility, Kovacs et al. demonstrated that Fas activation inhibited anti-
CD3-mediated Ca\textsuperscript{2+} influx in T cells without affecting Ca\textsuperscript{2+} release from internal stores (52).

We found that ionomycin can protect cells from SLF plus blocker induced cell death, supporting our contention that specific blockade of Ca\textsuperscript{2+} influx is required for apoptosis induction. The observation that higher levels of ionomycin restores cell death shows that both extremes of Ca\textsuperscript{2+} influx can lead to apoptosis. In both cases however, cell activation is required since only minimal cell death is observed in the absence of SLF. As observed in other cell systems (53, 2), I found that IL-3 or bcl-2 overexpression protected cells from death induced by high concentrations of ionomycin, highlighting the difference between apoptosis caused by SLF plus Ca\textsuperscript{2+} influx blockade and apoptosis caused by 'Ca\textsuperscript{2+} overload'. One possible mediator of high Ca\textsuperscript{2+} death may be the Ca\textsuperscript{2+}-dependent phosphatase calcineurin, which has been shown to potentiate apoptosis in T cells (54) and B cells (55).

The observation that caspase inhibitors protect cells from apoptosis induced by SLF stimulation plus Ca\textsuperscript{2+} influx blockade, coupled with the demonstration that PARP, a nuclear target of caspases is cleaved by this treatment indicates that this form of apoptosis is effected through activation of a caspase cascade. However the specific molecular events responsible for activating this cascade remain to be identified. SLF stimulation mobilizes Ca\textsuperscript{2+} through activation of the enzyme PLC-\textgamma\textsuperscript{(56, 57)}. It is therefore likely that PLC activation is a minimal requirement. In agreement with this hypothesis, other signals that activate PLC such as stimulation of the FceRI receptor with IgE plus antigen and stimulation with Substance P also combine with Ca\textsuperscript{2+} influx blockers to induce apoptosis. In addition, the PLC-\textgamma\textsuperscript{inhibitor oleic acid prevents SLF plus blocker induced apoptosis of 32D-kit cells, and KitYF728 32D cells which fail to activate PLC-\textgamma\textsuperscript{are resistant to induction of apoptosis with SLF and blockers.}
This study has identified a role for Ca\textsuperscript{2+} influx in apoptosis and has characterized an apoptotic pathway mediated by PLC-activating signals such as SLF, Substance P or IgE plus Ag in combination with Ca\textsuperscript{2+} influx blockade. Although PLC activation has been demonstrated to be required for this form of apoptosis, the specific downstream events of PLC-activation responsible for apoptosis remain to be identified. In Chapter 5, a proposed mechanism for this apoptotic pathway will be described.
References:


beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell.* 81:801-9.


CHAPTER V

Summary and Future Directions.
Summary

In this thesis, the role of each PI3-kinase and PLC-γ in the context of SLF-stimulated c-Kit internalization as well as SLF-mediated mitogenic and survival signals is addressed. In order to answer these issues, two mutant receptors, KitYF719 and KitYF728 which contain mutated binding sites for PI3-kinase and PLC-γ respectively, were expressed in IL-3-dependent, c-Kit negative cell lines. In addition, inhibitors of both PI3-kinase activity and Ca²⁺ influx were used to evaluate the role of some of the second messengers generated by PI3-kinase and PLC-γ.

Given the importance of receptor internalization in the regulation of mitogenic signals, our first aim was to understand the mechanism for c-Kit internalization. In Chapter 2, a role for both PI3 kinase activity as well as Ca²⁺ influx in SLF-stimulated c-Kit internalization was identified. Specifically, in the absence of both PI3-kinase activity and Ca²⁺ influx, c-Kit receptors failed to internalize. However, if only PI3-kinase activity was inhibited, receptors internalized but exhibited an altered endocytic pattern where receptors were found to be localized close to the plasma membrane. This result suggested that in the absence of PI3-kinase activation, there was a defect in a later endocytic step. In addition, in the absence of both PI3-kinase activity and Ca²⁺ influx, SLF-stimulated c-Kit no longer co-immunoprecipitated with clathrin molecules. Therefore, this study identified some of the requirements for both the first SLF-stimulated internalization events as well as a role for PI3-kinase in a later endocytic step.

The identification of signaling requirements for c-Kit internalization and endocytosis prompted the evaluation of the biological function of this process. Ligand-stimulated receptor internalization often results in the lysosomal degradation of receptor/ligand complexes (1). Therefore, receptor internalization is considered to be a mechanism for...
limiting the amount of signal delivered to a cell. In agreement with this concept, a non-
internalizing EGFR mutant has been demonstrated to induce cellular transformation (2).
However, there are a number of examples where defects in receptor internalization correlate
with decreased mitogenic signaling (3-5). In Chapter 3 of this thesis, wild-type and mutant
KitYF719 and KitYF728 receptors were tested for their ability to respond to "non-
internalizing" stimuli, specifically membrane-bound SLF and plate-bound anti-c-Kit
antibodies. Stimulation with soluble SLF did not reveal any differences between the wild-
type and mutant receptors. However, KitWT and KitYF719 32D cells but not KitYF728
cells responded to stimulation with membrane-bound SLF or plate-bound anti-c-Kit
antibodies. Given that the KitYF728 32D cells do not mobilize Ca2+ and likely do not
activate PLC-γ, this experiment therefore implicates PLC-γ in mSLF-mediated mitogenic
signaling.

Furthermore, an examination of the Kit+ isoform, which demonstrates a defect in
endocytosis compared to the Kit isoform, revealed a correlation between receptor
internalization and mitogenesis. Specifically, stimulation of Kit+ expressing cells induced
a sub-mitogenic response compared to Kit expressing cells. Therefore, this work
highlighted an important function for PLC-γ and also described a correlation between
receptor internalization and mitogenesis.

The observation in Chapter 2 that Ca2+ influx plays a role in receptor internalization
prompted an examination of the requirement for Ca2+ in SLF-mediated cell survival
signals. As discussed in the Introduction, SLF is an important survival factor for a variety
of cell types including mast cells. In addition, the mechanism underlying SLF-mediated
survival is different from that supported by other cytokines such as IL-3 as SLF fails to
upregulate the Bcl-2 anti-apoptotic protein. In Chapter 4, stimulation of either mast cells or
c-Kit positive myelomonocytic cells with SLF and Ca2+ channel blockers together resulted
in apoptosis rather than cell survival. Incubation of these cells with IL-3 and Ca\textsuperscript{2+} channel blockers, however, failed to induce apoptosis. Apoptosis induced by SLF and Ca\textsuperscript{2+} channel blockers resulted in the cleavage of the caspase substrate PARP and could be inhibited by caspase inhibitors. However, Bcl-2 overexpression failed to protect cells from SLF and Ca\textsuperscript{2+} channel blocker induced apoptosis.

One of the key differences between SLF and IL-3 mediated signals is that SLF-stimulation of c-Kit results in the activation of PLC-\(\gamma\) whereas stimulation of the IL-3 receptor fails to induce the activation of PLC-\(\gamma\), suggesting a role for PLC-\(\gamma\) in this apoptotic process. This hypothesis was confirmed by several approaches. Stimulation of the PLC-\(\gamma\) activating FceRI while blocking Ca\textsuperscript{2+} influx also induced apoptosis. In addition, PLC-\(\gamma\) inhibitors protect c-Kit positive cells from apoptosis induced by SLF and Ca\textsuperscript{2+} channel blockers. Finally, stimulation of KitYF728 32D cells with SLF in the presence of Ca\textsuperscript{2+} channel blockers does not result in apoptosis. Therefore, this Chapter revealed a requirement for Ca\textsuperscript{2+} influx in SLF-mediated survival signals. In addition, this Chapter characterized a PLC-\(\gamma\)-dependent mechanism of apoptosis induced by activating signals such as SLF along with blockade of Ca\textsuperscript{2+} influx.

**Future Directions**

*What are the Ca\textsuperscript{2+} dependent molecule(s) which are involved in SLF-mediated receptor internalization?*

The observation that Ca\textsuperscript{2+} influx plays a role in c-Kit internalization in the absence of PI3-kinase activity reveals that there are likely Ca\textsuperscript{2+}-dependent proteins involved in this process. Ca\textsuperscript{2+} ions as well as several Ca\textsuperscript{2+}-binding proteins have been implicated in receptor movement. Specifically, clathrin light chains are stabilized by Ca\textsuperscript{2+} and formation
of clathrin lattices is facilitated by elevations in intracellular Ca\(^{2+}\) (6, 7). Thus, blocking Ca\(^{2+}\) influx may result in a less stable clathrin coated pit, thus preventing receptor internalization. In addition, there are a number of Ca\(^{2+}\) binding proteins which are activated upon increases in intracellular Ca\(^{2+}\) concentrations. Annexin VI is a Ca\(^{2+}\)-dependent protein which is required for the budding of clathrin-coated pits, a process which is essential for receptor internalization (8). Calpains are Ca\(^{2+}\)-dependent enzymes which are implicated in the stripping of clathrin coats from clathrin-coated pits so that they may fuse with early endosomes (9). Inhibition of Ca\(^{2+}\) influx upon SLF-stimulated c-Kit internalization may therefore prevent the function of calpains or annexins, thus impairing internalization.

Calmodulin is a Ca\(^{2+}\)-dependent molecule which induces the activation of calcineurin. Calmodulin molecules interact with clathrin triskelions, thus suggesting a role for this molecule in receptor internalization (10). The use of specific inhibitors of calmodulin have revealed a role for calmodulin in endosome fusion (11). Preliminary data from our lab has indicated that inhibition of calmodulin with a specific inhibitor, W13 (a naphthalene sulfonamide related to the previously mentioned inhibitor W7), in conjunction with inhibition of PI3-kinase activity also results in an impairment of c-Kit internalization. Importantly, the combination of the non-active isomer W12 with PI3-kinase inhibition does not result in a defect in c-Kit internalization. However, the impairment observed under these conditions is not absolute, therefore suggesting that there are other Ca\(^{2+}\)-dependent proteins which also play a role in c-Kit internalization.

A model for the role of both Ca\(^{2+}\) and Ca\(^{2+}\)-dependent proteins as well as PI3-kinase activity in c-Kit internalization can be constructed. It is possible that Ca\(^{2+}\) ions and Ca\(^{2+}\)-dependent proteins such as calmodulin are required to stabilize early clathrin structures. In the presence of PI3-kinase activity, however, these structures are fused with early
endosomes and subsequently later endosomal compartments. In the absence of Ca\textsuperscript{2+} ions, it is postulated that the early clathrin structures are unstable and susceptible to disassembly, however the contents of these structures can still shuttled down an endocytic pathway via PI3-kinase. However, in the absence of both PI3-kinase and Ca\textsuperscript{2+} ions, the receptor/ligand complexes in the early clathrin structures are not internalized and remain exposed to the extracellular milieu. This model is represented schematically in Figure I. Future work will be required to determine the identity of other Ca\textsuperscript{2+}-dependent proteins involved in this process which cooperate with PI3-kinase.

What is the mechanism for SLF plus Ca\textsuperscript{2+} channel blocker induced apoptosis?

In Chapter 4, a requirement for PLC-\(\gamma\) in SLF plus Ca\textsuperscript{2+} channel blocker-induced apoptosis was determined. Given that PLC-\(\gamma\) activation leads to both the release of Ca\textsuperscript{2+} from internal stores as well as the activation of PKC, it is therefore possible that one or both of these events are required for the induction of apoptosis upon Ca\textsuperscript{2+} influx blockade. Two previous findings correlate with the possibility that release of Ca\textsuperscript{2+} from internal stores in the absence of Ca\textsuperscript{2+} influx causes apoptosis: Engagement of mIgM with multivalent antigen or with F(ab\textsuperscript{2})\textsubscript{2} fragments of anti-IgM antibodies normally leads to a strong activation of PLC-\(\gamma\) resulting in the production of IP\textsubscript{3}, Ca\textsuperscript{2+} mobilization, Ca\textsuperscript{2+} influx and subsequent proliferation of the B cell (12). Over 10 years ago, the effects of co-aggregating mIgM with Fc\textsubscript{y}RII (CD32) were documented. Specifically, co-aggregation of mIgM and CD32 using whole antibody results in mobilization of Ca\textsuperscript{2+}, however Ca\textsuperscript{2+} influx is completely inhibited (13). This was thought to represent a mechanism for “desensitizing” B cells, thus curtailing an antigen-specific B cell response (14). Furthermore, co-aggregation of CD32 with mIgM on the surface of B cells with whole
Figure 1. Model of PI3-kinase and Ca^{2+} dependent steps in c-Kit receptor internalization.

This model depicts an early Ca^{2+}-dependent internalization step followed by later PI3-kinase-dependent steps. Importantly, in the absence of both Ca^{2+} influx and PI3-kinase activity, receptors/ligand complexes fail to become internalized.
Figure 1.

A) PI3-kinase activity and presence of Ca$^{2+}$ ions.

B) No PI3-kinase activity and presence of Ca$^{2+}$ ions.

C) No PI3-kinase activity and absence of Ca$^{2+}$ ions.
antibody was found to result in growth arrest or apoptosis (15). Thus, there is a correlation between a block in Ca^{2+} influx and apoptosis. The same correlation is observed in human thymocytes where CD3-mediated Ca^{2+} influx was found to be ablated in the case where the Fas antigen was concomitantly aggregated on these cells using specific monoclonal antibodies. Again, release of Ca^{2+} from internal stores was found to be unaffected (16).

It is important to note that in these cases, signals mediated by either CD3 or mIgM aggregation normally induce the release of Ca^{2+} from internal stores. The same is true for stimulation with SLF, IgE plus Ag or Substance P. A blockade of Ca^{2+} influx in the context of Ca^{2+} mobilization may therefore be responsible for inducing an apoptotic program, perhaps by prohibiting the refilling of these stores. Baffy et al. have investigated the repartitioning of intracellular Ca^{2+} upon withdrawal of IL-3 from the IL-3 dependent 32D cell line. Removal of IL-3 from cultures of 32D cells led to DNA fragmentation and apoptosis. This programmed cell death was found to be associated with decreased Ca^{2+} concentrations in thapsigargin-sensitive, non-mitochondrial internal stores, but an increase in mitochondrial Ca^{2+} concentration. Re-addition of IL-3 to the factor deprived 32D cells resulted in Ca^{2+} replenishment of thapsigargin-sensitive stores and rescue of these cells from apoptosis. In addition, treatment of IL-3 deprived 32D cells with Ca^{2+} ionophores also rescued cells from apoptosis. Taken together, these results suggest that an emptying of thapsigargin-sensitive internal Ca^{2+} stores results in apoptosis, and replenishment of these stores rescues cells from apoptosis (17). It is therefore possible that stimulation with SLF, Substance P or IgE plus Ag which leads to the emptying of Ca^{2+} from IP3-sensitive stores, in combination with Ca^{2+} influx blockade, causes apoptosis by a similar mechanism.
Furthermore, Baffy et al. demonstrated that 32D cells were protected from IL-3 withdrawal induced death by overexpression of Bcl-2. Interestingly, Bcl-2 overexpression in these cells prevented the observed decreases of Ca\(^{2+}\) levels in thapsigargin-sensitive internal stores. These authors therefore concluded that the mechanism of Bcl-2 protection was to inhibit the release of Ca\(^{2+}\) from internal stores. In addition, Lam et al. have directly demonstrated that Bcl-2 inhibits thapsigargin-mediated Ca\(^{2+}\) release from the endoplasmic reticulum (18). If the mechanism responsible for SLF plus Ca\(^{2+}\) channel blocker induced apoptosis is a failure to replenish emptied internal Ca\(^{2+}\) stores, and if Bcl-2 blocks Ca\(^{2+}\) release from these stores, overexpression of Bcl-2 should protect 32D-Kit cells from apoptosis. However, we find that overexpression of Bcl-2 in 32D-Kit cells incubated with SLF and Ca\(^{2+}\) channel blockers has no effect on cell survival. One explanation for this discrepancy may be that these Bcl-2 expressing 32D-Kit cells are not expressing the anti-apoptotic protein in the appropriate cellular location. Indeed, Zhu et al. have observed that expression of Bcl-2 in the mitochondria versus the endoplasmic reticulum membranes has different effects depending on the cell types (19). Therefore, an examination by fluorescence microscopy of the subcellular location of Bcl-2 in 32D-Kit cells overexpressing Bcl-2 is required.

In order to test the hypothesis that failure to replenish emptied internal stores leads to apoptosis, Ca\(^{2+}\) measurements in different subcellular locations would be required. These measurement would then be correlated with the induction of apoptosis. In addition, the recent observation that release of cytochrome c from the endoplasmic reticulum causes apoptosis raises the question of whether not only release of internally stored Ca\(^{2+}\) but also cytochrome c is involved in SLF plus Ca\(^{2+}\) channel blocker induced death (20). Therefore, measurements of cytosolic cytochrome c before and after treatment with SLF and Ca\(^{2+}\) channel blockers may be performed. These experiments may therefore assist in clarifying the confusions regarding the role of Ca\(^{2+}\) physiology in programmed cell death.
The involvement of internal Ca\textsuperscript{2+} store emptying in apoptosis is likely not the only event required for the initiation of apoptosis. The description of caspases in Chapter 1 reveals a complex interaction between these molecules which may be affected by the release of cytochrome c from internal organelles. Release of Ca\textsuperscript{2+} from these organelles may also modulate caspases, and as further characterization of these molecules emerges, a role for Ca\textsuperscript{2+} may be revealed.

How would KitYF728 expressing cells respond to their environment in vivo?

The observation that the W\textsuperscript{o} allele produces a c-Kit receptor that is unable to activate PLC-\gamma but retains the ability to activate PI3-kinase, suggests that PLC-\gamma mediated signals may be critical in vivo for c-Kit mediated activities such as hematopoiesis (21). In Chapter 3, KitYF728 32D cells were found to be unable to respond to membrane-bound SLF (mSLF) although their response to soluble SLF (sSLF) was unimpaired. Stimulation of KitYF728 cells does not result in Ca\textsuperscript{2+} influx, thus confirming that these receptors likely do not activate PLC-\gamma. Thus, taken together, although PLC-\gamma signals are not required for response to sSLF, these signals are required for response to the more physiologically relevant mSLF. Therefore, defects in PLC-\gamma signaling may result in observable phenotypes in vivo.

Expression of c-Kit in 32D cells was previously shown to enhance the leukemogenic potential of the 32D cells when injected into mice. In fact, recovery and culture of these neomycin-resistant cells revealed, in some cases, factor-independent growth (22). If PLC-\gamma signals are important for c-Kit signaling in vivo, then c-Kit-dependent leukemogenesis may be impaired in the case of KitYF728 cells. To test this hypothesis, uninfected, KitWT and KitYF728 32D could be injected into syngeneic hosts and leukemic burden would be
evaluated based on size of spleen and lymph nodes as well as enumeration of recovered
cells in semi-solid agar in the presence of G418. In addition, the ability to grow in culture
in the absence of added factor will be evaluated. Our prediction is that KitYF728 32D
cells, unlike KitWT cells, will be unable to induce leukemogenesis. This would therefore
confirm a previously unappreciated role for c-Kit PLC-γ-mediated signals in vivo.

Why does SLF-stimulation of Kit+ expressing cells induce a sub-mitogenic response?

The observation that Kit+ expressing 32D cells are sub-mitogenic compared to KitWT cells
remains to be fully explained. As demonstrated, Kit+ cells exhibit an endocytic defect.
Fluorescence microscopy analysis of both Kit+ cells as well as KitYF719 cells (see
Chapter 2), reveal that their distributions are remarkably similar. Specifically, following
ligand-stimulated internalization, these receptors localize close to the plasma membrane. In
addition, a comparison of Kit+YF728 32D mutant cells (shown in Chapter 3) and
KitYF719/YF728 32D double mutants 32D cells (Chapter 3 and work in progress) reveals
that both cell types have profound defects in receptor internalization and fail to respond
mitogenically to sSLF. Given that the YF719 mutation abolishes PI3-kinase binding to c-
Kit, these similarities suggest that the Kit+ isoform may not properly activate PI3-kinase.

While it is true that we and others have demonstrated successful SLF-stimulated
recruitment of the p85 subunit to the Kit+ receptor, (Chapter 3 and (23)), it remains to be
demonstrated whether stimulation of the Kit+ receptors results in activation of downstream
PI3-kinase targets such as Akt. Thus, there may be a correlation between receptor
endocytosis and the activation of PI3-kinase targets such as Akt. This is also supported by
the observation that p85 remains associated with endocytic vesicles, suggesting a signaling
function for p85/PI3-kinase in this particular intracellular compartment (24). This
hypothesis is illustrated in Figure 2.
Figure 2. Relationship between sub-cellular localization of c-Kit receptors and PI3-kinase activation.

In this model, internalized c-Kit receptors interact with recruited p85/p110 complexes. However, only in the case where receptors are subsequently routed into endosomes, such as with KitWT but not Kit+ receptors, will downstream elements of the PI3-kinase pathway become activated.
Figure 2.

- D3 phosphoinositides
- Akt
- PKC
- (Kit+)
- cell survival
- p70 S6 kinase activation
- PKC isoform (ζ) activation
In order to test this hypothesis, both KitWT and Kit+ receptors would be stimulated with SLF and cellular Akt would be immunoprecipitated and measured in vitro for kinase activity. If it is true that later endocytic steps are required for activation of downstream PI3-kinase targets, Akt precipitated from stimulated Kit+ 32D cells should be catalytically inert whereas Akt from KitWT 32D cells should be active. This observation would explain why stimulation of Kit+YF728 receptors with sSLF is not mitogenic. Without both downstream PI3-kinase signals and PLC-γ signals, these cells remain unresponsive to ligand.

An alternative strategy to examine the activity of either Kit or Kit+-associated PI3-kinase activity would be to evaluate the location of D3 phosphoinositides in stimulated Kit or Kit+ expressing cells. Green Fluorescence Protein (GFP) has been used in conjunction with PH domains to evaluate the location of PI3-kinase lipid products in stimulated cells (25). As described in Chapter 1, D3 phosphoinositides bind to the PH domains of a variety of intracellular proteins such as Akt. Using a recombinant Akt PH domain fused to GFP, the location of these lipids could be evaluated. In Kit+ cells, if a defect in Akt activation is observed, a correlating alteration in D3 phosphoinoside location may be what is responsible for this defect. These experiments would further confirm a requirement for receptor endocytosis in PI3-kinase downstream signals.

It is also possible that the very first steps of receptor internalization may be required for p85 recruitment to the c-Kit receptor. In Chapter 3, immunoprecipitation of c-Kit from Kit+YF728 32D cells stimulated with SLF revealed very little associated p85, although the receptor was tyrosine phosphorylated. In addition, the Kit+YF728 receptor was found to not internalize. Thus, a model for PI3-kinase activation in which recruitment requires the first internalization steps and activation of downstream elements requires subsequent endocytic steps would be tested. Preliminary data reveal that KitWT 32D cells stimulated
by plate-bound anti-c-Kit antibodies, which we have shown does not induce receptor
internalization (Chapter 3), results in tyrosine phosphorylation of these receptors.
However, under these circumstances, we have not been able to co-immunoprecipitate p85
with the tyrosine phosphorylated KitWT receptors (work in progress). This suggests that
receptor activation may be insufficient for p85 recruitment and that an initial internalization
step may be required. Further work needs to be done to confirm this hypothesis.

Therefore, these experiments will attempt to reveal the internalization/endocytic
requirements for PI3-kinase recruitment and activation. Given the confusion in the
literature as to whether PI3-kinase is necessary or sufficient for a number of biological
outcomes, a consideration of the subcellular localization of this enzyme under different
circumstances may be revealing.

Is there a role for soluble SLF in vivo?

The observation that Sl<sup>d</sup> mice, which only generate sSLF but not mSLF, exhibit the same
phenotype as Sl mice which do not generate SLF at all, suggests that the relevant
physiological form of SLF is the membrane-bound configuration (26). In vitro
experiments have demonstrated that mSLF but not sSLF supports the survival of the
murine myeloid cell line FDC-P1. Soluble SLF was found to support this cell line only in
combination with other cytokines such as GM-CSF. In addition, it has been demonstrated
that mSLF but not sSLF supports the long term maintenance of hematopoietic precursors,
whereas sSLF only supports short term survival of these cells. In vivo experiments have
also evaluated the differences between mSLF and sSLF. Kapur et al. have assessed the
effect of either membrane-bound or soluble forms of SLF expressed as transgenes in Sl<sup>d</sup>
mice. These authors demonstrated that only mSLF is able to support erythropoiesis in
vivo. Thus, these experiments suggest that mSLF is the relevant physiological form of the c-Kit ligand.

The mechanistic reason for why sSLF fails to stimulate these activities has not been identified, but may simply be due to adhesion between c-Kit positive cells and mSLF expressing fibroblasts or stromal cells (27). This adhesion would allow the developing c-Kit positive cells to receive differentiation, survival and growth signals from the neighboring stromal cells as well as synergistic signals from other factors in the developmental microenvironment. If our hypothesis that "non-internalizing" signals such as mSLF fail to induce the recruitment and/or activation of the PI3-kinase pathway is true, this would explain the frequent requirement for synergy between SLF and other growth factors which activate the PI3-kinase pathway such as IL-3. These PI3-kinase-mediated signals may therefore be critical for the development of precursor cells.

However, incubation of c-Kit positive cells in vitro with sSLF induces a strong mitogenic response, and injection of sSLF in vivo results in mast cell proliferation and maturation (28, 29). Thus, although sSLF may not function very well in vivo in the context of stromal cell-dependent development, it is indeed mitogenic. In order to further understand the in vivo role for sSLF, Kunisada et al. expressed SLF transgenes in mouse epidermal keratinocytes in order to evaluate the effect of expression of membrane vs soluble SLF on dermal mastocytosis. Expression of the wild type form of SLF which generates both mSLF and sSLF resulted in profound mastocytosis and epidermal pigmentation. In contrast, expression of a transgene cDNA which produced only mSLF resulted in local melanin production but failed to induce mastocytosis. Therefore, an overproduction of sSLF by keratinocytes may be a potential cause of mastocytosis disease. In addition, although sSLF is unable to promote differentiation of various precursors in vitro and in vivo, sSLF appears to be more effective than mSLF at inducing the proliferation of dermal
In Chapter 1, the role of mast cells in the immune system was discussed and several examples where mast cells play a protective role in immunity against bacteria were listed. In situations of acute sepsis such as those described by Prodeus et al., a strong proliferative burst of mast cells may be required to produce quantities of TNF-α in order to control the peritoneal infection (31). The recent identification of a mast cell produced chymase which specifically cleaves mSLF into its soluble form suggests the possibility of a positive feedback loop (32). Specifically, in situations such as peritonitis, mast cells will rapidly degranulate releasing a variety of mediators such as pre-formed neutrophil-recruiting TNF-α in addition to chymases which will cleave the mSLF on neighboring fibroblasts. The sSLF which is proteolytically generated would therefore induce potent proliferation of resident mast cells. The signals mediated by sSLF may be more potent than those mediated by mSLF if it is true that sSLF but not mSLF activates the PI3-kinase pathway. Thus, a strong amplification mechanism whereby sSLF is generated would assist in controlling the infection or sepsis. This hypothesis is represented schematically in Figure 3.

This hypothesis is not easily testable, however, with the recent identification of the specific chymase involved in mSLF cleavage, a chymase knock-out mouse could be constructed. Thus, chymase deficient mice, if viable, would be tested for their ability to resolve peritoneal infections. Alternatively, peritoneal injections of recombinant chymase may result in an improved response to peritoneal infections or sepsis. These two experimental approaches would further clarify the role of mast cells in immunity.

Mast cells have been often associated with responses which are deleterious to the host such as allergy and anaphylaxis. In this thesis, a role for mast cells in protective immunity...
against bacteria and parasites has been described and a role for c-Kit-mediated signals in
mast cell responses has been discussed. The experiments proposed in this Chapter should
lead to a greater understanding of c-Kit-mediated signals. Understanding these signals may
reveal potential therapeutic strategies for either controlling or enhancing the proliferation of
c-Kit positive cells.
Figure 3. The role of sSLF in peritoneal infections.

Resident peritoneal mast cells, when they come in contact with lipopolysaccharide positive bacteria, release stored mediators from intracellular granules. Among these mediators are TNF-α which strongly recruits neutrophils, as well as chymases which cleave resident mSLF into sSLF. sSLF then acts on peritoneal mast cells to induce further proliferation and degranulation.
Figure 3.

Peritoneal Cavity

Chymase Cleavage

mSLF

sSLF

c-Kit

stored granules

Mast Cell

Bacterial Contact

Released Mediators
- TNFα
- Chymases
- Histamine
- PDG2 etc.

Proliferation
References


