INTERLEUKIN-3 SIGNAL TRANSDUCTION: PURIFICATION AND CHARACTERIZATION OF THE MURINE INTERLEUKIN-3 RECEPTOR

by

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ABSTRACT

Murine interleukin-3 (mIL-3) is a potent hemopoietic growth factor that is produced primarily by activated T lymphocytes and stimulates the proliferation and differentiation of pluripotent stem cells and committed myeloid and early lymphoid progenitors. In order to gain some insight into its mechanism of action, we set out to purify and characterize its cell surface receptor. To monitor this purification, an assay capable of detecting detergent solubilized mIL-3R's was devised. With this assay, a simple two step purification protocol, involving anti-phosphotyrosine Sepharose and biotinylated mIL-3/streptavidin agarose, was developed to purify the mIL-3R. This protocol was based, in part, on a previous observation in our laboratory that the mIL-3R becomes tyrosine phosphorylated upon ligand binding. Two forms of the receptor were obtained using this procedure; a tyrosine/serine phosphorylated 140 kD form that was greater that 98% pure, and a less pure, serine phosphorylated 120 kD form. Alkaline phosphatase treatment, chymotrypsin digestion and Western analysis using antibodies to the N-terminus of the 120 kD form established that, apart from phosphorylation differences, these two proteins were identical. Intriguingly, the 140 kD tyrosine/serine phosphorylated mIL-3R was exquisitely sensitive to proteolysis whereas the 120 kD receptor was not. This proteolysis was also shown to occur in intact cells in response to mIL-3 and took place rapidly at 37°C in the presence of lysosomal inhibitors. These observations suggest that this mIL-3 stimulated proteolysis occurs at the cell surface and could play an important role in the mechanism of action of mIL-3.

N-terminal amino acid sequencing and amino acid composition analysis of the purified mIL-3R showed it to be identical to an mIL-3 binding protein subsequently cloned by expression in COS cells. Examination of this sequence revealed no consensus kinase domains, indicating the tyrosine phosphorylation of the mIL-3R must be mediated by an associated kinase. Studies directed towards the identification and purification of this mIL-3R associated tyrosine kinase, as well as the characterization of other mIL-3R associated proteins that we

have identified, are presented. Our results to date have led us to propose a model of mIL-3R induced signal transduction that may also apply, to some extent, to other members of the hemopoietic receptor superfamily.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
β-ME	β-mecaptoethanol
B-mIL-3	Biotinylated murine interleukin-3
BSA	bovine serum albumin
c/mL	cells/mL
CNBr	cyanogen bromide
Con A	Concanavalin A
DMS	dimethyl suberimidate
DSS	disuccinimidyl suberate
DTT	dithiothreitol
Ep	erythropoietin
FCS	fetal calf serum
GAP	GTPase activating protein
gst	glutathione-S-transferase
IL-x	interleukin-x (x= 1-12)
IL-xR	interleukin-x receptor (x=1-12)
KLH	keyhole limpet hemocyanin
mGM-CSF	murine granulocyte-macrophage colony stimulating factor
mIL-3	murine interleukin-3
NP40	Nonidet P40
OG	octylglucoside
PBS	phosphate buffered saline
PI3-K(inase)	phosphatidylinositol 3-kinase
PLCγ	phospholipase Cγ
PMSF	phenylmethylsufonyl flouride
PSB	phosphorylation solubilization buffer
SA	streptavidin agarose
SDS	sodium dodecyl sulfate
SF	steel factor
SH2	src homology domain 2
TBS	20 mM Tris-Cl, 0.15M NaCl, pH 7.4
TBST	20 mM Tris-Cl, 0.15M NaCl, pH 7.4, 0.05% Tween 20

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CHAPTER I

INTRODUCTION

A. HEMOPOIESIS

Hemopoiesis is the process by which a pluripotent, hemopoietic stem cell proliferates and differentiates to generate all the functional end cells circulating in the blood stream. Since blood cells have a limited life span they must be continuously replaced throughout life. In addition to maintaining this steady state, the hemopoietic system must also be capable of responding to hematologic or immunologic insults by rapid production of appropriate cells. Control of this process is achieved by a complex regulatory system consisting of direct cell-cell interactions as well as soluble regulatory molecules.

1. Cells of the Hemopoietic System

The hemopoietic system can be functionally divided into a hierarchy of four cellular compartments characterized by decreasing self-renewal capability and increasing differentiative state.[1] At the top of this hierarchy (Figure 1) are the pluripotent hemopoietic stem cells which are characterized by extensive self-renewal capacity and unrestricted differentiation potential. These cells differentiate to give the more committed progenitors, of both myeloid and lymphoid lineages, which are more limited in their differentiative capacity. Both stem cells and committed progenitors lack distinct morphological characteristics and can only be detected by their functional properties. These progenitors divide and differentiate further to give rise to the morphologically identifiable, immediate precursors of the mature blood cells.

Stem cells, in the adult, are primarily located in the bone marrow, having migrated there from developmentally earlier sites of hemopoiesis in the embryonic yolk sac and fetal liver.[2] Totipotent stem cells are characterized functionally by pluripotentiality, self-renewal capacity

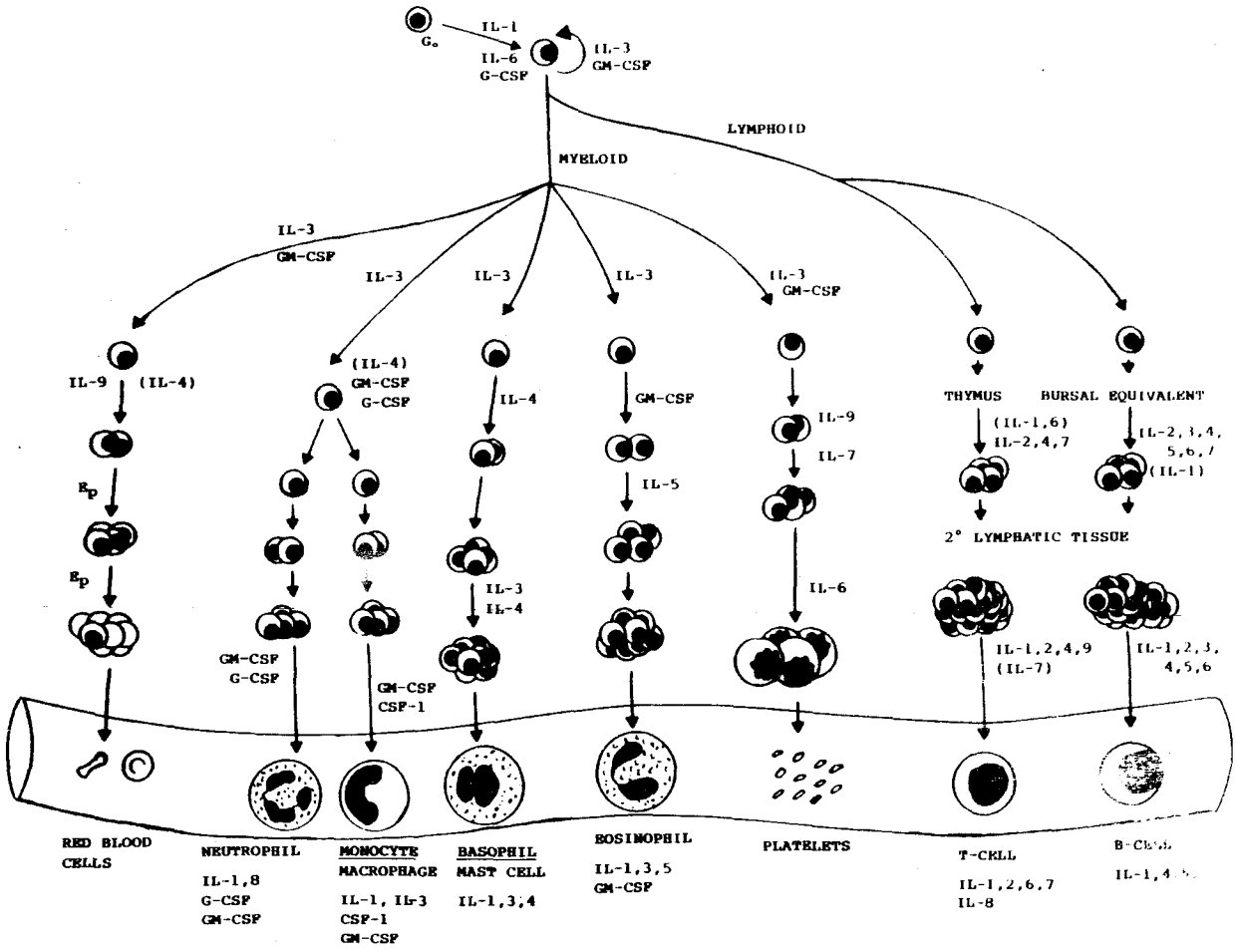


Figure 1. A schematic of the hemopoietic hierarchy. Also shown are some of the hemopoietic growth factors and where they are thought to act (from reference 32)

and high proliferative potential.[3] In the murine system, the existence of totipotent, lymphomyelopoietic stem cells was shown in experiments examining the long term hemopoietic reconstitution of lethally irradiated mice by genetically marked bone marrow cells.[4,5] In humans, evidence for a similar cell, with both lymphoid and myeloid potential, derives principally from studies of patients with hematologic disorders. For example, the Philadelphia chromosome (Ph[1]), a diagnostic, cytogenetic abnormality associated with chronic myelogenous leukemia (CML), is observed in both myeloid and B-lymphoid tissues of most CML patients.[6,7] In addition, examination of the restriction fragment length polymorphisms within the X-linked hypoxanthine phosphoribosyl transferase gene of a patient after allogeneic bone marrow transplantation revealed long term monoclonal hemopoiesis of donor origin.[8]

Since stem cells are morphologically unremarkable, the study of stem cells has depended on assays based on their biological characteristics. The first technique for measuring murine pluripotent stem cells, the spleen colony assay,[9] was based on the finding that intravenous injection of bone marrow cells into lethally irradiated, histocompatible mice, gave rise to macroscopic splenic nodules of hemopoietic tissue. Cytological[10] and chromosomal[11] markers showed that each nodule was derived from a single pluripotent cell. However, although some of the cells (referred to as CFU-S or Colony Forming Unit-Spleen) detected by this assay appear to be capable of hemopoietic reconstitution of lethally irradiated mice, several lines of evidence suggest that most cells detected by spleen colony assays are not capable of long term hemopoietic repopulation and thus are not equivalent to the most primitive toti-potent stem cell.[12] A more recent assay for this more primitive cell has been developed based on competitive long term reconstitution of lethally irradiated mice.[13] Similar *in vivo* assays for human stem cells are obviously not available. However, two *in vitro* assays have been reported that detects very primitive human hemopoietic cells with stem cell properties.[14,15]

With these assays, various questions have been examined. Of particular interest is what influences stem cell self-renewal versus differentiation. Two theories regarding how this decision is made have been suggested.[16] In the instructive model, cells become committed to

a particular lineage as a consequence of instructive, microenvironmental signals. In contrast, the stochastic model proposes that commitment to differentiation is a random process determined by probabilities intrinsic to the stem cell. According to this second model, external regulatory factors merely permit the growth and amplification of a committed cell. Indeed, evidence for this random commitment of progenitor cells has come from experiments in which paired daughter cells are separated by micromanipulation.[17] Despite being placed into identical culture conditions, the daughter cells develop independently down different lineages in a stochastic manner. However, aspects of the first, deterministic, model may also be correct since extrinsic factors may change the stem cell's intrinsic probability of commitment to a specific lineage.[1]

In contrast to stem cells, the committed progenitors within the next compartment are in active cell cycle. In addition, these more developed progenitors have a diminished capacity for self-renewal and proliferation and are committed to specific lineages. However, like stem cells, progenitors do not possess morphologically distinct characteristics. They can only be identified by the differentiated progeny they produce when cultured *in vitro*. To assay for progenitors,[3] cells are suspended in semi-solid medium (such as agar, methylcellulose or plasma clot) supplemented with serum and hemopoietic growth factors (see below). The lineage specificity of a progenitor can then be recognized by the colonies of morphologically recognizable progeny they produce.[3] A progenitor that produces a colony consisting of granulocytes and macrophages is termed a colony forming unit-granulocyte/macrophage (CFU-GM). Similarly, progenitors giving rise to granulocytes, macrophages or megakaryocytes are called CFU-G, CFU-M or CFU-Meg respectively. Three types of erythroid progenitors have also been defined[18] based on colony size, composition and time of maturation. The most primitive progenitors produce multi-clustered erythroid colonies, or "bursts" and are called BFU-E (burst forming unit erythroid). These are further classified as either primitive of mature depending on the number of colonies (>8 or 3-8 respectively) in the burst. Progenitors which give rise to single or paired clusters of erythroblasts are called CFU-erythroid (CFU-E). In addition to these

unipotent or bipotent myeloid progenitors, cells have been detected which produce mixed colonies of granulocytes, erythrocytes, macrophages and megakaryocytes (CFU-GEMM).[18] Lymphoid progenitors have also been described.[19]

The committed progenitors differentiate further to produce cells of the quantitatively largest hemopoietic compartment. These cells are morphologically recognizable and consist of two classes. The first contains precursor cells, such as myeloblasts and normoblasts, which have restricted proliferative potential. These terminally differentiate to produce the second class of fully differentiated, mature cells which are then released into the circulation to carry out their designated functions.

2. Control of Hemopoiesis

During normal hemopoiesis, the levels of each cell type is maintained within strict limits. However, during stresses such as blood loss or infection, the hemopoietic system rapidly responds by producing the necessary cells. This exquisite regulation is mediated by two interacting systems of stromal and humoral control.

2.1 Stromal control of hemopoiesis

Hemopoietic cells exist in intimate contact with a specialized microenvironment consisting of stromal cells and extracellular matrix (ECM) molecules. The stromal cell population includes fibroblasts, endothelial cells, adipocytes, osteoblasts and osteoclasts.[16] Fixed marrow macrophages, although they are derived from hemopoietic stem cells, are also considered part of the stroma. These stromal cells produce and maintain a lattice-work of extracellular proteins which include collagen types I and IV, glycosaminoglycans, fibronectin, laminin[16] and hemonectin.[20] Hemopoietic cells nestle in this network and bind to the extracellular matrix through specific adhesion receptors.[21]

The contribution of the stroma to hemopoietic regulation is suggested by a number of observations. Firstly, the cellular composition of spleen cell colonies varies with their location in the spleen. Granulocytic colonies tend to form along splenic trabeculae while erythroid or mixed lineage colonies localize to the splenic capsule.[22] Secondly, examination

of bone marrow ultrastructure, similarly revealed specific localizations for certain cells such as CFU-GEMM.[23] Evidence for the supportive nature of the stoma is also observed *in vitro*. When bone marrow cells are cultured for extended periods of time, stromal cells establish an adherent layer of cells and ECM at the bottom of the long term culture dish. Hemopoietic cells can be maintained on this stromal layer and differential localization of primitive progenitors on this layer has been observed.[24] In addition, the mIL-3 dependent cell line, FDCP-mix, will proliferate and differentiate when grown on an irradiated stromal layer in the absence of any exogenously added growth factor.[25]

One of the ways by which stromal cells might influence hemopoiesis is through local production of humoral factors. In fact, as discussed in the next section, stromal cells such as fibroblasts, endothelial cells and macrophages can produce soluble growth regulatory molecules. Intriguingly, the manner in which the stroma presents these factors may be an important aspect of stromal regulation of hemopoiesis. Some humoral factors such as interleukin-3 (IL-3),[26] granulocyte/macrophage colony stimulating factor (GM-CSF)[27] and leukemia inhibitory factor (LIF)[28] are bound and sequestered by glycosaminoglycans in the ECM. The binding of factors by the ECM may serve to store, localize or regulate the supply of factors that act on hemapoietic cells. In addition, at least two growth factors which were originally characterized as soluble molecules, macrophage colony stimulating factor (M-CSF)[29] and Steel factor (SF)[30] are also synthesized as bioactive, membrane bound forms. Importantly, at least in the case for SF, the membrane bound form is absolutely essential for normal hemopoietic development, ie., mice with a Steel mutation (Sl^d) that results in the sequences encoding the transmembrane and intracellular regions to be deleted suffer from macrocytic anemia even though these mice produce soluble SF.[30] Melanogenesis and gametogenesis are also affected since SF is involved in these pathways during development as well. These findings indicate that one of the roles of the stroma is to present SF, and perhaps other molecules, as a cell bound protein. However, it is important to keep in mind that the stoma may also serve other purposes which are not yet as well characterized. For example, the

binding of hemopoietic cells by the stroma may limit access to soluble factors, or regulate the release of cells into the circulation.

2.2 Humoral control of hemopoiesis

The initial identification of humoral factors that could modulate hemopoiesis came from studies of the *in vitro* culture requirements of hemopoietic cells. Hemopoietic cells will not grow, or form colonies of recognizable cells as described above, unless the culture is supplemented with conditioned media from a variety of tissues or cell lines.[31] Biochemical purification of these colony stimulating activities (CSF's, Table 1) led to the identification of glycoproteins defined by their ability to support the growth of certain colony types. Subsequently, many more cytokines (Table 1) which act on the hemopoietic system, but do not necessarily support colony formation, were discovered using different types of assays. Importantly, a number of negative regulatory proteins have also been identified (Table 2).

As can be seen from tables 1 and 2, a large number of factors act on hemopoietic cells. This number continues to increase as new sources are tested for activities or new assays for analyzing hemopoietic cells are developed. None of these cytokines, except for IL-6, G-CSF and LIF, share significant primary sequence homology. However, the cytokines that act on the hemopoietic system can be summarized as follows:

1. They are all glycoproteins. The carbohydrate is typically not required for bioactivity, but glycosylation does contribute to *in vivo* stability.
2. They are extremely potent, with activities in the pM range. The notable exception is SF, which as discussed above, probably exists physiologically as a membrane bound form.
3. Many factors, especially IL-3, are not lineage restricted and act on many cell types. As a general rule of thumb, the earlier discovered factors (the lower numbered interleukins) have the widest range of activities.
4. Many cytokines have overlapping activities. However, their *in vivo* functions may be quite distinct. For example, IL-3 and GM-CSF both support the growth of

granulocyte/macrophage precursors, but whereas IL-3 is produced during an immune response by activated T cells, GM-CSF may be produced constitutively by stromal cells.

5. Most cells respond to more than one cytokine. The ability of most hemopoietic cells to respond to more than one factor is the basis of the intricate cytokine networks observed *in vivo*[43] which confers great complexity and specificity to the regulation of hemopoiesis.

Not listed in Tables 1 and 2 are indirect acting factors, such as platelet derived growth factor (PDGF), which stimulate stromal cells to produce factors, such as IL-1, which then act directly on hemopoietic cells.

Murine interleukin-3 (mIL-3), the subject of this thesis, is a 14-30 kD glycoprotein, that is produced by activated T cells[44] or IgE cross-linked mast cells.[45] Recently the mRNA for mIL-3 was detected in neural tissue,[46] however, whether protein is actually produced is not clear. Murine IL-3 supports the proliferation and differentiation of pluripotent stem cells as well as a variety of committed myeloid and lymphoid progenitors. The action of mIL-3 on primitive cells, however, is restricted to those cells which are already in cycle; thus the ability of IL-6 and SF to bring quiescent cells out of G_0 is believed to be the basis of the synergy observed between mIL-3 and IL-6[47] or SF[48,49] in many systems. Murine IL-3 also exerts actions on more mature cells. For example, it has been shown to induce thy-1 antigen expression on committed myeloid cells[51] and stimulate mature macrophage cytoxicity. [50]

Since the physiological sources of mIL-3 are likely restricted to activated mast and T cells, this factor is probably not involved in the regulation of normal, steady state hemopoiesis. In fact, of the growth factors listed in Table 1, only GM-CSF, SF, IL-7 and CSF-1 have been shown to be constitutively produced. As noted above, GM-CSF, SF and CSF-1 are intimately associated with the stoma as membrane or ECM bound forms. These observations suggest that steady state hemopoiesis is controlled predominantly by stromal elements while humoral factors become important during times of hematologic or immunologic stress.

Table 1. Hemopoietic Growth Factors

Factor	Abbreviation	Cellular Source	Cellular Targets
erythropoietin	Ep	renal peritubular cells	erythroid
granulocyte CSF	G-CSF	macrophages, endothelial cells fibroblasts	neutrophils
granulocyte/ macrophage CSF	GM-CSF	activated T cells endothelial cells fibroblasts	granulocytes macrophages early progenitors
macrophage CSF	M-CSF (CSF-1)	fibroblasts endothelial cells	monocyte/macrophages
leukemia inhibitory factor	LIF	stromal cells	early progenitors
steel factor	SF	fibroblasts	mast cells early progenitors
interleukin-1	IL-1	macrophages	co-stimulator of T cells and progeni
interleukin-2	IL-2	activated T cells	B and T cells
interleukin-3	IL-3	activated T cells activated mast cells	most myeloid lineages
interleukin-4	IL-4	activated T cells	B,T and mast cells
interleukin-5	IL-5	activated T cells	B and T cells eosinophils
interleukin-6	IL-6	activated T cells	B and T cells early progenitors
interleukin-7	IL-7	stromal cells	T and pro B cells
interleukin-9	IL-9	activated T cells	myeloid cells progenitors
interleukin-10	IL-10	activated T cells	mast, B cells
interleukin-11	IL-11	stromal cells	granulocytes, macrophages megakaryocytes
interleukin-12	IL-12	activated B cells	T cells

references:[3,32-38]

Table 2. Hemopoietic Inhibitory Factors

Factor	Abbreviation	Cellular Source	Cellular Targets
interleukin-1 receptor antagonist	IL-1ra	macrophages	IL-1 responsive cells
macrophage inflammatory protein-1α	MIP-1α	macrophages	early progenitors
tumour growth factor β	TGFβ	platelets	myeloid cells early progenitors
interferon γ	IFNγ	T cells	most mature cell types

References:[39-42]

B. HEMOPOIETIC GROWTH FACTOR RECEPTORS

Murine IL-3, as described above, possesses a wide range of activities depending on the responding cell. The activity that we chose to investigate was the ability of mIL-3 to support proliferation. Since the first step in growth factor action is the ligand induced activation of its cell surface receptor, the first step in studying mIL-3 signal transduction is the study of its receptor.

At the time the studies described in this thesis were initiated, the colony stimulating factor-1 receptor(CSF-1R) and c-kit were the only hemopoietic receptors which had been characterized at the molecular level. Both of these are receptors with intrinsic tyrosine kinase activity. During the past two years, however, many hemopoietic growth factor receptors have been cloned and it has become apparent that although the hemopoietic growth factors are not related in primary structure, many of their receptors are. Indeed, many of the cytokine receptors are now classed together in the hemopoietin receptor superfamily. Thus, the two major classes of receptors present on hemopoietic cells are the intrinsic tyrosine kinase receptors and the members of the hemopoietin receptor superfamily.

1. Intrinsic Tyrosine Kinase Receptors

Many cell surface receptors resemble allosteric enzymes with an external regulatory, ligand binding domain and an intracellular, catalytic domain. Examples of these receptors include the activin[52] and the *Drosophila*, daf-1[53] receptors which contain an intrinsic serine kinase and the atrial natriuretic receptor[54] which harbours an intrinsic guanylate cyclase activity. However, the most common and best studied receptors with intrinsic enzymic activity are those with tyrosine kinase activity. These include the receptors for EGF, PDGF and insulin. Among the hemopoietic growth factors, at least three utilize tyrosine kinase receptors.

1.1 Structure of tyrosine kinase receptors

Tyrosine kinase receptors have been well characterized because many of the earliest discovered polypeptide growth factors, such as epidermal growth factor (EGF), PDGF and insulin, possess receptors of this type. Based on structural features, all tyrosine kinase

receptors can be clasified into three groups (Figure 2).[55] The first group consists of receptors with only a single polypeptide chain. The extracellular, ligand binding domain of these receptors contain two cysteine rich repeats. The EGF receptor (EGFR), the neu proto-oncogene and the *Drosophila sevenless* protein are examples of this first group. In addition, the product of the *erb-B2* oncogene encodes a truncated version of the EGFR. The second class of receptors include those for insulin and insulin-like growth factor 1 (IGF-1). These receptors are heterotetrameric with two α and β disulfide bonded subunits. The α subunits compose the ligand binding site, and each contain one of the cysteine rich repeats also found in the type I receptors, while the kinase domain is located on the β chains. The PDGF receptor (PDGFR) typifies the third class of receptors. These receptors are single chain polypeptides with immunoglobulin (Ig) like repeats in the external domain. Comparison of the catalytic domain of this class of receptors with those of the other two, revealed that an insert region separates the ATP binding site from the phosphotransferase domain. The sequence of this kinase insert (KI) domain is quite heterogeneous for different growth factor receptors in this class. However, for the same receptor, the KI region is very well conserved among different species.[56] Other members of this last class of tyrosine kinase containing receptors include the hemopoietic receptors CSF-1R,[57] *c-kit*[58] and *flk-2*.[59]

The CSF-1R, expressed on cells of the mononuclear phagocyte lineage,[60] is the cellular homologue of the transforming gene of the McDonough strain of feline sarcoma virus, *v-fms*.[61] The activated *v-fms* differs from the cellular gene, *c-fms*, by having a point mutation in the extracellular domain and a short cytoplasmic truncation.[62] Both of these alterations contribute to the constitutively activated kinase activity, and thus the transforming potential of *v-fms*. The *c-kit* protein is the receptor for SF.[63] Approximately 10% of all bone marrow cells and most hemopoietic progenitors express *c-kit*.[58] A transforming version of *c-kit*, *v-kit*, is found in the Hardy-Zuckerman-4 strain of feline sarcoma virus and differs from *c-kit* by deletions within both the ligand binding and transmembrane region. The *c-kit* gene is allelic with the murine W (white spotting) locus. Mice with mutations at the W locus exhibit

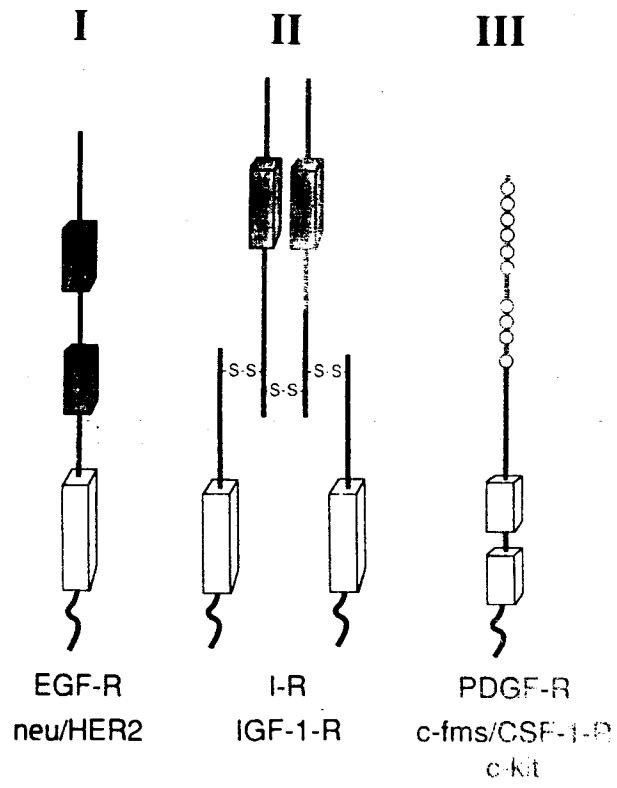


Figure 2. The three classes of tyrosine kinase containing receptors. Shaded boxes represent the cysteine rich domains; clear boxes designate the kinase domains, which are split by an insert region in receptor class III. The circles represent the conserved cysteines in the Ig domains of the third receptor. (from reference 55)

defects in hemopoiesis, melanogenesis and gametogenesis; these defects resemble those observed in mice with mutations at the Steel locus. Complementation analysis with reconstitution experiments and finally with the purification of the Steel gene product[63] showed that the *c-kit* protein is the receptor for the Steel ligand. The most recently discovered tyrosine kinase receptor in hemopoietic cells is *flk-2*. Unlike, *c-fms* or *c-kit*, *flk-2* was not discovered by its homology to any viral oncogenes. Instead, *flk-2* was identified during a deliberate search for tyrosine kinases that are expressed in hemopoietic cells.[59] Although, *flk-2* has only been characterized to date at the DNA level, the fact that its expression is restricted to stem cell and progenitor populations makes it and its, as yet, unknown ligand potentially important in hemopoietic regulation.

1.2 Signalling from receptors with intrinsic tyrosine kinase activity

From extensive studies with the classical tyrosine kinase receptors, ie., the EGFR, insulinR and PDGFR, a model by which these receptors transduce signals across the membrane has been proposed. Specifically, the binding of a ligand to the external domain results in the activation of the cytoplasmic tyrosine kinase domain.[64] This activation, which reflects an increase in the V_{max} of the phosphorylation reaction,[65] is an essential step in the receptor signalling since receptors with defective kinase domains can not signal.[55,66] Accompanying receptor activation is autophosphorylation of the receptor itself.

In an early model of receptor action, the binding of a ligand to the external domain was thought to induce a conformational change that is transmitted through the transmembrane region to activate the kinase. However studies with chimeric receptors[67,68] or receptors with altered transmembrane domains[69] suggested that the transmembrane segment does not function in this manner. A large body of evidence now supports a mechanism in which receptor activation occurs as a result of ligand induced oligomerization. For example, certain anti-insulinR antibodies mimic the effects of insulin and this insulinometic property depends on antibody divalency.[70] Also, the isolated ectodomains of the EGFR have been observed to undergo dimerization upon the addition of EGF.[71,72] In the case of the insulin and EGF

receptors, dimerization may be the result of ligand induced conformational changes. However, the ligands for PDGFR, CSF-1R and *c-kit* are dimeric so one ligand molecule may bridge and thus bring together two receptor molecules.[73-75] The involvement of receptor clustering has been proposed for the basis of the constitutive kinase activity of the oncogenic version of *neu*. Oncogenic *neu* differs from the normal cellular version by a point mutation in the transmembrane domain and this mutation apparently causes oncogenic *neu* to exist as aggregates in the absence of ligand stimulation.[76]

One result of receptor clustering is the transphosphorylation of other receptor molecules.[77] Indeed, the demonstration that receptor autophosphorylation is primarily an intermolecular,[78,79] rather than an intramolecular, reaction further strengthens the dimerization model of receptor activation. Recent studies suggest that receptor autophosphorylation is a key event in receptor signalling. Several proteins implicated in signal transduction, including the $p74^{raf}$, phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3-K) and *ras* GTPase activating protein (GAP), have been shown to physically associate with tyrosine phosphorylated receptors.[80-82] The function of these proteins in signal transduction will be discussed in later sections. Since the initial demonstration that $p74^{raf}$ binds the PDGF receptor,[83] the number of reports showing association of receptor and signalling proteins has grown exponentially (Table 3).

Table 3. Molecules Associated with Intrinsic Tyrosine Kinase Receptors

	PI3-K	GAP	PLCγ	$p74^{raf}$
EGFR	+	-	+	+
PDGFR	+	+	+	+
InsulinR	+	?	-	-
CSF-1R	+	+	-	-
c-kit	+	?	+	?

References in text. Association is designated with a + or - sign. The ? refers to cases where association is not known.

Intriguingly, with the exception of $p74^{raf}$, the proteins which bind to intrinsic tyrosine kinase receptors all contain SH2 domains (Src Homology region 2).[84] SH2 domains are approximately 100 amino acids in length and were first described as regions of homology in cytoplasmic tyrosine kinases such as src and were subsequently found in other, non-kinase proteins involved in signal transduction. The SH2 domains of PLCγ, PI3-K and GAP bind to specific phosphotyrosines in the appropriate receptor.[80,84,85] PLCγ and GAP each have two adjacent SH2 domains and these act synergistically in binding.[86] The raf polypeptide does not have an SH2 motif, so it either associates with a protein that does contain an SH2 domain or binds receptors through an alternate mechanism. It is important to note, however, that although many early intermolecular interactions associated with signal transduction are mediated through SH2 domains, not all may be. For example, GAP has been shown to associate with 190 and 62 kD tyrosine phosphorylated proteins in mitogen activated or transformed cells.[84] Although pp62 binds to the GAP SH2 motif, pp190 binds some other region in the molecule.[85] In addition, although PLCγ binds tightly to tyrosine phosphorylated PDGFR's through its SH2 domains, PLCγ is loosely associated, presumably through an SH2 independent mechanism, with the receptor prior to activation (see below).

The structural requirements in the receptor for the binding of signalling proteins has also been studied. Phosphorylation of tyr-706 in the kinase insert region of the CSF-1R is important for PI3-K binding. Similarly, tyr-857 in the PDGFR appears to be specific for GAP association.[87] Interestingly, deletion of tyr-809 in the human CSF-1R specifically uncouples the CSF-1R to the pathway leading to the elevation of *c-myc* gene expression and impairs the mitogenic response to CSF-1. This suggests that a protein specific to the signalling pathway leading to *c-myc* elevation associates with the CSF-1R through tyr-809[88] since all other early cellular signalling pathways appear to be intact. This finding also illustrates the importance of the discovery that receptor tyrosine phosphorylation followed by association with other proteins

is a key event in signal transduction. Namely, mutagenesis of specific tyrosines in the receptor may allow identification of proteins which link receptors with intracellular signalling pathways.

2. The Hemopoietin Receptor Superfamily

Unlike the receptors with intrinsic tyrosine kinases, the other receptors on hemopoietic cells are normally present only in low numbers. Because of this low abundance, most of the members of the hemopoietin superfamily, with the exception of the mIL-3R (this work) and the IL-4R,[89,90] were isolated using cDNA cloning and COS cell protein expression systems.[91] In this method, COS cells are transfected with pools of cDNA's from an appropriate source and the COS cells allowed to express the proteins encoded by the cDNA. Pools that contain the cDNA coding for the receptor of interest are identified by screening the transfected COS cells with either an anti-receptor antibody, if available, or with labelled ligands. The human IL-6R α subunit was the first to be cloned in this manner. The EpR and IL-2R β chain were isolated a couple of years later. In the past year, however, more than 10 hemopoietin superfamily receptors have been cloned (Figure 3C). Thus considerable information regarding the structure of this family of receptors has recently been accumulated. However, knowledge of the signalling mechanisms utilized by these receptors is still at a nascent stage.

2.1 Structure of the hemopoietin receptors

Some of these receptors consist of two subunits, one or both of which may belong to the hemopoietin superfamily, while others only have one. The common feature of all members of the superfamily is a 210 amino acid module in the extracellular domain.[92] This module (Figure 3A) is characterized by a W-S-X-S-W (single letter codes, X=any amino acid) sequence near the C-terminal and a motif of four spatially conserved cysteines $CX_{9\text{-}20}$-CXW$X_{22\text{-}36}CX_{8\text{-}25}$C near the N-terminal. A fibronectin type III domain is also found in the C-terminal region which has led to speculation that these receptors are evolutionarily derived from primitive adhesion molecules.[93] From an analysis of the primary amino acid sequence, Bazan[94] has suggested that the extracellular hemopoietin domains fold into a

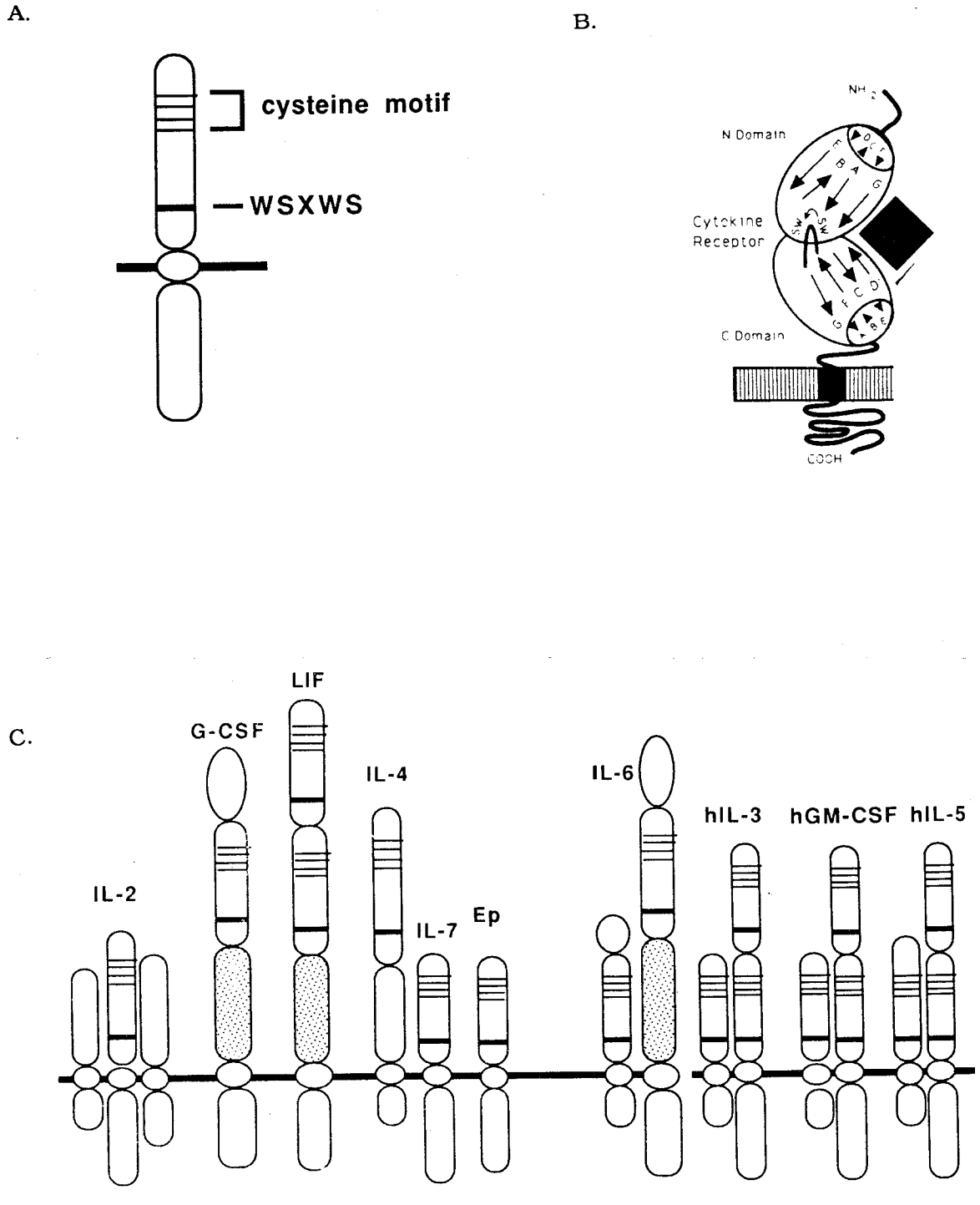


Figure 3. Structure of the hemopoietin receptors. The hemopoietin consensus domain is shown in 3A. Figure 3B shows the barrel model for receptor folding; the black box represents a ligand.[94] The members of the hemopoietin receptor superfamily are displayed in 3C. In Fig. 3C, the stippled segment represents a contactin domain.

double barrel configuration, with each barrel consisting of seven β strands (Figure 3B). The ligand binding site is predicted to be located in the crevice between the two barrels. This model has been substantiated, to some extent, by the finding that replacement of amino acid residues in the putative hinge region between the two barrels, greatly diminishes ligand binding.[95] The WSXWS motif has also been shown to be important in ligand binding.[96]

Analysis of the ligand binding characteristics of the hemopoietin receptors in native, hemopoietic cells revealed that while some, ie. the G-CSF receptor, exhibit only one high affinity class, others, such as the Ep receptor, exhibit both high and low affinity binding. However, not all of the cloned receptors, when expressed in an ectopic cell such as the fibroblast COS cell, demonstrate the same ligand binding characteristics as the intact receptor on hemopoietic cells. This anomaly suggests that either, the receptor protein is modified in hemopoietic cells in a manner that can not occur in non-hemopoietic cells or that the receptor contains additional protein subunits present in hemopoietic cells which are absent in COS cells. Thus the hemopoietin receptors that have been cloned, thus far, can be divided into two groups based on whether the cloned molecules display native ligand binding characteristics. Examples of receptors for which all of the components contributing to ligand binding have been isolated include the receptors for G-CSF, GM-CSF, IL-4, IL-5, IL-6, IL-7 and <u>human</u> IL-3. In contrast, the receptor structures for erythropoietin (Ep), leukemia inhibitory factor (LIF) IL-2 and <u>murine</u> IL-3 are still under investigation. The following section summarizes our knowledge, to date, of the hemopoietin receptors. The receptors for which the structure has been "solved", including the ones for G-CSF, IL-4, IL-6 and IL-7, will be discussed first. Following these will be the "unsolved" receptors for erythropoietin, leukemia inhibitory factor and IL-2. Finally, the murine interleukin-3 receptor will be discussed together along with the murine IL-5, GM-CSF and human IL-3, GM-CSF and IL-5 receptors. These last receptors are treated together, even though the murine IL-3R is the only one of the group for which the native structure is not yet known, because they are inextricably linked by the way they were cloned.

2.1.1 Hemopoietin receptors with solved structures

Granulocyte-colony stimulating factor receptor (G-CSFR). Although normal hemopoietic cells and cell lines express a single high affinity class (120 pM) of G-CSFR's, solubilization of this receptor generates an additional low (3 nM) affinity class. However, sucrose density gradient centrifugation has shown that the high affinity receptors are dimers which dissociate to give low affinity binding monomers.[97] The mouse 130 kD G-CSFR was cloned by expression with radiolabelled ligand and expresses high affinity binding when expressed in COS cells.[98] In addition to the typical hemopoietin receptor domain, the G-CSFR also has an N-terminal, Ig like domain which is not involved in G-CSF binding or biological activity[99] and a contactin domain near the transmembrane region. A related human protein with 63% amino acid identity has been isolated. Interestingly, in human cells, two mRNA splice variants of the G-CSFR exist. One contains a 27 amino acid insertion in the cytoplasmic domain which may alter its signalling characteristics, while the second encodes a truncated form of the receptor that retains only the N-terminal ligand binding domain. In fact, the G-CSFR is only one of many hemopoietin receptors that can exist as soluble molecules.

Interleukin-4 receptor (IL-4R). Only one high (20-80 pM) affinity class of IL-4R has been observed on hemopoietic cells.[100] Initial cross-linking experiments suggested that ^{125}I labelled IL-4 bound to two proteins with apparent molecular weights of 80 and 150 kD. However, the lower molecular weight form was shown to be a proteolytic fragment of the larger species.[101] Murine[102,103] and human[100] IL-4R's have been isolated which possess 50% homology at the amino acid level. Analysis of the requirements for signalling demonstrated that the terminal 400 amino acids of the cytoplasmic domain can be deleted with no effect on activity.[92] Like the G-CSFR, a mRNA species that encodes a soluble IL-4 binding domain has been described.[102]

Interleukin-6 receptor (IL-6R). Both subunits of the IL-6R belong to the hemopoietin receptor superfamily. The 80 kD α chain has an N-terminal Ig domain in addition to the hemopoietin receptor module and binds IL-6 with low affinity.[104] The 130 kD β subunit

resembles the G-CSFR, with one copy of the hemopoietin domain and a membrane proximal contactin domain.[105] Reconstitution of the α with the β chain produces high affinity binding.[106] Interestingly, the α and β chains do not exist as preformed complexes and only associate, through interactions of their extracellular domains, in the presence of ligand.[106]

Interleukin-7 receptor (IL-7R). Unlike the G-CSF and IL-4 receptors, the IL-7R exhibits two affinity classes on hemopoietic cells. Since the cloned 80 kD murine protein[90] also produces two affinity classes when expressed in COS cells, the high affinity class is thought to be a result of dimerization. The human and mouse receptors share 64% amino acid homology; a soluble form of the IL-7R has also been detected.[90]

2.1.2 Hemopoietin receptors with unsolved structures

Erythropoietin receptor (EpR). The cDNA encoding a 65 kD murine EpR was cloned from an erythroleukemia cell line[107] and a human homologue was isolated which is 82% identical to the murine protein at the amino acid level.[108] Although the EpR is the best studied of the hemopoietin receptors, probably because it was one of the earliest to be cloned,[107] there is some disagreement in the literature as to whether there are one or two affinity classes of receptors.[109] When expressed in fibroblasts, the EpR exhibits one high affinity (200 pM) class. However, chemical cross-linking studies with ^{125}I-Ep have revealed two bands, one corresponding to the 65 kD cloned EpR polypeptide, and another with an apparent weight of 100 kD. Cross-linking experiments with normal EpR bearing cells also yields similar, though not identical, bands. These experiments suggests that the EpR complex may contain additional proteins.

Mutagenesis of the cytoplasmic domain of the EpR has mapped a region likely to be important for mitogenesis to a membrane proximal region.[111] Provocatively, this region has a high degree of homology with the domain of the IL-2R β subunit[112] that has been shown to be important in signalling.

Leukemia inhibitory factor receptor (LIFR). The native LIFR displays low (1 nM) and high affinity (400 pM) binding. Since the 80 kD, LIF binding protein that was cloned binds LIF

with low affinity when expressed in COS cells, a second subunit has been postulated that is required for high affinity binding.[113] The 80 kD protein has two copies of the hemopoietin domain, and like the G-CSFR and IL-6R β chain, has a contactin domain near the transmembrane region. In fact the G-CSFR the 80 kD LIFR and the IL-6 β chain are the most closely related among the members of the hemopoietin receptor superfamily. This relationship is even more intriguing given the fact that the ligands for these three receptors also share some sequence similarity and exhibit similar spectra of activity on hemopoietic cells.[114]

Interleukin-2 receptor (IL-2R). From a comparison of the IL-2 binding characteristics of activated and resting T cells, it has long been postulated that the IL-2R consists of two subunits.[92] The 55 kd α subunit was cloned early in the history of cytokine receptors because of the availability of the Tac antibody and was shown to bind IL-2 with low affinity (10 nM) when expressed in COS cells. This 55 kD subunit does not belong to the hemopoietin receptor family and is not present on resting T cells. The other subunit, called the β subunit, is constitutively produced on T cells and binds IL-2 with intermediate affinity. Activation of T cells induces expression of the α subunit and together the two form a complex that binds IL-2 with high affinity (10 pM). Subsequently, a 75 kd β subunit was isolated. Unexpectedly, when this β chain was expressed in COS cells, it bound IL-2 with very low affinity (100 nM) and not with the expected intermediate affinity. However, when the β subunit was expressed in an oligodendroglioma cell line, it exhibited the expected intermediate affinity (2 nM) binding characteristics. These results have led to the speculation that the IL-2R actually consists of three subunits, ie., in resting T cells, the β exists as a complex with the putative γ subunit and together the two are responsible for intermediate affinity binding. This γ chain is absent from COS cells but is present in oligodendroglioma cells. Biochemical evidence for a 100 kD γ chain has been reported.[115,116]

2.1.3 The receptors for interleukin-3, GM-CSF and interleukin-5

Murine interleukin-3 receptor (mIL-3R). The mIL-3R is qualitatively very different from the human IL-3R (hIL-3R). For example, although mIL-3 is able to downmodulate surface

expression of the murine GM-CSF and G-CSF receptors, neither mGM-CSF, mG-CSF or other ligands can compete with mIL-3 for binding to the mIL-3R.[117] In contrast, in the human system, IL-3, IL-5 and GM-CSF compete with each other for binding to their receptors.[92,118]

An mIL-3 binding protein was cloned by COS cell expression[119] using an antibody that was believed to recognize the mIL-3R because of its ability to partially inhibit mIL-3 binding to mIL-3R expressing cells.[120] This 120 kD mIL-3 binding protein, called Aic 2A, has two copies of the hemopoietin receptor domain and when expressed in COS cells, binds mIL-3 with low affinity.[119] Because of the low affinity binding, a second subunit has been hypothesized to exist.[92] Interestingly, a second protein, called Aic 2B, with 91% sequence identity to Aic 2A was isolated during the cloning of Aic 2A.[121] This second protein is expressed in similar amounts to Aic 2A in all mIL-3 dependent cells tested. However, when expressed by itself in COS cells, Aic 2B does not bind mIL-3 or any other known ligand.

Murine interleukin-5 and GM-CSF receptors (mIL-5R and mGM-CSFR). These two receptors are discussed together since they share a common subunit responsible for high affinity binding. Both mIL-5 and mGM-CSF bind to normal hemopoietic cells with high and low affinities.[92] In 1990, a 60 Kd mIL-5 binding protein was cloned[122] which binds mIL-5 with low affinity when expressed in COS cells. This protein is a member of the hemopoietin superfamily. However, when this 60 kD protein was expressed in an mIL-3 dependent cell which normally does not bind mIL-5, high and low affinity mIL-5 affinity binding was observed. This suggested that a protein present in the mIL-3 dependent line could be the second subunit of the mIL-5R complex. This theory was confirmed when high and low affinity mIL-5 binding was reconstituted by co-expression, of the 60 kD mIL-5 binding protein and Aic 2B in COS cells.[106,123] Several lines of evidence suggest that Aic 2B also serves as the β subunit for the mGM-CSFR.[124]

Human interleukin-3, interleukin-5 and GM-CSF receptors. In an attempt to clone the hIL-3R, a human cDNA library was screened with probes based on the mIL-3 binding protein, Aic 2A. Only one protein was isolated,[125] but it did not bind any known factor when

expressed in COS cells. However, when co-expressed with the previously described low affinity receptors for hIL-5[126] or hGM-CSF,[127] high affinity binding of the appropriate ligand was observed.[126,127] Thus the human IL-5 and GM-CSF receptors share a common subunit that confers high affinity binding to the low affinity, ligand specific receptor subunits. Soluble forms of the hIL-5[126] and hGM-CSF[128] α subunits have also been described.

It had long been speculated that the receptors for human IL-3 and GM-CSF shared some common receptor component since their ligands showed a complex pattern of cross-competition.[118,129] Specifically, hIL-3 displayed only one class of high affinity binding sites and hGM-CSF was able to effectively compete with hIL-3 for binding to those sites. In contrast, hGM-CSF had both high and low affinity sites and hIL-3 was able to compete with GM-CSF for binding to these high affinity sites. From these observations, Kitamura suggested that the hIL-3R complex shares a β subunit with the hGM-CSFR and both hGM-CSF and hIL-3 receptor complexes have unique ligand specific α subunits.[130] However, although the hGM-CSF α subunit alone can bind to hGM-CSF with low affinity, the hIL-3 α subunit by itself can not bind hIL-3 at all. This model was confirmed with the expression cloning of the hIL-3R α subunit in COS cells.[130] The expression cloning was successful only because, the COS cells were co-transfected with the cDNA encoding the hGM-CSFR β subunit. The hIL-3R α subunit, like the hIL-5 and hGM-CSF α subunits are members of the hemopoietin receptor superfamily.

Other members of the hemopoietin superfamily. The *v-mpl* oncogene of the myeloproliferative murine leukemia virus, is capable of immortalizing hemopoietic progenitors and resembles a hemopoietin receptor.[131] The ligand for this putative receptor is not known. The prolactin and growth hormone receptors are also considered members of the hemopoietin superfamily although they lack the usual W-S-X-W-S motif.[92]

2.2 Signalling from the hemopoietin receptors

Studies of the mechanism by which the hemopoietin receptor family transmits signals across the membrane are only just beginning since most of the receptors have only been

recently cloned. However, from the work reported so far, it appears that the hemopoietin receptors may utilize mechanisms similar to the intrinsic tyrosine kinase receptors.

Dimerization, which is important in tyrosine kinase receptor signalling, may also be involved in hemopoietin receptor activation. For example, intact anti-prolactin receptor antibodies can mimic the effects of prolactin, but monovalent fragments can not.[132] Secondly, it has recently been shown that one molecule of growth hormone binds to two receptor molecules.[133] Thirdly, an activating point mutation in the EpR extra-cellular domain was shown to be a replacement of an arginine by a cysteine[110] and analysis of this mutation showed that replacement of this arginine by any amino acid except for cysteine did not activate the receptor. Furthermore, non-reducing SDS-PAGE suggested that dimerization through disulfide bonding was involved in receptor activation.

After dimerization, tyrosine kinase receptors autophosphorylate and become associated with signalling proteins. Although members of the hemopoietin receptor family do not have intrinsic tyrosine kinase domains, many become phosphorylated on tyrosine residues upon ligand binding. This phosphorylation must be mediated by an associated tyrosine kinase and both the growth hormone[134] and IL-2 receptors have been shown to be associated with tyrosine kinases.[135] Phosphorylation of the IL-2R on tyrosines following ligand binding may be the signal for the observed association of PI3-K,[136] a molecule better known for its association with intrinsic tyrosine kinase receptors.

Therefore, although structurally very unlike the better characterized tyrosine kinase receptors, the hemopoietin receptors may use many of the same signalling pathways. The following section discusses some of the proteins described in many systems to be involved in signal transduction.

C. PROTEINS INVOLVED IN SIGNAL TRANSDUCTION

The binding of a growth factor to its cell surface receptor induces many intracellular changes including: changes in ion fluxes,[137,138] cytoskeletal reorganization,[56] and protein phosphorylations.[139] These early signals lead to changes in gene expression[140] and

eventually culminate with the initiation of DNA synthesis and mitosis.[141] Some of the proteins involved in these changes have been characterized and they are discussed below.

1. GTP binding proteins

The members of the GTP binding protein superfamily, all of which bind and hydrolyze GTP, are involved in regulating information flow and ensuring fidelity of specific macromolecular interactions in diverse processes ranging from photoreception and hormone action to intracellular vesicle transport and ribosomal function.[142] Two members of this family that are implicated in signal transduction are the heterotrimeric G proteins and the low molecular weight, ras related GTP binding proteins. The mechanism of action of the heterotrimeric G proteins are well understood. In contrast, the regulators and function of the *ras* like proteins are not well characterized and our knowledge of *ras* protein function derives mostly from comparison of these proteins with the more familiar members of the GTP binding protein superfamily.

1.1 G proteins

The term G protein is reserved for the signal transducing GTP binding proteins composed of a 40 - 45 kD α, a 35 - 36 kD β, and 8 - 10 kD γ subunit.[143] Both the α and γ subunits are post-translationally modified, the α with a myristate and the γ with a geranylgeranyl moiety, which appears to mediate the observed association of the trimeric complex with the plasma membrane or plasma membrane proteins.[144] Almost 20 G proteins have now been described,[145] and they differ from each other primarily in their α subunits. The α subunit, which contains the GTP binding site and GTPase activity, is subject to ADP ribosylation, with different α's being differentially susceptible to ribosylation by *Vibrio Cholera* and *Bordetella Pertussis* toxins. Originally, the few G proteins that were known, were classified by their toxin sensitivity and by their action on the downstream effector molecules, adenylate cyclase and GMP phosphodiesterase.[146] However, in the wake of the discovery of multiple α's and the multiplicity of effector molecules,[145] G proteins are now categorized by sequence homologies within the α subunit.

In the basal state, G proteins exist as a GDP bound, trimeric complex in association with a receptor.[145] Upon activation, the receptor stimulates exchange of α bound GDP for GTP and this reduces the affinity of the G-protein for the receptor. In the absence of receptor stimulation, the nucleotide exchange rate is very slow. The β and γ subunits, which form a tightly associated heterodimer, dissociate from the GTP bound, activated α subunit to allow interaction of the activated α with downstream effector molecules. These effector molecules include diverse proteins such as adenylate cyclases, phospholipases and ion channel components.[147] GTP hydrolysis, catalyzed by an intrinsic GTPase activity, deactivates the α subunit and it reassociates with the βγ subunits. Reassembly is required for reassociation with the receptor.[145] The βγ subunits, important as regulators of α subunit activity, were once also postulated to have a direct mediator role. For example, adenylate cyclase inhibitory G proteins were thought to exert their effects by releasing βγ's which would bind to and inactivate adenylate cyclase stimulatory G proteins (G_S).[146] However, this model has fallen into disrepute since inhibition has been shown to occur in G_S deficient cells and because inhibition does not exhibit competitive kinetics.[145]

G proteins have been shown to couple receptors, including receptors for light, chemotactic factors and neurotransmitters, to downstream events in over 100 systems.[145] Most of these receptors are opsin proteins which span the membrane seven times. G proteins interact with the third cytoplasmic loop of these integral membrane proteins;[148] however, single membrane spanning receptors may also couple to G proteins.[149] The importance of G proteins in signal transduction is evidenced not only by their wide ranging expression and usage, but also by the discovery that oncogenesis and viral subversion of host cell metabolism can proceed through G protein pathways. For example, the *gsp* and *gip2* oncogenes implicated in neuroendocrine tumours are GTPase deficient G protein α subunits.[150] Also, the CMV genome encodes three seven-times-membrane-spanning, G protein coupled receptors that might, when expressed, modify host cell signalling to suppress anti-viral effects.[151]

1.2 The *ras* related GTP binding proteins

The mechanism of action of the *ras* family of GTP binding proteins is less well understood. *Ras* (rat sarcoma) was first identified as the transforming gene of the Harvey and Kirsten rat sarcoma viruses.[152] The *ras* oncogenes, Ha-*ras*, K-*ras* and N-*ras*, are the most frequently encountered oncogenes in human tumours with an overall incidence of approximately 30%. The discovery that this viral oncogene was a mutated version of a normal cellular gene suggested that the normal homologue might be important in controlling mitogenesis.

Ras encodes a 21 kD GTP binding protein with an intrinsic GTPase activity in its N terminal functional domain.[153] Transforming activity, and thus presumably normal activity of the non-oncogenic form of $p21^{ras}$ requires addition of both a farnesyl and palmitic acid to the C terminal end of the protein.[153] By analogy with the heterotrimeric G proteins, the GTP bound molecule is the active form. Oncogenic *ras* proteins typically have point mutations at codons 12, 13 and 59 and these result in both decreased intrinsic GTPase activity and refractoriness to regulatory proteins which normally enhance the GTPase activity of *ras* proteins.[154] *Ras* belongs to a family of approximately 30 members[155-157] which includes: *rap1* (K-*rev*-1) which appears to antagonize *ras* activity; the *rho* subfamily, which is involved in cytoskeletal regulation; the *rab*'s, which may control intracellular vesicle transport and *ran*, which links completion of DNA synthesis to mitosis. The yeast *RAS*1 and *RAS*2 proteins are also members of this family. *Ras* itself is implicated in cell growth and signal transduction since IL-2,[158] IL-3[158] GM-CSF,[158] PDGF,[159,160] serum[161] and insulin[162] increase the ratio of GTP to GDP bound $p21^{ras}$ in responsive cells. *Ras* expression will also induce differentiation in rat pheochromocytoma cells[163] and meiotic maturation of Xenopus oocytes.[164]

In mammalian cells, neither the upstream regulators or the downstream effector molecules by which $p21^{ras}$ mediates these actions have been well defined. Tyrosine kinases are apparently upstream of $p21^{ras}$ since tyrosine kinase oncogenes can not transform

$p21^{ras}$deficient cells.[165] Moreover, studies with antibodies to phospholipase Cγ (PLCγ) suggest $p21^{ras}$ acts upstream of this molecule.[165] *Ras* expression also been shown to activate a kinase which phosphorylates and activates *c-jun*, a component of the AP-1 transcription factor.[166] However, no direct connection has been demonstrated for $p21^{ras}$ with any of these signalling proteins. In *S. cerevisiae*, the *ras* signalling pathway is, perhaps, slightly more defined. The yeast *RAS*1 and *RAS*2 proteins activate adenylate cyclase through a cyclase associated protein (CAP) in response to external signals.[167] However, the coupling of $p21^{ras}$ to adenylate cyclase in yeast has not been conserved in mammalian cells. On the other hand, recent data which link CAP to an actin and phosphatidylinositol 4,5-bisphosphate (PIP_2) binding protein called profilin, suggest certain aspects of *ras* function have been conserved in evolution. It has long been suspected that regulation of adenylate cyclase is not the sole function of the yeast *ras* proteins since *RAS* deletions are lethal whereas adenylate cyclase null mutations are not;[167] the discovery that CAP is a bifunctional protein which couples $p21^{ras}$ to profilin and PIP_2 as well as to adenylate cyclase confirmed this suspicion.[168] Importantly, this second $p21^{ras}$ effector pathway may have been conserved in higher organisms. As mentioned earlier, PLCγ is a downstream molecule in the mammalian[165] $p21^{ras}$ pathway, and as will be discussed below, PLCγ is regulated by profilin sequestration of PIP_2 in mammalian cells.[169] The finding that $p21^{ras}$ action in yeast involves profilin, along with the observation that $p21^{ras}$ action in mammalian cells involves PLCγ,[165] suggests the intriguing possibility that profilin may be an intermediate mediator if not a direct effector of $p21^{ras}$ in eucaryotes. Regardless of the role of profilin, the discovery that the yeast and mammalian $p21^{ras}$ pathways are more similar than initially thought, may allow use of powerful yeast genetics to dissect $p21^{ras}$ action in mammalian cells.

1.3 Regulators of *ras*

By analogy with better known GTP binding proteins, $p21^{ras}$ can be regulated by switching between the GDP and GTP bound state. One report showed that *ras* becomes tyrosine phosphorylated in response to GM-CSF in normal murine hemopoietic progenitor

cells.[170] However the effect of this phosphorylation on *ras* activation is not known. Better characterized are the factors which affect $p21^{ras}$ intrinsic GTPase activity and factors that catalyze exchange of bound GDP for GTP.

1.3.1 GTPase activating protein as a regulator of *ras*

Normal and oncogenic forms of $p21^{ras}$ differ in their ability to hydrolyze GTP. However, the magnitude of this difference can not be accounted for by the slight differences in their intrinsic GTPase activities.[171] This discrepancy was resolved by the discovery of a GTPase activating protein (GAP) that binds to both normal and oncogenic $p21^{ras}$ but is able to stimulate the GTPase activity only of the normal *ras* protein.[172] GAP, a 120 kD protein, is widely expressed in all tissues[173] and is homologous to the yeast *IRA*1 and *IRA2* (Inhibitory regulator of *ras*/cAMP pathway) proteins.[174] GAP's for other members of the *ras* family have also been isolated, including GAP's for *rap1*,[175] *rho*,[176] *ral A*,[177] and *rab* 3A[178] proteins. The neurofibromatosis type 1[179,180] and *bcr* genes[181] also encode functioning GAP activity. Interestingly, a GTPase inhibitory protein (GIP) for *ras* has also been described.[182] Consistent with an upstream regulator model of GAP action is the observation that GAP can suppress the transformation induced by c-*ras* over-expression.[183] In addition, phorbol ester stimulation of T cells apparently elevates $p21^{ras}$-GTP levels by inhibiting GAP activity.[184]

Interest in GAP was heightened recently by the finding that GAP may provide the molecular basis for the observed connection between tyrosine kinases and $p21^{ras}$. GAP becomes tyrosine phosphorylated in cells stimulated with EGF,[185,186] PDGF[187,188] or transformed with *fps*,[189] *src*[189] or the *erbB*-2[190] tyrosine kinases and is physically associated with activated EGF,[82] PDGF[87] and CSF-1[191] receptors. Association of GAP with tyrosine kinases occurs via SH2 interactions as discussed earlier. Over-expression of GAP inhibits *src* transformation,[192] presumably by acting on $p21^{ras}$, since none of the $p56^{src}$ induced tyrosine phosphorylations are inhibited. Interestingly, the catalytic C-terminal region of GAP is more efficient in suppressing *src* transformation than the full length protein, suggesting that $p56^{src}$ phosphorylation of the GAP N-terminal domain may regulate GAP's

activity on $p21^{ras}$.[192] However, no *in vitro* correlation of tyrosine phosphorylation and GAP activity has been demonstrated. Moreover, in some systems, the stoichiometry of tyrosine phosphorylation is quite low with most of the phosphorylation occurring on serines residues.[186] An alternate model of GAP regulation involves sequestering GAP into an inactive complex.[186] In mitogen stimulated or tyrosine kinase transformed fibroblasts, GAP becomes associated with two proteins with molecular weights of 62 and 190 kD. Comparison of free GAP to the form bound to the 190 kD protein, showed that the complex had diminished *ras* GTPase stimulating activity.[186] An attractive hypothesis for the function of GAP receptor association is a similar sequestration of GAP, thus resulting in accumulation of active $p21^{ras}$-GTP complexes. This may not be the whole story however. Certain lipids, the levels of which change during mitogenic stimulation, like arachidonic acid and phosphatidylinositol phosphate[193] are also capable of inhibiting GAP activity *in vitro*. The same lipids which inhibit GAP activate GIP.[182] Perhaps these multiple layers of regulation allow fine tuning of $p21^{ras}$ regulation.

Because the function of $p21^{ras}$ is not known, GAP may be actually a target of $p21^{ras}$ action instead of, or in addition to, being an upstream $p21^{ras}$ regulator.[171] Genetic and biochemical evidence have shown that GAP interacts with the effector domain of $p21^{ras}$;[194] regions of $p21^{ras}$ required for oncogenicity are also those which interact with GAP.[195] The best evidence that GAP is a target for $p21^{ras}$ comes from experiments examining the control of K^+ channels by muscarinic receptors.[196] Addition of recombinant GAP, $p21^{ras}$ or GAP-$p21^{ras}$ complexes to isolated atrial membranes inhibited K^+ flux through activated channels. The effect of free GAP was dependent on the presence of endogenous $p21^{ras}$ since neutralizing antibodies to $p21^{ras}$ abrogated this effect. Intriguingly, the C-terminal domain of GAP, which contains the GTPase activating domain and is sufficient to suppress *ras* mediated cellular transformation[192] and also stimulate *ras* GTPase activity[197] but was not sufficient for channel blocking. These observations suggest that GAP-$p21^{ras}$ complexes uncouple the K^+

channels from muscarinic receptors and that the GAP polypeptide contains important activities outside of its GTPase activating domain.

1.3.2 Nucleotide diphosphate kinases as regulators of *ras*

A downstream effector role for GAP is attractive since an upstream regulator model would predict a futile cycle in which GAP, unless deactivated by a mitogenic stimulus, keeps $p21^{ras}$ in a GDP bound state by catalyzing hydrolyis of bound GTP. A more conservative model of $p21^{ras}$ regulation involves control of the rate with which GDP is replaced by GTP. In this scenario, $p21^{ras}$ is in a GDP bound state until agonist stimulation signals exchange of GTP for GDP. Two mechanisms exist for this replacement: direct phosphorylation of $p21^{ras}$ bound GDP by a nucleotide diphosphate kinase, or stimulation of the rate at which GDP is exchanged for GTP. The action of a nucleotide diphosphate kinase on protein bound GDP has always been a theoretical possibility.[198] The simplest interpretation of the site of action of these kinases suggest that they act on free nucleoside diphosphates which would result in greater pools of nucleoside triphosphates. Exchange of bound nucleotide diphosphates for triphosphates would then give the appearance of direct phosphorylation of the original protein bound nucleotide diphosphate. Although direct phosphorylation has been difficult to prove experimentally because nucleotide exchange has been difficult to control for, a recent report suggests that purified nucleotide diphosphate kinase catalyzes the phosphorylation of GDP bound to ARF (adenosine diphosphate ribosylation factor).[199] It is too early, at this point, to predict the relevance of this mechanism to the control of $p21^{ras}$ guanine nucleotide phosphorylation state.

1.3.3 Nucleotide exchange proteins as regulators of *ras*

Far better characterized are the proteins which catalyze the exchange of *ras* bound GDP for GTP. A number of nucleotide exchange proteins have been described for various members of the *ras* family including ones for *ras*[200,201] *rho*,[202] and *ran*.[203] The *ras* family proteins have very low rates of nucleotide dissociation in the absence of these catalysts,[200] an essential feature if $p21^{ras}$ is controlled by the regulated release of GDP. Support for this model has

been accumulating. It has been recently proven that the yeast CDC25 gene product, which acts upstream of the *ras* proteins, is a nucleotide exchange protein.[204] In *Drosophila*, a nucleotide exchange protein has been recently implicated in the *ras* signalling pathway from the *sevenless* tyrosine kinase receptor.[205] These provocative results suggest that $p21^{ras}$ may be controlled by agonist stimulation of nucleotide exchange, in a manner very similar to receptor regulation of the heterotrimeric G proteins.

Regulation of $p21^{ras}$ at the level of nucleotide exchange does not preclude regulation at the level of GTP hydrolysis by GAP, ie. both mechanisms may be operative. However, it is becoming increasingly apparent that GAP has functions other than stimulating GTPase activity since two of the known GAP's contain activities in addition to their GTPase activating domain. *Ras* GAP has SH2 domains important to its interaction with tyrosine kinases and other proteins; the interaction, directly or indirectly, uncoupling the muscarinic receptor from K^+ channels.[196] The *bcr* protein contains, in addition to its GAP domain, a region which binds SH2 sequences and a serine/threonine kinase activity.[410] Perhaps the GTPase stimulating activities of these molecules are important as downstream signal terminators of these other functions rather than as upstream regulators of $p21^{ras}$.

2. Protein Kinase C and Inositol Phosphate Metabolism

Protein kinase C (PKC) is involved in the regulation of many cellular metabolic processes including endocrine and exocrine tissue secretion, smooth muscle contraction, steroidogenesis, ion channel fluxes, cAMP dependent phosphorylations, receptor desensitization, and gene expression.[206] The importance of PKC in growth control is suggested by the fact that PKC is the site of action of tumour promoting phorbol esters[207] and by the discovery that the retroviral *akt* oncogene codes for a PKC analogue.[208] PKC was originally described as a protein kinase activity that could be activated *in vitro* by limited proteolysis with calpain.[206] Further characterization of this activity showed that the physiological activator of PKC, diacylglycerol (DAG), increases the enzyme's affinity for its cofactors, Ca^{2+} and phosphatidylserine.[209,210] In the classically described PKC activation pathway, agonist induced receptor activation

stimulates hydrolysis of membrane PIP_2 which generates diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (InP_3). DAG remains in the membrane to stimulate PKC while InP_3 binds to cognate receptors on the ER membrane[211,212] to liberate Ca^{2+} stores. This picture has been complicated by the discovery of new regulators of PKC activity[213,214] and by the cloning of multiple PKC isozymes.[215] In addition, although agonist controlled phosphatidylinositol (PI) hydrolysis is intimately involved in PKC activation because of the generation of DAG and InP_3, other metabolites of PI turnover have been implicated in signal transduction.[216]

2.1 Protein kinase C

Several PKC isozymes have been isolated which have been categorized into five groups.[215] Although there is much inter-group variation, the members within each group are very homologous and highly conserved between species. The various isozymes differ in their distribution, the α group being widely expressed, while the β and γ isoforms are highest in endocrine and brain tissues, respectively. The δ and ε forms have not as yet been well characterized. The α,β and γ isozymes are similar in structure with five variable and four constant domains.[217,218] The C-terminal half of the enzyme contains the catalytic site, the N-terminal portion has the regulatory domain with its cysteine rich, zinc finger motif essential for DAG binding.[210] The δ and ε isozymes which are independent of Ca^{2+}, lack the C2 region in the N-terminal domain which may be important in Ca^{2+} binding. In all PKC's, a region between the catalytic and regulatory domains is a unique region with high affinity for the catalytic site of the enzyme.[219] This pseudosubstrate region appears to be important in regulating PKC activity.[220] PKC activators such as DAG may induce a conformational change that shifts the pseudosubstrate domain out of the catalytic site to allow access of substrates.

2.1.1 Regulators of PKC

The variable regions determine co-factor requirements and substrate specificity of the different isozymes. For example, the α isozyme is the most dependent on DAG for activation, the β form exhibits considerable activity in the absence of Ca^{2+}, and the γ can be activated *in vitro* by arachidonic acid without Ca^{2+} or phospholipid. Although co-factor requirements for

the δ,ε and η are not as well characterized, these isozymes, unlike the α, β and γ forms, do not recognize histone as a substrate.[206] Moreover, phorbol esters bind to and activate the various isozymes to varying degrees.[210] The different isozymes of PKC also differ in their sensitivity to proteolysis.[210] Proteolysis may be important in PKC regulation. Specifically, calpain preferentially cleaves the activated form of PKC[221] in a region between its N-terminal regulatory and catalytic domain to generate a potentially active fragment referred to as PKM which might be important in persistent PKC activation. However, this PKM fragment may be broken down further since it is often difficult to detect, either immunologically or by activity measurements. The generation of PKM may therefore be involved in PKC downregulation rather than persistent PKC activation. The distinct differences among the isozymes, however, suggests that a cell's response to PKC activating signals depends on the particular combination of isozymes expressed.

Other regulators of PKC include unsaturated fatty acids, such as arachidonic, oleic or linoleic acid, which synergize with DAG to allow activation of PKC when Ca^{2+} levels are low.[213] These fatty acids can be produced by receptor mediated activation of phospholipase A_2.[214] Sphingolipids, on the other hand, are inhibitory and may be responsible for maintaining PKC in a basal, inactivated state in the presence of cellular levels of DAG.[222] However, PKC may never actually be exposed to DAG generated during normal housekeeping processes because of compartmentalization. For example, a cardinal sign of PKC activation is the translocation of this enzyme from the cytosol to the plasma membrane. Recent studies have also shown that some PKC molecules become associated with the detergent insoluble cytoskeleton. Activated PKC binds to cytoskeleton associated proteins called RACKs (Receptors for Activated C-Kinase); this association alters the subcellular localization of PKC and thus may be involved in its regulation.[223] The different PKC isozymes display differing RACK binding efficiencies.[223] PKC may also translocate to the nucleus[224,225] in response to nuclear DAG production[226] and act on nuclear targets.

2.1.2 Targets of PKC

Various targets of PKC action have been described and receptor desensitization is one important consequence of PCK activation. Specifically, the intrinsic tyrosine kinase activities of the EGF and insulin receptors are inhibited, while the beta-adrenergic and glucagon receptors become uncoupled from G-proteins upon PKC phosphorylation.[227] PKC phosphorylation of the EGFR was also once believed to be responsible for decreasing its affinity for EGF. However, site-directed mutagenesis of the residues involved showed that these phosphorylations were important only in receptor internalization.[227] The effect of PKC on EGFR binding affinity is probably mediated indirectly, for example, through phosphorylation of other proteins which associate with the receptor. Another prominent PKC substrate is a cytosolic "80 kD" protein which is phosphorylated in a PKC dependent manner in a wide range of cells in response to many factors.[228] This almost ubiquitous marker of PKC activation was cloned and the cDNA found to encode a 32 kD polypeptide. Since this protein is myristylated, which gives it an aberrant mobility on SDS-PAGE, and contains a high alanine content, it was named MARCKS for Myristylated, Alanine Rich C-kinase Substrate. MARCKS contains a binding site for calmodulin,[229] an ubiquitous Ca^{2+} dependent regulatory protein that binds to and regulates cellular proteins. PKC phosphorylation of MARCKS releases MARCKS sequestered calmodulin and may allow activation of calmodulin dependent processes. MARCKS also has an actin binding site which suggests that it may be involved in the cytoskeletal rearrangements observed upon PKC activation.[230] Transcription factors are another important target of PKC action. PKC activates NF-κB[225] via releasing free, active NF-κB from an inactive NF-κB/ IkB complex by phosphorylation of the IκB inhibitory protein.[231] PKC also phosphorylates and activates AP-1 and AP-2.[231] In the case of NF-κB, PKC acts on a cytosolic, inactive complex to generate an active factor that enters the nucleus.[231] However, PKC itself may translocate into the nucleus to phosphorylate factors *in situ*.[224,225] Regardless of the subcellular location, the action of kinases such as PKC on transcription factors is an important, intermediate link from early receptor to later nuclear events.

2.2 Phospholipase C

PKC is a downstream effector, rather than a receptor associated signal transduction molecule since its activation depends on upstream production of regulators such as DAG and Ca^{2+}. DAG production is controlled by agonist sensitive phospholipase C's and much has been learned recently about the role of phospholipases in signal transduction, including the exciting discovery that one isozyme of PLC may directly couple tyrosine kinase receptors to downstream effectors such as PKC.

Phosphatidylinositol specific phospholipases are classified into five groups based on primary structure.[232,233] They share two regions of significant homology which probably contain the catalytic domain. However, outside of these domains, the sequences are highly variable. Of the different types of PLC's, only PLCβ_1, PLCε and PLCγ appear to be involved in signal transduction. PLCβ_1 and PLCε[233] but not PLCγ[234] are regulated through G proteins[233] of the pertussis toxin insensitive,[235] G_q class.[234] In the case of PLCβ_1, the G protein activates by reducing PLCβ_1's Ca^{2+} requirement.[236] Interestingly, a specific growth factor can activate different types of PLC in different cell types. EGF, for example, stimulates inositol phosphate metabolism through a pertussis toxin sensitive PLC in hepatocytes but activates a G protein independent PLC in A431 cells.[237]

PLCγ, the only isoform with SH2 domains, has been the subject of considerable attention because of its physical association with intrinsic tyrosine kinase receptors. As with other SH2 containing proteins, these domains are dispensable for *in vitro* enzymic activity.[238] These domains are required, however, for binding to ligand activated, tyrosine phosphorylated receptors.[239,240] Activation of PLCγ in intact cells is accompanied by tyrosine phosphorylation of the enzyme itself, although this phosphorylation does not increase catalytic activity, since replacement of the relevant tyrosine with phenylalanine has no effect on *in vitro* activity.[241] Tyrosine phosphorylation is essential, however, both for interaction with profilin, a protein which sequesters PIP_2 *in vivo*, and subsequent cleavage of profilin bound PIP_2.[169] These observations are consistent with a model in which EGF,[239] PDGF,[240] nerve growth

factor (NGF)[242] and fibroblast growth factor (FGF)[242] stimulate the receptor intrinsic tyrosine kinase that phosphorylates receptor associated PLCγ. Tyrosine phosphorylated PLCγ then catalyses DAG and InP_3 production from profilin bound PIP_2. That PLCγ is associated with the receptor prior to receptor autophosphorylation, in a presumably SH2 independent manner, comes both from the observation that PLCγ phosphorylation occurs at 4°C[243-246] and from immunoprecipitation experiments.[245]

DAG, in some systems, can also be generated from phosphatidylcholine (PC) hydrolysis[247] via an agonist sensitive PC specific PL.[248] Significantly, DAG from this source does not result in proteolysis and down-regulation of PKC, either because the signal that activates PKC is different or because the fatty acid composition of the PIP_2 and PC derived DAG's differ.[249] As a consequence, PKC activation can be long term, and this sustained PKC activation is important for stimulation of AP-1 enhancer activity in macrophages[250] and for activation of resting human T cells.[251]

It is not clear how Ca^{2+} mobilization occurs or whether it is required in systems where DAG is derived from PC. However, in the more common PIP_2 pathway, Ca^{2+} is released from intracellular endoplasmic reticulum (ER) stores. Two ER InP_3 receptors have been cloned and shown to consist of a calcium channel domain joined to a cytoplasmic ligand binding domain through a coupling domain.[252,253] The two receptors are the least homologous in the binding domain and have different affinities for InP_3.[253] The coupling domain is subject to cAMP dependent kinase phosphorylation, suggesting that it may have a regulatory role in controlling the function of the ion channel.[254] Ca^{2+} release may therefore dependent on InP_3 receptor expression in a particular cell type as well as the activation state of other signalling pathways.

2.3 Phosphatidylinositol 3-kinase and other PIP_2 metabolites

Although the best studied product of PIP_2 metabolism is InP_3, other metabolites may also be important in signalling. Inositol 1,3,4,5-tetrakisphosphate, for example, may be responsible for promoting the influx of external Ca^{2+}[255] and inositol 1,4-bisphosphate may activate a low specific activity form of DNA polymerase α.[216] However, much interest has

focussed on inositol polyphosphates that are phosphorylated on the D-3 position of the inositol ring because of the discovery that the enzyme responsible for this modification, phosphatidylinositol 3-kinase (PI3-K), which associates with tyrosine phosphorylated growth factor receptors.[139]

PI3-K was first described as an enzyme activity associated with cytoplasmic tyrosine kinases.[256] Intriguingly, PI3-K associates only with transforming versions of *src* and not normal *src*.[257] This association of PI3-K with these tyrosine kinases is thought to recruit PI3-K to the plasma membrane and thus allow access substrates.[258] Plasma membrane recruitment may also be the purpose of PI3-K association with the ligand activated, intrinsic tyrosine kinase receptors for insulin,[259] CSF-1,[260] PDGF[261] and EGF.[262] PI3-K consists of subunits with molecular weights of 110 and a 85 kD[263] and it is the 85 kD subunit which contains two SH2 domains[264,265] and mediates the association of PI3-K with tyrosine phosphorylated receptors.[266]

Of interest are the recent observations that PI3-K also associates with activated IL-2R's[136] and $p21^{ras}$,[267]. As discussed earlier, the first observation is important since it demonstrates that some of the signalling proteins that bind to intrinsic tyrosine kinase receptor may also associate with receptors that lack tyrosine kinase domains. The way in which the second observation, although potentially very exciting, fits into the known scheme of signal transduction is not clear.

3. Tyrosine Kinases

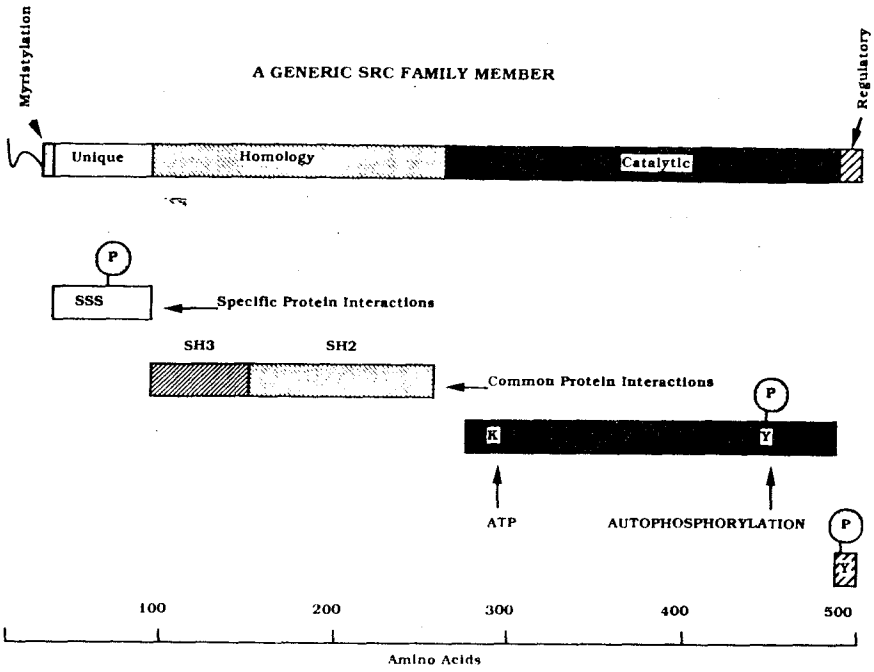
The importance of tyrosine kinases in signal transduction is demonstrated by their many manifestations in the form of viral oncogenes[268] and growth factor receptors. Tyrosine kinases can be classified as those which are transmembrane receptors, which were discussed earlier, and the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases can further be divided into *src*,[268] *abl*[269] and *fps*[270] families based on sequence homologies. Of these, the *src* family of tyrosine kinases are the best characterized.

3.1 Structure of the *src* family of tyrosine kinase

The overall structure of a *src* kinase is shown in figure 4A. The N-terminus is myristylated and the recognition sequence for this modification is found in the first 7-10 amino residues.[271] $p60^{src}$ must be membrane associated in order to exerts its action and myristylation is necessary, although not sufficient, for this to occur.[272] The association with the membrane may be necessary to bring $p60^{src}$ in contact with certain substrates and appears to be mediated by specific $p60^{src}$ receptors.[273,274] The next 75 residues are highly divergent among different members of the *src* family[275] and may determine substrate specificity and subcellular localization. The C terminus, on the other hand, contains a negative regulatory domain. Phosphorylation of a C-terminal tyrosine, tyr-527 in $p60^{src}$, inhibits tyrosine kinase activity as shown by phosphatase[276] and site directed mutagenesis experiments.[277,278] Members of the *src* family can also be activated *in vivo*[275] by the association with middle T antigen in polyoma virus infected cells. Since the region required for middle T binding is also the region required for *in vivo* phosphorylation at tyr-527, the binding of middle T is believed to activate $p60^{src}$ by preventing phosphorylation of tyrosine 527 by a regulatory kinase. The receptor intrinsic tyrosine kinases may have analogous C terminal regulatory domains.[62,279]

The SH1 region contains the tyrosine kinase domain which is conserved in all tyrosine kinases and homologous to serine/threonine (Ser/Thr) catalytic domains.[280] The major site of autophosphorylation, tyrosine 416 in $p60^{src}$ is located in the catalytic domain, and phosphorylation at this residue activates kinase activity.[277,281] In contrast, SH2 and SH3 domains are found in proteins other than tyrosine kinases. The SH3 domain is thought to be involved in cytoskeletal interactions.[84] However, attention has focused on the SH2 domains because of their role in mediating interactions between tyrosine kinase receptors and signalling proteins.

A.



B.

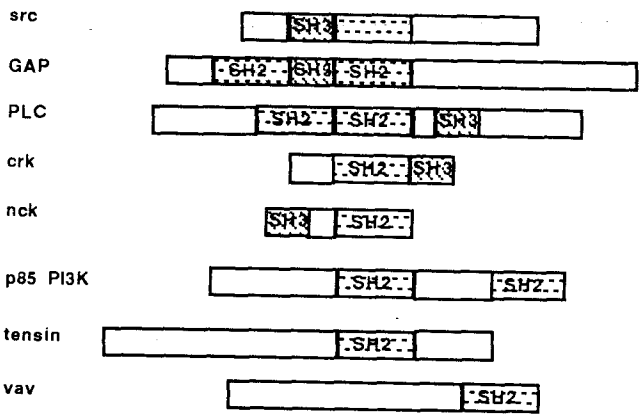


Figure 4. Panel 4A shows the structure of a *src* family tyrosine kinase (from reference 280). Proteins with SH2 and SH3 domains are shown in Panel 4B.

As mentioned earlier, the SH2 domains have been found in a number of proteins (Figure 4B). Many of these proteins: PLCγ, PI3-K and *ras* GAP, have been shown to be involved in signal transduction by associating with tyrosine phosphorylated proteins through SH2 domain mediated interactions. The presence of SH2 domains in other proteins[84] : nck, tensin and vav, may therefore suggest that these proteins are also important in signalling. However, regardless of whether these three do participate in signal transduction, the SH2 domains do provide potential sites for protein/protein interactions.

The role of SH2 domains in $p60^{src}$ have been extensively studied and they are thought to serve either in regulation of kinase activity or in substrate recognition. Both of these models are based on the tyrosylphosphoprotein binding function of the SH2 domains. In the regulator model, the SH2 domain is postulated to bind to the C terminal, negative regulatory phosphotyrosine and thus mask the tyrosine kinase domain. In support of this model, limited proteolysis of $p60^{src}$ suggests that $p60^{src}$ changes its conformation upon activation.[282] In addition, the observation that point mutations in the SH2 domain activate the transforming potential of $p60^{src}$, without any detectable change in the phosphorylation state of the C-terminal tyrosine, is also consistent with this model. This model also suggests a mechanism by which the *crk* oncogene, which does not encode a tyrosine kinase, transforms cells. The *crk* protein,a 47 kD polypeptide consisting almost entirely of SH2 and SH3 domains,[283] associates with $p60^{src}$ in an SH2 dependent manner.[284] The binding of $p47^{crk}$ SH2 to the terminal phosphotyrosine, in the place of the intramolecular $p60^{src}$ SH2, would open up the tyrosine kinase domain. Release of endogenous tyrosine kinases from negative regulatory control would account for the increases in tyrosine phosphorylation level in *crk* transformed cells.[283] Not mutually exclusive with its role in regulating tyrosine kinase activity, is a model in which the SH2 domain mediates interaction with regulatory proteins or substrates. Various mutations have been described in the SH2 domain that decrease the transforming potential of various kinases[285,286] or change the cellular range of the transforming protein[287] without altering its

in vitro kinase activity. These observations suggest that interaction with cellular substrates is impaired in these SH2 mutants.

3.2 Targets of tyrosine kinase action

Activation of a tyrosine kinase results in phosphorylation of many cellular proteins. Many of these substrates have not been characterized further than by molecular weight. However, several have been identified. For example, $p60^{src}$ activation correlates with phosphorylation of vinculin, talin, erzin, calpactin, and lipocortin II.[280] The physiological effects of these phosphorylations are not known. Despite the difficulty in proving direct consequences of tyrosine phosphorylation, the involvement of cytoplasmic tyrosine kinases in signalling cascades is not doubted. For example, $p60^{src}$ is thought to be involved in EGF signalling because over expression of *c-src* in fibroblasts potentiates the mitogenic response to EGF.[288] The discovery of SH2 and SH3 mediated interactions has opened up the possibility that tyrosine phosphorylation serves mainly to direct protein traffic.

3.3 Association of tyrosine kinases with receptors

Many cell surface receptors, including the hemopoietin receptors, lack intrinsic tyrosine kinase domains yet they become tyrosine phosphorylated upon ligand binding. Recently, very exciting work has shown that several of these receptors are physically associated with *src* family tyrosine kinases (Table 4).

TABLE 4: Tyrosine Kinases Associated with Cell Surface Receptors

Receptor	tyrosine kinase	Reference
IL-2R β chain	lck	135
T cell antigen receptor (TcR)	fyn	289
B cell antigen receptor	lyn	290
CD4/CD8	lck	291,292
basophil/mast cell IgE F_c receptor	lyn, yes	293
platelet glycoprotein CD36	fyn, yes, lyn	293

These kinases become activated upon ligand stimulation of the receptor, resulting in phosphorylation of the receptor on tyrosines residues.

The structural features important in these interactions has been investigated for CD4/CD8 and the IL-2R. A membrane proximal +-+-X-C-X-C-(P) (where + is any basic amino acid) sequence on both the CD4 and CD8 molecules appear to be important in the binding of $p56^{lck}$.[294] A similar cysteine sequence in the bovine metallothionein I protein is known to interact with zinc. Since 1,10-ortho-phenanthroline, a zinc chelator, disrupts the association of $p56^{lck}$ with CD4 or CD8, formation of a zinc coordination complex may be involved in the binding of $p56^{lck}$ to CD4/CD8.[294] In contrast, the region in the IL-2R β chain important in $p56^{lck}$ association is characterized by an abundance of acidic amino acids.[135] This region contains the two tyrosines (residues 355 and 358), phosphorylated by $p56^{lck}$, but phosphorylation of these residues is not required for $p56^{lck}$ binding. Interestingly, the region in $p56^{lck}$ important for association with CD4/CD8 differs from the region involved in binding to the IL-2R. The N-terminal unique region, which distinguishes different members of the *src* family, is involved in $p56^{lck}$ association with CD4/CD8,[294] whereas a region in the N terminal half of the SH1 domain mediates interaction with the IL-2R β chain.[135]

In addition to being associated with receptors lacking intrinsic tyrosine kinase domains, members of the *src* family of tyrosine kinases have also been shown to associate with the PDGF receptor.[295] Three *src* family kinases, $p60^{src}$, $p60^{fyn}$ and $p60^{yes}$ become activated in PDGF stimulated cells and co-precipitate with the PDGFR.[295] These kinases may serve to phosphorylate substrates that the PDGFR is not capable of phosphorylating.

4. Serine/threonine Specific Kinases

Serine/threonine kinases are far more common than tyrosine specific kinases and many, including protein kinase C and the cyclic nucleotide dependent kinases are thought to play key roles in signal transduction. However, of all the serine/threonine kinases in the cell, only three have been shown to be controlled by both tyrosine and serine/threonine phosphorylation, suggesting perhaps these kinases play a pivotal role in the signal transduction cascade by

integrating information from both tyrosine and serine/threonine kinase pathways. One of these is the cyclin dependent kinases which will be discussed in the following section on cell cycle control: the other two are the Raf and MAP kinases.

4.1 Raf-1 kinase

Raf-1 the normal cellular homologue of *v-raf*, the transforming gene of murine moloney sarcoma virus,[296] belongs to a family of closely related cytosolic 74 kD serine/threonine kinases. Interestingly, a transmembrane, receptor-like *raf* homologue has been detected in C. elegans.[53] Other members of this family include *A-raf*, *B-raf*, the *raf-2* pseudogene, and the avian v-mil. While *Raf-1* mRNA is ubiquitously expressed in all proliferating cells the distribution of A- and B-*raf* is mainly restricted to urogenital and brain tissues, respectively.[298] The structure of $p74^{raf-1}$ is reminiscent of other serine/threonine kinases since it has an N-terminal regulatory domain and a C-terminal catalytic domain.[297] Interestingly, the N-terminal regulatory region contains a cysteine rich region similar to PKC suggesting, perhaps, similar small ligand regulation. Truncation of the N-terminal region, as in *v-raf*, results in activation of the transforming potential of *raf-1*.

$p74^{raf-1}$ has been studied intensely because its activity is upregulated in mitogen stimulated cells. IL-3,[299,300] GM-CSF,[299,300] CSF-1,[301] PDGF,[302] insulin,[303] and EGF[304] all stimulate $p74^{raf-1}$ kinase activity. In all cases, $p74^{raf-1}$ kinase activation is accompanied by serine phosphorylation of the $p74^{raf-1}$ protein itself. The kinase responsible for this modification has not as yet been identified but it is not believed to be PKC since $p74^{raf-1}$ activation can occur in PKC downregulated cells. Tyrosine phosphorylation also accompanies activation in some cases and this phosphorylation is most convincingly demonstrated in the mouse IL-3, GM-CSF[299] and PDGF[302] systems where considerable phosphotyrosine can be detected. However, in the remaining systems, tyrosine phosphorylation is of very low stoichiometry or not detectable. This has raised the question of whether tyrosine phosphorylation is essential for $p74^{raf-1}$ activation. In support, *in vitro* phosphorylation of $p74^{raf-1}$ with recombinant, baculovirus PDGFR tyrosine kinase has been

shown to increase $p74^{raf-1}$ kinase activity six fold.[83] It is possible that tyrosine phosphorylation of $p74^{raf-1}$ is very transient in some cells, but nonetheless is necessary for serine phosphorylation which then is itself sufficient for activation. One group,[301] however, suggests that in vivo activation of $p74^{raf-1}$ does not require tyrosine phosphorylation at all. This group points out possible deficiencies in antibody specificity which makes interpretation of certain data difficult in studies[302] which do show $p74^{raf-1}$ tyrosine phosphorylation. The question of whether tyrosine phosphorylation is indeed important in regulating the kinase activity of $p74^{raf-1}$ waits to be resolved by site directed mutagenesis of the tyrosine residue(s) thought to be involved.

$p74^{raf-1}$ is also notable among signal transduction molecules because it is one of the four proteins which have been shown to associate with ligand activated, tyrosine phosphorylated receptors. $p74^{raf-1}$ associates with the PDGFR[83,302] and the EGFR[304] and this association may allow it to be phosphorylated by the tyrosine kinase intrinsic to these receptors. However, $p74^{raf-1}$ does not associate with either the CSF-1[301] or the insulin[303] receptors even though both of these contain intrinsic tyrosine kinases and $p74^{raf-1}$ does become stimulated in cells incubated with ligands for these receptors.

The placement of $p74^{raf-1}$ downstream of tyrosine kinase receptors in the signal transduction cascade, whether $p74^{raf-1}$ physically associates with the receptor or not, is apparently conserved evolutionarily, ie. the *Drosophila* homologue, *D-raf*, acts downstream of torso, a transmembrane tyrosine kinase similar to the PDGFR.[305] $p74^{raf-1}$ may also act downstream of $p21^{ras}$, or alternatively, use a pathway independent of *ras* since *v-raf* is able to transform *ras* deficient cell lines.[296,298] $p74^{raf-1}$ may be an upstream regulator of transcription factors such as those responsible for regulating *c-fos*.[306] In this regard it is interesting to note that PDGF or TPA stimulation of quiescent cells results in rapid redistribution of *raf* protein from a uniform cytoplasmic distribution to a perinuclear location.[298]

4.2 MAP kinase

MAP-2 kinase is a mitogen activated 42 kD serine/threonine kinase originally assayed *in vitro* with, and thus named for, microtubule associated protein-2 as a substrate.[307] However, with the recent discovery that MAP-2 kinase was actually one member of a family[308] and because of its increasingly apparent importance and wide range of activities in many signalling systems, "MAP" kinase is now considered an acronym for Mitogen Activated Protein kinase. However, several synonyms, such as "ERK" (Extra-cellular signal Regulated Kinase), exist.[308]

MAP kinases are stimulated in various cells in response to many factors, including IL-3,[309] EGF,[310] NGF,[311] FGF,[311] insulin[312] and TcR ligation.[313] In contrast to raf kinase, many substrates for MAP kinases have been described. One of the major *in vivo* substrates is the microtubular network, the phosphorylation of which may lead to disassembly.[314] Other substrates include other kinases important in signal transduction, namely, the 90 kD S6 kinase[311] (or $pp90^{rsk}$,[315]) and the EGFR. MAP kinase mediated phosphorylation of S6 kinase has been shown to increase S6 kinase activity in response to insulin, indicating that MAP kinase acts as a downstream effector in a kinase cascade initiated by the insulin receptor tyrosine kinase.[312] MAP phosphorylation of the EGFR, on the other hand, apparently decreases EGFR kinase activity[316] and thus may be involved in a negative regulatory feedback loop responsible for EGFR desensitization. MAP kinase has also been shown to act late in the signal transduction cascade by phosphorylating and activating the transcription factor c-jun.[317]

Activation of MAP kinase is accompanied by phosphorylation on both tyrosine and threonine residues and both are required for MAP kinase activity.[318] Because most kinases described to date phosphorylate substrates on either tyrosine or serine/threonine residues, but not both, the requirement for both phosphorylations suggests MAP kinases may integrate signals from two upstream kinases. Efforts directed towards purifying and identifying the upstream kinases have led to some controversy.[319,320] One group for example believes that MAP kinase is phosphorylated and activated by a MAP kinase kinase that is, surprisingly,

capable of phosphorylating both tyrosine and threonine residues.[320] Krebs' group, on the other hand, describes the upstream molecule as a MAP kinase activator[319] that does not have intrinsic kinase activity. This second interpretation has been strengthened by the unexpected observation that, although phosphorylation of exogenous substrates appears to be still restricted to serine/threonine residues, MAP kinases can autophosphorylate on both tyrosine and threonine residues.[321]

The possibility that MAP kinase is capable of autophosphorylating on both tyrosine and threonine residues casts in doubt any theory that the MAP, raf and cyclin dependent kinases occupy pivotal, integrative positions in signal transduction which is based only on the observation of both serine/threonine and tyrosine phosphorylations. These kinases are interesting, nevertheless, since they have many actions and functions in many signalling systems. The possibly shared ability[321] for autophosphorylation on both tyrosine and serine/threonine residues may indicate a unique method of regulation, the significance of which remains to be discovered.

5. Phosphatases

Protein phosphorylation is regulated not only by kinases, but by phosphatases as well. As with protein kinases, the phosphatases involved in intracellular signal transduction can be divided into tyrosine[322] and serine/threonine specific[323] classes. However, unlike the kinases, the two classes are not evolutionarily related, as evidenced by differing catalytic domain structure.[322]

5.1 Tyrosine specific phosphatases

Tyrosine specific phosphatases are a fairly recent discovery[324] and have been implicated in the mechanism of action of at least one of the interleukins (IL-4).[325] A role in signal transduction for tyrosine phosphatases is also suggested by the discovery of a tyrosine specific phosphatase containing two copies of SH2 domains.[326] Like the tyrosine specific kinases, tyrosine phosphatases are found in both cytoplasmic and transmembrane forms.[322] The transmembrane tyrosine phosphatases are especially intriguing because they resemble

receptors[322] and one of these transmembrane tyrosine phosphatases, CD45, appears to be essential in the action of the *src* family of tyrosine kinases.[327-330]

CD45 is a molecule originally identified as an antigenic marker of leukocytes. Different splice variants of CD45, which are expressed on different subsets of leukocytes, generate different N-terminal, external, putative ligand binding domains which may suggest each variant may respond differently to an external stimulus. Like all transmembrane tyrosine phosphatases, the cytoplasmic region of CD45 contains two phosphatase catalytic domains which may possess different specificities.[324] The action of CD45 has been implicated in TcR[327-330] and B cell antigen receptor[331] activation of receptor associated *src* family kinases. As discussed above, TcR ligation results in activation of a receptor associated $p56^{lck}$ kinase. However, like other members of the *src* family, $p56^{lck}$ is inhibited by the phosphorylation of its carboxy terminal tyrosine (tyr-505 in $p56^{lck}$).[275] Activation of $p56^{lck}$ requires dephosphorylation of this residue and CD45 may be the phosphatase responsible since T cells deficient in CD45 are impaired in TcR signalling.[329] Furthermore, CD45 phosphatase treatment of $p56^{lck}$ *in vitro* activates $p56^{lck}$ kinase activity[328] and overexpression of CD45 *in vivo* results in decreased phosphorylation of $p56^{lck}$.[327] The CD45 tyrosine phosphatase, which may be regulated by serine phosphorylation,[330] has also been similarly implicated in B cell antigen receptor activation of lyn.[331]

5.2 Serine/threonine specific phosphatases

Numerous Ser/Thr specific phosphatases have been described[323] and some of these may be involved in hemopoietic signal transduction pathways. For example, CD2 mediated T cell activation is associated with the dephosphorylation of a 19 kD protein[332] and a phosphatase may be involved in antagonizing the PKC mediated phosphorylation of CD3.[333] Also, unexpectedly, the immunosuppressants cyclosporin and FK506 were recently shown to act by suppressing the activity of a Ca^{2+}/calmodulin dependent phosphatase called calcineurin.[334] Thus, like tyrosine phosphatases, Ser/Thr specific phosphatases may also be important in signalling.

6. Signalling Pathways Implicated in the Mechanism of Action of IL-3

Murine IL-3, like all other hemopoietic growth factors, is absolutely required for cell growth and survival. Cells deprived of mIL-3 quickly lose viability and die by a process of programmed cell death referred to as apoptosis.[335,336] Early studies investigating the biochemical basis of mIL-3 action showed that mIL-3, like many growth factors, stimulated glucose uptake[337,338] and ATP elevation[339] in target cells. Eventually, similar to that observed in many other growth factor systems,[140] these initial events lead to elevation of *c-myc* and *c-fos* expression.[340,341] Studies directed towards further dissecting the mIL-3 signalling pathway have thus far concentrated on protein kinase C activation and protein tyrosine phosphorylation events.

With respect to protein kinase C, the literature contains many apparently conflicting results. In the majority of studies, mIL-3 was observed to activate PKC activity.[342-345] However, whereas activation of PKC was accompanied by its translocation to the plasma membrane in some instances,[344-346] no change in localization was detected in others.[342] Similarly, while some studies have implied that diacylglycerol is generated through the classical inositol phosphate pathway,[346] others have suggested that DAG may be derived from an alternate source[343,347] such as PC.[348] This variability may, to some extent, be attributed to differences intrinsic to the cells studied. Different cells, as discussed above, may possess a different complement of PKC and PLC isozymes. These differences may also account for the fact that phorbol esters can support the growth of some mIL-3 dependent cells[349,350] but have only modest effects on others.[351]

There is less variation amongst cell lines when it comes to mIL-3 stimulated tyrosine phosphorylation events. A role for tyrosine phosphorylation in mIL-3 action was first suggested by the fact that v-abl could confer growth factor independence on a mIL-3 dependent cell line via a non-autocrine mechanism.[352] Subsequently, mIL-3 was shown to rapidly induce tyrosine phosphorylations in a number of different cell lines.[351,353-356] In all cases, a 140 kD tyrosine phosphorylated protein, shown to be the mIL-3R,[357,358] was observed. The

identity of other notable tyrosine phosphorylated proteins, with apparent molecular masses of 90-100 kD,[351,354] 70 kD[353,355,356] and 56 kD[354,356] are not yet known.

A logical next step in furthering our knowledge of mIL-3 signal transduction, is to characterize these phosphoproteins, especially with regard to their relationship to the mIL-3R. The studies described in this thesis focuses on these phosphoproteins and the earliest biochemical events that occur following growth factor stimulation. However, it is important to keep in mind that the biochemistry set into motion by growth factor stimulation must eventually interact with intrinsic cell cycle controls.

D. CELL CYCLE CONTROL

There are three points in the cell cycle at which progression can be controlled: at the entrance into G1, the G1/S transition or at the G2/M boundary. The first two of these checkpoints are subject to control by external growth stimulatory or inhibitory signals, while the third is apparently subject to an intrinsic cellular program.

Much of our understanding of mammalian cell cycle control comes from studies in simpler systems such as that encountered in the Xenopus oocyte during maturation or yeast cell division. In xenopus, the G_2/M transition is controlled by an activity called M-phase Promoting Factor (MPF),[359] originally assayed by its ability to induce maturation upon injection into oocytes. Purification and biochemical characterization of this activity, which can be isolated from any mitotic, eucaryotic cell, showed the catalytic component of the multi-subunit complex to be identical to a 34 kd serine/threonine kinase originally identified in yeast. In yeast, study of the cell cycle is facilitated by the ease of isolation of conditional cell division cycle (cdc) mutants which are blocked, when grown in non-permissive conditions, at various points in the cell cycle. The 34 kd kinase is the gene product of the fission yeast cdc2 gene; a mutation at cdc2 results in M phase arrest.[360,361] The cdc2 kinase is essential for cell cycle progression through mitosis and its importance is suggested by its ubiquitous expression and high degree of conservation in all eucaryotic cells.[362] In fusion yeast, the CDC28 gene encodes an homologous kinase. In humans, a cdc2 homologue was isolated by

complementation of the cdc2 mutation in fission yeast,[363] and shown to be essential for mitosis with the use of either temperature sensitive mutants[364] or microinjection of neutralizing antibodies.[365]

The activity of cdc2 kinase fluctuates within the cell cycle, being highest at the onset of mitosis[362,366] and undetectable outside of M phase. However, cdc2 protein levels remain constant throughout the cycle and its activity is regulated by phosphorylation and association with cyclin proteins.[367] Towards the beginning of G_2 phase, unphosphorylated cdc2 kinase becomes phosphorylated on threonine residues and associated with cyclins.[366-368] This association targets cdc2 for tyrosine phosphorylation[369] and nuclear localization.[368] The inactive cdc2/cyclin complex is then activated at the onset of mitosis by cdc25 phosphatase mediated[370] dephosphorylation of phosphotyrosine residues.[371] The activated cdc2 kinase may then phosphorylate histones, nuclear lamins and other substrates responsible for chromosome condensation, nuclear envelope breakdown and mitotic spindle formation.[362] Cyclin proteolysis[366,367] at metaphase/anaphase via an ubiquitin dependent pathway[372] then inactivates the cdc2 kinase.

Cyclins were originally described in marine invertebrates as proteins that cyclically accumulate to high levels in interphase and undergo abrupt destruction at the end of mitosis.[373] In addition to these mitotic cyclins, G_1 specific cyclins (CLN1, CLN2, CLN3, and CLN4) which mediate the G_1/S transition have since been isolated in fusion yeast.[374,375] The levels of two of these, CLN1 and CLN2, undergoe cell cycle fluctuation as expected, with highest levels at late G_1. CLN4 has only just been discovered and little is known about its level of expression.[375] However, the level of CLN3 remains constant through the cell cycle.

Intriguingly, G_1 cyclin expression is regulated through a positive feedback loop.[376,377] Transcription of CLN1 and CLN2 requires the action of CLN3/CDC28 kinase activity, possibly through the phosphorylation and activation of transcription factors SWI4 and SWI6[377] which control cyclin gene expression. CLN3, the only G_1 cyclin not to undergo cycle dependent changes in abundance, is necessary as an upstream activator but is not sufficient in itself for

cell cycle progression since G_1/S transition requires the presence of CLN1, CLN2 or CLN4. The feedback loop is closed by the ability of any of the synthesized cyclins to positively regulate its own transcription. This positive feedback mechanism is an effective way to convert an initial signal to a large increase in cyclin protein and may also be the basis for the irreversibility of S phase commitment.

In higher eucaryotes the cycle is complicated by the presence of many more cyclins. Several human cyclins have been isolated[378-381] which have been classified into five types according to sequence homology or function.[380] Cyclins A and B are considered mitotic cyclins since highest levels exist at M phase. However, the abundance of cyclin A rises earlier in the cell cycle than cyclin B,[382] implying it possesses a function outside of M phase. Indeed, differences exist between the two with respect to the kinase they prefer. In humans, it has recently been shown that there are at least two different cdc2-like kinases.[382,383] Cyclin B complexes with the original cdc2, which can also be referred to as Cyclin Dependent Kinase-1 (CDK-1), while cyclin A but not cyclin B associates with the newly cloned CDK-2.[384,385] The histone H-1 kinase activity of cyclin A/CDK-2 is not as efficient as cyclin B/cdc2,[382,384] suggesting different substrates and functions. As well, cyclin A differs from cyclin B in that it is sequestered by E1A in adenovirus infected cells whereas cyclin B is not.[382,384] Cyclins C, D and E, on the other hand, are G_1 cyclins. Cyclin C mRNA is highest during early G_1 while cyclin E is highest at late G_1. Expression of the D type cyclins is more complex since they exhibit both tissue specific expression[386,387] and cell specific regulation. Cyclin D1 (also called CYL1) is, at present, of great interest because it is the first cyclin whose expression has been shown to be under growth factor regulation.[386] Specifically, in monocytes, CSF-1 is required for cell cycle progression; in its absence cells arrest at G_1 and die. Cyclin D1 mRNA and protein levels in G_1 arrested monocytes are low, but the addition of CSF-1 induces cyclin D1 mRNA during late G_1. Continued presence of CSF-1 through S phase is required for mRNA stability. Cyclin D1 was independently isolated as a *bcl1* linked gene from HeLa cells.[387] In these cells, cyclin D1 mRNA is induced following S phase and degraded at the G_1/M boundary.

Perhaps the differences in cyclin expression reflect cell cycle control differences between CSF-1 dependent monocytes and factor independent HeLa cells.

Targets for the cyclin dependent kinases are not yet well defined.[226] Nuclear lamins[388] and histone H1 are two potential substrates since they undergo cell cycle specific phosphorylation coincident with the activation of mitotic cdc2 kinase. Phosphorylation of these proteins may initiate the nuclear envelope disassembly and chromosome condensation observed at mitosis. Other proteins have also been implicated as substrates since they also exhibit cycle dependent phosphorylations mediated by cdc2 kinase. These include $p60^{src}$ [389,390] $p150^{abl}$ [391] and the transcription factor encoded by *oct 1*.[392] These phosphorylations may regulate their activity or alter substrate specificity in a cycle specific manner.

Recently, however, considerable attention has focussed on the cycle specific regulation of the retinoblastoma (Rb) gene product. The Rb susceptibility gene encodes a 100 kd nuclear protein that functions as a negative regulator of cell growth.[393] Loss of Rb function correlates with development of certain human tumours. The Rb protein is phosphorylated in a cell cycle dependent manner with maximal phosphorylation during S-phase, reduced phosphorylation after and no phosphorylation during G_1.[394] This phosphorylation is believed to be mediated by cdc2 kinase.[395] Elegant microinjection studies have shown that it is the unphosphorylated form which suppresses cell proliferation by restricting cell cycle progression at a specific point in G_1[396]

Active, unphosphorylated, Rb protein inhibits transcription of several cellular early response genes including *c-fos* and *c-myc*. Initially, it was believed that Rb exerts this suppressive action by direct interaction with DNA promoter regions. However, evidence now suggests that Rb does not bind directly to DNA, but interacts with and regulates transcription factors such E2F[397,398] and DRTF1.[399,400] The association of E2F and Rb has been rigorously studied. Free, or Rb uncomplexed E2F has higher transcriptional activity than Rb bound E2F. In quiescent, serum starved cells or cells synchronized at G_1, E2F is sequestered

by unphosphorylated Rb.[397] Cell cycle progression is associated with dissociation of this complex. This complex can also be disrupted by the transforming proteins of several DNA tumour viruses including adenovirus E1A, SV40 and polyoma virus large T and human papilloma virus E6. The conserved regions in these viral transforming proteins essential for transformation are also those which are required for interaction with the Rb protein, suggesting that these oncogenes stimulate cell proliferation by sequestering Rb and thus inactivating its growth suppressive activity. Efforts have now turned towards identifying the normal cellular homologue of the DNA viral oncogenes which mediate the normal transition from Rb bound E2F to active E2F.[395] These predicted cellular Rb binding proteins must then be integrated into a model in which disruption of Rb/E2F complexes occurs with cycle dependent changes in phosphorylation of the Rb protein.

Another piece to add to this puzzle is the observation that E2F complexes with cyclin A during S phase. It is therefore tempting to speculate that during the G_1/S transition, as more cyclin A accumulates,a cyclin A dependent kinase associates with the G_1 Rb/E2F complex and the resulting phosphorylation of Rb by the CDK then releases Rb. Indeed, in studies with DRTF1,[400] a transcription factor probably identical to E2F, Rb and cyclin A have been detected in the same DRTF1 complex.[399] Adenovirus E1A will also disrupt cyclin A/E2F complexes.[398,399,401] However, the role of cyclin A in the E2F complexes is open to speculation. Perhaps the association with E2F targets the CDK to S-phase specific targets, or perhaps the association with cyclin A changes the specificity of E2F. The effect of the combination is, however, not a simple global activation of E2F since cyclin A/E2F complexes have lower transcriptional activity than free E2F.[398] Considerable evidence points to the the importance of Rb and its interaction with transcription factors, cyclins and CDK's, not only in cell cycle control but, as will discussed in the next section, in growth factor responses as well.

1. Integration of Growth Factor Control With Cell Cycle Control

Until very recently, work on growth factor control and cell cycle analysis have progressed independently. Progress in our understanding of the cell cycle has been achieved primarily via

deletion mutants in simple organisms such as yeast where genetic manipulations are relatively simple. Growth factor studies have, on the other hand, concentrated on accessible early biochemical changes in mammalian cells. Thus, control of the cell cycle was essentially divided into two solitudes. Lately this dichotomy has been disappearing as rapid progress is being made from both ends of the cycle. We are beginning to to able to integrate growth factor control mechanisms with cell cycle events.

An example of this kind of integration is the inhibition by TGFβ of *c-myc* transcription.[402] TGFβ also inhibits G_1/S cdc2 kinase activity[403] and Rb phosphorylation.[403] These observations suggest a model of TGFβ action in which TGFβ inhibition of G_1/S cdc2 kinase results in failure to phosphorylate directly, or indirectly, the Rb protein, a phosphorylation which ordinarily undergoes cell cycle oscillation. Failure to inactivate Rb allows persistence, perhaps, of an inactive Rb/E2F complex so that *c-myc* is not transcribed. Consistent with this model is the abrogation of TGFβ mediated inhibition of *c-myc* (which contains an E2F binding motif in its promoter region) transcription when cells are transfected with Rb binding proteins such as E1A.[404]

A more concrete instance of a growth factor pathway feeding into cell cycle control is the recent, direct demonstration that certain growth factors activate cyclins or CDK's directly. CSF-1, as mentioned earlier, induces the synthesis of cyclin D protein. The continued presence of CSF-1 during G_1 is required for persistence of cyclin D. Removal of CSF-1 and thus cyclin D at any time before G_1/S commitment results in cycle arrest. More intriguing is the involvement of CDK's very early in the signal transduction cascade. Both EGF[405] and NGF[406] stimulate activation of a proline directed protein kinase (PDPK). Biochemical characterization of PDPK has shown it to be a cyclin A/cdc2 complex.[405] This surprising observation suggests that certain CDK's act much earlier than the G_1 restriction point and a possible role for this factor responsive complex in cell division control will be discussed below. In fact, a role outside of cell division altogether has been hypothesized for $p34^{cdc2}$ since

platelet activating factor apparently activates cdc2 histone H1 kinase activity in platelets, a cell incapable of undergoing division.[407]

Work in growth factor and cell cycle control has progressed sufficiently such that our knowledge of the two fields may soon become integrated completely. An important concept that has emerged from studies so far, is the high degree of conservation of fundamental growth and cycle control mechanisms from yeast to man. Because of this conservation, there are certain observations made in yeast that might by integrated with certain growth factor responses in mammalian cells. For example, in fusion yeast the G_1 transition is mediated by expression of four CLN genes. Upregulation of the three oscillating cyclins is mediated by the activation of CLN3. However, since the protein level of CLN3 does not oscillate through the cycle, it must be controlled post-translationally. In mammalian cells, an analogous system may exist; G_1/S commitment may also be initiated by the activation of a constitutively expressed cyclin. Indeed, although cyclin D1 induction in the first round through the cell cycle requires CSF-1, in subsequent cycles there is a basal, constant level throughout the cycle. Genetic complementation analyses demonstrate that CLN3 is regulated in yeast by the product of the FUS3 gene.[408,409] Very intriguingly, FUS3 encodes a Ser/Thr kinase homologous to the mammalian MAP kinases.[307] It is very tempting to speculate that growth factor stimulation of a MAP kinase activates the basally expressed cyclin which then initiates the cyclin transcription positive feedback loop. Alternatively, instead of cyclin regulation by MAP kinase activation similar to the FUS3 kinase signalling system in yeast, mammalian cells could utilize a cyclin/CDK complex which is more directly coupled to growth factor stimulation. PDPK is exactly such a complex. One can envisage a signal transduction cascade in which growth factor stimulation activates either MAP kinase or PDPK, which then, in a manner analogous to the events described in yeast, elevates cyclin levels by phosphorylating transcription factors which regulate cyclin gene expression. These models are readily testable and the results could be very interesting and may potentially provide a direct path from receptor activation to the initialization of a cell's intrinsic cell cycle machinery.

E. THESIS OBJECTIVE

At the time the work described in this thesis was initiated, relatively little was known about the receptors for the hemopoietic growth factors. The best characterized receptor, an intrinsic tyrosine kinase receptor, was the one for CSF-1. No member of the hemopoietin receptor superfamily had yet been cloned. Indeed, studies into the mechanism of action of the interleukins (and there were only four at this time), had only just begun with the recent availability of recombinant growth factors. Thus a logical first step in understanding the mechanism of action of mIL-3 was the purification and characterization of its cell surface receptor. Once this was accomplished, the mIL-3R could then be studied to see if it utilized signalling mechanisms similar to those used by better characterized receptors, such as those for CSF-1 and EGF. Specifically, the thesis objectives were to:

1. Develop an assay for detecting the detergent solubilized mIL-3R.
2. Purify the mIL-3R in sufficient amounts for amino acid sequencing.
3. Conduct biochemical characterization studies of the purified receptor.
4. Investigate the signalling pathways used by this receptor, especially with respect to the kinase responsible for tyrosine phosphorylation of the mIL-3R.

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CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Materials

Streptavidin-agarose beads and protein grade Nonidet P-40 (NP40) were purchased from Calbiochem. [^{32}P]-orthophosphate (50 mCi/mL; carrier free) in acid free solution, Na[^{125}I] (100 mCi/mL; carrier free) and ^{3}H-Thymidine (2 Ci/mmole) were from ICN Biochemicals. Expre^{35}S^{35}S protein labeling mix (11.5 nCi/mL) was from NEN. Co-bind plates were from Micromembranes, Inc (Newark, NJ). All other reagents were purchased from the Sigma Chemical Company unless otherwise indicated.

2. Growth Factors, Antibodies and cDNA's

Purified *E. coli* derived recombinant mIL-3[1] was generously supplied by Biogen, Geneva, Switzerland. Purified recombinant murine SF was provided by Immunex, Seattle, Washington. Polyclonal rabbit serum raised against GAP, p90^{fps}, p56^{hck} and p97^{vav} were kindly provided by Drs. Tony Pawson (Samuel Lunenberg Research Centre, Toronto), Peter Greer (Samuel Lunenberg Research Centre), Roger Perlmutter (University of Washington, Seattle) and Mariano Barbacid (Bristol-Meyers Squibb, Princeton, NJ), respectively. Affinity purified rabbit polyclonal antibodies against cyclin A and PSTAIRE were the kind gifts of Drs. Fred Hall (University of Southern California, Los Angeles) and Steven Pelech (University of British Columbia, Vancouver), respectively. Affinity purified rabbit α-phosphotyrosine antibodies were obtained from Upstate Biotechnology, Inc.(UBI, Lake Placid, NY) and purified mouse monoclonal α-phosphotyrosine antibody 4G10 was generously provided by Dr. Brian Druker (Dana Farber Institute, Boston, Ma) and both antibodies were used at 2 μg/mL in Western analyses. Monoclonal α-phosphotyrosine 1G2 was purified using protein A agarose from hybridoma supernatants and coupled to CNBr Sepharose (Pharmacia) according to standard

procedures. Antibodies against PLCγ and MAP kinase were from UBI. The AIC 2A cDNA was kindly provided by Dr. Atsushi Miyajima of DNAX, California. *E. coli* transformed with pGEX vectors containing the SH2 domains of GAP, PLCγ and PI3-K were generously supplied Dr. Tony Pawson.

3. Cells

The mIL-3 dependent cell line, B6SUtA, generously provided by Dr. J. Greenberger, (University of Massachusetts, Worchester, MA) was derived from a Friend virus infected culture of C57Bl.S bone marrow cells.[2] The clone, $B6SUtA_1$, was obtained by plucking an individual B6SUtA colony growing in methylcellulose. These cells were propagated in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 u/mL penicillin G, 100 μg/mL streptomycin and either 5% pokeweed mitogen stimulated spleen cell conditioned medium or 3 ng/mL COS cell derived murine granulocyte-macrophage colony stimulating factor (mGM-CSF). The mIL-3 dependent cell line FDC-P1 was also kindly supplied by Dr. Joel Greenberger and propagated in the same medium as the $B6SUtA_1$ cells. COS-7 cells were grown in DMEM containing 5% FCS. Normal mouse bone marrow cells were isolated from the femurs and tibia of B6xC3H (F1) mice, treated with ammonium chloride to lyse the mature red blood cells and washed 2x with Hank's Balanced Salt Solution (HBSS).

4. Preparation of Polyclonal Rabbit Serum Against mIL-3R Peptides

Rabbit anti-serum to the amino terminal 15 amino acids of the AIC 2A product[3] (which corresponds exactly to the amino terminus of the mIL-3R that we purified[4]) and the terminal 15 amino acids of AIC 2A were produced by immunizing rabbits with peptide conjugated to keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant followed by monthly boosts with peptide-KLH conjugate in incomplete Freund's adjuvant. This antiserum was purified on an amino terminal peptide affinity column and used at 1.0 μg/mL in Western analyses. Specificity for the mIL-3R was established by comparing its reactivity with AIC 2A transfected and non-transfected COS cells.

B. GENERAL BIOCHEMICAL TECHNIQUES

1. Protein Determination

Protein concentrations were determined using the Coomassie Brilliant Blue G-250 dye-binding technique,[5] the soluble silver binding assay,[6] and densitometric analysis of silver stained gels.

2. Radio-iodinating Proteins for SDS-PAGE Analysis

Protein samples, usually 50 µL, were buffered to pH 7.5 with 0.1 M Tris-Cl. 100 µCi of $Na^{125}I$ was added and the sample made 100 µg/mL with chloramine T. After incubating at 23° for 20 min, the reaction was terminated with 200 µg/mL Na bisulfite and 10 mM NaI. Unincorporated ^{125}I was removed by centrifugation through a 1 mL Sephadex G-25 (Pharmacia) column equilibrated with 1% SDS in PBS.

3. One and Two Dimensional Gel Electrophoresis

Samples for one-dimensional sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) were adjusted to 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and boiled for 2 min prior to electrophoresis on one-dimensional 7.5% or 5-15% gradient SDS-polyacrylamide gels, as described by Laemmli.[7] Two dimensional gel analysis, isoelectrofocusing in the first and SDS-PAGE in the second dimension, was performed with the Bio-Rad Protean 2D cell according to the manufacturer's instructions. ^{14}C-labelled molecular weight markers were from Amersham and consisted of myosin (200 kD), phosphorylase-b (97.4 kD) , bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD) and lysozyme (14.3 kD).

4. Western Blotting

Proteins were electrophoresed on 7.5% polyacrylamide gels at 100 V for 12 h and then electrotransferred to Immobilon-P membranes at 100 V for 2 h at 23°C. Blots were blocked for 8 h at 23°C with 20 mM Tris-Cl, 0.15M NaCl, pH 7.4 (TBS) containing 5% BSA, and 0.02% NaN_3, and then incubated for 2 h with the appropriate first antibody in TBS with 0.05% Tween 20 (TBST). Blots were then washed 3 X 5 min with TBST, incubated with 200,000 cpm/mL of ^{125}I-goat anti-rabbit IgG or ^{125}I-goat anti-mouse IgG (Jackson Immunoresearch Labs, Inc.,

West Grove, PA) in TBST for 1 h at 23°C, washed 3 times with TBST, air dried and subjected to autoradiography.

5. Phosphoamino Acid Analysis

The analysis of phosphoamino acids was based on the method of Hunter and Sefton.[8] Phosphoproteins of interest were localized by autoradiography of unfixed gels dried onto cellulose acetate sheets. Phosphoproteins were electro-eluted from gel slices into 0.1 M $NaHCO_3$, 0.05% SDS, dialyzed against 0.01% SDS in distilled water and lyophilized. Residues were dissolved in 300 µL of 6 M HCl, boiled for 1.5 h at 100°C under N_2 in sealed tubes, diluted with 1 mL distilled water and lyophilized a further 2 times. The dried residues were then dissolved in 50 % EtOH containing 1 mg/mL phosphoserine, phosphothreonine and phosphotyrosine standards and applied to cellulose thin- layer plates. The plates were wetted evenly with a solution consisting of 1:10:189 pyridine/acetic acid/water and electrophoresed at 100 V for 40 min at 23°C. The ^{32}P-labelled phosphoamino acids and the standards were detected by autoradiography and ninhydrin staining, respectively.

C. PURIFICATION AND DERIVITIZATION OF GROWTH FACTORS

1. $B6SUtA_1$ Cell Proliferation Assay for mIL-3 and mGM-CSF

To assay mIL-3, mGM-CSF or SF, $B6SUtA_1$ cells were washed twice with RPMI 1640 and then incubated at 2 X 10^5 cells/mL with 10% FCS in RPMI in the presence of the test sample in a total volume of 0.1 mL in Linbro U-shaped microtitre plate wells. After 18-24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, 20 µL of ^3H-thymidine stock containing 50 µCi/mL (2 Ci/mmole) in RPMI 1640 was added to each well to give a final ^3H-thymidine concentration of 1 µCi/well. After a further 2 h incubation at 37°C, the well contents were harvested onto glass fiber filters using an LKB 1295 cell harvester and the filters counted on an LKB Betaplate scintillation counter.

2. Production and Purification of COS cell Derived mIL-3 and mGM-CSF

The coding region of mIL-3 was assembled from cDNA and genomic mIL-3 clones obtained from Biogen and expressed in COS cells using a pAX expression vector.[9] An mGM-

CSF vector was similarly constructed using a cDNA clone obtained from Dr. N. Gough.[10] Murine IL-3 and mGM-CSF were purified from serum free supernatants by sequential phenyl Sepharose and Sephadex G-75 gel filtration chromatography. Preparations were >95% pure as assessed by SDS-PAGE and autoradiography of ^{125}I-labeled material.

3. Biotinylation of COS Cell Derived mIL-3

In preliminary experiments biotinylated mIL-3 (B-mIL-3) was prepared as described previously[11] with NHS-LC-Biotin (Pierce). In later experiments, to avoid the batch to batch variability in biological activity of NHS-LC-biotinylated mIL-3, B-mIL-3 was prepared from COS cell derived mIL-3 using biotin-X-hydrazide (Calbiochem). Purified mIL-3 was made pH 5.5 with 0.5 M sodium acetate and oxidized with 10 mM sodium periodate. After 1 h at 23°C, the periodate was removed by gel filtration through a Sephadex G-25 (Pharmacia) column equilibrated with 0.1 M sodium acetate, 0.05% Tween 20, pH 5.5. The oxidized mIL-3 was then made 2 mM in biotin-X-hydrazide and allowed to react for 5 h at 23°C. B-mIL-3 was separated from free biotin by gel filtration through a Sephadex G-25 Sephadex column equilibrated with PBS containing 0.05% Tween 20.

4. Iodination of mIL-3

Aliquots of recombinant, *E. coli* derived mIL-3 containing 4 μg of growth factor were resuspended in a total volume of 100 μL of 0.1 M Na phosphate, pH 7.2, containing 10% dimethyl sulfoxide and 100 μg/mL polyethylene glycol 4000. To this was added 1 mCi of Na^{125}I, followed by 20 μL of freshly prepared 1 mg/mL chloramine T. After incubating for 20 min at 23°C, 20 μL each of freshly prepared 3 mg/mL Na metabisulfite and 0.1 M NaI were added. Free ^{125}I was then removed by gel filtration on a 10 mL G-25 Sephadex column equilibrated with 0.05% Tween 20 in PBS. The void volume was collected, made 0.1% in BSA and 0.02% and NaN_3 and stored at 4°C. Following iodination, mIL-3 retained >95% of its biological activity as assessed by the B6SUtA$_1$ ^{3}H-thymidine incorporation assay. Specific activity was determined by self-displacement analysis according to the method of Calvo.[12]

D. IN VIVO ISOTOPIC LABELLING OF B6SUtA$_1$ CELLS

1. Labelling Cells with ^{32}P-Orthophosphate

To prepare ^{32}P-labeled B6SUtA$_1$ cells, the cells were washed twice with phosphate free RPMI 1640 and incubated at 2 x 10^6 c/mL in phosphate-free RPMI 1640 containing 3 ng/mL mGM-CSF and 0.25 mCi/mL of carrier-free [^{32}P]-orthophosphate for 2 h at 37°C. The cells were then washed twice with PBS, resuspended at 7.5 x 10^7 c/mL in PBS containing 0.1% ovalbumin, 50 μM sodium orthovanadate (Na_3VO_4), 0.02% sodium azide (NaN_3) for growth factor stimulation.

2. Labelling Cells with ^{35}S-Methionine

To prepare ^{35}S-labelled B6SUtA$_1$ cells, the cells were first grown in RPMI 1640 containing 10% FCS and 3 ng/mL mIL-3 to downregulate mIL-3R levels.[13] The cells were then washed twice with 37°C methionine-free DMEM and incubated at 1 X 10^6 c/mL in methionine-free DMEM supplemented with 100 μM methionine, 50 μCi/mL Expre^{35}S^{35}S protein labelling mix, 10% FCS (dialyzed against PBS) and 3 ng/mL mGM-CSF (to upregulate mIL-3R levels[13]), for 6 h at 37°C. The cells were then washed twice in PBS and resuspended at 7.5 x 10^7 c/mL in PBS containing 0.1% ovalbumin, 50 μM Na_3VO_4, 0.02% NaN_3 for growth factor stimulation.

E. PURIFICATION OF THE mIL-3R

1. mIL-3R Solubilization

B6SUtA$_1$ cells or B6SUtA$_1$ cell membranes were suspended at 2 X 10^7 c/mL or 2 mg/mL respectively in 0.5% NP40 in PBS, or other buffers as indicated, containing 0.2 mM phenylmethyl sulfonylfluoride (PMSF), 100 KIU/mL aprotinin and 2 μg/mL leupeptin. Samples were then agitated for 1 h at 4°C before being centrifuged at 100 000 X g for 1 h to remove insoluble material.

2. Soluble Receptor Assay

Detergent solubilized samples, usually 50 μL, were incubated with 30 nM ^{125}I-mIL-3 for 2 to 6 h at 4°C and then transferred to 5 mL polystyrene tubes containing 4 mL of 0.1 % NP40 in TBS and 15 μl (packed volume) of Concanavalin A (Con-A) Sepharose beads. The beads were

rocked at 4°C for 6 h, washed twice with TBS containing 0.1% NP40 and counted in a Beckman 5500 gamma counter.

3. Purification of the mIL3-R From Intact B6SUtA$_1$ Cells

B6SUtA$_1$ cells, propagated in 3 ng/mL mGM-CSF to upregulate mIL-3 receptors,[13] were washed twice in PBS and resuspended at 7.5 x 10^7 c/mL in PBS containing 0.1% ovalbumin, 50 μM sodium orthovanadate, 0.02% sodium azide and 200 nM B-mIL-3. After incubating for 4 h at 4°C, the cells were washed twice with ice-cold PBS and resuspended at 2 X 10^7 c/mL with 0.5% NP40 in 50 mM Hepes, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 4 mM EDTA, 2 mM PMSF, 100 KIU aprotinin, 2 μg/mL leupeptin (pH 7.4) (phosphorylation solubilization buffer, **PSB**) and solubilized by shaking for 1 h at 4°C. Insoluble material was removed by centrifuging for 1 h at 100,000 x g. The supernatant was incubated batchwise with α-phosphotyrosine Sepharose overnight at 4°C. The beads were then poured into a column and washed with phosphorylation buffer containing 0.1% NP40 to elute the 120 kDa mIL-3R and other non-tyrosine phosphorylated proteins. The tyrosine phosphoproteins were then eluted with PSB containing 40 mM phenylphosphate and 0.1% NP40. Both the α-phosphotyrosine bound fraction, containing the 140 kDa mIL-3R and the unbound fraction containing the 120 kD mIL-3R were applied separately to streptavidin-agarose (SA) columns and washed sequentially with PSB containing 0.1% NP40, with 20 mM sodium citrate, 0.15 M NaCl (pH 6.0) containing 0.05% NP40, and then with 0.05% NP40, 20 mM sodium citrate, 0.15 M NaCl (pH 3.5). The low pH fractions were neutralized with 2 M Tris-Cl (pH 7.5).

4. Determination of the Amino Acid Sequence and Composition of the 120 kD mIL-3R

The unbound fraction from the α-phosphotyrosine Sepharose column was chromatographed through a SA column to recover non-tyrosine phosphorylated, B-mIL-3 bound mIL-3Rs. These 120 kD mIL-3Rs were then eluted as above, made 5% with SDS and β-ME, heated at 60°C for 15 min and applied to an SDS gel consisting of a 2% agarose stacking gel and an acrylamide separating gel (7.5% T, 0.4% C) that had been dialyzed for 48 h against

50 μM reduced glutathione, 375 mM Tris-Cl, 0.1% SDS (pH 8.8) in order to reduce amino terminal blocking. Following electrophoresis for 5 h at 40 mA with 100 μM thioglycolate in the cathodic buffer, the gel was equilibrated for 15 min in electrotransfer buffer (0.5 mM DTT, 10 mM Caps (pH 10), 10% MeOH) before electrotransfer to an Immobilon-P membrane (Millipore) at 90 V for 2 h at 23°C. The blot was stained for 5 min with 0.1% Coomassie R-250, destained in 10% acetic acid, 50% methanol, rinsed well with distilled water and air-dried. The 120 kDa band was excised and sent to the University of Victoria Protein Microchemistry Centre for sequencing on an Applied Biosystems 470A gas phase sequencer. A portion of the same band was also acid hydrolyzed and analyzed using an Applied Biosystems Model 420A Derivatizer-Analyzer System to obtain amino acid composition data.

5. Displacement Analysis With the 120 kD mIL-3R

The purified 120 kDa mIL-3R (prepared in 0.1 M glycine buffer, pH 3.0, instead of citrate buffer, since the latter inhibits N-glycanase) was supplemented with 0.2 M Tris-Cl, pH 8.0, and 10 mM phenanthroline and incubated with or without 30 U/mL N-glycanase (Genzyme). The samples were then incubated with 30 nM ^{125}I-mIL-3 at 4°C. After 2 h, 1 μM unlabelled mIL-3 was added and, at various times, duplicate aliquots were applied to 1 mL A1.5m (Bio-Rad) columns equilibrated with PBS containing 0.1% NP-40. The void volume containing ^{125}I-mIL-3/mIL-3R complexes was collected and counted in a Beckman 5500 gamma counter.

6. Scatchard Analysis of the mIL-3R on Intact Cells

Cells were washed twice in PBS and resuspended at either 2 X 10^5 cells/80 μL (cell lines) or 3 X 10^6 cells/80 μL (normal mouse bone marrow) in PBS containing 1% BSA, 0.1% gelatin, 0.02% NaN_3. The cells were then added to 0.5 mL microfuge tubes containing 20 μL of 2 fold dilutions of ^{125}I-mIL-3, starting at 10 nM, in the presence or absence of a 20 fold excess of unlabelled mIL-3. After incubating 2 h at 4°C, the sample was layered over 250 μL of 2:3 dioctyl:dibutyl phthalate and microfuged for 2 min at 14 000 rpm. The tubes were then frozen on dry ice and the cell pellets clipped into gamma tubes for counting in a Beckman 5500 counter. To examine the effect of carbohydrate on receptor binding affinity, B6SUtA$_1$ cells,

growing exponentially in 3 ng/mL mIL-3, were washed twice in RPMI and grown for 6 h with 10 μg/mL tunicamycin in 3 ng/mL mGM-CSF, 10% FCS in RPMI 1640. These cells were then subjected to Scatchard analysis as described.

F. ANALYSIS OF THE PROPERTIES OF THE TYROSINE PHOSPHORYLATED mIL-3R

1. Purification of Tyrosine Phosphorylated and Tyrosine Unphosphorylated mIL-3R's

B6SUtA$_1$ cells propagated in 3 ng/mL mGM-CSF to upregulate mIL-3Rs,[13] were washed twice in PBS and resuspended at 7.5 x 10^7 c/mL in PBS containing 0.1% ovalbumin, 50 μM Na_3VO_4, 0.02% NaN_3 and 200 nM B-mIL-3. After incubating for 4 h at 4°C, the cells were washed twice with ice-cold PBS and resuspended at 2 x 10^7 c/mL with 0.5% NP40 in PSB and solubilized by shaking for 1 h at 4°C. Insoluble material was removed by centrifuging for 1 h at 100,000 x g. The supernatant was incubated batchwise with α-phosphotyrosine Sepharose overnight at 4°C. The beads were then poured into a column and washed with PSB containing 0.1% NP40 to recover the 120 kDa mIL-3R and other non-tyrosine phosphorylated proteins. The tyrosine phosphoproteins were then eluted with PSB containing 40 mM phenylphosphate and 0.1% NP40. Both the α-phosphotyrosine bound fraction, containing the 140 kDa mIL-3R and the unbound fraction containing the 120 kDa mIL-3R were applied separately to streptavidin agarose columns and washed sequentially with PSB containing 0.1% NP40, with 20 mM sodium citrate, 0.15 M NaCl (pH 6.0) containing 0.05% NP40, and then with 0.05% NP40, 20 mM sodium citrate, 0.15 M NaCl (pH 3.5). The low pH fractions were neutralized with 2 M Tris-Cl (pH 7.5). Typically, 10 - 20% of the total mIL-3R preparation was tyrosine phosphorylated.

2. Phosphatase Treatment of the Tyrosine Phosphorylated mIL-3R

Tyrosine phosphorylated mIL-3R's were purified as described on page 92 except that underivatized mIL-3 was used instead of B-mIL-3 and the streptavidin step was omitted. The α-phosphotyrosine bound, phenyl phosphate eluate was chromatographed through a Sephadex G-25 column equilibrated with 20 mM imidazole, pH 7.2, 0.15 M NaCl, 0.1 mM EDTA and 0.1% β-mercaptoethanol to remove the phosphate and protease inhibitors. Receptor

preparations were then incubated 16 h at 37°C with or without each of the following phosphatases: 100 U/mL T cell specific phosphatase (truncated form, kindly provided by Dr. Deborah Cool, University of Washington, Seattle, WA), 1 U/mL each of the catalytic subunits of the serine phosphatase 1 and 2A (kindly provided by Dr. Nick Tonks, Cold Spring Harbour Laboratory, NY), or 1 U/mL calf intestinal alkaline phosphatase (Boehringer Mannheim). The incubation mixtures were supplemented to 2 mM EDTA for the tyrosine phosphatase samples and to 1 mM $MgCl_2$ and 2 mM $ZnCl_2$ for the alkaline phosphatase samples. Receptor integrity was then analyzed by SDS-PAGE and Western blotting with affinity purified anti-N terminal mIL-3R antibody as described above.

G. ANALYSIS OF mIL-3R INDUCED TYROSINE PHOSPHOPROTEINS

1. Preparation of NP40 Detergent Lysates from Factor Stimulated B6SUtA$_1$ Cells

B6SUtA$_1$ cells were washed twice with RPMI 1640, resuspended at 2 X 10^5 cells/mL in 0.1% BSA in RPMI 1640 and incubated at 37°C for 3 h. The cells were then pelleted and resuspended at 2 X 10^7 cells/mL in 0.1% BSA, RPMI 1640 equilibrated to 4°C or 37°C. Growth factors were then added to give a final concentration of 30 ng/mL mIL-3, 5 ng/mL mGM-CSF or 20 ng/mL SF. For the 4°C growth factor stimulations, the cells were pelleted after the appropriate time by centrifuging for 4 min at 2000 rpm in an Eppendorf microcentrifuge 5415. For the 37°C stimulations, 20 volumes of ice cold PBS were added to stop the reaction before pelleting the cells by centrifuging for 5 min at 600 X g in a Beckman TJ-6 centrifuge. The cells were then resuspended at 2 X 10^7 cells/mL in 0.5% NP40 in PSB and solubilized for 1 h at 4°C. Insoluble material was pelleted by a 20 min centrifugation at 12 000 X g and the supernatants taken for study. If the supernatants were to be analyzed directly by SDS-PAGE, the samples were made 4% in SDS and 5 % in 2-ME prior to boiling for 2 min at 100°C.

2. Immunoprecipitation and SH2 Precipitation of Cellular Proteins

Detergent lysates, prepared as described above, were incubated with first antibody, α-phosphotyrosine-Sepharose (100 μg 1G2/20μL packed beads/mL of lysate) or SH2 Sepharose

(20 μL packed beads/mL lysate, prepared as described below). After 2 h at 4°C, protein A beads were added to the soluble antibody samples and incubated an additional 2 h. All beads were then washed three times with 0.1% NP40 in PSB and either boiled in SDS-PAGE sample buffer (protein A and SH2 beads) or eluted in 90 μL of 5 mM phenyl phosphate, 0.1% NP40 in PSB (α-phosphotyrosine Sepharose).

3. mIL-3R Tyrosine Kinase Assay

Co-bind plates were incubated for 2 h at 23°C with 10 μg/mL BSA in PBS, washed four times with PBS and then incubated with 0.1 mg/mL NHS-LC-Biotin (Pierce). After 1 h at 23°C, the plates were blocked with 1% BSA in PBS. The BSA blocked plates were then incubated with 10 μg/mL SA in PBS for 1 hr at 23°C. B-mIL-3R complexes were prepared by incubating B6SUtA$_1$ plasma membranes (4 mg/mL) with 200 nM B-mIL-3 for 2 h at 4°C. The membranes were washed once with PBS and solubilized at 4 mg/mL with 0.5% NP40 in PBS containing 0.2 mM PMSF, 100 KIU/mL aprotinin and 2 μg/mL leupeptin for 1 h at 4°C. Insoluble material was then pelleted by microfuging 20 min at 12 000 X g, and the solubilized B-mIL-3/mIL-3R complexes were incubated with the biotin/BSA treated Co-bind plates. After 2 h at 4°C, the plates were washed 4 times with 0.1% NP40 in PBS.

Samples to be assayed, usually 100 μL, were added to the wells in duplicate and supplemented with 10 mM $MgCl_2$, 5 mM ATP, 2 mM $NaVO_4$, 0.2 mM PMSF, 100 KIU/mL aprotinin and 2 μg/mL leupeptin. The plates were incubated overnight at 4°C, washed 3 times with 0.1% NP40 in PSB and incubated a further 2 h at 4°C with 1 μg/mL ^{125}I-1G2 α-phosphotyrosine. After 2 h, the plates were washed 3 X with 0.1% NP40 in PSB and eluted with 1% SDS. The SDS eluates were transferred to gamma tubes for counting as above.

4. Sephadex G150 Fractionation of mIL-3 Induced Tyrosine Phosphoproteins

Tyrosine phosphorylated proteins, from B6SUtA$_1$ cells stimulated for 10 min at 4°C with mIL-3, were purified from 8 X 10^7 cells using α-phosphotyrosine Sepharose as described above. The 500 μL phenyl phosphate eluate, was applied to a 30 cm X 0.3 cm Sephadex G150

column equilibrated with 0.1% NP40, 2 mM $NaVO_4$, 50 mM Hepes, pH 7.5. The column was developed at 2 mL/h and 0.5 mL fractions were collected.

5. Preparation of SH2 Sepharose

E. coli, transformed with pGEX (Pharmacia) alone or pGEX containing inserts coding for the SH2 domains of GAP, PLCγ or PI3-K, were cultured overnight at 37°C in LB broth supplemented with 100 μg/mL ampicillin. The stationary phase cultures were then diluted 10 fold with fresh 100 μg/mL ampicillin in LB broth and grown for a further 2 h at 37°C. Isothio-β-D-thiogalactopyranoside was then added to induce production of the glutathione-S-transferase (gst) fusion proteins. After 4 h, the cells were harvested by centrifugation and washed once with PBS. The cell pellets were resuspended in 20 volumes of 1% NP40 in PSB containing 10 mM DTT, 0.2 mM PMSF, 2 μg/mL leupeptin and 100 KIU/mL aprotinin, and then sonicated with 3 X 5 sec bursts at 30% power on a Biosonik III sonicator (Bronwell Scientific, Rochester, NY). Insoluble material was pelleted with a 20 min, 12 000 x g centrifugation and the supernatants incubated with glutathione-agarose. After 15 min, the beads were washed extensively with PSB containing 0.1% NP40, 1 mM DTT followed by PBS containing 1 mM DTT. The proteins were then eluted with 15 mM reduced glutathione, 5 mM DTT, 50 mM Tris Cl (pH 7.5), desalted by gel filtration on a Sephadex G-25 column equilibrated with 1 mM DTT, PBS and coupled to CNBr Sepharose at 1 mg protein/mL swollen beads. In preliminary experiments, GAP SH2-gst fusion proteins were also coupled directly to glutathione-agarose with 10 mM dimethyl suberimidate (DMS) according to standard procedures.

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CHAPTER III

PURIFICATION OF THE mIL-3 RECEPTOR

A. INTRODUCTION

Murine IL-3[1] is a potent hemopoietic growth factor that is produced primarily by activated T lymphocytes and stimulates the proliferation and differentiation of pluripotent stem cells and committed myeloid and early lymphoid progenitors.[1] Characterization studies of its cell surface receptor using ^{125}I-mIL-3, various crosslinking agents and intact mIL-3 responsive cells have suggested the presence of two mIL-3 binding proteins with apparent molecular masses of approximately 140 kD and 70 kD,[2] the former apparently being cleaved to the latter upon mIL-3 binding by a receptor associated protease.[2,3] Studies in our laboratory, and others, have shown that the higher molecular weight form contains two N-linked carbohydrate moieties,[2] and becomes tyrosine phosphorylated following mIL-3 binding (3,4). As a step towards purifying the receptor for more detailed characterization, we have developed an assay to monitor detergent solubilized receptors that exploits the differential ability of unglycosylated ^{125}I-mIL-3 and receptor bound ^{125}I-mIL-3 complexes to bind to Con A-Sepharose beads. With this assay, we have optimized solubilization conditions for the receptor and this, in turn, has made possible its purification from intact cells using a simple two step procedure involving B-mIL-3, SA and α-phosphotyrosine-Sepharose beads. Two forms of the receptor were obtained; a tyrosine phosphorylated 140 kD form which was purified to apparent homogeneity and a non-tyrosine phosphorylated 120 kD form. Alkaline phosphatase treatment, chymotrypsin digestion and Western analysis of the 2 forms (using antibodies that were subsequently developed against the N- and C- termini of the AIC 2A cDNA product[4]) established that, apart from phosphorylation differences, these two proteins were identical. Sequence and amino acid composition analysis of the 120 kD receptor indicated that it was very similar, if not identical to the 120 kD product[5] of the AIC 2A cDNA recently cloned by Itoh

et al.[4] Western analysis using antibodies against the amino- and carboxy-termini of the predicted AIC 2A product and chymotryptic maps comparing our purified mIL-3R with the AIC 2A protein also suggested identity. However, a comparison of the ^{125}I-mIL-3 binding properties of our 120 kD and 140 kD purified mIL-3R preparations with the binding properties of the AIC 2A cDNA product expressed on 3T3 and COS cells suggested that the purified mIL-3R preparations had the same high affinity as the mIL-3R in intact cells whereas the AIC 2A product displayed a substantially lower affinity for ^{125}I-mIL-3.

To investigate this discrepancy in affinity, we examined the contribution that mIL-3R glycosylation makes to mIL-3 binding. Inhibition of normal mIL-3R glycosylation in B6SUtA$_1$ cells with tunicamycin resulted in loss of high affinity binding. Furthermore *in vitro* removal of carbohydrate from purified mIL-3Rs with N-glycanase also produced low affinity binding. These results suggest that proper glycosylation of the mIL-3R may be required for high affinity binding to mIL-3.

B. RESULTS

1. Development of an Assay for Solubilized mIL-3Rs

As a first step in purifying the mIL-3R, it was necessary to develop an assay to monitor its purification. The final form of this assay was based in part on the results of preliminary experiments involving DSS crosslinking of ^{125}I-mIL-3 to mIL-3 dependent cells before and after solubilization with different detergents. These results demonstrated that receptors solubilized with 0.5% NP40 or 2% octylglucoside retained their ability to bind ^{125}I-mIL-3 while receptors solubilized with various concentrations of CHAPS or deoxycholate did not. Then, based on our previous finding that the mIL-3R is a glycoprotein,[2] we tested various Sepharose bound lectins for their ability to bind DSS crosslinked complexes containing unglycosylated E coli derived ^{125}I-mIL-3 and mIL-3Rs. Our results suggested that the lectins Con-A and wheat germ agglutinin were equally capable of binding to these ^{125}I-mIL-3R complexes. The final form of this "lectin assay", described in chapter II, was found to be mIL-3R specific and gave a linear dose response with increasing solubilized mIL-3Rs (Figure 5). With this assay we were

able to further optimize solubilization conditions in order to obtain solubilized mIL-3Rs from mIL-3 dependent cells in high yield and with a similar K_D, ie., 1-5 nM, to that observed with intact mIL-3 dependent cells (Figure 6).

2. Purification of the mIL-3R

From previous studies, we found that a subline of B6SUtA cells, which we have designated B6SUtA$_1$, was capable of expressing 100,000 mIL-3Rs/cell when propagated in mGM-CSF.[6] This level of expression is approximately 10-20 times higher than that found on typical mIL-3 dependant cell lines.[1] These cells were therefore used as starting material for the purification of the receptor. Standard chromatographic techniques involving ion exchange, gel filtration, hydrophobic interaction, isoelectric focussing, reverse phase high performance liquid chromatography failed to give reasonable yields or significant purification of the mIL-3R. Moreover, affinity chromatography techniques in which mIL-3 was covalently linked to various activated matrixes, such as Affi-gel 10, Affi-gel 15, Affi-gel Hz, CNBr activated Sepharose and carbodimide-aminohexyl Sepharose all gave poor yields. However, an initial purification of the mIL-3R was made possible by incubating mIL-3R bearing cells or plasma membranes from these cells with B-mIL-3, solubilizing the membrane proteins with NP40 or octylglucoside and then chromatographing the solubilized material through a SA column. After washing at neutral pH, mIL-3R activity could be eluted at pH 3.5 (Figure 7A). Although greatly enriched for the receptor, these preparations still contained several other proteins as evidenced by 2D O'Farrell gels of pH 3.5 eluates from cells prepared in the presence and absence of excess unlabeled mIL-3 (Figure 7B).

To further purify the mIL-3R we took advantage of our previous finding that the mIL-3R becomes tyrosine phosphorylated upon binding mIL-3.[7] Biotinylated mIL-3 was first allowed to bind to B6SUtA$_1$ cells in the presence of sodium orthovanadate and sodium azide to inhibit tyrosine phosphatases and receptor internalization, respectively. The cells were then solubilized in NP40 and the phosphorylated B-mIL-3R complexes allowed to bind to α-phosphotyrosine Sepharose. The bound complexes were eluted with phenylphosphate and put

directly onto a SA column. The pH 3.5 eluate from this SA column yielded a 140 kD band on SDS-polyacrylamide gels. This protein represented more than 98% of the total protein present, as assessed by SDS-PAGE of either silver stained, iodinated, or ^{35}S-labelled preparations (Figure 8). In addition, the α-phosphotyrosine-Sepharose unbound fraction, which contained from 2 to 10 times the mIL-3 binding activity of the bound fraction, was chromatographed through SA as well and this less pure mIL-3R fraction displayed a prominent 120 kD band upon SDS-PAGE (Figure 8). A flow chart depicting the purification of these two mIL-3 binding species is shown in Figure 9 and a summary of the purification of the 140 kD species is given in Table 5.

3. Characterization of the Purified mIL-3R

Preliminary characterization studies revealed that the highly purified 140 kD mIL-3R possessed an isoelectric point of approximately 5.4 and migrated identically on SDS-PAGE under reducing and non-reducing conditions suggesting that disulfide bridges, if present, did not significantly restrain this receptor from assuming a random coil conformation in the presence of SDS (data not shown). The less pure 120 kD mIL-3R also migrated identically on SDS-PAGE under reducing and non-reducing conditions and had a slightly more basic isoelectric point (i.e. approximately 5.6). N-glycanase studies suggested that both species contained 10 kD of N-linked carbohydrate (shown for the 120 kD mIL-3 in Figure 10) confirming earlier studies using ^{125}I-mIL-3, DSS crosslinkers and intact cells.[2] The 20 kD difference in apparent molecular mass between the two receptor preparations appeared to be due solely to differences in the level of phosphorylation, since alkaline phosphatase digestion reduced both bands to the same molecular mass of 115 kD (Figure 11). The relationship of these two proteins was studied further by comparing their chymotrypsin digestion patterns before and after alkaline phosphatase treatment. Following alkaline phosphatase digestion, complete identity was observed on SDS polyacrylamide gels (Figure 12). In addition, affinity purified rabbit anti-sera which was raised against the N- and C- termini of the AIC 2A product (see below), not only recognized both receptor preparations on Western blots, but, more

importantly, only detected the 120 kD protein in unstimulated B6SUtA$_1$ cells. On addition of mIL-3, the antibodies detected a new band at 140 kD and a reduced amount of the 120 kD species (Figure 13). This provided strong evidence that the 140 kD mIL-3R was a tyrosine-phosphorylated derivative of the 120 kD mIL-3R. Scatchard analysis with the two purified mIL-3R preparations indicated that their affinity for mIL-3 was 2-5 nM, similar to that observed in intact B6SUtA$_1$, normal mouse bone marrow and mIL-3 dependent FDC-P1 cells (Figure 14).

Further studies comparing the two receptor preparations revealed a marked difference in their stability. The tyrosine phosphorylated receptor rapidly degraded on storage, even at 4°C (Chapter IV). We therefore used the 120 kD species to obtain sequencing and amino acid composition data. Although this receptor preparation contained a few contaminating proteins, 2-D gel analysis yielded, as expected, a very similar pattern to that obtained with SA purified total cell extracts (Figure 7B) and demonstrated that the 120 kD receptor was the only protein at this molecular mass. This made possible the sequencing of the amino terminus of this protein from a preparative one dimensional SDS polyacrylamide gel and yielded x-glu-val-thr-glu-glu-glu-x-thr-val-pro-leu-lys-thr-leu-glu-x-tyr-asn-asp. This sequence was confirmed by a second purification and sequence analysis. To address the possibility that the mIL-3R in the 120 kD band might have been N-blocked and that a contaminant with an identical isoelectric point and molecular mass had been sequenced, we determined the amino acid composition of the 120 kD band (Table 6). The calculated amount of protein present suggested very little amino terminal blockage.

A search of the NBRF protein sequence data bank using the FASTA program of the University of Wisconsin Genetics Computer Group[8] revealed this to be a previously unreported sequence. However, during the preparation of this manuscript, Itoh et al[4] reported the expression cloning of a gene encoding a protein reactive with a monoclonal antibody that partially inhibited mIL-3 binding to mIL-3R bearing cells. The amino terminal amino acid sequence we report here corresponds exactly with their predicted amino terminal sequence.

The proline and serine rich content of the mIL-3R we have purified also closely resembles the predicted amino acid composition of Itoh et al's protein encoded by the AIC 2A cDNA (Table 6).

4. Comparison of the Purified mIL-3R with the Product of the AIC 2A cDNA

To determine whether our purified mIL-3R was identical to the AIC 2A cDNA product which has a predicted molecular mass of only 95 kD,[4] we compared the apparent molecular masses of the receptors isolated from B6SUtA$_1$ cells with that of 3T3 cells transfected with the AIC 2A cDNA. Side by side comparisons using SDS-PAGE and Western analysis with the N-terminal antibody to the predicted AIC 2A sequence revealed that the AIC 2A product had an identical apparent molecular mass to the 120 kD mIL-3R (data not shown) Addition of mIL-3 to the cells resulted in the appearance of the 140 kD mIL-3R species in B6SUtA$_1$ cells but not in the transfected 3T3 cells, consistent with Itoh et al's finding that mIL-3 does not elicit any tyrosine phosphorylation events in these transfected cells.[4] We subsequently prepared affinity purified rabbit antiserum to the predicted C-terminal 15 amino acids of the AIC 2A cDNA and obtained identical results, further confirming the relatedness of our 140 kD and 120 kD mIL-3R species and their identity with the AIC 2A product (data not shown). Chymotryptic peptide map comparisons of the 120 kD mIL-3R with the AIC 2A protein expressed in COS cells, also suggested identity (Figure 15).

However, a major difference between the AIC 2A product and our purified mIL-3R preparations was in their affinity for mIL-3. Scatchard analyses with the purified 140 kD and 120 kD species as well as with intact normal mouse bone marrow and mIL-3 dependent FDC-P1 cells all yielded K_D's of 1-5 nM (Figure 14A). On the other hand, the AIC 2A product displayed a substantially lower affinity (~200 nM) when expressed on 3T3 cells or COS cells (Figure 14D). A low affinity for the AIC 2A product has also been reported by Itoh et al[4] who showed that this was due to a more rapid off rate than that for mIL-3Rs on mIL-3 dependant cell lines (t1/2 or 4 min vs 2 h). This could suggest that another subunit is required for high affinity binding. However, the fact that purified preparations of our mIL-3Rs exhibit high

affinity binding suggests only one chain is sufficient and perhaps the difference in affinity might be due to post-translational modifications.

5. Effect of mIL-3R Glycosylation on mIL-3 Binding

To examine the role of carbohydrate in mIL-3R function, B6SUtA$_1$ cells were treated with tunicamycin for 6 h and the receptor number and affinity determined by Scatchard analysis. As shown in Figure 16A, a 6 h tunicamycin treatment decreased the molecular mass of the bulk of the mIL-3R population by 10 kD. The total amount of receptor protein was also reduced, reflecting the inhibitory effect of tunicamycin on protein synthesis. Scatchard analysis indicated two affinity classes on tunicamycin treated cells, the high affinity class corresponding to that observed on control untreated B6SUtA$_1$ cells while the low affinity class had a K_D similar to that of COS cells transfected with the AIC 2A cDNA (Figure 16B)

The generation of low affinity binding with tunicamycin treatment suggests that carbohydrate may be important for mIL-3 binding. Alternatively, tunicamycin could be affecting the synthesis of a putative second subunit or the loss of carbohydrate may disrupt the assembly of the mIL-3R complex. To rule out these two possibilities, the carbohydrate was removed from the purified 120 kD mIL-3R *in vitro* with N-glycanase. As the removal of carbohydrate made use of the lectin assay impossible, ^{125}I-mIL-3 binding had to be assessed using gel filtration columns to separate receptor bound from free ^{125}I-mIL-3. In our hands, this technique proved too insensitive for Scatchard analysis but cold displacement studies provided an estimate of the mIL-3 dissociation rate. As Figure 17 shows, N-glycanase treatment of the mIL-3R dramatically increased the rate of dissociation.

C. DISCUSSION

Based on our previous finding that mIL-3 induces the tyrosine phosphorylation of the mIL-3R,[7] we have devised a simple two step procedure to purify this cell surface protein utilizing B-mIL-3, streptavidin-agarose and α-phosphotyrosine-Sepharose beads. With this purification strategy, both a highly purified 140 kD tyrosine phosphorylated and a less pure, more stable 120 kD non-tyrosine phosphorylated preparation were obtained. The relatedness

of these 2 proteins was investigated using alkaline phosphatase, chymotrypsin and two different antisera and our results suggest that the 140 kD protein is the tyrosine phosphorylated form of the 120 kD protein. The physical properties of both the tyrosine phosphorylated receptor and its non-tyrosine phosphorylated precursor are consistent with the properties of the bonafide mIL-3R, established using crosslinking studies with ^{125}I-mIL-3 and various intact mIL-3 dependent cell lines.[2,9] Scatchard analyses carried out with the partially purified 120 kD and highly purified 140 kD forms of the receptor reveal a single affinity class of 1-5 nM, identical to the value obtained with intact B6SUtA$_1$ cells.

From amino terminal amino acid sequence and composition analysis, molecular mass data as deduced from SDS-PAGE (under both reducing and non-reducing conditions) and Western analysis results, we also conclude that the receptor we have purified is very similar, if not identical, to the protein encoded by the AIC 2A cDNA recently cloned by Itoh et al.[4] However, according to these authors, their AIC 2A cDNA clone yields a low affinity mIL-3 binding protein with a K_D of 17.9 ± 3.6 nM at 4°C and 5.7 ± 1.0 nM at 37°C when stably transfected into L cell fibroblasts. The low affinity of the AIC 2A protein for mIL-3 may indicate that more than one subunit is required for high affinity binding as has been shown for the human IL-3, GM-CSF and IL-5 receptors. In the human system, the IL-3, GM-CSF and IL-5 receptors consist of unique α subunits but share a common β subunit. The shared subunit forms the basis of the cross-competition observed between the three ligands. The mIL-3R, like the human IL-3R, could be composed of two subunits as well. However, the mIL-3R appears to be qualitatively different from its human counterpart in several respects: There is no cross-competition between mIL-3, mGM-CSF and mIL-5; the AIC 2B[10] product does not confer high affinity binding for mIL-3 to AIC 2A[5] and; the mIL-3R is a 140 kD protein, not 60-80 kD as has been reported for the hIL-3R[11] and the α subunits of the hGM-CSF[12] and mIL-5R.[13] Moreover, our Scatchard plots indicate that the mIL-3R on intact cells, plasma membranes, solubilized membranes or as a purified molecule exhibits the same ligand affinity. Thus the mIL-3R may be more like the IL-4R, which is a 140 kD protein closely related in structure to

the mIL-3R and capable of high affinity binding in the absence of a second subunit.[14,15] To help resolve the difference in affinity between our purified mIL-3R and the AIC 2A product, we investigated whether glycosylation of the receptor could play a role. Consistent with this hypothesis we found that tunicamycin treatment of B6SUtA, cells resulted in the generation of two mIL-3R affinity classes. Furthermore, *in vitro* N-glycanase treatment of purified mIL-3Rs increased the dissociation rate of mIL-3. These observations suggest, as has been described for the insulin receptor,[16] and the basic fibroblast growth factor receptor[17] that carbohydrate structure may be important for high affinity binding. To further study the role of glycosylation it would be of interest to express the extracellular domain of the AIC 2A cDNA in both fibroblasts and mIL-3 dependent cell lines in order to compare the binding characteristics of their soluble products.

Table 5. Purification of the 140 kD mIL-3R

Step	Total Protein (mg)	Total Binding Activity (pmol)	Specific Activity (pmol/mg)	Overall Yield (%)
1. Solubilized B6SUtA1 cells	375	57	0.13	100
2. α-phosphotyrosine-Sepharose phenylphosphate eluate	30	-----**	-----	-----
3. Streptavidin agarose pH 3.5 eluate	<0.15	8	>9100	14

* This typical purification began with 3 X 10^9 mIL-3 deprived B6SUtA$_1$ cells and the number in brackets refers to total protein.

** The phenylphosphate eluate could not be assayed since the mIL-3R's at this stage, being already bound with B-mIL-3, were not available for ^{125}I-mIL-3 binding.

Similar results were obtained in two separate purifications.

Table 6. Comparison of Amino Acid Compositions

Amino Acid	Number of Residues	
	Predicted*	Determined**
ala	35	47
arg	30	41
asx	66	62
cys	17	-
glx	104	87
gly	54	73
his	20	18
ile	29	31
leu	85	101
lys	37	37
met	12	14
phe	24	26
pro	102	104
ser	101	109
thr	40	39
trp	18	-
tyr	31	29
val	51	62

* Calculated from the AIC 2A cDNA sequence reported by Itoh et al.[4]

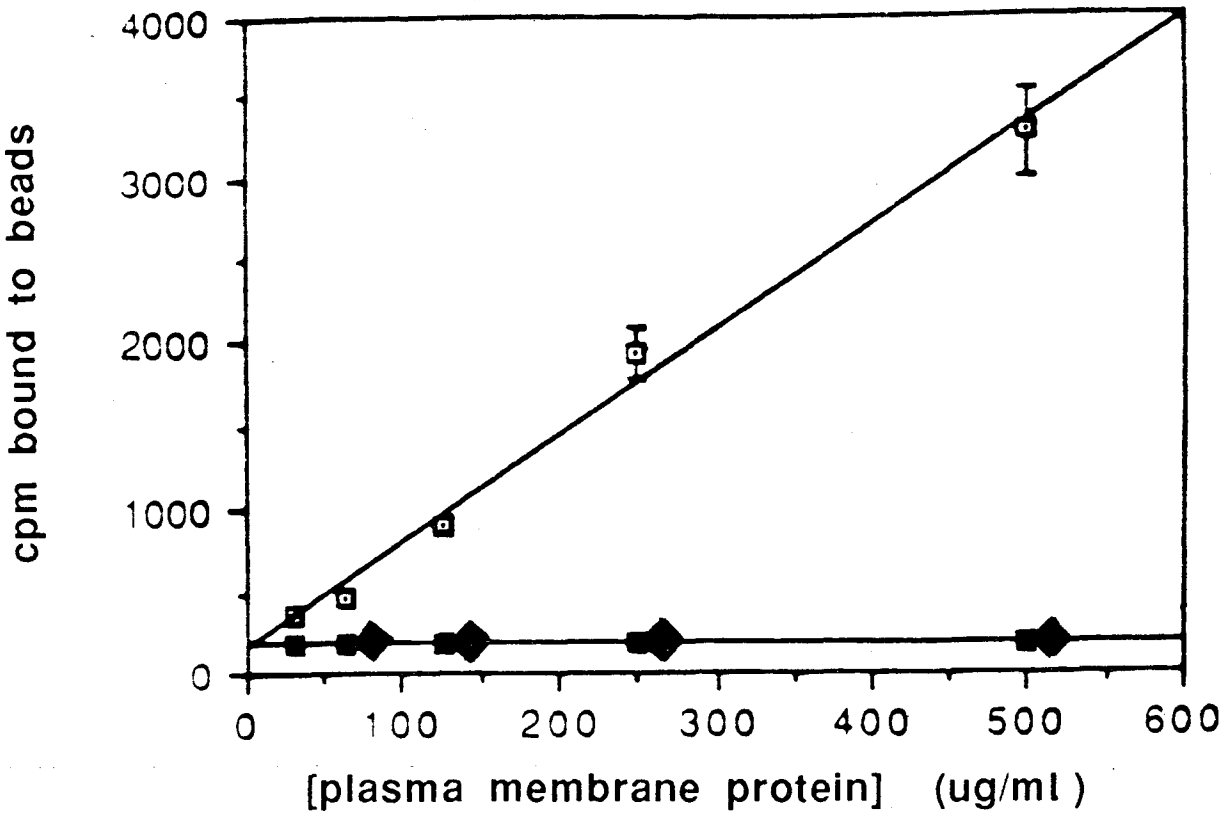


Figure 5. Specificity and sensitivity of the lectin assay for the solubilized mIL-3R. NP40 solubilized B6SUtA$_1$ (⊡) or P815 (mIL-3R negative[8] (♦) plasma membranes were incubated with ^{125}I-mIL-3 in the presence (■) or absence (⊡,♦) of a 20 fold excess of unlabelled mIL-3 for 2 h at 4°C. Con-A Sepharose beads (15 µl packed volume) were then added and the mixture rocked at 4°C for 6 h before the beads were washed twice with 0.1% NP40 in TBS and counted in a Beckman 5500 gamma counter. Similar results were obtained in three separate experiments.

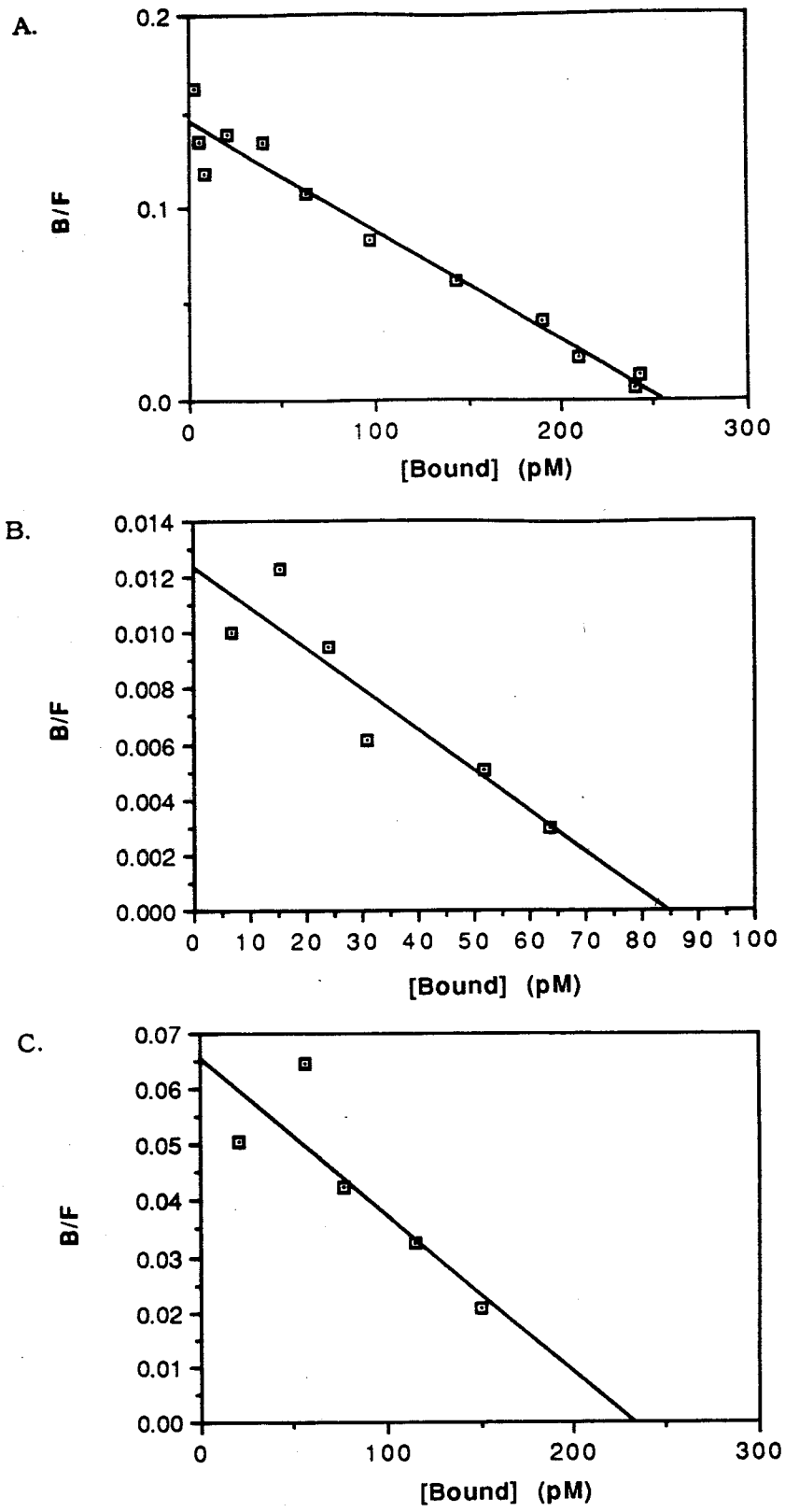


Figure 6. Scatchard analyses were performed with (A) intact cells as described earlier[2] or with (B) B6SUtA$_1$ plasma membrane proteins solubilized in 2% octylglucoside in PBS or (C) 0.5% NP40 in PBS. All 3 samples gave K_D's between 1 and 5 nM. Similar results were obtained in three separate experiments.

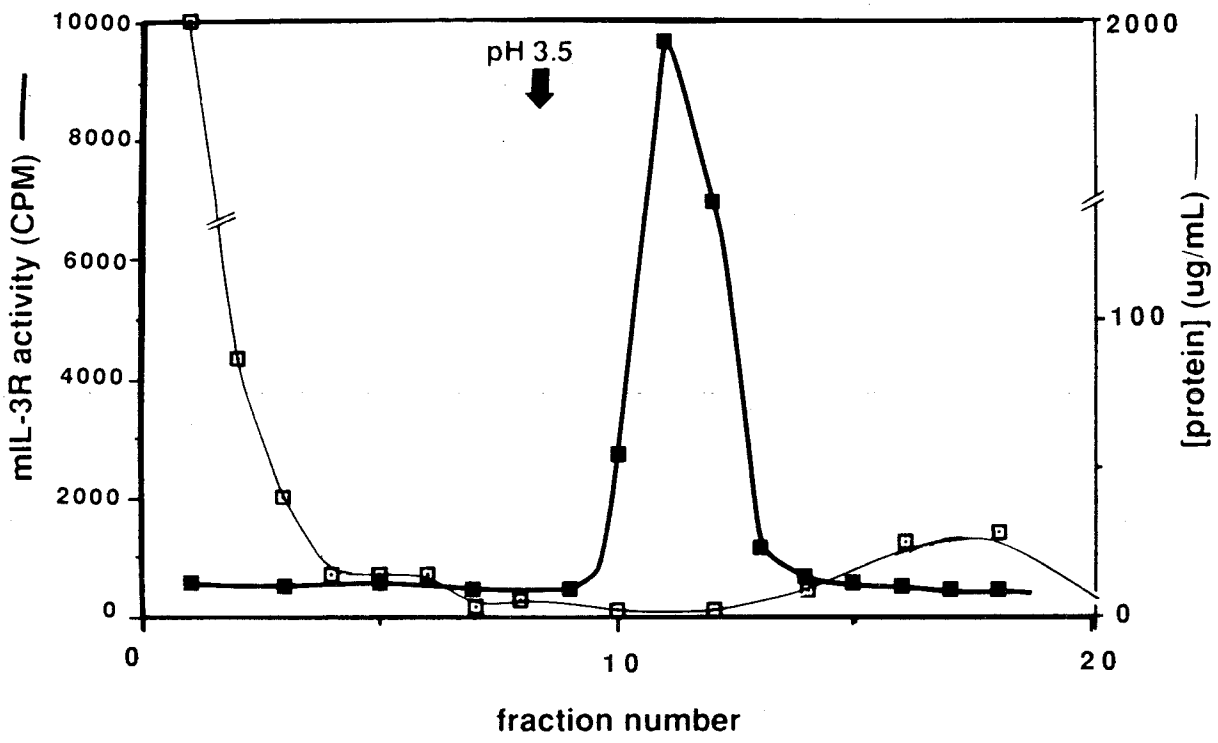


Figure 7A. Streptavidin-agarose elution profile of the mIL-3R. B-mIL-3 was incubated with B6SUtA$_1$ cells for 2 h at 4°C and the membranes solubilized with 0.5% NP40 and applied to SA columns. mIL-3R activity was monitored using the lectin assay and protein concentrations were determined with the Coomassie Dye binding assay[18] and the silver-binding assay.[19] Similar results were obtained in ten separate experiments.

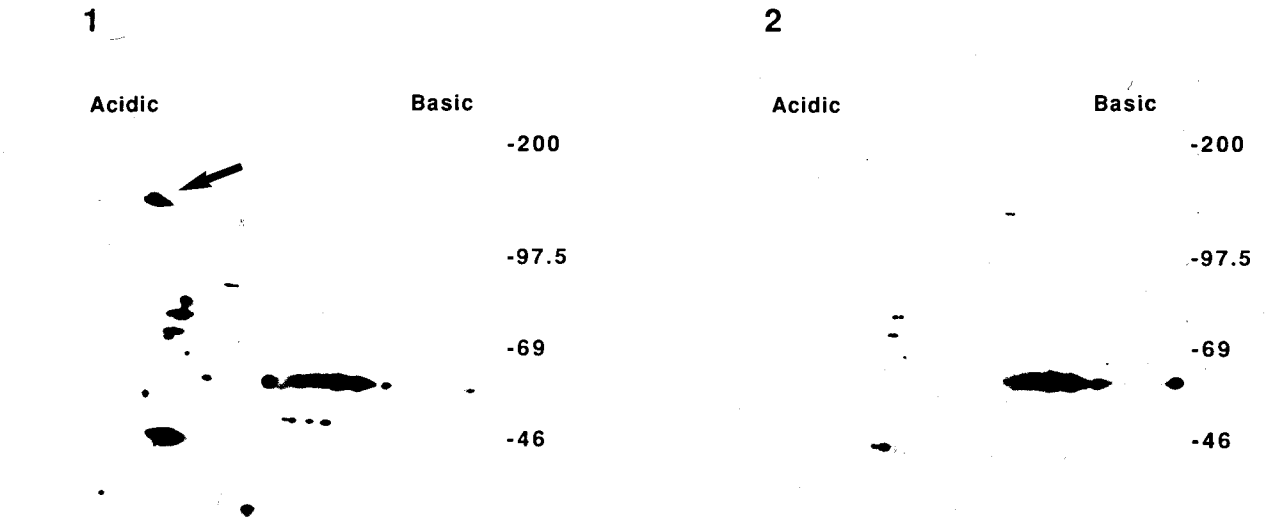


Figure 7B. Two dimensional O'Farrell gels of the SA purified mIL-3R. B-mIL-3 was incubated with B6SUtA$_1$ cells in the presence (2) and absence (1) of a 20 fold excess of unbiotinylated mIL-3 for 2 h at 4°C. The cells were then solubilized with 0.5% NP40 in PBS and incubated with SA. After washing the beads, bound proteins were eluted at pH 3.5. An mIL-3 specific protein at approximately 140 kD (see arrow) was consistently observed when eluants were radioiodinated and subjected to 2-D SDS-PAGE[17] and autoradiography. Similar results were obtained in three separate experiments.

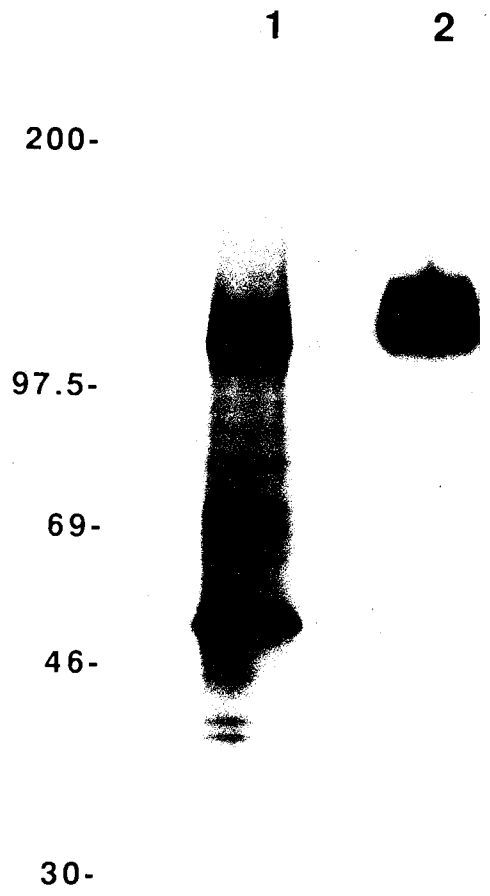


Figure 8. SDS-PAGE of the purified mIL-3R from ^{35}S labelled B6SUtA$_1$ cells. The SA eluates of the α-phosphotyrosine Sepharose bound (lane 2) and unbound fractions (lane 1) were subjected to SDS-PAGE and fluorography. Simliar results were obtained in five separate experiments.

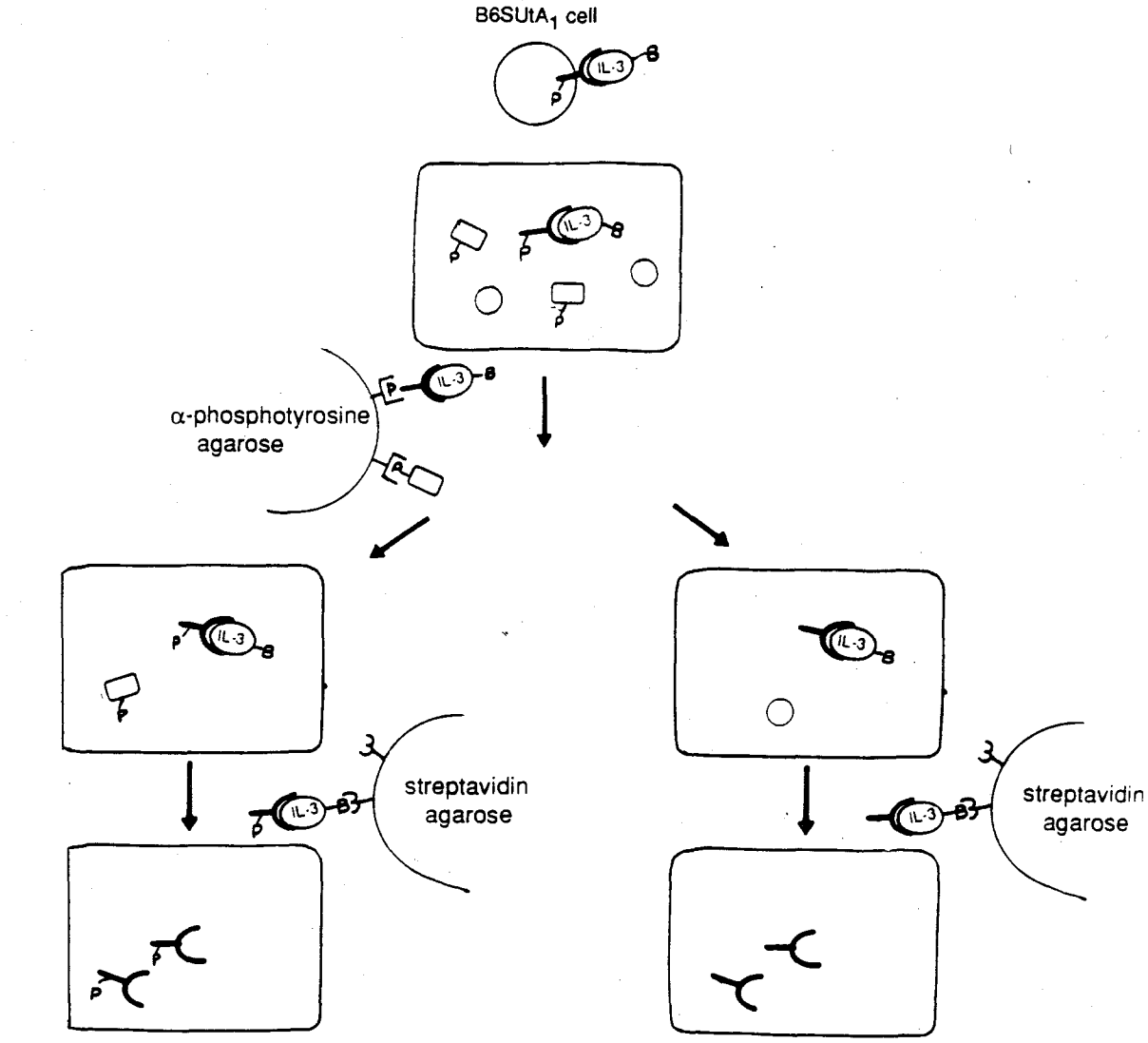


Figure 9. Flow diagram showing the purification of the mIL-3R. Both highly purified 140 kD tyrosine phosphorylated and less pure 120 kD tyrosine-unphosphorylated mIL-3R species were obtained.

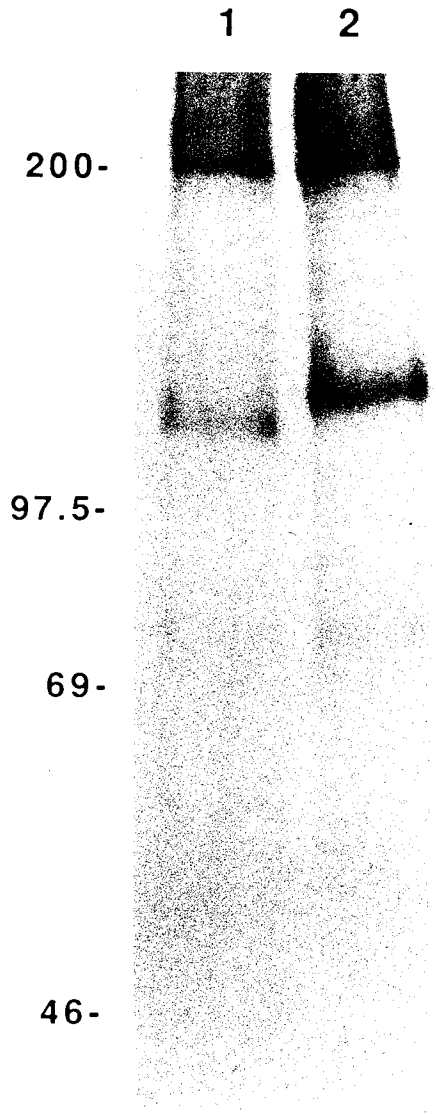


Figure 10. N-glycanase digestion of ^{32}P-labelled mIL-3R's. B6SUtA$_1$ cells were equilibrated with ^{32}P-orthophosphate as described in chapter II. ^{32}P-labeled 120 kD mIL-3Rs were purified from the α-phosphotyrosine unbound fraction as described in Chapter II, denatured by boiling 2 min in 0.5% SDS, 0.1 M β-mercaptoethanol and diluted into 0.1 M sodium phosphate buffer (pH 8.6) containing 10 mM 1,10 phenanthroline and a 7 fold excess of NP40. The sample was then incubated in the presence (lane 1) and absence (lane 2) of 5 U/ml of N-Glycanase (Genzyme) for 16 h at 37°C. Both samples were then made 4% in SDS and 5% in β-mercaptoethanol, separated by SDS-PAGE and subjected to autoradiography. Similar results were obtained in two separate experiments.

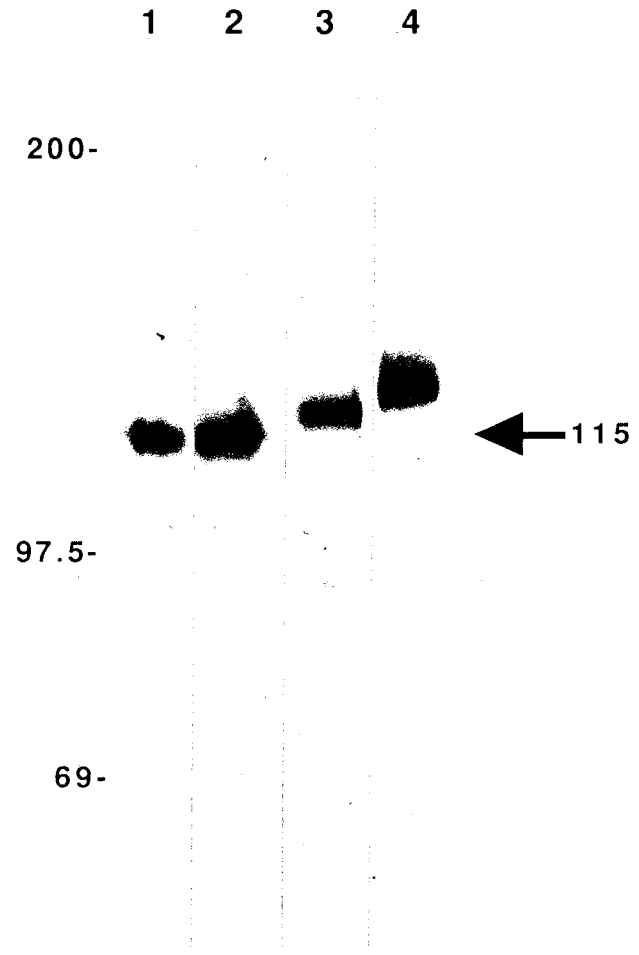


Figure 11. Effect of phosphorylation on apparent molecular mass. The mIL-3R, SA purified from either the α-phosphotyrosine unbound or bound fraction, was radioiodinated subjected to SDS-PAGE and electroeluted from the gel. The electroeluates from the unbound (lanes 1 and 3) and the bound (lanes 2 and 4) fractions were incubated with (lanes 1 and 2) or without (lanes 3 and 4) 0.07 U/ml alkaline phosphatase (Boehringer Mannheim) in 100 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, for 2 h at 37°C and analyzed by SDS-PAGE and detected by autoradiography. Similar results were obtained in two separate experiments.

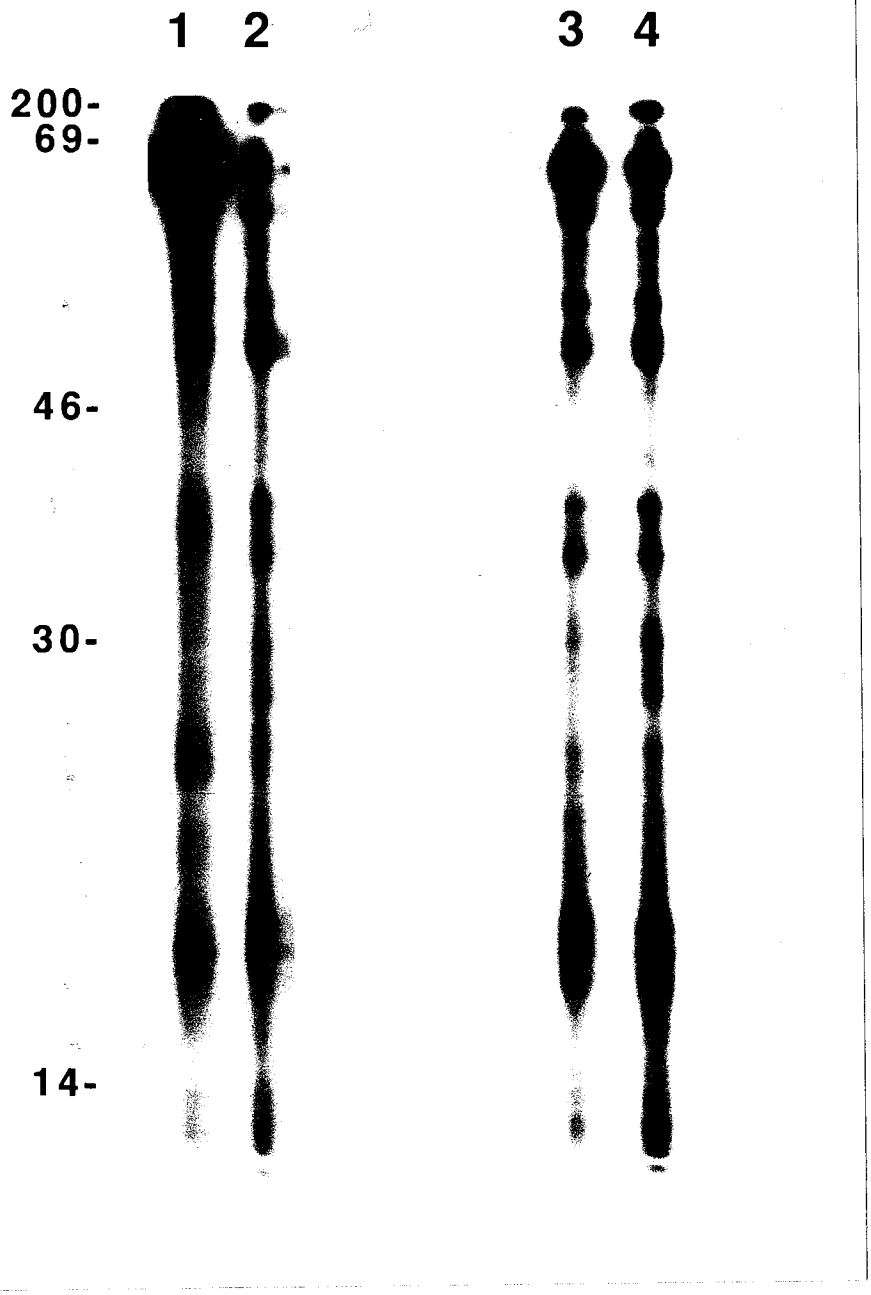


Figure 12. Chymotryptic maps of the tyrosine phosphorylated 140 kD (lanes 1 and 3) and the tyrosine unphosphorylated 120 kD (lanes 2 and 4) proteins before (lanes 1 and 2) and after (lanes 3 and 4) alkaline phosphatase digestion. Receptor preparations were radiolabelled and treated, or not, with alkaline phosphatase & subjected to SDS-PAGE as in figure 11. The receptor bands were excised, placed in the wells of a 15% SDS-polyacrylamide gel & digested *in situ* with 10 μg/ml chymotrypsin according to the method of Cleveland.[20] Fragments were visualized by autoradiography. Similar results were obtained in three separate experiments.

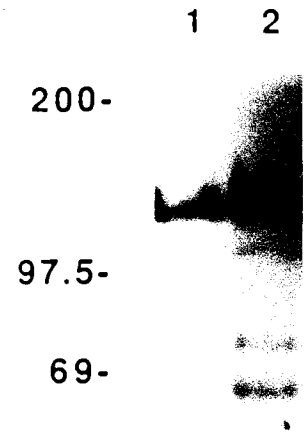


Figure 13. Western analysis of plasma membranes from B6SUtA$_1$ cells exposed to control buffer (lane 1) or 30 nM mIL-3 for 15 min at 37^{o}C. The Immobilon blot was probed with affinity purified rabbit antibody to the amino-terminus of the AIC 2A product as described in Chapter II. Similar experiments were obtained in five separate experiments.

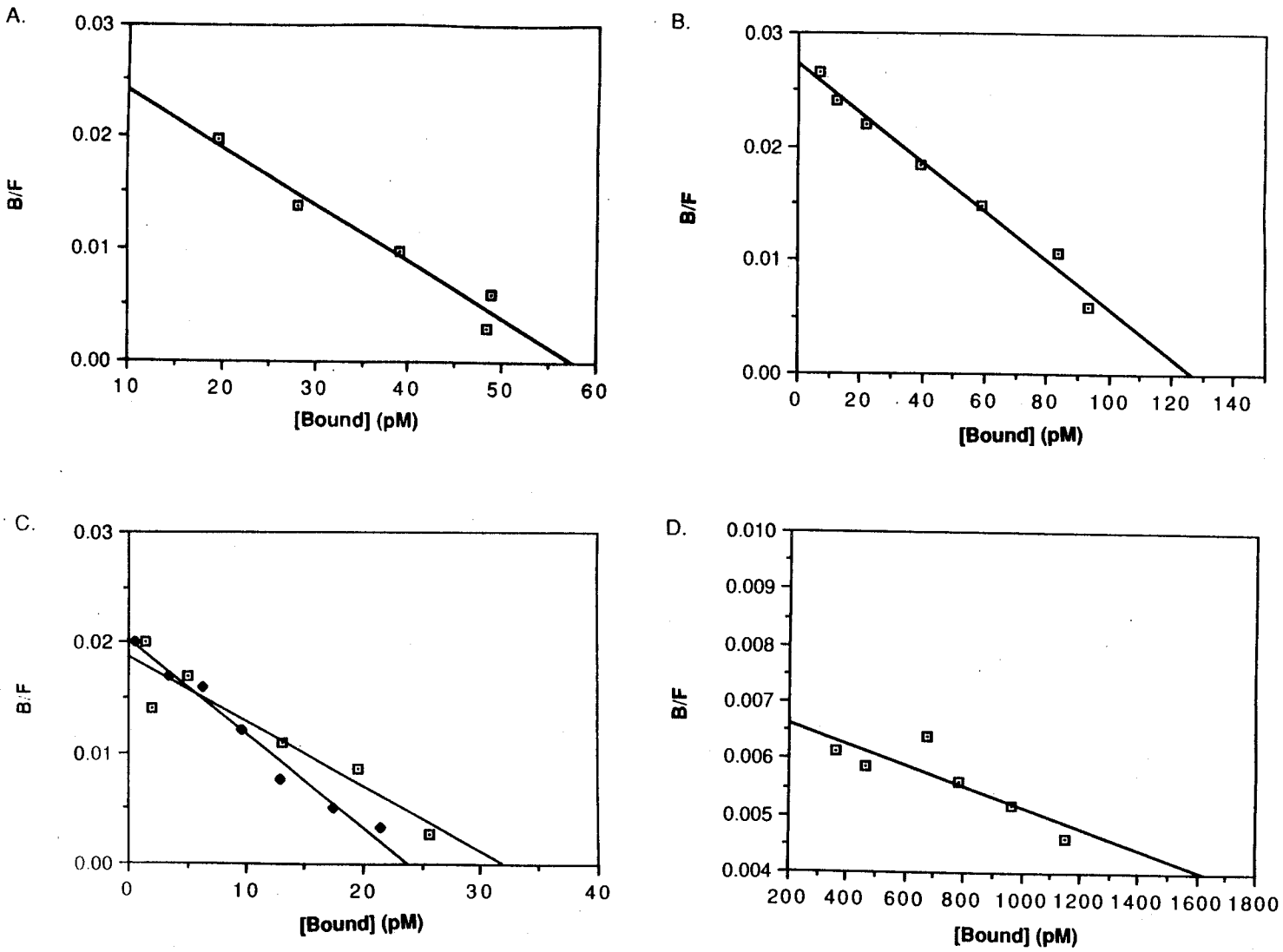


Figure 14. Scatchard analyses of the purified 140 kD (A) and 120 kD (B) mIL-3R species as well as intact normal mouse bone marrow cells (C,□) and FDC-P1 cells (C,♦) and COS cells transfected with the AIC 2A cDNA (D).

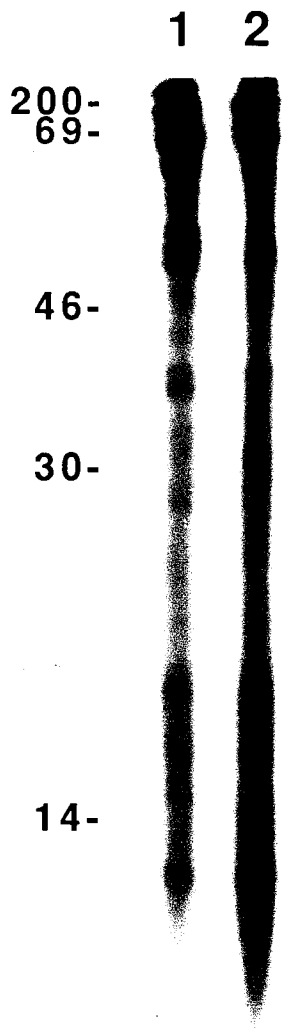
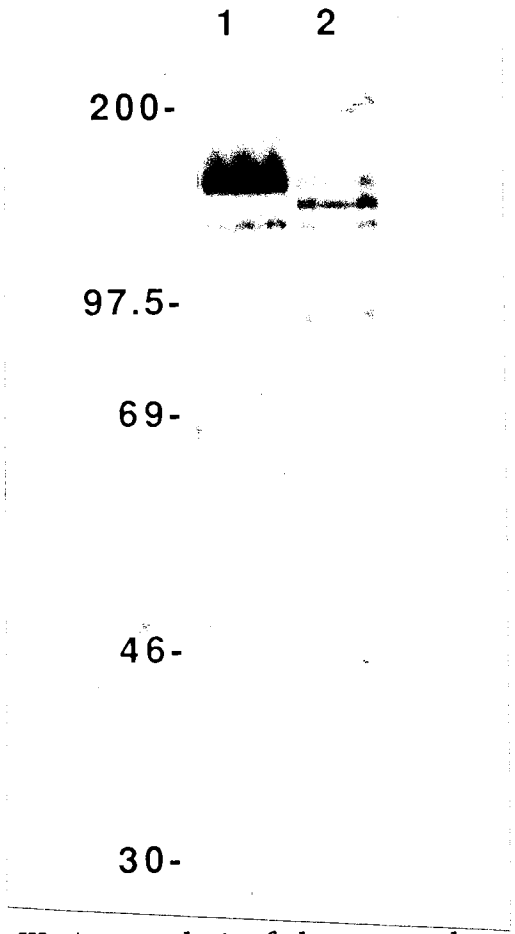


Figure 15. Cymotryptic map of the purified 120 kD mIL-3R and the AIC 2A protein. The Aic 2A protein was expressed in COS-7 cells using the DEAE-Dextran method and purified using B-IL-3/SA. Peptide maps were then prepared of the 120 kD mIL-3R (lane 1) and the purified AIC 2A protein (lane 2) as described in figure 12.

A.



B.

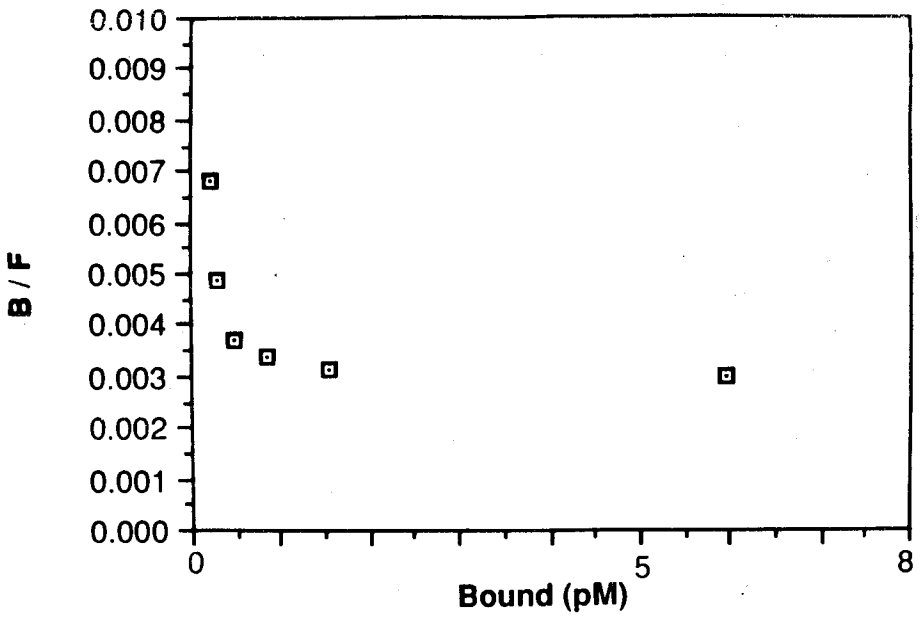


Figure 16A. Western analysis of plasma membranes from B6SUtA$_1$ cells following tunicamycin treatment. Cells were grown for 6 h in RPMI containing 3 ng/mL GM-CSF, & 10% FCS with (lane 2) or without (lane 1) 10 µg/ml tunicamycin. Plasma membranes were then prepared and subjected to Western analyses with affinity purified rabbit antibody to the amino-terminus of the AIC 2A product, as described in Chapter II.

Figure 16B. Scatchard analysis of B6SUtA$_1$ cells following tunicamycin treatment. Tunicamycin treated cells were washed and used for Scatchard analysis as described in Chapter II. Similar results were obtained in three separate experiments.

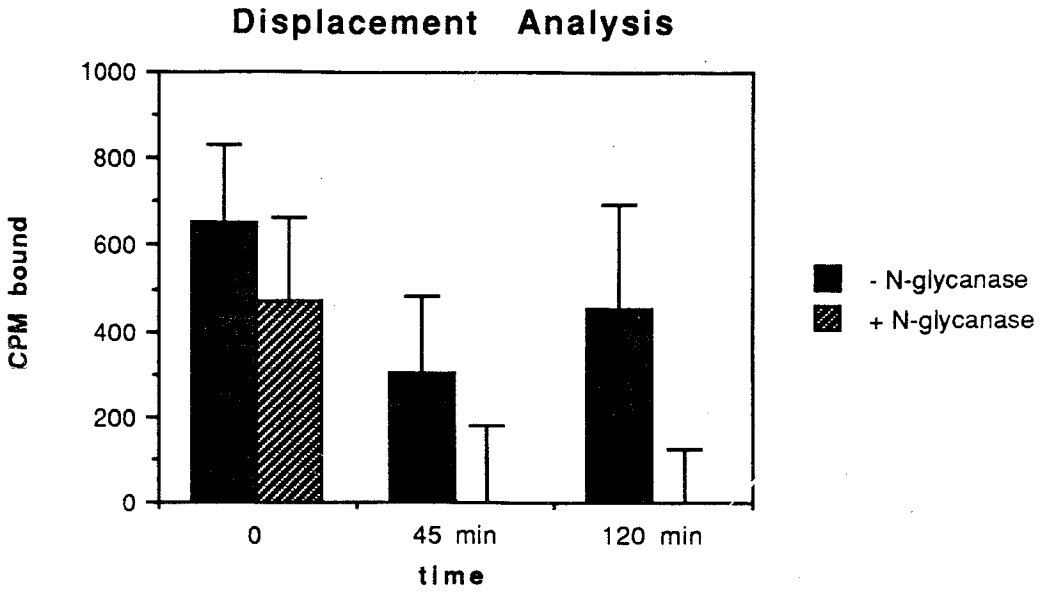


Figure 17. Displacement kinetics of ^{125}I-mIL-3 from the purified 120 kD mIL-3R. The receptor preparation was incubated in the presence and absence of N-glycanase. ^{125}I-mIL-3 was then added and, after 2 h, excess unlabeled mIL-3 was included to displace the receptor bound ^{125}I-mIL-3, as described in Chapter II. Data points represent means (n=2) +/- SEM. The significance of the difference between the 0 time point and the 45 and 120 min time points, in the N-glycanase treated sample, has a p value < 0.05.

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CHAPTER IV

TYROSINE PHOSPHORYLATION OF THE mIL-3 RECEPTOR INCREASES ITS SUSCEPTIBILITY TO CLEAVAGE

A. INTRODUCTION

As described in chapter III, we have purified the 140 kD protein to apparent homogeneity on the basis of its ability to bind mIL-3.[1] In addition, we have established the likely identity of the 140 kD protein with the mIL-3 binding protein, Aic 2A, recently cloned by Itoh et al.[2] through amino acid sequence and composition analysis, Cleveland maps and its ability to react with antisera to the amino and carboxy termini of the Aic 2A protein.

The AIC 2A cDNA product belongs to a unique family of growth factor receptors that includes the β chain of the IL-2 receptor (IL-2R) and the receptors for IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF, LIF and erythropoietin.[3,4] The members of this hemopoietic growth factor receptor superfamily are characterized by conserved cysteines and Trp-Ser-X-Trp-Ser motifs in their extracellular domains, and an exceptionally high proline and serine content in their cytoplasmic domains.[3] None of these receptors possess intrinsic tyrosine kinase domains and their mechanisms of action are, as yet, unknown. However, although these receptors do not appear to possess tyrosine kinase activities, at least three of them, the mIL-3R,[5,6] the EpR[7,8] and the β chain of the IL-2R,[9] become tyrosine phosphorylated upon ligand binding. We have shown previously that mIL-3 induces tyrosine phosphorylation of its receptor[5] and have thus been able to use immobilized α-phosphotyrosine coupled with biotinylated mIL-3 and streptavidin agarose to purify the tyrosine phosphorylated form of the mIL-3R to homogeneity as described in Chapter III. During the course of these studies we noted that different mIL-3R preparations differed in their susceptibility to proteolysis and that this differential susceptibility appeared to correlate with the tyrosine phosphorylation state of the mIL-3R being isolated. We now show that tyrosine phosphorylation of the mIL-3R is the signal for its own

cleavage. This generation of a receptor fragment may be important to the mechanism of action of mIL-3.

B. RESULTS

1. Purification of Tyrosine Phosphorylated and Tyrosine Unphosphorylated mIL-3Rs

The 140 kD tyrosine phosphorylated and the 120 kD tyrosine unphosphorylated mIL-3R's were purified from B6SUtA$_1$ cells that were grown under conditions which allowed expression of approximately 100,000 mIL-3Rs/cell.[10] The two step purification procedure involved B-mIL-3, streptavidin agarose and α-phosphotyrosine Sepharose, as previously described in Chapter III. As shown in Figure 18A when ^{32}P-labeled B6SUtA$_1$ cells were subjected to this purification procedure only 1 band was observed in both the tyrosine phosphorylated and the non-tyrosine phosphorylated preparations, as assessed by SDS-PAGE. This is consistent with our previous data in which we observed only a 140/120 kD doublet following B-mIL-3 stimulation of ^{32}P-labeled B6SUtA$_1$ cells and subsequent streptavidin agarose purification.[5] The tyrosine phosphorylated mIL-3R had an apparent molecular mass 20 kD higher than the tyrosine unphosphorylated form. Phosphoamino acid analysis confirmed that the two forms of the receptor differed in phosphotyrosine content (Figure. 18B). Previous studies using alkaline phosphatase, Cleveland maps and antisera to the amino terminal of the mIL-3R demonstrated that these two proteins were otherwise identical.[1]

2. Effect of Tyrosine Phosphorylation on the Stability of the mIL-3R

To compare the stability of the two receptor preparations, they were stored at 4°C and, at various times, aliquots were either assayed for ^{125}I-mIL-3 binding activity or directly radioiodinated and subjected to SDS-PAGE. The results clearly demonstrated that the non-tyrosine phosphorylated receptor was unaffected by storage at 4°C, since it retained its full ability to bind ^{125}I-mIL-3 (Figure. 19A) and remained intact as a 120 kD protein (Figure. 19B). In contrast, the tyrosine phosphorylated receptor rapidly lost mIL-3 binding activity and this correlated kinetically with the disappearance of the 140 kD mIL-3R moiety. Since addition of protease inhibitors could prevent this (Figure. 19B), it seemed likely that this instability was

due to proteolysis. To determine whether this sensitivity to proteolysis was an intrinsic property of the tyrosine phosphorylated mIL-3R (due to tyrosine phosphorylation induced conformational alterations which allow protease binding and cleavage) or a secondary effect due to the possible presence of proteases or protease inhibitors in one but not the other receptor preparation, mixing experiments were performed. Tyrosine phosphorylated and tyrosine unphosphorylated mIL-3Rs were combined and their ability to bind ^{125}I-mIL-3 examined (Figure. 19B). The resulting drop in activity corresponded to the level of tyrosine phosphorylated receptor present in the mixture, indicating that if a protease co-purified with the tyrosine phosphorylated mIL-3R, it was not capable of degrading the tyrosine unphosphorylated receptor. A more rigorous mixing experiment was then performed with ^{35}S-labelled and unlabelled receptor preparations. ^{35}S-labelled tyrosine phosphorylated and tyrosine unphosphorylated mIL-3Rs were purified from B6SUtA$_1$ cells equilibrated with ^{35}S-methionine and mixed with either tyrosine phosphorylated or tyrosine unphosphorylated, unlabelled mIL-3R preparations. As shown in Figure. 20, the ^{35}S-labelled tyrosine phosphorylated mIL-3R degraded to 70 kD fragments and this degradation was not inhibited by the presence of unlabelled, non-tyrosine phosphorylated mIL-3R. Conversely, the ^{35}S-labelled tyrosine unphosphorylated mIL-3R did not undergo proteolysis even in the presence of unlabelled tyrosine phosphorylated mIL-3R. These results strongly support the conclusion that tyrosine phosphorylation of the receptor causes an intrinsic change in the receptor which greatly increases its susceptibility to proteolysis. These results further suggest the intriguing possibility of a specific protease that becomes associated with (or is already associated with) the mIL-3R, since it apparently co-purifies with the tyrosine phosphorylated receptor.

3. Receptor Degradation Requires Both Tyrosine and Serine Phosphorylation

To investigate the phosphorylation requirements for receptor cleavage, mIL-3Rs were purified from mIL-3 stimulated B6SUtA$_1$ cells with α-phosphotyrosine Sepharose. Receptors isolated using this procedure contain both phosphotyrosine and phosphoserine residues (see Figure 18B). This receptor preparation was incubated at 37°C for 16 h in the presence or

absence of a tyrosine specific phosphatase, a mixture of serine phosphatases or alkaline phosphatase, as described in Chapter II. As shown in Figure 21, receptor degradation was markedly inhibited by all three phosphatases, suggesting that both serine and tyrosine phosphorylation contribute to the proteolytic sensitivity of the 140 kD tyrosine phosphorylated mIL-3R. Alternatively, it is conceivable that the protease responsible for the cleavage is itself activated by tyrosine and serine phosphorylation and that dephosphorylation inactivates it. However, this is not consistent with our previous mixing studies. The p70 cleavage product was not observed in the phosphatase experiments shown in Figure 21 because of the lengthy incubation period and the less pure receptor preparation used (i.e. streptavidin agarose was not employed). This resulted in the further fragmentation of the initial cleavage product.

4. Degradation of the mIL-3R in Intact Cells.

To examine whether mIL-3R cleavage also occurs in intact cells, B6SUtA$_1$ cells were incubated at 37°C with mIL-3 for various times and plasma membranes were prepared and electrophoresed in SDS-polyacrylamide gels. Western analysis was then carried out using an affinity purified antibody raised against a peptide corresponding to the N-terminal 15 amino acids of the mIL-3R.[1,2] In the absence of mIL-3 (Figure. 22, time 0) only the 120 kD unphosphorylated mIL-3R (lower arrow) was detected. However, in the presence of mIL-3, the 140 kD phosphorylated species (upper arrow) appeared. Consistent with earlier studies,[11] the 140 kD band was maximal within 10 min of incubation with mIL-3 and then began to decline by 30 min. As can also be seen in Figure. 22, this decline was accompanied by the appearance of three bands at approximately 70 kD. These three bands might reflect some secondary processing, or heterogeneity in the 140 kD mIL-3R (perhaps due to differential glycosylation) or in the cleavage site. By 60 min, the 140 kD species was barely visible and the 70 kD bands more prominent. Identical results were obtained when whole cell lysates were analyzed instead of plasma membranes, although the blots contained additional bands because of cross-reactivity with cellular proteins (data not shown). Interestingly, treatment of these cells with the phorbol ester TPA, instead of mIL-3, stimulated, exclusively, the serine and/or threonine

phosphorylation of the mIL-3R (Figure. 23A, lane 6) (i.e. no tyrosine phosphorylation was detected by Western analysis using α-phosphotyrosine antibodies (Figure. 23B, lane 6). This phosphorylation was suggested by the increase in apparent molecular mass of the receptor (Figure. 23A) No breakdown to 70 kD fragments was observed under these conditions, consistent with our in vitro findings that both tyrosine and serine phosphorylations are required for cleavage. Also shown in Figure 23A is the effect of various inhibitors on mIL-3R stimulated mIL-3R cleavage. Chloroquine did not prevent mIL-3R cleavage (lane 3), further establishing that this cleavage reaction occurs at the cell surface and not in lysosomes. Genistein, an inhibitor of certain tyrosine kinases, also did not prevent mIL-3R cleavage (Figure 23B) but it also did not inhibit mIL-3R receptor tyrosine phosphorylation, suggesting that it does not affect the tyrosine kinase responsible for phosphorylating the mIL-3R. This inhibitor did, however, inhibit the tyrosine phosphorylation of several other, constitutively phosphorylated proteins (Figure. 23B, lane 3). Interestingly, staurosporine, originally described as a protein kinase C inhibitor,[12] did prevent the mIL-3 stimulated mIL-3R cleavage (Figure. 23A, lane 5) but, in our hands, also inhibited mIL-3R tyrosine phosphorylation (Figure. 23B, lane 4). These observations are consistent with our hypothesis that tyrosine phosphorylation of the mIL-3R is essential for cleavage.

C. DISCUSSION

Our previous studies[13] and those by others,[6] using ^{125}I-labeled mIL-3, intact cells and various crosslinking agents, established that mIL-3 binds to both a 140 kD and a 70 kD species. Upon incubation at 37^{o}C, we found that the intensity of the 140 kD crosslinked band decreased and the 70 kD band increased, suggesting conversion of the p140 to the p70. Based on these and other data showing that the p70 could be generated with both plasma membrane and solubilized membrane preparations,[13] we proposed that a protease closely associated with the mIL-3R was responsible for this cleavage. This interpretation was strengthened by carrying out crosslinking studies with our purified 140 kD mIL-3R preparations.[14] Specifically, as reported in the present study, a slow conversion of the 140 kD receptor to a 70 kD fragment

was noted in the absence of cross-linkers. However, this conversion was markedly accelerated when the crosslinker DSS and ^{125}I-mIL-3 were added, suggesting that crosslinking altered the conformation of the mIL-3R so as to make it more susceptible to cleavage. We confirmed that it was DSS and not mIL-3 binding that accelerated the cleavage by using unlabeled mIL-3 and ^{32}P-labeled 140 kD mIL-3Rs incubated in the presence and absence of DSS. Only when DSS was present did the ^{32}P-140 kD mIL-3R rapidly disappear (data not shown).

In the present study, both our *in vitro* and *in vivo* data are consistent with a model of mIL-3 action in which the mIL-3 induced tyrosine and serine phosphorylation of its cell surface receptor leads to receptor cleavage. Very recent studies in our laboratory indicate that these events also occur readily at 4°C in intact cells, suggesting, perhaps, that the tyrosine and serine kinases and the protease are all closely associated with the mIL-3R (data not presented). This would be consistent with our observation that the highly purified tyrosine phosphorylated 140 kD mIL-3R undergoes cleavage, and suggests that the protease remains physically associated during the purification procedure. The amino-terminal 70 kD fragment (i.e. identified using an anti N-terminal mIL-3R antibody) likely contains the transmembrane region of the mIL-3R since it is detected in Western blots of plasma membrane preparations and because of its likely identity with the 70 kD cross-linked species seen on intact cells and plasma membranes.[13]

Studies to date to examine the fate of the tyrosine and serine phosphorylated C-terminal fragment have been hindered by the lack of an anti-C terminal mIL-3R antibody that recognizes the mIL-3R well in Western blots and by the inability to observe ^{32}P-labelled fragments following 4^{o}C incubation of ^{32}P-labelled 140 kD mIL-3Rs. The latter could be due either to dephosphorylation or to further *in vitro* hydrolysis of this C-terminal fragment to peptides that electrophorese with the dye front on our 7.5% polyacrylamide gels. We are currently investigating the possible biological function(s) of the generated C-terminal fragment(s) by expressing cDNAs corresponding to the intracellular domain in mIL-3 dependent 32D cells.

The identity of the protease responsible for receptor cleavage is unknown. One possibility is that the receptor itself contains an intrinsic protease, as has been hypothesized for the insulin receptor.[15] The protease activity of the insulin receptor is postulated to be present as an inactive zymogen until activated by the binding of insulin and this may explain the insulinometic effect of trypsin. We have, however, found batch to batch variation in protease sensitivity with our purified 140 kD mIL-3R preparations, consistent with the notion that the receptor is acted upon by an exogenous protease that copurifies to varying extents. A potential candidate for this mIL-3R specific protease may be calpain since it has been shown recently to be involved in signal transduction. Specifically, in neutrophils stimulated with f-met-leu-phe it cleaves the Ca^{2+} and lipid dependent protein kinase C to the Ca^{2+} and lipid independent protein kinase M.[16]

The function of the proteolytic cleavage of the mIL-3R is, of course, unknown at present. Receptor proteolysis could release a cytoplasmic receptor fragment that serves as part of a mitogenic signal transducing pathway. Alternatively, the proteolysis may result in receptor down-regulation, analogous to phorbol ester stimulated cleavage and down-regulation of the colony stimulating factor-1 receptor.[17] One possible mechanism, in this regard, is that mIL-3 binding induces a conformational change in its receptor such that the associated tyrosine kinase becomes activated and phosphorylates the receptor and other receptor associated proteins (which then act as second messengers to stimulate mitogenesis). The tyrosine phosphorylated mIL-3R then becomes a substrate for the associated serine kinase and this subsequently leads to receptor cleavage and shutting down of the mitogenic signal. A third alternative is that generation of mIL-3R fragments may not play any role in mIL-3 induced signal transduction. Instead, receptor cleavage may be secondary and incidental to the activation of the associated protease. The activated protease might then play an important role in the propagation or regulation of the mitogenic signal by acting on mIL-3R associated proteins. To gain further insights into the role of mIL-3R cleavage it would be of interest to

explore the biological consequences of preventing tyrosine phosphorylation and/or cleavage using site directed mutagenesis of the AIC 2A product.

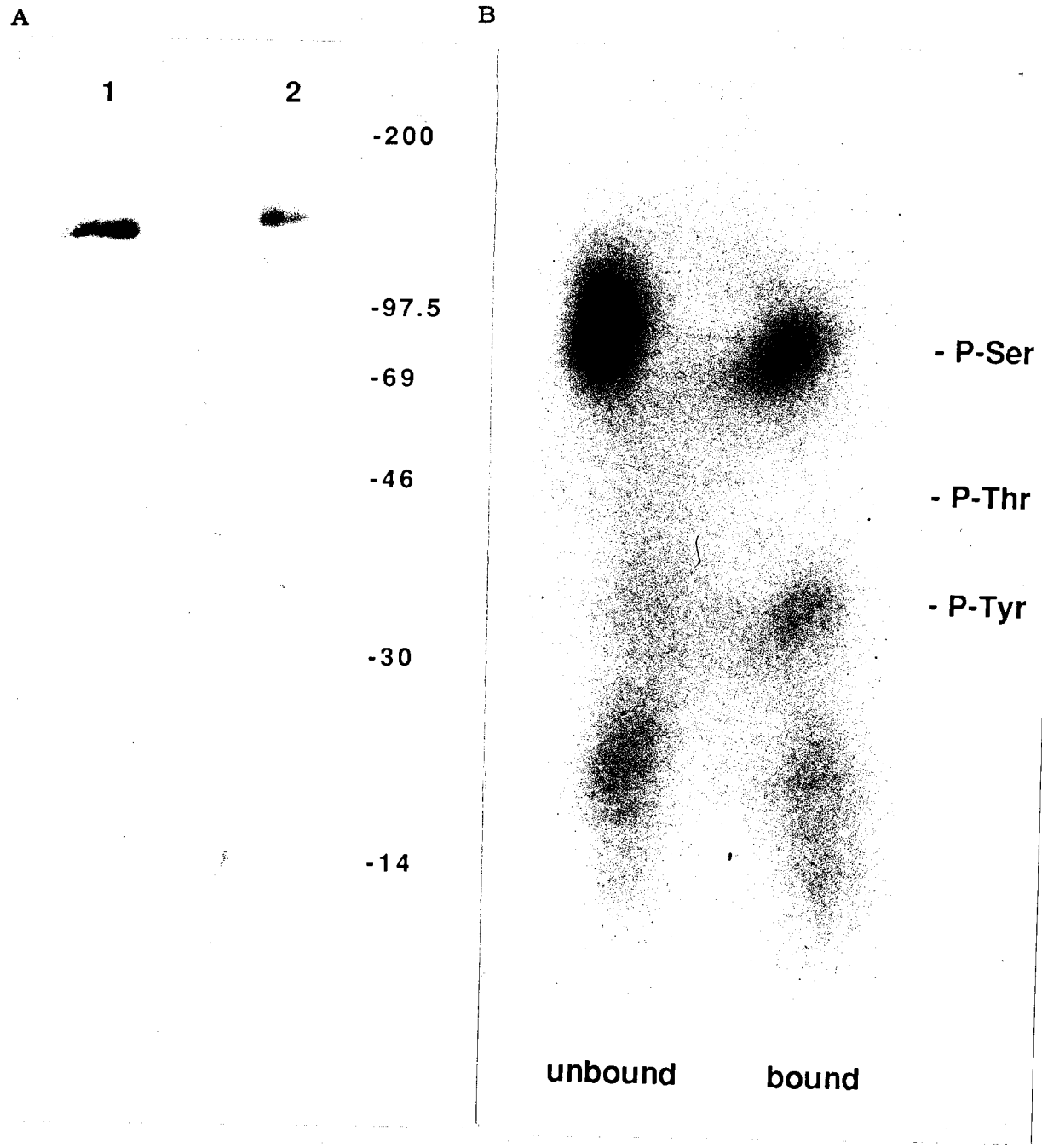

Figure 18. Tyrosine phosphorylation of the mIL-3R increases its apparent molecular mass. ^{32}P-labeled tyrosine phosphorylated (panel A, lane 2) and tyrosine unphosphorylated (panel A, lane 1) mIL-3Rs were purified from 1 x 10^8 ^{32}P-labeled B6SUtA1 cells, as described in Chapter II. To compare the phosphoamino acid content of the two preparations, they were electrophoresed on SDS-polyacrylamide gels as shown in (A) and, the bands excised and subjected to phosphoamino acid analysis (B). In panel B, three times more tyrosine unphosphorylated (unbound) than tyrosine phosphorylated (bound) mIL-3R's were analyzed. Similar results were obtained in two separate experiments.

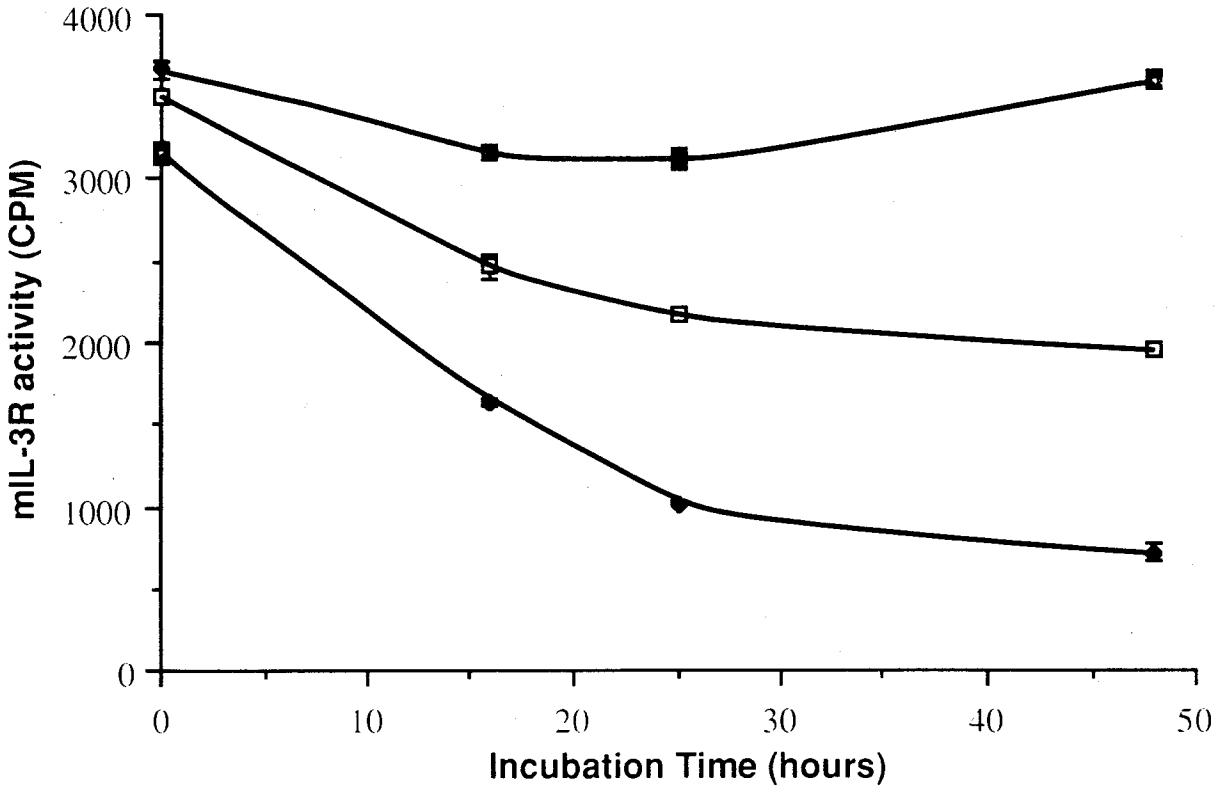

Figure 19. Tyrosine phosphorylation of the mIL-3R increases its susceptibility to proteolysis. Both tyrosine phosphorylated (♦) and tyrosine unphosphorylated (■) mIL-3R preparations and a mixture of the two (▫), were incubated for the indicated times at 4°C and either assayed for mIL-3 binding activity (A) in the lectin mIL-3R assay[1] or directly radioiodinated using the chloramine T procedure previously described[13] and analyzed by SDS-PAGE and autoradiography (B , on following page). For the lectin mIL-3R assay results, non-specific binding (which was determined using a 20 fold excess of unlabeled mIL-3) represented approximately 10 % of total binding and has been subtracted. In B a mixture of protease inhibitors (0.5 mM PMSF, 1μg/ml leupeptin and 100 KIU/ml aprotinin) was also added to an aliquot of the tyrosine phosphorylated mIL-3R at the beginning of the incubation. The level of protein in the non-tyrosine phosphorylated preparation was approximately 10 fold that in the tyrosine phosphorylated preparation. Similar results were obtained in two separate experiments.

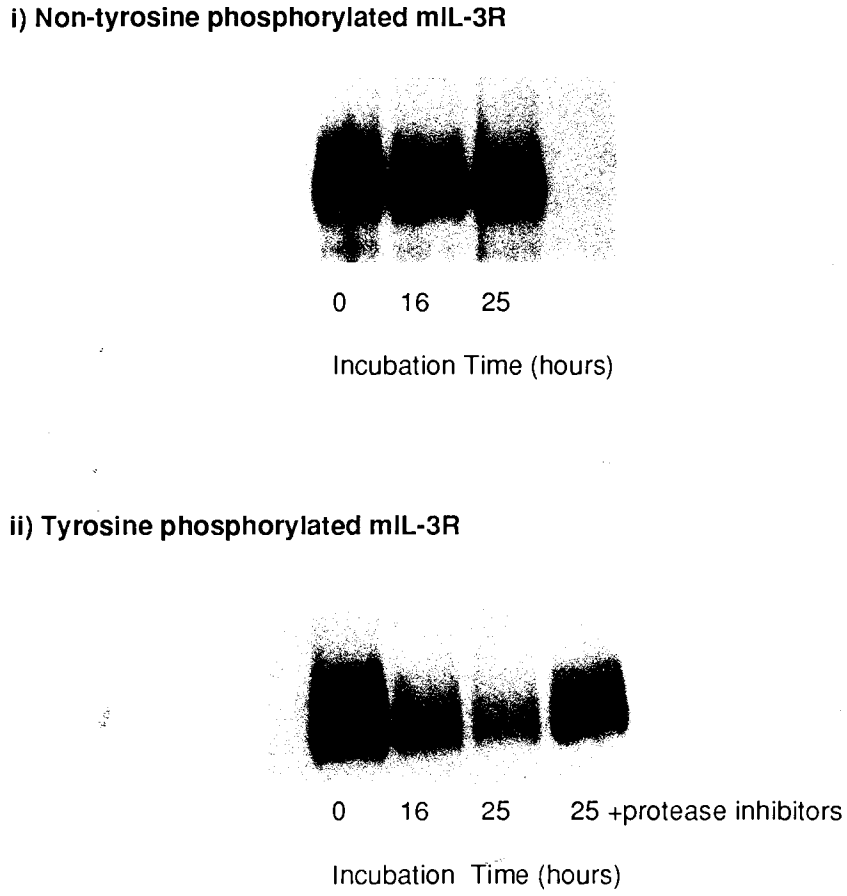

Figure 19B. Tyrosine phosphorylation of the mIL-3R increases its susceptibility to proteolysis. Legend on previous page.

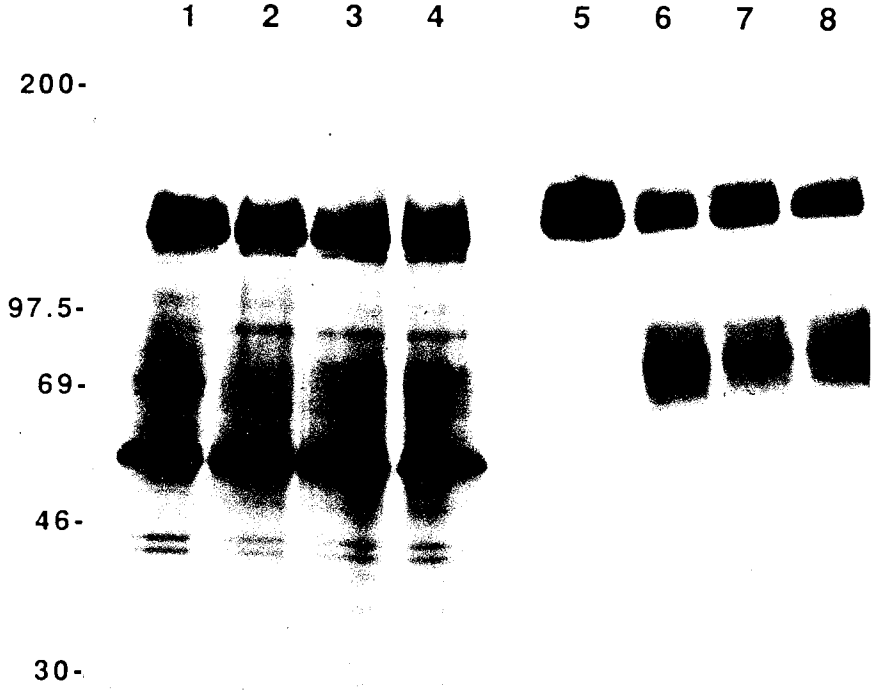

Figure 20. Increased proteolytic susceptibility of the tyrosine phosphorylated mIL-3R is an intrinsic property of the tyrosine phosphorylated receptor. ^{35}S-labelled, tyrosine phosphorylated (lanes 5-6) and tyrosine unphosphorylated (lanes 1-4) mIL-3R's were purified as described in Chapter II from B6SUtA$_1$ cells equilibrated with ^{35}S-methionine and stored at -20°C (lanes 1 and 5) or incubated at 4°C for 24 h with either no additions (lanes 2,6), or with unlabelled tyrosine phosphorylated mIL-3R (lanes 3,7) or unlabelled tyrosine unphosphorylated mIL-3R (lanes 4,8). The level of protein in the ^{35}S-labeled and unlabeled preparations were identical so that the total amount of protein was approximately equal in lanes 3 and 8. The ^{35}S-labeled bands, with apparent molecular masses ranging from 40 to 90 kD in lanes 1 through 4 simply reflect the fact that the 120 kD mIL-3R preparation is less pure than the 140 kD mIL-3R preparation. Proteins were visualized by autoradiography. Similar results were obtained in three separate experiments.

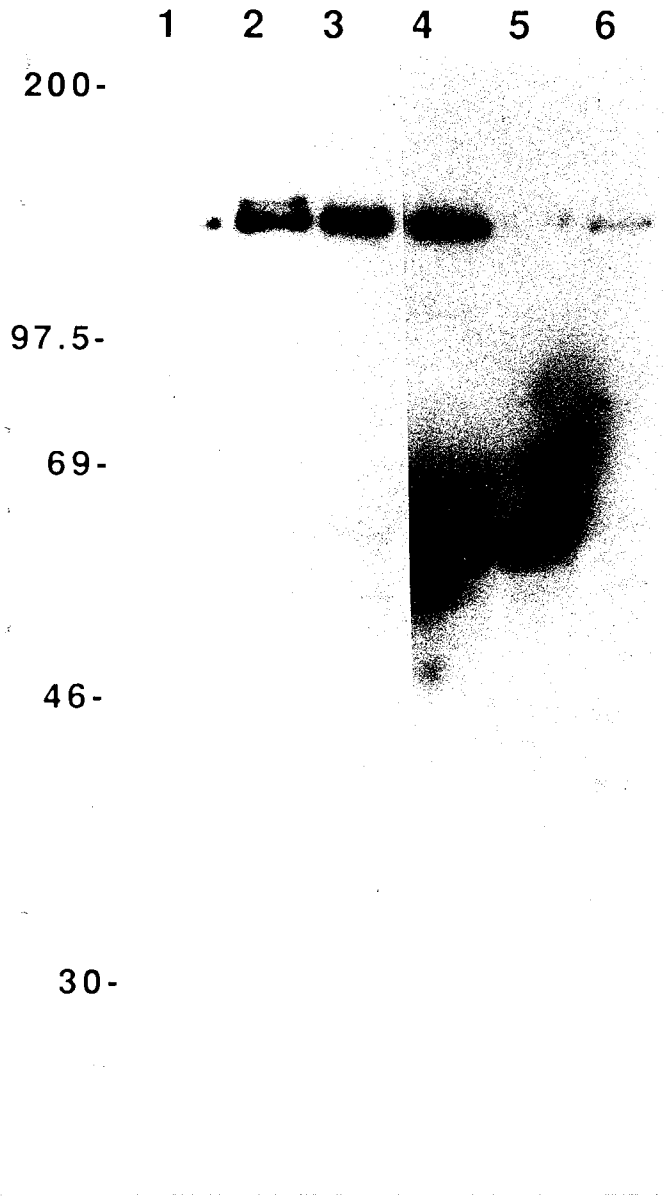

Figure 21. Effect of phosphatase treatment on receptor integrity. Aliquots of a 140 kD mIL-3R preparation were incubated with three different phosphatases (or phosphatase control buffers) for 16 h at 37°C and than subjected to Western analysis using affinity purified anti-N terminal mIL-3R antibody. Lane 1, no addition; 2, 100 U/ml T cell specific tyrosine phosphatase; 3, 1 U/ml each of the catalytic subunits of serine phosphatase 1 and 2A; 4, 1 U/ml of calf intestinal alkaline phosphatase; 5, tyrosine phosphatase buffer containing 2mM EDTA; and 6, alkaline phosphatase buffer containing 1 mM $MgCl_2$ and 2 mM $ZnCl_2$. Similar results were obtained in three separate experiments.

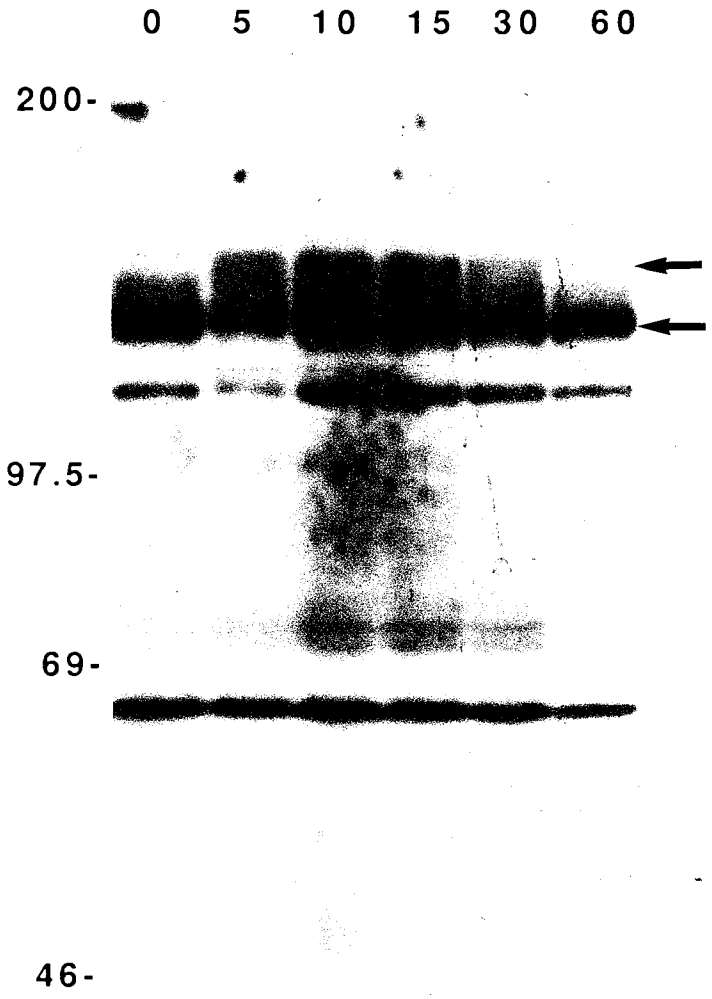

Figure 22. Degradation of the mIL-3R in intact cells. B6SUtA$_1$ cells were incubated at 37°C with 30 nM mIL-3. At the indicated times (in minutes) the incubation was stopped, plasma membranes were prepared and processed for Western analysis with anti-mIL-3R antibody as described in Chapter II. Similar results were obatined in four separate experiments.

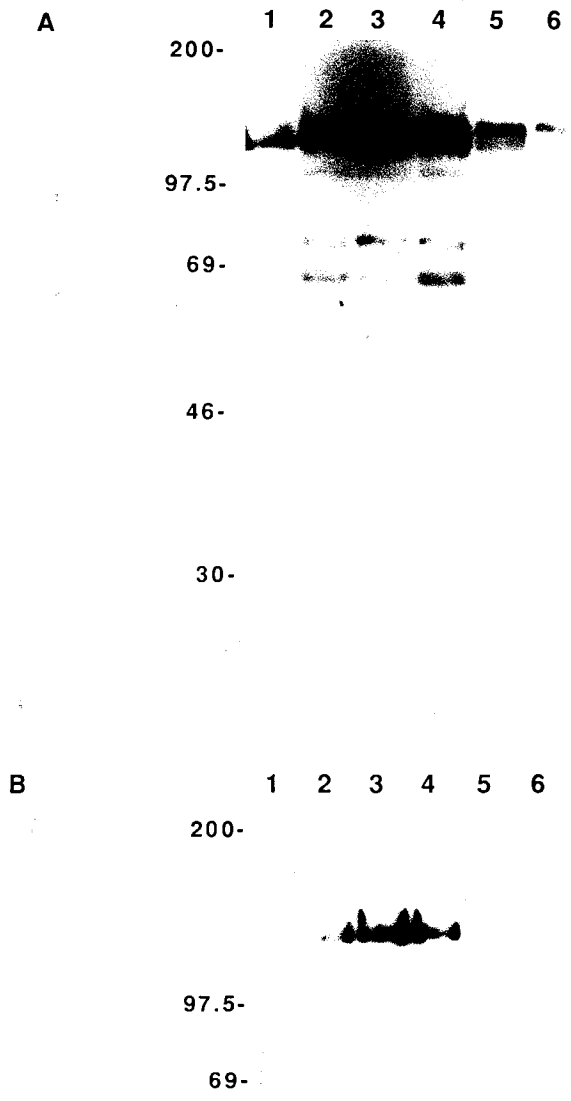

Figure 23A. Effect of various inhibitors on mIL-3R cleavage. B6SUtA$_1$ cells were incubated for 30 min in 10% FCS/RPMI with no addition (lane 1,2,6), 1 nM chloroquine (lane 3), 2 mM genistein (lane 4), or 0.3 μM staurosporine (lane 5). The cells were then stimulated for 15 min with 30 nM mIL-3 (lanes 2,3,4,5) or 100 nM TPA (lane 6). Plasma membranes were prepared, solubilized in SDS-PAGE sample buffer, separated by SDS-PAGE and subjected to Western analysis with polyclonal rabbit α-phosphotyrosine antibody. Similar results were obtained in three separate experiments.

Figure 23B. Effect of kinase inhibitors on mIL-3R tyrosine phosphorylation. B6SUtA$_1$ cells were treated as described in Figure 23A. The cells were then pelleted, solubilized with NP40 and immunoprecipitated with 1G2 α-phosphotyrosine Sepharose. The immunoprecipitates were separated by SDS-PAGE and subjected to Western analysis with polyclonal rabbit α-N terminal mIL-3R antibody. Similar results were obtained in two separate experiments.

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CHAPTER V

IDENTIFICATION OF PROTEINS POTENTIALLY ASSOCIATED WITH THE mIL-3R

A. INTRODUCTION

The binding of many hemopoietic growth factors to their receptors induces the tyrosine phosphorylation of several cellular substrates[1,2] including the receptor itself.[3,4] In the case of CSF-1 and SF, the cellular phosphorylations are initiated by the activation of a receptor intrinsic tyrosine kinase. However, although at least three members of the hemopoietin receptor superfamily, ie. the mIL-3R,[4,5] EpR[6] and IL-2R,[3] become tyrosine phosphorylated upon ligand binding, none of the hemopoietin receptor superfamily members possess a kinase domain similar to any known tyrosine kinases. It is possible that these receptors possess a novel tyrosine kinase domain that is not homologous to previously characterized kinases. A precedent for this has recently been reported, namely, the *bcr* protein which is a serine/threonine kinase with no obvious homology to other known kinases. Alternatively, receptor tyrosine phosphorylation could be mediated by a receptor associated tyrosine kinase. Several members of the *src* family of tyrosine kinases have been shown recently to associate with and phosphorylate cell surface molecules. Examples include the $p56^{lck}$ kinase which binds to the cytoplasmic tails of CD4 or CD8 and becomes activated upon crosslinking of these molecules,[7,8] and the $p60^{fyn}$ kinase, which is associated with the TcR and becomes activated upon TcR ligation.[9] Recently, $p56^{lck}$ has also been shown to associate with the IL-2R, and be responsible for tyrosine phosphorylation of the IL-2R β chain following IL-2 stimulation.[10]

Since we and others[11] have been unable to detect tyrosine kinase activity in purified preparations of the mIL-3R, it is likely that tyrosine phosphorylation of the receptor is mediated by an associated kinase. We therefore undertook studies directed at identifying and characterizing this putative kinase, as well as any other receptor associated proteins that may be involved in mIL-3 induced signal transduction.

B. RESULTS

1. Comparison of Tyrosine Phosphorylations Induced by mIL-3, mGM-CSF and SF

Most tyrosine kinases autophosphorylate upon activation, so as a first step in identifying the mIL-3R tyrosine kinase, we examined the tyrosine phosphorylations induced by mIL-3. As shown in Figure 24, at 37°C , mIL-3 (lane 6) stimulated the tyrosine phosphorylation of several proteins in B6SUtA$_1$ cells including ones at 140, 130, 95, 70, 56, and 32 kD. The 140 kD band we had previously shown to be the mIL-3R.[4] Interestingly, mGM-CSF (lane 7) and SF (lane 8) also induced the tyrosine phosphorylation of 56 and 32 kD proteins. Although further work (ie. 2D gels, tryptic maps) is needed to establish that these two tyrosine phosphoproteins are identical, this result does suggest that these two proteins may be components of a common signalling pathway utilized by all three growth factors. The 95 kD protein was tyrosine phosphorylated in response to both mIL-3 and mGM-CSF (lanes 6 and 7) as observed previously[2] and may represent a common signal transduction intermediate. A slower migrating 97 kD protein was induced by SF (lane 8). In order to determine whether these phosphorylations were receptor proximal in the signalling cascade, we also looked at the phosphorylations induced at 4°C. At this temperature, membrane and cytoskeletal movements are minimal so interactions should occur only between molecules which are already closely associated and events which are further down in the signalling cascade should not take place at 4°C.[12] As shown in Figure 24, the mIL-3 induced phosphorylations after 10 min at 4°C (lane 2) were identical to those observed following 5 min at 37°C (lane 6). The rapid induction kinetics at 4°C suggests that the mIL-3 activated tyrosine kinase may be associated with the mIL-3R before ligand binding. In addition to the receptor, the other substrates of this kinase, i.e. the other proteins tyrosine phosphorylated in response to mIL-3 also appear to be receptor associated. Interestingly, the SF induced tyrosine phosphorylation of the 150 kD protein, perhaps the *c-kit* tyrosine kinase, was more intense at 4°C (lane 4) than at 37°C (lane 8). This may reflect dephosphorylation of labile tyrosine phosphates at 37°C.

The kinetics of phosphorylation were then examined in more detail in order to try to distinguish the first autophosphorylation event of the activated tyrosine kinase from subsequent phosphorylations of exogenous substrates by this activated enzyme. As shown in Figure 25, phosphorylation of all proteins as well as the mIL-3R occurred, remarkably, within 2 min at 4°C. However, the kinetics of phosphorylation and dephosphorylation of each protein differed, with the phosphorylation of pp56 and pp32 continuing to increase with time at 4°C whereas the level of phosphorylation of the 140, 90 and 70 kD proteins peaked at 2 min and declined rapidly thereafter. By 15 min, approximately half of the original tyrosine phosphorylation of the 140, 90 and 70 kD proteins remained. Although these results were very interesting, they did not allow us to clearly distinguish a defined sequence of phosphorylation events and so did not allow assignment of any one of these proteins as a possible tyrosine kinase.

2. Identity of the mIL-3 Stimulated Tyrosine Phosphoproteins with Known Proteins

To examine whether any of the mIL-3 stimulated tyrosine phosphorylated proteins were identical to tyrosine phosphorylated signal transduction intermediates previously described in the literature, antibodies to potential candidates were obtained for analysis. Given that the molecular weight of a major protein tyrosine phosphorylated in response to mIL-3, mGM-CSF and SF was 56 kD, it was tempting to speculate that this protein might be a member of the *src* family. However, pp56 did not react with antiserum to $p56^{hck}$ (data not shown), a *src* family member present predominantly in hemopoietic cells.[13] Moreover, other investigators have shown that other *src* family members, ie. *lck, src and lyn* , are not involved in mIL-3 stimulated signal transduction.[6] However, this does not rule out the possibility that pp56 is a *src* family member since a number of new src family members have recently been identified using less stringent oligonucleotide screening techniques (Dr. J. Ihle, personal communication).

Because of a recent report[20] showing that the activation of a cdc2 kinase/cyclin A complex is an early event in in the response to PDGF, immunoblotting was carried out with antibodies to cdc2 (αPSTAIRE) and cyclin A (αCHLA-4) following immunoprecipitation of

tyrosine phosphoproteins from mIL-3 stimulated cells (Figure 26A). As Figure 26A shows, the αCHLA-4 reactive band did not line up with the mIL-3 stimulated pp56. In contrast, the αPSTAIRE reactive band aligned exactly with pp32. However, the αPSTAIRE reactive protein did not appear to be pp32 since its tyrosine phosphorylation level appeared to drop rather than increase upon mIL-3 stimulation.

A potential candidate for the identity of the pp95 was the *fps* kinase. However, although immunoprecipitation with αfps precipitated a 95 kD band, it did not undergo any changes in its phosphorylation state with mIL-3 (Figure 26B). In the same experiment, proteins were immunoprecipitated from mIL-3 treated and untreated cells with αGAP anti-serum, both as a control for the αfps immunoprecipitation and to see if GAP became tyrosine phosphorylated in response to mIL-3. A 120 kD band, probably GAP, was precipitated from both control and mIL-3 stimulated cells, suggesting the tyrosine phosphorylation state of GAP does not change with stimulation. Of interest, however, is the co-immunoprecipitation of a 95 kD protein from mIL-3 stimulated cells. This is especially interesting since GAP has been shown recently to associate with tyrosine kinases[14] and so this associated 95 kD protein, which is most likely identical to the mIL-3R associated 95 kD phosphoprotein, may be a tyrosine kinase.

GAP associates with tyrosine phosphorylated proteins through its two SH2 domains. Many proteins, in addition to GAP have now been described which contain SH2 domains,[14] including the 97 kD proto-oncogene *vav* . Since this protein has recently been shown to be tyrosine phosphorylated under certain conditions, we investigated whether it could be the 95 kD protein tyrosine phosphorylated in response to mIL-3 and mGM-CSF or the 97 kD protein tyrosine phosphorylated in response to SF. As Figure 26C shows, $p97^{vav}$ appeared to be constitutively tyrosine phosphorylated in $B6SUtA_1$ cells (lanes 1,5), although the addition of either mIL-3 or SF increased the level of tyrosine phosphorylation slightly. Thus the mIL-3 stimulated pp95 is not $p97^{vav}$. However, we did find that it was the 97 kD protein tyrosine phosphorylated in response to SF, both in $B6SUtA_1$ cells and in the human myeloblastic cell line, MO7E (15 and data not shown). Interestingly, upon longer exposure of the autoradiogram

in Figure 26C, a smaller band just below $p97^{vav}$ could be seen in the mIL-3 and mGM-CSF lanes which might represent either a protein co-precipitating with activated $p97^{vav}$ or a $p97^{vav}$ degradation product (Figure 26D).

3. Effect of Various Kinase Inhibitors on mIL-3 Stimulated Tyrosine Phosphorylations

Within the limitations of analysis, it did not appear that the mIL-3R associated tyrosine kinase nor any of the mIL-3 stimulated tyrosine phosphoproteins were previously isolated protein. Thus to further characterize the mIL-3R tyrosine kinase, the effect of genistein and staurosporine on mIL-3 induced tyrosine phosphorylations were examined. Genistein is a specific tyrosine kinase inhibitor while staurosporine is a serine/threonine kinase specific inhibitor at low concentrations and an inhibitor of tyrosine kinases at high concentrations. As shown in Figure 27, genistein (lane 4) did not affect mIL-3 stimulated tyrosine phosphorylation. Thus the mIL-3R associated kinase is genistein insensitive. In contrast, the mIL-3R tyrosine kinase was profoundly inhibited by high concentrations of staurosporine (lane 5). Interestingly, TPA (lane 6), which is slightly mitogenic for $B6SUtA_1$ cells in the absence of mIL-3,[2] did not induce tyrosine phosphorylations similar to mIL-3. However, in separate experiments in which TPA was added to $B6SUtA_1$ cells and immunoblotting carried out with antibodies against the mIL-3R, it appeared that this phorbol ester caused the mIL-3R to migrate more slowly in SDS polyacrylamide gels (Chapter III, Figure 23A, Lane 6). This suggests that protein kinase C may be the serine/threonine kinase responsible for serine phosphorylation of the mIL-3R. Lastly, chloroquine, a lysosomal inhibitor was tested for its effect on mIL-3 stimulated tyrosine phosphorylation events (lane 3). Interestingly, there was no obvious effect suggesting that internalization of the mIL-3R may not play a role in regulating, ie. limiting, the levels of tyrosine phosphorylation (at least not within 10 min at 37°C).

4. Development of an Assay for the mIL-3R Associated Tyrosine Kinase

To facilitate identification and purification of the mIL-3R associated tyrosine kinase, an assay was developed to detect mIL-3R specific tyrosine kinase activity. In this assay, B-mIL-3/mIL-3R complexes were isolated from $B6SUtA_1$ cell plasma membranes, as described in

Chapter II, in a buffer not containing phosphatase inhibitors. Preliminary experiments showed that when no precautions were taken to inhibit tyrosine phosphatases, the tyrosine phosphorylation of the mIL-3R quickly declines to very low levels. These receptor complexes were immobilized on Co-bind plates as described in Chapter II. Samples were then incubated with the immobilized receptors in the presence of ATP and Mg^{2+}. After this incubation, the wells were washed and tyrosine phosphorylation detected with ^{125}I-labelled α-phosphotyrosine antibodies. A summary of the assay procedure is shown in Figure 28. The specificity of this assay is shown in Figure 29. As a source of mIL-3R tyrosine kinase, tyrosine phosphorylated proteins were purified with α-phosphotyrosine Sepharose from $B6SUtA_1$ cells stimulated with mIL-3 for 10 min at 4°C. As Figure 29 shows, when all components of the assay were present (+ATP), approximately 500 cpm were associated with the well in this particular experiment. Omission of ATP (-ATP) or tyrosine kinase sample (buffer) decreased the amount of counts bound. Omission of the mIL-3R, by adding a 20 fold excess of unbiotinylated mIL-3 during the B-mIL-3 isolation of mIL-3R's from plasma membranes, also decreased the level of tyrosine phosphorylation detected (+ cold mIL-3). Thus this assay appears to be capable of detecting tyrosine specific phosphorylation of the mIL-3R.

With this assay we confirmed that the mIL-3R specific tyrosine kinase activity was in the α-phosphotyrosine bound fraction of $B6SUtA_1$ cells stimulated with mIL-3. Figure 30 compares the mIL-3R tyrosine kinase activity isolated with α-phosphotyrosine Sepharose from stimulated and unstimulated $B6SUtA_1$ cells. Considerably more activity was present in the sample from mIL-3 stimulated cells, suggesting that one of the tyrosine phosphorylated proteins, or a non-tyrosine phosphorylated protein physically associated with one of these proteins, was the mIL-3R kinase. In an attempt to determine which of these proteins was the tyrosine kinase, samples were fractionated on a Sephadex G150 column. The eluted fractions were analyzed with this tyrosine kinase assay and the results plotted in Figure 31. Four peaks of activity were consistently observed, suggesting that either there are four distinct tyrosine

kinases, or that one kinase can associate to varying degrees with potential regulatory molecules.

C. DISCUSSION

As was previously reported from our laboratory, several proteins in the mIL-3 dependent murine hemopoietic cell line, B6SUtA$_1$, become tyrosine phosphorylated upon mIL-3 stimulation.[2] However, these earlier studies were extended in the present work by the demonstration that almost all the tyrosine phosphorylations observed at 37°C are also seen at 4°C, the only exception being the minor tyrosine phosphorylated 42 kD protein which we (data not shown) and others[16] have identified as MAP kinase. The 4°C studies were conducted in an attempt to identify the earliest tyrosine phosphorylation events, and thus potentially identify the mIL-3R specific tyrosine kinase. However, similar to results obtained from studies in the human GM-CSF system reported by Okuda et al,[17] most of the phosphorylations observed at 37°C were also seen at 4°C. This suggests either that our working hypothesis (that at 4°C, phosphorylations can only occur if proteins are already associated before ligand binding) is incorrect, or that all the observed phosphorylated proteins are indeed already receptor associated. We favour the latter interpretation since phosphorylation occurs by 2 min at 4°C; the rapid kinetics makes it unlikely that these phosphorylated substrates diffuse together. In addition, many receptor systems, notably the intrinsic tyrosine kinase receptors and the *src* family associated receptors such as the IL-2R and the TcR have been shown to exist in preformed complexes with signalling proteins.[9,18] The physiological significance of the phosphorylation events we observe at 4°C is further supported by the fact that not all phosphorylations take place at 4°C. Phosphorylation and activation of p74$^{raf\text{-}1}$,[17] and MAP[16] kinases do not occur at 4°C. An interesting corollary to this last observation is that p74$^{raf\text{-}1}$ and MAP may act further downstream in the signalling cascade than the processes regulated by the phosphorylation events observed at 4°C.

The five major mIL-3 stimulated phosphoproteins do not appear, within the limits of our analysis, to be p90^{fps}, p97^{vav}, cyclin A, p56^{hck} or p34^{cdc2}. However, the tyrosine

phosphorylation state of $p34^{cdc2}$ decreased following 10 min of mIL-3 stimulation at 4°C. This observation suggests that $p34^{cdc2}$ may have a role early in mIL-3 signal transduction since tyrosine dephosphorylation of $p34^{cdc2}$ occurs during activation of the kinase in M-phase.[19] Furthermore, a proline directed protein kinase activity (PDPK) has been shown to be activated early in PDGF stimulated signal transduction.[20] This PDPK was subsequently shown to consist of a cdc2/cyclin A complex.[21] Perhaps a cdc2 kinase activity has a similar role in the mIL-3 system.

Another possible identity for pp32 is the *ras* guanine nucleotide exchange protein (GEF).[22,23] The levels of *ras* GTP increase in cells in response to mIL-3[24] and this may be a consequence of GAP inhibition. However, there is increasing evidence that accelerated nucleotide exchange may be important in mediating cellular responses to growth factors.[25] Nucleotide exchange proteins may potentially be regulated by phosphorylation.[22] Thus it is interesting to speculate that mIL-3 may regulate *ras* GTP levels by regulation, through tyrosine phosphorylation, of a guanine nucleotide exchange protein.

One of the mIL-3 stimulated tyrosine phosphoproteins is likely to be the mIL-3R tyrosine kinase itself since most tyrosine kinases autophosphorylate upon activation. To identify the kinase, we developed an assay specific for tyrosine phosphorylation of the mIL-3R. In order to make the assay specific for tyrosine phosphorylation, we monitored phosphate incorporation using labelled α-phosphotyrosine antibodies instead of ^{32}P-ATP. A similar tyrosine kinase assay was recently reported which utilized synthetic tyrosine containing peptides as a substrate.[26] However, since our assay uses purified mIL-R's as the substrate, it is more specific for the mIL-3R tyrosine kinase. The major limitation of this assay is that it does not distinguish between the action of an mIL-3R tyrosine kinase and the presence of an ATP dependent association of tyrosine phosphorylated proteins with the mIL-3R. However, both possibilities are of interest to the study of mIL-3 induced signal transduction so this lack of specificity does not disqualify the utility of this assay. The assay is simple and allows the rapid

screening of multiple samples, e.g., column fractions. However, once an activity is isolated, it must be analyzed for its ability to phosphorylate the mIL-3R on tyrosine residues.

Four peaks of mIL-3R kinase activity were detected following Sephadex G150 gel filtration chromatography. This suggests that there are either four kinases (or mIL-3 associating proteins) or that the putative kinase is associated with other proteins which may serve as regulatory subunits. The resolution of these possibilities awaits further purification of these mIL-3 induced tyrosine phosphoproteins.

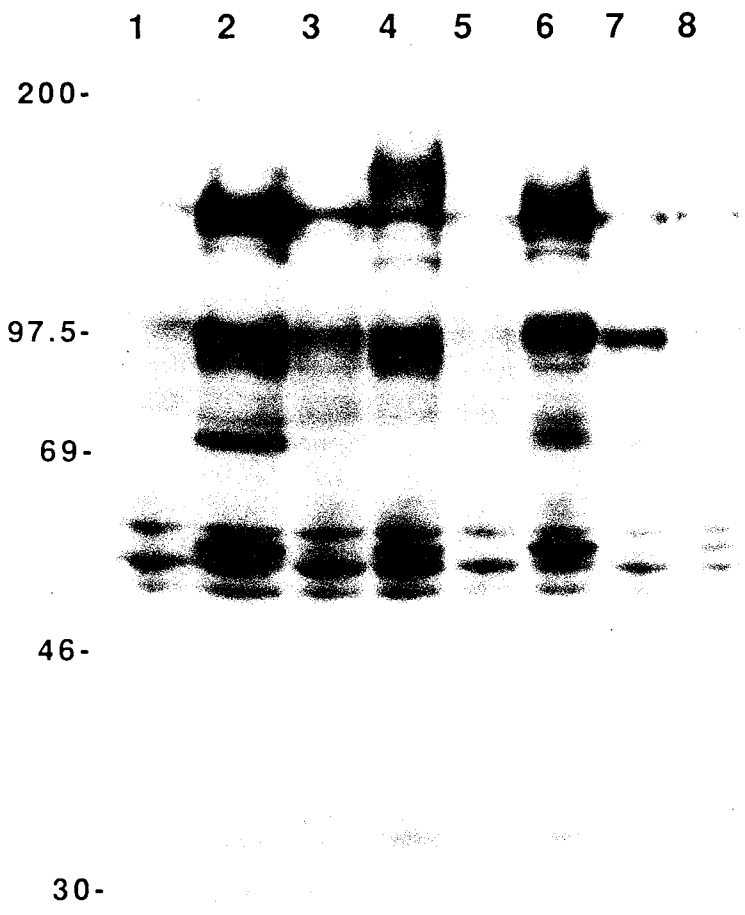

Figure 24. Comparison of tyrosine phosphorylations induced by mIL-3, mGM-CSF and SF in B6SUtA$_1$ cells. Cells were incubated for 10 min at 4°C (lanes 1-4) or 5 min at 37°C (lanes 5-8) with control buffer (1,5), mIL-3 (2,6), mGM-CSF (3,7) or mSF (4,8). Cells (1 X 10^6 per lane) were then solubilized with 0.5% NP40 as described in Chapter II and subjected to Western analysis with α-phosphotyrosine Ab 4G10. Similar results were observed in three separate experiments.

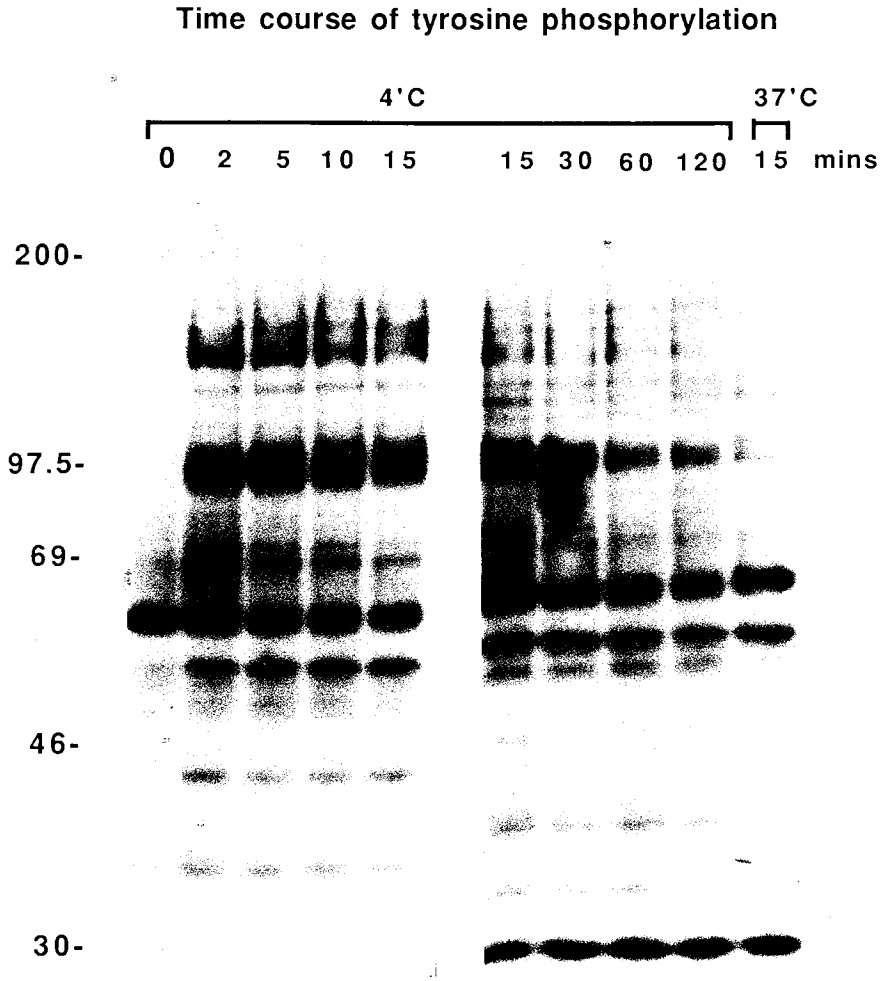

Figure 25. Time course of mIL-3 stimulated phosphorylations at 4°C. B6SUtA$_1$ cells were stimulated for the indicated times at 4°C and solubilized in 0.5% NP40 as described in Chapter II. The cell lysates (2 X 10^6 cells/sample) were immunoprecipitated with α-phosphotyrosine Sepharose and the phenyl phosphate eluates analyzed by Western blotting with rabbit polyclonal α-phosphotyrosine Ab. For comparison, one sample (far right) was stimulated for 10 min at 37°C. Similar results were obtained in two separate experiments.

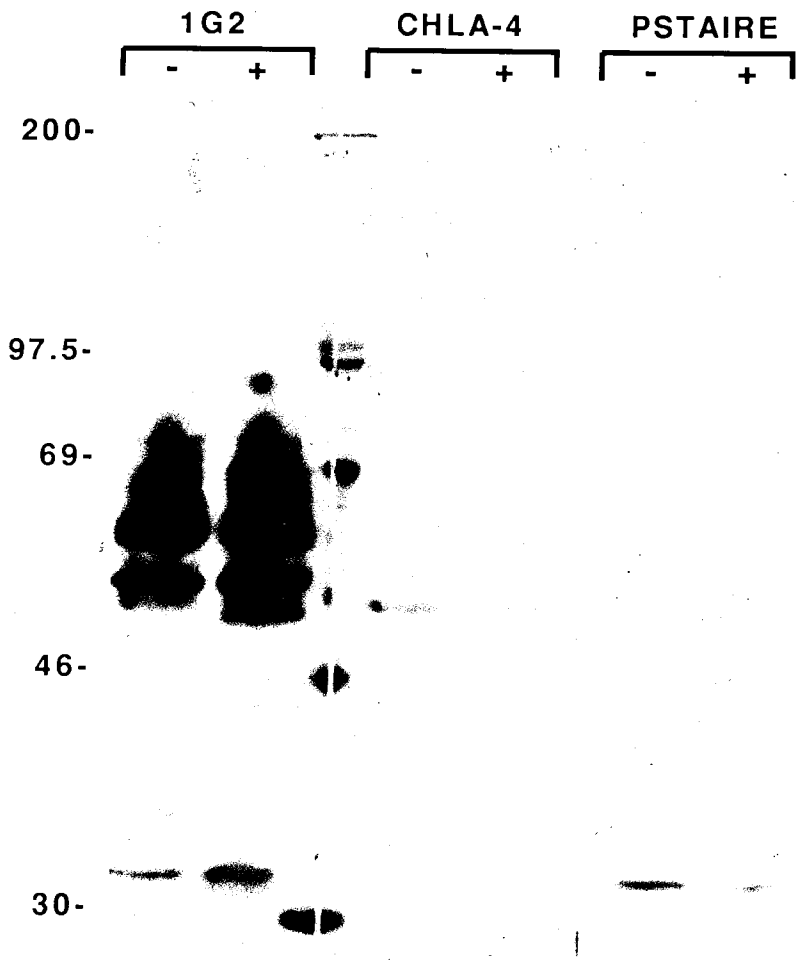

Figure 26A. Comparison of pp56 and pp32 with cyclin A and cdc2 kinase. B6SUtA$_1$ cells were stimulated (+) or not (-) with mIL-3 for 10 min at 4°C. Tyrosine phosphorylated proteins were purified as described in Chapter II and separated by SDS-PAGE and electrotransferred onto Immobilon. The membrane was cut and immunoblotted with α-phosphotyrosine Ab 4G10, α-CHLA4 or α-PSTAIRE and developed with the appropriate ^{125}I labelled second antibody. Similar results were obtained in three separate experiments.

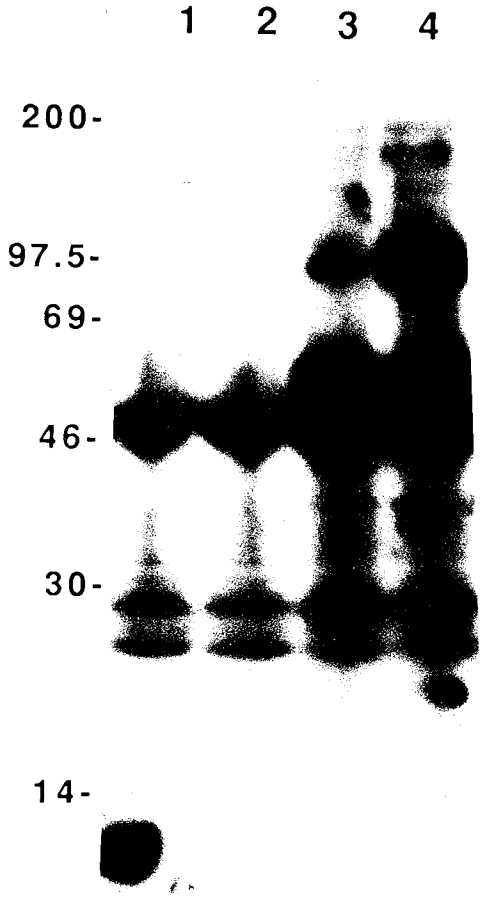

Figure 26B. α-fps and α-GAP immunoprecipitation from mIL-3 treated B6SUtA1 cells. Cells were stimulated (lanes 2 and 4) or not (lanes 1 and 3) with mIL-3 and solubilized in 0.5% NP40 as described in Chapter II. The cell lysates were incubated with α-fps (lanes 1 and 2) or α-GAP (lanes 3 and 4) (both at 1/200 dilution) rabbit anti-serum for 2 h and Protein A Sepharose was added for 2 h. The beads were washed and boiled in SDS-PAGE sample buffer and the proteins subjected to Western analysis with rabbit polyclonal α-phosphotyrosine Ab. Similar results were obtained in three separate experiments.

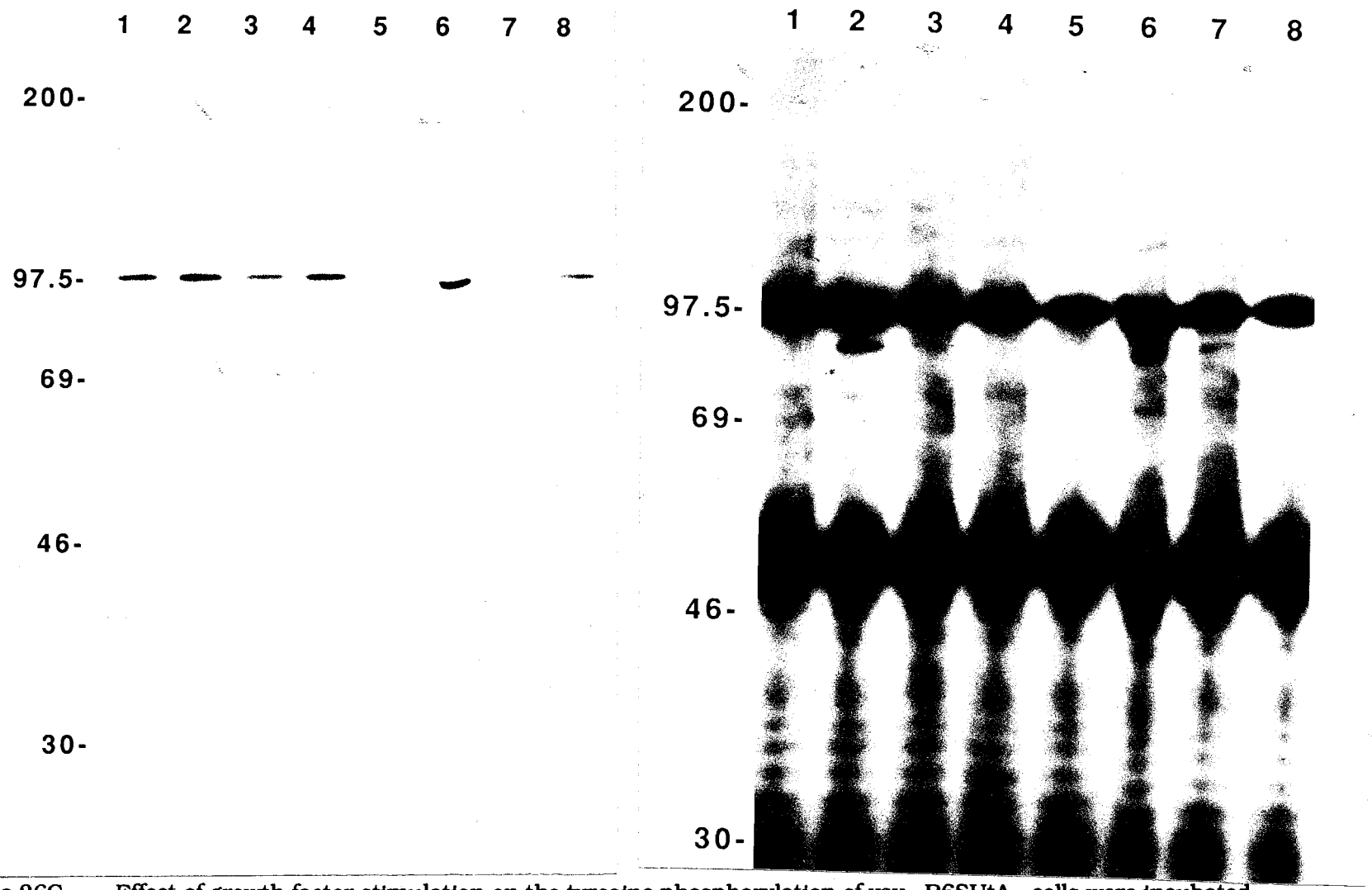

Figure 26C. Effect of growth factor stimulation on the tyrosine phosphorylation of vav. B6SUtA$_1$ cells were incubated for 10 min at 4°C (lanes 1-4) or 5 min at 37°C (lanes 5-8) with control buffer (1,5), mIL-3 (2,6), mGM-CSF (3,7) or mSF (4,8). Cells (2 X 10^6 cells/sample) were solubilized in 0.5% NP40 as described in Chapter II. The lysates were incubated with α-vav rabbit serum (1/500 dilution) for 2 h, protein A Sepharose was then added and incubated for a further 2 h. After washing the beads, proteins were eluted by boiling in SDS-PAGE sample buffer and subjected to Western analysis with α-phosphotyrosine (4G10). Similar results were observed in three separate experiments.

Figure 26D. A 10 X longer exposure of the Western blot from Figure 26C.

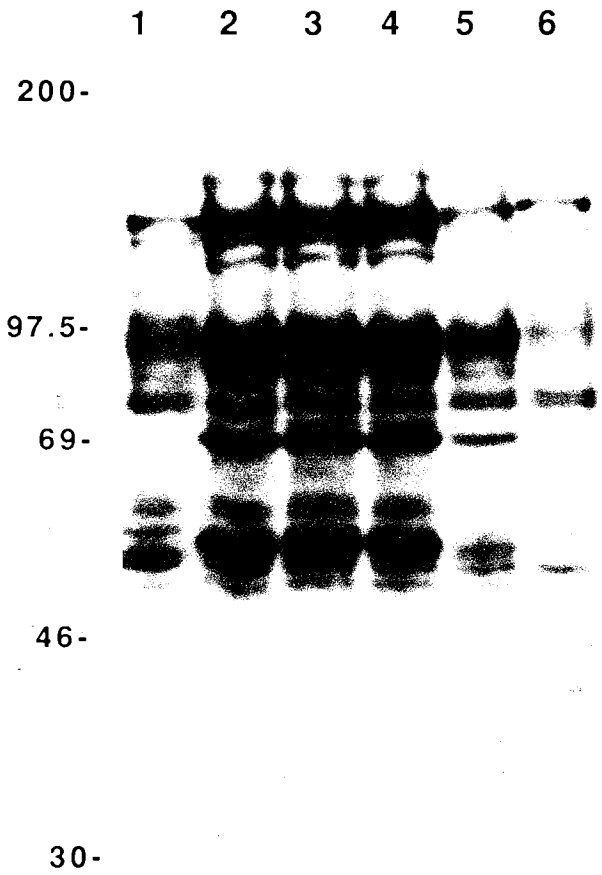

Figure 27. Effect of various inhibitors on mIL-3 stimulated tyrosine phosphorylations. B6SUtA$_1$ cells (2 X 10^7 cells/sample) were incubated with 100 μM chloroquine diphosphate (lane 3), 2 mM genistein (lane 4), 1 μM staurosporine (lane 5) or control buffer (lanes 1,2 and 6) for 30 min at 37°C. mIL-3 (lanes 2-5) or TPA (100 μM, lane 6) was then added, and the cells incubated a further 10 min. Samples were then processed as in Figure 24 and analyzed by immunoblotting with α-phosphotyrosine (4G10). Similar results were obtained in three separate experiments.

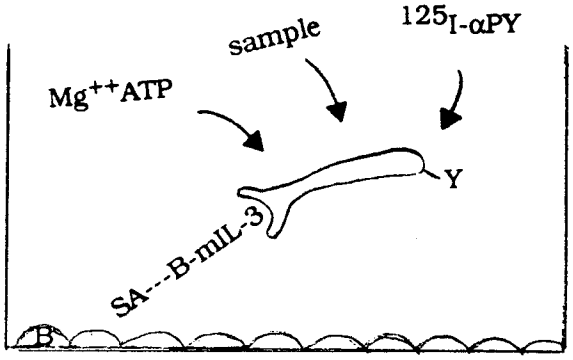

Figure 28. Scheme of mIL-3R tyrosine kinase assay. B-mIL-3/mIL-3R complexes were prepared in 0.5% NP40/PBS as described in Chapter II and immobilized onto Co-bind plates modified with biotinylated gelatin. Samples to be assayed are added to the wells, in triplicate, and made 10 mM ATP and 10 mM $MgCl_2$. The plates were incubated 12-16 h at 4°C, washed with 0.1 % NP40/PSB and incubated 2 h with 10^5 cpm/mL (50 ng/mL) ^{123}I labelled goat anti-mouse Ig. After washing away unbound antibody, the contents of the well were eluted with 1% SDS and counted.

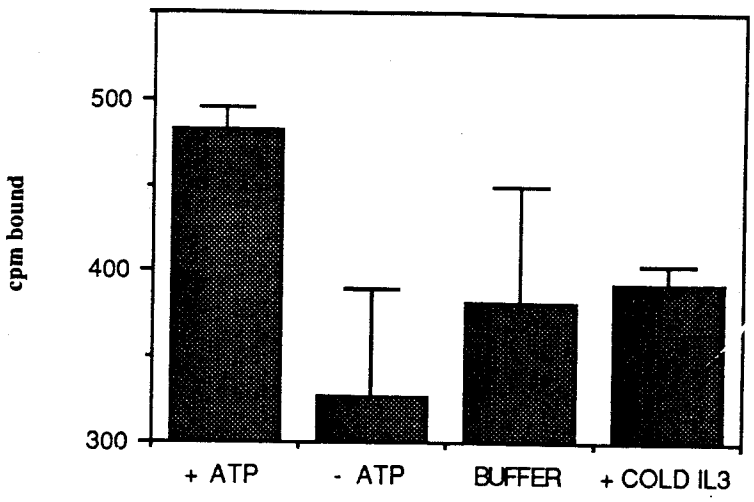

Figure 29. Specificity of the mIL-3R tyrosine kinase assay. B-mIL-3/mIL-3R complexes were immobilized to Co-bind plates as described in Chapter II. In one set of wells (+ cold mIL-3), a 20 fold excess of unbiotinylated mIL-3 was included with B-mIL-3 in the preparation of mIL-3R complexes in order to control for non-specific binding of non-mIL-3R proteins to B-mIL-3. The wells were then incubated with the kinase sample and ATP/$MgCl_2$. ATP was omitted from some wells (-ATP); control buffer was substituted for the kinase sample in others (buffer). Tyrosine phosphate incorporation was measured as described above. Data points represent means (n=3) +/- SEM. Similar results were observed in two separate experiments.

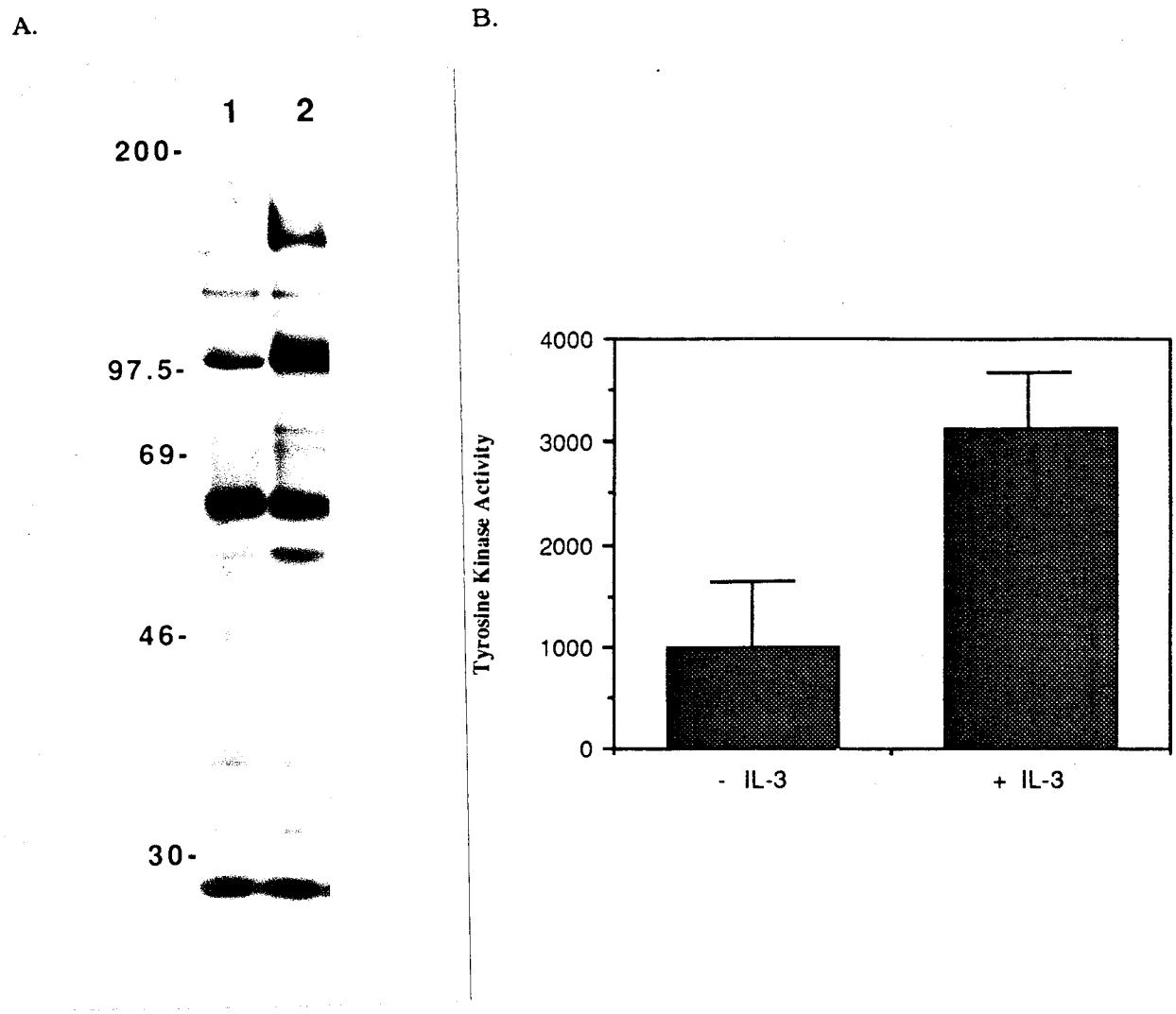

Figure 30. mIL-3R tyrosine kinase activity is present in the α-phosphotyrosine bound protein fraction from mIL-3 stimulated B6SUtA$_1$ cells. Tyrosine phosphorylated proteins were purified from mIL-3 unstimulated (panel A, lane 1) and stimulated (panel A lane 2) cells with α-phosphotyrosine as described above and analyzed for activity in the mIL-3R tyrosine kinase assay. The protein sample was visualized by α-phosphotyrosine Western (Figure 30A, also shown in Figure 32) and the tyrosine kinase activity compared in Figure 30B. In panel B, data points represent means (n=2) +/- SEM. Similar results were seen in two separate experiments.

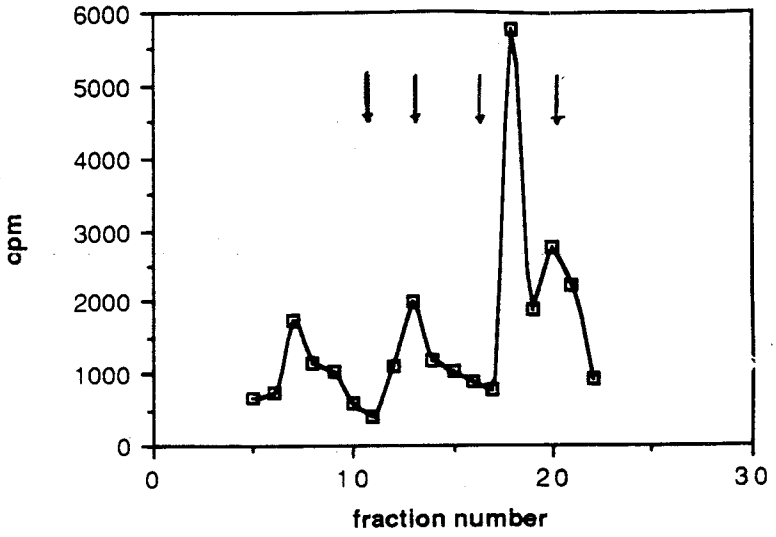

Figure 31. Sephadex G150 fractionation of mIL-3 induced tyrosine phosphoproteins. α-phosphotyrosine Sepharose purified proteins from mIL-3 stimulated B6SUtA$_1$ cells were separated on a 10 mL G150 sephadex size exclusion column equilibrated with 0.1% NP40/2 mM Na_3VO_4/50 mM Hepes, pH7.5. Fractions, 0.5 mL each, were collected and assayed for tyrosine kinase activity. Arrows mark the elution positions of (from left to right) thyroglobulin (670 kD), Ig (158 kD), ovalbumin (44 kD) and myoglobin (17 kD). Similar results were obtained in two separate experiments.

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CHAPTER VI

DEVELOPMENT OF STRATEGIES TO PURIFY mIL-3R ASSOCIATED PROTEINS

A. INTRODUCTION

As discussed in Chapter V, we have identified several proteins which could be potentially associated with and mediate mIL-3R signal transduction. To further characterize these proteins and determine their possible roles in mIL-3 stimulated signal transduction, we investigated various protocols to purify them to homogeneity. From our initial attempts at fractionating the mIL-3 induced phosphoproteins, and from our experience with the purification of the mIL-3R itself, we concluded that standard chromatographic techniques would probably be inefficient and result in yields too low to obtain amino acid sequence data. We therefore examined various affinity purification protocols. For pragmatic reasons, we focused our attention on the purification of the 95 kD tyrosine phosphoprotein which co-precipitated with GAP. Since GAP often binds proteins through its SH2 domains, we reasoned that an affinity column consisting of the SH2 domain of GAP might allow for the rapid purification of pp95. We also decided to focus on pp95 because, as mentioned in Chapter V, it may be a signal transduction intermediate common to mIL-3, mGM-CSF and SF. Sorensen et al[1] for example, first described a cytosolic 95 kD protein that became tyrosine phosphorylated in response to both mIL-3 and mGM-CSF stimulated B6SUtA$_1$ cells. Others have also reported a similar phosphoprotein induced by mIL-2, mIL-3, mGM-CSF and Ep in cells responsive to these factors.[2,3] In the human system, a protein with a very similar molecular weight was reported to be the major tyrosine phosphorylated protein observed in response to hIL-3 and hGM-CSF.[4,5] These observations, along with the fact that pp95 undergoes phosphorylation within 2 minutes at 4°C, suggested that pp95 might be an important molecule in signal transduction.

B. RESULTS

1. The use of B-mIL-3 to purify mIL-3R associated proteins.

Biotinylated mIL-3 was successfully used in the affinity purification of the mIL-3R as discussed in Chapter III. To determine if B-mIL-3/SA could also be used to purify mIL-3R associated proteins, B6SUtA$_1$ cells were stimulated with B-mIL-3 at 4°C for 5-15 min (Figure 32), and were then solubilized and incubated with SA. For comparison, tyrosine phosphoproteins were immunoprecipitated with α-phosphotyrosine Sepharose. As Figure 32 shows, the only tyrosine phosphorylated protein isolated with B-mIL-3 was the receptor itself. Thus, the other tyrosine phosphoproteins induced by mIL-3, which can be seen in the anti-PY lanes, did not co-purify with the mIL-3R. There are several possible explanations for this observation. For example, the NP40 solubilization conditions might have been too harsh to allow survival of an intact complex. However, when we tried lower NP40 concentrations and different detergents (ie., OG and Chaps) they did not yield results different from those shown in Figure 32 (data not shown). It is also conceivable that, because of steric hindrance, the SA might only be able to bind B-mIL-3/mIL-3R complexes from which receptor associated proteins have dissociated. Lastly, it is possible that the tyrosine phosphorylated proteins, putatively associated with the mIL-3R, might have remained associated with the mIL-3R upon cell lysis but became dissociated, because of high k_D's, during the SA column washing steps.

2. Association of mIL-3 Induced Tyrosine Phosphoproteins With SH2 domains

Recently, GAP and other SH2 domain containing proteins have been shown to bind tightly, via their SH2 domains, to various tyrosine phosphorylated proteins.[6] We therefore explored the possibility of using the SH2 domain of GAP to purify pp95. Specifically the SH2 domain of GAP was expressed in *E. coli* as a glutathione S-transferase (gst) fusion protein (see Chapter II), purified on a glutathione agarose column, and immobilized to Sepharose beads. These beads were then tested for their ability to bind pp95. As shown in Figure 33A, control beads containing only the gst portion of the fusion protein did not bind any tyrosine phosphorylated proteins from mIL-3 stimulated B6SUtA$_1$ cells. The GAP SH2-gst fusion

protein beads, however, bound several tyrosine phosphorylated B6SUtA$_1$ proteins, including the pp140, pp95, pp70, and pp56 tyrosine phosphorylated proteins induced by mIL-3. The 140 kD protein was shown to be the mIL-3R by immunoblotting duplicate samples with an α-N terminal mIL-3R antibody (Figure 33B). The presence of all four mIL-3 induced tyrosine phosphoproteins could suggest that they all independently bind GAP SH2. This, however, is probably unlikely. Alternatively, this apparent co-precipitation could simply be due to insufficient washing of the beads and that the beads are not specifically binding proteins at all. However, this is not likely, since the GAP SH2 beads bound mIL-3R only from mIL-3 stimulated cells (Figure 33B). A third possibility is that only one of these proteins actually binds to the GAP SH2 and the other proteins are simply associated with it. In this case, more stringent washing should remove all but one phosphoprotein. A fourth possibility is that the four phosphoproteins specifically associate *in vivo* with SH2 domains of different proteins, eg. domains of PLCγ or the PI3-kinase p85 subunit, but they can also bind with lower affinity to the SH2 domain of GAP.

To investigate these possibilities, gst fusion proteins of the SH2 domains of PLCγ and the p85 subunit of PI3-kinase were immobilized to beads. The binding of mIL-3 induced tyrosine phosphoproteins was then analyzed as before, except with more extensive washes. Figure 34 shows that, as before, no proteins bound to the control gst beads. However this time, after subjecting the beads to more washes, only the 140 kD mIL-3R bound to GAP SH2. Gratifyingly, the other SH2 domains had different specificities; the C-terminal SH2 domain of the p85 subunit of PI3-kinase specifically bound pp95. This dramatic finding not only suggests a powerful new technique for purifying proteins that are tyrosine phosphorylated in response to mIL-3 stimulation but also hints at specific protein/protein interactions that may occur *in vivo* as the result of mIL-3R activation.

3. Hemopoietic Growth Factor Receptors and GAP SH2 Domains

Our finding that the mIL-3R bound to the SH2 domain of GAP begged the question of whether other hemopoietic growth factor receptors could do the same. As a preliminary test,

B6SUtA$_1$ cells were stimulated with mIL-3, mGM-CSF or SF, solubilized as usual and incubated with GAP SH2 beads. The bound proteins were then analyzed by immunoblotting with α-phosphotyrosine antibodies. As shown in Figure 35, a tyrosine phosphorylated band (lane 3) slightly higher in molecular weight than the phosphorylated mIL-3R (lane 2) was specifically precipitated from mGM-CSF stimulated cells. This protein had a molecular weight highly reminiscent of the β-subunit of the murine GM-CSF.[7] An even larger protein, with the same molecular weight as the *c-kit* protein,[8] was purified from SF treated cells. Further work is needed to establish that these are indeed the receptors for mGM-CSF and SF and to determine the generality of this phenomenon within the hemopoietic system.

C. DISCUSSION

As discussed in Chapter V, our working hypotheisis is that the proteins which become tyrosine phosphorylated in response to mIL-3 at 4°C are mIL-3R associated proteins. In a survey of purification techniques that might be used to purify these proteins, the mIL-3R was found to bind the SH2 domain of GAP while pp95 associated with the SH2 domain of the PI3-kinase p85 subunit. Thus these SH2 affinity matrices should prove highly useful in purifying both the mIL-3R and pp95. More importantly, the binding of the mIL-3R and pp95 to these SH2 domains suggests that GAP and PI3-kinase might be involved in mIL-3 induced signal transduction. Although it is well established that proteins bearing SH2 domains can bind to growth factor receptors containing intrinsic tyrosine kinase domains, it has only recently been shown that the IL-2R is associated with both p56^{lck},[9] and PI3-kinase.[10] This is not entirely surprising since the IL-2R β chain has been shown to become tyrosine phosphorylated following IL-2 binding.[11]

From our observations, a model of mIL-3 signal transduction can be proposed in which the inactive mIL-3R is associated with a 95 kD protein and both of these become phosphorylated on tyrosines upon mIL-3 binding. GAP may then bind to both the phosphorylated mIL-3R and pp95 may through SH2 and non-SH2 dependent interactions, respectively. pp95 also binds to PI3-K in an SH2 dependent manner. If this model is correct

then one might expect to see mIL-3R's as well as the pp95 in α-GAP immunoprecipitates. However, only pp95 was observed. To explain this discrepancy, one could postulate that the binding of tyrosine phosphorylated mIL-3R's to GAP could make the GAP epitopes inaccessible to α-GAP. In support of this, perhaps, is the fact that we observe binding of a 150 kD tyrosine phosphorylated protein, most likely $p150^{c\text{-}kit}$ to GAP SH2 domains (Figure 35). However, neither Rottapel et al[8] nor Miyazawa[12] could detect a 150 kD tyrosine phosphorylated protein in α-GAP immunoprecipitates. If the 150 kD protein is $p150^{c\text{-}kit}$, then the failure of these investigators to co-precipitate pp150 might be due, as mentioned above, to the blocking of epitope(s) recognized by α-GAP antiserum. For example, it is possible that the association of this large, detergent solubilized, SF receptor molecule with GAP prevents precipitation with α-GAP because of steric hindrance or epitope masking. While it may not be possible at this time to fit the data we have gathered using the SH2 domains of GAP and PI3-K with our α-GAP results, our preliminary findings suggest models that can be readily tested.

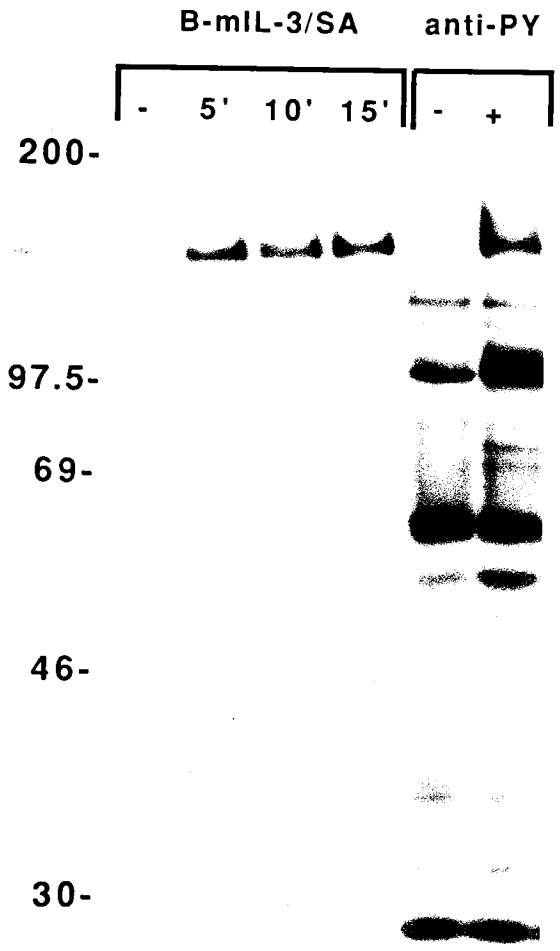

Figure 32. B-mIL-3/streptavidin agarose precipitation of tyrosine phosphorylated proteins from B6SUtA$_1$ cells. For the B-mIL-3/SA lanes, B6SUtA$_1$ cells were incubated with B-mIL-3 for the indicated times, or with a 20 fold excess of unbiotinylated mIL-3 (-) for 10 minutes and solubilized in 0.5% NP40/PSB as described in chapter II. Streptavidin-agarose was then added to precipitate the B-mIL-3 bound proteins. For the anti-PY lanes, B6SUtA$_1$ cells were stimulated (+) or not (-), with mIL-3, solubilized and the phosphoproteins precipitated with α-phosphotyrosine as described in the legend to Figure 25. Similar experiments were obtained in three separate experiments.

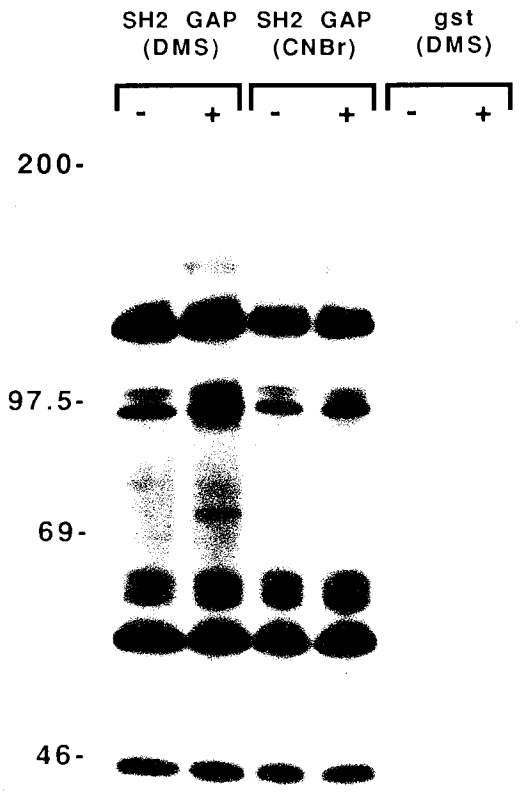

Figure 33A. Binding of B6SUtA$_1$ cell proteins to the SH2 domain of GAP. Gst fusion proteins were immobilized to Sepharose using the DMS or CNBr methods as described in chapter II. The beads were then incubated with cell lysates, prepared as described in the legend to figure 25, from mIL-3 stimulated (+) and unstimulated (-) B6SUtA$_1$. After washing, the beads were boiled in SDS-PAGE sample buffer and subjected to Western analysis with α-phosphotyrosine Ab (4G10) (Figure 33A) or α-N terminal mIL-3R Ab (Figure 33B, on following page).

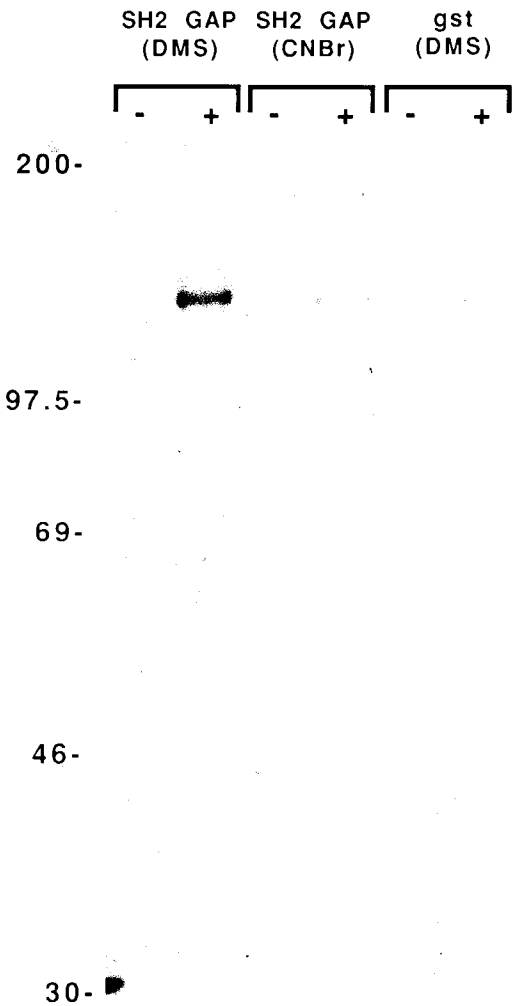

Figure 33B. Binding of B6SUtA$_1$ cell proteins to the SH2 domain of GAP. Legend on previous page.

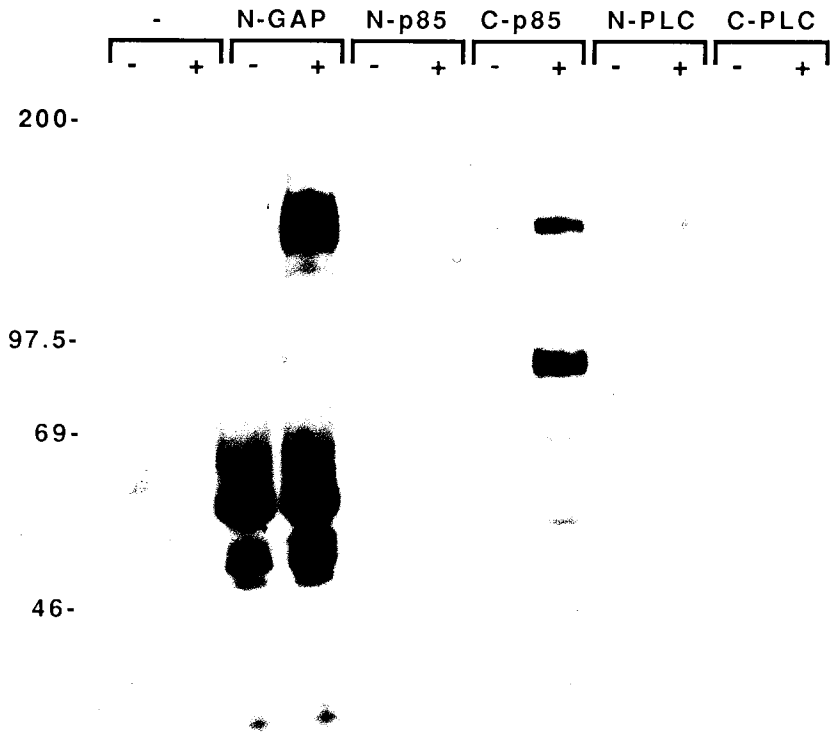

Figure 34. Binding of B6SUtA$_1$ cell proteins to various SH2 domains. Gst fusion proteins of the N-terminal GAP (N-GAP), N-terminal and C-terminal PI3-kinase p85 subunit (N-p85 and C-p85), N-terminal and C-terminal PLCγ (N-PLC and C-PLC) or the underivitized gst protein (-) were coupled to CNBr activated Sepharose as described in chapter II. The beads were then incubated with mIL-3 stimulated (+) and unstimulated (-) B6SUtA$_1$ cell lysates as described in Figure 25. Bound proteins were then analyzed by immunoblotting with α-phosphotyrosine Ab (4G10). Similar results were observed in two separate experiments.

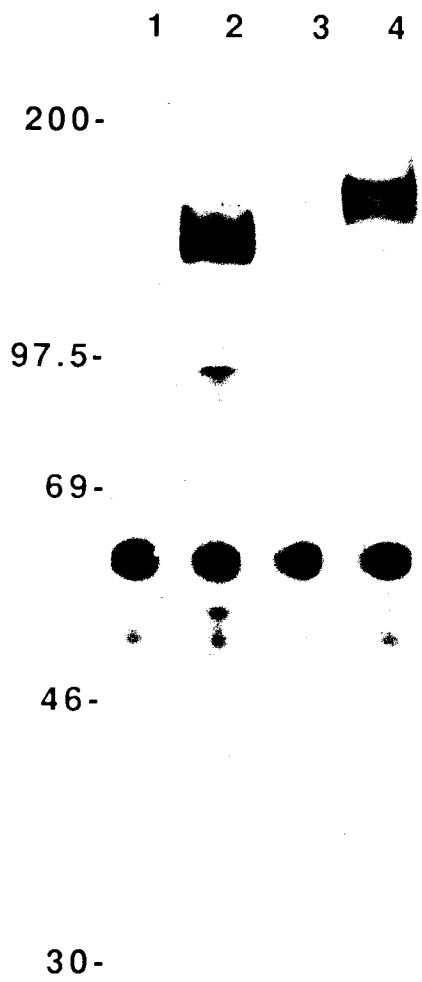

Figure 35. Binding of mIL-3, mGM-CSF and SF induced tyrosine phosphoproteins to GAP SH2 domains. B6SUtA$_1$ cells were stimulated with control buffer (lane 1), mIL-3 (2), mGM-CSF (lane 3) or SF (lane 4) for 10 min at 4°C. Cell lysates were prepared and incubated with GAP SH2 beads and bound proteins analyzed as described in the legend to Figure 33A.

D. REFERENCES

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CHAPTER VII

SUMMARY AND CONCLUSIONS

A. Purification of the mIL-3R

In order to purify the mIL-3R, a lectin assay based on Con A beads and unglycosylated ^{125}I-mIL-3, was developed that could detect detergent solubilized mIL-3R's. With this assay, solubilization conditions that optimized retention of mIL-3R binding ability were determined. None of a range of standard chromatographic techniques which were tested gave satisfactory increases in purity or substantial yields of mIL-3R. Similarly, none of the standard methods of preparing immobilized ligand affinity columns yielded an mIL-3 column that bound mIL-3R's with good efficiency. However, we found that mIL-3 could be biotinylated without loss of bioactivity and that this B-mIL-3 could be used in a simple two step procedure to the purify the mIL-3R to apparent homogeneity. This two step protocol involved B-mIL-3, α-phosphotyrosine Sepharose and streptavidin agarose and took advantage of the fact that the mIL-3R becomes phosphorylated on tyrosine residues upon mIL-3 binding to intact B6SUtA$_1$ cells. N-terminal amino acid sequencing of the purified mIL-3R revealed it to be identical to the Aic 2A protein subsequently cloned by expression in COS cells using an antibody which partially inhibits mIL-3 binding to mIL-3R positive cells.[1]

As assessed by the lectin assay, both the NP40 and OG solubilized mIL-3R's displayed high affinity binding equal to that detected on intact cells. Scatchard analysis of semi-purified and pure mIL-3R preparations suggested that high affinity binding was not lost upon purification of the 120 - 140 kD mIL-3R. This result is not readily reconciled with the observation that the Aic 2A protein exhibits low affinity binding when expressed in fibroblasts. Examination of the contribution of carbohydrate to mIL-3 binding affinity showed that the deglycosylated mIL-3R possessed lower binding affinity that the properly glycosylated molecule. Thus it is possible that glycosylation differences between hemopoietic cells and fibroblasts may

partially explain the differences in affinity. However, many other members of the hemopoietic receptor family consist of two subunits, and both are required for high affinity binding.[2] As well, in chemical cross-linking experiments with ^{125}I-mIL-3, two proteins with molecular weights of 120 and 70 kD are consistently seen. Although we have shown previously that the higher molecular weight protein undergoes proteolysis to generate a 70 kD fragment,[3] it is possible that a portion of the 70 kD labelled band seen on cross-linking gels is due to another subunit that associates with the 120 kD molecule to produce high affinity binding. In this case, our high affinity estimate of the purified 140 kD molecule might be due to an artefact of the lectin assay or to aggregation of solubilized mIL-3R's. Aggregation of receptors in other systems has been reported to increase their affinity for their ligands.[4,5] Further study, including, the isolation of the putative second mIL-3R subunit, is needed to resolve the issue of whether the mIL-3R is composed of more than one ligand binding subunit.

B. Tyrosine Phosphorylation of the mIL-3R Increases its Susceptibility to Proteolysis

During the course of the purification of the mIL-3R, we noticed that, the serine phosphorylated mIL-3R was very stable while the tyrosine/serine phosphorylated 140 kD form rapidly degraded. Closer examination of this phenomenon using specific phosphatases revealed that both tyrosine and serine phosphorylations were required for degradation to occur. Moreover, this proteolysis of the mIL-3R was also found to occur in intact cells in response to mIL-3. Agents that inhibited the tyrosine phosphorylation of the mIL-3R inhibited mIL-3R cleavage, whereas agents that did not affect tyrosine phosphorylation were unable to alter cleavage. These observations are consistent with a model in which mIL-3 binding to its receptor results in both the tyrosine and serine phosphorylation of the mIL-3R. This converts the mIL-3R into a substrate for a receptor associated protease which cleaves the mIL-3R to generate a 70 kD membrane associated molecule and release a cytoplasmic fragment. The generation of these fragments may be involved in receptor downregulation and/or propagating the mitogenic signal initiated by mIL-3 binding. Determination of the proteolytic site, followed

by site directed mutagenesis of this region to prevent proteolysis will allow testing of these possibilities.

C. Identification of mIL-3R Associated Proteins

The mIL-3R does not contain an intrinsic tyrosine kinase domain, although it does become phosphorylated on tyrosine residues upon mIL-3 stimulation. Thus at least a tyrosine kinase, and also an mIL-3R protease, become associated with the mIL-3R. A kinetic examination of mIL-3 induced tyrosine phosphorylations showed, remarkably, that phosphorylation of the mIL-3R and four other proteins, with apparent molecular weights of 95, 70, 56 and 32 kD, occur by 2 min at 4°C. This observation suggests that the kinase is associated with the mIL-3R and its other substrates prior to mIL-3 binding. One of these mIL-3 induced tyrosine phosphoproteins, pp95, co-immunoprecipitates with GAP. Since GAP associates with tyrosine kinases, this association makes pp95 a good candidate for the mIL-3R specific tyrosine kinase.

Since tyrosine kinases associate with GAP through GAP SH2 domains, an affinity matrix consisting of GAP SH2 domains was tested for its suitability in purifying pp95. Unexpectedly, we discovered that the mIL-3R bound tightly to GAP SH2 domains whereas pp95 did not. This observation suggests that the association of pp95 with GAP might occur through an SH2 independent mechanism. However, pp95 did bind to an affinity matrix constructed of the SH2 domains of the p85 subunit of PI3-kinase. Further work is needed to determine whether the pp95 binding *in vitro* to the p85 SH2 domains reflects an *in vivo* association with PI3-kinase. However, these preliminary results are intriguing in the light of the recent report of the association of PI3-kinase with $p21^{ras}$.[8] One could envisage a model in which ras co-precipitates with PI3-kinase because of the association of pp95 with GAP and PI3-kinase, through SH2 independent and dependent mechanisms, respectively. Further experiments are required to test this model, and may yield insights into the mechanism of action of mIL-3.

D. A Model for mIL-3R Signal Transduction

Based on the studies reported in this thesis and on previous work carried out in our laboratory, the following model of mIL-3 induced signal transduction events is proposed (Figure 36). The ligand unoccupied, inactive receptor (lower left hand corner of Figure 36) is a 120 kD glycoprotein that is associated via its cytosolic domain with at least four other signalling molecules with apparent molecular weights of 95, 70, 56 and 32 kD. Upon mIL-3 binding, the receptor becomes phosphorylated on tyrosines and serines and the tyrosine/serine phosphorylated receptor becomes cleaved to release a cytoplasmic fragment. The mIL-3R associated proteins also become phosphorylated on tyrosines and serines and are released from the mIL-3R. One of these proteins, pp95, becomes associated with both GAP and PI3-kinase. The association of pp95 with GAP may attenuate GAP's ras GTPase stimulatory activity and thus account for the accumulation of ras GTP that is observed in cells stimulated with mIL-3.[6] The association of pp95 with PI3-kinase may similarly modulate the activity of this kinase. Also shown in Figure 36, is a 42 kD protein, MAP kinase, which we and others[7] have shown to become tyrosine phosphorylated and activated in response to mIL-3. However, this reaction occurs only at 37°C, suggesting perhaps that this is a late event in the signal transduction cascade. Although many aspects of this model are still somewhat speculative, it does provide an interesting basis for further experimentation.

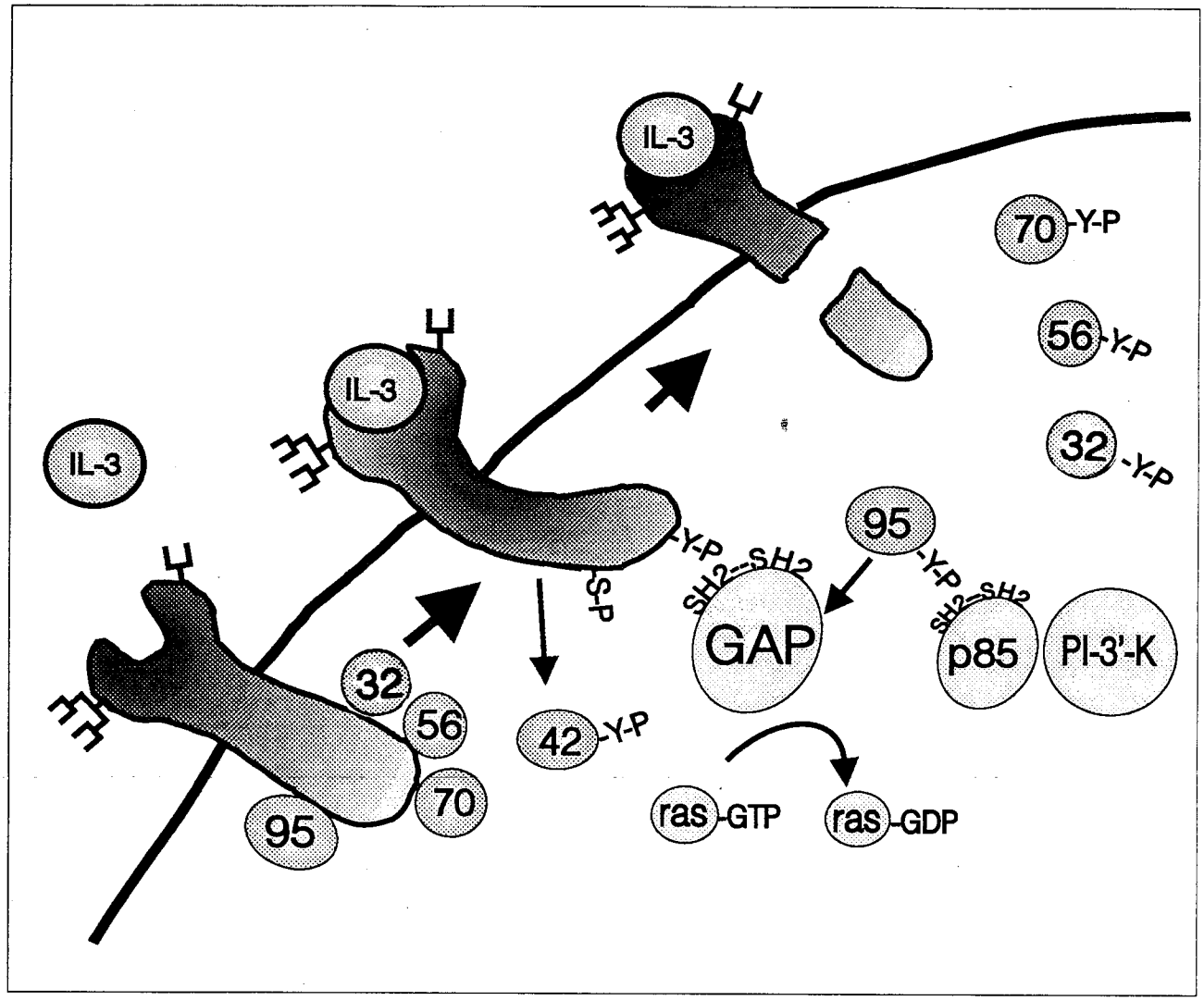

Figure 36. Model of mIL-3R mediated signal transduction

E. REFERENCES

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