PROTEIN-TYROSINE KINASE SIGNALLING PATHWAYS

IN

NORMAL HEMATOPOIESIS AND LEUKEMOGENESIS

by

Lorri Jane Puil, M.D.

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Institute of Medical Science,
in the University of Toronto

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PROTEIN-TYROSINE KINASE SIGNALLING PATHWAYS IN NORMAL HEMATOPOIESIS AND LEUKEMOGENESIS
Doctor of Philosophy (1998)

Lorri Jane Puil, M.D.
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ABSTRACT

The reversible phosphorylation of proteins on tyrosine residues modulates protein function and is central to the regulation of hematopoiesis. Consistent with the emerging role of phosphotyrosine in normal hematopoietic processes, gain- or loss-of-function mutations in genes encoding protein-tyrosine kinases (PTKs) have been identified in a number of human hematological disorders. A well characterized example of an activating mutation is fusion of the abl and bcr genes which produces a chimeric Bcr-Abl protein with deregulated Abl kinase activity. The bcr-abl fusion gene is the result of a reciprocal t(9;22) translocation and occurs in chronic myelogeneous leukemia and a subset of acute lymphoblastic leukemia.

Identification of the targets of normal and transforming PTKs is necessary for understanding both normal signal transduction pathways and the means by which these pathways are deregulated during cellular transformation. In this thesis, proximal targets of the Bcr-Abl oncoproteins are characterized. Src homology 2 (SH2) and Src homology 3 (SH3) domains are protein interaction domains which recognize phosphotyrosine-containing and proline-rich motifs respectively, and are present in an otherwise diverse group of signalling molecules. In Chapters 2 and 3, a number of SH2/SH3-containing enzymes and adaptor molecules, along with their associated proteins, are implicated in bcr-abl-mediated transformation. These include the 120 kDa Ras GTPase activating protein (p120-Gap), the Gap-associated proteins p62 and p190, Grb2, mSos1, Shc, Shc-associated p140/p145, Vav, the p85 regulatory subunit of phosphatidylinositol 3'-kinase and phospholipase C-
Subsets of these proteins are substrates of Bcr-Abl (or a downstream PTK) and/or form stable complexes with Bcr-Abl *in vivo*. A number of the identified targets are potential regulators of Ras and provide mechanisms whereby Bcr-Abl may be directly linked to Ras signalling pathways. These data are consistent with a prominent role for Ras in PTK-mediated transformation. In Chapter 4, some of these proteins, notably Shc, Shc-associated p140/p145, Grb2 and Vav, are demonstrated to participate in cytokine-stimulated pathways which control the survival, proliferation or activation of normal primary hematopoietic cells. Taken together, these data support the notion that leukemogenesis involves the constitutive stimulation of signalling molecules that are normally transiently activated in cells responding to cues within the hematopoietic microenvironment.

The structural basis for the interaction of PTKs with some of their targets was also investigated. These data suggest that SH2-mediated interactions are important in hematopoietic cell signalling. In Chapter 3, the binding of Grb2 to Bcr-Abl is demonstrated to be mediated by the Grb2 SH2 domain which recognizes an autophosphorylated sequence, p.Tyr\textsubscript{177}-Val\textsubscript{178}-Asn\textsubscript{179}-Val\textsubscript{180}, located within the N-terminal *bcr*-encoded sequence common to all isoforms of Bcr-Abl. The Bcr element of the Bcr-Abl fusion proteins thus contributes to the target specificity of the activated Abl kinases. This is a novel function of the Bcr moiety which has not previously been defined, and provides new insight into the potential mechanisms whereby fusion of the *abl* and *bcr* genes is oncogenically activating.
For the Yukon bear
ACKNOWLEDGEMENTS

I would like to thank Keith Withers, Mike Moran, Jane McGlade, Louise Larose and Venus Lai for encouraging discussions. The National Cancer Institute of Canada provided financial support in the form of a postdoctoral fellowship. Catherine Whiteside of the Institute of Medical Science assisted in facilitating the completion of regulatory requirements for this thesis.

This thesis is dedicated to Barry, the many friends and relatives who helped along the way, and the patients I have known who have faced their own personal tragedies with dignity, courage and hope. Once again I would like to express the wish that the scientists doing basic medical research never lose sight of the human faces behind their goals.
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<th>Definition</th>
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<tbody>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myelogeneous leukemia</td>
</tr>
<tr>
<td>A-MuLV</td>
<td>Abelson murine leukemia virus</td>
</tr>
<tr>
<td>Bcr</td>
<td>breakpoint cluster region</td>
</tr>
<tr>
<td>BMMC</td>
<td>bone marrow-derived mast cells</td>
</tr>
<tr>
<td>cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelogeneous leukemia</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor-1</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>FLMC</td>
<td>fetal liver-derived mast cells</td>
</tr>
<tr>
<td>Gap</td>
<td>GTPase activity protein</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol (1,4,5) trisphosphate</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrate-1</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine based inhibitory motif</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBC</td>
<td>myeloid blast crisis</td>
</tr>
<tr>
<td>MDS</td>
<td>myelodysplastic syndrome</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated/extracellular kinase kinase</td>
</tr>
<tr>
<td>NRTK</td>
<td>nonreceptor tyrosine kinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol (4,5) bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PTK</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PTP</td>
<td>protein-tyrosine phosphatase</td>
</tr>
<tr>
<td>R</td>
<td>receptor</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SF</td>
<td>steel factor</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>S/TK</td>
<td>serine/threonine kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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INTRODUCTION

The reversible phosphorylation of proteins on tyrosine residues modulates protein function and is central to the regulation of hematopoiesis. Protein-tyrosine kinases (PTKs) catalyze the transfer of the γ-phosphate of magnesium adenosine triphosphate (ATP) to suitable substrates in a covalent modification that is reversed by protein-tyrosine phosphatases (PTPs) (1-3). Consistent with the emerging role of phosphotyrosine in normal hematopoietic processes, gain- or loss-of-function mutations in genes encoding PTKs have been identified in a number of human hematological disorders including leukemia (4-10). An understanding of the mechanisms whereby PTKs regulate both normal hematopoiesis and leukemogenesis requires identification of the intracellular targets of activated PTKs and elucidation of the means by which PTKs interact with such targets. Studies undertaken for this thesis address these issues.

In the next section, normal hematopoiesis is reviewed. This is followed by an overview of the role that PTKs play in hematopoiesis and a more detailed discussion on the activated Abl tyrosine kinases which are important in the pathogenesis of the Philadelphia chromosome positive (Ph +ve) leukemias. Lastly, features of proximal PTK targets and the Ras signalling pathways are summarized, and an outline of the thesis presented.

1.1. NORMAL HEMATOPOIESIS

During vertebrate embryogenesis, stem cells giving rise to embryonic and fetal/adult hematopoiesis arise from common progenitor cells which migrate from the ventral mesoderm into the yolk sac (11,12). A subset of these cells then migrate into the intraembryonic aorta, genital ridge and mesonephros (AGM) region. Yolk sac embryonic stem cells giving rise to "primitive" hematopoiesis are a distinct population of cells which constantly cycle, predominantly give rise to the erythroid lineage and undergo apoptosis during fetal life (11). In contrast, fetal/adult stem cells
which initiate "definitive" hematopoiesis in the AGM region may be maintained in G₀ for long periods of time. During embryonic/yolk sac hematopoiesis, hematopoietic stem cells (HSC) and vascular progenitors form blood islands, allowing blood cells to enter the circulation as soon as terminal differentiation occurs. Some evidence exists that the hemangioblast is a common precursor of both hematopoietic and vascular cells during blood island formation (13). Waves of hematopoietic activity from the dorsal (AGM) population of cells later colonize the fetal liver, spleen and bone marrow (14).

Stem cells and their progeny must develop in the bone marrow throughout the lifetime of an individual in order to maintain basal levels of hematopoiesis and to meet acute demands imposed by stresses such as infection. This requires a balance between stem cell self renewal, the commitment of progenitor cells to a particular cell lineage, the differentiation of progenitors into mature specialized effector cells and programmed cell death. Pluripotent stem cells which are relatively few in number give rise to both myeloid and lymphoid progenitors (15-19). The sequential clonal dominance of one or a few stem cells may occur (15,16). Long term reconstituting multipotent progenitors can be enriched on the basis of cell surface markers and represent .005-.01% of bone marrow cells (19). In the mouse, these cells are Thy-1<sup>lo</sup>Lin<sup>−</sup>Mac-1<sup>−</sup>CD4<sup>−</sup>Sca-1<sup>+</sup> (where lin represents lineage specific markers other than Mac-1 and CD4) and are dull when stained with rhodamine 123 (Rh123). Human hematopoietic stem cells are Thy-1<sup>+</sup>Lin<sup>−</sup>CD34<sup>+</sup> and also Rh123<sup>dull</sup>. However, a correlation between surface marker expression and stem cell function is specific to the tissue and conditions examined (19). The immediate precursor of hematopoietic stem cells (HSC) has not been characterized and the conditions which allow long term maintenance or self renewal of HSC are unknown.
Cells of all lineages arise from pluripotent stem cells which exhibit extensive self renewal properties as well as the ability to commit to different cell lineages. The sites of action of some of the known cytokines are illustrated. Early acting synergistic factors that enhance the entry of dormant primitive progenitors into the cell cycle are depicted in square brackets. Adapted from (19).

Abbreviations: APC, antigen presenting cell; bas, basophilic; BFU, burst forming unit; CFU, colony forming unit; CSF, colony-stimulating factor; E, erythroid; Eo, eosinophilic; Epo, erythropoietin; meg, megakaryocytic; GM, granulocytic-monocytic; FL, ligand for Flk2/Flt3 receptor; IL, interleukin; SF, Steel factor; Tp, thrombopoietin
A potential scheme of lineage differentiation is depicted in figure 1-1 (20-22). At the single cell level, one model proposes that the differentiation of stem cells and progenitor cells is a stochastic process which represents a unidirectional continuum or hierarchy of cells, with differentiation accompanied by a progressive loss in proliferative capacity (18,20,22). Others have suggested that the stem cell pool is organized into discrete subpopulations with distinct self renewal capabilities (19). The commitment of HSC involves either a restriction in lineage potential or an active commitment to a particular lineage, and is not yet fully understood (18). Some models of hematopoiesis include a role for environmental cues such as cytokines in modulating the genetic changes that accompany lineage commitment (23). Indeed, the ligand-activated G-CSF receptor stimulates lineage-specific maturation events by inducing neutrophil-specific genes (24). However, other data suggest that the primary role of hematopoietic growth factors in differentiation is enabling rather than inductive (25). While lineage fidelity is assumed in traditional models of hematopoiesis, the existence of biphenotypic and bilineage leukemias as well as the demonstration that pre-B cells can differentiate into macrophages, suggest that under special circumstances such fidelity is not rigidly adhered to (3,26,27).

1.1.1. Hematopoietic growth factors

A large family of glycosylated proteins or cytokines regulates the survival, proliferation, differentiation and function of blood cells (28-30). With the exception of erythropoietin (Epo), most cytokines function in a paracrine or autocrine fashion and are produced by cells within the adult bone marrow. Cytokine production is regulated at multiple levels including transcription and the rate of mRNA turnover (31-33). Different subsets of cytokines may be involved in the regulation of basal and acute stress hematopoiesis (28). Most cytokines exhibit both pleiotropy (multiple biological effects) and redundancy (shared effects).
Some factors, particularly combinations of interleukin (IL)-1, IL-3, IL-4, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and Steel factor (SF), have a broad range of activity and act on early stem cells as well as mature cells, whereas others are more restricted and have lineage predominant effects (figure 1-1) (28,29,34-37). IL-3, GM-CSF and IL-4 support the proliferation of multipotential progenitors only after they exit G0. Other cytokines such as IL-6, IL-11, IL-12, G-CSF and SF trigger the cycling of dormant primitive progenitors and can synergize with IL-3 (18). Enhancement of cell survival by cytokines involves the suppression of apoptosis and is distinct from the ability to stimulate DNA synthesis and cell proliferation (38-40).

Growth factors such as SF may be synthesized as membrane-anchored proteins which can then be regulated to control the generation of soluble ligand (41-45). The availability of soluble cytokines may also be regulated by binding to the extracellular matrix, serum proteins, co-ligands or co-receptors (46). In some cases, membrane-bound factors elicit different biological effects than their soluble counterparts (43,44,47,48).

1.1.2. Ligand-receptor interactions and the cytokine receptor superfamily

Before an individual cell can respond to a stimulus in its microenvironment, the signal must be transmitted across the cell membrane into the cytosol. This process is initiated by the binding of ligand to specific receptors on the cell surface. Potential ligand-receptor interactions in hematopoiesis are summarized in figure 1-2.

Hematopoietic growth factor receptors which have a single transmembrane domain may be distinguished by the presence or absence of an intracellular tyrosine kinase domain. Many receptors lacking intrinsic kinase activity, including the majority of interleukin receptors, belong to the cytokine receptor (R) superfamily (49,50). These receptors are distinguished by the cytokine receptor homology (CRH) domain, an extracellular region of homology of approximately 200-250 amino acids (figure 1-3). Class I cytokine receptors include receptors for growth hormone (GH),
Growth factors may be soluble, membrane-bound on a cell surface, or attached to the extracellular matrix. The classic interaction between receptor and ligand involves activation of a cell surface receptor by interaction of its extracellular domain with soluble ligand. If ligand is membrane-bound, bidirectional signalling may occur, as recently demonstrated in non-hematopoietic cells (740), or such an interaction may have inhibitory effects. Soluble forms of receptors have been described for a subset of hematopoietic growth factor receptors including c-Kit (736-739); these are generated by alternative mRNA splicing or by proteolytic cleavage. Soluble receptors can act as antagonists by sequestering ligand or may function as ligand carriers and prolong the ligand's serum half-life. The IL-6R α subunit in its soluble form can promote the biological effect of IL-6 by inducing homodimerization of gp130.
Cytokine-receptor interactions

a. one soluble partner

receptor expressing cell

ligand expressing cell

b. both partners "anchored"

ECM

c. two soluble partners

Legend:
- receptor
- ligand
- extracellular domain of receptor
- serum binding protein
Figure 1-3. Hematopoietic growth factor receptors.

The structural features of selected receptors belonging to the Ig or cytokine R superfamilies are shown schematically. Also shown is the IL-2R α subunit which belongs to a unique family of proteins distinguished by the presence of a short consensus motif termed the sushi domain. The IL-3, IL-5 and GM-CSF receptors share a βc subunit. The IL-2R γc subunit is also shared by multiple receptors including receptors for IL-4, IL-7, IL-9 and IL-15. IL-2R β is utilized by the IL-15R. The IL-4R α subunit is shared by the IL-13R and an additional form of the IL-4R lacks a γc subunit but contains an IL-13R α subunit. The box 1 and box 2 motifs are juxtamembrane regions of homology shared by many cytokine receptors: box 1 is defined as PXP preceded by hydrophobic residues whereas box 2 contains a cluster of hydrophobic residues followed by positively charged amino acids. The I4R motif (PLX₄NPXYXSXSD) is found in the α subunit of the IL-4 R and in the insulin and IGF receptors. Although depicted as monomers, receptors for PDGF, IL-1, G-CSF, Epo and thrombopoietin form homodimers.

Abbreviations: C, cysteine; CRH, cytokine receptor homology; CNTF, ciliary neurotrophic factor; FN, fibronectin; Ig, immunoglobulin; IL, interleukin; LIF, leukemia inhibitory factor; Osm, oncostatin M; R, receptor
IL-2 (the β and γ chains), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, Epo, G-CSF, GM-CSF, leukemia inhibitory factor (LIF) and the thrombopoietin receptor, c-Mpl (50). The Class I CRH domain contains 4 spatially conserved cysteine residues along with an invariant tryptophan in its amino terminal half, and a highly conserved Trp-Ser-X-Trp-Ser (WSXWS) motif (where X is any amino acid) located just outside the transmembrane domain. Based on the structure of the hGH and prolactin receptors, the Class I CRH domain is composed of two fibronectin (FN) III-like domains which form β-barrels (51,52). Loops at the end of the barrels bind growth factor. The four conserved cysteines are buried in the core of the N-terminal domain and form two disulfide bridges. The WSXWS motif in the carboxy-terminal domain is located away from all binding interfaces, and its primary function may be structural, contributing to proper receptor folding and the ability to exit the endoplasmic reticulum (53). An effect of mutations in this motif on ligand binding in the IL-3 R βc subunit (54) may be due to structural destabilization.

Tyrosine residues which are phosphorylated following receptor activation are located in the cytoplasmic tails of many Class I receptor subunits. The intracellular portion of these receptors is variable in length and not well conserved. In the juxtamembrane portion, some receptors share weak regions of homology separated by a spacer region of 12-49 amino acids, termed Box 1 and Box 2 (49,55). For several cytokine receptors, the Box 1 motif has been shown to be essential for mitogenesis and the recruitment of Jak PTKs (49,55-62). Residues within the spacer region and/or Box 2 may also be important for biological responses (58,63,64). In some cases, distinct portions of the cytoplasmic domains of receptor subunits are required for functions other than cell proliferation (24,40,62-66). For example, G-CSF-mediated differentiation requires the distal portion of the G-CSF R cytoplasmic domain (24).

Ligands for the cytokine R superfamily have a similar four-helical bundle motif (52,67). Most of these ligands are monomeric; however, IL-5 is a disulfide-linked dimer. The receptors for
GH, prolactin, G-CSF, Epo and thrombopoietin form homodimers upon ligand binding (52, 53). The prototype for ligand-homodimer association is the GH R which binds to two assymmetrical sites, Site 1 and Site 2, on GH (51). Interaction is sequential with one receptor contacting Site 1 and another receptor subsequently interacting with Site 2. Ligand binding brings together the membrane-proximal halves of the C-terminal domains of the receptors, thereby stabilizing the complex. A similar interaction may occur between the intracellular portions of the receptors. Based on mutant studies, dimerization is required for intracellular responses. Dimer interface homology regions have been identified in the Epo and thrombopoietin receptors, suggesting a common mechanism for the initiation of intracellular signalling (52, 68).

Other Class I cytokine receptors have multiple subunits and function as heterodimers or hetero-oligomers. These receptors can be subclassified into three major groups: 1) receptors sharing the signal transducing component gp130, 2) the IL-3, IL-5 and GM-CSF receptors which share a common β subunit (βc or gp140), and 3) receptors sharing the IL-2 Rγc subunit (figure 1-3) (50, 69). These receptors have specific α ligand binding subunits and exhibit different affinity binding classes, with high affinity binding of ligand requiring interaction with two or more R subunits. The stoichiometry of active ligand-receptor complexes is not yet known for some receptors such as the IL-3 R (50). It is also unclear whether oligomerization is invariably induced by ligand or whether some receptors exist as oligomers in the unstimulated state (70). Whatever the mechanism, ligand binding induces interaction with effector proteins and triggers downstream signalling events. Depending on the particular system, the cytoplasmic domain of the α subunit may or may not contribute to propagation of the signal into the cytosol (49, 71). For example, a soluble secreted form of IL-6Rα bound to IL-6 can stimulate signalling indicating that gp130 homodimerization is sufficient for transmitting the signal into the cytosol (72).
The sharing of common receptor subunits may explain why some cytokines exhibit functional redundancy. In some cell systems, however, receptors that share a common subunit elicit different biological responses, suggesting that receptor specific subunits have a role in determining the outcome of a signalling event (66). It has been suggested that the extracellular domain(s) of the receptor may influence the specificity of the signal generated (73).

Class II cytokine receptors include receptors for interferon (IFN) α/β, IFN-γ and IL-10. These receptors share one pair of conserved cysteines but lack a definitive WSXWS motif. Some differences exist in domain orientation and ligand binding for Class II receptors but the overall domain architecture of these receptors is identical to Class I (74).

The physiological roles of a number of cytokines and/or their receptor subunits have now been studied by targeted gene disruption in mice (50). In several instances, the phenotype of receptor -/- mice is more severe than ligand -/- mice indicating that multiple ligands activate the same receptor and generate a functionally similar signal. While data from animal models generally correlate with tissue culture studies, exceptions exist. For example, IL-3 is critical for hematopoiesis in vitro but mice lacking the βc R subunit have limited hematological abnormalities, notably reduced numbers of circulating eosinophils due to the loss of IL-5, and pulmonary alveolar proteinosis secondary to loss of GM-CSF and malfunction of alveolar macrophages. The relatively mild nature of the hematopoietic phenotype observed is attributed to the presence of an IL-3-specific β subunit (AIC2B) in mice and the ability of GM-CSF to perform functions redundant with IL-3 (75). However, double mutant IL-3 -/- βc -/- mice show no additional abnormalities, and have normal hematopoietic recovery after 5-fluorouracil (76). The precise role of IL-3 in vivo therefore remains to be defined.

Mutations in the genes encoding certain cytokine receptors are implicated in human hematological disorders such as X-linked severe combined immunodeficiency (the IL-2 R γc
subunit), familial benign erythocytosis (Epo R) and severe congenital neutropenia (G-CSF R) (77,78). Deregulated expression or activating mutations in cytokine receptors or their cognate ligands may also be involved in the pathogenesis of hematological malignancies including leukemia (79-81).

1.2. PROTEIN-TYROSINE KINASES

PTKs play a pivotal role in propagating signals from the external environment to the cell nucleus, and induce biochemical cascades which modulate cytoskeletal architecture, interactions with the extracellular matrix, cell metabolism and ultimately gene expression and DNA synthesis (82). A large number of biochemical and genetic studies now support the notion that PTKs regulate many aspects of hematopoiesis such as cell growth or differentiation, apoptosis, receptor internalization and specialized effector functions (83-103). Based on structure and subcellular localization, PTKs may be classified into two groups, transmembrane receptor-tyrosine kinases (RTKs), and non-receptor tyrosine kinases (NRTKs) which localize to the cytoplasm and nucleus (104-107). A subset of PTKs in both groups have expression largely restricted to the hematopoietic system. Moreover, some widely expressed PTKs exhibit hematopoietic specific expression of certain isoforms as a consequence of alternative splicing (108,109).

1.2.1. The catalytic domains of protein-tyrosine kinases (SH1)

The catalytic domains of PTKs are approximately 260 amino acids in length, share homology with other eucaryotic protein kinases, and can be divided into 11 conserved subdomains which are separated by regions of lower conservation (1,110). Scattered throughout the catalytic core are highly conserved residues that are critical in protein folding, binding of nucleotide or in mediating catalysis. The crystal structure of the unphosphorylated kinase domain of the insulin receptor (IR) has confirmed that PTK kinase domains share a general topology with
serine/threonine kinases (S/TKs), consisting of a small N-terminal lobe and a larger C-terminal lobe with the domain interface forming the active site and the ATP-binding site (110-112). The N-terminal lobe is formed by subdomains I-IV and is critical for the binding of ATP; a Gly-X-Gly-X-Gly-X-Val motif in subdomain I and a conserved lysine in subdomain II anchor and orient ATP by interacting with the non-transferable phosphate groups. The C-terminal lobe includes subdomains VI-XI and is primarily responsible for binding the substrate and initiating phosphotransfer. A catalytic loop in subdomain VI (HRDLAARN) contains an invariant Asp residue which is presumed to act as the catalytic base, removing a proton from the substrate hydroxyl group during phosphotransfer. Residues in the catalytic loop and subdomain VIII are specifically conserved in either the serine/threonine or tyrosine kinase families and thus may play a role in recognition of the correct hydroxyamino acid residue (110).

In the inactive IR, the two kinase lobes are rotated away from each other (111). An extended sequence in the large lobe which connects subdomains VII and VIII is termed the activation loop (residues 1150-1179) and contains 3 Tyr residues which are sites of autophosphorylation during kinase activation. In other RTKs, a single Tyr is generally found in this location, analogous to Tyr 416 of c-Src which increases catalytic activity when phosphorylated. In the IR kinase, one of the tyrosines, Tyr 1162, sits in the active site; cis-autophosphorylation is prevented by occupation of the ATP binding site by other residues within the activation loop. The positioning of these residues thus represses kinase activity and excludes exogeneous substrate from gaining access into the active site. A conformational change upon ligand binding presumably removes this inhibition and allows phosphorylation of the tyrosines in the activation loop, increasing kinase activity. This mechanism of autoinhibition may be common to many PTKs. Activation may also be facilitated by interactions of p.Tyr in the activation loop with catalytically important residues, as suggested by the crystal structure of the activated Lck kinase domain (113).
The level of intrinsic substrate specificity for PTKs is generally less than for S/TKs (110). The substrate preference of a number of PTKs was recently investigated in vitro using a partially degenerate phosphopeptide library (114). Consistent with previous studies (110), most PTKs phosphorylated peptides with Glu or Asp residues at specific sites N-terminal to the Tyr. However, selectivity was dominated by hydrophobic residues at key positions. RTKs selected peptides with Glu at the -1 position relative to Tyr and a large hydrophobic residue at +1, while NRTKs selected substrates with Ile or Val at -1 and Glu, Gly or Ala at +1. All of the PTKs under study selected a hydrophobic residue at the +3 position. The sequences selected by the NRTKs and RTKs corresponded to Group I and Group III Src Homology 2 (SH2) ligand specificities respectively (see section 1.2.3.1.). Indeed, some NRTKs such as Abl selectively phosphorylated sites recognized by their own SH2 domains. Substrate selectivity may also be influenced by multiple other variables such as competition for a limited number of targets, localization of targets, the specificity and activity of PTPs, and either intrinsic or extrinsic kinase regulatory mechanisms (96,115). External regulatory mechanisms may involve heterologous phosphorylation by other kinases such as protein kinase C (PKC) (116). These regulatory mechanisms allow for the rapid integration of a number of signals that may simultaneously impinge on the cell.

1.2.2. Receptor tyrosine kinases

RTKs contain a large, glycosylated extracellular domain that binds ligand, a single hydrophobic membrane-spanning region which plays a role in receptor dimerization, and a cytoplasmic region that includes a juxtamembrane region, a catalytic domain and a carboxy-terminal tail of variable length. Subclasses of RTKs are defined by additional structural features in the extracellular domain and the presence or absence of a kinase insert in the cytoplasmic domain (46,104,105). To date, 14 subfamilies have been identified (figure 1-4) (46). Many of these receptors are at present "orphan" receptors as their ligands have not yet been identified. RTK
Figure 1-4. Receptor tyrosine kinases. Schematic representation of selected subfamilies of receptor tyrosine kinases. Adapted from (42).
ligands may be soluble, bivalent monomers (eg. epidermal growth factor (EGF)), or dimers held together by covalent interactions (eg. CSF-1) or non-covalent forces (eg. SF). Fibroblast growth factor (FGF) is monomeric but is oligomerized by binding heparan sulfate on the cell surface (117). A subset of ligands are membrane-bound. For example, ligands for the eph R subfamily are active only as transmembrane or glycosylphosphatidylinositol (GPI)-anchored molecules (118,119). Different ligands may bind more than one RTK or vice versa, thereby increasing the diversity of possible responses.

With the exception of the insulin R which exists as a disulfide linked α2β2 heterodimer in the resting state, binding of ligand is thought to induce dimerization of the cognate receptor (120). Dimerization may occur between identical receptors, between isoforms of the same receptor or between members of the same subfamily. Evidence for receptor dimerization includes the demonstration that kinase inactive mutant forms of a receptor suppress signalling by the wildtype R. *Trans*-autophosphorylation of the cytoplasmic tails of the dimer pair is initiated either by allosteric activation or due to the juxtaposition of the cytoplasmic domains which allows phosphorylation by low basal levels of kinase activity. RTK activation independent of ligand may also occur through inactivation of phosphatases or due to NRTK activity (121). As mentioned in section 1.2.1, an autophosphorylation site in many RTKs corresponds to the major autophosphorylation site, Tyr 416, within the kinase domain of the cytosolic tyrosine kinase c-Src and is important for activation of kinase activity (122-125). Specific sites of autophosphorylation within the cytoplasmic region but outside of the kinase domain, bind intracellular proteins, particularly proteins containing Src Homology (SH) 2 domains (126). The cytoplasmic region thus defines an array of activities or target specificities that are unique to each RTK. Heterodimerization may result in different autophosphorylation states than homodimerization, thus contributing to the diversity of signalling (120).
1.2.2.1. Receptor tyrosine kinases in hematopoiesis

Several members of the platelet derived growth factor (PDGF) R subclass such as c-Kit, c-Fms, Fetal liver kinase (Flk)-1 and Flk-2/Flt-3 participate in hematopoiesis (127-130). The PDGF R subclass is distinguished by the presence of 5 or 7 Ig-like repeats in the extracellular domain and a kinase insert (figure 1-4). The kinase insert is a non-conserved hydrophilic sequence of up to 100 amino acid residues that is incorporated into a loop in the catalytic domain. While the kinase insert may be important for substrate recognition, it is not required for catalytic activity and is dispensable for at least some biological functions (131).

The c-Kit R is expressed on a broad range of hematopoietic cells, particularly multipotential stem cells and committed progenitor cells (132). Anti-Kit antibody, when injected in vivo, causes a reduction in progenitor cells and mature myeloid and erythroid cells in the bone marrow of adult mice, suggesting a role for SF/c-Kit in the maintenance of normal basal, or steady state, hematopoiesis (133). In humans, early CD34+ and myeloid progenitor cells are increased in the bone marrow following administration of SF (132). In culture, SF enhances both the recruitment and the proliferation of primitive murine hematopoietic cells in response to other cytokines (132), and is sufficient, but not essential, for the maintenance of human longterm culture-initiating cells, candidate stem cells that share many of the features of longterm repopulating murine stem cells (134). In the absence of other cytokines, SF may selectively promote the survival of primitive cells (132). Myeloid progenitor cell growth and differentiation in response to cytokines such as IL-3 is also enhanced by SF, and SF synergizes with Epo to stimulate erythropoiesis, particularly the growth of BFU-E (132,135). Activated c-Kit can bypass the requirement for Epo in Epo-dependent cell lines by interacting with and phosphorylating the cytoplasmic tail of the Epo R (136). In a murine model, SF/c-Kit is required for optimal erythropoiesis in response to acute hemolysis (137). This effect may be in part related to the ability of SF/c-Kit to modulate the homing of progenitor
cells to the spleen, the predominant site of acute erythropoiesis (137). SF also plays a role in lymphopoiesis and is required for early murine thymocyte development in vivo. IL-7-mediated proliferation of pre-B cells in culture is enhanced by addition of SF although an in vivo role in B cell development has not been defined (138,139). Based on human cell culture studies, SF may promote the differentiation of natural killer cells and the amplification of dendritic cell progenitors from CD34+ bone marrow cells (140,141).

In the mouse, the dominant white spotting (W) and Steel (Sl) loci encode c-Kit and SF respectively (142,143). Homozygous, severe loss-of-function W or Sl alleles are lethal during embryogenesis, a phenomenon which has been attributed to severe anemia (144). In heterozygotes, hematopoietic abnormalities include macrocytic anemia, a deficiency of mast cells and a decreased number of early hematopoietic cells such as CFU-S (128,144). c-Kit is also expressed in primordial germ cells, melanoblasts and the interstitial cells of Cajal in the intestine; defects in melanogenesis, gametogenesis and intestinal pacemaker activity are therefore also present in W and Sl mutant mice (128,145). In the central nervous system, c-Kit is involved in processes of learning and memory (146). The membrane-bound form of SF is biologically active in vivo (47,48,147) and is expressed contiguously with c-Kit during embryogenesis and in the adult (148,149). In the embryo, this distribution of ligand and receptor likely contributes to the migration of hematopoietic stem cells, melanoblasts and germ cells (148).

The CSF-1 R (c-Fms) is expressed predominantly in the monocyte-macrophage lineage, and is required for the survival, proliferation and differentiation of these cells (129). Flk-1 is found in fetal liver cells enriched for early hematopoietic cells, and also in endothelial cells and their precursors (127,150). Targeted disruption of murine Flk-1 results in absence of yolk sac blood islands and deficiencies in hematopoietic progenitors (151). Expression of Flk-2/Flt-3 is largely confined to primitive hematopoietic cell populations, including a subset of stem cells (152). A
ligand for Flk-2/Flt-3 (FL) has been recently identified and in common with SF and CSF-1, is a type 1 transmembrane protein (42,153). FL is active on several types of primitive hematopoietic cells in synergy with other growth factors and alone, and also potentiates the differentiation of progenitor cells into monocytes and possibly basophils in culture (154). Flk2 R -/- mice exhibit only a relatively mild defect in B lymphopoiesis (155).

Other RTKs that are expressed in hematopoietic cells include the insulin and insulin-like growth factor (IGF) receptors, Met (HGF-R), Tie1, Tie2 (Tek), Ryk (Mrk), Hek, Eck and Ron (Stk) (156-161). Several of these receptors are expressed in cell populations enriched for stem cells (160). Ron, a member of the Met family, is broadly expressed in hematopoietic cells, and is the receptor for macrophage-stimulating protein (MSP) which induces responsiveness of macrophages to chemoattractants (160,162). Other tyrosine kinase receptors which may regulate aspects of hematopoiesis include the basic FGF R (163).

1.2.3. Non-receptor tyrosine kinases

The classic prototypes for NRTKs include c-Src, c-Fps and c-Abl; the structural features of these and more recently identified subclasses (107) are depicted in figure 1-5. The majority of NRTKs are associated with the inner plasma membrane or cytoskeleton. The tyrosine kinases c-Abl, Fer, Fgr, Fes, Rak, Weel and Jak 1 are also found in the nucleus (164-171). Other PTKs, notably c-Src, can enter the nucleus under special circumstances (170). Structural features of NRTKs that are not found in RTKs include SH2, SH3 and pleckstrin homology (PH) domains. These domains are also found in other signalling proteins (see figure 1-8) and are discussed below.

1.2.3.1. The Src Homology 2 domain

SH2 domains are conserved non-catalytic regions of approximately 100 amino acids which are present in all NRTKs except for the Jak, Fak and Ack subclasses. The ZAP 70/Syk and Shark subclasses contain two of these domains (172-174). Sequence alignment of known SH2 domains
Figure 1-5. Non-receptor protein-tyrosine kinases.

10 subclasses of non-receptor PTKs are depicted. The largest subclass, the Src family, is characterized by a unique amino terminus, SH2 and SH3 domains, a catalytic domain that contains a major autophosphorylation site (Y416), and a short carboxy terminal tail that contains a negative regulatory site (Y527). Y416 and Y527 are numbered according to the prototype, c-Src. Other subclasses vary in the length of their amino or carboxy termini, the presence or absence of SH2, SH3 and PH domains and the presence or absence of a negative regulatory site (Y) in the carboxy terminus. The Tec homology region contains a proline-rich sequence. Not drawn to scale.

Abbreviations: h, human; m, murine; N, amino; BD, binding domain; NTS, nuclear translocation signal; myr, myristylation sequence; P, pleckstrin homology domain; TH, Tec homology; Pro, proline-rich
Example
Src, Fyn, Lck
Hck, Blk, Fgr
Yes, Yrk, Lyn
Fps/fes
53-64
Abl, Arg
92-98
Btk, Itk, Tec
62-77
Zap 70, Syk
70
CsK
Csk
50
Jak 1, Jak 2,
Jak3, Tyk2,
Hopscotch
130
Fak
125
Shark
86
Ack
120

Legend:

- \( \text{SH2} \)
- \( \text{SH3} \)
- \( \text{PH} \)
- \( \text{kinase} \)
has identified five well conserved sequence motifs that are separated by more variable sequence elements (175). SH2 sequence identity between mammalian Src, Fps/Fes and Abl proteins is approximately 30%. SH2 domains recognize phosphotyrosine within the context of surrounding, particularly C-terminal, amino acids. These interactions can be mimicked in vitro using isolated SH2 domains and phosphopeptides containing 5-10 amino acid residues (176-180). Interaction with a phosphopeptide of optimal sequence occurs with relatively high affinity, exhibiting $K_d$s that are variously estimated at 10-500 nM. High affinity phosphopeptides bind rapidly but also dissociate relatively quickly allowing for rapid exchange (176).

SH2 domains generally comprise a central $\beta$-sheet flanked by two $\alpha$-helices (181) and can be classified according to the identity of the amino acid at the $\beta D5$ position which is critical in determining ligand specificity (179,181). Group I SH2 domains include the Src, Abl and ZAP 70 subclasses and contain a Tyr or Phe at $\beta D5$. This group preferentially binds the consensus motif p.Tyr-hydrophilic-hydrophilic-Pro/Ile from a phosphopeptide library degenerate at the +1 to +3 positions carboxy terminal to the phosphotyrosine (179,180). For example, the Src SH2 domain selects the high affinity ligand p.Tyr-Glu-Glu-Ile. The topology of Group I SH2 domains has been described as a two-holed socket engaging a two-pronged plug (the ligand) (182,183). One conserved pocket, consisting of residues from $\alpha A$, $\beta B$, $\beta D$ and the BC loop, binds phosphotyrosine and is lined by basic residues. Two conserved arginines at the $\alpha A2$ and $\beta B5$ positions interact directly with the phosphate group. Arg $\beta B5$ is part of the highly conserved FLVRES motif and is located at the base of the pocket where it forms a bidentate ion pairing with the phosphate group. The $\alpha A2$ arginine and another basic residue (Lys $\beta D6$) also interact with the aromatic ring of the tyrosine. A second small hydrophobic pocket interacts with the ligand's +3 hydrophobic residue (Ile in the case of Src SH2) while the +1 and +2 Glu residues are extended along the surface of the SH2 domain. The hydrophobic pocket is made up of residues from the EF and BG loops and serves to
anchor and orient the phosphopeptide on the SH2 domain surface. While well conserved residues make up the p.Tyr binding pocket, more variable residues interact with the +1 to +3 residues, thus determining peptide specificity (183). Substitution of a single variable SH2 residue at the EF1 position in the +3 binding pocket of the Src SH2 domain can alter its ligand specificity and biological activity to that of the C. elegans Sem-5 SH2 domain (184).

Other SH2 domains with a Cys at the BD5 position (Group III according to the classification proposed by Cantley and co-workers (179)) have a peptide binding surface which is an extended shallow hydrophobic groove that binds predominantly hydrophobic residues in the +1 to +5 positions following p.Tyr (185). This group includes the SH2 domains of the p85 regulatory subunit of phosphatidylinositol-3'-kinase (PI3K), phospholipase C (PLC)-γ1 and the tyrosine phosphatase Shp-2 (186-188). Some evidence exists that N-terminal peptide residues may also participate in binding (185,189).

Several signalling proteins, including the ZAP-70 and Syk NRTKs, contain two adjacent SH2 domains. The crystal structure of the tandem SH2 domains of ZAP-70 bound to a phosphopeptide containing two p.Tyr-X-X-Leu motifs has been solved (190). The two p.Tyr binding pockets line up in a co-linear fashion and both the C- and N-terminal SH2 domains contribute residues to the second p.Tyr binding pocket. A coiled-coil between the two SH2 domains may provide stability to the ligand-bound structure. The distance between the tandem SH2 domains and their orientation to each other is markedly different in the phosphatase Shp-2, suggesting that these parameters contribute to binding specificity (191).

Less commonly, SH2 interactions may involve the recognition of phosphoserine or phosphothreonine residues (192-195). The affinity of phosphoserine/threonine-dependent interactions is estimated to be less than those involving phosphotyrosine (189,194) and the physiological relevance of such interactions remains to be established.
The p85 PI3K and Src SH2 domains also bind phosphatidylinositol (3,4,5) trisphosphate (PIP₃), a product of PI3K activity (196). Phospholipid competes with phosphotyrosine for binding, but a residue of the Src SH2 which is essential for p. Tyr binding (Arg βB5) is not required to bind phospholipid, suggesting overlapping but not identical binding sites. Phospholipids may provide a negative feedback mechanism to dissociate PI3K from activated RTKs. Another mechanism whereby SH2 function may be regulated is phosphorylation within the domain itself which may decrease affinity for ligand (197).

### 1.2.3.2. The Src Homology 3 domain

One or more copies of a distinct non-catalytic regulatory region of approximately 50 amino acid residues, the SH3 module, is often found adjacent to SH2 domains and may also occur independently in proteins involved in subcellular localization, cytoskeletal organization and the regulation of guanine nucleotide binding proteins (179,198-209). The Src, Abl, Btk and Csk subclasses of tyrosine kinases contain a single SH3 domain.

Proteins which bind Abl SH3 include 3BP-1, 3BP-2, Abi-2 and AAP1 (210-213). Other proteins that bind to a subset of PTK-derived SH3 domains include the p85 subunit of PI3K, the microtubule-associated GTPase dynamin, paxillin, CDC42 GTPase activating protein, the actin-associated protein AFAP-110, Sam 68 (a substrate of mitotically active c-Src) and the Wiskott-Aldrich syndrome protein (206,214-219). The Tec homology region in Btk and other members of the Tec family can also bind PTK-derived SH3 domains (220). In some instances, binding to a PTK SH3 domain activates the ligand or facilitates its phosphorylation (215,218,221,222).

NMR or X-ray crystallographic analyses of a number of SH3 domains have indicated that these domains consist of two β sheets containing 3 or 2 antiparallel strands arranged at right angles to each other (223-229). A hydrophobic region made up of conserved aromatic side chains forms a relatively flat surface patch for ligand and is flanked by two variable charged loops termed the RT
and n-Src loops (228). SH3 ligands are proline-rich sequences of approximately 10 amino acids in length which adopt a left-handed helical conformation of three residues per turn, termed a polyproline type II helix (PPII) (223,230-232). This was first demonstrated for 3BP1 which has an Abl SH3-binding site of APTMPPLPP (230). The core consensus ligand motif is X₁-Pro₀-pro₁-X₂-Pro₃ where X tends to be an aliphatic residue. Each X-Pro interacts with hydrophobic pockets formed by conserved aromatic SH3 residues. Binding specificity is conferred by the non-proline ligand residues and the two variable SH3 loops (233-235). The Src SH3 domain contains a highly conserved Asp residue (Asp 99) which interacts with Arg at the P₋₃ position in a third, more variable, pocket (232). In the Abl SH3 domain, a threonine is present at the equivalent site and binds Met or Tyr (230). SH3 ligands are pseudosymmetrical and can potentially bind in either an N→C-terminal or C→N-terminal orientation (defined as Class I and Class II ligands respectively) (236,237). Isolated SH3 domains bind ligand with dissociation constants in the range of 5-100 μM.

The WW or WWP protein interaction domain can compete with Abl SH3 for binding to the same ligand by forming largely hydrophobic contacts with proline residues (238). WW motifs are approximately 28 amino acids in length and are present in a diverse group of proteins such as formins, Yes PTK-associated protein (YAP 65), ubiquitin protein ligases and IQGap1 (239). By modulating SH3-ligand interactions, WW motifs may regulate the function of SH3-containing proteins.

A role for SH3 domains in the pathogenesis of several human diseases is now evident. For example, chronic granulomatous disease (CGD) is characterized by recurrent and persistent bacterial and fungal infections, and results from an inability of phagocytes to manufacture microbicidal oxidants (240). The assembly of an active NADPH oxidase which generates superoxide, a precursor of antimicrobial oxidants, is dependent on SH3-mediated interactions (241,242). Mutations in the SH3 domain of the Btk tyrosine kinase may be involved in the
pathogenesis of X-linked agammaglobulinemia (243). Although other contacts are also made, an interaction between proline-rich motifs in an HIV-1 protein Nef and the SH3 domain of the NRTK Hck may play a role in regulating the ability of HIV to infect primary cells (244). The latter interaction is also instrumental in increasing Hck kinase activity (245).

1.2.3.3. PTK SH2 and SH3 domains regulate kinase activity and substrate recognition

In NRTKs, SH2 and SH3 domains perform dual roles, functioning in cis to regulate kinase activity and in trans to interact with cellular factors including substrates (198,245-249). In c-Src and other Src-like PTKs, Csk-mediated phosphorylation of a Tyr residue in the carboxy terminal tail (Tyr 527 in chicken c-Src) inhibits kinase activity (250). Both the SH2 and SH3 domains are required for this inhibition (249). The recent solving of the crystal structures of human c-Src and Hck has provided evidence that the role of p.Tyr 527 in kinase regulation is to interact with the SH2 domain on the opposite side of the molecule from the kinase active site (251,252). This may allow the region between the SH2 and kinase domains to adopt a PPII helical conformation which is recognized by the SH3 domain. As a consequence, the RT loop of the SH3 domain is positioned such that it contacts the small lobe of the kinase domain. Src is thus held in an inactive conformation in which the ATP- and peptide-binding lobes are more closely apposed than in the active kinase structure, and the SH2 and SH3 domains are tied up in intramolecular interactions. Activation of c-Src may occur through destabilization of the inactive conformation by dephosphorylation of Tyr 527 or by the binding of high affinity ligands to SH2 and SH3 binding domains. Phosphorylation at Tyr 416 by another PTK may also contribute to activation (253). Once Src is activated, both SH2 and SH3 domains may contribute to the recognition of Src substrates such as AFAP-110 and Sam 68 (216,217).

The mechanisms governing kinase regulation of NRTKs which lack a Tyr 527 equivalent may vary depending on the subclass. The NRTK Syk, a member of the ZAP-70 subclass,
undergoes catalytic activation upon binding of phosphopeptide (254). This may occur either through de-repression of an intramolecular inhibitory mechanism or by allosteric activation. In Btk, a member of the Tec family, the SH3 domain undergoes an intramolecular interaction with the Tec Homology region, a proline-rich sequence which lies between the PH and SH3 domains (255). Phosphorylation of a tyrosine residue within the SH3 domain may disrupt this interaction allowing the SH3 domain to engage kinase substrates.

1.2.3.4. The Pleckstrin Homology domain

The PH domain was originally identified as an internal repeat of approximately 100 amino acids in pleckstrin, the major protein kinase C (PKC) substrate in platelets (256). One or two PH domains are found in a number of proteins involved in intracellular signalling or cytoskeletal organization (257,258). In common with the structure of SH2 and SH3 domains, the 3-dimensional structures of several PH domains suggest that the N and C termini are apposed, aiding in the formation of a compact structure which can be inserted into different host proteins (126). PH structure consists of a barrel framework of 7 $\beta$ strands capped by a C-terminal amphipathic $\alpha$ helix (257). Physiological ligands for PH domains may include phosphoinositide lipids (259-265) and the $\beta\gamma$ subunits of heterotrimeric G proteins (266,267). Binding to $\beta\gamma$ subunits involves the C-terminal $\alpha$ helix and residues extending beyond the C-terminus of the PH domain and may not be a property of the domain itself. Positively charged residues clustered in the N-terminal half at the top of the $\beta$ barrel bind the phosphate groups of phospholipids such as PIP$_3$, inositol (1,4,5) trisphosphate (IP$_3$) and PI (4,5) bisphosphate (PIP$_2$) (264). PH-containing proteins may be targets of PI3K since this enzyme produces some of the phospholipids which bind PH domains (261). It is likely that PH domains provide a membrane-tethering function (268-270). A PH domain at the amino terminus of a PTK may have a membrane-localizing function analogous to the myristylation sequence in the Src family. The Btk PH domain binds PIP$_3$, inositol (1,3,4,5) tetrakisphosphate (IP$_4$) (271) and also
PKC (272). Mutations in the PH domain of human Btk have been reported in X-linked agammaglobulinemia (273) and show reduced IP₄ binding activity (271). Mutations within the PH domain of mouse Btk also impair B cell development (274).

1.2.3.5. Non-receptor tyrosine kinases in hematopoiesis

Non-receptor tyrosine kinases function as the catalytic subunits for cytokine receptors and either constitutively associate with receptor subunits or are induced to associate upon ligand binding (49). In analogy to RTKs, ligand results in local aggregation of the associated PTKs and activation of the kinase domains by trans phosphorylation. The activated NRTKs subsequently phosphorylate receptor components along with a variety of cellular substrates which are recruited to the activated receptor complex. Cytosolic PTKs including the Src and Jak families also associate with RTKs such as the CSF-1 R, providing a potential mechanism for amplification of the incoming signal (275). In mature specialized cells, several cytosolic PTKs may act sequentially in order to activate effector functions. This is best characterized in the responses of B and T lymphocytes to antigen, as summarized below.

Antigen-mediated activation of T cells requires the co-ordinated activity of PTKs and the transmembrane PTP CD45 (276). Lck constitutively associates with the cytoplasmic tails of the co-accessory molecules CD4 and CD8 through its unique N-terminus while Fyn or other Src-like PTKs interact constitutively with the TCR-ξ-CD3 complex (277-280). Receptor ligation results in aggregation and phosphorylation of multiple immunoreceptor tyrosine-based activation motifs (ITAMs) located in the ξ and CD3 chains of the TCR (281-283). ITAMs (also known as antigen recognition activation motifs or ARAMs) are consensus motifs consisting of Tyr-X-X-Leu-X₆-8-Tyr-X-X-Leu and are also found in components of the B cell antigen R and the FcR family (276,284,285). ITAMs are responsible for transducing signals initiated by the cross linking of antigen receptors and may also be required for receptor internalization and antigen presentation.
Phosphorylation of the two conserved tyrosines within an ITAM is critical for signal transduction, as is the spacing between the tyrosine residues (281). The specific context of the ITAM may determine downstream events (292,293). Phosphorylated ITAMs in the ξ chain of the TCR-CD3 complex provide binding sites for the tandem SH2 domains of ZAP 70, as described in section 1.2.3.1. Following recruitment of ZAP 70 to the TCR-CD3 complex, Tyr 493 of the activation loop of ZAP 70 is phosphorylated by Lck, thereby increasing ZAP 70 kinase activity which can then contribute to the activation of downstream molecules (283).

A second signal delivered by an accessory cell is required for full activation of a T cell blast and the production of IL-2 in response to antigen (294). This may be provided by engagement of CD28 on the T cell with its ligand B7-1 (CD80) on the antigen presenting cell. One study suggests that ligand-induced phosphorylation of CD28 and its subsequent association with the p85 PI3K SH2 domains is required for co-stimulatory activity (295) although this remains controversial (296).

In B cells, the N-terminal regions of Lyn and Fyn bind to a specific sequence within the non-phosphorylated ITAM sequence of the resting Ig α subunit (297,298). Antigen-induced receptor aggregation induces ITAM phosphorylation presumably by the low basal activity of the PTKs. This results in a high affinity association between the ITAMs and the PTK SH2 domains leading to enhanced PTK activity (299). ITAM phosphorylation also leads to Syk recruitment and activation (299,300).

A mechanism for termination of signalling by the B cell R involves the cytosolic SH2-containing tyrosine phosphatase Shp-1 (previously known as PTP1C, HCP or SH-PTP1). FcyRIIB, the low affinity receptor for the Fc portion of IgG, inhibits B cell activation when co-aggregated with the B cell R by immune complexes. The inhibitory function of FcyRIIB depends on the phosphorylation of an immunoreceptor tyrosine-based motif (ITIM) in its cytoplasmic tail which contains Tyr-X-X-Leu (301,302). Shp-1 binds to the B cell R in its resting state (303) and is
recruited to the phosphorylated FcγRIIB ITIM upon receptor activation. This interaction increases Shp-1 phosphatase activity and the response to antigen is abrogated. Another B cell co-receptor, CD22, also inhibits B cell activation through interaction of an ITIM with Shp-1 (304). ITIMs are also found in the cytotoxic lymphocyte antigen 4 (CTLA-4) which can inhibit T cell activation, in killer cell inhibitory receptors (KIRs) which inhibit the cytotoxic activity of NK cells as well as T cells, in gp49B1 and mast cell function-associated antigen which (in addition to FcγRIIB) inhibit mast cell mediator release in response to IgE, and in ILT3 which can inhibit activation of monocytes and dendritic cells (305). The ITIMs in these receptors may recruit Shp-1, the related tyrosine phosphatase Shp-2 and/or the 5'-inositol phosphatase Ship (305). The receptors for IL-3, Epo and SF do not contain ITIMs but also bind Shp-1 (306-308). Indeed, genetic studies have implicated Shp-1 in the regulation of c-Kit and the CSF-1 R (309-311). Deletion of the Shp-1 binding site on the Epo R occurs in benign erythrocytosis and may increase sensitivity to Epo through deregulation of the Jak2 PTK (75,308).

1.3. HEMATOLOGICAL DISORDERS AND PTKS

PTKs which have recently been implicated in human hematological disorders are summarized in Table 1-1. In a specific disease, a PTK may have reduced or increased activity or undergo aberrant expression.

In leukemogenesis, an abnormal hematopoietic stem cell proliferates or expands uncontrollably, and no longer has the capacity to differentiate or otherwise respond appropriately to normal regulatory processes (4). In the Philadelphia chromosome positive (Ph+) leukemias, a characteristic chromosomal translocation results in the production of a constitutively active Bcr-Abl tyrosine kinase which plays a key role in the pathogenesis of these disorders. The c-abl proto-oncogene, and the role of its activated variants in Ph+ leukemia, are described in the next sections.
Table 1-1. Tyrosine kinases implicated in human hematological disorders

<table>
<thead>
<tr>
<th>PTK</th>
<th>Class</th>
<th>Gene abnormality</th>
<th>Disease</th>
<th>Comments</th>
<th>Selected Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Abl</td>
<td>NRTK</td>
<td>t(9;22)(q34.1;q11.1)</td>
<td>CML</td>
<td>activation due to fusion with Bcr sequences</td>
<td>See text</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ph+ ALL</td>
<td>oligomerization domain of Bcr required</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ph+ AML</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Abl</td>
<td>NRTK</td>
<td>t(9;12;14)(q344;p13;q22)</td>
<td>acute leukemia</td>
<td>production of Tel-Abl fusion protein tel-encoded HLH domain may induce oligomerization of Abl and activation of Abl kinase domain in a manner similar to Bcr</td>
<td>See text</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1148)</td>
</tr>
<tr>
<td>ZAP 70</td>
<td>NRTK</td>
<td>loss-of-function point mutations</td>
<td>selective T cell deficiency</td>
<td>ZAP 70 is required for TCR signalling</td>
<td>(744)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(745)</td>
</tr>
<tr>
<td>Lck</td>
<td>NRTK</td>
<td>t(1;7)(p34;q34)</td>
<td>T-ALL, lymphoma</td>
<td>CD8+ cells not produced CD4+ peripheral cells do not produce IL-2 in response to Ag</td>
<td>(7)</td>
</tr>
<tr>
<td>Jak3</td>
<td>NRTK</td>
<td>loss-of-function point mutations</td>
<td>severe combined immunodeficiency</td>
<td>Jak3 associates with IL-2R γc subunit and other cytokine R subunits</td>
<td>(8)</td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphocytic leukemia; CML, chronic myelogeneous leukemia; CMML, chronic myelomonocytic leukemia; NRTK, non-receptor tyrosine kinase; RTK, receptor tyrosine kinase
Table 1-1. Tyrosine kinases implicated in human hematological disorders

<table>
<thead>
<tr>
<th>PTK</th>
<th>Class</th>
<th>Gene abnormality</th>
<th>Disease</th>
<th>Comments</th>
<th>Selected Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alk</td>
<td>NRTK</td>
<td>t(2;5)(p23;q35)</td>
<td>Non-Hodgkin's lymphoma</td>
<td>Alk is an orphan RTK (insulin R family) normally expressed in the nervous system fused with nucleophosmin</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(748)</td>
</tr>
<tr>
<td>Btk</td>
<td>NRTK</td>
<td>loss-of-function point mutations</td>
<td>X-linked agammaglobulinemia</td>
<td>impaired B cell development</td>
<td>(747)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(748)</td>
</tr>
<tr>
<td>Bmx</td>
<td>NRTK</td>
<td></td>
<td>AML</td>
<td>role in leukemia is not defined</td>
<td>(749)</td>
</tr>
<tr>
<td>HEK</td>
<td>RTK</td>
<td></td>
<td>leukemia</td>
<td>member of the Eph family? aberrant expression in a subset of pre-B and T leukemic cell lines</td>
<td>(158)</td>
</tr>
<tr>
<td>c-Fms</td>
<td>RTK</td>
<td>activating point mutations</td>
<td>AML</td>
<td>detected at low frequency</td>
<td>(750)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(751)</td>
</tr>
<tr>
<td>Flk2/Fit3</td>
<td>RTK</td>
<td></td>
<td>AML</td>
<td>high levels of expression may reflect cell of origin and role in leukemia is not established</td>
<td>(752)</td>
</tr>
<tr>
<td>Fit4</td>
<td>RTK</td>
<td></td>
<td>AML</td>
<td>variable expression in AML role in leukemia is not defined</td>
<td>(753)</td>
</tr>
</tbody>
</table>
Table 1-1. Tyrosine kinases implicated in human hematological disorders

<table>
<thead>
<tr>
<th>PTK</th>
<th>Class</th>
<th>Gene abnormality</th>
<th>Disease</th>
<th>Comments</th>
<th>Selected Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF R β</td>
<td>RTK</td>
<td>t(5,12)(q33;p13)</td>
<td>CMML</td>
<td>fusion of tyrosine kinase domain of PDGF R with Ets-like protein Tel</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tel-encoded HLH domain may induce oligomerization and activation of kinase analogous to ligand-induced dimerization</td>
<td></td>
</tr>
<tr>
<td>c-Kit</td>
<td>RTK</td>
<td>activating point mutations</td>
<td>systemic mastocytosis</td>
<td>D816V alters Kit substrate specificity, resulting in rapid degradation of</td>
<td>(754)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asp 816 → Val</td>
<td>urticaria pigmentosa</td>
<td>phosphatase Shp-1</td>
<td>(755)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asp 52 → Asn</td>
<td>primary myelofibrosis</td>
<td>V560G cause spontaneous dimerization</td>
<td>(756)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Val 580 → Gly</td>
<td>CML¹</td>
<td>wildtype c-Kit is highly expressed in AML; expression may reflect cell of origin and role in AML is not established</td>
<td>(757)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asp 820 → Gly</td>
<td></td>
<td></td>
<td>(758)</td>
</tr>
<tr>
<td>Axl/Ufo</td>
<td>RTK</td>
<td></td>
<td>CML</td>
<td>role in leukemia is not yet defined</td>
<td>see text</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>may normally be expressed in stromal and myelomonocytic cells</td>
<td></td>
</tr>
<tr>
<td>DTK</td>
<td>RTK</td>
<td></td>
<td>AML</td>
<td>DTK is similar to Axl</td>
<td>(759)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>role in leukemia is not yet defined</td>
<td></td>
</tr>
<tr>
<td>Insulin R</td>
<td>RTK</td>
<td>t(1;19)(q23;p13)</td>
<td>pre-B leukemia</td>
<td>may involve fusion protein (large novel mRNA detected)</td>
<td>(760)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>enhanced autophosphorylation and response to insulin detected</td>
<td></td>
</tr>
</tbody>
</table>

¹ one patient reported
13.1. The c-abl proto-oncogene

Originally identified as the cellular homologue of the transforming gene of the Abelson murine leukemia virus (A-MuLV), c-abl is highly conserved in eucaryotic cells (314). Mammalian c-abl encodes a 145 kDa protein that contains a variable amino terminal region, SH3 and SH2 domains, a kinase domain and a large, unique carboxy terminal region that includes three nuclear localization signals, sites for phosphorylation by PKC and Cdc2, proline-rich motifs, a DNA-binding region and a region which binds actin (figure 1-6) (314-317). The carboxy terminal tail is encoded by a single exon, and distinguishes the Abl subclass from other NRTKs. Type 1a and 1b c-Abl vary at their extreme amino termini due to alternative splicing of different 5' exons, which are initiated from separate promoters, to a set of 10 common exons (318). Exon 1a is located 19 kb 5' of the common exons, while exon 1b is more than 200 kb upstream of 1a (319). The predominant form of Abl, c-Abl 1b, contains a potential myristylation site at its amino terminus. In the mouse, mRNA levels of type I (equivalent to human type 1a) and type IV (equivalent to human type 1b) c-abl are differentially regulated (320).

13.2. Drosophila Abl

Drosophila Abl (D-Abl) shares >80% sequence similarity with mammalian c-Abl in the kinase, SH2 and SH3 domains (321,322). Little homology exists in the carboxy terminal tails of mammalian and D-Abl although both are large and proline-rich. The Drosophila abl gene is cytosolic and expressed in a variety of tissues and at various times during development (323). By mid-embryogenesis, D-Abl is most abundant in the axons of the developing CNS system and at sites of attachment of somatic muscle fibres to the body wall. Loss-of-function mutations in abl result in lethality at the pupal stage; a small fraction of flies survive to adulthood and exhibit abnormal retinal cell development (roughened eyes), sterility and decreased longevity (323).
Figure 1-6. Schematic representation of c-Abl and its oncogenic variants.

The 145 kDa c-ab1 protein (a), the 160 kDa product of the Abelson murine leukemia virus oncogene (b), the 110 kDa Hardy-Zuckerman feline sarcoma virus oncogene (c), and the 210 and 185 kDa isoforms of Bcr-Abl (d and e respectively) are illustrated. Sequences derived from genes other than abl are shaded. c-Abl contains a variable N-terminal exon of 26 or 45 amino acids. The larger exon 1 contains a potential myristylation site (myr). The unique carboxy terminal tail of c-Abl contains 3 nuclear translocation signals, a pentalysine motif (K5) and two other basic sequences, KRFLR and PRKR (not shown), along with regions which bind DNA or actin. The DNA-binding region contains 3 high mobility group (HMG)-like domains which are related to the HMG of some DNA-binding proteins and bind A/T-rich sequences. The Rb-binding site is within the ATP binding lobe of the kinase domain, and proline-rich sequences in the carboxy terminal tail bind c-Crk and p53. The variable N-terminal exon 1 is replaced by gag-encoded sequences in murine and feline v-Abl and by bcr-encoded sequences in Bcr-Abl.

Abbreviations: BD, binding domain; K5, pentalysine motif; myr, myristylation; NTS, nuclear translocation signal; PH, pleckstrin homology; oligo, oligomerization domain
Axonal localization in *Drosophila*, providing functions necessary for adult viability and eye development, is dependent on the D-Abl carboxy terminal tail but does not require kinase activity (324). Although the kinase domains of D-Abl and mammalian c-Abl are interchangeable, the carboxy terminal tails are not, suggesting that this region performs different functions in mammals and flies.

Functions of D-Abl are masked by redundant processes or second site modifiers which include the genes for disabled (*dab*), failed axon connections (*fax*), prospero and fasciculin I (325-327). When one of these genes is mutated, D-Abl kinase activity is essential for normal axonal development in the central nervous system (322,325,327). The gene enabled (*ena*) is a dominant genetic suppressor of mutations in *d-abl* (328). Both the *dab* and *ena* gene products are PTK substrates, and the Ena protein interacts with the SH3 domain of c-Abl (325,328). Mammalian genes encoding proteins with homology to Dab and Ena have recently been identified (329-332).

1.3.3. Mammalian c-Abl

Murine *c-abl* mRNA is expressed widely throughout gestation, and in all adult tissues surveyed (333). Two mutations introduced into the mouse germ line by homologous recombination have indicated that c-Abl function is not required for embryogenesis (334,335). One of these mutations, *abl* m1, results in expression of a truncated protein that retains kinase activity but lacks the carboxy terminal 385 amino acids 3' to the pentalysine nuclear localization signal. Similar phenotypic abnormalities were observed in homozygotes expressing *abl* m1 and a null mutation, with developmental abnormalities in spleen, cranium and eye, lymphopenia, thymic atrophy and reduced postnatal viability in approximately half of the animals. These results suggest that the carboxy terminal tail contributes to the normal function of mammalian c-Abl as is the case for D-Abl. The relatively mild effects of *c-abl* loss-of-function mutations on mouse development may reflect the
presence of functionally redundant processes, perhaps provided by the related PTK Arg (336). Mammalian Dab homologues may also mask Abl requirements (329).

Mammalian c-Abl is found in the nucleus as well as in the cytoplasm (164,165,337). The kinase activity of nuclear c-Abl is regulated during the cell cycle by its interaction with the retinoblastoma protein (Rb) (314,338,339). Rb is a nuclear protein which contains at least 3 independent protein-binding sites including a binding region for the E2F transcription factor (the A/B pocket) and an Mdm-2/c-Abl binding site (the C pocket). Rb inhibits G1/S transition by binding multiple transcription factors and repressing or enhancing transcription from specific promoters (339). The proper assembly of Rb-mediated protein complexes is required for promotion of cell cycle arrest (340). In quiescent and early G1 cells, unphosphorylated Rb binds to the ATP-binding lobe of c-Abl and inhibits Abl kinase activity (341). Other proteins such as E2F simultaneously bind Rb. At G1/S transition, the phosphorylation of Rb by cyclin-dependent kinases results in the release of bound proteins. The release of c-Abl correlates with activation of the Abl kinase domain. Although c-Abl does not have direct transcriptional activating properties, it can enhance transcriptional activity in a manner which requires both kinase activity and DNA binding (314). It has been proposed that partial phosphorylation of Rb on selective sites that regulate C pocket binding may result in the release of c-Abl before complete disruption of the Rb protein complex, and allow activated c-Abl to phosphorylate other Rb-bound proteins prior to their release (341). This may be one mechanism whereby c-Abl participates in the regulation of promoters.

One nuclear substrate of c-Abl is the carboxy terminal repeated domain (CTD) of the largest subunit of RNA polymerase (pol) II (342). The CTD of RNA Pol II is required for the transition from initiation to the elongation phase of transcription; the regulation of transcription elongation may be an important control mechanism for mitogen-inducible gene expression (343). The tyrosine phosphorylation of CTD correlates with stimulation of promoter activity (344). The CTD is also
required for efficient mRNA processing (345). Although it is accepted that c-Abl binds DNA, it is controversial whether c-Abl binds sequence-specific sites (346-348). Recent data suggest that c-Abl binds A/T-rich sequences by regions in the carboxy tail which share homology to the high mobility group I domain found in some DNA-binding proteins (348). During G2/M transition, phosphorylation of c-Abl on serine/threonine residues by cdc2-cyclin B inactivates the DNA binding function of Abl (314,338,340,346).

Nuclear c-Abl has the ability to function as either a positive or negative regulator of cell growth depending on the cell system (341,349-351). Overexpression of c-Abl type 1b causes cell cycle arrest in rodent fibroblasts, an event which requires kinase activity, nuclear localization and a functional SH2 domain (349). A kinase-defective form of c-Abl accelerates the re-entry into the cell cycle following addition of serum to quiescent cells and enhances transformation by c-myc, ras, v- abl and v-fms (349). One interpretation of this finding is that kinase-defective c-Abl functions as a dominant negative mutant. Both Rb and p53 have been implicated in c-Abl-mediated cytostasis (350,352,317). c-Abl enhances p53 transcriptional activity and binds p53 by a proline-rich sequence located in the Abl carboxy-terminal tail (350). Abl has been reported to contribute to p53-dependent downregulation of cdk2 and G1 growth arrest in response to ionizing radiation (351,353), although another study failed to reveal any checkpoint defects in abl -/- cells responding to such a stress (354). It is not clear whether overexpressed c-Abl's cytostatic properties reflect a physiological function as other studies suggest a positive role for c-Abl in cell growth (355). For example, c-Abl induces c-myc expression which is generally associated with cell growth. Ablm mutant mice do not exhibit a higher incidence of tumours than normal mice nor has a deficiency of Abl activity been associated with human tumours, as might be expected if c-Abl is a tumour suppressor. Furthermore, overexpression of both kinase-active and kinase-defective c-Abl abrogate Rb-induced growth arrest in human osteosarcoma (Saos-2) cells (352). This effect, along with the
ability to accelerate transition from quiescence to S-phase or to co-operate with c-Myc in transforming Rat-1 fibroblasts, is dependent on the binding of c-Abl to Rb. It has been proposed that occupation of the C pocket by overexpressed c-Abl might prevent assembly of the appropriate growth-suppressing Rb protein complexes (352).

During stress responses to agents such as ionizing radiation, cisplatinum and mitomycin C, c-Abl stimulates the Jun N-terminal kinase (Jnk) (see section 1.5.1.5) (353). p38 Mapk activation is also induced by c-Abl in response to a subset of DNA-damaging agents which include cisplatinum and 1-β-D-arabinofuranosyl-cytosine (ARA-C) (356). The ataxia telangiectasia mutant (ATM) protein kinase and the DNA-dependent protein kinase are serine/threonine kinases which have recently been implicated in the phosphorylation and activation of c-Abl in response to ionizing radiation or other DNA-damaging agents (354,357,358).

In the cytosol, the myristylated form of murine c-Abl associates with actin stress fibers (359-361). Distinct regions in the carboxy terminal tail of Abl bind to F-actin and its monomeric counterpart, G-actin (361). These domains co-operate to bundle F-actin filaments in vitro suggesting that c-Abl may promote assembly of actin locally in cells. c-Abl competes with gelsolin, the actin severing protein, for binding to F-actin. The F-actin binding site is within the carboxy terminal 30 residues of Abl and is homologous to a consensus motif in actin-crosslinking proteins (314). Although D-Abl and mammalian c-Abl are least similar in their carboxy tails, the actin-binding region is nonetheless conserved. Cellular adhesion to fibronectin induces translocation of c-Abl from the nucleus to focal contacts, coinciding with activation of the Abl kinase (362). Active c-Abl is later found in the nucleus suggesting that c-Abl participates in integrin-mediated regulation of gene expression and cell cycle progression.

c-Abl is also found in association with the cytosolic molecular adaptor c-Crk which complexes with proline-rich sequences in the carboxy tail of c-Abl by virtue of its SH3 domain
Abl-mediated phosphorylation of a tyrosine residue present in c-Crk II (but absent from v-Crk and c-Crk I) may normally restrain Crk II function (363). Transformation by v-Crk is dependent on intracellular tyrosine phosphorylation which may be mediated by c-Abl (315).

1.3.4. Activated variants of Abl

The tyrosine kinase activity of c-Abl is normally tightly regulated *in vivo*, and tyrosine phosphorylation of c-Abl itself is not detectable in asynchronous mammalian cells (364). As described above, nuclear c-Abl is in part inhibited by its interaction with Rb. However, additional regulatory mechanisms must exist since the entire pool of c-Abl is normally inhibited including the cytosolic fraction. Several mechanisms that activate c-Abl without structurally altering the gene (213,364,365) can be explained by a model in which mammalian *trans* acting inhibitory factors normally regulate c-Abl. This model is also consistent with the findings that deletion or mutation of the SH3 domain causes oncogenic activation of the myristylated form of murine c-Abl, and that the presence or absence of the SH3 domain does not affect the kinase activity of purified c-Abl *in vitro* (366-368). Candidates for inhibitors of c-Abl which bind the SH3 and/or carboxy terminus of Abl are Abi-1, Abi-2, AAP1 and Pag, a protein with anti-oxidant properties (212,213,369,370). An alternate model proposes that an intra- or intermolecular interaction between the SH3 domain and binding motifs in the carboxy terminal tail induces an inactive conformation (314). Consistent with the latter model, an in-frame deletion of a proline-rich stretch of amino acids in the carboxy terminal tail activates fibroblast transforming potential (371).

Several spontaneously arising mechanisms which alter the amino terminal region of c-Abl enhance kinase activity *in vivo*. These include replacement of N-terminal sequences with *gag*-encoded sequences that include an N-myristylation signal in the *v-abl* oncogene product of the Abelson murine leukemia virus (A-MuLV) or the Hardy-Zuckerman 2 feline sarcoma virus (HZ2-FSV), and replacement of the N-terminus by human *bcr*-derived sequences in Bcr-Abl fusion
proteins (figure 1-6) (372). In the oncogene product of A-MuLV the SH3 domain is deleted, whereas in HZ2-FSV and Bcr-Abl, the SH3 region is juxtaposed with foreign sequence. Although additional mutations are found in both A-MuLV and HZ2-FSV (372-374), the common underlying basis for activation may be the disruption of the negative regulatory function of the SH3 motif. Oncogenic Abl proteins are largely localized to the cytoplasm and possess constitutive tyrosine kinase activity which is essential for transformation (167,168,375,376); this alteration in subcellular location may be important in bringing together signalling proteins that are essential for transformation.

An Abi-2 mutant protein which is no longer able to bind Abl SH3 but can still interact with the C-terminus activates the transforming capacity of c-Abl, suggesting that some mechanisms of activation may involve alterations in Abl-binding proteins (213). The potential contribution of altered Abl regulatory proteins to human malignancies has not yet been assessed.

1.4. THE BCR-ABL ONCOPROTEINS IN Ph+ LEUKEMIAS

The Ph chromosome was initially detected in CML and represents a shortened chromosome 22, the product of a reciprocal translocation t(9;22)(q34.1;q11.1) (372). This translocation results in juxtaposition of abl exons 2-11 from chromosome 9 with 5' sequences derived from the bcr gene on chromosome 22. The Ph chromosome is detected in over 95% of adults with CML, and a similar abnormality is seen in 20% of adult acute lymphoblastic leukemia (ALL), 2-5% of childhood ALL and 1-2% of AML (378-382).

In all Ph+ leukemias, the breakpoint in the abl gene may occur within a wide region of the first intron between exons 1a and 2, or between exons 1b and 1a (383). Transcription is initiated from the bcr promoter and pre-mRNA processing results in the splicing of bcr sequences to the common abl exon 2. All of the isoforms of Bcr-Abl therefore have identical abl-derived sequences.
Although an abl promoter is nested within the bcr-abl transcriptional unit in the majority of Ph translocations, transcription from this promoter appears compromised either as an immediate consequence of rearrangement, or due to later progression of allele-specific methylation (384).

Depending on the precise breakpoint within the bcr gene, a chimeric transcript encoding a 210 (p210) or 185 (p185) kDa fusion protein is usually produced. Bcr-abl mRNA appears to be more stable than endogeneous abl mRNA (385). In the majority of CML patients, the breakpoint in the bcr gene is localized to the major breakpoint cluster region (M-bcr), a specific 5.8 kb region that spans exons 12-16 (previously known as exons b1-b5). Two-thirds of CML patients exhibit a breakpoint between exons 13 and 14 (e13a2 or b2a2 junction) while most of the remaining breakpoints are between exons 14 and 15 (e14a2 or b3a2 junction) (384). These breakpoints result in production of a 210 kDa chimeric protein which contains 927 or 902 bcr-derived amino acids (386). The presence of the 25 additional amino acids encoded by exon 14 (b3) may be associated with a shorter duration of chronic phase although this remains controversial (387,388). In two-thirds of adult Ph+ ALL, breakpoints fall upstream of M-bcr, within the 3' end of the first intron between the two alternative exons e2' and e2, a region referred to as m-bcr (383,389,390). This leads to an e1a2 junction and production of the smaller 185 kDa form of Bcr-Abl which contains 426 bcr-derived amino acid residues encoded by exon 1 only. In addition to the major p210-encoding transcripts, variable amounts of alternatively spliced transcripts showing an e1a2 junction were recently demonstrated in CML patients at diagnosis by a sensitive PCR technique (391). The biological and clinical significance of this latter finding has not yet been established. A 3rd bcr breakpoint which is downstream of M-bcr, designated μ, has been described and results in a 230 kDa fusion protein (e19a2 junction). The third breakpoint is rare and may be preferentially associated with neutrophilic CML, a less aggressive disease, or marked thrombocytosis (386,392-395).
Bcr-abl transcripts are found in a proportion of otherwise normal individuals particularly in an older age group, similar to the distribution of CML itself, but the significance of this is uncertain (396). The causes and mechanisms leading to the 9;22 translocation are unknown. A high density of Alu repeats has been noted in both genes (383). Such elements at or near translocation breakpoints may trigger translocation due to the formation of hairpin loop structures. However, no consistent breakpoint features have been identified (383).

The roles of p210 and p185 Bcr-Abl in the pathogenesis of leukemia are supported by tissue culture studies, transgenic models and reconstitution experiments using bone marrow infected with recombinant retroviruses encoding Bcr-Abl (397-403). Experimental models also correlate with the clinical impression that p185 Bcr-Abl has greater oncogenic potential (404-406). Cell culture studies suggest that promotion of cell survival (suppression of apoptosis), acquisition of growth factor independence and tumorigenicity are distinct events in p210 Bcr-Abl-related pathology (407-411), although the anti-apoptotic activity of Bcr-Abl in primary cells is controversial (412,413). In vitro, p185 Bcr-Abl exhibits greater tyrosine kinase activity than p210 as measured by autophosphorylation and the phosphorylation of exogeneous substrates (414). Both p210 and p185 Bcr-Abl have a similar target cell spectrum which is similar but not identical to that of v-Abl (415-417). The transformation efficiencies of p210 and p185 Bcr-Abl are generally less than v-Abl. A permissive cell context is required for v-Abl to transform NIH 3T3 cells (418), and for p210 Bcr-Abl to abrogate anchorage-dependence in such cells (411). Permissive (P) NIH 3T3 cells exhibit different G1 cyclin/cdk activities in response to phorbol ester than do non-permissive (N) cells which undergo growth arrest in the presence of v-abl (419). N- and P-NIH 3T3 cells do not differ in p53 or Rb status and the precise molecular basis for the difference in susceptibility to activated Abl is not yet known.
1.4.1. **Chronic myelogeneous leukemia**

CML is a clonal disorder that arises in a multipotential hematopoietic stem cell (420); consequently, *bcr-abl* rearrangement may be detected in lymphocytes as well as in myeloid cells (421,422). Recent evidence for a transplantable CML SC population includes studies on xenogeneic recipients of transplanted CML cells and retroviral marking of autografts (423). The initial chronic phase (CP) of CML is characterized by discordant maturation, resulting in expansion of the myeloid compartment, typically granulocytes (424). Primitive hematopoietic cells, although exhibiting *bcr-abl* rearrangement, are less affected than more mature cells in CML CP and do not display a corresponding expansion (425,426). This may be secondary to differential expression of *bcr-abl* in mature and immature cells (425). In CP, the expanded myeloid cells appear morphologically normal and are capable of undergoing terminal differentiation but exhibit subtle functional abnormalities, dysplasia and abnormal ultrastructure (426). Based on cell labelling studies, mature CML granulocytes have a longer life span in the circulation but have a diminished capacity for survival and proliferation, and require exogenous growth factor in culture (427). Normally, primitive cells are quiescent when in contact with stroma via fibronectin receptors. Ph+ progenitors may not express the adhesion molecule LFA-3, adhere less well to the stromal layer of longterm bone marrow cultures and are less responsive to stromal-derived regulatory signals (428-431). Abnormalities in β1 integrins are also detected (432,433). Treatment of CML progenitors with IFNα may correct the defects in adhesive properties by acting on both Ph+ progenitor and stromal cells (432,434,435). The interaction of Ph+ macrophages with other stromal elements may diminish the growth of normal hematopoietic progenitors, thus allowing a selective expansion of malignant progenitors (436). The suppression of normal hematopoiesis contributes to the clinical manifestations of CML CP. IFNα's action may in part be due to removal of the suppressive effect on Ph- cells (437).
At least a partial hematological remission may be achieved in 70-80% of chronic phase CML patients with IFNα but this is not necessarily accompanied by a cytogenetic response (438,386). Cytogenetic responses and duration of survival may be increased by the addition of cytarabine (439). The chronic phase of CML lasts an average of 4-6 years before lapsing into a terminal blast crisis or acute phase (AP) that resembles ALL or AML but is more resistant to therapy (440). Extremely rare cases of spontaneous remission have been reported (441).

Blast cells, which are immature cells blocked in differentiation, accumulate in AP. The phenotype of the blast cells may reflect any of the lineage potentials of the stem cell involved (378,442). Unlike CP stroma, CML AP stroma display spontaneous induction of cytokines (443). Blast crisis may be preceded by an ill defined accelerated phase during which the disease becomes less easily controlled with agents such as IFNα, busulfan or hydroxyurea which effectively reduce cell numbers in CP (4). The inability to predict accurately when evolution into acute phase will occur for an individual patient remains a therapeutic problem. A recent animal model which may prove useful for the study of CML progression indicates that both normal and Ph+ leukemic cells from CML patients can engraft the bone marrow of sublethally irradiated SCID mice (444).

Chromosomal rearrangement is an early event in the course of CML and is present at diagnosis in the majority of CML patients. It remains an issue of debate whether the Ph translocation is the first event or occurs in a subclone (445). This is of considerable therapeutic importance since Ph- cells may be abnormal. A number of studies have not shown a consistent change in bcr-abl mRNA and protein levels during progression to AP, although a recent study reported a rise in mRNA levels in peripheral blood cells preceding AP (424,446). Progression is accompanied by cytogenetic clonal evolution suggesting that expression of p210 Bcr-Abl confers some selective advantage over normal stem cells, but is insufficient for induction of blast crisis. Four relatively frequent non-random changes are: duplication of the Ph chromosome, trisomy 8,
isochromosome 17q and trisomy 19 (447). The chance of disease progression is apparently the same each year following diagnosis, suggesting that only one further rate-limiting stem cell mutation is required for progression although multiple chromosomal changes are frequently observed (448). Cytogenetic evolution may result in increased myc expression or decreased expression of the tumour suppressor genes p53, Rb, p16\textsuperscript{INK4a} or AML1-EVI-1 (447,449-452). Homozygous deletion of p16 may have a particular association with lymphoid transformation (452).

In a transfection-tumorigenicity assay using the DNA from a CML patient in lymphoid acute phase, an oncogene termed lbc has been detected; lbc encodes a 47 kDa protein which contains a PH domain and promotes guanine nucleotide exchange on the GTPase Rho (453,454). A role for lbc in progression of CML has not yet been established. Another gene identified in transfection-tumorigenicity studies using DNA from two CML patients (455,456) and from a patient with a Ph- chronic myeloproliferative disease (457) is axl (also known as UFO or ark). The Axl RTK is expressed in a wide range of cells including bone marrow stroma and myelomonocytic cells (458), and can transform fibroblasts when overexpressed (455). In K562 cells, axl mRNA is upregulated during differentiation (456,457). A ligand for Axl is Gas6, a vitamin K-dependent relative of protein S which was originally identified as the product of a gene expressed during cell growth arrest (459-461). While initial studies implicated Gas6 in the negative regulation of cell proliferation, Gas6-Axl interactions may have restricted mitogenic activity during serum starvation (459). The murine homologue of Axl induces cell aggregation by homophilic interactions (462).

1.4.2. Ph+ acute leukemias

De novo Ph+ ALL is a distinct, rapidly aggressive clonal disease that appears to affect a more mature or restricted stem cell than CML, and responds differently to therapy (440). In adults, Ph+ ALL comprises 10-25% of all ALL cases whereas in children, approximately 5% of ALL cases are Ph+ (379,381,382). The p185 Bcr-Abl isoform is expressed in the majority of Ph+ ALL cases
in children and in two-thirds of adult cases. The production of a 185 kDa Bcr-Abl protein has also rarely been reported in AML (380) and in CML where it may be associated with a predominant monocytic component (463).

In Ph+ ALL, the bcr-abl rearrangement may be detected only in lymphoid cells or in both lymphoid and myeloid cells, suggesting heterogeneity in the population of stem cells that this disease may affect (464,465). A subset of Ph+ ALL patients that express p210 Bcr-Abl may have undergone an undetected chronic phase and have CML in lymphoid blast crisis. CML AP and Ph+ ALL may be indistinguishable at presentation but with successful chemotherapy, CML patients revert to chronic phase, whereas Ph+ ALL patients will exhibit eradication of the Ph chromosome to undetectable levels.

1.4.3. The bcr gene product

The bcr gene is widely expressed and encodes a major cytosolic species of 160 kDa (figure 1-7) (466,467). Alternate 1st and 2nd exons exist (383). The first exon, encoding 426 amino acids, is serine-rich and encodes a novel serine/threonine kinase activity (468). In its central region, Bcr shares homology with Dbl, Vav and yeast CDC24 (469). The Dbl-homology domain catalyzes guanine nucleotide exchange for CDC42Hs, RhoA, Rac1 and Rac2 but not Rap-1a or H-Ras (470). The central region of Bcr also contains a PH domain. The carboxy terminal region of Bcr shares homology with RhoGap, the p85 subunit of PI3K and chimerin, and exhibits GTPase promoting activity specific for CDC42Hs and Rac1 (471,472). Rac-induced membrane ruffling in Swiss 3T3 cells is abrogated by microinjection of full length Bcr, consistent with Bcr's function as a RacGap (473). A regulatory role for Bcr in the respiratory burst of neutrophils has been identified and is also consistent with Bcr's RacGap activity (474). Bcr is not required for normal hematopoietic cell development (474), possibly due to the presence of functionally redundant proteins such as Abr (475). The 210 kDa form of Bcr-Abl contains approximately two thirds of the bcr-encoded
Figure 1-7. Structural features of the bcr gene product.

Exon 1 of bcr encodes 426 amino acids which contain a coiled-coil tetramerization domain, a novel serine/threonine (S/T) kinase activity and two serine-rich regions termed box A and box B. The N-terminal region, particularly the tetramerization domain, enhances binding of the Abl carboxy terminal tail to actin. Box A and B overlap with regions implicated in binding to the Abl SH2 domain (amino acid residues 192-242 and 298-413). Two pairs of cysteines (C) which are important for S/T kinase activity are shown. The Dbl homology (H) region encodes a guanine nucleotide exchange domain for members of the Rho family such as Rac, and the rhoGap domain possesses Gap activity for Rac and CDC42Hs. Arrows illustrate the sites of the breakpoints in p210 and p185 Bcr-Abl. Also shown is the breakpoint for p230 Bcr-Abl. Another rare variant breakpoint which produces a 203 kDa protein lacking Abl exon 2 (including the N-terminal 17 amino acids of the SH3 domain) is not shown.

Abbreviations: C, cysteine; H, homology; Gap, GTPase activity protein; S/T, serine/threonine; PH, pleckstrin homology
sequence, including the S/TK activity, the Dbl-like GEF domain and the PH domain (figure 1-6). p185 Bcr-Abl contains the N-terminal 426 bcr-derived amino acid residues which include the S/TK activity. The RacGap domain is not present in either p210 or p185 Bcr-Abl but those residues essential for RacGap activity are retained in the 230 kDa variant (383).

1.4.4. Activation of the Abl kinase domain by Bcr sequences

Bcr sequences encoded by exon 1, but not those essential for S/TK activity, are required for activation of the Abl kinase domain (192,476). Amino acid residues 1-63 of Bcr comprise a coiled-coil oligomerization domain which mediates the formation of homotetramers (476). Tetramerization may promote intermolecular phosphorylation which is important for activation. The tel-encoded helix-loop-helix (HLH) domain may serve as an oligomerization domain in a manner analogous to Bcr in the recently described Tel-Abl fusion protein which occurs in rare cases of acute leukemia (477-480) and has been reported in one case of CML (1149).

Fusion with Bcr sequences, particularly the first 63 amino acids, also enhances the ability of Abl to bind actin, an event which is independent of tyrosine kinase activity and the presence of the SH2 or SH3 domains (359,360). Mutations in the abl-encoded F-actin binding domain decrease the transforming potential and ability of Bcr-Abl to abrogate IL-3 dependence without altering protein stability or kinase activity (359). Actin association may allow Bcr-Abl to interact with adhesion receptors or cytoskeletal proteins, enabling the efficient interaction of Bcr-Abl with critical substrates. This may explain the altered cell adhesion properties of Ph+ cells and the inability of bone marrow stromal cells to adequately inhibit expansion of these cells in vivo. Cytosolic localization is dependent on the presence of bcr-derived amino acids but does not require interaction with actin. Consistent with the localization of Bcr-Abl to the cytosol, the DNA binding domain is dispensable for transforming activity (314).
Two nonconsecutive serine/threonine rich regions within exon 1 of Bcr bind Abl SH2 \textit{in vitro}, in a phosphoserine/threonine-dependent manner (192,193). Deletions encompassing the more 3' SH2-binding region or both SH2-binding regions abolishes transformation of fibroblasts suggesting that binding to Abl SH2 is activating (192). However, the precise mechanism whereby SH2-binding Bcr sequences contribute to activation of the Abl kinase domain is not known.

\section*{1.4.5. Expression of \textit{c-myc} is required for transformation by Bcr-Abl}

The transforming properties of p185 Bcr-Abl are dependent on expression of \textit{c-myc} (481). Myc is a short-lived basic region-HLH protein which regulates transcription and the expression of cell cycle regulators (482). Expression of c-Myc is tightly regulated at both transcriptional and post transcriptional levels, and is rapidly increased by mitogens (483,484). Myc binds another HLH protein, Max, and recognizes distinct and specific DNA consensus sequences which repress or activate transcription (485). The repression function of Myc is necessary for inhibition of cell differentiation and for co-operation with activated Ras in the transformation of primary fibroblasts (486). Myc is required for G\textsubscript{0}-G\textsubscript{1} transition, and also may have a role in S-G\textsubscript{2} transition (486). Expression of \textit{c-myc} is also required for some forms of apoptosis which occur under conditions of suboptimal cell proliferation such as the withdrawal of IL-3 (487). Myc-dependent apoptosis requires dimerization with Max. Translocation, amplification or overexpression of \textit{c-myc} has been implicated in the evolution of CML (449).

\section*{1.5. TARGETS OF PROTEIN-TYROSINE KINASES}

The response of a cell to increased tyrosine kinase activity depends on the array of targets available as well as the integration of other concurrent stimuli. Proximal or immediate targets of PTKs may be broadly defined as proteins or other molecules whose biological function is rapidly altered prior to the signal reaching the nucleus where gene expression is regulated. The recent
characterization of early targets of activated RTKs in non-hematopoietic cells has revealed that many of these proteins contain one or more protein interaction domains. SH2, SH3 and PH domains were described in section 1.2.3. The importance of SH2 and/or SH3 domains in signal transduction has been demonstrated by genetic studies in Drosophila and C. elegans (see section 1.5.1.3.), and by the identification of mutations which are associated with human disease. More recently identified protein interaction domains include the WW module which recognizes proline-rich sequences, and the phosphotyrosine-binding (PTB) or phosphotyrosine interaction domain. PTB domains are approximately 160 amino acids in length and are found in proteins such as IRS-1, Shc and Fe65, a protein homologous to viral integrases (238,239,488,489). In contrast to SH2 domains which recognize phosphotyrosine largely in the context of C-terminal residues, PTB domains recognize a core consensus sequence Asn_{3}-Pro_{2}-X_{1}-p.Tyr which adopts a β-turn structure (488,490-493). Additional N-terminal residues are also important as the Shc PTB domain prefers a hydrophobic residue at -5 and IRS-1 favours a patch of hydrophobic residues at positions -6/-7/-8 (492,493). In some cases, recognition of a β-turn structure may occur without the presence of phosphotyrosine (489,494). PTB domains exhibit slower, longer-lived interactions than SH2 domains (495,496) and have a topology which is remarkably similar to PH domains despite the lack of primary sequence homology (497,498).

Proteins containing SH2, SH3, PTB and/or PH modules include a diverse group of enzymes (figure 1-8) (126,200,201). One well characterized enzyme in this group is PLC-γ1. PLC-γ1 hydrolyzes PIP_{2} and other phosphatidylinositols to produce the second messengers diacylglycerol (DAG) and IP_{3}. DAG and IP_{3} activate PKC and mobilize Ca^{2+} respectively, resulting in pleiotropic effects (499).

A second group of proteins which possess one or more protein interaction domains do not exhibit readily apparent catalytic activity. These include Nck, Crk, Shc, Grb2, Stats (signal
Figure 1-8. Signalling proteins which contain SH2, SH3, PH or PTB domains.

Representative targets of RTKs which contain SH2, SH3, PH and/or PTB domains are illustrated schematically, and their general functions indicated. These proteins are divided into those with intrinsic enzymatic activity (a), and those without known catalytic activity (b). Many proteins in the latter group function as adaptors or docking proteins to link tyrosine kinases to downstream targets. Several members of the Crk family are shown. In v-Crk, point mutations (depicted by "x") occur in addition to replacement of the N-terminus with gag-encoded sequences. Grb3-3 is a naturally occurring isoform of Grb2 which has an incomplete SH2 domain. X and Y indicate the catalytic region of PLC-γ. The PH domain in PLC-γ is split.

Abbreviations: Calb, Ca^{2+}-dependent phospholipid binding region; CH, collagen homology; GEF, guanine nucleotide exchange factor
SH3  SH2  SH1
Src  tyrosine kinase
pl20 GAP  Ras regulator
PLC-γ  phospholipase C
Shp-1  tyrosine phosphatase
Vav  GEF

SH3  SH2  SH1
p85α  regulatory subunit of PI3K
Shc  Ras regulator
Grb2  Ras regulator
Grb3-3  ? negative regulator of Ras

SH2  SH3  PH

Legend
SH2  SH3  PH

Src tyrosine kinase
p120 GAP Ras regulator
PLC-γ phospholipase C
Shp-1 tyrosine phosphatase
Vav GEF

p85α regulatory subunit of PI3K
Shc Ras regulator
Grb2 Ras regulator
Grb3-3 ? negative regulator of Ras
c-Crk I adaptor function
c-Crk II adaptor function
v-Crk oncogene
CrkL adaptor function
Nck adaptor function
IRS-1 docking protein
STATs transcription regulator
transducers and activators of transcription) and the p85 non-catalytic subunit of PI3K (126,200,500). p85 functions as an adaptor and binds to activated receptors by virtue of its two SH2 domains, and to the PI3K catalytic subunit through its inter-SH2 region (501). Allosteric activation of the catalytic subunit of PI3K results in the phosphorylation of the D3 position of the inositol ring of phospholipids. Products of PI3K activity such as PIP₃ function as second messengers and initiate secondary signalling cascades which have multiple effects including potential roles in regulating apoptosis, cell proliferation, cell differentiation, protein trafficking and cytoskeletal organization. Other proteins such as Nck, Crk and Grb2 are composed almost exclusively of SH2 and SH3 domains and also function as molecular adaptors to create multi-protein signalling complexes that link activated RTKs to downstream effectors. The importance of adaptor molecules in mitogenic signalling is supported by the finding that overexpression of some of these molecules or their truncated versions is transforming (502-505). In the case of v-Crk and Crk I (but not Nck), transformation is associated with increased cellular phosphotyrosine.

1.5.1. Consequences of RTK interactions with SH2-containing targets

Signalling proteins interact with autophosphorylated RTKs such as the PDGF or EGF receptor via their SH2 and/or PTB domains. These domains recognize specific, generally non-overlapping phosphotyrosine sites within the non-catalytic regions of the receptor's cytoplasmic domain (figure 1-9) (200). Variations on this theme are utilized by other receptors (506-510). For example, a master docking protein which contains multiple phosphotyrosine sites may bridge the receptor and its effectors (figure 1-9).

The interaction of SH2-containing proteins with activated receptors or their docking proteins has several potential consequences. Recruitment to the inner plasma membrane may allow a target such as PLC-γ1 or PI3K access to substrates or other downstream effector molecules. Association with a receptor may also facilitate the target's phosphorylation, or modulate kinase activity toward
**Figure 1-9. Variations on a common theme: mechanisms of signalling by receptor tyrosine kinases and receptors associated with cytosolic PTKs.**

a. RTKs autophosphorylate and directly bind SH2-containing proteins. The PDGF receptor is shown as a prototype. Some of the phosphotyrosine motifs that bind to SH2-containing proteins are illustrated.

b. The tail of the hepatocyte growth factor/Scatter factor R (Met) contains two closely spaced autophosphorylation sites (Y^{1349}VHVNATY^{1356}VNV). This sequence functions as a master docking sequence for multiple SH2-containing proteins and also binds the docking protein Gab1.

c. The insulin/IGF-1 receptors utilize a major substrate IRS-1, which has multiple p.Tyr sites, as a docking protein rather than directly engaging the majority of SH2-containing targets (488). Other potential docking proteins are Cas, the Cas-related Sin/Efs, Gab1 and *Drosophila* Dos (741-743). Y^{960} on the receptor is required for efficient phosphorylation of IRS-1 and binds the PTB domain of IRS-1.

d. Cytokine, antigen and interferon receptors which lack intrinsic catalytic activity associate with NRTKs either constitutively or subsequent to engagement of the receptor by ligand. Activation of the NRTK upon ligand binding results in phosphorylation of both receptor subunits and downstream effectors. The phosphorylated receptor may then engage other SH2-containing proteins. Some cytokine receptors such as IL-2, IL-4, IL-7 and IL-15 may utilize the docking proteins IRS-1 and IRS-2.

All receptors are depicted in a monomeric state. However, the PDGF and Met receptors form dimers upon ligand stimulation and the insulin R consists of a heterodimer of disulfide linked subunits (see figure 1-4). As illustrated in figure 1-3, many cytokine receptors are multi-subunit complexes which also oligomerize upon ligand engagement.
other substrates (342,511). Either partner may also be protected from dephosphorylation (512).

A target's biological activity may be directly altered by phosphorylation on tyrosine residues (513,514). In addition, Shp-2 (515,516), Syk (254) and PI3K (518-520) show enhanced catalytic activity when their SH2 domains bind to phosphopeptides corresponding to either receptor or insulin receptor substrate-1 (IRS-1) binding sites. Such an effect may be due to a conformational change in the SH2 domain(s) which is transmitted to the catalytic domain, or more likely, due to a re-orientation of the SH2 domain in relation to the rest of the protein.

The potential function of SH2 domains in receptor signalling is well illustrated by the Stat pathway (500). Stat SH2 domains bind to phosphorylated sites on activated cytokine receptors (or RTKs) thus recruiting the cytosolic Stats to receptor complexes where they are phosphorylated on a single tyrosine residue by the Jak kinases (or other PTKs). High affinity reciprocal SH2-p.Tyr interactions between Stat proteins ensue, resulting in homo- or heterodimers which then translocate into the nucleus where they bind DNA response elements and function as transcription regulators.

1.5.2. Ras signalling pathways

A major pathway by which RTKs and cytokine receptor-associated NRTK activity stimulate cell growth or differentiation involves the guanine nucleotide binding protein p21ras (521-529). In mammalian cells three Ras proteins are expressed (H-ras, N-ras, K-ras), which undergo post translational modification and localize to the inner surface of the plasma membrane (529). Ras proteins cycle between a GDP-bound inactive form and a GTP-bound active state which have different conformations (530,531). A wide range of stimuli induces the transient accumulation of GTP-bound Ras (532). Microinjection of neutralizing anti-Ras antibodies or expression of DN Ras mutants prevents tyrosine kinase-induced DNA synthesis, morphological transformation, differentiation or motility depending on the particular cell system (521,522,524,526,528,533). DN
Ras also prevents apoptosis in serum-deprived PC12 cells, likely by preventing attempts to re-enter the cell cycle which normally induces apoptosis in the absence of trophic support (534).

Ras is required for cells to leave G0 and pass through G1/S transition of the cell cycle (535,536). A requirement for Ras has been demonstrated at multiple points in G1 (535-537). A major link between Ras and the cell cycle machinery is the ability of Ras to induce cyclin D expression and downregulation of the cdk inhibitor p27kip1 (537,538). This results in cdk activation, phosphorylation of Rb and release of Rb-bound E2F transcription factors which can then modulate gene transcription. The growth inhibitory effect of Ras in Schwann cells has recently been shown to be due to the induction of expression of the cdk inhibitor p21Cip1/Waf1 by a p53-dependent mechanism (539). The inability of activated Ras to transform primary fibroblasts without a cooperating oncogene may be related to cdk inhibitor expression.

1.5.2.1. Regulators of Ras proteins

Ras proteins are regulated by GTPase activators, guanine nucleotide exchange factors and potentially, guanine nucleotide dissociation inhibitors (540,541). An intrinsic GTPase activity slowly hydrolyzes GTP to GDP, thus returning Ras to its inactive GDP-bound state. RasGaps stabilize the transition state of the phosphoryl transfer reaction by supplying a catalytic residue, termed the arginine finger, into the active site of Ras and greatly increase the intrinsic GTPase activity, thereby negatively regulating Ras (542). Mammalian Gaps such as p120-Gap, neurofibromin (Nf-1) and other more recently identified Gaps (543-549) share homology in their catalytic domains with other Gaps such as IRA-1 and IRA-2 in Saccharomyces cerevisiae, but also have unique structural features outside of their catalytic domains that suggest they perform other distinct functions. Both p120-Gap and Nf-1 act on H-Ras, N-Ras, K-Ras and the related R-Ras, and also bind to Rap proteins but do not stimulate their GTPase activities (529).
Two alternatively spliced products of Nf-1 have been identified (550). Type I Nf-1 is more highly expressed in proliferative cells whereas Type II, which contains an insert in the GTPase activating domain, is expressed to greater levels in differentiated cells. Autosomal dominant inherited or sporadic loss-of-function mutations of Nf-1 cause the disease neurofibromatosis type I (551,552). Neurofibromatosis-1 patients have multiple neurofibromas and other benign tumours and are at increased risk for certain malignancies, particularly tumours of the peripheral nerve sheath (552). Targeted disruption of Nf-1 in mice leads to cardiac malformations and neural crest deformities in homozygotes and is lethal in utero (553). In heterozygote mice, an increase in neural tumours and myeloid leukemia occurs (554). Increased Ras.GTP is found in some but not all human Nf-1 tumour cell lines, and in murine Nf-1 +/- Schwann cells (555-558). Some tumours from patients with neurofibromatosis exhibit loss of heterozygosity resulting from a somatic mutation in the second Nf-1 allele (559,560). Young children with neurofibromatosis 1 are predisposed to myelodysplastic (MDS) and myeloproliferative syndromes particularly juvenile CML (a disease which is distinct from Ph+ CML and does not involve the abl or bcr genes). As adults with neurofibromatosis 1 are not prone to preleukemia or AML, Nf-1 activity appears to be more important in the regulation of myeloid cells in neonates and young children. Somatic mutations of Nf-1 have been described in sporadically occurring human tumours that are not derived from neural crest tissue (552,559,561). The mechanism whereby loss-of-function Nf-1 mutations promote growth presumably varies depending on whether Ras.GTP is normally growth inhibitory or growth stimulatory in the cell type giving rise to the tumour.

The 120 kDa Ras Gap protein (p120-Gap) possesses an N-terminal hydrophobic region, two SH2 domains with an intervening SH3 module, a central domain with homology to the Ca^{2+}-dependent phospholipid binding (Calb) domains of PKC and synaptotagmin, a PH domain and a carboxy terminal catalytic region (figure 1-8) (543,544,562). The PH and/or Calb domain may
regulate interactions of p120-Gap with phospholipids and hence the plasma membrane. An alternatively spliced 100 kDa variant of Gap (Type II) which lacks 180 amino acids of the amino terminal hydrophobic region is expressed in placenta and is induced in lymphoid and myeloid leukemic cell lines upon differentiation (543,563). Although Type II Gap has similar in vitro activity as p120-Gap, it does not exhibit the same biological activity (564). While the carboxy terminal region of p120-Gap is sufficient for catalytic activity, the amino terminal region modulates Gap activity in vitro (565). Inactivating mutations of p120-Gap, in contrast to Nf-1, appear to be rare in a wide range of tumours (566).

Co-expression of p120-Gap and Nf-1 occurs in hematopoietic as well as other cell types (540). Nf-1 and p120-Gap are differentially regulated and tissue specific differences in the consequences of their activity may exist (564,567-575). Both Nf-1 and p120-Gap undergo phosphorylation on serine and threonine, and p120-Gap is tyrosine phosphorylated in response to growth factors (551,576,577). Phosphorylation does not appear to modulate the catalytic function of either p120-Gap or Nf-1 in vitro, but may regulate other functions of these molecules. A major in vivo phosphorylation site in p120-Gap, Tyr 460, is located immediately adjacent to the carboxy terminal SH2 domain (578) and may modulate the biological activity of the Gap SH2-SH3-SH2 region (579).

The observation that p120-Gap and Nf-1 bind to the effector region of Ras (see next section) and that a number of biologically inactive mutants of oncogenic Ras do not bind either protein led to the proposal that these proteins may simultaneously function as downstream effectors of Ras (580,581). p120-Gap inhibits the opening of atrial membrane K+ channels in a Ras-dependent manner by uncoupling muscarinic receptors from heterotrimeric G proteins (582). The N-terminus of p120-Gap alone can achieve this effect in the absence of Ras (583). Binding of Ras to the p120-Gap catalytic domain was therefore proposed to induce a conformational change that allows the N-
terminal SH2-SH3-SH2 region to interact with putative downstream targets (583-585). Indeed, the N-terminal region of p120-Gap can co-operate with a non-myristylated v-Src mutant in inducing cellular transformation (586) and also modulates the actin cytoskeleton (587). Other studies in mammalian cells also support a role for p120-Gap as a downstream effector (588-595). However, inactive Ras proteins containing certain point mutations in the effector region still associate with p120-Gap and Nf-1, suggesting that additional effectors must exist (596,597). Furthermore, overexpression of full length p120-Gap suppresses focus formation and morphological transformation of fibroblasts by v-src or overexpressed wildtype Ras (598-600). A number of other studies in mammalian cells are also most consistent with a predominant function of p120-Gap as a negative regulator of Ras (572,601,602). In *S. cerevisiae* where the Ras effector is adenylate cyclase, genetic studies indicate that IRA-1 and IRA-2 function solely as negative regulators (603). Genetic analysis also suggests that the role of *Drosophila* Gap1 is restricted to negative regulation of Ras (604).

Positive regulation of Ras is mediated by GEFs such as CDC25 and SDC25 in *S. cerevisiae*, Ste6 in *S. pombe*, Son of Sevenless (Sos) in *Drosophila*, the mammalian brain-specific CDC25<sup>BM</sup> (Ras GRF), Ras GRF2 and the mammalian homologues of Sos (605-614). These proteins share a region of homology of approximately 380 amino acids designated the CDC25 domain which is responsible for promoting guanine nucleotide exchange. In cells, the ratio of GTP to GDP is high, and as the affinity of Ras for GDP and GTP is similar, any free p21<sup>ras</sup> will bind GTP. GEFs bind the inactive form of Ras and promote GDP dissociation, the rate-limiting step in Ras activation (540). Genetic evidence in *Drosophila* indicates that the GEF Sos participates in the signalling pathways of the Sevenless, EGF and Torso RTKs which are important in development (605,607,615). Expression of Sos in rat fibroblasts induces transformation as does expression of the CDC25 domain of SDC25 in NIH 3T3 cells (616-618).
In the mouse, cDNAs encoding two proteins related to Sos, mSos1 and mSos2, were isolated by the hybridization of a *Drosophila* gene probe to a mouse cDNA library under conditions of low stringency (608). mSos1 and mSos2 are 67% identical and share the highest degree of homology in their central GEF domain. The lowest degree of similarity is in their carboxy terminal 270 amino acids which are 41% identical. Mammalian Sos proteins are widely expressed throughout development and in adult tissue, and are found in all hematopoietic lineages. An additional GEF designated C3G has weak activity for mammalian Ras and is also widely expressed (619). Other potential regulators of Ras inhibit the activity of GEFs and are termed guanine nucleotide dissociation inhibitors (540,541).

1.5.2.2. Structural analysis of Ras proteins and potential effectors of Ras

Ras proteins contain a nucleotide binding region, an effector domain and a carboxy terminal CAAX motif which is required for membrane attachment. GTP binding induces conformational changes in Ras which are localized to two regions of primary structure designated switch 1 and switch 2, corresponding to amino acid residues 30-37 and 59-76 respectively (620,621). Switch 1 overlaps with the genetically defined effector region which interacts with downstream targets (amino acid residues 32-40) (530,620,621). Mutations in this region inhibit the biological activity of oncogenic Ras without altering guanine nucleotide binding or intrinsic GTPase activity (588,589). A number of proteins bind overlapping sequences within the switch 1 and switch 2 regions of Ras.GTP, and are therefore candidate effectors of Ras. In addition to p120-Gap and Nf-1, these include the serine/threonine kinases c-Raf-1, MEKK1 and PKC ζ, the p110 catalytic subunit of PI3K and members of the Ral guanine nucleotide dissociation family (540,622-628). Distinct targets bind Ras in yeast (626). Studies on effector loop mutants of activated H-ras which show selective loss of binding to a subset of these proteins, suggest that multiple effector interactions and diverging pathways contribute to Ras-mediated transformation of mammalian cells.
These and other studies have revealed the presence of at least two distinct pathways downstream of Ras: the Raf/mitogen-activated protein kinase (Mapk) pathway which is associated with cell growth or differentiation, and a second pathway resulting in activation of Rho GTPases which alter the actin cytoskeleton (see sections 1.5.1.5 and 1.5.1.6) (629,631,632).

Transformation by Ras proteins can be induced by overexpression of normal Ras or by mutational activation. Oncogenic forms of Ras occur in approximately one-third of human tumours including AML and MDS (532). Activating structural mutations either prevent Gap/Nf-1-stimulated GTP hydrolysis or accelerate GDP/GTP exchange (621,542). Other mutants of Ras have been identified that act in a dominant negative fashion to block normal Ras activation. These mutants form a complex with Ras GEFs but have reduced affinity for GTP and may be defective in displacement of GEFs by GTP (621,633).

1.5.2.3. Molecular adaptors link receptor tyrosine kinases to Ras

Two recently described proteins, encoded by the src homology and collagen (shc) and growth receptor bound (grb)-2 genes are implicated in the regulation of Ras signalling pathways and fall into the category of molecular adaptors (505,634-637). The human cDNA for Grb2 was cloned during a screen of cDNA expression libraries for proteins which bind to the tyrosine phosphorylated carboxy terminal tail of the EGF R (637). Grb2 is the mammalian homologue of C. elegans Sem-5 and Drosophila Drk (203,204), and is composed entirely of a single SH2 domain flanked by two SH3 domains (see figure 1-8). Genetic studies on C. elegans and Drosophila indicate that Sem-5 and Drk are essential for activation of the Ras pathway by RTKs and that the function of both the SH2 and SH3 domains is required (203-205,637). The SH3 domains of Grb2/Drk constitutively associate with proline-rich motifs in the C-terminal tail of Sos or its mammalian homologues while the Grb2 SH2 domain binds autophosphorylated receptors (231,612,638-640). Upon EGF stimulation, mSos1 is translocated to the EGF R by virtue of its
constitutive association with Grb2 (638-640). Blocking the interaction of the EGF R with Grb2 by specific phosphopeptides prevents EGF-induced stimulation of Ras nucleotide exchange whereas overexpression of Grb2 potentiates this response (639,640). Association with either Grb2 or the EGF R does not appear to change the intrinsic catalytic exchange activity of mSos1 (640). However, mSos1 derivatives lacking proline-rich SH3-binding sites but containing either myristylation or farnesylation signals that target mSos1 to the plasma membrane activate the Ras signalling pathway in the absence of external stimuli (641,642). This suggests that recruitment of mSos1 to the plasma membrane is sufficient to induce guanine nucleotide exchange.

The mammalian shc gene encodes three overlapping proteins of 46, 52 and 66 kDa which possess variable amounts of N-terminal sequence, a PTB domain, a central glycine and proline-rich region which is reminiscent of collagen (the collagen homology domain, CH1) and a C-terminal SH2 domain (figure 1-8) (505,643). p46\textsuperscript{shc} and p52\textsuperscript{shc} arise by differential use of translation initiation sites within a 3.4 kb shc mRNA, and are expressed in all mammalian cells that have been analyzed. A second transcript encodes the minor and more variably expressed 66 kDa protein as well as p52\textsuperscript{shc} and to a lesser extent, p46\textsuperscript{shc} (644). Shc proteins contain two domains which potentially mediate interaction with tyrosine phosphorylated proteins; the SH2 domain prefers sequences containing p.Tyr-(Gln/Leu/Ile/Tyr)-X-(Leu/Ile/Met) (180) whereas the Shc PTB domain recognizes ψ-Asn-Pro-X-p.Tyr motifs where ψ is a hydrophobic residue (490,491). The Shc PTB domain can also bind acidic phospholipids (497) but the physiological relevance of this is unknown as the isolated Shc PTB domain does not localize to the plasma membrane when microinjected into cells (646). Multiple potential SH3-binding sites are found in the CH1 region of Shc, together with a motif that mediates interactions with α and β-adaptins and might therefore regulate endocytosis (645). p66\textsuperscript{shc} contains an additional N-terminal region rich in glycine and proline residues (CH2) which also contains putative SH3-binding sites.
Overexpression of p46<sup>shc</sup> and p52<sup>shc</sup>, but not p66<sup>shc</sup>, induces transformation of NIH 3T3 cells (505,644) and stimulates neurite extension in PC12 pheochromocytoma cells (647), a response normally dependent on Ras activation (524). Shc-induced differentiation of PC12 cells is inhibited by expression of DN Ras indicating that Ras functions downstream of p42/p56 Shc (647). All three Shc proteins are phosphorylated on tyrosine and serine in response to growth factors such as EGF and insulin and in cells transformed by the v-Src or v-Fps tyrosine kinases, and concomitantly bind the SH2 domain of the Grb2 protein (505,647-650). Recently, Shc was shown to participate in signalling pathways activated by G-protein coupled receptors and Ca<sup>2+</sup> (651,652).

The cDNAs for two Shc-related proteins, designated ShcB and ShcC, have been cloned (653,654). Unlike Shc, these proteins have expression largely restricted to tissues of neural origin suggesting a role in cell type-specific signalling.

1.5.2.4. Regulation of the Ras GDP/GTP cycle by external stimuli

A number of stimuli induce a modest and transient increase in active p21<sup>ras</sup>.GTP complexes in hematopoietic cells, suggesting a role for Ras in transducing the signals initiated by hematopoietic growth or activation factors (575,655-663). For example, Ras activation correlates with the proliferative signals of CSF-1, IL-3 and GM-CSF and is essential for thymocyte development and the prevention of anergy (663-666). Either downregulation of Gap activity or an increase in guanine nucleotide exchange may underlie the increase in Ras.GTP depending on the cell type (575,655,667-671).

While Ras activity is rapidly increased upon serum stimulation of quiescent NIH 3T3 cells, improved techniques for the measurement of activated Ras have revealed that maximum activity occurs in mid G1 phase (approximately 5 hrs later) and is dependent on RNA and protein synthesis (535). Only the early phase of Ras activation is temporally correlated with activation of the Raf/Mapk pathway which is described below.
1.5.2.5. Pathways downstream of Ras

Erk1 and Erk2 are ubiquitously expressed cytosolic proline-directed serine/threonine kinases and the final participants in a triple kinase cascade which regulates translation, transcription and the cell cycle (figure 1-10) (672,673). Activation of the Mapk pathway is necessary for cell growth or differentiation in response to growth factors, v-Src or oncogenic Ras (674-676). To initiate this cascade, Ras-GTP interacts with two sites on the S/T kinase Raf and recruits Raf to the plasma membrane where it is activated by a poorly understood mechanism (677-684). 14-3-3 proteins constitutively bind Raf (685-687) and may have a role in stabilization of both the inactive and active conformations of Raf, or function as adaptors when bound to active Raf-1 (688). Of interest, a specific phosphoserine-dependent consensus binding motif for 14-3-3 proteins (689) is found in Bcr which interacts with Raf-1 in a 14-3-3 dependent manner (690). The tyrosine phosphorylation of Raf may be involved in its enzymatic activation (691-694). Indeed, the NRTK Jak2 associates with and activates Raf-1 in a Ras-dependent manner (695), and Raf-1 is tyrosine phosphorylated in v-abl-transformed cells (696).

Raf in turn activates the dual specificity Mapk kinases (termed Mapkk or Mek) which phosphorylate a Tyr-X-Thr motif in Erk1 and Erk2 (697,698). Other upstream regulators of Raf also exist (figure 1-10) (699-704). Once activated, Erk1 and Erk2 can translocate into the nucleus and enhance the activity of transcription factors to induce immediate-early gene expression (705,706). RNA Pol II CTD is also a substrate of nuclear Erks (707). The duration of Erk activation may determine nuclear translocation (1047) and whether cyclin D expression is subsequently increased (708). Erk1 and Erk2 also regulate the activity of a number of proteins in other cellular compartments such as PLA2 in the cytosol, and the rsk-encoded protein kinase which can itself translocate into the nucleus (705,706,709). Proteins which are upstream such as Mek, Raf, Sos and the EGF R are also phosphorylated by Erks (705,706,710,711). The phosphorylation
Figure 1-10. Ras signalling pathways.

Pathways downstream of mammalian Ras include the Mapk pathway and pathways leading to reorganization of the actin cytoskeleton. In addition to Raf-1, multiple other proteins directly interact with Ras and may function as effectors. These include Ras guanine nucleotide dissociation inhibitors, Mekk, PKCζ, p120-Gap/Nf-1 and the 110 kDa catalytic subunit of PI3K. PI3K is also a potential upstream regulator of Ras. Negative regulators of the Mapk pathway include cAMP and the phosphatases PP2A, Mkp-1 and Pac I. Although not shown, GTPases such as Ras pass through a nucleotide-free state when acted upon by GEFs.

Abbreviations: GEF, guanine nucleotide exchange factor; S/T, serine/threonine
of upstream targets may be involved in either negative or positive feedback mechanisms. A substantial portion of Erks is bound to the cytoskeleton suggesting a role in cytoskeletal reorganization (712).

At least two additional subgroups of mammalian Mapks exist, characterized by the Jnk/Stress activated protein kinase (Sapk) and the p38 kinases which are the mammalian homologues of the yeast kinase Hgl (713-715). These subgroups of Mapks may undergo activation by predominantly distinct stimuli (715). However, some overlap exists and Ras activates the Jnk cascade (716).

Recent studies have revealed several potential connections between the Ras-Mapk pathway and the Jak-Stat signalling pathway which is also widely utilized by cytokine receptors (500,717-721). The co-ordinated action of Jak-Stat and Raf/Mapk pathways may serve to maximally activate the transcription of immediate-early genes such as c-fos which contain response elements for both Stats and Erks (718).

1.5.2.6. Ras pathways are linked to the cytoskeleton

In addition to initiating DNA synthesis, mitogen-stimulated or oncogenic forms of Ras induce less well characterized pathways which lead to changes in cell morphology. These pathways involve activation of the guanine nucleotide binding proteins Rac and Rho (figure 1-10). Rho proteins control mitogen-responsive actin stress fiber formation and focal adhesions in fibroblasts as well as platelet aggregation, whereas Rac proteins regulate the respiratory burst of neutrophils and membrane ruffling (lamellipodia) (722-725). Ras-dependent membrane ruffling correlates with the ability of Ras mutants to activate PI3K (726,727). While PI3K is apparently upstream of Rac (728), some studies suggest that Rho lies on a different pathway in the induction of actin cytoskeletal rearrangements (726). In Swiss 3T3 cells, Rac appears to be upstream of Rho (722,723,729). Cross-talk occurs between Rac, Rho and a third Rho member, CDC42, although the precise links
are not fully defined. All three GTPases are required for progression through G1 in fibroblasts (727) and are essential for transformation by Ras (675,730,731). Rac is also necessary for v-abl-mediated transformation of NIH 3T3 cells (732). p65<sup>PAK</sup>, a target of Rac, is likely responsible for Rac-mediated activation of the Jnk/Sapk pathway (731). Multiple other potential targets of Rac and/or Rho have been identified (733). However, those effectors responsible for cytoskeletal organization remain an enigma (734).

1.6. OUTLINE OF THE THESIS

Identification of PTK targets is necessary for understanding both normal signal transduction pathways and the means by which these pathways are deregulated during cellular transformation. At the time this project was initiated, it was known that tyrosine kinase activity correlated with a number of hematopoietic processes, most notably transformation by the Bcr-Abl oncoproteins. However, those proximal targets of PTKs which are critical to the regulation of normal hematopoiesis or leukemogenesis were largely uncharacterized. Some studies had indicated that hematopoietic growth factors and oncogenic tyrosine kinases encoded by v-abl or v-fms could increase Ras-GTP levels (521,525). It was also known that v-myc co-operated with p210 Bcr-Abl in the transformation of fibroblasts (735) and subsequently it was reported that c-myc, which was thought to be downstream of Ras, was required for transformation by p185 Bcr-Abl (481). These data suggested that p21<sup>ras</sup> might play a crucial role in transformation initiated by the Bcr-Abl kinases. Evidence was also beginning to emerge in non-hematopoietic cells that SH2 and SH3 domains play fundamental roles in mediating interactions between signalling proteins. Studies were therefore undertaken to characterize the signalling pathways stimulated by the two common isoforms of Bcr-Abl, and in particular, to determine whether newly characterized SH2/SH3-containing regulators of Ras participated in such pathways. In Chapters 2 and 3, data are presented
that implicate a number of enzymes and adaptor molecules in Bcr-Abl-mediated transformation. Several of these proteins provide mechanisms whereby the Bcr-Abl oncoproteins may be directly linked to Ras. The identification of multiple regulators of Ras as targets of Bcr-Abl is consistent with a prominent role for p21\textsuperscript{ras} in \textit{bcr-abl}-mediated transformation. In Chapter 4, some of these proteins are also demonstrated to participate in cytokine-stimulated pathways which control the survival, proliferation or activation of normal primary hematopoietic cells. Taken together, these data support the notion that leukemogenesis involves the constitutive stimulation of signalling molecules that are normally transiently activated in cells responding to cues within the hematopoietic microenvironment. The structural basis for the interaction of PTKs with some of their targets was also investigated. These data suggest that SH2-mediated interactions are important in hematopoietic cell signalling, and that the Bcr element of the Bcr-Abl fusion proteins contributes to the target specificity of the activated Abl kinases. This is a novel function of the Bcr moiety which has not previously been defined, and provides new insight into the potential mechanisms whereby fusion of the \textit{abl} and \textit{bcr} genes is oncogenically activating.
CHAPTER 2

POTENTIAL TARGETS OF THE BCR-ABL ONCOPROTEINS INCLUDE THE RAS GTPase ACTIVATING PROTEIN p120-GAP AND ASSOCIATED PROTEINS

Attribution of data: all of the experiments described in this chapter were performed by myself.
2.1. ABSTRACT

The 210 and 185 kDa Bcr-Abl oncoproteins are chimeric proteins that possess deregulated Abl kinase activity and are strongly implicated in the pathogenesis of chronic myelogeneous leukemia (CML) and a subset of acute lymphoblastic leukemia (ALL). Understanding the mechanisms by which the Bcr-Abl proteins elicit their biological effects requires the identification of their intracellular targets. The guanine nucleotide binding protein p21\textsuperscript{ras} is essential for the mitogenic or oncogenic signals of a number of activated tyrosine kinases and is negatively regulated by GTPase activating proteins such as p120-Gap. p120-Gap interacts with GTP-bound Ras and stimulates an intrinsic GTPase activity which hydrolyzes GTP thereby converting Ras to its inactive, GDP-bound form. Our laboratory has recently demonstrated that p120-Gap and two associated proteins, p62 and p190, are phosphorylated on tyrosine in mitogen-stimulated fibroblasts and also in cells transformed by v-src, v-abl or v-fms. In this chapter, evidence is provided that p120-Gap and its associated proteins are targets of the common isoforms of Bcr-Abl. Bcr-Abl stably complexes with p120-Gap \textit{in vivo}, in an interaction which is likely mediated by the Gap N-terminal SH2 domain. Multiple p120-Gap-associated phosphoproteins, most notably p62 and p190, were readily detected in fibroblast or hematopoietic cell lines transformed by either Bcr-Abl protein. Furthermore, p62 was tyrosine phosphorylated in 8/9 primary acute phase CML or Ph+ ALL blast cell samples tested. These results suggest that p120-Gap and its associated proteins are targets relevant to the initiation or maintenance of the Ph+ leukemias. Other potential direct or indirect targets of Bcr-Abl include PLC-\gamma1 and PI3K, intermediaries in phospholipid metabolism, and the GEF Vav. The ability of Bcr-Abl to interact \textit{in vitro} with a subset of SH2 domains isolated from these additional targets suggests that a network of SH2-mediated interactions ultimately drives Bcr-Abl-mediated oncogenesis.
2.2. INTRODUCTION

The Bcr-Abl oncoproteins are chimeric proteins that possess deregulated Abl kinase activity and are strongly implicated in the pathogenesis of chronic myelogeneous leukemia (CML) and a subset of acute lymphoblastic leukemia (ALL) (389,390,398-401,415,761-763). Transformation by p185 or p210 Bcr-Abl induces the tyrosine phosphorylation of multiple intracellular proteins (414,764-768). However, the identity of the majority of these phosphoproteins is as yet unknown. Genetic and biochemical evidence exists that the small guanine nucleotide binding protein p21ras is required for oncogenesis or mitogenesis induced by a number of activated tyrosine kinases (521-523,525,526,769). Ras proteins cycle between an active GTP-bound conformation and an inactive GDP-bound form, and are regulated by several classes of proteins that include guanine nucleotide exchange factors and GTPase activating proteins (reviewed in: 540,541).

The 120 kDa GTPase activating protein p120-Gap is composed of multiple domains with distinct structural and functional properties; these include two N-terminal Src Homology 2 (SH2) domains with an intervening Src Homology 3 (SH3) motif, an N-terminal proline-rich segment, a more central calcium-dependent lipid binding domain (562) and a carboxy terminal domain which is related to sequences found in the products of the IRA1 and IRA2 genes of Saccharomyces cerevisiae and human neurofibromin 1 (545,583,770,771). The C-terminal region of p120-Gap binds to the active GTP-bound conformation of Ras and greatly accelerates GTP hydrolysis, thereby converting Ras to its inactive state (565,772). Although the C-terminal p120-Gap domain is sufficient for GTPase promoting activity, the N-terminal region may enhance such activity (565). The observation that p120-Gap interacts with a region overlapping with the Ras effector domain has led to the hypothesis that this protein may simultaneously be involved in signalling downstream of Ras (581,773). A number of studies in mammalian cells support such a role (584,588-595). In one
model, interaction with activated Ras is proposed to induce a conformational change that allows the p120-Gap N-terminal SH2-SH3-SH2 region to interact with downstream targets (583,774). Indeed, the isolated N-terminal portion of p120-Gap can direct interactions with cellular phosphoproteins in vivo (587). However, other studies in mammalian cells suggest that p120-Gap functions predominantly as a negative regulator of Ras since overexpression of full length p120-Gap or the C-terminal domain alone increases the relative amount of GDP-bound Ras and blocks transformation mediated by overexpressed c-ras or v-src (598-600).

In fibroblasts stimulated with platelet derived growth factor (PDGF) or epidermal growth factor (EGF), and in cells transformed by v-src, v-abl, v-fps or v-fms, p120-Gap is phosphorylated on tyrosine and serine residues and associates with two other phosphoproteins, a minor but highly phosphorylated 62 kDa species (p62) and a 190 kDa protein (p190) (577,775,776). The rat cDNA for p190 has recently been isolated and encodes a polypeptide which contains an N-terminal consensus sequence for guanine nucleotide binding and a C-terminal GTPase activating domain for members of the Rho/Rac family (777-779).

Non-transforming mutants of the cytosolic tyrosine kinases v-Src and v-Fps have been described that retain tyrosine kinase activity but fail to phosphorylate p120-Gap and p120-Gap-associated p62, suggesting that one or both of these proteins are biologically important substrates of oncogenic tyrosine kinases (246,780-782). In a recent comparison of the phosphoprotein profiles induced in Rat-1 fibroblasts acutely infected with recombinant retroviruses expressing either p185 or p210 Bcr-Abl, a 62 kDa phosphoprotein was reported to appear exclusively in whole cell lysates of cells expressing p185 Bcr-Abl (414). Transformation-defective variants of p185 Bcr-Abl, which retain autophosphorylation activity and activity toward some exogeneous substrates, failed to phosphorylate the 62 kDa protein suggesting that this unidentified protein might be critical for p185
Bcr-Abl's transforming properties (783). I have therefore investigated whether p120-Gap and its associated proteins are targets of the common isoforms of Bcr-Abl. Data presented in this chapter indicate that p120-Gap is a relatively inefficient substrate of p210 and p185 Bcr-Abl but stably complexes with both isoforms in vivo. Based on in vitro studies, this interaction is mediated by the p120-Gap [N]-SH2 domain. Multiple p120-Gap-associated phosphoproteins, most notably p62 and p190, are readily detected in fibroblast or hematopoietic cell lines transformed by either Bcr-Abl protein. Furthermore, tyrosine phosphorylated p62 was detected in association with p120-Gap in the majority of primary acute phase CML or Ph+ ALL leukemic blast cell samples tested. These results suggest that p120-Gap and its associated proteins participate in signalling pathways activated during Ph+ leukemogenesis.

A number of other SH2/SH3-containing signalling proteins are also identified as potential participants in Bcr-Abl signalling pathways. These proteins include intermediaries in phospholipid metabolism and the Rac guanine nucleotide exchange factor Vav. The ability of Bcr-Abl, or other phosphoproteins present in bcr-abl-transformed cells, to interact with a subset of SH2 domains isolated from these additional targets suggests that Bcr-Abl induces a network of SH2-mediated interactions which ultimately drives transformation.
2.3. MATERIALS AND METHODS

Cell culture

ALL A1, K562, SUP-B15, WL-1, and AML OCI 2 and AML OCI 3 cell lines were maintained in RPMI medium supplemented with 10-20% fetal bovine serum (FBS) depending on the particular cell line (784-787). AML OCI 2 and AML OCI 3 cell lines were provided by Dr. M. Minden (University of Toronto) and ALL A1 was provided by Dr. M. Freedman (University of Toronto). The SUP-B15 cell line was provided by Dr. S. Smith (University of Chicago) and the K562 cell line was obtained from the American Type Culture Collection (Rockville, Maryland). Dr. J. Ihle (St. Jude Research Hospital, Memphis) provided the WL-1 Ph+ ALL cell line. The IL-7 dependent pre-B lymphoid line, Clone H, was initially derived by single cell cloning of non-adherent cells from a long term bone marrow culture (786); Clone H cells infected with recombinant retroviral vectors encoding p210 Bcr-Abl, p185 Bcr-Abl or the cytokine IL-7 were a gift of Dr. O. Witte (UCLA) (788) and were maintained in RPMI medium supplemented with 5% FBS and 5x10^{-5} M 2-mercaptoethanol. Leukemic blast cells, provided by Dr. M. Minden (University of Toronto) and the Pediatric Oncology Group Tumor Bank (Memphis, Tennessee), were obtained from peripheral blood samples by Ficoll Hypaque fractionation and cryopreserved until use. Upon thawing, primary blast cells were maintained in RPMI medium supplemented with recombinant IL-3 (provided by Dr. S. Lymen, Immunex, Seattle) for 48-96 hours prior to lysis. Viable cells were determined by trypan blue exclusion.

Rat-1 fibroblasts were grown in Dulbecco modified Eagle medium (DME) containing 5% FBS. Rat-1 cells stably expressing p185 or p210 Bcr-Abl were a gift of Dr. O. Witte (UCLA). The establishment of these cells has been described (414,415). In brief, recombinant cDNA clones representing the complete coding sequence for p210 or p185 Bcr-Abl plus approximately 150 base
pairs of 5' untranslated region, were inserted into the EcoRI restriction site of the Moloney murine leukemia virus based vector pMV6 which contains the dominant selectable Tn5 neo gene (789). Upon co-transfection with a construct encoding Moloney helper virus and selection with G418, cell populations were isolated that stably express high levels of p210 (R1-p210) or p185 Bcr-Abl (R1-p185). Rat-1 cells were also stably infected with retroviruses encoding transformation-defective p185 Bcr-Abl in which bcr-encoded residues 3-426 were deleted by site-directed mutagenesis (R1-p185 Δ3-426) (787). R1-p210, R1-p185 and R1-p185 Δ3-426 cells were maintained in DME supplemented with 5% FBS and 50 μg ml⁻¹ of G418. Rat-2 cells expressing v-src were grown in DME with 5% FBS and have previously been described (790,791). All cells were cultured at 37°C in humidified incubators with 5% CO₂ (vol/vol).

Antibodies and immunoprecipitations

Polyclonal rabbit anti-p120-Gap antibodies were raised against amino acid residues 171-558 of human p120-Gap (577), and used for both immunoprecipitations and Western blotting. Anti-Bcr, anti-pEx 2/3 Abl and anti-pEx 5 Abl antibodies for immunoprecipitation have been described (192,761,792). Polyclonal rabbit anti-Shc antibodies were raised against a GST-SH2 fusion protein corresponding to amino acid residues 366-473 of human Shc, and the crude antiserum was affinity purified using the GST-Shc SH2 protein following preclearing with lysates of induced GST-expressing bacteria (505). Anti-Vav antibodies directed against amino acid residues 528-541 of murine Vav (793) and anti-Shp-1 antibodies (794) were a gift of Dr. S. Katzav (St. Jude Research Hospital, Memphis) and Dr. J. Matthews (Washington University, St. Louis) respectively. Anti-PLC-γ1 antibodies have been described (795). Confluent monolayers of cells, or a known number of viable cells in suspension, were lysed in 1 ml of ice-cold PLC lysis buffer (50 mM of N-2
hydroxyethyl piperazine-N'-2 ethane sulfonic acid (HEPES, pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μg ml⁻¹ of leupeptin, 10 μg ml⁻¹ of aprotinin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride and 1 mM phenylmethylene sulfonyl fluoride) and all subsequent steps carried out at 4°C. Cell lysates were centrifuged for 15 min at 10 000 g and the supernatant collected. For experiments involving Rat-1 fibroblasts, protein concentrations of the clarified supernatants were determined by a modified Lowry protein assay (Sigma) and similar quantities of protein in each sample used for immunoprecipitation or analysis as whole cell lysates. For immunoprecipitation, cell lysates were incubated with the appropriate antibody and 100 μl of 10% protein A-sepharose for 90 min at 4°C. The immune complexes were recovered, washed 3X in 20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate (HNTG) and following resuspension in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS (wt/vol), 0.5% β-mercaptoethanol (vol/vol)), heated for 3 min at 100°C.

**SDS-PAGE and Western blotting**

7.5, 8.25 or 10% polyacrylamide gels were cast using a 29.2:1 molar ratio of acrylamide and bis-acrylamide in 375 mM Tris (pH 8.8), 0.1% SDS, 0.11% N,N,N’N’-tetramethylenediamine (TEMED) and 0.04% ammonium persulfate (APS). Stacking gels consisted of 4.5% acrylamide:bis-acrylamide, 125 mM Tris (pH 6.8), 0.1% SDS, 0.2% TEMED and 0.03% APS. Proteins were resolved by running gels in a Bio-Rad minigel apparatus at 200 V constant voltage in 25 mM Tris, 192 mM glycine (pH 8.3), and 0.1% (wt/vol) SDS. After electrophoresis, gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol) and transferred to nitrocellulose with a semi-dry transfer apparatus at 0.8 mA·cm⁻² for 60 min (796).
For immunoblotting with all antibodies except anti-phosphotyrosine (p.Tyr), filters were blocked in Tris buffered saline (TBS) (20 mM Tris (pH 7.5), 150 mM NaCl) with 5% Carnation skim milk powder overnight at 4°C. Antibody incubations were carried out in TBS with 3% milk powder and 0.1% Tween 20 for 2 hrs at room temperature. The filters were subsequently washed 4X in TBS plus 0.1% Tween 20. To detect Abl proteins, a mouse monoclonal antibody directed against the carboxy terminal region of c-Abl was used at a concentration of 1 μg ml⁻¹ (792). Immunoblotting with polyclonal anti-Gap (577), anti-Vav (793), anti-PLC-γ1 (795) and anti-Shp-1 (794) antiserum was performed as previously described. To detect Shc proteins, blots were probed with affinity purified anti-Shc antibodies at a concentration of 2 μg ml⁻¹ for 2 hrs (505). For anti-p.Tyr immunoblotting, filters were blocked in TBS containing 5% bovine serum albumin and 1% ovalbumin, and incubated with affinity purified anti-p.Tyr antiserum (797,798). Anti-p.Tyr filters were subsequently washed twice in TBS, once in TBS with 0.05% Nonidet-P40, followed by 2 more washes in TBS.

Anti-p.Tyr and anti-Shc blots were probed with 2 μCi [¹²⁵I]-labelled protein A (35 μCi μg⁻¹, Amersham) in 10 mls blocking solution for 45 min and washed in the same regimens as described above. The filters were exposed to Kodak XAR-5 film at -70°C with an intensifying screen for 16-120 hrs. For anti-Abl, anti-p120-Gap and all other blots, a secondary incubation was carried out with horseradish peroxidase conjugated anti-immunoglobulin antiserum (Sigma) prior to development with chemiluminescent reagents (Amersham) and exposure to Kodak XRP-5 film for different times ranging from 1 sec to 10 min.
In vitro binding experiments with GST or TrpE fusion proteins

A restriction fragment from human p120-Gap, corresponding to the SH2-SH3-SH2 amino terminal region (residues 178-444, Gap-N) was subcloned into a pATH bacterial TrpE expression vector using engineered restriction sites as previously described (798). The carboxy terminal half of p120-Gap (Gap-C) including all residues required to stimulate p21 Ras GTPase activity was also subcloned into a pATH TrpE expression vector (800). Cultures of Escherichia coli strain RR1 transfected with the parental or derivative pATH expression vectors were grown and induced with indole acrylic acid (801). Collected cells were lysed with ice-cold PLC lysis buffer, sonicated and clarified by centrifugation at 15 000 g for 15 min. Supernatants were incubated with polyclonal anti-TrpE antiserum (raised against the N-terminal 323 residues of E. coli TrpE protein) and protein A-Sepharose beads. After incubation for 90 min, the immune complexes were washed 3X in HNTG, divided into aliquots and stored at -70°C until use. The different TrpE proteins were quantified by immunoblotting with monoclonal anti-TrpE antibodies (Oncogene Science) followed by incubation with anti-mouse immunoglobulin-alkaline phosphatase conjugates (Sigma). Antibody complexes were visualized by incubating blots for 5-10 min in 100 mM Tris HCl (pH 9.5), 100 mM NaCl and 5 mM MgCl₂ containing 6.6 μl of nitroblue tetrazolium (50 μg ml⁻¹ in 70% formamide) and 3.3 μl of 5-bromo-4-chloro-3-indolyl phosphate per ml (50 μg ml⁻¹ in 100% dimethyformamide) (Sigma). Similar quantities of each TrpE fusion protein were mixed with cell lysates for 90 min at 4°C. Cellular proteins bound to the immobilized fusion proteins were recovered by centrifugation, washed 3X in HNTG, resuspended in SDS sample buffer and boiled at 100°C for 3 min prior to analysis.

For GST fusion proteins, the p120-Gap [N]-SH2 and [C]-SH2 domains (corresponding to residues 181-276 and 351-442 of human p120-Gap respectively) were isolated by the polymerase
chain reaction (PCR) and subcloned into pGEX expression vectors as previously described (802,803). The GST-Crk SH2 fusion protein corresponded to residues 205-386 of p47^{gag-crk}, identical to the SH2 domain of c-Crk (781,804,805). GST PI3K [N]-SH2, [C]-SH2 and [N+C]-SH2 fusion proteins corresponded to residues 312-444, 612-722 and 312-722 of bovine p85α PI3K respectively (806). PLC-γ1 [N]-SH2 and [C]-SH2 corresponded to amino acid residues 547-659 and 663-759 respectively of bovine PLC-γ1 (193). For the Abl SH2 fusion protein, the DNA encoding the N-terminal region of murine type IV c-Abl was isolated by PCR beginning with the Stul restriction site and terminating at the 3' end of the SH2 domain. The Abl SH3 domain was subsequently deleted by PCR, and the fragment subcloned into a pGEX vector. The Vav SH2 domain (amino acid residues 621 to 717 of human Vav) was also isolated by PCR for subcloning into an appropriate pGex vector. GST-Vav SH2 and Abl SH2 fusion proteins were gifts of Dr. B. Margolis (New York University Medical Centre) and Dr. J. Wang (UCSD) respectively. Bacterial cultures expressing the pGex vectors were grown in LB and ampicillin 100 μg ml^{-1}, and induced with 1 mM isopropyl thiogalactopyranoside (IPTG) for 4 hr at 30° or 37°C depending on the solubility of the particular GST fusion protein. The induced bacteria were lysed at 4°C by sonication in PLC lysis buffer supplemented with 10 mM dithiothreitol, and the GST fusion proteins recovered from clarified lysates by glutathione-agarose (Pharmacia). The GST fusion proteins were stored at 4°C for up to one week or flash frozen and stored at -70°C until use. The amount of each GST fusion protein was determined by comparison of bands on a Coomassie Blue stained gel to a dilution series of bovine serum albumin; similar quantities of fusion proteins (approximately 5-10 μg) were incubated with cell lysates and binding experiments carried out as described for the trpE fusion proteins.
2.4. RESULTS

2.4.1. Expression of either isoform of Bcr-Abl induces the tyrosine phosphorylation of Gap-associated proteins in Rat-1 fibroblasts

Acute infection of Rat-1 fibroblasts with recombinant retroviruses encoding p210 or p185 Bcr-Abl induces morphological transformation and increased foci formation (414). Consistent with its characteristic association with a more rapidly aggressive acute leukemia (807), p185 Bcr-Abl exhibits higher specific activity than p210 Bcr-Abl in vitro and induces the growth of 100-fold more colonies in soft agar (389, 401, 404, 414). The tyrosine phosphorylation of a 62 kDa protein was recently reported to appear exclusively in whole cell lysates of Rat-1 cells transformed by p185 but not p210 Bcr-Abl (414). To ascertain whether this protein represents p120-Gap-associated p62 and to investigate whether p120-Gap or other Gap-associated proteins are substrates of Bcr-Abl, Rat-1 cells were stably infected with retroviruses encoding p185 (R1-p185) or p210 Bcr-Abl (R1-p210). Parental Rat-1 cells and cells expressing each isoform of Bcr-Abl were lysed in PLC lysis buffer and equivalent amounts of total cellular protein precipitated with polyclonal anti-Gap or pre-immune serum. Precipitated proteins were washed and separated by SDS-PAGE on a 7.5% polyacrylamide gel. Whole cell lysates which contained 25-fold less protein than the amount used for immunoprecipitation were also assessed. Following transfer to a nitrocellulose filter, the proteins were immunoblotted with anti-p.Tyr antibodies followed by[^125I]-protein A detection. The R1-p185 whole cell lysate contained greater total quantities of phosphorylated proteins than parental cells or cells transformed by p210 Bcr-Abl and a particularly prominent tyrosine phosphorylated 62 kDa band (figure 2-1a, lanes 7-9). In the anti-p120-Gap immunoprecipitates, a tyrosine phosphorylated 62 kDa protein was dramatically increased in both R1-p185 (lane 4) and R1-p210 cells (lane 6) when compared to parental cells (lane 2). These differences could not be attributed to
**Figure 2-1**

*Increased tyrosine phosphorylation of p120-Gap-associated p62 and p190 in Rat-1 fibroblasts expressing p185 or p210 Bcr-Abl.*

**a.** Lysates of parental Rat-1 fibroblasts (R1), R1-p185 and R1-p210 cells were immunoprecipitated with anti-p120-Gap (lanes 2, 4 and 6) or normal rabbit serum (C) (lanes 1, 3 and 5). The immune complexes were washed and the precipitated proteins electrophoresed through a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with anti-p.Tyr antibodies followed by $[^{125}]$-labelled protein A detection. Lanes 7-9 represent whole cell lysates. The positions of p62 and p190 are indicated by arrows. An 18 hr exp is shown.

**b.** Similar levels of p120-Gap are expressed in R1, R1-p185 and R1-p210 cells. Cell lysates containing comparable quantities of total protein were immunoprecipitated with anti-p120-Gap antibodies and the recovered proteins blotted with the same antiserum followed by horseradish peroxidase conjugated antibody directed against rabbit Ig and detection with enhanced chemiluminescence.
### a.

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<tr>
<td></td>
<td>R1-p210</td>
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</tbody>
</table>

**Blot:** αp.Tyr

### b.

**αGAP IPs**

1. R1
2. R1-p185
3. R1-p210

**p120 GAP**

1. 2
2. 3

**αGAP**
altered p120-Gap expression as similar amounts of total precipitable p120-Gap were present in all three cell populations (figure 2-1b).

A tyrosine phosphorylated p120-Gap-associated 190 kDa protein was also detected in cells transformed by either Bcr-Abl oncoprotein, but, if present, was below the level of detection in parental cells. An additional 130 kDa phosphoprotein which co-precipitated with p120-Gap from the bcr-abl-transformed cells was seen on longer exposures of the anti-p.Tyr blot but was less consistently observed than p190 and p62 in subsequent experiments (data not shown). p120-Gap itself was inefficiently phosphorylated on tyrosine in R1-p185 and R1-p210 cells and was detected only upon prolonged exposure of the autoradiogram (data not shown). This finding is consistent with previous observations that only a small fraction of total precipitable p120-Gap is phosphorylated on tyrosine in v-src-transformed cells (583,798,808).

2.4.2. Tyrosine phosphorylated proteins in Ph+ leukemic cell lines include p120-Gap-associated p62 and p190

To investigate whether p120-Gap-associated proteins are phosphorylated in the natural host cells transformed by Bcr-Abl, Ph+ leukemic cell lines were initially analyzed. The K562 cell line, originally established from a CML patient in myeloid blast crisis, expresses amplified p210 Bcr-Abl and retains characteristics of the erythroid as well as the myeloid lineage (785), whereas the SUP-B15 cell line is lymphoid and expresses p185 Bcr-Abl (786). In addition to bands corresponding to autophosphorylated Bcr-Abl, multiple p.Tyr-containing proteins were detected in the SUP-B15 and K562 whole cell lysates (figure 2-2a, lanes 5 and 6). Particularly prominent were proteins with apparent molecular weights of ~130-140, 120, 90-100, 75, 62 and 55 kDa in the SUP-B15 cells and ~140, 120, 90 and 55 kDa in the K562 cells. In the anti-Gap immunoprecipitates of both Ph+ cell
Figure 2-2

The tyrosine phosphorylation of p120-Gap-associated proteins in Ph+ leukemic cell lines.

a. Lysates containing $2 \times 10^7$ cells from the SUP-B15 or K562 Ph+ cell lines were immunoprecipitated with polyclonal anti-p120-Gap antibodies or pre-immune serum (C) as indicated. Following resolution by SDS-PAGE on a gel containing 7.5% polyacrylamide and transfer to nitrocellulose, the immunoprecipitated proteins were blotted with anti-p.Tyr antibodies followed by detection with $^{125}$I-labelled protein A (lanes 1-4). Lanes 5 and 6 show whole cell lysates of each cell line. The positions of p190, p120-Gap, p62 and a 55 kDa Gap-associated protein are shown. Molecular size markers are also indicated and also the location of the IgH chain which was located at the edge of the photograph. A 36 hr exposure of the autoradiogram is shown.

b. Pre-immune and anti-p120-Gap immunoprecipitates were blotted with anti-p120-Gap antibodies and $^{125}$I-labelled protein A to verify specific immunoprecipitation of p120-Gap.

c. $2 \times 10^7$ cells from a Ph- ALL cell line (HSC A1) and two Ph- AML cell lines (OCI 2 and 3) were immunoprecipitated with anti-Gap antibodies (lanes 1, 2 and 3 respectively) and the recovered proteins probed with anti-p.Tyr antibodies and $^{125}$I-labelled protein A. An anti-p120-Gap immunoprecipitate of v-src-transformed cells (v-src R2) (lane 4) was included as a positive control. An 18 hr exp is shown. On the original autoradiogram, tyrosine phosphorylated p190 and p120-Gap were detected in the v-src R2 immunoprecipitate.
lines, a 120 kDa phosphoprotein which may correspond to p120-Gap was detected upon prolonged exposure of the autoradiogram. However, this band may have represented in part a non-specifically precipitating protein since a minor phosphoprotein of similar mobility which was not immunologically related to p120-Gap was also precipitated by pre-immune serum (data not shown). In the reciprocal experiment, only minor amounts of total cellular p120-Gap were precipitated by anti-p.Tyr antibodies consistent with low levels of intrinsic p120-Gap phosphorylation or as a result of p120-Gap's co-precipitation with associated phosphoproteins (data not shown). As a 120 kDa phosphorylated band is prominent in the K562 and SUP-B15 whole cell lysates which contained 25-fold less protein, substrates of Bcr-Abl likely include additional polypeptides with similar mobilities to p120-Gap. Potential candidates for these proteins are 120 and 130 kDa proteins previously identified as substrates of v-Src (742,809-811) and the 120 kDa protein encoded by the proto-oncogene cbl (812).

A 62 kDa protein was the predominant p.Tyr-containing protein detected in the anti-Gap immunoprecipitate of SUP-B15 cells (figure 2-2a, lane 2). Lesser amounts of p62 but an additional 55 kDa phosphoprotein (p55) co-precipitated with p120-Gap from an equivalent number of K562 cells (lane 4). The differences in co-precipitating p62 could not be attributed to marked differences in p120-Gap expression (figure 2-2b). The 55 kDa protein was not recognized directly by the anti-Gap antibodies, and migrated more slowly than the phosphorylated IgH chain which was detected in both cell types. In both Ph+ cell lines, Gap-associated p190 was readily detected. Neither p62, p55 or p190 were detected in a Ph- ALL and two Ph- AML cell lines assayed under identical conditions (figure 2-2c) despite similar expression of p120-Gap (data not shown).

To confirm that p62 and p190 phosphorylation may be a direct consequence of Bcr-Abl expression in hematopoietic cells, p210 Bcr-Abl was ectopically expressed in the murine pre-B
lymphoid Clone H cell line (786). Phosphorylated p62 and p190 were detected only in the bcr-abl-expressing cells and not in the parental Clone H cells or in Clone H cells rendered growth factor independent by infection with a retroviral construct expressing IL-7 (data not shown). Interestingly, the autocrine production of IL-7 was not sufficient to transform these cells whereas expression of p185 or p210 Bcr-Abl resulted in clonal outgrowth and transformation (786) concomitant with the tyrosine phosphorylation of p62 and p190. A Gap-associated 55 kDa protein was not detected in the Clone H-p210 cells suggesting that the presence of such a protein may be restricted to myeloid cells.

2.4.3. The 55 and 62 kDa phosphoproteins interact with the amino terminal region of p120-Gap

p62 has previously been shown to interact with the p120-Gap N-terminal region in vitro, notably the [N]-SH2 domain (781,803). While p120-Gap [N]-SH2 alone has strong binding activity for p62, synergism for binding p.Tyr-containing proteins has been observed in the presence of both p120-Gap SH2 domains (791,799). The N-terminal SH2-SH3-SH2 region of p120-Gap (Gap-N) was therefore tested for its ability to recognize p55. Lysates of K562 or SUP-B15 cells were mixed with immobilized trpE fusion proteins containing Gap-N, the carboxy terminal half of p120-Gap (Gap-C) or the parental trpE protein alone, and the recovered proteins blotted with anti-p.Tyr antiserum. In the SUP-B15 cell lysate, a 62 kDa phosphoprotein specifically associated with the trpE fusion protein containing Gap-N (figure 2-3, lane 2). This protein co-migrated with p62 immunoprecipitated with anti-Gap antibodies from v-src-transformed cells (lane 9). In the K562 cell lysate only, a p.Tyr-containing 55 kDa protein was also detected in association with Gap-N but not Gap-C or GST alone. Although the precise p120-Gap residues required for p55 binding remain to be defined, these data are consistent with the possibility that interaction with p55 is mediated by
Figure 2-3

p62 and p55 interact with the amino terminal region of p120-Gap in vitro.

Lysates of SUP-B15 or K562 cells were mixed with immobilized trpE fusion proteins containing the amino terminal SH2-SH3-SH2 region of p120-Gap (Gap-N) (lanes 3 and 7), the carboxy terminal half of p120-Gap (Gap-C) (lanes 4 and 8) or trpE alone (lanes 2 and 6). Lysates were also immunoprecipitated with anti-p120-Gap antibodies (lanes 1 and 5). The protein complexes were recovered and immunoblotted with anti-p.Tyr antibodies followed by detection with $[^{125}]$-labelled protein A. An anti-p120-Gap immunoprecipitate of v-src transformed cells is included in the same gel to verify the relative migration position of p62 (lane 9). On the original autoradiogram, an additional 130 kDa p.Tyr-containing protein which binds p120-Gap-N can be detected in both the SUP-B15 and K562 cell lysate. Comparable quantities of each TrpE fusion protein were used, as assessed by anti-TrpE immunoblotting (not shown).
one or both Gap SH2 domains, in common with complex formation between p62 and p120-Gap.

The shc gene encodes overlapping proteins of 66, 52 and 46 kDa which are implicated in the positive regulation of Ras (505). p52\textsuperscript{shc} is a prominent tyrosine phosphorylated protein in K562 cells (L.P., M.Sc. thesis, University of Toronto, 1994) and migrates with a similar apparent molecular mass as the 55 kDa protein which co-precipitates with p120-Gap. To assess whether Gap-associated p55 represents a Shc protein, an anti-Gap immunoprecipitate of K562 cells was immunoblotted with affinity purified polyclonal anti-Shc antibodies. The anti-Shc antibodies did not recognize p55, nor did p120-Gap co-precipitate with Shc in the reciprocal experiment (data not shown). Gap-associated p55 is therefore distinct from p52\textsuperscript{shc} and may represent a breakdown product of p62, p62 which has undergone differential post-translational modification, or a novel p120-Gap-associated protein.

2.4.4. p120-Gap-associated p62 is phosphorylated on tyrosine in CML acute phase and Ph+ ALL primary blast cells

Primary acute phase mononuclear cells have been reported to display cellular patterns of tyrosine phosphorylation that are similar to those detected in established Ph+ cell lines (813). This suggests that proteins in leukemic cell lines which are substrates for Bcr-Abl are also substrates of the oncoproteins in primary cells. However, primary Ph+ leukemic blast cells obtained from bone marrow or peripheral blood are dependent on growth factors for survival and growth (427) whereas Ph+ cell lines are growth factor-independent and have likely undergone additional genetic alterations during their initial establishment or passage in culture. As these additional changes could conceivably alter the spectrum of targets available to the Bcr-Abl proteins, primary leukemic blast
cells were also analyzed for the presence of tyrosine phosphorylated p120-Gap and associated proteins. Blast cells were obtained by Ficoll Hypaque fractionation of peripheral blood from patients with CML, Ph+ ALL, Ph- ALL or Ph- AML, cryopreserved until use, then thawed and maintained for 48-96 hours in the presence of IL-3. Following cell lysis and immunoprecipitation with anti-Gap antibodies, the immune complexes were analyzed by anti-p.Tyr immunoblotting. Tyrosine phosphorylated p62 was readily detected in 8/9 Ph+ blast cell samples (Table 2-1 and figure 2-4), and in one patient sample, was present as a doublet (lane 4, figure 2-4). In the one Ph+ patient sample which lacked detectable p62, very few viable cells were obtained for assay (patient 9 in Table 1). A 62 kDa protein which co-migrated with p120-Gap-associated p62 was also the predominant protein precipitated by anti-p.Tyr antibodies in one CML myeloid blast crisis (MBC) sample that yielded sufficient cells to perform several immunoprecipitations (figure 2-4b; patient 1 in Table 1). Tyrosine phosphorylated p120-Gap was not detected in any of the samples. In patient 1's cells, significant amounts of p120-Gap were precipitated by anti-Gap antibodies but not by anti-p.Tyr antiserum suggesting that the fraction of p120-Gap which binds tyrosine phosphorylated p62 is small (figure 2-4b). In these experiments, the presence of a tyrosine-phosphorylated 55 kDa protein in the anti-Gap and anti-p.Tyr immunoprecipitates of MBC cells was somewhat obscured by the proximity of the phosphorylated IgH chain.

The p190 protein was less consistently observed in the Ph+ blast cells and was detected in 2/9 samples. The lower rate of detection of p190 may be attributed to the relatively low stoichiometry of tyrosine phosphorylation in p190 (781) and/or inefficient transfer of higher molecular weight proteins to the nitrocellulose filter. Neither p62 nor p190 were detected in 3 Ph- ALL or AML samples prepared under identical conditions.
Table 2-1

Assay of primary leukemic blast cells for the presence of tyrosine phosphorylated p62 and p190.

Ficoll Hypaque separated blast cells from patients in CML myeloid blast crisis (MBC) or lymphoid blast crisis (LBC), Ph+ ALL, 1° ALL and 1° AML, were cultured in the presence of IL-3 for 48-96 hours. All of the cells within a culture were lysed and immunoprecipitated with anti-Gap antibody followed by immunoblotting with anti-p.Tyr antibodies. The number of viable cells assayed for each sample is indicated. The presence (+) or absence (-) of tyrosine phosphorylated p62 and p190 is noted. p120-Gap itself was not detectably tyrosine phosphorylated in any of the samples.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myelogeneous leukemia; CML, chronic myelogeneous leukemia; LBC, lymphoid blast crisis; MBC, myeloid blast crisis; ND, not done.
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1 ^ provided by OCI/PMH  
2 ^ provided by St. Jude's/Pediatric Oncology Tumor Bank  
3 ^ ND, not done
Figure 2-4

Gap-associated p62 is phosphorylated on tyrosine in primary Ph+ leukemic blast cells.

a. Primary leukemic blast cells from two patients with myeloid blast crisis of CML (lanes 1 and 4), de novo Ph-AML (lane 2), Ph- ALL (lane 5) or Ph+ ALL (lane 6) (patients 2, 7, 11, 13 and 10 in Table 2-1 respectively), were obtained by Ficoll Hypaque fractionation of peripheral blood, and maintained in tissue culture for 48-96 hours. The entire cultures were lysed and immunoprecipitated with anti-p120-Gap antibodies, followed by immunoblotting with anti-p.Tyr antiserum and detection with $^{[125]}$-labelled protein A. SUP-B15 cells were also assayed (lane 3). Number of viable cells ($x10^5$) assayed per lane: 176 (lane 1), 192 (lane 2), 100 (lane 3), 23 (lane 4), 31 (lane 5) and 60 (lane 6).

b. $10^7$ viable primary leukemic blast cells from a patient with CML myeloid blast crisis (patient 1 in Table 2-1), were immunoprecipitated with anti-Gap, anti-p.Tyr or anti-Abl antibodies as specified, and the immune complexes subsequently immunoblotted with anti-p.Tyr antiserum followed by $^{[125]}$-protein A (lanes 1-3). Anti-p.Tyr and anti-Gap immunoprecipitates of patient 1's cells were also blotted with anti-Gap antiserum and developed with $^{[125]}$-protein A (lanes 4 and 5).
2.4.5. p120-Gap forms a stable complex with the Bcr-Abl oncproteins in vivo

Although not evident in figure 2-1a due to the relatively short exposure time of the autoradiogram, a phosphoprotein which co-migrated with p210 Bcr-Abl was detected in anti-Gap immunoprecipitates of R1-p210 or K562 cells suggesting that p210 Bcr-Abl stably complexes with p120-Gap in vivo (figure 2-5a). Furthermore, in some experiments close examination of the 190 kDa band in anti-Gap immune complexes from R1-p185 or SUP-B15 cells revealed the presence of a doublet with the upper band co-migrating with p185 Bcr-Abl (figure 2-5a and data not shown). (The faster migration of p190 compared to p185 Bcr-Abl is in agreement with Settleman and co-workers who reported that the migration of p190 was more compatible with a protein of molecular weight of 175 kDa, based on extensive comparison with proteins of known size (777). For clarity, the established nomenclature of p190 to refer to the p120-Gap-associated protein and p185 to refer to the isoform of Bcr-Abl characteristically associated with acute leukemia, will be retained.) To investigate whether the Bcr-Abl oncproteins form physical complexes with p120-Gap in vivo, anti-Gap immunoprecipitates of Rat-1, R1-p185 and R1-p210 cells were immunoblotted with anti-Abl antibodies (figure 2-5b). Anti-Abl immunoprecipitates of identical cell lysates were included as a positive control. Both p185 and p210 Bcr-Abl co-precipitated with p120-Gap (lanes 3 and 5) but not with pre-immune serum or an irrelevant antibody (data not shown). Endogeneous c-Abl which is tightly regulated and does not undergo detectable tyrosine phosphorylation in asynchronous mammalian cells (192) was not detected in association with p120-Gap. In the reciprocal experiment, p120-Gap was relatively inefficiently co-precipitated by antibody directed toward the carboxy terminal portion of Abl, but was readily detected using antibody raised against the N-terminal bcr-encoded sequences, or the region of Abl encoded by exons 2 and 3 (figure 2-5c). Co-
**Figure 2-5**

**Bcr-Abl oncoproteins form a stable complex with p120-Gap *in vivo*.

**a.** Lysates of R1, R1-p185, R1-p210 and K562 cells were immunoprecipitated with anti-p120-Gap or anti-Abl antiserum and immunoblotted with anti-p.Tyr antibodies followed by detection with $[^{125}\text{I}]-$labelled protein A. The positions of the Bcr-Abl proteins and p120-Gap-associated p190 are indicated by arrows. On the original autoradiogram, a 185-190 kDa doublet is distinguishable in the R1-p185 anti-p120-Gap immunoprecipitate.

**b.** R1, R1-p185 and R1-p210 cell lysates were immunoprecipitated with anti-Abl or anti-p120-Gap antibodies and immunoblotted with a monoclonal antibody directed against the carboxy terminal region of Abl. The filter was washed and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody followed by development with enhanced chemiluminescent reagents.

**c.** R1-p185 cells were lysed and immunoprecipitated with antibodies directed against the carboxy terminal (anti-pEx5) or the N-terminal portion (anti-pEx2/3) of Abl, the N-terminal 2-12 residues of Bcr, or p120-Gap. The immunoprecipitated proteins were subsequently blotted with anti-p120-Gap antibodies followed by a secondary incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibodies and development by enhanced chemiluminescence.
precipitation of p120-Gap and Bcr-Abl was not detected in the primary cell samples most likely due to the lesser quantities of cellular protein which were available for assay.

2.4.6. The N-terminal Gap SH2 domain is sufficient for association with Bcr-Abl

To further investigate the nature of the association between Bcr-Abl and p120-Gap, this interaction was reconstituted \textit{in vitro}. The p120-Gap [N]-SH2 domain was expressed as a GST fusion protein in bacteria, immobilized on glutathione-agarose beads and mixed with lysates of Rat-1, R1-p185 or R1-p210 cells. GST alone was included as a negative control. The isolated C-terminal p120-Gap SH2 domain was not tested as it had previously been shown to exhibit negligible independent binding activity for several other p.Tyr-containing proteins (data not shown) (781,799,803). Bound proteins were recovered, washed extensively and following separation by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Abl antibodies. p185 Bcr-Abl (figure 2-6, lane 6) and p210 Bcr-Abl (data not shown) specifically bound to p120-Gap [N]-SH2 whereas endogeneous c-Abl did not (lane 3). (The minor band in lane 6 which migrates in the vicinity of c-Abl likely represents a breakdown product of Bcr-Abl since it was detected by anti-p.Tyr antibodies (see figure 2-8)). These results suggest that the N-terminal p120-Gap SH2 domain mediates association with the activated Abl proteins in intact cells. A non-transforming p185 Bcr-Abl mutant lacking the majority of \textit{bcr}-derived amino acids (p185 \(\Delta3-426\)) also did not bind Gap [N]-SH2 (lane 9). A requirement for the amino terminal \textit{bcr} sequence may reflect its Abl kinase activating function (192,359,360,476) and/or the direct recognition of \textit{bcr}-encoded sequences by p120-Gap [N] SH2.

To determine whether p185 Bcr-Abl binds exclusively to p120-Gap [N]-SH2, SH2 domains isolated from other signalling proteins were also tested for their ability to interact with Bcr-Abl. As
Figure 2-6

The p120-Gap N-terminal SH2 domain is sufficient for the interaction with p185 Bcr-Abl in vitro.

The N-terminal SH2 domain of human p120-Gap was expressed in bacteria as a GST fusion protein, immobilized on glutathione-agarose beads, and mixed with lysates of parental Rat-1 cells (lane 3) or cells expressing wildtype p185 Bcr-Abl (lane 6). The p120-Gap [N]-SH2 binding properties of a transformation-defective p185 Bcr-Abl protein in which bcr-derived amino acid residues 3-426 were deleted (p185 Δ3-426) was also tested (lane 9). Lysates mixed with GST alone were included as negative controls (lanes 2, 5 and 8) and anti-Abl immunoprecipitates were also included to show the relative migration positions of the Abl proteins (lanes 1, 4 and 7). Bound proteins were recovered, separated by SDS-PAGE and following transfer to nitrocellulose, probed with anti-Abl antibody followed by antibody directed against mouse IgG and development with enhanced chemiluminescent reagents. The relative migration positions of p185 Bcr-Abl, p185 (Δ3-426) and c-Abl are depicted by arrows. The amounts of GST or GST fusion proteins were similar and between 5-10 μg as judged by Coomassie blue staining (not shown).
demonstrated in figure 2-7a, Bcr-Abl bound to the isolated C-terminal SH2 domain of the p85α subunit of phosphatidylinositol 3'-kinase (PI3K) but minimally to the N-terminal SH2 domain. Synergism between the two SH2 domains was detected when both p85α SH2 domains were expressed in the same GST fusion protein suggesting that cooperativity between SH2 domains may occur in the native molecule or that a functional conformation of [C]-SH2 depends on the presence of additional sequences. Bcr-Abl also bound to the isolated N- and C-terminal PLC-γ1 SH2 domains (figure 2-7b) and the Crk SH2 domain (figure 2-7c) but not to Vav SH2 (see figure 2-8b, lane 4). A minor fraction of Bcr-Abl was also detected in association with Abl SH2 upon prolonged exposure of the blot in figure 2-8b (data not shown).

2.4.7. p62 and a 130 kDa phosphoprotein in bcr-abl-transformed cells interact with p120-Gap [N]-SH2 and Abl SH2

To determine whether bcr-abl-transformed cells contain additional SH2-binding phosphoproteins, cellular proteins bound to GST fusion proteins corresponding to the p120-Gap [N]-SH2, Vav SH2 or Abl SH2 domain were immunoblotted with anti-p.Tyr antibodies. A prominent 130 kDa phosphoprotein in lysates of Clone H-p185 lymphoid cells specifically bound to the Gap [N]- and Abl SH2 domains but not to Vav SH2 or GST alone (figure 2-8a). In an anti-Abl blot of aliquots of the same samples, anti-Abl antibodies recognized a minor 130 kDa protein in the p120-Gap [N]-SH2 lane suggesting that this protein may in part represent a breakdown product of Bcr-Abl (figure 2-8b, lane 3). However, the Abl SH2-binding 130 kDa protein (figure 2-8a, lane 5) was not detected by anti-Abl antibodies (figure 2-8b, lane 5) indicating that this protein is distinct from Abl. Moreover, a similarly migrating 130 kDa phosphoprotein which bound p120-Gap [N]-SH2 was present in R1-p185 cells and was not recognized by anti-Abl antiserum (data not shown).
Figure 2-7

A subset of SH2 domains associates with p185 Bcr-Abl in vitro

a. Lysates of Clone H cells expressing p185 Bcr-Abl were mixed with GST fusion proteins corresponding to the isolated N- or C-terminal SH2 domains of the p85α isoform of PI3K or a fragment containing both SH2 domains together. GST alone was included as a negative control. Protein complexes were washed extensively, separated by SDS-PAGE and following transfer to nitrocellulose, blotted with anti-Abl antibodies followed by horseradish peroxidase-conjugated antibody against mouse IgG and development with chemiluminescent reagents.

b. In a similar experiment as in (a), Clone H cells expressing p185 Bcr-Abl were mixed with GST fusion proteins containing the isolated N- or C-terminal SH2 domain of PLC-γ1 or GST alone.

c. R1-p185 cell lysates were mixed with GST-Crk SH2 or GST alone and the recovered proteins subjected to anti-Abl immunoblotting as described in (a). GST-p120-Gap [N]- and PI3K [N]-SH2 fusion proteins were included as positive and negative controls respectively.
Figure 2-8

GST-Gap [N]-SH2 and Abl SH2 bind p62 and a 130 kDa phosphoprotein present in bcr-abl-transformed cells.

a. GST fusion proteins corresponding to the p120-Gap [N]-SH2, Vav SH2 or Abl SH2 domain, or GST alone, were mixed with lysates of Clone H cells expressing p185 Bcr-Abl. Bound proteins were recovered, separated by SDS-PAGE on a 7.5% polyacrylamide gel and following transfer to nitrocellulose, immunoblotted with affinity purified anti-p.Tyr antibodies followed by detection with $^{125}$I-labelled protein A. The positions of p130, Bcr-Abl and p62 are indicated by arrows. The amounts of GST or GST fusion proteins used in each sample were comparable, as judged by Coomassie blue staining (not shown).

b. Identical samples as in (a) were probed with anti-Abl antibodies followed by anti-mouse IgG and detection with chemiluminescence. Upon prolonged exposure to film, a small amount of Bcr-Abl was detected in the Abl SH2, but not the Vav SH2, lane (data not shown).
These SH2-binding proteins may correspond to 120-130 kDa proteins that associate with Src SH2 and are present in fibroblasts transformed by v-crk or v-src and in macrophages overexpressing c-fps (742,809-811,814). Indeed, a 130 kDa phosphoprotein from R1-p185 cells bound to the Crk SH2 domain (data not shown).

Variable amounts of a 62 kDa phosphoprotein from bcr-abl-transformed cells also bound to p120-Gap [N]-, Abl and Vav SH2 domains but not GST alone (figure 2-8a). In other experiments, these proteins co-migrated with p62 in anti-p120-Gap immunoprecipitates from v-src-transformed cells (data not shown). As p62 did not consistently co-precipitate with Bcr-Abl, it is likely that p120-Gap forms independent complexes with these two proteins in vivo and that the Abl SH2 domain in Bcr-Abl is either sequestered and unavailable for binding or forms only transient, low affinity complexes with p62.

2.4.8. Multiple SH2-containing proteins are phosphorylated in Ph+ cells

The in vitro binding studies presented above suggested that a number of SH2-containing proteins may participate in Bcr-Abl signalling pathways. As part of an initial survey to identify such proteins, lysates of Ph+ leukemic cell lines were immunoprecipitated with specific antibodies directed against the proteins Vav, Shc or the hematopoietic-specific tyrosine phosphatase Shp-1. Anti-Gap, anti-Abl and anti-p.Tyr immunoprecipitates were also included. Following protein separation by SDS-PAGE and transfer to nitrocellulose, immunoblotting with anti-p.Tyr antibodies was performed. In addition to p120-Gap-associated p62 and p190, p95 Vav was highly phosphorylated on tyrosine in Ph+ cells expressing p185 (figure 2-9a) or p210 Bcr-Abl (data not shown). The 52 and 46 kDa Shc proteins along with a Shc-associated 145 kDa protein were also prominent tyrosine phosphorylated proteins in the Ph+ cells, in agreement with data obtained.
Figure 2-9

Tyrosine phosphorylated proteins in Ph+ cells include Vav, PLC-γ1, Shc and Shc-associated p145

a. WL-1 Ph+ cells expressing p185 Bcr-Abl were lysed and immunoprecipitated with antibodies specific for Shc, Vav, p120-Gap, the tyrosine phosphatase Shp-1, Abl or phosphotyrosine. Normal rabbit serum (C) was included as a negative control. The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-p.Tyr antibodies followed by [125I]-labelled protein A. Precipitation of each protein was verified by specific immunoblotting (not shown). The figure shown is a composite of a 36 hr exp of lanes 1-5 and an 8 hr exp of lanes 6 and 7.

b. SUP-B15 cells expressing p185 Bcr-Abl were immunoprecipitated with anti-PLC-γ1, anti-p120-Gap or anti-Abl antibodies and immunoblotted with anti-p.Tyr antibodies and [125I]-protein A. The position of the 145 kDa PLC-γ1 protein is indicated. The 120 kDa protein co-precipitating with PLC-γ1 is non-specific as it was also present in pre-immune serum (not shown).
previously (L.P., M.Sc. thesis, University of Toronto, 1994). Tyrosine phosphorylation of the 68 kDa Shp-1 protein was not detected although small quantities of a co-precipitating 140 kDa protein were observed. Immunoblotting of duplicate samples with each specific antibody verified adequate precipitation of each protein analyzed (data not shown). In a similar experiment, the 145 kDa PLC-γ1 protein was also shown to be tyrosine phosphorylated in Ph+ cells expressing either isoform of Bcr-Abl (figure 2-9b and data not shown). A phosphoprotein which co-migrated with Bcr-Abl was consistently precipitated by anti-PLC-γ1 antibodies in several experiments suggesting that PLC-γ1 may also stably complex with Bcr-Abl. This result is consistent with the ability of the isolated PLC-γ1 SH2 domains to bind Bcr-Abl in vitro.
2.5. DISCUSSION

2.5.1. SH2-mediated interactions contribute to Bcr-Abl signalling

By mediating inter- or intramolecular protein interactions, SH2 domains play a pivotal role in signalling mechanisms activated by tyrosine kinases (reviewed in: 200). Individual SH2 domains bind with high affinity to specific tyrosine phosphorylated sequences (179), or rarely, serine/threonine phosphorylated sites (193). Several general types of SH2-mediated interactions may contribute to the transduction or regulation of signals initiated by the Bcr-Abl oncoproteins, as illustrated in figure 2-10a.

One potential set of interactions involves the SH2 domain intrinsic to the Bcr-Abl proteins themselves (815,816,179,192). Abl SH2 when isolated as a GST-fusion protein, interacts in vitro with two non-consecutive serine-rich sequences encoded by bcr (192). At least one of the SH2-binding regions is necessary for transformation. Serine/threonine phosphorylation of the Bcr sequence is apparently sufficient for the interaction with Abl SH2. Such an interaction has been postulated to activate the Abl kinase domain in vivo, perhaps by interfering with the binding of a negative regulator (192). However, the precise mechanism of Abl activation in cells, including the potential contribution of phosphotyrosine or phosphoryserine/threonine recognition by Abl SH2, is unknown. Consistent with the possibility that Bcr-Abl activation requires an interaction between Abl SH2 and bcr-encoded sequences, we detected a small amount of Bcr-Abl in association with GST-Abl SH2. The amount of Bcr-Abl which bound Abl SH2 was far less than that found in association with comparable quantities of the other SH2 domains tested such as p120-Gap [N]-SH2. This may reflect masking of the appropriate binding site by an intramolecular association with the intrinsic Abl SH2 domain.
Figure 2-10

SH2-mediated interactions in Bcr-Abl-transformed cells

a. Potential types of SH2-mediated, phosphorylation-dependent interactions following activation of the Abl kinase domain: 1) the SH2 domain of a downstream effector recognizes autophosphorylated Bcr-Abl, 2) the Abl SH2 domain recognizes a phosphorylated substrate, and 3) phosphorylation of a substrate by Bcr-Abl induces interaction of the target with the SH2 domain of another downstream effector. Interactions in cis or trans involving Abl SH2 and autophosphorylated motifs in Bcr-Abl are also possible (not shown).

b. p120-Gap forms multiple SH2-mediated complexes in Bcr-Abl-transformed cells.

Bcr-Abl, p62, p190, and p130 are shown as binding separately to p120-Gap as the p120-Gap [N]-SH2 is implicated in binding to all four molecules. In the intact p120-Gap molecule, the C-terminal SH2 domain may also contribute to binding as illustrated for p190. An additional 55 kDa protein present in at least some Ph+ cells also binds the N-terminus of p120-Gap (not shown). Events downstream of p62 and p130 are not yet established. Although not shown, the promotion of Ras.GTP formation by GEFs involves an intermediate state in which Ras is not bound to guanine nucleotide.
The predominant phosphoprotein in *bcr-abl*-transformed lymphoid cells which bound to the Abl SH2 domain *in vitro* was a soluble 130 kDa protein. This protein may correspond to p130 Cas, a recently characterized protein which associates tightly with v-Crk in *v-crk*-transformed cells and is also a target of v-Src and c-Fps (742,814). A 62 kDa protein which may correspond to p120-Gap-associated p62 also bound to the isolated Abl SH2 domain. Proteins of these molecular weights were variably detected in anti-Abl immunoprecipitates of *bcr-abl*-transformed cells. Complexes between p62 or p130 and Bcr-Abl may be transient due to higher affinity interactions with other polypeptides such as p120-Gap, or the Abl SH2 domain may be sequestered in the oncoprotein and unavailable for binding to exogeneous ligand.

Abl SH2 associates predominantly with a phosphoprotein of 65-68 kDa present in fibroblasts transformed by an activated form of murine type IV c-Abl (ΔSH3), and binds virtually all p.Tyr-containing proteins present in ΔSH3 c-Abl-transformed cells when such proteins are denatured and immobilized on a filter (366,815). The ability of mutant Abl SH2 domains to bind phosphotyrosine correlated with ΔSH3 c-Abl transforming potential (816). In affinity chromatography studies using a phosphopeptide library degenerate at the +1 to +3 positions carboxy terminal to the phosphotyrosine, the isolated Abl SH2 domain showed highest affinity for peptides containing p.Tyr-Gln-Asn-Pro with the most strongly selected feature Asn at the +2 position followed by proline at the +3 position (179). The Abl SH2 domain has also been reported to interact with a phosphopeptide corresponding to the sequence p.Tyr<sup>1086</sup>-His-Asn-Gln in the EGF receptor (817). It is not yet known whether proteins containing these motifs associate with Bcr-Abl *in vivo*. However, we have shown elsewhere that GST-Abl SH2 does not bind strongly to a *bcr*-encoded peptide containing the motif p.Tyr-Val-Asn-Val suggesting that residues other than the +2
Asn also influence binding (818). Furthermore, Asn at the +2 position may not be critical as Abl SH2 also recognizes motifs with a proline residue at this position (819).

2.5.2. p120-Gap is a target of the Bcr-Abl oncoproteins but not an efficient in vivo substrate of the Abl kinase domain

A second general type of SH2-mediated interaction which contributes to signalling by Bcr-Abl involves the recognition of autophosphorylated sites within Bcr-Abl by SH2 motifs in downstream signalling proteins. As described in Chapter 3, one such example is the association of Bcr-Abl with the molecular adaptor Grb2. The stable association of p120-Gap and Bcr-Abl is also likely representative of this type of interaction since the N-terminal SH2 domain of p120-Gap is sufficient for complex formation in vitro. Gap [N]-SH2 also binds a subset of autophosphorylated RTKs, p62, p190 and a p130 protein present in v-src-transformed fibroblasts (577,783,797). Two p120-Gap-binding sites which have been mapped consist of p.Tyr\textsuperscript{771}-Met-Ala-Pro in the kinase insert of the murine PDGF-β receptor and p.Tyr\textsuperscript{960}-Phe-Ser-Ala in the juxtamembrane region of the insulin receptor (820-822). These sequences have in common relatively hydrophobic residues at the +1 and +3 positions. In affinity chromatography studies, the p120-Gap [N]-, [C]- and [N+C]-SH2 domains preferentially bound phosphopeptides containing p.Tyr-Leu-Asp-Leu, p.Tyr-X-Tyr-Pro and p.Tyr-X-X-Pro respectively (G. Gish, Z. Songyang, T. Pawson and L. Cantley, unpublished observations). Experiments to delineate the precise p120-Gap binding site on the Bcr-Abl proteins, and to determine whether this is a direct or indirect interaction, are in progress.

Stable complex formation between p120-Gap and Bcr-Abl suggests that p120-Gap may be an early direct substrate of Bcr-Abl. However, we find that p120-Gap is only weakly phosphorylated on tyrosine in bcr-abl-transformed cells, despite the ability of purified p120-Gap to
function as a substrate of Bcr-Abl in vitro (A. Pendergast and O. Witte, personal communication). Inefficient phosphorylation in vivo may be due to competition from other substrates or preferential dephosphorylation of p120-Gap by cellular phosphatases such as Shp-1 or Shp-2. Phosphorylation of p120-Gap on tyrosine residues is also not dramatically increased following antigen receptor cross linking in the Jurkat T cell leukemic cell line or in normal primary human T lymphoblasts although p.Tyr-containing p190 and p62 are readily detectable (L.P., unpublished observations). Cytokines active on myeloid cells also do not elicit marked p120-Gap tyrosine phosphorylation (89,814,823-825). The functional significance of low phosphotyrosyl levels of p120-Gap remains to be determined. The ability of p120-Gap to enhance Ras GTPase activity is not altered following phosphorylation on tyrosine (781,826) but a major phosphorylation site, Tyr$^{460}$ of human p120-Gap, is located immediately adjacent to the carboxy terminal SH2 domain and may modulate the ability of the SH2-SH3-SH2 region to interact with potential downstream effectors such as p62 or p190 (578,808,826).

It is not known whether p120-Gap activity is regulated by complex formation with Bcr-Abl. Bcr-Abl does not directly modulate the GTPase promoting activity of purified baculovirus-derived p120-Gap in vitro (A. Pendergast, O. Witte and F. McCormick, personal communication) although such an effect in mammalian cells has not been ruled out. Association with Bcr-Abl may alter p120-Gap's subcellular localization and access to Ras, or modulate functions distinct from p120-Gap's ability to accelerate hydrolysis of Ras.GTP. For example, Bcr-Abl may displace p62 from p120-Gap complexes, allowing p62 to interact with other proteins. A study in which the association of p120-Gap with p62 was blocked by a phosphopeptide corresponding to the p120-Gap binding site on the PDGF receptor supports this possibility (820). Conversely, p120-Gap may modulate Bcr-Abl function. Although the in vitro autokinase activity of Bcr-Abl does not appear altered by
p120-Gap (A. Pendergast and O. Witte, personal communication), such an interaction could selectively affect the phosphorylation of an intracellular substrate, protect Bcr-Abl from dephosphorylation, or perturb in vivo regulatory mechanisms perhaps by influencing the ability of Bcr-Abl to interact with other proteins.

2.5.3. Multiple Gap-associated proteins are direct or indirect substrates of either isoform of Bcr-Abl

The presence of a 62-65 kDa phosphoprotein in acutely infected populations of Rat-1 fibroblasts was previously reported to correlate specifically with expression of the more highly oncogenic 185 kDa Bcr-Abl protein (414). However, the different transforming properties of p210 and p185 Bcr-Abl cannot be attributed to marked differences in their ability to utilize p120-Gap-associated p62 as a substrate, as p62 is highly phosphorylated on tyrosine following expression of either isoform. The fraction of cellular p62 which is phosphorylated by each isoform of Bcr-Abl could not be assessed due to the unavailability of an appropriate p62-specific antibody. Further studies will therefore be required to determine whether more subtle differences exist in the ability of p185 and p210 Bcr-Abl to phosphorylate p62 or otherwise regulate p62's biological activity.

p62 is phosphorylated on tyrosine by a number of hematopoietic stimuli (89,131,824,827,828, L.P., unpublished observations); in the human Jurkat T cell line, the extent of tyrosine phosphorylation of p62 in response to various stimuli is modulated by PKC activity (L.P., unpublished observations). The demonstration here and by others that p62 binds the SH2 or SH3 domains of a number of different proteins suggests that it may function as a docking protein and participate in diverse protein-protein interactions (781,829-832,811). Of interest, a 62 kDa protein which co-migrated with Gap-associated p62 was detected in anti-PLC-γ1 immunoprecipitates of the Ph+ ALL SUP-B15 cell line. In v-src-transformed cells, others have also reported that a protein
identical or highly related to p62 interacts with PLC-γ1, suggesting that p62 may mediate "cross-talk" between p120-Gap and PLC-γ1 signalling pathways (831).

Gap-associated p190 is also a direct or indirect substrate of Bcr-Abl. Our laboratory has previously determined that complex formation between p190 and p120-Gap is phosphorylation-dependent and inducible upon expression of v-Src or following stimulation with EGF (800). Association of p190 with p120-Gap reduces p120-Gap GTPase promoting activity 4-fold in vitro (800). Recently a reduction in cellular p120-Gap activity was reported to accompany expression of p210 Bcr-Abl in myeloid cells (595). Such a phenomenon may be secondary to the presence of p190/p120-Gap complexes.

The C-terminal portion of p190 exhibits GTPase promoting activity for the Rho/Rac subfamily of small guanine nucleotide binding proteins (778,473). The ability of p190 to regulate Rho family members suggest a key role in cytoskeletal organization since Rho and Rac are critical components of signalling pathways which link extracellular stimuli to the reorganization of polymerized actin (722,723). Indeed, the GTPase activating domain of p190 preferentially inhibits Rho-mediated stress fibre formation in Swiss 3T3 cells (473), and p190 is implicated in the disruption of cytoskeletal architecture induced by overexpression of the N-terminal region of p120-Gap (587). p190/p120-Gap complexes may therefore provide Bcr-Abl with a link to the control of cell morphology and adhesion.

My studies in primary leukemic blast cells suggest that p120-Gap-associated proteins, particularly p62, are relevant to the process of Ph+ leukemogenesis. The primary blast cells were assayed in the presence of IL-3 as adequate numbers of viable cells were not obtained from cryopreserved samples in the absence of growth factor. The relative contribution of IL-3 receptor-associated tyrosine kinase activity to the phosphorylation of p62 in these cells could therefore not be
determined. This may be of importance as cells expressing Bcr-Abl may have heightened sensitivity to the effects of exogeneous IL-3 (409,833). In addition, the autocrine production of IL-3 may play a role in conferring growth factor independence to factor-dependent myeloid cell lines when Bcr-Abl is expressed (833). In a preliminary experiment, tyrosine phosphorylation of p62 was increased by the addition of exogeneous IL-3 in 2/4 subclones of M07e cells ectopically expressing p210 Bcr-Abl (L.P., C. Sirard and J. Dick, unpublished observations). For primary hematopoietic cells, it is possible that a threshold level of activation of a particular target is required, and that optimal stimulation requires both exogeneous growth factor and the activated Abl kinase.

2.5.4. A network of SH2-mediated interactions participate in Bcr-Abl signalling pathways

I have demonstrated that a number of SH2-containing proteins in addition to p120–Gap are also potential targets of the Bcr-Abl oncoproteins, based on the ability of the SH2 domains of such targets to complex with Bcr-Abl and/or the tyrosine phosphorylation of these proteins in *bcr-abl*-transformed cells. These additional targets include PLC-γ1 and PI3K, proteins involved in phospholipid metabolism (501,834) and the hematopoietic-specific Vav protein which functions as a guanine nucleotide exchange factor for members of the Rho family (835). Vav has also been reported to function as a Ras GEF although this remains controversial (667,836-840). While *in vitro* recognition by an exogeneous SH2 domain may prove to be predictive of which proteins form stable physical complexes with Bcr-Abl, such an interaction does not necessarily predict whether a particular target will function as an efficient substrate of the Abl kinase domain (or a downstream PTK). The identification of these additional proteins as direct or indirect targets of Bcr-Abl indicates that multiple SH2/SH3-containing proteins participate in Bcr-Abl signalling pathways and provides the groundwork for future studies to determine which are Bcr-Abl's critical effectors.
CHAPTER 3

A GRB2 BINDING MOTIF ENCODED BY THE BCR GENE

LINKS THE BCR-ABL ONCOPROTEINS

TO THE RAS GUANINE NUCLEOTIDE EXCHANGE FACTOR mSOS1


Attribution of data: Jiaxin Liu and Gerald Gish participated in experiments shown in Figures 3-3, 3-4 and 3-6 respectively. All other experiments were performed in their entirety by myself.
3.1. ABSTRACT

The 185 and 210 kDa Bcr-Abl tyrosine kinases play important roles in the pathogenesis of chronic myelogeneous leukemia and a subset of acute lymphoblastic leukemia. Since activation of the guanine nucleotide binding protein p21\textsuperscript{ras} is critical for induction of DNA synthesis and morphological transformation by a number of oncogenic tyrosine kinases, we have analyzed the Bcr-Abl oncoproteins for their ability to interact with cytoplasmic proteins that mediate Ras activation. One such polypeptide is encoded by the growth receptor-bound 2 (grb2) gene and is composed of a single Src Homology 2 (SH2) domain flanked by two Src Homology 3 (SH3) domains. In work presented elsewhere, Grb2 has been shown to form a stable, phosphorylation-dependent complex with p185 and p210 Bcr-Abl \textit{in vivo}. In this chapter, the nature of the interaction between Grb2 and Bcr-Abl is further investigated. Evidence is provided that the binding of Grb2 to Bcr-Abl is mediated by the Grb2 SH2 domain which recognizes an autophosphorylated sequence, p.Tyr\textsuperscript{177}-Val\textsuperscript{178}-Asn\textsuperscript{179}-Val\textsuperscript{180} located within the N-terminal \textit{bcr}-encoded sequence common to all known isoforms of Bcr-Abl. The Ras guanine nucleotide exchange factor mSos1 is also shown to associate stably with p185 and p210 Bcr-Abl \textit{in vivo}. mSos1 promotes dissociation of GDP from Ras, thereby stimulating conversion of inactive GDP-bound Ras to the active GTP-bound state. Recruitment of mSos1 into complex formation with Bcr-Abl is most likely achieved through the constitutive association of the mSos1 carboxy terminal tail with the SH3 domains of Grb2. These results suggest that autophosphorylation within the Bcr element of Bcr-Abl creates a direct physical link to Grb2-mSos1 and potentially to the Ras pathway, and thereby modifies the target specificity of the Abl tyrosine kinase. This is a novel function of the \textit{bcr}-encoded sequence which has not previously been described.
3.2. INTRODUCTION

Activation of the guanine nucleotide binding protein p21\textsuperscript{ras} appears critical for the induction of DNA synthesis and morphological transformation by oncogenic tyrosine kinases such as v-src, v-fps and v-abl (521,522,528). Data presented in Chapter 2 implicate the p21\textsuperscript{ras} GTPase activating protein p120–Gap and its associated proteins in Bcr-Abl signalling pathways and suggest that Bcr-Abl, in common with other activated tyrosine kinases, is coupled to the Ras pathway. Indeed, tyrosine kinase-dependent accumulation of Ras in its active GTP-bound form was recently reported to occur upon expression of p210 Bcr-Abl in murine myeloid cells (841). In this chapter, the Bcr-Abl oncoproteins have been analyzed for interactions with Grb2 and mSos1, recently characterized positive regulators of Ras.

Grb2, the mammalian homologue of Caenorhabditis elegans Sem-5 and Drosophila melanogaster Drk, is composed entirely of a single SH2 domain flanked by two SH3 domains (637). Genetic evidence from C. elegans and Drosophila indicates that Sem-5 and Drk are crucial in coupling receptor tyrosine kinases to activation of the Ras pathway (203,205,606). In mammalian cells, Grb2 directly couples a subset of phosphorylated receptor tyrosine kinases, which it binds with its SH2 domain (637,647,668) to mSos1, a Ras guanine nucleotide exchange factor (GEF) (231,608,612,618,647,668). The SH3 domains of Grb2 constitutively associate with proline-rich motifs in the carboxy terminal tail of mSos1 allowing the association of mSos1 and Ras-GEF activity with the autophosphorylated tyrosine kinase receptor (231,612,638-640). These interactions apparently stimulate conversion of inactive GDP-bound Ras to the active GTP-bound form through recruitment of mSos1 to the plasma membrane (639). mSos1 promotes the release of GDP from Ras, allowing its replacement with the more abundant cellular pool of GTP. Although Grb2 associates with several activated receptor tyrosine kinases, it is not an efficient substrate for tyrosine
phosphorylation in either *Drosophila* or mammalian cells (204,637,647). Hence, Grb2 function is apparently regulated by the inducible binding of its SH2 domain to phosphotyrosine sites.

Grb2 co-precipitates with multiple tyrosine phosphorylated proteins in Ph+ leukemic cells or *bcr-abl*-transformed fibroblasts (L.P., M.Sc. thesis, University of Toronto, 1994). One of these proteins is the molecular adaptor encoded by the *src homology and collagen (shc)* gene. The *shc* gene encodes three isoforms of 66, 52 and 46 kDa which possess an alternatively spliced N-terminal sequence of variable length, a phosphotyrosine-binding (PTB) region, a more central SH2 domain and a carboxy terminal glycine/proline-rich region (505). All three isoforms of Shc are tyrosine phosphorylated and induced to associate with Grb2 in the presence of Bcr-Abl. The ability of p52*shc* and p46*shc* to transform fibroblasts and to induce Ras-dependent differentiation in PC12 pheochromocytoma cells when overexpressed suggests a key role in the regulation of cell growth (505).

Grb2 also forms a stable, phosphorylation-dependent complex with the autophosphorylated p185 and p210 Bcr-Abl oncoproteins *in vivo* (L.P., M.Sc. thesis, University of Toronto, 1994). *In vitro*, the Grb2 SH2 domain is sufficient for this interaction and binds directly to denatured Bcr-Abl proteins immobilized on a filter. In this chapter, the site of interaction of Bcr-Abl with the Grb2 SH2 domain is demonstrated to involve Tyr 177, located within the *bcr*-encoded sequence common to both isoforms of Bcr-Abl. Tyr 177 is shown to be an autophosphorylation site in both p185 and p210 Bcr-Abl. A phosphopeptide modelled on Bcr and containing p.Tyr 177, inhibits the interaction between Bcr-Abl and full length Grb2 or the isolated Grb2 SH2 domain *in vitro*. An asparagine at the +2 position carboxy terminal to the phosphotyrosine is also critical for recognition by the Grb2 SH2 domain. These studies suggest that the Bcr element of the Bcr-Abl oncoproteins contributes to the target specificity of the activated Abl kinases. This is a novel function of the *bcr-
encoded sequence which has not previously been described. Experiments reported in this chapter also show that the guanine nucleotide exchange factor (GEF) mSos1 complexes with Bcr-Abl \textit{in vivo}. Recruitment of mSos1 into a Bcr-Abl signalling complex is likely to occur by virtue of its constitutive association with the SH3 domains of Grb2. The interaction of Grb2 with Bcr-Abl may therefore directly couple Bcr-Abl to the Ras pathway.
3.3. MATERIALS AND METHODS

Cell culture

The K562 cell line was obtained from the American Type Culture Collection (Rockville, Maryland) (785). The Ph+ ALL cell lines SUP-B15 (786) and WL-1 were generously provided by Dr. S. Smith (University of Chicago) and Dr. J. Ihle respectively (St. Jude Research Hospital, Memphis). K562 and SUP-B15 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS). Rat-1 fibroblasts were grown in Dulbecco modified Eagle medium (DME) containing 5% FBS. Rat-1 cells stably expressing p185 or p210 Bcr-Abl (414) were maintained in DME supplemented with 5% FBS and 50 μg ml⁻¹ of G418, and were a gift of Dr. O. Witte (UCLA).

Immunoprecipitations and Western blotting

Immunoprecipitations were carried out as previously described (800). Cells were lysed in 1 ml of ice-cold PLC lysis buffer (50 mM of N-2 hydroxyethyl piperazine-N'2-ethane sulfonic acid (HEPES, pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μg ml⁻¹ of leupeptin, 10 μg ml⁻¹ of aprotinin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride and 1 mM phenylmethanesulfonfonyl fluoride). In some experiments as indicated, 1% Nonidet-P40 was substituted for Triton X-100. Cell lysates were centrifuged for 15 min at 10 000 g and the supernatant collected. In some experiments as specified, protein concentrations of the clarified supernatants were determined by a modified Lowry protein assay (Sigma) and similar quantities of total protein immunoprecipitated. Cell lysates were incubated and gently rocked at 4°C for 90 min with anti-Abl pEx5 antibody (761), anti-Grb2 50 antibody (637), anti-Shc antibody (505) or mSos1 antibody (608). 100 μl of 10%
protein A-sepharose were added to precipitate the immune complexes. The immune complexes were washed 3X with 20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate (HNTG), and heated in sodium dodecyl sulfate (SDS) sample buffer for 3 min at 100°C prior to protein separation by SDS-PAGE. Following gel electrophoresis, the separated proteins were transferred to nitrocellulose with a semi-dry transfer apparatus at 0.8 mA.cm⁻² for 60 min. For immunoblotting with Abl, Shc and Grb2 antibodies, filters were blocked in Tris buffered saline (TBS) (20 mM Tris.HCl, pH 7.5, 150 mM NaCl) with 5% Carnation skim milk powder. Antibody incubations were carried out in TBS with 3% milk powder and 0.1% Tween 20 and washes were performed in TBS with 0.1% Tween 20. To detect Abl proteins, a mouse monoclonal antibody directed against the carboxy terminal region of c-Abl was used at a concentration of 1 μg ml⁻¹ (792). Grb2 55 antibodies raised against the entire Grb2 molecule were used for Western blotting at a 1/200 dilution (637). Immunoblotting for mSos1 was performed as previously described (231). For anti-phosphotyrosine (p.Tyr) immunoblotting, filters were blocked in TBS containing 5% bovine serum albumin and 1% ovalbumin and incubated with affinity purified anti-p.Tyr antiserum (797). Washes were performed twice in TBS followed by 1 wash in TBS plus 0.05% Nonidet-P40 and 2 more washes in TBS. Blots were then probed with [¹²⁵I]-labelled protein A (35 μCi ml⁻¹, Amersham) in blocking solution and exposed to Kodak XAR-5 film. For anti-Abl and anti-Grb2 blots, a secondary incubation was carried out with horseradish peroxidase-conjugated anti-mouse immunoglobulin or anti-rabbit immunoglobulin antiserum (Sigma) respectively prior to development with chemiluminescent reagents (Amersham) and exposure to Kodak XRP-5 film. Anti-Vav antibodies for immunoprecipitation and Western blotting have been described (793).
Bacterial fusion proteins

Regions of Grb2, Abl and mSos1 were isolated by the polymerase chain reaction (PCR) and subcloned into pGex expression vectors (802). Full length Grb2 and Grb2 SH2 fusion proteins have been described (637). The Abl SH3 fusion protein corresponds to residues 929-1020 of p210 Bcr-Abl (842). For the Abl SH2 fusion protein, the N-terminal region of type IV murine c-Abl was isolated by PCR, beginning with the StuI restriction site and terminating at the 3' end of the SH2 domain. The Abl SH3 domain was subsequently deleted by PCR, and the fragment subcloned into a pGex vector. The GST fusion protein containing the carboxy terminal tail of mSos1 corresponded to amino acid residues 1037-1336 (711). Bacterial cultures expressing the pGex vectors were grown in LB containing ampicillin at 100 µg ml⁻¹, and induced with 1 mM isopropyl thiogalactopyranoside (IPTG) for 4 hours at 30° or 37°C, depending on the solubility of the particular protein. The induced bacteria were lysed by sonication in ice-cold PLC lysis buffer, and the glutathione-S-transferase (GST) fusion proteins recovered from clarified lysates by glutathione-agarose (Pharmacia). The amount of each GST fusion protein was determined by comparison of bands on a Coomassie Blue stained gel to a dilution series of bovine serum albumin; similar quantities of fusion proteins (5-10 µg) were incubated with cell lysates for 90 min at 4°C. SH2-binding proteins were recovered by centrifugation, washed 3X in HNTG, resuspended in SDS sample buffer and boiled for 3 min prior to analysis.

Two-dimensional trypsin/V8 protease maps of Bcr-Abl and Bcr proteins.

For analysis of in vitro autophosphorylated sites in the Bcr-Abl proteins, K562 and SUP-B15 cells were lysed in ice-cold buffer (0.1% Triton X-100, 5mM EDTA, 100 mM NaCl, and 100 KIU Trasylol in 10 mM sodium phosphate, pH 7.2) and the cell lysates centrifuged at 100 000 g for
1 hr. Supernatants were collected and the p210 or p185 Bcr-Abl proteins immunoprecipitated with anti-Abl monoclonal p6D antibody which was raised against a synthetic peptide corresponding to amino acid residues 51-64 of human c-Abl (843). The immune complexes were washed once each in ice-cold RIPA buffer (0.1% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 100 mM NaCl in 10 mM sodium phosphate, pH 7.2), wash buffer (0.1% Triton X-100, 100 mM NaCl in 10 mM sodium phosphate, pH 7.2) and 50 mM Tris HCl, pH 7.5. The pellets were resuspended in 50 μl of kinase assay mixture (20 mM HEPES (pH 7.2), 100 mM NaCl, 0.1% Triton X-100 and 10 mM MnCl2) and 10 μCi [γ-32P] ATP (3000 Ci mmol⁻¹, NEN) at 4°C to initiate the kinase reaction. After 10 min, the reaction was terminated by addition of 1 ml of RIPA buffer.

p160 Bcr was immunoprecipitated from K562 cells by antibody raised against a synthetic peptide corresponding to amino acid residues 1256-1271 of the C-terminal portion of Bcr. This results in co-precipitation of p210 Bcr-Abl which is not recognized directly by the antibody used (467,844). The 160 kDa Bcr protein was subsequently transphosphorylated by p210 Bcr-Abl in an in vitro kinase assay as described above (844).

The labelled proteins in the kinase reactions were separated by SDS-PAGE and the desired bands extracted from the gel and eluted as previously described (844). Performic acid oxidized proteins were first treated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin for 22 hours and then with V8 protease (Glu-C) for 22 hours under conditions that favour cleavage after glutamic acid residues (pH 7.8 ammonium bicarbonate) (844). Peptides were separated by two-dimensional electrophoresis and chromatography, sequenced by manual Edman degradation, and the elution position of [γ-32P]-labelled phosphotyrosine monitored (845). For analysis of peptides that bound to the Grb2 SH2 domain, a trypsin/V8 protease digest of in vitro labelled p210 Bcr-Abl was mixed with immobilized GST-Grb2 SH2 or GST-Abl SH2 fusion proteins for 90 min
at 4°C. Bound peptides were removed by centrifugation, and the remaining peptides analyzed by two-dimensional electrophoresis and chromatography. For phosphoamino acid analysis, individual peptides were eluted from the thin-layer plate by 20% acetonitrile and then treated with 6M HCl for 90 min at 110°C (846). The clarified supernatant was fractionated on thin-layer plates in the presence of phosphoserine, phosphothreonine and phosphotyrosine. Radioactive phosphoamino acids were detected by autoradiography and the plate subsequently treated with ninhydrin to reveal the position of the authentic phosphoamino acids.

**Phosphopeptide competition studies**

Phosphopeptides were synthesized by 9-fluorenyl methoxy carbonyl (Fmoc) chemistry according to published methods (847). The sequences of these synthetic phosphopeptides were: Bcr 177, DAEKPFp.Y177VNEFK, corresponding to amino acid residues 171-182 of human Bcr, with a carboxy terminal lysine added; Asn→Ala Bcr (Bcr Ala +2 or N179A), identical to the Bcr peptide except for substitution of alanine for asparagine at the +2 residue carboxy terminal to Tyr 177; Shc 317, ELFDDPsp.Y317VNVQNLDK corresponding to a Grb2 recognition site on the Shc protein (505, 848, 849); PDGFR 771, SSNp.Y771MAPYDNYK, corresponding to the p120-Gap recognition site of the PDGF-β receptor (850); PDGFR 1009, SSVLp.Y1009-TAVQP corresponding to the Shp-2 recognition site on the PDGF receptor (851). Immobilized GST-Grb2 SH2 proteins were prepared as described above, and mixed with lysates of R1-p185 or R1-p210 cells as a source of Bcr-Abl. Various concentrations of the phosphopeptides were added, and the lysates incubated at 4°C for 90 min. Proteins bound to Grb2 SH2 were recovered by centrifugation and washed 3X with HNTG. Following resuspension in SDS sample buffer, proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Abl antibodies.
**BIAcore analysis of phosphopeptides interacting with Grb2 SH2 or full length Grb2.**

The Pharmacia BIAcore instrument uses surface plasmon resonance to probe the change in refractive index in a flow cell which occurs secondary to the binding of molecules to immobilized ligand (177,852). For peptide immobilization to the sensor chip surface, carboxyl groups of the matrix were activated with 50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide for 3 min, and then a 0.5 mM solution of Bcr phosphopeptide in 50 mM HEPES (pH 7.5) and 2M NaCl was injected onto the matrix for 4 min with a flow rate of 5 µl min⁻¹ (178). GST fusion proteins containing full length human Grb2 or the Grb2 SH2 domain were resuspended in 50 mM HEPES (pH 7.5), 150 mM NaCl and 3.0 mM EDTA, and concentrated using a Centricon 10 microconcentrator device (Amicon). Solutions (100 µl) containing 1 µM GST-Grb2 or 1 µM GST-Grb2 SH2 and the indicated concentration of soluble peptide in 50 mM HEPES (pH 7.5), 150 mM NaCl and 3 mM EDTA, was injected across the surface containing immobilized Bcr phosphotyrosine peptide. The amount of GST-Grb2 or GST-Grb2 SH2 protein bound was estimated from the steady-state surface plasmon resonance signal at a fixed time following the end of the injection; the percentage bound relative to injection of GST-Grb2 SH2 alone was then calculated (853). After injection, the peptide surface was regenerated by dissociating remaining bound proteins with 2M Guanidine HCl.
3.4. RESULTS

3.4.1. Tyr$^{177}$ in Bcr-Abl is an autophosphorylation site recognized by the Grb2-SH2 domain

In lysates of bcr-abl-transformed Rat-1 fibroblasts (figure 3-1) and Ph+ leukemic cells (data not shown), Grb2 stably complexes with either isoform of Bcr-Abl. The Grb2 SH2 domain is sufficient for this interaction in vitro and directly recognizes denatured Bcr-Abl proteins on an immobilized filter (L.P., M.Sc. thesis, University of Toronto, 1994). SH2 domains bind with high affinity to specific tyrosine phosphorylated ligands with the specificity of ligand binding dictated by the amino acid residues in the vicinity of the phosphotyrosine, particularly residues located at the carboxy terminal +1 to +3 positions (179,854,855). These residues, together with the phosphorylated tyrosine, can make direct contact with the SH2 domain (183,856). When employed to screen a degenerate phosphopeptide library, the Sem-5 and Grb2 SH2 domains showed a strong preference for Asn at the +2 position, two residues C-terminal to phosphotyrosine (179,180). Indeed, the known Grb2 SH2-binding sites located on Shc, the epidermal growth factor (EGF) receptor and the insulin receptor substrate-1 (IRS-1), all have Asn at the +2 position (640,650,848,849) (figure 3-2). A search of the amino acid sequence of the Bcr-Abl proteins revealed a single potential Grb2-binding site, Tyr$^{177}$-Val$^{178}$-Asn$^{179}$-Val$^{180}$, located within the bcr-encoded sequence that is common to all known Bcr-Abl proteins (figure 3-2). This sequence, located N-terminal to the serine-rich regions of Bcr previously reported to bind Abl SH2 (192), is identical at the +1 to +3 positions to the Grb2 binding site found at Tyr 317 of the 52 kDa Shc protein. The presence of this motif, along with the demonstration that Bcr-Abl undergoes autophosphorylation on tyrosine residues within the N-terminal Bcr sequence (844,857) suggested that Tyr$^{177}$ might mediate the interaction with Grb2. Two approaches, in vitro phosphopeptide binding studies and analysis of proteolytic maps of autophosphorylated Bcr-Abl before and after
Figure 3-1

Bcr-Abl oncoproteins, but not endogeneous c-Abl, form complexes with Grb2 in vivo.

Lysates of Rat-1, R1-p185 or R1-p210 cells were immunoprecipitated with anti-Abl or anti-Grb2 antiserum, and subsequently immunoblotted with anti-Grb2 antibodies. The immune complexes were detected by horseradish peroxidase-conjugated anti-rabbit Ig antiserum and enhanced chemiluminescent reagents. The position of Grb2 is indicated by an arrow. In other experiments, Grb2 was not precipitated by pre-immune serum (data not shown).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Motifs</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>Val-Pro-Glu-p.Tyr&lt;sup&gt;1068&lt;/sup&gt;-Ile-Asn-Gly</td>
</tr>
<tr>
<td></td>
<td>Asn-Pro-Val-p.Tyr&lt;sup&gt;1086&lt;/sup&gt;-His-Asn-Gly</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Pro-Gly-Glu-p.Tyr&lt;sup&gt;895&lt;/sup&gt;-Val-Asn-Ile</td>
</tr>
<tr>
<td>SHC</td>
<td>Asp-Pro-Ser-p.Tyr&lt;sup&gt;317&lt;/sup&gt;-Val-Asn-Val</td>
</tr>
<tr>
<td></td>
<td>p.Tyr&lt;sup&gt;239&lt;/sup&gt;-p.Tyr&lt;sup&gt;240&lt;/sup&gt;-Asn-Asp-Phe-Pro</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Lys-Pro-Phe-p.Tyr&lt;sup&gt;177&lt;/sup&gt;-Val-Asn-Val</td>
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</tbody>
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Consensus (peptide library)  

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<tr>
<th></th>
<th>p.Tyr</th>
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<th>Asn</th>
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<tbody>
<tr>
<td></td>
<td>Val</td>
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<td></td>
<td>Phe</td>
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</table>

Figure 3-2. Grb2 binding motifs. The known or putative Grb2 binding sites on the EGF receptor, IRS-1, Shc and Bcr-Abl are listed. All of these motifs contain an asparagine at the +2 position. Also included is a consensus motif selected by the Grb2 SH2 domain from a partially degenerate phosphopeptide library consisting of the sequence GDGp,YX<sup>+</sup>XX<sup>2</sup>XX<sup>3</sup>SPLL where X represents any amino acid except Cys or Trp (180).
incubation with GST-Grb2 SH2, were taken to map the Grb2 binding site.

As an initial step in determining which Bcr-Abl phosphopeptides are recognized by Grb2, p210 Bcr-Abl was immunoprecipitated from K562 cells, autophosphorylated in an in vitro kinase assay using [γ-32P]-ATP, and digested with trypsin followed by V8 protease. Two-dimensional electrophoresis and chromatography revealed a number of phosphopeptides in the double digest (figure 3-3a). An identical proteolytic digest was incubated with either immobilized GST-Grb2 SH2 or Abl SH2 fusion proteins prior to analysis by two-dimensional electrophoresis and chromatography. The immobilized fusion proteins and any associated phosphopeptides were removed by centrifugation, and the remaining peptides analyzed. Two phosphorylated peptides, designated H and L, were specifically depleted from the Bcr-Abl trypsin/V8 protease digest by incubation with the immobilized GST-Grb2 SH2 fusion proteins (figure 3-3b) but not by GST-Abl SH2 which depleted another phosphopeptide (data not shown). Phosphoamino acid analysis of peptides H and L indicated that these peptides contained phosphotyrosine only, and not phosphothreonine or phosphoserine (data not shown). Manual Edman degradation of peptides H and L (partial V8) placed the phosphotyrosine at cycle numbers 14 and 15 respectively (figure 3-3c and d). In conjunction with the mobilities of the peptides in two-dimensional electrophoresis and chromatography, these results suggested the following peptide sequences: peptide H, GHGQPGADAEEKFPYP177VNVE; peptide L, KGHQPGADAEEKFPYP177VNVE. The underlined Glu (E) appeared resistant to cleavage by V8 protease.

These results indicate that Tyr 177 is indeed phosphorylated in p210 Bcr-Abl, and that peptides containing p.Tyr 177 are specifically recognized by the isolated Grb2 SH2 domain. Tyr 177 is also utilized by p185 Bcr-Abl as an autophosphorylation site in vitro (figure 3-4a).
Figure 3-3

Tyr 177 in p210 Bcr-Abl is an autophosphorylation site recognized by the Grb2 SH2 domain.

a. A two-dimensional tryptic/V8 protease map of p210 Bcr-Abl is shown. p210 Bcr-Abl was immunoprecipitated from K562 cells, labelled in an in vitro kinase assay using [γ-32P]-ATP, and subsequently digested with trypsin followed by V8 protease as described in Materials and Methods. Peptides were separated by two-dimensional electrophoresis and chromatography. Previously reported tryptic phosphopeptides are identified by number (844), and peptides produced by the action of both trypsin and V8 protease are identified by capital letters. The previously reported tryptic peptide #10 (844) probably contains peptide L. The origin is the small uniform spot in the lower lefthand corner.

b. Depletion of peptides H and L by immobilized Grb2 SH2 fusion proteins. The trypsin/V8 double digest of p210 Bcr-Abl shown in (a) was mixed with immobilized GST fusion proteins containing the Grb2 tides H and L.

c and d. Elution positions of [32P]-labelled phosphotyrosine by manual Edman degradation of peptides H (c) and L (d). The counts corresponding to each cycle number are the cpm remaining after the cycle.
Manuel Sequencing BCR Phosphopeptide Peptide GHGQPGADAEEKPFYVNVNE

Manuel Sequencing BCR Phosphopeptide Peptide KGHGQPGADAEEKPFYVNVNE
FIGURE 3-4

Tyr 177 is autophosphorylated in p185 Bcr-Abl and is transphosphorylated in p160 Bcr.

a. A two-dimensional tryptic/V8 protease digest of p185 Bcr-Abl is shown. p185 Bcr-Abl was immunoprecipitated from SUP-B15 cells, autophosphorylated *in vitro* with [γ-32P]-ATP, digested with trypsin and V8 protease, and the peptides resolved by two-dimensional electrophoresis and chromatography.

b. A two-dimensional tryptic/V8 protease map of p160 Bcr following transphosphorylation by p210 Bcr-Abl is depicted. Bcr was precipitated by antibodies directed against the carboxy terminal portion of Bcr. This has previously been shown to co-precipitate p210 Bcr-Abl through its association with Bcr. An *in vitro* kinase reaction was performed and following digestion of Bcr with trypsin and V8 protease, the Bcr peptides were analyzed by two-dimensional electrophoresis and chromatography.

Previously reported peptides are identified by number (844) and peptides produced by the action of both trypsin and V8 protease are identified by capital letters.
Furthermore, Tyr 177 is transphosphorylated in p160 Bcr (figure 3-4b) which oligomerizes with Bcr-Abl in cells (359,467,844,857).

3.4.2. The Grb2 SH2 domain specifically interacts with a bcr-encoded sequence containing p.Tyr^{177}-Val^{178}-Asn^{179}-Val^{180}.

The specificity of Grb2 binding to the Tyr 177 autophosphorylation site on Bcr-Abl was further investigated in vitro using synthetic phosphopeptides. A phosphopeptide (designated Bcr 177) corresponding to the bcr-encoded residues 171-182 and containing phosphotyrosine at position 177 was synthesized, along with phosphopeptides corresponding to one of the Grb2 recognition sites on Shc (Shc 317) (848,849) or the Shp-2 recognition site on the platelet derived growth factor (PDGF)-β receptor (PDGF R 1009) (851). Immobilized GST-Grb2 SH2 fusion proteins were incubated with R1-p210 cell lysates in the presence or absence of one of the phosphopeptides. After incubation, bound proteins were recovered, separated by SDS-PAGE and following transfer to nitrocellulose, immunoblotted with anti-Abl antibodies. Addition of the Bcr phosphopeptide, or the phosphopeptide corresponding to the Grb2 binding site on Shc, specifically inhibited the interaction between the immobilized Grb2 SH2 fusion proteins and Bcr-Abl (figure 3-5). Substitution of Ala for Asn at the +2 position in the Bcr peptide abolished the inhibition observed with the wildtype Bcr phosphopeptide, indicating that this residue is critical for the interaction with Grb2 SH2.

These results were confirmed using Biosensor technology (176,177,178). The Bcr phosphopeptide was immobilized to the sensor chip of a Pharmacia BIAcore instrument; soluble full length Grb2 or Grb2 SH2 fusion proteins bound to the Bcr phosphopeptide, as measured by surface plasmon resonance (figure 3-6). Binding of unphosphorylated peptides to SH2 domains in this system has previously been shown to be negligible (178). The affinities of various
Figure 3-5

Interaction of the Grb2 SH2 domain with Bcr-Abl is inhibited
by specific phosphopeptides in vitro.

a. Immobilized GST-Grb2 SH2 fusion proteins were incubated with lysates of R1-p210 cells, with or without 100 μM of synthetic phosphopeptide corresponding to one of the Grb2 binding sites on Shc (Shc 317) or the Shp-2 recognition site on the PDGF receptor (PDGF R 1009). Proteins bound to the immobilized GST-Grb2 SH2 fusion proteins were recovered and immunoblotted with anti-Abl antibodies followed by horseradish peroxidase-conjugated anti-mouse IgG and development with chemiluminescent reagents. GST alone was included as a negative control, and an anti-Abl immunoprecipitate was also included to indicate the relative migration position of p210 Bcr-Abl.

b. In a similar experiment to that described in (a) immobilized GST-Grb2 SH2 fusion proteins were incubated with lysates of R1-p210 cells in the presence or absence of a phosphopeptide corresponding to the Tyr177 site on Bcr (Bcr 177) or a phosphopeptide identical to the Bcr peptide but containing Ala substituted for Asn at the +2 position (Bcr Ala +2). Proteins bound to GST-Grb2 SH2 were immunoblotted with anti-Abl antibodies followed by secondary antibody and development with chemiluminescent reagents. An anti-Bcr immunoprecipitate was included to demonstrate the relative migration position of p210 Bcr-Abl.
Figure 3-6

The Grb2 SH2 domain specifically interacts with

the bcr-encoded sequence p.Tyr^{177}-Val-Asn-Val.

A Bcr phosphotyrosine-containing synthetic peptide corresponding to residues 171-182 of human Bcr, was immobilized to the sensor chip surface of a Pharmacia BIAcore instrument, and binding of GST-Grb2 fusion proteins to this peptide was monitored by surface plasmon resonance. The affinities of various phosphotyrosine-containing peptides for GST-Grb2 (solid symbols) or GST-Grb2 SH2 (open symbols) were assayed by their abilities to compete with this interaction. Soluble GST-Grb2 or GST-Grb2 SH2 proteins were mixed with varying concentrations of phosphotyrosine-containing peptides Bcr (O), Shc (☑), Asn→Ala Bcr (Δ), PDGFR 771 (□), or PDGFR 1009 (◊) and injected across the sensor chip surface containing the immobilized Bcr peptide. The sequences of these synthetic phosphopeptides are described in Materials and Methods. The amount of GST-Grb2 or GST-Grb2 SH2 bound to the immobilized peptide was estimated from the steady-state surface plasmon resonance signal at a fixed time following the end of the injection, and the percentage bound relative to injection of GST-Grb2 or GST-Grb2 SH2 alone was calculated. The maximal signal, obtained in the absence of competing peptide, was assigned a value of 100%.
phosphotyrosine-containing peptides for GST-Grb2 or GST-Grb2 SH2 were then measured by their abilities to compete with the interaction between the immobilized Bcr phosphopeptide and the soluble Grb2 fusion proteins. Soluble GST-Grb2 or GST-Grb2 SH2 proteins were mixed with varying concentrations of the phosphopeptides under test, and injected across the sensor chip surface containing the immobilized Bcr peptide. Addition of the soluble Bcr or Shc peptide specifically inhibited the binding of the Grb2 fusion proteins to the immobilized Bcr phosphopeptide whereas similar concentrations of the Asn<sup>179</sup>→Ala Bcr peptide (N179A) or the phosphopeptides corresponding to the p120-Gap (850) or the Shp-2 recognition sites on the PDGF receptor (851) did not. These results suggest that one function of the Bcr element of the Bcr-Abl oncoproteins is the provision of a site for tyrosine phosphorylation that can directly couple Bcr-Abl to Grb2 and hence to the Ras pathway, and that Asn at the +2 position is also critical for this interaction.

3.4.3. Recruitment of the GEF mSos1 into a complex with the Bcr-Abl oncoproteins

Grb2 possesses two SH3 domains which bind to proline-rich motifs within the C-terminal tail of the guanine nucleotide exchange factor mSos1 (231,503). Upon stimulation with EGF, the mSos1-Grb2 complex binds to the activated EGF receptor, an event that translocates mSos1 to the inner plasma membrane and enables mSos1 to stimulate the dissociation of GDP from Ras (668). Both mSos1 and the related protein mSos2 are widely expressed in hematopoietic cells regardless of cell lineage (608). Human counterparts to Sos have also been identified and may therefore participate in Ras activation in human leukemic cells (612). Significant quantities of mSos1 co-precipitated with Grb2 from lysates of parental Rat-1 cells; similar quantities of mSos1 were detected in association with Grb2 in <i>bcr-abl</i>-transformed cells (figure 3-7). To assess whether
Lysates of Rat-1 or R1-p210 cells were immunoprecipitated with anti-mSos1 or anti-Grb2 antiserum, and immunoblotted with anti-mSos1 antibodies followed by detection with $^{125}$I-labelled protein A. Normal rabbit serum (C) was included as a negative control. The position of mSos1 is indicated by an arrow.
IP: mSos1

Blot: anti-mSos1
mSos1 is recruited into complex formation with Bcr-Abl, Rat-1 or R1-p210 cell lysates were immunoprecipitated with anti-mSos1 or anti-Abl antibodies, and the precipitated proteins immunoblotted with anti-Abl antibodies. p210 Bcr-Abl but not endogeneous c-Abl, co-precipitated with mSos1 (figure 3-8a). Similar results were obtained with p185 Bcr-Abl which co-precipitated with mSos1 from R1-p185 cell lysates (figure 3-8b). A GST fusion protein corresponding to the proline-rich carboxy terminal tail of mSos1 bound p185 Bcr-Abl from an R1-p185 cell lysate, suggesting that this region is responsible for the interaction with Bcr-Abl (data not shown). The Abl SH3 domain binds short proline-rich motifs (858) and could potentially associate directly with the C-terminal tail of mSos1. However, a GST-Abl SH3 fusion protein did not bind efficiently to mSos1 in vitro suggesting that such an interaction does not contribute significantly to complex formation (data not shown). These results suggest that the association of mSos1 with Bcr-Abl occurs through the Grb2 adaptor protein. In common with Grb2, mSos1 was not significantly phosphorylated on tyrosine in bcr-abl-transformed cells (data not shown).

The 95 kDa Vav protein is expressed specifically in hematopoietic cells and has been reported to function as a Ras GEF in lymphocytes, an activity which correlates with induced tyrosine phosphorylation of Vav upon antigen receptor stimulation (667,836,838). In the previous chapter, Vav was noted to be highly phosphorylated on tyrosine in Ph+ cells. Although the putative function of Vav as a Ras GEF is controversial (839,840), this finding raises the possibility that Vav may function as a Ras GEF in bcr-abl-transformed cells. We therefore investigated whether Grb2 is capable of interacting with Vav in Ph+ cells. Significant amounts of Vav were not detected in association with Grb2 when WL-1 Ph+ ALL cells expressing p185 Bcr-Abl were lysed in the presence of 1% Nonidet-P40 and immunoprecipitated with anti-Vav antibody (figure 3-9).
Figure 3-8

Bcr-Abl oncoproteins complex with mSos1 in vivo.

a. Lysates of Rat-1 or R1-p210 cells were immunoprecipitated with anti-Abl or anti-mSos1 antibodies, and the immunoprecipitated proteins subsequently immunoblotted with anti-Abl antibodies followed by secondary antibody and detection with chemiluminescent reagents. Normal rabbit serum (C) was included as a negative control. The positions of p210 Bcr-Abl and p145 c-Abl are indicated.

b. R1-p185 cells were lysed, immunoprecipitated with anti-Abl or anti-mSos1 antibodies, and immunoblotted with anti-Abl antibodies followed by secondary antibody and detection with chemiluminescent reagents. The position of p185 Bcr-Abl is indicated.
a. R1-p210

b. R1-p185

IP:

p185 Bcr-Abl

C

C

C

1 2 3 4 5 6

p210 Bcr-Abl

cAbl

p145 c-Abl

Blot:

cAbl

a. R1-p210

b. R1-p185

IP:

p185 Bcr-Abl

C

C

C

1 2 3 4 5 6

p210 Bcr-Abl

cAbl

p145 c-Abl

Blot:

cAbl
Lysates of the Ph+ WL-1 cell line expressing p185 Bcr-Abl were immunoprecipitated with antibodies directed against Grb2 or Vav as indicated and identical samples immunoblotted with specific antibody or anti-p.Tyr antiserum. All blots were developed by $^{125}$I-labelled protein A. Pre-immune serum (C) was included as a negative control.
Co-precipitation of Vav with Grb2 was also not detected in the reciprocal experiment suggesting that Grb2 does not link Vav to the Ras pathway in these cells.
3.5. DISCUSSION

3.5.1. Autophosphorylation within the bcr-encoded sequence of Bcr-Abl creates a Grb2 SH2-binding site

Potential targets of Bcr-Abl may be regulated directly by tyrosine phosphorylation, or indirectly through the inducible association with tyrosine phosphorylated proteins such as the autophosphorylated Bcr-Abl kinases themselves. During a screen for proteins which associate with Bcr-Abl, the polypeptide Grb2 was found to associate with the Bcr-Abl tyrosine kinases in both human leukemic cells and bcr-abl-transformed Rat 1 fibroblasts. Grb2, which has the structure SH3-SH2-SH3, can function as a molecular adaptor by linking a subset of receptor tyrosine kinases and other phosphotyrosine-containing proteins to mSos1, a Ras-GEF (231,503,612,618, 639,650,668). Genetic data from C. elegans and Drosophila melanogaster suggest that the invertebrate homologues of Grb2 and mSos1 are crucial in coupling receptor tyrosine kinases to activation of the Ras pathway (203-205,606).

The association of Grb2 with receptor tyrosine kinases is normally transiently induced by receptor autophosphorylation, which creates a specific binding site for the Grb2 SH2 domain (200,231,637,668). However, Grb2 only binds to a subset of receptor tyrosine kinases upon growth factor stimulation (635) and has not previously been demonstrated to interact stably with cytosolic tyrosine kinases. We therefore investigated the nature of the interaction between Grb2 and Bcr-Abl. In vitro, the isolated Grb2 SH2 domain was sufficient for direct, phosphorylation-dependent binding to p210 and p185 Bcr-Abl (L.P., M.Sc. thesis, University of Toronto, 1994). Affinity chromatography studies with a degenerate phosphopeptide library have shown that the Sem-5 or Grb2 SH2 domain binds preferentially to phosphotyrosine followed at the +2 position by Asn (179,180). Examination of the sequences of p185 and p210 Bcr-Abl revealed a single potential
binding site for the Grb2 SH2 domain, Tyr^{177}-Val^{178}-Asn^{179}-Val^{180}, located within the N-terminal bcr-encoded sequence common to both p185 and p210 Bcr-Abl. Here it is demonstrated that Tyr 177 undergoes autophosphorylation in both of these isoforms of Bcr-Abl, and that a proteolytic peptide derived from Bcr-Abl which contains this site is specifically depleted by incubation with the isolated Grb2 SH2 domain. Furthermore, the interaction of Bcr-Abl with GST-Grb2 SH2 is selectively inhibited by a soluble p.Tyr-containing peptide representing the Tyr 177 phosphorylation site. The ability of this peptide to bind with high affinity to the Grb2 SH2 domain, and thereby block the interaction with Bcr-Abl is also dependent on Asn^{179} at the +2 position relative to phosphotyrosine.

The N-terminal Bcr sequence is implicated in Abl kinase activation and localization of Bcr-Abl to F-actin filaments, functions which are essential for transformation (192,359,360). Our findings suggest that Bcr sequences also contribute directly to interactions of Bcr-Abl proteins with their targets by providing autophosphorylation sites which allow Bcr-Abl to couple directly to SH2-containing signalling proteins. Consistent with the hypothesis that these Bcr-mediated interactions are important for Bcr-Abl transforming activity, a deletion mutant of p185 Bcr-Abl lacking residues 162-220 is impaired in its ability to transform primary bone marrow cultures (783). Taken together, these results suggest that inhibiting the interaction of Grb2 with p.Tyr 177 of Bcr-Abl represents a potential approach to reversing the transformed phenotype of Ph+ leukemic cells.

p185 and p210 Bcr-Abl are predominantly autophosphorylated on tyrosine on sequences encoded by bcr exon 1 (844). The bcr gene may therefore contribute additional as yet unidentified SH2-binding (or PTB-binding) sites for Bcr-Abl effectors. Furthermore, Bcr-Abl transphosphorylates full length Bcr on Tyr 177. This finding, coupled with the observation that Bcr oligomerizes with Bcr-Abl (857) suggests that Bcr itself may modulate signalling in Ph+ leukemia.
Bcr's structural features, notable serine/threonine kinase activity, Rac/CDC42Hs Gap domain and its Dbl-homology domain (468,470-472) predict multiple functions in intracellular signalling.

3.5.2. Multiple positive regulators of the Ras pathway are targets of the Bcr-Abl tyrosine kinases in vivo

The ability of Grb2 to stably complex with Bcr-Abl suggested that Bcr-Abl might associate with an activator of Ras guanine nucleotide exchange and indeed, data presented here indicate that mSos1 complexes with Bcr-Abl in vivo. The simplest explanation for this finding is that Grb2 binds directly to Bcr-Abl oncoproteins through its SH2 domain, thereby recruiting mSos1, which is constitutively associated with the Grb2 SH3 domains, into a heterotrimeric Bcr-Abl/Grb2-mSos1 complex (figure 3-10). A direct association between Bcr-Abl and the proline-rich tail of mSos1, although not ruled out, seems less likely since a GST-Abl SH3 fusion protein did not bind significant amounts of mSos1 protein.

Neither Grb2 nor mSos1 is significantly phosphorylated on tyrosine in bcr-abl-transformed cells, suggesting that their function is regulated by their physical association with phosphotyrosine-containing proteins. Since a fraction of Bcr-Abl localizes to F-actin filaments, a requirement for transformation (359,360), binding of Grb2-mSos1 to Bcr-Abl may relocalize Ras-GEF activity to the cortical cytoskeleton, providing access to Ras. In support of this possibility, relocalization of mSos1 to the plasma membrane by provision of a membrane localization site is sufficient for Ras activation (641,642). Alternatively, mSos1 activity may be stimulated by the interaction of Grb2 with Bcr-Abl.

Other proteins implicated in mediating Ras activation are the 66, 52 and 46 kDa proteins encoded by the shc gene (505,647). In common with Grb2 and mSos1, a fraction of Shc proteins
Fusion of bcr-encoded sequences with Abl results in activation of the Abl kinase domain and autophosphorylation of bcr-encoded Tyr177 in the N-terminal sequence. Phosphorylated Tyr177 and adjacent amino acids are recognized by the Grb2 SH2 domain which brings mSos1 to the vicinity of Bcr-Abl by virtue of the constitutive association of the Grb2 SH3 domains with mSos1.
also binds to the Bcr-Abl proteins (L.P., M.Sc. thesis, University of Toronto, 1994). The physical association of Shc polypeptides with Bcr-Abl is less striking than the Grb2/Bcr-Abl complex, but may serve to facilitate Shc phosphorylation as these proteins are highly phosphorylated on tyrosine residues in \textit{bcr-abl}-transformed cells. It is not yet clear whether the Shc proteins interact directly with Bcr-Abl. However, such an interaction may potentially occur through the N-terminal PTB domain of Shc as well as the Shc SH2 domain (643,859,860). In \textit{bcr-abl}-transformed cells, tyrosine phosphorylated Shc proteins associate with Grb2. Tyr$^{317}$ of Shc, which can bind to the Grb2 SH2 domain, is required for the induction of Ras-dependent events by overexpressed p52$^{\text{Shc}}$ and p46$^{\text{Shc}}$ (848). The formation of Grb2 complexes with Shc and with the Bcr-Abl oncoproteins may therefore act synergistically to enhance Ras activation and to enable Bcr-Abl proteins to transform hematopoietic cells.
CHAPTER 4

SH2-CONTAINING PROTEINS IN IL-3 AND STEEL FACTOR SIGNALLING PATHWAYS

Attribution of data: Anne Burkhardt participated in the experiments shown in Figures 4-8/4-9. All other experiments were performed in their entirety by myself.
4.1. ABSTRACT

The cytokine Steel factor (SF) is essential for normal hematopoietic development, and exerts its effects on a variety of hematopoietic cells including stem cells, multipotential progenitors and mature lineage-specific cells. Depending on the particular cell type, survival, proliferation, differentiation, chemotaxis or adhesion may be induced by the interaction of SF with its transmembrane receptor, the c-Kit tyrosine kinase. SF has previously been shown to induce autophosphorylation of c-Kit and its association with PI3K and PLC-γ1 but not p120-Gap. The SH2 domains of PI3K bind to the Kit kinase insert, suggesting that activated c-Kit, in common with other receptor tyrosine kinases, controls intracellular signalling pathways through interactions with specific SH2-containing proteins which recognize phosphotyrosine sites located within the non-catalytic regions of the receptor’s cytoplasmic tail. In this chapter, we have further characterized c-Kit signalling pathways and provide genetic evidence that the SH2-containing Shc and Vav proteins are direct or indirect substrates of c-Kit in primary hematopoietic cells. These proteins are also shown to be targets of IL-3-inducible tyrosine kinase activity. The participation of Shc and Vav in signalling pathways activated by hematopoietic growth factors, along with data presented in previous chapters, supports the notion that Bcr-Abl exerts its biological effects by the constitutive activation of targets which normally undergo transient activation in response to signals within the hematopoietic microenvironment. In quiescent primary mast cells, Shc and Vav appear to be differentially regulated as only the Vav protein displays low levels of constitutive phosphotyrosine which can occur in the absence of functional c-Kit. Preliminary studies to identify the IL-3-stimulated tyrosine kinase(s) in mast cells, and to determine whether cytosolic PTKs function downstream of c-Kit, are also presented.
4.2. INTRODUCTION

Oncogenes frequently encode activated variants of gene products that normally participate in mitogenic signalling pathways (861). This has led to the widely accepted concept that transformation results from the deregulation of intracellular signalling pathways that are usually tightly controlled by external stimuli. The study of PTK-induced signalling pathways in normal hematopoietic cells therefore complements the study of leukemic cells. In addition to providing insight into the regulation of normal hematopoietic development or function, such an analysis may enhance the understanding of the mechanisms whereby Bcr-Abl or other oncogenic tyrosine kinases exert their growth promoting effects and aid in the appropriate design of therapeutic strategies which specifically target the leukemic cell.

The cytokine Steel factor (also termed mast cell growth factor, stem cell growth factor or Kit ligand) supports the survival and growth of multipotential hematopoietic cells and committed progenitors, particularly in synergy with other cytokines such as interleukin-3 (IL-3), GM-CSF, IL-7 and erythropoietin (45). Other biological responses such as cell differentiation, adhesion and chemotaxis may also be induced by Steel factor (SF), depending on the particular cell type (862-867). In the mouse, the Steel (SI) locus on chromosome 10 encodes SF, and its receptor, c-Kit, is encoded by the Dominant White Spotting (W) locus on chromosome 5 (142,143,868,869). Hematopoietic abnormalities which result from loss-of-function (LOF) mutations at either loci include macrocytic anemia and a deficiency of mast cells (144,870). W mutant bone marrow cells also display defects in multilineage longterm repopulating cells when measured in an in vivo competition assay (871,872). The embryonic lethality of severe W and SI LOF mutations has been attributed to severe anemia, although other factors may contribute (310). Gain-of-function
mutations in the \textit{c-kit} gene have also been described and may have a role in leukemia or other hematological disorders such as systemic mastocytosis (10,754-757,874-876).

The receptor for SF, c-Kit, belongs to the type III RTK subfamily which includes receptors for platelet derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1). This subgroup is characterized by five immunoglobulin-like repeats in its extracellular ligand-binding domain and an intracellular kinase domain which is divided by a kinase insert of variable length into an ATP-binding region and a phosphotransferase domain (46,104). Ligand binding induces c-Kit dimerization which allows juxtaposition of the kinase domains, transphosphorylation and kinase activation (120). Two naturally occurring Kit isoforms, designated Kit\textsubscript{S} and Kit\textsubscript{L} or Kit A, are produced by alternative splicing and are co-expressed; the less abundant of these, Kit A, contains an additional four amino acids within the extracellular domain (877). The two isoforms may differ in their ability to transduce a mitogenic signal in response to soluble ligand when ectopically expressed in fibroblasts or COS cells (758,878, R. Blouin and A. Bernstein, unpublished observations). These differences are postulated to occur through different efficiencies in dimerization and/or internalization as both isoforms bind ligand with similar efficiency.

In contrast to c-Kit, the majority of cytokine receptors do not possess intrinsic enzymatic activity but recruit cytosolic tyrosine kinases whose activity is inducible upon binding of cognate ligand to the receptor. The high affinity IL-3 receptor is a heterodimer comprised of a unique \(\alpha\) subunit which binds ligand and a \(\beta_c\) subunit which is shared with the GM-CSF and IL-5 receptors (879). An additional IL-3 receptor-specific \(\beta\)-subunit (\(\beta_{\text{IL-3}}\)) in mice, but not humans, binds ligand with low affinity and is co-expressed on all IL-3-responsive cells (880). High affinity receptors containing \(\beta_c\) or \(\beta_{\text{IL-3}}\) subunits are thought to possess identical signalling properties \textit{in vivo} (881). The precise role of IL-3 and its receptors \textit{in vivo} is not well understood since mice lacking
expression of both the IL-3 ligand and the βe subunit do not have a readily discernable phenotype other than that attributed to IL-5 and GM-CSF-deficient signalling (882,883).

A useful model system in which to study receptor PTK or cytokine receptor-associated PTK activity is provided by the culture of primary mast cells (MC) from progenitor cells in bone marrow (BM) or fetal liver (FL) (884,885). Cultured mast cells remain growth factor-dependent and senesce, thus representing physiologically normal hematopoietic cells. Their analysis may therefore closely reflect signalling events in vivo. In BMMC, SF induces c-Kit autophosphorylation and its association with PI3K and PLC-γ1 but not p120-Gap (824,877). These results suggest that c-Kit, like other receptor tyrosine kinases, controls intracellular signalling pathways through interactions with specific SH2-containing targets (200). In this chapter, we have further characterized c-Kit signalling pathways in primary mast cells, and provide genetic evidence that the SH2-containing Shc and Vav proteins are direct or indirect substrates of c-Kit. The Shc proteins are molecular adaptors implicated in the positive regulation of Ras (647) and the hematopoietic-specific Vav protein was initially reported to be a Ras GEF (667, 836-838). More recent studies have indicated that Vav exhibits GEF activity for members of the Rho family, a finding more consistent with its Dbl-homology domain (835,839,840). In quiescent mast cells, Shc and Vav may be differentially regulated as only the Vav protein displays low levels of constitutive phosphorylation on tyrosine which can occur in the absence of functional c-Kit. We have also characterized the Src family PTKs which are expressed in BMMC and present preliminary studies to identify the IL-3-stimulated tyrosine kinase(s) in BMMC and to determine whether cytosolic PTKs function downstream of c-Kit.
4.3. MATERIALS AND METHODS

Mice. +/+, W\textsuperscript{41}/+ and W\textsuperscript{42}/+ mice in a C57BL/6 host background were purchased from Jackson Laboratory (Bar Harbour, Maine). W\textsuperscript{42}/+ or W\textsuperscript{37}/+ heterozygotes were crossed and the resulting W\textsuperscript{42} or W\textsuperscript{37} homozygous fetuses distinguished from their littermates by their anemic appearance. Embryos in which the syk gene has been disrupted by homologous recombination were obtained as previously described (100). Fetal livers from W\textsuperscript{42}/W\textsuperscript{42} and W\textsuperscript{37}/W\textsuperscript{37} embryos, or from Syk-/− and +/+ littermates, were taken at day 15.5 postcoitum. W\textsuperscript{41}/W\textsuperscript{41} homozygous adult mice were distinguished from their littermates by coat colour.

Cell culture and growth factor stimulations

Single cell suspensions derived from adult bone marrow or fetal liver were cultured in Iscove's modified Dulbecco medium (IMDM) supplemented with 5% FBS and conditioned medium from the myeloma cell line X63 Ag8-653 which carries a recombinant interleukin-3 (IL-3) cDNA expression vector (886). Conditioned medium was added to give a final concentration of 0.5 U of IL-3 per ml. After 7 days of incubation, the nonadherent cells were harvested and resuspended in IMDM with .05% bovine serum albumin (BSA), 1% FBS, 2 µg ml\textsuperscript{-1} Concanavalin A, 5 µg ml\textsuperscript{-1} transferrin, 5 µg ml\textsuperscript{-1} insulin and 0.5 U ml\textsuperscript{-1} IL-3. 80% of the medium was changed every 5-7 days and the cells maintained at a density of 5x10\textsuperscript{5} cells ml\textsuperscript{-1}. Homogeneous populations of mast cells were obtained after 4-8 weeks of culture. FDC-P1 cells (887) were a gift of Dr. M. Minden (University of Toronto) and were maintained in RPMI medium supplemented with 10% FBS and 1-5% X63 CM. M07e cells were also a gift of Dr. M. Minden and were grown in RPMI medium supplemented with 5637 conditioned medium and 20% FBS (888). NIH 3T3 cells infected with
retroviral constructs expressing Kit\textsubscript{S} or Kit\textsubscript{L} (Kit A) have been described (756). All cells were incubated at 37°C in a humidified incubator containing 5% CO\textsubscript{2} (vol/vol).

For growth factor stimulations, cultures of mast cells were washed once in phosphate buffered saline (PBS) and resuspended in IMDM plus 0.5% FBS and 1% BSA for 4-48 hours as indicated. Following growth factor deprivation, cells were stimulated with 100-250 ng ml\textsuperscript{-1} SF for varying lengths of time at 37°C. SF was generously provided by Immunex Corporation, Seattle, Washington. FDC-P1 cells were washed in PBS and resuspended in RPMI with 10% FBS but no IL-3. For IL-3 stimulations, 5% (vol/vol) conditioned medium from the X63 Ag8-653 myeloma cell line or 100 μg ml\textsuperscript{-1} purified recombinant IL-3 (provided by Immunex Corporation) was added to the cells.

**Immunoprecipitations and Western blotting**

Cells were briefly sedimented by centrifugation and following removal of supernatant, immediately lysed in PLC lysis buffer as previously described (798). All following steps were carried out at 4°C. Cell lysates were centrifuged at 13 000 rpm for 15 min to obtain clarified lysates. Rabbit polyclonal antibody directed against Shc (505), Vav (793) or Grb2 (647) and affinity purified anti-phosphotyrosine antibodies (797) were used for immunoprecipitations as previously described. Polyclonal anti-Kit antibodies were raised against a GST fusion protein corresponding to the kinase insert (824) or the carboxy terminal tail of murine c-Kit (amino acid residues 916-975). Monoclonal antibody directed against the extracellular domain of c-Kit was provided by Dr. S-I Nishikawa (Kumamoto University, Kumamoto, Japan). Antibodies directed against Syk (889) and each member of the Src family have been described (890) and were generously provided by Dr. J. Bolen (DNAX, Palo Alto, CA). Antibody and 100 μl of protein A-sepharose, or anti-mouse IgG
coupled to agarose were added to the cell lysates and the samples gently rocked for 90 min at 4°C. Immunoprecipitates were washed 3 times in HNTG (800), resuspended in SDS sample buffer and heated for 3 min prior to SDS-polyacrylamide gel electrophoresis (PAGE). Resolving gels contained 7.5, 10 or 12.5% polyacrylamide as specified. The separated proteins were transferred to nitrocellulose, blocked and incubated with specific primary antibodies for 90 min. After washing, the filters were probed with [125I]-protein A and exposed to Kodak XAR film with an intensifying screen at -70°C. Alternatively, filters were incubated with horseradish peroxidase-coupled antirabbit immunoglobulin and antibody binding subsequently detected by enhanced chemiluminescence reagents (Amersham). For anti-p.Tyr immunoblotting, affinity purified polyclonal anti-p.Tyr antibodies and [125I]-protein A or the monoclonal 4G10 antibody (Upstate Biotechnology) followed by horseradish peroxidase-conjugated anti-Ig antiserum and development by enhanced chemiluminescent reagents (Amersham) were used as specified. In some experiments, phosphotyrosine quantitation was carried out with a Molecular Dynamics Phosphorimager and IMAGEQUANT software provided by the supplier.

Bacterial fusion proteins

GST fusion proteins corresponding to the Shc SH2 domain have been described (649). Various portions of the c-Kit receptor, corresponding to the juxtamembrane region, the kinase insert or the carboxy terminal tail (amino acid residues 544-574, 685-763 and 916-975 of murine c-Kit respectively), were amplified by polymerase chain reaction from a cDNA encoding murine c-Kit receptor and subcloned into the bacterial expression vector pGEX-2t. All PCR products were sequenced. The DH5α E. Coli strain was transformed by the pGEX-2t plasmids and infected with a λB1 phage containing the Elk tyrosine kinase domain to allow tyrosine phosphorylation of the GST
fusion protein (797,824). Bacterial lysogens were isolated and grown at 30°C for 3 hr with 1 mM isopropyl-β-D-thiogalactoside (IPTG) to induce expression of both the GST fusion proteins and the Elk kinase. The tyrosine phosphorylation of the GST fusion proteins was verified by anti-p.Tyr immunoblotting. Similar quantities of the GST fusion proteins (10-20 µg) were mixed with cell lysates and mixing experiments carried out as described in Chapter 2.

Immune complex kinase assays

+/+ BMMC were growth factor-deprived, then stimulated with SF or IL-3 for 5 min. Unstimulated or stimulated cells were spun down and the cell pellets immediately flash frozen in liquid nitrogen and stored at -70°C until use. Upon thawing the cells were lysed in kinase buffer and [γ-32P]-ATP added for in vitro kinase reactions as previously described (891).
4.4. RESULTS

4.4.1. Multiple proteins are tyrosine phosphorylated in SF-stimulated +/+ mast cells

As an initial step in characterizing the targets of the c-Kit receptor in normal hematopoietic cells, homogeneous populations of mast cells were cultured from progenitors in the bone marrow of adult wildtype C57BL/6 mice and analyzed for the presence of tyrosine phosphorylated proteins. Cells were rendered quiescent by incubation in medium without growth factor for 48 hours, and then stimulated with recombinant soluble SF for 2 min at 37°C. Equivalent numbers of stimulated and unstimulated cells were lysed and immunoprecipitated with affinity purified polyclonal anti-p.Tyr antibodies (797) or anti-Kit antiserum raised against the kinase insert (824). The precipitated proteins were electrophoresed through an SDS gel containing 10% polyacrylamide, transferred to nitrocellulose and then immunoblotted with anti-p.Tyr antibodies. Little phosphotyrosine was detected in growth factor-deprived cells (figure 4-1). In accordance with previous findings, the mature glycosylated Kit isoforms, migrating at an apparent molecular mass of 145-160 kDa, were the principal tyrosine phosphorylated proteins detected in SF-stimulated cells (824,878). The broad smear of c-Kit observed is due to post translational modification including glycosylation and polyubiquitination (892). Other prominent phosphoproteins which were consistently detected in the anti-p.Tyr immunoprecipitates of SF-stimulated but not resting cells included discrete bands of approximately 85, 40 and 38 kDa and a relatively diffuse band of 55-60 kDa. Phosphorylation of the majority of the bands detected in whole cell lysates was maximal by 2 min and had declined by 10 min but was still present at 30 min with continued exposure to SF (data not shown).
Quiescent +/- BMMC were incubated with (+) or without (-) 250 ng ml⁻¹ of soluble recombinant SF for two min at 37°C, lysed and immunoprecipitated with polyclonal anti-Kit antiserum raised against the kinase insert (lanes 1 and 3) or affinity-purified polyclonal anti-p.Tyr antibodies (lanes 2 and 4). Following separation by electrophoresis through an SDS gel containing 10% polyacrylamide, the precipitated proteins were transferred to nitrocellulose and immunoblotted with anti-p.Tyr antibodies and [¹²⁵I]-protein A. The positions of c-Kit, p85, p60, p40 and p38 are indicated by arrows, and the positions of molecular weight markers are also indicated.
+/+ mast cells

IP:  αKIT  αPY  αKIT  αPY  M_r(K)

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Blot: αp.Tyr.
4.4.2. SF induces the tyrosine phosphorylation of p52\textsuperscript{shc} and p46\textsuperscript{shc} in BMMC

The \textit{shc} gene encodes three isoforms which differ only in the extent of their N-termini (505). \textit{p52} \textsuperscript{shc} and \textit{p46} \textsuperscript{shc} arise from the differential use of translation initiation sites within a 3.4 kb \textit{shc} mRNA whereas the 66 kDa protein is encoded by a distinct \textit{shc} transcript. To determine whether Shc proteins participate in signalling pathways activated by c-Kit, lysates of SF-stimulated or growth factor-deprived BMMC were immunoprecipitated with polyclonal anti-Shc antibodies. Following protein separation by gel electrophoresis and transfer to nitrocellulose, the immune complexes were probed with anti-p.Tyr or anti-Shc antiserum and developed with \textsuperscript{\(\text{125}\text{I}\)}-protein A. Both \textit{p52} \textsuperscript{shc}, the predominant Shc species, and \textit{p46} \textsuperscript{shc} were readily detected in BMMC (figure 4-2a, lane 1). A dramatic and rapid increase in the tyrosine phosphorylation of \textit{p52} \textsuperscript{shc} and \textit{p46} \textsuperscript{shc} was detected upon addition of SF (figure 4-2b, lanes 3 and 6), without a change in the level of total precipitable Shc (figure 4-2a, lane 2). The minor 66 kDa Shc species was detected in BMMC only when blots were developed with chemiluminescent reagents (data not shown) and was not analyzed in these experiments. However, SF-induced tyrosine phosphorylation of \textit{p66} \textsuperscript{shc} was readily detected in the growth factor-dependent megakaryocytic leukemic cell line M07e (888) (data not shown) indicating that all three Shc isoforms are inducibly phosphorylated in response to SF. In both M07e and BMMC, the intensity of SF-induced phosphorylation paralleled the level of expression of each isoform suggesting that all three isoforms are phosphorylated to similar levels (figure 4-2 and data not shown).

In SF-stimulated +/- BMMC, the peak level of \textit{p52} \textsuperscript{shc} tyrosine phosphorylation was reached by 2 min and had declined by 15 min but was still detected at 60 min with continued exposure to soluble Steel ligand (figure 4-3, lanes 9-12). This pattern of phosphorylation paralleled the autophosphorylation of c-Kit itself (lanes 1-4). The kinetics observed likely represent the combined
Figure 4-2

The tyrosine phosphorylation of Shc and Vav in response to Steel factor stimulation of +/- BMMC.

+/- BMMC were rendered quiescent by removal of growth factor for 48 hrs and then stimulated with 250 ng ml\(^{-1}\) SF for two min at 37\(^\circ\)C. Unstimulated or stimulated cells were lysed and immunoprecipitated with polyclonal antibodies directed against the kinase insert of c-Kit, Vav or Shc. The precipitated proteins were electrophoresed through an SDS gel containing 7.5% polyacrylamide and transferred to nitrocellulose. a. anti-Shc immunoprecipitates from SF-stimulated and unstimulated cells were blotted with anti-Shc antiserum followed by \(^{125}\text{I}\)-protein

b. anti-Kit (lanes 1 and 4), anti-Vav (lanes 2 and 5) and anti-Shc (lanes 3 and 6) immunoprecipitates were immunoblotted with anti-p.Tyr antibodies followed by \(^{125}\text{I}\)-protein A. The positions of c-Kit, Vav, the 46 and 52 kDa Shc proteins and a p140/p145 doublet associated with Shc are indicated by arrows. The positions of molecular weight markers are also indicated. A 72 hr exp is shown. c. anti-Vav immunoprecipitates were blotted with anti-Vav antibodies followed by a secondary incubation with horseradish peroxidase-conjugated anti-immunoglobulin antibodies and development with enhanced chemiluminescent reagents. Each lane represents approximately 2x10\(^7\) cells.
Figure 4-3

Kinetics of c-Kit, Vav and Shc tyrosine phosphorylation in response to Steel ligand.

+/+ BMMC were incubated with soluble 250 ng ml⁻¹ Steel ligand for 0 (lanes 1, 5 and 9), 2 (lanes 2, 6 and 10), 15 (lanes 3, 7 and 11) or 60 min (lanes 4, 8 and 12) at 37°C, then lysed and immunoprecipitated with antibodies to the c-Kit kinase insert (lanes 1-4), Vav (lanes 5-8) or Shc (lanes 9-12). The precipitated proteins were blotted with affinity purified anti-p.Tyr antibodies and [¹²⁵I]-protein A after protein separation by electrophoresis through an SDS gel containing 7.5% polyacrylamide and transfer to nitrocellulose. A 48 hr exp is shown.
+/+ mast cells

- p145 c-Kit
- p95 Vav
- p52 Shc

IP: αKit αVav αShc

αp.Tyr blot
effects of receptor-ligand internalization/degradation, and ligand-induced tyrosine phosphatase activity (305,892-895).

Genetic evidence for the notion that p52\textsuperscript{shc} and p46\textsuperscript{shc} are direct or indirect targets of c-Kit tyrosine kinase activity was provided by the analysis of mast cells derived from mouse embryos homozygous for severe loss-of-function \textit{W}/c-\textit{Kit} mutations. The \textit{W}\textsuperscript{42} mutation results in substitution of an invariant aspartate residue located within the conserved phosphotransfer active site in the distal portion of the split kinase domain (Asp\textsuperscript{790}$\rightarrow$Asn) (896). Structural analysis of cAMP-dependent protein kinase has indicated that this highly conserved residue serves as the catalytic base at the active site (897). Mutation of the analogous residue in the cytosolic c-Fps PTK abolishes kinase activity (1,799). In \textit{W}\textsuperscript{42}/\textit{W}\textsuperscript{42} mast cells, normal levels of a functionally inactive c-Kit protein are expressed at the cell surface (896). These cells cannot be maintained in tissue culture in the presence of SF, but develop from fetal liver progenitor cells when cultured in the presence of soluble IL-3. While the 52 and 46 kDa Shc proteins are expressed in \textit{W}\textsuperscript{42}/\textit{W}\textsuperscript{42} FLMC at levels comparable to those observed in +/+ cells (see figure 4-7a), neither Shc protein was tyrosine phosphorylated upon addition of SF (figure 4-4a, lanes 3 and 6). Similar results were obtained for FLMC cultured from mouse embryos homozygous for the \textit{W}\textsuperscript{37} mutation, another severe mutation (Glu\textsuperscript{582}$\rightarrow$Lys) which allows receptor expression at the cell surface but a selective reduction in the mature glycosylated form of c-Kit and total loss of kinase activity (data not shown) (878,898). In \textit{W}\textsuperscript{42}/+ heterozygous BMMC, SF stimulated sufficient dimerization and activation of the receptor encoded by the wildtype allele to induce the tyrosine phosphorylation of p52\textsuperscript{shc} (figure 4-4b, lanes 3 and 6).
Figure 4-4

Genetic evidence that Shc and Vav are targets of the c-Kit tyrosine kinase receptor.

a. W^{42}/W^{42} FLMC were rendered quiescent by growth factor deprivation for 36 hr, then incubated with or without SF for 2 min at 37°C. The cells were lysed and immunoprecipitated with antibodies to the kinase insert of c-Kit, Shc or Vav. The precipitated proteins were separated on an SDS gel containing 7.5% polyacrylamide, transferred to nitrocellulose and immunoblotted with anti-p.Tyr antibodies followed by [125I]-protein A. A 10 d exp is shown.

b. W^{42}+/ BMMC were assayed as described in (a). The autoradiogram shown is a 72 hr exp and was obtained in the same experiment as the +/- BMMC shown in figure 4-2.

c. W^{41}/W^{41} BMMC were assayed as in (a). A 48 hr exp is shown.

Each sample in a, b and c represents 2x10^7 cells.
\(W^{41}/W^{41}\) cells express a less severe mutation with residual Kit kinase activity (Val\(^{83}\)→Met) and also responded to SF by the phosphorylation of Shc on tyrosine (figure 4-4c, lane 6). The ability of the \(W^{41}\) receptor to induce phosphorylation of Shc correlated with the ability of \(W^{41}/W^{41}\) BMMC to survive in co-culture with fibroblasts as a source of SF, albeit at a lower level than wildtype cells (879,898).

**4.4.3. Shc-Grb2 complexes are detected in SF-stimulated cells**

The two Kit isoforms may differ in their ability to transduce signals upon ligand binding secondary to differences in dimerization efficiency and/or internalization (877,898, R. Blouin. X. Piao and A. Bernstein, unpublished observations). We therefore tested whether ligand activation of each Kit isoform elicits formation of a Shc-Grb2 complex. The inducible formation of such a complex is dependent on the phosphorylation of Tyr 239, Tyr 240 and/or Tyr 317 of Shc and is implicated in activation of the Ras pathway (231,505,848,899). NIH 3T3 cells infected with retroviral vectors encoding Kit\(_S\) or Kit\(_L\) (Kit A) (756) were stimulated with SF, and immunoprecipitated with anti-Grb2 antibodies followed by anti-Shc immunoblotting. All three isoforms of Shc co-precipitated with Grb2 in lysates of stimulated but not unstimulated NIH 3T3 cells expressing the predominant Kit isoform Kit\(_S\) (figure 4-5a). A modest amount of p52\(_{shc}\) also inducibly associated with Grb2 in SF-stimulated Kit\(_L\)-NIH 3T3 cells (figure 4-5b). In the reciprocal experiment, a fraction of Grb2 was detected in anti-Shc immune complexes (data not shown). Activation of either Kit isoform is therefore sufficient to modulate Shc-Grb2 complexes, although more subtle qualitative or quantitative differences may exist. In +/- BMMC, an inducible association of Shc with Grb2 was also detected (data not shown).
**Figure 4-5**

**Inducible association of Shc and Grb2 upon addition of SF.**

*a and b.* NIH 3T3 cells individually expressing the KitS (a) or KitL (b) isoform were growth factor-deprived for 36 hr, then stimulated with SF for 2 min at 37°C. The cells were lysed and immunoprecipitated with anti-Shc or anti-Grb2 antibodies, or pre-immune serum (C). The recovered proteins were immunoblotted with anti-Shc antibodies and ¹²⁵I-protein A. A 7d exp is shown.
4.4.4. Tyrosine phosphorylation of the 95 kDa Vav protein is stimulated by SF

The 95 kDa Vav protein has expression largely restricted to hematopoietic cells (900,901) and contains a number of structural motifs which suggest a key role in signal transduction (reviewed in 902). The ability of Vav to function as a target of c-Kit was therefore also examined. In +/- BMMC, a 3-fold increase in the tyrosine phosphorylation of Vav was detected after stimulation with SF for 2 min, as measured by scanning densitometry with a Phosphorimager (figure 4-2b, lanes 2 and 5). Increased phosphorylation was not accompanied by a change in the total amount of precipitable Vav (figure 4-2c). The kinetics of Vav tyrosine phosphorylation were rapid and matched those of p52\textsuperscript{shc} and c-Kit itself (figure 4-3). In other experiments, the phosphorylated Vav protein was clearly shown to migrate more slowly than the major 85 kDa phosphoprotein detected in anti-p.Tyr immunoprecipitates of SF-stimulated BMMC (data not shown). Increases in Vav phosphorylation were also detected in SF-stimulated \textit{W}\textsuperscript{d1}\textit{/W} and \textit{W}\textsuperscript{d2}/+ BMMC but not in mast cells homozygous for the \textit{W}\textsuperscript{d2} allele (figure 4-4a-c). Low levels of Vav tyrosine phosphorylation were consistently detected in quiescent mast cells expressing either wildtype (lane 2 in figure 4-2b) or \textit{W} mutant receptors (lane 2 in figure 4-4a-c). This was in contrast to the Shc proteins which did not exhibit detectable levels of phosphotyrosine in cells deprived of growth factor for more than 8-12 hours (lane 3 in figure 4-2b and 4-4 a-c). The differential phosphorylation of Vav and Shc when cells are growth factor-deprived may reflect the different ability of these proteins to function as substrates for a tyrosine kinase which is active in quiescent cells. Although the kinetics of dephosphorylation of these two proteins following addition of SF appears similar, an alternative explanation is that Vav and Shc have different susceptibilities to tyrosine phosphatases such as Shp-1, or different rates of protein turnover in resting cells. Spontaneous wildtype c-Kit dimerization
may have contributed to the somewhat higher and more variable levels of Vav tyrosine phosphorylation which were observed in lysates of unstimulated +/+ cells.

4.4.5. Phosphoproteins of 140-145 kDa co-precipitate with Shc in SF-stimulated BMMC

In SF-stimulated +/+ BMMC (figure 2-4b, lane 6) and W12+/+ BMMC (figure 4-4b, lane 6) anti-Shc antibodies precipitated low levels of a phosphorylated, sharply delineated 140/145 kDa doublet which was not recognized directly by the anti-Shc antibodies. SF-stimulated M07e cells also exhibited a prominent Shc-associated 140-145 kDa phosphoprotein (data not shown). Phosphoproteins of these molecular weights were not observed in anti-Grb2 or anti-Vav immune complexes from SF-stimulated +/+ cells. A similar Shc-associated protein, if present, was below the level of detection in W41/W41 BMMC when anti-p.Tyr blots were developed with [125I]-protein A (figure 4-4c, lane 6). The upper band of the Shc-associated doublet in +/+ and W12+/+ cells co-migrated with the lower portion of the broad autophosphorylated c-Kit band precipitated by anti-Kit antibodies. However, the lower band consistently migrated slightly faster than c-Kit suggesting that at least part of the doublet represents a novel polypeptide.

As the upper band of p140/p145 overlapped with the migration position of c-Kit, we investigated the possibility that c-Kit itself might associate with the Shc proteins. High affinity antibody which efficiently recognized denatured c-Kit was not available to determine directly whether c-Kit complexed with Shc. In the reciprocal experiment, Shc proteins were not detected in anti-Kit immune complexes precipitated from BMMC lysates by antibodies directed toward the kinase insert or the carboxy terminal tail of Kit (data not shown). However, p52shc and p46shc were observed in anti-Kit immune complexes
Figure 4-6

Interaction of Shc with c-Kit.

a. Shc is co-precipitated by antibody directed against the extracellular domain of c-Kit. Unstimulated +/- BMMC which had been growth factor-deprived for 4 hrs and factor-deprived cells which had been stimulated for 2 min with SF were lysed and immunoprecipitated with antibody directed against the extracellular domain of c-Kit. The recovered proteins were blotted with anti-Shc antibodies followed by horseradish peroxidase-conjugated protein A and the blot developed by chemiluminescent reagents.

b. A phosphoprotein corresponding to autophosphorylated c-Kit is recognized by the SH2 domain of Shc in vitro. NIH 3T3 cells ectopically expressing the Kit5 isoform were rendered quiescent by serum deprivation and then stimulated with 250 ng ml\(^{-1}\) SF. The stimulated cells were lysed and incubated with an immobilized GST fusion protein corresponding to the SH2 domain of Shc or GST alone. An aliquot of the identical cell lysate was also immunoprecipitated with anti-Kit kinase insert antibodies to show the corresponding position of c-Kit. The recovered proteins were subsequently immunoblotted with anti-p.Tyr antibodies followed by \(^{125}\text{I}\)-protein A (lefthand panel). The righthand panel indicates the relative quantities of GST-Shc SH2 and GST used, as measured by Coomassie blue staining. Comparison with BSA standards indicated that 5-10 \(\mu\)g of GST fusion protein was present in each sample.
a.  

\[ \begin{array}{c}
\text{+/+ MC} \\
\alpha\text{Kit IPs} \\
1 & 2 \\
\text{SF: 0' 2'} \\
\text{Blot: } \alpha\text{Shc}
\end{array} \]

b.  

\[ \begin{array}{c}
\text{IP or Mix: } \alpha\text{Kit, GST, Shc SH2} \\
c-\text{Kit} \\
1 & 2 & 3 \\
\text{Blot: } \alpha p\text{-Tyr}
\end{array} \]

\[ \begin{array}{c}
\text{GST, Shc SH2} \\
\text{Coomassie Blue}
\end{array} \]
precipitated by monoclonal antibody directed against the extracellular domain of Kit (figure 4-6b). (In this particular experiment, only a modest increase in p52 Shc association with c-Kit upon SF stimulation was observed, probably due to the relatively short growth factor deprivation time of 4 hours and residual tyrosine phosphorylation of Shc in the unstimulated cells (data not shown).) The 52 and 46 kDa Shc proteins were also readily detected in association with an activated form of c-Kit (Asp\textsuperscript{814}\rightarrow\text{Y}r) which is constitutively phosphorylated and spontaneously expressed in the P815 mastocytoma cell line (data not shown) (824,873). These results suggest that association of Shc and c-Kit can occur in intact cells.

The Shc SH2 domain selects peptides containing the sequence p.Tyr-(Ile/Glu/Tyr/Leu)-X-(Ile/Leu/Met) from a phosphopeptide library degenerate at the +1 to +3 positions carboxy terminal to the phosphotyrosine (180). Although a search of the sequence of murine c-Kit did not reveal any motifs which precisely conformed to the optimal motif recognized by Shc SH2, the isolated Shc SH2 domain, when expressed as a glutathione-S-transferase (GST) fusion protein, specifically bound to a broad 145 kDa phosphoprotein from SF-stimulated cells which co-migrated with autophosphorylated c-Kit (figure 4-6b). In an attempt to determine which portion of c-Kit mediated association with the Shc SH2 domain, the juxtamembrane region, kinase insert and cytoplasmic tail of murine c-Kit were expressed individually as bacterial GST-fusion proteins, phosphorylated \textit{in vitro} by the Elk tyrosine kinase (824) and mixed with lysates of SF-stimulated NIH 3T3 cells expressing c-Kits. In several experiments, significant amounts of full length Shc were not detected in association with any of the GST-fusion proteins, although the phosphorylated kinase insert specifically bound the p85\textalpha{} subunit of PI3K, consistent with prior demonstrations that PI3K binds to a specific motif within this region (data not shown) (824,903,904). While the inability to detect such an association may reflect the absence of a highly stable interaction \textit{in vivo}, it is conceivable
that the *in vitro* phosphorylation procedure did not result in adequate phosphorylation of the
physiologically relevant site(s) or that the appropriate conformation for association with Shc is
dependent on the intact c-Kit molecule. Alternatively, such an association may be indirect,
requiring proteins which have a restricted pattern of expression.

4.4.6. Shc and Vav are targets of IL-3 receptor-associated PTK activity

IL-3, in common with SF, transiently induces p21ras.GTP complexes (89). To test whether
IL-3 also elicits the tyrosine phosphorylation of the Shc and Vav proteins in primary hematopoietic
cells, +/+ and W42/W42 MC were assayed. When whole cell lysates of IL-3-stimulated +/+ BMMC
were analyzed at 0, 2, 15 and 30 min following addition of recombinant IL-3, maximal
phosphotyrosine levels occurred at 2 min (data not shown). In general, more modest levels of
cellular phosphotyrosine were induced in +/+ BMMC by 100 ng ml⁻¹ of IL-3 than by 100-250 ng
ml⁻¹ SF. This result is consistent with prior reports that maximal levels of cellular phosphotyrosine
in response to IL-3 require much higher amounts of IL-3 than the quantity required to support
proliferation which is in the range of 100 ng ml⁻¹ (905-907). Nevertheless, an IL-3 induced increase
in the tyrosine phosphorylation of p52^{shc} and p46^{shc} was clearly detected in both +/+ BMMC (data
not shown) and W42/W42 FLMC (figure 4-7a) following addition of 100 ng ml⁻¹ of IL-3. In the
particular experiment shown, a 4.5-fold increase in the tyrosine phosphorylation of p52/p46^{shc} was
measured by scanning densitometry. Kinetics studies performed in the IL-3-dependent
multipotential FDC-P1 myeloid cell line (888) indicate that Shc phosphorylation was maximal by 2
min and declined by 15 min but remained elevated for at least 60 min with continuous exposure to
IL-3 (figure 4-7b). During this time period, levels of p52^{shc} were unchanged (figure 4-7b and data
not shown). A Shc- associated p140/p145 phosphoprotein was not detected in IL-3-stimulated
Figure 4-7

She and Vav are targets of IL-3-induced PTK activity.

a. Quiescent W42/W42 FLMC were incubated with (+) or without (-) 100 ng ml⁻¹ purified recombinant murine IL-3 for 2 min, lysed and immunoprecipitated with anti-Shc antibodies. Following SDS-PAGE and transfer to nitrocellulose, the precipitated proteins were immunoblotted with anti-p.Tyr or anti-Shc antibodies followed by [¹²⁵I]-protein A. Each lane represents 2x10⁷ cells.

b. Kinetics of Shc phosphorylation in response to IL-3. 10⁷ FDC-P1 cells were deprived of growth factor for 48 hrs, then restimulated with 5% (vol/vol) conditioned medium containing IL-3 (X63 CM) for 0, 2, 15 or 60 min as indicated. The cells were lysed, immunoprecipitated with anti-Shc antibodies and immunoblotted with anti-p.Tyr antibodies followed by [¹²⁵I]-protein A (lefthand panel). In the middle panel, cells were incubated with or without 100 ng ml⁻¹ purified recombinant IL-3 for 2 min prior to assay. In the righthand panel, cells were incubated with or without X63 CM for 2 min, lysed and immunoprecipitated with anti-Shc antibodies followed by blotting with anti-Shc antibodies and [¹²⁵I]-protein A.

c. Growth factor-deprived FDC-P1 cells were stimulated with X63 CM for 0, 2, 15 or 60 min, lysed and immunoprecipitated with anti-Vav antibodies followed by immunoblotting with anti-p.Tyr antibodies and [¹²⁵I]-protein A. In the righthand panel, purified recombinant IL-3 was added to cells for 2 min prior to assay.
a.

**W^{42}/W^{42} mast cells**

 αShc IPs

<table>
<thead>
<tr>
<th>IL-3:</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blot:</td>
<td>αp.Tyr</td>
<td>αShc</td>
<td>p52 Shc</td>
<td>p46 Shc</td>
</tr>
</tbody>
</table>
b.

X63 CM

0' 2' 15' 60'

--- Vav ---

rIL-3

0' 2'

Blot: αp.Tyr

αp.Tyr
c.

**Blots:**

- αp.Tyr
- αShc
BMMC, FLMC or FDC-P1 cells (data not shown).

Increased tyrosine phosphorylation of Vav was also induced in BMMC in response to IL-3, but was less consistently observed than the phosphorylation of Shc in several experiments (data not shown). The ability to detect an increase in Vav phosphorylation may have been in part masked by the persistent phosphorylation of Vav in growth factor-deprived cells and the relatively low levels of phosphotyrosine elicited by IL-3. To confirm that Vav is phosphorylated in response to IL-3, the FDC-P1 cell line was similarly assayed. Upon stimulation with IL-3, a rapid and transient increase in Vav tyrosine phosphorylation was detected, with similar kinetics as the Shc proteins (figure 4-7c).

4.4.7. p53/p56 Lyn is the predominant Src-like tyrosine kinase expressed in BMMC

Members of the Src family associate with ligand-activated EGF, PDGF and CSF-1 receptors by virtue of their SH2 domains, and activate a pathway culminating in the transcription of c-myc; this pathway is required for initiation of DNA synthesis in response to EGF, PDGF or CSF-1 (275,908-911). The site in the intracellular portion of the PDGF and CSF-1 receptors which is recognized by Src family members is conserved in c-Kit suggesting that c-Kit may also utilize Src or related PTKs for signal transmission. As an initial step in determining whether cytosolic PTKs participate in c-Kit signalling pathways and in determining the identity of the IL-3-responsive tyrosine kinase(s) in primary mast cells, the range of Src-like tyrosine kinases which are expressed in BMMC was determined. $10^8$ exponentially growing mast cells were lysed, and equivalent aliquots immunoprecipitated with antibodies directed against the unique amino terminal sequence of Src, Fyn, Yes, Lyn, Blk, Hck, Fgr or Lck. In vitro kinase reactions were subsequently carried out in the presence of $[^{32}P]$-ATP (891). Although precise quantitation is not possible due to the
differences in the affinities of the anti-peptide antibodies used to precipitate each tyrosine kinase, p53/p56\text{\textsuperscript{lyn}} is clearly the predominant Src-like tyrosine kinase expressed in BMMC (figure 4-8a). Upon prolonged exposure of the autoradiogram, small amounts of Fyn and Src were also detected (data not shown).

As Lyn is expressed to high levels in BMMC, its ability to undergo alterations in activity in response to SF or IL-3 was investigated. In a preliminary experiment, a relatively high level of \textit{in vitro} autophosphorylating activity was detected in p53/p56\text{\textsuperscript{hm}} immunoprecipitated from BMMC deprived of growth factor for 36 hours. A slight increase in the kinase activity of Lyn, along with increased phosphorylation of a 60 kDa co-precipitating protein, was induced by SF but not IL-3 (data not shown). Using the same standardized \textit{in vitro} kinase conditions, analogous increases in kinase activity were not detected in Src or Fyn. Consistent with the possibility that Lyn may participate in c-Kit signalling pathways, other preliminary experiments revealed a modest increase in the tyrosine phosphorylation of p53/p56\text{\textsuperscript{hm}} in response to SF (figure 4-8b), unaccompanied by marked changes in total precipitable Lyn (data not shown).

Several other NRTKs which do not belong to the Src family, notably Fer, Fps, Btk and Jak2, are expressed in BMMC (data not shown) and were also investigated for their potential role in SF-induced signalling pathways. In preliminary experiments, increases in the kinase activities of Fer or Btk were not detected in response to SF. Indeed, Fer activity appeared to decrease in both SF- and IL-3-stimulated BMMC compared to unstimulated cells (data not shown). In other experiments, neither Jak2 nor c-Fps was precipitated by anti-phosphotyrosine antibody from SF-stimulated cells although both Jak2 and c-Fps were readily detected in BMMC (data not shown).
**Figure 4-8**

p53/p56^{ly} is the predominant Src-like tyrosine kinase expressed in BMMC.

a. $10^8$ exponentially growing +/- BMMC were lysed and equivalent aliquots immunoprecipitated with antibodies directed against the unique N-terminus of Src, Fyn, Yes, Lyn, Blk, Hck, Fgr or Lck. *In vitro* kinase reactions were performed in the presence of [$\gamma$-$^{32}$P]-ATP using standardized conditions as described in Materials and Methods. Following gel electrophoresis, the separated proteins were exposed to film for 4 hr.

b. A modest increase in Lyn phosphotyrosine is induced by SF in BMMC. Lysates of unstimulated +/- BMMC or cells which had been exposed to SF for 5 min were lysed and immunoprecipitated with anti-Lyn antibodies. The recovered proteins were immunoblotted with anti-p.Tyr antibodies followed by horseradish peroxidase-conjugated protein A and the blot developed with enhanced chemiluminescent reagents. On the original autoradiogram, a 53/56 kDa doublet is present in lane 2.
a. +/+ mast cells

Src family tyrosine kinases

\[ \text{ctrl} \quad \text{src} \quad \text{fyn} \quad \text{yes} \quad \text{lyn} \quad \text{blk} \quad \text{hck} \quad \text{fgr} \quad \text{ick} \]

92.5
69
46

p53/p56 Lyn

in vitro kinase reactions

b. SF: 0' 5'

p56 Lyn

1 2

Anti-p.Tyr blot
4.4.8. **SF-induced phosphorylation of Shc and p140/p145 occurs in the absence of the Syk tyrosine kinase**

The cytosolic tyrosine kinase Syk is implicated in the phosphorylation of Shc in response to activation of FcεR1 receptors in the RBL-2H3 leukemic mast cell line (912) and is essential for degranulation and MAPK-dependent phospholipase A₂ (PLA₂) production in response to FcεR1 cross-linking (913,914). As SF may enhance certain IgE-mediated biological responses in mast cells (866,915,916), we investigated whether Syk might participate in c-Kit signalling pathways. In a preliminary experiment, *in vitro* kinase reactions revealed an increase in the phosphorylation of a 40 kDa doublet (p40) precipitated by anti-Syk antibodies from SF-stimulated but not IL-3-stimulated or resting BMMC (figure 4-9a). p40 likely represents a proteolytic fragment of p72\textsuperscript{Syk} since this PTK is highly susceptible to proteolysis and was originally isolated as a 40 kDa polypeptide (917). Indeed, a doublet of this size is recognized directly by anti-Syk antibodies in anti-Syk immunoprecipitates from +/+ FLMC but is absent from FLMC derived from embryos in which the *syk* gene has been disrupted by homologous recombination (100) (data not shown). The increase in soluble phosphorylated p40 which was detected in SF-stimulated cells is unlikely to represent increased proteolysis since precipitable p40 levels decreased in these cells compared to unstimulated cells in 3/3 experiments (figure 4-9b). In addition, we had observed a 40 kDa phosphoprotein in anti-p.Tyr immunoprecipitates of SF-stimulated but not unstimulated cells. These results suggested that Syk may participate in c-Kit signalling pathways. To further explore this possibility, the phosphoprotein profile in SF-stimulated Syk -/- FLMC was compared to wildtype FLMC derived from a normal littermate. Syk -/- FLMC grew as well as their normal counterparts in IL-3. Upon stimulation with SF, no major differences were apparent in the total cellular phosphoprotein pattern of +/+ and Syk -/- FLMC (figure 4-9c). In particular, the 40 kDa
Figure 4-9. Analysis of the potential role of p72<sup>Syk</sup> in SF signalling pathways in primary mast cells.

a. Anti-Syk immunoprecipitates from unstimulated +/- BMMC or cells stimulated for 5 min with SF or IL-3 were subjected to <i>in vitro</i> kinase reactions in the presence of [γ-<sup>32</sup>P]-ATP. A 40 kDa doublet displaying increased phosphorylation in response to SF is indicated by an arrow. A 4 hr exp is shown.

b. Unstimulated or SF-stimulated MC were immunoprecipitated with anti-Syk antibodies, separated by SDS-PAGE and following transfer to nitrocellulose, immunoblotted with anti-Syk antibodies. The blot was subsequently developed with horseradish peroxidase-conjugated protein A and developed with enhanced chemiluminescent reagents. The positions of p72<sup>Syk</sup> and p40 are indicated by arrows.

c. Cellular phosphoprotein profiles in unstimulated and SF-stimulated Syk -/- and +/- FLMC. FLMC were growth factor-deprived for 36 hrs, then stimulated for 5 min with SF. Whole cell lysates of unstimulated and stimulated cells were electrophoresed through an SDS gel containing 10% polyacrylamide. Following transfer to nitrocellulose, the proteins were blotted with 4G10 monoclonal anti-p.Tyr antibody followed by detection with horseradish-peroxidase conjugated anti-IgG and enhanced chemiluminescent reagents.

d. Syk is not essential for the tyrosine phosphorylation of Shc and Shc-associated p140/p145 in primary mast cells. Unstimulated and SF-stimulated +/- and Syk -/- FLMC were lysed, and immunoprecipitated with anti-Shc antibodies. The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose and blotted with 4G10 monoclonal antibody followed by secondary antibody and development with chemiluminescent reagents.
a. +/+ mast cells
   \[\alpha\text{Syk IPs}\]
   growth factor:
   \[\text{in vitro kinase reactions}\]

b. +/+ mc
   \[\alpha\text{Syk IPs}\]
   \[\text{p72 Syk}\]
   \[\text{p40}\]
   \[\text{SF: O' 2'}\]
   \[\alpha\text{Syk blot}\]
phosphoprotein detected in whole cell lysates of SF-stimulated +/+ cells was also present in Syk -/- cells. SF-stimulated Syk -/- FLMC also contained at least some tyrosine phosphorylated Shc and Shc-associated p140/p145, indicating that the Syk PTK is not essential for SF-induced phosphorylation of these proteins (figure 4-9d).
4.5. DISCUSSION

Activation of the c-Kit receptor by its ligand Steel factor, plays a key role in the development of the hematopoietic system during embryogenesis and adult life (451,918). Here we show that Kit targets in primary murine mast cells include Shc and Vav, two SH2-containing proteins that are implicated in the regulation of guanine nucleotide binding proteins. We also show that Shc and Vav are substrates of the tyrosine kinase activity associated with the IL-3 receptor, substantiating prior suggestions that IL-3 and SF stimulate overlapping sets of phosphoproteins (906,907). Following initiation of these studies, several other groups reported the tyrosine phosphorylation of Shc or Vav in SF- or IL-3-stimulated growth factor-dependent hematopoietic cell lines (920-924,940). One group also reported the phosphorylation of p52/p46 Shc in BMMC stimulated by IL-3, SF or GM-CSF (923). Our studies confirm and extend these findings by providing genetic evidence in murine mast cells that both Vav and Shc are direct or indirect targets of the c-Kit receptor. Genetic evidence is also presented that the cytosolic Syk tyrosine kinase, which is responsible for increases in Shc phosphorylation following ligation of the FceR1 receptor in the mast cell leukemia line RBL 2H3 (912), is not essential for the phosphorylation of either Shc or its associated p140/p145 protein in SF-stimulated BMMC. However, it remains to be established whether quantitative differences exist in the tyrosine phosphorylation of these proteins in the presence or absence of Syk.

The identity of the non-receptor tyrosine kinases which are activated by IL-3 in primary mast cells and responsible for Shc or Vav phosphorylation are not yet known. Cytosolic tyrosine kinases which have been implicated in IL-3 signalling pathways in different cell lines include Lyn, Fyn, Hck, Fps/Fes, Jak2 and Tec although some of these studies have not been confirmed by others
Our preliminary studies suggest that Jak2, but not c-Fps, Lyn or Fyn, participates in the response to IL-3 in BMMC (L.P., unpublished observations).

It is now evident that the Shc proteins are rapidly phosphorylated on tyrosine in response to a variety of hematopoietic stimuli (929-935). All three isoforms of Shc are also constitutively phosphorylated on tyrosine in the presence of the Bcr-Abl oncoproteins in Ph+ leukemias (818) and in the presence of activating c-Kit mutations in the RBL-2H3 leukemic cell line and in P815 mastocytoma cells (L.P., unpublished observations). While the precise function of Shc in mitogenesis and transformation remains under intense investigation, several lines of evidence suggest that the Shc proteins play a key role in activation of the Ras pathway. Overexpression of the 46 and 52 kDa Shc proteins induces transformation of NIH 3T3 cells, a phenomenon which requires Tyr$^{317}$ (505,848). p52/p46 Shc overexpression also causes Ras-dependent neurite extension in the pheochromocytoma cell line PC12 (647) and increases Ras-dependent responsiveness to GM-CSF in the growth factor-dependent TF-1 myeloid cell line (930).

Shc inducibly associates with Grb2 in SF-stimulated cells. It has been proposed that the Shc proteins provide an alternate route to the Ras pathway, particularly for those receptor and cytosolic tyrosine kinases that do not bind stably to Grb2. In BMMC, only a minor fraction of Grb2 co-precipitates with c-Kit, and the ability to detect such an association is in part dependent on the precipitating c-Kit antibody (L.P., unpublished observations). A GST fusion protein containing full length Grb2 has been reported to bind directly to activated c-Kit (894). However, despite the presence of two potential Grb2 binding sites located in the kinase insert and carboxy terminal tail of c-Kit, we have been unable to demonstrate that phosphorylated GST fusion proteins containing either of these regions bind significant amounts of Grb2 from SF-stimulated cell lysates using Triton X-100 detergent lysis conditions (L.P. and L. Larose, unpublished observations).
The identity of the 140-145 kDa phosphoprotein which co-precipitates with Shc in SF-stimulated BMMC has not yet been established. We did not detect substantial amounts of a p140/p145 polypeptide in association with Shc in BMMC or M07e cells stimulated with IL-3. However, it is possible that such proteins may be detected by more sensitive anti-p.Tyr detection methods or other methodological variations such as different detergent lysis conditions. Shc-associated 140-150 kDa phosphoproteins have recently been reported in a variety of hematopoietic cell lines stimulated by antigen, CSF-1, erythropoietin, IL-3, GM-CSF or SF (919,929,930,937-939). Indeed, p140/p145 in SF-stimulated BMMC co-migrates with the Shc PTB-binding, phosphorylated doublet in BAL17 B lymphocytes stimulated by cross linking of the IgM receptor (L.P., unpublished observations). It is not yet clear how many different proteins are represented by p140/p145 as different groups have reported different in vitro binding properties of these proteins and variable expression in non-hematopoietic cells (929,930,937-941). Recently, the cDNA for a Shc-associated 145 kDa 5'-phosphatase was independently cloned by several groups (939-941). This protein, termed Ship, contains an SH2 domain, two consensus PTB-binding sites, proline-rich motifs and an ATP/GTP binding site as well as motifs with homology to inositol polyphosphate-5-phosphatases. Alternatively spliced Ship variants of 130 and 110 kDa have been reported; the 110 kDa form lacks an SH2 domain (938). Overexpression of Ship is growth inhibitory in FDC-P1 cells but does not affect fibroblast growth (939). The targets of Ship phosphatase activity are not yet precisely defined, but may include phosphatidylinositol (3,4,5) trisphosphate, a PI3K product, and inositol (1,3,4,5) tetraphosphate (940). Ship binds ITIMs in the FcγRIIB receptor and may thus have a role in inhibiting IgE-triggered degranulation (941,942). Association between Ship and Shc is mediated by the Shc PTB domain (859,936,939). Ship has also been reported to bind the Grb2
SH3 domain (940) but its ability to bind Grb2 in vivo is not well established and here we did not detect p140/p145 in anti-Grb2 immunoprecipitates of BMMC.

Shc binds a number of other known proteins including several activated receptors (505,933,944,945,947-950). Although a protein co-migrating with autophosphorylated c-Kit associates with the Shc SH2 domain in vitro, anti-Kit immunoblotting of anti-Shc immunoprecipitates revealed at best only a weak band corresponding to c-Kit (L.P., unpublished observations). The available anti-c-Kit antibodies were not highly efficacious in Western blotting. While small amounts of Shc protein occasionally co-precipitated with c-Kit, most consistently in the presence of an activating c-Kit mutation in P815 cells, it does not appear that such a complex is highly stable in BMMC. This finding is in agreement with other groups who did not detect significant co-precipitation of these two proteins in Western blotting or when in vitro kinase reactions were performed on Shc immune complexes from SF-stimulated M07e cells (920,921). It is possible that in myeloid cells Shc associates predominantly with other proteins such as Ship.

In common with Shc, the 95 kDa Vav protein has been implicated in Ras activation. In B and T lymphocytes, engagement of antigen receptors leads to the tyrosine phosphorylation of Vav which has been reported to correlate with Vav's ability to promote guanine nucleotide exchange on Ras (667,836-838). Other groups have argued that Vav is not a Ras GEF but promotes guanine nucleotide exchange on members of the Rho GTPase family, a finding more consistent with Vav's homology to Dbl (835,839,840,951). Vav also contains a pleckstrin homology domain, a region with similarities to the diacylglycerol binding site of PKC and a carboxy terminal SH2 domain flanked by two SH3 domains in an arrangement reminiscent of the Grb2 protein. Truncation of the N-terminal portion of Vav results in oncogenic activation but leaves all of the aforementioned domains intact, suggesting that deletion of a regulatory region is responsible for activation of
transforming potential (791,952). Vav is required for the development of B and T lymphocytes in vivo and for the response of mature lymphocytes to antigen (953-955). Homozygous Vav -/- embryonic stem cell clones differentiate into myeloid lineages normally, suggesting that the Vav protein is not required for development of myeloid cells per se (956,957). However, subtle as yet undetermined functional abnormalities may exist in the ability of vav -/- myeloid cells to perform specialized effector functions.

The studies presented here and by others indicate that Vav, like Shc, is rapidly phosphorylated on tyrosine residues in response to activation of a structurally diverse group of hematopoietic receptors that include antigen and immune complex receptors, co-stimulatory receptors, the IL-2 receptor, Flk2/Flt3 and the IFNα receptor in addition to the receptors for IL-3 and SF (958-962). Increased tyrosyl phosphorylation of Vav also occurs in response to CSF-1 stimulation of FDC-P1 cells ectopically expressing the human CSF-1 receptor (L.P., unpublished observations) although others have not detected Vav tyrosyl phosphorylation in response to CSF-1 stimulation of the murine Bac1.2F5 cell line (962). Vav therefore functions as a direct or indirect substrate of several members of the PDGF receptor subclass which are normally expressed in specific hematopoietic lineages. Although the Vav SH2 domain stably interacts with autophosphorylated c-Kit in vitro (L.P., unpublished observations), a stable complex between Vav and c-Kit does not form in SF-stimulated BMMC.

The Vav protein is constitutively phosphorylated on tyrosine in quiescent cells expressing either kinase-inactive or wildtype c-Kit. A tyrosine kinase distinct from c-Kit is therefore responsible for the basal level of phosphotyrosine observed. Vav is also constitutively phosphorylated in freshly isolated double positive CD4+CD8+ and single positive human thymocytes (963). It will be of interest to determine whether Vav is phosphorylated at different
sites \textit{in vivo}, depending on the presence or absence of growth factor, and whether basal tyrosine phosphorylation represents a mechanism for the regulation of Vav activity.

The predominant Src-like tyrosine kinase expressed in BMMC is p53/p56\textsuperscript{lyn}. While further studies are required to confirm a role for Lyn in SF-signalling pathways, preliminary studies revealed a modest increase in Lyn phosphotyrosine in response to SF. Intriguingly, both Lyn and Syk, which was also identified as a potential target of c-Kit in preliminary studies, lie downstream of the FceR1 receptor (914,964-966). Following FceR1 ligation, Lyn is responsible for the phosphorylation of FceR1 receptor subunits whereas Syk is required for the phosphorylation of Shc, PLC-\textgreek{g}1 and Vav and indeed, has been reported to stably complex with Vav (912,914,967). Complex formation between Syk and Vav was not detected in SF-stimulated mast cells (L.P., unpublished observations), and our initial results indicate that at least some Shc and p140/p145 phosphorylation can occur in the absence of Syk. However, given the ability of SF to enhance IgE-stimulated release of inflammatory mediators from mast cells (866,916), it will be of interest in future studies to address whether other known participants in FceR1 signalling pathways are targets of SF-induced Syk or Lyn activity.
Acknowledgements

Shirley Vesely provided excellent technical assistance in the initial establishment of some of the primary mast cell cultures, and the Syk -/- embryos were provided by Alec Cheng. Antibodies were generously provided by J. Bolen, S. Katzav and S. Nishikawa, and L. Larose provided GST fusion proteins.
CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS
5.1. The molecular mechanisms of transformation by Bcr-Abl

The use of IFNα in the treatment of CML has resulted in the ability to induce at least partial hematological remission in the majority of chronic phase patients (386,438). However, the only curative therapy presently available for CML is high-dose chemotherapy followed by allogeneic bone marrow transplantation (BMT). Even when a suitable donor is available, transplant-related mortality and morbidity are significant, particularly in older patients. Furthermore, cure is only possible when BMT is performed during chronic phase when patients are relatively asymptomatic. The prognosis for Ph+ ALL is even more severe. Understanding the molecular events that underlie these leukemias may 1) lead to novel and more widely applicable *in vivo* methods for the disruption of mitogenic signalling to the cell nucleus, 2) provide more effective *in vitro* purging techniques for therapy involving autologous stem cell transplantation, or 3) aid in determining the most appropriate timing, as well as type, of therapy.

In this thesis, a number of SH2/SH3-containing proteins are implicated in Bcr-Abl signalling pathways (Table 5-1). To put these studies in context, at the time this project was initiated, none of the biochemical pathways activated by Bcr-Abl were known, and p120-Gap and its associated proteins were the first immediate targets of the Bcr-Abl oncoproteins to be identified (968). The involvement of p120-Gap, Grb2, Shc and mSos1 in pathways activated by Bcr-Abl is consistent with the notion that the guanine nucleotide binding protein p21ras is an important mediator of the growth promoting and/or transforming properties of Bcr-Abl. During the course of these studies, several other groups reported additional data supporting such a role for Ras (841,969-971). Indeed, studies with dominant negative (DN) interfering mutants of Ras have indicated that
Table 5-1. Summary of proteins identified as proximal targets of the Bcr-Abl oncoproteins

<table>
<thead>
<tr>
<th>Proximal target</th>
<th>Function</th>
<th>p.Tyr detected</th>
<th>Complexes with Bcr-Abl in vivo</th>
<th>Site of Interaction</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>p120-Gap</td>
<td>Negative regulator of Ras and possible effector</td>
<td>+/-</td>
<td>+</td>
<td>Gap [N]-SH2</td>
<td>On target</td>
</tr>
<tr>
<td>p62</td>
<td>NK</td>
<td>+</td>
<td>+/-</td>
<td>NK</td>
<td>SH2</td>
</tr>
<tr>
<td>p55</td>
<td>NK</td>
<td>+</td>
<td>NK</td>
<td>NK</td>
<td></td>
</tr>
<tr>
<td>p190</td>
<td>GTPase Rho GAP</td>
<td>+</td>
<td>NK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p130</td>
<td>NK</td>
<td>+</td>
<td>NK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shc (66, 52 and 46 kDa)</td>
<td>Molecular adapter positive regulator of Ras</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td>p140/p145</td>
<td>NK</td>
<td>+</td>
<td>NK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-1. Summary of proteins identified as proximal targets of the Bcr-Abl oncoproteins

<table>
<thead>
<tr>
<th>proximal target</th>
<th>function</th>
<th>p.Tyr detected</th>
<th>complexes with Bcr-Abl in vivo</th>
<th>site of interaction on target</th>
<th>site of interaction on Bcr-Abl</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grb2 (27 kDa)</td>
<td>molecular adapter positive regulator of Ras</td>
<td>-</td>
<td>+</td>
<td>SH2</td>
<td>bcr-encoded Tyr 177 [N]- and [C]-SH3</td>
<td>Grb2 has potential interactions with multiple other proteins in addition to mSos1 and Shc (see appendix 5-1)</td>
</tr>
<tr>
<td>mSos1 (150 kDa)</td>
<td>Ras GEF</td>
<td>-</td>
<td>+</td>
<td>proline-rich C-tail</td>
<td>interaction is most likely indirect via Grb2 since minimal binding of mSos1 is detected to Abl SH3</td>
<td>potential interactions with multiple other proteins which may function as adapters (see text)</td>
</tr>
<tr>
<td>PLC-γ1 (145 kDa)</td>
<td>produces second messengers DAG and IP3 by hydrolysis of phospholipids</td>
<td>+</td>
<td>+</td>
<td>PLC-γ1 SH2 ([N] and [C])</td>
<td>NK</td>
<td>PLC-γ1 associates with a 62 kDa phosphoprotein in Ph + cells</td>
</tr>
<tr>
<td>Vav (95 kDa)</td>
<td>Rac/Rho GEF</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td>Vav SH2 does not bind Bcr-Abl in vitro consistent with the lack of stable complex formation in vivo</td>
</tr>
<tr>
<td>PI3K (85 kDa)</td>
<td>phosphorylates D3 position of inositol phospholipids to produce second messengers</td>
<td>+/−</td>
<td>+</td>
<td>[C]-SH2 (synergistic binding in the presence of [N]-SH2)</td>
<td>NK</td>
<td>p210 Bcr-Abl contains a bcr-encoded YXXM motif which conforms to the consensus motif for the p85 PI3K SH2 domains but p185 Bcr-Abl; also binds PI3K suggesting either a unique site of interaction or an indirect association</td>
</tr>
<tr>
<td>Bcr (160 kDa)</td>
<td>Rac GAP Rac GTPase S/T kinase</td>
<td>+</td>
<td>+</td>
<td>oligomerization domain4</td>
<td>oligomerization domain4</td>
<td>Bcr-Abl trans phosphorylates Bcr on Tyr 177 Bcr can associate with Grb2 in vitro multiple tyrosine phosphorylation sites in exon 1 may provide additional SH2- or PTB-binding sites</td>
</tr>
</tbody>
</table>

1 based on published studies with c-Abl, proline-rich motifs in carboxy terminal tail are likely binding sites (315)
2 based on the detection of a phosphoprotein in PLC-γ1 immunoprecipitates which co-migrates with Bcr-Abl
3 unpublished observations
4 McWhirter and Wang, 1991 (359)
Ras is essential for bcr-abl-mediated oncogenesis (971). Some studies have suggested that altered cell survival, anchorage independence, diminished growth factor requirements and transformation are distinct bcr-abl-mediated events (407-411). While studies on IL-3 signalling pathways suggest a role for Ras in an anti-apoptotic pathway (972,973), the precise nature of the biological event which requires Ras in bcr-abl-mediated transformation is unresolved (410,974,975).

The significance of Ras regulatory proteins in human oncogenesis is only now emerging. A deficiency in the GTPase activating protein neurofibromin predisposes neurofibromatosis type I patients to juvenile chronic myelogeneous or myelomonocytic leukemia (JCML) (a disease which is distinct from Ph+ CML) (559,560). Myeloid progenitors from JCML patients display an increased sensitivity to GM-CSF but not IL-3 or G-CSF suggesting that Nf-1 may have a specific role in negatively regulating GM-CSF signalling pathways (976,977). A mutation in Nf-1 has also been reported in myelodysplastic syndrome (MDS) (559). In contrast, no mutations in p120-Gap were detected in a small series of patients with MDS or preleukemia, although such mutations have been reported in a small number of basal cell carcinomas (566).

N-ras mutations occur in approximately 1-5% of CML acute phase cases studied and are relatively rare in CML chronic phase, in contrast to other hematological malignancies (978,979). Such mutations may not confer a significant growth advantage to Ph+ cells which already have constitutive stimulation of Ras pathways due to altered regulatory mechanisms. Similarly, this may explain why activating Ras mutations are restricted to cases of JCML which are not associated with neurofibromatosis-1 (980). However, it is not clear why spontaneously arising Ras mutations do not induce syndromes similar to CML or Ph+ ALL in the absence of Bcr-Abl. This may reflect a requirement for additional Ras-independent pathways, as suggested by recent complementation studies (410,981,982).
In general, the activated Abl tyrosine kinases may regulate the biological activity of their targets by one of several mechanisms: 1) conformational changes and allosteric activation may occur as a consequence of non-covalent interaction with Bcr-Abl, 2) increased phosphorylation on tyrosine may directly activate a target's function or alter its interactions with other downstream molecules, and 3) contact with Bcr-Abl may alter the target's subcellular localization and facilitate access to substrates or other critical effectors.

5.1.1. The role of p120-Gap and Grb2 in Bcr-Abl signalling

Chapters 2 and 3 provide evidence that a network of SH2-mediated interactions is activated in response to Bcr-Abl. Two of the proteins recruited into complex formation with Bcr-Abl are p120-Gap and Grb2. The function of p120-Gap as an inhibitor of Ras activity is well established whereas its potential role as a downstream effector remains controversial, is not precisely defined and may depend on cell context. Studies on fibroblast cells cultured from gap -/- embryos (573) suggest that the predominant function of p120-Gap in these cells is to downregulate Ras (983). Microinjection of anti-p120-Gap antibody or p120-Gap [N]-SH2 into fibroblasts does not alter the mitogenic response to PDGF (984). While one antisense study has implicated p120-Gap as a positive signal transducer in hematopoiesis (595), gap antisense has also been reported to inhibit K562 cell growth by a nonspecific mechanism which does not involve decreased expression of the p120-Gap protein (985). The isolated catalytic domain of p120-Gap inhibits transformation by Bcr-Abl when co-expressed (971). Intriguingly, a recent study suggests that p120-Gap may function as an effector for Ras-mediated activation of Jnk, possibly through its link with p190 (986)

Gap-associated p62 was identified as a particularly prominent phosphoprotein in CML acute phase and Ph+ ALL primary blast cells, suggesting that this protein is important in the process
of Ph+ leukemogenesis. During the preparation of this thesis, human and murine cDNA sequences of a 62 kDa protein which associates with p120-Gap were reported (987,988). The function of p62^dok^ is unknown but the presence of a putative PH domain and a number of potential SH2- and SH3-binding sites suggest a role as a docking protein.

In Chapter 3, a direct interaction of Bcr-Abl with Grb2 was demonstrated to occur through the recognition of a bcr-encoded motif by the Grb2 SH2 domain. This interaction is likely responsible for recruitment of the GEF mSos1 into complex formation with Bcr-Abl. The isolated Grb2 SH3 domains also interact with Bcr-Abl in vitro and indeed, synergistic binding occurs in the presence of all three grb2-encoded domains (L.P., unpublished observations). However, the biological significance of the association of the SH3 domains with Bcr-Abl is unclear since these domains co-operate to bind mSos1.

Although the association between Grb2 and Sos was originally reported to be constitutive in fibroblasts (231,618,638), more recent studies have suggested that this association is inducible under certain conditions (989-992). Furthermore, the binding of mSos1 to Grb2 may alter the affinity of the Grb2 SH2 domain for various phosphopeptides (993) and thereby modulate Grb2's interactions with other proteins. mSos1 tyrosine phosphorylation is not detectable in bcr-ABL-transformed cells, consistent with other studies indicating that growth factor-stimulated phosphorylation of mSos1 is limited to serine and threonine (711,992,994). Such phosphorylation depends on Mapk and other as yet unidentified serine/threonine kinases, and may attenuate nucleotide exchange by inhibiting complex formation with activated PTKs, Shc and/or Grb2 (231,699,987,994-996). This negative feedback mechanism is presumably altered in the presence of a constitutively active PTK such as Bcr-Abl.
The immediate consequences of the Bcr-Abl/Grb2-mSos1 interaction are not yet established. Recruitment of Grb2-Sos to the cortical cytoskeleton, the predominant location of Bcr-Abl, may facilitate access to Ras. Such a mechanism was originally proposed to explain how activated RTKs promote Ras guanine nucleotide exchange (641,642), but more recent data have suggested a more complex model in which repression of Sos catalytic activity by the Sos C-terminal tail is relieved by RTK/Grb2-Sos complex formation and a second signalling event required for catalytic activity is transmitted through the Sos N-terminus (998-1002). Complex formation with Bcr-Abl may regulate mSos catalytic activity by an allosteric effect or by modulating mSos phosphorylation on serine/threonine. It will be of interest to compare the catalytic activity of purified Grb2-mSos1 complexes in the presence or absence of Bcr-Abl (or a synthetic peptide modelled on the p.Tyr 177 site), and also to determine whether mSos1 is a substrate for the serine/threonine kinase domain of Bcr. Binding of the Grb2 SH2 domain to Bcr-Abl could also alter the ability of the Grb2 SH3 domains to bind mSos1 or other proteins. This seems less likely, however, since peptides mimicking either the EGF receptor Tyr 1068 or the Shc Tyr 317 phosphotyrosine binding site do not alter Grb2's affinity for Sos peptides in vitro (1003,1004).

5.1.1.1. Do alterations in Grb2 expression contribute to progression?

Grb2 maps to chromosome 17q22 (1005). A frequent chromosomal change during CML progression is the formation of an isochromosome 17 which involves duplication of 17q and loss of 17p. Increased gene dosage of Grb2 as a result of this chromosomal aberration may increase Grb2 protein levels and enhance complex formation with mSos1, as demonstrated for a subset of breast carcinoma cells (1006). If this occurs in Ph+ cells, the magnitude of Ras activation or other downstream events may increase and could conceivably contribute to progression.
A number of as yet unidentified phosphoproteins interact with Grb2 in bcr-abl-transformed cells or SF-stimulated BMMMC including a 36 kDa protein (818 and L.P., unpublished observations). Studies on the stoichiometry of Grb2/Bcr-Abl complexes, and the characterization of these additional proteins may provide further clues as to the role of Grb2 in bcr-abl-mediated transformation. The cDNAs for two proteins which bind the SH2 domain of Grb2 in hematopoietic cells have recently been cloned. One of these encodes a 34-36 kDa SH2-containing protein designated Lnk (1007,1008). Lnk binds the SH2 domain of Grb2 and may couple the TCR to the phosphatidylinositol pathway since it also binds PLC-γ1 (1009). The second protein, SLP-76, is a 76 kDa protein which also contains an SH2 domain at its carboxy terminus (1010). SLP-76 binds the Grb2 SH3 domains and also interacts with Vav. A third protein which interacts with Grb2 and is expressed in hematopoietic cells is the serine/threonine kinase Hpk which interacts with the Grb2 SH3 domains (1011). Additional proteins which interact with the SH3 or SH2 domains of Grb2 in various cell systems are rapidly being identified. Grb2-mediated interactions within an individual cell may be complex as different splicing variants of Grb2 exist which vary in their binding properties as well as their biological function (1012,1013). Furthermore, the recent isolation of Grap, a protein with 59% homology to Grb2, suggests the presence of a family of Grb2-related proteins (1014). Grap is expressed predominantly in spleen and thymus and also interacts with Bcr-Abl, mSos1 and Shc (1014,1015).

5.1.2. Is Grb2 the critical link between Bcr-Abl and Ras?

Following my identification of the Grb2 SH2 binding site in Bcr-Abl, mutation of Tyr 177 to Phe was reported to reduce p185 Bcr-Abl transforming activity in fibroblasts (969,981,1016). Pendergast et al. also initially reported that loss of this site rendered Bcr-Abl incapable of
transforming primary pre-B lymphoid cells (969). However, this latter finding was not confirmed in subsequent studies (410,981,971). DN forms of Grb2, which lack either the N- or C-terminal SH3 domain, inhibit Ras activation and transformation by Bcr-Abl in Rat-1 fibroblasts and also inhibit the tumorigenicity of K562 cells in vivo (1017). These data suggest that Grb2 is indeed essential for bcr-abl-mediated transformation but that a requirement for direct binding to Bcr-Abl may depend on the host cell type. It is not yet known how such an association affects transformation in hematopoietic stem cells, the natural host cells of Bcr-Abl.

5.1.3. Shc provides Bcr-Abl with an alternate route to Ras

The ability of Shc to complex with Grb2-mSos1 may provide an alternate route to Ras, particularly for activated PTKs that do not bind directly to Grb2. This alternate path to Ras may have particular importance in some hematopoietic cells. Indeed, overexpression of p52/p46 Shc has now been shown to restore fibroblast transforming properties to Y177F Bcr-Abl and to enhance the rate of hematopoietic cell transformation by wildtype Bcr-Abl although this was not shown to be directly due to increased Ras.GTP levels (982).

Each isoform of Shc may have distinct functions (505,644,1018,1019) and the precise role of Shc proteins in hematopoietic processes may vary depending on the particular stimulus and cell type (930,935,950,1020-1027). Some of these functions may involve Ras-independent events (1027). Like Bcr-Abl, Shc binds F-actin suggesting a role in cytoskeletal organization (1028). In non-hematopoietic cells, ligation of a subset of integrins induces Shc phosphorylation and association with Grb2, independent of p125 Fak; studies with DN interfering mutants (Y317F) suggest a role for Shc in integrin-mediated Mapk activation, cell survival and cell cycle progression (1029). It is possible that c-Abl normally participates in this response (362). Constitutive
phosphorylation of Shc in bcr-abl-transformed cells may therefore contribute to the abnormal responses of Ph+ cells to the extracellular matrix, and premature release of progenitor cells. Confirmation of the function of Shc proteins in Ph+ leukemogenesis requires additional studies, for example, the use of DN proteins to determine whether Shc is essential for Ras-dependent events.

Mammalian p52\textsuperscript{shc} contains at least three \textit{in vivo} tyrosine phosphorylation sites, Tyr 239, Tyr 240 and Tyr 317 (849,899). All three residues contribute to Grb2 binding but only Tyr 239 and Tyr 240 are conserved in Drosophila Shc (849,899,991,1030). Signals generated from Shc, involving Tyr 239/240, induce c-myc expression and contribute to the suppression of apoptosis by IL-3 in Ba/F3 cells (1022). Such a pathway, which is proposed to be distinct from that involving Ras, may play a role in bcr-abl-mediated suppression of apoptosis. Immobilized Shc sequences containing p.Tyr 239/240 residues bind a number of proteins present in lysates of v-src-transformed cells (849). These additional proteins are candidates for intermediaries in this as yet uncharacterized pathway.

Shc binds tyrosine phosphorylated proteins through its N-terminal PTB domain which recognizes tyrosine phosphorylated sites in the consensus sequence ψ-X-Asn-Pro-X-p.Tyr (where ψ is a hydrophobic residue) or by its SH2 domain which preferentially recognizes p.Tyr (Gln/Leu/Ile/Tyr)-X-(Leu/Ile/Met) motifs \textit{in vitro} (179,491,643,859,860,1030). Each of these domains may therefore be involved in functionally distinct interactions. Despite the lack of motifs which precisely conform to either PTB- or SH2-consensus binding motifs for Shc, a small amount of Shc co-precipitates with Bcr-Abl (818). The nature of this interaction, and whether other proteins reside in the same complex, merits further evaluation. Of interest, a phosphotyrosine-independent interaction between v-Abl and Shc was recently reported to occur via the N-terminus of Shc and the Abl SH2 domain (1031).
The predominant phosphoprotein which bound Shc in both growth factor-stimulated normal primary hematopoietic cells and Ph+ leukemic cells was a 140/145 kDa doublet. This protein may correspond to Ship, a newly characterized 145 kDa inositol 5'-phosphatase which binds to the PTB domain of Shc (938-940,943). Overexpression of Ship inhibits cell growth induced by CSF-1 and IL-3 in FDC-P1 cells (939). Ship binds phosphorylated ITIMs and is involved in negative signalling generated by receptors such as FcγRIIb (941,1033). Ship also binds to the ITAMs of the β and γ subunits of the FceR1 receptor although its role in FceR1 signalling is not yet defined (1111). The phosphorylation of Ship on tyrosine does not alter phosphatase activity in vitro (940), and the functional significance of Shc-Ship complexes is also not known. In one model, Ship is proposed to decrease Ras activation by competing with Grb2 for Shc (942,1033). However, others have reported that Ship also binds Grb2 (1034,1035). In Ph+ leukemic cells, phosphorylated p140/145 is not readily detected in anti-Grb2 immunoprecipitates suggesting that Shc forms separate complexes with each of these proteins. As a 145 kDa phosphoprotein is detected in anti-Abl immunoprecipitates of Ph+ cells, it is possible that Ship binds Bcr-Abl. Indeed the Ship SH2 domain has been reported to recognize the motif p.Tyr217-Val-Asn-Val in Shc (1035), which is identical to the Tyr 177 site in Bcr-Abl. Ship also maps to chromosome 2q37, a site of reported chromosomal aberrations in CML (1036).

5.1.4. Other potential targets of Bcr-Abl include the GEF Vav

The hematopoietic-specific 95 kDa Vav protein, which is found both in the cytosol and in the nucleus, is implicated as a target of Bcr-Abl based on its high levels of phosphorylation on tyrosine residues in Ph+ cells. Although Vav has now been reported to interact with a number of NRTKs via its SH2 domain (962), stable complexes with Bcr-Abl are not formed in vivo, consistent
with the inability of the Vav SH2 domain to recognize Bcr-Abl in vitro. The ability of Vav to promote guanine nucleotide exchange on Rac-1 is dependent on tyrosine phosphorylation and leads to activation of Jnk, a Mapk reported to be downstream of Rac (727,835,1037-1040). Activation of Jnk may occur in a Ras-dependent manner in response to Bcr-Abl (1041). Vav may thus contribute to Jnk stimulation in bcr-abl-transformed hematopoietic cells. However, the precise relationship between Vav and Ras has not been delineated (840,1042).

5.1.5. The specificity of the Abl kinase and Abl SH2 domains

The Abl SH2 domain is required for leukemogenesis in vivo but may not be required for focus formation in culture or for abrogation of IL-3 dependence and transformation in Ba/F3 cells (360,983,1043,1044). In Chapter 2, GST-Abl SH2 was found to bind predominantly to 62 and 130 kDa phosphoproteins present in lysates of bcr-abl-transformed cells. These proteins may correspond to p120-Gap-associated p62 and p130 Cas (1045).

The c-Abl kinase preferentially phosphorylates peptides similar to those recognized by its own SH2 domain, notably Ile-Tyr-X-X-Pro motifs (114,179). It is not known whether fusion with Bcr modifies the substrate selectivity of the Abl kinase domain. However, some of the newly identified targets of Bcr-Abl such as p130 Cas, p62dek and Rin1 do indeed contain Tyr-X-X-Pro motifs (987,1045,1046).

The Abl SH2 domain may facilitate phosphorylation of substrates by one of two mechanisms. Binding of phosphorylated substrate by the SH2 domain may facilitate release of the product from the active site and increase the turnover rate for substrates containing a single phosphorylation site. For substrates containing multiple potential phosphorylation sites, this interaction may allow the PTK to act processively (511,819). Processive phosphorylation may also
be facilitated by a trans acting SH2, as shown for c-Abl and the adaptor molecule Crk (511). The substrate specificity of an activated Abl kinase may therefore be altered depending on the local availability of trans acting processivity factors or ligands which bind Abl SH2. It is of interest to note that, in common with Crk SH2, p120-Gap [N+C]-SH2 recognizes Tyr-X-X-Pro motifs (1147) raising the possibility that p120-Gap could be a trans processivity factor for Bcr-Abl in vivo. This possibility would depend on the ability of regions outside the p120-Gap SH2 domains to bind Bcr-Abl.

5.1.6. Do different target specificities account for the biological differences of p185 and p210 Bcr-Abl?

A secondary goal of my studies was to determine whether the different biological properties of p185 and p210 Bcr-Abl could be attributed to unique target specificities. A subset of tyrosine analogues (tyrphostins) discriminate between p185 and p210 Bcr-Abl in vitro, suggesting that different physiological substrates may exist for each isoform (1047). No consistent differences were detected in the ability of p185 and p210 Bcr-Abl to associate with, or phosphorylate, the downstream effectors identified in this thesis including p120-Gap-associated p62. However, subtle qualitative or quantitative differences in the tyrosine phosphorylation or activation of these targets may have gone undetected. For example, the extent and duration of Ras-dependent Erk activation may account for different biological outcomes following RTK stimulation in PC12 or 32-D cells (1048-1051). These different responses may also correlate with different levels of Grb2-Sos1 complexes which occur immediately following differentiating or mitogenic stimuli (990). It is also possible that differences in target specificities were not detected in bcr-abl-transfected cell lines since p210-expressing cell lines may more closely resemble blast crisis (and hence p185-mediated
disease) rather than chronic phase CML disease (1052). It will therefore be of particular importance to extend the primary cell work reported here to include chronic phase cells although the low level of \( bcr-abl \) expression and high levels of proteases in mature granulocytes pose certain technical challenges (1053).

5.1.7. The roles of Bcr and \( bcr \)-derived domains in the Ph+ leukemias and normal hematopoiesis

I have demonstrated that \( bcr \)-derived sequences encoded by exon 1 contribute to the target specificity of the Bcr-Abl oncoproteins by providing an autophosphorylation site for the binding of the Grb2 SH2 domain. These data are evidence for a hitherto unrecognized function of the Bcr moiety. Additional phosphotyrosine sites exist in the Bcr portion of Bcr-Abl (1054), suggesting that other SH2- or PTB-containing targets may also bind to this region. Furthermore, the extra \( bcr \)-derived sequences retained by p210 (or p230) Bcr-Abl may bind additional proteins and contribute to the unique biological properties of the larger fusion protein(s). Deregulation of \( bcr \)-encoded domains in reciprocal Abl-Bcr fusion proteins may also modulate the leukemic phenotype although the existence of such proteins has not yet been confirmed (1060-1062).

Bcr oligomerizes with Bcr-Abl and undergoes \textit{trans} phosphorylation on Tyr 177. Indeed, Bcr itself can interact with full length Grb2 (L.P., unpublished observations; 1054). Although expression of full length Bcr is not required for \( bcr-abl \)-mediated transformation (1063), Bcr could conceivably modulate signalling by Bcr-Abl or vice versa. Cytokines or other upstream signals which normally regulate Bcr activity are unknown despite attempts by several groups to identify such stimuli (L.P., unpublished observations; A. Pendergast, personal communication). Recently, Bcr was reported to be a substrate for the NRTK c-Fes when both proteins are overexpressed (1054) (see also section 5.2). The serine/threonine kinase, GTPase activating and GEF activities exhibited
by Bcr, along with the presence of a PH domain, suggest multiple roles in signal transduction. Furthermore, Bcr can interact with a subset of SH2 domains derived from diverse proteins (193,1055, L.P., unpublished observations) suggesting the possibility of such interactions in vivo. Although Bcr-SH2 interactions can occur independently of tyrosine phosphorylation, they are enhanced by the presence of phosphotyrosine (1055,1056). Moreover, the serine/threonine kinase activity of Bcr has recently been shown to be regulated by phosphorylation on tyrosine (1057). The physiologically relevant substrates of the Bcr S/T kinase domain are not yet known. An in vitro substrate of Bcr is Bap-1, a member of the 14-3-3 family (1058). An association between Bcr and membrane-associated (active) Raf is mediated by another 14-3-3 protein, 14-3-3β, suggesting a potential role for Bcr in Ras-linked pathways (1059).

5.2. Bcr-Abl signalling pathways overlap with cytokine-induced pathways in normal primary hematopoietic cells

In Chapter 4, I presented evidence that Shc, Shc-associated p140/p145, Grb2 and Vav participate in signalling pathways activated by signals that normally function to regulate hematopoiesis. The majority of these studies were carried out in normal primary cells and support the notion that Bcr-Abl constitutively activates signalling proteins which are transiently stimulated by regulatory signals in the hematopoietic microenvironment. An autocrine loop induced by Bcr-Abl could account for the observed overlap in targets (833). However, primary CML colony progenitors generally remain growth factor-dependent (1064,1065) and the majority of proteins phosphorylated on tyrosine in response to IL-3 or Bcr-Abl are apparently different as assessed by two-dimensional immunoblotting (1065). While common targets may reflect overlapping kinase specificities, an alternative possibility is that Bcr-Abl stimulates cytosolic PTKs normally associated
with cytokine receptors (1067), or activates the cytoplasmic tails of RTKs such as c-Kit (1068). Regardless of mechanism, the sharing of signalling intermediates has implications for potential therapy based on exploitation of the signalling properties of Bcr-Abl.

5.3. Additional proximal targets of abl- and bcr-encoded proteins

Since initiation of this project, others have reported a number of additional proteins which interact with, or are substrates of, c-Abl, Bcr or Bcr-Abl. These are summarized in Appendix 5-2, and a few are discussed below. Much of the reported in vivo data involves cell lines co-transfected with Bcr-Abl and the protein of interest, and must therefore be interpreted with caution since they may involve nonphysiological amounts of protein in cells which are not necessarily representative of the in vivo targets of Bcr-Abl.

Abi-1, Abi-2, AAP1 and Pag are candidates for in vivo regulators of c-Abl (212,213,369,370). While Abi-1 and Abi-2 are reported to be substrates for v-Abl and c-Abl respectively, AAP1 does not appear to function as a substrate for either cellular or oncogenic Abl. AAP1 directly inhibits c-Abl kinase activity by binding to the Abl SH3 domain; a minor degree of inhibition of p210 Bcr-Abl kinase activity towards an exogeneous substrate was also observed (212). The potential role that altered function or expression of Abi-1, Abi-2 or AAP1 may play during the course of Ph+ leukemias remains to be explored.

A number of cytoskeletal proteins are substrates of Bcr-Abl (1068). These proteins, which include tensin, talin, p125 Fak and RAFTK (related adhesion focal tyrosine kinase), co-localize with Bcr-Abl in focal adhesion-like structures and could play a role in the altered adhesive properties of Ph+ cells. In addition to p125 Fak (1069,1070), the NRTK p92 fes has been reported to undergo a modest increase in tyrosine phosphorylation and enzymatic activity in p210 Bcr-Abl-transformed...
cells (1071). p92 fes is a hematopoietic specific PTK whose expression increases with myeloid differentiation (1072). In response to IL-3, GM-CSF, or Epo, Fes has been reported to increase in activity and co-precipitate with the activated receptor (927,1072). However, others have not detected a significant increase in p92 fes tyrosine phosphorylation in a variety of cell lines in response to GM-CSF, IL-3 or other hematopoietic growth factors (1071, L.P., unpublished observations). In transgenic mice expressing the human c-fes gene under its own promoter (1073), no significant abnormalities were detected in the number or growth of myeloid and B lymphoid progenitor cells (L.P., unpublished observations). The precise function of p92 fes in normal hematopoiesis or leukemogenesis therefore remains to be established. The Src-like PTKs Lyn and Hck have also been recently implicated in Bcr-Abl signalling (1067).

The 120 kDa proto-oncogene Cbl (for Casitas B-lineage lymphoma) is tyrosine phosphorylated in response to a number of hematopoietic stimuli (1075-1081) and has variously been reported to associate with PI3K, Grb2, Shc, CrkL, focal adhesion proteins, 14-3-3 proteins, NRTKs and c-Kit (1075,1081-1086). Although Cbl has features suggestive of a transcription factor, the proto-oncogene product localizes to the cytoplasm. In its oncogenic form, Cbl produces B cell lymphomas and is found in the nucleus as well as the cytoplasm. Oncogenic forms of c-Cbl are tyrosine phosphorylated indicating that a PTK participates in transformation by Cbl. Cbl associates with both c-Abl and activated Abl proteins and is tyrosine phosphorylated in K562 cells or v-abl-transformed cells (812). The C. elegans sli-1 gene is a homologue of the amino terminal half of Cbl and binds directly to the EGF R (1087,1088). Recent genetic experiments suggest that Sli-1 is a negative regulator of the Ras pathway activated by the C. elegans EGF R homologue, Let-23 (1088).
Tyrosine phosphatases may play positive or negative regulatory roles in hematopoiesis (1089). The SH2-containing tyrosine phosphatase Shp-2 (previously known as Syp, PTP1D, PTP2C and SHPTP2) associates with p210 Bcr-Abl (1090). The motif preferred by the Shp-2 SH2 domains is most similar to the Tyr 177 site for Grb2 binding (180). It is not yet known whether Shp-2 shares this site with Grb2 and whether Shp-2 limits Bcr-Abl signalling or is a positive signal transducer. Shp-2 has previously been postulated to link Grb2-Sos with the PDGF R, providing a link to Ras (1091).

5.3.1. Multiple molecular adaptors may lead to Ras in Bcr-Abl-transformed cells

In addition to Grb2, the molecular adaptors Crk, CrkL and Nck may associate with mSos1 and/or C3G, another GEF which displays weak activity for Ras and greater activity for Rap (363,619,1092-1096). These adaptors can also interact with proline-rich motifs in the carboxy terminal tail of Abl by virtue of their SH3 domains (363) and CrkL has been identified as a prominent tyrosine phosphorylated protein in Ph+ cells (1098,1099). Elimination of the CrkL binding site reduces Bcr-Abl transforming activity in fibroblasts (1100) although mutation of this site does not significantly alter transforming activity in myeloid cells perhaps due to an indirect association of CrkL with Bcr-Abl (1101). This may occur through Cbl (1085). Like Grb2, the gene dosage of both Crk and CrkL may be altered during CML progression. Increased copy number of CrkL, which maps centromeric to Bcr on chromosome 22q11 (1102), may occur secondary to duplication of the Ph chromosome while Crk maps to chromosome 17p (1103) and may be lost during the formation of an isochromosome 17. Changes in the levels of these molecular adaptors may contribute to progression by as yet unknown mechanisms.
5.4. Transcriptional activation of genes in response to Bcr-Abl

Genes which are transcriptionally activated in response to Bcr-Abl include myc, bcl-2, a ras-like gene kir and Ras-responsive or Jun-responsive elements (1104,1105). Induction of bcl-2 expression by Bcr-Abl is Ras-dependent (1105). Kir was initially isolated due to its expression in pre-B cells transformed by wildtype Bcr-Abl but not in cells rendered growth factor-independent by a non-transforming mutant in which Phe is substituted for Tyr 1294 in the kinase domain (1104). Kir is almost identical to gem, an immediate early gene transiently expressed and phosphorylated on tyrosine residues in mitogen-stimulated T cells (1106). In yeast, Kir functions upstream of the Ste20 kinase in activating Mapk cascades suggesting a role in cell growth (1107). Induction of kir expression may require the Grb2 binding site as well as Tyr 1294 (1104). A role for Kir has not been elucidated as its overexpression neither transforms fibroblasts nor enhances transformation mediated by Bcr-Abl (1104).

One major signalling pathway leading to transcriptional events in hematopoietic cells is the Jak-Stat pathway. This pathway was initially described for interferons (IFNs) α/β and γ, and involves the tyrosine phosphorylation of latent cytoplasmic proteins termed Stats (for signal transducers and activators of transcription) by the Jak family of cytosolic tyrosine kinases (reviewed in 500, 1108). Stats translocate into the nucleus where they bind DNA consensus motifs. Specificity in the ligand-receptor signalling events is thought to result in differential activation of Stat proteins which can then act at different DNA binding sites. The relationship between Jak-Stat events and the Ras signalling pathway has not been thoroughly defined although it appears that Jaks are required for cytokine-stimulated activation of Erks (718,1109). DN forms of Stat-5 inhibit the IL-3 induced expression of several genes such as pim-1, and partially inhibit c-fos but not c-myc (1110). Some groups have reported the constitutive phosphorylation of Stat-1 and Stat-5 in Bcr-
Abl-transformed cells (1111,1112) although this has not been confirmed by others (1113). One group has suggested that Bcr-Abl may directly activate Stats without Jak intermediaries (1112).

5.4. Future work

Avenues for continuation of the work presented in this thesis have been discussed in some of the previous sections and include further analysis of primary Ph+ cells since these cells exhibit different growth properties than established growth factor-independent leukemic cell lines which may have undergone cytogenetic evolution during initial selection in culture (427). Another immediate priority is the characterization of the sites of interaction of Bcr-Abl with targets such as p120-Gap. First, phosphorylation dependence and the direct or indirect nature of these interactions must be established. Several approaches can then be taken to map the recognition motifs of proteins interacting directly with Bcr-Abl, including the techniques used to determine the Grb2 SH2 binding site. The binding properties of p.Tyr 360 or other recently identified phosphorylation sites in Bcr-Abl can readily be screened with synthetic phosphopeptides modelled on these sites and a panel of SH2 domains.

The identification of the precise sites where p120-Gap and other SH2-containing proteins bind may allow the generation of mutant Bcr-Abl proteins that selectively lack the ability to interact with a particular protein. The biological properties of the mutants generated can then be directly analyzed to determine which proteins are critical effectors of Bcr-Abl. While Grb2 binding sites identified to date are more or less specific, other phosphotyrosine sites, for example, on the hepatocyte growth factor receptor, may bind to several proteins (506,1114,1115). As one criticism of such studies is the possibility that an unidentified interacting protein may be the critical or salient feature of a particular binding site, selective complementation studies to rescue transformation with
downstream effectors should be performed (981). Additional approaches to determine the role of a specific protein in transformation by Bcr-Abl include the microinjection of neutralizing antibody or specific peptides to inhibit a particular protein and/or interaction, and the creation of dominant negative interfering mutants (1017,1116). A particularly definitive approach in addressing whether a specific protein is essential to the transmission of signals by Bcr-Abl is to determine whether modified fibroblasts or hematopoietic cells, derived from embryonic stem cells in which a particular target gene has been disrupted, can undergo transformation in response to Bcr-Abl. Grb2 -/-, p120-Gap -/-, Shp-2 -/- and Vav -/- cells are now available for such studies.

The potential involvement of multiple pathways which culminate in Ras activation requires further investigation to determine whether such pathways are functionally redundant or whether multiple proteins are necessary to achieve a threshold level of Ras activation to permit signal propagation. In addition, Ras has multiple potential effectors and the relative importance of these effectors in bcr-abl-transformed cells has not been established. One approach to addressing these issues is to co-express DN forms of each potential Ras regulator or effector with Bcr-Abl and to determine the effect. In this manner, Tanaka et al. showed that Ras-dependent activation of Erk-1 by an activated form of c-Abl was inhibited by Grb2 or Crk I DN mutants whereas Erk-1 stimulation of the same cell line in response to EGF was inhibited only by DN Grb2 (1116).

While p62 has now been cloned, its function and the consequences of its association with p120-Gap remain to be determined. The function of Gap-associated p55 and its relationship to p62 also remains to be established. Other proteins which bind p120-Gap (p130), Shc (p140/p145) and Grb2 (p36) may correspond to known proteins which can now be identified by specific probes.

The isolation and characterization of additional proteins which bind to phosphorylated N-terminal Bcr sequences may provide further insight into the role of Bcr in Ph+ leukemogenesis.
Techniques which can be used for such studies include a modified yeast hybrid system in which an active PTK is expressed (939,1117). Other questions to be addressed include the mechanism whereby Bcr activates Abl kinase activity. In addition to the key role of oligomerization (359,369,476), it has been hypothesized that binding of Abl SH2 to phosphoserine-rich bcr-encoded regions activates the Abl kinase (192). However, a different mechanism of activation may occur as a Bcr peptide phosphorylated on Ser 354 inhibits Abl kinase activity whereas the same peptide phosphorylated on Tyr 360 does not (1054). The normal functions of Bcr in hematopoiesis and other cell systems also remain to be fully investigated and may provide further insight into the potential role of Bcr in Ph+ leukemogenesis.

Continued work on IL-3 and SF-signalling pathways includes further evaluation of potential NRTKs which may function downstream of c-Kit and identification of the PTK(s) activated by the IL-3 receptor in primary cells. As these pathways are further elucidated, it will be possible to determine if hematopoietic growth factor signalling diverges from pathways induced by Bcr-Abl.

5.4.1. The rational design of anti-leukemic reagents for the Ph+ leukemias: peptidomimetics

A model for Bcr-Abl signalling is presented in Figure 5-1, along with potential sites that may be amenable to intervention. These sites include SH2-, SH3- and PTB-mediated interactions. Antagonists which inhibit these interactions are particularly attractive candidates for development as therapeutic or purging agents due to the short and specific recognition sequences with which these domains interact. The design of small organic molecules or peptidomimetics that mimic phosphopeptide binding sites is feasible (1118,1119). As the rate of dissociation is high for SH2 domains, a peptidomimetic could compete with the natural ligand in vivo (1120). The appropriate
design of such reagents will require knowledge of critical non-redundant signalling events and the specificity of the targeted event combined with detailed structure/function data.
Figure 5-1. Summary of Bcr-Abl signalling pathways.

a. Activation of the Abl kinase by fusion with Bcr sequences. Bcr-encoded amino acid residues 1-63 comprise a coiled-coil oligomerization domain which is required for transformation and may facilitate activation of the Abl kinase domain by the formation of homotetramers. Serine/threonine-rich regions of Bcr which bind Abl SH2 are also implicated in Abl kinase activation although their precise role is unclear. Binding of an inhibitor to the Abl SH3 motif and/or the carboxy terminal tail may be prevented by Bcr sequences; several candidate inhibitors have been identified (see text). The Bcr first exon, particularly the tetramerization domain, also enhances the F-actin binding function of the Abl carboxy terminal tail. Localization to F-actin is required for transformation. Activation of the Abl kinase domain results in autophosphorylation of tyrosine residues located in both the abl- and bcr-encoded sequences.
b. Model of signalling events initiated by Bcr-Abl. Autophosphorylation recruits effector molecules which complex with Bcr-Abl. Phosphorylation sites include *bcr*-encoded Y177 and Y360, and Y793 (or Y1294) in the Abl kinase domain which corresponds to Y416 of c-Src. Binding of Grb2 to p.Y177 recruits mSos1, which is associated with the Grb2 SH3 domains, into a heterotrimeric complex with Bcr-Abl, thereby activating guanine nucleotide exchange on Ras. Phosphorylation of the Shc proteins by Bcr-Abl (or a downstream PTK) induces Shc/Grb2-mSos1 complexes which also link to the Ras pathway. Three tyrosine residues on Shc are known to contribute to Grb2 binding and are depicted. Shc also complexes with p140/p145, a phosphoprotein which likely corresponds to the inositol 5' phosphatase Ship which binds the PTB domain of Shc. The CH1 and CH2 regions of Shc may also bind proteins, particularly those containing SH3 domains (not shown).

p120-Gap also complexes with Bcr-Abl through at least one of its SH2 domains. Complex formation with Bcr-Abl may inhibit p120-Gap activity *in vivo*, and thus contribute to elevated Ras.GTP levels, although such an effect has not been demonstrated *in vitro*. The association of p120-Gap with the RhoGap p190 may also decrease p120-Gap activity. p120-Gap also associates with p62 which may provide docking sites for other proteins such as PLC-γ1. The formation of multiple p120-Gap complexes may thus integrate or link several divergent pathways.

Additional routes to Ras which may exist in *bcr-abl*-transformed cells are 1) Bcr-Abl/Grap-mSos1 complexes, 2) CrkL-mSos1 complexes, 3) Shp-2/Grb2-mSos1 complexes, and 4) activated PI3K. Ras.GTP stimulates the Erk and Jnk kinase cascades. Hpk1, a Ste20-like serine/threonine kinase which is expressed in hematopoietic cells, is depicted as upstream of Jnk although Hpk1 activation has not yet been shown in Ph+ cells. Hpk1 can also associate with
Grb2 (not shown). Both p120-Gap (see text) and Grb2 (1146) have also been linked to Jnk activity in non-hematopoietic cells. Since Jnk may be downstream of Rac, phosphorylated Vav may contribute to Jnk activation by promoting guanine nucleotide exchange on Rac. Activated Erks and/or Jnks translocate to the nucleus leading to phosphorylation of transcription factors and altered gene expression. Modulation of gene expression may also occur due to stimulation of Stat pathways although the activation of Stats in Ph+ cells is controversial. It is also possible that phosphorylated Vav contributes to altered gene expression through its potential association with nuclear proteins.

Expression of bcl-2 occurs downstream of Ras. Cyclin D expression is also increased in response to Ras.GTP, and may lead to increased myc expression through modulation of cdk activity and release of Rb-bound E2F transcription factors (inset). However, the precise relationship of c-myc to Ras is not well understood since myc overexpression apparently does not rescue transformation by mutant Bcr-Abl proteins which cannot bind Grb2 (981).

Bcr-Abl is linked to the cytoskeleton by its localization to F-actin and by multiple downstream targets such as p190 RhoGap, Vav, PI3K and Shc which binds actin and participates in cell adhesion-mediated events. In addition, several cytoskeletal proteins such as paxillin, vinculin and talin are substrates of Bcr-Abl. The participation of these proteins in Bcr-Abl signalling pathways may contribute to the altered adhesive properties exhibited by Ph+ cells and the inability to respond appropriately to stromal regulation.

Although not shown, multiple other SH2-containing proteins stably complex with Bcr-Abl including PLC-γ1 and PI3K. PLC-γ hydrolyzes PIP2 to produce the second messengers DAG and IP3 which activate PKC and Ca^{2+} flux respectively. PI3K phosphorylates the inositol ring of phospholipids at the D3 position. The PI3K product PI(3,4,5)P3 plays a significant role
as a second messenger and can bind SH2 domains itself although its precise function is not well characterized. Amplification of the proximal signals from Bcr-Abl may occur by the phosphorylation and activation of other cytosolic PTKs or through the phosphorylation of the cytoplasmic tails of RTKs or cytokine receptors.
c. Potential sites of intervention in Bcr-Abl signalling pathways. Approaches to inhibition of expression of the Bcr-Abl kinases themselves include the use of antisense oligonucleotides (1121), ribozyme-mediated cleavage of bcr-abl transcripts (1122,1123) and zinc finger DNA-binding proteins (1124). The AG112 and AG957 tyrphostins, synthetic PTK inhibitors modelled on the benzylidene moiety of the natural inhibitor erbstatin inhibit Bcr-Abl kinase activity (1047,1125,1126). Such inhibitors can discriminate between normal and transforming Abl proteins (1047). Another recently described inhibitor, CGP57148B, a 2-phenylaminopurimidine derivative, selectively inhibits c-Abl and Bcr-Abl (1127,1128). The potential therapeutic value of PTK inhibitors may depend on the ability to develop agents which display a high degree of selectivity for Bcr-Abl.

Peptidiornimetics may be used to inhibit protein-protein interactions mediated by SH2, SH3 or PTB domains (1129). DN interfering forms of an effector may be designed to contain an isolated protein interaction domain or to be catalytically inactive. Farnesyl transferase inhibitors were designed to inhibit the post translational modification which allows Ras proteins to localize to the cell membrane (1130). Since FT inhibitors revert the malignant phenotype of Ras-transformed cells without affecting normal cell growth, the precise mechanism of action of these agents has been in question. Recently, it was demonstrated that FT inhibitors alter actin stress fiber organization and interfere with Rho activity which is essential for Ras transformation (1131,1132). Other relatively specific inhibitors can be used to target potential Ras effectors such as Jnk (1133). Antisense can also be used to target Bcr-Abl effectors.
Ph chromosome

- Inhibition of expression
  - Ribozymes
  - Anti-sense oligonucleotides
  - Zinc finger DNA-binding proteins

Bcr-Abl

- Inhibition of activity
  - Inhibition of oligomerization
  - PTK inhibitors
  - Antibodies

- Inhibition of protein-protein interactions
  - Peptidomimetics
  - DN proteins (Isolated protein interaction domains)
  - Antibodies

SH2, SH3 and PTB-mediated interactions

- Inhibition of Ras function
  - DN Ras
  - Farnesyltransferase inhibitors

Ras

- Inhibition of effectors
  - Specific inhibitors
  - DN forms of effector (eg. catalytically inactive proteins)
  - Antibodies

Other effectors

- Inhibition of Ras effectors
  - Effectors
Appendix 5-1a. Summary of recently identified proximal targets of the Bcr-Abl oncoproteins (see also Table 5-1)

<table>
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<tr>
<th>target (mol wt)</th>
<th>function</th>
<th>p.Tyr</th>
<th>complexes with Bcr-Abl in vivo</th>
<th>potential site of interaction on target</th>
<th>potential site of interaction on Bcr-Abl</th>
<th>comments</th>
<th>selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grb2</td>
<td>molecular adaptor</td>
<td>-</td>
<td>+</td>
<td>SH2</td>
<td>Tyr 177</td>
<td>see Table 5-1</td>
<td>Pull et al., 1994 (818)</td>
</tr>
<tr>
<td>mSos1</td>
<td>as GEF</td>
<td>-</td>
<td>+</td>
<td>likely indirect via Grb2</td>
<td>indirect via Grb2</td>
<td>see Table 5-1</td>
<td>Pull et al., 1994 (818)</td>
</tr>
<tr>
<td>Shc</td>
<td>molecular adaptor</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>NK</td>
<td>see Table 5-1</td>
<td>Pull et al., 1994 (8180)</td>
</tr>
<tr>
<td>p120-Gap</td>
<td>negative regulator of Ras and possible effector</td>
<td>+/-</td>
<td>+</td>
<td>SH2</td>
<td>NK</td>
<td>see Table 5-1</td>
<td>data herein</td>
</tr>
<tr>
<td>Shp-2 (68 kDa)</td>
<td>tyrosine phosphatase</td>
<td>+</td>
<td>+</td>
<td>Shp SH2-[N]</td>
<td>NK&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>forms ternary complex with Bcr-Abl and Grb2 co-precipitates with p85 PI3K</td>
<td>Tauchi et al., 1994 (894) Tauchi et al., 1997 (1134)</td>
</tr>
<tr>
<td>CrkL (39 kDa)</td>
<td>molecular adaptor</td>
<td>+</td>
<td>+</td>
<td>CrkL SH3</td>
<td>Abl C-tail</td>
<td>indirect association with Bcr-Abl also reported (?via Cbl) CrkL also complexes with paxillin, p85 PI3K, p130 Cas, C3G, Sos, c-Abl, DOCK 180 and Hef1</td>
<td>Heaney et al., 1997 (1101) deJong et al., 1985 (1079) Oda et al., 1994 (1098) Salgia et al., 1995 (1069) Ribon et al., 1996 (1085)</td>
</tr>
<tr>
<td>Grap (27 kDa)</td>
<td>molecular adaptor</td>
<td>-</td>
<td>+</td>
<td>Grap SH2</td>
<td>NK&lt;sup&gt;3&lt;/sup&gt;</td>
<td>shares homology with Grb2</td>
<td>Feng et al., 1996 (1014)</td>
</tr>
</tbody>
</table>

<sup>1</sup> NK, not known
<sup>2</sup> *bcr*-encoded oligomerization domain has been implicated but this may reflect its kinase activation function (Tauchi et al., 1997) (1134)
<sup>3</sup> based on homology to Grb2, a probable site is *bcr*-encoded p.Tyr<sup>177</sup>.
Appendix 5-1a. Summary of recently identified proximal targets of the Bcr-Abl oncoproteins (see also Table 5-1)

<table>
<thead>
<tr>
<th>Proximal target (mol wt)</th>
<th>Function</th>
<th>p.Tyr detected</th>
<th>Complexes with Bcr-Abl in vivo</th>
<th>Potential site of interaction</th>
<th>Comments</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crk (39 kDa)</td>
<td>Molecular adaptor</td>
<td>+/-</td>
<td>+/-</td>
<td>Crk SH3, Abl C-tail</td>
<td>Crk can bind C3G in addition to Sos</td>
<td>Sattler et al., 1996 (1045), Ribon et al., 1996 (1085)</td>
</tr>
<tr>
<td>Fes (92 kDa)</td>
<td>NRTK</td>
<td>+</td>
<td>-</td>
<td>see^4</td>
<td>Kinase activity increased</td>
<td>Ernst et al., 1994 (1071)</td>
</tr>
<tr>
<td>FAK (125 kDa)</td>
<td>NRTK found in focal adhesions</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Kinase activity increased</td>
<td>Salgia et al., 1995 (1069), Gotoh et al., 1995 (1070)</td>
</tr>
<tr>
<td>Lyn (53/56 kDa)</td>
<td>NRTK</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>Kinase activity increased</td>
<td>Danhauser-Riedl et al., 1996 (1067)</td>
</tr>
<tr>
<td>Hck (59 kDa)</td>
<td>NRTK</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>Kinase activity increased</td>
<td>Danhauser-Riedl et al., 1996 (1067)</td>
</tr>
<tr>
<td>IL-3 RβC (130 kDa)</td>
<td>Cytokine R subunit shared by IL-5 and GM-CSF receptors</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>Jak2 also phosphorylated in this study but not confirmed by others</td>
<td>Wilson-Rawls et al., 1996 (1139)</td>
</tr>
<tr>
<td>c-Kit (145 kDa)</td>
<td>RTK</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>Kinase activity increased</td>
<td>Hallek et al., 1996 (1068)</td>
</tr>
</tbody>
</table>

^4 2 fes domains, amino acids 1-347 and the fes SH2 domain, have been reported to bind full length Bcr (Maru et al., 1995) (1055).
### Appendix 5-1a. Summary of recently identified proximal targets of the Bcr-Abl oncoproteins (see also Table 5-1)

<table>
<thead>
<tr>
<th>Target</th>
<th>Function</th>
<th>p.Tyr detected</th>
<th>Complexes with Bcr-Abl in vivo</th>
<th>Potential Site of Interaction</th>
<th>Comments</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat 1,3,5</td>
<td>transcription factors</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Others have not confirmed the phosphorylation of Stats in Ph+ cells (see text)</td>
<td>Carlsson et al., 1996 (1111)</td>
</tr>
<tr>
<td>(91-95 kDa)</td>
<td>(81-95 kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ilaria and van Etten, 1996 (1112)</td>
</tr>
<tr>
<td>Cbl</td>
<td>proto-oncogene with features reminiscent of</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>Interaction is apparently not dependent on phosphorylation of Cbl</td>
<td>Andoniou et al., 1994 (812)</td>
</tr>
<tr>
<td>(120 kDa)</td>
<td>transcription factors but cytosolic function NK</td>
<td></td>
<td></td>
<td></td>
<td>Cbl is also reported to complex with p85 PI3K, Crkl and Grb2</td>
<td>Sattler et al., 1996 (1138)</td>
</tr>
<tr>
<td>c-Raf-1</td>
<td>MAPKK</td>
<td>NK</td>
<td>-</td>
<td></td>
<td>Downregulation by antisense is growth inhibitory in Ph+ lines</td>
<td>Skorski et al., 1993 (1140)</td>
</tr>
<tr>
<td>(74 kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Raf-1 interacts with full length Bcr via 14-3-3β</td>
<td>Braselman and McCormick, 1995 (690)</td>
</tr>
<tr>
<td>Bap-1</td>
<td>14-3-3 protein dimerizes and may bridge proteins</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>A substrate of both the bcr- and abl-encoded kinases in Bcr-Abl</td>
<td>Reuther et al., 1994 (1058)</td>
</tr>
<tr>
<td>(30 kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14-3-3ζ also binds but is a poor substrate</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix 5-1a. Summary of recently identified proximal targets of the Bcr-Abl oncoproteins (see also Table 5-1)

<table>
<thead>
<tr>
<th>Target</th>
<th>Function</th>
<th>p.Tyr detected</th>
<th>Complexes with Bcr-Abl in vivo</th>
<th>Potential site of interaction</th>
<th>Comments</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rin1 (90 kDa)</td>
<td>originally identified by its ability to bind and interfere with activated Ras in yeast</td>
<td>+</td>
<td>+</td>
<td>proline-rich motifs pYXXP</td>
<td>Abl SH3, Abl SH2. Rin1 also interacts with 14-3-3 proteins.</td>
<td>Afar et al., 1997 (1046) Han et al., 1997 (1141)</td>
</tr>
<tr>
<td>Cas (130 kDa)</td>
<td>nuclear and cytosolic protein, function unknown, originally isolated as v-Crk substrate</td>
<td>+/-</td>
<td>+</td>
<td>pYXXP5</td>
<td>Abl SH2. Association with Bcr-Abl may also be indirect via CrkL. Also associates with Crk, paxillin, and Fak. Association with tensin disrupted in Ph+ cells.</td>
<td>Salgia et al., 1996 (1045) Mayer et al., 1995 (511)</td>
</tr>
<tr>
<td>paxillin (68 kDa)</td>
<td>focal adhesion protein</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>NK. Interactions with other cytoskeletal proteins altered in the presence of Bcr-Abl.</td>
<td>Salgia et al., 1995 (1069)</td>
</tr>
<tr>
<td>vinculin (116 kDa)</td>
<td>focal adhesion protein</td>
<td>+</td>
<td>+</td>
<td>NK</td>
<td>NK.</td>
<td>Salgia et al., 1995 (1069)</td>
</tr>
</tbody>
</table>
### Appendix 5-1a. Summary of recently identified proximal targets of the Bcr-Abl oncoproteins (see also Table 5-1)

<table>
<thead>
<tr>
<th>target</th>
<th>function</th>
<th>p.Tyr</th>
<th>complexes with Bcr-Abl in vivo</th>
<th>potential site of interaction on target on Bcr-Abl</th>
<th>comments</th>
<th>selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>talin</td>
<td>focal adhesion protein</td>
<td>+</td>
<td>NK</td>
<td></td>
<td></td>
<td>Salgia et al., 1995 (1069)</td>
</tr>
<tr>
<td>tensin</td>
<td>focal adhesion protein</td>
<td>+</td>
<td>NK</td>
<td></td>
<td>interacts with Fak and Cas</td>
<td>Salgia et al., 1995 (1069)</td>
</tr>
<tr>
<td>actin</td>
<td>cytoskeletal component</td>
<td>+</td>
<td></td>
<td></td>
<td>F- and G-actin binding domains</td>
<td>McWhirter and Wang, 1991 (359)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>van Etten et al., 1994 (361)</td>
</tr>
<tr>
<td>Hef1 (110 kDa)</td>
<td>Cas-like protein</td>
<td>+</td>
<td>+</td>
<td>pYXXP</td>
<td>indirect association via CrkL, associates with Abl SH2 in vitro, phosphorylated in response to integrin ligation expression restricted to lymphocytes</td>
<td>deJong et al., 1997 (1149)</td>
</tr>
</tbody>
</table>

5 based on studies with c-Abl (Mayer et al., 1995) (511)
### Appendix 5-1b

**Recently identified targets of c-Abl**

<table>
<thead>
<tr>
<th>Target</th>
<th>association with c-Abl reported (either in vivo or in vitro)</th>
<th>p.Tyr reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abi-1</td>
<td>+</td>
<td>+</td>
<td>Shi et al., 1995 (369)</td>
</tr>
<tr>
<td>Abi-2</td>
<td>+</td>
<td>+</td>
<td>Dai and Pendergast, 1995 (213)</td>
</tr>
<tr>
<td>AAP1</td>
<td>+</td>
<td>-</td>
<td>Zhu and Shore, 1996 (212)</td>
</tr>
<tr>
<td>CrkL</td>
<td>+</td>
<td>+</td>
<td>ten Hoeve et al., 1994 (1096)</td>
</tr>
<tr>
<td>Crk</td>
<td>+</td>
<td>+</td>
<td>Feller et al., 1994 (363) Ren et al., 1994 (315)</td>
</tr>
<tr>
<td>Nck</td>
<td>+</td>
<td>+</td>
<td>Ren et al., 1994 (315)</td>
</tr>
<tr>
<td>actin</td>
<td>+</td>
<td></td>
<td>van Etten et al., 1994 (361) McWhirter and Wang, 1991 (359)</td>
</tr>
<tr>
<td>3BP-1</td>
<td>+</td>
<td></td>
<td>Cicchetti et al., 1992 (210) Cicchetti et al., 1995 (211)</td>
</tr>
<tr>
<td>3BP-2</td>
<td>+</td>
<td></td>
<td>Cicchetti et al., 1992 (210)</td>
</tr>
<tr>
<td>RNA Pol II CTD</td>
<td>+</td>
<td>+</td>
<td>Baskaran et al., 1996 (342,344)</td>
</tr>
<tr>
<td>Shd</td>
<td>+</td>
<td></td>
<td>Oda et al., 1997 (1098)</td>
</tr>
<tr>
<td>p53</td>
<td>+</td>
<td></td>
<td>Goga et al., 1995 (350) Yuan et al., 1996 (351)</td>
</tr>
</tbody>
</table>
Recently identified targets of c-Abl

<table>
<thead>
<tr>
<th>Target</th>
<th>association with c-Abl reported (either in vivo or in vitro)</th>
<th>p.Tyr reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>+</td>
<td></td>
<td>Welch and Wang, 1995 (340,341)</td>
</tr>
<tr>
<td>disabled</td>
<td>+</td>
<td>+/-</td>
<td>Howell et al., 1997 (329)</td>
</tr>
<tr>
<td>Pag (MSP23)</td>
<td>+</td>
<td></td>
<td>Wen et al., 1997 (370)</td>
</tr>
<tr>
<td>ATM(^1)</td>
<td>+</td>
<td></td>
<td>Shafman et al., 1997 (357) Baskaran et al., 1997 (354)</td>
</tr>
<tr>
<td>ArgBP2</td>
<td>+</td>
<td>+</td>
<td>Wang et al., 1997 (1143)</td>
</tr>
<tr>
<td>DNA-PK(^2)</td>
<td>+</td>
<td>+</td>
<td>Kharbanda et al., 1997 (358)</td>
</tr>
<tr>
<td>p85 PI3K</td>
<td>+</td>
<td>+</td>
<td>Yuan et al., 1997 (1144)</td>
</tr>
<tr>
<td>enabled</td>
<td>+</td>
<td>+</td>
<td>Gertler et al., 1995 (328)</td>
</tr>
<tr>
<td>amphiphsin-like protein (ALP1)</td>
<td>+</td>
<td></td>
<td>Kadlec and Pendergast, 1997 (1150)</td>
</tr>
<tr>
<td>Pr60 gag of murine AIDS defective virus</td>
<td>+</td>
<td></td>
<td>Dupraz et al., 1997 (1145)</td>
</tr>
</tbody>
</table>

\(^1\)ATM, ataxia telangiectasia mutant protein

\(^2\)DNA-PK, DNA-dependent protein kinase
### Appendix 5-1c

**Recently identified targets of Bcr**

<table>
<thead>
<tr>
<th>Target</th>
<th>stable association reported (either in vivo or in vitro)</th>
<th>probable interaction site on Bcr</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bap-1(^1)</td>
<td>+</td>
<td>Bcr exon 1</td>
<td>Reuther et al., 1994 (1058)</td>
</tr>
<tr>
<td>14-3-3β</td>
<td>+</td>
<td>Box B serine-rich region</td>
<td>Braselmann and McCormick, 1995 (1059)</td>
</tr>
<tr>
<td>Raf-1</td>
<td>indirect via 14-3-3β</td>
<td></td>
<td>Braselmann and McCormick, 1995 (1059)</td>
</tr>
<tr>
<td>Grb2</td>
<td>+</td>
<td>Tyr 177</td>
<td>L.P., unpublished observations</td>
</tr>
<tr>
<td>CDC42Hs</td>
<td></td>
<td>Dbl homology (GEF) domain</td>
<td>Hart et al., 1991 (470)</td>
</tr>
<tr>
<td>RhoA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rac</td>
<td></td>
<td>Rac GAP domain</td>
<td>Chuang et al., 1995 (471)</td>
</tr>
<tr>
<td>CDC42Hs</td>
<td></td>
<td></td>
<td>Diekmann et al., 1991 (472)</td>
</tr>
</tbody>
</table>

\(^1\) substrate for Bcr serine/threonine kinase activity
REFERENCES


244. Saksela K, Cheng G and Baltimore D (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J 14, 484.


923. Welham M, Duronio V, Leslie K, Bowtell D and Schrader J (1994). Multiple hemopoietins with the exception of interleukin-4, induce the modification of Shc and mSos1, but not their translocation. *J Biol Chem* 269, 21166.


