THE ROLE OF SRC FAMILY TYROSINE KINASES IN BCR-ABL SIGNAL TRANSDUCTION AND CHRONIC MYELOGENOUS LEUKEMIA

by

Matthew Brian Wilson

Bachelor of Science in Biology, University of Scranton, 1998

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This dissertation was presented

by

Matthew Brian Wilson

It was defended on

December 13, 2004

and approved by

Baskaran Rajasekaran, PhD.

Martin C. Schmidt, PhD.

Richard Steinman, MD/PhD.

Jack Yalowich, PhD.

Thomas E. Smithgall, PhD.
Dissertation Director
The hallmark of chronic myelogenous leukemia (CML) is the Philadelphia chromosome, which arises from the reciprocal translocation of the c-abl proto-onogene on chromosome 9 and the bcr locus on chromosome 22. This translocation results in the expression of a 210 kDa fusion protein (Bcr-Abl) with constitutive tyrosine kinase activity that is responsible for CML pathogenesis. Bcr-Abl activates several signaling proteins important for the proliferation and survival of myeloid progenitors, including the Src family kinases Hck and Lyn, the Stat5 transcription factor and upstream components of the Ras/Erk pathway. Previous work from our laboratory found that kinase-defective Hck blocks Bcr-Abl-induced transformation of DAGM myeloid leukemia cells to cytokine independence, suggesting that activation of the Src kinase family may be essential to oncogenic signaling by Bcr-Abl.

Chapter II explores the contribution of Src kinases to Bcr-Abl signaling \textit{in vivo}, using selective Src family kinase inhibitors. Inhibition of Src family kinases in Ph+ CML cell lines resulted in growth arrest, induction of apoptosis and blocked Stat5 and Erk activation downstream. These data implicate the Src kinase family in Stat5 and Erk activation downstream of Bcr-Abl, and identify myeloid-specific Src kinases as potential drug targets in CML.

In Chapter III, I investigated the biochemical interactions between myeloid Src family members and Bcr-Abl. Hck, Lyn and Fyn each bind the kinase domain, C-terminal tail, and SH3/SH2 region of Bcr-Abl and strongly phosphorylated the Bcr-Abl SH3-SH2 protein \textit{in vitro}. Seven phosphorylated tyrosine residues were identified and substitution of these residues with
phenylalanine in the context of full-length Bcr-Abl blocked transformation of TF-1 myeloid cells to cytokine independence. The position of several of these tyrosines in the crystal structure of c-Abl and transformation defect of the Bcr-Abl mutant suggest that phosphorylation by Src kinases may impact Bcr-Abl autoregulation and downstream oncogenic signaling. Taken together, these data firmly establish an important role for Src family tyrosine kinases in Bcr-Abl-mediated oncogenic signaling and implicate Src kinases as a promising therapeutic target for chronic myelogenous leukemia.
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PREFACE

When I began my PhD studies in 1998 I knew almost nothing about the process of genuine hypothesis driven research and how to approach issues as complex as the cell. As I write this today, I feel confident to finally call myself a scientist. This evolution would never have been possible without the guidance and support of numerous individuals. First and foremost, I would like to thank Dr. Thomas E. Smithgall for his guidance and particularly his patience. Under his mentorship, I was allowed to grow into the burgeoning scientist I am today and I can never thank him enough for everything he has given me. I would also like to thank my committee members, Dr. Martin Schmidt, Dr. Jack Yalowich, Dr. Richard Steinman and Dr. Baskaran Rajasekaran for their time, support and advice.

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1. Introduction
1.1.  Src family kinases

1.1.1.  Overview

The c-Src tyrosine kinase was originally identified as the non-transforming cellular homologue of v-Src, the oncogene encoded by the chicken Rous sarcoma retrovirus (1-3). The key discovery of c-Src as the first “proto-oncogene” earned Harold Varmus and J. Michael Bishop the 1989 Nobel Prize in Physiology or Medicine. While c-Src is the prototype, other members of the Src family include: Hck, Lyn, Fgr, Fyn, Yes, Blk, Lck, Src, and Yrk (4). Although some Src members are ubiquitously expressed, Hck, Lyn, Fgr, Lck, and Blk, are restricted to a few hematopoietic cell types. All members of the Src family exhibit common structural features that beget their role in cell signaling (Figure 1; discussed below).

1.1.2.  Structure and Regulation

Each Src family member encodes a 9 to 12 amino acid region required for membrane attachment, a 40 to 70 amino acid unique N-terminal unique domain, followed by Src-homology 3 (SH3) and Src-homology 2 (SH2) domains, a catalytic tyrosine kinase domain, and a negative regulatory “tail” region (Figure 1A). Src family kinases are primarily localized to the cytoplasm linked to the intracellular membranes of the plasma membrane, endosomes and secretory vesicles. They connect to the membranes via an N-terminal myristate moiety, salt bridges between basic amino acids in the N-terminus and phosphates of the lipid backbone, and noncovalent interactions with integral or associated membrane proteins (5, 6). This association with intracellular membranes appears to be critical for normal growth-factor signaling of the Src family kinases (7). The unique domain of the Src kinases exhibits very little sequence homology
between members and it is hypothesized that this domain confers functional specificity via unique member-specific protein/protein interactions (8).

Following the unique domain are the SH3 and SH2 domains (Figure 1A). Found in a wide variety of catalytic and noncatalytic proteins, the SH3 and SH2 regions are modular protein domains capable of binding specific amino acid target sequences. Both domains were originally identified in c-Src and are highly conserved among all Src family members (9). The SH2 domain encompasses about 100 amino acids and starts with a very short β strand (βA). An α-helix (αA) follows, which forms part of the phosphotyrosine-binding site. A continuous β-meander formed by strands B, E and F make up the core of the domain. These strands form two β-sheets, which are connected by the long strand βD. Strand F leads into helix αB, which is followed by a loop of variable length (βG). The final strand βG is part of the β-sheet formed by strands B-D (Figure 1B) (10, 11).
Figure 1: Structural organization and activation of Src family kinases

A) A representative model of Src family kinases is shown (c-Src). The unique domain (N) is shown in white, the SH3 domain in red, the SH2 domain in blue, the SH2-kinase linker in orange, the N-lobe of the kinase in light blue, the C-lobe in purple and the negative regulatory tail in green. Phosphorylation of tyrosine Y416 facilitates activation of the kinase, while phosphorylation of Y530 by Csk negatively regulates catalytic activity. B) The crystal structure of the autoinhibited form of Hck is shown (numbering for c-Src is shown for consistency) (12). Src family kinases are down-regulated by two intramolecular interactions involving 1) the SH3 domain and a proline rich region in the SH2-kinase linker, and 2) engagement of phospho-Y530 with the SH2 domain. C) Disruption of either intramolecular interaction can stimulate Src family kinase activity.
The primary function of the SH2 domain is to bind target proteins containing a phosphorylated tyrosine residue. From a structural perspective, the phospho-peptide binds to a relatively flat surface formed by the hydrophobic core of the αA and αB helices and a portion of the central β-sheet. Three positively charged amino acids, including a strictly conserved arginine within the βD region (R175 in c-Src), form critical interactions with the target phospho-peptide. Specifically, R175 falls within the highly conserved FLVRES motif of SH2 domains (13) and functions to form hydrogen bonds with the oxygen atoms of the phosphate (Figure 1B) (10). Highlighting the importance of R175, Mayer and Baltimore showed that a mutation of this residue to lysine completely blocked the SH2—phosphotyrosine interaction in vitro (14). The other two conserved positive amino acids, Arg155 (R155) and Lys203 (K203) form a hydrogen bond with the target phosphate and interact with the face of the aromatic ring of tyrosine, respectively (10) (Figure 1B).

Mutagenesis studies involving the SH2 domains of GAP (GTPase activating protein) and the p85 subunit of phosphotidylinositol-3-kinase (PI3K) with the PDGF receptor demonstrate that only a short phosphotyrosine containing peptide is required for binding (15). Specifically these studies showed that in addition to the phosphotyrosine, the residue at the +3 position was critical for binding (15, 16). Unlike the highly conserved phosphotyrosine pocket described above, the binding cleft for the +3 residue is variable among SH2 containing proteins and may provide substrate specificity. Moreover, subsequent studies have shown that the Src family kinases have a strong affinity for phospho-peptides containing the “YEEI” motif particularly when compared to peptides that only contacted the SH2 domain through the phosphotyrosine (17).
The SH3 domain comprises about 60 amino acids and in the case of Src family kinases, like many other non-receptor protein-tyrosine kinases, plays a dual role in autoregulation and protein/protein interactions (Figure 1B). SH3 domains bind proline sequences that adopt a polyproline type II (PPII) helical conformation. The PPII helix is an extended left-handed helical structure with three residues per turn and an overall shape resembling a triangular prism (18). A combination of steric and hydrogen-bonding properties of proline-rich motifs is thought to contribute to its preference for this secondary structure (18, 19). The SH3 domain itself consists of two antiparallel β sheets at right angles to one another. Within this fold are two variable loops, referred to as the RT and the n-Src loops (20). When bound, the proline-rich peptide ligand adopts a PPII helix conformation (21, 22) and the ridges of the PPII helix insert into a complementary pair of grooves on the SH3 surface (Figure 1B). These surface grooves are defined by a series of nearly parallel, well-conserved aromatic residues. In addition, hydrogen-bonding donors are well positioned to recognize ligand backbone carbonyl moieties (23). The ligand itself consists of two variable—usually hydrophobic—amino acids flanked by two required proline residues (PxxP). Each aromatic groove of the binding pocket recognizes an xP dipeptide. The variable RT and n-Src loops lie adjacent to the PPII binding sites and interact with residues flanking the PxxP motif forming a tertiary binding pocket (24).

The catalytic domain of Src family kinases encompasses approximately 300 amino acids and has a bi-lobal structure (Figure 1A-B). The smaller N-lobe contains a five-stranded β sheet and one critical α helix (αC). The larger C-terminal lobe is comprised of mostly α helices and primarily interacts with the target peptide. ATP binds at the interface between the two lobes. There are two segments of the kinase domain that are essential for proper catalytic activity: the “catalytic segment” and the “activation loop” (Figure 1B). The catalytic segment consists of a
highly conserved glycine rich strand-turn-strand motif and contains several amino acids critical for proper orientation of the α and β phosphates of ATP. Numerous studies have show that mutation of a critical lysine (K295 in c-Src) in the catalytic segment renders the kinase catalytically inactive (25). Adjacent to this region is the more variable “activation loop,” which contains a tyrosine residue important for activation of the kinase (Y416 in c-Src; Figure 1B). This segment is defined as the region between the highly conserved Asp-Phe-Gly (“DFG”) and Ala-Pro-Glu motifs (404 to 432 in c-Src). Phosphorylation of this tyrosine facilitates a series of conformational changes directly leading to kinase activation (see below for more detail).

The C-terminal tail of Src family kinases contains a regulatory tyrosine residue (Y530 in c-Src) that becomes phosphorylated by C-terminal Src kinase (CSK; Figure 1) (26). The phosphorylated tail tyrosine is capable of binding to its own SH2 domain and thereby renders the kinase inactive (Figure 1C) (12). Deletion of this regulatory tail tyrosine (as in v-Src) or mutation of the tyrosine to a phenylalanine residue (Y530F) results in a deregulated, constitutively activated tyrosine kinase. Consequently, the active Src family kinases phosphorylate various target proteins leading to transformation and oncogenesis (27, 28).

The crystal structure of the active form of Lck (29) and inactive form of Hck (12) and Src (30) have provided invaluable insight into the structural mechanism of Src kinase auto-regulation and activation. As predicted from biochemical results, the down-regulated structures of Src and Hck showed the SH2 domain engaged with the regulatory tail phosphotyrosine (Figure 1B-C). Not predicted from existing data at the time was the observation that the SH3 domain of the Src kinases bound an internal PPII helix present in the linker between the SH2 and kinase domains (Figure 1B-C). The SH2 and SH3 intramolecular interactions exert their influence on the catalytic activity of the kinase via the distal face of the kinase domain, rather than by blocking
the catalytic binding cleft. Engagement of the SH3-linker and SH2-tail in the inactive crystal structures of Hck (Figure 1B) (31) and Src (30) results in displacement of the critical αC helix in the N-terminal lobe compared to the active conformation of Lck (29) and PKA (32, 33). This leads to the disruption of the ion pairing between the invariant Lys295 and Glu310. Both residues are strictly conserved and even found in a very distantly related bacterial kinase (34). In the structures of Src and Hck Glu310 is present on the αC helix, and when inactive, the helix swings outward and away from the protein core and prevents association with Lys295. As a result, Lys295 is no longer able to precisely orient the α and β phosphates of ATP and catalytic transfer cannot take place. Phosphorylation of the activation loop has a profound effect on the position of the αC helix and thus kinase activity. In the down-regulated structures of c-Src and Hck the n-terminal region of the activation loop is well ordered and prevents the outward swing of αC (Figure 1B). This conformation is stabilized through the interaction of Arg385 and Tyr382 on the activation loop with the displaced Glu310 residue. When phosphorylated, Tyr416 forms a salt bridge with the guanidinium group of R409 and a water-bridged interaction with the side chain of R385 of the N-lobe rather than associating with Glu310. This results in a charge neutralization, thus allowing the αC helix to swing outward promoting catalytic activity (35).

The orientation of the critical αC helix is also influenced by the binding of the PPII helix with the SH3 domain. When bound and inactive, the side chain of Trp260 is buried in a hydrophobic binding pocket created by the αC helix and a flanking region of the N-terminal lobe. Displacement of the PPII-SH3 interaction by a substrate protein disrupts this hydrophobic binding pocket, the Typ260 side chain is released, and the αC helix swings outward promoting kinase activation. The importance of the negative regulatory effects of the SH3-PPII interaction has been demonstrated by our laboratory and others. For example, a PPII helix in the HIV
protein Nef strongly binds to the SH3 domain of Hck, disrupts the intramolecular PPII—linker interaction, and potently activates the kinase (Figure 1C) (36, 37). Moreover, work from our laboratory has also shown that a double alanine substitution of the PxxP motif (AxxA) in the SH2-kinase linker of Hck potently activates tyrosine kinase and transforming activities in Rat-2 fibroblasts (38).

In cells, activation of Src-family members can occur through several mechanisms which all rely on the structural observations discussed above. Activation by receptor tyrosine kinases (RTK), such as the c-kit receptor and PDGF receptor, may involve recruitment of the Src kinase to the autophosphorylated receptor via the Src SH2 domain. Subsequent activation may then occur by displacement of the negative regulatory tail and/or phosphorylation of the activation loop tyrosine in the kinase domain by the RTK (8, 39). Activation of Src-related proteins by non-catalytic cytokine receptors, such as the receptors for IL-2, IL-3, IL-6, GM-CSF, Epo, and others is less clear. Ligand binding may induce complex formation between the receptor and the membrane bound Src proteins (40). This places the Src proteins in close proximity, potentially leading to trans-phosphorylation of the activation loop tyrosine and subsequent kinase activation (41). As mentioned previously, there is strong evidence that Src proteins can also become activated by disruption of the intramolecular interaction between the polyproline helix in the linker and SH3 domain (36-38) (Figure 1C). This can involve any of a growing list of physiological substrates for Src family kinases, including p130Cas, N-Wasp, Stat3 and RasGAP [reviewed in (42)]
1.1.3. Role in hematopoietic cells

Although Src kinases are activated by multiple cytokines and growth factors, their direct contribution to cytokine signal transduction and their role in growth and differentiation is not well understood. Some of the best evidence for Src function has come from mouse knockout studies. For example, mice with a deletion in the Hck gene exhibit a defect in phagocytosis, although hematopoiesis proceeds normally (43). The activity of Lyn kinase was increased in macrophages from Hck-deficient mice, suggesting a functional redundancy among Src-family members. Additional evidence for Src-family redundancy comes from studies with mice lacking both Fgr and Hck. These mice develop macrophages that are unable to respond to infection with *Listeria monocytogenes* (43) and neutrophils with defective integrin signaling (44, 45). Mice deficient in Lyn kinase have a reduced number of mature B-cells and aberrant B cell signaling (46). Lck knockout mice have reduced numbers of thymocytes (47), whereas those lacking Fyn have reduced T-cell receptor responsiveness (48).

Src-related kinases exert their effects by activating several downstream pathways involved in growth regulation, differentiation, migration, and adhesion. Hck has been linked to signaling for various hematopoietic cytokines, including IL-3 (interleukin-3), GM-CSF (granulocyte-macrophage colony stimulating factor), and LIF (leukemia inhibitory factor) (49-52). (53). For example, G-CSF (granulocyte colony stimulating factor) and GM-CSF were unable to stimulate DNA synthesis in DT40 cells lacking Lyn (54). Other Src members, such as Fyn, Lyn and Lck, have also been linked to cytokine signal transduction (49, 55-57). Src family kinases have also been shown to recruit/activate signaling molecules, including Ras, PI3K, focal adhesion kinase (FAK), and STATs (signal transducers and activators of transcription). Src proteins can directly phosphorylate these downstream effectors (FAK) (58) or phosphorylate an
adapter protein, such as Cbl (59) or Shc (60), linking Src family kinases to various downstream signaling pathways. Specific examples are discussed below.

1.1.4. Mitogenic signaling

There is a substantial body of literature implicating Src family kinases in a multitude of mitogenic signaling pathways. This section will focus on a few key pathways common to Src family kinases and Bcr-Abl in hematopoietic cells (see section 1.2.7. for an introduction to Bcr-Abl signaling). Loss of Shc phosphorylation in Lyn-deficient DT-40 B-cells and the coprecipitation of Shc and Lyn suggest a role for Src-related kinases in Ras activation and mitogenic signaling (61). Analysis of these same Lyn-deficient cells showed a drastic reduction in Cbl phosphorylation and PI3K activation (62). Lck and Fyn can tyrosine phosphorylate CD28 in vitro, resulting in recruitment of PI3K and Grb2 via their SH2 domains. The regulation of CD28 mediated co-stimulation of T cells by Lck and Fyn, therefore, plays a critical role upstream of the T cell receptor signaling cascade (63). Overexpression of Fyn increases Sos activity, which works by converting inactive GDP-Ras to active GTP-Ras (53).

Several lines of evidence have implicated a role for Src-related kinases in STAT activation and cellular transformation (64). A role for v-Src family kinases in STAT activation was suggested from a study examining Src-transformed NIH3T3 cells. Stat3 was constitutively phosphorylated in cells transfected with the constitutively active v-Src oncogene and v-Src could bind and phosphorylate Stat3 in vitro (65, 66). Stat5 phosphorylation was reduced in response to c-Src antisense RNA or treatment with a Src-selective inhibitor in K562 and F-36P cells and coexpression of Src and Stat5 in COS cells resulted in Stat5 activation (67). Lastly, v-Src
induced colony formation was inhibited by dominant negative mutants of Stat3, indicating that Stat3 activation is required for v-Src mediated transformation (68).

1.1.5. Role in cancer

The majority of current evidence indicating a role of the Src family kinases in cancer comes from studies involving c-Src, although many Src family members have been implicated in various malignancies [reviewed in (6)]. Most mutations of Src family kinases in cancers involve alterations in enzymatic activity rather than alterations at the gene level (69). There are some notable exceptions, however. Several groups have detected a deletion in the long arm of chromosome 20 as a consistent nonrandom abnormality in the myeloid malignancies, non-lymphocytic leukemia and polycythemia and part of this region of chromosome 20 encodes for c-Src (70-72).

In the majority of malignancies, Src family kinases exhibit increased expression indicating an oncogenic role in disease initiation and progression. c-Src was found to have elevated kinase activity compared to normal controls in a series of colon cancer cell lines (73) and in 60% of all lung cancers (74). Similarly, a study of 72 breast cell lines and tumor samples showed that Src kinase activity was elevated in 100% of the samples (75). Stat3 was shown to be constitutively activated in breast carcinoma cell lines overexpression Src family kinases (76). Various other malignancies exhibit overexpression of Src family kinases, including neuroblastomas (77), ovarian cancer (78), and skin cancer (79). Sequences related to the human c-Yes gene were amplified in a single primary gastric cancer out of 22 cases that were examined (80) and elevated c-Yes activity was observed in premalignant legions of the colon (81). There is also evidence in a fish model that Fyn may contribute to melanoma formation (82).
1.2. Chronic Myelogenous Leukemia

1.2.1. Hematopoiesis

Hematopoiesis is a complex biological process in which hematopoietic stem cells can undergo self-renewal as well as differentiation into myeloid and lymphoid progenitor cells [reviewed in (83)]. These committed progenitor cells then go on to form the mature adult cells which carry out the basic functions of the immune system and erythropoiesis. During development, hematopoiesis takes place in the aorta-gonadal-mesonephros (AGM) region, yolk sac, and fetal liver. In adults, hematopoiesis takes place in the bone marrow. In each microenvironment, “support” cells provide a local concentration of cytokines and form an extracellular matrix which provides signaling cues that initiate cell division, migration, differentiation and apoptosis. These cells include fibroblasts, endothelial cells, adipose tissue, osteoblasts, and cells of hematopoietic origin such as macrophages. Once these progenitor cells reach maturation they migrate to secondary hematopoietic organs or circulate throughout the bloodstream.

Numerous growth factors and cytokines and their downstream effectors have been implicated in the process of stem cell maturation. These factors include c-kit ligand, several interleukins, such as IL-1, IL-3, IL-6, and IL-11, G-CSF, LIF, thrombopoietin, and the FLT3 ligand [reviewed in (84)]. Loss of this strict regulation can lead to various hematopoietic malignancies.
1.2.2. **CML Overview**

Chronic myelogenous leukemia (CML) has an incidence of 1 to 10 cases per 100,000 people per year and accounts for approximately 15 to 20 percent of all leukemias (85, 86). The median age for CML onset is in the fifth decade, but all age groups are affected, particularly individuals over 60 years of age. CML is a myeloproliferative disorder characterized by the clonal expansion of transformed hematopoietic progenitor cells. Patients typically present with elevated number of neutrophils. Ultimately, the disease results in increased myeloid, erythroid, megakaryocytic and B-lymphoid cell types in the peripheral blood and myeloid hyperplasia in the bone marrow. Most patients also present with thrombocytosis, fatigue, weight loss, abdominal fullness, bleeding, splenomegaly, leukocytosis, and anemia. CML progresses from a relatively benign chronic phase to a rapidly fatal blast crisis within three to five years of initial onset (87, 88). The blast phase is defined by the presence of 30 percent or more leukemic cells in the peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blast cells. In contrast to cells in the chronic phase, which retain the ability to differentiate, cells present during blast crisis fail to differentiate and resemble the myeloblasts or lymphoblasts found in patients with acute leukemias.

1.2.3. **The Philadelphia Chromosome**

Interestingly, CML was one of the first malignancies for which a single genetic mutation was identified as the primary causative agent. In 1960, Nowell and Hungerford identified a consistent genetic abnormality in patients with chronic granulocytic leukemia (89). Cytogenetic analysis of these patients revealed the presence of a shortened 22nd chromosome, which they dubbed the “Philadelphia Chromosome” (Ph1 or Ph) for the city in which the discovery was
made (89). In 1973, Rowley further characterized the Ph chromosome as a translocation between the chromosome 9 and chromosome 22 [t(9;22)(q34;q11)] using quinacrine fluorescence and Giemsa staining (90). In forming the Ph chromosome, sequences of the c-abl protooncogene are translocated from chromosome 9 (91) to a 5.8 kb region on chromosome 22 termed the “break point cluster region” (BCR) (92) (Figure 2; discussed below).

Breakpoints in the abl gene can occur anywhere within a 5′ segment that extends for over 300 kilobases (kb). Most commonly the breakpoint occurs within an intron between the first two alternative first exons of Abl. In the Bcr-Abl fusion, however, mRNA processing results in the bcr sequences directly fused to exon 2 (a2) of Abl. The breakpoints in the bcr gene are found within three defined regions, most often in the 5.8 kb breakpoint cluster region described by Groffen et al. (92). This region contains five exons (e12 to e16; originally termed b1 to b5) and most breakpoints occur immediately downstream of e13 or e14 (originally termed b1 to b5). The hybrid mRNA transcripts of the Bcr-Abl fusion have an e13a2 (b2a2) or e14a2 (b3a2) junction resulting in an 8.5 kb sequence that encodes a 210 kDa fusion protein (p210 Bcr-Abl) (93). The p210 form of Bcr-Abl is present in up to 95 percent of all cases of CML (94), 20-50 percent of cases of adult B-lineage acute lymphoblastic leukemia (ALL) (95) and about five percent of cases of acute myeloid leukemia (AML) (Figure 2) (96).
Figure 2: Structural organization of c-Abl, c-Bcr, and Bcr-Abl p210 and p185

Sequences from c-Bcr fuse to c-Abl sequences as shown. Depending on the breakpoint, two different forms of Bcr-Abl are created, p210 and p185. See text for details regarding c-Bcr, c-Abl and Bcr-Abl domain structure and organization. *Myr = myristate, Dbl=region homologous to the Dbl exchange factor, Oligo=coiled-coil oligomerization domain, SH2-B=P-Tyr independent SH2 binding domain, GAP=Rac GTPase activating protein domain, CAP=3' regulatory region of c-Abl, SH3=Src homology 3 domain, SH2=Src homology 2 domain, Kinase=c-Abl tyrosine kinase domain.*
In two-thirds of patients with Ph-positive (Ph+) ALL and in a rare subset of CML and acute myelogenous leukemia (AML), the breakpoint in \textit{bcr} occurs in a region upstream of the major break point region known as the minor breakpoint cluster region (97-99). The hybrid mRNA consists of sequences that are approximately 7 kb in length in which exon e1 from \textit{bcr} is fused to exon a2 of \textit{abl} resulting in the production of a 190 kDa protein (p185- or p190- Bcr-Abl; Figure 2). The mechanism responsible for the t(9:22)(q34;q11) reciprocal chromosomal translocation is unknown (100). Although the generation of the Ph chromosome is widely considered a random event, other non-random mechanisms have been postulated. For example, Surralles \textit{et al.} suggest a link between chromatin structure, DNA repair, and chromosomal “hot spots” with a high-probability of rearrangement (101). Epidemiologic studies have shown that ionizing radiation is a risk factor for CML (102, 103) and that high-dose irradiation can lead to the \textit{in vitro} formation of Bcr-Abl (104). Furthermore, when human lymphocytes are irradiated with neutrons, translocations between chromosomes 9 and 22 are 11 times higher than the predicted frequency (105) due in part to the topographical proximity of chromosome 9 and 22 in the nucleus (106) (107). In contrast, no evidence for sequences known to effect translocational frequency, such as Alu sequences or Chi-like octomers, has been found near the Bcr-Abl rearrangement (108).

Considerable data, including \textit{in vivo} transgene experiments in mice and \textit{in vitro} studies with hematopoietic cell lines, implicate Bcr-Abl as the primary etiologic factor in disease onset. Specifically, McLaughlin \textit{et al.} stably transformed murine bone marrow cells with a retrovirus containing the p210 form of Bcr-Abl. When infected bone marrow was plated under conditions for long term culture of B-lymphoid (109) or myeloid lineage cells (110), cells expressing high amounts of p210 dominated the culture and resulted in the clonal outgrowth of immature
lymphoid cells. Moreover, Bcr-Abl rendered the IL-3 dependent FDC-P1 myeloid cell line cytokine independent, providing an initial clue into a possible mechanism for transformation (111). Further evidence implicating Bcr-Abl as the causative factor in CML disease onset came from the elegant work of Daley, Van Etten, and Baltimore in 1990. Bcr-Abl p210 was expressed in hematopoietic stem cells of murine bone marrow via retroviral-mediated transfer and these cells were used to reconstitute the marrow of irradiated mice. These mice developed a myeloproliferative disorder (MPD) with features of human CML, showing convincingly that the Bcr-Abl fusion protein was responsible for disease initiation (112). In 1991, work from Szczylik and colleagues highlighted the importance of Bcr-Abl in disease maintenance by demonstrating that anti-sense oligonucleotides specific for Bcr-Abl inhibited Bcr-Abl-mediated leukemic cell proliferation (113).

1.2.4. Bcr

The breakpoint cluster region gene, *bcr*, has been mapped to chromosome 22q11 (89, 90) and codes for two alternative transcripts either 4.5 kb or 7.0 kb in size (114). The *bcr* gene codes for either a 130 kDa or 160 kDa cytoplasmic protein (Figure 2) (115) ubiquitously expressed in mammals with the highest concentration of mRNA in the brain and hematopoietic cells (116-118). Bcr is expressed primarily in the early stages of myeloid differentiation, and levels are reduced significantly as cells mature to polymorphonuclear leukocytes (119). Studies with mice with a targeted deletion in the *bcr* locus have yielded several insights into the *in vivo* function of Bcr (120). Exposure of *bcr*-null mutant mice to gram-negative endotoxin leads to severe septic shock and increased tissue injury by neutrophils. Neutrophils of *bcr* (-/-) mice show a pronounced increase in reactive oxygen metabolite production upon activation and are more
sensitive to priming stimuli. Bcr also plays a role in Rac-mediated superoxide production in leukocytes (120). Other studies have implicated Bcr in various signal transduction pathways, particularly those regulated by G proteins (121). Lastly, Bcr can bind the SH2 domain of Bcr-Abl and inhibit its kinase activity through a negative-feedback mechanism (122).

The structure of Bcr is complex and contains several functional domains that play a role in cell signaling (Figure 2). The N-terminus of Bcr contains a coiled-coil oligomerization domain, which is characterized by a heptad repeat of hydrophobic residues between amino acids 28 and 68 (123). The coiled-coil domain is required for constitutive activation of the Bcr-Abl fusion protein (discussed below) (123) and mutations within the domain abolish Bcr-Abl tyrosine kinase activity and block the interaction between Bcr and Bcr-Abl (123).

The Bcr protein contains a serine/threonine kinase domain in its first exon (124, 125). Bcr can autophosphorylate on serine and threonine residues, as well as transphosphorylate casein and histones in vitro (125). c-Bcr can also phosphorylate Bap-1, which is a member of the 14-3-3 family of proteins (126) and form a 14-3-3-mediated complex with Raf in vivo (127). There are a few key SH2-binding sites in the serine/threonine kinase domain of Bcr, the most notable being tyrosine 177 (Y177), which when phosphorylated binds the SH2 domain of Grb2, providing a link to downstream Ras activation (128). Interestingly, phosphoserine and phosphothreonine, but not phosphotyrosine residues in this region can bind to the SH2 domain of c-Abl (Figure 2) (129).

The central portion of the protein contains a region with distinct homology to Cdc24/Dbi, Cdc25 and Vav guanine nucleotide exchange (GEF) factors (Figure 2) (130, 131). GEFs act as GDP-GTP exchange factors for Ras-like G proteins and play a role in cytoplasmic and cytoskeletal organization, cell polarity and cell-cycle control (132). Furthermore, using a yeast-
2-hybrid screen, the DNA repair protein, xeroderma pigmentosum group B (XPB), was found to associate with the Cdc24/Dbh homology domain of Bcr (133) and Bcr-Abl (134), indicating that Bcr and/or Bcr-Abl may play a role in DNA repair.

A GAP (GTPase activating protein) domain with activity for the Ras-related GTP-binding protein Rac is present in the C-terminus of the protein (Figure 2) (135). This region contains structural similarity to RhoGAP (136) and the GAP-related neuronal protein, chimaerin (137).

Work from our laboratory has implicated Bcr in Fps/Fes non-receptor tyrosine kinase signaling. Coexpression of Bcr with the non-receptor tyrosine kinase Fps/Fes in Sf-9 cells resulted in stable Bcr-Fes protein complex formation and tyrosine phosphorylation of Bcr (138). Phosphorylation of Y177 in Bcr by Fes induced its association with the Grb-2/Sos guanine exchange factor complex and downstream Ras activation (138, 139) but suppressed Bcr-mediated serine/threonine phosphorylation of Bap-1 (139, 140). Furthermore, Fes-mediated phosphorylation of Bcr enhanced Bcr association with the SH2 domains of Abl and the p85 subunit of PI3K (140). Lastly, Laurent and Smithgall recently demonstrated that Fes and Bcr cooperate to activate the Rac and cdc42 GTPases as part of a novel pathway regulating neuronal differentiation in PC12 cells (141).

1.2.5. c-Abl

The Abl family of non-receptor tyrosine kinases includes c-Abl and Arg (abl-related gene). c-Abl was originally identified as the normal human counterpart to the v-Abl oncoprotein of the Abelson murine leukemia virus (Figure 2) (142-144). The mammalian c-abl gene is localized to chromosome 9q34 (145), is ubiquitously expressed (119) and has two alternative 5′
exons that encode for either a 6.5 kb (1b) or 5 kb mRNA (1a) and a ~150 kDA protein (146, 147). Like members of the Src family of tyrosine kinases, c-Abl contains a tyrosine kinase domain, an SH3 domain, and an SH2 domain (Figure 2). These domains are highly conserved between nearly all tyrosine kinases, with 42% sequence homology between c-Src and c-Abl, as well as similarities in domain arrangement and spacing (148). However, c-Abl contains several features that distinguish it from Src family kinases. c-Abl shuttles between the nucleus and cytoplasm because it contains three nuclear localization signals (NLS) and a nuclear export signal (NES) (149, 150). Unlike Src family kinases, c-Abl can bind F-actin (151, 152), G-actin (152), and chromatin on the DNA (153). These functions are associated with a long C-terminal tail not found in Src kinases (Figure 2).

c-Abl interacts with a wide range of cellular proteins similar to Src family kinases, including cell-cycle regulators, other kinases, signaling adaptor molecules, transcription factors, and phosphatases and its function often depends on subcellular localization [reviewed in (154) and (155)]. The mouse c-abl locus has been targeted by homologous recombination resulting in generation of either an abl -/- null mutant (156) or C-terminal tail truncation with intact kinase activity (157). Mice with the homozygous null for Abl became runted and died 1 to 2 weeks after birth. In addition, many showed thymic and splenic atrophy, T and B cell lymphopenia and abnormal head, and eye development (156, 157).

1.2.5.1. Structure and regulation

Splicing of two alternative 5′ exons in the primary ABL1 transcript generates two messenger RNAs that encode proteins that differ in their N-terminal sequences (147). The N-terminus of the 1b variant of c-Abl is myristoylated, whereas the 1a variant is 19 amino acids
shorter and does not undergo this modification. Following the myristate signal are the SH3 and SH2 domains, which like Src family kinases play a role in substrate interaction and autoregulation (Figure 2) (14, 158). Following the SH2 domain is a short linker region, followed by the bi-lobal tyrosine kinase domain. Although the SH3, SH2, and kinase region of c-Abl is homologous in structure and function to the Src family kinases, the N-terminal and C-terminal tail regions of c-Abl are distinct. The N-terminus consists of a ~80kDa negative regulatory “cap” region, which contains part of the SH3 domain (Figure 2).

The C-terminus of Abl comprises a long extension, which contains various protein—protein interaction motifs and localization signals, such as the NLS and NES mentioned above (Figure 2). The C-terminal tail of c-Abl also contains several PPII helix sequences, which have been shown to bind the SH3 domains of Crk and Grb2 (159). Various other proteins bind this region, including ATM (ataxia-telangiectasia, mutated) (160), p53 (161), Rb (162), and the carboxy-terminal domain of RNA polymerase II (163). In contrast to the cap region, the C-terminal tail does not appear to be required for autoregulation of the kinase (164).

Like all tyrosine kinases, the activity of c-Abl is tightly regulated in cells and dysregulation can often lead to cellular transformation. Deletion of portions of the N-terminal regulatory region has been found to release the transformation potential of c-Abl in vivo (165). Several direct and indirect lines of evidence suggest that c-Abl is regulated by a cellular inhibitor, which interacts with the cap region of the molecule (166). For example, the stress-response activated protein, Pag (PrxI, MSP23) was shown to bind and suppress c-Abl kinase activity in an SH3-dependent fashion (167). Work from other groups has also shown that both pRb (162) and F-actin (168) can inhibit c-Abl function in cells through binding of the kinase domain and carboxy terminus, respectively.
Recent comprehensive analysis of the cap region and c-Abl regulation by Pluk and colleagues suggests an auto-inhibitory role for the cap rather than binding to a cellular inhibitor in trans (164). Specifically, deletion of the first 81 amino acid residues of c-Abl was sufficient to fully activate the kinase activity of purified c-Abl in vitro, demonstrating that regulation is an intrinsic property of the molecule (164). A growing body of literature now points to a cis mode of autoregulation of c-abl involving portions of the SH3, SH2, the SH3-SH2 connector, SH2-kinase domain linker, and N-terminal myristate of c-Abl (169, 170). Barilá and Superti-Furga were the first to show a Src-like mechanism of autoregulation involving the SH3 domain and the SH2—kinase linker region (169). Mutation of proline 242 and 249 to glutamate in the SH2—kinase linker or reciprocal mutation in the SH3 domain of Abl activated the kinase (169). This mechanism was confirmed with the solving of the crystal structure of the autoinhibited form of c-Abl (Figure 3) (170). Structural analysis shows that the SH3-connector-SH2 region of c-Abl forms a “clamp” which packs tightly against the distal face of the kinase domain preventing catalytic function (170). Specifically, the SH3 interacts with a PPII helix in the SH2-kinase linker region at the N-terminal lobe of the kinase domain much like Src family kinases (Figure 3). Whereas in Src kinases the PPII helix is comprised of a PxxP motif, the second proline in c-Abl is replaced with a tyrosine residue (Y245; PxxY). Mutation of either the proline (P242) or reciprocal sequences in the SH3 domain required for peptide binding disrupted the negative auto-regulation of c-Abl (169). Brasher and Van Etten demonstrated that phosphorylation of tyrosine 245 can enhance kinase activity of c-Abl by up to 50% by potentially disrupting the SH3—linker interaction (Figure 3) (171). Deletion of the Abl SH3 domain stimulated kinase activity in vivo confirming the SH3 domain’s role in auto-inhibition (14). Similarly, Brasher et al. analyzed sequences within the SH3 domain of c-Abl using a random mutagenesis strategy (172). Several
residues in or near the SH3 domain of c-Abl were found to be important for kinase activity and cellular transformation (172). As expected, mutations that disrupted SH3 peptide binding function resulted in increased kinase activity and cellular transformation. Interestingly, mutation of Ser140 in the SH3-SH2 connector did not affect SH3—peptide binding but still activated tyrosine kinase activity and transforming ability most likely through disruption of the association of Pag with c-Abl (172) or c-Abl autoregulation. In contrast, the RT loop of the Abl SH3 domain is insensitive to mutational activation, suggesting that this region does not play a role in autoregulation of the kinase (172).

The SH2 domain of Abl most likely plays role controlling kinase activity and transformation both by modulating binding of downstream substrate proteins (14) and through an auto-regulatory mechanism discussed below. Unlike Src family kinases in which the SH2 binds a regulatory tail phosphotyrosine, the SH2 domain of c-Abl tightly packs against the C-terminal lobe of the kinase domain through a series of hydrogen bonding interactions (discussed in detail below). The eight residues of the SH3-SH2 connector form a rigid connector that dynamically couples the SH3 and SH2 domains forming the basis for a regulatory “clamp” (Figure 3) (170). Like Src family kinases, binding of external substrates to the SH2 domain of c-Abl can disrupt the closed conformation and activate the kinase (173).
Figure 3: Crystal Structure of c-Abl

The c-Abl crystal structure reveals an autoregulatory mechanism involving an SH3-SH2 clamp and myristoyl switch (170, 173). c-Abl is inhibited by a negative regulatory SH3-SH2 clamp, which packs tightly against the distal face of the kinase domain causing a conformation change in the ATP-binding pocket. Two intramolecular interactions govern this negative regulation. 1) The SH3 domain interacts with a poly-proline helix in the SH2-kinase and 2) myristate binds within the C-lobe of the kinase, causing a conformation “switch” in the αI′ helix. This switch allows the SH2 domain to pack tightly against the C-lobe, inhibiting c-Abl kinase activity. Phosphorylation of Tyr-412 in the activation loop and Tyr-245 in the SH2-kinase linker are required for full catalytic activity.
Two of the most interesting features observed in the crystal structure of the autoregulated form of c-Abl, which also distinguish it from Src family kinases, are the SH2 docking mechanism and “myristoyl switch” (Figure 3) (170, 173). For SH2 docking to occur the kinase domain must be in the open conformation and a specific conformational switch in the C-lobe of the kinase domain must take place. In the Src family kinase Hck, the down-regulated form of the kinase is correlated with rotation of the critical $\alpha_C$ helix, which disrupts the positioning of several residues critical for ATP orientation and substrate binding (Figure 2B) (31). In contrast, the kinase domain of the inactive form of c-Abl remains in an open conformation similar to that seen in the crystal structure of active Lck (29). A conformational change in the conserved Asp-Phe-Gly (DFG) anchor region of the activation loop reorients residues critical for coordination of ATP, rendering the kinase inactive. Importantly, this inactivation does not require rotation of the $\alpha_C$ helix thereby allowing the kinase domain to remain in an open conformation, facilitating SH2 docking.

Docking of the SH2 domain also requires a conformation switch in the C-terminal lobe of the kinase domain. It is apparent from the crystal structure of c-Abl that an $\alpha$-helix at the far C-terminal portion of the kinase domain ($\alpha_I$) blocks SH2 packing (Figure 3) (173). However, the crystal structure of c-Abl combined with a myristoylated peptide corresponding to the N-terminal myristoylated sequence of Abl yielded an intriguing result (173). The myristoyl group binds into a deep hydrophobic pocket in the C-terminal lobe of the kinase domain and disrupts the $\alpha_I$ helix, creating a new helix, $\alpha_I'$ and as a result allows the SH2 domain to tightly dock against the C-terminal lobe of the kinase domain (Figure 3). This close association between the SH2 domain and the C-lobe of the kinase of Abl is stabilized by a series of interactions between the aromatic rings of Tyr158 in the SH2 domain and Tyr361 in the kinase domain. Work presented in
Chapter III will show that Tyr158 is phosphorylated by Hck, potentially disrupting this interaction. Binding of the myristoyl group into the C-lobe of the kinase domain is also critical for the autoregulation of the 1b form of c-Abl. Hantschel and colleagues demonstrated that forms of c-Abl 1b that lacked the myristoylation moiety exhibited constitutive tyrosine kinase activity but that myristoylation did not affect protein localization (173). In contrast to c-Abl 1b, the 1a variant, which is missing the myristoylation signal sequence, does not require the myristate group for autoregulation (174). The precise mechanism of c-Abl 1a regulation has not yet been determined.

In addition to the SH3—SH2 clamp and myristate binding, phosphorylation of c-Abl also plays a key role in modulating kinase activity. The auto-inhibited form of c-Abl exhibits no autophosphorylation even when overexpressed, while activated mutants of the protein are heavily phosphorylated (169, 175). Numerous studies have implicated a specific role for individual tyrosine residues in modulating c-Abl kinase activity and downstream signaling, particularly Y412 and Y245 (Figures 2-3), which have been studied extensively. Reynolds and colleagues examined v-Abl tyrosine phosphorylation both in vitro and in rat FRE 3A cells and identified a phospho-peptide corresponding to Y412 and either Y245 or Y272 (176). Like Src kinases, when the tyrosine on the activation loop is unphosphorylated the loop folds back into the active site and prevents proper ATP orientation and substrate binding (Figure 1B) (177). Dorey and colleagues showed that tyrosine phosphorylation of endogenous Abl is linked to a concomitant increase in kinase activity and requires the presence of Y412 (178). As mentioned previously, tyrosine phosphorylation of Y245 in the SH3—kinase linker is required for full activation of c-Abl most likely through disruption of the SH3—linker PxxY association or stabilization of the active form of the kinase (171). Several recent studies have elucidated a role
for Src family tyrosine kinases in *trans* phosphorylation of Y412 and Y245 on c-Abl both *in vitro* and in cells (178-181) and are discussed in detail in section 1.2.7.4.

Several other tyrosine residues have been implicated in controlling c-Abl and the oncogenic fusion protein Bcr-Abl (discussed below) in terms of kinase activity and transformation potential (174, 182, 183). Y276 is located in a highly conserved nucleotide-binding P-loop that contacts the phosphate group of ATP in the kinase domain and has been found in forms of Bcr-Abl resistant to the kinase inhibitor, imatinib (184). Substitution of tyrosine 272 for phenylalanine (Y272F) in c-Abl stimulates kinase activity, transforms the Ba/F3 cell line to cytokine independence (184, 185) and desensitizes c-Abl to inhibition by imatinib (184). Currently it is unknown whether Y272 is phosphorylated in *trans* or is a site of c-Abl/Bcr-Abl autophosphorylation. Four sites of phosphorylation have been determined in the SH3—linker—kinase domain interface: S94, Y283, Y245 and Y134. Mutation of S94 in the RT-loop of the SH3 domain to arginine results in decreased transformation potential compared to wild-type c-Abl (172). S94 and Y283 are juxtaposed to each other on the kinase and SH3 domains, respectively, and phosphorylation of either would be predicted to disrupt SH3—linker assembly and c-Abl autoregulation. Y245 is located on the SH2—kinase linker and was discussed previously. Y134 is a conserved residue in SH3 domains that is directly involved in binding PxxP ligands (186) and phosphorylation of this residue is predicted to disrupt the intramolecular interaction between the Abl SH3 domain and the SH2—kinase linker. Lastly, two phosphorylation sites in the “southern face” of c-Abl have been identified: Y204 in the SH2 domain and Y488 in the C-terminal lobe of the kinase domain. No structural or functional roles have been identified for either tyrosine, however, Hantschel and Superti-Furga hypothesize that both residues could be involved in the interface with the long c-terminal tail of c-Abl (174).
Bcr-Abl is a constitutively activated tyrosine kinase; therefore it has been assumed that the regulatory elements found in c-Abl do not impact Bcr-Abl activity. However, Hantschel and Superti-Furga propose that this model of Bcr-Abl is an oversimplification and that Bcr-Abl retains a partial level of regulation from c-Abl (174). In support of this, recent evidence shows that mutation of numerous residues that affect c-Abl regulation also impact Bcr-Abl kinase activity and sensitivity to imatinib (187). Azam et al. used a non-biased random mutagenesis screen to uncover mutations in Bcr-Abl that confer imatinib resistance (187) and mapped these residues onto the crystal structure of the autoinhibited form of c-Abl (170). As expected, many of the mutations affect Bcr-Abl sensitivity to imatinib though alteration of kinase domain residues that directly contact the inhibitor. Interestingly, several residues conferring imatinib resistance identified by Azam and colleagues (187) are located in the interface between the kinase domain and the SH3-SH2 regulatory clamp and are identical to residues that dysregulate c-Abl kinase activity (14, 164, 169, 172, 173). For example, mutation of Tyr89 to Asp/His/Asp in the SH3 domain of Abl resulted in imatinib resistance, presumably by disrupting the SH3-linker interaction and thus stabilizing the active form of the kinase (187). This strongly suggests that mutations that disrupt the autoinhibition of c-Abl result in imatinib resistance of Bcr-Abl and implies that mechanisms governing c-Abl regulation are retained in Bcr-Abl (174). Work presented in Chapter III shows that several tyrosines located in the Abl kinase/SH3-SH2 interface, including Tyr89, are phosphorylated by Hck, Lyn and Fyn in vitro.

1.2.6. Bcr-Abl overview

Depending on where the breakpoint is located, several different forms of the Bcr-Abl fusion protein are generated, each leading to a related but distinct disease phenotype. CML
results from the 210 kDa form of Bcr-Abl (p210), while acute lymphocytic leukemia (ALL) is associated with a 185 kDa form (p185) (97, 188) (Figure 2; discussed above).

Creation of the p210 Bcr-Abl fusion protein via chromosomal translocation juxtaposes several protein domains essential for its transforming potential and constitutive tyrosine kinase activity (Figure 2). The N-terminal 426 amino acids of Bcr encoded by exon 1 are present in both the p210 and p185 forms of Bcr-Abl (Figure 2). This region contains the coiled-coil oligomerization domain (described above), which is essential for Bcr-Abl tetramerization and tyrosine kinase activation (123). The crystal structure of the Bcr-Abl oligomerization domain was recently solved and revealed that two monomers associate in antiparallel dimers that, in turn, associate to form a tetramer (189). Both p185 and p210 contain the serine/threonine kinase domain (125) and the p210 form also contains the GEF/Dbl-like homology domain (130), which may play a role in selecting the myeloid phenotype observed in CML compared to the lymphoid phenotype of Ph+ ALL (190). In addition to the coiled-coil motif, Bcr-Abl also requires Y177—which links Bcr-Abl to Ras activation downstream—for full transforming ability (128) (191) (discussed in Section 1.2.7.4.).

From c-Abl, Bcr-Abl retains the SH3 domain, which is important for negative regulation of Abl kinase activity and substrate interaction. For example, the activation defect caused in Bcr-Abl by deletion of the coiled-coil domain could be restored by deletion of either the entire SH3 domain or by point mutations that disrupt binding of the SH3 domain to the SH2—kinase linker (169, 192). As expected, Bcr-Abl with an SH3 domain deletion still transforms 32Dcl3 murine cells to cytokine independence. Interestingly, mice expressing this SH3 mutant do not efficiently develop a leukemic phenotype, and cells from these mice exhibit altered adhesion and invasive properties indicating a dual role for the SH3 domain in kinase regulation and
downstream substrate interaction (193). Bcr-Abl with a deletion in the SH2 domain is capable of inducing a fatal MPD in mice, although mice exhibit an increase in disease latency and an altered phenotype (194). Bcr-Abl also retains the Abl tyrosine kinase domain, which is essential for Bcr-Abl transformation (195), and C-terminal tail sequences for nuclear localization, DNA binding, and actin binding (Figure 2) (196). Unlike c-Abl, which shuttles between the cytoplasm and the nucleus, Bcr-Abl is localized exclusively in the cytoplasm bound to the actin cytoskeleton (119, 151).

Although Bcr-Abl is essential for onset and maintenance of the neoplastic condition, progression of the disease to blast crisis requires additional mutations. These secondary genetic abnormalities are nonrandom and include duplication/increased expression of the Ph chromosome (197), trisomy of the 8th chromosome, and deletions or mutations in the p16 and/or p53 tumor suppressor genes (198-200). Mutations in p53 have particular relevance since it has been shown that loss of p53 function occurs in up to 30% of CML patients during disease progression (201). Moreover, Skorski and colleagues showed that immune-deficient p53-null mice reconstituted with Bcr-Abl-transduced bone marrow developed an aggressive, blast-like disease phenotype compared to their p53+ counterparts (200).

1.2.7. Bcr-Abl signaling

Bcr-Abl constitutively activates numerous signal transduction pathways that are normally regulated by growth factors and cytokines in hematopoietic cells. Activation of these pathways can lead to growth factor independence, increased proliferation, altered differentiation, and resistance to apoptosis. Several of the major signaling pathways are discussed below.
1.2.7.1. Ras/MAPK

Ras was originally identified as an oncogene and belongs to a family of monomeric GTP-binding signaling proteins thought to play an essential role in transducing and amplifying growth signals inside the plasma membrane (202-204). Activated Ras binds to the serine/threonine kinase Raf-1 (205), recruiting it to the plasma membrane where it is activated through tyrosine phosphorylation (206). Activated Raf-1 then initiates a signaling cascade via the mitogen-activated protein kinase (MAPK) pathway. The Ras/Raf/Erk (extracellular-signal-regulated kinase) signaling pathway can affect proliferation and/or apoptosis via downstream transcription factor targets including NF-kB, CREB, Ets-1, AP-1 and c-Myc. Erk can directly phosphorylate and activate Ets-1, AP-1 and c-Myc. Alternatively, Erk can phosphorylate and activate a downstream kinase target RSK, which then phosphorylates and activates transcription factors, such as CREB. These transcription factors then induce the expression of genes important for cell cycle progression such as cdks, cyclins, and Bcl-family genes [reviewed in (207)].

Bcr-Abl is linked to downstream Ras signaling through a variety of adaptor proteins and the activation of Ras is thought to block apoptosis in Bcr-Abl transformed myeloid cells (208). Ras activation in Bcr-Abl-expressing cells can stimulate two different MAPK pathways, including Erk (209) and Jun kinase (210). When phosphorylated, Y177 in the Bcr portion of Bcr-Abl can directly interact with the Grb-2/Sos guanine nucleotide exchange factor complex, providing a link to Ras activation (128, 208, 211, 212). Pendergast and colleagues demonstrated that Bcr-Abl complexes with Grb2 in vivo and that this association was mediated by the direct interaction of the Grb2 SH2 domain with phospho-Y177 (128). Mutation of Y177 to phenylalanine (Y177F) abolished Grb2 complex formation and blocked Bcr-Abl-induced Ras activation. Highlighting the requirement for Y177 in Bcr-Abl oncogenic signaling, the Y177F
mutant is unable to transform Rat fibroblasts (128) or efficiently induce a CML-like myeloproliferative disease (MPD) in mice (213). Bcr-Abl can also activate Ras through the Shc adaptor protein, which links many growth factor and cytokine receptors to Grb-2/Sos (212). The association of Bcr-Abl with the Shc/Grb2 complex depends on the kinase activity of Bcr-Abl (214). Lastly, Bcr-Abl can activate the Ras/Erk pathway through binding and phosphorylation of the CrkL-C3G adapter complex, resulting in induction of c-fos and elk-1 transcription factor gene expression (215).

1.2.7.2. STAT signaling

Stat (signal transducers and activators of transcription) proteins are a seven member family of cellular proteins that function as both cytoplasmic signaling molecules and nuclear transcription factors [reviewed in (216)]. Stats are phosphorylated by receptor tyrosine kinases or various cytoplasmic kinases such as Jaks or Src family kinases in response to numerous cytokines and growth factor signals (216-218). Tyrosine phosphorylation of Stat proteins is necessary for both homo- or hetero-dimerization and subsequent transport into the nucleus.

In Ph+ cells, Bcr-Abl induces constitutive activation of Stat transcription factors, particularly Stat5 (219-226). Separate work from Ilaria and Van Etten and Carlesso and colleagues showed that 32D, Ba/F3 and TF-1 hematopoietic cells transformed with Bcr-Abl exhibited constitutive Stat5 activation and to a lesser extent, activation of Stat1 and Stat3 (219, 227) and this activation required Bcr-Abl kinase activity (219). Similarly, Shuai and colleagues demonstrated that cell lines derived from CML patients and mouse bone marrow cells transformed with Bcr-Abl exhibited constitutive Stat5 activation independent of GM-CSF signaling or Janus kinase (JAK) activity (221). Ba/F3 cells transformed with a dominant-negative mutant of Stat5 exhibited decreased cell proliferation and increased sensitivity to
apoptosis via cytarabine or hydroxyurea (223). Stat5 however is not required for leukemia induction in mice, raising questions as to the requirement of Stat5 for CML disease progression (228). Despite this observation, constitutive Stat5 activation is consistently observed in CML patients (221) and may play a role in progression from the chronic phase to the accelerated phase and blast crisis (229). Stat5 activation induces transcription of several genes necessary for the growth and anti-apoptotic effects observed in CML cells, including Bcl-X\textsubscript{L} and cyclin-D1 (224, 225).

Although Stat5 was thought to be directly phosphorylated and activated by Bcr-Abl, new evidence has cast doubt on this hypothesis. The SH3 and SH2 domains of Bcr-Abl are necessary for the activation of Stat5 in 32Dcl3 cells, but Bcr-Abl and Stat5b do not coprecipitate suggesting that they do not interact directly (220). In collaboration with our laboratory, Klejman and colleagues found that Bcr-Abl-mediated activation of Stat5b was dependent on Hck (230). Bcr-Abl interacts with Hck through its SH3 and SH2 domains resulting in activation of Hck. Hck then phosphorylates Stat5b on Y699, which is required for Stat5b activation. Inhibition of Hck results in a decrease in Stat5b activation and down-regulates expression of the Stat5b target proteins, A1 and pim-1 (230).

1.2.7.3. Src family kinases

Although Bcr-Abl possesses a constitutively active tyrosine kinase domain, recent work suggests that it may initiate signaling by activating other non-receptor tyrosine kinases, including members of the Fps/Fes (discussed above) and Src families (231-234). Danhauser-Riedl and colleagues were the first to identify a functional interaction between the Src family kinases and Bcr-Abl (231). They observed that both Hck and Lyn showed increased kinase activity in
murine 32D cells transformed with Bcr-Abl compared to wild-type untransformed cells (231). Moreover, both Hck and Lyn coprecipitated with Bcr-Abl indicating that these proteins form a complex in cells (231). Activity of Hck and Lyn was also increased in the Ph+ cell lines, K562, BV173, LAMA84, but not the Ph- cell line, JOSK-M (231). A follow up study by the same group demonstrated that the Src family kinase Hck could phosphorylate Bcr-Abl on Y177 (232), which has been shown to link Bcr-Abl to the Ras pathway through Grb2 binding (128). Interestingly, the interaction and activation of Hck did not require Bcr-Abl kinase activity (232), suggesting Bcr-Abl may activate Src family kinases through displacement of the autoinhibitory SH3—PPII linker intramolecular interaction in Hck (12, 29, 31) similar to that of HIV-1 Nef (36, 37).

Src family kinases have also been found to act as signaling intermediates in between Bcr-Abl and various downstream signaling pathways. For example, Bcr-Abl triggers G-protein-coupled receptor CXCR4 signaling through up-regulation of Lyn resulting in activation of PI3K and the loss of chemotactic regulation by SDF (stromal-derived factor)-1 (235). In contrast to the findings of Warmuth et al. (232), Bcr-Abl-mediated activation of Lyn was dependent on Bcr-Abl kinase activity (235) in this context. Lastly, as mentioned previously, the Bcr-Abl-mediated activation of Stat5b in hematopoietic cells requires Hck (230).

Hu and colleagues recently addressed the question of the role of Src family kinases in the murine MPD model of chronic phase CML (236) and B-cell acute lymphocytic leukemia (B-ALL) (237) (238). Bcr-Abl activated Lyn, Hck, Src, Blk, Fgr and Lck in a mouse pre-B-cell leukemia line and Hck, Lyn and Fgr in primary cells from a mouse with Bcr-Abl-induced B-ALL (158). To test the requirement of these Src kinases in the mouse model, a Bcr-Abl retrovirus was used to transduce bone marrow from mice lacking the myeloid expressed Src family kinases,
Hck, Lyn and Fgr. The Hck/Lyn/Fgr-deficient marrow cells were still able to induce a CML-like syndrome but not a B-ALL syndrome in the reconstituted host mice, suggesting that the Src family kinases are necessary for B-ALL but not CML initiation (158). Moreover, a Src-selective inhibitor was able to impair growth of ALL cells in vitro and in B-ALL mice, but was ineffective in blocking CML progression (158). From these data the authors conclude that Src family kinases are not required to induce CML in mice, but instead are important for B-ALL and potentially progression to CML blast crisis (238).

Work published by our laboratory and contained in this dissertation presents data contrary to this viewpoint. We previously showed that Bcr-Abl can bind to the SH2 and SH3 domains of Hck through kinase-dependent and kinase-independent mechanisms, respectively (239). Furthermore, several regions of the Abl portion of Bcr-Abl were found to bind Hck, Lyn and Fyn through the Abl SH2, SH3, and kinase domains as well as the distal portion of the Abl C-terminal tail [(239); Chapter III]. Lastly, a kinase-defective mutant of Hck blocked Bcr-Abl-induced transformation of the murine myeloid cell line DAGM to cytokine independence, suggesting that Src family kinase activation may be necessary for Bcr-Abl transformation signaling (239). This issue regarding the conflicting role of Src family kinases in CML is discussed further in Chapter IV.

1.2.7.4. Src kinase-mediated phosphorylation of c-Abl and Bcr-Abl

Several studies have linked Src family kinases to c-Abl regulation and signaling and could potentially influence Bcr-Abl kinase activity, downstream oncogenic signaling and sensitivity to the Bcr-Abl inhibitor, imatinib (discussed below). Plattner et al. were the first to identify a role for Src family kinases in c-Abl signaling (179). They demonstrated that the kinase
activity of c-Abl increased 10- to 20-fold by the presence of constitutively activated v-Src in mouse Ba/F3 hematopoietic cells and 10T1/2 fibroblasts (179). Conversely, the Src-mediated elevation in c-Abl activity was blocked by a kinase-defective mutant of c-Src. The Src kinase-mediated increase in c-Abl kinase activity directly correlated with phosphorylation of c-Abl by the Src family kinases. Specifically, coexpression of activated mutants of either c-Src or c-Fyn with a kinase-defective mutant of c-Abl resulted in phosphorylation of Y412 in Abl (179) in cells. Dorey et al. showed that activated c-Src can phosphorylate Y412 and enhance the ability of Abl to phosphorylate the downstream substrate, c-Jun (178). A similar study by a separate group showed that Src kinase activity was necessary for c-Abl activation in NIH3T3 cells and that activated c-Src phosphorylated c-Abl on both Y412 and Y245 and dual phosphorylation was required for c-Abl function (180). Furthermore, Hck can desensitize Abl to inhibition with imatinib through an activating phosphorylation of Y412 in vitro (240) (181). Importantly, imatinib can only bind the ATP-binding cleft of the down-regulated form of Abl/Bcr-Abl but not the active form. Therefore, phosphorylation of residues that activate the kinase result in imatinib resistance (177) (discussed in 1.2.9.1).

1.2.7.5. Other signaling pathways

Several other signaling pathways are activated by Bcr-Abl and play various roles in oncogenic signaling and CML disease progression. Constitutive phosphorylation of the SH2- and SH3-containing adapter protein CrkL (241) was observed in blastic CML cell lines and neutrophils from patients with chronic phase CML but not in normal cells (242-244). CrkL links Bcr-Abl to several signal transduction pathways necessary for cellular transformation. For instance, CrkL has been shown to link Bcr-Abl to paxillin, which could contribute to the
adhesion defects observed in Bcr-Abl transformed cells (245). CrkL may also provide a link to several small-G-protein signaling pathways including Ras (215) (246, 247).

The SH2 domain of Bcr-Abl directly binds to the p85 subunit of PI3K, which leads to downstream activation of Akt and subsequent survival signaling (248, 249). Other studies have shown that Bcr-Abl can activate the PI3K pathway by forming a multimeric complex with PI3K, p120Cbl, and CrkL (250). Activation of Akt by PI3K leads to phosphorylation of the forkhead transcription factor, FKHRL1 (251), ultimately resulting in the proteasome-mediated degradation of the cell cycle regulator, p27 (252, 253). Akt also influences Bcr-Abl-mediated survival by phosphorylating and inactivating the pro-apoptotic protein, Bad (249).

Hematopoietic cells containing the Ph chromosome display a reduced capacity to bind to stromal layers, fibronectin, and other extracellular matrix (ECM) proteins including laminin and collagen (254, 255). Salgia et al. observed that Bcr-Abl induces various adhesive and cytoskeletal abnormalities in hematopoietic cell lines such as spontaneous motility, membrane ruffling, or filopodia formation (256). Bcr-Abl has been shown to diminish the function of the β1-integrin in CML cells, which may partially explain this phenotype (257). Bcr-Abl has also been shown to bind and phosphorylate numerous cytoskeletal proteins, including F-actin (151), focal adhesion kinase (258), paxillin, and others (245, 259).

1.2.8. Classical treatment

Despite the advent of selective tyrosine kinase inhibitors directed towards Bcr-Abl, the only known curative treatment for CML is allogeneic bone marrow transplantation (BMT). Patients who have an HLA-identical donor and who are younger than 40 years exhibit a 60-70% 10 year survival rate (260, 261). However, only 15-30% of patients with CML are candidates for
allogeneic BMT due to lack of an HLA-matched donor or age limitations. Moreover the effectiveness of BMT is limited by transplant-related complications such as infection, bleeding, and graft-versus-host disease. It is believed that the therapeutic benefit of allogeneic BMT is not entirely due to the radiation and chemotherapy treatment given during the conditioning phase prior to stem-cell infusion. Instead, the curative potential relies on the T-cell-mediated phenomenon of graft-versus-leukemia effect (262, 263).

Before the discovery of selective tyrosine kinase inhibitors of Bcr-Abl, interferon therapy was the best treatment option for patients who were not candidates for BMT. In several randomized clinical trials, the cytogenetic response rate was higher and survival was better in patients undergoing long term interferon treatment compared to treatment with busulfan or hydroxyurea (264-267). Interferon therapy is associated with a number of cytotoxic side effects, particular in older patients and only 5% to 10% of patients have sustained complete disappearance of the Ph chromosome (268).

1.2.9. **Imatinib mesylate (Gleevec/STI-571)**

Because Bcr-Abl is necessary for the initiation and maintenance of the CML phenotype and is not found in normal cells, it represents an ideal target for specific small-molecule drug therapy. The novel tyrosine kinase inhibitor, imatinib\(^1\), potently inhibits Bcr-Abl kinase activity, induces apoptosis in Ph\(^+\) cells, and reduces tumor formation by Bcr-Abl-transformed cells *in vivo* (269). Imatinib is a 2-phenylaminopyrimidine that acts as a competitive inhibitor for the ATP binding pocket within the kinase region of Bcr-Abl (240). Like many tyrosine kinase inhibitors, imatinib mesylate was formerly designated CGP-57148 then STI-571 and is now commercially marketed as Gleevec. The inhibitor is referred to as imatinib in this dissertation.
imatinib takes advantage of small sequence variations within the highly conserved ATP binding cleft as well as the different conformations between the active and inactive forms of the kinase.

Schindler and colleagues recently solved the crystal structure of abl complexed with imatinib (177). When imatinib is bound in the ATP-binding cleft, Abl assumes a downregulated conformation with the activation loop projecting inward into the substrate binding core. The pyridinyl group of imatinib is inserted underneath the αC helix in the N-terminal lobe of the kinase and it straddles the highly conserved DFG motif in the activation loop of Abl. Several residues in this region of the kinase confer inhibitor specificity for towards imatinib. The drug forms critical hydrogen bonds with Met318, Thr315, Lys271, Glu286, and Asp381, and various van der Waals interactions with Tyr253, Leu370, Phe382, Met290, and Ile313 of Abl, and the aromatic rings of the inhibitor (240). Although both Src family kinases and Abl are inactive when the unphosphorylated activation loop is directed inward, there is no displacement of the αC helix in Abl. This results in a conformation of the Abl DFG motif permissive to binding of the drug. In contrast, the conformation of the DFG motif in Src family kinases blocks drug access to the ATP binding site. Interestingly, phosphorylation of the activation loop tyrosine in Abl (Y412) destabilizes the inactive, closed conformation of the kinase, alters the structure of the DFG motif and, thus, interferes with drug binding. Therefore, only the down-regulated, unphosphorylated form of Abl, and by extension Bcr-Abl, is sensitive to imatinib treatment.

Imatinib was first used in 1998 to treat patients who were either refractory to or who could not tolerate interferon alpha (270). Numerous studies have shown that imatinib selectively inhibits Ph+ leukemia cells, leaving normal cells largely unaffected (269, 271, 272). Although the drug can cause nausea, headache, rashes, fluid retention, clinically significant cytopenia, and other side effects, these problems are less significant compared to interferon alpha therapy (270).
A phase I study with imatinib showed that 51 of 53 patients with chronic phase disease exhibited complete hematological remission after 265 days (273). A larger phase II study with 532 patients in late-chronic phase CML demonstrated similar results (274). In this study, imatinib induced a cytogenetic response in 60% of the 454 patients with confirmed chronic phase CML and complete hematologic response in 95% of patients (274). A phase II study with 181 patients with confirmed diagnoses of accelerated phase CML displayed no disease progression over an estimated 12 months and an overall survival rate of up to 78% with a 600 mg dose of imatinib (275). A similar phase II study was performed with patients with a confirmed blast crisis diagnoses. In this study, 52% of patients given 600mg imatinib displayed at least a partial hematologic response with 31% displaying a sustained hematologic response. Median survival of patients enrolled in the study was 6.9 months (276). A recent study compared the efficacy of imatinib compared to interferon combined with low-dose cytarabine in newly diagnosed chronic-phase CML. Nineteen months after treatment initiation, 87.1% of patients receiving imatinib displayed major cytogenetic response compared to 34.7% in the group receiving interferon plus cytarabine (277).

1.2.9.1. Imatinib resistance

Although imatinib has shown promise in chronic phase CML, patients with more advanced disease often develop resistance to the drug and relapse (278). Bcr-Abl-independent mechanisms of imatinib resistance include drug sequestration by alpha-1-acid glycoprotein (279, 280) or active drug efflux by the multidrug resistance 1 (MDR-1) gene (281). Another Bcr-Abl-independent mechanism for imatinib resistance may be overexpression of Src family kinases. A recent study by Donato et al. showed Ph+ K562 cells selected for imatinib resistance as well as
patients with advanced CML treated with imatinib display increased expression and activity of Lyn (282). Inhibition of Lyn in the imatinib resistant CML cells blocked proliferation, while imatinib had no effect, suggesting an important role for Lyn in both imatinib resistance and CML progression (282). Moreover, a dual inhibitor for Bcr-Abl and the Src family kinases was able to efficiently kill imatinib resistant cells, indicating that imatinib resistant Ph+ cells may rely on Src family kinases for disease maintenance (283).

Two types of Bcr-Abl-dependent mechanisms of resistance have been identified: Ph chromosome amplification (284, 285) and Bcr-Abl point mutations (184, 187, 284). A study by Gorre et al. using clinical material from patients resistant to imatinib showed that out of 9 patients, 6 had a single amino acid substitution in the kinase region of Bcr-Abl which rendered it refractory to drug treatment (284) (discussed in detail in the following section). Three of the 9 patients developed resistance through progressive Bcr-Abl gene amplification (284). Similarly, an analysis by Weisberg and Griffin found that Bcr-Abl protein levels are increased 10-fold in Bcr-Abl-transformed Ba/F3 cells selected for resistance to imatinib (286).

The most interesting and clinically relevant form of resistance appears to be STI-resistant point mutations in the Bcr-Abl sequence. Gorre et al. described the first mutation linked to imatinib resistance in a cohort of relapsed CML patients (284). They found that the mutation of threonine 324 to isoleucine (T334I) prevented imatinib from binding in the ATP-binding pocket, but preserved ATP binding and catalytic function. T334 makes a critical hydrogen bond with the drug, but when this residue is replaced by the bulkier isoleucine side chain the ATP binding cleft is narrowed leading to steric hindrance of the phenylaminopyrimidine group of the drug. Separate work published by Shah et al. and Branford et al. uncovered two interesting facets of imatinib-resistant Bcr-Abl point mutants: 1) drug resistant mutations can preexist in patients and
predate exposure to imatinib (287), and 2) certain mutations, such as those in the P-loop, can adversely affect clinical outcome of the disease compared to other point mutations (287) (288). Since the initial discovery of the T334I mutation, many other point-mutants in the ATP-binding pocket have been described, including F378V, F336L, V308A, E274K, E274V, Y272F, Y272H, G269E, and Q271R/H (184, 187, 284, 287, 289, 290). Each of these mutations either altered the ATP-binding cleft, changed the flexibility of the “P-loop” or shifted the equilibrium of the kinase conformation to favor the activated state, which naturally resists drug binding (240, 291). Emergence of imatinib drug resistance underscores the need for development of new drugs and discovery of additional therapeutic targets such as Src family kinases.
1.3. Dissertation Hypothesis

Bcr-Abl is the primary etiologic agent in CML development. It has been shown to constitutively activate multiple signaling pathways normally associated with the growth and differentiation of hematopoietic cells. Recent work suggests that Bcr-Abl may also initiate signaling by activating members of the Src-family of tyrosine kinases (231-234). Previous data from our group have demonstrated that Bcr-Abl-mediated cytokine independent proliferation of myeloid cells is blocked by a kinase-defective Hck mutant and identified regions of each protein that bind the other \textit{in vitro} (239). Therefore, I hypothesize that activation of Src-family kinases contributes to the diversity of Bcr-Abl signaling pathways, ultimately facilitating CML progression. The role of Src-family kinases in Bcr-Abl signaling and CML progression was tested with the following specific aims:

1.4. Specific Aims

Aim 1. Determine the biological role of the Src-related kinases in Bcr-Abl-mediated oncogenic signaling in myeloid cells. Our laboratory has shown that activation of Hck is required for Bcr-Abl-mediated cytokine independence (239). In this Aim, I investigated the role the myeloid expressed Src family kinases in Bcr-Abl signaling using a pharmacological inhibitor of these kinases. I predicted that inhibition of Src kinases would block cytokine independent growth similar to kinase-defective Hck. I investigated the downstream signaling events in these cells to determine how Src family members contribute to Bcr-Abl signaling. Specifically, Ras/MAPK and STAT activation status was characterized in the presence of a Src family kinase inhibitor. These results of this Aim demonstrated how Src family kinases contribute to Bcr-Abl oncogenic signaling and cellular transformation in hematopoietic cells.
Aim 2. Identify and characterize the mechanism of interaction between the Src family tyrosine kinases and Bcr-Abl. I identified the specific regions in the Abl portion of the protein that interact with the myeloid-expressed Src kinases (Hck, Lyn, Fyn, and Fgr) by coexpressing various regions of Abl fused to GST with the Src kinases in a co-precipitation assay. I then investigated the biochemical outcome of the interaction between Src family kinases and Bcr-Abl via mass spectrometry and western blotting. The physiologic relevance of this Src family kinase-mediated phosphorylation of Bcr-Abl was tested by introducing mutations into the relevant regions in the context of the full-length Bcr-Abl sequence in hematopoietic cells and measuring cytokine independent proliferation. I predicted that Src family kinases phosphorylate Bcr-Abl downstream and modulate Bcr-Abl kinase activity and transformation potential. The results of this Aim further elucidated the mechanism by which Bcr-Abl and Src family kinases communicate with one another and the biological outcome of this communication.
Selective Pyrrolo-pyrimidine Inhibitors Reveal a Necessary Role for Src Family Kinases in Bcr-Abl Signal Transduction and Oncogenesis

Matthew B. Wilson, Steven J. Schreiner, Hyun-Jung Choi, Joanne Kamens\(^1\), and Thomas E. Smithgall

Department of Molecular Genetics and Biochemistry, University of Pittsburgh
School of Medicine, Pittsburgh, Pennsylvania 15261

\(^1\)Abbott Bioresearch Center, 100 Research Drive, Worcester, MA 01605
2.1. Abstract

Chronic myelogenous leukemia (CML) is defined by the presence of the Philadelphia (Ph) chromosome, which results in the expression of the 210 kDa Bcr-Abl tyrosine kinase. Bcr-Abl constitutively activates several signaling proteins important for the proliferation and survival of myeloid progenitors, including the Src family kinases Hck and Lyn, the Stat5 transcription factor and upstream components of the Ras/Erk pathway. Recently, we found that kinase-defective Hck blocks Bcr-Abl-induced transformation of DAGM myeloid leukemia cells to cytokine independence, suggesting that activation of the Src kinase family may be essential to oncogenic signaling by Bcr-Abl. To investigate the contribution of Src kinases to Bcr-Abl signaling in vivo, we used the pyrrolo-pyrimidine Src kinase inhibitors PP2 and A-419259. Treatment of the Ph+ CML cell lines K-562 and Meg-01 with either compound resulted in growth arrest and induction of apoptosis, while the Ph− leukemia cell lines TF-1 and HEL were unaffected over the same concentration ranges. Suppression of Ph+ cell growth by PP2 and A-419259 correlated with a decrease in Src kinase autophosphorylation. Both inhibitors blocked Stat5 and Erk activation, consistent with the suppressive effects of the compounds on survival and proliferation. In contrast, the phosphotyrosine content of Bcr-Abl and its endogenous substrate CrkL was unchanged at inhibitor concentrations that induced apoptosis, blocked oncogenic signaling, and inhibited Src kinases. These data implicate the Src kinase family in Stat5 and Erk activation downstream of Bcr-Abl, and identify myeloid-specific Src kinases as potential drug targets in CML.
2.2. **Introduction**

Chronic myelogenous leukemia (CML) is a human malignancy that affects hematopoietic progenitor cells and accounts for 15% of all adult leukemias (85). The clinical course of CML progresses through three phases, becoming more resistant to treatment in each successive phase. The initial chronic phase is associated with the clonal expansion of multiple myeloid lineages that retain the potential for normal differentiation. Within 4-6 years, patients enter an accelerated phase in which immature blast cells begin to accumulate in the blood. Patients inevitably progress to blast crisis, which is characterized by the rapid and uncontrolled expansion of bone marrow progenitor cells and is ultimately fatal.

The cytogenetic hallmark of CML is the Philadelphia chromosome (Ph$^+$), which arises from a translocation between the c-abl locus on chromosome 9 and the bcr locus on chromosome 22 (90, 292). The Ph$^+$ translocation results in the expression of Bcr-Abl, a 210 kDa oncogenic fusion protein with constitutive tyrosine kinase activity. Bcr-Abl transforms both fibroblasts and hematopoietic cells in culture and causes a CML-like condition in mouse models, providing strong evidence that it is directly responsible for disease initiation (109, 112, 293).

Bcr-Abl has been shown to constitutively activate numerous signal transduction pathways that are normally regulated by growth factors and cytokines in hematopoietic cells. Activation of these pathways can lead to growth factor independence, increased proliferation, altered differentiation, and resistance to apoptosis. For example, tyrosine phosphorylated Bcr-Abl can directly interact with the Grb-2/Sos guanine nucleotide exchange factor (GEF) complex, providing a link to Ras/Erk activation (128, 208, 211, 212). Bcr-Abl can also activate Ras through the Shc adaptor protein, which links many growth factor and cytokine receptors to Grb-2/Sos (212). A related adaptor protein, CrkL, is constitutively phosphorylated in Ph$^+$ cells and
may also provide a link to several small-G-protein signaling pathways including Ras (246, 247). Bcr-Abl directly binds to the p85 subunit of phosphatidylinositol 3-kinase (PI-3K), which leads to activation of Akt downstream and subsequent survival signaling (248, 249). Bcr-Abl also induces constitutive activation of Stat transcription factors, particularly Stat5 (219-226). Stat5, in turn, up-regulates transcription of several genes necessary for the growth and anti-apoptotic effects observed in CML cells, including Bcl-X\textsubscript{L} and cyclin-D1 (224, 225).

Although Bcr-Abl possesses a constitutively active tyrosine kinase domain, recent work suggests that it may initiate signaling by activating other non-receptor tyrosine kinases, particularly members of the Src family. Bcr-Abl binds both Hck and Lyn following expression in the murine 32D myeloid cell line and in primary CML cells, and this association correlates with increased activity of these myeloid Src family members (231). Moreover, Hck phosphorylates Bcr-Abl on tyrosine 177 (Y177) within the Bcr region, providing a docking site for the Grb2 SH2 domain and thus a possible link to the Ras pathway (232). Recent work from our laboratory has shown that Bcr-Abl can bind to the SH2 and SH3 domains of Hck through kinase-dependent and kinase-independent mechanisms, respectively (239). Furthermore, several regions of the Abl portion of Bcr-Abl were found to bind Hck, Lyn and Fyn through the Abl SH2, SH3, and kinase domains as well as the distal portion of the Abl C-terminal tail [(239); Chapter III]. Lastly, a kinase-defective mutant of Hck blocked Bcr-Abl-induced transformation of the murine myeloid cell line DAGM to cytokine independence, suggesting that Src family kinase activation may be necessary for Bcr-Abl transformation signaling (239).

Because Bcr-Abl is necessary for the initiation and maintenance of the CML phenotype and is not found in normal cells, it represents an ideal target for specific small-molecule drug therapy. The novel tyrosine kinase inhibitor, STI-571 (Gleevec; imatinib mesylate), potently
inhibits Bcr-Abl kinase activity, induces apoptosis in Ph\(^+\) cells, and reduces tumor formation by Bcr-Abl-transformed cells *in vivo* \(269\). STI-571 is a 2-phenylaminopyrimidine that acts as a competitive inhibitor for the ATP binding pocket within the kinase region of Bcr-Abl \(240, 294\). Previous studies have shown that STI-571 selectively inhibits Ph\(^+\) leukemia cells, leaving normal cells largely unaffected \(269, 271, 272\). A phase I study with STI-571 showed that 51 of 53 patients with chronic phase disease exhibited complete hematological remission after 265 days \(273\). A larger, more recent study confirmed these initial findings, showing that the drug induces high rates of cytogenetic and hematological responses in chronic phase CML patients \(274\). Recent phase II studies show that STI-571 is also effective in CML accelerated phase and blast crisis, although some individuals relapse with drug-resistant disease \(275, 276\) (see below). These studies provide important evidence that targeting an oncogenic signaling protein with a highly selective inhibitor is very effective in the management of cancer.

Although STI-571 has shown promise in chronic phase CML, patients with more advanced disease can develop resistance to the drug and relapse. Initial findings by Gorre *et al.* \(284\) showed that patients resistant to STI-571 had either a single amino acid substitution in the kinase domain of Bcr-Abl which rendered it unable to bind to the drug, or developed resistance through progressive Bcr-Abl gene amplification. More recent studies have reported a wider range of Abl kinase domain mutations, many of which map to the ATP binding region \(184, 295\). Other groups have found that Bcr-Abl gene and protein levels are increased in cells resistant to STI-571 \(286\) and that plasma proteins in a murine model can inactivate the drug \(279\).

The identification of additional therapeutic targets remains critical in improving the long-term survival of patients with CML, particularly those in the accelerated phase or blast crisis.
Our recent work suggests that Src family kinases play a role in Bcr-Abl-mediated transformation and may serve as potential drug targets for blocking CML progression. In the present study, we show that the Src kinase inhibitors PP2 and A-419259 block Ph⁺ leukemia cell proliferation and induce apoptosis, but do not affect the growth or survival of Ph⁻ myeloid cell lines. Moreover, we show that these cellular effects are due to disruption of Src family kinase activity and not direct inhibition of Bcr-Abl. Finally, we observed that inhibition of Src family kinases correlates with the suppression of Ras/Erk signaling and Stat5 activation. These data provide new evidence that Src kinases expressed in myeloid cells, such as Hck, Lyn, Fyn and Fgr, are essential intermediates coupling Bcr-Abl to Stat and Ras/Erk signaling and represent rational targets for anti-CML therapy either alone or in combination with STI-571.

2.3. Results

2.3.1. The Src family kinase inhibitors, PP2 and A-419259, block proliferation and induce apoptosis in Ph⁺ cells

Recent studies have shown that a large percentage of patients in the blast crisis phase of Ph⁺ leukemia develop resistance to the anti-CML drug and Bcr-Abl inhibitor STI-571, highlighting the need for additional therapeutic approaches (296). Previous work from our laboratory and others suggests that the Src family of tyrosine kinases plays an essential role in Bcr-Abl-mediated transformation and CML progression and may prove to be an attractive therapeutic target (231, 232, 239). To test this hypothesis, we used the pyrrolo-pyrimidine Src kinase inhibitors, PP2 and A-419259. PP2 was initially described as a potent inhibitor of the Src family of tyrosine kinases (297), with lower activity toward Jak2 and the EGF receptor kinase
and virtually no activity toward Ser/Thr kinases. To determine the effects of Src inhibition on
CML cell growth, the Ph\textsuperscript{+} myeloid leukemia cell lines K-562 (298, 299) and Meg-01 (300) were
treated with various concentrations of PP2 and cell proliferation was measured using a 96-well
plate-based assay. Figure 4 shows that incubation with PP2 resulted in a dose-dependent
decrease in cell proliferation in both K-562 and Meg-01 cells over the four-day incubation
period. Specifically, K-562 cell proliferation was inhibited by PP2 with an IC\textsubscript{50} of
approximately 10 \(\mu\)M (Figure 4A). Similarly, PP2 inhibited cellular proliferation of Meg-01
cells with an IC\textsubscript{50} between 3 and 10 \(\mu\)M (Figure 4B).

Since PP2 has been reported to inhibit other tyrosine kinases at higher concentrations
(297, 301), we sought to confirm these results with a more specific Src family kinase inhibitor.
A-419259 is a second-generation pyrrolo-pyrimidine designed to enhance selectivity towards the
Src family relative to other cytoplasmic tyrosine kinases, similar in design to those reported by
Arnold, et al. (302). Importantly, A-419259 exhibited greater than 1000-fold selectivity towards
Lyn and Lck compared to c-Abl in an \textit{in vitro} kinase assay (Table 1), suggesting that this
compound would allow us to assess the effect of Src family kinase inhibition on CML cell
growth without direct effects on Bcr-Abl. Surprisingly, A-419259 inhibited the growth of the
same CML cell lines with a 30- to 100-fold increase in potency compared to PP2. Specifically,
A-419259 inhibited K-562 cells with an IC\textsubscript{50} between 0.1 and 0.3 \(\mu\)M (Figure 4C), and Meg-01
proliferation with an IC\textsubscript{50} of approximately 0.1 \(\mu\)M (Figure 4D).
Table 1: Comparison of IC50 values of A-419259 for the Src family members Src, Lck, and Lyn with c-Abl and PKC

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src</td>
<td>0.009</td>
</tr>
<tr>
<td>Lck</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Lyn</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Abl</td>
<td>3.0</td>
</tr>
<tr>
<td>PKC</td>
<td>&gt; 33</td>
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</tbody>
</table>
Figure 4: The pyrrolo-pyrimidine Src family kinase inhibitors PP2 and A-419259 block Ph+ leukemia cell proliferation in a dose-dependent fashion.

K-562 (A, C) and Meg-01 (B, D) cells were treated with the indicated concentrations of PP2 (A, B) or A-419259 (C, D) over the course of four days. On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and Methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU) +/- S.D. Growth curves were repeated at least three times with comparable results and a representative experiment is shown.
To determine if Src inhibitor-induced growth arrest was specific to CML cells, the Ph\(^-\) GM-CSF-dependent monocytic precursor cell line, TF-1 (303), and the Ph\(^+\) erythroleukemic cell line, HEL (304), were tested in the same assay. PP2 had no effect on the proliferation of TF-1 cells at 10 µM, although growth was slightly diminished in the presence of 30 µM PP2. However, inhibition was only observed at this high PP2 concentration and was significantly less than that of either Ph\(^+\) cell line (Figure 5A). PP2 had no effect on HEL cell proliferation over the entire concentration range (Figure 5B). A-419259 was also without effect on either TF-1 or HEL cell growth at the highest dose tested (1 µM; Figures 5C and 5D, respectively).

We next investigated whether the dose-dependent inhibition of Ph\(^+\) cell proliferation by Src family kinase inhibitors was associated with the induction of apoptosis. To answer this question, K-562 (Ph\(^+\)) and TF-1 (Ph\(^-\)) cells were treated in parallel with various concentrations of each Src inhibitor over the course of four days and apoptosis was measured using fluorescence-activated cell sorting assays for annexin-V binding and caspase-3 activation. Measurable induction of apoptosis in K-562 cells was evident 24 hours after incubation with either inhibitor (data not shown) and increased over the 3-day time course. Figure 6 shows the results of PP2 and A-419259 treatment of K-562 cells after 72 hours. Treatment with PP2 increased the apoptotic population in a dose-dependent fashion to a maximum of 67% at the highest dose tested (30 µM; Figure 6A, top). The caspase-3 activation assay correlated well with these findings, ranging from 8% in untreated cells to 48% at 30 µM (Figure 6A, bottom). A-419259 also potently induced apoptosis in K-562 cells beginning at 0.1 µM and increasing in a dose-dependent manner (Figure 6B). In contrast, neither compound induced appreciable apoptosis in TF-1 cells at 72 hours, although there was a small increase in the apoptotic population at 30 µM PP2 (Figure 7). This finding is consistent with the small growth suppression observed with this
high PP2 concentration (Figure 7A), and may result from the non-specific inhibition of Jak2 or other tyrosine kinases linked to the GM-CSF receptor in this cell line.
Figure 5: PP2 and A-419259 do not markedly affect proliferation of Ph- myeloid leukemia cells.

TF-1 (A, C) and HEL (B, D) cells were treated with the indicated concentrations of PP2 (A, B) or A-419259 (C, D) over the course of four days. On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and Methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU) +/- S.D. Growth curves were repeated at least three times with comparable results and a representative experiment is shown.
Figure 6: PP2 and A-419259 induce apoptosis in Ph+ leukemia cell lines.

K-562 cells were incubated for 72 hours with the indicated micromolar concentrations of PP2 (A) or A-419259 (B) and apoptosis was measured by either annexin-V binding, or caspase-3 activity using flow cytometry as indicated. Percent apoptotic cells are shown above the bar in each panel. Experiments were performed at least twice and produced comparable results in each case; a representative assay is shown.
Figure 7: PP2 and A-419259 do not induce apoptosis in Ph- myeloid leukemia cells.

TF-1 cells were incubated for 72 hours with the indicated micromolar concentrations of PP2 (A) or A-419259 (B) and apoptosis was measured by either annexin-V binding, or caspase-3 activity using flow cytometry as indicated. Percent apoptotic cells are shown above the bar in each panel. Experiments were performed at least twice and produced comparable results in each case; a representative assay is shown.
2.3.2. PP2 and A-419259 inhibit Src family kinase activity in Ph\(^+\) leukemia cells at concentrations that causes growth arrest and apoptosis

We next determined the effects of PP2 and A-419259 on Src family kinase activity in the CML cell lines shown to respond to these inhibitors. Cells were treated for 20 hours with the same inhibitor concentrations used in the proliferation and apoptosis assays. Lysates from control and treated cells were then probed with an antibody specific for the active, tyrosine-phosphorylated forms of myeloid Src family members. Using recombinant Src family kinase proteins, we found that this antibody specifically recognizes the autophosphorylated activation loop peptide sequence found in Hck, Lyn, Fyn, and Fgr, the predominant members of the Src family expressed in myeloid cells (data not shown). Therefore, this antibody allowed us to assay the effects of the inhibitors on the entire population of Src family kinases within the cells simultaneously. Figure 8A shows that PP2 inhibited Src kinase autophosphorylation in both Ph\(^+\) cell lines (K-562 and Meg-01) with an IC\(_{50}\) between 3 and 10 \(\mu\)M, while A-419259 blocked kinase activation between 0.1 and 0.3 \(\mu\)M. This dose-response for Src kinase inhibition closely correlates with the effects of both agents on CML cell growth and survival (Figures 4 and 6).

As a control, Ph\(^-\) TF-1 cells were treated with the same concentrations of PP2 and A-419259 under identical conditions (Figure 8A). Although both compounds inhibited Src family kinase autophosphorylation in TF-1 cells with about the same potency observed in the Ph\(^+\) leukemia cell lines, the growth suppressive and apoptotic effects were strictly limited to the Ph\(^+\) leukemia cells. These results strongly suggest that CML cells are dependent on Src family kinase activity for growth and survival, whereas cytokine-dependent cells such as TF-1 are not.

We next determined the effects of PP2 and A-419259 on Bcr-Abl phosphotyrosine content in treated and untreated CML cells. K-562 and Meg-01 cells were treated overnight with...
the same concentrations of PP2 or A-419259 used in the proliferation and apoptosis assays. Lysates from the cells were then probed using an anti-phosphotyrosine antibody, which readily detected the 210 kDa Bcr-Abl phospho-protein. Figure 8B shows that inhibitor concentrations that caused growth arrest and apoptosis had little impact on Bcr-Abl phosphotyrosine content. PP2 inhibited cell proliferation in K-562 cells with an IC$_{50}$ of approximately 10 µM (Figure 4A). Bcr-Abl remains tyrosine phosphorylated to nearly the same extent at this inhibitor concentration compared to untreated control cells when Bcr-Abl protein levels are taken into account (Figure 8B, top left). Similarly, Meg-01 cell growth was inhibited by PP2 with an IC$_{50}$ between 3 and 10 µM, and Bcr-Abl remains tyrosine phosphorylated within this dose range (Figure 8B, bottom left). At the highest concentration of PP2 tested (30 µM), Bcr-Abl phosphotyrosine content was reduced compared to untreated cells. Similar results were obtained with A-419259, although a small reduction in Bcr-Abl phosphotyrosine content was observed at lower concentrations of this compound. However, whether these effects were due to direct inhibition of Bcr-Abl kinase activity or inhibition of trans-phosphorylation of Bcr-Abl by Src kinases remains to be determined (see Discussion).
Figure 8: PP2 and A-419259 inhibit Src family kinase activity but not Bcr-Abl tyrosine phosphorylation at concentrations that inhibit cell growth and induce apoptosis.

A) The Ph⁺ cell lines K-562 and Meg-01 and Ph⁻ cell line TF-1 were incubated with the indicated concentrations of PP2 (left) or A-419259 (right) for 20 h. Cell lysates were prepared and probed with an antibody specific for the autophosphorylated, active forms of Src family kinases via immunoblotting (PY-Src). Duplicate blots were probed for Lyn expression as a loading control (Lyn). B) K-562 and Meg-01 cells were treated with PP2 (left) or A-419259 (right) and Bcr-Abl phosphotyrosine content was measured via immunoblotting with an anti-phosphotyrosine antibody (P-Tyr) or an Abl antibody as a loading control (Abl). Experiments were repeated at least three times with similar results and a representative experiment is shown.
The Bcr region of Bcr-Abl is phosphorylated on Tyr 177 and possibly other non-regulatory sites (128, 211, 305), and these phosphorylation events may be due to transphosphorylation by Hck in addition to Bcr-Abl autokinase activity (232). Thus, changes in overall Bcr-Abl phosphotyrosine content may not necessarily reflect direct actions of PP2 or A-419259 on the Bcr-Abl kinase domain in vivo. Therefore, we investigated tyrosine phosphorylation of the CrkL adaptor protein as an additional measure of Bcr-Abl kinase activity in the inhibitor-treated cultures. CrkL is a direct substrate for Bcr-Abl in CML cells and serves as useful endogenous reporter of Bcr-Abl tyrosine kinase activity (247, 284, 306). When phosphorylated by Bcr-Abl, CrkL migrates more slowly on SDS-polyacrylamide gels, providing a rapid assay for its phosphorylation status (284). Using this method, we probed the lysates from inhibitor-treated K-562 and Meg-01 cells for phosphorylation-induced changes in CrkL migration. Interestingly, 10 µM PP2 did not affect the levels of phospho-CrkL in either Ph⁺ cell line, suggesting that Bcr-Abl remained active in the presence of this inhibitor. At 30 µM PP2, CrkL phosphorylation was reduced in both cell lines, although the levels of phosphorylated CrkL remained higher than those observed following direct inhibition of Bcr-Abl with STI-571 (Figure 9A). Similarly, A-419259 did not affect CrkL phosphorylation in either cell line at the fully growth-inhibitory concentration of 0.3 µM (Figure 9B), although phosphorylation was partially reduced at the highest concentration tested (1.0 µM). Most importantly, CrkL remained significantly phosphorylated in both Ph⁺ cell lines in the presence of PP2 and A-419259 concentrations that caused growth arrest and induction of apoptosis, suggesting that the growth-inhibitory and pro-apoptotic effects of these compounds are primarily due to the inhibition of Src family kinases. However, we cannot exclude the possibility that high concentrations of these compounds may inhibit both Src kinases and Bcr-Abl, or that inhibition
of Src kinases indirectly reduces CrkL phosphorylation by affecting Bcr-Abl kinase activity (see Discussion). Treatment of K562 cells with STI-571 resulted in only a modest decrease in phosphorylated CrkL (Figure 9A-B, top panel). One possible explanation for this observation is that CrkL could be a downstream target of additional cellular kinases in K562 cells. Therefore, treatment with STI-571 would not inhibit these other kinases and CrkL would remain partially phosphorylated.
Figure 9: Phosphorylation of the endogenous Bcr-Abl substrate CrkL is not affected by growth-inhibitory concentrations of PP2 or A-419259.

The Ph\(^+\) leukemia cell lines K-562 and Meg-01 were treated for 20 h with the indicated concentrations of PP2 (A) or A-419259 (B), and cell lysates were probed for CrkL by immunoblotting. Cells were also treated with the Bcr-Abl inhibitor STI-571 (5 µM) as a positive control. The upper, slower migrating band represents the tyrosine phosphorylated form of CrkL (P-CrkL), while the lower band represents the unphosphorylated form (CrkL) (284). Experiments were repeated at least 3 times with comparable results.
2.3.3. A-419259 inhibits proliferation of myeloid cells following transformation with Bcr-Abl

Data presented so far demonstrate that CML-derived leukemia cell lines are sensitive to growth arrest and apoptosis induced by PP2 and A-419259, whereas Ph’ cell lines are not. This observation suggests that transformation by Bcr-Abl may sensitize myeloid cells to the anti-proliferative and apoptotic effects of Src kinase inhibitors. To test this idea, we transformed the IL-3-dependent myeloid leukemia cell line DAGM with a p210 Bcr-Abl retrovirus (DAGM/Bcr-Abl) or a drug selection marker as a negative control (DAGM/Neo). As observed previously (212, 239), the DAGM/Bcr-Abl cells grew in the absence of IL-3, exhibited increased cellular protein tyrosine phosphorylation, and demonstrated enhanced Stat5 tyrosine phosphorylation (data not shown). Both populations of DAGM cells were then incubated with A-419259 and proliferation was measured over a four-day period either with or without IL-3. As shown in Figure 10, A-419259 strongly inhibited DAGM/Bcr-Abl cell proliferation in the absence of IL-3 with an IC\textsubscript{50} between 0.1 and 0.3 \( \mu \)M. This result is strikingly similar to that observed with K-562 and Meg-01 CML cells (Figure 4). Interestingly, when the DAGM/Bcr-Abl cells were incubated with the same A-419259 concentrations in the presence of IL-3, the effect of the inhibitor was partially reversed. Finally, A-419259 had no effect on the growth of the untransformed DAGM/Neo cells, consistent with results obtained from HEL and TF-1 cells. These results suggest that Bcr-Abl-induced proliferation and survival of DAGM cells requires Src kinase activity, whereas IL-3-dependent growth and survival does not.
Figure 10: Transformation with Bcr-Abl sensitizes DAGM myeloid leukemia cells to growth arrest by A-419259.

The IL-3-dependent myeloid leukemia cell line DAGM was transformed to cytokine independence by infection with a recombinant Bcr-Abl retrovirus (DAGM/Bcr-Abl). DAGM cells infected with a retrovirus carrying only a drug selection marker served as a negative control (DAGM/Neo). DAGM/Bcr-Abl cells were treated with the indicated concentrations of A-419259 in the absence (A) or presence of IL-3 (B) and proliferation was measured over the course of four days. The DAGM/Neo control population was similarly treated in the presence of
IL-3 (C); these cells undergo rapid apoptosis in the absence of IL-3 (data not shown). On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and Methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU) +/- S.D. Growth curves were repeated at least three times with comparable results and a representative experiment is shown.
We next assessed Src kinase activity in the DAGM cells. DAGM/Bcr-Abl cells were treated overnight with various concentrations of A-419259 either in the presence or absence of IL-3 and cell lysates were probed for activated Src family kinases with phosphospecific antibodies as described in Figure 8. Incubation with A-419259 caused a partial inhibition of Src kinases at 0.1 µM and nearly complete inhibition at 0.3 µM (Figure 11A, top). In contrast to the Src kinases, only modest changes in Bcr-Abl phosphotyrosine content were observed at these A-419259 concentrations, although both the expression and phosphorylation of Bcr-Abl were diminished at 1 µM (Figure 11A, bottom). Nearly identical patterns of Src family kinase inhibition were observed in the presence of IL-3 (Figure 11B), suggesting that the compensatory effects of IL-3 on cellular sensitivity to the inhibitor were not due to Src reactivation. Similarly, Bcr-Abl phosphorylation in the presence of IL-3 was nearly identical to that of cells grown without cytokine (Figure 11B, bottom). As a control, Src family kinase activity was measured in the DAGM/Neo cells after incubation with A-419259. The activated Src kinases present in these cells were partially inhibited at 0.3 µM and displayed complete inhibition at 1 µM (Figure 11C). These results show that A-419259 inhibits the Src kinases present in both DAGM cell populations, but the growth-suppressive effect of this inhibitor is limited to DAGM cells expressing Bcr-Abl in the absence of IL-3.
Figure 11: A-419259 inhibits Src kinase activity but not Bcr-Abl autophosphorylation at growth-inhibitory concentrations in Bcr-Abl-transformed DAGM cells.

DAGM/Bcr-Abl cells were treated with the indicated concentrations of A-419259 in the absence (A) or presence (B) of IL-3, and cell lysates were prepared and probed with an antibody specific for the autophosphorylated, active forms of Src family members via immunoblotting (PY-Src). Duplicate blots were probed for Lyn expression as a loading control (Lyn). Bcr-Abl phosphotyrosine content was determined by immunoblotting with an anti-phosphotyrosine
antibody (P-Tyr) and with an Abl antibody as a loading control (Abl). C) DAGM/Neo cells were treated with A-419259 in the presence of IL-3 and lysates were probed with Src phosphospecific and Lyn antibodies as in part A. Each experiment was repeated at least twice with comparable results.
2.3.4. **PP2 and A-419259 block Stat5 and Erk activation in Ph\(^+\) leukemic cells**

We next used PP2 and A-419259 to assess the contribution of the Src kinase family to the activation of growth and survival pathways downstream of Bcr-Abl. Several recent studies have shown that constitutive activation of Stat5 contributes to Bcr-Abl-induced leukemogenesis through up-regulation of Bcl-X\(_L\) and cyclin-D1, and possibly other survival and proliferative genes (224-226). Moreover, other work has implicated small G protein/MAP kinase pathways in Bcr-Abl signaling, including the Ras/Erk (128, 211, 212, 307) and c-Jun kinase (Jnk) pathways (210). Other studies have shown that Src family kinases can also activate these pathways directly in other cellular contexts (60, 64, 308, 309). Therefore, we focused our attention on Stat5 and Ras/Erk signaling as possible effectors for Src kinases downstream of Bcr-Abl.

An electromobility shift assay (EMSA) was used to examine DNA binding of activated Stat5 (193). K-562 cells were treated overnight with concentrations of either PP2 or A-419259 shown above to cause growth arrest and induce apoptosis. Nuclear extracts were prepared and incubated with a radiolabeled DNA probe containing the Fc\(\gamma\)R1 promoter GAS consensus sequence for Stat5 binding. Both PP2 and A-419259 potently inhibited Stat5/DNA complex formation (Figure 12A). PP2 partially inhibited complex formation at 3 µM, with complete inhibition at 10 µM, while A-419259 disrupted the DNA/Stat5 complex partially at 0.1 µM and completely at 0.3 µM. Figure 12B shows that both compounds also inhibited Stat5 tyrosine phosphorylation, consistent with the gel-shift data. Importantly, inhibition of both the DNA-binding and tyrosine phosphorylation of Stat5 correlates very well with inhibition of Src family kinases but not Bcr-Abl. This result supports the idea that Src kinases couple Bcr-Abl to Stat5 activation *in vivo*. Neither compound affected Stat5 DNA binding or tyrosine phosphorylation in
Ph§ TF-1 cells, suggesting that Src kinases are not involved in Stat5 activation in response to GM-CSF treatment in this cell line (data not shown).

As mentioned above, Stat5 regulates transcription of several genes involved in deregulated survival of CML leukemia cells. Bcl-X\textsubscript{L} is a member of the Bcl-2 family of anti-apoptotic genes and has been shown to be up-regulated by Stat5 in Ph\textsuperscript{+} cells (224). Since the Src inhibitors blocked Stat5 activation, we predicted that one or more members of the Bcl-2 family, particularly Bcl-X\textsubscript{L}, would also be affected. To test this prediction, K-562 cells were treated overnight with either PP2 or A-419259 and gene expression was determined using an RNase protection assay with probes specific for Bcl-X\textsubscript{L} and other members of the Bcl-2 gene family. Both PP2 and A-419259 caused a dose-dependent decrease in Bcl-X\textsubscript{L} expression in K-562 cells with concentrations of compound that correlated well with Stat5 inhibition (Figure 12C). Moreover, the effects of the inhibitors appeared to be limited to Bcl-X\textsubscript{L} expression and did not affect Bcl-2 (data not shown). Neither inhibitor had an effect on Bcl-2 family gene expression in TF-1 cells, consistent with the lack of inhibitor action on Stat5 activation in this cell line (data not shown).
Figure 12: PP2 and A-419259 inhibit Stat5 activation and Bcl-XL expression in CML cells.

K-562 cells were treated with the indicated concentrations of PP2 and A-419259 for 20 h. A) Nuclear extracts were prepared and tested for the presence of activated Stat5 by electromobility shift assay (EMSA) using a $^{32}$P-labeled probe based on the $\gamma$-activation sequence (GAS) as described under Materials and Methods. To test probe specificity, reactions were incubated in the presence (+) or absence (-) of a 100-fold molar excess of unlabeled oligonucleotide probe. B) Stat5 tyrosine phosphorylation was assessed by immunoprecipitation of Stat5 from clarified cell lysates and immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Aliquots of the
immunoprecipitates were also blotted with anti-Stat5 antibodies to insure equivalent loading in each lane (Stat5).  C) To analyze Bcl-X\textsubscript{L} expression, total RNA was isolated and subjected to ribonuclease protection assay using a Bcl-2 family probe set as described under Materials and Methods. The intensity of the Bcl-X\textsubscript{L} signals were measured by storage phosphor imaging and normalized to levels of the GAPDH housekeeping gene. Normalized Bcl-X\textsubscript{L} levels are presented as percent of maximum signal observed in the absence of inhibitor treatment. The experiment was repeated twice and the mean Bcl-X\textsubscript{L} levels +/- S.D are indicated.
To determine if the Src inhibitors affected constitutive activation of the Ras pathway in CML cells, we examined Erk activation using phosphospecific antibodies. K-562 cells were treated overnight with each Src inhibitor, and the cellular lysates were probed for activated Erk using phosphospecific antibodies. Growth inhibitory concentrations of both Src inhibitors caused a dose-dependent decrease in Erk phosphorylation, suggesting that down-regulation of this pathway may contribute to the growth suppressive actions of these compounds as well (Figure 13).
Figure 13: PP2 and A-419259 inhibit the Ras/Erk pathway in CML cells.

K-562 cells were treated with the indicated concentrations of PP2 (top) or A-419259 (bottom) for 20 hours. Cell lysates were prepared and analyzed for the presence of active Erk by immunoblotting with phosphospecific antibodies (P-Erk). Duplicate blots were probed with antibodies to Erk2 protein as a loading control (Erk). Experiments were repeated three times with comparable results.
2.4. Discussion

Previous work from our laboratory and others has implicated the Src tyrosine kinase family in Bcr-Abl-mediated transformation of myeloid cells (231, 232, 239). In the present study, we have demonstrated a requirement for Src kinase activity in Bcr-Abl transformation and oncogenic signal transduction using a pharmacological approach. The Src-selective pyrrole-pyrimidines PP2 and A-419259 blocked Ph+ CML cell growth and induced apoptosis in a dose-dependent manner, but had little or no effect on Ph− myeloid cells. Growth arrest and apoptosis correlated with inhibition of Src kinase activity at drug concentrations that did not markedly affect Bcr-Abl kinase activity. Transformation of IL-3-dependent myeloid cells with Bcr-Abl sensitized them to the effects of A-419259, while the untransformed parent cells were unaffected. The anti-proliferative actions of both inhibitors correlated with a decrease in Stat5 and Erk activation, but did not impact the tyrosine phosphorylation of the direct Bcr-Abl substrate, CrkL. These results strongly support the hypothesis that Src family kinases are necessary for Bcr-Abl signal transduction as well as the proliferation and survival of CML cells.

The anti-proliferative actions of Src-selective inhibitors on K-562, Meg-01, and DAGM/Bcr-Abl cells are very similar to those observed previously following direct inhibition of Bcr-Abl with STI-571 in Ph+ cells (269). Therefore, it was critical to show that the effects of PP2 and A-419259 are due to Src inhibition and not non-specific effects on Bcr-Abl. Although no in vitro data have been reported for PP2 with Abl kinases, other studies have shown that the closely-related inhibitor PP1 exhibits only modest (10-fold) selectivity for Src kinases compared to c-Abl in vitro (310) (J. Kamens, unpublished data). PP2 differs from PP1 by a substitution of a chlorine atom for a methyl group and both have a similar inhibition profile against Src, Lck, Jak2, and the EGF receptor (297). In addition, Abl shares some of the kinase domain residues
proposed to determine PP1 selectivity in the Hck-PP1 co-crystal structure (31). Despite these caveats, we found that concentrations of PP2 that inhibited Src family kinases and induced growth arrest and apoptosis in CML cells did not markedly reduce Bcr-Abl phosphotyrosine content or levels of phospho-CrkL, suggesting that Src kinases are the target for this compound.

A-419259 is one of a group of second generation pyrrolo-pyrimidines recently developed to exhibit improved selectivity towards the Src kinase family (302). This compound was chosen for our study because it exhibited greater than 1000-fold selectivity towards the leukocyte Src family members Lck and Lyn compared to c-Abl \textit{in vitro} (Table I). A-419259 also inhibited Hck and Lyn with equal potency in immune-complex kinase assays (data not shown), indicating that Hck may be a target for this compound \textit{in vivo} as well. Hck has been strongly implicated as a Bcr-Abl signaling partner in several previous studies (230-232, 239). A-419259 inhibited CML cell proliferation and induced apoptosis with 30- to 100-fold greater potency than PP2. Importantly, concentrations of A-419259 responsible for these anti-CML effects also inhibited Src family kinases but did not markedly affect Bcr-Abl or CrkL tyrosine phosphorylation, again suggesting that Src kinases are the primary target for this drug in CML cells. Together with the PP2 data, these findings establish a necessary role for Src family kinases in Bcr-Abl signaling.

At the highest concentrations of PP2 and A-419259 tested (30 µM and 1 µM, respectively), reductions in Bcr-Abl and CrkL tyrosine phosphorylation were observed in CML cell lines. At least two explanations are possible for this observation. First, pyrrolo-pyrimidines may directly bind and inhibit the Abl kinase domain at higher concentrations. This may be particularly true for PP2, because the Abl and Hck kinase domains share residues important for binding of the related compound PP1 in the Hck crystal structure (31). A second possibility is that inhibition of Src kinases may indirectly affect Bcr-Abl phosphotyrosine content and kinase.
activity. Previous work has shown that Src family kinases can trans-phosphorylate Bcr-Abl, particularly on the Grb2 SH2 domain binding site in the Bcr-derived portion of the molecule (232). More recently, Src family kinases have been shown to trans-phosphorylate c-Abl within its activation loop and SH2-kinase linker region, suggesting that Src kinases may regulate Bcr-Abl kinase activity as well (179, 180). Thus, selective inhibitors for Src family kinases may block this transphosphorylation event in vivo, subsequently reducing Bcr-Abl phosphotyrosine content or kinase activity. Experiments to address these possibilities are currently underway.

Studies with the cytokine-dependent cell line DAGM suggest that transformation with Bcr-Abl sensitizes myeloid cells to Src inhibitors. A-419259 potently inhibited the proliferation of Bcr-Abl-transformed DAGM cells in the absence of IL-3 with an IC\textsubscript{50} similar to that observed with the K-562 and Meg-01 CML cell lines (0.1 - 0.3 µM). This finding supports the general conclusion that Bcr-Abl requires Src kinases for growth and survival signal transduction. Interestingly, incubation of A-419259-treated DAGM/Bcr-Abl cells with IL-3 partially rescued the cells from the growth-inhibitory effects of this compound. IL-3 treatment did not reactivate Src kinases, however, suggesting that Src activation by IL-3 is not required for proliferation or survival in this cell line. Similar results were obtained with the DAGM/Neo control cells as well as GM-CSF-dependent TF-1 cells, in which proliferation and survival were unaffected despite a complete block of Src kinase activity by the inhibitor. This observation helps to validate Src family kinases as CML drug targets, because it implies that Src activation is only crucial to the proliferation and survival of Bcr-Abl transformed cells and not normal myeloid progenitors. However, the finding that IL-3 can rescue Bcr-Abl-transformed DAGM cells from the toxic effects of A-419259 (Figure 10) may influence the effectiveness of selective Src kinase inhibitors when used as stand-alone agents in CML.
Data presented in this report strongly suggest that Src family kinases may couple Bcr-Abl to various downstream effector molecules, facilitating CML disease progression. Both PP2 and A-419259 blocked Stat5 and Erk activation at concentrations that inhibited Src family kinases but did not affect Bcr-Abl or CrkL tyrosine phosphorylation. Our results agree with recent evidence suggesting a dominant and non-redundant role for Stat5 in mediating resistance of CML cells to apoptosis, while both Ras/Erk and Stat5 signaling drive proliferation (226). We also showed that PP2 and A-419259 blocked expression of the anti-apoptotic Stat5 target gene Bcl-XL in CML cells, but had no effect on Stat5 activation and Bcl-XL expression in the Ph− cell line, TF-1.

Despite mounting evidence that Src kinases are involved in Bcr-Abl-mediated activation of Stat5, the exact mechanism is not clear. The simplest mechanism for Stat5 activation in Ph+ CML cells is direct tyrosine phosphorylation by Bcr-Abl. However, Nieborowska-Skorska et al. were unable to demonstrate a direct association of Stat5 with Bcr-Abl (220). These authors also found that the SH2 and SH3 domains of Bcr-Abl are necessary for Stat5 activation in myeloid cells. Interestingly, this region of Bcr-Abl binds tightly to Hck and other myeloid Src family members in vitro (239). Furthermore, Hck and Lyn can bind and phosphorylate Stat5 both in Sf-9 insect cells and in vitro (311) (S. Schreiner and T. Smithgall, unpublished data). A general role for Stats in oncogenesis has emerged in recent years, and the Src kinase family may have a prominent role as an intermediate coupling upstream tyrosine kinases to Stat activation in several cancers including CML (312, 313).

Numerous studies have demonstrated that Ras activation is critical to Bcr-Abl-mediated transformation and that inhibition of Ras or its downstream effectors can block Ph+ proliferation (128, 211, 212, 307, 314, 315). Data presented here show that the Src kinase inhibitors PP2 and
A-419259 can block Erk activation at concentrations that do not affect Bcr-Abl kinase activity. Several possible mechanisms of Ras/Erk activation in CML have been suggested, including direct interaction with the Grb-2/Sos complex through the Grb2 SH2 domain binding site at Tyr 177 (128, 211) or through the Shc (212) or CrkL adaptor proteins (306, 316). Previous studies have shown that Hck phosphorylates Bcr-Abl on Tyr 177 in a heterologous expression system (232), predicting that Src inhibition would suppress Bcr-Abl interaction with Grb2 in CML cells. However, we did not detect a decrease in Bcr-Abl/Grb-2 complex formation in the presence of either PP2 or A-419259 (data not shown). This result suggests that Bcr-Abl autokinase activity may also contribute to the phosphorylation of this site in CML cells.

In summary, data presented here validate the Src family kinases expressed in myeloid cells as alternative targets for CML drug therapy, particularly in patients shown to be refractory to treatment with STI-571. Treatment of CML patients, especially those in blast crisis, with a combination of Src and Bcr-Abl inhibitors could provide a dramatic therapeutic benefit. Furthermore, like combinatorial therapy, the rational design of compounds that have dual specificity for both kinases could also provide similar benefit and would potentially prevent drug resistance and subsequent disease relapse. Such an agent has recently been reported by Huang, et al. and is a very potent inhibitor of CML cell growth (317). Future work will address the role Src kinases play in CML progression in vivo and the effects of Src inhibition on Bcr-Abl signaling and oncogenic activity in a whole-animal model of CML.
2.5. Materials and methods

2.5.1. Cell culture & proliferation assays

The K-562 (298) and Meg-01 (300) cell lines, both derived from Ph⁺ CML patients in blast crisis, as well as the Ph⁻ erythroleukemia cell line HEL (304) were purchased from the American Type Culture Collection (ATCC). K-562 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and 50 µg/ml gentamycin. Meg-01 cells were cultured in Vitacell modified RPMI 1640 (ATCC), supplemented with 10% FCS and 50 µg/ml gentamycin. The human GM-CSF-dependent myeloid leukemia cell line TF-1 (303) was obtained from the ATCC and grown in RPMI 1640 supplemented with 10% FCS, 50 µg/ml gentamycin, and 1 ng/ml of recombinant human GM-CSF. DAGM murine myeloid leukemia cells (a gift from Dr. Owen Witte, Howard Hughes Medical Institute, UCLA) were cultured in RPMI 1640 supplemented with 10% FCS, 50 µg/ml gentamycin, and 0.5 ng/ml recombinant IL-3. Concentrated stock solutions of PP2 (5 mM; Calbiochem) and A-419259 (2 mM; Abbott Biotech) were prepared in DMSO and stored at -20°C. Cellular proliferation was measured using the Live/Dead growth assay (Molecular Probes) according to the manufacturer's protocol. This assay employs calcein-AM, a fluorogenic esterase substrate that is taken up by viable cells and hydrolyzed intracellularly, releasing a green fluorescent product. Briefly, 10⁴ cells were plated per well in 96-well plates for each day of a four-day time course. Various concentrations of PP2, A-419259 or vehicle control were added to the wells (5 wells per concentration per day) and the plates were incubated at 37°C. At each time point, one plate was centrifuged at 1,500 X g for 10 min to pellet the cells. Cells were washed with phosphate buffered saline (PBS), and calcein-AM was added to each well to a final concentration of 1 µM. Plates were incubated in the dark at room
temperature for 1 h. The plates were then read at 485 nm/530 nm (excitation/emission) using a SpectraMax Gemini XS fluorescent plate reader and data were analyzed with SoftMax Pro software (Molecular Devices).

2.5.2. **Apoptosis Assays**

To measure apoptosis, K-562 and TF-1 cells were treated with PP2, A-419259 or the vehicle control for 72 hours at 37°C. Apoptosis was assessed by determining cell-surface annexin-V binding and intracellular caspase-3 activity. For annexin-V binding, K-562 and TF-1 cells were incubated with annexin-V-FITC (BD Pharmingen) according to manufacturer's protocol. Briefly, 10⁶ cells were centrifuged at 1,000 x g for 10 min, and washed twice with cold 1X PBS. Cells were then resuspended in annexin-V binding buffer and 10⁵ cells were pelleted and incubated with 5 µl annexin-V-FITC and 5 µl propidium iodide (50 µg/ml) for 15 min. Apoptosis was measured using a FACSCalibur flow cytometer (Becton-Dickinson) set for two-color acquisition, and data were analyzed using CellQuest software. For the caspase-3 assay, 10⁶ cells were centrifuged at 1,000 x g for 5 min and media was completely removed. Sixty µl of 10 µM PhiPhiLux reagent (OncoImmumun, Inc.) and 5µl FCS were added to the cell pellet and incubated at 37°C for 45 minutes. PhiPhiLux is a profluorescent protease peptide substrate that upon cleavage by intracellular caspase-3 emits green fluorescence at 530 nm. Following incubation, cells were washed once with ice-cold flow cytometry dilution buffer provided with the reagent. The cells were resuspended in 1 ml of the same buffer for FACS analysis.

2.5.3. **Transformation of DAGM cells with Bcr-Abl Retroviruses**

The pMig-Bcr-Abl/GFP retroviral vector was generously provided by Dr. Warren Pear, University of Pennsylvania. This vector encodes the 210 kDa form of Bcr-Abl followed by an internal ribosomal entry sequence (IRES) and the GFP coding sequence under the control of the
murine stem cell virus long-terminal repeat (318). The control pSRαMSVtk neo retroviral construct was provided by Dr. Owen Witte, Howard Hughes Medical Institute, UCLA (319). To make retroviral stocks, subconfluent 100 mm dishes of 293T cells were co-transfected with each retroviral vector and an ecotropic packaging vector using a calcium phosphate procedure described elsewhere (36). Viral supernatants were collected at 48-, 72-, and 96-h post transfection, pooled, filtered with 0.45 μm filters, and stored at -80°C. DAGM cells were stably infected as follows: 10^6 cells were incubated with 5ml of viral supernatant in the presence of 4mg/ml polybrene. To increase viral uptake, cells were centrifuged during infection at 2,400 rpm for 3 h at room temperature (239). DAGM cell populations infected with the pMig-Bcr-Abl/GFP retroviral vector were observed for expression of GFP by fluorescence microscopy, cytokine independent outgrowth, and expression of Bcr-Abl protein by immunoblotting. For cytokine independent outgrowth, cells were washed free of cytokine and resuspended in media lacking IL-3. Proliferation was measured using the Live/Dead assay as described above over the course of 10 d. To confirm expression of Bcr-Abl protein, lysates were prepared from 10^7 cells and probed with anti-Abl (8E9, BD-Pharmingen) or anti-phosphotyrosine (PY99; Santa Cruz) antibodies on immunoblots as described below. Control populations were prepared by infecting DAGM cells with a retrovirus carrying only the neo drug resistance marker and selecting with G-418 at 800 μg/ml for 7-10 days in medium plus IL-3.

2.5.4. **Analysis of protein expression and tyrosine kinase activity**

To monitor Src kinase activity, 10^7 cells were collected by centrifugation, washed once with PBS, and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with the protease inhibitors aprotinin (25 μg/ml),
leupeptin (25 µg/ml) and PMSF (1 mM) and the phosphatase inhibitors NaF (10 mM), and Na$_3$VO$_4$ (1 mM). Clarified lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted with an antibody to the phosphorylated form of the Src activation loop (Y-418; BioSource International) or an anti-Lyn polyclonal antibody (Santa Cruz) to normalize for loading. Bcr-Abl phosphorylation was determined by probing replicate immunoblots with the anti-phosphotyrosine and anti-Abl monoclonal antibodies. CrkL phosphorylation was assessed by determining the relative levels of the unphosphorylated and phosphorylated forms of the protein following visualization on immunoblots with an anti-CrkL polyclonal antibody (H-62, Santa Cruz) as described (284). Erk activation was measured using an anti-phospho-Erk monoclonal antibody (E-4; Santa Cruz) as well as an anti-Erk2 polyclonal antibody (C-14; Santa Cruz) as a loading control. To assess Stat5 tyrosine phosphorylation, $10^7$ cells were lysed in RIPA buffer, and Stat5 was immunoprecipitated with 2 µg of the Stat5a polyclonal antibody (L-20; Santa Cruz) and 25 µl protein G-Sepharose (50:50 w/v slurry; Amersham-Pharmacia Biotech) by rotation at 4°C overnight. Beads were then collected and washed three times with ice-cold RIPA buffer, resuspended in a final volume of 25 µl, combined with an equal volume of 2X SDS sample buffer and heated for 5 min at 95°C. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted with a Stat5 monoclonal antibody (Transduction Laboratories) or the PY-99 anti-phosphotyrosine antibody. All immunoreactive bands were visualized with goat-anti-rabbit secondary antibodies conjugated to alkaline phosphatase with NBT-BCIP as colorimetric substrate (Southern Biotechnology Associates).

2.5.5. Electromobility shift assays (EMSA)

Following treatment with various concentrations of Src inhibitors for 20 h, K-562 or TF-1 cells ($10^7$) were centrifuged, washed once with PBS, and nuclear extracts were prepared as
described by Skorski, et al. (193) Stat5 DNA-binding activity was assessed using a double-stranded DNA probe based on the γ-activation sequence (GAS) (193). The probe was prepared by combining 200 pmoles of the complementary single-stranded GAS oligos (5'-AGCTTGTATTTCCCAGAAAAGGG-3' and 5'-TCCCTTTTCTGGGAAATAC-3') with 6.6 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA), heating to 70° C, and cooling slowly overnight. For labeling, 1µl of the duplex GAS oligo (20 pmol) was added to 12 µl H2O, 4 µl Labeling Mix dATP (0.1 mM dCTP, dGTP, dTTP in 50 mM Tris-HCl, pH 7.5, 50 mM MgCl2, 250 mM NaCl, 25 mM β-mercaptoethanol; AP Biotech), 2 µl [α-32P]dATP (2,000 Ci/mmol; New England Nuclear), and 1 µl DNA polymerase I Klenow fragment (0.5 U/µl, Gibco-BRL). Following incubation for 1 h at room temperature, the reaction was diluted with 20 µl H2O and unincorporated nucleotides were removed using a G-25 Sephadex spin column (AP Biotech) according to the manufacturer's instructions. For each gel-shift reaction, 40,000 cpm of the radiolabeled GAS oligo was combined with 4 µl H2O, 0.2 µl 0.5 M HEPES, pH 7.9, 0.2 µl 50% glycerol, 0.2 µl 50 mM DTT, 1 µl poly dI-dC (1µg/µl), and 2.5 µl BSA (2.0 µg/µl, Sigma). Nuclear extracts (5 µl containing equal amounts of protein) were combined with 4 µl of H2O (3 µl for cold competitor experiments; see below), and 1 µl of 10X incubation buffer (100 mM HEPES, pH 7.9, 500 mM KCl, 10 mM EDTA). Control reactions for binding specificity were run in parallel and contained 1 µl of a 100-fold molar excess of non-radiolabeled probe. The binding reaction was started by the addition of 10 µl of the labeled probe mixture and incubated at 30° C for 30 min. Reactions were quenched by transferring them to ice, and Stat5-GAS complexes were resolved on 5% non-denaturing polyacrylamide gels pre-run for 30 min at 100 volts in 0.25X TBE buffer. Gels were fixed with 10% acetic acid/10% methanol, rinsed with H2O, dried, and radiolabeled bands were visualized by autoradiography.
2.5.6. **RNase protection assay (RPA)**

For the RPA assay, $10^7$ K-562 or TF-1 cells were treated for 20 h with either PP2 or A-419259. Following incubation, total cellular RNA was isolated from each cell line using an RNA isolation kit (ToTALLY RNA, Ambion Inc.) according to the manufacturer's instructions. To measure expression of Bcl-X$_L$, a multi-probe set containing cDNA templates of Bcl-2 family members and GAPDH as an internal control was utilized according to the manufacturer's protocol (BD PharMingen). Briefly, $^{32}$P-labeled riboprobes of defined length were generated using T7 RNA polymerase and 50 ng of the DNA template in the presence of 150 µCi of [α-$^{32}$P]UTP (New England Nuclear). Template DNA was digested with RNase-free DNase, followed by precipitation of labeled RNA. Five µg of total cellular RNA was mixed with 7.5 x $10^5$ cpm of the $^{32}$P-labeled riboprobe in hybridization buffer (40 mM PIPES, 1mM EDTA, 0.4 M NaCl, and 80% formamide) and incubated for 5 min at 90° C followed by 12 h at 56° C. The hybridized RNA duplexes were then treated with RNase A and RNase T1, followed by proteinase K digestion. RNase-resistant RNA duplexes were extracted with phenol and precipitated by the addition of equal volumes of 4 M ammonium acetate and 2 volumes of ethanol. Labeled RNA samples were resolved on 6% urea denaturing gels and visualized by autoradiography and PhosphorImager analysis (Molecular Dynamics). The relative expression of Bcl-X$_L$ was normalized to the signal corresponding to GAPDH expression.

2.5.7. **In vitro kinase assays**

*In vitro* kinase assays were performed on His$_{(6)}$-tagged Lck (residues 62-509) and full-length c-Abl purified from Sf-9 cells, and commercial sources of Lyn, Src (Upstate Biotechnology) and PKC (Calbiochem). Lck, Lyn, Src and Abl activities were measured with an ELISA-based assay. Flat bottom 96-well ELISA plates were incubated with a 200 µg/ml
solution of Poly(Glu,Tyr) 4:1 substrate in phosphate buffered saline (PBS) for 1 hour at 37°C and then washed with PBS containing 0.1% Tween-20 (PBS-T). Inhibitor dilutions were added to the washed plates already containing the appropriate enzyme in kinase assay buffer (250 mM Mopso, pH 6.75, 10 mM MgCl$_2$, 2 mM MnCl$_2$, 2.5 mM DTT, 0.02% BSA, 2 mM Na$_3$VO$_4$, 5% DMSO, 5 µM ATP). After incubation at room temperature for 20 min, plates were washed 3 times with PBS-T and plate-bound phosphotyrosine was detected with an anti-phosphotyrosine-HRP antibody conjugate and subsequent color development with K-Blue reagents (Neogen Corporation). All assays were optimized to use the least amount of enzyme necessary for a reproducible signal-to-noise ratio. Specifically, the amount of each kinase per reaction was as follows: Src, 0.2 ng; Lck, 12 ng; Lyn, 0.1 ng; and Abl, 0.8 ng.

PKC activity was measured with a radioactive kinase assay. Enzyme (100 ng) and substrate peptide were incubated in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 2 mM DTT, 1 mM EGTA, 10 mM ATP, 8 µM peptide, 5% DMSO and [γ-$^{33}$P]ATP. Reactions were stopped with 10 µl 5 mM ATP in 75 mM phosphoric acid. The reaction was spotted on phosphocellulose filters and washed with in 75 mM phosphoric acid. Incorporation of radiolabel was quantified by liquid scintillation counting.
2.6. Acknowledgments

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3. Chapter III

Src Family Tyrosine Kinases Phosphorylate Bcr-Abl in the SH3-SH2 Region and Modulate Transforming Activity

Matthew B. Wilson, Fadi Abdi¹, James Hochrein², John Engen², Anthony Schiavone and Thomas E. Smithgall

Department of Molecular Genetics and Biochemistry, University of Pittsburgh
School of Medicine, Pittsburgh, Pennsylvania 15261

and

¹Applied Biosystems, Framingham, MA 01701
²University of New Mexico, Department of Chemistry, Albuquerque, NM 87131
3.1. **Abstract**

The hallmark of chronic myelogenous leukemia (CML) is the Philadelphia chromosome, which arises from the reciprocal translocation of the *c-abl* proto-oncogene on chromosome 9 and the *bcr* locus on chromosome 22. This translocation results in the expression of a 210 kDa fusion protein (Bcr-Abl) with constitutive tyrosine kinase activity that is responsible for CML pathogenesis. Recently, we demonstrated that selective inhibition of myeloid Src family kinase activity (Hck, Lyn, Fyn, and Fgr) induces growth arrest and apoptosis in Bcr-Abl-transformed cells. These and other data provide compelling evidence that oncogenic transformation by Bcr-Abl requires Src family kinase activity. In the present study, we investigated the biochemical interactions between myeloid Src family members and Bcr-Abl. We found that Hck, Lyn, Fyn and Fgr each bind the kinase domain, C-terminal tail, and SH3/SH2 region of Bcr-Abl. Furthermore, Hck, Lyn and Fyn strongly phosphorylated recombinant, purified Abl SH3-SH2 protein *in vitro*. To identify the phosphorylation sites, the phosphorylated Abl SH3-SH2 protein was analyzed by MALDI-TOF mass spectrometry. Seven phosphorylated tyrosine residues were identified: Y89 and Y134 in the SH3 domain, Y147 in the SH3-SH2 connector, Y158, Y191 and Y204 in the SH2 domain, and Y234 in the SH2-kinase linker. We found that Hck, Lyn and Fyn phosphorylate these sites in full-length Bcr-Abl *in vitro*. Lastly, mutation of all seven residues in the context of full-length Bcr-Abl blocked transformation to cytokine independence of TF-1 myeloid cells. The position of several of these tyrosines in the crystal structure of c-Abl and transformation defect of the Bcr-Abl mutant suggests that phosphorylation by Src kinases may impact Bcr-Abl autoregulation and downstream oncogenic signaling.
3.2. Introduction

The cytogenetic hallmark of chronic myelogenous leukemia is the presence of the Philadelphia Chromosome (Ph\(^1\) or Ph) translocation (89). The Ph chromosome is a shortened 22\(^{nd}\) chromosome that results from a reciprocal translocation between \(c-abl\) on chromosome 9 and \(bcr\) on chromosome 22 [t(9;22)(q34;q11)] (90) [reviewed in (320)]. The Ph translocation results in the expression of Bcr-Abl, a 210 kDa oncogenic fusion protein with constitutive tyrosine kinase activity. The p210 form of Bcr-Abl is present in up to 95 percent of all cases of CML (94), 20-50 percent of cases of adult B-lineage acute lymphoblastic leukemia (ALL) (95) and about 5 percent of cases of acute myeloid leukemia (AML) (96). Considerable data, including in vivo transgene experiments in mice (112) and in vitro studies with hematopoietic cell lines (109-111), implicate Bcr-Abl as the primary etiologic factor in disease onset.

The constitutive activity of Bcr-Abl can result in the phosphorylation and activation of many cellular substrates, initiating a diverse set of signaling pathways affecting the growth and differentiation of hematopoietic cells. Specifically, the Grb-2/Sos guanine nucleotide exchange factor complex can bind to phosphotyrosine 177 in the Bcr region of Bcr-Abl, leading to the activation of Ras (128). Bcr-Abl can also activate Ras via Shc, an adapter protein that couples the receptors for many growth factors and cytokines to the Grb2/Sos complex (212). Bcr-Abl can associate with and phosphorylate the Crk-L adapter (306), stimulate PI-3K/Akt signaling via p85 (248), and activate STAT transcription factors (219, 227). All of these signaling pathways involve components with SH2 and SH3 domains and are dependent upon interactions with phosphotyrosine or proline-rich docking sites, respectively.

Although Bcr-Abl possesses a constitutively active tyrosine kinase domain, recent work suggests that it may initiate signaling by activating other non-receptor tyrosine kinases, including
members of the Fps/Fes (138-141, 234, 321), Janus (JAK; (322) and Src kinase families (231-
234). Danhauser-Riedl and colleagues showed that Bcr-Abl can bind and activate the Src family
members Hck and Lyn in several Ph+ cell lines (231) and that Hck could phosphorylate Bcr-Abl
on Y177 (232), which links Bcr-Abl to the Ras pathway through Grb2 binding as described
above (128). Interestingly, the interaction and activation of Hck did not require Bcr-Abl kinase
activity (232), suggesting Bcr-Abl may activate Src family kinases through displacement of the
autoinhibitory SH3—PPII linker intramolecular interaction (12, 29, 31). In support of this, work
from our group demonstrated that the SH3 and SH2 domains of Hck can bind Bcr-Abl through
kinase independent and kinase dependent mechanisms, respectively (239). Furthermore, we
showed that a kinase defective mutant of Hck blocked transformation of the DAGM myeloid cell
line to cytokine independence by Bcr-Abl (239). Additionally, we showed that a Src family
selective inhibitor was able to induce apoptosis in the Ph+ blastic cell lines, Meg-01 and K562
(323). The growth defect was due to Src kinase inhibition rather than direct inhibition of Bcr-
Abl and resulted in down-regulation of both Stat5 and Erk activation (323). Klejman and
colleagues also found that Bcr-Abl-mediated activation of Stat5b was dependent on Hck (230).
In support of our previous findings, inhibition of Hck in this context resulted in a decrease in
Stat5b activation and down-regulated expression of the Stat5b target proteins, A1 and pim-1
(230).

Currently, it is unknown if Src family kinases transphosphorylate full-length Bcr-Abl and
how tyrosine phosphorylation affects its transforming potential. In the present study, we identify
a consistent mode of Abl binding across several different myeloid expressed Src family kinases
involving the Abl SH3-SH2 region. Unexpectedly, Hck, Lyn and Fyn transphosphorylated Abl in
the SH3-SH2 region on seven different tyrosine residues. Moreover, Hck phosphorylated full-
length Bcr-Abl on a subset of these residues \textit{in vitro}. Mutation of these seven tyrosines in the context of full-length Bcr-Abl significantly diminished its ability to transform TF-1 myeloid cells to cytokine independence but did not significantly alter autophosphorylation or SH3-SH2-dependent binding to signaling partners compared to wild-type Bcr-Abl. These data suggest that Src family kinase-mediated phosphorylation of tyrosine residues located in the Bcr-Abl SH3-SH2 region is critical for the Bcr-Abl transforming signal. The position of these seven tyrosines in the crystal structure of c-Abl suggest that transphosphorylation by Src family kinases may significantly alter c-Abl conformation and activity.

3.3. Results

3.3.1. Myeloid expressed Src family kinases bind Abl in a similar fashion

Previously, we found that Hck can bind the SH3-SH2, kinase, and far C-terminal tail domains of Abl \textit{in vitro} (239). In the present study, we extended these findings to include the other myeloid expressed Src family kinases, Lyn, Fyn and Fgr. Each of these Src family members was coexpressed in Sf-9 insect cells along with a series of Abl GST fusion proteins (Figure 14A). The GST fusion proteins were precipitated and analyzed for bound Src family kinases by immunoblotting. Interestingly, Hck (Figure 14B), Lyn (Figure 14C), Fyn (Figure 14D) and Fgr (not shown) each exhibited a nearly identical pattern of binding to the GST fusion proteins involving the SH3-SH2, kinase, and far C-terminal tail domains of Abl.
Figure 14: Bcr-Abl interacts with Hck, Lyn, & Fyn through common mechanisms

Bcr-Abl GST fusion proteins (A) bind Hck (B), Lyn (C), Fyn (D), and Fgr (not shown). Hck, Lyn, and Fyn were each individually expressed in Sf-9 insect cells along with the GST Abl fusion proteins shown in A. The proteins were precipitated using GSH-agarose beads and bound Src family kinases were visualized via immunoblotting (Bound). The levels of each GST fusion protein captured (GST) and input Src family kinase (Lysate) are also shown. Each binding experiment was repeated at least twice with comparable results. A representative experiment is shown above.
To further characterize the interaction between the Src family kinases and the Abl regulatory region, we examined the contribution of the isolated Abl SH3 and SH2 domains to the binding event. Baculoviruses containing GST fused to the isolated Abl SH3 or SH2 domain were constructed and coexpressed with the various myeloid expressed Src kinases in Sf-9 cells (Figure 15A). As expected, Hck (Figure 15B), Lyn (Figure 15C) and Fyn (Figure 15D) each strongly associated with the SH3-SH2 region of Abl compared to a GST negative control. On the other hand, binding to the individual SH3 or SH2 domains of Abl differed among the Src family members. Hck and Fyn strongly bound the SH2 domain of Abl at levels comparable to that of the combined SH3-SH2 region, but neither bound the isolated SH3 domain (Figure 15B, 2D). In contrast to Hck and Fyn, Lyn associated with both the isolated SH3 and SH2 domains of Abl (Figure 15C). These results indicate that Src family kinases may play overlapping but not identical roles in Bcr-Abl signaling involving the SH3-SH2 region.
Figure 15: Hck, Lyn, & Fyn bind the Bcr-Abl SH3-SH2 region

Bcr-Abl GST fusion proteins of the SH3, SH2 and SH3-SH2 region (A) bind Hck (B), Lyn (C) and Fyn (D). Hck, Lyn, and Fyn were each individually expressed in Sf-9 insect cells along with the GST Abl fusion proteins shown in A. The proteins were precipitated using GSH-agarose beads and bound Src family kinases were visualized via immunoblotting. The experiment was repeated 3 times with comparable results. A representative experiment is shown above.
3.3.2. Myeloid expressed Src kinases transphosphorylate the SH3 and SH2 domains of Abl on multiple tyrosines

We next sought to understand the functional consequence of the interaction between the Src kinases and the Abl SH3 and SH2 domains. Surprisingly, when the western blots from the binding assay in Figure 15 were probed with an anti-phosphotyrosine antibody, the Abl SH3-SH2 region exhibited strong levels of phosphorylation when coexpressed with Hck, Lyn or Fyn (data not shown). To confirm this observation, we expressed the Abl GST-SH3, GST-SH2, GST-SH3-SH2 and a GST control in *E. coli* and purified the protein using glutathione agarose. Equimolar amounts of purified GST or the Abl GST fusion proteins were combined with recombinant Hck, Lyn or Fyn in an *in vitro* kinase assay. Figure 16 shows that Hck, Lyn and Fyn each strongly phosphorylated the Abl GST-SH3-SH2 region. However, there were distinct differences in the patterns of phosphorylation of the isolated Abl SH3 and SH2 domains by the Src kinases. Hck strongly phosphorylated the Abl SH3 domain, but phosphorylation of the SH2 domain was only slightly above background GST levels (Figure 16). Similarly, Lyn also phosphorylated the SH3 domain of Abl above background, but phosphorylation of the SH2 domain was greatly reduced in comparison (Figure 16). In contrast, Fyn phosphorylated both the SH3 and SH2 domains of Abl, but to a much lower extent than the combined SH3-SH2 protein (Figure 16). Interestingly, the pattern of phosphorylation of the isolated Abl SH3 and SH2 domains does not correlate well with the pattern of binding observed in Figure 15. For example, Hck did not coprecipitate with the Abl SH3 domain (Figure 15B), yet Hck strongly phosphorylates this domain (Figure 16) suggesting that the interaction between the Abl SH3 and Hck is transient.
Figure 16: Src family kinases phosphorylate the Abl SH3-SH2 region in vitro

The Abl SH3-SH2-, SH3-, and SH2- GST fusion proteins were purified along with GST from bacterial cells and incubated with purified recombinant Src family kinases in vitro with ATP (500 µM). The protein was then separated by SDS-PAGE and visualized with an anti-phosphotyrosine antibody to detect trans-phosphorylation (top) or an anti-GST to confirm equal amounts of purified Abl protein in each reaction (bottom).
We next identified the sites of Src family kinase-mediated Abl SH3-SH2 phosphorylation using MALDI-TOF mass spectrometry. To eliminate interference from the GST moiety, the SH3-SH2 region was reconstructed with a hexa-histidine tag at the C-terminus (His$_6$-Abl 3+2) instead of GST and the His$_6$-Abl 3+2 construct was expressed in E. coli. The His$_6$-Abl 3+2 was purified using affinity chromatography with a Ni$^{2+}$ chelating column and equimolar amounts of the purified protein were incubated with recombinant Hck, Lyn or Fyn in the presence of ATP (Figure 17A). Consistent with the results with the Abl GST fusion proteins in Figure 16, Hck, Lyn and Fyn transphosphorylated His$_6$-Abl 3+2. The individual tyrosine residues in His$_6$-Abl 3+2 phosphorylated by the Src family kinases were determined by MALDI-TOF MS analysis of tryptic digests of each phosphorylated His$_6$-Abl 3+2 protein. Out of 10 possible tyrosines in the His$_6$-Abl 3+2 protein, seven were phosphorylated above background levels (summarized in Figure 17B). Tyrosine 89 (Y89) in the SH3 domain of Abl exhibited the highest levels of phosphorylation by Hck (98.8%), Lyn (85%) and Fyn (86.5%) compared to the other tyrosine residues. Moreover, Hck selectively phosphorylated a higher percentage of each individual tyrosine compared to Lyn or Fyn. For instance, 60% of the tryptic peptides containing tyrosine 191 (Y191) were phosphorylated by Hck, compared to 6% and 15% by Lyn and Fyn, respectively (Figure 17B). This could explain the stronger overall phosphorylation of His$_6$-Abl 3+2 by Hck compared to Lyn and Fyn (Figure 17A). Tyrosine 112 in the Abl SH3 domain and tyrosines 186 and 193 in the SH2 domain exhibited no phosphorylation by Hck, Lyn or Fyn (data not shown). Figure 17C shows the seven phosphotyrosines mapped onto a molecular model of the recombinant Abl SH3-SH2 protein based on the recently solved c-Abl crystal structure (170). Intriguingly, four tyrosine residues—Y89 and Y134 in the SH3 domain as well as Y158 and Y191 in the SH2 domain—lie along the interface between the SH3-SH2 and the Abl kinase.
domain (Figure 17C). Since c-Abl kinase activity is down-regulated, in part, through a tight
docking of the SH3-SH2 “clamp” onto the distal face of the kinase domain, phosphorylation of
residues along this interface could influence kinase activity (see Discussion).
Figure 17: Src family kinases phosphorylate the Bcr-Abl SH3-SH2 region on multiple tyrosine residues

A) The Abl SH3-SH2 region was engineered to include a C-terminal 6-Histidine repeat moiety (His\textsubscript{6}-Abl 3+2). The construct was expressed in bacteria and purified using metal-chelating chromatography and incubated alone (Con) or with recombinant Hck, Lyn or Fyn and ATP \textit{in vitro}. The phosphorylated His\textsubscript{6}-Abl 3+2 proteins were separated by SDS-PAGE and visualized by immunoblotting with an anti-phosphotyrosine antibody (P-Tyr) or an anti-His antibody to confirm equal amounts of purified protein in each reaction. B) The His\textsubscript{6}-Abl 3+2 proteins from part A were digested to completion with trypsin and analyzed by MALDI-TOF mass spectrometry. Seven tyrosine residues out of a possible 10 exhibited phosphorylation
above background and the extent of phosphorylation with each kinase is shown. C) The seven phosphorylated tyrosines were mapped to the published crystal structure of the SH3-SH2-Linker domain of c-Abl. SH3 residues are shown in red (Y134, Y89), the SH3-SH2 connector residue in gray (Y147), the SH2 residues in turquoise (Y158, Y191, Y204) and the SH2-kinase linker residue in yellow (Y234). The remainder of the structure (kinase domain) is rendered in grey.
3.3.3. **Hck phosphorylates full-length Bcr-Abl in vitro**

We next sought to determine whether the Src family kinases could phosphorylate the Abl SH3-SH2 region within the context of the full-length Bcr-Abl protein. To accomplish this, a baculovirus containing the coding sequence of full-length p210 Bcr-Abl was engineered with a hexa-histidine tag on the C-terminus to facilitate purification. We used the kinase-dead form of Bcr-Abl in which the strictly conserved lysine within the kinase domain is replaced with arginine. The kinase-dead form of Bcr-Abl is unable to undergo autophosphorylation, thus allowing for the characterization of only transphosphorylation by Src family kinases. The kinase-dead His-tagged Bcr-Abl (210 KR-His$_6^6$) was purified and incubated alone or with purified recombinant Hck plus ATP *in vitro* (Figure 18A). As expected, 210 KR-His$_6^6$ incubated with Hck showed a high degree of phosphorylation compared to background levels (Figure 18A). The phosphorylated 210 KR-His$_6^6$ protein was then digested with trypsin and analyzed by liquid chromatography-coupled MALDI-TOF mass spectrometry. Figures 18B-D show the mass spectra from 210 KR-His$_6^6$ incubated alone (top spectrum) or with Hck (bottom spectrum) for the tryptic peptides containing Y134 (Figure 18B), Y147 (Figure 18C) and Y158 (Figure 18D). For each of the three tryptic peptides, incubation with Hck resulted in an 80 Da shift in molecular weight corresponding to the covalent addition of a phosphate group. The identity and phosphorylation state of the Y134, Y147 and Y158 peptides were confirmed via sequential MS/MS analysis (parent ion peptide sequence shown in Figure 18B-D; MS/MS data not shown).
Figure 18: Full-length Bcr-Abl phosphorylation site mapping by LC-MALDI-TOF mass spectrometry

A) Full-length kinase-dead Bcr-Abl with a 6-His tag was expressed in Sf-9 insect cells and purified using a Ni2+ chelating chromatography column. The protein was then incubated with purified recombinant Hck \textit{in vitro} and a portion analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine and anti-Abl antibodies. The remainder of the phosphorylated sample was digested with trypsin and subjected to liquid chromatography-coupled MALDI-TOF mass spectrometry. Representative spectra for the peptides containing Y134 (NGQGWVPSNYITPVNSLEK; B), Y147 (HSWYHGPVSR; C) and Y158 (NAAEYLLSSGINGSFLVR; D) are shown. The top spectrum in each pair shows the peak
corresponding to the mass of the unphosphorylated peptide while the bottom spectrum contains the corresponding phospho-peptide (B-D). The amino acid sequence and phosphorylation state of all six peptides was confirmed in subsequent MS/MS experiments (data not shown).
The results of the MS analysis of the purified full-length 210 KR-His\(_6\) protein are summarized in Table 2. Parent ions corresponding to six out of the seven SH3-SH2 tyrosine phospho-peptides observed with the His\(_6\)-Abl 3+2 construct (Figure 17) were also found in the full-length protein, and three of these were present in sufficient abundance to permit confirmation by MS/MS sequence analysis as described above. A parent ion corresponding to the tryptic phospho-peptide containing Y191 (V\(^\text{HYR}\)) could not be found and low abundance prevented the amino acid sequence confirmation of the three remaining candidate peaks (Y89, Y204 and Y234; Table 2). As an internal control for the system, we surveyed the data set for the phosphorylated tryptic peptides containing known sites of c-Abl and Bcr-Abl transphosphorylation by Src family kinases, including Y245 and Y412 in the Abl portion of the protein and Y177 in the Bcr portion of Bcr-Abl (Table 2). Parent ions of phosphorylated tryptic peptides for Y177 and Y412 were found and the amino acid sequence was confirmed via sequential MS/MS analysis. In contrast, a parent ion for the tryptic peptide containing phosphorylated Y245 could not be located despite strong reactivity of this site with a phosphospecific antibody (see below). Although Y412 has long been considered a site for Bcr-Abl autophosphorylation, these data suggest Y412 may also be a site of transphosphorylation by Hck. Lastly, mass peaks corresponding to the phosphorylated tryptic peptides for Y134, Y147 and Y204 were found in TF-1 myeloid cells stably expressing kinase-dead Bcr-Abl. Unfortunately, the low concentration of purified protein prevented amino acid sequence confirmation of these peptides (data not shown).
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</table>

*Numbering from human Bcr-Abl protein; all other numbering based on c-Abl crystal structure.

Table 2: Hck phosphorylates SH2-SH3 sites in full-length Bcr-Abl

The table summarizes the mass spectrometry data for the tyrosine phosphorylated residues in the Abl SH3 and SH2 domains as well as the previously described Y177 and Y412 autophosphorylation sites. For each tryptic peptide the theoretical mass and the observed mass of a candidate peak ion are given. Three phospho-peptides identified in the Abl SH3-SH2 MS analysis (Y147, Y158, Y134) were also identified in the full-length Bcr-Abl protein (shaded in green) and their identity was confirmed via MS/MS sequence analysis. The intensities of the remaining peaks were too low to permit further analysis. The Y177 and Y412 phospho-peptides were also found in the full-length protein and the sequence was confirmed via MS/MS analysis providing and internal control for the assay.
To confirm and extend the findings that the known sites of autophosphorylation are also sites of Hck transphosphorylation, we used an immunoblotting strategy with phosphospecific antibodies. Specifically, full-length kinase-dead Bcr-Abl p210 was expressed alone or coexpressed in Sf-9 insect cells along with Hck, Lyn or Fyn. Wild-type Bcr-Abl was also expressed alone to measure autophosphorylation. Wild-type and kinase-dead Bcr-Abl proteins were immunoprecipitated and probed with antibodies specific for phospho-Y177, phospho-Y245 and phospho-Y412 (Figure 19). As expected, wild-type Bcr-Abl underwent autophosphorylation on all three sites (Figure 19A). Interestingly, Hck, Lyn and Fyn also phosphorylated Y177, Y245 and Y412 within kinase-dead Bcr-Abl, suggesting that each of these putative autophosphorylation sites may also be transphosphorylated by Src family kinases (Figure 19A).
Figure 19: Myeloid Src kinases phosphorylate full-length Bcr-Abl on linker, loop and Grb2 binding sites

A) Full-length wild-type (Active) and kinase-dead Bcr-Abl were expressed in Sf-9 insect cells alone (Control) or with Hck, Lyn or Fyn. The cells were lysed 48-h post infection and Bcr-Abl was immunoprecipitated using an anti-Abl antibody. The captured protein was then separated by SDS-PAGE and immunoblotted with antibodies specific for the Grb-2 binding site (PY-177), the linker tyrosine (PY-245), the loop tyrosine (PY-412) or Abl (Bcr-Abl). B) The activity of the various Src family kinases was measured by probing the lysate with an antibody specific for the active form of the kinases (P-Src). C) The location of the linker and loop tyrosine residues are shown in the context of the crystal structure of the c-Abl SH3-SH2-kinase domain “core” (170).
3.3.4. Mutation of the seven SH3-SH2 tyrosine phosphorylation sites reduces Bcr-Abl transforming potential

To test the contribution of the seven SH3-SH2 tyrosine residues to Bcr-Abl function in a relevant biological context, we engineered a mutant Bcr-Abl protein containing a tyrosine to phenylalanine substitution at each of the seven phosphorylation sites for Src family kinases identified in Figure 17 (Bcr-Abl 210-7YF). A recombinant retrovirus containing the 210-7YF construct was then used to stably transduce GM-CSF-dependent TF-1 human myeloid cells (TF-1/210-7YF). The transformed phenotype of TF-1/210-7YF cells was compared to uninfected TF-1 cells (TF-1 parental) and TF-1 cells transformed with wild-type Bcr-Abl (TF-1/210-WT) in a soft-agar colony-forming assay. Cells were plated in the presence or absence of GM-CSF and colony formation was quantified 10-14 days later by Giemsa staining followed by colony counting. The stained colonies are shown in Figure 20A and the colony numbers in Figure 7B. As expected, TF-1 parental cells exhibited a moderate level of colony-forming activity in the presence of GM-CSF, but formed virtually no colonies in the absence of GM-CSF (Figure 20B). The TF-1/210-WT cells formed colonies in the presence or absence of GM-CSF, confirming previous data showing wild-type Bcr-Abl transforms various myeloid/monocyte cell lines to cytokine independence (Figure 20B). Interestingly, TF-1/210-7YF cells formed colonies in the presence of GM-CSF, but were unable to do so in the absence of cytokine (Figure 20B). These data suggest that phosphorylation of one or more of the seven tyrosine residues in the SH3-SH2 region is required for the full activity of the Bcr-Abl.

To characterize the TF-1/210-7YF cells at the biochemical level, the cell lysates were probed with antibodies specific for several key Bcr-Abl phosphotyrosines (Figure 20C). Compared to TF-1 cells stably transformed with kinase-dead Bcr-Abl, the TF-1/210-7YF cells
exhibited enhanced overall tyrosine phosphorylation, as well as increased site-specific phosphorylation of Y177, Y245 and Y412 (data not shown). These data suggest that Bcr-Abl 210-7YF is similar to WT Bcr-Abl from a phosphorylation standpoint despite the fact that it is unable to transform TF-1 cells to cytokine independence.
Figure 20: TF-1 210-7YF cells revert back to a cytokine dependent phenotype

A Bcr-Abl mutant was constructed containing tyrosine to phenylalanine (YF) mutations of the seven tyrosine residues phosphorylated by Src family kinases in the Abl SH3-SH2 region \textit{in vitro} (210-7YF). A recombinant 210-7YF retrovirus was used to stably transduce human myeloid GM-CSF-dependent TF-1 cells. Uninfected parental TF-1 cells (p) and TF-1 cells stably transduced with wild-type Bcr-Abl (210-WT) served as negative and positive controls, respectively. A) Cells were plated in triplicate in soft agar in either the presence (+) or absence (-) of GM-CSF. Cells were stained with Giemsa and colony formation was assessed after 10-14 days. B) The results were quantitated using a densitometer and colony counting software. The number of colonies represent the mean of three replicates and error bars indicate the standard
error of the mean. C) Cellular lysates of the various TF-1 cell populations were immunoblotted with a general anti-phosphotyrosine antibody (P-Tyr), three Bcr-Abl phosphospecific antibodies (PY177, PY245 and PY412), anti-Abl (Bcr-Abl) and anti-actin as a loading control.
It is possible that the mutation of the seven tyrosines to phenylalanine caused the SH3-SH2 region of Bcr-Abl to misfold, thus explaining the transformation defect of the TF-1/210-7YF cells. To test this possibility, we created baculoviruses for expression of the wild-type and 7YF Abl SH3-SH2 regions as GST fusion proteins. Hck strongly binds the SH3-SH2 region of Abl [Figure 215B; (239)] and work from Skorski et al. shows that the SH2 domain of Bcr-Abl binds the p85 regulatory subunit of PI-3K (248). Therefore, the association of 3+2-YF with both Hck and p85 compared to that of 3+2-WT provided a useful tool for testing the functionality of the 3+2-7YF domain. The GST 3+2-WT or 3+2-7YF proteins were coexpressed in Sf-9 cells along with either Hck or p85 and association was measured by coprecipitation (Figure 21). The Abl 3+2-7YF protein bound both Hck (Figure 21A) and p85 (Figure 21B) to the same extent as 3+2-WT, strongly suggesting that the mutant SH3-SH2 region folds properly and retains the ability to interact with downstream effectors. Lastly, Hck-mediated tyrosine phosphorylation of 3+2-7YF was reduced to background levels in comparison to 3+2-WT (data not shown). These controls suggest that the effect of the Phe substitutions is due to lack of phosphorylation rather than an interaction defect.
Figure 21: The Bcr-Abl SH3-SH2 7YF mutant retains Hck and p85 binding activity

Hck (A) and the p85 subunit of phosphatidylinositol-3’-kinase (B) were coexpressed with GST, Abl SH3-SH2 wild type (3+2 WT) or SH3-SH2 7YF (3+2 7YF) GST fusion proteins in Sf-9 cells. The GST fusion proteins were captured with GSH-agarose beads and bound Hck or p85 was visualized via immunoblotting. The levels of the captured GST proteins were visualized using an anti-GST antibody (GST) and input levels of Hck or p85 in the cell lysate were probed with antibodies specific for each protein (Lysate).
3.4. Discussion

Previous work from our group demonstrated that Hck can bind the SH3-SH2 region, the kinase domain, and far C-terminal tail of Bcr-Abl (239). Here, we have extended those findings to include the myeloid expressed Src family kinases, Lyn, Fyn and Fgr, and show that they bind Bcr-Abl though a mechanism similar to Hck. Hck, Lyn and Fyn strongly associate with the SH3-SH2 region of Bcr-Abl, but differ in their binding to the isolated SH3 and SH2 domains. Hck and Fyn each show a strong affinity for the SH2 domain but do not bind the SH3 domain. In contrast, Lyn interacts with both the SH3 and SH2 domains. In addition to Src family kinases, several key signaling proteins in CML oncogenesis bind Bcr-Abl through the SH3 and SH2 domains. Activation of the PI-3K pathway requires binding of p85 to the Bcr-Abl SH2 domain (248), while activation of Stat5 is dependent on both an intact SH2 and SH3 domain (220). In the case of Stat5, the SH2-SH3 region of Bcr-Abl does not directly bind Stat5 but instead interacts with Hck, which acts as a signaling intermediate and directly phosphorylates Stat5 (230). Since we show that Lyn and Fyn can also bind the SH3 and SH2 region of Bcr-Abl, it stands to reason they might function like Hck and act as a bridge between Bcr-Abl and Stat5 or other downstream signaling pathways. Indeed, work from our laboratory has shown that Lyn and Fyn can bind, phosphorylate and activate Stat3 in vitro (324). Moreover, inhibition of all the myeloid expressed Src family kinases using a Src-selective inhibitor blocked Stat5 activation and induced apoptosis in CML cells (323). However, the reasons for differential binding to the isolated SH3 and SH2 domains and the individual functional role of Lyn, Fgr and Fyn in Bcr-Abl-mediated signaling remain unclear.

One novel role for the various Src family kinases in Bcr-Abl signaling is demonstrated in the present work. We show for the first time that Hck, Lyn and Fyn transphosphorylate Bcr-Abl
in the SH3-SH2 region on seven different tyrosine residues (Figure 17). Moreover, Hck phosphorylates full-length Bcr-Abl on at least a subset of these residues (Figure 18) and we have identified candidate peptides for several of these sites in TF-1 cells expressing kinase-dead Bcr-Abl (data not shown). Although Bcr-Abl is a constitutively activated tyrosine kinase, Hantschel and Superti-Furga have recently proposed that Bcr-Abl retains at least a partial level of regulation from c-Abl [reviewed in (174)]. Therefore, to understand how phosphorylation of the SH3-SH2 region of Bcr-Abl potentially impacts its signaling and/or kinase activity, the structure and regulation of c-Abl must be considered. The crystal structure of c-Abl shows that the SH3 domain forms an intramolecular interaction with polyproline type-II helix within its own SH2-kinase linker near the N-lobe of the kinase domain, much like Src family kinases. The SH2 domain docks on the distal surface of the C-lobe through a novel switch mechanism governed by myristoyl binding. The eight residues of the SH3-SH2 connector form a rigid connector that dynamically couples the SH3 and SH2 domains forming the basis for a regulatory “clamp” (170).

Like Src family kinases, binding of external substrates or specific mutations within the SH3 or SH2 domains of c-Abl can disrupt the clamp and activate the kinase (164, 169, 173). Several of the tyrosine residues phosphorylated by the Src family kinases, including Y89, Y134, Y158 and Y191 are located along the interface between the regulatory clamp and the distal surface of the c-Abl kinase domain (Figure 17). A key interaction is made by the aromatic ring of Y158 of the SH2 domain, which stacks with the aromatic ring of Y361 within the kinase domain. Furthermore, the hydroxyl group of Y158 forms a hydrogen bond with the backbone carbonyl group of Asn-393 of the kinase domain (170). Interestingly, mutation of Y158 to glutamate in Abl resulted in nearly a four-fold stimulation of kinase activity compared to wild-
type c-Abl (173). Similarly, phosphorylation of one or more of these sites by Src family kinases in cells could potentially disrupt kinase regulation and have a profound effect on c-Abl and, and therefore, Bcr-Abl.

Are the regulatory interactions revealed in the c-Abl crystal structure retained in Bcr-Abl? Recent evidence shows that mutations of numerous residues that affect c-Abl regulation also impact Bcr-Abl kinase activity and sensitivity to imatinib. Azam et al. used a non-biased random mutagenesis screen to uncover mutations in Bcr-Abl that confer imatinib resistance (187) and mapped these residues onto the crystal structure of the autoinhibited form of c-Abl (170). Several residues conferring imatinib resistance are identical to residues that impair c-Abl negative regulation (14, 164, 169, 172, 173). This is fundamentally important since imatinib only binds the inactive form of the Bcr-Abl kinase domain (240). Therefore, mutations that disrupt the negative regulation of c-Abl result in imatinib resistance of Bcr-Abl. This strongly implies that mechanisms governing c-Abl autoinhibition are retained in Bcr-Abl (174). Importantly, several of these activating mutations are located within the SH3 or SH2 domain of Abl or within the kinase domain and form reciprocal contacts with the SH3-SH2 region. Direct confirmation of the hypothesis that Bcr-Abl retains negative regulatory elements from c-Abl comes from the recent work of Smith et al. (192). They showed that the Bcr-Abl kinase inactivation and the leukemogenesis defect caused by the loss of the Bcr-Abl oligomerization domain could be rescued by a point mutation disrupting the intramolecular interaction of SH3 with the SH2-kinase domain linker (192). These data provide direct proof that c-Abl SH3-mediated negative regulation is at least partially retained in Bcr-Abl.

The cumulative analysis of these recent data regarding c-Abl and Bcr-Abl structure and regulation suggests that Bcr-Abl exists in a dynamic equilibrium between the active and inactive
states in cells. Phosphorylation of Y89 and Y134 by Src family kinases could potentially shift this equilibrium towards the active conformation through disruption the SH3/SH2-kinase domain interaction or via stabilization of the active form of the kinase. Indeed, Azam et al. (187) found that mutation of Y89 to D/H/N in Bcr-Abl resulted in imatinib resistance. This strongly suggests that Y89 plays a role in Bcr-Abl autoregulation and that disruption of Y89 by mutation or potentially via phosphorylation as observed here may activate or at least stabilize the active conformation of Bcr-Abl.

In the present study, we found that Hck, Lyn and Fyn phosphorylate full-length Bcr-Abl on Y177 in the Bcr region, and on Y245 and Y412 in the Abl region. Previous work from other groups has shown that Hck can phosphorylate Y177 (232), linking Bcr-Abl to Ras activation downstream (128). Y245 and Y412 in c-Abl have been shown to be sites of both autophosphorylation (171) and transphosphorylation by Src family kinases (179) (178) (discussed below) and phosphorylation has a profound effect on c-Abl kinase activity (171). It is also noteworthy that we showed tyrosine 245 was phosphorylated by Hck, Lyn and Fyn using a phosphospecific antibody but were unable to locate this phospho-peptide using mass spectrometry. This highlights an important point that several technical constraints, including protein concentration, extent of tryptic digestion, recovery of individual peptides from LC, and chemical properties of the peptides themselves can influence the identification of particular phospho-peptides. Therefore, the inability to locate a particular tyrosine phosphorylated tryptic peptide in Bcr-Abl is not conclusive proof of its absence and additional detection methods, such as phosphospecific antibodies are required.

Several studies have linked Src family kinases to c-Abl regulation and signaling and could potentially influence Bcr-Abl kinase activity, downstream oncogenic signaling and
sensitivity to imatinib. Plattner et al. demonstrated that the kinase activity of c-Abl increased 10- to 20-fold by the presence of constitutively activated v-Src in mouse Ba/F3 hematopoietic cells and 10T1/2 fibroblasts (179). The Src kinase-mediated increase in c-Abl kinase activity directly correlated with phosphorylation of c-Abl by Src or Fyn (179). Dorey et al. showed that activated c-Src can phosphorylate Y412 and enhance the ability of Abl to phosphorylate the downstream substrate, c-Jun (178). A similar study by a separate group provided evidence that Src kinase activity was necessary for c-Abl activation in NIH3T3 cells and that activated c-Src phosphorylated c-Abl on both Y412 and Y245 and dual phosphorylation was required for c-Abl function (180). Brasher and Van Etten demonstrated that phosphorylation of tyrosine 245 can enhance kinase activity of c-Abl by potentially disrupting the SH3/SH2-kinase linker interaction and that dual phosphorylation of Y245 and Y412 is necessary for full catalytic activation (171). Lastly, Hck can desensitize Abl to inhibition with imatinib through an activating phosphorylation of Y412 in vitro (240) (181). When taken together with our work, these data predict that Src family kinases may enhance Bcr-Abl kinase activity or stabilize the active conformation of the kinase via transphosphorylation. Whether tyrosine Y245 and Y412 are predominantly sites of auto- or transphosphorylation in a cellular milieu is unknown and may depend on several factors, including the stage of disease and requires further investigation.

To better understand the biological role of the seven SH3-SH2 region tyrosines, we created a Bcr-Abl mutant with a phenylalanine substitution at each of the seven sites and measured its ability to transform cytokine dependent myeloid cells. Mutation of these residues in Bcr-Abl significantly diminished its ability to transform TF-1 cells to cytokine independence but did not significantly alter autophosphorylation or binding to Bcr-Abl SH3-SH2-dependent binding partners compared to wild-type Bcr-Abl. These data suggest that Src family kinase-
mediated phosphorylation of tyrosine residues located in the Bcr-Abl SH3-SH2 region are critical for the transformation potential of Bcr-Abl. As mentioned previously, the position of these seven tyrosines along the interface between the SH3-SH2 regulatory clamp and the Bcr-Abl kinase domain suggests that transphosphorylation by Src family kinases may significantly impact kinase conformation and catalytic activity. Since the regulatory clamp mechanism of c-Abl depends on tight docking of the SH3-SH2 against the distal kinase surface, it stands to reason that disruption of this interaction would enhance or stabilize the active form of the kinase. For instance, it has been established that deletion of the SH3 domain or mutations that abrogate ligand binding can potentiate both c-Abl and Bcr-Abl activity (14, 192). Similarly, phosphorylation of Y245 in the SH2/kinase-linker enhances c-Abl kinase activity by disrupting SH3-mediated negative regulation (171). Therefore, mutation of the tyrosine residues along the interface prevents potentially activating phosphorylation from taking place, shifting the Bcr-Abl equilibrium towards the inactive state.

Two potential caveats of our approach of mutating all seven tyrosine residues are apparent. First, the seven phenylalanine substitutions could alter the folding or stability of the SH3-SH2 region resulting in a loss of function. The data presented in Figures 20 and 8 argue against this possibility. We showed that the mutant SH3-SH2 region still associates with two independent downstream partner proteins, p85 and Hck, similar to that of the wild-type SH3-SH2 region (Figure 20). The full-length Bcr-Abl 210-7YF mutant in TF-1 cells also appears to be biochemically similar to Bcr-Abl wild-type in terms of phosphorylation of Y412, Y245, and Y177 (Figure 21). In contrast, kinase-dead Bcr-Abl exhibits significantly less tyrosine phosphorylation at these sites compared to Bcr-Abl 210-7YF (data not shown). The second caveat is that the individual tyrosine residues may have opposing functions in Bcr-Abl regulation.
and that these effects may be masked by mutation of all seven residues. We can not exclude this possibility and the role of the individual tyrosines will be the subject of future investigations.

3.5. Materials and Methods

3.5.1. Co-precipitation of Abl Domains and Src family kinases in Sf-9 Cells

Sf-9 binding assays were performed as described previously (239). The coding sequences for the human Abl SH3-SH2 region (Gly$^{57}$-Thr$^{224}$) and kinase domain (Tyr$^{215}$-Ile$^{489}$) were amplified by polymerase chain reaction and subcloned into the baculovirus transfer vector pVL-GST (325). The SH3-SH2 region was further subdivided into the SH3 (Gly$^{57}$-Ser$^{126}$) and SH2 (Ser$^{121}$-Thr$^{224}$) domains and subcloned into the pVL-GST vector. The C-terminal region of Abl was similarly amplified as a series of four sequences encoding residues Pro$^{480}$-Gly$^{638}$ (CT1), Arg$^{639}$-Leu$^{813}$ (CT2), Ile$^{801}$-Ala$^{993}$ (CT3), and Gly$^{994}$-Arg$^{1130}$ (CT4), which were subcloned into the same vector. The resulting pVL-GST constructs were used to create recombinant baculoviruses for the expression of these Abl regions as GST fusion proteins. Sf-9 cells (2.5 × 10$^6$) were co-infected with each of the GST-Abl baculoviruses (or a GST baculovirus as a negative control) and Hck, Lyn, Fyn or Fgr baculoviruses. Forty-eight hours post infection, the cells were lysed in 1.0 ml of Hck lysis buffer, and GST fusion proteins were precipitated with glutathione-agarose beads (Sigma; 20 µl of a 50% w/v suspension). The precipitates were washed three times with 1.0 ml of RIPA buffer, and bound proteins were eluted by heating in SDS-PAGE sample buffer. Proteins were resolved on duplicate SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Associated Hck, Lyn and Fyn were visualized by immunoblotting with kinase isoform-specific polyclonal antibodies (Santa Cruz).
The amount of precipitated GST-Abl fusion protein present in each reaction was determined by immunoblotting with anti-GST antibodies (Santa Cruz). Equivalent expression of the Src kinases in each culture was verified by immunoblot analysis of the clarified cell lysates. All immunoreactive bands were visualized with goat-anti-rabbit secondary antibodies conjugated to alkaline phosphatase with NBT-BCIP as colorimetric substrate (Southern Biotechnology Associates).

3.5.2. **Bcr-Abl GST fusion protein purification and in vitro kinase assay**

For the Bcr-Abl domain phosphorylation experiments, *Escherichia coli* strain DH5α was transformed with the GST-fusion vector, pGEX-2T, containing the Bcr-Abl SH3, SH2, SH3-SH2 regions described above. GST fusion protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and recombinant fusion proteins were isolated from clarified cell extracts with glutathione-agarose beads. The protein was dialyzed overnight against 20 mM Hepes pH 7.4 and 1 mM β-mercaptoethanol. The concentration of each protein was determined on Coomassie-stained gels by two-dimensional laser densitometry using bovine serum albumin as a standard. Each purified GST fusion protein or GST alone (380 pmol) was incubated with recombinant purified Hck-YEEI (326), Lyn (Upstate Biotechnology) and Fyn (Upstate Biotechnology) along with 500 µM ATP. The ratio of substrate to kinase was at least 20:1 for each kinase. The reaction was carried out at 30 °C for 30 minutes (50 mM Hepes pH 7.4, 10 mM MgCl₂, 500 µM ATP; 50 µl total reaction volume) and quenched by freezing in a dry ice ethanol bath. An aliquot of the phosphorylated sample was analyzed by SDS-PAGE followed by western blotting with anti-GST (Santa Cruz) or anti-phosphotyrosine (PY99, Santa Cruz) monoclonal antibodies.
3.5.3. **Bcr-Abl His\textsubscript{6} 3+2 and full-length Bcr-Abl kinase-dead protein purification**

The Bcr-Abl SH3-SH2 region (described above) was subcloned into the pET-14b expression vector (Novagen) resulting in the addition of an N-terminal hexa-histididine repeat. BL21 pLysS bacterial cells were transformed with the vector and induced with IPTG. The His\textsubscript{6} 3+2 protein was purified from clarified cell extracts using an HiTrap metal-chelating column charged with Ni\textsuperscript{2+} (Amersham). The purified protein was phosphorylated with recombinant Hck YEEI, Lyn and Fyn as described above.

Full-length kinase-dead Bcr-Abl was purified as follows. A hexa-histididine tag was engineered onto the C-terminus of a kinase-defective mutant of Bcr-Abl using PCR. The resulting construct was subcloned into the pVL-1393 baculovirus transfer vector (BD Biosciences). Sf-9 cells (1 L) were infected with 100 ml of virus and harvested 72 hours later and lysed with a Tris-based lysis buffer (20 mM Tris, pH 8.3, 10% glycerol, 5 mM β-mercaptoethanol, 20 mM imidazole, 500 mM NaCl). The protein was purified, quantitated using a Bio-Rad GS-710 densitometer and BSA protein standards, and approximately 8 pmol was phosphorylated by Hck YEEI *in vitro* as described above prior to SDS-PAGE and MS analysis.

3.5.4. **Mass Spectrometry**

His\textsubscript{6} 3+2 or kinase-dead Bcr-Abl p210 was purified as described and phosphorylated *in vitro* with purified Src kinases. For the full-length Bcr-Abl sample, the phosphorylated protein was digested overnight with trypsin. The tryptic fragments were separated by reverse-phase capillary HPLC, fractions mixed online with MALDI matrix and automatically spotted on a MALDI plate at 20 second intervals with a Probot robot (144 spots total for full-length Bcr-Abl).
MALDI-TOF spectra were collected on all fractions with an Applied Biosystems 4700 proteomics analyzer. A theoretical tryptic digest of each protein was prepared using BioLynx software. Mass peaks for most peptides were identified in the MALDI spectra and the sequence of these peptides was verified with MS/MS sequencing of the parent ion where possible.

3.5.5. **Sf-9 Bcr-Abl/Src family kinase coexpression**

To assess Bcr-Abl phosphorylation, wild-type and kinase-dead full-length Bcr-Abl were coexpressed in Sf-9 cells along with Hck, Lyn or Fyn. Cells were harvested 48 h later, lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), and Bcr-Abl was immunoprecipitated with 4 µg of the c-Abl monoclonal antibody (8E9; BD-Pharmingen) and 25 µl protein G-Sepharose (50:50 w/v slurry; Amersham Biotech) by rotation at 4°C overnight. Beads were then collected and washed three times with ice-cold RIPA buffer, resuspended in a final volume of 25 µl, combined with an equal volume of 2X SDS sample buffer and heated for 5 min at 95°C. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted with antibodies specific for phospho-Y177, phospho-Y245, phospho-Y412 (BioSource), Abl (BD-Pharmingen) and actin (Chemicon; loading control).

3.5.6. **TF-1 cell culture**

The human GM-CSF-dependent myeloid leukemia cell line TF-1 (303) was obtained from the ATCC and grown in RPMI 1640 supplemented with 10% FCS, 50 µg/ml gentamycin, and 1 ng/ml of recombinant human GM-CSF.
3.5.7. Bcr-Abl 210-7YF mutant

A 1121 base-pair oligonucleotide spanning the SH3-SH2 region of Bcr-Abl and containing seven tyrosine to phenylalanine substitutions (Y89F, Y134F, Y147F, Y158F, Y191F, Y204F, Y234F) flanked by HindIII (5′) and BsrGI (3′) restriction sites was commercially synthesized (DNA 2.0). The SH3-SH2 7YF oligonucleotide was subcloned into wild-type Bcr-Abl using the corresponding HindIII and BsrGI sites in Bcr-Abl (nucleotides 2610-3730 in Bcr-Abl). The entire coding region of the mutant Bcr-Abl construct was then inserted en mass into the pMSCV-neo retroviral vector (Clonetech). As a control, Bcr-Abl p210 wild-type was also subcloned into the pMSCV-neo retroviral vector.

3.5.8. Transformation of TF-1 cells with Bcr-Abl Retroviruses

To make retroviral stocks, subconfluent 100 mm dishes of 293T cells were co-transfected with each retroviral vector and an amphotropic packaging vector using a calcium phosphate procedure described elsewhere (36). Viral supernatants were collected at 48-, 72-, and 96-h post transfection, pooled, filtered with 0.45 µm filters, and stored at -80°C. TF-1 cells were stably infected as follows: 10^6 cells were incubated with 5 ml of viral supernatant in the presence of 4 mg/ml polybrene. To increase viral uptake, cells were centrifuged during infection at 2,400 rpm for 3 h at room temperature (239). Cell populations were selected for neomycin resistance with 800 µg/ml G418 for 10-14 days. For the colony forming assays, 2000 cells were plated in soft-agar in the presence or absence of GM-CSF. Ten to 14 days later the colonies were stained with Giemsa and quantitated using densitometry and colony counting software. To analyze Bcr-Abl WT or 7YF protein expression, 10^7 cells were collected by centrifugation, washed once with PBS, and lysed in ice-cold RIPA buffer supplemented with the protease inhibitors aprotinin (25
µg/ml), leupeptin (25 µg/ml) and PMSF (1 mM) and the phosphatase inhibitors NaF (10 mM), and Na$_3$VO$_4$ (1 mM). Clarified lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and blotted with antibodies specific for Abl phospho-Y177, phospho-Y245, phospho-Y412 (BioSource), c-Abl (BD-Pharmingen) or actin (Chemicon) to normalize for loading.
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Chapter IV – General Discussion
Previous work from our laboratory and others has implicated the Src tyrosine kinase family in Bcr-Abl-mediated transformation of myeloid cells (231, 232, 239). Based on these data, I hypothesized that Src family kinases (SFK) are essential for Bcr-Abl-mediated oncogenesis and CML progression.

Work from our lab published in 2000 demonstrated that a kinase-defective mutant of Hck could block cytokine independent outgrowth of Bcr-Abl transformed cells (239). This result suggested for the first time that loss of Src family kinase activity could prevent Bcr-Abl-mediated transformation in a fashion similar to direct inhibition of Bcr-Abl itself and provided a key starting point for my project. To extend these findings, I used two pan-SFK inhibitors, PP2 and A-419259. This strategy had the added advantage of inhibiting all the Src kinases within a myeloid cell and provided a clinical rationale for targeting SFKs in patients with CML. There were a few potential pitfalls with employing this approach. The crucial issue was the need for inhibitors that selectively blocked SFK activation without interfering with Bcr-Abl kinase activity. This is underscored by the fact that amino acid sequence of the kinase domain of SFKs and Bcr-Abl are over 45% homologous. Moreover, several key residues in the ATP binding pocket are strictly conserved between the two kinases (31). The availability of A-419259 for this project work provided me with the necessary reagent to overcome this issue. A-419259 is one of a group of second generation pyrazolo-pyrimidines developed to exhibit improved selectivity towards the Src kinase family (302). This compound was the ideal inhibitor for the project because it exhibited greater than 1000-fold selectivity towards the myeloid expressed SFK members Lck and Lyn compared to c-Abl \textit{in vitro}. Importantly, concentrations of A-419259 responsible for the anti-CML effects also inhibited Src family kinases but did not markedly
affect Bcr-Abl activity, suggesting that Src kinases are the primary target for this drug in CML cells.

It is noteworthy to mention that at the highest concentrations of PP2 and A-419259 tested, reductions in Bcr-Abl and CrkL tyrosine phosphorylation were observed in CML cell lines. At least two explanations are possible for this observation. First, the simplest explanation is that these inhibitors may directly bind and inhibit the Abl kinase domain at higher concentrations. A second, more intriguing possibility is that inhibition of Src kinases may indirectly affect Bcr-Abl phosphotyrosine content and kinase activity. I was particularly excited about exploring the issue of whether SFKs may lie upstream of Bcr-Abl signaling in CML cells. Up to this point, there seemed to be a consensus in the field that SFK were simply another downstream signaling substrate of Bcr-Abl and there was very little evidence that SFK could directly phosphorylate Bcr-Abl. The lone exception was the work of Warmuth et al. that showed Hck could phosphorylate Bcr-Abl on Y177 in the Bcr region, providing a link to downstream Ras signal transduction (232).

A thorough survey of the c-Abl literature revealed that SFKs have been implicated upstream of c-Abl signaling both in vitro and in cells (see Chapter 3 Discussion). To summarize, numerous studies have convincingly shown that various SFKs can phosphorylate c-Abl on Y412 and Y245 and this phosphorylation correlates with a significant increase in c-Abl kinase activity in vitro and in fibroblast and hematopoietic cell lines (171, 179, 180). Of particular clinical significance, Hck can desensitize Abl to inhibition with imatinib by activating the kinase via phosphorylation of Y412 in vitro (181, 240). These data implicated SFKs upstream of c-Abl signaling and strongly suggested that SFKs could also phosphorylate Bcr-Abl, thus modulating
its activity. It was still unclear, however, whether SFKs could phosphorylate full-length Bcr-Abl or on what sites.

The data in Chapter 3 show for the first time that SFKs can indeed phosphorylate Bcr-Abl and that mutation of these residues can block Bcr-Abl-mediated transformation. In summary, I showed for the first time that Hck, Lyn and Fyn transphosphorylated Abl in the SH3-SH2 region on seven different tyrosine residues. Moreover, Hck phosphorylated full-length Bcr-Abl on a subset of these residues \textit{in vitro}. To better understand the biological role of the seven SH3-SH2 region tyrosines, I created a Bcr-Abl mutant with a phenylalanine substitution at each of these sites and measured its ability to transform cytokine dependent myeloid cells. Mutation of these seven tyrosines in the context of full-length Bcr-Abl significantly diminished its ability to transform TF-1 myeloid cells to cytokine independence but did not significantly alter autophosphorylation or binding to Bcr-Abl SH3-SH2-dependent binding partners compared to wild-type Bcr-Abl. These data suggest that Src family kinase-mediated phosphorylation of tyrosine residues located in the Bcr-Abl SH3-SH2 region are critical for the Bcr-Abl transforming signal.

How might phosphorylation of the SH3-SH2 region of Bcr-Abl affect the kinase? The position of these seven tyrosines in the crystal structure of c-Abl suggest that transphosphorylation by Src family kinases may significantly alter c-Abl conformation and activity (170). Briefly, c-Abl is maintained in the off conformation through autoregulation involving tight binding of the SH3-SH2 regulatory clamp to the distal surface of the Abl kinase domain. The clamp closely associates with the N-lobe of the kinase domain through binding of the Abl SH3 domain with a PPII helix in SH2-kinase linker and an N-terminal myristoyl switch that regulates SH2/C-lobe interaction [(170, 173); discussed in detail in Chapter 1].
Several lines of evidence show that Bcr-Abl retains several of the key regulatory features found in c-Abl (187) (192). Bcr-Abl clearly exists in a dynamic equilibrium between the on and off states in cells. Because several of the tyrosines I identified lie along this interface, Src kinase-mediated phosphorylation may disrupt the tight packing of the regulatory clamp and, therefore, stabilize the active open form of the kinase. For example, a key set of interactions is made by the aromatic ring of Y158 of the SH2 domain, which stacks with the aromatic ring of Y361 within the kinase domain. Mutation of Y158 to glutamate in Abl resulted in nearly a four-fold activation of kinase activity compared to wild-type c-Abl (173). Similarly, phosphorylation Y158 in cells by SFKs could potentially disrupt kinase regulation and have a profound effect on c-Abl and, and therefore, Bcr-Abl. Likewise, phosphorylation of Y89 and Y134 by Src family kinases could potentially shift this equilibrium towards the active conformation through disruption of the SH3 interaction with the SH2-kinase linker. In support of this, Azam et al. (187) found that mutation of Y89 to D/H/N in Bcr-Abl resulted in imatinib resistance. This strongly suggests that Y89 plays a role in Bcr-Abl autoregulation and that disruption of Y89 by mutation or potentially via phosphorylation may activate or at least stabilize the active conformation of Bcr-Abl. Brasher and Van Etten demonstrated that phosphorylation of tyrosine 245, which is located in close proximity to both Y89 and Y134, can enhance kinase activity of c-Abl by up to 50% by potentially disrupting the SH3—linker interaction (171). Consequently, it is reasonable to hypothesize that phosphorylation of Y89 and/or Y134 by SFKs might work via the same mechanism leading to stabilization of the active Bcr-Abl kinase domain. This point is particularly relevant from a clinical perspective since imatinib will only bind and inhibit the “off” form of Bcr-Abl (177). Therefore, the prediction is that inhibition of Src kinases would push the dynamic equilibrium of Bcr-Abl activation towards the off conformation, thus
sensitizing Bcr-Abl to imatinib treatment. It is noteworthy that several “dual specificity”
inhibitors to both SFKs and Bcr-Abl have potent nanomolar sensitivity towards CML cells,
compared to the low micromolar sensitivity of imatinib (327) and also inhibit imatinib-resistance
Ph+ cells (283).

The question remains: How does inhibition of SFK affect Bcr-Abl and block downstream
activation of Stat5, Erk and potentially other oncogenic signaling pathways, ultimately resulting
in apoptosis of Ph+ myeloid cells? The cumulative evidence presented in Chapters 2 and 3 of
this dissertation and work from other groups suggests that inhibition of SFKs may both directly
and indirectly block Bcr-Abl-mediated signal transduction. In the direct paradigm, Bcr-Abl
activates SFKs, which then directly phosphorylate downstream effectors such as Stat5.
Therefore, treatment of cells with A-419259 blocks SFK activation, which in turn are then
unable to phosphorylate Stat5, causing apoptosis. Evidence supporting the direct paradigm was
provided by Klejman and colleagues, who in collaboration with our group found that Bcr-Abl-
mediated activation of Stat5b was dependent on Hck (230). Specifically, Bcr-Abl directly
interacts with Hck, which then directly phosphorylates and activates Stat5b. They also found
that inhibition of Hck results in a decrease in Stat5b activation and down-regulates expression of
the Stat5b target proteins, A1 and pim-1 (230), further validating the findings in Chapter 2 in this
dissertation.

Inhibition of SFK could result in the downstream inactivation of Bcr-Abl-mediated
oncogenic signaling through an indirect mechanism. In this model, Bcr-Abl directly activates
SFKs in cells, which in turn phosphorylate Bcr-Abl in the SH3-SH2 region on several key
regulatory tyrosines. Phosphorylation of these residues stabilizes the active form of Bcr-Abl,
pushing the dynamic equilibrium in favor of the on state of the enzyme (discussed above). Src
kinase-mediated phosphorylation of one or more of these tyrosines in Bcr-Abl could also lead to the recruitment of SH2-containing signaling proteins important for maintaining the transformed phenotype. Therefore, inhibition of SFKs using pharmacological inhibitors or dominant-negative mutants indirectly blocks downstream activation of substrates by preventing Src-mediated phosphorylation of Bcr-Abl.

The answer is that SFK most likely play a dual role in both directly and indirectly facilitating Bcr-Abl oncogenic signaling. For instance, Hck could directly bind, phosphorylate and activate Stat5 and also stabilize the active conformation of Bcr-Abl through phosphorylation of one or more tyrosine residues in the SH3-SH2 region or kinase domain. This dual model of signaling in CML where SFKs are both downstream substrate and upstream modulators of Bcr-Abl is especially clinically relevant. In this scenario, inhibition of Src kinases within a CML cell would not only block activation of direct Src target proteins, but also inhibit full activation/stabilization of Bcr-Abl kinase. The challenges associated with elucidating the potential dual role of SFKs in Bcr-Abl-mediated signal transduction and possible future approaches are detailed below.

Dissecting the direct paradigm for SFK in Bcr-Abl signaling is complicated by the fact that both SFK and Bcr-Abl share a wide range of substrates. For example, Bcr-Abl and SFKs have each been shown to phosphorylate Shc (62, 328), Dok (329, 330), and various cytoskeletal proteins (331, 332). If the dual role hypothesis of Src kinases in Bcr-Abl signal transduction is correct, pharmacological inhibitors or dominant-negative mutants will not be useful in differentiating between Src-mediated and Bcr-Abl-mediated pathways since blockage of one pathway will undoubtedly alter the other. One intriguing solution to this problem is provided by the work of Kevan Shokat at the University of California San Francisco. His group has devised a
method for tagging the direct substrates of a given tyrosine kinase by engineering the kinase domain to accept an orthogonal ATP analogue that is not a substrate for any wild-type protein kinase (333). Interestingly, Liu et al. created a chimeric v-Abl protein that contained a space-creating mutation in the N-lobe of the kinase domain, which allowed for the catalytic transfer of phosphate from a unique ATP analogue to Abl-specific peptides (334). The same strategy could be employed to create a Bcr-Abl mutant with a modified ATP-binding cleft that would accept an ATP analogue not recognized by wild-type SFKs. Conversely, a mutant Src family kinase could be engineered and compared to wild-type Bcr-Abl. This strategy would allow us to distinguish between Src-specific or Bcr-Abl-specific substrates.

The question of the indirect role of SFK in downstream Bcr-Abl signaling is further complicated by the possibility that several of the sites I identified as being targets for SFK transphosphorylation could also be sites of Bcr-Abl autophosphorylation. Data in Chapter 3 show that wild-type Bcr-Abl can autophosphorylate on Y177, Y245 and Y412 in vitro, yet these sites are also transphosphorylated by Hck, Lyn and Fyn in the same system using a Bcr-Abl kinase-dead mutant. Moreover, it is unknown if any of the novel SFK sites I identified in the SH3-SH2 region are also sites of autophosphorylation. The stability of wild-type Bcr-Abl was significantly lower than kinase-dead Bcr-Abl preventing analysis of autophosphorylation sites using the same MS approach. This problem will eventually be addressed through the generation of phosphospecific antibodies raised against the sites in the SH3-SH2 region of Bcr-Abl.

The generation of phosphospecific antibodies will also address the related question of whether one or more of the seven tyrosines in the Bcr-Abl SH3-SH2 region are phosphorylated by SFKs in Ph+ myeloid cells. I attempted to address this question by stably transducing TF-1 myeloid cells with a His-tagged kinase-defective mutant of Bcr-Abl. This resulted in a modest
expression of Bcr-Abl that was significantly lower than expression in Sf-9 insect cells as expected. Attempts at purifying His-tagged kinase-dead Bcr-Abl protein from TF-1 cells had limited success and as a result I was not able sequence several candidate peptides. Currently, obtaining a pure, concentrated sample of Bcr-Abl from myeloid cells presents a daunting challenge, but I am confident this problem will be overcome by improvements in purification strategies and enhanced detection sensitivity in next generation mass spectrometers. Similar to the issue of distinguishing between auto- and transphosphorylation, a better approach will be the application of phosphospecific antibodies to the putative SFK phosphorylation sites in myeloid cells. For instance, TF-1 cells stably expressing wild-type or kinase-dead Bcr-Abl could be incubated alone or with SFK-selective inhibitor such as A-419259, and immunoblots of cellular lysates probed with SH3-SH2 region phosphotyrosine-specific antibodies. The prediction would be that sites of SFK transphosphorylation would exhibit diminished reactivity with the various antibodies in the presence of A-419259 compared to the untreated cells.

An addition layer of complexity is that the potential direct and indirect roles played by SFKs in Bcr-Abl signaling described above may also be context dependent. This point is illustrated by work by Hu and colleagues, which recently addressed the question of the requirement of Src family kinases in disease initiation and progression in the murine model of chronic phase CML and B-cell acute lymphocytic leukemia (B-ALL) (238). In this context, Bcr-Abl activated Lyn, Hck, Src, Blk, Fgr and Lck in a mouse pre-B-cell leukemia line and Hck, Lyn and Fgr in primary cells from a mouse with Bcr-Abl-induced B-ALL (158). To test the requirement for these Src kinases in the mouse model, a retrovirus containing Bcr-Abl was used to transduce bone marrow from mice lacking the myeloid expressed Src family kinases, Hck, Lyn and Fgr. The Hck/Lyn/Fgr-deficient marrow cells were still able to induce a CML-like
syndrome but not a B-ALL syndrome in the reconstituted host mice, suggesting that the Src family kinases are necessary for B-ALL but not CML initiation. Moreover, a Src-selective inhibitor was able to impair growth of ALL cells in vitro and in B-ALL mice, but was ineffective in blocking CML progression (158). From these data the authors conclude that Src family kinases are not required to induce CML in mice, but instead are important for B-ALL and potentially progression to CML blast crisis (238).

In contrast to the mouse model of chronic phase of CML, Src family kinases seem to play an important role in the context of human CML progression to blast crisis and imatinib resistance. For example, a recent study by Donato et al. showed Ph+ K562 cells selected for imatinib resistance as well as patients with advanced CML treated with imatinib display increased expression and activity of Lyn (282). Inhibition of Lyn in the imatinib resistant CML cells blocked proliferation, while imatinib had no effect, suggesting an important role for Lyn in both imatinib resistance and CML progression (282). Moreover, a dual inhibitor for Bcr-Abl and the Src family kinases was able to efficiently kill imatinib resistant cells, indicating that imatinib resistant Ph+ cells may rely on Src family kinases for disease maintenance (283).

In summary, data presented in this dissertation show a necessary role in Bcr-Abl-mediated oncogenic signaling and validate the myeloid expressed SFKs as alternative targets for CML drug therapy, particularly in patients shown to be refractory to treatment with imatinib. Treatment of CML patients, especially those in blast crisis, with a combination of Src and Bcr-Abl inhibitors could provide a dramatic therapeutic benefit by blocking activation of both SFKs and Bcr-Abl as well as altering the activation state of Bcr-Abl. Furthermore, like combinational therapy, the rational design of compounds that have dual specificity for both kinases could also provide similar benefit and would potentially prevent drug resistance and subsequent disease
relapse. Several dual specificity reagents have recently been reported and have proven to be very potent inhibitors of CML cell growth (283, 327). Future work in this project should focus on dissecting both the direct and indirect roles of SFKs in downstream Bcr-Abl signaling as well as the validation and extension of these data through alternative strategies, including phosphospecific antibodies and ATP analogue protein engineering. The observation that Bcr-Abl 210-7YF was unable to induce TF-1 cytokine independent growth is certainly intriguing, but the question of how each individual tyrosine might contribute to this phenotype remains unanswered. Finally, the work presented expands the current knowledge regarding the role of Src family kinases in Bcr-Abl signaling and CML progression. The continuation of this project is essential for a complete understanding of the molecular basis for CML disease initiation and progression as well as improvement of the prognosis of patients, particularly those in terminal blast crisis.
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