MECHANISMS OF BCR-ABL- MEDIATED ONCOGENESIS

by

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Abstract

Chronic myelogenous leukemia (CML) is a disease of the hematopoietic stem cell. Its genetic hallmark, the Philadelphia chromosome, is the result of a reciprocal translocation between chromosomes 9 and 22. This translocation results in the fusion of c-Bcr to c-Abl sequences, creating the hybrid fusion gene Bcr-Abl. The Bcr-Abl gene encodes a cytoplasmic tyrosine kinase with significantly elevated activity that is necessary and sufficient for transformation of hematopoietic and nonhematopoietic cell lines. Investigations into its molecular mechanism have revealed that many cytoskeletal and signaling proteins are constitutively tyrosine phosphorylated in Bcr-Abl-expressing cells and that some of these proteins form a complex with Bcr-Abl. Moreover, numerous signaling pathways are inappropriately activated. Clinically, CML is characterized by an expansion of the myeloid compartment resulting in the accumulation of myeloid precursors in the bloodstream. These combined clinical and molecular findings have led to speculation that defects may exist at the level of the cytoskeleton and thus contribute to the premature release of CML progenitors from the bone marrow. To investigate the role of two, activated, signaling pathways in the morphological defects associated with Bcr-Abl-mediated transformation, we examined the changes in the cytoarchitecture following inhibition of these pathways in a fibroblast model. Our findings suggest that inhibition of Abl kinase but not PI3-kinase can significantly reverse the cytoskeletal abnormalities

inherent in Bcr-Abl-expressing cells, as well as restore normal adhesive properties. Thus, activated Abl kinase appears to play a pivotal role in regulating the cytoskeleton in these cells. To broaden our understanding of other signaling pathways important in the pathogenesis of CML, several other signaling components previously identified to interact with Bcr-Abl were examined. In a comparative analysis of the adaptor proteins Grb2 and Grap, we demonstrate direct binding of these proteins to a similar site on Bcr-Abl (Y177). However, Grb2 displayed a 2-fold higher affinity for Bcr-Abl than Grap. Thus, Grb2 probably serves a more physiologically relevant function in Bcr-Abl-mediated transformation through its ability to activate Ras. Finally, investigation into the role of the proto-oncogene c-Cbl in Bcr-Abl-mediated transformation was examined. Previous work in our lab had demonstrated that tyrosine phosphorylated Cbl could bind directly to Bcr-Abl. In the present study, we found that the initial interaction of Cbl with Bcr-Abl requires the presence of Grb2. These findings support a model whereby Grb2 is the major protein that mediates an initial interaction between Cbl and Bcr-Abl. Following this initial interaction, Cbl can become tyrosine phosphorylated, interact with Bcr-Abl directly, and engage in subsequent signaling events required for Bcr-Abl function.

Introduction

Chronic Myelogenous Leukemia

In 1845 a Scottish physiologist named John Hughes Bennett reported the first case of Chronic Myelogenous Leukemia (CML) in an article entitled: 'Case of hypertropy of the spleen and liver in which death took place from suppuration of the blood' [Bennett 1845]. The following year, in a classic paper, the famous German pathologist Rudolf Virchow ascribed a similar clinical case of splenic enlargement and "weisses blut" (white blood) not to a suppurative (infectious) process but rather to a novel disease entity [Virchow 1846]. Later, Virchow would coin the word leukemia for this disease.

Despite Virchow's efforts to define CML as a disease of the blood, as recently as the 1940's the theory that CML may be caused by a virus or other infectious agent was still published in a leading textbook of medicine [Clarkson et al., 1997]. Finally, in 1960, the underlying genetic basis of CML was revealed in a landmark study by Nowell and Hungerford at the University of Pennsylvania [Nowell and Hungerford 1960]. These researchers described the presence of an abnormally small chromosome in the CML bone marrow, one in which the long arm was missing. This minute chromosome, designated the Philadelphia chromosome, was significant for it was the first example of a specific cytogenetic abnormality associated with a human cancer. Its identification

paved the way not only to diagnose CML microscopically, but to verify the somatic mutation theory of cancer proposed earlier in the century [Boveri 1914].

Subsequent to Nowell and Hungerford's pivotal discovery was the seminal observation made by Rowley. In 1973, she demonstrated that the small size of the Philadelphia chromosome was not because a portion of it had been deleted but rather a chromosomal translocation had occurred: a portion of chromosome 22 was translocated to the long arm of chromosome 9 (t9;22) [Rowley 1973].

While it had been assumed that the translocation was reciprocal, it was not until the 1980's that the theory of a balanced translocation between chromosomes 9 and 22 was verified along with molecular details of what genes were involved. During this period, it was shown that exons 2-11 of the cytoplasmic tyrosine kinase c-abl gene are transposed from their normal location on chromosome 9 (q34.1) to the major breakpoint cluster region (M-bcr) of the c-Bcr gene on chromosome 22 (q11.1). [Bartram et al., 1983; Prakash and Yunis 1984; Groffen et al., 1984]. Whereas the breakpoint in the c-abl gene can occur anywhere within a 300-kb segment at its 5'end (in general, there are three breakpoints) the breakpoint locations within the c-Bcr gene occur either between exons 2 or 3 or between exons 3 and 4 [Melo 1996] (Figure 1). Thus, the result of the reciprocal translocation is the formation of a Bcr-Abl fusion gene on chromosome 22 and an Abl-Bcr fusion gene on chromosome 9.

The Bcr-Abl fusion gene transcribes a novel 8.5 kb mRNA that in turn encodes a 210 kDa fusion protein with elevated tyrosine kinase activity [Shtivelman et al., 1985; Konopka et al., 1984]. A related Bcr-Abl fusion gene with a different breakpoint is found in cases of a more aggressive leukemia, acute lymphoid leukemia (ALL). In this case, the breakpoint occurs upstream on chromosome 22 within the minor breakpoint cluster region (m-bcr) and retains only exon 1 of c-Bcr sequences that fuses with exons 2-11 of c-Abl [Chissoe et al., 1995] (Figure 1). The result is a smaller Bcr-Abl transcript that generates a 185 kDa protein and possesses even greater tyrosine kinase activity than p210Bcr-Abl [Lugo et al., 1990].

While its etiology still remains unknown, CML is thought to be an acquired defect since the Philadelphia chromosome is not found in all cells of the body and no studies have proven a genetic link. The only known environmental risk factor for CML is large doses of ionizing radiation. This was documented in epidemiological studies of survivors of the Hiroshima and Nagasaki atomic bomb explosions where the incidence of CML was significantly increased [Lange et al., 1954].

Clinically, CML is recognized as a malignant disease of the primitive hematopoietic stem cell and is characterized by an abnormal expansion of myeloid (primarily granulocytic) progenitors and precursors in the blood and bone marrow [Verfaillie 1998]. The Philadelphia chromosome is found in more than 90% of CML patients. CML accounts for approximately 15 percent of leukemias in adults and has an incidence of 1 to 2 cases per 100,000 people per year [Faderl et al., 1999]. While the median age of

diagnosis is 45 to 55 years of age, 12 to 30 percent of the patients are 60 years or older.

Most individuals present in the "chronic phase" of the disease when there is an increase in circulating mature granulocytes that are terminally differentiated and are generally, functional [Champlin and Golde 1985]. Clinical symptoms such as fatigue, weight loss, anemia, splenomegaly and leukocytosis begin to appear [Epstein 1999]. For reasons not well understood within three to five years the disease progresses to "blast crisis." During this phase, defined by the presence of 30% or more immature blast cells in the bone marrow or peripheral blood, additional cytogenetic abnormalites develop such as trisomies and an extra Philadelphia chromosome. In contrast to the chronic phase, these immature blast cells have lost the ability to terminally differentiate. The prognosis of patients is poor since the blast cells that predominate in the blood are unable to carry out their normal functions such as transporting oxygen, killing microorganisms, and maintaining hemostasis [Clarkson and Strife 1993].

While the underlying genetic abnormality in CML, the Bcr-Abl oncogene, is well established, the cause of granulocyte expansion is not clear. Several theories have been proposed. One model focuses on the adhesive defects of CML hematopoietic progenitors [Verfaillie 1992]. Normally, strict regulation of proliferation and differentiation of hematopoietic progenitors occurs in close contact within the bone marrow microenvironment [Verfaillie 1992]. This process involves interactions of the progenitors with stromal cells, immobilized growth regulating cytokines, and extracellular matrix

(ECM) components such as fibronectin. Consequently, the adhesion of normal progenitors to these components serves not only to anchor them until they are functionally mature but to inhibit their proliferation as well.

In contrast, CML progenitors have been shown to display defective adhesive interactions with the bone marrow stroma and fibronectin *in vitro* [Gordon et al., 1987; Eaves et al., 1986; Verfaillie 1992]. Based on these *in vitro* data it has been speculated that reduced adhesion of primitive cells in CML patients might facilitate their premature release from the bone marrow, and in the absence of regulatory and developmental cues from the bone marrow stroma, continue to grow unrestrained in the blood. How exactly the Bcr-Abl oncoprotein might cause reduced adhesion of progenitors is unknown; however, two potential mechanisms include changes in the cytoarchitecture or the reduced affinity of the adhesion receptors on Philadelphia chromosome+ (Bcr-Abl-expressing) cells.

An alternative model that attempts to provide a comprehensive explanation for the initial phase of CML is the "discordant maturation" model [Clarkson and Strife 1993]. According to this theory, the elevated tyrosine kinase activity of Bcr-Abl profoundly changes the pattern of protein phosphorylation within the cell at a particularly susceptible stage of development. Thus, the normal regulatory pathways of proliferation and maturation of stem cells is perturbed, leading to altered hematopoiesis. This model suggests that Bcr-Abl expression leads to an increase in the number of cell divisions and inhibition of programmed cell death (apoptosis) [Clarkson and Strife 1993]. A

deregulated apoptotic signaling pathway in Bcr-Abl expressing cells could partially account for the abnormal expansion of the myeloid compartment.

While the mechanisms to explain the early stage of CML may differ, both the discordant maturation model and the pathological adhesion model directly attribute the cancerous properties of this disease to the oncoprotein Bcr-Abl. As previously mentioned, the Bcr-Abl oncoprotein is the result of the fusion of portions of the c-Bcr and the c-Abl genes. The c-Abl gene is an example of a proto-oncogene, a cellular gene that when mutated is converted into an oncogene and, acquires transforming abilities (as defined by its ability to promote cancer-like properties in cultured cells or cancer in animals). Thus while c-Abl's normal activity as a tyrosine kinase (transfers phosphate from ATP to tyrosine residues on other proteins) is usually tightly regulated, when Bcr sequences are fused to it, this activity becomes unregulated. The result is an abundance of tyrosine phosphorylated proteins within the cell. These events profoundly disrupt normal signal transduction pathways by the constitutive activation of signaling molecules and the creation of large multimolecular complexes. Examples of signaling pathways that have been identified as dysregulated in Bcr-Abl-expressing cells are the Ras, mitogen-activated protein (MAP) kinase, PI3-kinase and JAK/STAT pathways [Reviewed in Raitano et al., 1997]. Which of these pathways is ultimately the most critical is unknown; it is conceivable that each may contribute to different aspects of leukemogenesis as posited in the three models.

The following sections will examine the normal cellular functions of the c-Bcr and c-Abl proteins and review what is presently known about the major oncogenic versions of c-Abl: v-Abl and Bcr-Abl. This will provide a background for the discussion of the adaptor/signaling proteins that interact with Bcr-Abl and which may play a pivotal role in mediating downstream signals from Bcr-Abl: Grb2, Grap, Cbl, and PI3-kinase. Finally, the consequences of Bcr-Abl's association with the cytoskeleton and the implications for CML will be evaluated.

c-Bcr

First identified through its association with the Philadelphia chromosome, the Breakpoint cluster region (Bcr) gene encodes a cytoplasmic protein of approximately 160kDa [Nowell et al., 1960; Priest et al., 1980]. Bcr is widely expressed in most tissues and during embryogenesis [Fioretos et al., 1995]. Structural features of Bcr suggest a role for this protein in the regulation of the cytoskeleton and cellular signaling (Figure 2).

At the far N-terminus of Bcr are 63 amino acids which comprise an oligomerization or coiled-coil domain; these sequences allow Bcr to form homo-dimers, trimers and tetramers [McWhirter and Wang 1991]. In the central part of the molecule is a Dbl-homology domain, which encodes a guanine nucleotide exchange factor (GEF) activity for the small GTP-binding proteins Rho, Rac and CDC 42 (Cerione and Zheng, 1996). If this domain were active, formation of stress fibers, focal adhesions, and membrane ruffling could presumably result, though experiments to address this question have not

been reported. This domain is present in p210Bcr-Abl but absent in p185 Bcr-Abl, suggesting it is dispensable for transformation.

At the far C-terminus of Bcr is a GTPase-activating domain for the small GTP binding protein Rac; this sequence is not present in either p185Bcr-Abl or p210Bcr-Abl [Ridley and Ellis 1993]. Thus, Bcr has the potential to regulate Rac-related cytoskeletal functions such as membrane ruffling and pinocytosis or the ligand-mediated oxidative burst in neutrophils [Deikmann et al. 1994]. Indeed, while Bcr null mice are generally healthy and display no obvious defects in hematopoietic cell development, neutrophils from these mice exhibit a pronounced increase in oxidative burst; exposure to endotoxin in these mice is fatal.

Recent work on the signaling capabilities of c-Bcr has revealed a novel serine/threonine kinase domain in the first exon of Bcr [Maru and Witte et al., 1991]. Upon its tyrosine phosphorylation at positions 328 and 360 the transkinase activity of Bcr is severely reduced; however, its autophosphorylation remains unaffected [Liu et al., 1996; Wu et al., 1998]. In Bcr-Abl-expressing cells these tyrosines are phosphorylated, and thus the serine/threonine kinase activity of Bcr is downregulated. While few substrates of Bcr have been identified, a Bcr-associated protein 1 (Bap-1) which is phosphorylated by Bcr and binds the serine/threonine kinase domain has been cloned [Reuther et al., 1994]. This protein, a member of the 14-3-3 family, has been implicated in cell cycle regulation and may function to regulate Bcr.

Finally, both Bcr-Abl and c-Bcr can be phosphorylated at position Y177 by activated Abl kinase, and like Bcr-Abl phosphorylated at Y177, wild-type Bcr can bind the adaptor protein Grb2 [Pendergast et al., 1993; Puil et al., 1994]. These findings raise the possibility that Bcr could function to activate Ras, though experiments have not been conducted to examine this.

The c-Abl Protein Tyrosine Kinase

c-Abl is a member of the non receptor tyrosine kinase family and is the normal cellular homologue of v-Abl. It is a multidomain protein of approximately 145 kDa that is expressed in all tissues [Westin et al., 1982]. Within its structure are a multitude of signal transduction modules: an SH1 domain (tyrosine kinase); an SH2 domain which binds phosphotyrosine residues; an SH3 domain which binds proline-rich sequences; a nuclear localization signal; a DNA-binding domain; and an actin-binding domain (Figure 3).

Abl not only possesses the ability to interact with a variety of proteins but it can localize either to the nucleus or to the cytoplasm. Two isoforms of human c-Abl have been identified which differ in their N-terminus sequence and subcellular localization. Isoform Ia is cytoplasmic and does not contain any post-translational modifications. In contrast, isoform Ib is targeted to the nucleus and is myristoylated on an N-terminus glycine [Ben-Neriah et al., 1986; Shtivelman et al., 1986].

Abl's overall biological function has been examined through targeted disruptions of the c-Abl gene in mice. Two independent groups examined the phenotype of c-Abl knock-out mice and reported normal fetal development but high neonatal mortality rates, suggesting that a functionally redundant gene might compensate for the c-Abl deletion [Schwartzberg et al., 1991; Tybulewicz et al., 1991]. (Indeed, a close relative of c-Abl, the abl-related gene, arg, has been cloned but not extensively studied [Kruh et al., 1990].) Surviving animals display pleiotropic defects including increased susceptibility to infections (attributed to a B-cell lymphopenia), reduced fertility, and developmental abnormalities in the spleen, cranium and eye. Thus, while c-Abl's role in post-fetal development is clearly important, delineating what its normal physiological function has been a difficult task.

In contrast to its oncogenic variants Bcr-Abl and v-Abl, the basal *in vivo* tyrosine kinase activity of c-Abl is low [Pendergast et al., 1991]. The following section highlights recent findings on how the processes of integrin-mediated adhesion, cell cycle progression to S phase, and DNA damage, can enhance c-Abl kinase activity, thus providing a better understanding of its overall function within the cell [Raitano et al., 1997].

When fibroblasts are plated on fibronectin, a nuclear to cytoplasmic shuttling of c-Abl in response to adhesion can be observed by confocal immunofluorescence microscopy and cell fractionation experiments [Lewis et al., 1996]. The levels of nuclear c-Abl diminish, while at the same time a transient co-localization of c-Abl with cytoplasmic focal adhesion proteins is observed. Moreover, this adherence induced redistribution of

c-Abl from the nucleus to the cytoplasm correlates with an increase in Abl kinase activity as assessed by an *in vitro* kinase assay. Detachment of cells from the extracellular matrix reduces this activity three to five fold. Furthermore, nuclear to cytoplasmic shuttling of c-Abl is dependent on a functional nuclear export signal (NES) and it has been shown that cells treated with leptomycin B, an inhibitor of nuclear export, accumulate nuclear c-Abl [Taagepera et al., 1998]. While these findings are an interesting phenomenon, the significance of c-Abl shuttling, and whether for example Abl is shuttling alone, or with other proteins, remains to be determined.

Early studies on the function of c-Abl in fibroblasts concluded that c-Abl was a negative regulator of growth since its overexpression caused growth inhibition [Sawyers et al., 1994]. However, more recent reports suggest c-Abl is critical for cell growth since exposure of primary bone-marrow-derived CD34+ cells with c-Abl antisense oligomers reduced the proportion of cells in S phase [Rosti et al., 1995]. While these different findings may be attributable to different cell types or over-expression vs. antagonism of expression, c-Abl's kinase activity has been shown to increase during transition to S phase [Welch et al., 1995]. Interestingly, this increase correlates with the dissociation of c-Abl and the growth inhibitory protein, retinoblastoma (Rb) [Wong et al., 1995]. It has been proposed that Rb's binding may serve to regulate the kinase activity of c-Abl in G1; Rb binds to the ATP-binding lobe in the SH1 (kinase) domain of c-Abl. Upon Rb's phosphorylation in S phase by Cdk-cyclin complexes, Rb and c-Abl dissociate and c-Abl becomes activated [Welch 1993]. A mutant c-Abl, unable to bind Rb, is not cell cycle regulated. One of Abl's substrates in the nucleus is the carboxy-terminus repeated

domain (CTD) of RNA polymerase II [Duyster et al., 1995]. This finding, and the fact that c-Abl has a DNA binding domain, suggests that c-Abl may function to modulate gene transcription and act as a transcriptional coactivator [Raitano et al., 1997].

Upon exposure to some forms of DNA damage, c-Abl kinase activity has been shown to increase. Because cells normally die under these circumstances, these findings suggest a possible role for c-Abl in the induction of apoptosis (programmed cell death)[Kharbanda et al., 1995; Liu et al., 1996]. Indeed, in response to radiation or chemotherapy, an upstream kinase, the ataxia-telangiectasia protein (ATM), has been demonstrated to specifically activate c-Abl by phosphorylating it on serine residues [Baskaran et al., 1997; Shafman et al., 1997; Yuan et al., 1996]. Phosphorylated c-Abl then binds the tumor suppressor p53, thereby enhancing its transcriptional activity. However, despite its association with c-Abl, p53 has never been demonstrated to be directly phosphorylated by c-Abl [Goga et al., 1995; Banin et al., 1998; Canman et al., 1998].

Until recently, it was believed that p53 was the primary proapoptotic mechanism available to the cell. In the past several years, relatives of p53 that could also induce apoptosis in response to DNA damage were identified. One protein, p73, was recently shown to be the direct substrate of activated c-Abl when cells are treated with either cisplatin or γ irradiation [Gong et al., 1999; Agami et al., 1999; Yuan et al., 1999]. Proof of substrate specificity was demonstrated in c-Abl-deficient cells; phosphorylation of p73 did not occur [Gong et al.,1999]. More convincingly, activation of p73 and protection

from apoptosis was shown to require active Abl kinase. From these experiments, a novel pro-apoptotic pathway requiring c-Abl has emerged.

Oncogenic forms of c-Abl: v-Abl

The first oncogenic variant of c-Abl identified was v-Abl, a cytoplasmic phosphoprotein of approximately p160 kDa (Figure 4). This protein, a chimera of viral and cellular sequences, contains myristoylated gag polyprotein sequences at its N-terminus fused to non-M-MuLV sequences [Witte et al., 1978; Reynolds et al., 1978]. The non-viral sequences are derived from the cellular gene c-Abl with a deletion at the amino terminus including the SH3 domain; thus, v-Abl retains the kinase domain of c-Abl and has protein tyrosine kinase activity. However, relative to the low but detectable protein tyrosine kinase activity of c-Abl, v-Abl is characterized by its high kinase activity. [Prywes et al., 1985].

The oncogene v-Abl and its proto-oncogene c-Abl were discovered through studies of RNA tumor viruses that caused leukemia in mice. The Abelson murine leukemia virus (A-MuLV) was originally isolated in 1970 after inoculating a glucocorticosteroid-treated BALB/C mouse with Moloney murine leukemia virus (M-MuLV) [Abelson et al., 1970]. While generally mice infected with M-MuLV developed T-cell leukemia, one mouse developed a distinctive pathology, a lymphosarcoma, which did not involve the thymus and developed quite rapidly. Extracts of the tumor from this mouse were passaged into

other mice, which, after a short latent period, successfully recapitulated the phenotype seen in the original mouse.

At the time progress towards understanding this novel virus was limited to the characterization of the tumors it caused in mice. Then, in 1975, two researchers demonstrated that A-MuLV could morphologically transform an immortalized mouse embryo fibrobast cell line, NIH3T3 [Scher and Siegler 1975]. Significantly, another group was developing an *in vitro* lymphoid transformation system for studying Ab-MuLV, and, eventually, cell lines from Abelson tumors were isolated and plated directly *in vitro* [Sklar et al., 1974]. Thus, two new assay systems were made available that could be used as tools to study the virus.

The search and eventual discovery of *in vitro* kinase activity associated with Ab-MuLV was prompted by reports that immunoprecipitates from the avian Rous sarcoma virus (RSV), contained protein kinase activity [Collett et al., 1978; Witte et al., 1979]. This paved the way to identify specific sequences from mutant or truncated Ab-MuLV proteins which were required for transformation of fibroblasts and which retained *in vitro kinase* activity [van de Ven et al., 1980]. Interestingly, and in contrast to the RSV protein, an Ab-MuLV encoded protein was shown to phosphorylate itself rather than the RSV substrate, immunoglobulin heavy chain [Rosenberg 1988].

The subsequent molecular cloning of the Abelson viral genome in 1980 was a milestone, for it provided the opportunity to decipher cellular from viral sequences [Goff

et al., 1980]. Using unique regions from subcloned restriction fragments of the Ab-MuLV, a set of genomic fragments containing the exons of the c-Abl gene homologous to the viral sequences were defined [Wang et al., 1984]. Homologous c-Abl sequences were quickly identified in other species from nematode to human [Goddard et al., 1986; Shtivelman et al., 1986].

Identification of transformation-essential sequences was facilitated by the *in vitro* assay established earlier in the NIH3T3 cells. Transformation was demonstrated to require tyrosine kinase activity, which was mapped to a 50-60 kDa segment in the central portion of a p120 v-Abl gene product [Rosenberg and Witte 1988]. Unequivocal proof that this sequence of v-Abl was a tyrosine kinase came when it was cloned into E. coli and used in expression studies to demonstrate the generation of tyrosine kinase products [Wang et al., 1985].

Transformation of cells with A-MuLV or v-Abl is accompanied by a significant increase in proteins that are tyrosine phosphorylated. In addition to the Abl kinase, the Abl SH2 domain is also essential for fibroblast transformation; mutagenesis of this domain abrogates transformation [Rosenberg and Witte 1988; Afar et al., 1994; Pendergast et al., 1993]. This suggests not only that binding of proteins to the SH2 domain is important in the uncontrolled growth of v-Abl but that several domains can contribute to transformation.

Expression of v-Abl in hematopoietic cells has demonstrated that v-Abl can induce growth in the absence of growth factors [Cook et al., 1985; Pierce et al., 1985]. In fibroblast cells transformed by v-Abl have decreased requirements for serum and display anchorage independent growth properties [Rees and Goff 1988; Renshaw et al., 1992]. These findings implicate v-Abl in activating a mitogenic pathway. One mechanism v-Abl may employ to accomplish factor-independent myeloid cell proliferation is binding and activating the transcription factor, E2F-1 [Birchenall-Roberts et al., 1997]. Through binding of E2F-1 it has been proposed that v-Abl may regulate E2F-1 transcriptional activity and thus, possibly the cell cycle.

Studies of the cellular and murine viral forms of Abl have determined two major structural alterations associated with the activation of the gene to its oncogenic form: a myristylation signal contributed by the viral gag sequences and the deletion of the SH3 domain of Abl [Rosenberg and Witte 1988]. N terminus myristyl group permits association with the membrane (Bcr-Abl does not contain such a sequence). Though not required to transform hematopoetic cells, this post-translational modification is essential for the efficient transformation by v-Abl of fibroblasts possibly by targeting PI3-kinase [Wang 1993; Varticovski et al., 1991]. Deletion of the entire SH3 domain or point mutations within this domain of c-Abl have been shown to activate the oncogenic potential of c-Abl [Mayer and Baltimore 1994].

Oncogenic forms of c-Abl; Bcr-Abl

As mentioned previously, the other major oncogenic form of c-Abl is Bcr-Abl. Associated with the human diseases CML and ALL the Bcr-Abl fusion gene encodes a 210 kDa and p185 kDa tyrosine kinase respectively. Both are constitutively active and both can transform hematopoietic and nonhematopoietic cell lines (Figure 5).

Structural and functional similarities exist between the v-Abl and Bcr-Abl oncogenes, yet there remain important differences as well. For instance, both v-Abl and Bcr-Abl are fusion proteins. However, rather than fusion of N-terminus viral sequences to c-Abl, Bcr-Abl contains N-terminus cellular Bcr sequences fused to c-Abl sequences. As a result of the Bcr-Abl fusion, the Rac-GAP sequence of c-Bcr and the myristoylation site in c-Abl are both removed. In addition, the SH3 domain is retained in Bcr-Abl but not in v-Abl. In p185Bcr-Abl the Dbl sequence of Bcr is removed as well. One novel feature of Bcr-Abl is its ability to oligomerize, unlike either c-Abl or v-Abl [McWhirter et al., 1993]. Moreover, while c-Abl may reside in either the nucleus or cytoplasm, Bcr-Abl and v-Abl, are exclusively cytoplasmic [Van Etten et al., 1989].

While the fusion of Bcr to Abl sequences is required for the elevated tyrosine kinase activity associated with the Bcr-Abl fusion protein. Two specific regions of Bcr have been identified as playing an important role in promoting transformation: amino acids 1-63 and amino acids 176-242. Amino acids 1-63 of Bcr-Abl are identical to amino acids 1-63 of the c-Bcr protein, predicting a coiled-coil oligomerization motif containing an amphipathic alpha helix. This sequence has been shown to be essential for the transforming function of Bcr-Abl oncoproteins; tetramerization of Bcr-Abl correlates with

both the activation of the tyrosine kinase and Bcr-Abl's association with the cytoskeleton via actin binding [McWhirter et al., 1991]. Three mechanisms have been proposed to explain how oligomerization of the Bcr-Abl molecule might promote kinase activation: (1) intermolecular crossphosphorylation of tyrosine kinase domains; (2) actin crosslinking by the clustering of actin-binding domains and (3) activation of an SH2 binding site in Bcr (Y177) [McWhirter et al., 1991].

Other researchers examining the significance of the coiled-coil motif have shown that it plays a role in Bcr-Abl's interactions with downstream signaling molecules such as the adaptor protein Grb2 and the SH2-containing phosphotyrosine phosphatase SHP-2 [Tauchi et al., 1997]. Deletion of the oligomerization domain prevented both tyrosine phosphorylation and coimmunoprecipitation with SHP-2, and coimmunoprecipitation with Grb2. Hence, oligomerization provides one mechanism for Bcr-Abl to physically interact with its substrates.

In examining the contribution of N-terminus Bcr sequences to transformation, it was demonstrated that fusion of Bcr with Abl sequences unmasks an actin-binding consensus motif in the C-terminus of Abl [McWhirter and Wang 1993]. In K562 cells, a CML cell line, Bcr-Abl and F-actin co-localize, as demonstrated by immunofluorescence microscopy. Deletion of the actin-binding domain in Bcr-Abl or of Bcr sequences abolishes binding to actin, reduces the ability of Bcr-Abl to transform fibroblasts, and abrogates cytokine dependence in Ba/F3 cells[McWhirter and Wang 1993]. The implications of these findings are that the stable association of Bcr-Abl with the actin

cytoskeleton is of paramount importance, analogous to the N-myristoylation in v-Abl. Thus, the subcellular localization of the Bcr-Abl molecule can govern its access to specific substrates and thereby is a critical component in determining its oncogenic potential.

Another important region of Bcr was first mapped to amino acids 176-242 and later, tyrosine 177. Upon phosphorylation of this residue, a docking site for SH2 domain-containing proteins such as the adaptors Grb2 and Grap is provided. Binding of Grb2 to Bcr-Abl may activate the small GTP-binding protein Ras through mSOS, and, activated Ras is directly implicated in the disruption of normal growth control. However, different laboratories have reached inconsistent conclusions about the requirement of Grb2 binding to Bcr-Abl in activating Ras and promoting transformation. In Rat-1 fibroblasts, Grb2 binding to Bcr-Abl was found to be essential for transformation [Pendergast et al., 1993]. Yet, in hematopoietic cells, including primary mouse bone marrow cells, Grb2 binding to Bcr-Abl appeared to be dispensable [Goga et al., 1995; Cortez et al., 1995]. These conflicting findings are not atypical, for they illustrate how examining Bcr-Abl function in hematopoietic and fibroblast transformation assays can yield varying results, thus reinforcing the need to use more than one method of analysis to assess mutations. (Additional discussion of Grb2 and Bcr-Abl is on pages 29-34.)

The contribution of Abl sequences in Bcr-Abl has also been extensively examined. The SH3 domain of Bcr-Abl confers binding to proteins containing proline-rich sequences (PxxP). While its role is still uncertain, the SH3 domain has been implicated in the

negative regulation of Abl kinase, possibly through an intramolecular folding mechanism [Pendergast et al., 1991]. Thus, deletion of the SH3 domain in the myristoylated form of c-Abl is sufficient to render it transforming [Franz et al., 1989; Jackson and Baltimore 1989]. Recently two Abl SH3-binding proteins, 3BP1 and 3BP2, that may serve an inhibitory function have been identified; 3BP1 can act as a GTPase-activating protein for Rac and 3BP2 contains a pleckstrin homology domain and an SH2 domain [Ren et al., 1993; Cicchetti et al., 1992]. However, no direct inhibition of tyrosine kinase activity by these proteins has been demonstrated. In contrast another Abl-binding protein, Abi-2, has been shown to interact with the SH3 domain, suppress Abl kinase activity, and hence regulate transforming activity [Dai and Pendergast et al., 1995].

Recent studies using immunofluorescence support the theory of an inhibitory role for the SH3 domain of Bcr-Abl. A Bcr-Abl ΔSH3 mutant was demonstrated to localize to the cytosol, as opposed to wild-type Bcr-Abl that associates primarily to the actin cytoskeleton/membrane fraction. Both Bcr-Abl and the ΔSH3 mutant transformed primary mouse bone marrow cells. However, when cells were injected into severe combined immunodeficient (*scid)* mice, the Bcr-Abl ΔSH3 mutant displayed reduced leukemogenic potential; mice died of leukemia after 17 to 45 weeks as opposed to 6 to 9 weeks in wild-type [Skorski et al., 1998]. Overall, these findings suggest that while the SH3 domain may not display a significant inhibitory function in conventional transformation assays, its deletion may be very important in upregulating Abl kinase activity in a mouse model.

Downstream of the SH3 domain in Bcr-Abl is an SH2 domain. This domain contains a highly conserved FLVRES motif which mediates binding to phosphotyrosine-containing peptides [Mayer et al., 1992]. For example, autophosphorylated Bcr sequences within Bcr-Abl or phosphorylated c-Bcr can bind the SH2 domain of Abl [Pendergast et al., 1991]. In addition, the Abl SH2 domain has been shown to interact with signaling proteins such as Shc and c-Cbl [Goga et al., 1995; Bhat et al., 1997]. Early work examining the requirement of the SH2 domain in transformation demonstrated that point mutations in this region impaired both phosphotyrosine binding and *in vivo* transforming activity, suggesting that interactions of the SH2 domain with downstream effectors, such as c-Cbl, may play a pivotal role in leukemogenesis [Mayer et al.,1992]. Subsequent studies that deleted the entire SH2 domain (ΔSH2Bcr-Abl) resulted in slightly different findings than those using the point mutants. In fibroblasts, a decrease in transformation was reported and in myeloid cells the SH2 domain was not required for induction of growth factor independence [Mayer et al., 1992; Ilaria et al., 1995; Oda et al., 1995].

The tyrosine kinase domain is the defining feature of Bcr-Abl. It is directly responsible for autophosphorylation and the phosphorylation of a large number of cellular proteins. A partial list of phosphorylated proteins in Bcr-Abl-expressing cells include signaling proteins such as PI3-kinase [Varticovski et al., 1991], CRKL [ten Hoeve et al., 1994; Oda et al., 1994; Nichols et al., 1994], and c-Cbl [Andoniou et al.,1994; Sattler et al.,1996] as well as the focal adhesion proteins paxillin, vinculin, tensin and p125 FAK [Salgia et al.,1995; Gotoh et al.,1995]. It has not been established whether the phosphorylated proteins are necessarily the direct substrates of the Abl kinase; rather,

they may be substrates of other cellular kinases inducibly activated in Bcr-Abl-expressing cells [Danhauser-Riedl et al., 1996].

To demonstrate unequivocally the importance of activated Abl kinase, an Abl kinase inactive mutant p210Bcr-Abl (K1174R) was generated. This mutant, unable to bind ATP, is non-transforming in both fibroblasts and hematopoietic cells [Pendergast et al., 1993]. More recently a selective inhibitor of the Abl kinase, CGP57148, was reported [Druker et al., 1996]. Treatment of cells with CGP57148 resulted in a specific killing of Bcr-Abl-expressing but not normal cells and inhibition of colony formation. Furthermore, in mice injected with Bcr-Abl expressing cells and treated with CGP57148, reduced tumor formation was observed. These findings demonstrate the absolute requirement of active Abl kinase for Bcr-Abl-mediated transformation.

Downstream of the kinase domain of Bcr-Abl is a proline-rich domain. This region provides a docking site for proteins containing SH3 domains. One prominently tyrosine phosphorylated adaptor protein which directly binds to this domain is CrkL [Oda et al., 1994; Heaney et al., 1997; Bhat et al., 1997]. Interestingly, when a Bcr-Abl mutant that contains a deletion in the proline-rich domain was generated, CrkL was still found to coimmunoprecipitate with Bcr-Abl [Bhat et al., 1997]. This suggested another protein could indirectly link CrkL to Bcr-Abl. Indeed, CrkL contains an SH2 domain that binds phophorylated c-Cbl, and phosphorylated c-Cbl binds Bcr-Abl. Thus, CrkL can associate with Bcr-Abl in either a direct or indirect manner. Additional proteins which have been

identified to bind the proline-rich region of Bcr-Abl are c-Crk I, c-Crk II, and Nck [Feller et al., 1994; Ren et al., 1994].

Examining the Biological Effects of Bcr-Abl Expression: Murine Models

To gain a greater understanding of the biology of Bcr-Abl and CML a variety of transgenic and syngeneic transplant mouse models have been constructed. Unfortunately, all fall short of thoroughly recapitulating the chronic phase of CML. Rather, they appear to more resemble the blast crisis phase as is seen in the acute forms of leukemia. Nevertheless, certain progress has been made.

The early animal models for CML used syngeneic transplants of hematopoietic stem cells infected by a retrovirus carrying the p210Bcr-Abl gene into lethally irradiated mice [Daley et al., 1990; Daley and Ben-Neriah, 1991]. In these reports, approximately 50% of transplant recipients developed a myeloproliferative syndrome similar to CML within a few months. Some mice also developed tumors of macrophage origin or acute lymphoid leukemia (ALL), resulting in leukemic blasts infiltrating the peripheral blood, spleen, and bone marrow. A second group, using a similar methodology also attempted to reproduce a CML-like syndrome in mice [Kelliher et al., 1990]. The central finding from this study was that only a small number of animals (15%) developed a myeloproliferative-like syndrome similar to the chronic phase of CML, specifically, an expansion of Bcr-Abl infected granulocytic cells. Both studies, however, were unable to transplant the Bcr-Abl-induced-myeloproliferative disorder observed in the original donor

into secondary recipients. This suggests that the transplantable stem cells may not have been transduced. In a later study, the secondary transfer was performed on mice six months after the transplant to ensure stable engraftment of the stem cell clones. In this case, some of the secondary recipients developed tumors and a myeloproliferative disorder, though not resembling CML. More recently two independent groups using a murine stem cell retroviral vector have reported improvements in the model in which a higher frequency of animals developed a myeloproliferative disorder with a shorter latency period [Pear et al., 1998; Zhang and Ren 1998]. Other *in vivo* assays include injecting Bcr-Abl transformed cells into mice (often nude or *scid*) to measure tumor frequency or histopathological changes.

An alternative approach that has been used to develop an animal model for CML is the introduction of Bcr-Abl cDNA into the germline to generate transgenic animals. When the p185Bcr-Abl cDNA regulated by a metallothionin promoter was introduced into embryonic stem cells, most animals developed an acute B-lymphocytic leukemia after a brief period of latency [Voncken et al., 1992]. In contrast, a p210Bcr-Abl cDNA regulated from a BCR promoter resulted in 100% intrauterine lethality [Voncken et al., 1995]. However, when a metallothionin promoter-regulated p210Bcr-Abl cDNA was used, development proceeded normally and approximately 40% of the animals quickly developed either an acute T-lymphocytic leukemia, T-lymphoblastic lymphoma, or acute myeloid leukemia. While neither the p185Bcr-Abl nor p210 Bcr-Abl transgenics developed a CML like syndrome, the different diseases they produce suggest that the

additional sequences contained in p210Bcr-Abl could play a role in these distinct clinical outcomes, similar to what is seen the human diseases [Verfaillie 1998].

The most recent strategy to develop a mouse transplant model has been construction of a human/mouse chimera [Sirard et al., 1996]. Experiments were undertaken using Philadelphia chromosome-positive bone marrow or peripheral blood from CML patients, in either chronic or blast phase, engrafted into nonobese diabetic (NOD), *scid* mice. While these cells successfully engrafted, the coexisting progenitors that do not express Bcr-Abl (Philadelphia chromosome-negative) engrafted preferentially. These findings suggest that the growth of normal cells is favored in this model. Thus, this model will not be of significant utility in understanding CML pathogenesis until further refined.

Examining the Biological Effects of Bcr-Abl Expression: Tissue Culture Models

To gain a greater understanding of how the Bcr-Abl molecule may contribute to oncogenesis, a number of *in vitro* assays have been developed. While these assays obviously cannot recapitulate the disease, they can assist in defining the structural requirements for transformation in Bcr-Abl, other than the kinase domain. This section will give an overview of the three most frequently used assays: morphological transformation of fibroblasts; the abrogation of interleukin (IL-3) dependence; and the induction of long-term survival of bone marrow cells in culture.

Most types of normal cells require attachment to a substrate in order to grow, and if denied anchorage, will undergo apoptosis. It has long been appreciated that transformed cells, in contrast to normal ones, are anchorage-independent for growth [Folkman and Moscana, 1978]. The Rat-1 fibroblast transformation assay was designed to exploit the anchorage-independent property associated with malignant cells, and provide a quantitative measurement of transformation potency [Lugo and Witte 1989]. This single step assay measures the number of fibroblast foci in soft agar per number of cells plated (usually 10^4) after an acute infection with a recombinant retrovirus expressing Bcr-Abl proteins. In the initial study describing this technique, a direct comparison of p185Bcr-Abl and p210Bcr-Abl established the higher transformation potency of p185Bcr-Abl; the frequency of macroscopic foci infected with p185Bcr-Abl after two weeks was approximately 100 fold higher than p210Bcr-Abl[Lugo and Witte 1989]. This was a significant finding given p185Bcr-Abl's association with the more aggressive form of leukemia, ALL.

The *in vitro* transformation of immature bone marrow cells was designed to assess the growth-promoting effects of Bcr-Abl [Young and Witte1988; McLaughlin et al., 1987]. In this assay, a long-term B-lymphoid culture containing a range of B cells at different stages of differentiation or fresh bone marrow harvested from the femurs of young mice, are infected with a recombinant retrovirus containing the Bcr-Abl cDNA. After four to six weeks the number of nonadherent cells are counted. Cultures infected with Bcr-Abl display a much higher density of large, immature, lymphoid cells, reflecting the growth stimulus conferred by Bcr-Abl. Further, the culture is demonstrably more acidic. The

major drawback of this assay, as opposed to the Rat-1 assay, is that the transformed lymphoid cells may have accumulated secondary oncogenic events since they represent clonal outgrowths from the mass population of infected cells. This appears to be the case since the p210Bcr-Abl-expressing clones displayed a wide variation of tumorigenicity when inoculated into mice and examined three months later [Young and Witte1988; McLaughlin et al., 1987].

The abrogation of interleukin (IL-3) dependence assay exploits the growth factor dependence of hematopoietic cell lines [Daley and Baltimore 1988; Daley et al., 1992]. As in the previous assays, retroviral constructs encoding Bcr-Abl are used to infect different cell lines such as: (1) the bone marrow-derived, IL-3 dependent, Ba/F3 cell line; (2) the murine myeloid, IL-3 dependent 32D cell line; and (3) the human megakaryocytic, GM-CSF dependent, Mo7e cell line. To test for abrogation of IL-3 dependence, the IL-3 is removed two days after infection and live cells are counted at different time points. For example, the Ba/F3 cells will fail to proliferate and will die quickly in the absence of an exogenous source of IL-3; however, Ba/F3 cells expressing wild-type Bcr-Abl will not. (Normally, to propagate these cells, conditioned medium from the WEHI-3B cell line is generated to provide this source.) Thus, Bcr-Abl provides a mitogenetic stimulus in these cells, allowing them to bypass the normal proliferative signaling pathway.

Given that CML is a hematopoietic disease, the use of Bcr-Abl-expressing hematopoietic cell lines should theoretically be more instructive than studying fibroblasts

in a tissue culture setting. However, there is no indication that chronic phase CML progenitors display growth factor independence, as seen in the Bcr-Abl-expressing hematopoietic cell lines [Bedi et al., 1994; Clarkson and Strife 1993]. In contrast, Bcr-Abl-expressing fibroblasts are growth factor dependent and anchorage-independent. This phenotype better resembles the phenotype of CML progenitors: their obvious drawback being that Bcr-Abl is not naturally found in this lineage.

The Role of Adaptor Proteins in Bcr-Abl Signaling

Central to unraveling the mechanisms by which Bcr-Abl may cause leukemia is an understanding of how other signaling molecules bind to Bcr-Abl to generate a multi-molecular complex. As a protein tyrosine kinase, Bcr-Abl phosphorylates and associates with numerous proteins within the cell. Upon their tyrosine phosphorylation, these proteins can be recognized by specific motifs in other molecules referred to as SH2 domains. These latter proteins are one example of "adaptors" or "molecular adhesives." Their function is to generate networks of signaling molecules within the cell, without the requirement of enzymatic activity.

Src-Homology 2 (SH2) domain-containing proteins interact specifically with the phospho-tyrosyl residue of activated receptor or cytoplasmic phosphoproteins. These domains consist of approximately 100 amino acids that structurally comprise a deep binding pocket and a relatively flat binding surface. Basic residues within the pocket, including an invariant arginine, contact the negatively charged phosphate group and tyrosyl ring [Cohen et al., 1995]. The binding surface, which is generally more variable,

confers specificity by recognizing amino acids C terminus to the phosphotyrosine. For instance, Grb2 contains an SH2 domain that recognizes the phosphopeptide ligand pTyr-Val-Asn-Val [Songyang et al., 1993]. In contrast, the Src tyrosine kinase recognizes its partner, FAK, by the sequence pTyr-Ala-Glu-Ile [Songyang et al., 1993].

Adaptor proteins also frequently contain Src-homology 3 (SH3) domains. In contrast to SH2 domains, these domains are approximately 60 amino acids in length and mediate binding to a proline-rich consensus sequence (PxxP). Structurally, SH3 domains are quite different than SH2 domains; they are generally flat and fold into five antiparallel ß strands that pack to form two perpendicular ß sheets [Cohen et al., 1995]. Though less well studied than SH2 domains, SH3 domains have been shown to have distinct binding specificities, defined by a hydrophobic patch which consists of conserved aromatic residues flanked by two charged and variable loops forming a pocket for ligand-binding [Cohen et al., 1995]. Thus, the Abl SH3 domain binds poorly to Src-specific ligands and Src does not bind well to Abl-specific peptides [Rickles et al., 1994].

Though recent evidence has demonstrated that SH3 motifs may also target specific proteins to the cytoskeleton and thereby play a role in cellular localization, in general the major function of SH3 domains is to act as a protein module: that is to aggregate other proteins and generate intracellular signaling cascades [Bar-Sagi et al., 1993; Skorski et al., 1998].

Grb2

The Growth factor-receptor binding protein 2 (Grb2), is the human homologue of the *Caenorhabditis elegans* gene, *sem-5*, a protein required for *let-23* receptor tyrosine kinase signaling to Ras [Clark et al., 1992]. Grb2 is considered the prototype adaptor protein consisting entirely of one SH2 domain flanked by two SH3 domains (Figure 6). These domains play a critical role in coupling receptor and non receptor tyrosine kinases to Ras activation.

Grb2 was first identified and cloned by virtue of its SH2 domain binding to the phosphorylated cytoplasmic domain of the epidermal growth factor receptor [Lowenstein et al., 1992]. Its full signaling capabilities were later illuminated when its SH3 domain was shown to bind constitutively to proline-rich sequences of mSOS, the guanine nucleotide exchange factor for Ras [Egan et al., 1993; Rozakis-Adcock et al., 1993]. Thus, upon activation of the EGF receptor, the SH2 domain of Grb2 binds phosphorylated EGFR at the membrane, bringing with it mSOS and allowing mSOS access to its substrate, Ras [Gale et al., 1993]. Conversion of the inactive GDP-bound form of Ras to its active GTP-bound form by mSOS can provide the stimulus for both normal mitogenic and differentiation signals of receptor tyrosine kinases and transformation by oncogenic tyrosine kinases as well [Boguski and McCormick 1993].

Grb2 is now established as a universal adaptor protein in many tyrosine kinase signaling pathways that lead to Ras activation. In Bcr-Abl transformed cells, Grb2 and mSos were shown to form a stable complex with Bcr-Abl as assessed by

coimmunoprecipitation and GST-binding assays [Puil et al., 1994; Pendergast et al., 1993]. Previous work deciphering the Grb2's SH2 domain binding site on EGFR, Shc and IRS-1 was instrumental in mapping the binding site of Grb2 to sequences encoded in exon 1 of Bcr. Those early studies indicated that Grb2 had a binding preference for an asparagine at the pY+2 position [Songyang et al., 1993]. Thus, when the Bcr-Abl sequence was examined, a potential Grb2–binding site was clearly apparent: tyrosine177-valine178-asparagine179-valine180.

To demonstrate that the phosphorylation of tyrosine 177 of Bcr was necessary for binding of Grb2 to Bcr-Abl, a point mutation in Bcr-Abl substituting a phenylalanine in place of tyrosine (Y177F) was generated and transfected into two different cell lines [Pendergast et al., 1993]. This mutant was unable to bind to Grb2 by coimmunoprecipitation. To assess the physiological relevance of this mutation on Bcr-Abl-induced transformation Rat-1 fibroblasts and hematopoietic cells were transfected with either wild-type Bcr-Abl or the Y177F mutant. In these experiments, the Y177F Bcr-Abl protein failed to transform the hematopoietic (mouse bone marrow) cells and displayed a reduced capacity to transform fibroblasts compared to wild-type [Pendergast et al., 1993]. The authors concluded from these findings that Grb2 binding to Bcr-Abl was a requirement for activation of Ras. However, subsequent studies examining the identical point mutation in Bcr-Abl (Y177F) contradicted some of the earlier findings [Goga et al., 1995].

In these studies, consistent with the previous study, mutation of tyrosine 177 to phenyalanine (Y177F) in Bcr-Abl impaired fibroblast transformation activity. However this mutant successfully transformed primary bone marrow cells (despite a similar *in vitro* transformation assay being used) and rendered hematopoietic cell lines growth factor independent. Furthermore, *scid* mice injected with the Y177F mutant developed approximately the same number of tumors with nearly the same latency, as in mice receiving wild-type Bcr-Abl. These findings prompted the authors to argue that alternative routes to Ras activation, in the absence of Grb2 binding to Bcr-Abl, must exist. Indeed, Grb2 can bind other phosphorylated proteins such as the adaptor molecule SHC which, when phosphorylated, binds Bcr-Abl [Tauchi et al., 1994]. Hence additional mechanisms are available to activate Ras in the absence of direct Grb2 binding to Bcr-Abl.

Additional studies support a direct connection between Bcr-Abl and Ras through Bcr-Abl's association with Grb2. Thus, inhibition of Ras activation correlates with inhibition of Bcr-Abl-mediated transformation. Supporting a role for Grb2's activation of Ras through its association with mSOS are studies using mutant forms of Grb2 which lack either amino or carboxy terminus SH3 domains [Gishizky et al., 1995]. In these studies, Grb2 SH3 deletion mutants (the domain which binds mSOS) inhibited growth of Bcr-Abl-expressing Rat-1 cells in soft agar, decreased GTP loading of Ras, and induced K562 cells to differentiate.

Outside the context of Bcr-Abl, Grb2's biological function in embryonic development has been determined through the generation of Grb2 null mice [Cheng et al., 1998]. These studies reveal the necessity of Grb2 during embryogenesis, particularly for differentiating endodermal cells and their formation into an epiblast. Interestingly, mutant blastocysts resemble mouse embryos lacking the adhesion receptor, ß1 integrin. Replacement of the C terminus proline-rich sequences of SOS with the Grb2 SH2 domain significantly rescued these defects in Grb2-deficient embryonic stem cells, supporting Grb2's fundamental role as an adaptor linking tyrosine kinases to mSOS.

There is also evidence suggesting that Grb2 links receptor tyrosine kinase signaling to proteins which regulate the cytoskeleton. Cellular localization studies on Grb2 have demonstrated that in the absence of exogenous factors, Grb2 is localized primarily to the cytosol of fibroblasts [Bar-Sagi et al. 1993]. However, in response to epidermal growth factor, when cells are induced to ruffle their membranes, Grb2 translocates to the membrane ruffles (lamellipodia). To demonstrate this, GST-Grb2 was microinjected into EGF-stimulated cells, stained with GST antibodies, and subsequently examined by immunofluorescence. Localization to the membrane ruffles required the SH3 domain of Grb2. Indirect evidence of Grb2's interaction with proteins regulating the cytoskeleton was demonstrated through microinjection studies; microinjection of Grb2 antibodies inhibited EGF-induced membrane ruffling [Matuoka 1993]. More recently Rac, the GTPase responsible for regulating membrane ruffling in response to growth factor stimulation, was shown by immunofluorescence to co-localize with Grb2, and inhibition

of Ras and EGF-induced membrane ruffling could be observed with specific inhibitors of the Grb2 SH2 domain [Gay et al., 1999].

Grap

Five years after the cloning of Grb2, a protein with significant homology and similar structure (SH3-SH2-SH3) was identified [Feng et al., 1996; Trub et al., 1997]. This protein, Grap (Grb2-related adaptor protein), shares approximately 59% amino acid sequence identity to Grb2, with the greatest homology found in the N-terminus SH3 domain (Figure 6). Interestingly, despite these similarities, the tissue distribution of Grap mRNA and protein is distinct. While Grb2 is expressed ubiquitously, Grap expression patterns are predominant in lymphocytic and hematopoietic tissues. This finding has led to speculation that while Grb2 can function ubiquitously, lymphocytic signaling may require additional components to activate downstream pathways; hence, Grap could serve as an immune cell-restricted adaptor [Rudd et al., 1999].

Given their similar domain architecture, initial studies focused on whether the binding specificities of Grap to phosphoproteins were similar to Grb2's. Indeed, the SH2 domain of Grap, like the SH2 domain of Grb2, can bind the ligand-activated receptors for stem cell factor and erythropoietin, though with less apparent binding affinity [Trub et al., 1997]. Similar to Grb2, Grap can also interact constitutively with mSos via Grap's SH3 N-terminus domains. Other proteins that were shown to associate with Grap in T cells include Shc, Sam 68, and dynamin but not PI3-kinase or c-Cbl [Trub et al., 1997].

Mice homozygous for a null mutation in the Grap gene do not apparently display any physical deformities [Cheng et al.,1998]. These preliminary findings neither assessed nor addressed a requirement for Grap when Grap null animals are immunologically challenged. While further biological studies of these animals await completion, a null mutation in the mouse Grb2 gene has shed light onto the potential functional redundancy of these two proteins. Embryonic stem cells isolated from Grb2 homozygous mice, which do not express Grap, display normal proliferation though they are defective in endodermal differentiation. Introduction of Grap cDNA into these cells partially rescues the differentiation defect of these cells. These findings suggest that Grap can substitute for Grb2 in this developmental setting, most likely by linking an unidentified phoshotyrosine-containing protein to a Grap-SOS complex.

One of the proteins Grap was reported to co-immunoprecipitate with was Bcr-Abl [Feng et al., 1996]. Furthermore, like Grb2, this association with Bcr-Abl was via the SH2 domain of Grap. These findings suggested the possibility that in leukemic cells, Grap could serve as a conduit to Ras activation through its association with mSOS, similar to Grb2. However, to more clearly define their relative roles in leukemogenesis would require determining whether Grap bound Bcr-Abl at the same site as Grb2 (tyrosine 177 of Bcr-Abl) and determining the relative binding affinities of these two proteins for Bcr-Abl.

<u>c-Cbl</u>

Cbl (Casitas B-lineage lymphoma) is a ubiquitously expressed cytoplasmic adaptor molecule that can both positively and negatively regulate cellular signaling pathways (Figure 6). It was initially identified as the cellular homologue of the transforming gene v-cbl, of the Casitas-NS-1 retrovirus, which was isolated from a mouse in the Lake Casitas area of California [Langdon et al., 1989]. The viral oncogene contains 355 amino acids of the amino terminus of cellular Cbl fused to viral gag-encoding sequences [Blake et al., 1991]. Cell-free extracts of the Cas-NS-1 retrovirus induce a host of hematopoietic neoplasms in mice including B- and T- cell lymphomas and erythroid and myeloid leukemias [Frederickson et al., 1984]. Interestingly, tumors induced by v-Cbl are phenotypically and histologically similar to those induced by v-Abl.

Unlike v-Cbl which fractionates to both the nuclear and cytoplasmic fractions, c-Cbl is localized exclusively to the cytoplasm and may associate with the cytoskeleton [Blake et al., 1993]. Both c-Cbl and v-Cbl contain a novel SH2 domain at the N-terminus of the molecule as well as numerous tyrosine phosphorylation sites [Meng et al., 1999]. In addition Cbl possesses a putative leucine zipper, a zinc-finger-like motif, and a proline-rich motif [Blake et al., 1991]. Thus, Cbl contains a plethora of protein-protein interaction domains: an SH2 domain capable of associating with tyrosine phosphorylated proteins; proline-rich sequences which may bind SH3 domain-containing proteins; and numerous tyrosines which when phosphorylated can link to SH2 containing proteins. Finally, like other adaptor molecules, Cbl lacks any known catalytic function.

possibilities for Cbl in generating downstream signals is a formidable task given its complex structure and the many proteins it can interact with. Nevertheless, general principles for Cbl's function in signal transduction can be gleaned from seminal experiments performed in both the nematode and in mammalian cells.

A role for Cbl as a negative regulator of tyrosine kinases was first revealed in studies in the nematode *Caenorhabditis elegans.* Genetic examination of vulval development identified a gene, *sli-1*, that suppressed mutations in *Let-23*, the EGF receptor homologue [Yoon et al., 1995]. Sli-1 was later identified to be the homologue of human Cbl. Subsequent mammalian studies confirmed an interaction between the N-terminus of c-Cbl (containing the conserved SH2 domain) with the EGF receptor. Moreover, binding was dependent on the extent of tyrosine phosphorylation of the receptor, consistent with its binding to an SH2 domain [Bowtell et al., 1995]. Recently the Cbl gene was disrupted by two independent groups [Naramura et al.,1998; Murphy et al.,1998]. The findings from these reports support Cbl's role as a critical negative regulator in tyrosine kinase signaling and proliferation; an increase in constitutive ZAP-70 kinase phosphorylation was detected as well as tissue hyperplasia.

In Bcr-Abl-expressing cells, c-Cbl is constitutively tyrosine phosphorylated [Andoniou et al., 1994; de Jong et al., 1995]. Tyrosine phosphorylated Cbl coimmunoprecipitates with Bcr-Abl and the adaptor molecules PI3-kinase, Grb2, and CrkL. (However its association with Grb2 is observed even when Cbl is not tyrosine phosphorylated.) [de Jong, 1995; Andoniou, et al., 1994; Ribon et al., 1996; Sattler et al., 1996; Bhat et al.,

1997]. The interaction between Cbl and Bcr-Abl requires an active Abl kinase and Cbl tyrosine phosphorylation. However, the kinase responsible for phosphorylating Cbl in Bcr-Abl-expressing cells has not been ascertained; it may or may not be Bcr-Abl [Bhat et al., 1997].

While significant progress has been made in understanding how these proteins interact by mapping their binding sites, less is known about how the Bcr-Abl protein complex is assembled. Specifically, do certain adaptor molecules assist in a coordinated, orderly progression to generate the multi-molecular Bcr-Abl complex akin to the discrete steps in creating a transcriptional apparatus? Or, is the association of Cbl with Bcr-Abl independent of a preassembly of proteins bound to the Bcr-Abl molecule?

p85 Subunit of PI3-Kinase

Phosphoinositide 3-kinase (PI3-kinase) activity was first demonstrated by coimmunoprecipitation with the activated PDGF receptor and the polyomavirus transforming protein (polyoma middle t/pp60$^{c\text{-}src}$ complex) [Whiman et al. 1985; Kaplan 1986]. These complexes, which were immunoprecipitated with an anti-phosphotyrosine antibody, possessed increased polyphosphoinositide lipid kinase activity; specifically phosphorylating the substrates phosphatidylinositol (PI), PI 4-P,and PI 4,5-P_2 on position 3' of the inositol ring to generate PI-3-phosphate, PI-3,4-bisphosphate, and PI-3,4,5-trisphosphate respectively [Whitman 1988]. Since this initial discovery many

laboratories have demonstrated PI3-kinase activity in other growth factor-stimulated and oncogenically transformed cells, such as Bcr-Abl and v-src [Cantley et al., 1991].

Purification of PI3-kinase revealed a heterodimeric enzyme consisting of a p85 regulatory and p110 catalytic subunit [Carpenter et al., 1990]. Two isoforms have been isolated; p85 alpha is expressed in all tissues and p85ß is restricted to the brain and lymphoid tissue. The p85 regulatory subunit is classified as an adaptor for it contains one SH3 and two SH2 domains and lacks any intrinsic enzymatic activity (Figure 6). The specificity of the SH2 domain of PI3-kinase to its phosphopeptide partner was determined by its binding to distinct sequences within polyoma middle t as well as in the PDGF receptor. This work demonstrated a requirement for a phosphotyrosine followed by the residues Met/Val-X-Met sequences [Cantley et al., 1991]. Thus, the interaction of the SH2 domain of the p85 regulatory subunit with a tyrosine phosphoylated receptor provides a mechanism to transduce tyrosine kinase signals to the p110 catalytic subunit resulting in its activation and increasing p110's proximity to its lipid substrates.

PI3-kinase has been implicated in the regulation of a myriad of cellular processes. They include: inhibition of apoptosis, cytoskeletal rearrangements, mitogenesis, oncogenesis, and vesicle trafficking [Vanhaesebroeck et al., 1997; Carpenter and Cantley 1996; Varticovski et al., 1994]. While impaired vesicle trafficking has not been reported in Bcr-Abl transformed cells, inhibition of apoptosis, aberrant cytoskeletal structures, abnormal mitogenic signaling, and oncogenesis all characterize the Bcr-Abl phenotype. Hence,

the contribution of PI3-kinase to these processes has been under extensive investigation.

Early studies examining the activation of PI3-kinase in cells which express oncogenic variants of Abl yielded insight into the relationship between the cellular ratios of PI-3,4-bisphosphate and PI-3,4,5-trisphosphate and transformation [Varticovski et al., 1991]. In these experiments, PI3-kinase was shown to associate (coimmunoprecipitate) only with the activated forms of Abl: v-Abl, Bcr-Abl and gag/Bcr-Abl. However, association alone did not automatically confer transformation; rather, cellular PI3-kinase lipid products were the best predictor of the ability of the abl oncogene variants to transform fibroblasts. Interestingly, this study concluded that despite the phosphorylation of PI3-kinase and its association *in vivo* with Bcr-Abl, cells expressing Bcr-Abl fail to either accumulate PI3-kinase products *in vivo* or to transform NIH3T3 cells. (Neither Bcr-Abl-expressing Rat-1 fibroblasts nor hematopoietic cells were examined in this analysis.)

While the previously mentioned findings implied that PI3-kinase might not be important for transformation by Bcr-Abl, subsequent reports using different cell lines and methodologies have. Using a mouse, hematopoietic, temperature sensitive cell line for Bcr-Abl, Jain and colleagues demonstrated the activation of PI3-kinase and the formation of PI3-kinase lipid products (by HPLC) at the permissive but not at the restricted temperature [Jain et al., 1996]. Moreover, activation of PI3-kinase activity did not require PI3-kinase association with Bcr-Abl. Therefore, while the tyrosine kinase

activity of Bcr-Abl was essential for activation of PI 3-kinase and the generation of its lipid products, the association of these two molecules did not appear to be so.

Subsequent to the identification of PI3-kinase as a major player in Bcr-Abl-mediated transformation was the pivotal discovery of a novel PI3-kinase effector, Akt, that could mediate protection from apoptosis [Klippel et al., 1997]. Akt is a proto-oncogene, serine/threonine kinase. Characterization of this molecule revealed a pleckstrin homology (PH) domain capable of binding lipids, providing a link to PI3-kinase lipid products and Akt activity [Franke et al., 1997]. Subsequent work in Bcr-Abl-expressing cells has shown that inhibition of the PI3-kinase/Akt pathway by the PI3-kinase inhibitor wortmannin (an inhibitor of the p110 subunit) or by specific antisense oligonucleotides (directed against the p85 subunit), impaired Bcr-Abl mediated proliferation [Skorski et al., 1995; Skorski et al., 1997].

In addition to its established role in transformation, PI3-kinase and its lipid products have recently been implicated in regulating the actin cytoskeleton [Carpenter and Cantley 1996]. When Swiss 3T3 cells are stimulated with PDGF, association of PI3-kinase with the Rho family members, Rac and CDC42, is detected in immunoprecipitates [Tolias and Carpenter 1995]. Further, this association of PI3-kinase is GTP dependent. These interactions present indirect evidence of a pathway linking PI3-kinase to the changes in the cellular architecture. In support of that evidence are experiments performed to assess changes in the cytoarchitecture of mammary epithelial cells transfected with constitutively active PI3-kinase, Rac and Cdc42. [Keely et al.,

1997]. Profound perturbations of the normal polarized epithelial cell morphology were observed as well as an increase in motility and spreading in cells transfected with each of the constructs. Interestingly, addition of LY294002, a pharmacological inhibitor of PI3-kinase, dramatically reduced the increased spreading and motility observed in every case. These data suggest that PI3-kinase is downstream of both Rac and CDC42 in the regulation of the cytoskeleton.

Recently, a specific integrin, α6B4, has been demonstrated to promote changes in the cytoarchitecture and invasiveness of carcinoma by signaling through a PI3-kinase pathway. Using breast and colon carcinoma cells, this study identified PI3-kinase activity as a prerequisite for the formation of motility-based structures such as lamellae [Shaw et al., 1997]. While cellular carcinoma requires invasion as a defining event and leukemogenesis does not, the motile phenotype associated with activated PI3-kinase in cellular carcinoma makes plausible the argument that activated PI3-kinase in Bcr-Abl-expressing cells may contribute to the motile phenotype observed in Bcr-Abl-expressing cells.

The mouse p85 gene, recently knocked out by two independent groups, both reported defects in B cell function and development [Fruman et al.,1999; Suzuki et al.,1999]. While most animals died shortly after birth, those that survived displayed a reduction in B cell numbers, decreased B cell proliferative responses and diminished serum immunoglobulin. Interestingly this phenotype resembles the phenotype of X-linked

immunodeficiency or Btk -/- animals. (Btk is a downstream effector of PI-3,4,5-trisphosphate.)

Bcr-Abl and the Cytoskeleton

Through its interactions with adaptor proteins Bcr-Abl may not only profoundly disrupt normal signaling pathways but also potently perturb the overall physical organization of the cell, namely the cytoskeleton. Structural changes in cells containing the Philadelphia chromosome might explain the premature release of myeloid progenitors from the bone marrow of patients with CML. A wealth of new research examining the role of the cytoskeleton has provided fresh insight into changes in adhesive properties, motility, and cytoarchitecture in Bcr-Abl-expressing cell lines and CML progenitor cells. To put these findings in context, the components of the cytoskeleton and its regulators will be briefly discussed.

The cytoarchitecture is defined by two major components: the actin cytoskeleton and focal adhesions. Studies performed primarily in a fibroblast cell model system have demonstrated that the actin cytoskeleton is a highly dynamic structure, important not only in maintaining cellular shape but also motility, chemotaxis, cell division, endocytosis and secretion [Devreotes and Zigmone, 1988; Bretscher, 1991]. Through associations with specialized actin-binding proteins and regulated changes in polymerization and depolymerization, actin filaments can be organized into three distinct structures: (1) Actin stress fibers, which are long bundles of filaments that traverse the

cell and link focal adhesion complexes to the extracellular matrix; (2) Lamellipodia (or membrane ruffles) which are a highly compact meshwork of actin which define the leading edge of motile cells; and (3) Filopodia, which are short bundles of actin which protrude from the cell surface.

The other major component of the cytoskeleton is the focal adhesions. First identified by electron microscopy (Abercrombie et al., 1971), these multi-molecular protein complexes comprise specialized areas of the cell where the adhesion receptors, integrins, span the membrane and make contact with the extracellular matrix on the outside, and specific focal adhesion proteins on the inside (Lo et al. 1994). A number of structural and enzymatic proteins have been identified as focal adhesion components such as vinculin, focal adhesion kinase (FAK), tensin, talin, and paxillin. Further, many of these focal adhesion proteins are inducibly tyrosine phosphorylated upon cellular adhesion and contain actin-binding domains and adaptor motifs such as SH2 domains. Thus, these proteins are positioned to transmit signals from the outside of the cell (through integrins) to the inside of the cell (through their tyrosine phosphorylation and various protein-protein interaction domains).

Examination of the morphological response of cells to extracellular factors and oncogenic viruses has provided valuable insight into the cytoskeleton and its regulators. An almost complete disassembly of the actin cytoskeleton is observed in Swiss 3T3 fibroblasts made quiescent overnight by serum starvation [Ridley and Hall 1992]. However, when serum or its active constituent lysophosphatidic acid (LPA) is readded,

actin stress fibers rapidly reform. During the past few years, members of the Rho GTPase family have been shown to play a key regulatory role in mediating a signal transduction pathway from the LPA receptor to stress fiber formation, and the PDGF receptor to lamellipodia formation. In fact, microinjection of activated Rho or Rac is sufficient to generate stress fibers and lamellipodia in the absence of exogenous LPA and PDGF, respectively [Nobes and Hall 1995].

As in serum-starved fibroblasts, a dearth of stress fibers can also be observed in cells transformed by the Rous sarcoma virus [Parsons and Weber 1989]. Cells infected with this virus, which encodes the tyrosine kinase oncogene v-Src, exhibit a cytoarchitecture with few focal adhesions and stress fibers, even in the presence of growth factors. In contrast to normal cells, these fibroblasts are rounder, adhere poorly to the extracellular matrix (ECM), and exhibit increased phosphotyrosine levels of focal adhesion proteins. Although the mechanisms responsible for inducing this phenotype may differ from those in Bcr-Abl, examination of the biochemical and cytoskeletal consequences of oncogenic transformation by this prototype, cytosolic tyrosine kinase has assisted in providing a benchmark in which to compare the cytoskeletal defects associated with Bcr-Abl.

The cytoskeletal studies of Bcr-Abl have focused on three general areas: (1) the role of integrins in modulating adhesive properties of hematopoietic cells; (2) cytoarchitectural changes in Bcr-Abl expressing cells; and (3) interactions of Bcr-Abl with focal adhesion proteins. The context of this body of work is hematopoiesis, a process whereby normal progenitors anchor themselves to the extracellular matrix of the bone marrow through

integrins and a variety of other receptors, and are released into the bloodstream upon their differentiation [Verfaillie 1997]. One prominent hypothesis to explain the abnormal circulation of CML progenitors which permits their premature release is a defect in integrin function in Bcr-Abl-expressing cells. However, experimental findings to support this hypothesis have yielded conflicting results depending on the cell type used and duration and type of adhesion assay employed examined.

In three hematopoietic cell lines expressing Bcr-Abl, Bazzoni and colleagues reported a marked increase in integrin-mediated adhesion to the extracellular matrix protein fibronectin (a component of bone marrow stroma) compared to normal cells [Bazzoni et al., 1996]. These studies, performed in a 30-minute assay, concluded that Bcr-Abl stimulates adhesion by activating the integrins VLA-4 and VLA-5. (The cell-surface numbers of these receptors remained unchanged.) In contrast, reported a pronounced reduction in adherence of CML progenitors to bone marrow stroma [Gordon et al., 1987]. These latter findings were confirmed by another group who reported decreased adhesion to both bone marrow stroma and fibronectin and increased adhesion to other extracellular matrix molecules, laminin and collagen type IV [Verfaillie 1992]. While conditions in the latter study were slightly different, allowing the cells to adhere for a longer period of time, the authors attributed the altered adhesion status of these CML progenitors to a reduced functional state of the integrins. Integrins, which can exist in either low or high affinity state can be modulated by cytokines, activating antibodies, or an inside-to-outside signaling mechanism [Verfaillie 1997]. While signaling is generally thought of as unidirectional (an outside stimulus generating internal signals), in this

situation, Bcr-Abl, through a direct or indirect interaction with the cytoskeleton, may interfere with integrin-dependent adhesion through an inside-to-outside signaling mechanism.

A recent report attempted to mitigate some of the conflicting data concerning the relationship between adhesion and motility in Bcr-Abl-expressing cell lines, as well as examine the subcellular localization of Bcr-Abl in migrating cells [Skourides et al., 1999]. In this study, 32D myeloid cells expressing Bcr-Abl were reported to have increased adhesion to fibronectin coated cover slips compared to their normal counterparts (a conventional adhesion assay was not performed). This enhanced adhesion correlated with an in increase in migration by time-lapse video microscopy, and an increase in the frequency of motility-based structures lamelipodia and filopodia. Interestingly, migrating cells stained for Bcr-Abl revealed a polarized distribution; Bcr-Abl co-localized with actin fibers at the rear of the cell. In contrast, Bcr-Abl co-localized to ill-characterized vesicle-like structures (neither lysosomes nor endosomes) in non-migrating cells. Deletion of the actin-binding domain increased its localization to these vesicle structures but had no demonstrable effect on adhesion. While this report does not reconcile the findings of the decreased adhesion of primary CML progenitors they do illustrate that an increase in adhesion can cause an increase in motility in these cell lines. Further, they demonstrate the importance of the actin-binding domain of Bcr-Abl for its association with distinct intracellular compartments, where presumably it affords greater access to its many substrates that contribute to induce transformation.

Enhanced motility of Bcr-Abl-expressing cells has been documented in other cell lines and CD34+ primary bone marrow cells from CML patients [Salgia et al., 1997]. Using time-lapse video microscopy, BaF3 cells expressing Bcr-Abl displayed increased membrane ruffling and filopodia, indicators of cell movement. Moreover, while untransformed cells were sessile for long periods of time, Bcr-Abl-expressing cells were persistently motile. Consistent with the findings in BaF3 cells, fibroblasts expressing Bcr-Abl displayed a profoundly, disorganized cytoskeleton when examined by phase contrast or immunofluorescence microscopy for actin [Salgia et al., 1997]. Interestingly, overexpression of Grb2 in these Bcr-Abl-expressing fibroblasts possessed an even greater number of filopodia and increased ruffling of the membrane, suggesting a role for Grb2 in accentuating the activating signals to the cytoskeleton.

To analyze the focal adhesion components of the cytoskeleton on a molecular level, Salgia and colleagues examined the status of tyrosine phosphorylation of focal adhesion proteins in cells expressing Bcr-Abl [Salgia et al., 1995]. A large number of cytoskeletal and focal adhesion proteins were constitutively tyrosine phosphorylated, a very similar pattern to what has been reported for v-Src transformed cells. Furthermore, in Bcr-Abl expressing cells the focal adhesion protein paxillin was constitutively associated with vinculin, focal adhesion kinase, tensin and talin. These data provide compelling evidence of Bcr-Abl's ability to potently disrupt normal protein-protein interactions and thereby signaling pathways emanating from the focal adhesions.

Bcr-Abl's potential to significantly impair the cytoskeleton is perhaps best exemplified by reports on the consequences of its physical association with actin. At its C- terminus Bcr-Abl possesses an F-actin binding domain [McWhirter and Wang 1993]. Association with actin, enhanced by an oligomerization domain contained in the first 63 amino acids of Bcr, retains Bcr-Abl in the cytoplasm and excludes it from the nucleus. Interestingly, Bcr-Abl mutants, which are defective in actin association, exhibit significantly reduced transforming efficiencies, as assessed by IL-3 dependence in a lymphoid cell line and growth in soft agar of Rat-1 fibroblasts.

Until recently no studies have specifically examined the connection between small GTP-binding proteins and regulation of the cytoarchitecture by Bcr-Abl. A recent report has shed some light on the role of Rac and its effect on motility [Skorski 1998]. In this analysis a dominant negative Rac (N17) was tested for its ability to reduce the hypermotility of Bcr-Abl expressing cells in an invasion chamber assay and in *in vivo* homing. These experiments demonstrated that N17Rac reduced the invasive potential of Bcr-Abl, and minimally reduced homing to the bone marrow. However, as previously mentioned, leukemogenesis is not characterized by invasion so while these findings are interesting, the relevance of these experiments to this disease is somewhat uncertain. A more instructive analysis on the effects of dominant negative Rac on Bcr-Abl-mediated hypermotility might have been through the use of time-lapse video microscopy, adhesion assays, and examination of cytoarchitectural changes.

Thesis Aims

This thesis has two major aims. The first aim is to expand our scientific understanding of the influence of activated Abl kinase and activated PI3-kinase in contributing to changes in the cytoarchitecture and adhesive properties in Bcr-Abl-expressing cells. The second major aim is to broaden our basic knowledge of the molecular mechanisms available to this oncogene through a detailed dissection of its interactions with the adaptor proteins Grb2, Grap and c-Cbl. These latter studies examine two basic questions. One, given the overall homology between the adaptor proteins Grb2 and Grap, do they bind to similar regions of the Bcr-Abl molecule and with similar affinities? Second, is there a specific requirement for Grb2 to bring c-Cbl into a macromolecular complex containing Bcr-Abl?

Together, I hope these findings not only elevate our general knowledge base of what is required for Bcr-Abl-mediated oncogenesis but also assist in the design of better therapies for patients with Bcr-Abl-induced diseases.

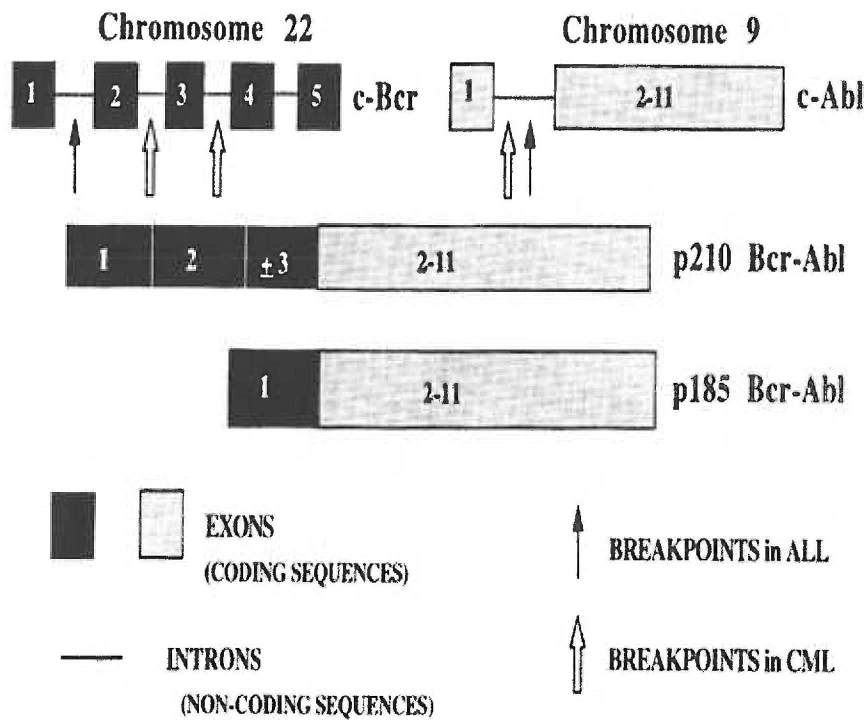

Figure 1

Kolibaba and Druker, 1997

Structure of c-Bcr (p160)

Figure 2

Adapted from Raitano et al. 1997

Structure of c-Abl (p145)

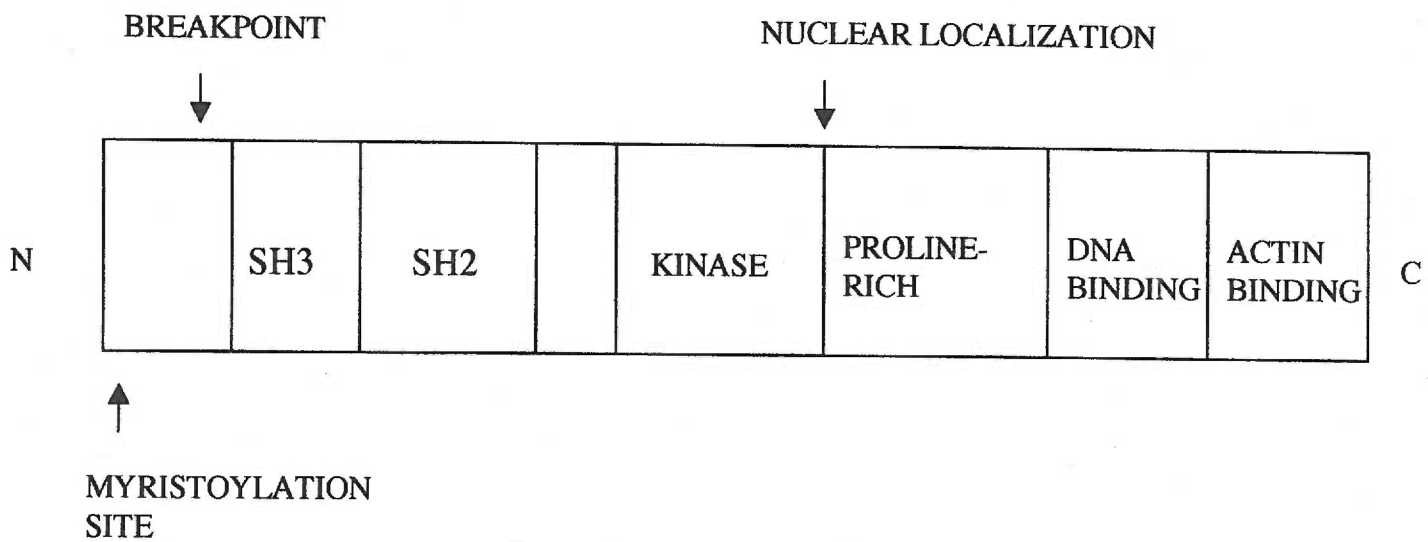

Figure 3

Adapted from Raitano et al. 1997

Structure of v-Abl (p160)

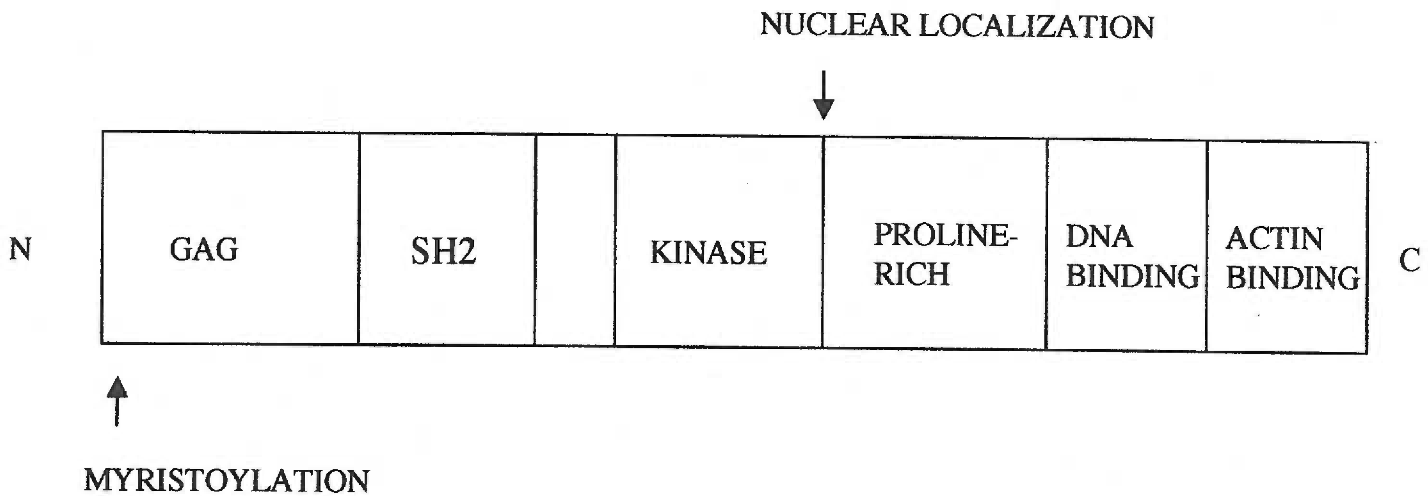

Figure 4

Adapted from Raitano et al. 1997

Note deletion of SH3 domain.

Structure of Bcr-Abl (p210)

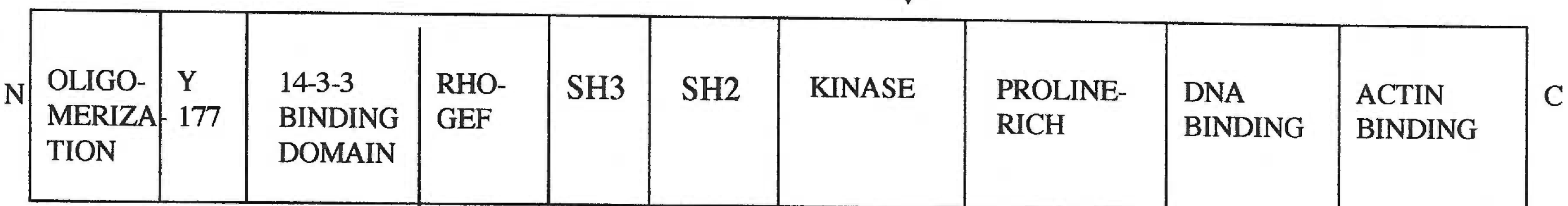

Figure 5

Adapted from Raitano et al. 1997

Note p185 Bcr-Abl lacks Rho-Gap

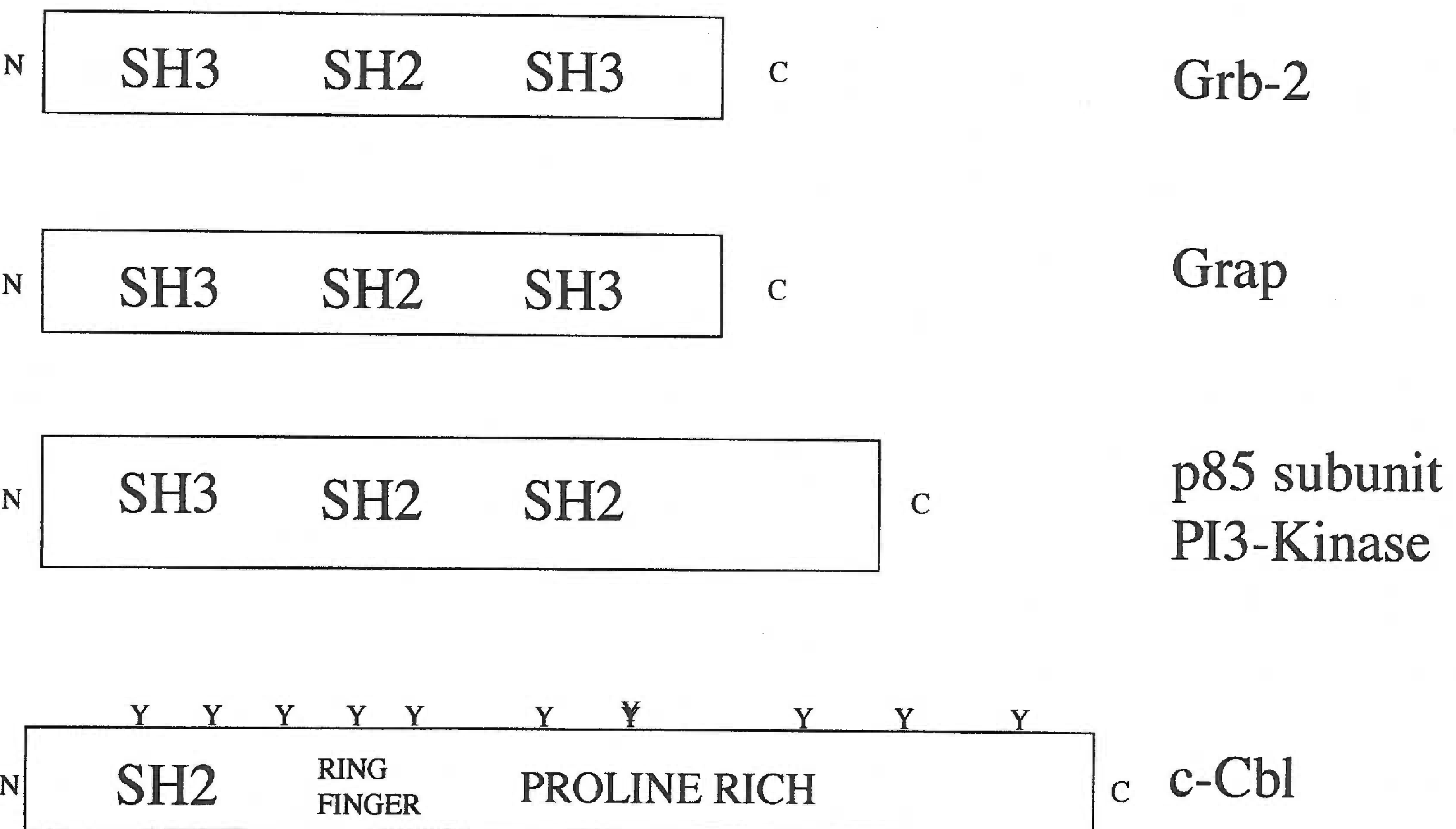

Figure 6

CHAPTER II

RESULTS

ABL Kinase and PI3-Kinase Linkage to the Cytoskeletal Defects in Bcr-Abl Transformed Cells

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Abstract

Chronic myelogenous leukemia (CML) is characterized by a massive expansion of myeloid progenitors and premature release of these cells into the bloodstream. Bcr-Abl, the causative abnormality of this disease, has been reported to increase proliferation, decrease apoptosis, and alter adhesive properties of myeloid progenitors. The altered adhesive properties have been postulated to contribute to the premature release of progenitor cells from the bone marrow. Further, Bcr-Abl is known to associate with and phosphorylate a variety of cytoskeletal proteins, suggesting a molecular mechanism for the induction of an adhesion defect. Using a fibroblast model of Bcr-Abl expression, significant defects in the cytoskeletal architecture can be observed. Specific changes include loss of stress fibers and focal adhesions and these alterations correlate with an adhesive defect. The contribution of Abl kinase and PI3- kinase to these abnormalities was investigated using specific inhibitors of the Abl and PI3-kinases. Our studies demonstrate that Bcr-Abl tyrosine kinase but not PI3-lipid kinase activity are required for the maintenance of cytoskeletal rearrangements resulting from Bcr-Abl expression. Further, inhibition of Abl kinase restored normal adhesive properties to the Bcr-Abl-expressing cells demonstrating the contribution of Bcr-Abl kinase activity to abnormal cytoskeletal function.

Introduction

Chronic myelogenous leukemia (CML) is characterized by a reciprocal translocation between the long arms of chromosomes 9 and 22 [Nowell and Hungerford 1960; Rowley 1973]. This gene rearrangement results in the production of a novel oncoprotein, p210Bcr-Abl, a constitutively active tyrosine kinase [Groffen et al., 1984; Heisterkamp et al., 1985; Ben-Neriah et al., 1986] One of the most prominent clinical features of CML is the accumulation of myeloid progenitors in the bloodstream. This is thought to arise from the premature release of progenitor cells from the bone marrow, possibly reflecting a defect in adherence. Bcr-Abl is known to induce multiple cytoskeletal abnormalities which may account for its ability to alter adhesion [Renshaw et al., 1995; Salgia et al., 1997; McWhirter and Wang 1993; Verfaillie et al., 1997].

Bcr-Abl is a cytoplasmic protein and associates with the cytoskeleton through a COOH terminal actin-binding domain [McWhirter and Wang 1993; Van Etten et al., 1994; Van Etten et al., 1989; Wetzler et al., 1993]. This subcellular localization and the tyrosine kinase activity of Bcr-Abl permit it to phosphorylate and to complex with specific intracellular signaling proteins such as PI3- kinase [Varticovski et al., 1991], CRKL [ten Hoeve et al., 1994; Oda et al., 1994; Nichols et al., 1994], and c-CBL [Andoniou et al., 1994; Sattler et al., 1996] as well as the focal adhesion proteins paxillin, vinculin, tensin and p125FAK [Salgia et al., 1995; Gotoh et al., 1995]. Salgia, et. al. demonstrated that Bcr-Abl-expressing cells have increased staining for filamentous actin and an enhanced rate of formation and retraction of actin-containing protrusions such as pseudopodia and filopodia [Salgia et al., 1997]. Further, Bcr-Abl-

phenotype. In contrast, Renshaw, et. al, have described a fibroblast cell line, P3T3, that are susceptible to transformation by p210Bcr-Abl. The Bcr-Abl expressing derivative of this cell line is anchorage independent for growth but remains growth factor independent, thus more closely resembling the phenotype seen in CML cells. Lastly, the significantly larger size of fibroblasts as compared to myeloid cells allows a more detailed analysis of cytoskeletal structure.

Materials and methods

Cells and Cell Culture

The Rat-1 and Rat-1 p185 fibroblast cell lines were generously provided by Charles Sawyers (UCLA). Rat-1 p185 is a derivative of Rat-1 fibroblasts transfected with and selected for expression of p185Bcr-Abl [Lugo and Witte 1989]. P3T3 fibroblasts, a subclone of NIH3T3 fibroblasts, were selected for susceptibility to transformation by Bcr-Abl [Renshaw et al., 1992] and were a kind gift of Jean Y. J. Wang. The P3T3 Bcr-Abl cells used in this study express the p210 form of Bcr-Abl. V-src transformed Rat-1 fibroblasts were provided by Karen Rodland (OHSU). All cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, L-glutamine and penicillin-streptomycin and cultured at $37^{O}C$ in 10% CO_2.

Inhibitors

CGP57148, an Abl tyrosine kinase inhibitor, was provided by E. Buchdunger (Novartis Pharm, Inc.). LY294002, a PI3-kinase inhibitor, was obtained from Calbiochem. Cells were incubated with either CGP57148 (1 μM) dissolved in sterile phosphate buffered saline (PBS), LY294002 (10 uM) dissolved in DMSO, or in DMSO of equivalent concentrations (vehicle control) for 4 hours prior to analysis by either immunofluorescence, phase microscopy, immunoblotting, or adhesion assays.

Immunoblotting

Six well plates containing $1x10^5$ cells were lysed in a buffer containing 20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 μg/mL aprotinin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylfluoride. Lysates were precleared by centrifugation (6000 x g) for 10 minutes and protein concentrations determined using the Bradford assay (BioRad). For immunoblotting, 100 μg of each sample was boiled in SDS sample buffer and separated on a 10% SDS-PAGE gel. Proteins were transferred onto PVDF (Immobilon-P, Millipore) membranes in a buffer containing 25 mM Tris, 192.5 mM glycine, 20% MeOH for 4 hours at 0.45 Amps. Following transfer, residual binding sites on the membrane were blocked with either 5% non-fat dry milk or 2.5% gelatin (BioRad) in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) for 60 min at 25^oC. Membranes were washed once with TBST (TBS containing 0.05% Tween-20 (USB)) and incubated overnight at room temperature with either an anti-phosphotyrosine antibody (4G10) at 1.5 μg/ml in TBST, or anti-AKT or anti-phospho-AKT rabbit serum (1:1000) purchased from New England Biolabs. Tyrosine phosphorylated proteins were detected using an alkaline phosphatase conjugated

anti-mouse antibody. AKT and phospho-AKT proteins were detected using an alkaline phosphatase conjugated anti-rabbit antibody. Both immunoblots were developed with NBT/BCIP (Promega) as described [Kanakura et al., 1990].

Immunofluorescence and phase microscopy

Cells plated onto glass coverslips in 6-well dishes were allowed to adhere overnight. After treatment with either LY294002 or CGP57148, fibroblasts were fixed with picric acid paraformaldehyde (PAPF), followed by three washes with PBS. After 3 washes with PBS/.1% saponin the coverslips were incubated with an anti-phosphotyrosine (4G10) monoclonal antibody at a 1:100 dilution for one hour at room temperature. Coverslips were then washed three times with PBS/.1% saponin and incubated with both FITC conjugated anti-mouse antibody (Cappell, 1:200) and rhodamine-phalloidin (Molecular Probes 1:100) for one hour at room temperature. Coverslips were then washed three times with PBS/.1% saponin and mounted onto microscope slides using the Slowfade Light Antifade Kit (Molecular Probes). For immunofluorescence, images were viewed with a Zeiss Axiophot microscope at a 40x magnification and photographed using Kodak Ektachrome 400 film. For phase microscopy, a Zeiss IM photomicroscope was employed at a 32x magnification.

Cell adhesion assays

To evaluate cell adhesion, 5 x 10^5 fibroblasts were plated in triplicate in 6-well cluster plates in DMEM supplemented with 10% calf serum. Cells were then treated with either 10 uM LY294002, 1 uM CGP57148, or vehicle control for 4 hours at 37° and

10% CO_2. Following this incubation, cells were washed three times with PBS to remove all nonadherent cells, trypsinized and counted. The following formula was used to calculate the percent adhesion: the number of fibroblasts adhering to the plate after trypsinizing divided by the total input of fibroblasts ($5x10^5$) X 100.

Results

Bcr-Abl expression alters the cytoarchitecture of Rat-1 and P3T3 fibroblasts

Previous investigations of myeloid cells expressing Bcr-Abl have demonstrated increased F-actin staining , however, given the small size of these cells, detailed analysis of the cytoskeletal architecture has not been possible [Salgia et al., 1997]. For this reason, two fibroblast cell lines expressing Bcr-Abl were used for our analysis: P3T3 and Rat-1 fibroblasts. P3T3 fibroblasts, a subclone of NIH3T3 fibroblasts, were selected for susceptibility to transformation by Bcr-Abl [Renshaw et al., 1992]. Both the Rat-1 p185 and P3T3 Bcr-Abl cell lines display anchorage independent growth but not factor independence [Renshaw et al., 1995; Lugo and Witte 1989].

To characterize the phenotypic effects of Bcr-Abl expression on the cytoskeleton, phase contrast microscopy of Bcr-Abl transformed Rat-1 and P3T3 fibroblasts was performed (Figure 1A and 2A, top panels). In contrast to their parental counterparts, both Bcr-Abl transformed cell lines were morphologically altered. The P3T3 Bcr-Abl cells were rounder, smaller and adhered less avidly to the culture flask, while the Rat-1 p185 fibroblasts were spindly and lacked the “cobblestone” appearance of their

parental counterparts. To investigate whether structural abnormalities could account for these phenotypic differences, actin filaments and focal adhesions of these cells were examined by immunofluorescence (Figures 1A and 2A, middle and bottom panels). As compared to parental cells, Bcr-Abl expressing cells appeared somewhat brighter when stained with rhodamine phallodin for F-actin (Figures 1A and 2A, middle panels). This intensity of staining could reflect differences in cell shape in these cells. However, Bcr-Abl transformed fibroblasts contained considerably fewer actin stress fibers as detected using rhodamine-phalloidin (Figures 1A and 2A, middle panels). When examined by indirect immunfluorescence with an anti-phosphotyrosine monoclonal antibody, transformed cells had markedly fewer focal adhesions (Figures 1A and 2A, bottom panels). Identical results were obtained with an antibody directed against vinculin (data not shown). Further, there was a pronounced change in the staining pattern of focal adhesions with the anti-phosphotyrosine and anti-vinculin antibodies from a punctate distribution in the parental fibroblasts to a highly diffuse pattern in Bcr-Abl transformed cells (Figures 1A and 2A, bottom panels and data not shown). It should be noted that the parental Rat-1 fibroblasts contained fewer focal adhesions than the P3T3 cells, however, in both cells lines, Bcr-Abl expression consistently altered the number and pattern of the focal adhesions. These results indicate that Bcr-Abl alters the assembly of a normal cytoskeletal network by disrupting actin stress fiber formation and reducing the number of focal contacts.

Evaluation of the role of Abl and PI3-kinase in contributing to the cytoskeletal abnormalities in Bcr-Abl-expressing cells

To elucidate the role of two major signaling pathways that may contribute to the morphologic defect in the cytoskeleton of Bcr-Abl transformed fibroblasts, cells were treated with either a potent PI3-kinase inhibitor, LY294002 [Okada et al., 1995] or the Abl protein tyrosine kinase inhibitor, CGP57148 [Druker et al., 1996]. CGP57148 has been shown to inhibit the Abl, platelet-derived growth factor (PDGF) receptor, and c-kit tyrosine kinases with an IC_{50} for Abl of 0.3 µM. No other kinases tested were inhibited by this compound including epidermal growth factor receptor, insulin receptor, insulin-like growth factor-1 receptor, fibroblast growth factor receptor, colony stimulating factor-1 receptor, FLT-3, src family members, csk, syk, or Jak kinases [Druker et al., 1996]. To confirm the specificity of this inhibitor, Rat-1 cells transformed by v-Src were treated with CGP57148. No changes in morphology as assessed by phase microscopy and immunofluorescence were observed (Figure 3).

Parental Rat-1 and P3T3 fibroblasts and their Bcr-Abl-expressing derivatives were treated with CGP57148 and LY294002. In parental P3T3 fibroblasts no morphological changes were observed with either inhibitor (Figure 4). Identical results were obtained with parental P3T3 cells (data not shown). In contrast, CGP57148 treatment of the Bcr-Abl expressing cells partially to fully reverted cells to their parental phenotype (Figures 1B and 2B). By phase contrast microscopy, Rat-1 p185 fibroblasts treated with CGP57148 assumed a more cuboidal, "cobblestone" shape similar to the appearance of the parental cells (compare Figures 1A and B, top panels). LY294002 treatment did not result in any reversion to the parental phenotype (compare Figures

1A and B, top panels). Most prominently, CGP57148 treatment of Rat-1 p185 cells resulted in increased numbers of actin stress fibers and a significant increase in focal adhesions (Figure 1B, middle panels) whereas the cytoskeleton in the LY294002 treated cells was unchanged (Figure 1B, bottom panels).

By phase microscopy, CGP57148 treatment of P3T3 Bcr-Abl cells resulted in flattening and extending of the cells towards the parental morphology (compare Figures 1 and 2B, top panels). The effects of treatment with LY294002, as assessed by phase microscopy, were negligible (compare Figures 1 and 2B, top panels). Furthermore, cells treated with CGP57148 but not LY294002 treatment resulted in restoration of qualitatively normal numbers of focal adhesions (Figure 2B, bottom panels). Rhodamine-phallodin staining also showed that CGP57148 treatment of the P3T3 Bcr-Abl fibroblasts resulted in a near-normal pattern and number of stress fibers (compare Figures 1B and 2B, middle panels). LY294002 treatment of these cells had no effect on restoring stress fibers or increase the amount of cortical actin (compare Figures 1B and 2B, middle panels). These results underscore the importance of Bcr-Abl tyrosine kinase in orchestrating the disruption of the cytoarchitecture.

CGP57148 treatment decreases levels of tyrosine phosphorylation in Bcr-Abl transformed fibroblasts to parental levels

Having described the cytoskeletal changes induced by treatment of Bcr-Abl-expressing cells with CGP57148 and LY294002, experiments were performed to confirm the biochemical effects of these compounds. It is well established that the

increased tyrosine kinase activity of the Bcr-Abl oncogene results in a significantly elevated number and intensity of tyrosine phosphorylated proteins. To assess the effects of CGP57148 and LY294002 on tyrosine phosphorylation, whole cell lysates of parental and Bcr-Abl transformed Rat-1 and P3T3 cells were immunoblotted with an anti-phosphotyrosine antibody (Figures 5A and B). As expected, neither inhibitor altered the pattern or intensity of tyrosine phosphorylation in the parental cell line, and LY294002 did not have any discernible effect on tyrosine phosphorylation in the Bcr-Abl-expressing cell line. However, in both the Rat-1 p185 and P3T3 Bcr-Abl cell lines, CGP57148 treatment resulted in a significant reduction in the pattern and intensity of tyrosine phosphorylation, to a level comparable to that of parental cells (Figures 5A and data not shown). No change in the pattern of tyrosine phosphorylation was observed in a v-src transformed Rat-1 cell line treated with CGP57148 (Figure 5C), confirming the specificity of this compound for Abl tyrosine kinase.

LY294002 inhibits PI3-kinase activity in Bcr-Abl transformed fibroblasts

To analyze the inhibition of PI3-kinase activity by LY294002, immunoblotting for phospho-AKT, a downstream effector of PI3-kinase, was performed on parental and Bcr-Abl transformed Rat-1 and P3T3 cells. This method was chosen over PI3-kinase assays because LY294002 binds reversibly to PI3-kinase and hence, inhibition cannot be accurately assessed. In this assay, whole cell lysates treated with LY294002 significantly reduced activated, phosphorylated, AKT levels in both parental and Bcr-Abl transformed cells (Figure 6). As expected, CGP57148 also inhibited phospho-AKT, however to a lesser extent than cells treated with the PI3-kinase

inhibitor. Endogenous AKT protein levels remained unchanged(Figure 6). These experiments confirm the biochemical inhibition of PI3-kinase activity with LY294002 and suggest that reducing activated PI3-kinase is not sufficient to rescue the transformed morphology of Bcr-Abl-expressing fibroblasts.

CGP57148 treatment increases adhesion of Bcr-Abl transformed fibroblasts

One hypothesis which may account for the accumulation of myeloid precursors in the blood of patients with CML is a defect in adherence of these cells in the bone marrow [Renshaw et al., 1995; Salgia et al., 1997; McWhirter and Wang 1993; Verfaillie et al., 1997]. To examine the functional consequences of Bcr-Abl expressing cells treated with LY294002 or CGP57148, we performed short-term attachment assays on cells treated with either compound. As illustrated in Figure 7, untreated Bcr-Abl-expressing P3T3 and Rat-1 cells adhered less avidly as compared to their parental counterparts. Furthermore, in the parental cell lines, neither inhibitor significantly altered the number of adherent cells. In contrast, in the Bcr-Abl-expressing fibroblasts CGP57148 substantially increased the percent of cells remaining attached after a 4 hour incubation period. A small increase in attachment of cells treated with LY294002 above the control was also observed in the P3T3 Bcr-Abl-expressing cells. These results provide further support that activated Abl tyrosine kinase activity plays an important role in preventing cells from firmly anchoring to a substratum, which correlates with the diminished number of both actin stress fibers and focal adhesions observed in these cells.

Discussion

Previous studies using myeloid cells have demonstrated that expression of Bcr-Abl results in increased F-actin staining [Renshaw et al., 1997]. However, due to their small size, it has not been possible to determine the precise organization of the F-actin in these cells. Specifically, it could not be determined whether the cytoskeleton has a normal or abnormal architecture. Using fibroblasts which are considerably larger than myeloid cells, we have been able to demonstrate that Bcr-Abl expression results in a significant perturbation of the cytoskeletal architecture. Consistent with the morphological changes typical of other transformed cells, Bcr-Abl-expressing cells contained decreased numbers of stress fibers and fewer focal adhesions. This suggests that although Bcr-Abl expressing cells may have an increased content of F-actin, its organization in these cells is distinctly abnormal.

The consequences of these cytoskeletal abnormalities may be relevant to the clinical finding of premature release of myeloid progenitors into the bloodstream in CML patients. CML progenitors are known to express normal levels of ß1 integrins, however, these cells demonstrate significantly reduced ß1 integrin adhesion to fibronectin [Verfaillie et al., 1992; Gordon et al., 1987]. Engagement and clustering of ß1 integrins results in association of the cytoplasmic tail of the ß1 integrins with cytoskeletal proteins such as αactinin, talin, vinculin, tensin and paxillin, and induces the formation of focal adhesions [Burridge et al., 1988; Clark and Brugge 1995]. Many of these same proteins are known to be substrates of Bcr-Abl and present in a complex with Bcr-Abl [Salgia et al., 1995; Salgia et al., 1996]. Our data demonstrates

cells revert to near-normal, suggesting that a kinase-independent effect of Bcr-Abl is possible. This could be due to the ability of Bcr-Abl to complex with focal adhesion proteins, inhibiting or disrupting their normal function.

PI3-kinase lipid products are known to be involved in a variety of signaling pathways, including pathways regulating the organization of the cytoskeleton. Alterations in the cytoskeleton and adhesion induced by several receptor tyrosine kinases, a G protein-coupled receptor and integrin ligation have been demonstrated to be dependent on activation of PI3- kinase [Hawkins et al., 1995; Kotani et al., 1994; Wennstrom et al., 1994; King et al., 1997; Ma et al., 1998]. Further, expression of a constitutively active PI3-kinase induces actin reorganization in the form of Rac-mediated lamellipodia and focal complexes, and Rho-mediated stress fibers and focal adhesions [Reif et al., 1996].

This presents a paradox for in Bcr-Abl-expressing cells PI3-kinase is activated, yet stress fibers and focal adhesions are significantly decreased. Therefore, activation of PI3-kinase in Bcr-Abl expressing cells is not sufficient to induce either stress fibers or focal adhesions. Thus, our data, from LY294002-treated cells, is consistent with the existence of a PI3-kinase independent pathway that results in Rho activation leading to stress fiber formation. Possible explanations for this apparent contradiction are that even though PI3-kinase is activated, it may be inappropriately localized in Bcr-Abl transformed cells, unable to access its lipid substrates. Similarly, Bcr-Abl may sequester PI3-kinase thereby preventing it from activating or inhibiting downstream effectors that regulate cytoskeletal organization. Lastly, Bcr-Abl may activate or inhibit other pathways that counteract and predominate over PI3-kinase's effects on the

cytoskeleton. Thus, we propose that in Bcr-Abl expressing cells the dominant pathway governing the overall regulation of the cytoskeleton is activated Abl, rather than PI3-kinase. This, however, does not preclude PI3-kinase from playing a significant role in other signaling events such as cell survival, perhaps through the phsophorylation of BAD. Nevertheless, our data supports a negligible role for PI3-kinase in contributing to the cytoskeletal architecture we have described in two Bcr-Abl transformed cell lines.

While the mechanism whereby PI3-kinase results in cytoskeletal alterations remains unknown, the fibroblast model used in these studies should be of use in further dissecting the signaling pathways involved in the Bcr-Abl-induced cytoskeletal abnormalities. Nevertheless, our studies demonstrate that Bcr-Abl kinase activity but not PI3-kinase activity are required for the induction of cytoskeletal changes resulting from Bcr-Abl expression in a pathway leading to these cytoskeletal rearrangements.

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Figure Legends

Figure 1. A. Bcr-Abl expression in Rat-1 fibroblasts results in significant morphological changes. Top panel. Phase microscopy of Rat-1 (parental) and Rat-1 p185 cells . Middle panel. Rhodamine-phalloidin staining of actin stress fibers in fixed Rat-1 and Rat-1 p185 cells. Bottom panel. Phosphotyrosine staining of focal adhesions in Rat-1 and Rat-1 p185 cells. Bar represents 10 μm.

B. Treatment with the Abl kinase inhibitor, CGP57148 but not the PI3-kinase inhibitor, LY294002, results in reassembly of the normal cytoarchitecture in Bcr-Abl expressing fibroblasts. Phase microscopy (top panel), rhodamine-phalloidin staining (middle panel) and phosphotyrosine staining (bottom panel) of Rat-1 p185 cells treated with either CGP57148 (10 μM) or LY294002 (10 uM) for 4 hours. Bar represents 10 μm.

Figure 2. P3T3 cells expressing Bcr-Abl were analyzed as in Figure 1. A. Top panel. Phase microscopy of P3T3 (parental) and P3T3 Bcr-Abl cells. Middle panel. Rhodamine-phalloidin staining of P3T3 and P3T3 Bcr-Abl cells. Bottom panel. Phosphotyrosine staining of focal adhesions in P3T3 and P3T3 Bcr-Abl. Bar represents 10 μm.

B. Phase microscopy (top panel), rhodamine-phalloidin staining (middle panel) and phosphotyrosine staining (bottom panel) of Rat-1 p185 cells treated with either CGP57148 (10 µM) or LY294002 (10 uM) for 4 hours. Bar represents 10 µm.

Figure 3. Treatment with the Abl kinase inhibitor CGP57148 does not alter the morphology of v-Src transformed Rat-1 fibroblasts. (A) Phase microscopy of cells treated with or without CGP57148. (B) Cells were fixed and rhodamine-phalloidin staining of actin stress fibers was performed. (C). Phosphotyrosine staining of focal adhesions. Bar represents 10 µm.

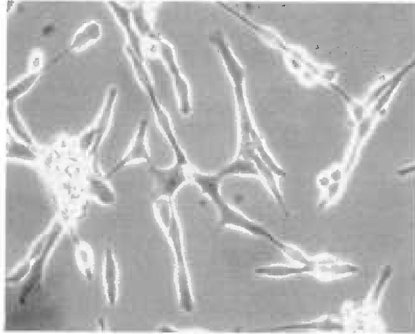
Figure 4. Treatment of parental P3T3 fibroblasts with CGP57148 does not alter overall morphology, extent of stress fiber formation, or focal adhesions. Cells were treated with CGP57148 (10 µM) and then either viewed by phase microscopy (A), or stained with rhodamine phalloidin (B) or an anti-phosphotyrosine antibody (C) and examined by immunofluorescence. Bar represents 10 µm.

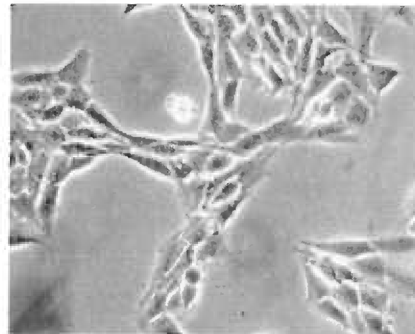
Figure 5. Inhibition of tyrosine phosphorylation by CGP57148 in Bcr-Abl transformed fibroblasts. Untreated cells or cells treated with CGP57148 (10 µM) or LY294002 (10 uM) for 4 hours, were analyzed by anti-phosphotyrosine immunoblotting. Molecular weight markers are indicated on the left. (A) Approximately 100 µg of whole cell lysates (WCL) of Rat-1(parental) and Rat-1 p185 Bcr-Abl or (B) P3T3 and P3T3 Bcr-Abl were assessed.(B) WCL of Rat-1 v-Src fibroblasts untreated or treated with CGP57148.

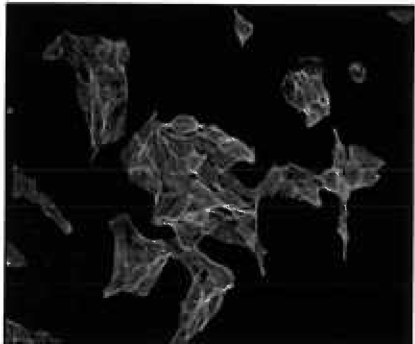
Figure 6. Inhibition of phosphorylated AKT in parental and Bcr-Abl expressing fibroblasts. Phosphorylated Akt (Top) and total Akt (Bottom) levels of whole cell lysates were assessed in Rat-1, Rat-1 p185, P3T3 and P3T3 Bcr-Abl fibroblasts after treatment with either CGP57148 (1 μM), LY294002 (10 uM), or vehicle control for 4 hours.

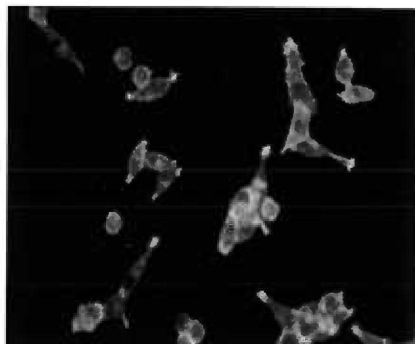
Figure 7. Increased adhesion of Bcr-Abl transformed fibroblasts following treatment with CGP57148. Cells were trypsinized, plated, and then treated with either 1 um CGP57148, 10 uM LY294002 or vehicle control and tested for attachment after 4 hours. (A) Parental P3T3 and Bcr-Abl P3T3 fibroblasts. (B) Parental Rat-1 and Rat-1 p185 fibroblasts. Results are the mean and standard deviation from three experiments.

Rat-1 **Rat-1 p185$^{bcr\text{-}abl}$**









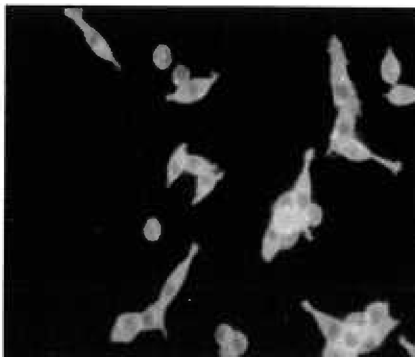
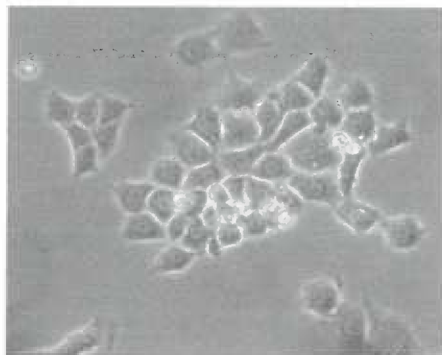
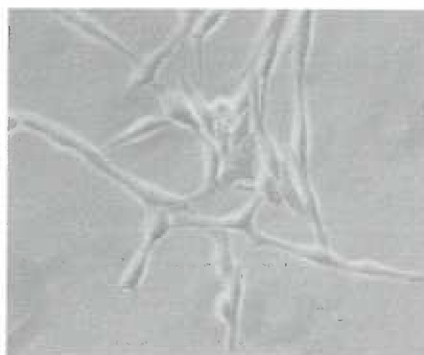


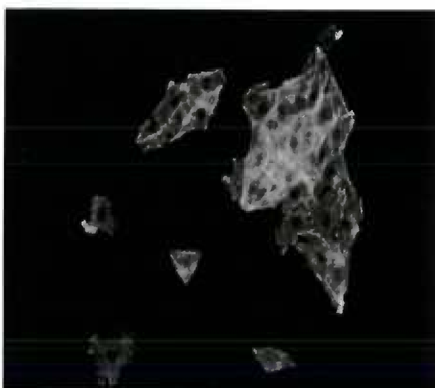
Figure 1A

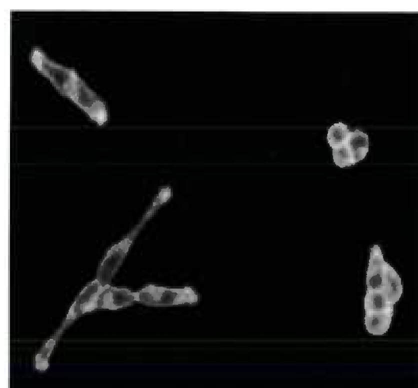
Rat-1 p185$^{bcr\text{-}abl}$

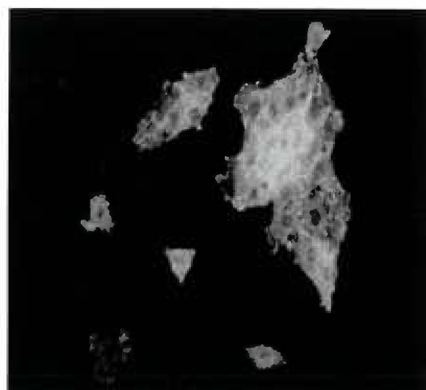
CGP57148 **LY294002**











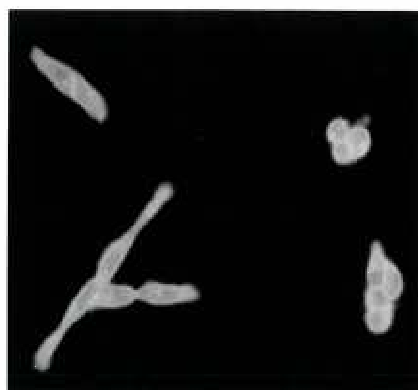


Figure 1B

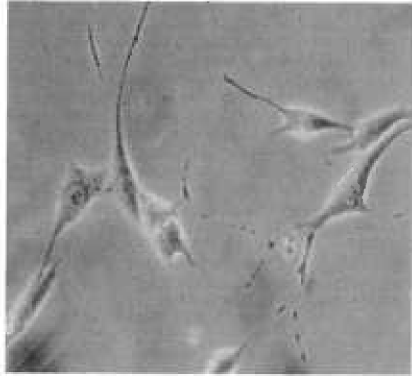
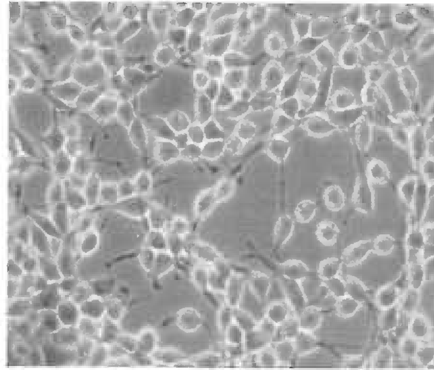
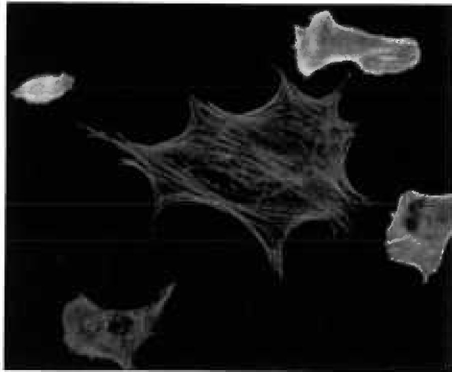
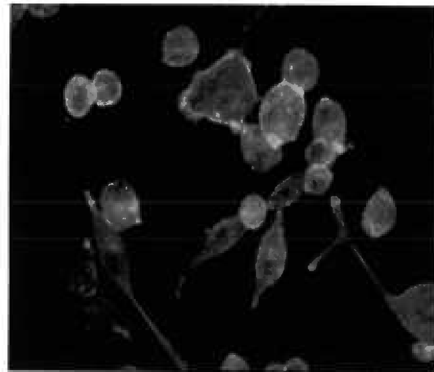
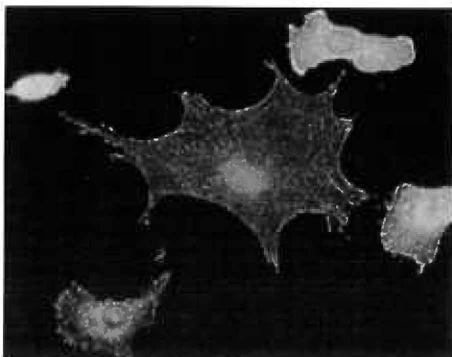
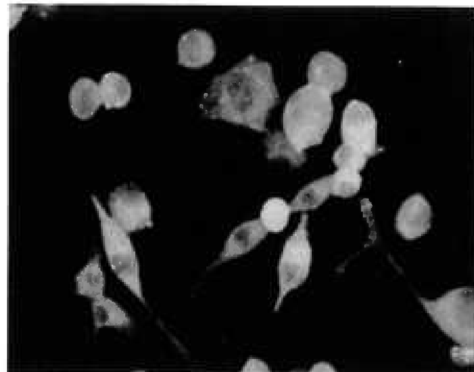
P3T3	P3T3 p210$^{bcr\text{-}abl}$
	
	
	

Figure 2A

P3T3 Bcr-Abl

CGP57148 | LY294002

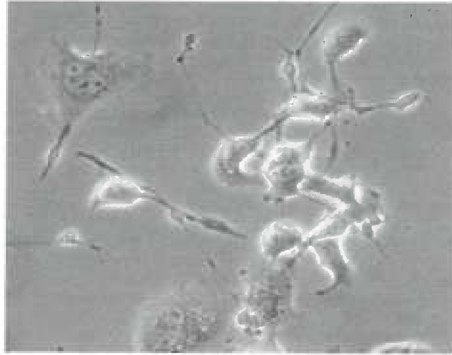
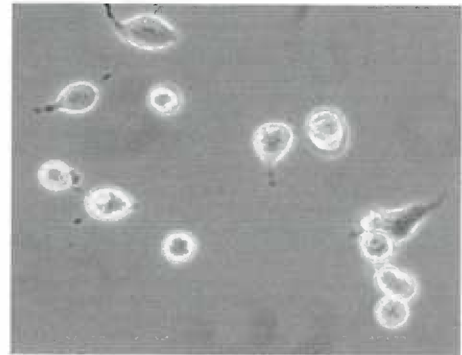
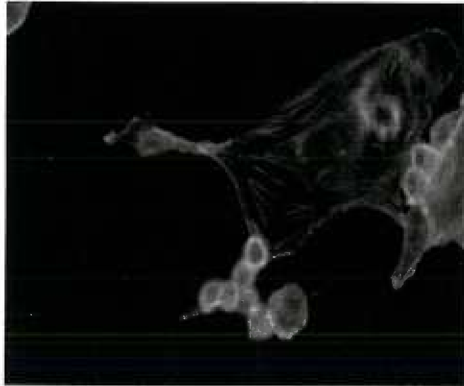
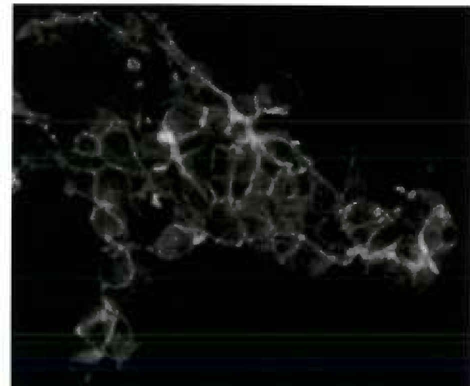
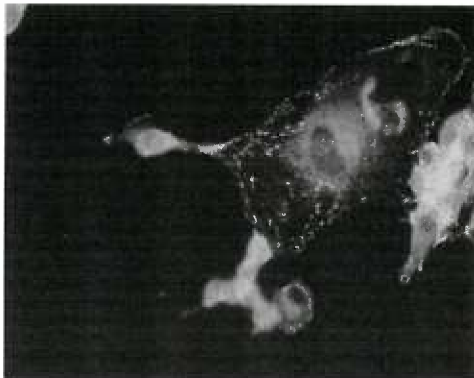
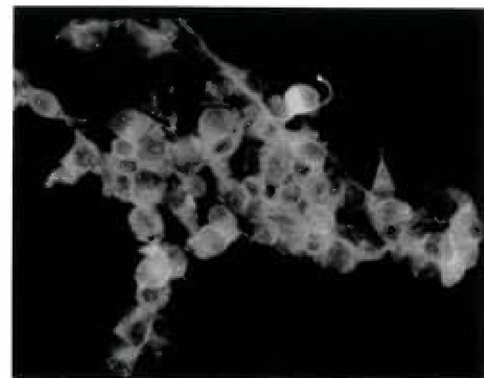
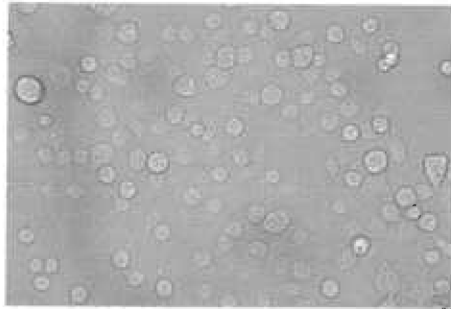
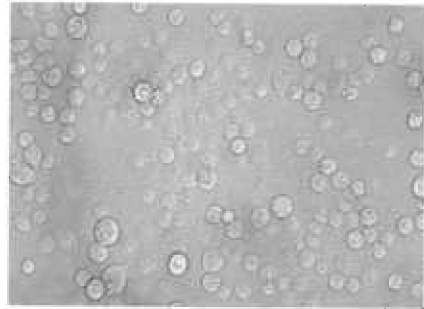
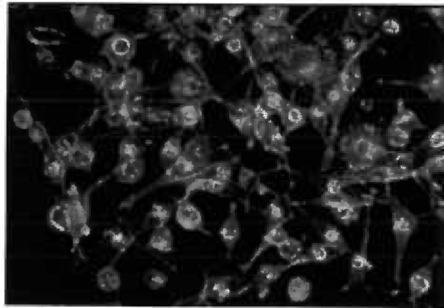
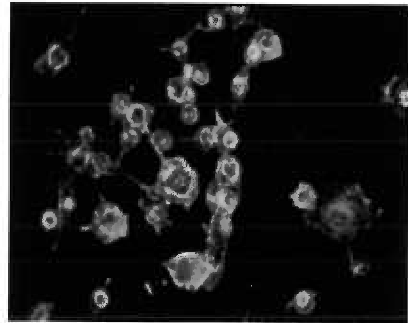







Figure 2B

Rat-1 p60$^{v\text{-}src}$

Control | **CGP57148**

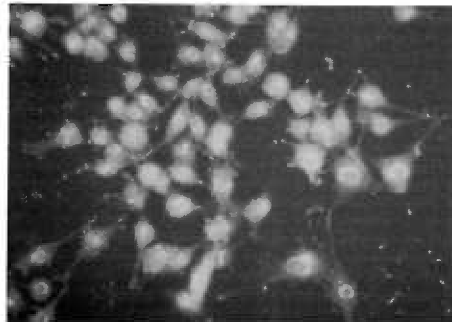
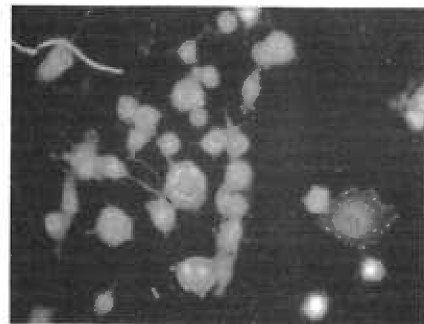



Figure 3

Rat-1

CGP57148 LY294002

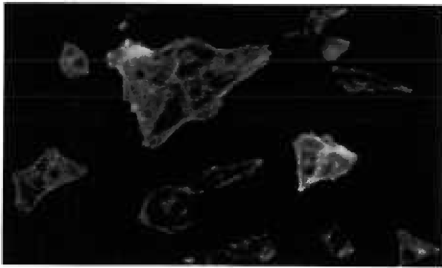
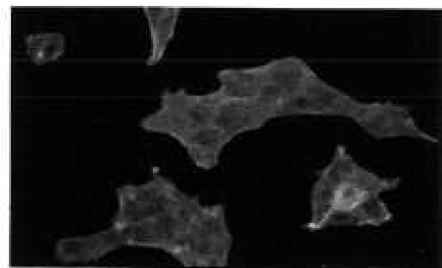
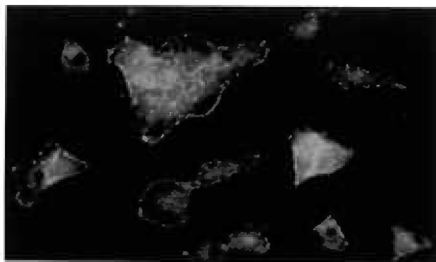
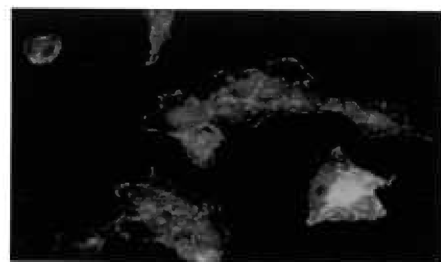





Figure 4

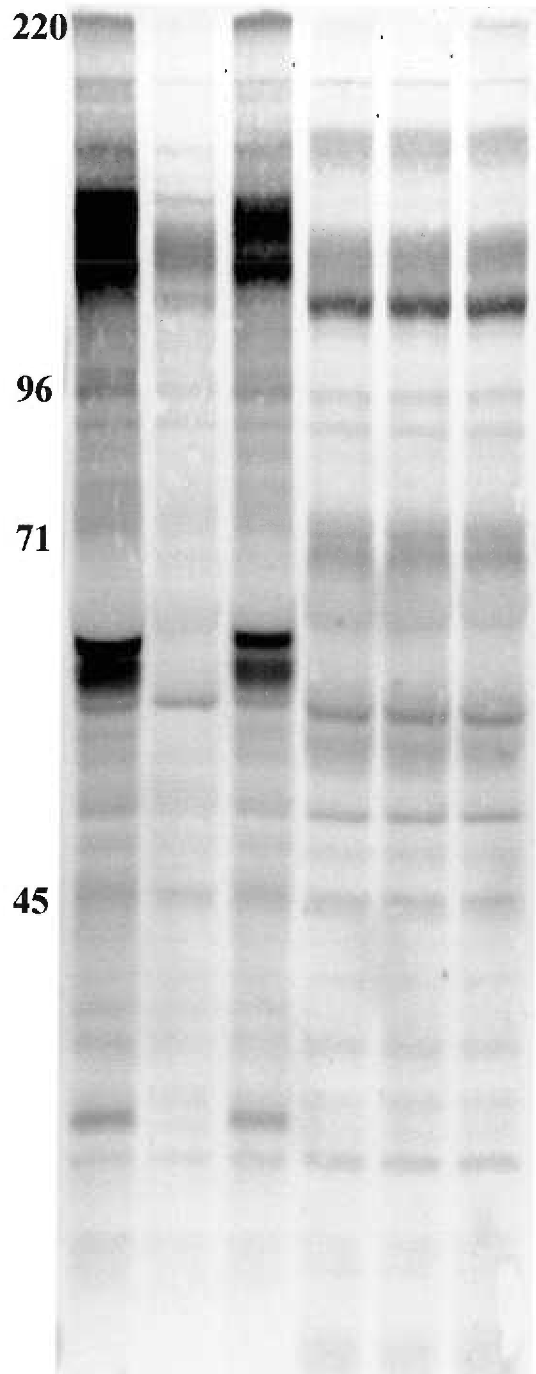
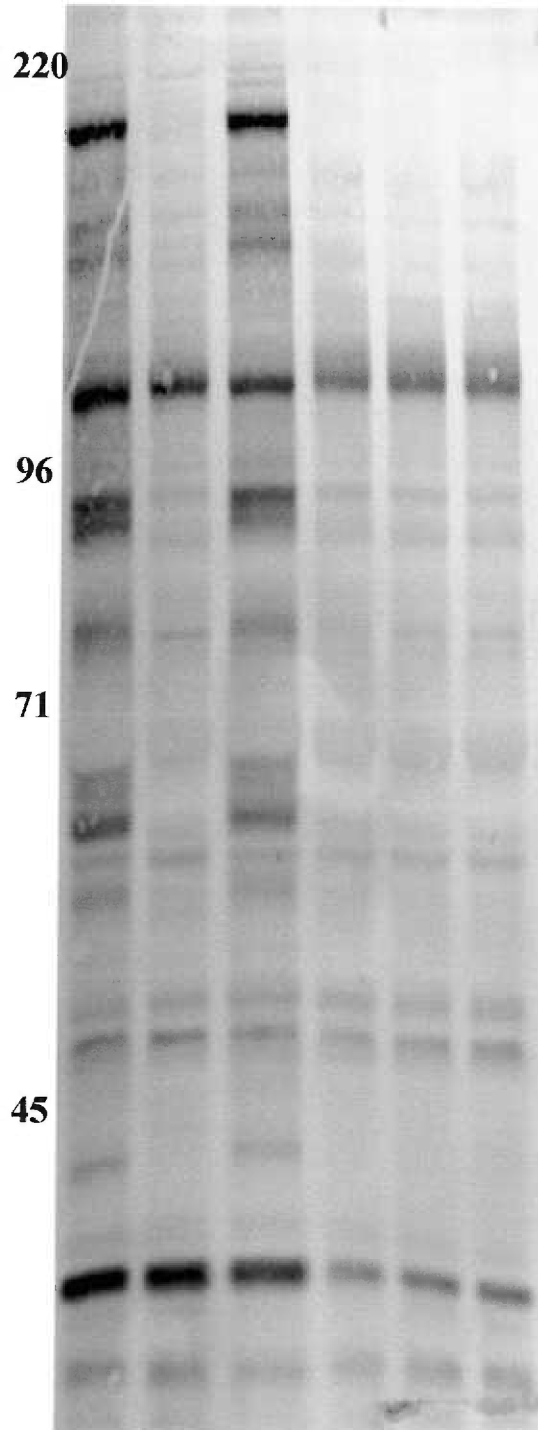


Figure 5A

P3T3 p210$^{bcr\text{-}abl}$ P3T3

Ctl CGP LY Ctl CGP LY



WCL
Blot: Anti-pTyr

Figure 5B

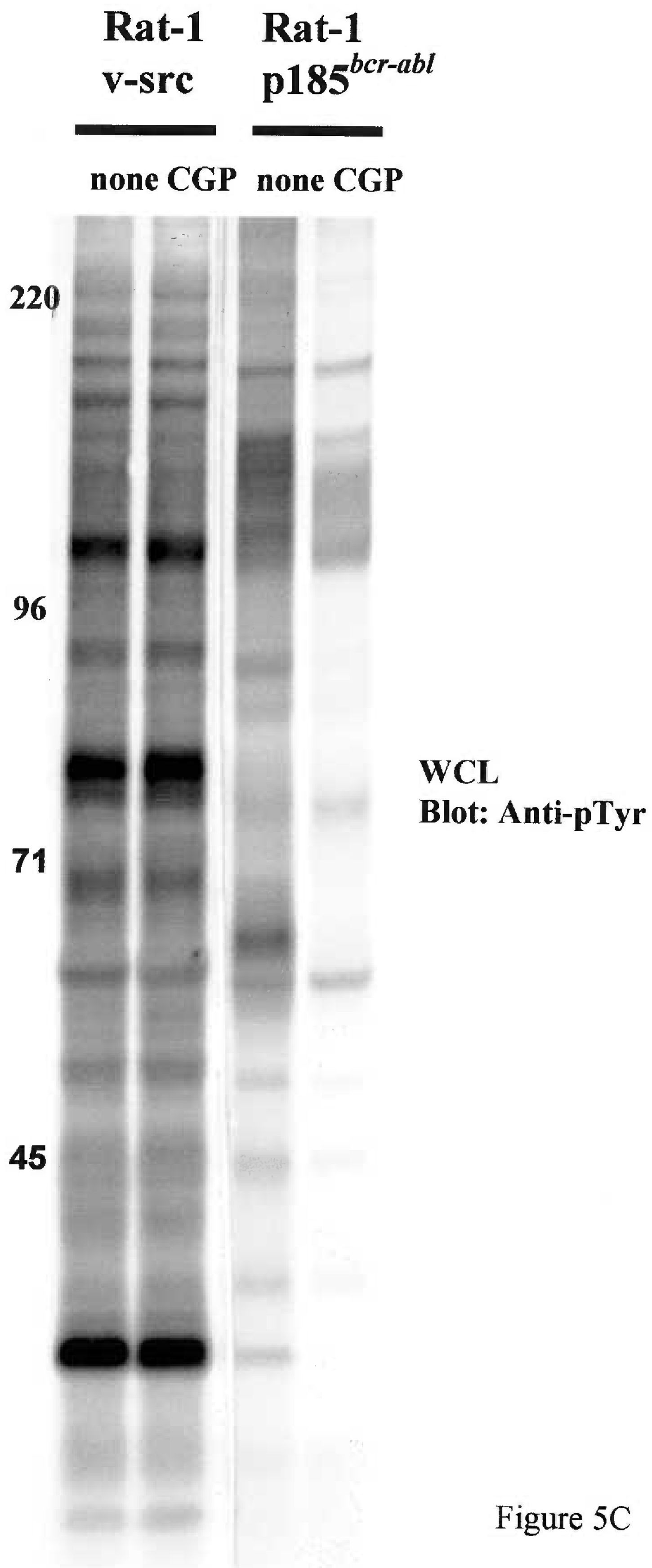


Figure 5C

	Rat-1 p185			Rat-1			P3T3 p210			P3T3		
Phospho-Akt												
Akt												
CGP57148	-	+	-	-	+	-	-	+	-	-	+	-
LY294002	-	-	+	-	-	+	-	-	+	-	-	+
Vehicle Control	+	-	-	+	-	-	+	-	-	+	-	-

Figure 6

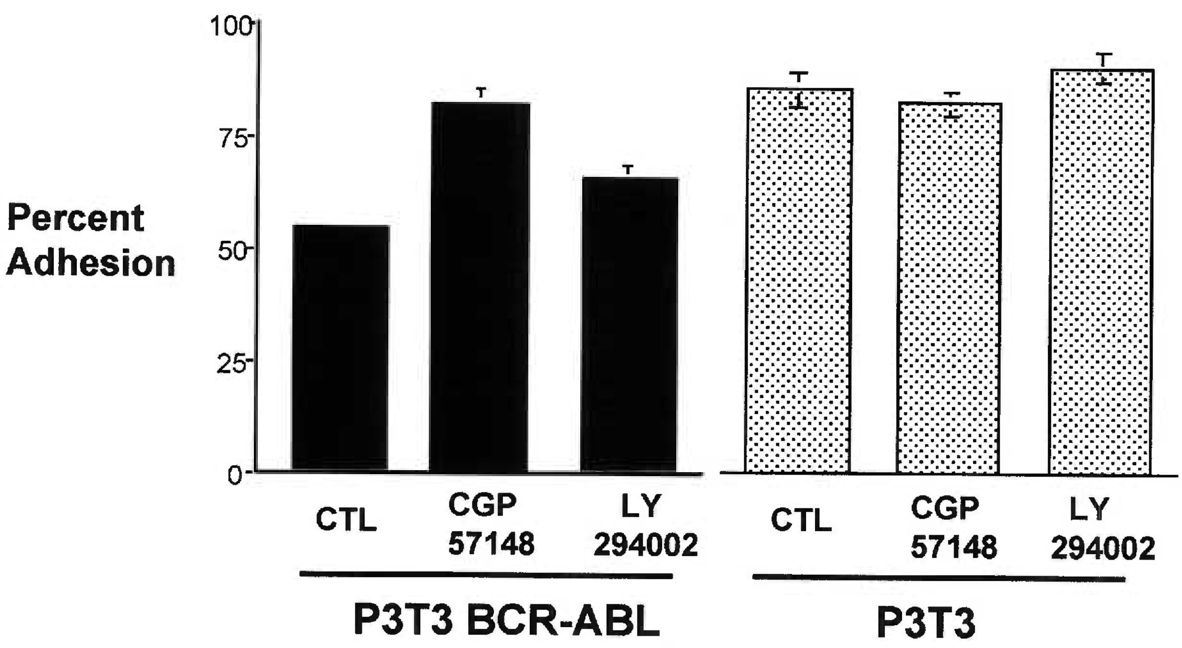


Figure 7A

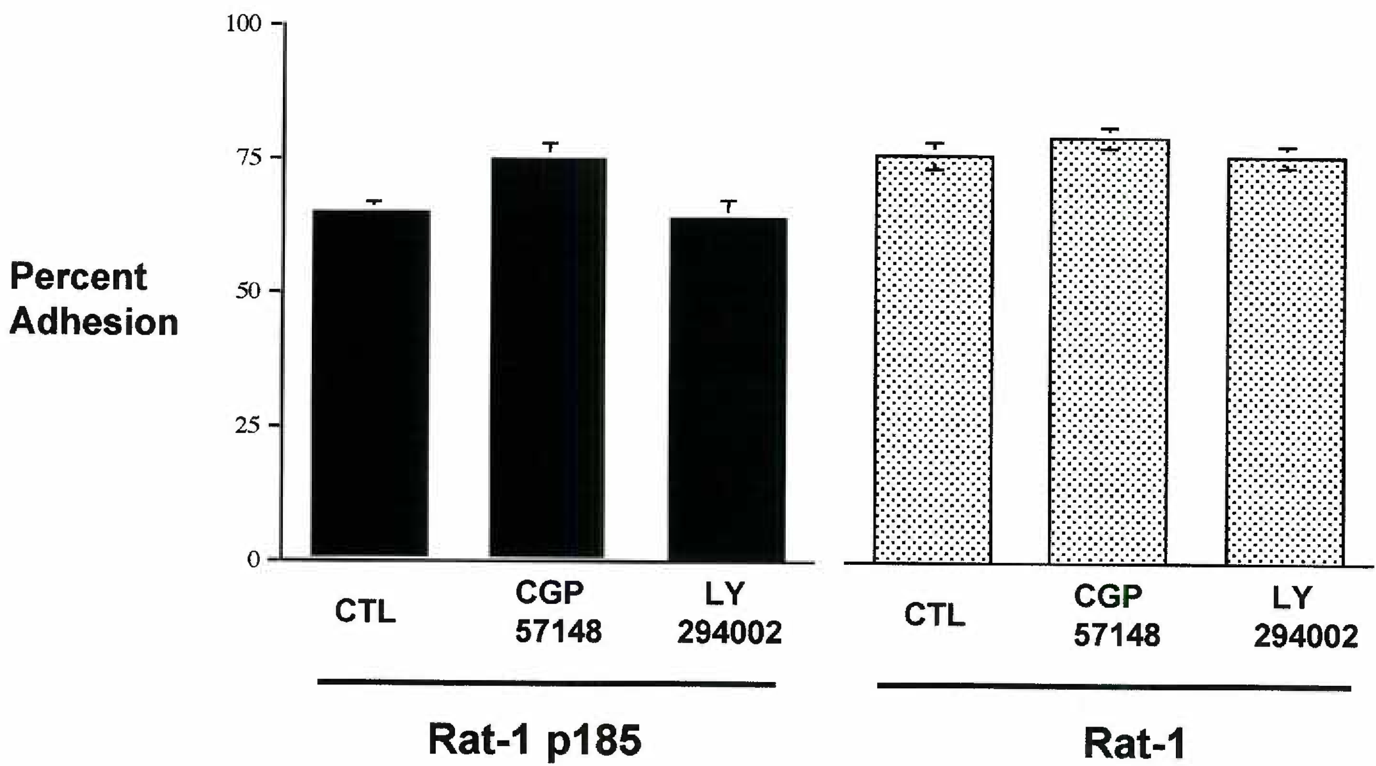


Figure 7B

Abstract

Grbw2 and Grap (Grb2 related adaptor protein) are highly homologous, SH3-SH2-SH3 domain-containing adaptor proteins. To better understand their potential physiological relevance in chronic myelogenous leukemia (CML), a comparative analysis of their interactions with Bcr-Abl, the causative agent of this disease was performed. An initial examination of expression in myeloid cells from patient samples demonstrated equivalent levels of Grb2 and Grap. In a yeast two-hybrid analysis, both Grb2 and Grap associate directly with Bcr-Abl at tyrosine 177. In Bcr-Abl expressing cells, Grap, like Grb2, associated with Bcr-Abl, as determined by coimmunoprecipitation. To investigate the relative affinities of Grb2 and Grap for Bcr-Abl, a split-hybrid analysis, where disruptions of protein-protein interactions can be selected, was performed. In these experiments, Grb2 disrupted the interaction of Grap with Bcr-Abl. However, Grap could not disrupt the interaction between Grb2 and Bcr-Abl, suggesting Grb2 has a greater affinity. To quantitate these interactions, surface plasmon resonance and GST-binding assays were performed. In these experiments the affinity of Grb2 for Bcr-Abl is approximately two times higher than the affinity of Grap for Bcr-Abl. As Grb2 and Grap expression levels are similar in hematopoietic cells, these findings suggest that the interaction of Grb2 with Bcr-Abl is of greater physiological relevance.

Introduction

The Bcr-Abl chimeric oncogene is generated by a reciprocal translocation between chromosomes 9 and 22 [Groffen et al., 1984; Heisterkamp et al., 1985; Ben-Neriah et al., 1986]. This t(9;22) translocation fuses *bcr* sequences upstream of the second exon of c-*abl* and generates one of two Bcr-Abl fusion proteins, p185 and p210. The p210 form of Bcr-Abl is seen in 95% of patients with chronic myelogenous leukemia while the p185 form is more commonly associated with acute lymphocytic leukemia [Shtivelman et al., 1985; Clark et al., 1988]. Both of these chimeric proteins are capable of inducing leukemias in mice, thereby implicating them as the cause of these diseases [Scott et al., 1991; Kelliher et al., 1990; Daley et al., 1990].

Bcr-Abl is a constitutively active tyrosine kinase and has elevated protein tyrosine kinase activity, as compared to c-ABL[Kanopka et al., 1984; Lugo et al., 1990]. Numerous substrates of, and pathways activated by, the Bcr-Abl tyrosine kinase have been identified. One of the critical pathways implicated in transformation by Bcr-Abl is the ras pathway [Mandanas et al., 1993; Cortez et al., 1995]. Tyrosine 177 in the BCR first exon has been shown to be phosphorylated and mediates binding to Grb2, a protein that functions to link activated tyrosine kinases to a ras nucleotide exchange factor, SOS. The interaction between Grb2 and Bcr-Abl occurs by the SH2 domain of Grb2 binding to tyrosine 177 of Bcr-Abl. Mutation of this tyrosine to phenylalanine results in a Bcr-Abl protein that transforms fibroblasts poorly and does not activate ras [Puil et al., 1994; Pendergast et al., 1993].

Recently, a Grb2 related adaptor protein, Grap, was cloned. Grap shares 59% amino acid identity and the same SH3-SH2-SH3 domain structure as Grb2, with the highest homology in the N-terminal SH3 domain [Feng et al., 1996]. Grap associates with mSos through its N-terminal SH3 domain, and with ligand-activated receptors for stem cell factor and erythropoietin through its SH2 domain [Feng et al., 1996]. Further, Grap was reported to coimmunoprecipitate with Bcr-Abl and, using GST fusion proteins, the SH2 domain of Grap was found to bind Bcr-Abl [Feng et al., 1996].

As Grap is predominantly expressed in hematopoietic cells this suggests that Grap, like Grb2, may be an important link to the ras pathway in cells transformed by Bcr-Abl and, given their similar structure, one may be preferentially utilized over another. To examine this possibility, expression levels of Grb2 and Grap in primary hematopoietic cells derived from patients with CML were examined and found to be similar. These findings prompted us to investigate whether the relative affinities of Grb2 or Grap for Bcr-Abl could be distinguished thereby allowing a determination of the relative importance of these two proteins as links from Bcr-Abl to ras.

Material and Methods

Cells and Cell Culture - K562 cells, a Bcr-Abl-positive leukemic cell line, were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C, 5% CO_2. Peripheral blood from a patient with chronic myelogenous leukemia in the stable phase of the disease was obtained by venipuncture after obtaining informed consent. Mononuclear cells were obtained by density gradient centrifugation using Ficoll-Hypaque [Oda et al., 1994].

Antisera - Rabbit polyclonal antisera against GST-Grap was used as described [Feng et al., 1996]. The anti-Grb2 polyclonal antibody used for immunoblotting and the anti-Grb2 mouse monoclonal (SC 1-68) used for immunoprecipitations were purchased from Santa Cruz Biotechnology. The anti-Abl antibody 8E9 was generously provided by Jean Wang (UCSD).

Immunoprecipitation and immunoblotting - Cells were lysed in NP40 lysis buffer (20mM Tris, pH8.0, 1mM EDTA, 150 mM NaCl, 1% NP40, 10% glycerol) containing 10ug/ml aprotinin, 1mM Na_3VO_4, and 1mM phenylmethylsulfonyl fluoride. Equal amounts of whole cell lysates were immunoprecipitated with either 2 ug Grb2 or 10 ul Grap antisera followed by a 2 h incubation with protein A-Sepharose. Following SDS-PAGE and transfer of proteins onto a PVDF membrane (Immobilon-P, Millipore) for 4 h at .45 Amps (in a buffer containing 25mM Tris, 192.5 mM glycine and 20% MeOH), residual binding sites were blocked in 5% non-fat milk in TBS (10mM Tris, pH 8.0, 150 mM NaCl) for 1 h at 25°. The blots were incubated overnight at 4° with either Grb2

or Grap antisera. Antibody reactions were detected using alkaline phosphatase (Promega).

Yeast two-hybrid assay - Bcr-Abl, Bcr-Abl with a tyrosine to phenylalanine substitution at tyrosine 177 (Y177F), or a tyrosine kinase defective mutant of Bcr-Abl were subcloned into pBTMa as in-frame LexA-DNA binding domain fusion proteins. Grap and Grb2 were expressed as VP16 activation domain fusion proteins. As a control, full length Crkl was also cloned into pVP16. Mating was done as described [Vojtek et al., 1993; Oda et al., 1996] and analyzed for growth on histidine plates in the absence of tryptophan and leucine to select for yeast containing the two plasmids.

Yeast split-hybrid- p185 Bcr-Abl and Grb2 or Grap were subcloned into pBTMa such that p185 was expressed as a LexA-DNA binding domain fusion protein, and Grb2 or Grap were contained in the same plasmid as VP16 activation domain fusion proteins [Baskaran et al., 1996]. To accomplish this, Grb2 and Grap were subcloned as VP16 activation domain proteins into the plasmid pAD4. BamH I fragments containing the ADH promoter and termination sites were then placed into the Pvu II site of pBTMa. pBTMa contains a *TRP* selectable marker. An EcoR I partial digest of this plasmid was performed and an EcoR I fragment containing p185 Bcr-Abl was ligated into this plasmid.

A second yeast expression plasmid was generated that would express the full length Grap or Grb2 proteins without fusion to a DNA binding domain or VP16 activation domain. To accomplish this, pVP16 was modified by excising the VP16

activation domain. pVP16 contains a *LEU2* selectable marker. Grb2 and Grap were subcloned into this modified plasmid (pFS).

The pBTMa and a pFS plasmids were cotransformed into the yeast strain Y1584 and selected for growth on plates lacking tryptophan and leucine in the presence or absence of histidine. The yeast strain Y1584 carries a HIS gene under the control of a *tet* operator, and a *tet* repressor downstream of a LexA operator [Shih et al., 1996].

Production and purification of Bcr-Abl - Bcr-Abl with an amino terminal hemagglutinin antibody tag (12CA5) and a hexa-histidine tag was expressed in Sf9 cells as a recombinant baculovirus [Bhat et al., 1997]. Bcr-Abl was purified over Ni-NTA-Agarose (Qiagen), eluted with 500 mM imidazole and dialyzed against Tris-buffered saline as described [Bhat et al., 1997].

GST binding assays - GST, GST-Grb2 and GST-Grap constructs were transformed into E.coli DH-5α and induced to express protein with the addition of 0.5mM IPTG to exponentially growing cells. Following sonication of bacterial lysates, GST-fusion proteins were immobilized on glutathione sepharose beads (Pharmacia, Piscataway, NJ). 250 ng of Bcr-Abl, purified over Ni-NTA-Agarose (Qiagen), was added to the GST-fusion proteins and incubated at $4^{o}C$ overnight. Bound proteins were washed three times with phosphate-buffered saline, boiled in SDS sample buffer and separated by SDS-PAGE on a 10% gel, followed by silver staining. Gels were scanned with a Lumi-Imager (Boehringer Mannheim).

Surface Plasmon Resonance - GST, GST-Grb2 and GST-Grap were expressed, purified over glutathione Sepharose beads (Pharmacia), and eluted with glutathione as described [Heaney et al., 1997]. GST, GST-Grb2, and GST-Grap were covalently attached to carboxymethyl dextran coated cells (Iasys Affinity Sensors, Paramus, NJ) as follows: 200 µl of PBS with 0.05% Tween 20 (PBST) was equilibrated in a cuvette in the Iasys Affinity Sensor for 20 min. The PBST was replaced with 200 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), 58 mM NHS (N-hydroxysuccimide) in water for 7 minutes to activate the dextran surface. This was washed 3 times with PBST and left for 1 min after the final wash. The cuvette was then washed 3 times with 10 mM Na Acetate (pH 6.5) and left for 1 min after the final wash. Grb2 was diluted to 10 ng/µl in 10 mM NaAc pH 5.4. 200 µl was applied to the cuvette and binding was monitored in the instrument. Immobilization was allowed to proceed to 2500 arc sec. The chamber was washed 3 times with PBST as above. Then, 200 µl of ethanolamine pH 8.5 was added and left for 2 min, washed with PBST and left for 2 min after the final wash. Before use, the cuvette was washed with 10 mM HCl and then three times with PBST and either used immediately or stored in this solution. Grap was immobilized in a similar fashion, except the concentration was 20 ng/µl and 10 µl of 10 mM HCl was added to the 10 mM NaAc solution to achieve the desired response of 2500 arc sec. GST was immobilized in the same fashion except the concentration was 25 ng/µl and 3 µl of 10 mM HCl was added to achieve immobilization.

For analysis of Bcr-Abl binding, the chamber was evacuated and 100 µl of PBST added. Bcr-Abl, purified over Ni-NTA-Agarose as above, was added at various concentrations as determined by Bradford assay (Biorad), and response was

determined to analyze equilibrium binding. Binding was allowed to proceed until complete and additional Bcr-Abl was added. Concentration bound is determined by the equation: C_{bound} = (response)/(815000 x MW). Account is taken of the dilution of immobilized sites. The molecular weight of Bcr-Abl is 210 kDa.

Results

Expression of Grap and Grb2 in myeloid cells from CML patients

In contrast to Grb2 which is ubiquitously expressed, Grap is expressed primarily in cells of hematopoietic and lymphocytic origin. As both Grap and Grb2 can link activated tyrosine kinases to ras, this suggested that Grap may be the important link for Bcr-Abl to ras in CML, a disease of the myeloid lineage. To begin to address this question, the relative levels of expression of Grb2 and Grap were analyzed in primary mononuclear cells isolated from a patient with CML. As seen in Figure 1, immunoblot analysis of these lysates with either Grap or Grb2 antisera demonstrated equivalent expression levels.

Grap and Grb2 associate with Bcr-Abl in a yeast two-hybrid system

Previous reports have identified tyrosine 177 of Bcr-Abl as the requisite binding site for Grb2. To confirm this observation *in vivo* and to examine whether Grap, like Grb2, also required this residue for its association with Bcr-Abl, the yeast two-hybrid system was employed. In this system, p185 was expressed as a LexA-DNA binding domain fusion protein. Kinase active Bcr-Abl protein was expressed by analysis of phosphotyrosine immunoblots of yeast lysates (data not shown). By expressing Grb2 as a VP16 activation domain fusion protein, a direct interaction between Bcr-Abl and Grb2 was observed (Figure 2). In contrast, no interaction was detected using Bcr-Abl with a mutation of tyrosine 177 to phenylalanine or a tyrosine kinase inactive mutant of Bcr-Abl. Similar results were obtained by expressing Grap as a VP16 activation

domain fusion protein (Figure 2). Thus, both Grap and Grb2 can bind directly to tyrosine 177 of Bcr-Abl. As a positive control for Bcr-Abl expression, binding of all Bcr-Abl constructs to Crkl was observed, as previously described [Heaney et al., 1997].

Interaction of Grap and Grb2 with Bcr-Abl

Having established that expression levels of Grb2 and Grap in primary mononuclear cells were similar and that both proteins interacted with Bcr-Abl at the identical residue, this suggested that the relative affinities of Grap versus Grb2 for Bcr-Abl would determine the relative importance of these proteins as links to the ras pathway. As expected, immunoprecipitation of Abl followed by immunoblotting with Grb2 or Grap antisera demonstrated a complex formation between Grap, Grb2 and Bcr-Abl (Figure 3a). Grb2 and Grap are distinguished by a small difference in mobility; the 23-kDa Grb2 migrates slightly faster than the 27 kDa Grap. Immunoprecipitation of Grap or Grb2 followed by immunoblotting for Abl also confirmed this interaction (Figure 3b). Interestingly, approximately twice as much whole cell lysate was required to immunoprecipitate Grap as Grb2 to attain a similar intensity in signal.

However, the Grap antibody, generated using a GST fusion protein, detected not only thrombin-cleaved GST-Grap but GST-Grb2 as well (Figure 3c). This gel was stripped and reprobed with a GST antibody to confirm complete cleavage of GST from Grap and Grb2 (data not shown). Other commercially available anti-Grap antibodies were also cross-reactive (data not shown). Thus, Grap precipitates would unavoidably, always contain some Grb2. Hence, quantitative experiments using these

antibodies to evaluate the relative affinities of Grb2 and Grap for Bcr-Abl would yield uninterpretable results.

Disruption of the Grap/Bcr-Abl complex by Grb2

To investigate the relative affinities of Grap and Grb2 for Bcr-Abl, a competitive binding assay was employed using a modification of the yeast split-hybrid system. The yeast split-hybrid system, designed to positively select for disruption of protein-protein interactions, uses the same two plasmids as for the yeast two-hybrid system but employs a modified yeast strain [Shih et al., 1996]. This strain carries a HIS3 gene activated by a *tet*-operator and a *tet*-repressor activated by a LexA operator. Interactions between a LexA-DNA binding domain fusion protein and a VP16 activation domain fusion protein results in the production of *tet*-repressor which blocks synthesis of histidine. In contrast, if the two proteins do not interact, *tet* repressor is not produced, and growth on plates lacking histidine can occur (Figure 4A).

The yeast split-hybrid screen was modified by placing LexA-p185 Bcr-Abl and VP16-Grb2 in the same plasmid with a trp selectable marker. Grap was cloned downstream of an ADH promoter into a second plasmid with a leu2 selectable marker [Baskaran et al., 1996]. In the yeast split-hybrid system, the interaction of LexA-p185 and VP16-Grb2 would prevent growth on plates lacking histidine (Figure 4B). However, if Grap was able to disrupt the interaction of Grb2 with Bcr-Abl, growth in the absence of histidine could occur (Figure 4D). As seen in Figure 5, Grap was not

capable of disrupting the interaction between Bcr-Abl and Grb2. In contrast, Grb2 did disrupt the interaction of Bcr-Abl and Grap.

Affinity of interaction of Bcr-Abl with Grap and Grb2

The above data suggested that the interaction between Bcr-Abl and Grb2 was of higher affinity than the interaction between Bcr-Abl and Grap. However, it is also possible that levels of expression in the yeast split-hybrid system could affect the interpretation of these experiments. To distinguish between these possibilities, the interactions of these proteins was analyzed *in vitro* using purified proteins.

Bcr-Abl was expressed in the baculovirus system, with an N-terminal hemagglutinin (12CA5) and a hexa-histidine tag [Bhat et al., 1997]. Lysates of Sf9 cells infected with this recombinant baculovirus were purified over Ni-NTA-Agarose and Bcr-Abl was eluted with 500 mM imidazole. Silver staining of eluted material demonstrated that Bcr-Abl was the major protein present in the eluate. Grap and Grb2 were expressed as GST-fusion proteins and immobilized on glutathione sepharose. Bcr-Abl protein was added to the beads and binding of Bcr-Abl was analyzed by SDS-PAGE followed by silver staining (Figure 6). Scanned gels were analyzed for the amount of Bcr-Abl bound compared to the amount of the GST-Grap or GST-Grb2 present. In these experiments Grb2 bound between 1.6 to 2 times as much Bcr-Abl than Grap.

To analyze the binding affinities of Grap and Grb2 for Bcr-Abl more precisely, surface plasmon resonance was employed. GST, GST-Grap, and GST-Grb2 were each covalently attached to carboxymethyl dextran coated cells (Iasys Affinity Sensors,

Paramus, NJ) with 200 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and 58 mM NHS (N-hydroxysuccimide). Bcr-Abl binding to GST binding was determined and plotted as shown (Figure 7a). The affinity of Bcr-Abl for GST is 1344 nM from double reciprocal plots of bound and free Bcr-Abl (Figure 7a).

As there was a low, but definite background binding of Bcr-Abl to GST, only the initial portions of the curve representing Bcr-Abl concentrations below 500 nM were used to calculate binding affinities. In three experiments, the Grb2 affinity ranged from 103 to 145 nM and the Grap affinity ranged from 233 to 357 nM. In each experiment the fold difference ranged from 2.0 to 2.5. Representative binding curves are shown in Figure 7b and c.

Discussion

Grap is a recently cloned adaptor protein which shares a high homology to Grb2 and binds a similar spectrum of proteins [Feng et al., 1996; Trub et al., 1997]. Using Grb2-deficient embryonic stem cells, Cheng et. al, demonstrated that expression of Grap partially restored the ability of these cells to undergo endodermal differentiation [Cheng et al., 1998]. Further, the predominant expression of Grap is in spleen, thymus, and peripheral blood leukocytes. This data suggests that Grap may be preferentially utilized as a link for activated tyrosine kinases to ras in hematopoietic tissues.

As Bcr-Abl expression is limited to hematopoietic cells and ras is one of the critical pathways activated in Bcr-Abl-expressing cells, this system is an ideal model to address the relative importance of Grap versus Grb2 in Bcr-Abl signaling. As shown

by the yeast two-hybrid data, both Grap and Grb2 bind directly to tyrosine 177 of Bcr-Abl. Using a modification of the yeast split-hybrid system, Grb2 was shown to disrupt the interaction of Bcr-Abl with Grap but Grap could not disrupt the interaction of Bcr-Abl with Grb2. Although these data suggest that the affinity of interaction between Grb2 and Bcr-Abl is higher than the affinity of Bcr-Abl for Grap, the yeast split-hybrid system is not designed for quantitative analyses. Thus, purified proteins were used to demonstrate that Bcr-Abl has an approximately 2-fold higher affinity for Grb2 than for Grap. This was confirmed by analysis of bound proteins using silver staining and by surface plasmon resonance.

The binding affinity of Bcr-Abl for Grb2 was between 103 and 145 nM and the affinity for Grap ranged from 233 to 357 nM. These values are similar to the values obtained by other groups in analyzing the affinities of SH2 domains with phosphotyrosine [Ladbury et al., 1995]. Although affinities were originally reported to be between 3.5 and 80 nM, these were likely to be higher than the true values due to avidity effects from dimerization of GST-fusion proteins used in these assays [Payne et al., 1993; Marengere et al., 1994]. To circumvent these problems, Ladbury, et. al., varied the concentration of phosphopeptide bound to the chip. This resulted in affinity constants closer to 300 nM to 4 uM [Ladbury et al., 1995]. Given these problems, the values presented in our study may not be representative of the true affinity of Bcr-Abl for Grap or Grb2. However, the relative affinities of Grap and Grb2 for Bcr-Abl are valid as experiments were performed in an identical fashion.

In mononuclear cells from patients with CML, the expression of Grap and Grb2 are nearly identical. These cells are the predominant cells expressing Bcr-Abl in human

disease. Given the higher affinity of Grb2 for Bcr-Abl as compared to Grap, our findings suggest that under physiological conditions more Grb2 is bound to Bcr-Abl. As both Grap and Grb2 bind to mSos, linking these proteins to the ras pathway, we propose that Bcr-Abl preferentially utilizes Grb2 over Grap as its link to activation of ras.

Acknowledgements

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Figure Legends

Figure 1. Immunoblot analysis of Grb2 and Grap. Peripheral blood from a patient with chronic myelogenous leukemia in the stable phase of the disease was obtained by venipuncture after obtaining informed consent. Mononuclear cells were obtained by density gradient centrifugation using Ficoll-Hypaque [Oda et al., 1994]. Equal amounts of 1% NP40 lysates were separated by SDS-PAGE on a 10% gel and immunoblotted with antisera to Grb2 or Grap. Grap migrates with a slightly higher mobility than Grb2 [Feng et al., 1996].

Figure 2. Grap and Grb2 interact directly with tyrosine 177 of Bcr-Abl.

A. Interactions between Grb2 or Grap and Bcr-Abl were examined using a yeast two-hybrid analysis [Vojtek et al., 1993; Fields and Song 1989]. Bcr-Abl or Bcr-Abl with a tyrosine to phenylalanine substitution at tyrosine 177 (Y177F) were subcloned into pBTMa as in-frame LexA DNA binding domain fusion proteins. Grap and Grb2 were expressed as VP16 activation domain fusion proteins. As a control, full length c-Crkl was also cloned into pVP16. Mating was done as described [Vojtek et al., 1993; Oda et al., 1996] and analyzed for growth on histidine plates in the absence of tryptophan and leucine to select for the two plasmids. Growth on plates lacking histidine is indicative of an interaction between the two proteins. 1. pBTM-p185 + pVP16-Grb2; 2. pBTM-p185 + pVP16-Grap; 3. pBTM-p185 + pVP16-Crkl; 4. pBTM-p185 + pVP16; 5. pBTM-p185Y177F + VP16-Grb2; 6. pBTMp185-Y177F+ pVP16-Grap; 7. pBTM-p185Y177F + VP16-Crkl; 8. pBTM-p185Y177F + pVP16. B. Summary of yeast two

hybrid interactions. Additional controls included a tyrosine kinase defective (KD) mutant of Bcr-Abl expressed as a LexA fusion protein and Crkl expressed as a VP16 activation domain fusion protein. pBTM-p185Y177F and p185KD interact with pVP16-Crkl indicating that the lack of interaction of Grap and Grb2 with this mutant is not due to lack of expression of the Bcr-Abl protein.

Figure 3. Association of Grb2 and Grap with Bcr-Abl in Bcr-Abl expressing myeloid cells.

A. K562 whole cell lysates were immunoprecipitated (I.P.) with an anti-Abl antibody and immunoblotted with either an anti-Grb2 or anti-Grap antibody. B. Lysates were immunoprecipitated with either an anti-Grb2 or anti-Grap antibody and immunoblotted with an anti-Abl antibody C. Bacterially expressed GST, GST-Grap, or GST-Grb2 were thrombin cleaved and then analyzed by anti-Grap immunoblotting. This immunoblot was stripped and reprobed with GST to confirm complete cleavage of the GST-Grb2 and GST-Grap fusion proteins (data not shown).

Figure 4. Yeast split-hybrid system. A. Association of proteins A and B recruits the VP16 transactivation domain to the LexA operator, activating synthesis of *tet* repressor. *Tet* repressor blocks expression of the HIS3 gene, preventing cell growth in the absence of supplemented histidine. B. A mutation disrupting the interaction between proteins A and B prevents formation of the activating complex and subsequent synthesis of the *tet* repressor. HIS3 is constitutively expressed and cells grow in the absence of supplemented histidine. Modified

yeast split hybrid system. Co-expression of a third protein which binds the LexA fusion protein with a higher affinity than does the VP16 fusion protein also disrupts the transcription activation complex and allows cell growth in the absence of supplemented histidine.

C. p185 is expressed as a LexA fusion protein and Grap as a VP16 fusion protein. The interaction of the two proteins assembles the transcription activating complex that allows synthesis of *tet* repressor, preventing HIS3 expression and growth in the absence of histidine. D. Grb2 simultaneously expressed in the same cells can disrupt the transcription activation complex by binding to p185 with higher affinity than does Grap. VP16 is no longer recruited to the LexA operator and *tet* repressor is not synthesized. HIS3 is produced and cells grow in the absence of supplemented histidine.

Figure 5. Grb2 disrupts the interaction of Bcr-Abl and Grap.

p185Bcr-Abl and Grb2 or Grap were subcloned into pBTMa such that p185 was expressed as a LexA DNA binding domain fusion protein and Grb2 or Grap were expressed as VP16 activation domain fusion proteins [Baskaran et al., 1996]. Grb2 and Grap were also subcloned into a yeast plasmid (pFS) under control of an ADH promoter in a plasmid with a leu2 selectable marker [Baskaran et al., 1996]. Plasmids were cotransformed into the yeast strain Y1584 which carries a HIS gene under the control of a *tet* operator and a *tet* repressor upstream of a LexA operator [Shih et al., 1996]. In this system, interaction of Bcr-Abl with Grb2 or Grap prevents growth on plates lacking histidine. 1. LexA-p185/VP16-Grb2 + pFS; 2. LexA-

p185/VP16-Grb2 + pFS-Grap; 3. LexA-p185/VP16-Grb2 + pFS-Grb2; 4. LexA-p185/VP16-Grap + pFS; 5. LexA-p185/VP16-Grap + pFS-Grap; 6. LexA-p185/VP16-Grap + pFS-Grb2.

Figure 6. Silver stain of purified Bcr-Abl binding to bacterially expressed Grb2 or Grap. Bcr-Abl with an amino terminal hemagglutinin antibody tag (12CA5) and a hexa-histidine tag was expressed in Sf9 cells as a recombinant baculovirus [Bhat et al., 1997]. Bcr-Abl was purified over Ni-NTA-Agarose, eluted with 500 mM imidazole, and dialyzed against Tris-buffered saline as described [Bhat et al., 1997]. An aliquot of purified material is in the far right lane. Grb2 and Grap were expressed as GST fusion proteins and immobilized on glutathione sepharose. Increasing amounts of the indicated fusion protein were incubated with Bcr-Abl protein, and bound protein analyzed by SDS-PAGE followed by silver staining [Beitner-Johnson and LeRoith 1995]. Gels were scanned with a Lumi-Imager (Boehringer Mannheim) and the amount of Bcr-Abl bound determined relative to the amount of Grap or Grb2 fusion protein. The far left lane demonstrates that no binding of Bcr-Abl to GST was seen at the concentrations of proteins used in these experiments.

Figure 7. Binding affinity of Grb2 and Grap for Bcr-Abl using surface plasmon resonance. GST, GST-Grb2, and GST-Grap were covalently attached to carboxymethyl dextran coated cells (Iasys Affinity Sensors, Paramus, NJ) with 200 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), 58 mM NHS (N-hydroxysuccimide) [Bedi et al., 1992]. Bcr-Abl, purified over Ni-NTA-Agarose

as in Figure 6, was added and binding was allowed to proceed until complete. Additional Bcr-Abl was added and the concentration of bound Bcr-Abl determined [Bennett and Hoffmann 1992]. The concentrations of bound Bcr-Abl $(\text{Bcr-Abl})_b$ and free Bcr-Abl $(\text{Bcr-Abl})_f$ obey the following relationship $[\text{Bcr-Abl}]_b = \frac{[\text{Bcr-Abl}]\,[\text{GST, GST-Grb2, or GST-Grap}]}{[\text{Bcr-Abl}]_f + K_d}$ where $[\text{Bcr-Abl}]_f$ is calculated from the difference between the total quantitiy applied and the quantity bound. Plots in the left panel are of free versus bound Bcr-Abl. The right panel shows double reciprocal plots of the same variable in which the slopes were used to calculate the affinity constant.

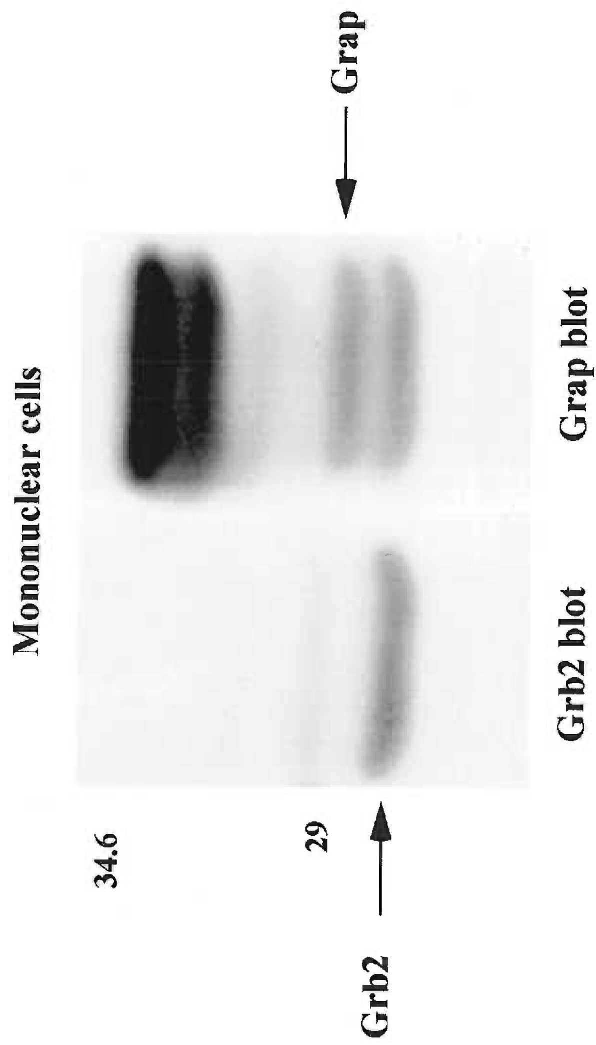
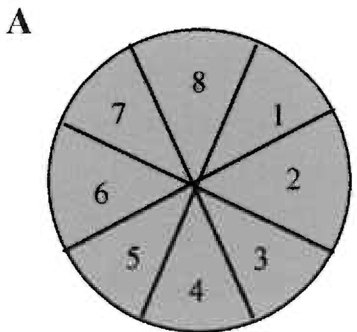
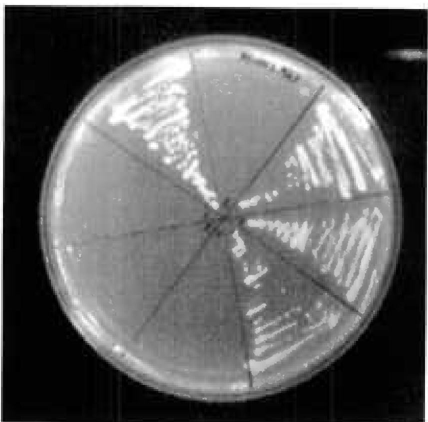


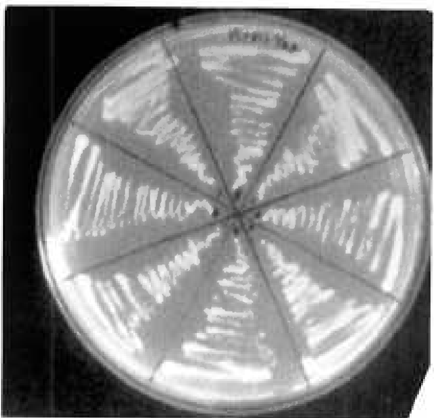
Figure 1

A





YC - HIS

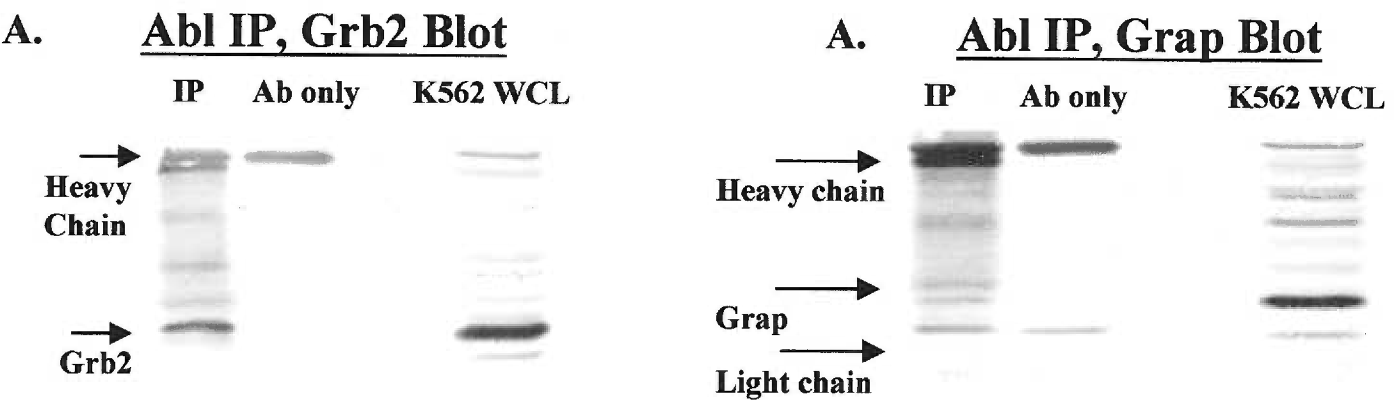


YC + HIS

B

LexA fusion construct	VP-16 fusion construct			
	VP-16	Grb2	Grap	CrkL
LexA-Lamin	-	-	-	-
p185BCR-ABL	-	+	+	+
p185BCR-ABL Y177F	-	-	-	+
p185BCR-ABL KD	-	-	-	+
pGrb2	-	ND	ND	-
pGrap	-	ND	ND	-

Figure 2 A ,B



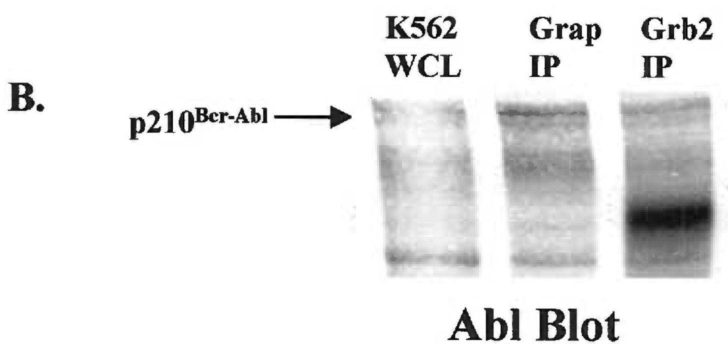


Figure 3 A, B

GST Fusion Proteins (thrombin cleaved)

Grap Blot

Figure 3C

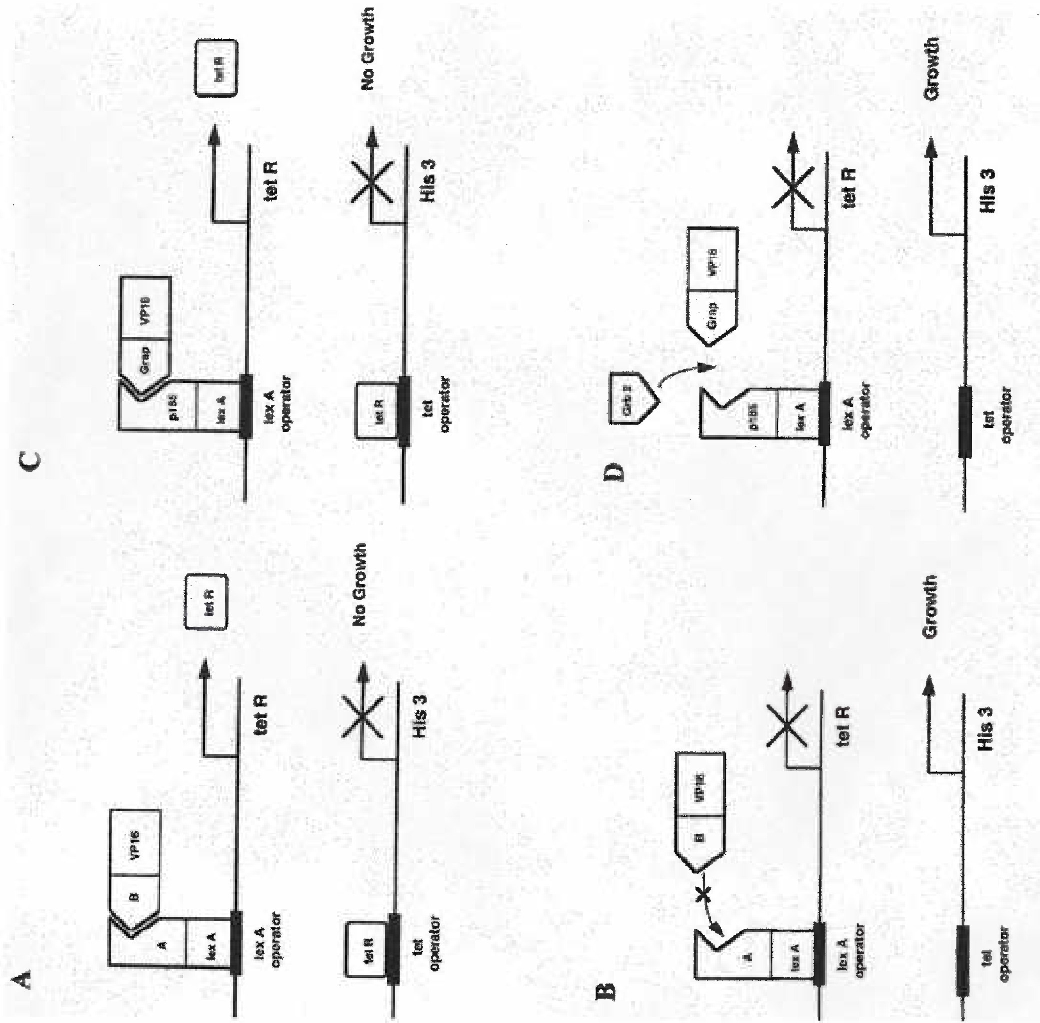
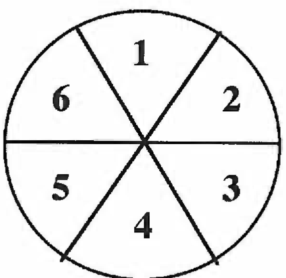
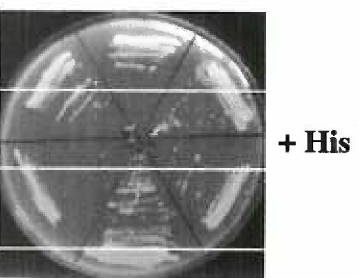


Figure 4





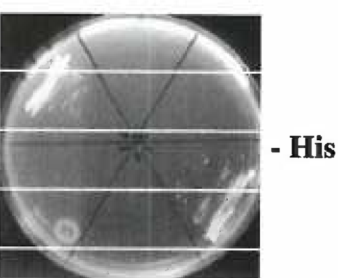


Figure 5

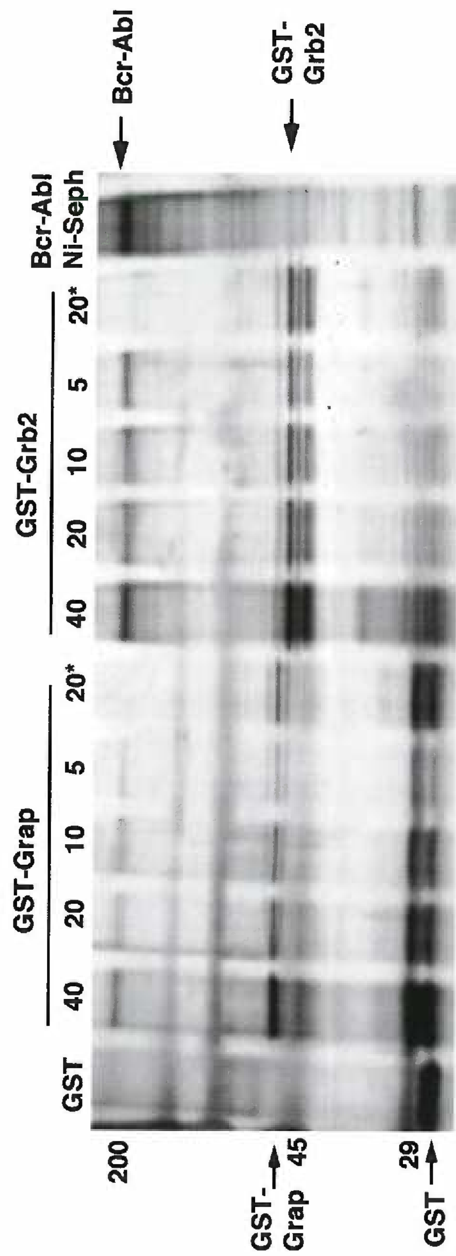


Figure 6

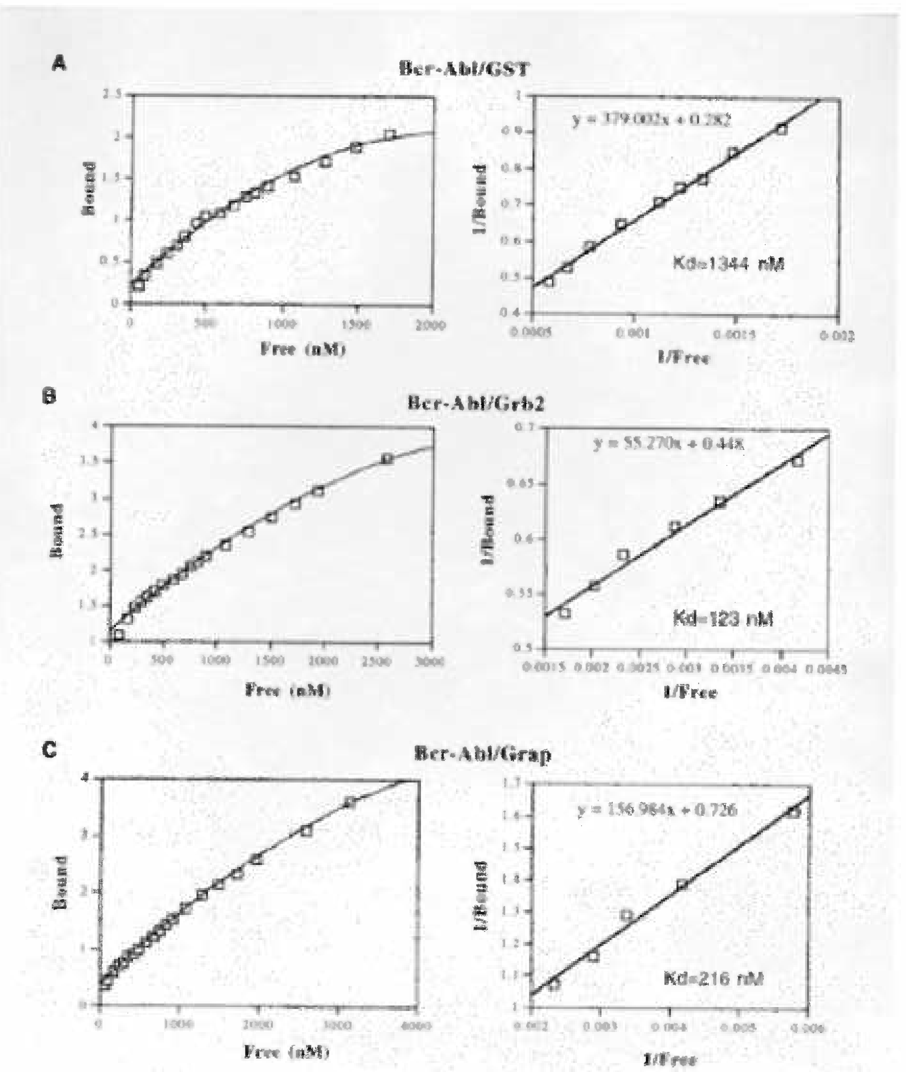


Figure 7

CHAPTER IV

RESULTS

Grb2 is the Major Mediator of the Interaction between Cbl and Bcr-Abl

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Abstract

Bcr-Abl is a constitutively active tyrosine kinase that is the causative molecular abnormality of chronic myelogenous leukemia (CML). Investigations into the molecular mechanism of action of Bcr-Abl have indicated that Bcr-Abl associates with numerous proteins resulting in activation of a multitude of signaling pathways. c-Cbl is one of the major proteins that interacts with Bcr-Abl, with Cbl linking Bcr-Abl to PI-3 kinase and cytoskeletal proteins that are critical to Bcr-Abl function. Previous experiments have demonstrated a direct association between the SH2 domain of Bcr-Abl and tyrosine phosphorylated Cbl, however, these results do not establish how Cbl and Bcr-Abl associate prior to Cbl becoming tyrosine phosphorylated. To broaden our understanding of the assembly of the Bcr-Abl complex, these studies addressed whether another protein may facilitate Cbl binding to Bcr-Abl. In a yeast two-hybrid analysis, no direct interaction between Bcr-Abl and Cbl was observed. However, Bcr-Abl did associate with Grb2, an adaptor protein which binds tyrosine 177 of Bcr-Abl. Additionally, Grb2 interacted with Cbl. Using a yeast three-hybrid assay and purified proteins, Grb2 mediated an interaction between Cbl and Bcr-Abl and this interaction was dependent on a functional Grb2 binding site. In cells expressing a mutation in the Grb2 binding site, the amount of Cbl associated with Bcr-Abl is significantly decreased. These findings

support a model whereby Grb2 is the major protein that mediates an initial interaction between Cbl and Bcr-Abl. Following this initial interaction, Cbl can become tyrosine phosphorylated and interact with the SH2 domain of Bcr-Abl, thus stabilizing the interaction of Cbl with Bcr-Abl and allowing interactions of Cbl with other signaling proteins required for Bcr-Abl function.

Introduction

Bcr-Abl is a constitutively activated tyrosine kinase that is the primary molecular abnormality in a variety of leukemias. Thus, 95% of chronic myelogenous leukemia patients express a 210 kDa Bcr-Abl fusion protein while 5-10% of patients with acute lymphoblastic leukemia express either the 210 kDa Bcr-Abl protein or a 185 kDa Bcr-Abl fusion protein [Faderl 1999]. These two fusion proteins differ in their size by the contribution of Bcr sequences to the fusion protein.

Investigations of the molecular mechanism of action of the Bcr-Abl fusion proteins have demonstrated that numerous proteins are tyrosine phosphorylated in cells expressing Bcr-Abl. The list of tyrosine phosphorylated proteins includes c-CBL [Andoniou et al., 1994; Sattler et al., 1996; de Jong et al., 1995], PI3-kinase [Varticovski et al., 1991], CRKL [Nichols et al., 1994; Oda et al., 1994; ten Hoeve et al., 1994], p62dok [Carpino et al., 1997; Yamanashi et al., 1997], p120rasGAP [Druker et al., 1992], SHC [Matsuguchi et al., 1994; Tauchi et al., 1994], as well as the focal adhesion proteins paxillin, vinculin, tensin and p125FAK [Salgia et al., 1995; Gotoh et al., 1995]. Besides being tyrosine phosphorylated in Bcr-Abl-expressing cells, most of these proteins have been shown to form a complex with Bcr-Abl, as demonstrated by coimmunoprecipitation studies. However, there is limited information about whether these proteins bind to Bcr-Abl directly or indirectly through other proteins.

Previous experiments have demonstrated complex formation in mammalian cells between Bcr-Abl and Cbl [Bhat et al., 1997; Salgia et al., 1996; Ribon et al., 1996; de Jong et al., 1995; Andoniou et al., 1994]. c-CBL, the cellular homolog of v-CBL, is the transforming protein of the Cas NS-1 retrovirus that induces pre-B cell lymphomas and myeloid leukemias in mice [Langdon et al., 1989]. c-CBL is a common substrate of tyrosine kinases and is tyrosine phosphorylated in a variety of signaling pathways associated with cellular proliferation or activation [Tanaka et al., 1995; Donovan et al., 1994; Fukazawa et al., 1995; Galisteo et al., 1995; Odai et al., 1995]. Cbl has several protein-protein interaction domains, including a novel SH2 domain [Meng et al., 1999], proline-rich sequences, and numeorus tyrosine phosphorylation sites [Miyake et al., 1997; Langdon et al., 1995].

Using lysates from Bcr-Abl-expressing cells, a direct association could be demonstrated between the SH2 domain of Bcr-Abl and Cbl in an *in vitro* gel overlay assay [Bhat et al., 1997]. For Cbl to interact directly with Bcr-Abl in vitro, tyrosine phosphorylation of Cbl was required [Bhat et al., 1997]. However, it is not clear whether Cbl and Bcr-Abl can interact prior to Cbl becoming tyrosine phosphorylated. We hypothesized that either Bcr-Abl and Cbl interact directly in an enzyme-substrate interaction or, that another kinase phosphorylates Cbl prior to its association with Bcr-Abl or, that another unidentified protein may bring Cbl to Bcr-Abl, thus allowing Cbl to become tyrosine phosphorylated. Experiments were devised to test these hypotheses using yeast protein-protein interactions systems and purified proteins.

Materials and Methods

Plasmid Constructions

The GST-Cbl 91, GST-Cbl 307, and GST-Cbl 366 constructs and a Cbl cDNA were generously provided by W.Y. Langdon (University of Western Australia). The GST-Cbl N terminal construct was created by excising an Nco1 to Nco1 fragment of Cbl and cloning it into pGEX-KG [Guan et al., 1991]. A schematic of the GST-Cbl constructs is depicted in Fig.2. GST-Grap was a gift from Gen-Sheng Feng (Indiana University School of Medicine) and GST-Grb2 was provided by B. Margolis (University of Michigan).

Yeast two-hybrid assay

Bcr-Abl(p185), Bcr-Abl with a tyrosine to phenylalanine substitution at tyrosine 177 (p185Y177F), and full length Cbl were subcloned into pBTMa as in-frame LexA-DNA binding domain fusion proteins. Full length Grap, Grb2, Cbl, and Crkl were expressed as VP16 activation domain fusion proteins. Analysis of interactions using the yeast two-hybrid assay were performed as previously described [Vojtek et al., 1993]. Interaction was determined by selection on plates lacking histidine.

Yeast three-hybrid

A yeast plasmid that co-expresses Bcr-Abl(p185) and Grb2 was initially created. VP16-Grb2 was digested with Cla I and religated, resulting in removal of VP16 sequences. The resulting plasmid expresses full length Grb2 under the control of a yeast promoter. This plasmid was partially digested with EcoR I and ligated to an EcoR I fragment containing either LexA-p185Bcr-Abl or LexA-p185Y177FBcr-Abl. The resulting plasmids were mated with either full length Cbl, CrkI, or lamin (negative control) which were expressed in frame fused to the acidic activation domain VP16. As in the yeast two-hybrid, positive interactions were determined by selection on plates lacking histidine (Figure 4a).

Antibodies

The mouse monoclonal anti-Abl antibody, 8E9, was a kind gift of J. Wang (University of California San Diego). The mouse monoclonal GST and Grb2(1-68) antibodies and rabbit polyclonal Grb2(C-23), Abl(K12), and Cbl (C-15) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Cbl rabbit polyclonal antibody R2 was generously provided by W.Y. Langdon and was used exclusively for immunoblotting. The anti-phosphotyrosine monoclonal antibody (4G10) was generated using KLH-phosphotyramine as the immunogen and was used as described [Kanakura et al., 1990; Druker et al., 1992]. The mouse monoclonal Grb2 antibody (3F2) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and used for immunoblotting.

Cell Lines

The 32Dc13 cell line was obtained from Joel Greenberger (University of Massachusetts Medical Center, Worchester, MA). pSRα or pSRα containing a full length p210Bcr-Abl and the p210Y177F mutant was introduced into 32Dc13 cells by electroporation as described [Oda et al., 1995]. Two independent single clones were isolated by cloning in soft agar. Cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc., Lake Placid, NY) and 15% WEHI-3B conditioned media as a source of interleukin-3. K562 cells are a p210Bcr-Abl-positive cell line derived from a CML patient in blast crisis [Lozzio and Lozzio 1975].

Immunoprecipitation and Immunoblotting

Cells were lysed in NP40 lysis buffer (20mM Tris, pH8.0, 1mM EDTA, 150 mM NaCl, 1% NP40, 10% glycerol) containing 10ug/ml aprotinin, 1mM Na_3VO_4, and 1mM phenylmethylsulfonyl fluoride. Equal amounts of whole cell lysates were immunoprecipitated with either anti-Cbl(C-15), anti-Grb2(C-23), or anti-Abl(K-12) antibodies followed by a 2 h incubation with protein A-Sepharose. Following SDS-PAGE and transfer of proteins onto a PVDF membrane (Immobilon-P, Millipore) for 4 h at .45 Amps (in a buffer containing 25mM Tris, 192.5 mM glycine and 20% MeOH), residual binding sites were blocked in either 2.5% gelatin (for anti-phosphotyrosine blots) or 5% non-fat milk in TBS (10mM Tris, pH 8.0, 150 mM NaCl) for 1 h at 25°. The

blots were incubated at room temperature with either an anti-Abl(8E9), anti-phosphotyrosine(4G10), or anti-Cbl (Clone 17) antibody. Antibody reactions were detected using alkaline phosphatase (Promega) or enhanced chemiluminescence (Pierce). All immunoprecipitation experiments were normalized for Abl protein to ensure equal loading.

Production and purification of Bcr-Abl -

Bcr-Abl with an amino terminal hemagglutinin antibody tag (12CA5) and a hexa-histidine tag was expressed in Sf9 cells as a recombinant baculovirus [Bhat et al., 1997]. Bcr-Abl was purified over Ni-NTA-Agarose (Qiagen), eluted with 500 mM imidazole and dialyzed against Tris-buffered saline as described [Bhat et al., 1997].

Gel Overlay Assays

Purified GST, GST-Cbl N terminal, GST-Cbl 91, GST-Cbl 306, GST-Cbl 366 and GST-Grb2 fusion proteins and nickel purified Bcr-Abl p210 were used in these assays. All GST-fusion constructs were expressed in *Escherichia coli* (DH5α) and induced with isopropylthio-β-D-galctosidase (IPTG). The GST fusion proteins were isolated from sonicated bacterial lysates using glutathione-Sepharose beads (Qiagen) and normalized for equivalent loading by Coomassie-stained gels or quantitated by immunoblotting for GST. The GST-Grb2 used for overlaying was purified by elution with glutathione as described [Heaney et al., 1997]. Proteins were separated by SDS-PAGE and

transferred to PVDF membranes which were blocked overnight in PBS with 0.05% Tween 20 (PBS-T) and 5% nonfat dry milk at 4°. The blots were washed twice in PBS-T followed by a 2 hour incubation (overlay) with either purified GST-Grb2 pre-incubated with nickel purified p210 or GST-Grb2 with nickel purified p210 (at 2ug/mL) in binding buffer (25 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.1% Tween 20, 2.5 mM EDTA, 20 mM NaF, 1% non-fat milk, 1 mM dithiothreitol, 10 ug/mL leupeptin, and 10 mg/mL aprotinin). Bound protein was detected with either an anti-GST, anti-Grb2, or anti-Abl antibody diluted 1:500 in binding buffer excluding milk, dithiothreitol, and protease inhibitors. Antibody reactions were developed using enhanced chemiluminescence (ECL). The blots were washed between each of the steps for one hour with PBS-T.

Results

Bcr-Abl and Cbl do not interact directly in a yeast two-hybrid analysis

Previous experiments have demonstrated a complex formation in mammalian cells between Bcr-Abl and Cbl [Bhat et al., 1997; Salgia et al., 1996; Ribon et al., 1996; de Jong et al., 1995; Andoniou et al., 1994]. A direct association could be demonstrated between the SH2 domain of Bcr-Abl interacting with tyrosine phosphorylated Cbl in an *in vitro* gel overlay assay [Bhat et al., 1997]. However, these results cannot explain how Cbl and Bcr-Abl asssociate prior to Cbl becoming tyrosine phosphorylated.

To investigate whether a direct interaction between Bcr-Abl and Cbl occurs *in vivo*, a yeast two-hybrid analysis was performed. In these experiments no interaction between Bcr-Abl and Cbl was observed (Figure 1). These results are consistent with our latter hypothesis that another protein may mediate an initial association between Bcr-Abl and Cbl. One of the candidates for mediating this association is Grb2, as it is known to bind through its SH2 domain to tyrosine 177 of Bcr-Abl and through its SH3 domain to proline-rich sequences of Cbl [Puil et al., 1994; Pendergast et al., 1993; Donovan et al., 1994; Fukazawa et al., 1995; Meisner et al., 1995; Odai et al., 1995]. As expected, an interaction between Bcr-Abl and Grb2 was detected in a yeast two-hybrid analysis, as determined by growth in the absence of histidine; whereas, a Bcr-Abl construct with a tyrosine to phenylalanine mutation in the Grb2 binding site (p185Y177F) abolishes this interaction (Figure 1). Similar results were obtained with Grap, a Grb2-related adaptor protein that has also been shown to interact with Bcr-Abl [Feng et al., 1996]. As a control for expression of the p185Y177F mutant, an interaction of Crkl which binds to a C-terminal proline-rich region of Bcr-Abl and this mutant [Heaney et al., 1997] was demonstrated (Figure 1). Lastly, a direct interaction between Grb2 and Cbl was observed. Despite the similarity of Grap and Grb2, no direct interaction was detected between Cbl and Grap (Figure 1). This is consistent with previous coimmunoprecipitation results [Trub et al., 1997].

Grb2 mediates an interaction of Cbl and Bcr-Abl in a yeast three-hybrid analysis

Results from our yeast two-hybrid analyses demonstrated that Bcr-Abl did not directly associate Cbl, yet could directly interact with Grb2. Additionally, Grb2 interacted specifically with Cbl. From these findings we hypothesized that Grb2 could serve to bring Cbl to Bcr-Abl. To test this hypothesis, we engineered a plasmid that expresses LexA-Bcr-Abl and Grb2. As Grb-2 is not expressed as a VP-16 fusion protein, the interaction between Bcr-Abl and Grb2 will not be scored by growth in the absence of histidine unless a VP16 fusion protein that interacts with either Grb2 or Bcr-Abl is provided. As shown in Figure 2a, VP-16-Cbl does interact with the LexA-Bcr-Abl/Grb2 construct and this interaction is dependent on a functional Grb2 binding site. Thus, a positive interaction was detected in matings between Cbl and a p185Bcr-Abl/Grb2 construct, whereas no interaction was detected when Cbl was mated with p185Bcr-Abl-Y177F/Grb2. As expected, when the p185Y177F mutant was mated to Crkl, growth was observed on plates in the absence of histidine. A summary of the yeast three-hybrid interactions is given in Figure 2b.

Cbl does not directly bind Bcr-Abl in vitro

The next series of experiments were designed to determine whether we could assemble the Bcr-Abl, Grb-2, Cbl complex in vitro using purified proteins.To confirm our findings in the yeast-two hybrid which indicated that Bcr-Abl and Cbl fail to associate in the absence of Grb2, gel overlay assays

were performed. In these experiments, four GST- Cbl fusion proteins, corresponding to the constructs illustrated in Figure 3, were separated by SDS-PAGE, probed with purified Bcr-Abl, and immunoblotted for Abl. As seen in Figure 4, no detectable interaction was observed between these two proteins. As a positive control for the gel overlay, binding of purified Bcr-Abl to GST-Grb2 was observed.

Direct Association of Grb2 with Cbl is mediated by two proline-rich domains of Cbl

We next examined the interction of purified Grb2 and Cbl. Previous studies examining the *in vitro* association between Cbl and Grb2 have mapped the binding of full length Cbl to the amino-terminal SH3 domain of Grb2 binding to the C-terminal proline-rich region of Cbl [Donovan et al., 1994; Meisner et al., 1995; Fukazawa et al., 1995; Donovan et al., 1996]. To examine this interaction in our system, the four GST-Cbl fusion proteins were separated by SDS-PAGE and probed with purified Grb2. Binding of Grb2 to Cbl was assessed by immunoblotting for Grb2. As shown in Figure 5, purified Grb2 recognized the two Cbl constructs containing the C-terminal proline-rich sequences, GST-Cbl 91, and GST-Cbl 366. No binding to the GST-Cbl 307 or the GST-Cbl N-terminal construct was observed. To confirm the specificity of this interaction, no direct binding of Grap to Cbl was detected, confirming our yeast two hybrid data (data not shown). Additional data in Figure 5 shows that

Grb2 binds directly to purified Bcr-Abl in this gel overlay assay, again confirming our yeast two hybrid data.

Cbl binds Bcr-Abl in the presence of Grb2 in vitro

The preceding results demonstrate that using purified proteins, Cbl and Grb2 interact directly, as do Grb2 and Bcr-Abl. However, no direct interaction between Bcr-Abl and Cbl is observed. To determine whether Grb2 could mediate an interaction between Bcr-Abl and Cbl, the experiment shown in Figure 4 was repeated. However, rather than overlaying GST-Cbl fusion proteins with Bcr-Abl, these GST-Cbl fusion proteins were overlaid with Bcr-Abl that had been preincubated with Grb2. This gel was then immunoblotted with Abl to detect binding of Bcr-Abl to the various GST-Cbl fusion proteins. In contrast to the results shown in Figure 4, binding of Bcr-Abl to Cbl is detected (Figure 6). Consistent with the results in Figure 5, binding is observed to the GST-Cbl fusion proteins to which Grb2 binds. Thus, an association of Bcr-Abl to Cbl was detected in the GST-Cbl 91 and GST-Cbl 366 fusion proteins but not in either the GST-Cbl 307 or GST-Cbl N-terminal constructs, in the presence of Grb2. These data independently confirm the findings from our yeast three-hybrid assay that the adaptor protein Grb2 serves to bring Cbl to Bcr-Abl.

Mutation of the Grb2 binding site in Bcr-Abl significantly diminishes the association of Bcr-Abl and Cbl

Having established the requirement for Grb2 in linking Bcr-Abl to Cbl in yeast and *in vitro,* we investigated this association in a more physiologically relevant setting; a Bcr-Abl-expressing cell line expressing the Y177F mutation. Cell lines from two independent clones expressing wild-type p210Bcr-Abl or p210Bcr-Abl with a Y177 mutation were analyzed in co-immunoprecipitation studies to determine the relative associations of Cbl, Bcr-Abl, and Grb2. The data presented in Figure 7 demonstrate that the amount of Grb2 associated with Bcr-Abl is significantly decreased in cells expressing the Bcr-AblY177F mutant. Similarly, the amount of Cbl associated with Bcr-Abl was significantly decreased in these cell lines (Figure 8).

Discussion

Bcr-Abl is known to associate with a variety of signaling proteins in vivo, resulting in the activation of a variety of signal transduction pathways. As a result of the constitutive tyrosine kinase activity of Bcr-Abl, numerous proteins have also been shown to be tyrosine phosphorylated in Bcr-Abl-expressing cells. Thus, the emerging view of the molecular mechanism of action of Bcr-Abl, is that Bcr-Abl exists as a multi-protein complex in cells, binding to, phosphorylating, and activating a number of substrates [Raitano et al., 1997; Faderl et al., 1999].

The view of Bcr-Abl as a multi-protein complex is supported by the finding that several proteins bind both directly and indirectly to Bcr-Abl. For example, Crkl, an SH2, SH3 domain-containing adaptor protein, binds through

its amino terminal SH3 domain to a proline-rich region in the C-terminus of Abl [Oda et al., 1994; Heaney et al., 1997]. Crkl can also bind directly to tyrosine phosphorylated Cbl through its SH2 domain [Bhat et al., 1997]. As tyrosine phosphorylated Cbl can also bind directly to Bcr-Abl, deletion of the direct binding site for Crkl in Bcr-Abl does not abolish Crkl binding to Bcr-Abl or tyrosine phosphorylation of Crkl in Bcr-Abl-expressing cells [Heaney et al., 1997]. These data, demonstrating both direct and indirect interactions, point out the difficulties in determining the contribution of an individual protein to Bcr-Abl transformation.

We previously demonstrated that tyrosine phosphorylated Cbl binds directly to the SH2 domains of Abl and Crkl [Bhat et al., 1997]. Thus, Cbl can also bind to Bcr-Abl through direct and indirect interactions. In the case of Crkl binding to Bcr-Abl, no tyrosine phosphorylation of Crkl was required for binding as this is an SH3 domain/proline-rich interaction. However, in the case of Cbl, we faced a dilemma, in that tyrosine phosphorylated Cbl was binding to Bcr-Abl and Crkl. Thus, it was unclear how Cbl became tyrosine phosphorylated prior to associating with Bcr-Abl. Possibilities include a direct enzyme-substrate interaction between Bcr-Abl and Cbl, another kinase phosphorylating Cbl prior to its association with Bcr-Abl, or a third protein mediating an initial interaction between Bcr-Abl and Cbl. In the case of another protein phosphorylating Cbl, either this kinase or another protein is still required to mediate an interaction between Bcr-Abl and Cbl.

We initially chose to analyze the interaction between Bcr-Abl and Cbl in a yeast two-hybrid assay as this system allows the detection of relatively low affinity interactions. Using this system, no direct interaction between Bcr-Abl and Cbl could be detected. These findings support the possibility that another protein mediates an initial interaction between Bcr-Abl and Cbl. Data presented in this manuscript demonstrates that tyrosine 177 of Bcr-Abl mediates binding of Grb-2 to Bcr-Abl, consistent with previous reports of the SH2 domain of Grb-2 binding to Bcr-Abl [Puil et al., 1994; Pendergast et al., 1993]. The association between Grb-2 and Cbl is mediated by two proline-rich regions of Cbl, also consistent with previous data demonstrating an interaction between the amino terminal SH3 domain of Grb-2 and C-terminal proline-rich regions of Cbl [Panchamoorthy et al., 1996; Meisner et al., 1995; Donovan et al., 1996; Rivero-Lezcano et al., 1994; Odai et al., 1995]. Thus, Grb-2 would be capable of simultaneously binding to Cbl and Bcr-Abl. Both the yeast three hybrid data and the data using purified proteins demonstrate that Grb-2 does mediate an interaction of Bcr-Abl and Cbl. These findings support a model whereby Grb2 mediates an initial interaction between Cbl and Bcr-Abl. Following this initial interaction, Cbl could become tyrosine phosphorylated and interact with the SH2 domain of Bcr-Abl, thus stabilizing the interaction of Cbl with Bcr-Abl and allowing interactions of Cbl with other signaling proteins required for Bcr-Abl function (Figure 9).

In cells expressing Bcr-Abl with a mutation in the Grb2 binding site, the amount of Cbl associated with Bcr-Abl is significantly decreased, but not

absent. This suggests that although Grb-2 is the major mediator of the interaction between Bcr-Abl and Cbl, either another protein can also mediate this residual interaction or that another kinase phosphorylates Cbl thereby permitting the association between Bcr-Abl and Cbl.

Interestingly, we find some specificity of interactions of proteins associating with Bcr-Abl. For example, Grb-2 and Grap are highly homologous and both bind to tyrosine 177 of Bcr-Abl. However, only Grb-2 is capable of binding Cbl. Thus, Grb-2, but not Grap is capable of linking Bcr-Abl to Cbl.

The Y177F mutant of Bcr-Abl is transformation defective in fibroblasts, but remains capable of inducing factor independent growth of myeloid cell lines [Cortez et al., 1995]. This mutation abolishes the majority of Grb-2 binding to Bcr-Abl. As Grb-2 is known to link Bcr-Abl to ras activation and ras activation is diminished in the Y177F mutant, this has been presumed to be responsible for the transformation defect of this mutant. However, our data also demonstrates a significant reduction in the amount of Cbl bound to the Bcr-Abl Y177F mutant. Thus, it is possible that the decrease in Cbl binding also contributes to the transformation defect of this mutant.

Acknowledgements

We are extremely grateful to Kara J. Johnson, Tsukasa Oda, Arun Bhat, Margaret Reis, and Lei Shen for all contributing substantially to this work and to Cheng-Sheng Guo for careful critiquing of the manuscript.

Figure Legends

Figure 1. **c-Cbl and Bcr-Abl do not interact directly in a yeast two-hybrid assay.** A. A yeast plasmid, pBTMa, which contains a selectable marker for tryptophan was engineered to express a LexA DNA binding domain fused to either p185$^{bcr\text{-}abl}$, p185$^{bcr\text{-}ablY177F}$, or Cbl. The yeast plasmid, pVP16, contains a selectable marker for leucine and a VP16 activation domain fused in frame to Grap, Grb2, Cbl, or CrkL. These constructs were transformed into a yeast strain containing his3 coding sequences driven by promoters fused to LexA DNA binding sites as a reporter strain. Yeasts were either grown on YC plates +his,-trp,-leu (YC + His) or YC plates without His (YC-His). 1. pBTM-Cbl + pVP16-Grb2; 2. pBTM-Cbl + VP16-Grap; 3. pBTM-Cbl + VP16; 4. pBTM-p185 + VP16-Cbl; 5. pBTM-p185 + VP16-Grb2; 6. pBTM-p185Y177F + VP16-Grb2; 7. pBTM-p185Y177F + VP16; 8. pBTM-p185Y177F + VP16-CrkL. B.Summary of yeast two-hybrid interactions. Additional controls included CrkL expressed as a VP16 activation domain fusion protein. pBTM-p185Y177F interact with pVP16-CrkL indicating that the lack of interaction of Cbl with this mutant is not due to lack of Bcr-Abl expression.

Figure 2. **Bcr-Abl and Cbl interact in a yeast three-hybrid in the presence of Grb2.** A. A yeast plasmid with contains a LexA DNA binding domain fused to Bcr-Abl and Grb2 expressed from an ADH promoter was mated to VP-16 Cbl or

CrkL. Controls include lamin and LexA p185Y177F/Grb2 which contains a tyrosine to phenylalanine mutation in the Grb2 binding site of Bcr-Abl. 1. pBTM-p185/Grb2 + VP16-Cbl; 2. pBTM-p185Y177F/Grb2 + pVP16-Cbl; 3. pBTM-lamin + VP16-Cbl; 4. pBTM-p185Y177F/Grb2 + VP16-CrkL. B.Summary of yeast three-hybrid interactions.

Figure 3. **Schematic of GST-Cbl Fusion Constructs used for Gel Overlay Assays.** The four GST-Cbl fusion constructs span the entire Cbl molecule and contain different domains. The numbers indicate the number of amino acids in each protein. GST-N-Cbl contains 220 amino acids.

Figure 4. **Bcr-Abl and Cbl do not directly interact in a gel overlay assay.** The four GST-Cbl fusion proteins depicted in Figure 3 were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with purified Bcr-Abl and binding detected with an Abl antibody. No binding was detected between the GST-Cbl fusion proteins and Bcr-Abl. The immunoblot was stripped and reprobed with GST to ensure equal loading of the indicated fusion proteins (data not shown). GST-Grb2 and purified Bcr-Abl were used as positive controls for the overlay and antibody binding, respectively. Bacterially expressed GST was used a negative control. The migration of Bcr-Abl and GST-Grb2 is indicated to the right of the panel. Migration of molecular weight markers is indicated on the left of the panel.

Figure 5. **Analysis of Grb2 binding to the proline-rich domains of Cbl expressed as GST fusion proteins.** GST-Cbl fusion proteins were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with bacterially expressed Grb2, and binding was detected with Grb2 antibody. The immunoblot was stripped and reprobed with GST to ensure equal loading of the indicated fusion proteins (data not shown). GST-Grb2 and purified Bcr-Abl were used as positive controls for the antibody binding and overlay, respectively. Bacterially expressed GST was used a negative control. The migration of GST-91 Cbl, GST-366 Cbl, GST-Grb2 and Bcr-Abl is indicated to the right of the panel.

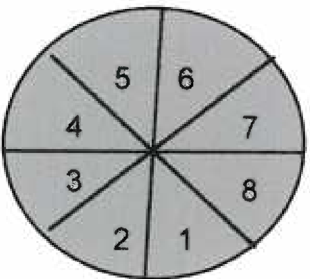
Figure 6. **Binding of Bcr-Abl to Cbl in the presence of Grb2.** GST-Cbl fusion proteins were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with purified Bcr-Abl that had been incubated overnight with bacterially expressed Grb2. Binding was detected with an Abl antibody. The immunoblot was stripped and reprobed with GST to ensure equal loading of the indicated fusion proteins (data not shown). GST-Grb2 and purified Bcr-Abl were used as positive controls for the gel overlay and antibody binding, respectively. Bacterially expressed GST was used a negative control. The migration of GST-91 Cbl, GST-366 Cbl, GST-Grb2 and Bcr-Abl is indicated to the right of the panel.

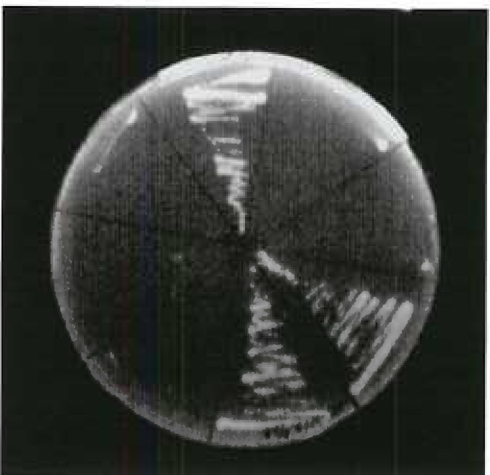
Figure 7. **Decreased association of Grb2 with Abl in Bcr-Abl expressing cell lines expressing a mutation in the Grb2 binding site of Bcr-Abl (Y177F).** Lysates from the indicated cells were immunoprecipitated (I.P.) with either an Abl (A) or Grb2 (C) antibody and immunoblotted with Grb2 or Abl, respectively. Immunoblots were stripped and reprobed with either Abl (B) or Grb2 (D) to ensure equal loading. The migration of Grb2 and Bcr-Abl is indicated to the right of the panel.

Figure 8. **Decreased association of Cbl with Abl in Bcr-Abl expressing cell lines expressing a mutation in the Grb2 binding site of Bcr-Abl (Y177F).** Lysates from the indicated cells were immunoprecipitated (I.P.) with either an Abl (A) or Cbl (C) antibody and immunoblotted with Cbl or Abl antisera, respectively. Immunoblots were stripped and reprobed with either Abl (B) or Cbl (D) to ensure equal loading. The migration of Cbl and Bcr-Abl is indicated to the right of the panel.

Figure 9. **Proposed Model: Grb2 mediates the major interaction between Cbl and Bcr-Abl.** (A). Prior to the tyrosine phosphorylation of Cbl, Cbl interacts directly with Grb2 and Grb2 interacts directly with Bcr-Abl. (B) Following the tyrosine phosphorylation of Cbl, Cbl binds directly to the SH2 domain of Bcr-Abl.

Abbreviations are 2 and 3, SH2 and SH3 domains; K kinase domain; Y tyrosine; pY phosphorylated tyrosine; PPP proline-rich domain.





YC - HIS

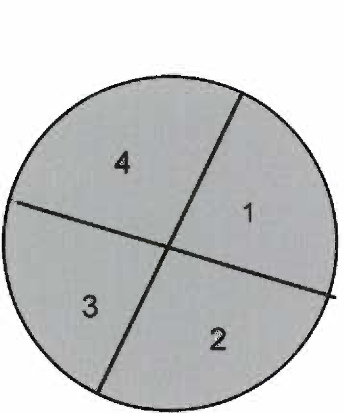
YC + HIS

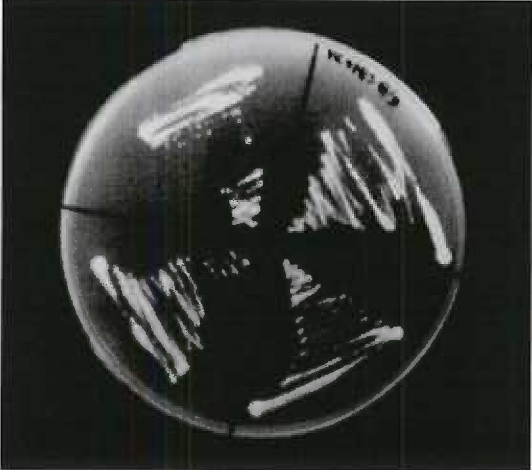
Figure 1A

VP-16 fusion construct

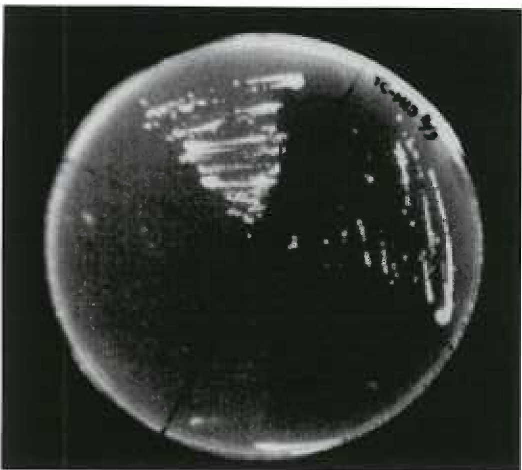
LexA fusion construct	Grap	Grb2	Cbl	CrkL	VP16
p185Bcr-Abl	+	+	-	+	-
p185Bcr-Abl Y177F	-	-	-	+	-
Cbl	-	+	ND	ND	-

Figure 1 B





YC + HIS

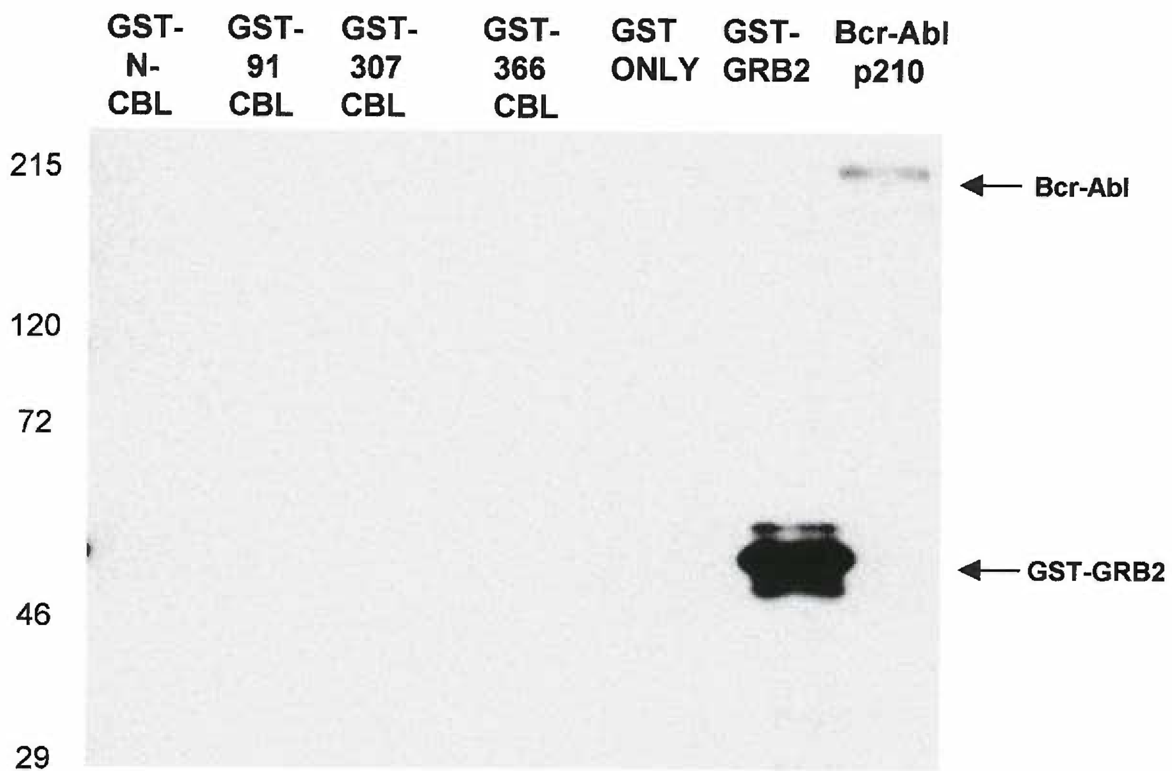


YC- HIS

Figure 2A

	VP16 fusion construct	
LexA fusion construct	Cbl	CrkL
p185Bcr-Abl/Grb2	+	+
p185Bcr-AblY177F/Grb2	-	+
Lamin	-	-

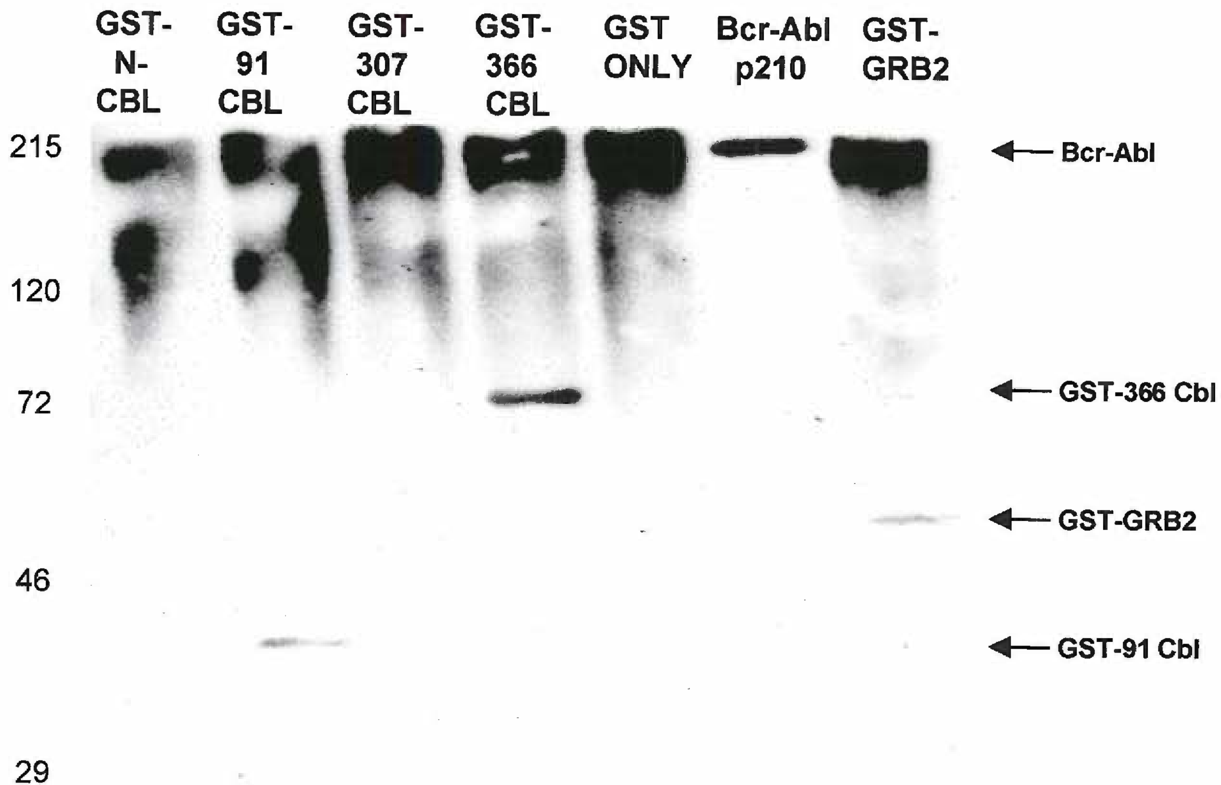
Figure 2B



GST-Cbl Fusion Proteins

Overlay: Bcr-Abl p210
Blot: anti-Abl

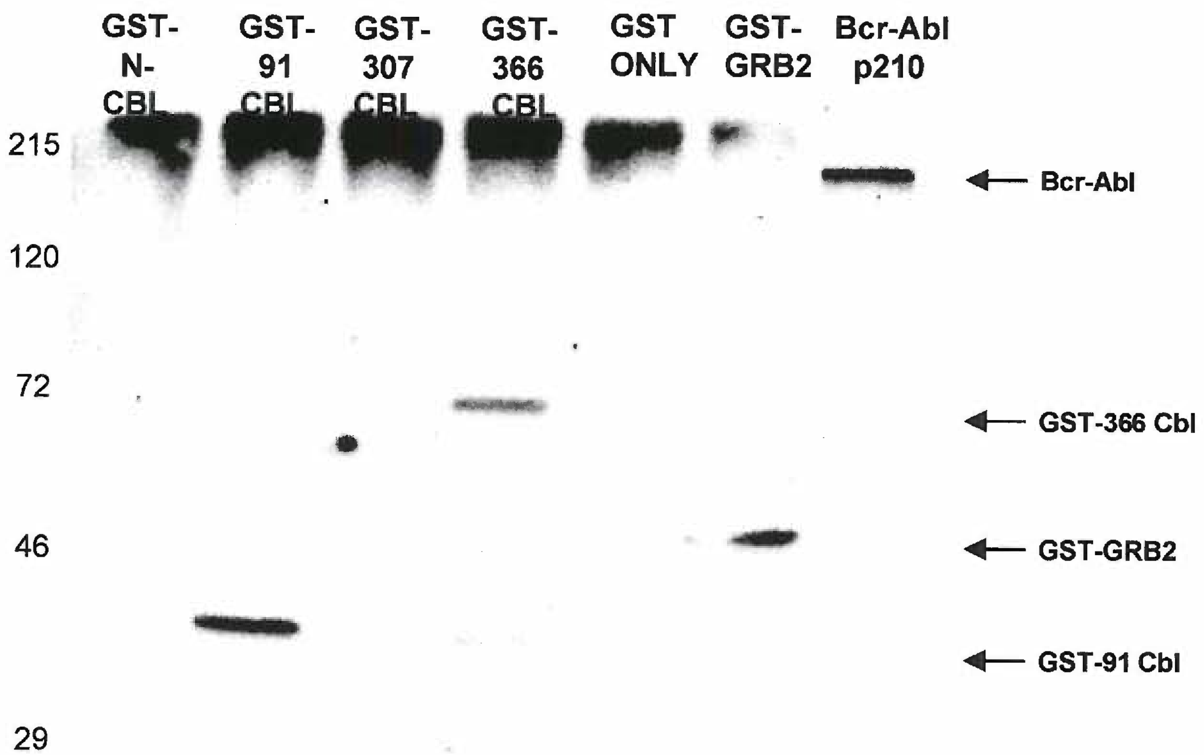
Figure 4



GST-Cbl Fusion Proteins

Overlay: GST-Grb2
Blot: anti-Grb2

Figure 5



GST-Cbl Fusion Proteins

Overlay: GST-Grb2 & Bcr-Abl p210
Blot: anti-Abl

Figure 6

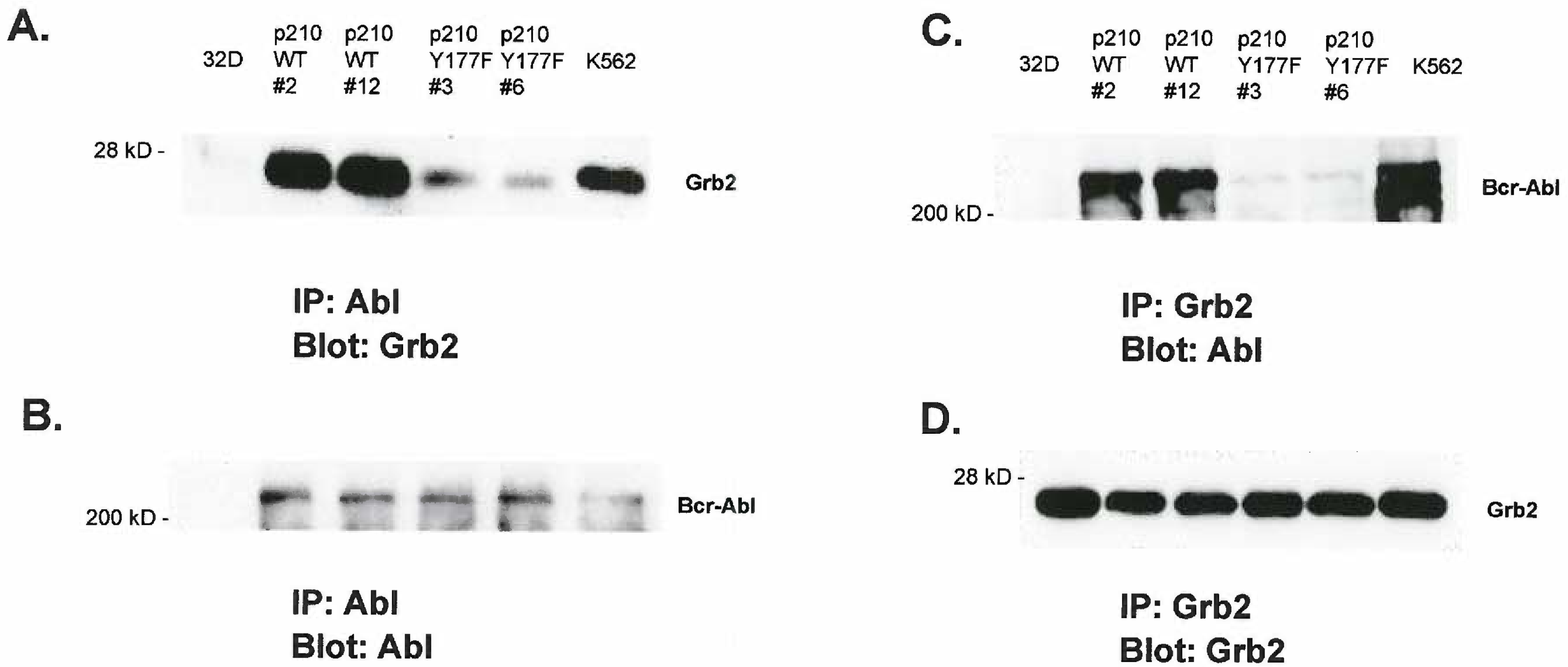


Figure 7

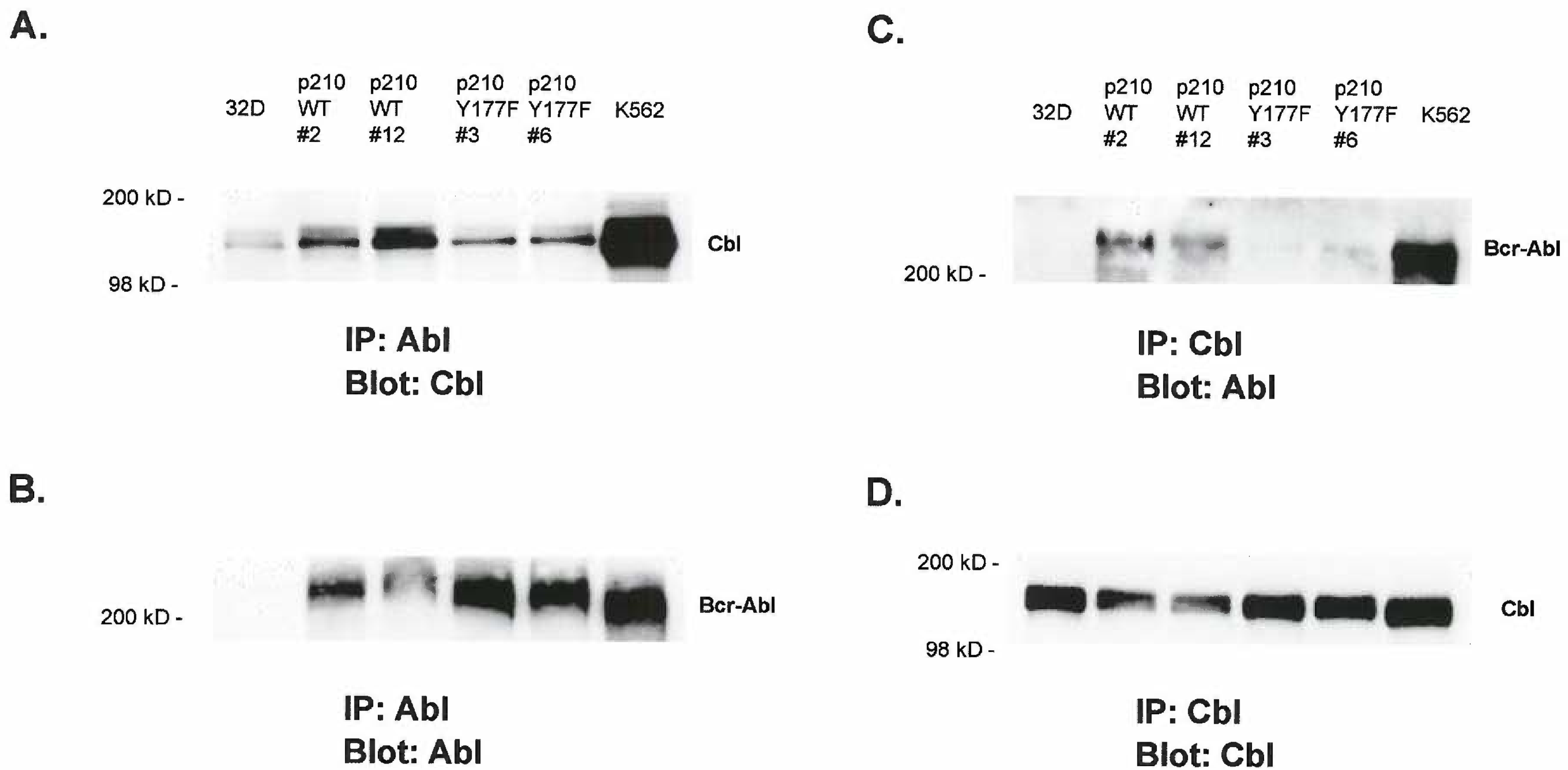
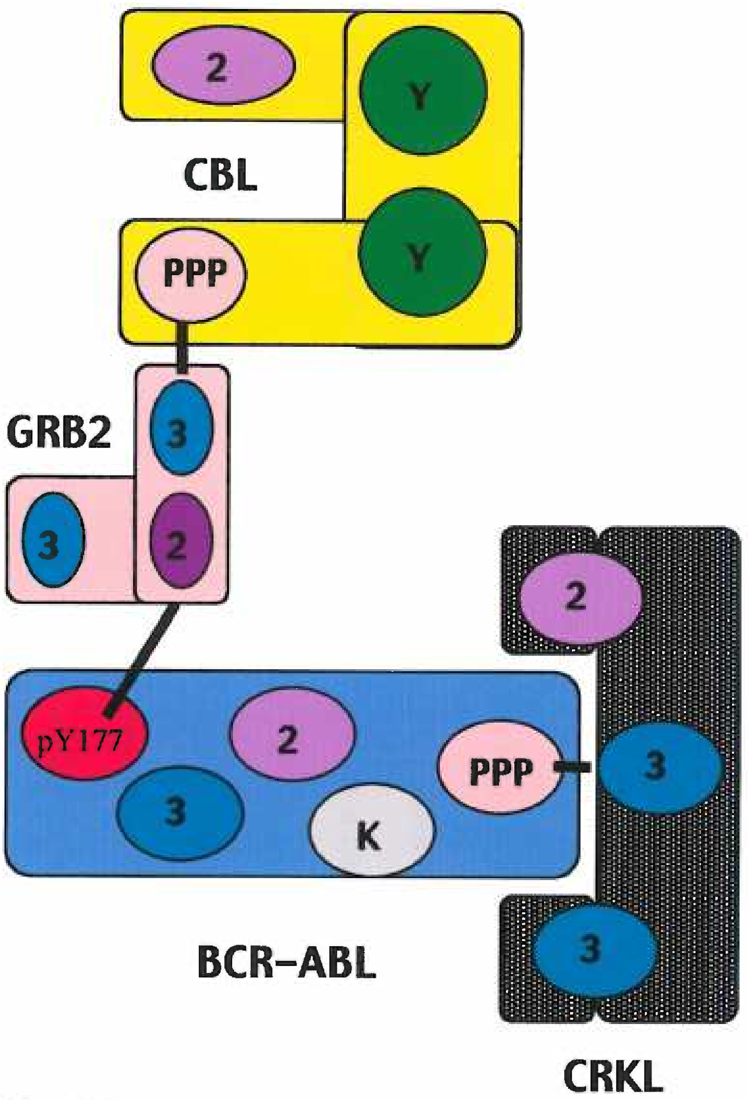


Figure 8



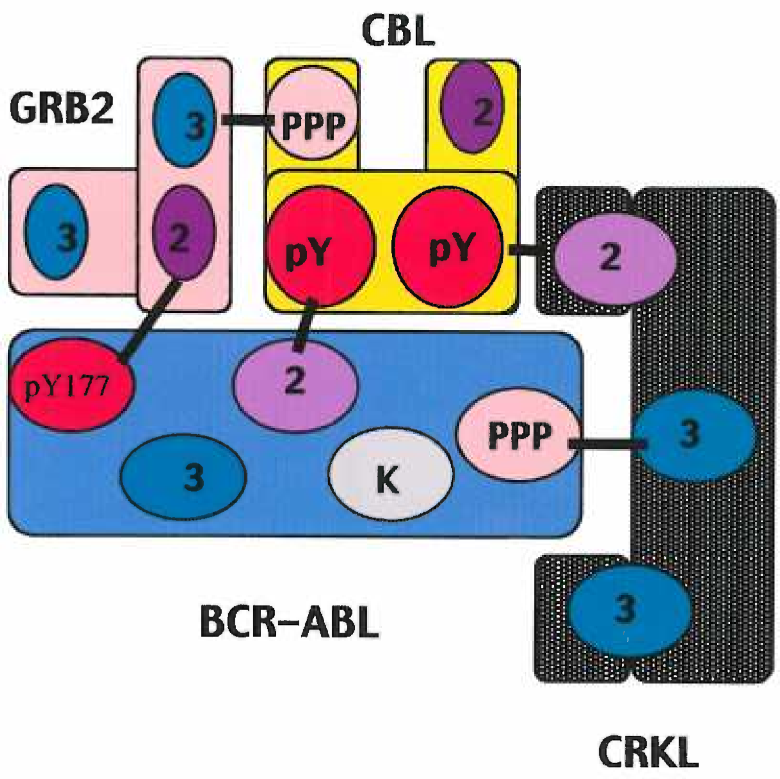


Figure 9

and their relationship to a specific disease entity, CML. Moreover, the inquiry into Bcr-Abl not only serves to improve our understanding of CML; it also serves as a more general paradigm for studying other oncogenes and their associated cancers. For example, the study of Bcr-Abl has elucidated much about the basic principles of how specific domains of proteins interact with other proteins, the requirement for tyrosine kinase activity to generate signaling cascades to the cytoskeleton, and how multi-molecular complexes in a cell can be assembled.

Examination of these fundamental cellular mechanisms serves as the foundation for this thesis. Thus, the findings pertaining to these processes will be presented. I will also outline important experiments that should be considered in the future. However, to put these findings and future experiments into the broader context of research into this disease, discussion of additional mechanisms that may contribute to the biological changes that result in leukemia is warranted.

The work in this thesis has focused on the hypothesis that defective adhesive interactions are responsible for the expansion of CML progenitors in the bloodstream. However, there is also considerable data to support two other mechanisms. These mechanisms include a decrease in programmed cell death (apoptosis) and an increase in proliferation. Thus, focusing on a singular mechanism (adhesion) to address the vast expansion of cells may overlook the complexities of the transformed phenotype. A more comprehensive approach, as described in the "discordant maturation" model [Clarkson and Strife 1993],

integrates the interrelationships of abnormal signaling events in CML cells. For instance, this hypothesis could help explain how inappropriate activation of the same effector (such as Ras) might deregulate not only the apoptotic and mitogenic pathways but also the adhesion pathways.

Numerous studies have demonstrated that cell lines expressing Bcr-Abl induce an anti-apoptotic signaling pathway. Hence, these cells live significantly longer in the absence of growth factors or in response to DNA damage, compared to cells not expressing Bcr-Abl [Laneuville et al., 1994; Bedi et al.,1994; Bedi et al., 1995; Cortez et al.,1996]. Consistent with the findings in cell lines, primary CML cells also display reduced apoptosis compared to normal cells: in the absence of cytokines primary CML cells grow 2 to 4 days longer than normal progenitors [Clarkson et al., 1997].

The downstream effectors of Bcr-Abl that protect cells from apoptosis are less clear. One study showed an increase in Bcl-2 protein expression in Bcr-Abl expressing-cells, and treatment of cells with anti-sense oligonucleotides to Bcl-2 diminished the transforming activity of Bcr-Abl [Sanchez-Garcia and Grutz, 1995]. However, these data have not been reproduced. Another report examined the requirement for the small guanine nucleotide-binding protein Ras in 32D cells [Cortez et. al,. 1996]. In this latter study, expression of a dominant negative Ras (Asn 17) in Bcr-Abl expressing cells successfully induced apoptosis in approximately 70% of the cells as meaured by DNA fragmentation. Subsequent

studies have examined potential downstream targets of Ras in the anti-apoptotic signaling pathway in Bcr-Abl expressing cells. Such pathways include PI3-kinase and its downstream target AKT [Skorski et al., 1995; Skorski et al., 1997]. Following the activation of AKT, the pro-apoptotic protein BAD would become phosphorylated and thus ultimately inhibit apoptosis by its inability to bind the anti-apoptotic protein BCL_{XL}.

More recent evidence on the role of the normal Abl protein may shed light on how expression of Bcr-Abl might cause an inhibition of apoptosis [Gong et al., 1999; Yuan et al., 1999; White and Prives 1999]. In response to infrared radiation or cis platinum (genotoxic stress), the Abl tyrosine kinase phosphorylates and stabilizes a recently identified p53 homologue, p73. Stabilization of this latter protein by phosphorylation has been shown to induce apoptosis. Thus, in this setting, the normal Abl tyrosine kinase behaves as an anti-oncogene. While unproven, these findings support the notion that in the absence of one allele of Abl, as is the case in CML, Bcr-Abl-expressing cells might be at a distinct disadvantage when exposed to stress-inducing agents by lacking an adequate apoptotic response.

In addition to the ability of activated Ras to inhibit apoptosis in Bcr-Abl-expressing cells, activated Ras has also been demonstrated to play a role in increased proliferative signals. One common read-out of Bcr-Abl's mitogenic ability is the rendering of cells' cytokine independent. Thus, transfection of a

dominant negative Ras or treatment of cells with anti-sense oligonucleotides against Ras (or Raf) has been demonstrated to abrogate Bcr-Abl-induced cytokine independence [Skorski et al.,1994; Sawyers 1993]. Downstream of Ras activation are the three mitogen activated protein kinase (MAPK) signaling pathways: ERK, JNK and p38. Reports in Bcr-Abl-expressing cells suggest that in contrast to the response of other tyrosine kinases, Bcr-Abl results in prefential activation of JNK over that of ERK or p38; however, the significance of this preference in regards to the overall cellular response and disease outcome is still speculative [Reviewed in Raitano et al., 1995].

Another potentially important signaling protein that might ultimately influence disease progression by inhibiting apoptosis or increasing proliferation is c-Myc. In a common model system, transfection of fibroblasts or 32D cells with wild-type Bcr-Abl results in transformation while defective Bcr-Abl mutants fail to transform. Overexpression of c-Myc can complement defective Bcr-Abl genes in these cell lines, demonstrating the importance of this downstream molecule in the transforming ability of Bcr-Abl [Afar et al., 1994]. Bcr-Abl expression has also been shown to promote cell cycle progression from G1 –phase to the S-phase by activating cyclin-D complexes. Similar to the overexpression of Myc studies, overexpression of cyclin-D in a cell line with a deletion in Bcr-Abl can complement this defect. While these studies suggest a proliferative advantage to cells endowed with Bcr-Abl, chronic phase CML progenitors do not display cytokine independence [Clarkson and Strife 1993; Bedi et al., 1994]. However, it

has been demonstrated that in contrast to normal cells, more CML cells are dividing. These findings suggest that if the proliferative signal provided by Bcr-Abl is not mediated through a growth-factor dependent signaling pathway, it must be through an alternate (possibly adhesion-mediated) pathway.

An additional mechanism that has been implicated in increased proliferation and the inhibition of apoptosis and that may contribute to CML by altering gene expression is the activation of the JAK-STAT pathway. In contrast to the highly regulated phosphorylation of the STAT proteins in response to cytokines in normal hematopoietic cells, in Bcr-Abl-expressing cells the STATs 1, 5 and 6 are constitutively phosphorylated [Illaria et al., 1996 Carlesso et al,. 1996]. Interestingly, activation of STAT 1 and 5 may occur in the absence of the prior phosphorylation of the JAK proteins. These findings, like those with c-Myc, suggest that Bcr-Abl may mimic the signals normally provided by growth factors and provide a mechanism to activate specific genes. Identifying what genes are differentially expressed in response to STAT activation will undoubtely yield greater insight into the process of Bcr-Abl mediated tranformation and the contributution of this pathway to leukemogenesis.

While inhibition of apoptosis and increased mitogenic signaling are two of the major processes besides adhesion which may result in the proliferation of the leukemic clone, recently the biological role of phosphatases in Bcr-Abl-expressing cells has come into question. In normal physiologic conditions, the

levels of tyrosine phosphoryled proteins are balanced by tyrosine kinases and tyrosine phosphatases. Thus, it has been hypothesized that in CML there may be an imbalance of phosphatases to down-regulate the aberrant tyrosine phosphorylation of proteins [Flint et al., 1997]. Recent reports have identified the protein tyrosine phophatase specific for Bcr-Abl, PTP1B. Interestingly, this protein is rapidly up-regulated in the presence in a Bcr-Abl-expressing cell line, can disrupt the Bcr-Abl/Grb2 complex, and reduce transformation when overexpressed [LaMontagne et al., 1998]. While these data suggest that PTP1B might serve as an important Bcr-Abl antagonist in cell lines, its physiologic relevance remains uncertain; PTP1B levels in CML patient samples have yet to be examined.

As the disease progresses from the chronic to the blast phase, usually 4 to 5 years following diagnosis, additional cytogenetic changes take place. These changes are characterized by the inability of the cells to terminally differentiate. While the precise reason for these changes are unknown (presumably owing to the multitude of molecular abnormalities) the consequences of cytogenetic mutations involving such proteins as p53, Ras, RB, c-myc are undoubtedly deleterious; such mutations are commonly seen in a variety of cancers. Thus, the chromosomal instability initiated by the presence of the constitutively active tyrosine kinase, Bcr-Abl, inexorably leads to an acute leukemia.

I. Studies on the Role of the Cytoskeleton

As previously mentioned, one of the most prominent features of CML is a disregulated hematopoiesis that results in a massive expansion of granulocytic progenitors and precursors in the bloodstream [Verfaillie 1998]. These clinical observations have led to the hypothesis that a defect in adherence of immature granulocytes may be responsible for their premature release from the bone marrow. Thus, a number of investigators have sought to examine the functionality of attachment receptors (integrins) on the cell surface of CML cells to determine whether these receptors may contribute to the premature release of cells from the bone marrow.

Hence, the point of departure for our studies was that if a defect in adherence did indeed exist, the origin of this defect would be the unique presence of the cytoplasmic Bcr-Abl oncoprotein. Further, given its known physical association with the cytoskeleton and focal adhesion proteins, Bcr-Abl is well positioned to influence these structural components. Thus, we set out to characterize the cytoskeletal architecture of two Bcr-Abl-expressing fibroblast cell lines. We then asked, if two distinct signaling pathways that Bcr-Abl is known to activate were inhibited, what impact would this have on the cytoarchitecture of Bcr-Abl-expressing cells? Could the abnormal cytoarchitecture we observed be restored to normal? In addition, we sought to determine whether changes in the cytoarchitecture correlated with the abnormal adhesive properties of Bcr-Abl expressing cells.

In contrast to normal cells, examination of the cytoskeleton of Bcr-Abl-expressing cells revealed a lack of discernible actin stress fibers and focal adhesions when stained with rhodamine-phalloidin and phosphotyrosine antibodies respectively. These findings were not that unexpected as a similar phenotype was observed in fibroblasts transformed by v-src, another oncogenic tyrosine kinase.

We next attempted to rescue the normal phenotype by interrupting two distinct but overlapping signaling pathways, the Abl kinase and PI3-kinase pathways. These two pathways were chosen because they both are inappropriately activated in Bcr-Abl-expressing cells. Moreover, activated PI3-kinase and its lipid products have been shown to be important in oncogenic transformation. Furthermore, it had been shown that in Bcr-Abl expressing cells, PI3-kinase associates with Bcr-Abl.

Having established biochemically that the two pharmocological inhibitors we would use in this endeavor, CGP57148 and LY29004, could successfully inhibit Abl and PI3-kinase respectively, we incubated Bcr-Abl-expressing cells with these compounds. Cells treated with the Abl kinase inhibitor had almost a full recovery of stress fibers and focal adhesions. In contrast, no apparent changes were observed in cells treated with the PI3-kinase inhibitor. These findings were further strengthened by our cellular adhesion assay results; treatment of cells with the Abl but not the PI3-kinase inhibitor significantly enhanced adhesion. To

test for specificity of CGP57148 for the ATP binding site of the Abl kinase, v-src transformed cells were exposed to CGP57148. In these experiments no phenotypic changes were observed, confirming the specificity of CGP57148 for Abl kinase.

Thus, even though PI3-kinase is inappropriately activated in Bcr-Abl-expressing cells and in other reports has been demonstrated to exert a role in regulating the cytoskeleton, in this study inhibition of this molecule did not visibly alter the cytoarchitecture. Our initial expectation from these experiments was that dampening its activation could reverse the transformed phenotype since parental cells possess a normal cytoskeleton and PI3-kinase is not inappropriately activated. However, this assumption may have been overly simplistic. In a recent article by Keely et al., these authors demonstrated that in transformed mammary epithelial cells PI3-kinase activation can induce cell motility and its inhibition disrupts actin polymerization [Keely et al., 1997]. While these findings may or may not reflect what is occurring in Bcr-Abl-expressing cells, they do underscore the complexity of deciphering precisely what signaling components might be regulating the cytoskeleton. In Bcr-Abl-expressing cells PI3-kinase may be miscompartmentalized or sequestered in some fashion. Thus, even when it is no longer inappropriately activated, it may be denied access to important effector molecules such as the Rho GTPases which direct cytoskeletal reorganization.

In contrast, we found that activated Abl kinase is the major regulator of the cytoskeleton. Exactly how the Abl kinase signals to the cytoskeleton remains to be determined. The results with CGP57148 demonstrate the unequivocal importance of activated Abl kinase as the cause of a disorganized cytoskeleton and reduced adhesive properties in Bcr-Abl-expressing cells. Despite the conclusive nature of these findings there are questions that remain unresolved about the exact signaling pathway Abl kinase may use to regulate the cytoskeleton.

Future Cytoskeletal Studies

The restoration of the normal cytoskeleton with the Abl kinase inhibitor demonstrates a proof of principle. That is, the fundamental problem with Bcr-Abl-expressing cells is activated Abl kinase. However, the inhibitor does not eliminate the Bcr-Abl protein from the cell; it simply binds the kinase and turns off kinase activity. Thus, the continued presence of Bcr-Abl may present a potential problem given its predilection for associating with the cytoskeleton through its actin-binding domain and other protein-protein interactions that are phosphorylation-independent (i.e. CrkL). These associations may be one explanation for the fact that restoration of the cytoskeleton was never fully complete.

While our experiments demonstrated how the normal morphology of cells could be rescued with CGP57148, a further and more complete biochemical characterization of the interactions of Bcr-Abl with cytoskeletal proteins after treatment with CGP57148 would yield greater physiological insight into kinase-independent associations. Specifically, does Bcr-Abl still associate with the cytoskeletal proteins vinculin, talin and paxillin? If these associations do still exist, they could disrupt cytoskeletal assembly and integrin engagement.

Ideally, to perform these experiments one would obtain CD34+ cells from the bone marrow of CML patients. From these cells an analysis of the interactions of Bcr-Abl with the cytoskeletal proteins would be performed, as well as a determination as to whether these proteins are tyrosine phosphorylated (previous reports in cell lines have indicated they are). These cells could then be treated with CGP57148 *in vitro* followed by a full assessment of Bcr-Abl's association with these cytoskeletal proteins by immunoprecipitating Bcr-Abl and immunoblotting with different cytoskeletal protein antibodies (and vice-versa). These studies would provide a more clear understanding of Bcr-Abl's linkage to proteins comprising the cytoskeleton and elucidate the requirement of kinase activity (phosphorylation) for these linkages. Moreover, they would provide a better foundation of knowledge on the physiological interactions of this aberrant protein in the absence of its catalytic function.

Given that inhibition of Abl kinase can restore to normal the cytoskeleton, it would be of enormous biological importance to ascertain Abl's downstream effectors in this signaling pathway. This is clearly a very open question. Hence I would begin by examining in Bcr-Abl transformed fibroblasts the role of three small GTPases that are well established in regulating the cytoskeleton. Specifically, microinjection experiments of dominant negative and constitutively activated constructs of Cdc42, Rac, and Rho, followed by phase contrast and fluorescence microscopy could be performed to establish their respective roles in this process. For example, if Rho is inactive in Bcr-Abl-expressing cells, it would be anticipated that microinjection of activated Rho would lead to stress fiber formation. This might suggest that Bcr-Abl is directly or indirectly maintaining Rho in an inactive form and further experiments could be devised to test this hypothesis.

II. Role of Grb2 Mediating an Interaction of Cbl with Bcr-Abl Studies

Bcr-Abl is known to interact with a variety of signaling proteins in vivo, resulting in the activation of a variety of signal transduction pathways. As a result of the constitutive tyrosine kinase activity of Bcr-Abl, numerous substrates have been shown to be tyrosine phosphorylated in Bcr-Abl-expressing cells. Thus, while the molecular mechanism responsible for Bcr-Abl transformation is not fully defined, the emerging view is that Bcr-Abl exists as a multi-protein complex in cells that can bind, phosphorylate, and activate a number of substrates.

One of most prominent substrates in Bcr-Abl-expressing cells is c-Cbl, the cellular homolog of the oncoprotein v-Cbl. This protein, tyrosine phosphorylated in Bcr-Abl-expressing cells, has also been identified as a key signaling molecule in many growth-factor proliferation pathways. Previous experiments in our laboratory demonstrated that tyrosine phosphorylated Cbl binds directly to the SH2 domain of Bcr-Abl [Bhat et al., 1997]. This presented a quandary. Prior to its tyrosine phosphorylation, how do Cbl and Bcr-Abl associate? Our hypothesis was that another protein, acting as an intermediary, could bring Cbl to Bcr-Abl and thus provide the opportunity for Cbl to directly bind Bcr-Abl once Cbl is phosphorylated.

To address this question we first determined whether Cbl and Bcr-Abl could bind directly in a yeast two-hybrid assay. In these experiments, no direct interaction was observed. One of the candidates we hypothesized for mediating an interaction between Cbl and Bcr-Abl was Grb2, as it is known to bind to Bcr-Abl through its SH2 domain and Cbl through its SH3 domain.

Given that it was plausible that Grb2 could mediate this interaction, this hypothesis was tested both in an *in vivo* assay and in an *in vitro* system using purified proteins. In a yeast three-hybrid assay we demonstrated that an interaction occurred between Bcr-Abl and Cbl only when Grb2 was present. Disruption of the Grb2 binding site in Bcr-Abl abrogated this interaction. Using

purified proteins in a gel overlay assay the *in vivo* findings were confirmed; binding of Bcr-Abl to Cbl required the presence of Grb2.

Finally, to fully test the requirement of Grb2 in bridging Cbl and Bcr-Abl we generated a Bcr-Abl-expressing cell line with a mutation in the Grb2 binding site (Y177F). In these experiments Cbl's association was dramatically decreased compared to wild-type association. However, it was not totally abolished. Thus, we concluded that Grb2 acts as the major, though not exclusive, mediator of the interaction between Cbl and Bcr-Abl.

Future Grb2 Interaction with Cbl Studies

If the direct interaction of Cbl with Bcr-Abl is dependent on the interaction of Grb2 with Bcr-Abl and Grb2 with Cbl, the question arises, what are the functional consequences if Grb2 can no longer serve in this capacity? While our results demonstrated that in the Y177F Bcr-Abl-expressing cells there is a reduction in the association of Bcr-Abl with Cbl, assessing the relevance of this decreased association with additional experiments would enhance its significance.

To initially examine this question, an analysis of Cbl's phosphorylation status could be performed in the Bcr-Abl-expressing cell line with the Grb2 binding site mutation. If Cbl is still phosphorylated, this would suggest that Bcr-Abl is not the kinase responsible for Cbl's phosphorylation and experiments would be performed to determine what kinase is phosphorylating Cbl (such as in vitro

kinase assays). If Cbl is not phosphorylated this would suggest that Bcr-Abl is the most likely candidate since disruption of the association between Grb2 and Bcr-Abl also renders Bcr-Abl unable to associate with Cbl, and thus presumably unable to phosphorylate Cbl as well.

To address the larger, more physiological question of Cbl and Grb2's direct role in Bcr-Abl-mediated transformation, cell lines from the recently reported Cbl and Grb2 null mice could be generated and studied. Specifically, is a Bcr-Abl-expressing,Cbl null fibroblast still transforming? This would be very interesting though problematic, for the Cbl knock-out is viable and suggests a possible redundancy in function by an unknown protein. Hence, the transformation phenotype might be predicted to be similar to wild-type. Furthermore, is a Bcr-Abl-expressing, Grb2 null fibroblast still transforming?

Finally, owing to the residual association of Bcr-Abl and Cbl in the Y177F cell line (single mutant) a complete abrogation of Cbl binding would require an additional deletions in the SH2 domain (double mutation) and in the proline-rich domain of Bcr-Abl (triple mutation). Because the SH2 domain is the sole direct binding site for Cbl, examining this double mutant would clarify Cbl's direct role in Bcr-Abl-mediated transformation. However, a triple mutation that totally abolishes Cbl binding to Bcr-Abl is required since tyrosine phosphorylated Cbl could still associate indirectly with Bcr-Abl through binding the SH2 domian of CrkL. (CrkL is a protein that binds Bcr-Abl through Bcr-Abl's proline-rich sequences.) These

mutants could be evaluated biochemically as outlined above, examined by conventional transformation assays, and then, if necessary, tested for leukemogenic potential in mice.

III. Studies on the Role of Grb2 and Grap Binding to Bcr-Abl

Three years ago, a Grb2 homologue, Grap, was cloned [Feng et al., 1996]. This protein was demonstrated to associate with Bcr-Abl [Feng et al., 1996]. Grap is found predominantly in hematopoietic tissues as opposed to Grb2 that is ubiquitously expressed. Grap is an adaptor protein with an SH2 domain flanked by two SH3 domains. It shares 59% homology with Grb2. Given that CML is a hematopoietic disease, the discovery of Grap raised the possibility that Grap could serve as the major pathway to Ras activation in Bcr-Abl-expressing cells. While a significant amount is known about Grb2's interactions with Bcr-Abl, relatively little was known about Grap. Hence, we performed experiments to explore the relative interactions of these adaptors with Bcr-Abl.

Our initial experiments examined the protein levels of Grap and Grb2 in primary cells from a CML patient. From an immunoblot analysis, we discovered equivalent expression levels. This suggested that the relative binding of these proteins to Bcr-Abl would determine their potential for downstream signaling (i.e. Ras activation). However, we first had to ascertain whether Grap bound the

same site as Grb2 in Bcr-Abl. Using a yeast two-hybrid system we demonstrated that Grap, like Grb2, bound Bcr-Abl at amino acid Y177.

Having established that binding of Grap to Grb2 to Bcr-Abl required the same site, and that these proteins are expressed at equivalent levels in the cell, we then sought to examine their relative binding to Bcr-Abl. To address this question, we employed a yeast split-hybrid analysis to assess whether Grb2 could "split" (disrupt) a favorable interaction between Grap and Bcr-Abl. Indeed it could. However, the reverse was not true; Grap could not disrupt a Grb2/Bcr-Abl interaction. From these experiments we concluded that the *in vivo* affinity of Grb2 for Bcr-Abl was greater than Grap for Bcr-Abl.

Two additional sets of experiments supported and extended the yeast two-hybrid findings that suggested that Grb2 has a greater affinity for Bcr-Abl compared to Grap. One was surface plasmon resonance and the other was a GST-binding assay. In each of these experiments purified proteins were used to analyze quantitatively the affinity of Grb2 and Grap for Bcr-Abl. The results from these experiments showed that Bcr-Abl displayed an approximately two-fold higher affinity for Grb2 than Grap. Thus, despite Grap being expressed at similar levels as Grb2 and being found primarily in hematopoietic tissues, Grap did not display a higher affinity for Bcr-Abl. Therefore, we concluded that Grap's ability to link Bcr-Abl to Ras was of less physiological relevance than Grb2.

Future Grb2/Grap Studies

The findings of the Grb2/Grap studies highlight the exquisite specificity of adaptor proteins. Despite their overall homology, Grb2's affinity for Bcr-Abl's phosphotyrosine was found to be significantly higher by three types of analyses: yeast split-hybrid, surface plasmon resonance, and GST-binding assays. These findings could be enhanced by the following experiments.

First, examine whether Grb2 binding to Bcr-Abl confers an increase in Ras activation and is thus, truly more physiologically relevant in terms of activating this key pathway linked to transformation. Our working assumption was that owing to Grb2's increased affinity for Bcr-Abl, it would. However this was not demonstrated experimentally. To examine this question, Bcr-Abl could be introduced into Grb2 and Grap null cell lines. After normalizing for existing Grap and Grb2 respectively, the level of Ras activation could be assessed by Ras GTP loading assays. Alternatively experiments could be performed to quantify the affinity of Grb2 and Grap for mSOS (the guanine nucleotide exchange factor for Ras) using surface plasmon resonance and GST-binding assays.

One of the drawbacks presented in this study was that an antibody that recognizes solely Grap, and does not cross-react with Grb2, was not available. Thus, developing a specific Grap antibody should be of high priority so that investigations into *in vivo* interactions between Grap with Bcr-Abl and Grb2 with

Bcr-Abl could be pursued (i.e. Grap/Grb2 immunoprecipitations followed by immunoblotting for Abl). Once this antibody is generated, and if it is determined that more Grap associates with Bcr-Abl *in vivo*, subcellular localization studies might be warranted to explain these results.

Finally, in our study we reported that the protein levels of Grap and Grb2 were equivalent when examined in a CML patient. This finding, and the conclusions we made based on this finding, would be significantly strengthened by performing this analysis on a larger sample of patients to demonstrate whether the same pattern exists across a broader spectrum of individuals.

Chapter VI

Conclusions

Ever since the discovery four decades ago of the genetic abnormality responsible for Chronic Myelogenous Leukemia, the Philadelphia chromosome, a profusion of basic and clinical research has been undertaken. Partly owing to its early discovery, this human malignancy is one of the most well studied of all human diseases and thus its molecular mechanisms have been extensively, though not exhaustively, examined. The central focus of the research, Bcr-Abl, is a fusion protein of approximately 210 kDa. Through its elevated tyrosine kinase activity this protein profoundly disrupts normal signaling events in the cell, ultimately

permitted this researcher. Ideally, and perhaps one day, there may be available a novel means to view (in a relatively noninvasive way) the maturing granulocytes in the bone marrow, and to finally “see” the cytoskeleton with a non-toxic fluorescent dye and ascertain its structure. Are focal adhesions visible? I doubt that day is too far off........

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