INTERACTIONS OF BCR-ABL WITH SIGNALING MOLECULES

by

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It is good to have an end to journey towards; but it is the journey that matters, in the end.

Ursula K. LeGuin

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List of Abbreviations

A-MuLV	Abelson's murine leukemia virus
ALL	Acute lymphocytic leukemia
CML	Chronic myelogenous leukemia
EGFR	Epidermal growth factor receptor
HZ2-FSV	Hardy-Zukerman-2 feline sarcoma virus
PDGF	Platelet derived growth factor
Ph.	Philadelphia chromosome
RTK	Receptor tyrosine kinase

Abstract

The Philadelphia chromosome, detected in virtually all cases of chronic myelogenous leukemia (CML), is formed by a reciprocal translocation between chromosome 9 and 22 that fuses Bcr encoded sequences upstream of exon 2 of c-Abl. The Bcr-Abl fusion creates a gene whose protein product, p210$^{bcr\text{-}abl}$, has been implicated as the cause of the disease. Although Abl kinase activity has been shown to be required for the transforming abilities of Bcr-Abl and numerous substrates of the Bcr-Abl tyrosine kinase have been identified, the requirement of most of these substrates for the transforming function of Bcr-Abl is unknown. To further our understanding of Bcr-Abl-induced transformation, two proteins CrkL and Cbl both reported to be important for Bcr-Abl function, were examined in 32D cells transformed to factor independence by p210$^{bcr\text{-}abl}$. We have mapped the site of interaction between CrkL and Bcr-Abl to the amino terminal SH3 domain of CrkL and the proline-rich region in the C-terminus of Abl. We also showed that the CrkL SH2 domain binds directly to phosphorylated Cbl. Phosphorylated Cbl also binds directly to the Abl SH2 domain. This suggests a model where Bcr-Abl interacts directly and indirectly with both CrkL and Cbl. That is, Bcr-Abl interacts directly through its SH2 domain with Cbl which in turn interacts with CrkL. Similarly, a proline-rich region of Bcr-Abl interacts directly with CrkL which in turn interacts with Cbl. Predictions from this model have been confirmed using Bcr-Abl mutants lacking the SH2 domain or the proline-rich region. We have demonstrated both loss of direct binding of Cbl to the SH2 domain

deletion mutant and loss of CrkL binding to the proline-rich region deletion mutant by various in vitro assays. However, in immunoprecipitation studies, binding of Bcr-Abl to both Cbl and CrkL through indirect interactions is preserved which correlates with the ability of both mutants to induce factor-independent growth of 32D cells. Thus, we cannot exclude a role for Cbl or CrkL in mediating Bcr-Abl transformation.

CHAPTER I

Introduction

"Cancer is an extraordinarily diverse disease; it arises in response to a multitude of environmental insults and inherited biases, and it is a disorder of that most complex attribute of living things-cell growth."

Varmus, 1985 [221].

Signaling and growth control

Normal cells need to adapt to a variety of chemical, hormonal or electrical changes in their environment. These environmental changes evoke responses such as cellular proliferation, contraction, secretion, metabolic adjustment and changes in gene expression. For these events to be possible, cells need to communicate external environmental changes to the inside of the cell. Receptors on the cell surface or in the cytoplasm of cells mediate these responses. Ligand binding to a receptor is not sufficient to induce these changes, receptors must somehow convey that a binding event has occurred. This information is passed through intracellular mediators and kinetic pathways that lie between ligand binding and the cellular response.

There are three main classes of signal transduction models for cell surface receptors bound with ligand: receptors can act as ion channels; receptors can interact with G proteins to produce other

signaling molecules termed second messengers; and receptors themselves can perform as enzymes [68] [156]. These three classes generally correspond to distinct time-scales for physiological responses in cells from fractions of seconds, seconds to minutes, and minutes to hours respectively.

Ion channels control passage of ions such as K^+ and Na^+ across the cell membrane. These ions typically regulate short time-scale behavioral phenomena, including the release of cell secretory vesicle contents and membrane electrical potential wave propagation. The main types of receptors using this mode of signal transduction are neurotransmitter receptors.

In the second class, receptors interact with and activate membrane-associated GTP-binding proteins. Activated G proteins may in turn act on other enzymes to induce the generation of small molecules called second messengers such as cAMP, cGMP, Ca^{2+}, and phospholipid metabolites with the cytoplasm. These molecules are then capable of either directly stimulating short-term responses or modulating intracellular enzymes activities for indirect regulation of long-term responses. Receptors using this mode of transduction are many and diverse, but typically regulate physiological responses occurring on the time scale of minutes, e.g., chemotactic peptide receptors.

In the third class, receptors with intrinsic enzymatic activity, ligand binding results in the covalent modification of the activity of the receptor by phosphorylation. The receptor then modulates activities of other intracellular enzymes to regulate long-term responses. Most receptors known to utilize this mode of transduction

are growth factor receptors acting as kinases and regulating physiological responses over a time scale of hours. This class of receptors can recruit other enzymatic proteins in the cytoplasm to mediate its responses. As most cellular responses are quite complex, multiple signaling pathways can be activated and quite often there is cross-talk between different signaling pathways, for example, between G protein-coupled receptors and receptor and non-receptor tyrosine kinase pathways [204] [126]. Due to the regulation of physiological responses and growth control mechanisms by these types of receptors, many oncogenic processes target these receptors and downstream mediators of responses to these receptors.

Oncogenesis

The events triggering transformation can be comparatively simple; often the expression of one or two genes, called oncogenes, is sufficient to transform a cell. An oncogene is any gene that encodes a protein able to transform cells in culture or induce cancer in animals. The word derives from the Greek *onkos*, meaning a bulk or mass; thus oncology is the scientific study of tumors. Oncogenes are derivatives of normal cellular genes. Cellular genes known to be progenitors of oncogenes are called proto-oncogenes. They are important for normal cellular processes, but they can be altered, often in very simple ways, to become oncogenes. Because most proto-oncogenes are important for cell growth and survival, they have been highly conserved. Most oncogenes derive from genes whose products act along normal cellular growth-controlling

pathways. Some oncogenes, are mutated versions of the corresponding proto-oncogenes. In other cases, the protein products of an oncogene and its proto-oncogene are the same; the oncoprotein causes cancer either by being present where it normally is absent or by being expressed at a level much higher than normal, or by loss of normal functional regulation i.e., constitutively active.

1) One of the largest groups of oncogenes discovered encode for constitutively activated tyrosine kinases. 2) Oncogenic tyrosine kinases include *v-abl* [189], *fms* [12] [234], EGFR [35] [102], *neu* [102] [235], *v-sis* [98] (PDGF), *v-fps* [142], *src* [183], and others. And 3) This data has implicated tyrosine kinases as important regulators of cellular proliferation.

Tyrosine Kinases

Two types of tyrosine kinases can be found in cells, the receptor tyrosine kinases (RTKs) and the non-receptor tyrosine kinases. All RTKs contain a large, extracellular ligand-binding domain, a single transmembrane region and a cytoplasmic portion with a conserved protein tyrosine kinase domain [89] [88]. In addition to the catalytic domain the cytoplasmic portion contains regulatory sequences that are subject to autophosphorylation and dephosphorylation by various exogenous kinases and phosphatases.

Non-receptor tyrosine kinases are usually found in the cytoplasm and can act as transducers of extracellular signals to downstream proteins involved in pathways that regulate cellular growth, activation, and differentiation. Many non-receptor tyrosine

kinases work in unison with transmembrane receptors, including receptors for peptide hormones [80][5][21], immune recognition [19][99][227], and hematopoietic cytokines [105][152][90][207][229]. These non-receptor tyrosine kinases are activated by extracellular events such as ligand binding to their associated receptors [16][207] whereas others are activated by events with no obvious link to a specific transmembrane receptor such as cellular adhesion, calcium influx, or cell-cycle dependent activation.

Growth factors induce oligomerization of their specific receptors, and oligomerization is important for transmembrane signaling [218]. This model was originally shown to be true for the EGFR [196]. It has since been confirmed for many other RTKs and other cytokine receptors. The general oligomerization model holds that inactive receptor monomers are in equilibrium with active receptor dimers, and that ligand binding induces dimerization and possibly a conformational change that results in the activation of the receptor [218]. There can be homodimers i.e., dimerization between two identical receptors or two kinds of heterodimers, 1) dimerization between different members of the same receptor family or 2) between a receptor and an accessory protein. Dimerization of RTKs is responsible for activation of their intrinsic protein kinase activity and for autophosphorylation [218]. The subunits of some RTKs, including the tetrameric insulin receptor, are covalently linked. Although these receptors exist as dimers or tetramers even in the absence of ligand, ligand binding is required for autophosphorylation and activation, presumably ligand binding results in a conformational change and activation of this receptor.

Activation of a non-receptor tyrosine kinase is similarly induced in response to the appropriate extracellular signal, however, dimerization may or may not be necessary for activation [85]. For example, JAK kinases are activated following ligand-induced dimerization or oligomerization [216][30][92][91] while members of the Src family of tyrosine kinases are maintained in an inactive state by phosphorylation of a specific tyrosine residue, tyrosine 527 [31]. Src can be activated by either dephosphorylation of this residue or by phosphorylation at other sites [210][32].

In the case of either receptor or non-receptor tyrosine kinases, activation of the tyrosine kinase initiates a cascade of phosphorylation that includes autophosphorylation, phosphorylation of cytoplasmic substrates, and recruitment of SH2 domain-containing proteins that bind to specific tyrosine phosphorylated residues. One important function of these autophosphorylation sites is to serve as binding sites for downstream signaling molecules. This recruitment step constitutes a mechanism to activate various cytoplasmic signaling pathways.

Two different classes of proteins associate with the cytosolic domain of activated RTKs 1) adapter proteins e.g., Grb2 or IRS1, which couple the activated receptor to other signaling molecules but have no intrinsic catalytic properties, and 2) enzymes involved in signaling pathways.

Alterations in any of the regulatory enzymes or proteins mediating interactions in the cascade of signaling events in these cells, could easily be envisioned as being detrimental to the cell. As noted, alteration in many of these proteins have been identified in

transforming retroviruses and a role for these proteins in human cancers has been suggested.

Cancer and Leukemia

Cancers are generally classified by their embryonic origin as they retain embryonic markers during the process of tumor formation. Malignant tumors are classified as carcinomas if they derive from endoderm or ectoderm and sarcomas if they derive from mesoderm. Leukemias, a class of sarcomas, grow as individual cells in the blood whereas the other tumors form solid masses. Leukemias are clonal malignancies of cells in blood and are characterized by acquired somatic mutations in hematopoietic stem cells.

There are specific chromosomal translocations associated with specific subtypes of leukemia. Thus, breakpoints of many mutant leukemic genes have been sequenced and identification of corresponding mutations found in proto-oncogenes, tumor suppressor genes, transcription factors, and/or tyrosine kinases has resulted in furthering our understanding of the genetic basis of these cancers. Highly conserved breakpoints characteristic of specific types of leukemia support a critical role for these chimeric gene products in leukemogenesis [120] [213] [110]. One of the best studied examples of leukemias with a specific translocation is Chronic Myelogenous Leukemia (CML).

Chronic Myelogenous Leukemia

CML is a neoplastic hematopoietic stem cell disorder that represents approximately 20% of cases of adult leukemia. The first clues to the genetic basis of this disease came in 1960 when Nowell and Hungerford showed that an abnormally small chromosome, the Philadelphia (Ph) chromosome, was a hallmark of the disease [159]. Rowley later showed that the Ph chromosome resulted from a translocation of the distal segment of the long arm of chromosome 22 to the distal portion of the long arm of chromosome 9 [t(9;22)(q34;q11)] [187] [7][79]. In more than 95% of CML cases, the Philadelphia chromosome is seen [79] [9] [84]. This reciprocal translocation results in a fusion gene that is transcribed into a novel chimeric mRNA of 8.5 kb [199] which encodes a hybrid 210kDa phosphoprotein called p210$^{bcr\text{-}abl}$ [108], an activated tyrosine kinase.

CML is characterized clinically by a biphasic course; in the chronic phase there is progressive accumulation of mature myeloid cells in the bone marrow and blood. In this stage leukemic cells retain the capacity to differentiate normally [28]. There seems to be a shift towards granulocyte differentiation at the expense of erythroid differentiation during this stage of the disease. After a median of 4-5 years, there is clonal evolution accompanied by additional cytogenetic abnormalities and a loss of the ability of the malignant clone to differentiate [45]. Some of the current work on this disease relates to deciphering the regulatory events that cause the shift towards acute leukemia.

The etiology of the disease is unknown. The disease is seen in all age groups and a peak incidence is seen in the fifth and sixth decades of life. The incidence of CML was significantly increased in survivors of the atomic bomb explosions in Hiroshima and Nagasaki who were exposed to high levels of radiation [117] [87] and in groups of patients given radiotherapy for Ankylosing Spondylitis [34], but for most patients radiation has no definite role in causation. No evidence suggests that toxic chemicals or viruses are risk factors for CML.

To understand the pathogenesis of CML, one of the simplest approaches has been to examine p210$^{bcr\text{-}abl}$, and the signaling pathways it activates. The following section will summarize the work done on the individual *c-abl* and *c-bcr* proto-oncogenes which together comprise the chimeric protein. The normal functions of c-Bcr and c-Abl are not known, though it seems that there may be a myriad of protein-protein interactions and pathways activated by these proto-oncogenes.

In considering the functions of the individual cellular oncogenes, one needs to keep in mind that there are a number of differences between the cellular oncogenes and the chimeric oncogene. Bcr-Abl is constitutively activated as a tyrosine kinase whereas c-Abl is tightly regulated during normal cell growth. *c-abl* is normally localized to the nucleus and cytoplasm [119]. c-bcr is normally localized to the cytoplasm in the G_0-G_1 phase of the cell cycle but translocates to the nucleus during mitosis [233]. Bcr-Abl, on the other hand, is cytoplasmic [29] [81]. Due to the differential localization and activity of these proteins, it can be envisioned that

the substrates and function of the cellular oncogenes versus the chimeric protein may be different.

The Abl family of protein tyrosine kinases

c-abl was originally defined as the cellular homolog of a transforming retrovirus, the Abelson murine leukemia virus (A-MuLV). The gene seems to be important through evolution, as homologous genes have been found in fruit flies *Dabl* [86], the nematode *Nabl* [70] and humans *c-abl*HS [201]. In humans there is another *abl*-related gene, *arg* [111], which is similar to *c-abl* but has not been well characterized.

The human *abl* genes *abl* and *arg* and c-abl from mouse are transcribed into two major transcripts which differ at their 5' ends. These two transcripts arise from splicing of two distinct 5' exons, which are transcribed from separate promoters, to a set of common exons in the *c-abl* gene [8]. The two transcripts produce two distinct products, types I and IV c-Abl proteins, which differ only at their N termini [8] [201] [111]. The c-Abl (IV) is myristoylated on the N-terminal glycine [94], while the c-Abl (I) protein is not myristoylated. Simple overexpression of either type I or type IV in NIH 3T3 cells does not result in transformation.

c-Abl domains

c-abl is a member of the non-receptor or cytoplasmic group of tyrosine kinases and contains *src* homology domains. Src homology

domains identified include the SH1 domain (the kinase), SH2 domain which binds phosphotyrosine residues, and SH3 domain which binds to proline-rich motifs, usually with the sequence PXXP. Both the Abl SH2 [155] [76] and the Abl SH3 domains [155] [76] have been crystallized.

The C-terminus of Abl is unique among cytoplasmic tyrosine kinases in that it is large and has been shown to contain a number of functional subdomains. These include nuclear localization signals [230] [220], proline-rich sequences important for adaptor protein binding [177], a DNA-binding domain [104], a p53 binding site [72] and an actin-binding domain [147]. c-Abl is expressed in all tissues examined [231] and can localize both to the nucleus and to the cytoplasm in cells [232] [220].

c-Abl deficient mice

Abl null mice were generated in 1991 by two independent groups. Two different disruptions in the c-Abl gene, a null mutation [215] and a deletion in the carboxy-terminal one-third of the protein [197] showed similar phenotypes. The C-terminal deletion construct contains an active kinase as compared to the null mouse, suggesting either that kinase activity may not be required for observed phenotype of these mice, that the unique C-terminus domain may be mediating an essential function of c-Abl or that the C-terminus is required for proper intracellular localization of the Abl protein. These Abl -/- mice showed high neonatal mortality rates and were more susceptible to infections. Because neonates were not impaired

in their development, it suggested that *c-abl* may not be required for cell proliferation during embryogenesis or that another gene, for example *arg*, might play a role at this stage of development and substitute for *c-abl*. However, half the mice were found to suffer from lymphopenia, indicating that *c-abl* may be critical for the proliferation of lymphocytes. These mice also showed atrophy of the spleen and thymus. B lymphocyte precursors were variably reduced in the bone marrow, and this defect was transferable via bone marrow transplantation. It is possible that Arg may be substituting for c-Abl during the initial phases of development, whereas c-Abl may take over during neonatal and subsequent development.

c-Abl cell cycle function and associated proteins

c-Abl has been found to be involved in some cell cycle functions and to associate with proteins essential for cell cycle regulation e.g., p53 and Retinoblastoma. Overexpression of c-Abl was shown to cause growth arrest in several cell systems [230] [72] [147], suggesting that c-Abl may perform a checkpoint function during the cell cycle [194].

c-Abl was shown to be activated by external DNA damaging stimuli e.g., ionizing radiation [101]. c-Abl appears to be necessary for the activation of the stress-activated protein kinase (SAPK), SAPK was not activated by exposure to ionizing radiation in *c-abl* null mice but this response was restored by introduction of wild-type c-Abl [101]. Therefore, c-Abl may play a critical role in pathways that are activated after a DNA damaging stimuli has been delivered to a cell.

c-Abl has also been shown to bind p53 in vitro and enhances p53 transcriptional activity when measured using artificial or natural promoters containing p53 DNA binding sites [72]. c-Abl and p53 also form a physical complex in vivo [238]. The tyrosine kinase activity of c-Abl was required for growth suppression but not for transcriptional activation of p53 [72]. Similarly, Rb can also form a complex with c-Abl [228] through its C-pocket domain which has been shown to inhibit its activity during G1. As cells transit from G1 to S phase, Rb becomes hyperphosphorylated resulting in dissociation of the complex and activation of c-Abl kinase. However, the role of Rb in growth arrest activity of c-Abl is unclear.

Wild-type c-Bcr

c-Bcr has structural features that suggest a role for c-Bcr in signal transduction. These include a novel serine/threonine kinase domain [129], an oligomerization domain [145], a Rho guanine-nucleotide exchange factor (Rho-GEF) homology domain [15], a calcium-dependent lipid binding site [15], a binding site for the 14-3-3 family member Bap-1 [180], and a Rac GTPase activating protein (Rac-GAP) domain [46]. The oligomerization domain, serine/threonine kinase domain and a stretch of serine/threonine phosphorylation sites are retained in all types of Bcr-Abl fusion proteins. The p210 form also contains the Rho-GEF homology domain of Bcr. Bcr knockout mice have a defect in neutrophil superoxide bursts [224], suggesting a role for Bcr in the anti-microbial function of myeloid cells.

Oncogenic forms of c-Abl

In an effort to better understand the oncogenic potential of *c-abl*, a number of groups have generated mutants of *c-abl*. Use of deletion [226] [74] and point mutants [219] [95] [41] of *c-abl* has lead to some ideas about the requirements for the oncogenic conversion of the *c-abl* proto-oncogene to a transforming gene.

The SH3 domain of *c-abl* is important in activating the kinase, deletion of this domain results in activation [226] [41]. The SH3 domain seems to be position sensitive and either deletion or subtle alteration of the position of this domain within the protein activates the kinase and the protein is fully transforming [138]. The SH3 domain is thought to be inhibitory due to binding of a protein that mediates the inhibitory effects [219] [36]. A potential candidate Abi-2 was cloned and found to interact with c-Abl. c-Abl bound this protein specifically via two distinct regions, the SH3 domain and the C-terminal sequences [36]. An Abi-2 mutant lacking Abl SH3 binding sequences but not the Abl C-terminal binding domain was found to activate the transforming capacity of c-Abl suggesting Abi-2 needs to interact via both regions to suppress c-Abl kinase.

Similarly, a point mutant of the SH3 domain, specifically P131L, converts c-Abl to a transforming protein. This corresponds to a loss of function mutation of the SH3 domain of Sem-5. *sem-5* is the *C.elegans* homolog of the *grb2* proto-oncogene [219]. Another mutant F420V, a mutation lying outside the SH3 domain resulted in transformation by myristoylated c-Abl [95]. This region was shown

to be generally conserved in tyrosine kinases [95]. This F420V mutant, however, was found not to be heavily tyrosine phosphorylated [95].

Oncogenic versions of Abl have been isolated from mouse, cat and humans. These Abl constructs are the *v-abl* oncogene of Abelson murine leukemia virus (A-MuLV), the *v-abl* oncogene of the Hardy-Zuckerman-2 feline sarcoma virus (HZ2-FSV), and the *bcr-abl* fusion gene in human leukemias from the Ph [185] [38] see Fig. 1. In these oncogenic proteins, Abl kinase is dysregulated and constitutively activated. Activation of the v-Abl tyrosine kinase requires both the deletion of the SH3 domain and fusion with the viral protein sequences. A-MuLV also has a SH3 domain deletion whereas the HZ2-FSV has a large carboxyl-terminal segment deletion; however, both have requirements for fusion with viral protein sequences [61] [94] [11]. Activation of the Bcr-Abl tyrosine kinase depends on Bcr amino acids [146] [154] [169].

p185*bcr-abl*/p210*bcr-abl*

The breakpoint on chromosome 9 that contains *c-abl* can occur within a large area at the 5' end of *c-abl*. The portion of Abl that is consistently retained in the fusion of the hybrid gene is exon 2 (a2) and all of the exons 3' of exon 2 (exons 2-11 [25]) [112]. *c-bcr* on chromosome 22 consists of 23 exons [25]. Historically, a confined region of the *bcr* gene about 5.8 kb [79] has been designated the major breakpoint cluster region (M-Bcr) [66]. M-Bcr includes five exons designated b1-b5 [66]. Following RNA transcription and

splicing, the hybrid gene can encode two potential junctions depending on whether Bcr exon b2 or b3 is joined to Abl a2. These junctions are designated as b2a2 or b3a2, depending on the presence or absence of exon b3. The difference in size between these two proteins is 25 amino acids [200]. These two products both code for a product called p210$^{bcr\text{-}abl}$. In another disease, ALL, chromosome 22 breakpoints take place within the first intron of Bcr [217]. In this case the resulting Bcr-Abl product is a fusion between the first exon of Bcr (b1) and Abl exon 2 (a2). This fusion product b1a2 (lacking two of the Bcr exons mentioned above, b2 or b3, results in the formation of a hybrid protein of 185kDa [22].

Bcr sequences in the fusion proteins p185$^{bcr\text{-}abl}$ or p210$^{bcr\text{-}abl}$ are essential for elevation of the tyrosine kinase activity [146] [154] [169]. The abl sequences of the *bcr-abl* genes are unchanged except for loss of the first exon, and this loss alone does not endow *c-abl* with the ability to transform cells [56] [61]. Bcr first-exon sequences potentate tyrosine kinase activation and transforming ability when they are fused to the second exon of *c-abl*. This occurs through dimerization of the chimeric oncogene [145] or by specifically interfering with the negative regulation of abl-encoded tyrosine kinase [146] [154]. The p185$^{bcr\text{-}abl}$ protein contains the first exon of *c-bcr* and has about 5 fold higher tyrosine kinase activity than p210$^{bcr\text{-}abl}$ [124]. This correlates with the former's much more frequent association with an acute rather than a chronic form of leukemia [48] [112] [27], and its greater transforming potency both in vitro [124] [39] [143] [144] and in animal experimental systems [40] [53] [83] [100] [38].

Assays of Bcr-Abl function

In vitro modeling of CML has been somewhat problematic as there is no single model that accurately reflects the disease. In an attempt to model transformation associated with the disease, four different assays have been used. These include growth factor independence, fibroblast focus formation, primary bone marrow colony formation, and transplantation into syngeneic mice.

Growth factor independence

For normal growth, certain cell lines require, in addition to serum, specific growth factors, the absence of which can result in cell death. Transformation by an oncogene can abolish the need for these growth factors. The oncogenic Abl proteins can abrogate the requirement of growth factors in lymphoid or myeloid cell lines. In this assay one assesses the shift to growth factor independence caused by the transfection of p210$^{bcr\text{-}abl}$ into a growth factor-dependent cell line [37] [113] [137] [127]. p210$^{bcr\text{-}abl}$ can abrogate the requirement for IL-3 in a pro-B cell line, BaF3 [41] and can convert an interleukin-3 (IL-3)-dependent murine myeloid cell line, 32D, or a GM-CSF-dependent human megakaryotic cell line, Mo7e, to growth factor independence [184][37].

Fibroblast focus formation

Normal fibroblasts require firm contact with the substratum for growth. If they are plated onto a surface to which they cannot adhere, they will not grow, although they can remain viable for long periods of time. Most transformed cell lines have lost the requirement for adherence; they grow without attachment to a substratum, as indicated by their ability to form colonies when suspended as single cells in agar. This characteristic correlates extremely well with the ability of transformed cells to form tumors; cells that have lost anchorage dependence generally form tumors with high efficiency when they are injected into animals that cannot immunologically reject them.

This ability of transformed cells to grow in agar has been exploited in the fibroblast transformation assay. In this assay, *bcr-abl* constructs transfected into a fibroblast cell line and transfectants are assessed for their ability to form colonies in soft agar. $p185^{bcr-abl}$ and the oncogene *v-abl* both readily transform NIH 3T3 and Rat-1 fibroblasts [39] [124], though $p210^{bcr-abl}$ transforms NIH 3T3 fibroblasts poorly and is less potent than $p185^{bcr-abl}$ in transforming Rat-1 cells [195].

Primary bone marrow colony formation

This assay is quite similar to the fibroblast transformation assay. $p210^{bcr-abl}$ has the ability to promote the outgrowth of myeloid cell lineages when introduced by retroviral infection into

cells plated in long term bone marrow cultures [143] [237]. This assay is quantitative as it assesses the number of myeloid colonies formed over time as compared to uninfected bone marrow cells.

Transplantation models

This assay uses the ability of retrovirally infected bone marrow cells carrying p210$^{bcr\text{-}abl}$ to cause a syndrome resembling chronic myelogenous leukemia in lethally irradiated syngeneic mice [40] [100] [69].

The procedure involves infecting harvested bone marrow cells of young BALB/c mice with retroviruses carrying p210$^{bcr\text{-}abl}$. These primary infected bone-marrow cells are then transplanted into lethally irradiated syngenic recipients. A CML-like myeloproliferative syndrome is seen usually after 9 weeks of infection [40]. This data strongly implicates p210$^{bcr\text{-}abl}$ as the cause of CML, providing, in fact, the strongest evidence for a protein tyrosine kinase as the etiologic agent in a human malignancy.

Structure-function aspects of Bcr-Abl

Studying Bcr-Abl mutants has led to the conclusion that certain domains of Bcr-Abl are important for transformation. Reviewed below is knowledge regarding transformation mediated by deletion or point mutations in the different domains of Bcr-Abl.

Bcr Exon 1

The first exon of Bcr is known to activate the tyrosine kinase activity of the Abl kinase [154] [146]. Four different activities have been attributed to this exon, of which three have been shown to be important in transformation. 1) there is a serine/threonine kinase whose activity is dispensable for transformation [169] [129]. 2) the N-terminal sequences of Bcr contain an oligomerization or coiled-coil oligomerization domain that has been shown to be important for transformation as deletion of these sequences results in a non-transforming phenotype [154] [147]. A rare translocation [96] in CML, Tel-Abl also appears to function in a similar manner since the dimerization motif in this protein, which is encoded by Tel sequences is required for transformation activity. Tel belongs to the Ets family of transcription factors [75]. This N-terminal domain of Bcr has also been shown to be important for activation of the Abl actin binding function. 3) interaction with a subset of phosphoserine/phosphothreonine-dependent binding proteins has been mapped to amino acids 192 to 413 of Bcr. Deletion of these amino acids has been shown to abolish interaction with these proteins and lead to a non-transforming phenotype [169] [153]. Finally 4) tyrosine 177 when phosphorylated binds to the SH2 domain of Grb2, a protein that links tyrosine kinases to the *ras* nucleotide exchange factor mSOS [170]. Thus mutation of this residue to a phenylalanine leads to a non-transforming phenotype in fibroblasts.

Bcr Exons 2 and 3

The second exon of Bcr bears homology to rho/rac guanine nucleotide exchange factor, however, this hasn't been documented experimentally [15]. These domains are not required for transformation, as p185 lacks both of these exons [124].

Abl SH3 domain

As discussed earlier, this domain seems to bind to an inhibitory protein. However, this domain is retained in Bcr-Abl, suggesting that the inhibitory function of the SH3 domain is circumvented in the Bcr-Abl fusion protein.

Abl SH2 domain

This domain in proteins is critical in mediating interactions with phosphotyrosine-containing proteins in cells [165] [107] and is important in fibroblast transformation, as deletion of this domain results in decreased transformation in these cells [145] [162]. A mutant of this domain and implications thereof will be discussed further in a following section.

Abl tyrosine kinase domain

Abl kinase activity is essential for its transformability as mentioned above [185] [2] [168]. The Bcr-Abl fusion products have

tyrosine kinase activity elevated several fold over the normal c-Abl gene product [42] [124] Fig. 1. Mutations of the conserved lysine in the ATP binding site abolishes transformation by Bcr-Abl. This has also been shown by use of temperature-sensitive mutants in the kinase domain, which require the tyrosine kinase activity for stimulation of cell growth [20] [103] [24].

Abl C-terminal domain

The unique C-terminus of Abl that distinguishes it from other tyrosine kinases contains multiple domains, including an actin-binding domain [220]. This actin-binding domain is though to be important in Bcr-Abl-mediated fibroblast transformation [147].

Bcr-Abl substrates and interacting proteins

The tyrosine kinase activity of the Abelson murine leukemia virus product, p160$^{v\text{-}abl}$, and Bcr-Abl fusion proteins is known to be necessary for cellular transformation [186]. This observation suggested that substrates of this kinase may be important in oncogenesis [109]. However, it has been difficult to identify the critical immediate target proteins which may be phosphorylated by p210$^{bcr\text{-}abl}$ and to define the ensuing protein interactions and cascade of signals which are activated.

Numerous proteins have been shown to interact with Bcr-Abl and many have been shown to be tyrosine phosphorylated in Bcr-Abl-expressing cells. These proteins include rasGAP [49], Grb2 [170,

172], Shc [136, 208], Fes [54], Syp [209], CrkL [161, 212] the 85 kDa subunit of phosphatidylinositol 3-kinase [222], Vav [135], c-Bcr [123], paxillin [192], and various other cytoskeletal proteins [191].

Although numerous substrates of the Bcr-Abl tyrosine kinase have been identified, the biological relevance of many of these protein substrates for the transformation function of Bcr-Abl is unknown and needs to be demonstrated to fully understand the mechanisms of disease [61] [146] [103] [178] [63]. However, it is likely that one or more of these substrates has a role in promoting the tumorigenic process initiated by Bcr-Abl. We have focused on the role of two of these, CrkL and Cbl.

c-Cbl

v-cbl (for **C**asitas **B**-lineage **L**ymphoma) is the transforming gene of the Cas NS-1 retrovirus that induces pre-B cell lymphomas and myelogenous leukemias in mice [115]. The sequence analysis of this retrovirus has shown that v-Cbl contains 355 amino acids of the amino terminus of its cellular homolog, c-Cbl, fused to viral gag sequences. v-Cbl is localized to the nucleus and cytoplasm of infected cells, whereas, c-Cbl encodes a 120kDa cytoplasmic protein [14]. Overexpression of c-Cbl does not transform fibroblasts and does not alter its subcellular localization [14]. The transforming potential of c-Cbl is activated by a 17-amino acid deletion found in a murine pre-B cell lymphoma cell line, 70Z/3, in an area corresponding to amino acids 366 to 382 in human Cbl [3]. The 70Z/3 mutant form of Cbl is localized to the cytoplasm, indicating that Cbl-induced transformation

can be mediated from the cytoplasm. Additionally, deletion of either of the two tyrosine residues in this area (Y368 and Y371) also promotes tumorigenesis [3]. These transforming variants of Cbl are heavily tyrosine phosphorylated, however, it is not clear if the 40kDa v-Cbl protein is tyrosine-phosphorylated. A schematic of Cbl is shown in Fig. 2.

Cbl is a common substrate of tyrosine kinases activated in immune recognition receptor signaling [164] [175] [65] [174] [149], after hematopoietic cytokine receptor stimulation [163] , after platelet stimulation [160] and integrin ligation [128], and is tyrosine-phosphorylated in a variety of cell-signaling pathways associated with proliferation or activation [149] [67] [17]. Additional evidence for the involvement of Cbl in signaling through tyrosine kinases has come from a genetic analysis of vulval induction during *C.elegans* development. In this system, *sli-1*, a *cbl* homolog, has been identified as a negative regulator of *let-23*, the EGF receptor homolog [236]. The N-terminus of c-Cbl, expressed as a GST-fusion protein, binds to the EGF receptor, as does v-CBL [17]. The extent of GST-N-Cbl binding correlated with the degree of tyrosine phosphorylation of the receptor, though Cbl has no SH2 domain. Additionally, the region of Cbl that binds to activated EGFR is highly conserved between Cbl and Sli-1 [236].

In Abl-transformed cells, Cbl is tyrosine-phosphorylated and coprecipitates with Abl [3] [43] [181] [193]. A truncated form of Cbl found in the HUT 78 T cell lymphoma line, HUT-Cbl, encodes the N-terminal 655 amino acids of c-Cbl. This truncated protein does not transform fibroblasts [14]. In Abl-transformed cells, HUT-Cbl is not

phosphorylated [4], raising the possibility that the sites of Cbl phosphorylation in Abl transformed cells are in the C-terminal domain. However, it is not clear that Abl interacts with this Cbl mutant. Tyrosine phosphorylated Cbl has also been shown to interact with CrkL, an SH2-SH3 domain adaptor protein. Consistent with this finding, HUT-Cbl does not interact with CrkL [4].

Therefore, Cbl is important in CML for a number of reasons. 1) v-Cbl is oncogenic [114], 2) v-Cbl induces a spectrum of hematologic neoplasms [1] similar to those induced by the Abelson murine leukemia virus, 3) it associates with Abl and is tyrosine-phosphorylated and 4) it associates with a variety of other signaling proteins, including CrkL, which have been shown to be important for Bcr-Abl transformation.

c-CrkL

The viral Crk (for CT10 regulator of kinases) protein, v-Crk is the transforming agent of the avian retrovirus CT10 and ASV-1. There are three genes related to Crk that have been cloned, c-Crk I, c-Crk II and c-CrkL (Crk Like). The proto-oncogene products of these Crk proteins belong to a family of proteins consisting primarily of SH2 and SH3 domains while lacking catalytic domains [134] [176]. Nck and Grb2 are two other members of this emerging family of so-called "adaptor proteins" [118] [121]. The widely expressed c-Crk-II protein and c-CrkL contains an amino-terminal SH2 domain followed by two SH3 domains. The c-Crk-I protein, which is found in embryonic lung cells, and v-Crk do not contain the second SH3

domain. In addition, v-Crk has an amino-terminal Gag region.

CrkL is only tyrosine-phosphorylated in peripheral blood cells of Ph-positive patients with an active Bcr-Abl protein [211] [161] [157]. CrkL is expressed in hematopoietic cells but is not constitutively tyrosine-phosphorylated in bone marrow [43] or peripheral blood [211] [161] [157]. In addition, stimulation of neutrophils with GM-CSF, tumor necrosis factor, lipopolysaccharide, 12-*O*-tetradecanoylphorbol-13-acetate, interleukin-1, fibroblast growth factor or insulin fails to evoke CrkL tyrosine phosphorylation [161] [157]. However, CrkL is one of the most prominent tyrosine-phosphorylated proteins in transgenic Bcr-Abl mouse material [43] and it associates with Bcr-Abl suggesting a distinctive role for this adaptor in leukemogenesis.

The v-Crk protein can transform fibroblasts. Overexpression of c-Crk I has been shown to transform fibroblasts, and cause massive tumor formation in nude mice; c-Crk II examined in the same assays induced altered cellular morphology, though, it was not transforming in fibroblasts and did not induce tumor formation in nude mice [130]. Overexpression of c-CrkL was shown to be transforming in fibroblasts [198]. Crk transformed cells contain numerous tyrosine phosphorylated proteins, and it has been suggested that v-Crk can activate cellular tyrosine kinases [140] [139].

v-Crk has also been shown to bind several phosphoproteins via its SH2 domain with high affinity. Three prominent Crk SH2-binding proteins are paxillin (p70), p130^{cas} (**C**rk-**a**ssociated **s**ubstrate) and c-Cbl (p120 mentioned above) [206]. All these proteins are strongly hyperphosphorylated in v-Src transformed cells suggesting that they

could be important in the transformation process of several oncogenes.

The SH3 domain of Crk has also been shown to bind proline-rich motifs of the GDP-GTP exchange factors Sos (p170) and C3G (p150). The binding of Crk SH3 [58] domain to mSos and C3G suggests a role for Ras or related proteins in v-Crk transformation [205] [59] [57]. c-Abl can form stable complexes with v-Crk and with overexpressed c-Crk-I [60] [177].

We embarked on studying the role of CrkL in CML for a number of reasons. 1) It was a unique tyrosine-phosphorylated protein in CML patients, 2) it associated with Bcr-Abl not only in patient neutrophils but also in Bcr-Abl transgenic mice, and 3) It associated with signaling proteins, including Cbl, suggesting an importance of these proteins in Bcr-Abl-mediated transformation.

In furthering our understanding of the disease we hypothesized that substrates of this kinase such as CrkL and Cbl may have an essential role in causing CML. To address this we have taken the approach of mapping the sites of interactions of CrkL and Cbl with Bcr-Abl, mutating these sites, and then assessing whether transformation was affected.

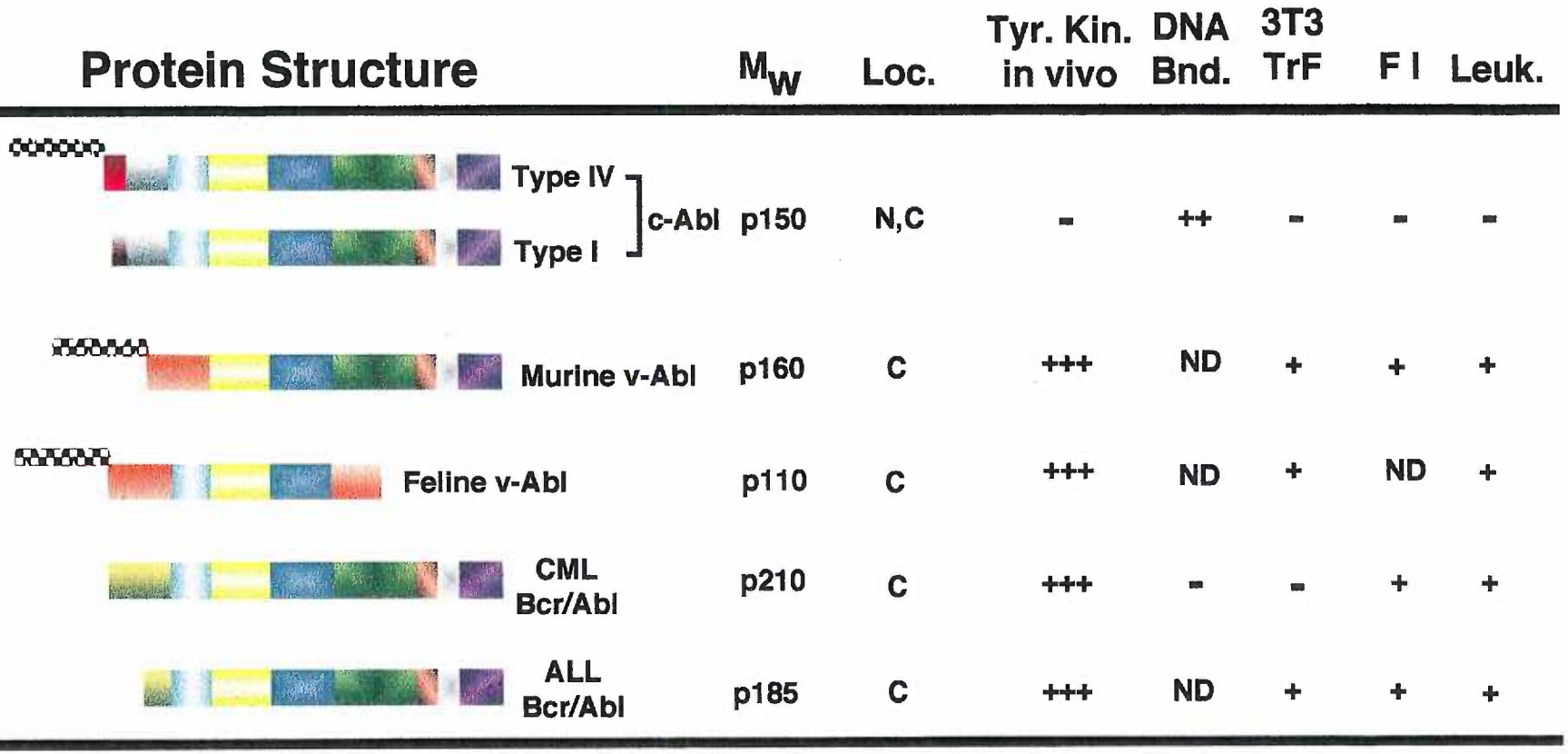

Figure 1. Characteristics of various Abl proteins.

Myristyl fatty acid | Variable first exon encoded region | SH3 domain | SH2 domain

SH1 domain | Nuclear translocation signal | DNA binding domain | Actin binding domain

Loc.=location N or C=nuclear or cytoplasmic; 3T3 TrF=transformation of NIH 3T3 cells; F I= factor-independence; Leuk.=initiation of leukemia in vivo; ND=not done.

Modified from Chung, SW & Wong, PM. 1995. *Oncogene* 10:1261-8

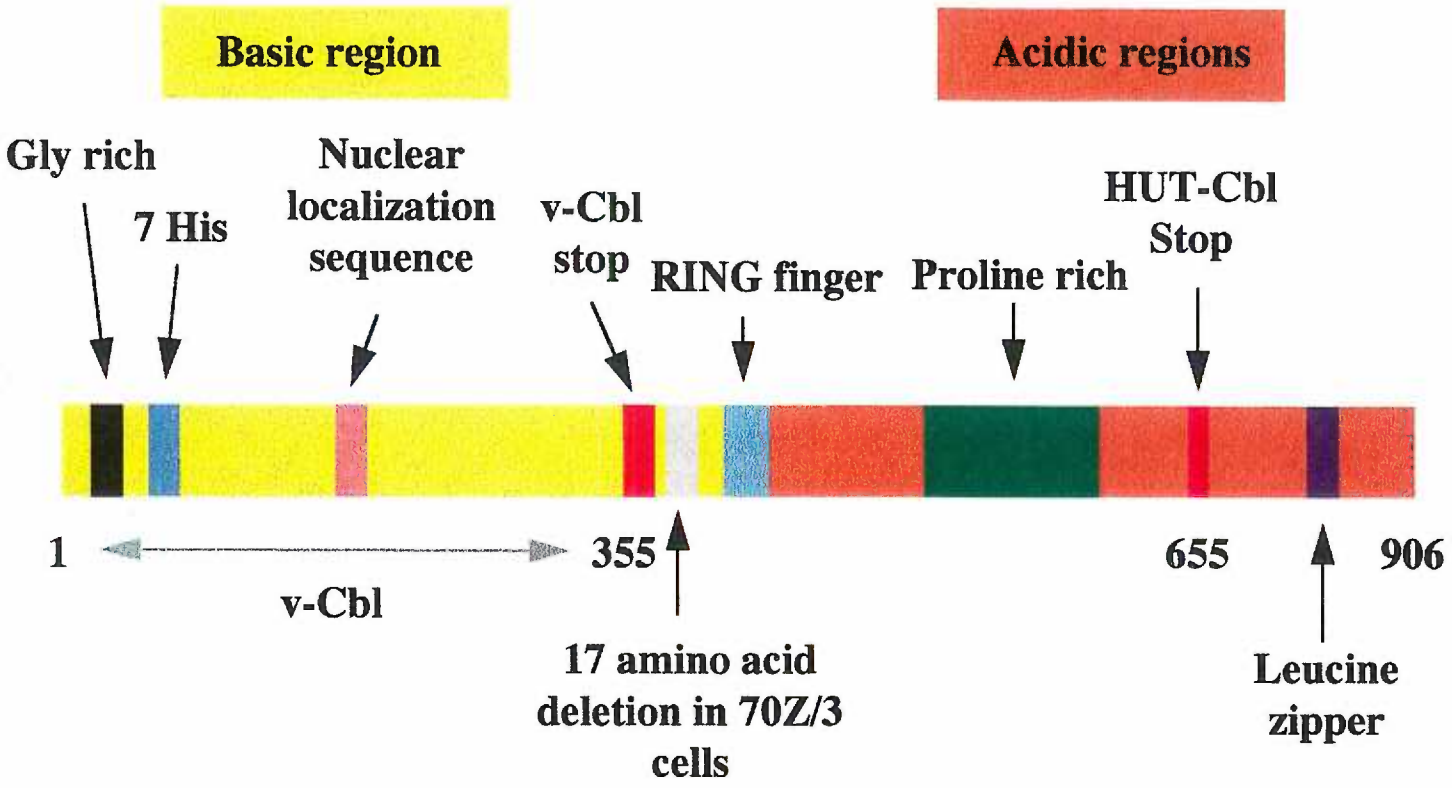

Figure 2. A composite map of Cbl

CHAPTER II

RESULTS

Direct Binding of CRKL to BCR-ABL is Not Required for BCR-ABL Transformation

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ABSTRACT

CRKL has previously been demonstrated to be a major tyrosine phosphorylated protein in neutrophils of patients with BCR-ABL positive chronic myelogenous leukemia and in cell lines expressing BCR-ABL. CRKL and BCR-ABL form a complex as demonstrated by coimmunoprecipitation and are capable of a direct interaction in a yeast two-hybrid assay. We have mapped the site of interaction of CRKL and BCR-ABL to the amino terminal SH3 domain of CRKL with a proline rich region in the C-terminus of ABL. The proline-rich region was mutated and the effect of this deletion on BCR-ABL transforming function was assayed. Our data demonstrate that this deletion does not impair the ability of BCR-ABL to render myeloid cells factor independent for growth. In cells expressing the proline-deletion mutation of BCR-ABL, CRKL is still tyrosine-phosphorylated and forms a complex with BCR-ABL as demonstrated by coimmunoprecipitation. Our data suggests that the interaction between CRKL and the proline-deletion mutant of BCR-ABL is an indirect interaction as CRKL does not interact directly with the proline deletion mutant of BCR-ABL in a gel-overlay assay or in a yeast two-hybrid assay. Thus, a direct interaction of CRKL and BCR-ABL is not required for CRKL to become tyrosine phosphorylated by BCR-ABL and suggests that CRKL function may still be required for BCR-ABL function through an indirect interaction.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell malignancy that constitutes approximately 20% of cases of adult leukemia. In more than 95% of patients with CML, a reciprocal translocation between chromosomes 9 and 22, known as the Philadelphia chromosome translocation is present [159, 187]. This balanced translocation juxtaposes the breakpoint cluster region (BCR) from chromosome 22 with the c-ABL proto-oncogene on the long arm of chromosome 9 [9, 79, 84]. The BCR-ABL fusion creates a gene whose protein product has tyrosine kinase activity increased several fold over the normal c-ABL gene product [39, 42, 124].

The BCR-ABL fusion protein has been shown to transform hematopoietic progenitor cells in bone marrow culture [125, 143, 237], to transform IL-3-dependent myeloid cell lines to growth factor independence [37, 113], and to cause a syndrome resembling CML in syngeneic mice [40, 100]. While ABL kinase activity has been shown to be required for transformation of myeloid cell lines by P210 BCR-ABL [124, 162], the mechanism by which transformation takes place is not yet known. Although numerous substrates of the BCR-ABL tyrosine kinase have been identified, the requirement of most of these substrates for the transforming function of BCR-ABL is similarly unknown. To assist in determining which of the substrates of BCR-ABL may be physiologically relevant for BCR-ABL function, we compared CML patient samples to normal controls for differences in tyrosine phosphorylated proteins. Through this analysis, we and others have demonstrated that the SH2-SH3 adaptor protein, CRKL, is

heavily tyrosine-phosphorylated in neutrophils of patients with Ph-positive CML [157, 161, 211].

CRKL is a 39kDa protein that is related to the CRK oncogene of the avian sarcoma virus, CT10 [140]. Two human homologs of CRK, in addition to CRKL, have been identified, termed CRK I and CRK II, that differ by the presence of a second SH3 domain in CRK II as a result of alternative splicing [134]. CRK II is most similar to CRKL in that both contain two SH3 domains. CRKL is not only tyrosine-phosphorylated in CML patient samples, but it is also tyrosine phosphorylated in cell lines that express BCR-ABL and is inducibly phosphorylated in a BCR-ABL temperature-sensitive mutant [20, 161, 212]. CRKL and BCR-ABL form a complex as demonstrated by coimmunoprecipitation and we have previously demonstrated direct association of CRKL with BCR-ABL sequences in the yeast two-hybrid system [161]. These studies suggest a significant role for this adaptor protein in BCR-ABL transformation.

Therefore, in the current study, we have mapped the direct binding site between CRKL and ABL. BCR-ABL mutants with a deletion of the CRKL binding site were constructed and analyzed for transforming abilities. Transforming function of BCR-ABL was preserved in this deletion mutant, but surprisingly, tyrosine phosphorylation of CRKL and persistent coimmunoprecipitation of CRKL with the BCR-ABL deletion mutant was observed. Although our data demonstrate that we have abolished direct interaction between BCR-ABL and CRKL, we show that CRKL is also capable of associating with BCR-ABL indirectly, through another protein. Thus, we can not exclude a significant role for CRKL in transformation by BCR-ABL via

indirect interactions.

MATERIALS AND METHODS

Generation of GST- fusion constructs and mutants. A schematic of the GST-CRKL fusion proteins is depicted in Figure 2. GST-CRKL SH2 was made by excising an Xba I to Ecl 136 II fragment from pGEX- CRKL [161] and replacing it with an Xba I to Rsa I fragment from the same plasmid. CRKL SH3 was obtained by PCR using the primers: 5'-ACAGCAGAAGATAACCTG-3' and 5'-GTATCTCGCCATGGCCTCAAG-3'. The PCR product was digested with Nco I and ligated into pGEX-KG that had been digested with EcoR I, filled in with Klenow and Nco I-digested. pGEX-CRKL SH3n was constructed by digesting pGEX-CRKL-SH3 with Hind III and religating. CRKL SH3c was constructed by PCR using the following primers: 5'-GTCTTTGCGAAAGCAATC-3' and 5'-GTATCTCGCCATGGCCTCAAG-3'. The PCR product was digested with Nco I and ligated into pGEX-KG prepared as for the CRKL SH2 construct. The intra SH3 domain was prepared by PCR using the primers: 5'-ACAGCAGAAGATAACCTG-3' and 5'-CCCAAGCTTTTCTGGATTGCTTTCGC-3'. The PCR product was digested with Hind III and ligated into Hind III-digested pGEX-KG. The FLVRES mutation, a substitution mutation of Arg to Lys at amino acid 39, was created by single-strand mutagenesis using the Amersham Sculptor mutagenesis system according to the manufacturer's instructions. Single-stranded DNA was made from CRKL cloned as an Xba I to Xho I fragment into pBD3 [51]. The oligonucleotide used for muatgenesis was: R-K39CRKL, 5-CCTCGTCAAGGATTCTTCCACC-3'. CRK II was obtained by RT-PCR from HeLa cells using the following

primers: 5'-GAGGCAGCCATGGCGGGCAAC-3' and 5'-AGATTGTTCCCATCTGTCAGC-3' and was cloned into pGEX-KG. All of the above constructs were sequenced to verify that no mutations had been introduced by the PCR reactions and that no second site mutations had been introduced during mutagenesis.

ABL deletion mutants. Deletion of two proline-rich sequences from the C-terminus of ABL (see Figure 1) was accomplished by PCR mutagenesis on a fragment of ABL corresponding to an Aat II to Bcl I fragment using the following primers: 5' ACCTCCAGGAGAGCTGCAGAG -3' and 5'-GGCCTGCAGCAAGGTAGTCAC-3' for deletion of the site referred to as P1 in Figure 1 and 5' - GAGCGAGGTCCCCCGGAGG -3' and 5'-AGACACGGCAGGCTCATGGTC - 3' for the site referred to as P2 in Figure 1. The double mutant containing a deletion of both sites was obtained by deleting the second site from a plasmid containing a deletion of the first site. The Aat II to Bcl I fragment was sequenced to ensure that no additional mutation had been obtained and was repackaged into full-length BCR-ABL in pUC9 that had been modified to remove the existing Aat II site [162]. The resulting BCR-ABL mutant was cloned into the retroviral vector pGD [40] (gift of G. Daley).

Antisera. Rabbit polyclonal antisera against CRKL were generated against a peptide corresponding to amino acids 204 to 225 of CRKL. This antisera was used exclusively for immunoblotting experiments. For other experiments, anti CRKL antibody was

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing p120 CBL, p145 C3G, and ABL proteins (24-11 and K-12) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The K-12 ABL antibody was used for some of the immunoprecipitation experiments and was found to more efficiently precipitate BCR-ABL than c-ABL. Antibodies recognizing the 85kDa subunit of PI-3 kinase, GRB2, SHC, p120 rasGAP and SOS were obtained from UBI (Lake Placid, NY). Anti p130 CAS was obtained from Transduction Laboratories (Lexington, Kentucky) The paxillin monoclonal antibody was a generous gift of Drs. J. Griffin and R. Salgia, Dana-Farber Cancer Institute, Boston, MA. The antiphosphotyrosine monoclonal antibody, 4G10, was generated using KLH-phosphotyramine as the immunogen and was used as described [50, 97].

Cell lines. The 32Dcl3 cell line [77] was obtained from Joel Greenberger, University of Massachusetts Medical Center, Worcester, MA. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (UBI, Lake Placid, NY) and 15% WEHI-3B conditioned media as a source of IL-3. Control retrovirus without insert, pGD, a full-length BCR-ABL, a tyrosine kinase-defective mutant of BCR-ABL [162] and the BCR-ABL deletion mutants were introduced into the 32Dcl3 cells by electroporation as described [20]. Two days after transfection, cells were selected in the presence of 1 mg/ml of G418 (Gibco) in the presence of 15% WEHI-3B conditioned media. Individual clones were selected after growth in soft agar and analyzed for IL-3 independent growth.

Immunoprecipitation and Immunoblotting. 32D, 32Dp210, and 32Dp210ΔP1,P2 cells were lysed in NP40 lysis buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP40, 10% glycerol) containing 10 μg/ml aprotinin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. Lysates were normalized in preliminary experiments by immunoblotting for c-ABL and these amounts were used for subsequent experiments. Normalized lysates were either immunoprecipitated with 25 μl of CRKL or ABL antisera followed by SDS-PAGE or were analyzed as whole cell lysates by SDS-PAGE. Proteins were transferred onto PVDF (Immobilon-P, Millipore) membranes in a buffer containing 25 mM Tris, 192.5 mM glycine, 20% MeOH for 4 h at 0.45 Amps. Residual binding sites were blocked by incubation in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) containing 3% BSA for 60 min at 25°C. The blots were incubated for 4 - 16 h at room temperature with primary antibody. Antibody reactions were detected using enhanced chemiluminescence (Pierce, Rockford, IL).

GST Binding Assays GST-fusion constructs were expressed in IPTG induced *E.coli* (DH-5α). The GST-fusion proteins were isolated from sonicated bacterial lysates using glutathione sepharose beads (Pharmacia, Piscataway, NJ). Coomassie-stained gels of the GST-fusion proteins were used to normalize for the expression of the various GST-fusion proteins. Between 2.5-5 μg of GST-fusion proteins were incubated with 50 μl of glutathione sepharose beads in bacterial lysis buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, [pH 7.3] containing 10 μg/ml aprotinin, 1 mM Na_3VO_4, 1 mM

phenylmethylsulfonylfluoride, and 0.1% ß-mercaptoethanol). The beads were washed 4 times in ice-cold PBS and incubated for 4 h with normalized cell lysates. The beads were washed three times with PBS, boiled in SDS-sample buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis.

Gel overlay assay. Purified GST, GST-CRKL, GST-CRKL SH3n, or GST-CRKL SH2 fusion proteins were used for this assay. PVDF membranes were blocked overnight at 4°C in PBS with 0.05% Tween 20 and 5% non-fat dry milk. The blots were washed twice in PBS with 0.05% Tween 20 (PBS-T) and incubated for 2 h at room temperature with either GST only or GST-CRKL fusion proteins (at 2 µg/ml) in binding buffer [25 mM Na-phosphate (pH 7.20), 150 mM NaCl, 0.1% Tween 20, 2.5 mM EDTA, 20 mM NaF, 1% non-fat milk, 1 mM dithiothreitol, 10 µg/µl leupeptin, and 10 µg/ml aprotinin]. Bound GST protein was detected by incubation with anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in binding buffer excluding milk, DTT and protease inhibitors. Antibody reactions were developed using enhanced chemiluminescence. The blots were washed for 1h in PBS-T between each of the steps.

Cell Proliferation Assays. MTT Assay. 2 X 10^4 cells were plated in quadruplicate into 96-well plates in RPMI 1640 medium with 10% FBS with or without 15% WEHI-3B conditioned media as a source of IL-3. Wells were assayed for MTT uptake at 24h intervals as described [151]. For each assay, controls were performed using

serial dilutions of 32D and 32Dp210 cells to ensure that assay was linear with respect to cell number over the time period examined. Results are presented as the mean absorbance at 570 nm (OD) +/- standard deviation.

Cell count assays. 2 X 10^5 cells were plated in media with or without 15% WEHI-3B conditioned media. Viable cells, as assayed by the exclusion of trypan blue, were counted at daily intervals.

Yeast two hybrid assay. Analysis of interactions using the yeast two hybrid system were performed as described [223]. The ABL construct included a Kpn I to Bcl I fragment of c-ABL corresponding to the SH2, tyrosine kinase and a small portion of the C-terminal domain fused in frame to the DNA-binding domain of LexA (Figure 1). The proline-deletion mutants described above and a kinase inactive version of ABL [162] were cloned into this ABL construct . A full length p185BCR-ABL (gift of O. Witte) was also expressed as a LexA fusion protein. Full length CRKL and the amino terminal SH3 domains of CRKL and CRK II were expressed in frame fused to the acidic activation domain VP16. Expression of each of these constructs was confirmed by immunoblotting of yeast lysates with the appropriate antisera. Interaction was identified by selection on plates lacking histidine and analyzed for ß-galactosidase production as described [223]. Controls included plasmid, Lex A, VP-16 and LexA-lamin controls.

RESULTS

Mapping the site of interaction between CRKL and ABL. We previously had identified CRKL and CRK II as proteins that interact with ABL in a yeast two-hybrid screen [161]. Several factors suggested that an SH3 domain of CRKL was interacting with a proline-rich sequence of ABL. All of the CRKL and CRK II clones that were sequenced clustered around the amino terminal SH3 domain of these proteins. Both CRKL and CRK II bound to a kinase-inactive mutant of ABL, suggesting that this was not a phosphotyrosine dependent interaction with the SH2 domain of CRKL. Ren, et al., had previously mapped the CRK II binding site to a proline-rich region just 3' of the kinase domain of ABL [177]. This proline-rich region was included in the construct that we had used for our yeast two-hybrid screen and is shown schematically in Figure 1.

To confirm that the amino-terminal SH3 domain of CRKL was interacting with ABL, various portions of CRKL were expressed as GST fusion proteins. These constructs are depicted in Figure 2. Lysates of BCR-ABL expressing cells were allowed to bind to these GST fusion constructs. These experiments demonstrated that both c-ABL and BCR-ABL bound to full length CRKL and that this binding was mediated predominantly by the amino-terminal SH3 domain of CRKL (Figure 3). On longer exposure of the gels, there appeared to be trace amounts of binding of BCR-ABL to the SH2 domain of CRKL. There was no binding of ABL or BCR-ABL to SH3c and in the construct containing both SH3c and SH3n (SH3), it appears that the presence of SH3c decreases the amount of ABL proteins that bind.

With the above information, two proline-rich sequences contained in the C-terminal region of c-ABL (referred to as P1 and P2 in Figure 1) were deleted individually or in combination. Each of these constructs was expressed as a fusion protein containing the DNA binding domain of LexA in a yeast plasmid and analyzed for interaction with either full-length CRKL or the amino terminal SH3 domain of CRKL, expressed in frame, fused to the acidic activation domain, VP16. As demonstrated in Figure 4, deletion of either proline-rich sequence abolished the interaction of ABL with CRKL.

Effects of the proline-deletion on BCR-ABL transformation. To analyze the effects of deletion of the site of interaction of CRKL with BCR-ABL, the proline-deletion mutants were repackaged into full length BCR-ABL. As the results of the deletion of each individual proline-rich region and the double mutant are identical, only the results of the double mutant, referred to as p210ΔP1,P2, will be presented. An IL-3-dependent murine cell line, 32D, was transfected with full-length p210BCR-ABL, p210ΔP1,P2, and control retrovirus and selected in the presence of neomycin and IL-3. Individual clones were selected after growth in soft agar and were analyzed for IL-3-independent growth. Figure 5 is representative of results obtained from 10 out of 10 clones expressing BCR-ABL and demonstrates that the p210ΔP1,P2 expressing cells are capable of IL-3 independent growth. Similarly, there was no defect in fibroblast transformation by this mutant (data not shown). Analysis of BCR-ABL kinase activity showed no difference between the proline deletion mutant and the full-length

version of p210BCR-ABL (data not shown).

Analysis of CRKL phosphorylation and interaction with BCR-ABL. Analysis of whole-cell lysate phosphotyrosine immunoblots of cells expressing the p210ΔP1,P2 mutant showed a pattern of tyrosine-phosphorylated proteins that was similar to that of cellular lysates obtained from cells expressing the full-length BCR-ABL. This included prominent tyrosine phosphorylation of a 39kDa protein that comigrated with the 39kDa protein that we had previously purified from K562 cells and had demonstrated to be CRKL [161]. That CRKL was tyrosine-phosphorylated in the p210ΔP1,P2-expressing cells was confirmed by antiphosphotyrosine immunoblotting of CRKL immunoprecipitates (Figure 6a). Further, CRKL and BCR-ABL remained capable of interacting as demonstrated by coimmunoprecipitation. Thus, CRKL immunoprecipitates from lysates of cells expressing the p210ΔP1,P2 mutant contain BCR-ABL and ABL (Figure 6c). Similar results for CRKL and BCR-ABL association were obtained using a p185 construct containing a similar deletion of the proline-rich sequences (data not shown). A kinase defective BCR-ABL also associates with CRKL, consistent with binding of CRKL to a region outside of the tyrosine kinase domain of ABL. However, there is no tyrosine phosphorylation of CRKL in cells expressing the kinase defective version of BCR-ABL.

Analysis of the CRKL and BCR-ABL interaction in the ΔP1,P2 mutant. The above data suggested that CRKL was binding to BCR-ABL through another protein or there was another direct

binding site for CRKL in full-length BCR-ABL. As the ABL construct that we had used initially in our yeast two-hybrid analysis only contained the following domains of Abl: SH2, kinase, and a portion of the C-terminus (Figure 1), it is possible that we missed an interaction with another portion of full length BCR-ABL. As p185BCR-ABL with a deletion of the P1,P2 region also interacted with CRKL, this suggests that the interaction site is not in the second or third exon of BCR, which constitutes the difference between these two molecules.

Three experiments were devised to help in distinguishing between direct and indirect interactions. Cellular lysates of the p210ΔP1,P2 were analyzed for binding to the various GST-CRKL constructs. As compared to full-length BCR-ABL, lysates from the proline-deletion mutant of BCR-ABL had minimal binding of BCR-ABL to the full length CRKL GST fusion constructs and no binding to the amino-terminal SH3 domain of CRKL (Figure 7). The 32Dp210 and 32Dp210ΔP1,P2 lysates were normalized for BCR-ABL expression prior to this analysis (data not shown). As can be seen from Figure 7, c-ABL from the p210ΔP1,P2 lysate remained capable of being removed from the lysate by the GST-CRKL fusion construct. Although this experiment suggests that the major site of direct interaction between BCR-ABL and CRKL is removed by the deletion of the proline-rich regions in the C-terminus of ABL, this experiment cannot distinguish direct from indirect interactions.

We next performed a gel overlay assay on ABL immunoprecipitates from 32Dp210 and 32Dp210ΔP1,P2 lysates with a GST-CRKL fusion construct. Immunoblots were detected with an anti-GST antisera. These experiments demonstrated that CRKL or

CRKL-SH3n bound to full-length BCR-ABL but not the ΔP1,P2 mutant (Figure 8a). Again, similar amounts of BCR-ABL were present in the ABL immunoprecipitates (Figure 8b). No binding to ABL was detected using CRKL-SH2 or GST alone (data not shown). c-ABL was not detected on these immunoblots as the antisera used for these experiments precipitated BCR-ABL more efficiently than c-ABL.

Finally, the ΔP1,P2 mutant was repackaged into p185BCR-ABL and expressed as a LexA fusion protein for analysis in the yeast two hybrid system. In this assay, CRKL and p185BCR-ABL were capable of an interaction. However, there was no interaction of CRKL with the ΔP1,P2 mutant (Figure 4).

Cellular interactions of CRKL. All of the above data suggested that the P1,P2 region mediated a direct interaction of BCR-ABL and CRKL. However, in cells expressing the ΔP1,P2 mutant, CRKL was still tyrosine-phosphorylated and associated with BCR-ABL, presumably through another signaling protein. Numerous proteins are known to be tyrosine-phosphorylated in BCR-ABL-expressing cells, and many of them have been found to be capable of forming a complex with BCR-ABL. While, many of the cellular interactions of CRK II have been determined, there is less information about the proteins that interact with CRKL [60, 212]. Therefore, we analyzed numerous proteins for interaction with CRKL and used the CRKL GST fusion constructs to determine the region of CRKL that mediated binding to these various intracellular proteins.

As an example, the pattern of phosphotyrosine containing proteins that bind to CRKL is shown in Figure 9a. As can be seen,

tyrosine-phosphorylated proteins of 210, 170, 135-140, 120, 68-70, 62, and 56 kDa are capable of binding to full-length CRKL. This is similar to the pattern of tyrosine-phosphorylated proteins seen in a CRKL immunoprecipitate (Figure 6a). Interestingly, some of these tyrosine phosphorylated proteins bind to the amino-terminal SH3 domain, particularly p210, p170, and p135-140, while the others bind, as expected, to the SH2 domain. Specific antisera were used to characterize the CRKL interactions. Thus, as previously seen, p210BCR-ABL binds to the amino-terminal SH3 domain of CRKL (Figure 3). Other proteins that bind to the amino-terminal SH3 domain include p170 SOS, p145 C3G, and p140 c-ABL (Table 1). Proteins that bind to the CRKL SH2 domain include p130 CAS, p120 CBL, and p68 paxillin (Figure 9b and Table 1). No proteins have been identified that bind to the C-terminal SH3 domain of CRKL and the 56 and 62kDa tyrosine-phosphorylated proteins that bind to CRKL have not been identified. Other proteins that were analyzed for binding to CRKL that are known to bind to BCR-ABL, but were negative for binding to CRKL include GRB2, the 85kDa subunit of phosphatidylinositol 3-kinase, p120 rasGAP, and SHC.

TABLE 1. Summary of Proteins That Bind to CRKL

Protein	Binding site of CRKL
BCR-ABL	SH3n
c-ABL	SH3n
C3G (145 kDa)	SH3n
SOS (170 kDa)	SH3n
*Paxillin (68kDa)	SH2
*CBL (120 kDa)	SH2
CAS (130 kDa)	SH2

* Indicates proteins that are also reported to bind to BCR-ABL

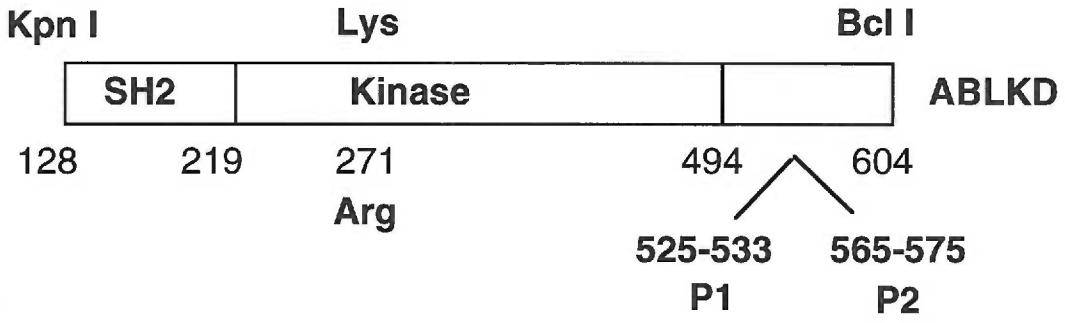


Figure 1. Schematic of Abl construct used in the yeast two hybrid screen. Numbers correspond to amino acids numbered according to c-Abl type Ia. Arg 271 corresponds to the substitution at this amino acid that yields a kinase defective Abl. P1 and P2 are two proline rich regions in the C-terminus of Abl.

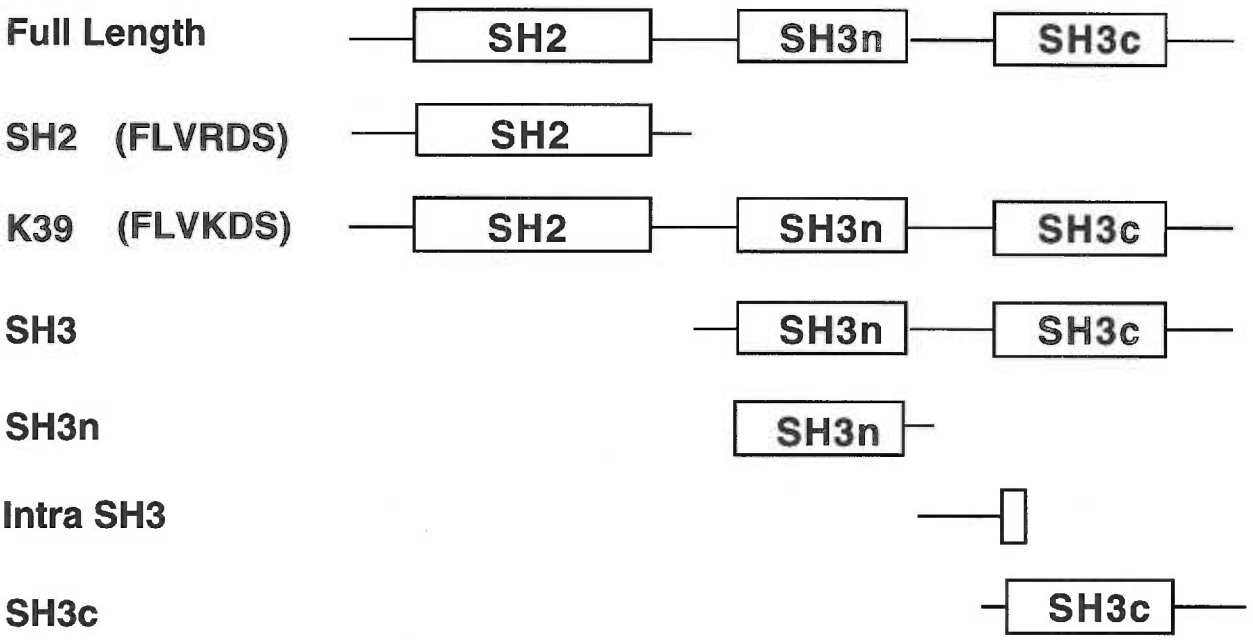


Figure 2. Schematic of CrkL constructs. Full length and various portions of CrkL were subcloned into pGEX-KG as described in the Materials and Methods section and are depicted in this figure.

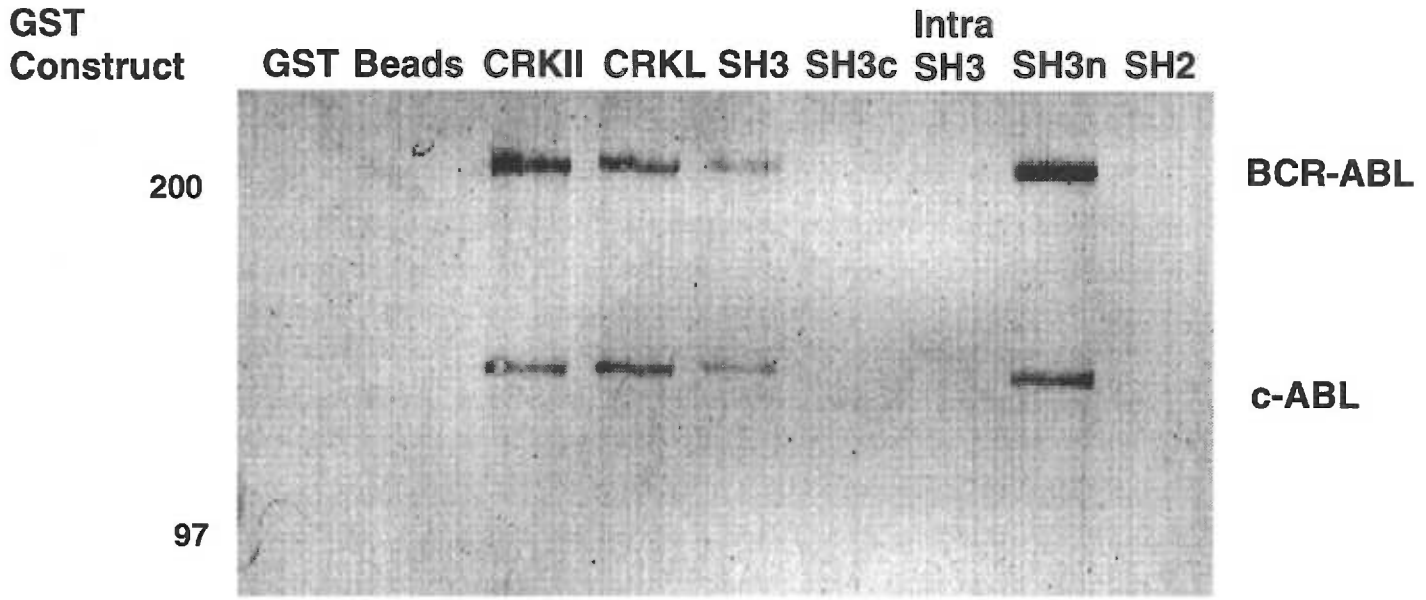


Figure 3. Binding of Abl proteins to bacterially expressed CrkL. Lysates from 1 x 10^7 32Dp210 cells were analyzed for binding to GST, glutathione beads (beads), or the various GST-CrkL constructs as described in the Materials and Methods. Bound proteins were separated by SDS-PAGE and transferred to PVDF membranes. Abl proteins were detected using the 24-21 Abl monoclonal antibody. Molecular weight markers are indicated on the left side of the panel.

LexA fusion construct	VP-16 fusion construct	VP-16	CRKL (full length)	CRKL-SH3n	CRK II-SH3n	AAP1	AAP2
LexA		-	-	-	-	-	-
Lex A-Lamin		-	-	-	-	-	-
ABL-WT		-	+	+	+	+	+
ABL-KD		-	+	+	+	+	-
ABL-P1		-	-	-	-	+	+
ABL-P2		-	-	-	-	+	+
ABL-P1,P2		-	-	-	-	+	+
p185BCR-ABL		-	+	+	+	+	+
p185BCR-ABL KD		-	+	+	+	ND	ND
p185BCR-ABL P1,P2		-	-	-	-	ND	ND

Figure 4. Binding of CrkL to Abl in the yeast two hybrid system. Abl and Bcr-Abl constructs and a lamin control were expressed as LexA-fusion proteins and analyzed for interaction with CrkL and Crk II expressed as VP-16 acidic activation domain fusion proteins. Two additional VP-16 constructs, AAP1 and AAP2, that were identified in our yeast two hybrid screen using the Abl kinase domain were used as controls. + indicates positivity for ß-galactosidase activity and growth on his^- plates. Abbreviations: Abl-WT refers to the SH2, kinase construct depicted in Figure 1; Abl-KD is a kinase defective version of Abl; P1, P2, are the deletion mutants of the proline rich regions depicted in Figure 1.

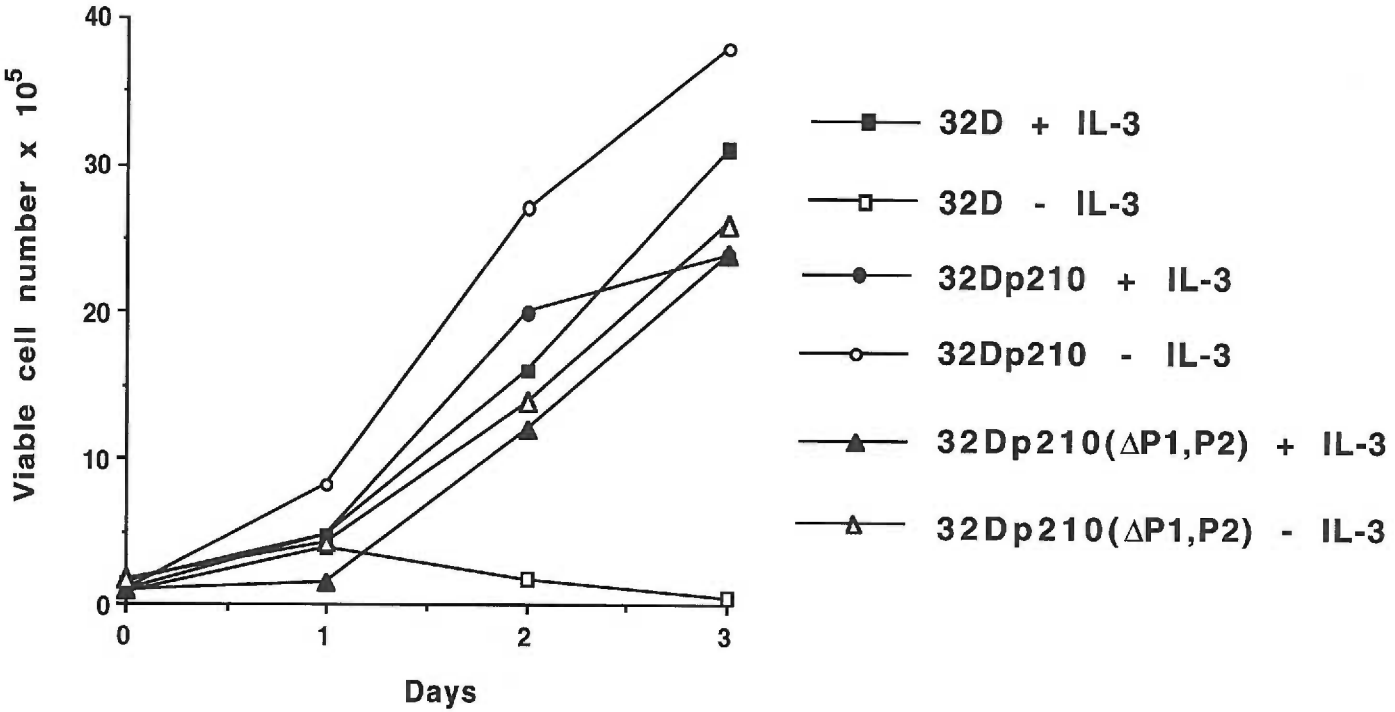


Figure 5. Proliferation assay of Bcr-Abl ΔP1,P2 mutant in 32D cells. 32D, 32Dp210, and 32Dp210ΔP1,P2 cells growing in the presence of IL-3 were washed three times with RPMI 1640 medium and plated at 1 x 10^5 cells/ml in regular growth media with or without exogenous growth factor. Each day, viable cells were counted as assessed by exclusion of trypan blue. The data presented is representative of four separate experiments. Similar results were obtained in MTT assays.

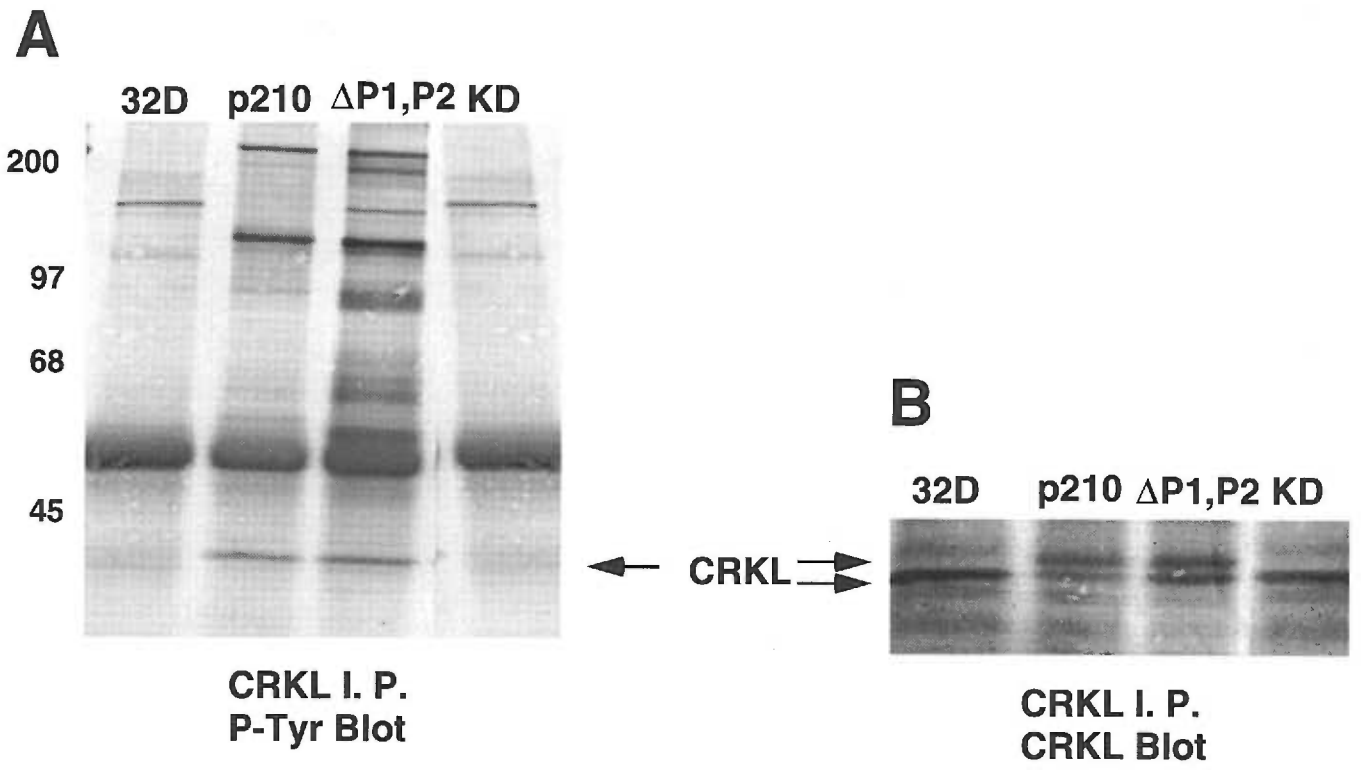
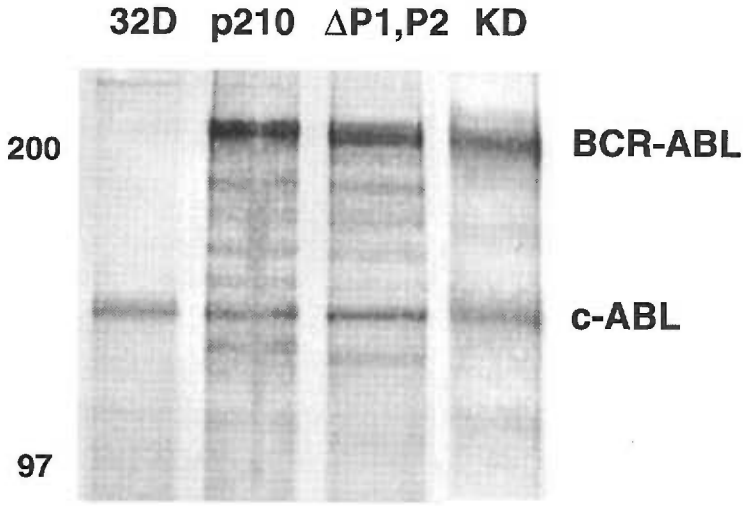


Figure 6. Tyrosine phosphorylation of CrkL and coimmunoprecipitation of Bcr-Abl and CrkL. Lysates of cells expressing the indicated constructs were (A) Immunoprecipitated with CrkL antisera and immunoblotted with 4G10, a monoclonal antiphosphotyrosine antibody; (B) Immunoprecipitated with CrkL antisera and immunoblotted with CrkL antisera; (C) Next page. Immunoprecipitated with CrkL antisera and immunoblotted with an Abl monoclonal antibody. KD refers to a tyrosine kinase defective mutant of Bcr-Abl. Molecular weight markers are indicated on the left side of the panel.

C



CRKL I.P.
ABL Blot

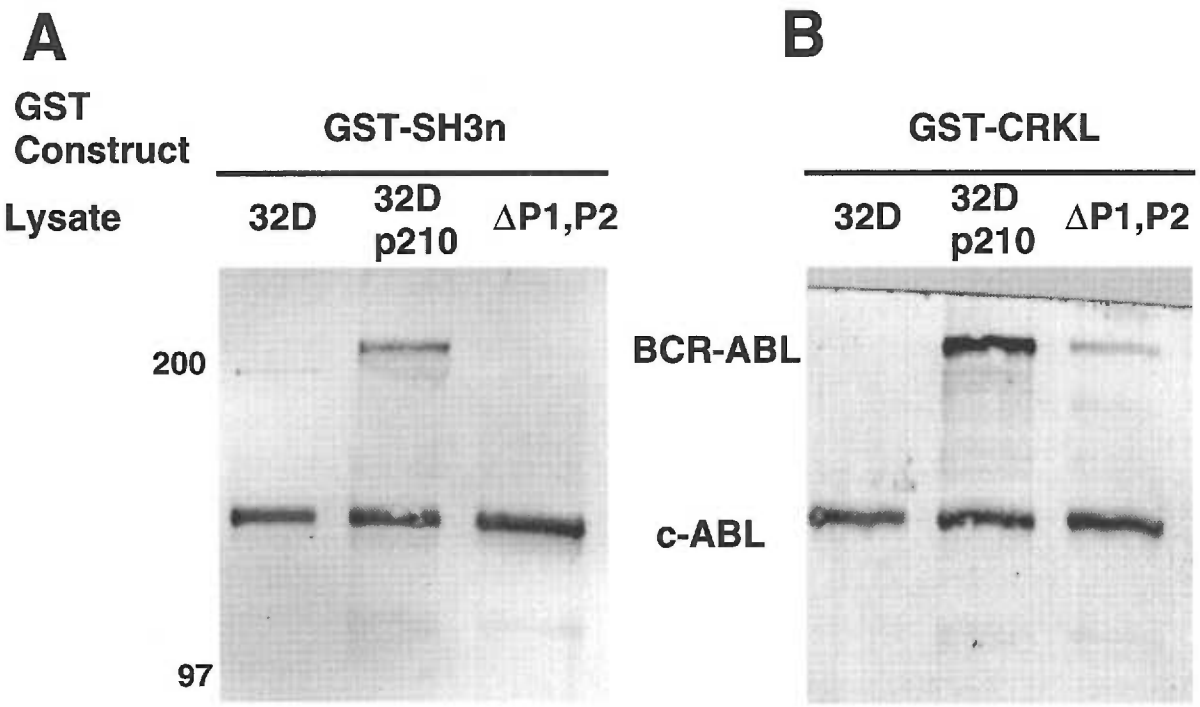


Figure 7. Binding of Bcr-Abl proteins to bacterially expressed CrkL. Lysates from 32D, 32Dp210, or 32Dp210ΔP1,P2 cells were analyzed for binding to (A) GST-CrkL or (B) GST-CrkL SH3n. Bound proteins were separated by SDS-PAGE and transferred to PVDF membranes. Abl proteins were detected using the 24-21 Abl monoclonal antibody. The 32Dp210 and 32Dp210ΔP1,P2 lysates were normalized for Bcr-Abl expression prior to this analysis. Molecular weight markers are indicated on the left side of the panel.

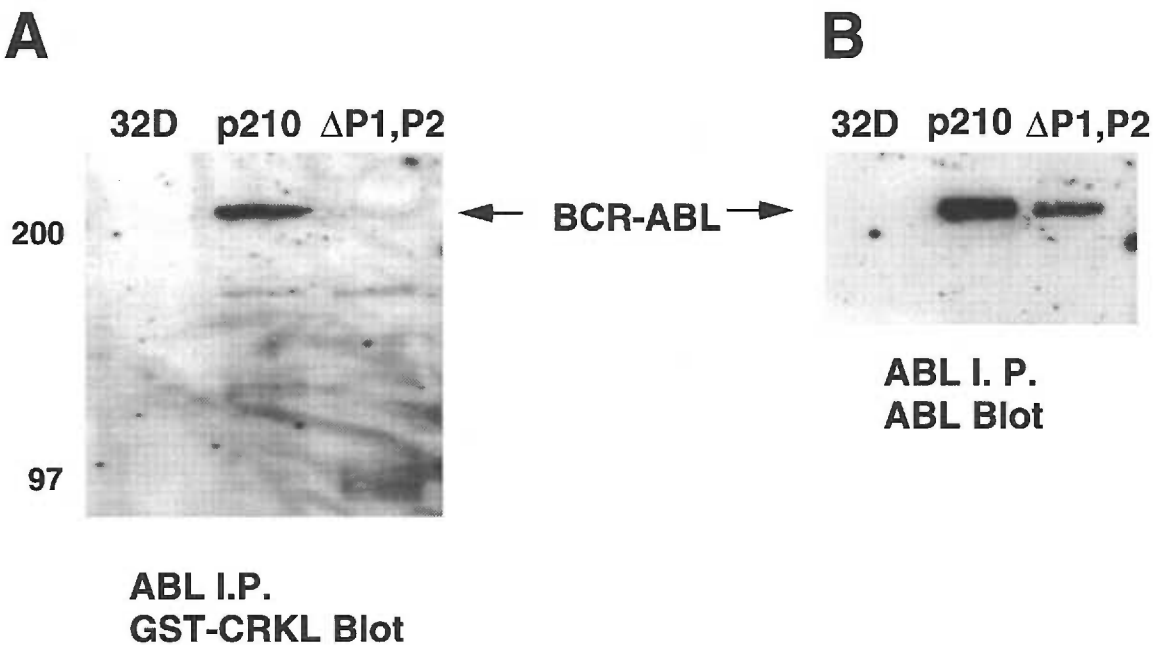


Figure 8. Gel overlay assay of CrkL binding to Bcr-Abl. Lysates of 32D, 32Dp210 and 32Dp210ΔP1,P2 cells were immunoprecipitated with Abl antisera K-12, separated by SDS-PAGE and transferred to PVDF membranes. Immunoblots were probed with (A) GST- CrkL and binding detected with a GST antibody or (B) Abl monoclonal antibody 24-21. Molecular weight markers are indicated on the left side of the panel.

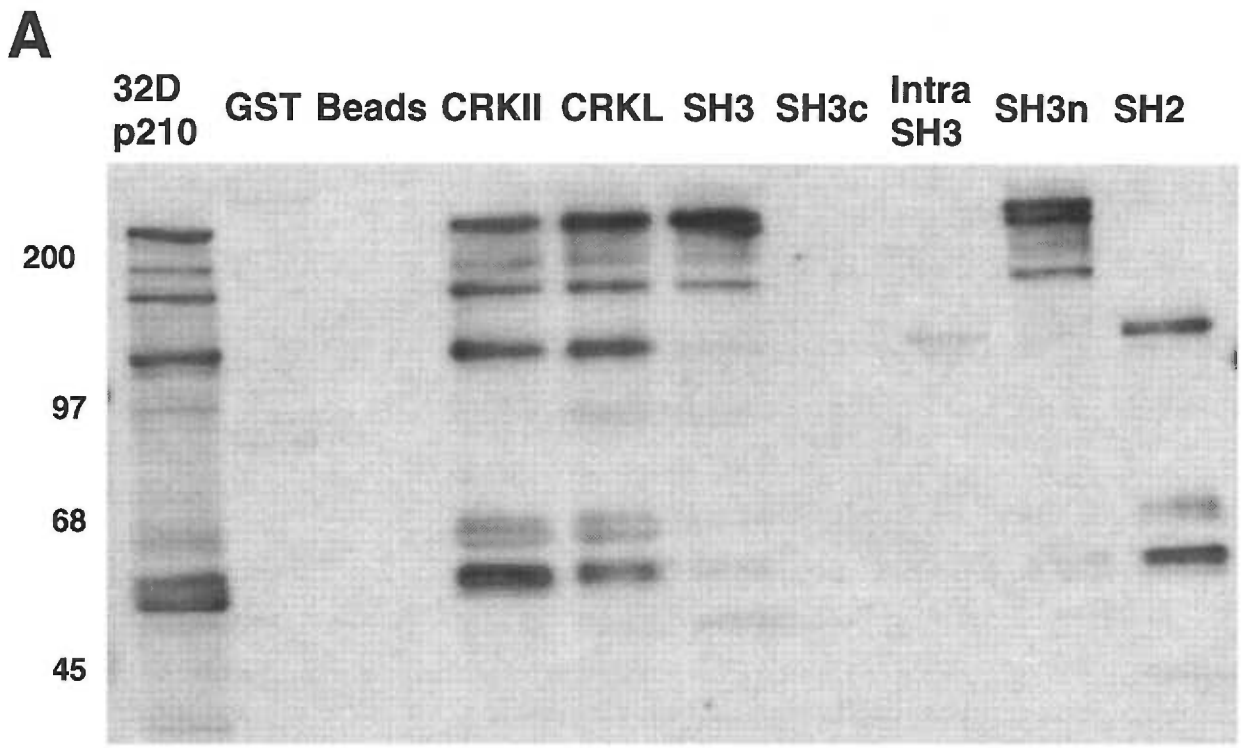
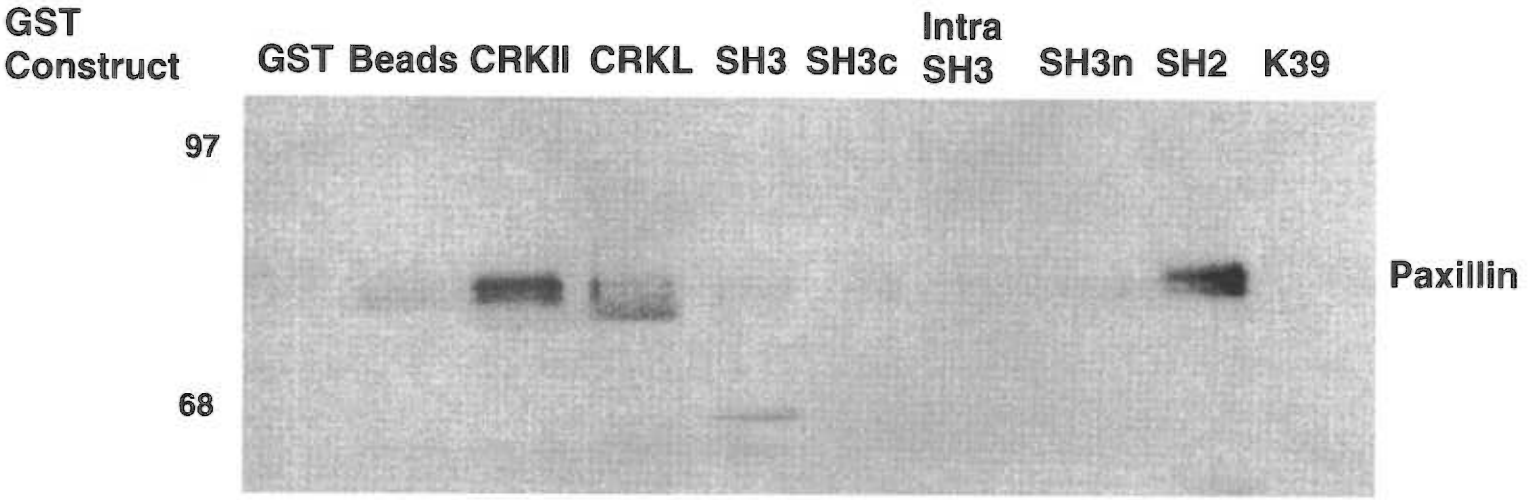


Figure 9. Binding of cellular proteins to bacterially expressed CrkL. Lysates from 1 x 10^7 32Dp210 cells were bound to GST, glutathione beads (beads), or the various GST-CrkL constructs as described in the Materials and Methods. Bound proteins were separated by SDS-PAGE and transferred to PVDF membranes. (A) Tyrosine phosphorylated proteins were detected with 4G10, a monoclonal antiphosphotyrosine antibody and (B) Next page. Paxillin was detected with an anti-paxillin monoclonal antibody. Molecular weight markers are indicated on the left side of each panel. The lane marked 32Dp210 in panel A is a whole cell lysate lane.

B


GST Construct
GST
Beads
CRKII
CRKL
SH3
SH3c
Intra SH3
SH3n
SH2
K39
97
68
Paxillin

DISCUSSION

In the current study, we have mapped the site of direct interaction of CRKL and BCR-ABL to the amino-terminal SH3 domain of CRKL with a proline-rich region in the C-terminus of ABL. Deletion of either proline-rich region abolished binding to CRKL, suggesting that both proline-rich sequences may be required for CRKL binding. Although our initial analysis of binding in the yeast two-hybrid system was performed with a fragment of ABL, we confirmed that there were no other sites of direct binding of CRKL to BCR-ABL by expressing full-length p185BCR-ABL and a p185BCR-ABL with the P1, P2 deletions fused to the DNA binding domain of LexA. This assay showed that there was no binding of CRKL to the p185ΔP1,P2 mutant. Additional evidence for the lack of a direct interaction with the deletion mutant was obtained using a gel-overlay assay.

Despite the lack of a direct interaction between BCR-ABL and CRKL in the ΔP1,P2 mutant of BCR-ABL, there was no defect in transforming ability of this mutant as assessed by growth factor independence of myeloid cells and fibroblast transformation. Similar data has been obtained independently by Senechal, et. al. [198]. Although our initial impression was that CRKL was not required for BCR-ABL function, analysis of the mutant cell lines revealed tyrosine phosphorylation of CRKL and persistent coimmunoprecipitation of CRKL with the proline deletion BCR-ABL mutant. Thus, we cannot exclude a significant role for CRKL in transformation by BCR-ABL via indirect interactions.

Numerous proteins have been shown to interact with BCR-ABL

and many have been shown to be tyrosine-phosphorylated in BCR-ABL expressing cells. Besides CRKL, these proteins include rasGAP [49], GRB2 [170, 172], SHC [136, 208], FES [54], SYP [209], CBL [3, 44, 181], the 85kDa subunit of phosphatidylinositol 3-kinase [222], VAV [135], c-BCR [123], paxillin [192], and various other cytoskeletal proteins [191]. These proteins and others are candidates for mediating the indirect interaction of CRKL with BCR-ABL. We analyzed the ability of several of these tyrosine phosphorylated proteins to interact with CRKL and found that p120 CBL, and p68 paxillin bind to the SH2 domain of CRKL, while binding to the amino-terminal SH3 domain of CRKL was demonstrated for p210 BCR-ABL. No binding of CRKL to other proteins that are known to associate with BCR-ABL was found including the 85kDa subunit of PI 3 kinase, GRB2, SHC, or p120 rasGAP.

Of the proteins that interact with CRKL, paxillin and CBL also interact with BCR-ABL. Either of these proteins or another unidentified protein could be responsible for mediating a ternary complex between BCR-ABL and CRKL in the proline-deletion mutant. In the case of CBL, several groups have demonstrated that c-CBL is tyrosine phosphorylated in BCR-ABL-expressing cells and that BCR-ABL and c-CBL are found in a complex as demonstrated by co-immunoprecipitation. [3, 44, 181]. The interaction of c-CBL and BCR-ABL has been shown to be dependent on tyrosine-phosphorylation of CBL [181]. Additional studies have shown that the ABL SH2 domain is capable of direct binding to tyrosine-phosphorylated c-CBL [193] and as shown here, the SH2 domain of CRKL is also capable of binding to c-CBL. Consistent with this, c-CBL immunoprecipitates from BCR-ABL

cells not only contain BCR-ABL, but also contain CRKL [193]. Thus, our current model is that tyrosine-phosphorylated c-CBL binds to the SH2 domain of ABL in BCR-ABL and through another tyrosine phosphorylated residue, binds the SH2 domain of CRKL. The interaction of CRKL and BCR-ABL can also occur directly through the amino terminal domain of CRKL binding to a proline-rich region of ABL.

A prediction of this model would be that BCR-ABL should be capable of binding to the GST-SH2 domain of CRKL by virtue of tyrosine-phosphorylated CBL binding to BCR-ABL and the SH2 domain of CRKL. This prediction was confirmed in our GST-fusion protein experiments. It would also be predicted that the GST-full length CRKL would still be capable of binding some BCR-ABL from the proline deletion, through the interaction of CBL with the SH2 domain of CRKL. Again, this prediction was borne out by the data.

However, it is possible that more complicated complexes occur or that proteins other than CBL mediate an indirect interaction between CRKL and BCR-ABL. For example, the SH2 domain of GRB2 is known to bind to Tyr 177 of BCR-ABL and the SH3 domain of CBL has been shown to bind to GRB2 [65]. As tyrosine-phosphorylated CBL binds to the SH2 domain of CRKL, it is possible that GRB2 binding to BCR-ABL allows CRKL to associate with BCR-ABL through this interaction with CBL. Given the multitude of complexes induced by BCR-ABL, combinations of mutants (e.g., Tyr 177 to Phe with a deletion of the proline-rich sequences in the C-terminus of ABL) will be required to determine the roles of various signaling proteins in BCR-ABL transformation. Alternatively, the ability to BCR-ABL to

transform cell lines generated from mice lacking various of these signaling proteins could also be informative.

ACKNOWLEDGMENTS

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CHAPTER III

RESULTS

Interactions of CBL with BCR-ABL and CRKL in BCR-ABL-Transformed Myeloid Cells

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ABSTRACT

The Philadelphia chromosome, detected in virtually all cases of chronic myelogenous leukemia (CML), is formed by a reciprocal translocation between chromosome 9 and 22 that fuses BCR encoded sequences upstream of exon 2 of c-ABL. The BCR-ABL fusion creates a gene whose protein product, p210BCR-ABL, has been implicated as the cause of the disease. Although ABL kinase activity has been shown to be required for the transforming abilities of BCR-ABL and numerous substrates of the BCR-ABL tyrosine kinase have been identified, the requirement of most of these substrates for the transforming function of BCR-ABL is unknown. In this study we mapped a direct binding site of the c-CBL proto-oncogene to the SH2 domain of BCR-ABL. This interaction only occurs under conditions where c-CBL is tyrosine-phosphorylated. Despite the direct interaction of c-CBL with the SH2 domain of BCR-ABL, deletion of the SH2 domain of BCR-ABL did not result in an alteration in the complex formation of BCR-ABL and c-CBL, suggesting that another site of direct interaction between c-CBL and BCR-ABL exists or that another protein mediates an indirect interaction of c-CBL and BCR-ABL. Since CRKL, an SH2, SH3 domain-containing adapter protein is known to bind directly to BCR-ABL and also binds to tyrosine-phosphorylated c-CBL, the ability of CRKL to mediate a complex between c-CBL and BCR-ABL was examined.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell malignancy that is associated with a reciprocal translocation between chromosomes 9 and 22, known as the Philadelphia chromosome translocation [188] [159]. This balanced translocation juxtaposes the breakpoint cluster region (BCR) from chromosome 22 with the c-ABL tyrosine kinase on the long arm of chromosome 9 [78] [84] [10]. The BCR-ABL fusion creates a gene whose protein product has been shown to transform hematopoietic progenitor cells in bone marrow culture [125] [143] [237], to transform IL-3 dependent myeloid cell lines to growth factor independence [37] [113], and to cause a syndrome resembling chronic myelogenous leukemia in syngeneic mice [100] [40]. The BCR-ABL tyrosine kinase activity is increased several-fold over the normal c-ABL gene product [39] [42] [124]. Although ABL kinase activity has been shown to be required for transformation of myeloid cell lines by BCR-ABL [124] [162], and numerous substrates of the BCR-ABL tyrosine kinase have been identified, the requirement of most of these substrates for the transforming function of BCR-ABL is unknown.

Two of the substrates of BCR-ABL on which we have focused are CRKL and c-CBL. CRKL is a 39kDa SH2, SH3 domain-containing adapter protein that is related to the CRK oncogene of the avian sarcoma virus, CT10 [140]. Two human homologs of CRK, in addition to CRKL, have been identified, termed CRK I and CRK II. CRKL is most similar to CRK II in that both contain two SH3 domains, whereas CRK I contains only one SH3 domain as a result of alternative splicing

[134]. We have previously demonstrated that the N-terminal SH3 domain of CRKL binds directly to a proline-rich region in the C-terminus of BCR-ABL and this direct binding can be disrupted by deletion of this region [82]. However, this deletion mutant of BCR-ABL remains transformation-competent, and CRKL is tyrosine-phosphorylated and binds to BCR-ABL through indirect interactions in cells expressing this deletion mutant [82].

One of the candidates for mediating indirect binding of CRKL and BCR-ABL is c-CBL. c-CBL is the cellular homolog of v-CBL, the transforming protein of the Cas NS-1 retrovirus that induces pre-B cell lymphomas and myeloid leukemias in mice [115]. 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 [206] [47] [65] [67] [163]. c-CBL is known to be tyrosine-phosphorylated in cells expressing activated ABL oncoproteins and has been shown to bind to CRK proteins when tyrosine-phosphorylated [44] [3] [181] [193]. In this study we have mapped a direct binding site of tyrosine-phosphorylated c-CBL to the SH2 domain of BCR-ABL. The effects of deletion of this domain on complex formation between c-CBL, BCR-ABL and CRKL are reported.

MATERIALS AND METHODS

Cells and cell culture. The 32Dcl3 cell line [77] was obtained from Joel Greenberger, University of Massachusetts Medical Center, Worcester, MA. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (UBI) and 15% WEHI-3B conditioned media as a source of IL-3. Sublines of 32Dc13 expressing p210BCR-ABL, the SH2 domain deletion of ABL (ΔSH2), a tyrosine kinase-inactive mutant of BCR-ABL, and a deletion of a proline rich region in the C-terminus of ABL that mediates direct binding of CRKL to ABL (ΔP1,P2) were generated as previously described [162] [82] [52]. K562 cells are a BCR-ABL positive leukemic cell line [122] that was cultured in RPMI 1640 supplemented with 10% FCS at 37°C, 5% $C0_2$.

Generation of Baculovirus constructs. Full-length p210BCR-ABL and the kinase domain of ABL were cloned into a modified version of the pBluBac vector (InVitrogen). This vector, pBluBac3C (gift of R. Maurer, Oregon Health Sciences University, Portland, OR) contains a 12CA5 antibody binding site, a hexa-histidine sequence for Ni-sepharose purification, a Factor X cleavage site and a BamH I cloning site. Full length BCR-ABL was modified to remove untranslated 5' sequences and was cloned in-frame into the BamH I site of pBluBac3C. The kinase domain of ABL was obtained by PCR using a BCR-ABL plasmid as the template. The 5' primer was : 5' CAGCGGATCCAAAGCGCAACAAGCCC 3' and the 3' primer was: 5' TACTAGGATCC*TTATCA*GGATTCCTGGAACATTGT 3'. The BamH I restriction site is underlined and stop codons are in italics. The PCR

product was digested with BamH I, ligated into pBluBac3C and sequenced to confirm that no errors were generated from the PCR amplification.

The full-length BCR-ABL and the ABL kinase domain were transfected into Sf9 cells as described (InVitrogen). Propagation, identification and isolation of plaques, generation of high-titer stocks, and infection of Sf9 cells for expression were performed as described [6]. Both the full-length BCR-ABL and the kinase domain were shown to be of correct predicted size and functional by an immune complex kinase assay. For large scale production of BCR-ABL and the ABL kinase domain, Sf9 cells were grown in Grace's insect media (Gibco), supplemented with 10% heat-inactivated fetal bovine serum, 0.33% yeastolate, 0.33% lactalbumin hydrolysate and 50 mg/ml gentamicin, at 27°C in spinner flasks to a density of 1-2 x 10^6 cells/ml. The cells were infected with plaque-purified virus at a multiplicity of infection of 2 and harvested 72 h post infection in the case of BCR-ABL and 48h post infection in the case of kinase domain infected cells.

Antisera. Rabbit polyclonal antisera against CRKL were generated against a peptide corresponding to amino acids 204 to 225 of CRKL. This antisera was used exclusively for immunoblotting experiments. For other experiments, anti CRKL antibody was purchased from Santa Cruz Biotechnology. Antibodies recognizing p120 CBL, ABL proteins (24-11 and K-12), and Anti-GST were purchased from Santa Cruz Biotechnology. The anti-hemagglutinin antibody 12CA5 was obtained from Boehringer Mannheim. The antiphosphotyrosine monoclonal antibody, 4G10, was generated using KLH-phosphotyramine as the immunogen and was used as

described [50] [97].

Immunoprecipitation and Immunoblotting. Cells were lysed in NP40 lysis buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP40, 10% glycerol) containing 10 mg/ml aprotinin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. The 32D lysates were normalized in preliminary experiments by immunoblotting for c-ABL and these amounts were used for subsequent experiments. Normalized lysates were either immunoprecipitated with 25 µl of CRKL or ABL antisera followed by SDS-PAGE or were analyzed as whole-cell lysates by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF, Immobilon-P, Millipore) membranes in a buffer containing 25 mM Tris, 192.5 mM glycine, 20% MeOH for 4 h at 0.45 Amps. Residual binding sites were blocked by incubation in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) containing 3% BSA for 60 min at 25°C. The blots were incubated for 4 - 16 h at room temperature with primary antibody. Antibody reactions were detected using enhanced chemiluminescence (Pierce). Sf9 lysates expressing ABL kinase or full-length BCR-ABL were bound to 12CA5 antibody at 4°C for 6-8 h with Protein A sepharose (Pharmacia), washed three times with ice-cold PBS and then incubated with 32D, 32Dp210, or K562 lysates for 4 h. The beads were then washed three more times with PBS before being boiled in SDS-sample buffer and analyzed by SDS-PAGE.

Glutathione-S-Transferase (GST) Binding Assays. The GST-CRKL constructs were previously described [82]. GST-ABL SH2, GST-ABL SH3, and GST-ABL SH2, SH3 were gifts from W. Haser (Dana-Farber Cancer Institute, Boston, MA). GST-fusion constructs were expressed

in isopropylthio-ß-D-galactosidase (IPTG) induced *E.coli* (DH-5α). The GST-fusion proteins were isolated from sonicated bacterial lysates using glutathione sepharose beads (Pharmacia). Coomassie-stained gels of the GST-fusion proteins were used to normalize for the expression of the various GST-fusion proteins. Between 2.5-5 µg of GST-fusion proteins were incubated with 50 µl of glutathione-sepharose beads in bacterial lysis buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, [pH 7.3] containing 10 µg/ml aprotinin, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 0.1% ß-mercaptoethanol). The beads were washed 4 times in ice cold PBS and incubated for 4 h with normalized cell lysates. The beads were washed three times with PBS and boiled in SDS-sample buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis.

Gel overlay assay. Purified GST, GST-ABL SH2, and GST-ABL SH3 fusion proteins were used for this assay. PVDF membranes were blocked overnight at 4°C in PBS with 0.05% Tween 20 and 5% non-fat dry milk. The blots were washed twice in PBS with 0.05% Tween 20 (PBS-T) and incubated for 2 h at room temperature with either GST only or GST-Abl SH2 or SH3 fusion proteins (at 2 mg/ml) in binding buffer [25 mM Na-phosphate (pH 7.20), 150 mM NaCl, 0.1% Tween 20, 2.5 mM EDTA, 20 mM NaF, 1% non-fat milk, 1 mM dithiothreitol, 10 µg/ml leupeptin, and 10 mg/ml aprotinin]. Bound GST protein was detected by incubation with anti-GST antibody diluted 1:500 in binding buffer excluding milk, DTT and protease inhibitors. Antibody reactions were developed using enhanced chemiluminescence. The blots were washed for 1h in PBS-T between each of the steps.

RESULTS

Tyrosine phosphorylation of CBL and association of CBL with BCR-ABL in BCR-ABL-expressing cells. Previous studies have demonstrated that p120 c-CBL is tyrosine-phosphorylated in cells expressing activated ABL oncoproteins, including BCR-ABL. Further, BCR-ABL and c-CBL form a complex as demonstrated by co-immunoprecipitation [44] [3] [181] [193]. These results were confirmed in 32D cells expressing p210BCR-ABL and in K562 cells, a BCR-ABL-positive cell line derived from a CML patient [122]. Figure 1 shows that c-CBL is tyrosine-phosphorylated in BCR-ABL expressing cells but not in control, IL-3-dependent 32D cells. A number of tyrosine phosphorylated proteins co-immunoprecipitate with c-CBL in BCR-ABL expressing cells, including proteins of relative molecular weight of 210, 170, 135-140, 68-70, and 56 kDa (data not shown). As shown in Figures 1B and C, BCR-ABL is present in c-CBL immunoprecipitates and c-CBL is present in ABL immunoprecipitates. The tyrosine phosphorylation of c-CBL and association with BCR-ABL is dependent on ABL kinase activity as it is not seen in cells expressing a tyrosine kinase-inactive mutant of BCR-ABL (Figure 3).

Localization of domains of BCR-ABL that interact with c-CBL. The data presented above demonstrated that c-CBL and BCR-ABL coimmunoprecipitate and this association requires tyrosine kinase activity of BCR-ABL. To assist in localization of a possible binding sequence of BCR-ABL to CBL, full-length BCR-ABL and the kinase domain of ABL were expressed in the baculovirus system, tagged with an N-terminal hemagglutinin (12CA5) epitope. 12CA5

immunoprecipitates from lysates of SF9 cells infected with these constructs were incubated with lysates from K562 cells and analyzed for c-CBL binding. As shown in Figure 2A, full-length BCR-ABL but not the kinase domain was capable of binding to c-CBL. No binding of c-CBL to the full-length BCR-ABL was seen using lysates from 32D cells, again suggesting that tyrosine phosphorylation of c-CBL is required for binding to BCR-ABL and that the binding of c-CBL to BCR-ABL requires a domain outside of the kinase domain of ABL.

A possibility raised by the data presented above is that c-CBL may be binding to the SH2 domain of ABL in a phosphotyrosine-dependent fashion. To test this, bacterially expressed GST-fusion proteins containing the ABL SH2 or SH3 domains were evaluated for their ability to bind c-CBL from cellular lysates. As seen in Figure 2B and 2C, the ABL SH2, but not the ABL SH3 domain was capable of binding CBL from BCR-ABL lysates, but not from 32D cells.

Although these experiments suggested that the SH2 domain of ABL was binding tyrosine-phosphorylated CBL, these experiments could not determine whether the interaction was direct or indirect. To assess whether the SH2 domain of ABL could bind directly to CBL, a gel overlay assay was performed. c-CBL immunoprecipitates from 32D and BCR-ABL-expressing cells were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with GST-ABL SH2 or SH3 domains and binding was detected with a GST antisera. The data presented in Figure 3 shows that equal amounts of c-CBL were immunoprecipitated from all cells examined and that the ABL SH2 domain binds directly to c-CBL. This binding of c-CBL to the ABL SH2 domain is only seen under conditions where c-

CBL is tyrosine phosphorylated (Figure 3).

In vivo interactions of c-CBL with a BCR-ABL mutant lacking the SH2 domain. We and others have previously constructed BCR-ABL mutants with a deletion of the SH2 domain. This mutant remains capable of rendering myeloid cells factor-independent for growth but is defective in fibroblast transformation assays [162] [93] [145] [33]. Myeloid cells expressing this mutant were examined for defects in c-CBL tyrosine-phosphorylation or BCR-ABL association. As shown in Figure 1, c-CBL is tyrosine-phosphorylated in cells expressing the SH2 domain mutant of BCR-ABL and also co-immunoprecipitates with BCR-ABL.

Association of CRKL and CBL. The data for the SH2 domain deletion of BCR-ABL and its interaction with c-CBL is reminiscent of data that we had obtained for CRKL and ABL interactions [82]. We previously mapped a direct binding site for the amino terminal SH3 domain of CRKL to a proline-rich region in the C-terminus of ABL. Deletion of this region in BCR-ABL did not result in an obvious transformation defect. In cells expressing this mutant (p210ΔP1,P2) CRKL remained tyrosine-phosphorylated and was present in a complex with BCR-ABL as demonstrated by co-immunoprecipitation. A variety of evidence suggested that another protein was mediating an indirect interaction of CRKL and BCR-ABL in this mutant cell line [82].

A possible explanation of this data is that CBL and CRKL form a complex in BCR-ABL-expressing cells. To determine whether CBL and CRKL interact, CRKL immunoprecipitates followed by CBL immunoblots or vice versa were performed. As shown in Figure 4,

these two proteins form a complex in cells expressing kinase active BCR-ABL, but not in control 32D cells or cells expressing a kinase-inactive BCR-ABL. Co-immunoprecipitation is also seen in cells expressing the ΔP1,P2 proline-deletion mutant of BCR-ABL and the SH2 domain deletion (Figure 4). Using various GST-CRKL constructs, we have shown that c-CBL binds to the SH2 domain of CRKL and this interaction is dependent on c-CBL tyrosine phosphorylation [82]. Using a gel overlay assay with GST-CRKL constructs, we find that the CRKL SH2 domain is capable of binding directly to c-CBL under conditions where c-CBL is tyrosine-phosphorylated (Figure 5).

In vitro analysis of ABL, CBL and CRKL complex formation using ABL mutants. The data presented thus far is consistent with a model of BCR-ABL interactions shown in Figure 6A. In this model, BCR-ABL interacts directly and indirectly with both CRKL and CBL. That is, BCR-ABL interacts directly through its SH2 domain with tyrosine-phosphorylated CBL and tyrosine-phosphorylated CBL also interacts with the SH2 domain of CRKL. Similarly a proline-rich region of BCR-ABL interacts directly with CRKL which in turn interacts with CBL. From this model, several predictions are possible. Using a GST-CRKL SH3 amino terminal domain, we would expect to see binding of BCR-ABL from lysates of cells expressing BCR-ABL. However, no binding should be seen using lysates of cells expressing the proline-rich deletion mutant of BCR-ABL (ΔP1,P2). As seen in Figure 7A, this is the observed result confirming this site as a binding site between BCR-ABL and the SH3 domain of CRKL. Consistent with a lack of binding of BCR-ABL from the ΔP1,P2 mutant to the SH3 domain of CRKL, no binding of CBL is seen when lysates

from 32Dp210ΔP1,P2-expressing cells are analyzed for binding to the GST-CRKL SH3 construct (Figure 6B and 7C). However, using the SH2 domain of CRKL, some binding of BCR-ABL was seen from cells expressing BCR-ABL or the ΔP1,P2 mutant (Figure 7B). This could be explained by our model with CBL binding to the SH2 domain of CRKL and presumably through another tyrosine residue also binding to BCR-ABL (Figure 6A). To confirm these findings, lysates of BCR-ABL cells were run over various GST-CRKL fusion proteins and analyzed for CBL binding (Figure 7C). In these experiments, the SH3 domain of CRKL bound to CBL from lysates of cells expressing full-length BCR-ABL. This would be expected as the SH3 domain of CRKL would interact with BCR-ABL which through its SH2 domain interacts with CBL. A prediction of this model would be that this interaction would be abolished if lysates from cells expressing the SH2 domain deletion of BCR-ABL were used in this experiment. As seen in Figure 7C, this prediction was confirmed as is modeled in Figure 6C.

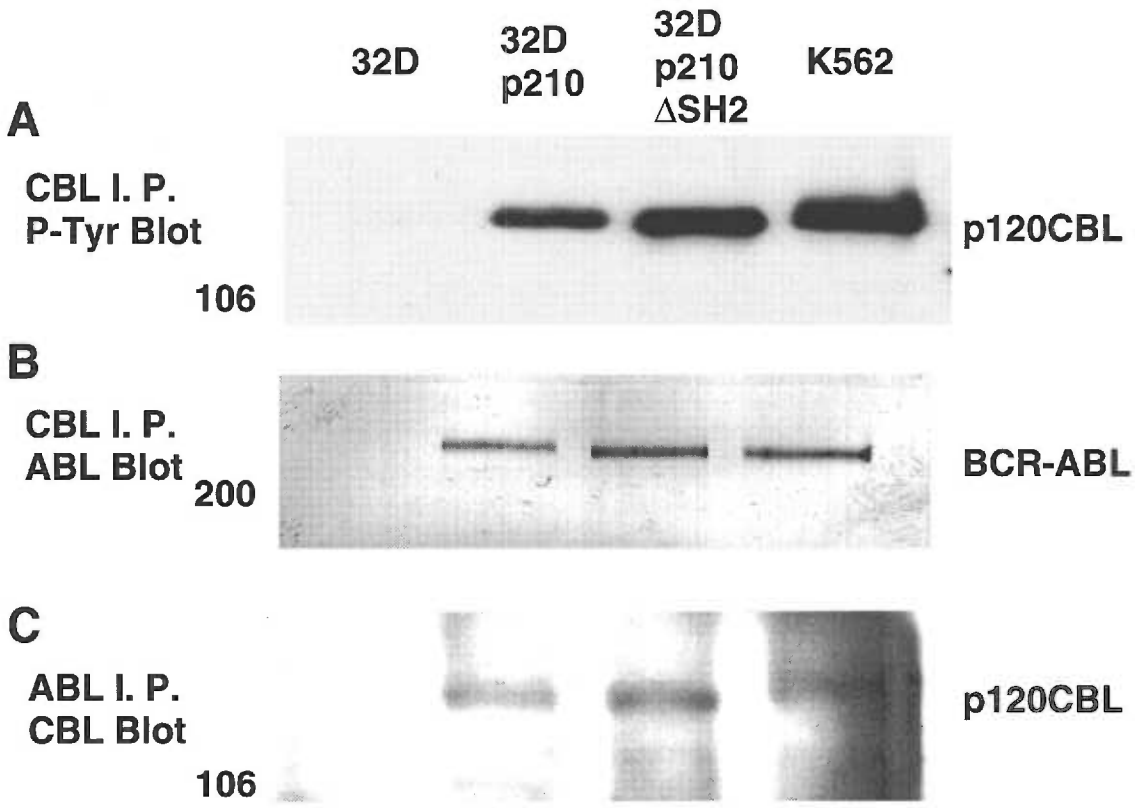


Figure 1. Cbl tyrosine phosphorylation and association with Bcr-Abl in Bcr-Abl expressing myeloid cells. Lysates from the indicated cells were immunoprecipitated with anti-Cbl (A and B) and immunoblotted with anti-phosphotyrosine (A) or anti-Abl (B). Immunoblots were stripped and reprobed with Cbl anti-sera and demonstrated that equal amounts of Cbl were present in the immunoprecipitates from each lysate (data not shown). For panel C, lysates were immunoprecipitated with a monoclonal Abl antisera and immunoblotted with Cbl antisera.

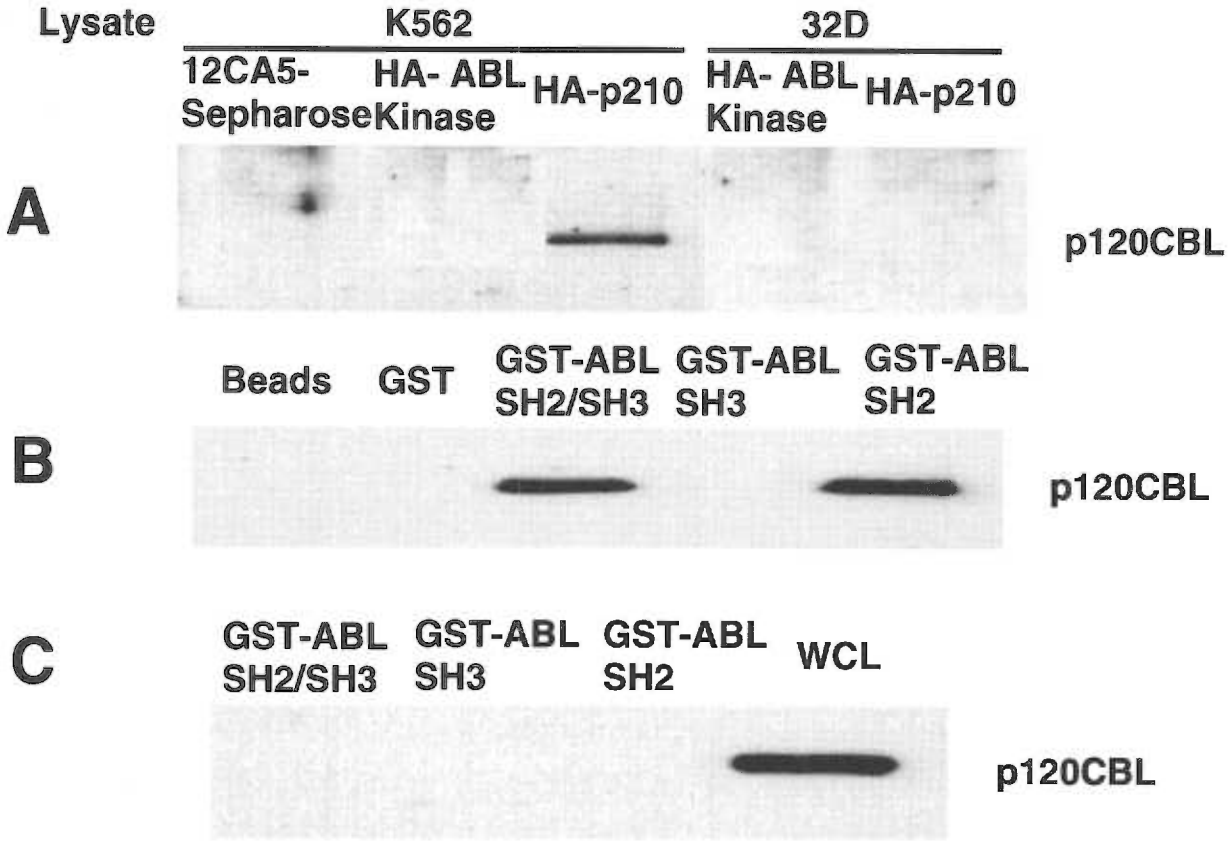


Figure 2. In vitro association of Cbl and Abl. (A) Abl kinase and Bcr-Abl were expressed as fusion proteins containing a 12CA5 antibody biding site. Lysates from K562 or 32D cells were run over columns containing 12CA5 antibody, or 12CA5 antibody bound to the kinase domain of Abl or full length Bcr-Abl. Cbl binding was detected by immunblotting with Cbl anti-sera. (B) and (C) Lysates of K562 or 32D cells were analyzed as in (A) for Cbl binding to the indicated fusion proteins. Glutathione beads and bacterially expressed GST were used as controls. WCL indicates whole cell lysate to determine the presence and migration of c-Cbl.

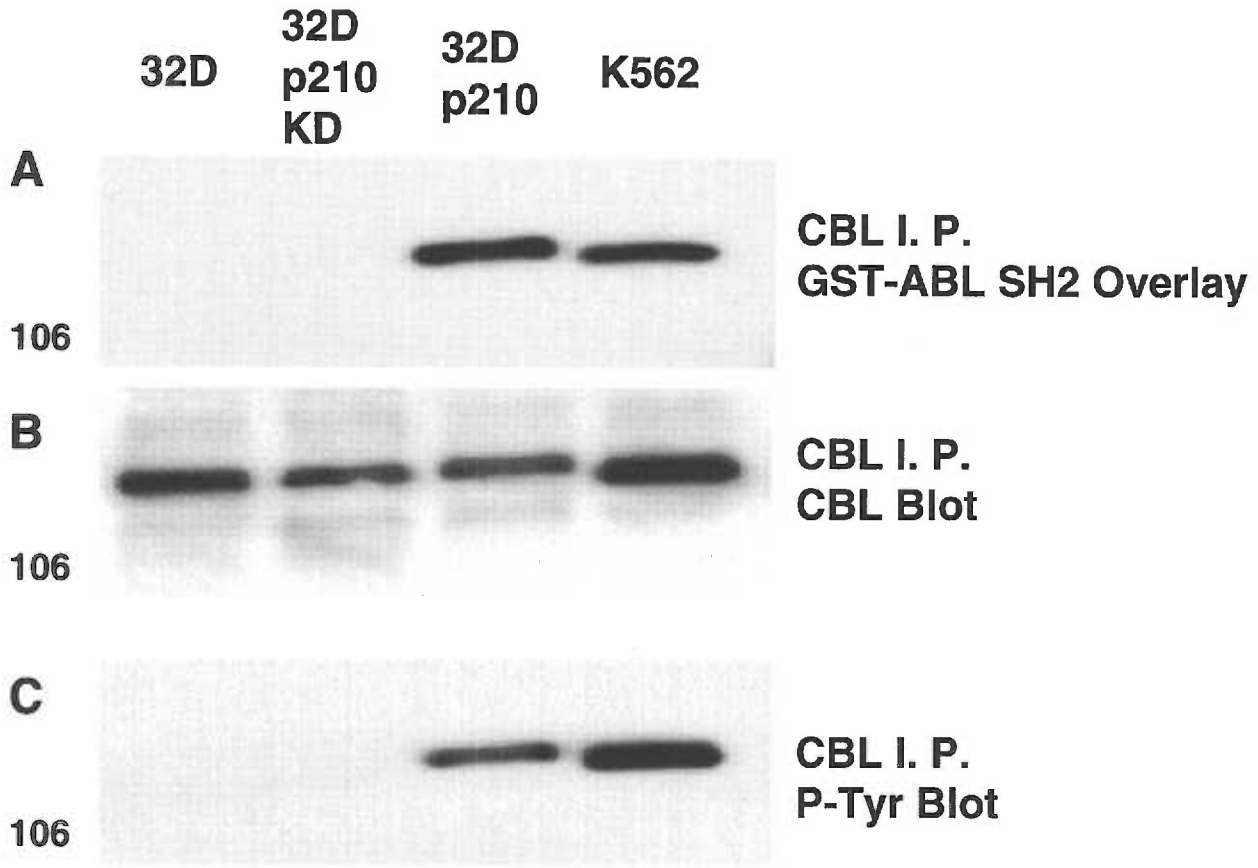


Figure 3. Direct binding of the Abl SH2 domain to tyrosine phosphorylated Cbl. Cbl immunoprecipitates from lysates of the indicated cells were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with bacterially expressed Abl SH2 domain and binding was detected with a GST antibody (A). No binding was detected using GST alone or the Abl SH3 domain (data not shown). The immunoblot was stripped and reprobed with Cbl antisera (B) or anti-phosphotyrosine (C).

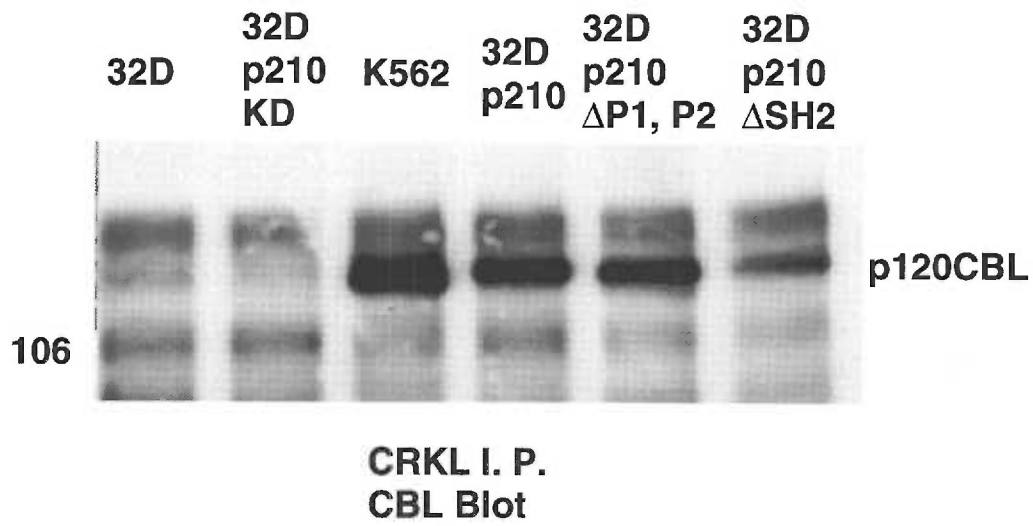


Figure 4. CrkL association with Cbl in Bcr-Abl expressing myeloid cells. CrkL immunoprecipitates from the indicated lysates were immunoblotted with Cbl antisera.

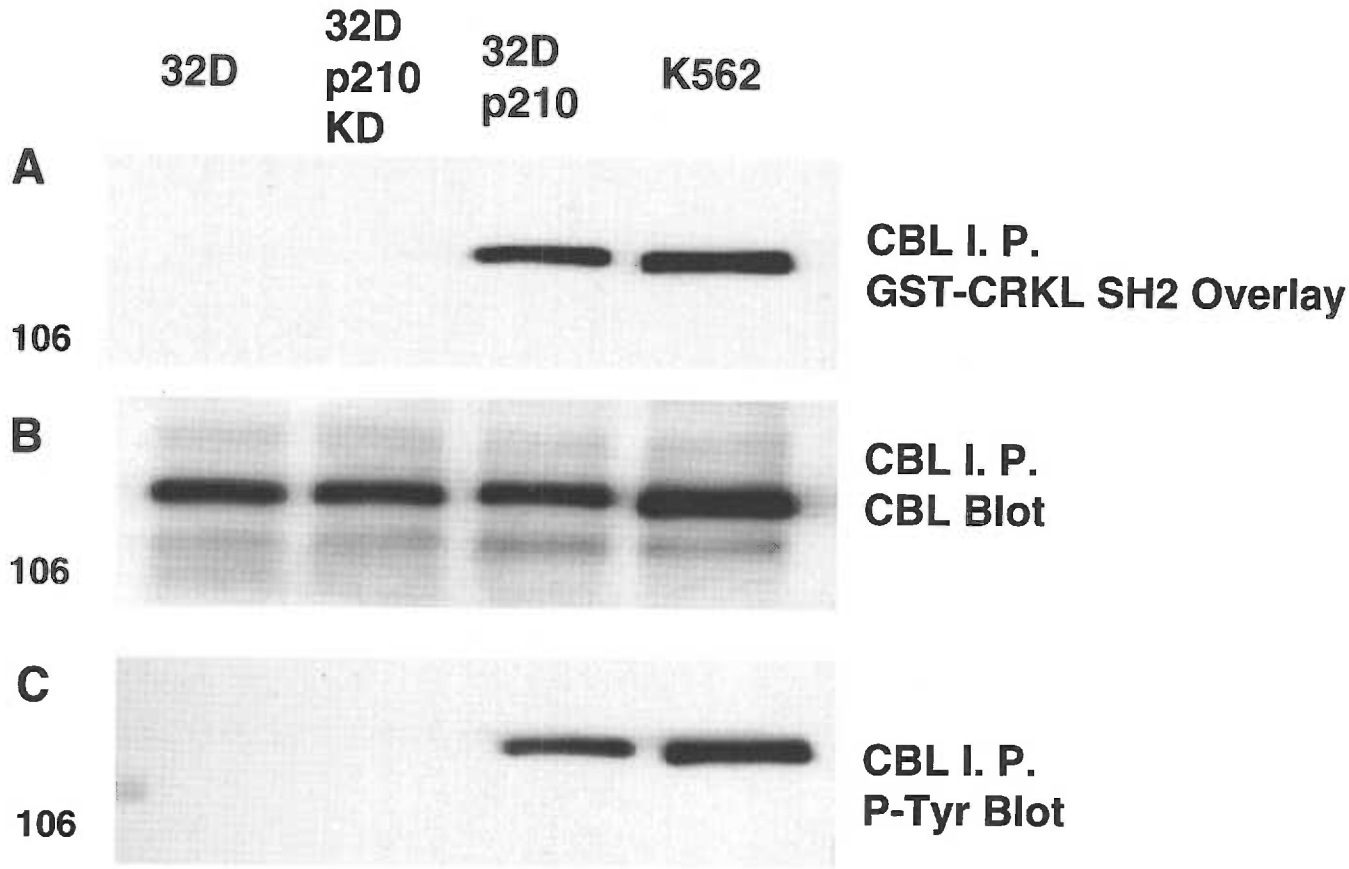


Figure 5. Direct binding of the CrkL SH2 domain to tyrosine phosphorylated Cbl. Cbl immunoprecipitates from lysates of the indicated cells were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with bacterially expressed CrkL SH2 domain and binding was detected with a GST antibody (A). No binding was detected using GST alone or the CrkL SH3 domain (data not shown). The immunoblot was stripped and reprobed with Cbl antisera (B) or anti-phosphotyrosine (C).

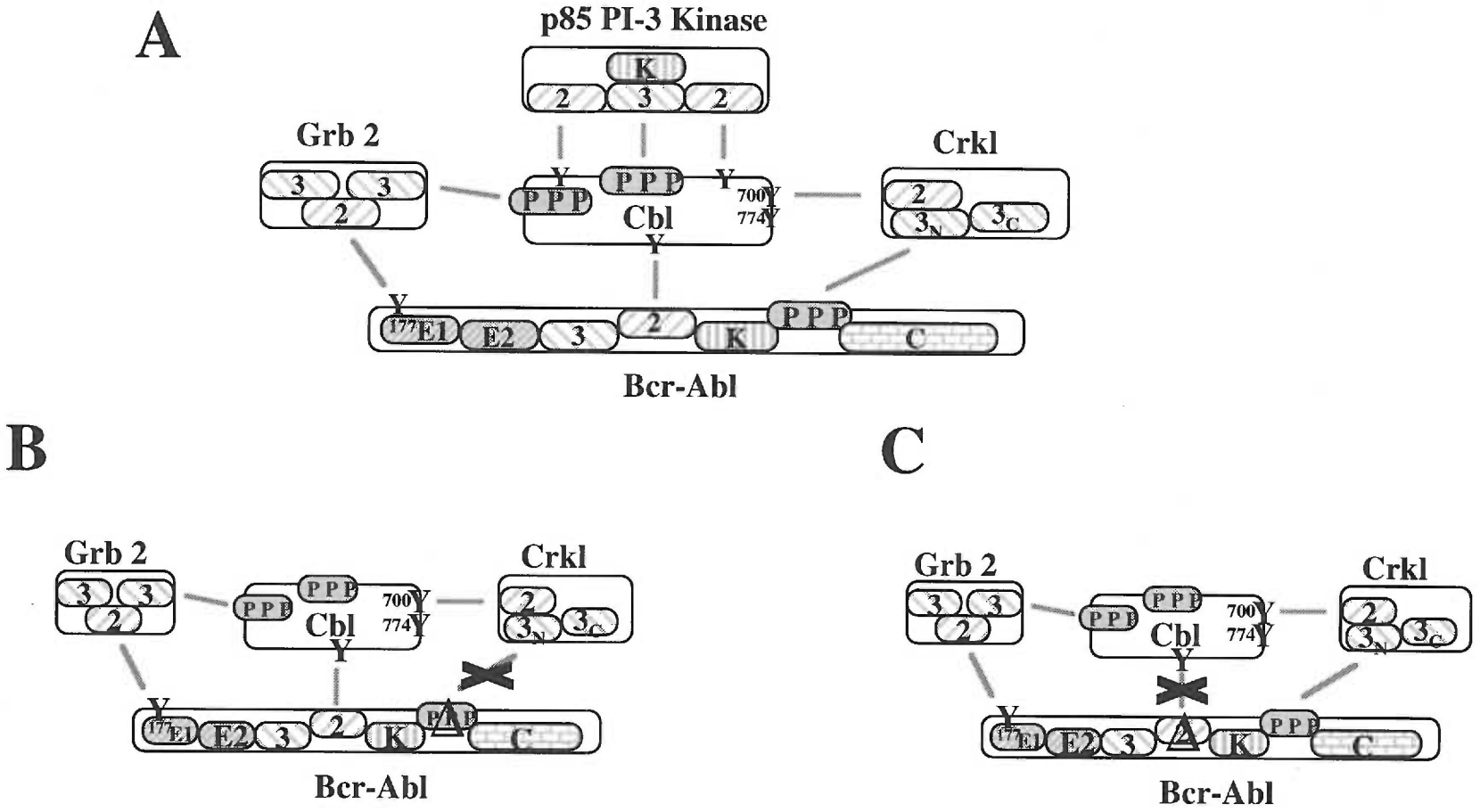


Figure 6. Model of Bcr-Abl interactions with signaling proteins. (A) Current model of Bcr-Abl interactions with signaling proteins. (B), (C) Model of interactions that occur with a deletion of a proline rich region that has been identified as a CrkL binding site of Bcr-Abl (B) or a deletion of the SH2 domain (C). Abbreviations are E1, E2: Exons 1 and 2; 2, 3: SH2 and SH3 domains; K: Kinase domain; Y: Tyrosine; PPP: Proline rich region; C: C-terminus; 3N, 3C: N and C-terminal SH3 domains.

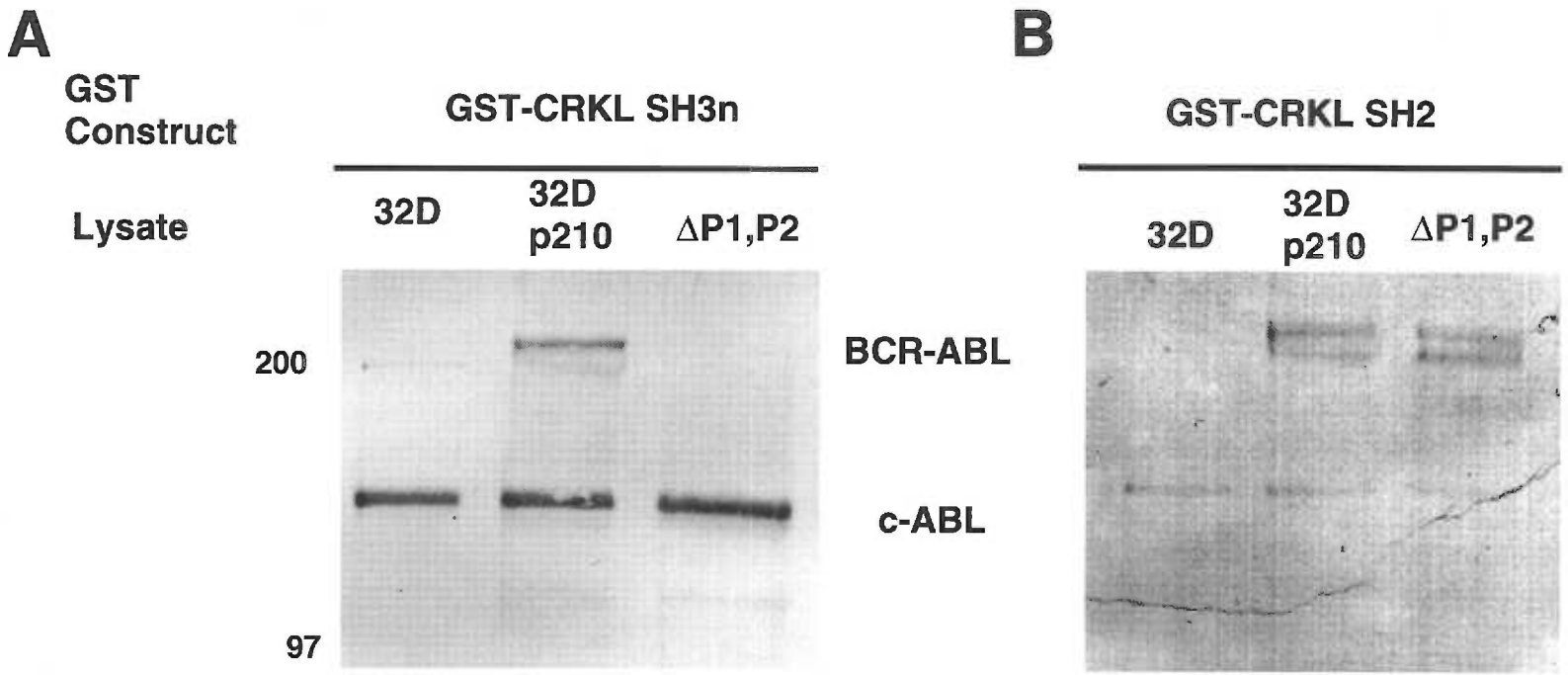
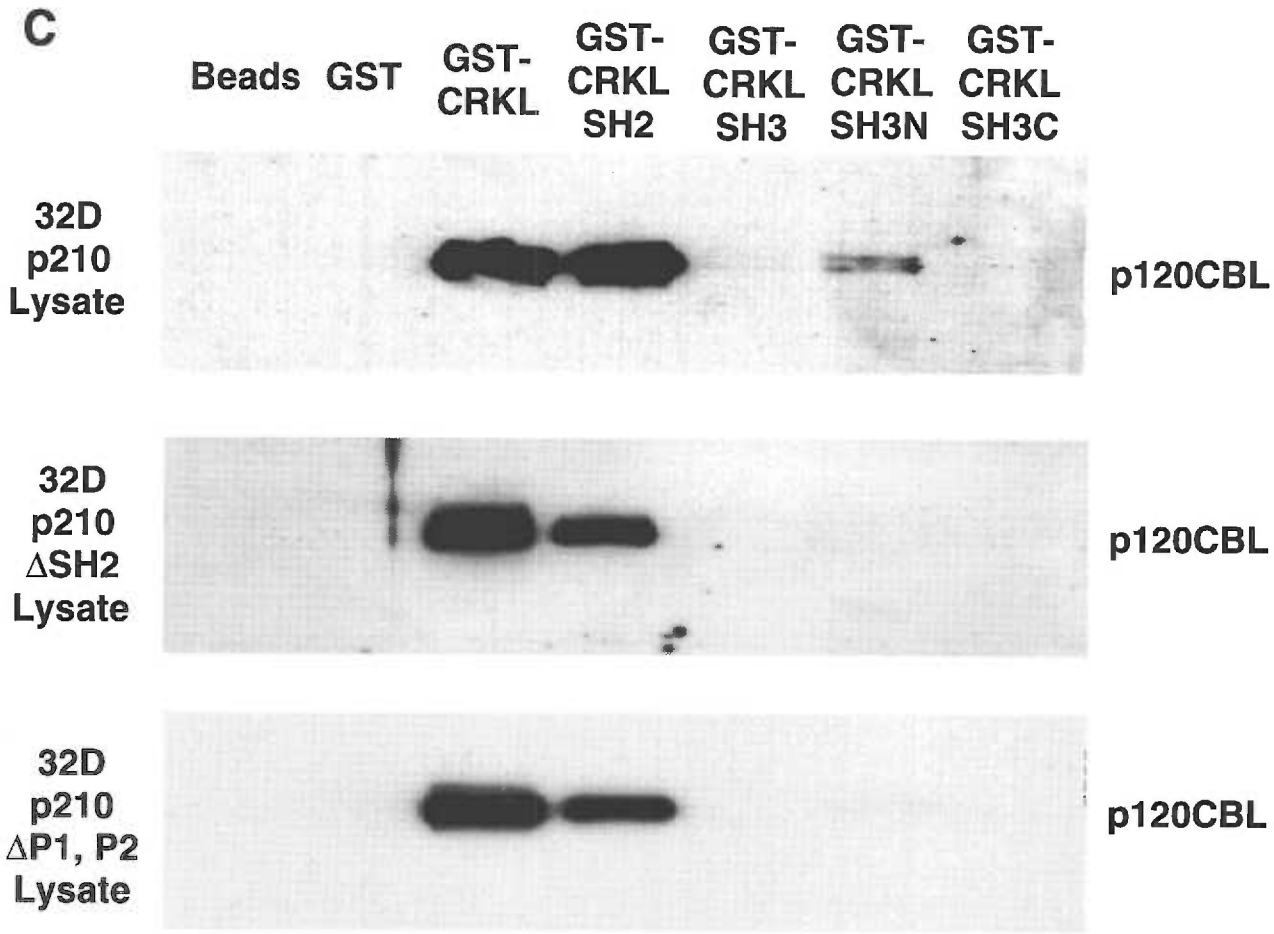


Figure 7. Binding of Bcr-Abl proteins to bacterially expressed CrkL. Lysates from 32D, 32Dp210, or 32Dp210ΔP1,P2 cells were analyzed for binding to (A) GST-CrkL SH3n or (B) GST-CRKL SH2. Bound proteins were separated by SDS-PAGE and transferred to PVDF membranes. Abl proteins were detected using the 24-21 Abl monoclonal antibody. The 32Dp210 and 32Dp210ΔP1,P2 lysates were normalized for Bcr-Abl expression prior to this analysis; (C) Next page. Lysates from 32Dp210, 32Dp210ΔSH2, or 32Dp210ΔP1,P2 cells were analyzed for binding to the indicated GST-CrkL construct. The GST-CrkL SH3 contains both the N and C-terminal SH3 domains of CrkL and consistently less binding to this construct is seen as compared to the N-terminal SH3 domain alone.


C
Beads
GST
GST-CRKL
GST-CRKL SH2
GST-CRKL SH3
GST-CRKL SH3N
GST-CRKL SH3C
32D p210 Lysate
32D p210 ΔSH2 Lysate
32D p210 ΔP1, P2 Lysate
p120CBL
p120CBL
p120CBL

DISCUSSION

In this manuscript, we have demonstrated a direct interaction between c-CBL and BCR-ABL. Our data shows that the kinase domain of ABL is not capable of binding to c-CBL, whereas a full-length BCR-ABL does, suggesting that a domain outside of the kinase is required for binding of c-CBL to BCR-ABL. The interaction of BCR-ABL and c-CBL only occurs under conditions where c-CBL is tyrosine-phosphorylated, implying that tyrosine-phosphorylated c-CBL may bind to the SH2 domain of ABL. Direct binding of the SH2 domain of ABL to c-CBL was confirmed using a gel-overlay assay and again this binding only occurred in lysates where tyrosine-phosphorylation of c-CBL has been demonstrated.

Despite the direct interaction of c-CBL with the SH2 domain of BCR-ABL, deletion of the SH2 domain of BCR-ABL did not result in an alteration in the complex formation of BCR-ABL and c-CBL. This suggests that another site of direct interaction between c-CBL and BCR-ABL exists or that another protein mediates an indirect interaction of c-CBL and BCR-ABL. Numerous proteins have been shown to interact with BCR-ABL and many have been shown to be tyrosine phosphorylated in BCR-ABL expressing cells. Besides c-CBL, these proteins include rasGAP [52], GRB2 [170] [172], SHC [208] [136], FES [54], SYP [209], CRKL [161] [212] the 85kDa subunit of phosphatidylinositol 3-kinase [222], VAV [135], c-BCR [123], paxillin [192], and various other cytoskeletal proteins [191].

In addition to binding to the SH2 domain of ABL, c-CBL was shown to bind to the SH2 domain of CRKL. c-CBL is also known to

bind to the SH2 domain of the 85kDa subunit of phosphatidylinositol 3-kinase, FYN, LCK, and phospholipase C-δ [65] [148] [164]. Binding of c-CBL to the SH3 domain of GRB2 has also been demonstrated, however, this association decreases when c-CBL is tyrosine-phosphorylated [65] [148] [18]. c-CBL has also been shown to co-immunprecipitate with paxillin [193]. Since many of these proteins also bind to BCR-ABL, they are candidates for mediating the indirect interaction of CBL with BCR-ABL. A model of the potential interactions of these proteins with each other and BCR-ABL is presented in Figure 6.

Consistent with this model, c-CBL immunoprecipitates from BCR-ABL cells not only contain BCR-ABL, but also contain CRKL [193]. As supported by our data, tyrosine-phosphorylated c-CBL binds to the SH2 domain of ABL in BCR-ABL and to the SH2 domain of CRKL. Thus, c-CBL interacts both directly and indirectly with BCR-ABL. That is, tyrosine phosphorylated CBL interacts directly with BCR-ABL by binding to the SH2 domain of ABL. CBL also interacts indirectly with BCR-ABL through CRKL. We have previously shown that a proline rich region of BCR-ABL is interacts directly with CRKL [82] and as shown here, the SH2 domain of CRKL interacts with c-CBL, thus allowing an indirect interaction of BCR-ABL and CBL.

A prediction of this model would be that BCR-ABL should be capable of binding to the GST- SH2 domain of CRKL by virtue of tyrosine-phosphorylated CBL binding to BCR-ABL and the SH2 domain of CRKL. This prediction was confirmed in our GST-fusion protein experiments. Similarly, the SH3 domain of CRKL bound CBL from lysates of cells expressing full-length BCR-ABL as the SH3

domain of CRKL binds to BCR-ABL which binds through its SH2 domain to c-CBL. This interaction was abolished in cells expressing the SH2 domain deletion of BCR-ABL as modeled in Figure 6C.

It is possible that more complicated complexes occur or that proteins other than CRKL mediate an indirect interaction between CBL and BCR-ABL. For example, the SH2 domain of GRB2 is known to bind to Tyr 177 of BCR-ABL and the SH3 domain of GRB2 has been shown to bind to CBL [65]. We have not seen any defect in tyrosine-phosphorylation of c-CBL in cells expressing the tyrosine to phenylalanine mutant of BCR-ABL that abolishes binding of BCR-ABL to GRB2 (data not shown). However, this mutant would be expected to bind c-CBL through an intact SH2 domain. As tyrosine-phosphorylated CBL also binds to the SH2 domain of CRKL, it is possible that GRB2 binding to BCR-ABL allows CRKL to associate with BCR-ABL through this interaction with CBL. Given the multitude of complexes induced by BCR-ABL, combinations of mutants (e.g., Tyr 177 to Phe with a deletion of the SH2 domain and a deletion of proline rich sequences in the C-terminus of ABL) will be required to determine the roles of various signaling proteins in BCR-ABL transformation. Results of these experiments may not allow an assessment of the requirement of an individual protein for transformation by BCR-ABL as these mutants might abolish complex formation of several proteins with BCR-ABL. Assessment of the role of individual proteins may require the use of cell lines generated from mice lacking various of these signaling proteins.

ACKNOWLEDGMENTS

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CHAPTER IV

Discussion

Chronic myelogenous leukemia (CML) is a malignancy of pluripotent hematopoietic stem cells [188] [159]. The disease is characterized by the presence of a specific chromosomal translocation which results in the production of a p210$^{bcr\text{-}abl}$ fusion protein [78] [84] [10]. The tyrosine kinase activity of this chimera has been shown to be essential for its transformation activity [124] [162] and p210$^{bcr\text{-}abl}$ has been reported to bind and/or phosphorylate a large number of proteins. Many of these proteins can be directly linked to signal transduction pathways based on defined roles in other systems, while others have no known function. It has been a major goal of the lab to identify p210$^{bcr\text{-}abl}$ substrates and determine their importance in transformations.

Role of CrkL in CML

We had shown that CrkL was a major tyrosine phosphorylated protein in neutrophils of patients with Bcr-Abl positive chronic myelogenous leukemia; it was present in cell lines expressing Bcr-Abl and was inducibly phosphorylated in a Bcr-Abl temperature-sensitive mutants [20, 161, 212]. We had also demonstrated direct association of CrkL with Bcr-Abl sequences in the yeast two-hybrid

system [161]. CrkL was also shown to be present in focal adhesions and interact with proteins present in these adhesions, including: p130^{cas}, p125fak, and p70paxillin. These observations together with the observation that CrkL could increase the tyrosine kinase activity of *src* related kinases suggested a significant role for this adaptor protein in Bcr-Abl transformation.

Crk was found to be the transforming oncogene present in two independent isolates of retroviruses CT10 and ASV-1 [140] [214]. The Crk oncoprotein belongs to a class of proteins called "adaptor proteins." These proteins mainly consist of SH2 and SH3 domains. A peculiar characteristic of transformation induced by v-Crk is an increase in the cellular content of phosphotyrosine-containing proteins, despite the fact that v-Crk is not a tyrosine kinase [140] [139]. A number of phosphotyrosine proteins coimmunoprecipitate with v-Crk [141] and this interaction is dependent on tyrosine-phosphorylation [132]. In an in vitro experiment using v-Src and v-Crk it was shown that v-Crk bound to v-Src after autophosphorylation of the latter, and dephosphorylation of v-Src abolished the binding to v-Crk [132] [131]. This binding subsequently was mapped to the Crk SH2 domain [133]. A number of proteins bind to Crk SH2 and SH3 domains and are discussed below.

Given that Crk transforms cells by activating tyrosine kinases this suggested several possible roles for CrkL in transformation by Bcr-Abl. One possibility is that CrkL binding to Bcr-Abl activates Abl. Another is that Bcr-Abl activates CrkL which in turn activates other tyrosine kinases. Lastly, it is possible that CrkL, through

binding to a variety of other proteins mediates an important effect of Bcr-Abl, such as proliferation, alteration in adhesion or inhibition of apoptosis.

Crk SH2 binding proteins

In *crk*-transformed cells, several proteins have been shown to be heavily phosphorylated and bind specifically to its SH2 domain. Major proteins are in the size ranges of p46-52kDa, 70kDa, 110kDa and 130kDa.

p46-52kDa proteins belong to the Shc family of adaptor proteins. This protein contains two domains capable of binding phosphotyrosines, a SH2 domain and a newly described non-SH2, phosphotyrosine binding (PTB) domain. Shc may link Crk and Crk like proteins CrkL to the *ras* pathway as Shc, when tyrosine-phosphorylated, binds to a Grb2-Sos complex which results in Ras activation [173] [167].

The 70kDa protein has been identified as paxillin [13]. Paxillin is a constituent of focal adhesions and binds to vinculin [26] [182]. Integrin engagement induces phosphorylation of paxillin at amino acid sequences that conform with the YXXP high-affinity binding site for the Crk SH2 domain [202].

The 130kDa protein is a novel molecule designated p130^{cas} (for crk associated substrate) [190]. p130^{cas} is phosphorylated by Src-family tyrosine kinases on tyrosine residues creating high-affinity binding sites for Crk SH2 upon integrin stimulation [158] [225]. p130^{cas} SH3 domain has been shown to bind to Fak [171],

thereby suggesting a role of CrkL in formation of focal adhesions.

The 120kDa protein is Cbl and will be further discussed below. Interactions of this protein with Bcr-Abl and CrkL were the subject of the second paper Chapter 3. The 110kDa protein has not been identified yet. Thus, the major Crk-binding proteins are involved in signaling by activation of the Ras pathway and may regulate cytoskeletal processes.

Crk SH3 binding proteins

Proteins that bind to the Crk SH3 domain are in the size range of 130, 135-145, 140, 160, 170, and p180 kDa. Some of these proteins have no known function and these include p130eps15, p160 and p180dock. The 140kDa protein is c-Abl which has been previously discussed (see introduction). In the chimeric protein, p210$^{bcr\text{-}abl}$, the CrkL $SH3_N$ domain binds to Bcr-Abl C-terminus proline-rich sequences as has been discussed previously and was the subject of the first paper, Chapter 2. p170 Sos and 135-145 kDa C3G are both guanine nucleotide exchange proteins and thus link Crk to the Ras or other GTP binding protein pathways.

One of the hallmarks of the disease CML is the expanded number of immature cells found in the bone marrow and blood of patients. These cells have been shown to have adhesion defects. They tend to adhere abnormally to glass, nylon and marrow matrix proteins [203]. They also have decreased motility and other chemotactic abnormalities [166]. They adhere less well to bone marrow stroma and are less responsive to stromal-derived

regulatory signals [203] [166]. It has been postulated that these adhesion defects in vivo could lead to increased proliferation of progenitor cells that are not under negative regulatory influences by adhesion to marrow stroma and could also lead these cells to prematurely leave the bone marrow.

Many of the CrkL-interacting proteins discussed above have been shown to interact with cytoskeletal proteins and are found in focal adhesions in cells including Paxillin, and Cbl.

Therefore, one can imagine in Bcr-Abl transformed cells that the CrkL interaction with Bcr-Abl kinase may lead to phosphorylation of many of these CrkL interacting proteins found in focal adhesion complexes. This may in part be responsible for the adhesion defect seen in CML myeloid progenitor cells. Other CrkL-interacting proteins such as Shc, C3G and Sos may link CrkL to the ras pathway suggesting a role for CrkL in mediating proliferative responses to Bcr-Abl.

Mapping of CrkL SH3(N) binding to Bcr-Abl

To determine the role of p39$^{c\text{-}crkl}$ in transformation by p210$^{bcr\text{-}abl}$, we used the following strategy, a) map the sites of interaction between p210$^{bcr\text{-}abl}$ and p39$^{c\text{-}crkl}$, b) mutate these sites and c) determine whether transformation was affected using tissue culture transformation assays.

Using the yeast two-hybrid assay and a gel-overlay assay we mapped the direct binding site of Bcr-Abl to the CrkL $SH3_N$ terminal domain. As full-length Bcr-Abl was used for these assays we

conclusively showed that the C-terminus prolines in Abl were the sole binding site for CrkL in Bcr-Abl. This was similar to data obtained by Baltimore's group [177] using CrkL related proteins Crk I and Crk II. They showed that these two proteins bound to c-Abl via proline-rich sequences found in the C-terminus of c-Abl.

We made a proline deletion mutant of Bcr-Abl and showed that this mutant was phenotypically similar to the full length p210$^{bcr-abl}$ i.e, it induced growth factor-independence, however, it did have a 50% reduction in capacity to transform fibroblasts [198]. We went on to show using GST CrkL domains and immunoprecipitations that this Bcr-Abl mutant was still able to associate with CrkL, suggesting CrkL was indirectly associating with Bcr-Abl. One of the proteins potentially mediating this indirect interaction is Cbl, as discussed below.

Curiously, CrkL Gst-SH3$_N$+SH3$_C$ was always less efficient at binding p210$^{bcr-abl}$ from transformed cells than Gst-SH3$_N$ alone. This suggested the SH3$_C$ domain may be inhibitory. Chicken c-Crk with two SH3 regions (similar to CrkL and Crk II) has been shown to be less transforming than a truncated version containing one SH3 when overexpressed in fibroblasts [176]. They also showed that Crk I with only one SH3 domain was more transforming than Crk II in hematopoietic cell lines, fibroblasts, and nude mice [134]. Therefore, it has been proposed by Matsuda [134] that the carboxyl-terminal SH3 regions of both chicken c-Crk and Crk-II proteins function as a negative modulator for transformation [134] . Our in vitro data is consistent with this data. The mechanism for this has not been fully elucidated but it could involve steric hindrance by the SH3$_C$ domain.

Role of Cbl in CML

Cbl and Abl are associated with induction of tumors with similar histology and spectrum of expression of cell surface molecules [1] [115] [71] [115]. Cbl is tyrosine phosphorylated in cells transformed by v-Abl or Bcr-Abl and Cbl co-immunopreciptates with v-Abl or Bcr-Abl in lysates from these cells [3]. Cbl not only interacts with CrkL, but also interacts with cytoskeletal proteins and Src family members. These observations suggested an important role for Cbl in CML.

The v-cbl oncogene was first identified in the acutely transforming Cas NS-1 retrovirus [115]. This retrovirus was isolated from an NFS/N mouse which developed a pre-B lymphoma following infection with the replication-competent Cas-Br-M murine leukemia virus. The Cas-Br-M murine leukemia virus induces mice to develop a wide spectrum of hematologic malignancies including T- and B-cell lymphomas, and myelogenous leukemias [62].

Cas NS-1 is a replication-defective virus which induces erythroid colony formation from hematopoietic precursors [106] and fibroblast transformation in vitro, and pre-B cell lymphomas and myeloid leukemias after inoculation in newborn mice [115]. Cloning and sequencing of Cas NS-1 revealed that it had been formed by recombination of Cas-Br-M with a mouse cellular oncogene, producing a 100 kD *gag-v-cbl* fusion protein, with 40 kD of this encoded by v-cbl.

Cas NS-1 is unique among fibroblast-transforming retroviruses

in that it induces B-lineage lymphomas in vivo and possesses no kinase activity. The only other transforming retroviruses found in nature that induce malignancies of B-cell lymphoid lineage are the Abelson murine leukemia virus and the Avian reticuloendothelosis virus. *v-abl* and *v-rel*, the oncogenes associated with these viruses, however, share no sequence homology with *v-cbl* [1][23] . Interestingly, the tumors induced by *v-abl* and *v-cbl* are phenotypically and histologically similar, the majority being pre-B cell lymphomas.

Lysates from cells separated into cytoplasmic and nuclear fractions and analyzed by immunoblotting show p120$^{c\text{-}cbl}$ to be localized in the cytoplasm in Jurkat [14] , transformed NIH 3T3 [14] HeLa [116], and Hep-2 cells [116], and this distribution was not altered by overexpression of *c-cbl* in NIH 3T3 cells [14, 116].

In contrast to p120 $^{c\text{-}cbl}$, p100 $^{gag\text{-}v\text{-}cbl}$ is distributed both in the nucleus and in the cytoplasm. In the cytoplasm it has been found to be associated with the cytoskeleton [116] [14]. Recently it was shown that integrin crosslinking in human B cells and in the MO7e promegakaryocytic cell line resulted in p120$^{c\text{-}cbl}$ phosphorylation. Disruption of the actin network by cytochalasin B resulted in lack of cbl phosphorylation [128] suggesting cbl may play a key role in integrin signaling in lymphoid and hematopoietic cells, similar to the role for CrkL discussed above.

v-src-tranformed cells, but not c-src-overexpressing cells, were found to have high levels of c-Cbl tyrosine phosphorylation [206]. Cbl is known to be a target of tyrosine phosphorylation upon stimulation through immune cell surface receptors, cytokine

receptors, and the EGF receptor. Via the EGF receptor this phosphorylation is transient and occurs in a dose dependent manner [64]. c-Cbl is known to bind to a number of proteins discussed below.

c-Cbl binding proteins

c-Cbl is known to bind to the SH2 domain of the 85 kDa subunit of phosphatidylinositol 3-kinase (PI3K), Fyn, Lck, and phospholipase C-δ [65] [148] [164]. Binding of c-Cbl to the SH3 domain of Grb2 has also been demonstrated [65] [148] [18] and c-CBL has been shown to co-immunprecipitate with paxillin [193].

Several of these proteins interacting with Cbl are members of the *src* family of kinases. As Cbl also binds CrkL and Crk proteins are known to activate tyrosine kinases, it would be possible that through the interaction of Abl and CrkL, that CrkL could activate the tyrosine kinases bound to Cbl. However, Cbl also binds to cytoskeletal proteins such as paxillin, suggesting that Cbl, like CrkL, could have a role in adhesion abnormalities characteristic of CML. As with CrkL binding to Bcr-Abl, we a) mapped the sites of interaction between p210$^{bcr\text{-}abl}$ and p120$^{c\text{-}cbl}$, b) mutated these sites and c) determined whether transformation was affected using tissue culture transformation assays.

Mapping of Cbl binding to Bcr-Abl

We mapped the binding site of Cbl to the SH2 domain of Abl and analyzed a mutant of Bcr-Abl lacking this direct c-Cbl binding

site. This Cbl binding site mutant was shown to be similar to full length p210$^{bcr\text{-}abl}$ in transforming hematopoietic myeloid cells i.e., these cells became factor-independent, however, it was defective in transformation of Rat fibroblasts [73] [162] [93] [145] [33].

Using a gel-overlay assay, we showed that the SH2 domain of Bcr-Abl was the sole binding site for c-Cbl in Bcr-Abl. We further showed that Cbl phosphorylation is required for binding to the SH2 domain of Abl and that CrkL also interacts directly with Cbl. Andoniou [4] showed that tyrosines 700 and 774 in the C-terminal of Cbl interacted with the SH2 domain of CrkL. Mutation of these two tyrosines to phenylalanine resulted in decreased association between CrkL and Cbl but did not abolish Cbl, and CrkL interaction.

The Model

Our data from Chapters 2&3 suggested a model that is shown in Fig. 6, chapter 3. In this model, Bcr-Abl interacts directly and indirectly with both CrkL and Cbl. That is, Bcr-Abl interacts directly through its SH2 domain with tyrosine phosphorylated Cbl and tyrosine phosphorylated Cbl also interacts with the SH2 domain of CrkL. Similarly a proline rich region of Bcr-Abl interacts directly with CrkL which in turn interacts with Cbl. This model was tested in vitro using GST constructs and the data from this is shown in Chap. 3, Fig. 7. Although the data generated fits this model, it is likely an oversimplification. Preliminary data with a Bcr-Abl mutant containing both a deletion of the SH2 domain and the CrkL binding site shows that both Cbl and CrkL are tyrosine phosphorylated and

associated with Bcr-Abl. A possible explanation for this data is that Grb2 may be an important mediator of indirect interactions between Cbl, CrkL and Bcr-Abl. Grb2 interacts with tyrosine 177 of Bcr and is known to interact with Cbl which in turn can interact with CrkL. Regardless, this data points out the complexity of Bcr-Abl signaling and complex formation.

This model does make it clear that we cannot fully test the hypothesis that CrkL or Cbl interactions are essential for Bcr-Abl mediated transformations unless we abolish completely the interaction of these molecules with Bcr-Abl. This may be a very difficult problem as many of the proteins interacting with Bcr-Abl, Cbl, CrkL and Grb2 also interact with each other and other proteins present in focal adhesions.

Future studies

To further define the role of Cbl, CrkL and Bcr-Abl in CML multiple deletion mutants will have to be made. These include, for example, deleting both the SH2 domain and the proline rich domain. The model (Fig. 6, Chap. 3) shows that Grb2 is capable of binding to Cbl and Bcr-Abl. Grb2 SH2 domain interacts with Tyr 177 on Bcr-Abl whereas the Grb2 SH3 domain interacts with Cbl. Mutants in Tyr177 in combination with mutants in the SH2 and proline-rich regions might also be required to analyze complex formation of Bcr-Abl with Cbl and CrkL. More conclusive results might be obtained from null phenotype mice or by generating dominant-negative mutants. However, CrkL function in null mice might be complemented by

other Crk family members.

Possible mechanism of model working in vivo

In vivo we favor a model in which a trimolecular complex involving at least Bcr-Abl, CrkL and Cbl occurs. In this model, CrkL would be thought of as being constitutively bound to Bcr-Abl via its SH3 domain. Low level transient tyrosine phosphorylation of Cbl through normal signaling events (e.g., GM-CSF or IL-3 stimulation) via a Src kinase family member would cause the association of CrkL and Cbl and a significant increase in tyrosine phosphorylation of Cbl by Bcr-Abl. Because the kinase activity of Bcr-Abl is dysregulated, Cbl would be constitutively tyrosine phosphorylated leading to a long-lasting activation of the signaling pathways normally controlled by cytokines.

Relationship to CML

As discussed CML is a disease of the pluripotent stem cells which results from acquisition of a highly consistent specific chromosomal translocation. Clinically, in the chronic phase CML cells may escape negative regulatory influences of the bone marrow stroma and may leave the marrow early and proliferate in the liver and spleen, accounting for the massive increase in circulating immature myeloid cells. The cells retain their capacity for differentiation and maturation but have increased motility and other chemotactic abnormalities [166]. They adhere less well to bone

marrow stroma and are less responsive to stromal-derived regulatory signals [203] [166]. More recently, it has been suggested that p210$^{bcr\text{-}abl}$ may function in part by inhibition of apoptosis or by inducing hyper-responsiveness to growth factor, resulting in the expansion of myeloid lineage cells [55].

The clinical manifestations presumably are a result of activation of multiple signaling pathways. These pathways could involve aberrant signaling from the cytoskeleton, apoptotic pathways and growth and differentiation pathways. Our model suggests many different signaling pathways may be targeted and activated by Bcr-Abl, including the Ras pathway and the PI3K pathways.

Grb2 an adaptor protein links tyrosine kinases to Ras. The SH2 domain of Grb2 binds to Bcr-Abl via tyrosine 177 of Bcr. Grb2 also binds to mSos (a RasGAP) leading to the activation of the Ras-MAPK pathways.

The altered adhesion seen in CML cells could result from dysregulation of integrin-mediated signaling pathways. Molecules important in this pathway e.g., Cbl and Paxillin, which can associate with the Bcr-Abl SH2 domain and CrkL which can associate via its SH3 domain have been found to be phosphorylated and present in focal adhesions.

Apoptotic pathways may also be involved in CML perhaps via CrkL and Cbl. Cbl is known to be involved in many cytokine mediated signaling pathways as discussed before.

One very important caveat from Bcr-Abl structure-function studies is that standard cell transformation assays, such as growth factor independence, may only measure a subset of the many cellular

events required for leukemogenesis. In vivo studies may be required to fully define the consequences of these mutations in hematopoietic cells. In fact, one such report has shown that point muations in the SH2 domain impairs leukemogenesis in mice [73].

Attempts to mirror the stable phase of CML in a tissue culture model has been problematic. p210$^{bcr\text{-}abl}$ transforms fibroblasts relatively poorly, as measured by focus formation or growth in soft agar. More recently however, a fibroblast cell line has been described, a variant of the NIH 3T3 cells, that is permissive (subtype P-3T3) or non-permissive (subtype N-3T3) for p210$^{bcr\text{-}abl}$ transformation as assessed by growth in soft agar [179]. Interestingly, the phenotype of the permissive cells that express p210$^{bcr\text{-}abl}$ is anchorage-independent but factor-dependent growth. Thus, these cell lines may allow for a dissection of the pathways utilized by Bcr-Abl to induce a defect in adherance of CML cells [179].

Other groups have attempted to use factor-dependent myeloid cell lines expressing p210$^{bcr\text{-}abl}$ as an in vitro model system for CML [37, 113, 127, 137]. This system has the advantage that the cells being used are similar to the cells in which p210$^{bcr\text{-}abl}$ typically is expressed. However, p210$^{bcr\text{-}abl}$ renders these cells lines factor independent for growth whereas myeloid cells from chronic phase CML patients are usually factor dependent [150]. Although factor-independent growth may not be an accurate indicator of Bcr-Abl function, these myeloid models have the advantage of examining Bcr-Abl in a context more closely resembling the cells in which Bcr-Abl is typically expressed.

Thus these models are used commonly because of ease of availability, they can easily allow evaluation of Bcr-Abl mutant for structure function analysis. They are also useful for identifying pathways critical for transformation or growth factor independence. Further, knowledge from in vitro models can be tested using patient material to determine relevance to the disease.

CHAPTER V

Conclusions

Chronic myelogenous leukemia (CML) is a malignancy of a pluripotent hematopoietic stem cell. A hallmark of the disease is the identification of a chimeric activated kinase, p210$^{bcr\text{-}abl}$.

The major goal of the lab has been to identify substrates of the kinase to determine the necessity of each of these proteins for transformation by Bcr-Abl. This is in the hopes of not only understanding the possible pathways that may be upregulated by these substrates but also understanding other oncogenic processes. This should eventually enable us to intervene at key regulatory points in the pathways so that transformation can be controlled in cancer patients.

Although much has been learned about CML in the last 20-30 years, many fundamental questions regarding how the p210$^{bcr\text{-}abl}$ fusion protein cause CML remain. Substrates of Bcr-Abl that are likely to be important to the leukemogenic process should be expressed in hematopoietic cell types and should be abnormally tyrosine phosphorylated. The adaptor molecule CrkL and Cbl meets these criteria [211].

Our observations further the understanding of the role of each of these proteins in CML signaling and allowed us to propose a working model as described in the discussion section incorporating our results and results of others.

CrkL and Cbl were found to be phosphorylated and present in both CML patient cells and in myeloid cells transformed by p210$^{bcr-abl}$. These proteins were shown to interact with each other and with Bcr-Abl. Mutants in the individual direct binding site for CrkL and Cbl in Bcr-Abl were made. Both these mutants were found to growth factor independence when transfected into myeloid cells but showed reduced fibroblast transformation. However, it was shown that CrkL and Cbl still interacted with each other and with Bcr-Abl in these mutants. Further, both were tyrosine-phosphorylated even though the direct binding sites were deleted. This suggested that complex indirect and direct interactions may occur in Bcr-Abl-transformed cells and further investigation of the model will have to await double, and triple binding site mutants.

Although some models of Bcr-Abl associated leukemogenesis invoke several distinct signaling pathways, it remains possible that only a single pathway leads to leukemogenesis in vivo. The similarities between the types of malignancies associated with Bcr-Abl, v-Abl and v-Cbl in animal models suggests that the pathways of these oncogenes converge or may be the same.

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