

Crkl Is Complexed with Tyrosine-phosphorylated Cbl in Ph-positive Leukemia*

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The deregulated tyrosine kinase activity of the Bcr/Abl protein has been causally linked to the development of Philadelphia (Ph) chromosome-positive leukemia in mice and man. Abnormally tyrosine-phosphorylated substrates of the Bcr/Abl kinase in Ph-positive cells are likely to contribute to leukemogenesis by interfering with normal signal transduction pathways. We have previously shown that the adaptor molecule Crkl is a major *in vivo* substrate for the Bcr/Abl tyrosine kinase, and it is thought to connect Bcr/Abl with downstream effectors. In the current study, a tyrosine-phosphorylated protein with a molecular mass of approximately 120 kDa was identified which binds only to the Crkl Src homology 2 (SH2) domain in cells, including Ph-positive patient material, containing an active Bcr/Abl protein. We demonstrate here that this protein is Cbl, originally discovered as an oncogene which induces B-cell and myeloid leukemias in mice. The Crkl SH2 domain binds specifically to Cbl. The Src homology 3 (SH3) domains of Crkl do not bind to Cbl, but do bind Bcr/Abl. These findings suggest the existence of a trimolecular complex involving Bcr/Abl, Crkl, and Cbl and are consistent with a model in which Crkl mediates the oncogenic signal of Bcr/Abl to Cbl.

It is generally accepted that the Bcr/Abl protein is responsible for the development of chronic myeloid leukemia (CML)¹ and a subset of acute lymphoblastic leukemia (ALL). Leukemic cells of such patients have a common molecular defect. This is the Philadelphia (Ph) chromosome, a hybrid chromosome consisting of fused parts of chromosomes 9 and 22, which produces a chimeric Bcr/Abl protein at the breakpoint on chromosome 22. The Abl segment of this protein has a deregulated tyrosine kinase activity (reviewed in Refs. 1 and 2).

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¹ The abbreviations used are: CML, chronic myeloid leukemia; SH2, Src homology region 2; SH3, Src homology region 3; GST, glutathione S-transferase; ALL, acute lymphoblastic leukemia; GAP, GTPase activating protein; GM-CSF, granulocyte-macrophage colony stimulating factor.

Proteins which can be directly or indirectly tyrosine-phosphorylated by Bcr/Abl include p93^{Fes}, p160^{Bcr}, p120^{Cbl}, p68^{paxillin}, p95^{Vav}, p52^{Shc}, p46^{Shc}, p67^{Syp}, p120^{RasGAP}, p190, and p62 (3–10). Substrates of Bcr/Abl which 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 meets these criteria (11).

Crkl is clearly expressed in hematopoietic cells but is not constitutively tyrosine-phosphorylated in bone marrow² or peripheral blood (11–13). In addition, stimulation of neutrophils with GM-CSF, tumor necrosis factor, lipopolysaccharide, 12-*O*-tetradecanoylphorbol-13-acetate, interleukin 1, fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, or insulin fails to evoke Crkl tyrosine phosphorylation (12, 13). However, in the CML cell line K562, Crkl is one of the most prominent tyrosine-phosphorylated proteins (14), and we have shown that it complexes with and is tyrosine-phosphorylated by Bcr/Abl (14). Significantly, Crkl is only tyrosine-phosphorylated in peripheral blood cells of Ph-positive patients with an active Bcr/Abl protein (11–13), which suggests a distinctive role for this adaptor in leukemogenesis.

Crkl consists of an SH2 and two SH3 domains in the absence of any catalytic domains (15). Adaptor proteins are thought to link tyrosine kinases with downstream effectors, and Crkl could be an important mediator of the leukemogenic activity of Bcr/Abl. Since SH2 and SH3 domains serve as docking sites for other proteins, it is critical to identify the binding partners of Crkl in Ph-positive cells. In the current study, we have identified a tyrosine-phosphorylated p120 protein which binds the Crkl SH2 domain but not its SH3 domains. Binding is restricted to cells containing a Bcr/Abl protein and apparently is the result of Bcr/Abl kinase activity. The identification of p120 as p120^{Cbl}, originally isolated as an oncogene associated with myeloid and lymphoid leukemias in mice (16), sheds light on signal transduction pathways likely to be affected in Ph-positive leukemia.

MATERIALS AND METHODS

Cells and Antibodies—Lysates of peripheral blood cells of patients have been described previously (11). Polyclonal rabbit anti-Cbl and anti-crkl were purchased from Santa Cruz Biotechnology. Crkl-specific antibodies (CH-16) recognizing the spacer region between the Crkl SH3 domains have been described (14) and were affinity-purified. Anti-phosphotyrosine (RC20) and anti-Cas monoclonal antibodies were from Transduction Laboratory; anti-Bcr (AB-2) and anti-phosphotyrosine antibodies (AB-2) were from Oncogene Science, Inc.

Preparation of GST-Fusion Proteins—GST-Crkl has been described previously (14). GST-Crkl SH2 was generated by inserting a 360-base pair *TaqI*-*RsaI* fragment of a Crkl cDNA into pGEX-2T. GST-Crkl SH3SH3 was obtained by cloning a blunt-ended 630-base pair *BsmI* fragment into pGEX-3X. GST-fusion proteins were expressed and purified as described (17), and glutathione was removed by dialysis against 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10% (v/v) glycerol.

Immunoprecipitation and Immunoblot Analysis—K562 cells were lysed in Triton-lysis buffer (25 mM sodium phosphate, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 μg/ml aprotinin and leupeptin, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride). 0.25–1.0 mg of lysate proteins were used for immunoprecipitates. Proteins on gels were transferred to Hybond-ECL nitrocellulose and visualized with ECL reagents (Amersham). Western blots were incubated

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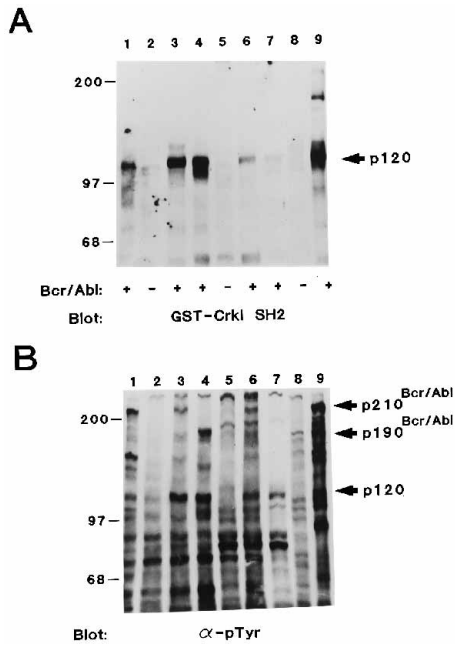


FIG. 1. The Crkl SH2 domain specifically binds to p120 only in cells containing an active Bcr/Abl P210 or P190 protein. A, far-Western blot analysis using GST-Crkl-SH2 as protein-binding probe. The presence or absence of an active Bcr/Abl in the samples is indicated with a - or +, respectively, beneath panel A. B, Western blot analysis of tyrosine-phosphorylated proteins in patient material and cell lines probed with α -Tyr(P) antibodies (OSI; AB-2). Lysates include those of the CML cell line K562, lane 1; Ph-negative ALL patient A0018, lane 2; P210-expressing blast crisis CML patient C1797, lane 3; Ph-positive, P190-expressing ALL patient A0055, lane 4; P210-negative CML chronic phase patient C2206, lane 5; P210-expressing CML chronic phase patient C2283, lane 6; P210-expressing CML blast crisis patient C1316, lane 7; NIH 3T3 cells, lane 8; NIH 3T3 cells stably transfected with a DNA construct encoding Bcr/Abl P210 (line 1F2), lane 9. The location of molecular weight markers is indicated to the left, that of P190^{Bcr/Abl}, P210^{Bcr/Abl}, and p120 to the right of the panels.

with antibodies as described (11, 14). Immunocomplex kinase reactions were performed as described previously (14). Gels containing immunocomplex kinase reactions were incubated for 2 h in 1 M KOH at 55 °C to specifically visualize phosphotyrosine-containing proteins.

In Vitro GST Binding Assays—Cell lysate proteins (0.5–1 mg), 10 μ g of GST-fusion proteins, and 25 μ l of glutathione-agarose beads were incubated in 1 ml of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM dithiothreitol, 0.1% Tween 20, 0.2% (w/v) bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 10 μ g/ml aprotinin and leupeptin at 4 °C for 3 h. Beads were washed four times with ice-cold 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.1% Tween 20, 1% Nonidet P-40. Far-Western assays were performed as described (14).

RESULTS

Crkl Binds a p120 in Cells Expressing an Active Bcr/Abl Protein—We initially compared Crkl protein binding in lysates from NIH 3T3 cells and NIH 3T3 cells stably transformed with BCR/ABL. Nontransfected NIH 3T3 fibroblasts contained no clearly detectable proteins binding to the Crkl SH2 domain on Far-Western blots (Fig. 1A, lane 8). However, transfection of these cells with Bcr/Abl P210 led to a marked increase in the total level of cellular phosphotyrosine (Fig. 1B, compare lanes 8 and 9) and the concomitant appearance of proteins of a molecular mass of approximately 120 kDa (p120) which bound to Crkl-SH2 (Fig. 1A, lane 9).

Ph-positive (expressing P210 or P190) or -negative peripheral blood cells from leukemia patients expressed different tyrosine-phosphorylated proteins (Fig. 1B). Of these, the Crkl-SH2 domain bound almost exclusively to the p120 and only in cells expressing an active Bcr/Abl protein. This included pa-

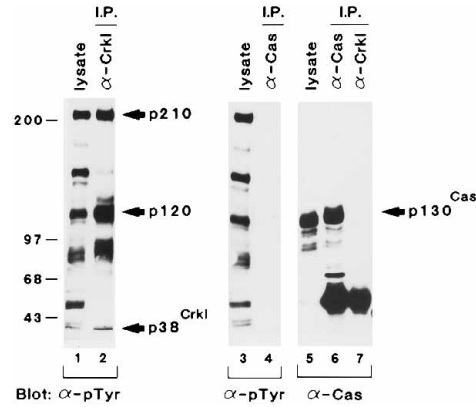


FIG. 2. The Crk-associated p130^{Cas} is not tyrosine-phosphorylated or in complex with Crkl in the CML cell line K562. Lysates of K562 were reacted directly with α -Tyr(P) antibodies (lanes 1 and 3) or with anti-Cas antibodies (lane 5). Extracts of K562 were first immunoprecipitated with α -Crkl (lanes 2 and 7) or α -Cas (lanes 4 and 6) antibodies, then probed with α -Tyr(P) or Cas antibodies as indicated.

tient samples of Ph-positive acute lymphoblastic leukemia expressing Bcr/Abl P190 (Fig. 1A, lane 4) and chronic myeloid leukemia samples expressing Bcr/Abl P210 (Fig. 1A, lanes 3, 6, and 7) (described in Ref. 11). Although the p120 was clearly detected in the Bcr/Abl expressing Ph-positive patient samples, the Crkl SH2 domain failed to detect the p120 in peripheral blood cells of patients not expressing Bcr/Abl (Fig. 1A, lanes 2 and 5). Similarly, the p120 was detected in involved spleens of BCR/ABL P190 transgenic mice but not in control spleens (not shown). K562, a CML cell line commonly used as a model for Ph-positive leukemia expressing P210, also contained the Crkl-SH2 domain-binding p120 phosphoprotein (Fig. 1A, lane 1).

To investigate whether the Crkl protein binds to p120 *in vivo*, tyrosine-phosphorylated proteins which associate with Crkl in K562 were examined. Although some anti-phosphotyrosine antibodies (OSI AB-2) detected Crkl as one of the most conspicuous phosphotyrosine-containing proteins in K562 (14), the RC20 antibodies (Signal Transduction) also detected additional prominent phosphotyrosine-containing proteins and had a lower affinity for Crkl (Fig. 2, lane 1). However, only a very limited subset of the tyrosine-phosphorylated proteins detected in K562 (Fig. 2, compare lanes 1 and 2) were co-precipitated with Crkl. This included P210^{Bcr/Abl}, in agreement with previous results (14), a group of 70–80-kDa phosphoproteins, and a p120 (Fig. 2, lane 2). We concluded that the tyrosine-phosphorylated p120 protein is uniquely associated with Crkl in Ph-positive cells, and that this is likely to be of relevance to leukemogenesis caused by Bcr/Abl. Therefore, experiments were initiated to identify p120.

Identification of the p120—The adaptor molecule Crk has a structure and primary sequence similar to that of Crkl (15), and overlap between the binding of Crk and Crkl to proteins on far-Western blots has been described (18). In *v-src* or *v-crk* transformed cells, a prominent phosphotyrosine-containing p130 is detected which binds to Crk. This protein, p130^{Cas}, contains various potential tyrosine phosphorylation sites (19). We therefore considered the possibility that the p120 is Cas. However, although p130^{Cas} was clearly expressed in K562 cells (Fig. 2, lane 5) and Cas antibodies were able to efficiently immunoprecipitate Cas from K562 extracts (lane 6), this protein contained no detectable phosphotyrosine (lane 4), nor did it co-immunoprecipitate with Crkl antisera (lane 7).

p120^{RasGAP} is another good candidate for p120, since it is tyrosine-phosphorylated in K562 cells (10). However, experiments similar to those described for p130^{Cas} failed to positively identify p120^{RasGAP} as the p120 protein (not shown).

p120^{Cbl} was originally identified as a retrovirally transduced oncogene which causes pre-B-cell lymphomas and myeloid leukemias in mice (16). Endogenous Cbl is tyrosine-phosphorylated in NIH 3T3 cells expressing *v-abl* and in K562 (5), suggesting it as a candidate for p120. p120^{Cbl} had a mobility very similar to that of the p120 immunoprecipitated with two different α -Crkl antisera from K562 (Fig. 3A), and both contained phosphotyrosine (Fig. 3A, top). Immunoblotting of the Crkl precipitates with α -Cbl antisera unambiguously identified the p120 as p120^{Cbl} (Fig. 3A, bottom). Mice transgenic for *BCR/ABL* P190 reproducibly develop lymphoblastic leukemia/lymphoma (20). Tyrosine-phosphorylated p120^{Cbl} was also co-immunoprecipitated with Crkl antisera from the spleens of leukemic transgenic Bcr/Abl mice (not shown).

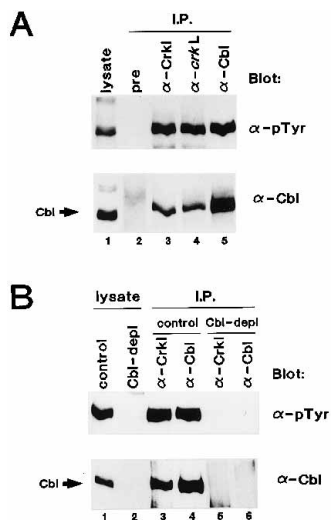


FIG. 3. p120^{Cbl} is in complex with Crkl and represents the 120-kDa tyrosine-phosphorylated protein band detectable in Crkl immunoprecipitates. A, co-immunoprecipitation of Cbl with Crkl antisera. Lane 1, Western blot of K562 lysate; lanes 2–5, immunoprecipitations from extracts of K562 using Crkl preimmune (lanes 2), α -Crkl CH-16 (lanes 3), α -Crkl *crkl*L (lanes 4), and α -Cbl (lanes 5) antisera. The blots were reacted with the antisera shown to the right of each panel. B, immunodepletion of p120^{Cbl} abrogates the detection of the Crkl-associated 120-kDa phosphoprotein. K562 lysate was subjected to two rounds of α -Cbl immunoprecipitation, and the resulting Cbl-depleted lysate (lane 2) was immunoprecipitated with either α -Crkl (lane 5) or α -Cbl (lane 6), as indicated. For comparison, control K562 lysate (lane 1) was immunoprecipitated with α -Crkl (lane 3) or α -Cbl (lane 4) antisera. Lysates and immunoprecipitates were blotted and incubated with α -Tyr(P) or α -Cbl antibodies, as indicated to the right.

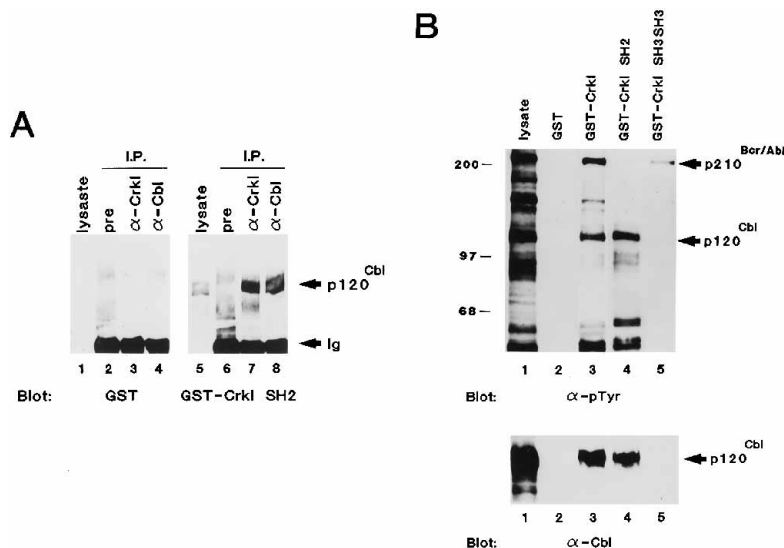
FIG. 4. The Crkl SH2 but not its SH3 domain stably binds to Cbl in K562. A, far-Western blot analysis of immunoprecipitates of Cbl. Lanes 1 and 5 contain K562 total cellular lysates. Proteins immunoprecipitated with control nonspecific antiserum (lanes 2 and 6), α -Crkl CH-16 (lanes 3 and 7), and α -Cbl (lanes 4 and 8) were blotted and reacted with GST (left panel) or GST-Crkl-SH2 as shown. The location of p120^{Cbl} and the immunoglobulin heavy chain (Ig) is as indicated. B, binding of Crkl subdomains to Cbl. Extracts in lanes 2–5 were incubated with GST (fusion) proteins as indicated. The locations of P210^{Bcr/Abl} and p120^{Cbl} are as shown. Blots were reacted with α -Tyr(P) antibodies (top panel) or α -Cbl antisera (bottom panel) as indicated.

In similar reciprocal experiments, we could detect Crkl in immunoprecipitates of K562 generated by α -Cbl antibodies, although Crkl co-precipitation was not as efficient as co-precipitation of Cbl by α -Crkl antisera. In addition, these Cbl precipitates also contained Bcr/Abl (data not shown). These results identify Cbl as the p120 protein.

To determine if all of the tyrosine-phosphorylated 120-kDa protein band in Crkl immunoprecipitates represents Cbl, K562 lysates were subjected to two rounds of immunoprecipitation with α -Cbl (Fig. 3B). This Cbl-depleted lysate was Western-blotted directly or was immunoprecipitated with α -Crkl or α -Cbl antisera. The two rounds of Cbl precipitation were sufficient to deplete the K562 lysate of tyrosine-phosphorylated Cbl (lanes 2 and 6, top and bottom), resulting also in a selective loss of all of the tyrosine-phosphorylated 120-kDa protein detected in the lysate and in the α -Crkl precipitate (lanes 2 and 5, top). From these results we conclude that p120^{Cbl} represents virtually all of the tyrosine-phosphorylated p120 associated with Crkl.

Crkl Binds to Cbl Only through Its SH2 Domain—To further confirm the identity of the Crkl SH2-binding p120 detected in Ph-positive cells as Cbl, proteins were immunoprecipitated from K562 with α -Crkl or α -Cbl antisera. When these precipitates were probed with Crkl SH2 on an immunoblot, p120^{Cbl} was clearly and specifically detected (Fig. 4A), whereas blotting to control GST protein detected no specific bands (Fig. 4A, left panel). Crkl SH2 bound only to p120^{Cbl} phosphorylated on tyrosine, as is demonstrated in the far-Western assay shown in Fig. 1, indicating that the SH2 interaction is phosphotyrosine-dependent.

Although the binding of Crkl to Cbl must involve the interaction of the Crkl SH2 domain and phosphotyrosine residues on Cbl, it was possible that Crkl could also interact with Cbl via its SH3 domains. This possibility is illustrated by Fyn and Lck, of which both SH2 and SH3 domains bind to Cbl from activated T-cells (21). To test this, bacterially expressed GST-Crkl and GST-Crkl SH2 and SH3 domains were used to pull down proteins from K562 extracts. The Crkl SH2 domain but not the SH3 domains precipitated the tyrosine-phosphorylated Cbl p120 protein (Fig. 4B, top and bottom panels) with an efficiency comparable to that of the entire Crkl protein (Fig. 4B, compare lanes 3 and 4). In agreement with previous results, the entire Crkl protein also bound to a P210 protein which was identified as P210^{Bcr/Abl} (not shown), and this interaction was mediated by Crkl SH3 domains (Fig. 4B, lane 5).



DISCUSSION

Crkl is one of the most prominent tyrosine-phosphorylated proteins in Ph-positive patient (11) and transgenic Bcr/Abl mouse material.² Because it is non-tyrosine-phosphorylated in normal bone marrow, this modification is leukemia-specific, and we are in the process of investigating the effect of tyrosine phosphorylation of Crkl on its cellular interactions. The current study demonstrates a second level of Crkl involvement: in Ph-positive leukemia, Crkl is bound to tyrosine-phosphorylated Cbl through its SH2 domain.

Although the phosphorylation status of Cbl has not been examined in detail in hematopoietic cell types, our results show that it is not constitutively tyrosine-phosphorylated in peripheral blood cells and are in agreement with results of others (21, 22). We therefore suggest that the constitutive tyrosine phosphorylation of Cbl in Ph-positive cells is abnormal. Transient tyrosine phosphorylation of Cbl has been demonstrated recently in normal hematopoietic signaling events: T-cell receptor activation of Jurkat cells and GM-CSF and Epo stimulation of UT-7 cells (21, 22). This shows that Cbl tyrosine phosphorylation is also part of normal signal transduction pathways.

The specific activity of the oncogene *v-cbl* in causing myeloid leukemias and lymphoblastic leukemia/lymphomas in mice also is in concordance with a defined normal role for Cbl in hematopoietic signaling. In addition, since removal of a defined region of 17 amino acids from Cbl causes it to become phosphorylated on tyrosine and to transform cells (5), a correlation appears to exist between myeloid/lymphoid cell types, Cbl phosphorylation, and leukemogenesis.

How does Cbl become phosphorylated? Andoniou *et al.* (5) showed that anti-Abl antiserum is capable of co-precipitating Cbl from K562 but did not show evidence for the mechanism through which this would occur. We have previously shown that Crkl and Bcr/Abl form complexes and demonstrate here that this is mediated through the Crkl SH3 domain. In the current study, we also demonstrate Crkl and Cbl binding through the Crkl SH2 domain. Although it remains a formal possibility that each of these molecules form independent bimolecular complexes with each other (Bcr/Abl + Crkl; Bcr/Abl + Cbl; Crkl + Cbl), we favor a model in which a trimolecular complex involving Bcr/Abl, Crkl, and Cbl is formed. In the most simple version of this model, Crkl is constitutively bound to Bcr/Abl via its SH3 domain. Low level transient tyrosine phosphorylation of Cbl through normal signaling events (*e.g.* GM-CSF stimulation) would cause an association of Crkl and Cbl and a significant increase of tyrosine phosphorylation of Cbl by Bcr/Abl. Because the kinase activity of Bcr/Abl is deregulated, Cbl tyrosine phosphorylation would be constitutively switched on, leading to a long-lasting activation of the signaling path-

ways normally controlled by cytokines. Interestingly, the results of Odai *et al.* (22) suggest that the GM-CSF signal transduction pathway in which Cbl appears to be involved is distinct from that of Ras.

Although some models of Bcr/Abl-associated leukemogenesis invoke several distinct signaling pathways, it remains very well possible that only a single pathway leads to leukemogenesis *in vivo*. The similarities between the types of malignancies associated with Bcr/Abl and *v-cbl* in animal models suggests that the pathways of these oncogenes converge, a possibility which can be currently investigated in complementation studies.

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