A Specific Need for CRKL in p210BCR-ABL–Induced Transformation of Mouse Hematopoietic Progenitors

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Abstract

CRKL (CRK-like) is an adapter protein predominantly phosphorylated in cells that express the tyrosine kinase p210BCR-ABL, the fusion product of a (9;22) chromosomal translocation causative for chronic myeloid leukemia. It has been unclear, however, whether CRKL plays a functional role in p210BCR-ABL transformation. Here, we show that CRKL is required for p210BCR-ABL to support interleukin-3–independent growth of myeloid progenitor cells and long-term outgrowth of B-lymphoid cells from fetal liver–derived hematopoietic progenitor cells. Furthermore, a synthetic phosphotyrosyl peptide that binds to the CRKL SH2 domain with high affinity blocks association of endogenous CRKL with the p210BCR-ABL complex and reduces c-MYC levels in K562 human leukemic cells as well as in mouse hematopoietic cells transformed by p210BCR-ABL or the imatinib-resistant mutant T315I. These results indicate that the function of CRKL as an adapter protein is essential for p210BCR-ABL–induced transformation. Cancer Res; 70(18); OF1–11. ©2010 AACR.

Introduction

Patients with chronic myeloid leukemia (CML) harbor a Philadelphia chromosome (Ph) due to a translocation between chromosomes 9 and 22 (1). The resulting BCR-ABL fusion gene gives rise to a p210BCR-ABL oncoprotein (2, 3). Forced expression of the p210BCR-ABL protein renders hematopoietic cells growth factor independent (4, 5) and induces proliferation of myeloid progenitors and bone marrow–derived B-lymphoid cells (6–9). Transplantation of p210BCR-ABL–transduced murine bone marrow cells into lethally irradiated syngeneic mice induces a CML–like myeloproliferative disorder, showing the leukemogenic potential of p210BCR-ABL in vivo (10–13). The tyrosine kinase activity of p210BCR-ABL is essential for its oncogenic potential both in vitro and in vivo (13, 14); hence, the ABL inhibitor imatinib has been a successful treatment for CML (15).

CRKL (CRK-like), a member of the CRK family of adapter proteins, consists of an NH2-terminal SH2 domain followed by two SH3 domains: SH3a and SH3c (16). Although related to CRK, CRKL is a distinct gene located on chromosome 22q11.21 (16). Overexpression of CRKL enhances p190BCR-ABL–induced transformation and leukemogenesis in fibroblasts and in a transgenic mouse model (17, 18). CRKL links tyrosine kinase substrates to downstream effectors containing SH3-binding motifs. CRKL is constitutively phosphorylated by BCR-ABL in neutrophils of CML patients, and the degree of CRKL phosphorylation is a marker of BCR-ABL kinase activity (19, 20).

Direct binding of CRKL to p210BCR-ABL is mediated by association of the CRKL SH3n domain with a proline-rich motif in ABL (21). However, a mutant BCR-ABL lacking the SH3-binding motif still transforms myeloid cells (21), suggesting that CRKL remains associated with the p210BCR-ABL complex by interactions with other proteins. Cell-penetrating peptides designed to bind with high affinity to the CRKL SH3n domain block the proliferation of primary CML blast cells (22). These SH3-binding peptides also bind to the CRK gene products with high affinity (23), identifying a commonly encountered difficulty in studies designed to provide definitive evidence for a role that CRKL may play in p210BCR-ABL–induced transformation.

We and others generated mouse mutants with targeted disruptions at the Crkl locus (24, 25). Crkl deficiency in 129/Sv and C57BL/6 backgrounds results in embryonic lethality that mimics 22q11 deletion syndrome, also known as DiGeorge syndrome (24). The phenotype seems to be sensitive to genetic background and disappears in a mixed genetic background involving 129/Sv and Black Swiss (25). Using this Crkl–insensitive background, it was shown that Crkl deficiency did not inhibit leukemogenesis caused by p190BCR-ABL in a mouse model for acute lymphoblastic leukemia (ALL; ref. 25). Disappearance of the embryonic phenotype, however, indicates that this genetic background provides an alternative mechanism that compensates for loss of Crkl.

Biological differences between p210BCR-ABL and p190BCR-ABL may also explain the results of the study mentioned above.
p190BCR-ABL causes Ph’ ALL, which is relatively refractory to imatinib therapy (26). A recent study shows that a significant subset of leukemia cells from p190BCR-ABL transgenic mice is insensitive to imatinib and quickly takes over the culture particularly when stromal cells are present (27). Because imatinib is still effective at blocking the kinase activity of p190BCR-ABL in these resistant cells, this phenomenon suggests a kinase-independent mechanism in p190BCR-ABL-induced leukemia. p190BCR-ABL can sustain survival and proliferation of leukemia cells in the presence of imatinib (thus without substantial ABL tyrosine kinase activity), which may explain the ability of p190BCR-ABL to induce leukemia in the transgenic model without Crkl. To directly address the requirement for CRKL for p210BCR-ABL transformation, we have therefore used hematopoietic progenitor cells from the fetal liver of Crkl-deficient mouse embryos that express a Crkl-sensitive genetic background. Our results are further supported by studies with a synthetic peptide that blocks association of CRKL with the p210BCR-ABL protein complex in mouse hematopoietic progenitor cells and in human K562 leukemia cells. The current study shows an essential role for CRKL in p210BCR-ABL-induced transformation of hematopoietic progenitor cells.

Materials and Methods

Mice

The Institutional Animal Use and Care Committees of the University of Chicago and the Oregon Health & Science University approved animal studies. Crkl−/− mice were described previously (24). All mice and embryos were examined in a mixed 129S4/SvJaeSor × C57BL/6J background. Embryos were derived by timed matings between Crkl−/− parents.

Cell lines

Certified BaF3 and K562 cells were obtained from the American Type Culture Collection and grown in the recommended culture medium. The BaF3 transfectant (expressing the p210BCR-ABL mutant T315I mutation) has been previously described (28). Transformed p210BCR-ABL B-lymphoid cells derived from wild-type (Wt) and Crkl-deficient fetal liver cells were generated as described below. None of the cell lines used in this study were cultured for longer than 6 months from initial purchase or characterization. No further authentication of cell line characteristics was done.

Retrovirus preparation

Bosc23 cells (28) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Hematopoietic progenitor cells were infected with p210BCR-ABL retrovirus or control green fluorescent protein (GFP) retrovirus generated by transfecting Bosc23 cells with MSCV-p210-ires-GFP (13) or empty MSCV-ires GFP vector, and the viral supernatant was harvested at 48 hours after transfection.

Myeloid and lymphoid outgrowth assays

Due to the lethal phenotype of Crkl−/− embryos at late gestation (24), the fetal liver was harvested as a source of hematopoietic progenitor cells. For colony-forming assays, Wt, Crkl−/−, or Crkl−/− cells harvested from fetal livers of embryonic day 13.5 (E13.5) mice were incubated overnight in prestimulation medium [Iscove’s modified Dulbecco’s medium, 15% FBS, 5% WEHI-conditioned medium, 1 μg/mL ciprofloxacin, murine interleukin (IL)-3 (6 ng/mL), IL-6 (10 ng/mL), and stem cell factor (50 ng/mL)]. Cells were transduced with p210BCR-ABL-expressing retrovirus in the presence of 2 μg/mL polybrene (Sigma-Aldrich) by two rounds of spinoculation (29), and 8 × 105 cells per 35-mm dish were plated in triplicate in Methocult M3234 medium (StemCell Technologies) with or without IL-3 (100 pg/mL). Colonies were scored at day 12.

Whitlock-Witte cultures were performed as described (8). Briefly, cells harvested from the fetal liver of E13.5 Wt, Crkl−/−, or Crkl−/− mouse embryos were infected with p210BCR-ABL or control retrovirus in lymphoid medium (RPMI 1640 plus 10% FBS and 0.05 mmol/L 2-mercaptoethanol) containing 8 μg/mL polybrene by two rounds of spinoculation. Approximately 5 × 105 cells were plated per 60-mm dish in triplicate. Cultures were fed every 3 days by adding 2 mL of lymphoid medium. Viable nonadherent cells were counted at day 14 after transduction, and viability was determined by trypan blue dye exclusion.

Analysis of cell surface markers

Immunophenotyping of p210BCR-ABL-transformed lymphoid cells expanded from Whitlock-Witte cultures as well as fresh fetal liver cells from E13.5 Wt, Crkl−/−, and Crkl−/− embryos was performed by fluorescence-activated cell sorting (FACS) using a FACSCalibur instrument (BD Biosciences). Whitlock-Witte culture-derived lymphoid cells transformed with p210BCR-ABL were stained with phycoerythrin (PE)-conjugated B220, Sca1, or c-Kit. For analysis of hematopoietic progenitor populations in fetal liver, lineage-committed (Lin+) hematopoietic cells were identified with the biotinylated mouse lineage antibody cocktail (BD Biosciences) and streptavidin-conjugated PE-Cy7 (Caltag Laboratories, Inc.). Lin− cells were then stained with the following antibodies: PE-conjugated c-Kit, allopelloerycin (APC)-conjugated Thy1.1, and FITC-conjugated Sca1. The percentage of stem cells in each fetal liver cell population was calculated by determining the number of Lin−, c-Kit−, Sca1−, and Thy1.1− cells. To evaluate the composition of B-cell progenitors in the fetal liver of Wt, Crkl−/−, and Crkl−/− mice, cells were stained with B220-APC, CD43-PE, IgM-APC, and IgD-APC. Based on immunophenotype, cells were characterized as pro-B (B220high, CD43+, IgM+, IgD−), pre-B (B220high, CD43+, IgM+, IgD+), immature B (B220moderate, CD43+, IgM+, IgD−), or mature B (B220high, CD43+, IgM+, IgDhigh) cells. Monoclonal antibodies raised against B220, Sca1, Thy1.1, c-Kit, and CD43 were purchased from BD Biosciences, and for IgM and IgD from Southern Biotechnology Associates.

Peptides, immunoprecipitation, and immunoblotting

The purity of synthetic peptides was >95%. The amino acid sequences used were as follows: pYELP peptide, ESVLPYELP; Crkl pY207 peptide, GIPEPAHIPYAQPTTTTLPA; and Crkl pY221 peptide, GGPEGPYTPAQTPNTPLPN. Control peptides included a tyrosine or...
phenylalanine residue in place of phosphotyrosine (pY). Co-immunoprecipitation, SDS-PAGE, and immunoblotting were performed using standard protocols. In addition to fetal liver–derived mouse lymphoid cells, we used the human CML blast crisis cell line K562 and murine cell lines BaF3-p210BCR-ABL or BaF3-p210BCR-ABL with T315I mutation. Cell lysates were prepared in lysis buffer [15% glycerol, 1% NP40, 50 mmol/L Tris (pH 7.4), 0.2 mol/L NaCl, 2.5 mmol/L MgCl2] including protease inhibitor cocktail (Roche), 1 mmol/L sodium orthovanadate, and 10 mmol/L NaF. For peptide inhibition experiments, phosphopeptide or control peptide was added to precleared cell lysates and incubated for 1 hour at room temperature. Stable association of CRKL or CRK with p210BCR-ABL was assessed by coimmunoprecipitation with mouse anti-ABL monoclonal antibody (clone 8E9; Santa Cruz Biotechnology) and anti-mouse IgG-conjugated Sepharose beads (Sigma), followed by SDS-PAGE and immunoblot analysis with rabbit anti-CRKL (C20) or anti-CRK antibody (Santa Cruz Biotechnology). Prior control experiments performed with isotype-matched, irrelevant antibody confirm the specificity of bands detected in the high-stringency anti-ABL immunoprecipitates.

To assess the biological effects of the phosphopeptide on BCR-ABL function, and to compare its activity to the ABL kinase inhibitor imatinib, PEP-1 (a 21-amino acid peptide carrier) was used to deliver phosphorylated or control peptides into the cell (30). The phosphorylated or control peptide was preincubated with PEP-1 for 1 hour at a molar ratio of 1:20. The peptide mix was then added to the cell culture and incubated for the indicated amount of time before washout and lysis. Imatinib, kindly provided by E. Buchdunger (Novartis), was prepared freshly as a 10 mmol/L stock solution in sterile PBS.

Immunoblots were performed according to a standard method and probed using specific antibodies. To probe phosphorylation at Y649 in signal transducer and activator of transcription 5A (STAT5A) and at Y699 in STAT5B, we used mouse monoclonal anti–phospho-STAT5 (clone ST5P-4A9; Invitrogen). Total STAT5 (STAT5A and STAT5B) was determined by rabbit anti-STAT5 (Santa Cruz Biotechnology). The BCL-X and MYC gene products were probed with rabbit anti-BCL-XL (Santa Cruz Biotechnology) and rabbit polyclonal anti-Myc (Upstate).

**Cell cycle analysis**

Cell cycle analysis was carried out by FACS. Cells were collected by centrifugation, washed with PBS, and permeabilized.
in 70% ethanol at −20°C for 30 minutes before DNA staining. Approximately 1 × 10^6 permeabilized cells were incubated with 50 μg/mL propidium iodide (Roche) or 3 μmol/L 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and 0.1 mg/mL RNase A in staining buffer [100 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 0.1% NP40] for 15 to 30 minutes. Stained cells were analyzed in a FACSCalibur or BD LSR II flow cytometer (BD Biosciences) using ModFit LT or FACSDiva software.

**Proliferation assays**

p210BCR-ABL–transformed Wt and Crkl−/− hematopoietic cells (5 × 10^4 per well) that were expanded from Whitlock-Witte cultures were plated into 24-well flat-bottomed plates in RPMI 1640 containing 5% FBS and 50 μmol/L 2-mercaptoethanol. Cells were counted daily for 3 days. Results represent counts obtained from triplicate wells performed with cells derived from three independent Whitlock-Witte cultures.

**Results**

CRKL is required for p210BCR-ABL–induced IL-3–independent myeloid outgrowth

BCR-ABL expression in hematopoietic progenitor cells results in growth factor–independent myeloid colonies in methylcellulose (6). To assess the requirement of CRKL in this process, fetal liver cells harvested from E13.5 Wt, Crkl−/+ or Crkl−/− embryos were infected with retrovirus that cotransduces p210BCR-ABL and GFP. Transduction efficiency of Wt, Crkl−/+, and Crkl−/− cells was similar, as comparable numbers of cells were GFP+ by FACS analysis following transduction (data not shown). Cells were seeded into methylcellulose in the presence or absence of IL-3. Growth factor–dependent and growth factor–independent colonies were counted on day 12. We found that p210BCR-ABL expression in Wt cells leads to the formation of cytokine-independent myeloid colonies of granulocyte-macrophage colony-forming unit (Figure 2).

![Figure 2](image-url)

**Figure 2.** The requirement for CRKL in generating p210BCR-ABL–B-lymphoid cells. A, fetal liver cells infected with p210BCR-ABL retrovirus were cultured under conditions favoring outgrowth of transformed B lymphocytes. Following incubation at 37°C for 14 d, viable cells were counted. Columns, average from three independent assays in triplicate with Wt, Crkl−/+, or Crkl−/− matched littersmates; bars, SD. *, P = 0.05; **, P = 0.0005, Crkl−/+ and Crkl−/− compared with Wt. B, the p210BCR-ABL–transformed lymphoid cells from Wt cultures were B220+, whereas Crkl−/− cells were largely B220−. Crkl−/− cells expressed markers of more primitive progenitors, such as c-Kit and Sca1. C, immunoblotting of BCR-ABL and CRKL in Wt and Crkl-deficient p210BCR-ABL–transformed lymphoid cells with anti-ABL and anti-CRKL antibodies, with actin as a loading control.
(CFU-GM), macrophage CFU (CFU-M), and granulocyte-erythrocyte-macrophage-megakaryocyte CFU (CFU-GEMM) lineages. In contrast, there was a 3-fold reduction in the number of growth factor–independent colonies formed by Crkl\(^{-/-}\) cells (Fig. 1A). The number of Crkl\(^{+/+}\) colonies was reduced by 2-fold, and there were significantly fewer cells formed by Crkl\(^{-/-}\) cells than Crkl\(^{+/+}\) cells, suggesting a dosage-sensitive effect of CRKL on p210 BCR-ABL–induced transformation (Fig. 1A). In the presence of IL-3, Wt, Crkl\(^{+/+}\), and Crkl\(^{-/-}\) cells gave similar numbers of myeloid colonies (Fig. 1B). There was no difference in the relative proportion of colonies of CFU-GEMM, CFU-GM, or CFU-M lineages formed by Wt, Crkl\(^{+/+}\), or Crkl\(^{-/-}\) cells (Fig. 1C). Importantly, all colonies were GFP\(^{+}\) by fluorescence microscopy and contained the provirus by PCR screening (data not shown). These data suggest that (a) Crkl deficiency does not compromise the generation of hematopoietic colonies in response to physiologic stimuli and (b) decreased myeloid colony formation in Crkl\(^{-/-}\) cultures is not due to decreased transduction efficiency of early myeloid progenitors in the absence of CRKL.

CRKL is required for p210\(^{BCR-ABL}\)–induced lymphoid outgrowth

To further explore the requirement of CRKL for transformation by p210\(^{BCR-ABL}\), we assessed outgrowth of Crkl\(^{-/-}\) lymphoid cells induced by p210\(^{BCR-ABL}\). Hematopoietic progenitor cells harvested from the fetal livers of 13.5-day-old Wt, Crkl\(^{+/+}\), and Crkl\(^{-/-}\) embryos were transduced with p210\(^{BCR-ABL}\) retrovirus and cultured under conditions favoring outgrowth of transformed lymphoid cells (8). A similar

Figure 3. Crkl deficiency does not affect distribution of stem cell and B-cell progenitor populations in fetal liver cells isolated from E13.5 Wt, Crkl\(^{+/+}\), and Crkl\(^{-/-}\) mouse embryos. FACS profiling of stem cells (Thy1.1\(^{+}\) and Sca1\(^{+}\)), pre-B cells (B220\(^{低}\)), pro-B cells (IgM\(^{低}\), CD43\(^{+}\)), immature (CD43\(^{低}\), IgM\(^{高}\)) B-cell progenitors revealed no significant differences in the distribution of cells from Wt, Crkl\(^{+/+}\), or Crkl\(^{-/-}\) embryos.
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Figure 4. pYELP peptide inhibits association of BCR-ABL and CRKL. A, a phosphotyrosyl peptide, pYELP, dissociates CRKL from the p210BCR-ABL complex in vitro. The 17-aa peptide pYELP or control peptides without phosphotyrosine (YELP and FELP) were added to cell lysates prepared from B-lymphoid cells derived from fetal liver transduced with Wt p210BCR-ABL—transduced Wt fetal liver. After 1-h incubation at room temperature, stable association of CRKL or CRK with p210 was assessed by coimmunoprecipitation (IP) with anti-ABL antibody followed by immunoblot (IB) analysis with anti-CRKL or anti-CRK antibody. B, pYELP peptide inhibits association of CRKL with p210BCR-ABL in vivo. B-lymphoid cells derived from Wt fetal liver transduced with p210BCR-ABL retrovirus cultured in Whitlock-Witte medium were incubated with pYELP or similar phosphopeptide corresponding to CRKL pY207 or CRK pY221 along with PEP-1 carrier peptide for 2 h before cell lysates were prepared. Association of CRKL and p210BCR-ABL was evaluated by coimmunoprecipitation.

percentage of Wt, Crkl+/−, and Crkl−/− cells were GFP+ following retroviral transduction with p210BCR-ABL (data not shown), but fewer cells were observed in Crkl+/− (P = 0.05) and Crkl−/− (P = 0.0005) cultures at day 14 after transduction compared with Wt cultures (Fig. 2A). We also observed fewer cells in Crkl−/− relative to Crkl+/− cultures, suggesting a dosage-sensitive effect of Crkl on p210BCR-ABL—induced lymphoid transformation. Consistent with previous reports (31), all cells emerging from Wt fetal liver expressed the B220 antigen; however, Crkl+/− cells were largely B220− and expressed markers of more primitive progenitors, such as c-Kit and Sca1 (Fig. 2B). There was no difference in p210BCR-ABL levels shown between these cells (Fig. 2C), indicating that poor outgrowth of cells from Crkl−/− fetal liver was not due to compromised expression of p210BCR-ABL.

These results suggest that p210BCR-ABL fails to induce B220− lymphoid cells in the absence of CRKL but could also be attributable to skewed progenitor populations in Crkl+/− and Crkl−/− cells compared with Wt cells. We therefore investigated by FACS whether the composition of hematopoietic progenitor cells differed between fetal liver cells derived from Wt, Crkl+/−, and Crkl−/− embryos. The percentage of stem cells (Lin−, c-Kit+, Sca1+, Thy1.1+) was the same in Wt, Crkl+/−, and Crkl−/− fetal liver cells (Fig. 3). Moreover, we found no difference in the percentage of pro-B (B220low, CD43−, IgM+, IgD−), pre-B (B220low, CD43+, IgM−, IgD−), immature B (B220moderate, CD43−, IgM+, IgD−), or mature B (B220high, CD43−, IgMhigh, IgDhigh) cells in the fetal liver of Wt, Crkl+/−, or Crkl−/− embryos (Fig. 3). We conclude that reduction in B-cell outgrowth from Crkl−/− and Crkl−/− fetal liver in Whitlock-Witte cultures was not due to a deficiency in B-cell progenitors. This finding is consistent with previous findings that there was no difference in the ability of hematopoietic progenitor cells derived from Crkl−/− fetal livers to reconstitute the bone marrow of irradiated Rag2−/− recipients compared with Wt with similar numbers of circulating B220− cells (32).

Phosphotyrosine-mediated interactions play a major role in association of CRKL with the p210BCR-ABL complex

Biological functions of adapter proteins such as CRKL rely on their ability to link partners through protein interaction domains (16). We previously showed that CRKL associates with fibroblast growth factor receptors, FGFR1 and FGFR2, through its SH2 domain (33). Based on a CRKL SH2 domain-binding motif identified in FGFR1, we synthesized a phosphotyrosyl peptide (pYELP) exhibiting high affinity for the CRKL SH2 domain but ∼30-fold weaker binding to the SH2 domain of CRK (34). Although the peptide sequence was based on an FGFR1 autophosphorylation site, pYELP peptide efficiently disrupted association of endogenous CRKL with the p210BCR-ABL protein complex when added to cell lysates from Wt cells (Fig. 4A). Conversely, this peptide had no inhibitory effect on association of CRK with the p210 protein complex (Fig. 4A). In the same experiment, control peptides without phosphotyrosine (YELP and FELP) did not affect association of CRKL with the p210BCR-ABL complex. Further, we observed that pYELP peptide also inhibited their association in vivo in a dose-dependent manner when the peptide is introduced into the cell along with a cell membrane penetrating peptide carrier PEP-1 (Fig. 4B; ref. 30). Of note, appropriate control experiments were performed with an isotype-matched, irrelevant antibody and these experiments confirmed the specificity of bands detected in the high-stringency anti-ABL immunoprecipitates (data not shown). In total, these results indicate that pYELP peptide can block the adapter function of CRKL in vitro and in vivo.

CRKL is constitutively phosphorylated at Y207 in leukemia cells expressing p210BCR-ABL (35). Y207 and Y221 in Crkl and CRK, respectively, are thought to play a role in regulating adapter functions through intramolecular associations (34, 36). Interestingly, however, phosphotyrosyl peptides based on the internal SH2 domain-binding motif in CRKL and CRK (pY207 or pY221 peptide, respectively) failed to dissociate these adapters from the p210BCR-ABL complex at concentrations up to 10 μmol/L (Fig. 4B; data not shown), providing evidence that CRKL functions as an adapter despite phosphorylation at Y207 in leukemia cells.
We used the ability of pYELP peptide to dissociate CRKL from the p210BCR-ABL complex in Wt B-lymphoid cells transformed by p210BCR-ABL. Treatment with either pYELP peptide or imatinib led to minor decreases in the number of cells in G0-G1, S, and G2, whereas the population in sub-G0-G1 was markedly increased (P < 0.01) in mouse B-lymphoid cells transformed by p210BCR-ABL in Whitlock-Witte culture (Fig. 5A). Treatment of K562 cells with either pYELP peptide or imatinib led to a significant increase in G0-G1 population (P < 0.01) and an increase in the sub-G0-G1 population (P < 0.01) accompanied by decreases in the S and G2 populations (P < 0.01; Fig. 5B). These results indicate that blockage of CRKL-mediated pathways or ABL catalytic activity had consistently similar effects in both cell types, manifested as increased apoptosis in p210BCR-ABL transformed B-lymphoid cells or as G1 arrest in K562 cells.

**CRKL is required for p210BCR-ABL-induced high BCL-\(\text{XL}\) and phospho-STAT5 levels**

p210BCR-ABL constitutively activates the transcription factor STAT5 (encoded by the Stat5A and Stat5B genes), which is required for transforming activity (37, 38). It has been reported that p210BCR-ABL promotes expression of the prosurvival factor BCL-\(\text{XL}\) through STAT5 (39). Given the adapter role of CRKL in BCR-ABL-mediated activation of STAT5 signaling cascades, we investigated whether the phenotypic defect observed in p210BCR-ABL transformed Crkl\(^{-/-}\) hematopoietic cells was a result of failure of BCR-ABL to activate STAT5 in the absence of CRKL. We observed a striking decrease in phospho-STAT5 in CRKL-deficient p210BCR-ABL-transformed cells relative to their Wt counterparts as well as a corresponding reduction in BCL-\(\text{XL}\) (Fig. 6A). Consistent with this, p210BCR-ABL-transformed Crkl\(^{-/-}\) cells proliferated significantly more slowly than transformed Wt cells (Fig. 6B). Next, we determined the effect of the blocking peptide pYELP on the phosphorylation of STAT5 (at Y694 or Y699 in STAT5A or STAT5B, respectively) in both mouse and human cells. On treatment of p210BCR-ABL-transformed lymphoid cells with pYELP peptide, phosphorylation of STAT5 was reduced in a dose-dependent manner, whereas control peptides had little effect (Fig. 6C, left). Similar effects of pYELP peptide were also observed in the human CML cell line K562 (Fig. 6C, right). In both cases, reduced phospho-STAT5 levels were again associated with a similar decrease in BCL-\(\text{XL}\) levels (Fig. 6C). Taken together, these results indicate an important role for CRKL in the cell survival network that p210BCR-ABL aberrantly activates.

**CRKL is required for p210BCR-ABL-induced high c-MYC levels**

The proto-oncogene c-MYC is required for BCR-ABL transformation (40) and plays an essential role in G1 to S transition in cell cycle (41). We therefore determined whether c-MYC levels were dependent on CRKL. We found that Crkl\(^{-/-}\) lymphoid cells expressing BCR-ABL had a striking reduction in c-MYC protein levels compared with Wt cells (Fig. 7A). This difference in Crkl-deficient lymphoid cells may again be

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**Figure 5.** pYELP peptide leads to G0-G1 cell cycle arrest with reduced cell survival. A, p210BCR-ABL-transformed mouse B-lymphoid cells isolated by Whitlock-Witte culture were treated with either pYELP, control peptide (CTR), or imatinib and cell cycle analysis was performed by DAPI staining using FACS 12 h after the treatment. B, K562 cells were treated with either pYELP or imatinib and cell cycle analysis was performed as described above. The percentage of cells in various stages of the cell cycle is shown to the right of cell cycle profiles. Columns, mean of four independent experiments; bars, SD. *, P < 0.01, significant difference from the control cells.
attributable to their primitive differentiation status (Fig. 2) rather than CRKL-dependent properties. To address this issue, we used pYELP peptide to dissociate CRKL from the p210BCR-ABL protein complex in Wt B-lymphoid cells transformed by p210BCR-ABL. As expected, treatment with the ABL inhibitor imatinib resulted in reduced c-MYC protein levels in B-lymphoid cells derived from Wt fetal liver by p210BCR-ABL (Fig. 7B). When pYELP peptide was introduced into transformed B-lymphoid cells using the carrier peptide PEP-1, c-MYC protein levels were efficiently reduced (Fig. 7B). In contrast, control peptides FELP or YELP without tyrosine phosphorylation had little effect. pYELP peptide also had a similar effect on c-MYC levels in the CML blast crisis K562 cells (Fig. 7C).

Emergence of imatinib-resistant CML cells leads to therapy failure in some patients treated with imatinib (42). Therefore, we tested the effect of pYELP peptide on BaF3 cells expressing p210BCR-ABL Wt or the p210BCR-ABL T315I mutant, which is insensitive to imatinib. We found that imatinib failed to reduce c-MYC levels in cells that express the p210BCR-ABL T315I mutant, whereas treatment with pYELP peptide was able to reduce c-MYC levels in these cells (Fig. 7D). These results provide evidence that signaling pathways mediated by CRKL play an essential role in the induction or maintenance of elevated levels of the oncprotein c-MYC in p210BCR-ABL-transformed cells.

Discussion

Since the discovery that CRKL is a major substrate of BCR-ABL in CML patient neutrophils (19, 20, 43), the role of this signaling protein in p210BCR-ABL leukemogenesis has been the focus of much attention. To address the role of CRKL in transformation by p210BCR-ABL, we analyzed the ability of p210BCR-ABL to transform hematopoietic progenitor cells derived from mice harboring a null mutation in the Crkl locus. In the current study, we used a Crkl-sensitive genetic background in which Crkl deficiency results in a lethal phenotype during late embryogenesis (24). On the contrary, although another Crkl knockout strain was used to assess the role of CRKL in p190BCR-ABL-induced leukemogenesis, the genetic background used was insensitive to CRKL as evidenced by disappearance of an overt phenotype in Crkl−/− homozygous mice (25).

We found that p210BCR-ABL–transduced Crtk−/− progenitors showed a marked decrease in growth factor–independent myeloid colony formation compared with its Wt counterparts. Likewise, we found that compared with Wt, transformation of Crtk−/− progenitor cells by p210BCR-ABL resulted in greatly reduced numbers of lymphoid cells under Whitlock-Witte conditions. BCR-ABL provides both proliferative and survival signals (44, 45), and a reduction in myeloid colony formation; failed outgrowth of B-lymphoid populations in Whitlock-Witte cultures indicates a reduced ability of p210BCR-ABL to promote survival and/or proliferation in Crkl-deficient cells.

Our results suggest that p210BCR-ABL–transformed mouse B-lymphoid cells are particularly sensitive to CRKL-dependent pathways for survival (Fig. 5). Consistent with our results using a CRKL SH2 blocking peptide, p210BCR-ABL–transformed Crtk−/− fetal liver cells outgrown from Whitlock-Witte cultures showed an abnormal cell cycle profile as well as reduced BCL-α levels compared with p210BCR-ABL–transformed Wt counterparts.

Figure 6. CRKL is required for p210BCR-ABL-induced STAT5-dependent downstream molecular cascades. A, cell lysates from p210BCR-ABL–transformed Crkl−/− lymphoid cells were immunoblotted with antibodies recognizing phospho-STAT5 (p-STAT5) and BCL-αL, with actin as a loading control. B, growth rates of BCR-ABL–transformed Wt and Crkl−/− cells. Cells were plated in triplicate, and viable cell counts were determined every 24 h by trypan blue exclusion. C, pYELP inhibits p210-induced upregulation of BCL-αL and tyrosine phosphorylation of STAT5. p210BCR-ABL–transformed mouse B-lymphoid cells isolated by Whitlock-Witte culture and K562 cells were treated with pYELP or control peptides (YELP and FELP). Cell lysates were prepared 12 h after peptide treatment. Immunoblots were probed with anti–BCL-αL, anti–phospho-STAT5 (Y694), or anti-STAT5 antibodies.
These CRKL-deficient lymphoid cultures, however, presented cell markers different from B-lymphoid cells (Fig. 2), thus making it difficult to directly interpret these results in light of a role that CRKL may play in p210BCR-ABL-induced transformation.

Anecdotal evidence for compromised survival included the inability of p210BCR-ABL-transduced Crkl−/− cells to recover from storage in liquid nitrogen or ultralow-temperature freezer, whereas Wt fetal liver counterparts transformed by p210BCR-ABL were easily reestablished from low-temperature storage. This was, however, an obstacle to further investigation of Crkl−/− cells after introduction of p210BCR-ABL by additional experimental manipulations such as transfection.

It has been reported that p210BCR-ABL induces expression of the antiapoptotic gene BCL-2 (39). One explanation for our observations above is that loss of CRKL as a transcriptional coactivator of STAT5 may reduce STAT5-dependent transcription of BCL-2, thus rendering susceptibility to apoptosis. Tyrosine phosphorylation at Y694 or Y699 in STAT5A and STAT5B, respectively, is essential for STAT5 dimerization required for its transcriptional activity (46). Thus, reduction of tyrosine phosphorylation of STAT5 at these sites in p210BCR-ABL-transformed cells on treatment with pYELP peptide likely contributes to increased apoptotic cell death.

CRKL is associated with STAT5 in p210BCR-ABL—expressing cells (47), and glutathione S-transferase fusion proteins carrying either the SH2 or the first of the two SH3 domains of CRKL were able to pull down STAT5 in lysates of cells expressing p210BCR-ABL (40, 48). As the tyrosine phosphorylation site of STAT5 is essential for dimerization required for its transcriptional activity, binding of the CRKL SH2 domain to this site would be counterproductive to the function of STAT5. Although it is possible that the SH3 domain may mediate their physical interaction in the cell, CRKL-STAT5 complexes are not found in cells that do not express p210BCR-ABL. Thus, interactions between CRKL and STAT5 may be stabilized in a tertiary protein complex in which CRKL SH2 domain may also play a role. It remains to be resolved how the CRKL-STAT5 complex is maintained in p210BCR-ABL—expressing cells.

Previous studies have indicated that c-MYC plays an essential role in p210BCR-ABL—mediated transformation (40, 48). The c-MYC oncprotein is overexpressed in many human neoplasms and has been found to regulate cell cycle (41). It is noteworthy that MYC is a putative STAT5 target gene (49, 50). However, p210BCR-ABL can transform STAT5A/STAT5B compound homozygous bone marrow cells (51), thus suggesting that c-MYC may be induced by STAT5-dependent and STAT5-independent pathways downstream of p210BCR-ABL. Our results indicate that disruption of CRKL SH2-mediated interactions results in a reduction of c-MYC protein levels in p210BCR-ABL—expressing cells. Therefore, CRKL is likely involved in multiple pathways. Future studies are warranted to elucidate more precise pathways that the adapter protein CRKL mediates in p210BCR-ABL—induced leukemogenesis. As CRKL SH2 blocking peptide can reduce c-MYC levels in the imatinib-resistant p210BCR-ABL mutant T315I, analysis of CRKL-mediated pathways may provide useful therapeutic targets for treatment of CML patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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