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Crkl Enhances Leukemogenesis in *BCR/ABL* P190 Transgenic Mice[1]

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ABSTRACT

The adapter protein Crkl has been implicated in the abnormal signal transduction pathways activated by the Bcr/Abl oncoprotein, which causes Philadelphia-positive leukemias in humans. To investigate the role of Crkl in tumorigenesis, we have generated transgenic mice that express human Crkl from the *CRKL* promoter. Western blot analysis showed a 4–6-fold overexpression of transgenic Crkl above endogenous crkl in two lines and increased constitutive complex formation between Crkl and C3G, an exchange factor for the small GTPase Rap1. This was associated with a significant increase in integrin-based motility of transgenic macrophages. Overexpression of Crkl was associated with increased incidence of tumor formation, and Rap1 was activated in a metastatic mammary carcinoma. The coexpression of Crkl and Bcr/Abl in mice transgenic for P190 *BCR/ABL* and *CRKL* markedly increased the rapidity of development of leukemia/lymphoma, decreasing the average survival by 3.8 months. These results provide direct evidence that Crkl plays a role in tumor development and is important in the leukemogenesis caused by Bcr/Abl.

INTRODUCTION

Ph[4]-chromosome positive leukemias in humans are characterized by the presence of an abnormal fusion protein, consisting of an NH_2-terminal Bcr moiety fused to the COOH-terminal part of the Abl tyrosine kinase. This protein exhibits a deregulated tyrosine kinase activity that aberrantly phosphorylates different substrates and is directly responsible for the development of leukemia (1, 2). Much effort has been directed toward examining the signal transduction pathways downstream of the deregulated kinase that lead to transformation. Although a growing number of proteins have been shown to bind to the Bcr/Abl protein or are tyrosine-phosphorylated by it when Bcr/Abl is expressed in cells, the physiological significance of this to the leukemia *in vivo* is not always evident.

In 1992, Freed and Hunter (3) described a M_r 41,000 protein that is specifically tyrosine phosphorylated in the leukemic cells of CML patients. The protein was subsequently identified by us as Crkl, an adapter that consists only of an SH2 and two SH3 domains (4). Crkl forms a constitutive complex with Bcr/Abl through the Crkl SH3 domain, which can bind to polyproline-rich regions within Abl (5). Crkl is specifically and constitutively tyrosine phosphorylated in human patient material containing a Bcr/Abl fusion protein but not in normal bone marrow or peripheral blood (6–8). We have established previously (9, 10) a line of transgenic mice to model the leukemia caused by Bcr/Abl P190 *in vivo*. The lymphoblastic leukemia/lymphoma that invariably develops in these mice is characterized by an active P190 Bcr/Abl kinase that tyrosine phosphorylates Crkl (11). In addition, Bcr/Abl phosphorylates tyrosine residues in Cas, Hef1, and Cbl, which subsequently can bind to Crkl via its SH2 domain in the leukemic cells of these mice (12, 13). Many studies (14–16) have shown that the normal cellular function of Crkl is closely associated with signal transduction through membrane-bound receptors for extracellular matrix, growth factors, and cytokines. One study (17) reported that overexpression of Crkl alone allows 293 epithelial cells to grow in soft agar, one characteristic of cellular transformation.

To examine the function of the Crkl-Bcr/Abl interaction, deletion mutants of Bcr/Abl have been made that lack the Crkl-binding sites. One study (17) reported that this mutant has an impaired ability to transform Rat2 fibroblasts. However, others have shown that it retains the ability to generate IL-3-independence in the murine myeloid 32D cell line. Because Crkl was still detected in complex with the Bcr/Abl mutant and became tyrosine phosphorylated, these results suggest that Crkl is an integral component of the transforming signal (18, 19). Antisense oligonucleotides against Crkl inhibited the growth of Ph-positive cell lines established from acute lymphoblastic leukemia and CML patients but not of a leukemia cell line from a Ph-negative patient (20). Cell-penetrating peptides that bind with high selectivity to the first SH3 domain of Crkl strongly inhibited the proliferation of primary blasts from 11 of 16 CML patients (21). Thus, the combined data suggest Crkl as a logical suspect for an important role in transducing the oncogenic signals of Bcr/Abl.

Because leukemogenesis caused by Bcr/Abl P190 in transgenic mice is an excellent *in vivo* model for the signal transduction perturbed by this oncogene, we wished to also address the role of Crkl in this process *in vivo*. To this end, we have generated *CRKL* transgenic mice that express the Crkl protein under control of the *CRKL* promoter. We report in this study that the overexpression of Crkl *in vivo* induces tumorigenesis and markedly enhances the development of lymphoblastic leukemia/lymphoma caused by Bcr/Abl P190. These data implicate Crkl in transduction of the leukemogenic signal of Bcr/Abl.

MATERIALS AND METHODS

***CRKL* Transgenic Construct.** Human genomic clone 70 (22) contains 12 kb of 5′ sequences, exon 1, and 6.6 kb of intron 1 of the *CRKL* gene. A 350-bp *Rsa*I fragment isolated from a *CRKL* cDNA (Ref. 4; GenBank accession no. X59656) was used as a probe to specifically isolate a phage clone, CR-4, containing *CRKL* exon 2 flanked by 6.5 kb of intron 1 and 9.5 kb of intron 2. *CRKL* is located on human chromosome 22 (4) and has been entirely sequenced; introns 1 and 2 are both around 15.5 kb (Ref. 23; sequence ref. NT-001454.20). To generate a transgenic DNA construct, a 7-kb *Sal*I-*Bam*HI fragment from CR-4, which included a *Sal*I site at the 5′ end from the phage polylinker and the 3′ *Bam*HI site located in exon 2, was ligated with a 0.78-kb *Bam*HI-*Eco*RI cDNA fragment, including exons 2, 3, and the 3′ untranslated region, into pSK digested with *Sal*Ix*Eco*RI. The insert was removed as a 7.7-kb *Sal*I-*Not*I fragment and ligated with a 1.2-kb *Sst*II-*Sal*I fragment from intron 1 in clone 70 into pSK digested with *Sst*II × *Not*I. The resulting insert was removed by digestion with *Sst*II × *Not*I. The 5′ promoter and exon 1 sequences were isolated on an 8-kb *Eco*RI-*Sst*II fragment that was subcloned into pSK

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[4] The abbreviations used are: Ph, Philadelphia; CML, chronic myeloid leukemia; RIPA, radioimmunoprecipitation assay; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; JNK, c-Jun-NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

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digested with *Eco*RI × *Sst*II; the insert was removed as an 8-kb *Kpn*I-*Sst*II fragment. The 8-kb *Kpn*I-*Sst*II fragment plus the 7.7-kb *Sst*II-*Not*I fragment were ligated into pSL1180 digested with *Kpn*I × *Not*I. To separate the insert from the vector, the plasmid was digested with *Kpn*I × *Mlu*I and purified via agarose gel electrophoresis as described (24). Transgenic mice were generated at the National Institutes of Child Health and Human Development Transgenic Mouse Development Facility at the University of Alabama at Birmingham. Four founder *CRKL* transgenics were obtained that were bred to B6CBAF1 mice (Jackson Labs). All of the four founders gave germ-line transmission of the transgene. Progeny was tested for transgene expression using Western blots of SDS-sample buffer lysates prepared from different tissues. The one-copy *CRKL* transgenic lines had a low level of transgenic Crkl expression. Subsequent generations that were used in this study were generated by breeding the five- and ten-copy *CRKL* transgenic mice to B6CBAF1 mice.

Mice and Pathology. All of the animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. *BCR/ABL* P190 transgenic mice have been described previously (10, 25, 26). A line of P190 *BCR/ABL* mice established from founder 623 has been bred to obtain animals that contained the transgene on both chromosomes. These mice were mated to the *CRKL* transgenic mice. Ensuing progeny was genotyped, which yielded genotypically matched siblings that were either P190 *BCR/ABL* only transgenics or P190 *BCR/ABL* × *CRKL* double transgenics. A defined cohort of animals of both genotypes was followed. Also, 5- and 10-copy *CRKL* only transgenics and their nontransgenic siblings were monitored.

Autopsies were performed as described (9, 10, 24, 25) on animals that appeared seriously ill. Involved tissues were dissected, and a portion was stored in formalin for routine histological analysis. Another aliquot was used to prepare protein lysates in Triton lysis buffer [25 mM sodium phosphate (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton-X100, 50 mM NaF, 10 μg/ml aprotinin and leupeptin, 1 mM Na_3VO_4, and 1 mM PMSF] or SDS-sample buffer. For analysis of protein levels (CrkII, Crkl, C3G, Cas/Hef1, and Cbl) in the Crkl-only transgenics, mice were sacrificed at 19 days of age. For analysis of Crkl-C3G immunocomplexes, protein lysates were precleared with protein A/G agarose beads (Life Technologies, Inc., Gaithersburg, MD). The supernatant, recovered after centrifugation, was immunoprecipitated with anti-Crkl monoclonal antibodies. Immune complexes were analyzed by SDS-PAGE and immunoblotting using anti-C3G rabbit polyclonal antibodies. For analysis of activated small GTPases, tissue samples were dissected and stored on ice. Tissues were homogenized in a Brinkmann Polytron (10 s; speed setting at 3–4) in an approximate volume of 1 ml of modified RIPA buffer (see below)/18–20 μg of tissue and then briefly homogenized in a straight-wall tissue grinder (Radnoti Glass Technology, Monrovia, CA). After an incubation for 20 min on ice, samples were centrifuged (4°C; 12,500 rpm; SS34; Sorvall), and the supernatant was frozen in aliquots on dry ice and stored at −80°C.

Macrophage Motility Assays. We isolated both bone marrow-derived and elicited peritoneal macrophages. The latter were obtained by injection of 3 ml of a 4% (w/v) solution of thioglycollate and by harvesting of the peritoneal macrophages 6 days later.

Short-term bone marrow-derived macrophage cultures were established essentially as described (27). In short, after isolation of bone marrow cells, 7×10^6 cells were incubated for 24 h in 5 ml of DMEM + 1 nM IL-3 + 0.44 nM CSF-1. Nonadherent cells were collected, Pronase-treated, washed in horse serum, and cultivated in 5 ml of DMEM +1 nM IL-3 + 0.44 nM CSF-1 for 2 days. After a second Pronase and horse serum treatment, cells were cultured for 2 days in DMEM + 4.44 nM CSF-1. Migration assays were performed for 8 h with 5×10^4 cells at 37°C and 8% CO_2 in Transwell plates (8-μm pores; Corning Coster Corp., Cambridge, MA). The bottom of each well was coated with 50 μl of a solution of 10 μg/ml fibronectin (Sigma Chemical Co., St. Louis, MO). Isolation of macrophages and migration assays were performed three times independently with two (elicited macrophages) or one mouse (bone marrow-derived macrophages)/genotype/experiment. Migration assays in each experiment were performed in triplicate. The results with the elicited macrophages were comparable with those of the bone marrow-derived macrophages, except that the absolute number of migrating macrophages in the latter samples was larger.

Cell Lines and Transfections. COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD). All of the tissue culture reagents were from Life Technologies, Inc. To obtain positive controls for detection of activated Ras or Rap1, COS-1 cells were transfected with DNA constructs encoding v-H-Ras or EGFP-V12Rap1 using DEAE/dextran. Mock-transfected COS-1 cells were used as negative controls. Cells were harvested 40–42 h after transfection and lysed in modified RIPA buffer [10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 1 PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] for the detection of activated small GTPases. Lysates were cleared by centrifugation, and the protein concentration was measured using the BCA protein assay (Pierce Chemical Co., Rockford, IL). All of the chemicals were from Sigma Chemical Co. or Life Technologies, Inc. NIH 3T3 cells were kept in DMEM supplemented with 10% FCS , grown until 80% confluency, and then incubated for 40 min with or without 0.4 M sorbitol before the preparation of protein lysates.

Assay for Activated GTPases. Plasmids encoding GST-RalGDS-RBD and GST-Raf1-RBD were obtained from Dr. H. Bos (University Medical Center, Utrecht, The Netherlands). GST-fusion proteins were expressed in *Escherichia coli*. GST-RalGDS-RBD was purified on glutathione agarose, then dialyzed to remove glutathione and stored at −80°C in 50 mM Tris-HCl (pH 8.0), 10% glycerol, and 0.01% NaN_3. GST-RalGDS (50 μg) was precoupled to 50 μl of a 50% slurry of glutathione agarose for 1 h at 4°C. Beads were washed three times in lysis buffer, after which the sample to be assayed was added. Bacterial lysates of GST-Raf1-RBD were obtained by sonication of the bacterial pellet on ice for 6 min in PBS + 0.5 mM DTT, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor. Triton-X100 was added to 1%, and the solution was mixed for 30 min at 4°C. After centrifugation (10 min; 12,500 rpm; Sorvall SS34; 4°C), the supernatant was collected and stored at −80°C after addition of 10% (v/v) glycerol. Bacterial lysate (250 μl) was incubated with 500 μl of a 33% slurry of glutathione agarose for 1 h at 4°C. Beads were washed three times with 1 ml of lysis buffer [50 mM Tris-HCl (pH 7.5), 10% glycerol, 1% NP40, 200 mM NaCl, and 2 mM $MgCl_2$]. Beads were resuspended in 500 μl of lysis buffer + inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor), aliquoted into 50-μl amounts, frozen on dry ice, and stored at −80°C.

Assays for GTP-bound Rap and Ras were essentially as described (28, 29). Briefly, lysates of COS-1 cells or of mouse tissues were thawed quickly and then placed on ice. Precoupled GST-Raf1-RBD or GST-RalGDS-RBD (50 μg) was incubated with 1 mg of tissue lysate or 5 μg (COS-1-transfected with v-H-Ras) or 100 μg (COS-1-transfected with EGFP-V12Rap1) of cell lysate in a final volume of 600 μl in modified RIPA buffer. After incubation, beads were washed three times in lysis buffer. The beads were boiled in SDS-sample buffer; protein samples were separated on a 15% SDS-PAA gel and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). In addition, 2–2.5% of each supernatant after pull-down was included to measure total (GTP- and GDP-bound) small GTPase levels.

Polyclonal rabbit pan-Ras antibodies (KAP-GP001E) were from StressGen Biotechnology Corp. Monoclonal Rap1 antibodies were from Transduction Laboratories (R22020). We obtained rabbit polyclonal antibodies to detect phosphorylated JNK, p38 MAPK, and p44/42 Erk1/2 from New England Biolabs. Rabbit polyclonal p38 MAPK (C-20) antibody, goat polyclonal ERK1 antibodies (K-23), and rabbit polyclonal C3G (C-19) antibody were from Santa Cruz Biotechnology. Peroxidase-conjugated goat antirabbit and goat antimouse antibodies were obtained from Bio-Rad (Hercules, CA). Immunocomplexes were detected by enhanced chemiluminescence and Hyperfilm-enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). When necessary, the filters were stripped using a Re-Blot kit (Chemicon International, Inc., Temecula, CA).

RESULTS

***CRKL* Transgenic Mice.** To generate a transgenic construct that efficiently expresses Crkl, we cloned segments of human genomic DNA that included *CRKL* exons 1 and 2 and joined these together with exon 3 and 3′ untranslated sequences in the form of a segment of *CRKL* cDNA. As shown in Fig. 1*A*, the transgenic construct included around 7 kb of sequences 5′ to *CRKL* exon 1 and around 8 kb of intron 1. We chose the *CRKL* promoter to control transcription of the transgene, because the *crkl* gene is abundantly expressed in hematopoietic cells (11).

Four founder animals were obtained with a transgenic copy number

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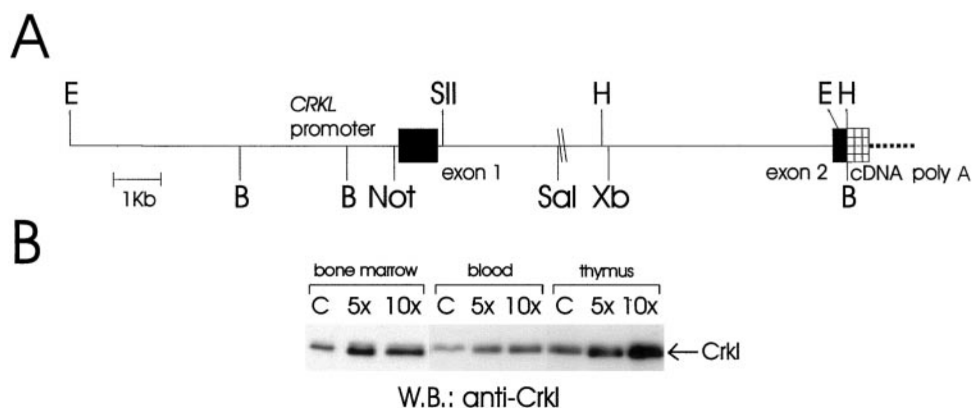

Fig. 1. *CRKL* transgenics. *A,* schematic representation of the *CRKL* transgenic construct. The exons are indicated as *boxes*. Restriction enzymes used include: *B*, *Bam*HI; *H*, *Hin*dIII; *E*, *Eco*RI; *Not*, *Not*I; *Sal*, *Sal*I; *SII*, *Sst*II; *Xb*, *Xba*I. *B*, Western blot analysis of Crkl expression in transgenic hematopoietic tissues. SDS-sample buffer lysates of bone marrow, blood, and thymus (10 μg of protein/lane) of a control mouse and of 5- and 10-copy transgenics (animals 5365, 5366, and 5423; age = 19 days) are shown which were immunoblotted and reacted with an anti-Crkl antiserum (monoclonal 2–2). W.B., Western blot.

of approximately <1 (a chimeric animal), 1, 5, and 10 (data not shown). All of the animals gave germ-line transmission of the transgene when bred with wild-type $B6CBAF_1$ mice. Both female and male mice of the 10-copy transgenic line (8740) had reproductive problems, which are currently under investigation. In brief, litters sired by transgenic males were severely reduced in size (average nest size, 3.4 pups; $n = 20$ nests), and female transgenics failed to nurse their offspring. Several female transgenic animals and wild-type siblings were timed-mated with a B6CBAF1 male. At 15.5–18.5 days of pregnancy and at birth, the size of the litters was determined. The average nest size for the transgenic group was 3.4 pups ($n = 11$ nests) compared with an average nest size of 6.0 pups ($n = 5$) for the wild-type group. However, the transgene was inherited in a normal Mendelian fashion, and there was no evidence of embryonic or postnatal lethality among the transgenic pups, which were also normal size. In addition, adult *CRKL* transgenics had no obvious external phenotype.

We analyzed expression of the transgene in different tissues of progeny of all of the four lines using Western blots. In all of the tissues analyzed, including heart, brain, kidney, spleen, liver, lungs, thymus, peripheral blood, and bone marrow, the expression level was correlated with the transgene copy number (data not shown). When levels of expression in different tissues within one line were compared with those of nontransgenics, the ratios mimicked those of endogenous *crkl*. As shown in Fig. 1*B*, Crkl protein was abundantly expressed in hematopoietic tissues including bone marrow, peripheral blood, and thymus. Expression levels in the 5- and 10-copy transgenics were approximately 4- and 6-fold that of the endogenous crkl protein in thymus. The expression of *CrkII*, a gene related to *Crkl*, was unaffected by Crkl overexpression, nor were differences found in the levels of Cas/Hef1 or Cbl (data not shown) in transgenics compared with nontransgenics.

Enhanced Motility of *CRKL* Transgenic Macrophages. Crkl has been implicated in integrin-mediated adhesion (30–32). Uemura and Griffin (30) demonstrated that in transfected hematopoietic BaF3 cells, overexpression of Crkl leads to increased cell migration. To investigate whether Crkl is involved in hematopoietic cell migration *in vivo*, we isolated peritoneal macrophages as well as bone marrow-derived monocyte/macrophage cells from 10-copy *CRKL* transgenics and from control nontransgenic siblings. Migration assays of these macrophages on fibronectin-coated Transwell plates showed that the haptotactic migration of both elicited (data not shown) and bone marrow-derived macrophages (Fig. 2*A*) on fibronectin was significantly increased.

Cotransfection of Crkl with the nucleotide exchange factor C3G in hematopoietic cells was shown to enhance the cell migration stimulated by Crkl overexpression (31). Therefore, we asked whether there was evidence for increased association of Crkl with C3G in the *CRKL* transgenics. Protein lysates were prepared from a 1- and a 10-copy *CRKL*/P190 *BCR/ABL* double transgenic, and Crkl was immunoprecipitated using monoclonal anti-Crkl antibodies. Western blotting with anti-C3G antiserum showed that the level of C3G was unaffected by Crkl overexpression (Fig. 2*B*, *lysates*). However, there were significantly more Crkl-C3G complexes in the 10-copy as compared with the 1-copy CRKL transgenic sample (Fig. 2*B*, *Crkl I.P.*). These results demonstrate that, *in vivo*, the involvement of Crkl and C3G in integrin-mediated motility in hematopoietic cells is biologically relevant.

Phenotype of *CRKL* Transgenics. Founders as well as the progeny of the 5- and 10-copy *CRKL* lines, including both transgenic and nontransgenic siblings of both sexes, were followed until the animals appeared seriously ill, at which point in time autopsies were performed. The major features found only in the *CRKL* transgenics are listed in Table 1. This analysis showed that the overexpression of Crkl protein was associated with an increased incidence of tumorigenesis in transgenic mice.

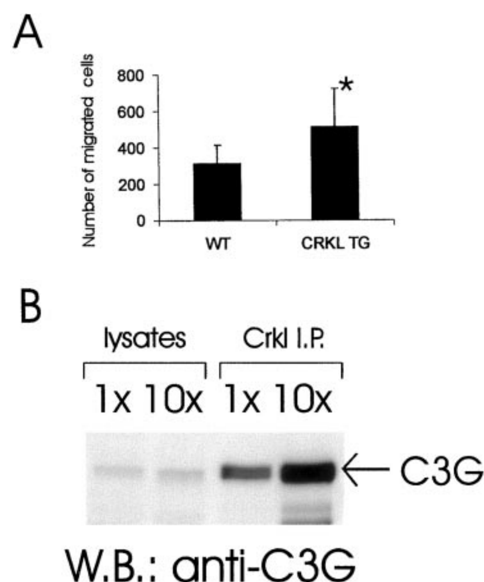

Fig. 2. Enhanced motility of *CRKL* transgenic macrophages. *A*, bone marrow-derived macrophages from control wild-type (*WT*) siblings or of 10-copy *CRKL* transgenic mice were assayed for haptotactic migration in fibronectin-coated Transwells. The results shown are the mean of three independently performed experiments. Each experiment included triplicate samples. The difference shown is statistically significant with a *P* of 0.05 (Wilcoxon's rank-sum test). *B*, immunoblot analysis of Crkl-C3G complex formation. Protein lysates (1.25 mg of lymphoma tissue) were immunoprecipitated with monoclonal anti-Crkl 2–2/5–6 antibodies and reacted with anti-C3G antibodies. W.B., Western blot.

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Table 1. *CRKL* transgenic mice

Identifier	Copy no.	Age at death	Diagnosis
8742	1×	19 mo	Germinal center lymphoma
8731	1×	22 mo	Primary pulmonary hypertension
5721	1×	23 mo	Adenoma of the lung
8746	5×	8 mo	Myeloid hyperplasia in bone marrow
5714	5×	19 mo	Lymphoma and/or osteomyelosclerosis with extramedullary hematopoiesis
8740	10×	19 mo	Congested seminal vesicle
5728	10×	10 mo	High grade anaplastic lymphoma
5632	10×	10 mo	Fibrosarcoma or malignant fibrous histiocytoma
5729	10×	17 mo	Mammary adenocarcinoma
5619	10×	26 mo	Adenocarcinoma

For example, 10-copy animal 5728 developed a large mesenteric lymphoma at the age of 10 months. Involvement of bone marrow, spleen, and liver (Fig. 3*A*) were seen. The animal was diagnosed with a high-grade anaplastic lymphoma. In animal 5632 (10-copy transgenic), we found two large s.c. tumors in close proximity to the spine at 10 months of age. Histology revealed a fibrosarcoma/malignant fibrous histiosarcoma (Fig. 3*B*). In another 10-copy animal, 5619, we found metastases in kidney, liver, spleen, and mesenteric lymph node (Fig. 3*C*) of an adenocarcinoma with unknown primarius at the age of 26 months. At 17 months, transgenic 5729 (10-copy animal) died of a mammary adenocarcinoma that was widely disseminated to other organs (Fig. 3, *D* and *E*).

Metastasis of mammary carcinomas is extremely rare in mice. Therefore, we investigated signal transduction processes in these tumors by preparing protein lysates from the primary tumor and a metastasis to the mesenteric lymph node. There was no detectable activation of the JNK pathway in these samples, as measured by the lack of signal with a JNK phosphospecific antibody (Fig. 4*A*). In contrast, both ERK1/2 and p38 MAPK were very markedly phosphorylated in the primary tumor and also in the metastasis (Fig. 4*A*). Then, we investigated the primary tumor for activation of small GTPases. Samples that were prepared as described in "Materials and Methods" were incubated with GST-Raf1-RBD to detect Ras in its GTP-bound form. As a positive methodological control, we transfected COS-1 cells with v-H-Ras, a mutated form of Ras that is constitutively GTP-bound. As shown in Fig. 4*B*, the transfected COS-1 cells expressed high levels of GTP-bound Ras, whereas no GTP-Ras was detectable in mock-transfected COS-1 cells. However, the mammary carcinoma did contain a small amount of active Ras in comparison with the transfectant. As described above, there is increased complex formation between Crkl and C3G, an exchange factor for the small GTPase Rap1 (33) in the *CRKL* transgenic mice. Therefore, we asked

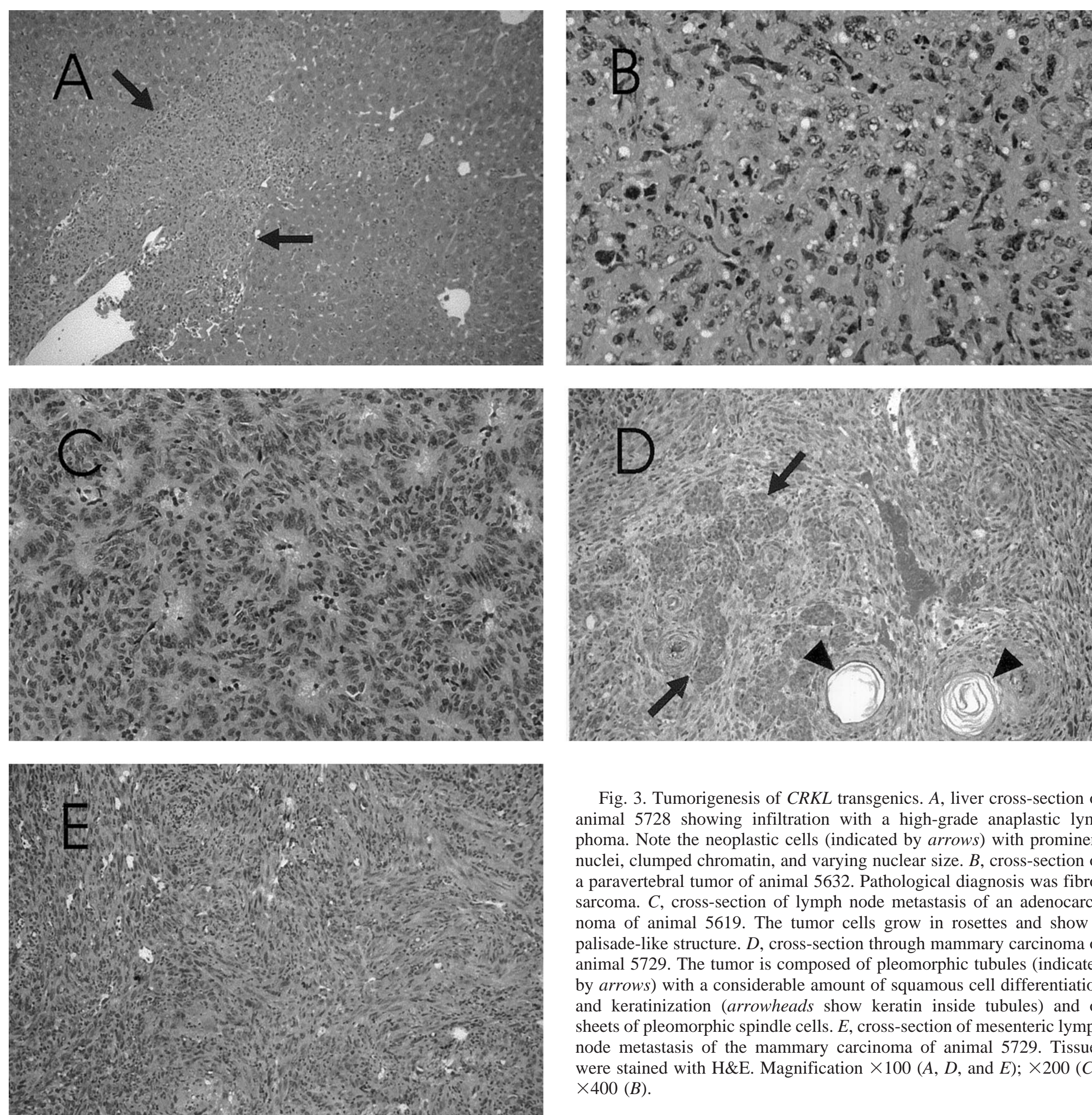

Fig. 3. Tumorigenesis of *CRKL* transgenics. *A*, liver cross-section of animal 5728 showing infiltration with a high-grade anaplastic lymphoma. Note the neoplastic cells (indicated by *arrows*) with prominent nuclei, clumped chromatin, and varying nuclear size. *B*, cross-section of a paravertebral tumor of animal 5632. Pathological diagnosis was fibrosarcoma. *C*, cross-section of lymph node metastasis of an adenocarcinoma of animal 5619. The tumor cells grow in rosettes and show a palisade-like structure. *D*, cross-section through mammary carcinoma of animal 5729. The tumor is composed of pleomorphic tubules (indicated by *arrows*) with a considerable amount of squamous cell differentiation and keratinization (*arrowheads* show keratin inside tubules) and of sheets of pleomorphic spindle cells. *E*, cross-section of mesenteric lymph node metastasis of the mammary carcinoma of animal 5729. Tissues were stained with H&E. Magnification ×100 (*A*, *D*, and *E*); ×200 (*C*); ×400 (*B*).

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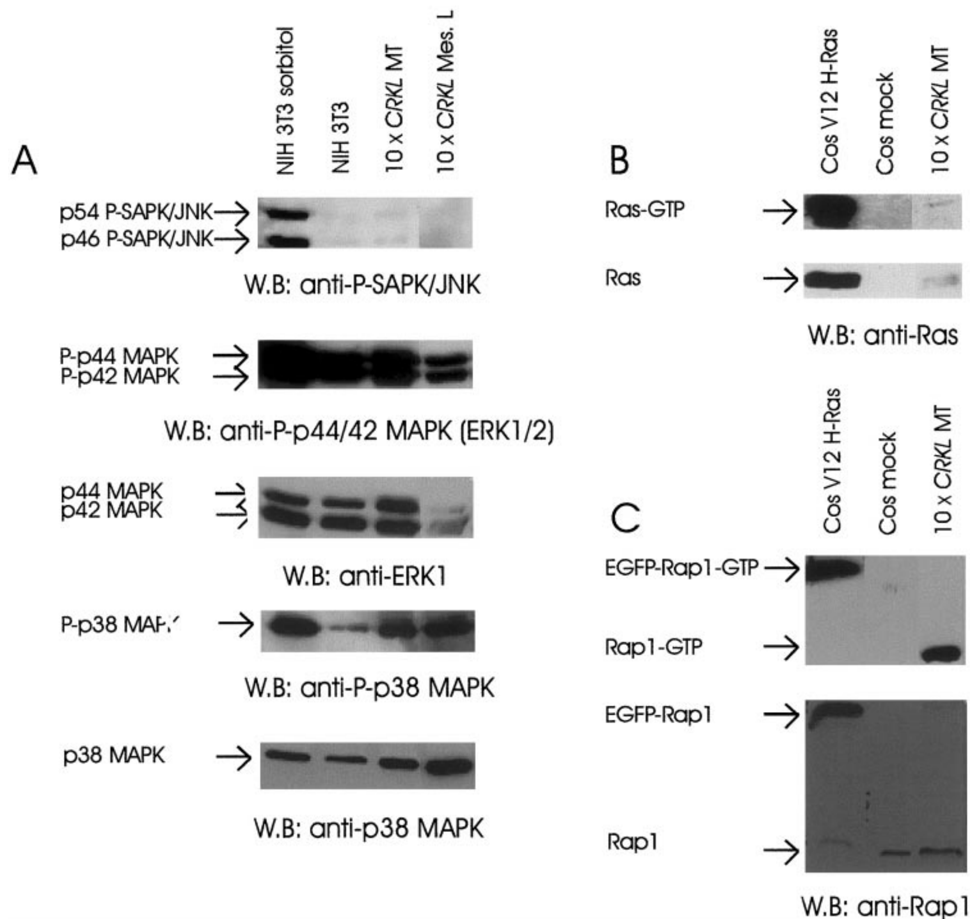

Fig. 4. Analysis of metastatic mammary carcinoma in 10-copy *CRKL* transgenic for activation of signaling pathways. *A*, protein samples (30 μg/lane) prepared in Triton-lysis buffer were run on a 12% SDS-PAA gel and reacted with the antibodies indicated underneath each panel. *B*, samples prepared in modified RIPA buffer were incubated with GST-Raf-RBD coupled to glutathione agarose. Precipitates were analyzed for the presence of GTP-bound Ras using a pan-Ras monoclonal antibody (*top panel*) or total (GDP- and GTP-bound) Ras (*bottom panel*), which represents 2% of the supernatant after the pull-down reaction. *C,* modified RIPA buffer lysates were incubated with GST-Ral-GDS to detect GTP-bound Rap1 (*top panel*). After pull-down, 2.5% of the supernatant was loaded to show the total (GDP- and GTP-bound) Rap1 levels in the sample (*bottom panel*). *Cos mock*, mock-transfected COS-1 cells; *10 ×* CRKL MT*,* mammary carcinoma of 10-copy *CRKL* transgenic; 10 × *CRKL Mes. L*, metastasis of mammary carcinoma to mesenteric lymph node. W.B., Western blot.

whether we could detect activated Rap1 in the mammary carcinoma. As positive and negative controls for Rap1 activation, we used lysates prepared from COS-1 cells transfected with EGFP-tagged V12Rap1 or from mock-transfected COS-1 cells. We could clearly detect GTP-Rap1 in the transfected COS-1 cells (Fig. 4*C*, *top panel*). Remarkably, the mammary carcinoma sample contained very high levels of GTP-bound Rap1.

Crkl Increases Speed of Leukemogenesis Caused by Bcr/Abl P190. To investigate whether Crkl expression would influence the development of lymphoblastic leukemia/lymphoma caused by Bcr/Abl P190, we bred the 5- and 10-copy *CRKL* transgenic founders with *BCR/ABL* P190 "homozygous" transgenics. The F_1 generation thus obtained consisted of genetically matched P190 *BCR/ABL* single transgenics and P190 *BCR/ABL* × *CRKL* double transgenic siblings. These were monitored for their entire life span for the development of tumors. All of the animals died of lymphoblastic leukemia/lymphoma typical of P190 transgenic mice. The cumulative mortality of these mice is shown in Fig. 5. The presence of the *CRKL* transgene markedly enhanced the rate with which leukemia/lymphoma developed in the P190 *BCR/ABL* transgenic mice. The average age at death of the P190 *BCR/ABL* transgenics was 413 days, whereas this was 300 days for the P190 *BCR/*ABL × *CRKL* transgenics.

We prepared lysates from end-stage involved tissue (lymphomas) to investigate whether the P190 tumors with or without overexpressed Crkl were significantly different in activation of the major MAPK pathways. As shown in Fig. 6*A*, there was no evidence that JNK was activated either in the P190 or in the P190 + Crkl-expressing lymphomas, although a positive control consisting of NIH 3T3 cells stimulated with sorbitol clearly showed that phosphorylated JNK could be detected. In contrast, the MAPKs p44 and p42 were phosphorylated in both types of lymphomas (Fig. 6*B*). Similarly, we could detect phosphorylated p38 MAPK in both samples (Fig. 6*D*). There were no detectable levels of GTP-bound Rap1 (data not shown).

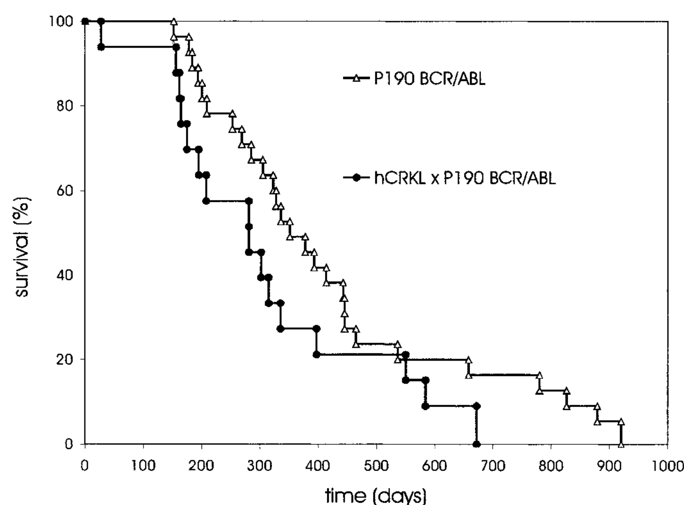

Fig. 5. The development of leukemia/lymphoma is enhanced by overexpression of Crkl in P190 *BCR/ABL* transgenic mice. The survival of P190 *BCR/ABL* only transgenics (n = 27; 11 males, 16 females) and that of P190 *BCR/ABL* × *CRKL* double transgenics (n = 16; 11 males, 5 females) is compared. The double transgenic group included both 5- and 10-copy *CRKL* transgenics. The average age of death for the P190 *BCR/ABL* × *CRKL* transgenic group was 300 days, whereas the P190 *BCR/ABL* transgenic animals had a mean survival time of 413 days.

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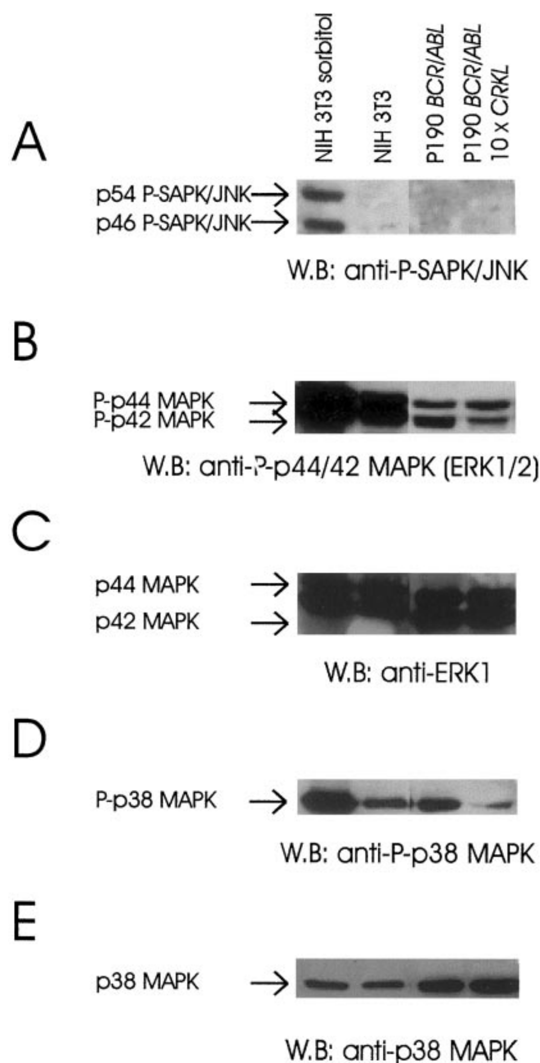

Fig. 6. Activation of Erk1/2 and p38 MAPK in P190 Bcr/Abl and P190 Bcr/Abl + Crkl-expressing lymphomas. Proteins in Triton-lysis buffer were analyzed on 12% SDS-PAA gels using the antibodies indicated below each panel. Each lane contains 30 μg of protein. The samples are indicated above the lanes in *Panel A* and include: *NIH 3T3 sorbitol*, NIH 3T3 cells treated with 0.4 M sorbitol; *NIH 3T3*, control nonsorbitol treated cells; *P190 BCR/ABL*, lymphoma of a P190 *BCR/ABL* transgenic; *P190 BCR/ABL 10 ×* CRKL, lymphoma of a P190 *BCR/ABL* × 10-copy *CRKL* transgenic. W.B., Western blot.

DISCUSSION

Previous data have provided indirect evidence that Crkl is involved in tumorigenesis. Crkl is related to Crk, the cellular homologue of the oncogene product v-Crk of the avian sarcoma retrovirus CT10. V-Crk is able to transform chicken embryonic fibroblasts and NIH 3T3 cells (34). Only one study (17) thus far has examined the possible transforming properties of Crkl and found that its overexpression in Rat-1 fibroblasts resulted in colony formation in soft agar. However, we have not been able to detect focus formation in NIH 3T3 cells transfected with wild-type Crkl nor were transfected cells able to grow in soft agar (35).

We have generated transgenic mice overexpressing Crkl to investigate the role of this adapter in cancer and found that several *CRKL* transgenics from distinct lines developed different types of tumors. However, there was a low incidence of tumorigenesis, and tumors that developed occurred later in life. Therefore, our findings do not support the concept that Crkl is oncogenic *in vivo* when overexpressed. Instead, it is more likely that other events lead to the onset of tumorigenesis and that overexpression of Crkl subsequently enhances this process.

How could Crkl contribute to tumor progression? Previous experiments (30–32, 36) using cell lines have linked Crkl and the related Crk to cell motility and integrin-mediated adhesion. The assembly of a complex between Crk and the scaffolding protein Cas serves as a molecular switch for the induction of cell migration of the human pancreatic carcinoma cell line FG-M (36). Uemura and Griffin (30) showed that overexpression of Crkl increased the spontaneous migration of murine pre-B Ba/F3 cells 2.8-fold. In the current study, we have found that, *in vivo*, *CRKL* transgenic macrophages show increased motility. In this context, it is of interest that all of the tumors that we have found thus far in the 5- and 10-copy *CRKL* transgenics are highly metastatic. Therefore, we suggest that the contribution of Crkl is to enhance the ability of the tumor cells to migrate from the site of the primary tumor to secondary locations.

There are several signaling pathways associated with Crkl that could transduce such signals. Crkl has been shown to form a constitutive complex with C3G in many cell types (37–39). This exchange factor for Rap1 and R-Ras stimulates the conversion of these small GTPases into their active, GTP-bound form (33, 40). R-Ras is involved in the regulation of integrin activation (41) and cell adhesion (42). Decreased adhesion is thought to promote cell invasion and tumor progression. C3G itself has also directly been implicated in cell motility, because the increased migration of BaF3 cells transfected with Crkl was enhanced even more when C3G was cotransfected into these cells (30). Hepatocyte growth factor/scatter factor induces motility and scattering of c-Met-expressing epithelial cell lines (43). Stimulation of a human embryonic kidney cell line with hepatocyte growth factor/scatter factor results in recruitment of Crkl to the c-Met receptor via a scaffolding complex and in the rapid activation of Rap1. Thus, the increased levels of constitutive complexes between C3G and Crkl that we observed in the *CRKL* transgenic tissue lysates could be a mechanism by which motility is enhanced. Similarly, the greatly increased levels of the GTP-bound form of Rap1 that we found in the highly metastatic mammary carcinoma could affect integrin-mediated adhesion and motility of these cells.

We examined the effect of Crkl *in vivo* on leukemogenesis caused by Bcr/Abl and found that it decreases the life span by an average of 3.8 months. Because mice have a maximal life span of around 36 months, this represents a very marked reduction in life expectancy. On a molecular level, we have examined possible activation of signaling pathways *in vivo* in the lymphomas but detected no differences between lymphomas of P190 only and P190 + Crkl-overexpressing transgenics. This is not entirely unexpected, because lymphomas represent end-stage metastatic disease, in which the Bcr/Abl-expressing cells have already accumulated all of the mutations they need to be fully oncogenic.

Previous experiments (44) have shown that Ras becomes activated in Bcr/Abl-transfected fibroblasts and hematopoietic cell lines, and a number of studies (45–48) have investigated the multiple pathways used by Bcr/Abl to activate Ras. In concordance with this, we have detected Ras activation in lymphomas of P190 *BCR/ABL* transgenic mice[5] and, in the current study, we find that downstream signaling mediators of Ras, ERK1/2, become phosphorylated both in tumors expressing P190 alone and in tumors expressing P190 and Crkl. Because this was also found in the mammary carcinoma of the *CRKL* transgenic mouse, phosphoryl-

[5] N. Heisterkamp, J. W. Voncken, D. Senadheera, B. Hemmeryckx, I. Gonzalez-Gomez, A. Reichert, P. K. Pattengale, and J. Groffen. The Bcr *N*-terminal oligomerization domain contributes to the full oncogenicity of Pi90 Bcr/Abl in transgenic mice, manuscript in preparation.

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ation of ERK1/2 may occur more commonly in tumorigenesis and may be a general indicator of increased mitogenesis. However, in contrast to what was reported for Bcr/Abl-transfected murine myeloid DAGM cells (48), we could not detect prominent signs of JNK activation in the P190 lymphomas. This may reflect differences in the cell type involved (myeloid *versus* lymphoid) or other differences between a cell line and an animal model.

In CML, patients invariably progress from chronic phase to blast crisis. A relatively large percentage of patients have a duplicated Ph-chromosome and, because of this, an extra copy of the *BCR/ABL* gene. Because *CRKL* is located on chromosome 22q11 proximal to the t(9;22) breakpoint, it remains on the Ph-chromosome and will also be duplicated in patients with an extra Ph-chromosome (4). Our current results suggest that in such patients, *CRKL* gene dosage may be a contributing factor in disease progression.

Hematopoiesis is a steady state process occurring in the bone marrow microenvironment, in which hematopoietic stem cells selectively adhere to the stroma and to the extracellular matrix. The proliferation, differentiation, and maturation of the progenitor cells in this environment are tightly regulated by growth factors, cytokines, and adhesion molecules expressed on stroma cells and the extracellular matrix (49). In CML patients, the hematopoietic stem cells are released prematurely from the bone marrow because of an apparent defect in the interaction with the microenvironment. Also, in contrast to their normal counterparts, CML progenitor cells continue to proliferate when in contact with bone marrow stroma (50). Compared with normal hematopoietic stem cells, stem cells from CML patients show decreased colony formation after adhesion to bone marrow stroma and/or fibronectin (51, 52). It is evident that motile cells will show diminished stable adhesion. Therefore, based on the current *in vivo* results, we speculate that the role of Crkl in disease progression of Bcr/Abl-positive leukemia is to decrease stable adhesion of the Bcr/Abl-positive cells to stroma and to increase motility.

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