

Deletion of the Src Homology 3 Domain and C-terminal Proline-rich Sequences in Bcr-Abl Prevents Abl Interactor 2 Degradation and Spontaneous Cell Migration and Impairs Leukemogenesis*

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The hematopoietic cells from patients with Bcr-Abl-positive chronic myelogenous leukemia exhibit multiple abnormalities of cytoskeletal function. The molecular events leading to these abnormalities are not fully understood. Previously we showed that Bcr-Abl elicits ubiquitin-dependent degradation of Abl interactor proteins. Because recent studies have suggested a role of Abl interactor proteins in the pathway that regulates cytoskeletal function, we investigated whether mutations in Bcr-Abl that interfere with the signaling to Abl interactor proteins affect its leukemogenic activity. We report here that the Src homology 3 domain and C-terminal proline-rich sequences of Bcr-Abl are required for its binding to Abl interactor 2 as well as for the induction of Abl interactor 2 degradation. Although the deletion of these regions did not affect the ability of the mutant Bcr-Abl to transform hematopoietic cells to growth factor independence, it abrogated its ability to stimulate spontaneous cell migration on fibronectin-coated surfaces. Furthermore, the mutant Bcr-Abl, defective in binding to Abl interactor 2 and inducing its degradation, failed to induce chronic myelogenous leukemia-like disease in mouse. These results are consistent with a role of Abl interactor proteins in the regulation of cytoskeletal function as well as in the pathogenesis of Bcr-Abl-induced leukemogenesis.

Bcr-Abl is generated by a reciprocal t(9;22)(q34;q11) chromosome translocation that fuses varying amounts of the *breakpoint cluster region* (*bcr*) gene on chromosome 22 with sequences upstream of the second exon of *c-abl* on chromosome 9. Depending on the amount of *bcr* sequences fused, three different Bcr-Abl fusion proteins with molecular masses of 185 kDa (p185^{Bcr-Abl}), 210 kDa (p210^{Bcr-Abl}), and 230 kDa (p230^{Bcr-Abl}) may be produced (1–3). Expression of Bcr-Abl is associated with greater than 95% of human chronic myelogenous leukemia (CML)¹ and ~20% of acute lymphocytic leukemia cases. This is an open access article under the [CC BY](http://creativecommons.org/licenses/by/4.0/) license.

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¹ The abbreviations used are: CML, chronic myelogenous leukemia; Abi, Abl interactor; BM, bone marrow; BMT, bone marrow transplantation; GST, glutathione S-transferase; SH, Src homology; WBC, white blood cell; IL, interleukin; wt, wild type.

(1–3). Mice transgenic for *Bcr-Abl* (4) or reconstituted with *Bcr-Abl*-transduced bone marrow (BM) cells (5–8) developed leukemia that recapitulated many aspects of human CML. The inducible expression of Bcr-Abl in transgenic mice demonstrated that Bcr-Abl is required for both induction and maintenance of the leukemia (9). Although these data provide strong evidence to support a direct role of Bcr-Abl in leukemogenesis, it remains unclear how the oncogenic activation of a single proto-oncogene induces malignancy *in vivo* with comprehensive changes in hematopoietic cell growth, differentiation, and homing. *In vitro* studies of Bcr-Abl-transformed cells suggest that the expression of Bcr-Abl promotes cell proliferation, enhances cell survival, and alters cell adhesion and migration (2, 3, 10–12). How the changes in these fundamental cellular processes in Bcr-Abl-expressing cells lead to the clinical phenotype of CML remains largely unknown.

Bcr-Abl proteins contain multiple domains important in interactions with other cellular proteins involved in the regulation of mitogenic and apoptotic pathways (2, 3). They also contain domains and motifs capable of binding to cytoskeleton proteins as well as the proteins involved in regulation of cell adhesion and migration (13–16). Previously we and others identified Abl interactor (Abi) proteins that bind to both the Src homology 3 (SH3) domain and the C-terminal proline-rich regions of Abl kinase through dual SH3-PXXP interactions (17, 18). Two highly related genes, *abi-1* and *abi-2*, were cloned. The corresponding protein products share overall 69% identity with the greatest homology observed in the N-terminal homeobox-like domain, proline-rich sequences, and the C-terminal SH3 domain (17, 18). In addition to the interaction with Abl kinase, Abi proteins also interact with other signaling molecules such as the *Abl-related gene* product Arg (19), epidermal growth factor receptor substrate Eps8 (20), the cytoskeleton protein spectrin (21), and the guanine nucleotide exchange factor Sos (22, 23). Although little is known about the function of Abi, recent studies suggested a role of Abi in the regulation of signal transduction mediated by small GTP-binding proteins. Abi-1 (also known as e3b1) forms a tricomplex with Eps8 and Sos-1 *in vivo* and regulates Rac-specific guanine nucleotide exchange factor activities *in vitro* (22). It was therefore proposed that Abi-1 is an important player in the transduction of signals from Ras to Rac, a pathway important in the regulation of cytoskeletal function. Consistent with this proposal, microinjection of fibroblasts with anti-Abi-1 antibodies resulted in abrogation of Rac-dependent membrane ruffling in response to platelet-derived growth factor stimulation (22).

The involvement of Abi proteins in signaling mediated by the wild type and oncogenic forms of Abl kinases is suggested by several lines of evidence. Abi proteins bind to c-Abl and are substrates of the Abl kinases (17, 18). Overexpression of Abi-1

potently suppresses the transforming activity of viral Abl (v-Abl) in NIH3T3 fibroblasts by inhibiting the v-Abl-stimulated extracellular signal-regulated kinase pathway (18, 23). More recently we reported that Bcr-Abl elicits the ubiquitin-dependent degradation of Abi-2 (24). Significantly, the expression of Abi-2 is lost in cell lines and bone marrow cells isolated from patients with aggressive Bcr-Abl-positive leukemia (24). These data are consistent with a hypothesis that the loss of Abi-2 may play a role in the progression of Bcr-Abl-positive leukemias. To test this hypothesis, we mapped the sequences in Bcr-Abl that are required for its interaction with Abi-2. This enabled the generation of mutant forms of Bcr-Abl defective in binding to Abi-2 and therefore allowed the evaluation of the effects of the mutations on Bcr-Abl-induced cellular transformation and leukemogenesis. In this report we show that the deletion of the SH3 domain and C-terminal proline-rich sequences in Bcr-Abl not only abrogates its interaction with Abi-2 but also prevents Abi-2 degradation, prevents spontaneous cell migration on a fibronectin-coated surface, and impairs leukemogenesis.

MATERIALS AND METHODS

Cell Culture—BaF3 cells and 32D cells were grown in RPMI medium containing 10% fetal bovine serum and 10% WEHI-3-conditioned medium as a source of IL-3. The primary bone marrow cells obtained from control and diseased BMT mice were cultured in minimum essential medium α medium containing 20% fetal bovine serum with or without added growth factors for 5 days before being subjected to Western blot analysis.

Retroviral Constructs—The retroviral vectors MIGR1 and MSCV were kindly provided by Dr. W. S. Pear (University of Pennsylvania, Philadelphia, PA) and Dr. R. G. Hawley (University of Toronto, Toronto, Canada), respectively. To construct retroviral vectors expressing p185^{wt} and p185^{ASH3}, the cDNA fragments encoding p185^{wt} and p185^{ASH3} (deletion of amino acids 414–519) were released from pGEMp185^{Bcr-Abl} and pGEMp185^{ASH3} (25), respectively, by restriction digestion with *EcoRI*. The purified cDNA fragments were then ligated to MIGR1 or MSCV at the *EcoRI* site. To generate retroviral vectors expressing p185^{AC} and p185^{ASH3AC}, pGEMp185^{Bcr-Abl} and pGEMp185^{ASH3} were digested with *AatII*, which released 5' cDNA sequences encoding amino acids 1–819 of p185^{Bcr-Abl} or amino acids 1–714 of p185^{ASH3}, respectively. The purified cDNA fragments were ligated to pGEMAb1 Δ SH3 Δ 544–637 (17) at the *AatII* site to replace 5' sequences of Abl. The resulting plasmids, designated pGEMp185^{AC} and pGEMp185^{ASH3AC}, respectively, were digested with *EcoRI*, which released the cDNA fragments encoding p185^{AC} and p185^{ASH3AC}. The released cDNA fragments were purified and ligated to MIGR1 and MSCV at the *EcoRI* site.

Generation of Retrovirus Stock—The retroviral packaging cell lines GP+E-86 and Bosc 23, kindly provided by Drs. A. Bank (Columbia University, New York, NY) and W. S. Pear (University of Pennsylvania), respectively, were used to generate retroviral stocks as described previously (26). The retroviral stocks were stored at –80 °C until use. To determine the titers of retroviral supernatants, a serial dilution was made and used to transduce NIH3T3 cells as described previously (27). After 10 days of selection with G418, the stable transfectants were counted, and titers were calculated.

Retroviral Transduction and Bone Marrow Transplantation—Stable mass populations of BaF3 cells expressing wild type and mutant forms of Bcr-Abl were generated by retroviral transduction as described previously (17). Retroviral transduction of mouse bone marrow cells was performed as described previously (27) with minor modifications. Briefly, 8–12-week-old male (C57BL/6J \times DBA/2J) F1 (BDF1) mice (Charles River Laboratories, Wilmington, MA) were treated with 5-fluorouracil (150 mg/kg of body weight) 4 days prior to marrow harvest. Marrow cells were incubated with retroviral supernatants (6×10^5 cells/ml) containing 15% fetal bovine serum, 6 μ g/ml Polybrene, 0.1% bovine serum albumin, 2.5 ng/ml IL-3 (PeproTech, Inc., Rocky Hill, NJ), 100 ng/ml IL-6, 100 ng/ml IL-11, and 100 ng/ml recombinant rat stem cell factor at 37 °C in 5% CO₂ for 3 days. The retroviral supernatants were replaced daily with fresh supernatants. The transduced BM cells were washed with phosphate-buffered saline and were injected into lethally irradiated (9.5 grays) syngeneic female mice (6–8 weeks old) through the tail vein at 4×10^5 cells/mouse.

Soft Agar Colony Formation Assay—Double layer agar cultures in 35-mm dishes were established as described previously (28). The min-

imum essential medium α medium supplemented with 20% fetal bovine serum was used for all cultures. Cultures were incubated at 37 °C in 5% CO₂ for 14 days after which colonies containing greater than 50 cells were scored using a dissecting microscope.

Pathology Examination—Two weeks after bone marrow transplantation, mice were monitored for disease development. Mouse peripheral blood samples were obtained under anesthesia by retro-orbital puncture. A differential count of white blood cells (WBCs) was performed using the Advia 120 Hematology System (Bayer Inc., Tarrytown, NY) with veterinary software for analysis of mouse blood. Disease development was judged by elevated WBCs as well as symptoms such as abnormal gait and labored breathing. Moribund animals were sacrificed by CO₂ asphyxiation and were examined for tumors or other visible abnormalities. Collection of spleens, livers, and bone marrow cells was performed immediately after sacrifice. All protocols used were approved by institutional review committees at the University of Colorado Health Sciences Center.

Antibodies and Immunoblotting—As described previously (17), anti-Abi-2 antibody was raised to a recombinant glutathione *S*-transferase (GST)-Abi-2 Δ 1–100 fusion protein. The antibody was affinity-purified by a standard technique (29). Immunoblots were performed as described previously (17) using anti-Abi-2 antibody, 8E9 anti-abl monoclonal antibody (PharMingen, San Diego, CA), and mixtures of antiphosphotyrosine antibodies PY20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PY69 (ICN Biomedicals, Inc.), and 4G10 (Upstate Biotechnology, Inc., Waltham, MA) as indicated in the text.

Binding Assays—Bosc 23 cells were transiently transfected with MSCV, MSCVp185^{wt}, MSCVp185^{ASH3}, MSCVp185^{AC}, and MSCVp185^{ASH3AC}. Two days after transfection, cells were lysed and incubated with GST or GST-Abi-2 fusion protein bound to glutathione-Sepharose beads as described previously (17). Bound proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with anti-Abl antibody, and visualized by the enhanced chemiluminescence detection system.

Cell Migration Assay—The spontaneous cell migration assay was performed as described previously (30). The inserts of Transwell plates (0.33-cm² growth area, 8- μ m pores; Corning Costar Corp., Cambridge, MA) were coated with human fibronectin (Sigma Chemical Co.). The bottom chambers of the Transwell plates contained 600 μ l of RPMI medium plus 1% bovine serum albumin. The BaF3 cells transduced with MIGR1 vector or MIGR1 containing cDNAs for wild type and mutant forms of Bcr-Abl were starved in RPMI medium containing 1% bovine serum albumin for 6 h. The cells were resuspended in RPMI medium containing 1% bovine serum albumin at a concentration of 1×10^6 cells/ml. One hundred microliters of cells were added into the inserts and allowed to migrate into the bottom chambers for 6–8 h.

RESULTS

Deletion of the SH3 Domain and C-terminal Proline-rich Sequences in Bcr-Abl Abrogates Its Interaction with Abi and Prevents the Induction of Abi Degradation—c-Abl binds to Abi proteins through dual SH3-PXXP interactions (17, 18). To determine whether the direct interaction is required for Bcr-Abl to induce Abi-2 degradation, retroviral vectors containing cDNAs for wild type (p185^{wt}) and mutant forms of p185^{Bcr-Abl} with deletions in the SH3 domain (p185^{ASH3}), C-terminal proline-rich sequences (p185^{AC}), or both SH3 and C-terminal proline-rich regions (p185^{ASH3AC}) were generated (Fig. 1A). The abilities of these wild type and mutant forms of p185^{Bcr-Abl} to bind to Abi-2 were tested by an *in vitro* binding assay. The p185^{wt} bound to Abi-2 because it was detected in GST-Abi-2 precipitates by anti-Abl Western blot analysis (Fig. 1B, lane 6). In addition to full-length p185^{wt}, proteins with faster mobility were also detected by the anti-Abl antibody in GST-Abi-2 precipitates. These are likely the truncated forms of Bcr-Abl that underwent degradation during incubation because they were also detectable in immunoprecipitates by anti-Abl antibody (data not shown). The deletion of the SH3 domain did not affect binding of p185^{ASH3} to Abi-2 (Fig. 1B, lane 9). The deletion of the C-terminal proline-rich sequences reduced, but did not abrogate, binding of p185^{AC} to Abi-2 (Fig. 1B, lane 12). In contrast, deletion of both the SH3 domain and C-terminal

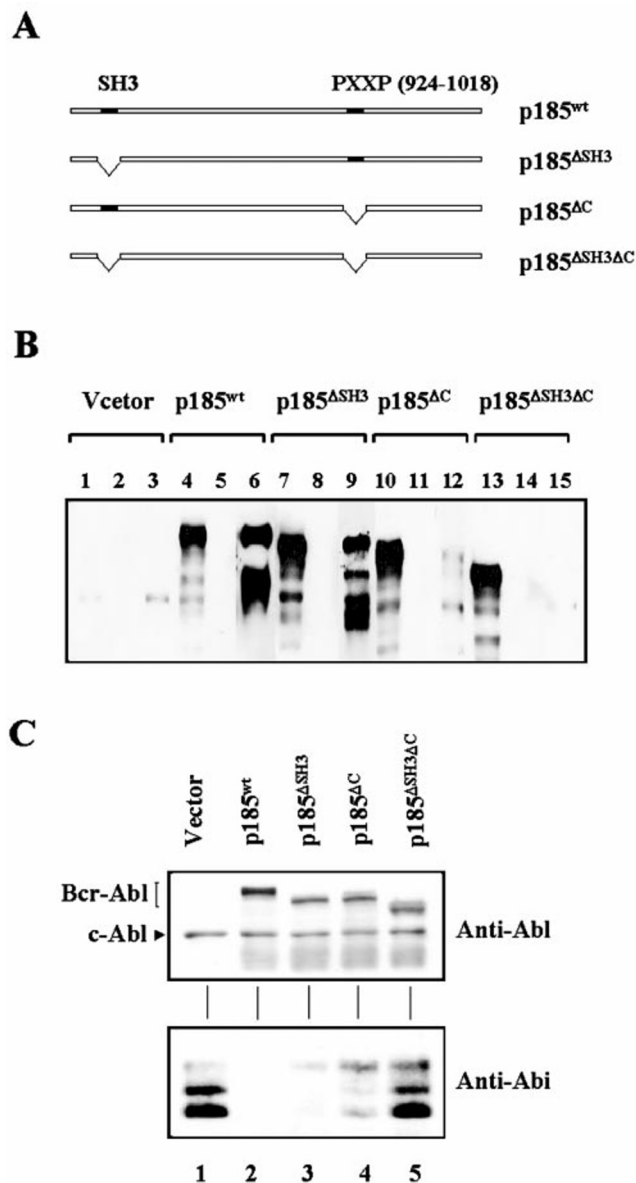


FIG. 1. Deletion of the SH3 domain and C-terminal proline-rich regions in Bcr-Abl abrogated interaction with Abi and prevented Abi degradation. *A*, schematic representation of wild type (p185^{wt}) and mutant forms of p185^{Bcr-Abl}. *B*, Abi-2 binds to the SH3 domain and C-terminal proline-rich regions of p185^{Bcr-Abl}. Bosc 23 cells transfected with the indicated retroviral constructs were lysed and incubated with either GST (lanes 2, 5, 8, 11, and 14) or GST-Abi-2 (lanes 3, 6, 9, 12, and 15). The bound proteins were subjected to Western blot analysis using anti-Abl antibody. A proportion (1/20) of that used for GST pull-down of total cell lysates was also included as a comparison (lanes 1, 4, 7, 10, and 13). *C*, deletion of the SH3 domain and C-terminal proline-rich regions in Bcr-Abl abolishes its ability to induce Abi degradation. BaF3 cells were transfected with MIGR1 vector (lane 1) or MIGR1 containing cDNAs for wild type and mutant forms of p185^{Bcr-Abl} as indicated (lanes 2–5). Total lysates from 1×10^6 cells were subjected to Western blot analysis using anti-Abl antibody (upper panel) or anti-Abi-2 antibody (lower panel) as indicated.

proline-rich sequences completely abolished the interaction between p185^{ΔSH3ΔC} and Abi-2 (Fig. 1*B*, lane 15).

These wild type and mutant forms of p185^{Bcr-Abl} were then introduced into the murine hematopoietic cell line BaF3 by retroviral transduction and tested for their abilities to induce Abi-2 degradation. BaF3 cells express Abi-2 that migrated on SDS-polyacrylamide gels as a major doublet and minor bands with apparent molecular masses of 60, 65, and 70 kDa, respectively (Fig. 1*C*, lane 1). The two bands with slower mobility are

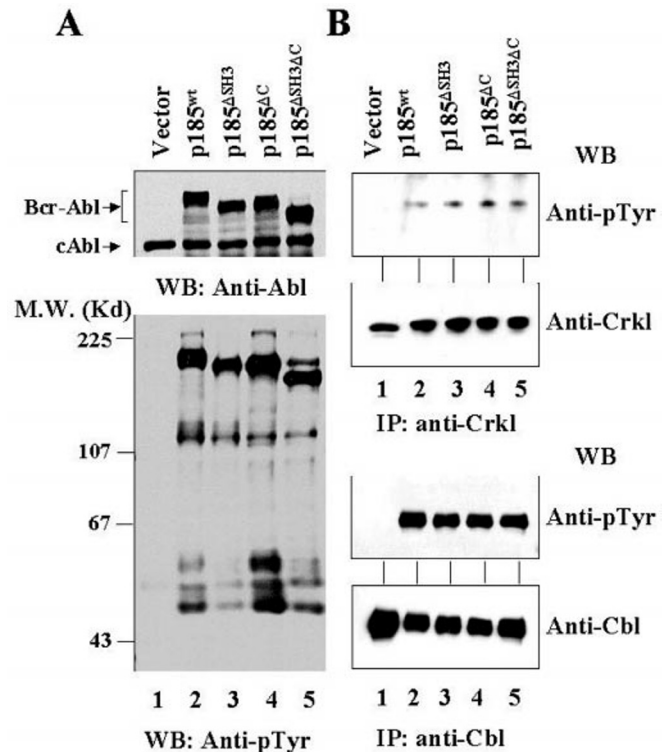


FIG. 2. The wild type and mutant forms of p185^{Bcr-Abl} are active tyrosine kinases. *A*, profiles of protein tyrosine phosphorylation in BaF3 cells transfected with control and Bcr-Abl-expressing retroviruses. Total lysates from 1×10^6 BaF3 cells transfected with indicated retroviruses were subjected to Western blot analysis with anti-Abl antibody (upper panel) or anti-phosphotyrosine (Anti-pTyr) antibodies. Arrows indicate the positions of wild type and mutant forms of p185^{Bcr-Abl} as well as the endogenous c-Abl. *B*, tyrosine phosphorylation of Crkl and c-Cbl upon Bcr-Abl transformation. 2×10^7 BaF3 cells transfected with the indicated retroviruses were lysed, immunoprecipitated (IP) with the indicated antibodies and immunoblotted (WB) with anti-Crkl, anti-Cbl, and anti-phosphotyrosine antibodies as indicated. *Kd*, kDa.

a reflection of the phosphorylation (31). As reported previously (24), expression of p185^{wt} in BaF3 cells induced degradation of Abi-2 (Fig. 1*C*, lane 2). In correlation with their binding capacities, p185^{ΔC} exhibited reduced ability to induce Abi-2 degradation (Fig. 1*C*, lane 4), whereas p185^{ΔSH3ΔC} was completely deficient in inducing Abi-2 degradation (Fig. 2*C*, lane 5). Similar results were observed in another murine hematopoietic cell line 32D (data not shown).

The Deletion Mutants of p185^{Bcr-Abl} Are Constitutively Active Tyrosine Kinases—The deletion of the SH3 domain and C-terminal proline-rich sequences does not affect the tyrosine kinase activity of the mutant forms of Bcr-Abl. This was demonstrated by the assessment of the protein tyrosine phosphorylation in BaF3 cells expressing the wild type and mutant forms of p185^{Bcr-Abl}. As would be expected, the wild type and mutant forms of p185^{Bcr-Abl} were all tyrosine-phosphorylated and were able to stimulate protein tyrosine phosphorylation in BaF3 cells (Fig. 2*A*).

The C-terminal proline-rich sequences deleted in p185^{ΔC} and p185^{ΔSH3ΔC} also contain a binding site for the adapter proteins Crk and Crkl (16, 32). In particular, Crkl is tyrosine-phosphorylated upon Bcr-Abl transformation and is believed to link Bcr-Abl to multiple downstream molecules (16). One of the molecules that associates with Crkl and Bcr-Abl is c-Cbl, a major tyrosine-phosphorylated protein in Bcr-Abl-expressing cells (15, 16). Therefore, we examined whether the deletion of the C-terminal proline-rich sequences in Bcr-Abl affects the tyrosine phosphorylation of Crkl and c-Cbl. Crkl and c-Cbl were

not tyrosine-phosphorylated in control BaF3 cells (Fig. 2B, lane 1). In contrast, the tyrosine phosphorylation of Crkl and c-Cbl was readily detected in BaF3 cells transformed by wild type as well as mutant forms of p185^{Bcr-Abl} (Fig. 2B, lanes 2–5).

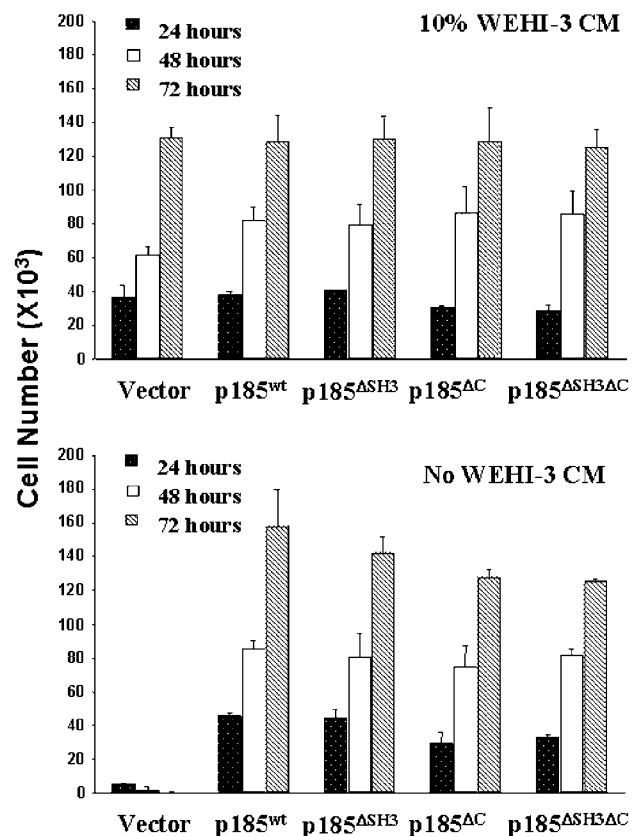
The SH3 and C-terminal Proline-rich Regions Are Not Required for Bcr-Abl to Transform Hematopoietic Cells to Growth Factor Independence—To determine whether the SH3 domain and C-terminal proline-rich sequences are required for Bcr-Abl to stimulate cell proliferation and survival, the mutant forms of p185^{Bcr-Abl} were tested for their abilities to transform BaF3 cells. Normal BaF3 cells require IL-3 for their proliferation and survival (17, 33), whereas cells expressing p185^{wt} were transformed and were able to proliferate in the absence of IL-3 (Fig. 3A). The deletions of the SH3 domain and C-terminal proline-rich sequences in p185^{Bcr-Abl} did not affect its ability to stimulate cell proliferation and survival because all three mutants, p185^{ΔSH3}, p185^{ΔC}, and p185^{ΔSH3ΔC}, were capable of transforming BaF3 cells to IL-3 independence (Fig. 3A).

Because hematopoietic stem cells are thought to be primary natural targets of Bcr-Abl, we tested if the deletions of the SH3 domain and proline-rich regions in Bcr-Abl affect its ability to transform mouse BM cells enriched for hematopoietic stem cells by 5-fluorouracil treatment. An *in vitro* soft agar assay (28) was performed to evaluate the colony-forming ability of the BM cells transduced with control retrovirus or retroviruses expressing the wild type and mutant forms of p185^{Bcr-Abl}. *In vitro* growth of BM cells requires a mixture of growth factors for optimal colony formation. Normal BM cells and the BM cells transduced with control retrovirus did not form colonies in agar culture in the absence of growth factors. In contrast, the BM cells transduced with wild type and mutant forms of p185^{Bcr-Abl} formed colonies in soft agar (Fig. 3B). It appeared that the p185^{ΔC} and p185^{ΔSH3ΔC} were more potent in transforming BM cells because their expression in BM cells induced 3- and 2-fold increases, respectively, in colony formation compared with p185^{wt} and p185^{ΔSH3}.

p185^{ΔSH3ΔC} Failed to Stimulate Spontaneous Migration of BaF3 Cells on Fibronectin-coated Surfaces—It was reported that the expression of Bcr-Abl in BaF3 cells stimulated spontaneous cell migration on fibronectin-coated surface (11). To determine whether the SH3 domain and C-terminal proline-rich regions of Bcr-Abl are required for this signaling pathway, we performed the Transwell cell migration assay (28). The spontaneous migration on fibronectin-coated membranes was examined in BaF3 cells expressing wild type and mutant forms of p185^{Bcr-Abl}. Consistent with the previous report (11), a 3.6-fold increase in spontaneous migration on fibronectin-coated membrane was observed in cells expressing p185^{wt} compared with that in control cells (Fig. 4). The expression of p185^{ΔSH3} and p185^{ΔC} also stimulated spontaneous migration but to a lesser extent compared with control cells. In contrast, no significant increase of spontaneous migration was observed in BaF3 cells expressing p185^{ΔSH3ΔC} (Fig. 4) compared with control cells.

The SH3 Domain and C-terminal Proline-rich Regions of Bcr-Abl Are Required for Bcr-Abl-induced Leukemogenesis—The findings that p185^{ΔSH3ΔC} is defective in inducing Abi-2 degradation and stimulating spontaneous cell migration prompted us to ask if these defects affect its leukemogenic activity *in vivo*. This was tested by performing bone marrow transplantation studies in mice. BM cells from 5-fluorouracil-treated donor mice were transduced with either control retrovirus or retroviruses containing cDNAs for wild type and mutant forms of p185^{Bcr-Abl}. To ensure that comparable transduction efficiencies were achieved with the different retroviruses, retroviral supernatants with approximately equivalent

A



B

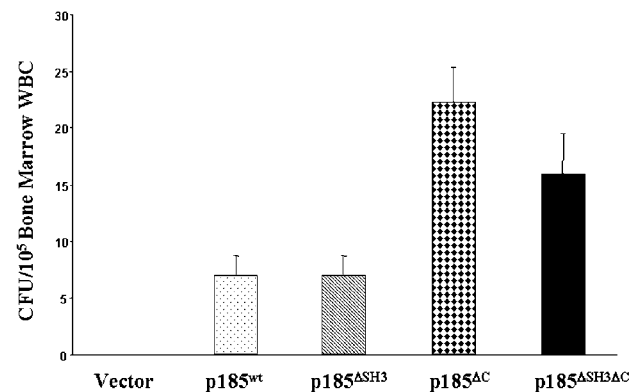
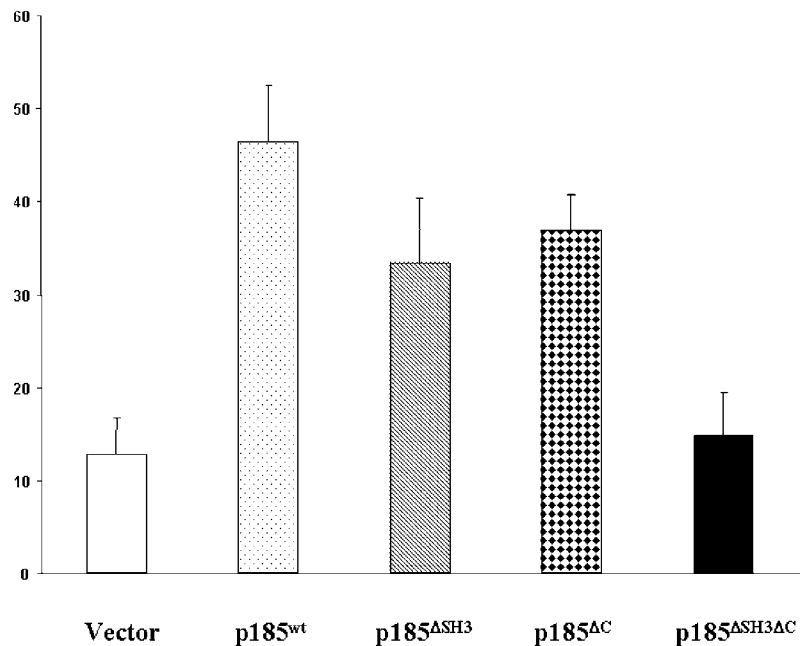


FIG. 3. The wild type and mutant forms of p185^{Bcr-Abl} transformed BaF3 cells and mouse bone marrow cells. A, factor-independent growth of BaF3 cells expressing wild type and mutant forms of Bcr-Abl. BaF3 cells transduced with the indicated retroviruses were grown in 96-well plates in the presence (upper panel) or absence (lower panel) of 10% WEHI-3-conditioned medium (WEHI-3 CM). Cells were counted at 24, 48, and 72 h. Cell numbers were presented as the average \pm S.D. of triplicate wells. B, colony formation of mouse bone marrow cells in soft agar. Mouse BM cells from 5-fluorouracil-treated mice were transduced with MSCV (Vector) or MSCV containing cDNAs for wild type and mutant forms of Bcr-Abl as indicated. 1×10^5 transduced cells were plated in soft agar and were counted for colony formation \sim 2 weeks later. The colony-forming unit (CFU) represents the average number \pm S.D. of agar colonies in triplicate plates.

lent titers were used. The levels of wild type and mutant p185^{Bcr-Abl} proteins expressed in transduced BM cells were determined by Western blot analysis (Fig. 5A, lanes 1–5). The

FIG. 4. Effects of the expression of wild type and mutant forms of p185^{Bcr-Abl} on cell migration. 1×10^5 BaF3 cells transduced with the indicated retroviruses were tested in the Transwell migration assay for spontaneous migration on a fibronectin-coated membrane. The vertical axis shows the percentage of migrated cells and is expressed as the average \pm S.D. of triplicate wells.



transduced BM cells were then transplanted into lethally irradiated syngeneic recipient mice. Consistent with previous reports (6–8, 34), the mice receiving BM cells transduced with p185^{wt} (p185^{wt} BMT mice) developed CML-like disease. These mice died in 4–6 weeks (Fig. 5B). Typically the mice had high WBC counts in their peripheral blood (2–6-fold higher than those in mice receiving BM cells transduced with control retrovirus, see Table I). The spleens of these mice were enlarged with weights 3–6 times greater than those of control animals (Table I). The leukemic disease that developed in these mice was caused by the expression of p185^{wt} because the mice receiving BM cells transduced with control retrovirus (control BMT mice) did not develop the disease. Furthermore, the expression of p185^{Bcr-Abl} was detected in the BM cells of the diseased mice (Fig. 5A, lanes 6–10). The mice receiving BM cells transduced with p185^{ASH3} (p185^{ASH3} BMT mice) or p185^{AC} (p185^{AC} BMT mice) also developed leukemia with the symptoms similar to those found in p185^{wt} BMT mice, although a slightly longer survival time was observed in p185^{ASH3} BMT mice (Fig. 5B). In contrast to p185^{wt} BMT mice, the mice receiving BM cells transduced with p185^{ASH3AC} (p185^{ASH3AC} BMT mice) survived longer (Fig. 5B). In two independent experiments that included 15 mice/group, 60% of p185^{ASH3AC} BMT mice survived 12 weeks or longer post-transplantation, whereas 100% of p185^{wt} BM mice died in 4–6 weeks post-transplantation. Striking differences in disease phenotype were also observed between p185^{wt} BMT mice and p185^{ASH3AC} BMT mice. Whereas most p185^{wt} BMT mice (greater than 80%) exhibited elevated peripheral WBC counts, only 3 of 11 p185^{ASH3AC} BMT mice analyzed developed peripheral WBC counts that were significantly higher than those of control BMT mice. In addition, all the p185^{wt} BMT mice developed massive splenomegaly, whereas only two of seven p185^{ASH3AC} BMT mice had slightly enlarged spleens (Table I). Interestingly, two p185^{ASH3AC} BMT mice that had high WBC counts in the peripheral blood (10- and 20-fold higher than those of control BMT mice) had no increase in spleen weight (Table I). The nature of the disease that developed in p185^{ASH3AC} BMT mice remains to be determined. However, the low incidence of elevated peripheral WBC counts and lack of splenomegaly, which are typically found in Bcr-Abl-positive leukemias, suggest that these mice may have died of a disease other than CML.

DISCUSSION

These studies demonstrated that a direct interaction between Bcr-Abl and Abi-2 is required for the induction of ubiquitin-dependent Abi-2 degradation. The deletion of the SH3 domain and C-terminal proline-rich regions of Bcr-Abl completely abolished the interaction of Bcr-Abl with Abi-2 and prevented Abi-2 degradation. Although the deletion of the SH3 domain and proline-rich regions had little if any effect on tyrosine kinase activity and the *in vitro* transforming activity of Bcr-Abl, it abrogated the ability of Bcr-Abl to stimulate spontaneous cell migration on a fibronectin-coated surface. Moreover, the deletion of these regions greatly impaired the ability of Bcr-Abl to induce CML-like disease in a murine bone marrow transplant model. Together these results are consistent with a role of Abi-2 degradation in the pathogenesis of Bcr-Abl-positive leukemia.

CML cells and the hematopoietic cell lines expressing Bcr-Abl exhibit multiple abnormalities in cytoskeletal function. How these abnormalities of cytoskeletal function relate to the clinical phenotypes of CML is not clear. The finding that the p185^{ASH3AC} is less leukemogenic in a murine BMT model lends support for a role of Bcr-Abl-induced abnormal cytoskeletal function in the pathogenesis of CML. The significant reduction in leukemogenic activity of p185^{ASH3AC} is not due to its failure to stimulate cell proliferation and survival because the mutant protein is equally if not more potent as wild type p185^{Bcr-Abl} in stimulating factor-independent growth of hematopoietic cell lines as well as mouse BM cells. Rather a defect in stimulating spontaneous cell migration, as evidenced by the Transwell migration assay, may be in part responsible. Notably, one pathological difference that distinguishes p185^{ASH3AC} BMT mice from p185^{wt} BMT mice is the lack of massive splenomegaly, which was found in all p185^{wt} BMT mice diagnosed (Table I) and in p185^{wt} and p210^{Bcr-Abl} BMT mice reported by other investigators (6–8, 34). Development of splenomegaly is believed to be the results of the massive accumulation and retention of both mature and immature myeloid cells in the spleen and is one of the most common phenotypes of CML patients (35). The observation that two p185^{ASH3AC} BMT mice developed high peripheral WBC counts with normal spleen weight is consistent with a hypothesis that the p185^{ASH3AC} is capable of

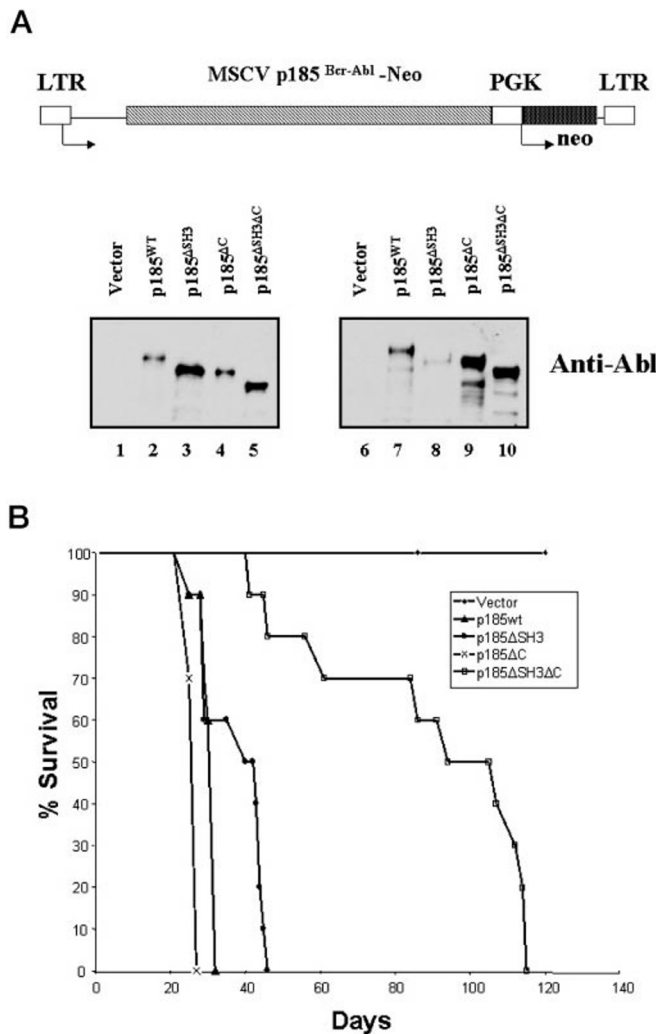


FIG. 5. Comparison of the disease development among the BMT mice. *A*, expression of the wild type and mutant forms of p185^{Bcr-Abl} in transduced mouse BM cells and BMT mice. *Upper panel*, schematic representation of the retroviral constructs used to transduce mouse BM cells. *Lower panel*, Western blot analysis of the BM cells transduced with the indicated retroviruses (lanes 1–5) or BM cells from the diseased BMT mice transduced with the indicated retroviruses (lanes 6–10). 1×10^6 cells were lysed and subjected to immunoblotting with the anti-Abl monoclonal antibody. *LTR*, long terminal repeat; *PGK*, phosphoglycerate kinase promoter; *neo*, neomycin phosphotransferase gene. *B*, survival of BMT mice receiving BM cells transduced with the indicated retroviruses. The data represent one of two independent experiments.

stimulating cell proliferation and survival but fails to induce the accumulation and retention of myeloid cells in the spleen. Further analysis of cytoskeletal function in primary hematopoietic cells from bone marrow, spleen, and peripheral blood of diseased p185^{WT} and p185^{ΔSH3ΔC} BMT mice should provide more information in this regard.

The complete abrogation of Bcr-Abl-stimulated spontaneous cell migration requires the deletion of both the SH3 domain and C-terminal proline-rich regions, suggesting a role for these regions in the regulation of cytoskeletal function. Although our data do not provide direct evidence of the mechanism(s) by which these motifs regulate cytoskeletal function, regulation of the signaling pathway from Ras to Rac via interaction with Abi proteins is likely involved. To date, only Abi family proteins have been found to interact with both the SH3 domain and C-terminal proline-rich sequences of Bcr-Abl. The inability of p185^{ΔSH3ΔC} to bind to Abi and to induce Abi-2 degradation together with the inability of p185^{ΔSH3ΔC} to stimulate sponta-

TABLE I

Features of disease in p185^{WT} and p185^{ΔSH3ΔC} BMT mice

Data are from two independent experiments with 15 BMT mice for p185^{WT} and p185^{ΔSH3ΔC} and 14 BMT mice for MSCV control retrovirus. The mice not presented here were those that died before analysis, and tissue degradation made it impossible for diagnosis.

Mouse	Latency ^a	WBC	Spleen weight	Liver weight
	days	$\times 10^3/\mu\text{l}$	g	
MSCV vector				
B1	— ^b	3.6	0.1	1.2
B2	— ^b	5.4	0.1	1.0
p185 ^{WT}				
A4	48	15	0.5	1.9
B1	32	12.7	0.4	1.2
B2	32	16.0	0.4	1.4
B3	32	5.7	0.3	1.0
B4	32	11.9	0.5	1.4
B5	32	11.5	0.5	1.0
B6	32	21.9	0.6	1.5
p185 ^{ΔSH3ΔC}				
B1	86	3.3	0.1	1.1
B2	94	4.0	0.1	1.0
B3 ^c	61	93.5	0.1	1.1
B4	116	9.6	0.2	1.3
B5 ^d	41	51.1	0.1	1.1
B6	116	2.2	0.3	1.6
B7	114	6.5	0.1	1.0

^a Latency is defined as the time post-BMT that mice died or became moribund.

^b —, control BMT mice survived >180 days without any disease observed.

^c Developed solid tumors.

^d Developed rear leg paralysis.

neous cell migration suggests a role for Abi proteins in Bcr-Abl-stimulated spontaneous cell migration. Cell migration involves coordinated and dynamic changes in the actin cytoskeleton and cell adhesion. The GTP-binding proteins Rho, Rac, and Cdc42 are known to play a crucial role in the regulation of these fundamental cellular processes (36, 37). In particular, Rac has been shown to function as a regulator of the integrin-dependent motility and invasiveness of epithelial tumor cells (38). Rac is activated in Bcr-Abl-transformed myeloid precursor 32Dcl3 cells, and its activity is required for Bcr-Abl-mediated leukemogenesis (39). 32Dcl3 cells co-expressing p210^{Bcr-Abl} and a dominant-negative Rac retained factor-independent growth but showed markedly reduced invasive properties and significantly impaired homing to marrow and spleen (39), a phenotype that is similar to that observed in p185^{ΔSH3ΔC}-expressing BaF3 cells and BMT mice. The involvement of Abi proteins in the regulation of signal transduction from Ras to Rac are suggested by recent findings that Abi-1 binds to Sos and regulates Rac-specific guanine nucleotide exchange factor activities (22). The function of Abi-2 is not clear. However, given the striking structural homology between Abi-1 and Abi-2, a similar function in the signal transduction mediated by small GTP-binding proteins may be predicted. It is also possible that Abi-2 may function as an antagonist of Abi-1 by competing with Abi-1 for complex formation with Sos and Eps8. Nonetheless, the fact that both Abi-1 and Abi-2 are substrates of Abl kinase and are capable of binding to the SH3 domain and C-terminal proline-rich sequences suggests that their functions may be regulated by activated Abl kinases. It is possible, for example, that the tyrosine phosphorylation of Abi-1 by Bcr-Abl may be required for activation of the Rac signaling pathway. Alternatively, the tyrosine phosphorylation and subsequent degradation of Abi-2 may be a critical step in activation of the Rac signaling. These two mechanisms do not have to be mutually exclusive. In support of this hypothesis, we have detected expression of both Abi-1 and Abi-2 in BaF3 cells. Although Abi-2 is sensitive to Bcr-Abl-induced ubiquitin-dependent degradation, Abi-1 is rel-

atively stable and is tyrosine-phosphorylated in p185^{wt}-expressing BaF3 cells.²

In addition to Abi proteins, the SH3 domain and C-terminal proline-rich regions of Bcr-Abl also interact with other molecules important in the control of cell proliferation, survival, and motility. The SH3 domain of Bcr-Abl, for example, may interact with Ras interactor 1 (40) and Pag (41), whereas the C-terminal proline-rich sequences interact with adapter proteins such as Nck, Crk, and Crkl (16, 32). Therefore, it is possible that the deletion of the SH3 domain and C-terminal proline-rich regions in Bcr-Abl also blocks the signal transductions mediated by these molecules and thereby affects the leukemogenic activity of the mutant Bcr-Abl. It seems unlikely, however, that the defect of p185^{ΔSH3ΔC} in stimulating spontaneous cell migration is because of its inability to transduce signal to Crkl. We have shown that Crkl is tyrosine-phosphorylated in BaF3 cells transformed by p185^{ΔSH3ΔC}. Despite the deletion of the Crkl binding site, p185^{ΔC} and p185^{ΔSH3ΔC} retain ability to form a complex with Crkl *in vivo* possibly through indirect interaction (42).³ The finding that p185^{ΔC} is capable of stimulating spontaneous cell migration and inducing CML-like disease in BMT mice is consistent with the results reported by other investigators that the direct binding of Crkl to Bcr-Abl is not required for Bcr-Abl transformation (42). Further mutation analysis in combination with the detailed analysis of pathology of BMT mice is required to determine the contribution of these signaling pathways in leukemogenesis induced by Bcr-Abl.

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³ Z. Dai, W. G. Schroeder, and I. K. McNiece, unpublished data.