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The SH2 Domain of P210$^{BCR/ABL}$ Is Not Required for the Transformation of Hematopoietic Factor-Dependent Cells

By Robert L. Ilaria Jr and Richard A. Van Etten

Src-homology region 2 (SH2) domains, by binding to tyrosine-phosphorylated sequences, mediate specific protein-protein interactions important in diverse signal transduction pathways. Previous studies have shown that activated forms of the Abl tyrosine kinase, including P210$^{BCR/ABL}$ of human chronic myelogenous leukemia, require the SH2 domain for the transformation of fibroblasts. To determine whether SH2 is also required for Bcr/Abl to transform hematopoietic cells, we have studied two SH2 domain mutations in P210$^{BCR/ABL}$: a point mutation in the conserved FLVRES motif (P210/R1053K), which interferes with phosphotyrosine-binding by SH2, and a complete deletion of SH2 (P210/ΔSH2). Despite a negative effect on intrinsic Abl kinase activity, both P210 SH2 mutants were still able to transform the hematopoietic factor-dependent cell lines Ba/F3 and FDC-P1 to growth factor independence. Unexpectedly, both mutants showed greater transforming activity than wild-type P210 in a quantitative transformation assay, probably as a consequence of increased stability of the SH2 mutant proteins in vivo. Cells transformed by both P210 SH2 mutants were leukemogenic in syngeneic mice, and P210/R1053K mice exhibited a distinct disease phenotype, reminiscent of that induced by v-Abl. These results demonstrate that while the Abl SH2 domain is essential for *BCR/ABL* transformation of fibroblasts, it is dispensable for the transformation of hematopoietic factor-dependent cell lines.

THE PHILADELPHIA chromosome is the result of a reciprocal translocation in which the c-*ABL* gene on chromosome 9 is truncated at its 5′ end by *BCR* sequences from chromosome 22, forming the chimeric gene *BCR/ABL*.[1,2] *BCR/ABL* has been implicated in a wide range of human leukemias, including virtually all cases of chronic myelogenous leukemia (CML), approximately 20% to 30% of adult acute lymphoblastic leukemia (ALL), and less commonly acute myelogenous leukemia.[3] The normal cellular function of the *BCR* gene is unknown, but c-*ABL*, the cellular homologue of the transforming gene of Abelson murine leukemia virus, encodes a non-receptor protein-tyrosine kinase. The normal c-Abl protein is unable to transform cells, even when over-expressed[4]; however, the fusion protein Bcr/Abl exhibits increased tyrosine kinase activity[5] and gains the ability to cause cellular transformation in several assays, including focus formation and soft agar colony growth in fibroblasts,[6] transformation of hematopoietic factor-dependent cell lines to growth factor independence,[7,8] transformation of bone marrow cells in vitro,[9] and induction of leukemia in mice.[10-12]

c-Abl, like many other proteins implicated in signal transduction, contains a Src homology region two (SH2) domain.[13] These domains are non-catalytic regions, approximately 100 amino acids in length, that bind with high affinity to specific amino acid sequences containing phosphorylated tyrosine residues.[14-16] The phosphotyrosine-binding function of SH2 is crucial for fibroblast transformation by *ABL*. A point mutation in the highly conserved FLVRES motif of the Abl SH2 domain, resulting in an arginine to lysine change at position 171 (R171K) in the phosphotyrosine binding site,[17] blocks binding of SH2 to phosphotyrosine-containing proteins in vitro and greatly decreases fibroblast transformation in vivo by activated c-Abl.[18] Complete deletion of the SH2 domain of activated c-Abl abolishes fibroblast transformation.[19] An analogous point mutation (R552L) in the SH2 domain of P185$^{BCR/ABL}$, the form of *BCR/ABL* most commonly seen in Philadelphia chromosome-positive ALL, impairs Rat-1 fibroblast transformation.[20] These studies demonstrate that activated forms of Abl require the SH2 domain for the transformation of fibroblasts.

Because *BCR/ABL* is associated with hematopoietic malignancies, it is important to determine whether the SH2 domain is also required for hematopoietic cell transformation and leukemogenesis by P210$^{BCR/ABL}$. A previous report suggested that the Abl SH2 domain may be dispensable for the transformation of a hematopoietic cell line.[21] We have generated P210$^{BCR/ABL}$ mutants containing either a FLVRES point mutation (P210/R1053K), or a complete deletion of the SH2 domain (P210/ΔSH2), and have examined their ability to transform the hematopoietic factor-dependent cell lines Ba/F3 (a murine pro-B lymphoid cell line[22]) and FDC-P1 (a murine early myeloid cell line[23]). Although P210/R1053K and particularly P210/ΔSH2 exhibit significantly decreased intrinsic kinase activity relative to P210$^{BCR/ABL}$, they were still able to transform Ba/F3 and FDC-P1 cells to growth factor independence. Unexpectedly, both P210 SH2 mutants exhibited a higher transforming activity than wild-type P210 in a quantitative transformation assay, and retained the ability to induce leukemia in syngeneic mice, although P210/R1053K mice exhibited a distinct disease phenotype.

MATERIALS AND METHODS

Construction of BCR/ABL SH2 Domain Mutants

The P210/R1053K mutant was constructed using site-specific mutagenesis using the MutaGene Phagemid system (Bio-Rad Labora-

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tories, Melville, NY), based on the method of Kunkel et al.[24] The mutagenic primer (5′ GCAGCTTCTTAGTGAAGGAGAGTG-AGAGTAGCCCGGGCCAGAGATCCATC 3′) was hybridized to a single-stranded, uracil-containing, P210$^{BCR/ABL}$ *Hin*dIII fragment contained in the vector pTZ19U. The primer contained a new *Hpa* II site to identify mutant clones. The P210/R1053K mutant was confirmed by dideoxy DNA sequencing, and the *Hin*dIII fragment was subcloned into the vector pGDΔRI, which is identical to the vector pGD,[10] but lacks the *Eco*RI site in the neomycin resistance gene cassette.

The P210/ΔSH2 mutant was generated through a series of restriction digests in which the *Hin*PI site at nucleotide #804 and the *Kpn* I site at #527[25] were blunt-ended and fused in-frame, resulting in the deletion of the SH2 domain from amino acid #1030 to #1120 in the K562 b3a2 cDNA. The mutation was verified by dideoxy DNA sequencing and restriction mapping was performed to confirm preservation of the *Kpn* I site. The mutant cDNA was subcloned into pGDΔRI for transfection.

Cells and Cell Culture

Ba/F3 and FDC-P1 cells were grown in liquid culture at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DME-high) containing 10% heat-inactivated fetal calf serum, penicillin/streptomycin, and 2 mmol/L glutamine. Where indicated, the medium was supplemented with growth factor in the form of either 5% to 10% (vol/vol) WEHI-3B-conditioned medium as a source of interleukin 3 (IL-3)[26] (Ba/F3 and FDC-P1 cells), or murine recombinant granulocyte macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA) at a concentration of 30 U/mL (FDC-P1 cells). BOSC-23 cells[27] were grown in DME-high containing 10% heat-inactivated fetal calf serum, penicillin/streptomycin, 2 mmol/L glutamine, and nonessential amino acids.

Gene Transfer

Retroviral infection. Ten micrograms of P210 or SH2 domain mutant DNA in the retroviral expression vector pGDΔRI,[10] which contains a gene encoding resistance to neomycin, was introduced into the retroviral packaging cell line BOSC-23 by calcium phosphate transfection, as described.[27] Twelve to 16 hours later, 6×10^6 Ba/F3 cells were gently added to each 10 cm BOSC-23 plate in the presence of 6 μg/mL hexadimethrine bromide (Polybrene; Sigma, St Louis, MO) and 10% WEHI-3B conditioned medium. The titers of the retroviral stocks did not differ significantly between P210 and the SH2 mutants, and was in the range that yields single copies of the *BCR/ABL* genome in recipient cells. After 36 hours of co-cultivation the Ba/F3 cells were removed, transferred to tissue culture flasks, and selected in the presence of growth factor in 1 mg/mL neomycin (absolute).

Electroporation. Because FDC-P1 cells were relatively resistant to BOSC-23 cell retroviral infection, electroporation was used. Approximately 8 to 12×10^6 exponentially growing FDC-P1 cells were washed twice in ice cold phosphate-buffered saline lacking calcium and magnesium chloride (PBS$^-$), and resuspended in 800 μL of PBS$^-$. All DNA was twice purified by buoyant density centrifugation in cesium chloride. Fifty micrograms of linearized P210 or SH2 domain mutant DNA in pGDΔRI was then added to the cells. Electroporation conditions were 220 volts and 960 μFarads (time constant: 17 to 22.5 msec), using a Bio-Rad Genepulser apparatus. After electroporation, the cells were placed on ice for 5 to 10 minutes and then allowed to recover in nonselective medium for 24 hours at 37°C. Selection was then performed in the presence of growth factor in 0.3 mg/mL neomycin (absolute) in a 75 cm^2 tissue culture flask. By day 9 to 11 a confluent population of neomycin resistant cells was obtained for subsequent cloning by limiting dilution.

Analysis of Clones for Growth Factor Independence

Neomycin resistant populations of Ba/F3 or FDC-P1 cells, containing P210 or one of the SH2 domain mutants, were cloned by limiting dilution into 96-well plates. Clones were isolated in the presence of growth factor and neomycin, with fresh medium added every 3 days to prevent any possibility of growth factor depletion. After approximately 10 days the wells were sufficiently confluent to be harvested. The cells were gently pelleted in 1.6 mL eppendorf tubes, washed twice in PBS$^-$, and distributed equally into parallel 24-well plates containing medium with or without supplemental growth factor. After 48 to 72 hours, the 24-well plates were scored by counting the number of clones capable of factor-independent growth. A growth factor-independent clone was defined as a clone that was able to proliferate both in the presence and absence of supplemental growth factor. Analysis of individual growth factor-independent clones by Southern blot revealed that the majority possessed a single copy of the *BCR/ABL* gene. The data were expressed as a ratio of the number of factor-independent clones divided by the total number of clones analyzed. The proportions obtained from hematopoietic factor-dependent cell lines containing the *BCR/ABL* SH2 domain mutants were compared with those containing wild-type P210, and analyzed using a χ^2 test for significance.

Autocrine Growth Analysis

(A) Conditioned media obtained from growth factor-independent populations of Ba/F3 and FDC-P1 cells expressing P210 or one of the SH2 mutants was concentrated 25- to 30-fold using centriprep-10 ultrafiltration (Amicon, Beverly, MA), and added to duplicate samples of 10^5 parental Ba/F3 or FDC-P1 cells at a concentration of 10% (vol/vol) in standard medium without growth factor. The viable cell count was determined daily. (B) Neutralizing monoclonal anti-IL-3 antibody (Genzyme, Cambridge, MA) was added to growth factor-independent populations of Ba/F3 or FDC-P1 cells expressing P210 or one of the SH2 mutants at a concentration (10 μg/mL) 10-fold higher than was required to cause growth arrest of Ba/F3 cells and death of FDC-P1 cells growing in 1% WEHI-3B-conditioned medium. Viable cells were counted daily and their growth rate compared with cells grown in the absence of neutralizing antibody.

RNA Analysis

Total RNA was prepared from populations of neomycin-resistant Ba/F3 cells transfected with P210 or the SH2 mutants, before and after selection for the growth factor-independent phenotype, using the guanidinium isocyanate method.[28] Equal amounts of total RNA were subjected to denaturing agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled DNA probe generated from a 1,100-bp *Cla* I fragment from the neomycin gene cassette contained in the pGDΔRI vector.[10]

In Vitro Kinase Assay

P210, P210/R1053K, or P210ΔSH2, contained in the vector PJ3Ω[29] were introduced into 293T cells[30] by transient transfection; alternatively, growth factor-independent Ba/F3 cells were used as the source of Bcr/Abl protein. Lysates were made and normalized by western blot for the amount of Bcr/Abl protein using the anti-Abl monoclonal antibody 19-84.[31] Equal amounts of Bcr/Abl proteins were subjected to immunoprecipitation with anti-GEX4 antisera (recognizing COOH-terminal Abl sequences[32]), and an immune complex kinase assay performed as previously described,[5] with enolase added as an exogenous substrate.[33] The proteins were then separated on a 5% to 9% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel, detected by autoradiography, and quantitated using a phosphorimager (GS-250; Bio-Rad Laboratories).

Leukemogenesis Assay

Polyclonal populations of Ba/F3 cells transformed by wild-type P210, P210/R1053K, or P210/ΔSH2 were propagated in liquid culture as described above, without a source of IL-3. Cells were washed twice in PBS^-, counted, and resuspended at a density of 10^5 cells per 0.5 mL PBS^-. Recipient BALB/c BYJ female mice (Jackson Laboratories, Bar Harbor, ME), between 6 and 8 weeks of age and having 17 to 20 g body weight, received 10^5 cells intravenously by tail vein injection. Injected mice were subsequently evaluated on a daily basis for weight loss, failure to thrive, splenomegaly, or hind-limb paralysis. Mice that appeared pre-morbid were killed. Hind-limb paralysis was scored if mice exhibited an inability to use hind limbs for ambulation on a smooth surface such as a countertop.

RESULTS

Abl SH2 Mutations Attenuate Bcr/Abl Kinase Activity In Vitro

To examine the effect of mutations in SH2 on the tyrosine kinase activity of P210, we expressed P210 and SH2 mutant proteins in 293T cells and performed an immune complex kinase assay. Addition of Bcr sequences to the NH_2-terminus of Abl increases the intrinsic tyrosine kinase activity of the protein.[34] Relative to c-Abl, P210 kinase activity was increased approximately six-fold, while P190, the form of Bcr/Abl most commonly associated with Philadelphia chromosome-positive ALL, was elevated almost ten-fold, consistent with previous observations[35] (Fig 1). The mutation disrupting the phosphotyrosine-binding function of P210, P210/R1053K, resulted in a modest decrease in kinase activity relative to P210, to 4.4-fold over c-Abl. However, complete deletion of the SH2 domain abrogated the elevated kinase activity of P210, essentially to the level of c-Abl (0.7-fold). The impact of these mutations on Bcr/Abl autophosphorylation paralleled their effects on the phosphorylation of the exogenous substrate enolase. Similar results were obtained when these proteins were immunoprecipitated from Ba/F3 cells (data not shown).

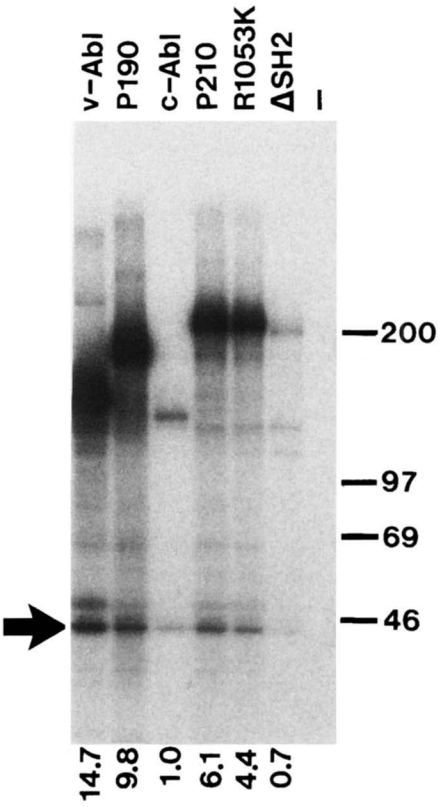

Fig 1. In vitro kinase analysis of $P210^{BCR/ABL}$ mutants. Cell lysates were made from 293T cells expressing either v-Abl, $P190^{BCR/ABL}$, murine type IV c-Abl, $P210^{BCR/ABL}$, the P210 SH2 FLVRES point mutant P210/R1053K, P210 lacking SH2 (ΔSH2), or cells transfected with vector alone (−). The lysates were normalized for Bcr/Abl protein and subjected to immune complex kinase assay as described in the text. Intrinsic kinase activity relative to c-Abl is depicted at the bottom. The position of the exogenous substrate enolase is indicated by the arrow. Molecular weight standards are shown at right.

Neither Phosphotyrosine-Binding nor Other Functions of SH2 Are Required for the Transformation of Factor-Dependent Hematopoietic Cell Lines by BCR/ABL

Quantitation of differences between wild-type and mutant forms of *BCR/ABL* in fibroblast transformation have relied on comparisons in focus formation or the number of colonies formed in soft agar. Because both Ba/F3 and FDC-P1 cells grow in liquid culture, we compared the transforming ability of the P210/R1053K and P210/SH2 mutations to wild-type P210 by the analysis of individual transfected clones. The neomycin-containing vector pGDΔRI, containing either wild-type P210, P210/R1053K, or P210/ΔSH2, was introduced into the factor-dependent cell lines Ba/F3 and FDC-P1 by helper-free retroviral infection or electroporation. After neomycin selection in the presence of growth factor, individual clones were isolated by limiting dilution and assessed for growth in medium with or without growth factor (WEHI-3B conditioned medium as source of IL-3 for Ba/F3 cells, and WEHI-3B or recombinant murine GM-CSF for FDC-P1 cells). Analysis of the site of chromosomal integration of proviral or plasmid DNA confirmed that independent clones were analyzed (data not shown). In parallel experiments where transfected cells were cloned directly in neomycin, the efficiency of recovery of neomycin-resistant clones was similar with either parental pGDΔRI vector alone, P210, or the SH2 mutants, suggesting there was no significant lethal or cytotoxic effect of transfection of Bcr/Abl in these cells.[36-38] Cells incapable of growth in the absence of IL-3 or GM-CSF generally died within 24 hours of growth factor deprivation. No Ba/F3 or FDC-P1 cells spontaneously acquired the ability to grow without growth factor (data not shown). Further, neither pGDΔRI vector alone nor a kinase-inactive mutant of Abl supported any factor-independent growth (data not shown), confirming that the tyrosine kinase activity of Abl is required for the transformation of factor-dependent hematopoietic cells.[39]

The ratio of growth factor-independent clones to total number of clones was calculated for each of the P210 mutants, and compared with wild-type P210 (Table 1). $P210^{BCR/ABL}$ conferred growth factor independence on approximately 25% and 23% of transfected Ba/F3 and FDC-P1 clones, respectively. Surprisingly, both SH2 domain mutants not only retained the ability to confer growth factor independence on these cells, but also exhibited approximately twice the transforming activity of wild-type P210;

Table 1. Effect of SH2 Mutations on GF Independence

Mutant*	Cell Type	No. of Clones GF−/GF+	Percent
P210/R1053K	Ba/F3	27/54	50.0†
P210/ΔSH2	Ba/F3	26/53	49.1†
P210	Ba/F3	13/53	24.5
P210/R1053K	FDC-P1	33/72	45.8†
P210/ΔSH2	FDC-P1	33/71	46.5†
P210	FDC-P1	16/71	22.5

* $P210^{BCR/ABL}$, P210 containing an FLVRES mutation (P210/R1053K), or P210 containing an in-frame deletion of the entire SH2 domain (P210/ΔSH2) were introduced into the hematopoietic factor-dependent cell lines Ba/F3 and FDC-P1 by retroviral gene transfer or electroporation. Individual clones were isolated by limiting dilution in the presence of growth factor (GF+), and then analyzed by the ability to grow in medium lacking supplemental growth factor (GF−).

† $P < .02$, for SH2 mutants compared to wild-type P210, using a χ^2 test comparing two proportions.

approximately 46% to 50% of clones containing the point mutation that severely impairs phosphotyrosine binding (P210/R1053K), and 47% to 49% of clones lacking the Abl SH2 domain entirely (P210/ΔSH2) were capable of growth factor-independent growth. The differences between P210 and the SH2 domain mutants were statistically significant ($P < .02$, using a χ^2 test comparing two proportions). In the case of the FDC-P1 cells, there was no significant difference in the ability of clones to grow in the absence of either IL-3 (WEHI-3B-conditioned medium) or GM-CSF (data not shown). Therefore, in contrast to fibroblast transformation, neither the phosphotyrosine-binding function of SH2 nor the SH2 domain itself was required for *BCR/ABL* to transform these hematopoietic factor-dependent cell lines to growth factor-independent proliferation.

To investigate the possibility that autocrine secretion of IL-3 was responsible for induction of factor-independence by P210 or the SH2 mutants,[7] we tested the ability of conditioned medium from transformed cells to support the growth of the parental Ba/F3 and FDC-P1 cells, and the effect of neutralizing anti-IL-3 antibodies on their growth. High concentrations of conditioned medium from Ba/F3 or FDC-P1 cells expressing P210/R1053K supported the growth of parental FDC-P1 cells, consistent with secretion of IL-3 or GM-CSF (data not shown). We were unable to detect similar growth-promoting activity in conditioned medium from cells expressing wild-type P210 or the P210 ΔSH2 mutant. However, neutralizing anti-IL-3 antibodies, added at levels tenfold higher than necessary to induce apoptosis of parental Ba/F3 or FDC-P1 cells in 1% WEHI-3B conditioned medium, did not affect the proliferation of factor-independent cells transformed by P210, P210/R1053K, or P210/ΔSH2 (data not shown). Therefore, autocrine production of IL-3 does not play a role in the induction of growth factor independence by P210 or the SH2 mutants, consistent with previous observations.[7,8]

Ba/F3 Cells Transformed by P210 SH2 Mutants Express Higher Levels of Bcr/Abl Protein, But Exhibit Similar Levels of Phosphotyrosine on Bcr/Abl

Polyclonal populations and individual clones of factor-independent cells expressing wild-type and mutant forms of $P210^{BCR/ABL}$ were analyzed by Western blot for levels of Bcr/Abl expression and pattern of protein tyrosine phosphorylation. Most of the clones that were incapable of factor-independent growth failed to express detectable Bcr/Abl protein. Some factor-dependent clones expressed low levels of Bcr/Abl, generally less than 5% of the amount of Bcr/Abl protein found in cells capable of factor-independent growth, and at about the same level as endogenous c-Abl (data not shown). This suggests that a threshold level of Bcr/Abl is necessary to overcome the requirement for growth factor, analogous to an effect documented for v-Src transformation of fibroblasts.[40] In factor-independent cells, the total level of Bcr/Abl protein was approximately twofold higher for both P210/R1053K and P210/ΔSH2 compared with wild-type P210 (Fig 2A); however, the level of phosphotyrosine on Bcr/Abl was more similar, perhaps due to the decreased kinase activity of the SH2 mutants (Fig 2B). Interestingly, the increased level of Bcr/Abl protein expression by the SH2 mutants was evident even before selection for growth factor independence (Fig 2C), while the relative levels of *BCR/ABL* mRNA were similar between the SH2 mutants and wild-type P210 (Fig 2D) before and after selection for growth factor independence. There was a general increase in expression of both Bcr/Abl mRNA and protein following selection for growth factor independence, consistent with a threshold requirement for transformation. These results suggest that the elevated expression of the Bcr/Abl SH2 mutant proteins may be due to increased protein stability. We could not demonstrate any significant decrease in the level of mutant or wild-type Bcr/Abl protein even after over 24 hours of treatment with cycloheximide (data not shown), consistent with the known long half-life of P210.[41]

The overall pattern of phosphotyrosinated proteins was quite similar between P210 and the SH2 domain mutants, except that R1053K-expressing clones frequently demonstrated decreased tyrosine phosphorylation of proteins migrating at 120 and 60 kd, which are likely p120 rasGAP (GTPase-activating protein)[42,43] and p62 rasGAP-associated protein,[44,45] respectively. These proteins have been shown to be tyrosine-phosphorylated in cells transformed by various tyrosine kinase oncogenes, including *BCR/ABL*.[46,47] Decreased tyrosine phosphorylation of p62 has also been reported in fibroblasts transformed by a *BCR/ABL* SH2 point mutant.[20] The overall pattern of Bcr/Abl expression and protein tyrosine phosphorylation seen in P210 and the SH2 domain mutations did not differ significantly between Ba/F3 and FDC-P1 cells (data not shown), suggesting that these findings are not peculiar to one hematopoietic lineage.

The P210/R1053K Mutant Exhibits a Distinct Disease Phenotype in an In Vivo Leukemogenesis Assay

The transformation of Ba/F3 cells to growth factor independence by P210 has been previously shown to correlate with their ability to form tumors after subcutaneous inoculation into athymic (nude) mice.[8] Because the Ba/F3 cell line was originally derived from BALB/c mice,[22] these animals provide a convenient in vivo model to explore $P210^{BCR/ABL}$-mediated leukemogenicity. Intravenous injection of 10^5 P210-transformed Ba/F3 cells causes death in all recipient

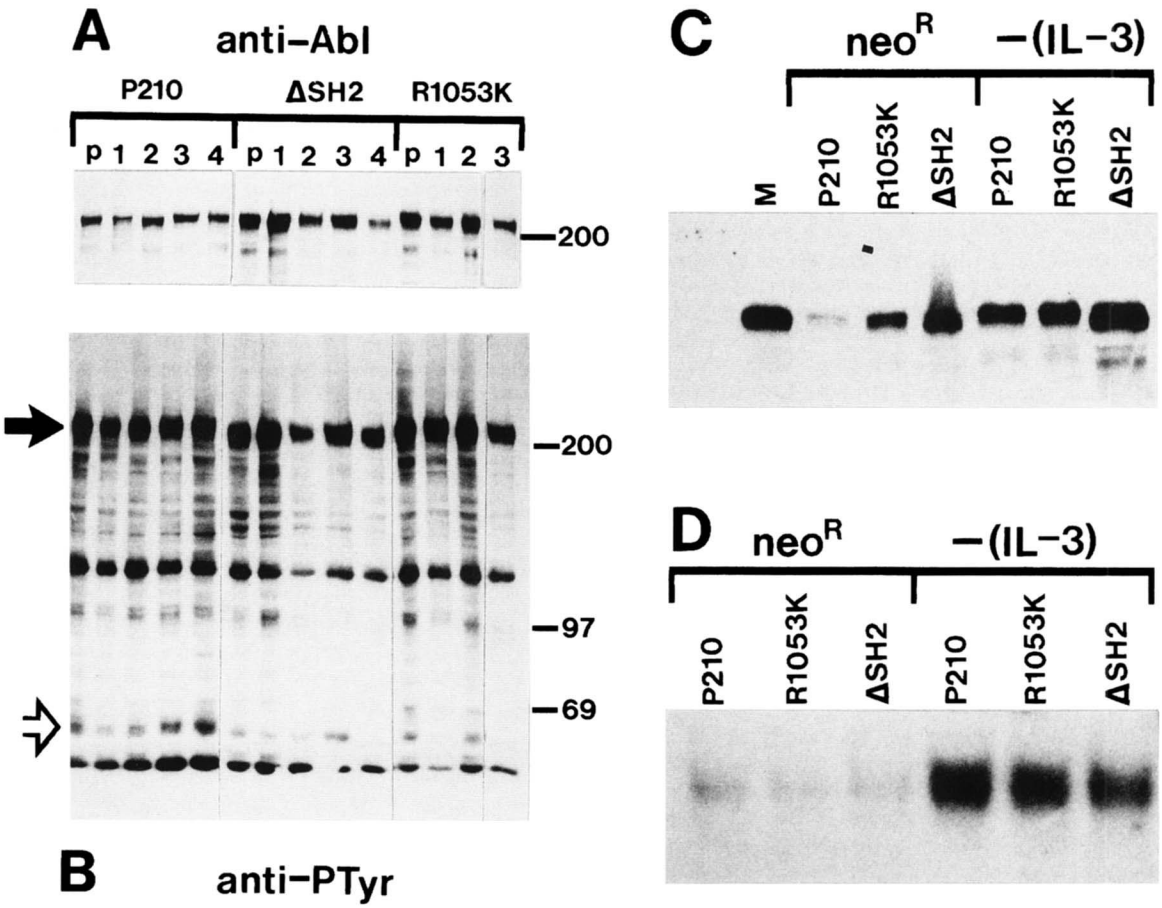

Fig 2. Analysis of protein and mRNA expression of P210 SH2 domain mutants. Protein lysates from populations (p) or individual clones (numbers) of factor-independent Ba/F3 cells transformed by P210, P210/ΔSH2, or P210/R1053K were studied by either anti-Abl (A) or anti-phosphotyrosine (B) antibody. The position of Bcr/Abl is indicated by the solid arrow; the presumptive p62 Ras-GAP-associated protein is indicated by the open arrow. Molecular weight standards are shown at right. Populations of transfected Ba/F3 cells were analyzed for expression of P210 protein (C) or mRNA (D) after selection for neomycin resistance in the presence of IL-3 (neo^R), or following selection for growth factor-independence by withdrawal of IL-3 (−IL-3). M indicates P210 protein size marker. Antibody used in C was anti-Abl monoclonal antibody 19-84. Probe used in the northern blot in D was a neomycin gene fragment, which hybridizes to the *BCR/ABL* mRNA produced from the pGD210 provirus.

BALB/c mice within 16 days (Van Etten et al, in preparation). Autopsy of diseased animals reveals an overwhelming leukemia, with diffuse replacement of bone marrow, liver, and spleen with cells that morphologically resemble the input Ba/F3 cells (data not shown). Injections of factor-dependent parental Ba/F3 cells have yielded no disease in recipient mice followed for up to 3 months.

The clinical manifestations or latency of *BCR/ABL*-associated illness did not differ significantly between mice that received Ba/F3 cells transformed by P210 or P210/ΔSH2, with all animals dying secondary to a diffuse leukemic process between days 15 and 16. In contrast, mice that received cells transformed by the SH2 point mutant P210/R1053K demonstrated a significantly longer latency, with all animals succumbing by day 26 (Fig 3). This was unlikely to be due to a difference in growth rate, because all three cell populations grew at similar rates in culture (data not shown). In addition to the longer latency period, mice receiving Ba/F3 cells transformed by P210/R1053K exhibited a distinct disease phenotype, with approximately two-thirds of animals developing a hind-limb paralysis syndrome before death. This condition, which is characteristic of adult BALB/c mice infected with Abelson murine leukemia virus and reflects compression of the lumbar spinal cord nerve roots due to leukemic involvement of the paraspinous lymph node chain,[48] was never observed in recipients of P210- or P210/ΔSH2-transformed Ba/F3 cells.

DISCUSSION

The interaction of SH2 domain-containing proteins with specific phosphotyrosinated sequences has been shown to be important for signal transduction involving both receptor and nonreceptor protein-tyrosine kinases. Previous studies have shown that the SH2 domain of activated forms of Abl is required for fibroblast transformation. We have studied two $P210^{BCR/ABL}$ SH2 domain mutants, P210/R1053K, which contains a point mutation interfering with phosphotyrosine-binding by SH2, and P210/ΔSH2, lacking the entire domain. In

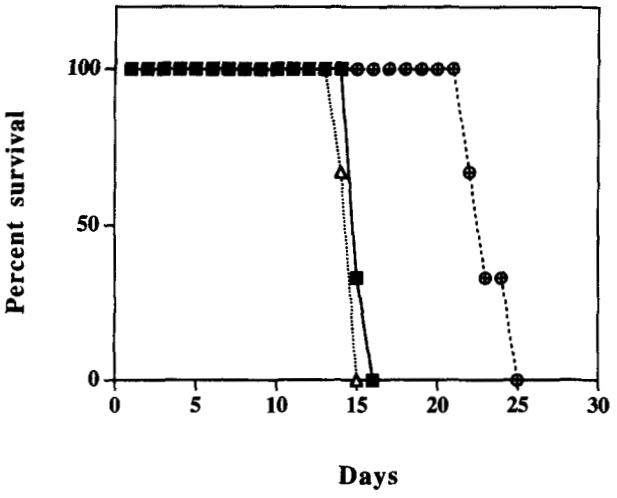

Fig 3. P210 SH2 mutant leukemogenesis assay. 10^5 Ba/F3 cells transformed either by (■) wild-type P210, (△) the SH2 domain point mutation P210/R1053K, or (⊕) the SH2 deletion mutant P210/ΔSH2 were injected into BALB/c BYJ female mice intravenously by tail vein injection. Survival was assessed from time of injection. The curve depicted represents one of three independent experiments, all of which obtained similar results.

contrast to the findings in fibroblasts, both SH2 mutants are more highly transforming than wild-type P210 in the hematopoietic factor-dependent cell lines Ba/F3 and FDC-P1, despite decreased intrinsic kinase activity. These results confirm a previous report that noted that a population of Ba/F3 cells transfected with an SH2-deleted form of *BCR/ABL* was able to grow in the absence of growth factor.[21] Others have also recently found FDC-P1 cells to be transformed by *BCR/ABL* lacking an SH2 domain.[49]

The tyrosine kinase function of Abl is required for Bcr/Abl transformation of hematopoietic factor-dependent cell lines[39] (data not shown). The mechanism by which Bcr activates the Abl kinase is unknown, but in fibroblasts may be related to its interactions with Abl SH2. Bcr first exon sequences have been shown to bind Abl SH2 in vitro in a phosphotyrosine-independent fashion, and deletion of these regions in Bcr abolished fibroblast transformation.[50] We have found that the tyrosine-binding function of SH2 (P210/R1053K) is not required for the activation of the Abl tyrosine kinase, while complete deletion of SH2 abrogated the activation of the Abl kinase by Bcr in vitro, consistent with this model. However, deletion of SH2 did not interfere with Bcr/Abl transformation of factor-dependent hematopoietic cell lines. These findings suggest that other functions of Bcr, such as oligomerization,[51] may be important for transformation of hematopoietic cells by *BCR/ABL*. A lack of correlation between Abl kinase activity in vitro and transformation ability in vivo has also been observed for SH3-deleted c-Abl, which is potent in cellular transformation despite the same kinase activity as c-Abl in vitro.[32,52] Although the P210/ΔSH2 mutant showed about sixfold lower in vitro autophosphorylation activity than wild-type P210, when isolated from transformed hematopoietic cells the degree of tyrosine phosphorylation of the ΔSH2 mutant was more similar to that of wild-type P210 (about twofold lower). This suggests that the tyrosine kinase activity of P210/ΔSH2 is higher in vivo than in vitro, or that the susceptibility of the ΔSH2 protein to tyrosine phosphatases in vivo is reduced.

Hematopoietic cells and fibroblasts have been shown to require distinct functions of Abl for transformation. Activated forms of Abl lacking the membrane-targeting myristoylation domain,[53] or containing a point mutation in an autophosphorylation site within the $P210^{BCR/ABL}$ kinase domain (Y1294F)[39] are defective in fibroblast transformation, but retain the ability to transform factor-dependent cell lines. Similarly, while the SH2 domain is essential for fibroblast transformation by Abl, it is dispensable for the *BCR/ABL* transformation of Ba/F3 or FDC-P1 cells. *BCR/ABL* signal transduction may involve different pathways, or emphasize particular mediators, depending on cell type. In fibroblasts the Abl SH2 domain may bind specific tyrosine-phosphorylated proteins that transduce signals required for transformation, while in hematopoietic factor-dependent cell lines this SH2-mediated signaling pathway is not required, or is regulated differently. Alternatively, the Abl SH2 domain might serve to protect phosphotyrosine sites on substrates from the action of phosphatases,[19] which might have different consequences in fibroblasts and hematopoietic cells.

Using a quantitative transformation assay we have found that both P210 SH2 domain mutants are actually more transforming than wild-type P210. There are several possible explanations for this finding. One possibility is that P210 SH2 mutants have decreased cytotoxicity. A growth inhibitory or toxic effect has been observed with expression of c-Abl, v-Abl, and Bcr/Abl in fibroblasts,[36-38] which may be dependent on SH2.[38] Therefore, mutations in SH2 may decrease this toxic effect of Bcr/Abl expression and allow increased recovery of transformed clones. However, we found no significant difference in the frequency of neomycin-resistant clones transformed with vector DNA alone or with wild-type or SH2 mutant forms of P210, suggesting that Bcr/Abl does not exhibit prominent toxicity in hematopoietic cells (data not shown). A more likely possibility is related to our observation that there is a threshold level of Bcr/Abl necessary for Ba/F3 transformation, analogous to Src transformation. We found that populations of Ba/F3 cells transfected with the SH2 mutants expressed higher levels of Bcr/Abl protein even before selection for growth factor independence, which could explain their greater transforming ability in this assay. Because P210 and the SH2 mutants had similar *BCR/ABL* mRNA levels by northern blot, this suggests a post-translational effect, such as increased stability of the SH2 mutant proteins, was responsible for the increased expression. We were not able to demonstrate any significant decrease in the level of mutant or wild-type Bcr/Abl proteins even with prolonged cycloheximide treatment, which probably reflects the relatively long half-life of P210, as has been noted by others.[41] The loss of SH2 function may affect the interaction of Bcr/Abl with another protein(s), which normally serves to regulate its stability.

The Abl SH2 domain is also not required for leukemogenicity of P210-Ba/F3 cells in vivo. Mice inoculated intravenously with Ba/F3 cells transformed by P210 of the ΔSH2

mutant die from an overwhelming leukemia by day 16, with infiltration of bone marrow, liver, and spleen. Interestingly, the SH2 domain point mutant P210/R1053K demonstrated a distinct disease phenotype, manifested by a greater latency of illness (death by day 25) and the frequent development of a syndrome of lower extremity paresis due to paraspinous lymph node infiltration, not seen in wild-type or P210/ΔSH2 disease, but reminiscent of classic disease induced by the Abelson virus. These findings raise the possibility that interference with the phosphotyrosine-binding function of $P210^{BCR/ABL}$ may affect cell homing in vivo. The possible mechanisms for this remain speculative, but may reflect alternative signal transduction pathways used by P210/R1053K, perhaps related to the differences in protein tyrosine phosphorylation of p120 rasGAP and p62 rasGAP-associated proteins seen on Western blot.

In summary, while the Abl SH2 domain is essential for *BCR/ABL* transformation of fibroblasts, it is dispensable for the transformation of hematopoietic factor-dependent cell lines. Transformation of fibroblasts may require a specific signal mediated by the Abl SH2 domain, which is not required for induction of growth factor independence in hematopoietic factor-dependent cell lines. Compared with wild-type P210, the P210/R1053K mutant demonstrated differences in both the pattern of protein tyrosine phosphorylation and leukemic phenotype in vivo. Whether these two phenomena are related, or reflect a physiologic difference between phosphotyrosine binding and other functions of Abl SH2, remains to be investigated. It will be important to determine whether the Abl SH2 domain is required for *BCR/ABL*-induced leukemia in humans. This issue is particularly relevant if the Abl SH2 domain is to be considered a target for rational drug design for the therapy of patients afflicted with these diseases. Experimental models that permit the analysis of P210 SH2 domain mutants in the context of early hematopoietic stem cells[10] may help resolve this issue.

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REFERENCES

1. Rowley JD: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290, 1973

2. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr,* on chromosome 22. Cell 36:93, 1984

3. Kurzrock R, Gutterman J, Talpaz M: The molecular genetics of Philadelphia chromosome-positive leukemias. N Engl J Med 319:990, 1988

4. Jackson P, Baltimore D: N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-*abl.* EMBO J 8:449, 1989

5. Konopka JB, Witte ON: Detection of c-*abl* tyrosine kinase activity in vitro permits direct comparison of normal and altered *abl* gene products. Mol Cell Biol 5:3116, 1985

6. Lugo TG, Witte ON: The *bcr-abl* oncogene transforms rat-1 cells and cooperates with v-*myc.* Mol Cell Biol 9:1263, 1989

7. Hariharan IK, Adams JM, Cory S: bcr-abl oncogene renders myeloid cell line factor independent: Potential autocrine mechanism in chronic myeloid leukemia. Oncogene Res 3:387, 1988

8. Daley G, Baltimore D: Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210 *bcr/abl* protein. Proc Natl Acad Sci USA 85:9312, 1988

9. McLaughlin J, Chianese E, Witte ON: In vitro transformation of immature hematopoietic cells by the P210 *bcr/abl* oncogene product of the Philadelphia chromosome. Proc Natl Acad Sci USA 84:6558, 1987

10. Daley GQ, Van Etten RA, Baltimore D: Induction of chronic myelogenous leukemia in mice by the $P210^{bcr/abl}$ gene of the Philadelphia chromosome. Science 247:824, 1990

11. Elefanty AG, Hariharan IK, Cory S: *bcr-abl,* the hallmark of chronic myeloid leukemia in man, induces multiple hematopoietic neoplasms in mice. EMBO J 9:1069, 1990

12. Kelliher MA, McLaughlin J, Witte ON, Rosenberg N: Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and bcr/abl. Proc Natl Acad Sci USA 87:6649, 1990

13. Sadowski I, Stone JC, Pawson T: A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of fujinami sarcoma virus $P130^{gag\text{-}fps}$. Mol Cell Biol 6:4396, 1986

14. Matsuda M, Mayer BJ, Fukui Y, Hanafusa H: Binding of transforming protein, $P47^{gag\text{-}crk}$, to a broad range of phosphotyrosine-containing proteins. Science 248:1537, 1990

15. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S: Oncogenes and signal transduction. Cell 64:281, 1991

16. Pawson T, Gish GD: SH2 and SH3 domains: From structure to function. Cell 71:359, 1992

17. Waksman G, Shoelson SE, Pant N, Cowburn D, Kuriyan J: Binding of a high affinity phosphotyrosyl peptide to the src SH2 domain: Crystal structures of the complexed and peptide-free forms. Cell 72:779, 1993

18. Mayer BJ, Jackson PK, Van Etten RA, Baltimore D: Point mutations in the *abl* SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo. Mol Cell Biol 12:609, 1992

19. Mayer BJ, Baltimore D: Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. Mol Cell Biol 14:2883, 1994

20. Afar DEH, Goga A, McLaughlin J, Witte ON, Sawyers CS: Differntial complementation of Bcr-Abl point mutants with c-myc. Science 264:424, 1994

21. McWhirter JR, Wang JYJ: An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of the Philadelphia chromosome-positive leukemias. EMBO J 12:1533, 1993

22. Palacios R, Steinmetz M: IL3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 41:727, 1985

23. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D: Growth of factor-dependent hematopoietic precursor cell lines. J Exp Med 152:1036, 1980

24. Kunkel TA: Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82:488, 1985

25. Fainstein E, Einat M, Gokkel E, Marcelle C, Croce CM, Gale RP, Canaani E: Nucleotide sequence analysis of human *abl* and *bcr-abl* cDNAs. Oncogene 4:1477, 1989

26. Lee JC, Hapel AJ, Ihle JN: Constitutive production of a unique lymphokine (IL 3) by the WEHI-3 cell line. J Immunol 128:2393, 1982

27. Pear WS, Nolan GP, Scott ML, Baltimore D: Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 90:8392, 1993

28. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

29. Morgenstern JP, Land H: A series of mammalian expression vectors and characteristics of their expression of a reporter gene in stably and transiently transfected cells. Nucleic Acids Res 18:1068, 1990

30. Dubridge RB, Tang P, Hsia HC, Phaik-Mooi L, Miller JH, Calos MP: Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 7:379, 1987

31. Schiff-Maker L, Burns MC, Konopka JB, Clark S, Witte ON, Rosenberg N: Monoclonal antibodies specific for v-*abl*- and c-*abl*-encoded molecules. J Virol 57:1182, 1986

32. Jackson PK, Paskind M, Baltimore D: Mutation of a phenylalanine conserved in SH3-containing tyrosine kinases activates the transforming ability of c-Abl. Oncogene 8:1943, 1993

33. Cooper JA, Esch FS, Taylor SE, Hunter T: Phosphorylation sites in enolase and lactate dehydrogenase utilized by protein kinases in vivo and in vitro. J Biol Chem 259:7835, 1984

34. Konopka JB, Watanabe SM, Witte ON: An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 37:1035, 1984

35. Lugo TG, Pendergast A, Muller AJ, Witte ON: Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 247:1079, 1990

36. Ziegler SF, Whitlock CA, Goff SP, Gifford A, Witte ON: Lethal effect of the Abelson murine leukemia virus transforming gene product. Cell 27:477, 1981

37. Renshaw MW, Kipreos ET, Albrecht MR, Wang JYJ: Oncogenic v-Abl tyrosine kinase can inhibit or stimulate growth, depending on the cell context. EMBO J 11:3941, 1992

38. Sawyers CL, McLaughlin JL, Goga A, Havlik M, Witte O: The nuclear tyrosine kinase c-Abl negatively regulates cell growth. Cell 77:121, 1994

39. Pendergast AM, Gishizky ML, Havlik MH, Witte ON: SH1 domain autophosphorylation of BCR/ABL is required for transformation but not growth factor independence. Mol Cell Biol 13:1728, 1993

40. Jakobovits EB, Majors JE, Varmus HE: Hormonal regulation of the rous sarcoma virus *src* gene via a heterologous promoter defines a threshold dose for cellular transformation. Cell 38:757, 1984

41. Dhut S, Chaplin T, Young BD: BCR/ABL and BCR proteins: Biochemical characterization and localization. Leukemia 4:745, 1990

42. Vogel US, Dixon RAF, Schaber MD, Diehl RE, Marshall MS, Scolnick EM, Sigal IS, Gibbs JB: Cloning of bovine GAP and its interaction with oncogenic *ras* p21. Nature 335:90, 1988

43. Trahey M, Wong G, Halenbeck R, Rubinfeld B, Martin GA, Lander M, Long C, Crosier WJ, Watt K, Koths K, McCormick F: Molecular cloning of two types of GAP complementary DNA from human placenta. Science 242:1697, 1988

44. Moran MF, Koch DA, Ellis C, England L, Martin GS, Pawson T: Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622, 1990

45. Wong G, Muller O, Clark R, Conroy L, Moran MF, Polakis P, McCormick F: Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell 69:551, 1992

46. Muller AJ, Young JC, Pendergast A, Pondel M, Landau NR, Littman DR, Witte ON: BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive leukemias. Mol Cell Biol 11:1785, 1991

47. Druker B, Okuda K, Matulonis U, Salgia R, Roberts T, Griffin JD: Tyrosine phosphorylation of rasGAP and associated proteins in chronic myelogenous leukemia cell lines. Blood 79:2215, 1992

48. Risser R, Potter M, Rowe WP: Abelson virus-induced lymphomagenesis in mice. J Exp Med 148:714, 1978

49. Mladenovic J, Mitchell D, Anderson SM: Requirement for both kinase activity and Src-homology 2 (SH2) domain for induction of cytokine secretion by BCR-ABL. Blood 84:381, 1994 (abstr, suppl 1)

50. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON: BCR sequences essential for transformation by the *BCR-ABL* oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell 66:161, 1991

51. McWhirter JR, Galasso DL, Wang JYJ: A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol 13:7587, 1993

52. Franz WM, Berger P, Wang JYJ: Deletion of an N-terminal regulatory domain of the c-*abl* tyrosine kinase activates its oncogenic potential. EMBO J 8:137, 1989

53. Daley GQ, Van Etten RA, Jackson PK, Bernards A, Baltimore D: Non-myristoylated Abl proteins transform a factor-dependent hematopoietic cell line. Mol Cell Biol 12:1864, 1992

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