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 Ab1 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/Ab1 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 phosphoryrosine-binding by SH2, and a complete deletion of SH2 (P210/ΔSH2). Despite a negative effect on intrinsic Ab1 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-Ab1. These results demonstrate that while the Ab1 SH2 domain is essential for BCR/ABL transformation of fibroblasts, it is dispensable for the transformation of hematopoietic factor-dependent cell lines.

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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 Laboratories).

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tories, Melville, NY), based on the method of Kunkel et al. The mutagenic primer (5′- GCAAGCTTCTAGTGAAAGGAGATGAGTAGCCCGCCAGATCCATC 3′) was hybridized to a single-stranded, uracil-containing, P210-neomycin HindIII 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 dyeodeoxy DNA sequencing, and the HindIII fragment was subcloned into the vector pGDARI, which is identical to the vector pGD, but lacks the EcoRI site in the neomycin resistance gene cassette.

The P210/ΔSH2 mutant was generated through a series of restriction digests in which the HindIII site at nucleotide #804 and the Kpn I site at #527 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 dyeodeoxy DNA sequencing and restriction mapping was performed to confirm preservation of the Kpn I site. The mutant cDNA was subcloned into pGDARI for transfection.

Cells and Cell Culture

Ba/F3 and FDC-P1 cells were grown in liquid culture at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DME-high) containing 10% heat-inactivated fetal calf serum, penicillin/streptomycin, and 2 mM 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) (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 were grown in DME-high containing 10% heat-inactivated fetal calf serum, penicillin/streptomycin, 2 mM glutamine, and nonessential amino acids.

Gene Transfer

Retroviral infection. Ten micrograms of P210 or SH2 domain mutant DNA in the retroviral expression vector pGDΔR1, which contains a gene encoding resistance to neomycin, was introduced into the retroviral packaging cell line BOSC-23 by calcium phosphate transfection, as described. Twelve to 16 hours later, 6 x 106 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 of 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 x 106 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 pGDΔR1 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 Gene pulser 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 cm2 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 chi square 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 centrifrip-10 ultrafiltration (Amicon, Beverly, MA), and added to duplicate samples of 105 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. Equal amounts of total RNA were subjected to denaturing agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a 32P-labeled DNA probe generated from a 1,100 bp C1a 1 fragment from the neomycin gene cassette contained in the pGDΔR1 vector.

In Vitro Kinase Assay

P210, P210/R1053K, or P210/ΔSH2, contained in the vector P317 were introduced into 293T cells 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. Equal amounts of Bcr/Abl proteins were subjected to immunoprecipitation with anti-GEX4 antisera (recognizing COOH-terminal Abl sequences), and an immune complex kinase assay performed as previously described, with enolase added as an exogenous substrate. 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).
TRANSFORMATION BY Bcr/Ab1, SH2 MUTANTS

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 10⁶ 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 10⁷ 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/Ab1 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 NH2-terminus of Ab1 increases the intrinsic tyrosine kinase activity of the protein. Relative to c-Ab1, P210 kinase activity was increased approximately six-fold, while P190, the form of Bcr/Ab1 most commonly associated with Philadelphia chromosome-positive ALL, was elevated almost ten-fold, consistent with previous observations (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-Ab1. However, complete deletion of the SH2 domain abrogated the elevated kinase activity of P210, essentially to the level of c-Ab1 (0.7-fold). The impact of these mutations on Bcr/Ab1 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).

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 pGĐΔRI, 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 pGĐΔRI vector alone, P210, or the SH2 mutants, suggesting there was no significant lethal or cytotoxic effect of transfection of Bcr/Ab1 in these cells. 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 pGĐΔRI vector alone nor a kinase-inactive mutant of Ab1 supported any factor-independent growth (data not shown), confirming that the tyrosine kinase activity of Ab1 is required for the transformation of factor-dependent hematopoietic cells.

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/R1053K 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;
factor-independent growth. The differences between P210 of the parental BaF3 and FDC-P1 cells, and the effect of these hematopoietic factor-dependent cell lines to growth case of the FDC-P1 cells, there was no significant difference test comparing cells expressing P210/R1053K supported the growth of pa-

IL-3 (WEHI-3B-conditioned medium) or GM-CSF (data not shown). We were unable to detect similar 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 cyclo-

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). Therefore, in contrast to fibroblast transformation, neither the phosphotyrosine-binding function of SH2 nor the SH2 domain entirely (P210/ASH2) were capable of growth in medium lacking supplemental growth factor (GF-).

Table 1. Effect of SH2 Mutations on GF Independence

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>Cell Type</th>
<th>No. of Clones</th>
<th>GF-/GF+</th>
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* P210BCWABL, 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-).

To investigate the possibility that autocrine secretion of IL-3 was responsible for induction of factor-independence by P210 or the SH2 mutants, 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). 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.

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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 < 0.02, using a χ² 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.

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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. Because the Ba/F3 cell line was originally derived from BALB/c mice, these animals provide a convenient in vivo model to explore P210BCWABL-mediated leukemogenicity. Intravenous injection of 10⁵ 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-Ab1 (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 (neo³), 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-Ab1 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 paraspinal lymph node chain, 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
Others have also recently found FDC-P1 cells to be transphosphotyrosine-independent kinase, while complete deletion of SH2 abrogated the activation of the ASH2 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,35 or containing a point mutation in an autophosphorylation site within the P210/c-Ab1 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 BaF3 or FDC-P1 cells. BCR/ABL signaling 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,36 which might have different consequences in fibroblasts and hematopoietic cells.

Using a quantitative transformation assay we have found that both P210/ΔSH2 domain mutants are actually more transforming than wild-type P210. There are several possible explanations for this finding. One possibility is that P210/ΔSH2 mutants have decreased cytotoxicity. A growth inhibitory or toxic effect has been observed with expression of c-Ab1, v-Ab1, and Bcr/Ab1 in fibroblasts,36,38 which may be dependent on SH2.39 Therefore, mutations in SH2 may decrease this toxic effect of Bcr/Ab1 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/Ab1 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/Ab1 necessary for BaF3 transformation, analogous to Src transformation. We found that populations of BaF3 cells transfected with the SH2 mutants expressed higher levels of Bcr/Ab1 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/Ab1 proteins even with prolonged cycloheximide treatment, which probably reflects the relatively long half-life of P210, as has been noted by others.39 The loss of SH2 function may affect the interaction of Bcr/Ab1 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 paraspinal 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<sub>BCR/ABL</sub> 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<sup>49</sup> may help resolve this issue.

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