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Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway

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The BCR/ABL oncogenic tyrosine kinase activates phosphatidylinositol 3-kinase (PI-3k) by a mechanism that requires binding of BCR/ABL to p85, the regulatory subunit of PI-3k, and an intact BCR/ABL SH2 domain. SH2 domain BCR/ABL mutants deficient in PI-3k activation failed to stimulate Akt kinase, a recently identified PI-3k downstream effector with oncogenic potential, but did activate p21 RAS and p70 S6 kinase. The PI-3k/Akt pathway is essential for BCR/ABL leukemogenesis as indicated by experiments demonstrating that wortmannin, a PI-3k specific inhibitor at low concentrations, suppressed BCR/ABL-dependent colony formation of murine marrow cells, and that a kinase-deficient Akt mutant with dominant-negative activity inhibited BCR/ABL-dependent transformation of murine bone marrow cells *in vitro* and suppressed leukemia development in SCID mice. In complementation assays using mouse marrow progenitor cells, the ability of transformation-defective SH2 domain BCR/ABL mutants to induce growth factor-independent colony formation and leukemia in SCID mice was markedly enhanced by expression of constitutively active Akt. In retrovirally infected mouse marrow cells, the BCR/ABL mutant lacking the SH2 domain was unable to upregulate the expression of c-Myc and Bcl-2; in contrast, expression of a constitutively active Akt mutant induced Bcl-2 and c-Myc expression, and stimulated the transcription activation function of c-Myc. Together, these data demonstrate the requirement for the BCR/ABL SH2 domain in PI-3k activation and document the essential role of the PI-3k/Akt pathway in BCR/ABL leukemogenesis.

Keywords: activation/Akt/BCR/ABL/leukemogenesis/PI-3k

Introduction

The *bcr/abl* chimeric oncogenes are generated from a reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome) which fuses a truncated *bcr* gene to sequences upstream of the second exon of c-abl (Epner and Koeffler, 1990). *bcr/abl* genes encode the constitutively active p210 and p185 BCR/ABL tyrosine kinases (Shtivelman *et al.*, 1986; Clark *et al.*, 1988) which play essential roles in the pathogenesis of chronic myelogenous leukemia (CML) and Philadelphia[1] (Ph[1]) acute lymphoblastic leukemia (Lugo *et al.*, 1990). The expression of cytoplasmic BCR/ABL proteins (Dhut *et al.*, 1990) is associated with the activation of several downstream effector molecules (Cortez *et al.*, 1995). Among them is phosphatidylinositol-3 kinase (PI-3k) (Varticovsky *et al.*, 1991; Skorski *et al.*, 1995) which is also required for the proliferation of Ph[1] cells but not normal hematopoietic cells (Skorski *et al.*, 1995). However, there has been no evidence indicating that PI-3k plays an essential role in BCR/ABL leukemogenesis. PI-3k was discovered as an activity that phosphorylates phosphoinositols at the D-3′ position of the inositol ring and produces novel phosphoinositides (Whitman *et al.*, 1988; Carpenter and Cantley, 1990). Purified PI-3k was shown to be a heterodimer consisting of a 85 kDa (p85) regulatory subunit and a 110 kDa (p110) catalytic subunit (Shibasaki *et al.*, 1991). The mechanism of PI-3k activation is not fully understood, but the association of p85 with activated tyrosine kinases is thought to provide a signal sufficient for the activation of the p110 catalytic subunit.

The role of PI-3k in transducing tyrosine kinase signals is well established (Coughlin *et al.*, 1989; Wages *et al.*, 1992), but the activation of PI-3k by tyrosine kinases may involve different mechanisms, as suggested by the fact that its interaction with tyrosine kinases may be mediated by a variety of interacting domains. PI-3k can interact with activated tyrosine kinase directly via the p85 SH2 domain and phosphorylated tyrosine residues in the kinase (Hu *et al.*, 1992), or via tyrosine kinase SH3 domains and the proline-rich region of p85 (Liu *et al.*, 1993; Pleiman *et al.*, 1994), and also indirectly via other proteins such as RAS, Shc, CRKL, Grb-2, GAP or c-Cbl (Harrison-Findik *et al.*, 1994, 1995; Rodriguez-Viciana *et al.*, 1994; Sattler *et al.*, 1996) which may also influence PI-3k activation. Once activated, PI-3k may affect several downstream targets including RAS (Hu *et al.*, 1995), Akt (Franke *et al.*, 1995) and S6 kinase (Cheatham *et al.*, 1994; Chung *et al.*, 1994).

Although it has been shown that BCR/ABL activates PI-3k, the mechanism(s) of its activation, the effects on the putative downstream effectors, and the role of PI-3k-dependent pathway(s) in BCR/ABL-induced leukemic transformation have remained unclear. We show here that the mechanism of activation of PI-3k by BCR/ABL involves not only association of p85 with BCR/ABL but also an intact BCR/ABL SH2 domain, and that the Akt serine/threonine kinase, but not RAS or S6 kinase, is a

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downstream effector of PI-3k in BCR/ABL-expressing cells. Most importantly, we provide the first direct demonstration that the activation of the PI-3k/Akt pathway is essential for BCR/ABL-mediated leukemogenesis *in vitro* and *in vivo*.

Results

BCR/ABL activation of PI-3k

To investigate the mechanisms and functional consequences of PI-3k activation by BCR/ABL, we first identified BCR/ABL mutants that were defective in PI-3k activation. The following mutants were used: the kinase-deficient, transformation-defective mutant K1172R (Pendergast *et al.*, 1993); the ΔSH2 BCR/ABL mutant which lacks the entire SH2 domain (aa 1030–1120) (Ilaria and Van Etten, 1995); the SH2 FLVRES motif mutant R1053L (FLVLES) which, like the ΔSH2 mutant, fails to bind motifs containing phosphorylated tyrosines (Afar *et al.*, 1994); the ΔSH3 BCR/ABL mutant which lacks the entire SH3 domain (aa 959–1020) (Skorski *et al.*, 1997); the ΔSH3/ΔSH2 mutant which lacks both the SH3 and SH2 domains (aa 959–1020 and aa 1030–1120); the Y1370F mutant which contains a single amino acid substitution in the YELM motif that is recognized by the SH2 domain of p85 (Songyang *et al.*, 1993); the Y177F mutant which is deficient in Grb-2 binding (Pendergast *et al.*, 1993); and the Δ176–426 mutant which lacks a segment required for intramolecular interaction with the SH2 domain of ABL and for Grb-2 and 14-3-3 binding (Pendergast *et al.*, 1991, 1993; Reuther *et al.*, 1994).

After transfection of growth factor-dependent 32Dcl3 murine myeloid precursor cells (Greenberger *et al.*, 1983), clones with the highest expression of wild-type (WT) and mutant BCR/ABL proteins were selected (Figure 1A) and examined for complex formation between BCR/ABL and the p85 subunit of PI-3k and for the ability to activate the p110 catalytic subunit. BCR/ABL–p85 complex formation was assayed in anti-p85 immunoprecipitates by SDS–PAGE followed by Western blotting with an anti-ABL antibody, and PI-3k enzymatic activity was determined in anti-phosphotyrosine (anti-P.Tyr), anti-p85 and anti-BCR/ABL immunoprecipitates from lysates of cell clones expressing BCR/ABL proteins or carrying the empty vector, as described (Skorski *et al.*, 1995).

As expected, binding of p85 to WT BCR/ABL (Figure 1B, upper panel) correlated with activation of PI-3k (Figure 1B, lower panel), whereas lack of binding to the K1172R kinase-deficient mutant was associated with absence of PI-3k activation (Figure 1B). The ability of the ΔSH3, Y1370F and Y177F BCR/ABL mutants to form a complex with p85 was also accompanied by PI-3k activation indistinguishable from that induced by WT BCR/ABL (Figure 1B). The interaction of p85 with the Δ176–426 mutant was strongly impaired (Figure 1B, upper panel) and this correlated with a proportional reduction in PI-3k activation (Figure 1B, lower panel). The defect in the interaction of Δ176–426 BCR/ABL with the PI-3k p85 subunit may reflect either the lack of a specific motif in the mutant protein or conformational changes, since the deletion involves a region of BCR that interacts with the SH2 domain in the ABL segment of BCR/ABL (Pendergast *et al.*, 1991). Thus, in accordance with previous findings on the mechanisms of PI-3k activation by activated tyrosine kinases (Pleiman *et al.*, 1994; Liu *et al.*, 1993; Kapeller and Cantley, 1994), our results indicate that PI-3k activation by BCR/ABL is dependent on complex formation between the p85 subunit of PI-3k and BCR/ABL, and on BCR/ABL tyrosine kinase activity.

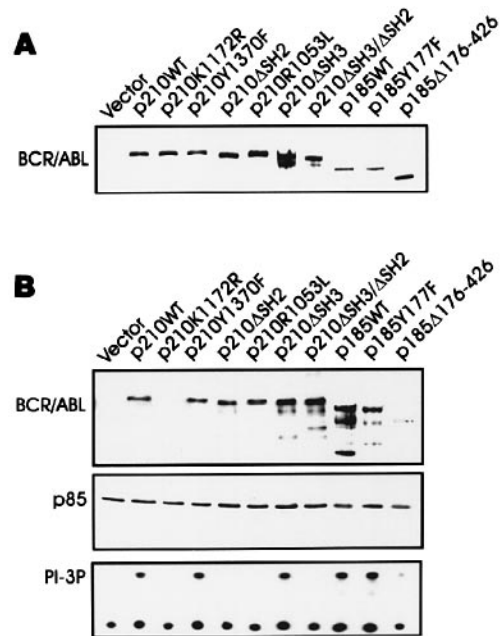

Fig. 1. (**A**) Expression of BCR/ABL proteins in 32Dcl3 cell clones. 32Dcl3 cells were electroporated with the pSRα constructs encoding the indicated BCR/ABL proteins. Three clones with the highest expression of BCR/ABL proteins were selected for further studies. Western blots show expression of wild-type or mutant BCR/ABL protein in representative clones. (**B**) Interaction with and activation of PI-3k by BCR/ABL mutants. Experiments were performed using serum- and growth factor-starved (5 h) 32Dcl3 cells. Upper panel, coimmunoprecipitation of PI-3k with various BCR/ABL proteins. Anti-PI-3k (p85) immunoprecipitates from 32Dcl3 transfectants expressing wild-type or mutant BCR/ABL were analyzed by SDS–PAGE followed by Western blotting with anti-ABL antibody. Middle panel, Western blotting detection of p85 in anti-PI-3k immunoprecipitates. Non-immune serum did not precipitate any BCR/ABL or p85 proteins (not shown). Lower panel, PI-3k activity in 32Dcl3 transfectants expressing various BCR/ABL proteins. PI-3k was assayed in anti-phosphotyrosine immunoprecipitates using [γ-^{32}P] adenosine triphosphate and phosphatidylinositol as a substrate (Skorski et al., 1995). Levels of BCR/ABL proteins were identical in immunoprecipitates from wild-type and SH2 domain mutant BCR/ABL-expressing cells (data not shown). ^{32}P-labeled phosphatidylinositol-phosphate (PIP) were resolved by thin layer chromatography and visualized by autoradiography. Results represent three independent experiments from individual clones. Similar results were obtained when PI-3k was assayed in anti-p85 and in anti-BCR/ABL immunoprecipitates (data not shown).

Like WT BCR/ABL, the two SH2 domain mutants ΔSH2 and R1053L formed a complex with p85 (Figure 1B, upper panel). However, both mutants failed to stimulate PI-3k catalytic activity in 32Dcl3 growth factor-dependent cells (Figure 1B, lower panel) and in BaF3 cells (not shown). Thus, BCR/ABL activation of PI-3k is dependent on complex formation with p85 and on the integrity of the FLVRES motif of the BCR/ABL SH2 domain.

The mechanism by which the FLVRES motif in the SH2 domain of BCR/ABL is required for PI-3k activation is unknown, but most likely involves binding to other phosphorylated protein(s), since the FLVRES motif inter-

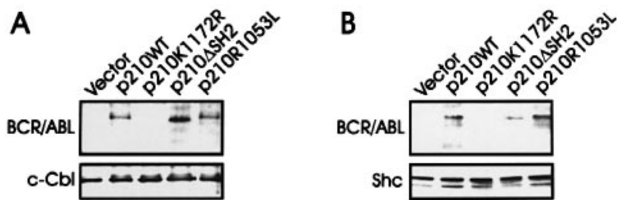

Fig. 2. Interaction of c-Cbl and Shc with wild-type and mutant BCR/ABL proteins. c-Cbl (**A**) and Shc (**B**) were immunoprecipitated from cells expressing BCR/ABL proteins and analyzed by SDS–PAGE followed by Western blotting with anti-ABL, anti-c-Cbl or anti-Shc antibodies, respectively.

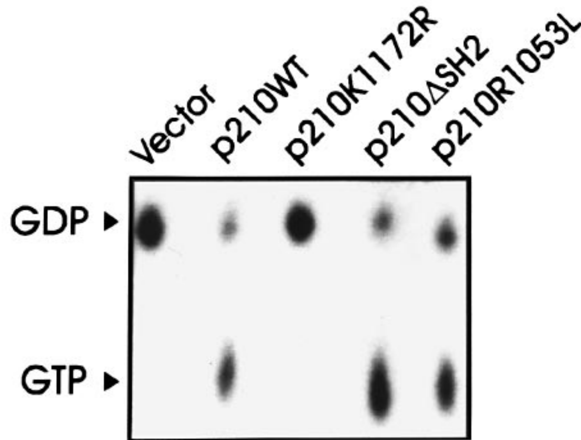

Fig. 3. RAS activation by wild-type and mutant BCR/ABL proteins. A standard GTP-bound assay of RAS activation was performed as described (Skorski et al., 1994) using serum- and growth factor-starved BCR/ABL-transfected 32Dcl3 cells. Results represent three independent experiments using individual cell clones.

acts with peptides containing phosphorylated tyrosine (Mayer *et al.*, 1992). One of the major tyrosine phosphorylated proteins associated with p85 and BCR/ABL is that encoded by the c-cbl protooncogene (c-Cbl) (Sattler *et al.*, 1991). However, in cells transfected with the ΔSH2 or FLVRES BCR/ABL mutant, c-Cbl was phosphorylated on tyrosine (not shown), formed a complex with p85, and was also detected in a complex with mutant BCR/ABL proteins (Figure 2A). Shc proteins are other tyrosine phosphorylated proteins detectable in complexes with both p85 and BCR/ABL (Tauchi *et al.*, 1994). However, these proteins are also present in complexes with the SH2 mutants of BCR/ABL and p85 (Figure 2B), which again argues against an essential role for these proteins in PI-3k activation by BCR/ABL.

BCR/ABL activates RAS which has been shown to bind and activate p110 (Rodriguez-Viciana *et al.*, 1994). This raised the possibility that the activation of PI-3k by BCR/ABL might be mediated by RAS. Thus, we measured the ratio of GTP/GDP-bound RAS in 32Dcl3 cells expressing wild-type or mutant BCR/ABL proteins. The PI-3k activation-deficient ΔSH2 and R1053L BCR/ABL mutants efficiently increased the relative ratio of GTP-bound RAS (Figure 3), suggesting that PI-3k and RAS are independently regulated by BCR/ABL, and that RAS activation is not sufficient for PI-3k activation by BCR/ABL.

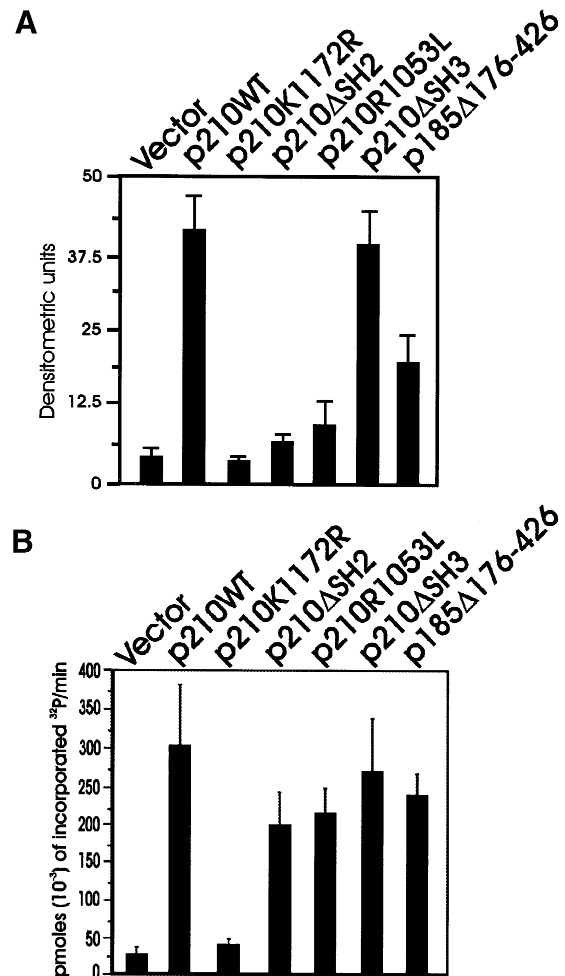

Fig. 4. Activation of Akt serine/threonine kinase and S6 kinase by wild-type and mutant BCR/ABL. Experiments were performed using serum- and growth factor-starved cells. (**A**) Akt activity was measured by phosphorylation of histone H2B used as a substrate. Equal amounts of Akt proteins were immunoprecipitated in each sample (data not shown). (**B**) S6k enzymatic activity was examined by an immune complex *in vitro* kinase assay using an S6k assay kit according to the protocol suggested by the manufacturer's (UBI, Lake Placid, NY). Results represent three independent experiments using individual cell clones.

Activation of PI-3k downstream effectors in BCR/ABL-expressing cells

Earlier studies have shown that the activation of the serine/threonine protein kinase Akt and p70 S6 kinase (S6k) is dependent on PI-3k (Cheatham *et al.*, 1994; Chung *et al.*, 1994; Franke *et al.*, 1995). We therefore examined whether PI-3k activation by BCR/ABL was followed by activation of Akt and S6k. Indeed, activation of PI-3k by WT BCR/ABL, ΔSH3 BCR/ABL and Δ176–426 BCR/ABL correlates with Akt activation, whereas the kinase-deficient K1172R BCR/ABL and the ΔSH2 and R1053L BCR/ABL PI-3k activation-deficient mutants failed to stimulate Akt enzymatic activity (Figure 4A). Additional evidence that Akt activation is due to BCR/ABL-mediated stimulation of PI-3k came from the observation that wortmannin, a PI-3k specific inhibitor at low concentrations (Powis *et al.*, 1994), inhibited Akt activation in 32Dcl3 cells expressing wild-type BCR/ABL (not shown). In contrast to Akt, S6k was activated by the PI-3k activation-deficient BCR/

Table I. Effect of wortmannin on the clonogenic activity of BCR/ABL-expressing mouse marrow cells

Dose (nM)[a]	IL-3⁻	IL-3⁺
0	30 ± 10[b]	166 ± 28
62.5	10 ± 2	87 ± 13
125	3 ± 2	43 ± 9
250	1 ± 1	30 ± 9
500	0 ± 0	21 ± 5

[a]Methylcellulose colony formation, in the absence or in the presence of a threshold concentration (0.1 U/ml) of recombinant murine IL-3 from mouse bone marrow cells infected with the retrovirus carrying wild-type BCR/ABL and treated with wortmannin (Sigma) at the indicated concentrations. Wortmannin was added to the cells (10^5/0.1 ml) at 0, 12 and 24 hrs of culture because of its instability (Woscholski *et al.*, 1994). Cells were plated in methylcellulose immediately after adding the last dose of wortmannin.
[b]Values are mean ± SD of duplicate cultures from two independent experiments.

ABL SH2 domain mutants (Figure 4B), consistent with the results of an earlier study suggesting the existence of a PI-3k-independent pathway in S6k regulation (Downward, 1994).

Akt activity is essential for BCR/ABL-mediated leukemogenesis in vitro

We have shown previously that the expression and the activity of PI-3k is essential for the growth of CML primary cells (Skorski *et al.*, 1995). To demonstrate that PI-3k activity is also important for BCR/ABL-dependent leukemogenesis, murine bone marrow cells were infected with the retrovirus carrying wild-type BCR/ABL and treated with wortmannin. Clonogenic assay in methylcellulose revealed that wortmannin markedly inhibited the colony-forming ability of BCR/ABL-infected marrow cells (Table I). Based on this finding and the results in Figure 4 supporting the role of Akt as a PI-3k downstream effector in BCR/ABL-expressing cells, we asked whether Akt activity is required for BCR/ABL leukemogenesis. In mouse marrow cells coinfected with BCR/ABL- and hemagglutinin (HA)-tagged Akt-containing retroviruses (Figure 5, upper panel), we tested whether the kinase-deficient dominant-negative Akt K179M mutant (Dudek *et al.*, 1997) inhibits transformation of hematopoietic progenitor cells by wild-type BCR/ABL and whether the impaired transforming potential of BCR/ABL SH2 domain mutants can be rescued by overexpression of constitutively active Akt E40K mutant (Ahmed *et al.*, 1997). Infection of mouse bone marrow cells with a retrovirus encoding wild-type BCR/ABL induced the formation of a high number of colonies in the presence of threshold concentration of recombinant murine IL-3 (0.1 U/ml) and also of a few colonies in the absence of IL-3 (Figure 5, lower panel). Coinfection of marrow cells with c-Akt or the constitutively active Akt E40K mutant moderately increased BCR/ABL-dependent colony formation (Figure 5, lower panel); in contrast, coinfection with the dominant-negative kinase-deficient Akt K179M mutant inhibited colony formation induced by wild-type BCR/ABL by ~50% (Figure 5, lower panel). Upon infection of mouse marrow progenitor cells with retroviruses carrying BCR/ABL SH2 domain mutants (ΔSH2 and R1053L), no hematopoietic colonies formed in methylcellulose in the

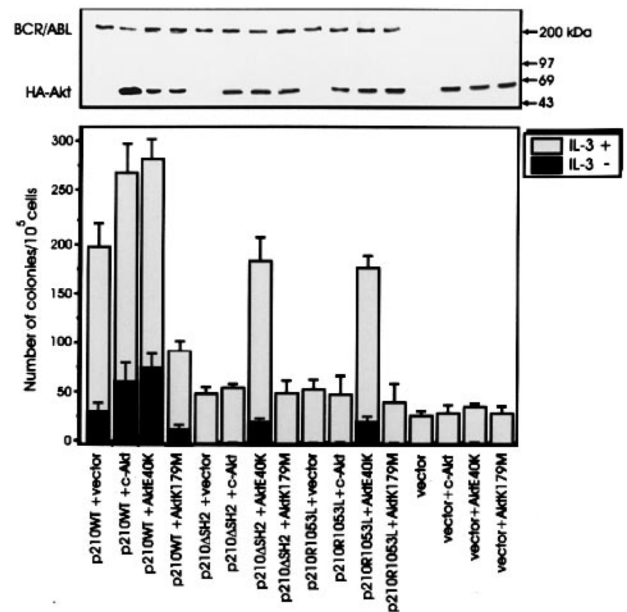

Fig. 5. Akt is essential for BCR/ABL-dependent transformation of murine bone marrow cells. Upper panel, expression of BCR/ABL and HA–Akt proteins in mouse marrow cells coinfected with BCR/ABL or Akt and the insert-less vector or with BCR/ABL and Akt retroviruses. Lower panel, methylcellulose colony formation from mouse marrow cells infected with BCR/ABL and/or Akt retroviruses and cultured in the absence or in the presence of threshold concentration (0.1 U/ml) of recombinant murine IL-3. Results represent three independent experiments and are expressed as the mean ± SD.

absence of IL-3 and only few colonies developed when cultures were supplemented with threshold concentrations (0.1 U/ml) of IL-3 (Figure 5, lower panel). However, coinfection of marrow cells with the BCR/ABL SH2 domain mutants and with a retrovirus carrying a constitutively active Akt E40K mutant induced extensive colony formation as indicated by size (not shown) and number of methylcellulose colonies (Figure 5, lower panel). Morphological examination (Giemsa staining) of cytospin preparations from methylcellulose cultures of marrow cells infected with wild-type BCR/ABL or coinfected with BCR/ABL SH2 domain mutants and Akt E40K mutant did not reveal obvious differences. The majority of the cells exhibited features of differentiation in the granulocyte–macrophage lineages, whereas ~10% of the cells were still blast-like (not shown). The kinase-deficient Akt K179M mutant and the wild-type c-Akt were unable to rescue the transformation-deficient phenotype of SH2 domain BCR/ABL mutants (Figure 5, lower panel). Moreover, infection of marrow cells only with the retrovirus carrying the constitutively active Akt E40K mutant did not yield growth factor-independent colonies (Figure 5, lower panel). Thus, Akt cooperates with BCR/ABL in transformation of hematopoietic cells.

Akt is essential for BCR/ABL-mediated leukemogenesis in vivo

To determine whether Akt plays an essential role in BCR/ABL-mediated leukemogenesis *in vivo*, mice were injected with marrow cells expressing both wild-type BCR/ABL and the dominant-negative kinase-deficient Akt K179M mutant. In addition, because *in vivo* leukemogenesis of BCR/ABL-infected mouse marrow cells is impaired by

Table II. Detection of BCR/ABL transcripts in the peripheral blood of SCID mice injected with BCR/ABL- and Akt-expressing marrow cells

Cells injected[a]	Weeks[b]		
	3	6	9
p210 WT + vector	6/6[c]	6/6	6/6
p210 WT + Akt K179M	0/4	0/4	2/4
p210 ΔSH2 + vector	0/6	0/6	3/6
p210 ΔSH2 + c-Akt	0/4	0/4	2/4
p210 ΔSH2 + Akt E40K	4/4	4/4	4/4

[a]Bone marrow cells were infected with the wild-type (WT) or the ΔSH2 BCR/ABL retrovirus together with the insert-less pSRα vector, c-Akt or Akt mutants, and injected (1×10^6) i.v. into pre-irradiated SCID mice.
[b]At 3, 6 and 9 weeks, the presence of BCR/ABL transcripts in peripheral blood leukocytes was examined by RT–PCR.
[c]Number of positive mice/number of mice per group.

the absence of a functional SH2 domain (Afar *et al.*, 1995; Goga *et al.*, 1995), we tested whether expression of the constitutively active Akt E40K mutant might rescue the defective leukemogenic potential of ΔSH2 BCR/ABL-infected marrow cells in SCID mice.

Marrow cells from 5-FU-treated mice were simultaneously infected with supernatant containing wild-type or ΔSH2 BCR/ABL viruses together with supernatant containing viruses carrying the hemagglutinin (HA)-tagged c-Akt or the constitutively active (E40K) or the dominant-negative kinase-deficient (K179M) form of Akt, or the insert-less virus. Expression of BCR/ABL and HA–Akt proteins was detected by SDS–PAGE followed by Western blotting with anti-ABL and anti-HA11 antibody, respectively (not shown). Infected cells (10^6) were injected into pre-irradiated SCID mice, and leukemia development was monitored by assessing the presence of BCR/ABL transcripts in peripheral blood leukocytes (PBL) collected from the tail vein 3, 6 and 9 weeks post-injection. BCR/ABL mRNA transcripts were detectable in all mice 3 and 6 weeks after injection of cells coinfected with wild-type BCR/ABL and the empty virus or ΔSH2 BCR/ABL and Akt E40K, but not after injection of cells infected with wild-type BCR/ABL and the dominant-negative kinase-deficient Akt K179M, or ΔSH2 BCR/ABL and the empty virus, or ΔSH2 BCR/ABL and c-Akt (Table II). Nine weeks after cell inoculation, low levels of BCR/ABL transcripts also became detectable in two of the four mice injected with wild-type BCR/ABL and Akt K179M mutant, in three of the six mice injected with ΔSH2 BCR/ABL and the empty virus, and in two of the four mice injected with ΔSH2 BCR/ABL and c-Akt.

Twelve weeks after injection of retrovirally infected cells, various organs obtained from control SCID mice or mice injected with bone marrow cells expressing BCR/ABL, BCR/ABL and Akt K179M, ΔSH2 BCR/ABL, ΔSH2 BCR/ABL and c-Akt or ΔSH2 BCR/ABL and Akt E40K were evaluated by visual inspection and light microscopy (Figure 6). Control SCID mice had mild splenomegaly with extramedullary hematopoiesis consisting of orderly maturing erythroid, megakaryocytic, and some myeloid cells. Injection with BCR/ABL-expressing marrow cells resulted in massive splenomegaly in all six mice tested. Hematoxylin and eosin (H&E)-stained sections demonstrated extensive involvement of the spleens by a myeloproliferative disorder either chronic, CML-like, or acute, consistent with acute myelogenous leukemia (AML). Accordingly, spleens in four out of six mice were extensively involved by blasts which displayed a low degree of myeloid maturation. Leder stain confirmed the myeloid origin of the blasts (not shown). Bone marrows were characterized by extensive CML-like myeloproliferation (granulocytic hyperplasia with various degree of left shift in maturation) or an overt AML. While abnormal, the bone marrows usually demonstrated a higher degree of myeloid maturation than other organs. AML also involved livers and, less frequently, kidneys and lungs. Mice injected with marrow cells expressing both wild-type BCR/ABL and kinase-deficient dominant-negative Akt K179M mutant had only mild splenomegaly and bone marrow showed CML-like morphology with variable left shift in myeloid maturation, but not AML. While AML or CML-like disease involved spleens, the extent of involvement was much lower as compared with spleens from mice injected with marrow cells expressing wild-type BCR/ABL only (Figure 6). Also, only one tumor nodule was found in the liver, and the kidneys and the lungs were free of leukemia. These findings support the conclusion that the leukemogenic properties of BCR/ABL are, to a certain degree, mediated by Akt.

In contrast to wild-type BCR/ABL, mice injected with marrow cells expressing ΔSH2 BCR/ABL usually demonstrated only mild to moderate splenomegaly. Although evidence of AML was found in spleen and bone marrow in three of six mice (the remaining three mice developed CML-like disease), the involvement was less extensive; focal kidney involvement was noted only in one mouse, whereas liver and lungs were free of leukemia in all six mice. These data indicate that the loss of the SH2 domain decreases the leukemogenic capability of BCR/ABL as determined by a lower degree of splenomegaly, the inability to grow efficiently in non-hematopoietic organs and, apparently, to undergo blastic transformation. To determine whether the constitutively active Akt can substitute for the deleted SH2 domain, four mice were injected with marrow cells expressing both ΔSH2 BCR/ABL and Akt E40K. The extent of splenomegaly and leukemic involvement of various organs was similar to that observed in mice injected with cells expressing wild-type BCR/ABL. The only subtle difference between the wild-type and ΔSH2 BCR/ABL plus Akt E40K-expressing cells was the apparently more mature feature of acute myeloid leukemia in the latter. As expected, replacing Akt E40K with c-Akt failed to enhance the leukemogenic potential of the ΔSH2 mutant. Similarly to ΔSH2 BCR/ABL alone, the ΔSH2 BCR/ABL plus c-Akt-expressing cells produced no massive splenomegaly or involvement of non-hematopoietic organs, and only rarely underwent blastic transformation in all four mice tested.

Akt activates c-Myc and Bcl-2 expression

BCR/ABL proteins carrying the R→L mutation in the FLVRES motif of the SH2 domain are defective in transformation (Afar *et al.*, 1995) and in Akt activation (Figures 4 and 5), but can be complemented by c-Myc overexpression in fibroblasts (Afar *et al.*, 1994).

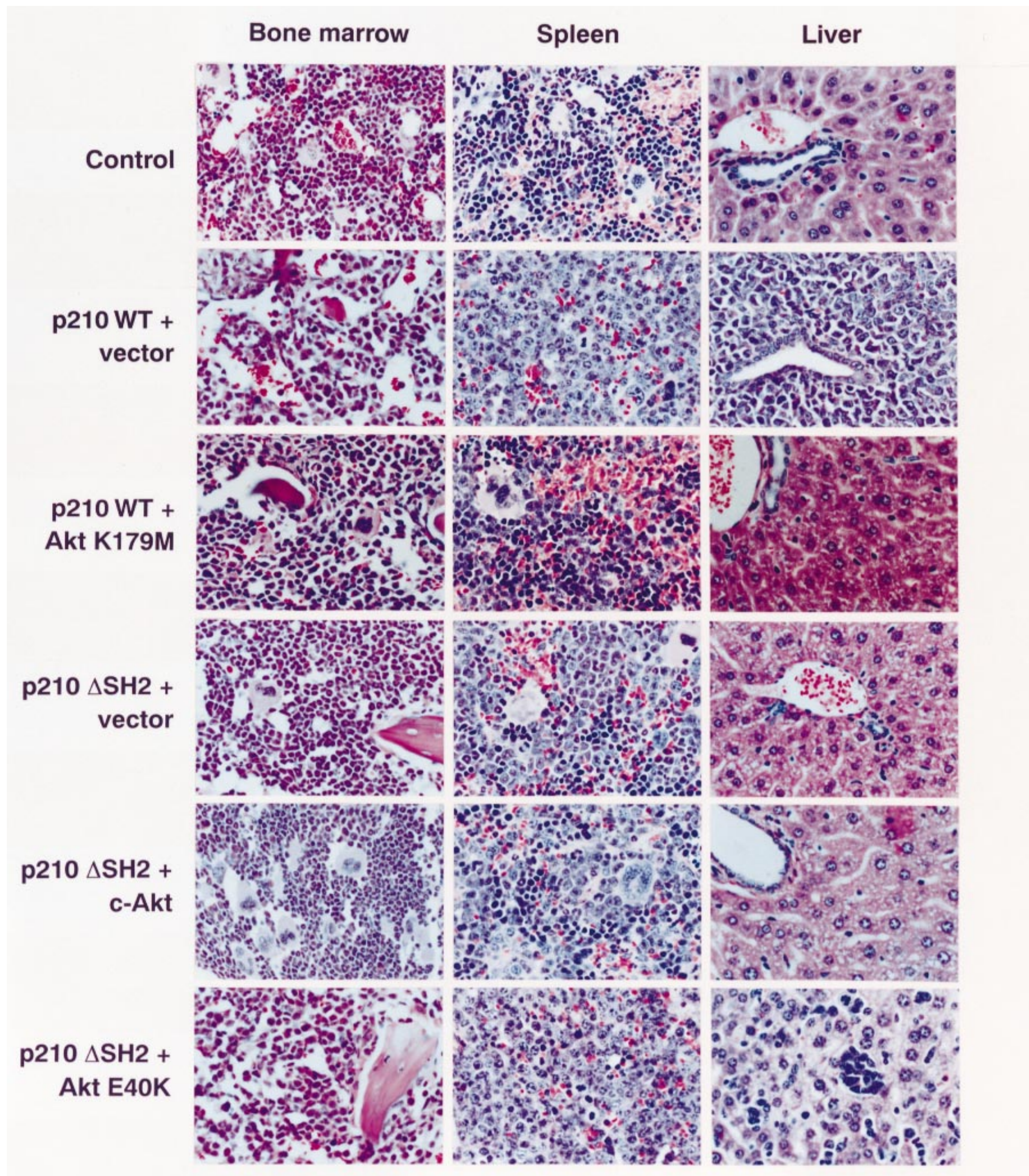

Fig. 6. Histologic findings in mice injected with BCR/ABL- or BCR/ABL/Akt-expressing marrow cells. Tissue sections from various organs were fixed in phosphate buffered formalin, embedded in paraffin block, and stained with hematoxylin/eosin (H&E). These are representative morphologic findings in the bone marrow, spleen, and liver (600× magnification) from groups of four to six mice of each phenotype. The BCR/ABL and Akt mutants are the same as those described in Table I. Age-matched, non-injected SCID mouse served as a control.

Since ΔSH2 BCR/ABL failed to stimulate PI-3k/Akt and constitutively active Akt complemented this mutant, we asked whether expression of constitutively active Akt stimulates the expression and/or the function of c-Myc. Western blot analysis of c-Myc expression in murine bone marrow cells expressing BCR/ABL proteins revealed, 8 h after starvation from growth factors, high levels of c-Myc in cells expressing wild-type, but not ΔSH2 BCR/ABL (Figure 7). Interestingly, coexpression of the dominant-negative Akt K179M mutant suppressed the enhanced c-Myc levels induced by wild-type BCR/ABL (Figure 7). Expression of the constitutively active Akt E40K mutant was sufficient to upregulate c-Myc expression in marrow cells, regardless of the coexpression of ΔSH2 BCR/ABL (Figure 7). To determine whether the Akt-dependent increased expression of c-Myc also enhances its trans-activating function, we carried out transient transfection assays in hamster fibroblasts using a reporter plasmid (p-MMBS-SV1-Luc) (Gu *et al.*, 1993) in which a single copy of the Myc–Max binding site is linked upstream of a minimal SV40 promoter/luciferase reporter gene; compared with the promoter construct lacking the Myc–Max binding site, luciferase activity was enhanced ~8- and 25-fold by cotransfection with the constitutively active v-Akt and Akt E40K mutant, respectively (Figure 8). This effect was dependent on a functional Akt enzyme, since

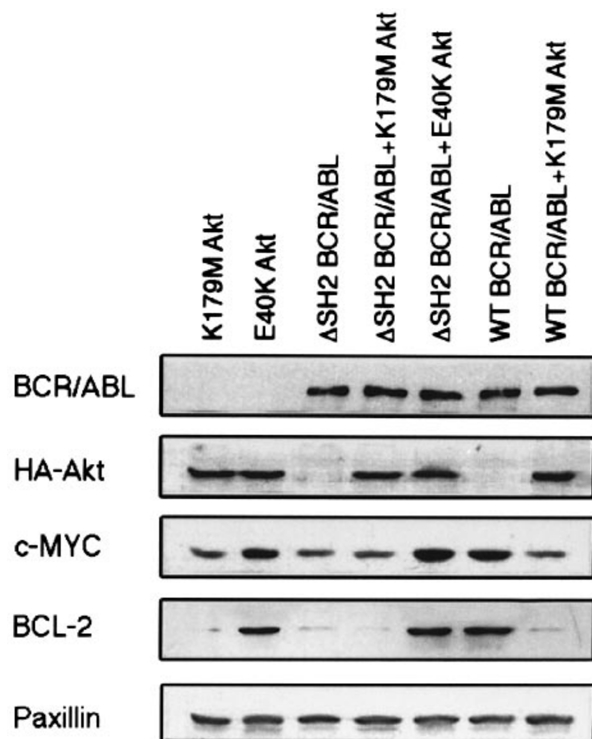

Fig. 7. Akt is essential for upregulation of c-Myc and Bcl-2 expression. Expression of the indicated proteins was detected by SDS–PAGE and Western blotting in growth factor-starved marrow cells infected with the indicated viruses carrying BCR/ABL and/or Akt mutants.

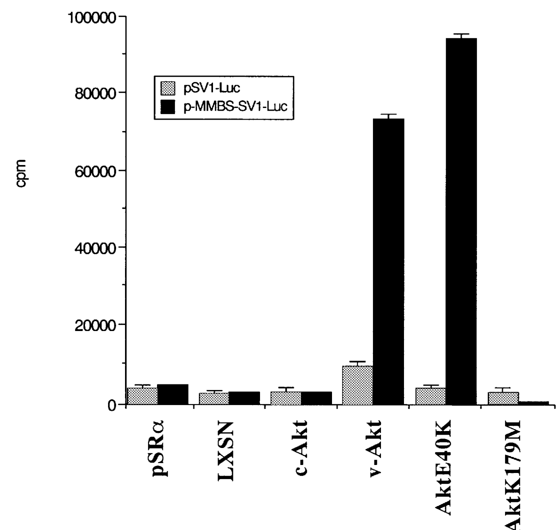

Fig. 8. Constitutively active Akt enhances the transactivation ability of c-Myc. Luciferase assays in Tk⁻ts13 hamster fibroblasts transiently transfected with plasmids containing c-Akt or Akt mutants and the reporter gene promoter SV1-luciferase (pSV1-LUC) or p-MMBS-SV1-LUC (containing a Myc–Max binding site upstream of the SV40 promoter/luciferase construct). Transfection efficiency was normalized by measuring β-galactosidase activity. Results (mean ± SD) represent five independent experiments.

the kinase-deficient Akt K179M mutant and also c-Akt, which is inactive under conditions of serum-deprivation, were unable to enhance luciferase activity driven by the Myc–Max binding site (Figure 8).

A similar enhancement of luciferase activity was observed in cotransfection assays in COS-7 cells using the constitutively active form of the p110 subunit of PI-3k (Hu *et al.*, 1995), but not using the kinase-deficient derivative (not shown).

Since ΔSH2 BCR/ABL is also defective in the stimulation of Bcl-2 expression in murine bone marrow cells (Figure 7), as well as in 32Dcl3 cells (unpublished observations), we tested whether Akt E40K enhances Bcl-2 expression. Indeed, when expressed in marrow cells, Akt E40K increased Bcl-2 level after IL-3 starvation (Figure 7), and also restored Bcl-2 expression in cells coinfected with ΔSH2 BCR/ABL. Of interest, the dominant-negative Akt K179M mutant suppressed the BCR/ABL-dependent increase in the levels of Bcl-2 (Figure 7).

Discussion

BCR/ABL oncoproteins transform hematopoietic cells by activation of multiple pathways (Cortez *et al.*, 1995; Goga *et al.*, 1995) that allow their growth factor-independent proliferation and survival (Mandanas *et al.*, 1992; Bedi *et al.*, 1994; McGahon *et al.*, 1994: Sirard *et al.*, 1994), and their enhanced motility and trafficking (Gordon *et al.*, 1987; Verfaille *et al.*, 1992). Most of the BCR/ABL-dependent signaling pathways are also activated by the cytokines that enable the growth and differentiation of normal hematopoietic progenitor cells (Matulonis *et al.*, 1993; Wisniewski *et al.*, 1996). PI-3k is a typical example of a signaling protein which can be activated by both BCR/ABL and growth factors. However, while the PI-3k pathway is required for the proliferation of BCR/ABL-expressing cells, it is dispensable for normal hematopoietic cell growth (Skorski *et al.*, 1995). This feature led us to investigate the mechanism(s) of BCR/ABL activation of PI-3k and to assess the role of pathway(s) activated by the PI-3k in BCR/ABL leukemogenesis.

Mechanisms of PI-3k activation by BCR/ABL

The association of activated tyrosine kinases with the p85 subunit of PI-3k is essential for triggering the catalytic activity of the p110 subunit (Kapeller and Cantley, 1994). Using 32Dcl3 myeloid precursor cells transfected with various BCR/ABL mutants, we found that an intact SH2 domain is required for BCR/ABL-dependent activation of PI-3k. The inability of SH2 domain mutants to activate PI-3k was unexpected because these mutants continue to exhibit tyrosine kinase activity and form a complex with PI-3k. Most likely, the association BCR/ABL-PI-3k and the activity of PI-3k is mediated by distinct domains from different proteins. In support of this hypothesis, the Y1370F BCR/ABL mutant which is defective for *in vitro* interaction with the p85 subunit (Jain *et al.*, 1996), formed a complex *in vivo* with PI-3k and was competent for PI-3k activation (Figure 1). Moreover, the ΔSH3 BCR/ABL mutant was also competent for PI-3k activation and formed a complex with immunoprecipitated p85 (Figure 1), even though the SH3 domain of BCR/ABL might be required for *in vitro* interaction with p85 as demonstrated for c-Abl (Kapeller *et al.*, 1994).

The inability of the ΔSH2 BCR/ABL mutant to activate PI-3k might depend in part on defective signaling via

tyrosine phosphorylated protein(s), because mutation in the FLVRES motif (R1053L) of the BCR/ABL SH2 domain, which is essential for interaction with tyrosine phosphorylated proteins (Mayer *et al.*, 1992), also prevented activation of PI-3k. Previously identified proteins interacting with both BCR/ABL and PI-3k, such as RAS, c-Cbl, Shc and Grb-2, most probably are not sufficient for PI-3k activation because they are activated by, or remain in complex with, ΔSH2 BCR/ABL. Experiments to identify BCR/ABL SH2 domain-interacting proteins involved in PI-3k activation are now in progress.

PI-3k-dependent Akt activation is required for BCR/ABL leukemogenesis

The SH2 domain BCR/ABL mutants that were defective in PI-3k activation were also unable to activate the Akt serine/threonine kinase (Figure 4A), but not other PI-3k downstream effectors such as S6 kinase (Figure 4B) and RAS (Figure 3). On the other hand, all the BCR/ABL mutants able to activate PI-3k also activated Akt. Moreover, the PI-3k inhibitor wortmannin (Powis *et al.*, 1994) suppressed PI-3k and Akt activities in 32Dcl3 cells expressing BCR/ABL and abrogated colony formation by bone marrow cells infected with BCR/ABL. Thus, Akt appears to be the primary target of PI-3k in the signaling pathway activated from the SH2 domain of BCR/ABL.

The essential role of Akt in BCR/ABL-mediated leukemogenesis was established by the experiments demonstrating that the kinase-deficient Akt K179M mutant, which acts in a dominant-negative manner (Dudek *et al.*, 1997; Kennedy *et al.*, 1997), inhibited BCR/ABL-induced transformation of bone marrow cells *in vitro* and suppressed leukemia development in mice. The residual leukemogenic potential of wild-type BCR/ABL in the presence of the dominant-negative Akt mutant is most likely due to Akt-independent mechanisms of transformation, although we cannot exclude incomplete suppression of Akt activation in cells coexpressing wild-type BCR/ABL and K179M Akt mutant. Consistent with the important role of Akt in BCR/ABL leukemogenesis, the constitutively active Akt E40K mutant rescued the defective transformation mediated by BCR/ABL SH2 mutants (ΔSH2 and R1053L) *in vitro*. The importance of Akt as a signal transducer from the SH2 domain of BCR/ABL established in the *in vitro* experiments was confirmed *in vivo* using retrovirally infected bone marrow cells injected into SCID mice. Compared with wild-type BCR/ABL, cells expressing ΔSH2 BCR/ABL have markedly decreased leukemic potential as demonstrated by decreased tumor burden, only occasional involvement of non-hematopoietic organs, and diminished frequency of blastic transformation. Coexpression of the constitutively active Akt E40K, but not of c-Akt, restored the leukemogenic properties of ΔSH2 BCR/ABL *in vivo*. In summary, Akt appears to be the only target of PI-3k we know to date, whose activation is obligatory following PI-3k activation by BCR/ABL, and which is required for the BCR/ABL-mediated leukemogenic transformation of hematopoietic cells.

Potential mechanisms of Akt requirement in BCR/ABL leukemogenesis

A possible explanation for the Akt-mediated rescue of the transforming ability of ΔSH2 BCR/ABL mutants might rest in the Akt-dependent induction of c-Myc and Bcl-2 expression. BCR/ABL upregulates both c-Myc (Pendergast *et al.*, 1993) and Bcl-2 (Sanchez-Garcia and Grütz, 1995); however, deletion of the SH2 domain prevents such induction. Although the SH2 domain of BCR/ABL is not required for the transformation of hematopoietic growth factor-dependent cell lines (Ilaria and Van Etten, 1995; Oda *et al.*, 1995), *in vivo* leukemogenesis of BCR/ABL-infected murine bone marrow cells is impaired by the absence of a functional SH2 domain (Afar *et al.*, 1995; Goga *et al.*, 1995). The ΔSH2 BCR/ABL mutant reportedly protects growth factor-dependent cell lines from apoptosis induced by growth factor deprivation (Cortez *et al.*, 1995); however, in our studies using BCR/ABL-infected mouse marrow cells, ~35% of the cells expressing the ΔSH2 mutant undergo apoptosis (our unpublished data). This somewhat enhanced susceptibility to apoptosis probably reflects an altered equilibrium between pro- and anti-apoptotic signals in BCR/ABL-expressing cells. For example, compared with cells expressing wild-type BCR/ABL, Bcl-2 levels are decreased in cells expressing ΔSH2 BCR/ABL, whereas Bax levels remain unchanged (not shown). Thus, the relative ratio between Bax and Bcl-2 increases, which favors pro-apoptotic signaling via formation of Bax–Bax homodimers (Oltvai *et al.*, 1993). The rescue of the transformation potential of ΔSH2 BCR/ABL by Akt is likely to depend in part on restoration of anti-apoptotic signaling, because both PI-3k and Akt have been reported to prevent or to inhibit apoptosis (Yao and Cooper, 1995; Ahmed *et al.*, 1997; Dudek *et al.*, 1997; Kauffman-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Kulik *et al.*, 1997). Since Akt also activates c-Myc and rescues the growth factor-independent colony formation of hematopoietic progenitors expressing ΔSH2 BCR/ABL, the restoration of signals that promote proliferation is likely to be important for the Akt-dependent enhancement of the leukemogenic potential of ΔSH2 BCR/ABL. The ability of constitutively active E40K Akt to enhance the expression of Bcl-2 and c-Myc is not unprecedented, since similar effects were observed in growth factor-dependent BAF/3 cells transfected with a truncated IL-2 Rβ defective in transducing activation signals for c-Myc and Bcl-2 expression (Ahmed *et al.*, 1997). Interestingly, overexpression of both Bcl-2 and c-Myc in BAF/3 cells is sufficient to induce progression through the cell cycle (Miyazaki *et al.*, 1995; Ahmed *et al.*, 1997). However, expression of constitutively active Akt does not appear to enhance Bcl-2 expression in fibroblasts (Kennedy *et al.*, 1997); this raises the possibility of cell-type-specific mechanisms underlying the Akt-regulated increase in Bcl-2 levels. One such mechanism could involve autocrine production of hematopoietic growth factors that would be consistent with the ability of IL-3 to induce Bcl-2 expression (Otani *et al.*, 1993; Rinaudo *et al.*, 1995) and the observation that ΔSH2 BCR/ABL is defective in the ability to stimulate autocrine production of IL-3 (Anderson and Mladenovic, 1996).

Enhanced expression of Bcl-2 and c-Myc, *per se*, cannot explain entirely the phenotype of marrow cells infected with SH2 domain BCR/ABL mutants and rescued by expression of the constitutively active E40K Akt mutant. Bcl-2 and c-Myc expression is enhanced in marrow cells infected with Akt E40K only, yet these cells are not growth factor-independent in methylcellulose colony formation

assays (Figure 5) and are not leukemogenic in SCID mice (data not shown). Presumably, coexpression of ΔSH2 BCR/ABL, which retains most of the properties of wild-type BCR/ABL, including tyrosine kinase activity provides transforming signals that are complemented by the over-expression of c-Myc and Bcl-2.

In conclusion, this study lends support to the importance of the PI-3k/Akt pathway in BCR/ABL or leukemogenesis, and raises the possibility that interference with this pathway might be a rational therapeutic strategy in the treatment of Ph[1] leukemias and, perhaps of other malignancies characterized by oncogenic activation of tyrosine kinases.

Materials and methods

Retroviral constructs

The pSRαMSVtkneo-p210BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$ wild-type (WT) was obtained by replacing the *Eco*RI–*Bsr*GI fragment in the pSRαMSVtkneop185 BCR/ABL retroviral construct (gift of Dr C.Sawyers, UCLA, Los Angeles, CA) with that of the p210 BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$ cloned in the sp65 plasmid (gift of Dr E.Canaani, Weizman Institute, Israel). The p210 BCR/ABL (K1172R) kinase-deficient mutant was obtained from Dr C.Sawyers (UCLA, Los Angeles, CA). The Y1370F mutation in the YELM motif of p210 BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$ was generated by oligonucleotide site-directed mutagenesis (TAT→TTT mutation) and cloned into the *Bsr*GI–*Bcl*I fragment of pSRαMSVtkneo-p210BCR/ABL$^{(bcr\ exon\ 3/abl\ exon2)}$. The p210ΔSH3 BCR/ABL mutant (ΔSH3) lacks the BCR/ABL SH3 domain from amino acids 959 to 1020 of p210BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$. The p210ΔSH2 BCR/ABL mutant (from Dr R.Van Etten, Harvard Medical School, Boston, MA) was obtained in the pGD210 vector and subsequently cloned into the pSRαMSVtkneo-p210 BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$ by replacing the wild-type *Eco*RI–*Bsr*GI fragment with that containing the ΔSH2 deletion (ΔSH2 mutant). The p185 BCR/ABL FLVRES mutant (R522L) was obtained from Dr C.Sawyers and the *Kpn*I–*Bsr*GI fragment containing the mutation was cloned into the p210BCR/ABL$^{(bcr\ exon\ 3/abl\ exon\ 2)}$ (R1053L mutant). Y177F and Δ176–426 p185 BCR/ABL mutants, cloned in pSRαMSVtkneo vector, were obtained from Dr A.M.Pendergast (Duke University Medical Center, Durham, NC). In the pSRαMSVtkneo vector, wild-type and mutant BCR/ABL cDNAs were under the control of the long terminal repeat (LTR) of the murine sarcoma virus (MSV), and the neomycin resistance gene (neo) under the herpes simplex virus thymidine kinase (*tk*) promoter (Muller *et al.*, 1991). Hemagglutinin (HA)-tagged wild-type c-Akt, oncogenic v-Akt, and kinase-deficient Akt K179M mutant (Franke *et al.*, 1995) were cloned into the pSRαMSVtkneo vector (c-Akt, v-Akt) or into the LXSN vector (Akt K179M). The Akt E40K mutant contains a single amino acid mutation in the pleckstrin homology (PH) domain of c-Akt. This mutation enhances the basal Akt kinase activity (Ahmed *et al.*, 1997). The Akt E40K mutant was cloned into the pSRαMSVtkneo vector.

Electroporation of 32Dcl3 cells

Constructs were electroporated into 32Dcl3 growth factor-dependent murine myeloid cells growing in IMDM-CM [Iscove's modified Dulbecco medium supplemented with 10% FBS, 2mM L-glutamine, penicillin/streptomycin (100 μg/ml each) and 15% WEHI-conditioned medium (WEHI-CM) as a source of IL-3]. BCR/ABL-expressing clones were obtained after selection in G418-containing medium (1 mg/ml) and were maintained in IMDM-CM.

Immunoprecipitation and Western blot analyses

A rabbit anti-p85 PI-3k antibody (UBI, Lake Placid, NY) was used for immunoprecipitation as described (Skorski *et al.*, 1995). Cell lysates or immunoprecipitates were electrophoresed on SDS–polyacrylamide gels and Western blotting was performed with anti-ABL (Oncogene Sci., Uniondale, NJ), anti-p85, anti-Myc (Oncogene Sci.), anti-Bcl-2 (Oncogene Sci.), anti-hemagglutinin (HA11 epitope) (BabCo, Richmond, CA) or anti-paxillin (Zymed Laboratories, Inc., San Francisco, CA).

Preparation of viral stocks

Helper-free retroviral stocks were prepared by transient hyper-expression in COS-1 cells of vectors carrying wild-type or mutants of p210 BCR/ABL$^{(b3/a2)}$, the HA-tagged wild-type or mutants of c-Akt (HA–Akt), and the pSV-Ψ^{-}- E-MLV packaging vector (obtained from Dr C.Sawyers) as described (Skorski *et al.*, 1996). Viral titers were quantitated in infected Rat-2 cells by measuring expressed proteins. Viral stocks were adjusted to give approximately the same infection efficiency.

Infection of bone marrow cells with BCR/ABL and/or Akt viruses

Bone marrow cells from C57BL/6TacfBR mice (The Jackson Laboratory, Bar Harbor, ME) treated with 5-fluorouracil (5-FU) (150 mg/kg body weight) 6 days before cell harvest were infected with BCR/ABL and/or Akt viruses, or the insert-less virus in the presence of recombinant IL-3, Kit ligand and IL-6 as described (Skorski *et al.*, 1996). Expression of BCR/ABL and/or Akt proteins was confirmed in SDS–PAGE followed by Western blotting with anti-ABL and anti-HA11 monoclonal antibodies, respectively.

Clonogenic assay

Clonogenic assays were performed 72 h post-infection in MethoCult H4230 semisolid medium (Stem Cell Technologies, Vancouver, Canada) containing 1 mg/ml of G418 in the absence or in the presence of threshold concentrations of recombinant murine IL-3 (rmuIL-3, Genetics Institute, Cambridge, MA) as described (Skorski *et al.*, 1996).

Enzymatic assays

All enzymatic assays were performed on serum- and growth factor-starved cells after a 5 h incubation in IMDM supplemented with 0.1% bovine serum albumin (BSA), 2 mM L-glutamine and penicillin/streptomycin (100 μg/ml).

PI-3k was asssayed in anti-P.Tyr and anti-p85 immunoprecipitates using [γ-^{32}P]adenosine triphosphate and phosphatidylinositol as a substrate (Skorski *et al.*, 1995). ^{32}P-labeled phosphatidylinositol-phosphate (PIP) was resolved by thin layer chromatography and visualized by autoradiography.

Akt was immunoprecipitated from starved cells, and incubated with [γ-^{32}P]ATP and histone H2B as a substrate (Franke *et al.*, 1995). Reaction mixtures were electrophoresed in SDS–PAGE, transferred onto nitrocellulose membranes (Protran) and exposed to X-ray film.

S6k enzymatic activity was examined by an immune complex *in vitro* kinase assay using the S6k assay kit according to the manufacturer's protocol (UBI, Lake Placid, NY).

RAS activation was determined by measuring GTP-bound RAS as described (Skorski *et al.*, 1994) using serum- and growth factor-starved cells.

Luciferase assay

Tk^{-}ts13 hamster fibroblasts were cotransfected by the calcium-phosphate precipitation method with the luciferase plasmids (pSV1-Luc and p-MMBS-SV1 Luc) and the empty vectors or vectors containing c-Akt, v-Akt, the constitutively active Akt E40K, or the kinase-deficient K179M Akt at a 1:5 ratio. 36 h after transfection, cells were washed and serum-starved for 24 h in DMEM containing 0.1% BSA only. Luciferase assays were performed as suggested by the manufacturer (Promega Corp., Madison, WI).

Leukemogenesis in SCID mice

C57BL/6-SCID-SzJ mice (The Jackson Laboratory) received total body irradiation (350 rads) and 1 day later were injected i.v. with 10^6 bone marrow cells expressing the indicated BCR/ABL and/or Akt proteins. To assess the development of leukemia, total RNA was isolated (Chomczynski and Sacchi, 1987) from 10^5 peripheral blood mononuclear cell suspensions 3, 6 and 9 weeks later. BCR/ABL transcripts were detected by RT–PCR followed by Southern blotting as described (Skorski *et al.*, 1997) using the following primers: 5′ primer: AAGATGATGAGT-CTCCGGGC, 3′ primer: CGTCAGGCTGTATTTCTTCCA and the probe spanning the b3/a2 junction-region: AGAGTTCAAAAGCCCTTC. To demonstrate that the RT–PCR was equally efficient in each sample, 10^2 32Dcl3 transfectants carrying a ΔSH3ΔSH2 BCR/ABL mutant were added to each cell sample before RNA extraction. Because RT–PCR was performed with reagents used in excess and only 10^2 cells expressing ΔSH3ΔSH2 BCR/ABL mutant were added to the samples, it is unlikely that ΔSH3ΔSH2 BCR/ABL mRNA was a competitor for the BCR/ABL mRNA isolated from mouse tissues. The expected lengths of PCR products are: wild-type BCR/ABL=800 bp, ΔSH2 BCR/ABL=521 bp, ΔSH3ΔSH2 BCR/ABL=328 bp. β-actin was also detected in each sample to demonstrate comparable quality of the isolated RNA. Twelve weeks after inoculation of the cells mice were sacrificed and organs were analyzed for the presence of leukemia. For pathological examin-

ation, tissue sections from bone marrow, spleen, liver, lungs, kidney and brain were fixed in phosphate-buffered formalin and embedded in paraffin blocks. Two levels from each block were cut and slides were stained with hematoxylin/eosin (H&E). In addition, selected slides were stained for chloroacetate esterase (Leder stain) to confirm myeloid differentiation of the blasts.

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