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Progressive changes in the leukemogenic signaling in BCR/ABL-transformed cells

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Our previous study indicated that BCR/ABL SH2 domain and BCR/ABL SH3 domain + SH2 domain complex are required for immediate activation of the phosphatidylinositol-3 kinase PI-3k)→ Akt serine/threonine kinase pathway and of the signal transducer and activator of transcription 5 (STAT5), respectively, in hematopoietic cells. We show here that the defect in activation of PI-3k/Akt by BCR/ABL ΔSH2 mutant (SH2 domain deleted) and of STAT5 by BCR/ABL ΔSH3 + ΔSH2 mutant (SH3 and SH2 domains deleted) is not permanent and both Akt and STAT5 could be 're-activated' by *in vitro* culture. This phenomenon was responsible for increased resistance to apoptosis, growth factor-independent proliferation and leukemogenesis in SCID mice. Incubation of cells with BCR/ABL tyrosine kinase inhibitor STI571 abrogated the 're-activation' of Akt or STAT5 by BCR/ABL SH3 + SH2 mutants in some clones, in the others Akt and STAT5 activation became independent on BCR/ABL kinase activity. The immediate upstream activators of Akt and STAT5 such as PI-3k and Jak-2 were also activated. In addition, the common β subunit of IL-3/IL-5/GM-CSF receptor was tyrosine phosphorylated in the clones in which 're-activation' was dependent on the BCR/ABL kinase activity. These results suggested that 're-activation' of Akt and STAT5, in the absence of functional BCR/ABL SH3 + SH2 domains, may be achieved by two different mechanisms: (i) BCR/ABL kinase-dependent activation of alternative pathway(s) and (ii) additional genetic changes stimulating Akt and STAT5 independently of BCR/ABL. *Oncogene* (2000) **19,** 4117–4124.

Keywords: BCR/ABL; signaling; Akt; STAT5

Introduction

BCR/ABL oncogenic tyrosine kinase induces: resistance to apoptosis (Bedi *et al*., 1994; McGahon *et al*., 1994), growth factor independence (Sirard *et al*., 1994), alterations in cell–cell and cell–matrix interactions (Verfaille *et al*., 1992; Skorski *et al*., 1998a) and leukemogenesis (Daley *et al*., 1990; Heisterkamp *et al*., 1990; Kelleher *et al*., 1990). This phenotype is associated with enhanced expression/activation of several effectors (Raitano *et al*., 1997; Sattler and Salgia, 1997) such as Ras (Skorski *et al*., 1994; Sawyers *et al*., 1995), Rac (Skorski *et al*., 1998b), Raf-1 (Skorski *et al*., 1995a), PI-3k (Varticovski *et al*., 1991; Skorski *et al*., 1995c, 1997a), Akt (Skorski *et al*., 1997a; Neshat *et al*., 2000), Bcl-2 (Sanchez-Garcia and Grutz, 1995), nuclear factor (NF)-κB (Reuther *et al*., 1998), CRKL (Senechal *et al*., 1996; Bhat *et al*., 1997), and STAT5 (Carlesso *et al*., 1996; Frank and Varticovski, 1996; Ilaria and VanEtten, 1996; Shuai *et al*., 1996; Chai *et al*., 1997; Nieborowska-Skorska *et al*., 1999; DeGroot *et al*., 1999; Sillaber *et al*., 2000; Horita *et al*., 2000). These proteins are stimulated by various functional domains/motifs of BCR/ABL (Pendergast *et al*., 1993a, b; Afar *et al*., 1994; Goga *et al*., 1995; Cortez *et al*., 1995; Anderson *et al*., 1996; Skorski *et al*., 1997a; Salomoni *et al*., 1998; Nieborowska-Skorska *et al*., 1999). Our previous studies demonstrated that signaling from SH2 or SH3 + SH2 domain of BCR/ABL could be responsible for the induction of PI-3k/Akt (Skorski *et al*., 1997a) and STAT5 (Nieborowska-Skorska *et al*., 1999), respectively. PI-3k and Akt activated by signaling from the BCR/ABL SH2 domain were required for transformation and leukemia formation in mice (Skorski *et al*., 1997a), and contributed also to the induction of anti-apoptotic pathways (Skorski *et al*., 1995c, 1997a). STAT5 stimulated by signaling from the BCR/ABL SH3 + SH2 complex was essential for the induction of cell cycle progression, protection from apoptosis, transformation and development of leukemia in SCID mice (Nieborowska-Skorska *et al*., 1999). In the present study we have demonstrated that, although Akt and STAT5 are not activated immediately in hematopoietic cells expressing BCR/ABL SH3 and/or SH2 mutants, they became activated after prolonged *in vitro* cell culture. Appearance of activated Akt or STAT5 enhanced or induced resistance to apoptosis, growth factor independence and leukemogenic ability in cells expressing BCR/ABL ΔSH2 mutant or BCR/ABL ΔSH3 + ΔSH2 mutant, respectively. Identification of the ability of leukemia cells to modulate intrinsic signaling pathways may contribute to better understanding of the molecular events occurring during the disease progression and may allow designing of more rational anti-leukemia treatments at the molecular level.

Results

To determine if the defects in Akt and STAT5 activation in hematopoietic cells expressing BCR/ABL SH3 and SH2 mutants are permanent or only temporary, 32Dcl3 parental cells and freshly established clones expressing BCR/ABL wild-type, BCR/ABL ΔSH2 or BCR/ABL ΔSH3 + ΔSH2 mutants were cultured in the presence of IL-3 and the enzymatic

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activity of Akt, the DNA binding ability of STAT5 and the expression of BCR/ABL proteins were examined periodically.

The level of BCR/ABL wild-type and BCR/ABL mutant proteins remained stable in the clones during the time of experiment (Figures 1a and 2a and data not shown). Activation of neither Akt nor STAT5 was significantly changed in cells expressing BCR/ABL wild-type during *in vitro* culture (data not shown). Also, we did not detect activation of these signaling proteins in parental 32Dcl3 cells during the time of experiment (Figures 1a and 2a).

Akt became gradually stimulated in three out of five clones expressing BCR/ABL ΔSH2 mutant (Figure 1a). This phenomenon was accompanied by an increase of resistance to apoptosis induced by growth factor withdrawal (Figure 1b). Cells expressing BCR/ABL ΔSH2 mutant, which initially were only partially susceptible to apoptosis, became completely resistant. Two clones in which Akt was not 're-activated', remained partially susceptible to apoptosis (data not shown), implicating 're-activation' of Akt in acquired resistance to apoptosis in clones expressing BCR/ABL ΔSH2 mutant. 'Re-activation' of Akt was also associated with increased potential of growth factor independent proliferation of these cells (Figure 1c). The influence of 're-activated' Akt in cells expressing BCR/ABL mutant on their leukemogenic potential in SCID mice was also examined. Mice injected with cells expressing wild-type BCR/ABL died after 3–6 weeks; in contrast mice injected with cells expressing BCR/ABL ΔSH2 mutant died after 11–17 weeks (Figure 1d). Mice inoculated with cells expressing BCR/ABL ΔSH2 mutant with 're-activated' Akt succumbed to leukemia in 6–9 weeks, faster than those injected with cells expressing BCR/ABL ΔSH2 mutant without 're-activation' of Akt.

STAT5 DNA-binding ability, initially not activated by BCR/ABL ΔSH3+ΔSH2 mutant, was recovered in three out of five BCR/ABL ΔSH3+ΔSH2-positive clones during *in vitro* culture (Figure 2a). The clones with 're-activated' STAT5 became partially resistant to apoptosis (Figure 2b); in comparison survival of clones in which STAT5 was not 're-activated' and of freshly established clones expressing BCR/ABL ΔSH3+ΔSH2 mutant, was completely dependent on IL-3. This result indicated that the acquired resistance to apoptosis in clones expressing BCR/ABL ΔSH3+ΔSH2 mutant is due to 're-activation' of STAT5. Again, this phenomenon was associated with growth factor independent proliferation (Figure 2c) and with restoration of the leukemogenic potential of these clones (Figure 2d). SCID mice injected with cells expressing BCR/ABL ΔSH3+ΔSH2 mutant, in which STAT5 was 're-activated', succumbed to leukemia after 9–14 weeks; in comparison cells expressing BCR/ABL wild-type induced lethal disease in 7–9 weeks, and cells expressing BCR/ABL ΔSH3+ΔSH2 mutants without 're-activated' STAT5 did not induce leukemia after 5 months of observation.

To confirm that 're-activation' of Akt and STAT5 had an essential impact on the acquirement of leukemogenic phenotype of BCR/ABL ΔSH2 and BCR/ABL ΔSH3+ΔSH2 clones, respectively, these cells were infected with retroviruses carrying Akt or STAT5 dominant negative mutants (DNMs). Infection with the Akt DNM or the STAT5B DNM retroviruses increased the susceptibility to apoptosis induced by growth factor withdrawal (Figure 3a,c). In addition, survival time of mice inoculated with cells infected with the Akt DNM or STAT5 DNM was significantly longer in comparison to that after injection of cells infected with empty virus (Figure 3b,d). Altogether, these results confirmed that 're-activation' of Akt and STAT5 contributed to the rescue of leukemogenic phenotype of cells expressing BCR/ABL ΔSH2 or ΔSH3+ΔSH2 mutants.

Next, we determined if 're-activation' of Akt or STAT5 in cells expressing BCR/ABL mutants is due to the changes in signaling from the mutants or to the additional genetic alterations. For this reason, Akt and STAT5 activation were assessed after inhibition of BCR/ABL tyrosine kinase activity using STI571, a synthetic inhibitor of the ABL kinase (Druker *et al*., 1996). Inactivation of BCR/ABL tyrosine kinase activity caused inhibition of 're-activated' Akt and

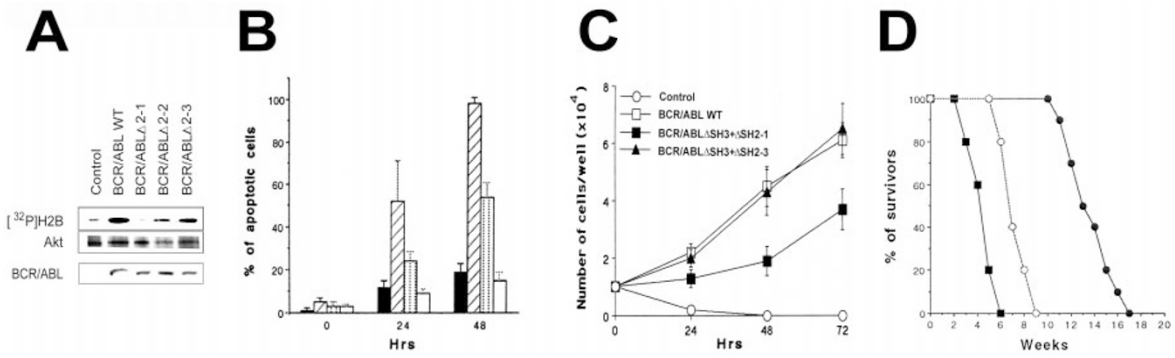

Figure 1 'Re-activation' of Akt by BCR/ABL ΔSH2 mutant (**a**) 32Dcl3 parental cells cultured for 4 months (Control), cells expressing BCR/ABL wild-type cultured for 1 week (BCR/ABL WT) and cells expressing BCR/ABL ΔSH2 mutant (BCR/ABL ΔSH2) cultured for 1 week (1), 2 months (2) or 4 months (3), were examined for Akt activity after starvation from IL-3 and serum for 5 h. Histone H2B was used as a substrate in the *in vitro* Akt kinase assay (upper panel). Immunoprecipitated Akt was detected by Western blot with anti-Akt antibody (lower panel). (**b**) Control (□), BCR/ABL WT (■), BCR/ABL ΔSH2-1 (▨) and BCR/ABL ΔSH2-3 (▩) cells were cultured without IL-3. Apoptotic cells were counted at the indicated time using *in vitro* apoptosis detection kit. (**c**) Living cells were counted in the same conditions using Trypan blue exclusion test. (**d**) SCID mice were injected i.v. with 10^6 of BCR/ABL WT (■), BCR/ABL ΔSH2-1 (●) or BCR/ABL ΔSH2-3 (○) cells on day 0. Terminally ill mice were sacrificed and analysed for the presence of leukemia. Results are representatives for three of five clones expressing BCR/ABL ΔSH2 mutants

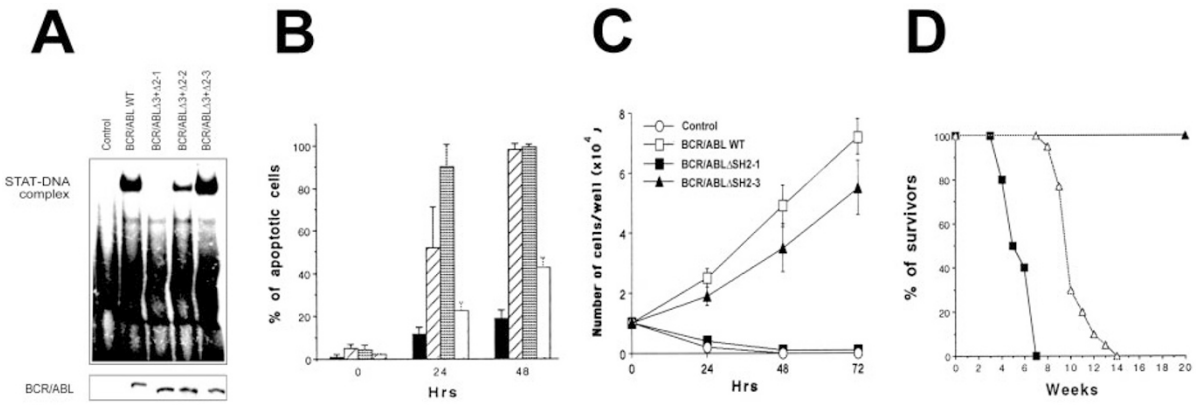

Figure 2 'Re-activation' of the stimulation of STAT5 DNA-binding activity by BCR/ABL ΔSH3+ΔSH2 mutant. (**a**) 32Dcl3 parental cells cultured for 4 months (Control), cells expressing BCR/ABL wild-type cultured for 1 week (BCR/ABL WT), and cells expressing BCR/ABL ΔSH3+ΔSH2 mutant (BCR/ABL ΔSH3+ΔSH2) cultured for 1 week (1), 3 months (2) or 5 months (3), were examined for STAT5 DNA-binding activity after starvation from IL-3 and serum for 5 h. GAS oligonucleotide labeled with γ-^{32}P was used as a probe in EMSA assay. (**b**) Control (□), BCR/ABL-WT (■), BCR/ABL ΔSH3+ΔSH2-1 (▨) and BCR/ABL ΔSH3+ΔSH2-3 (▤) cells were cultured without IL-3. Apoptotic cells were counted at the indicated time using *in vitro* apoptosis detection kit. (**c**) Living cells were counted in the same conditions using Trypan blue exclusion test. (**d**) SCID mice were injected i.v. with 10^6 of BCR/ABL WT (■), BCR/ABL ΔSH3+ΔSH2-1 (▲) or BCR/ABL ΔSH3+ΔSH2-3 (△) cells on day 0. Terminally ill mice were sacrificed and analysed for the presence of leukemia. Results are representative for three of five clones expressing BCR/ABL ΔSH3+ΔSH2 mutant

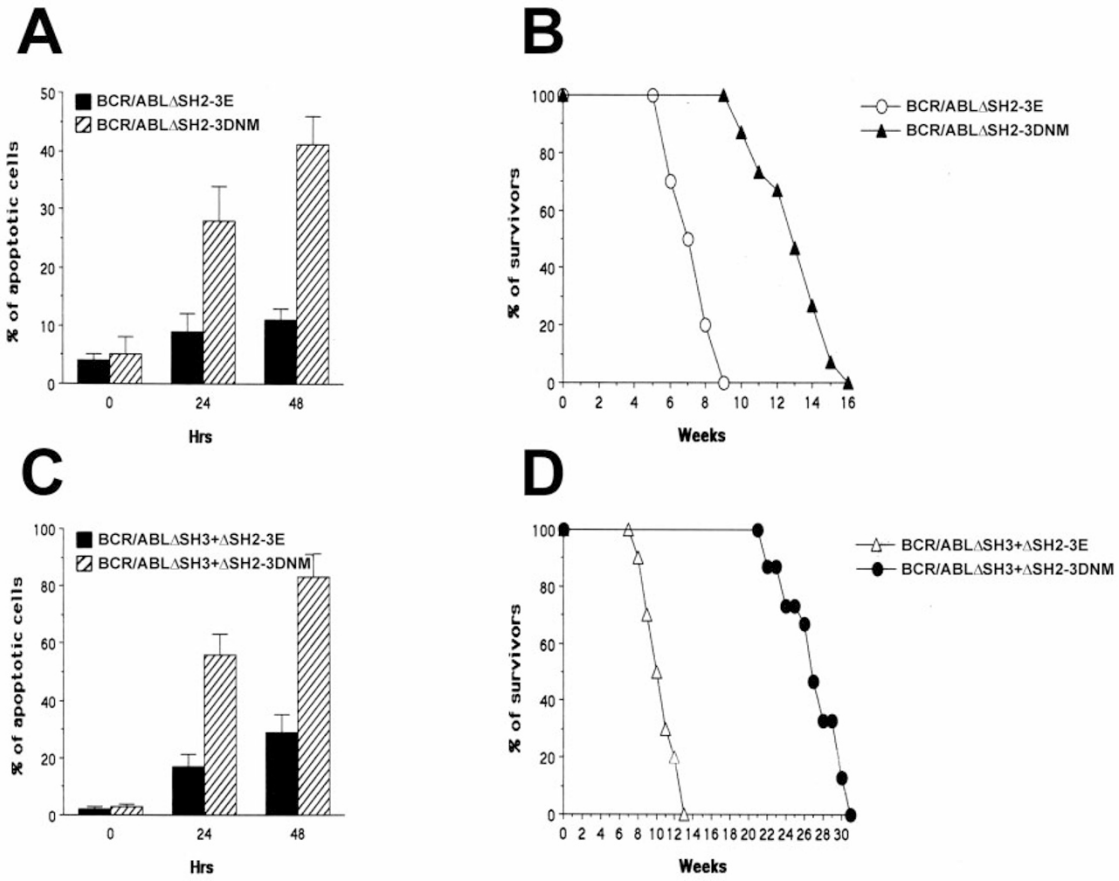

Figure 3 Dominant negative mutants (DNMs) of Akt and STAT5 abrogated transformation in cells expressing BCR/ABL SH3+SH2 mutant with 're-activated' Akt and STAT5. Cells expressing BCR/ABL ΔSH2 mutant (**a,b**) or BCR/ABL ΔSH3+ΔSH2 mutant (**c,d**) with 're-activated' Akt or STAT5, respectively, were infected with retroviruses carrying Akt K179M mutant or ΔSTAT5 mutant and examined for apoptosis in growth factor-free medium (**a,c**) and for leukemogenesis in SCID mice (**b,d**) as described before

STAT5 only in some clones, in others Akt and STAT5 remained in an activated stage (Figure 4a,c, respectively). In the absence of ABL kinase activity the former clones were more susceptible to apoptosis induced by growth factor withdrawal than the latter one (Figure 4b,d). STI571 inhibited BCR/ABL kinase activity in all clones as confirmed by the lack of detection of phosphorylated proteins in total cell lysates analysed by SDS–PAGE followed by Western blotting with anti-P. Tyr antibodies (data not shown).

'Re-activation' of Akt and STAT5 was associated with activation of their direct upstream stimulators such as PI-3k and Jak-2 (Figure 5a,b, respectively). Moreover, 're-activation' of Akt and STAT5, which was dependent on the BCR/ABL kinase activity, was accompanied by the phosphorylation of the β common subunit of IL-3/IL-5/GM-CSF receptor (Figure 5c).

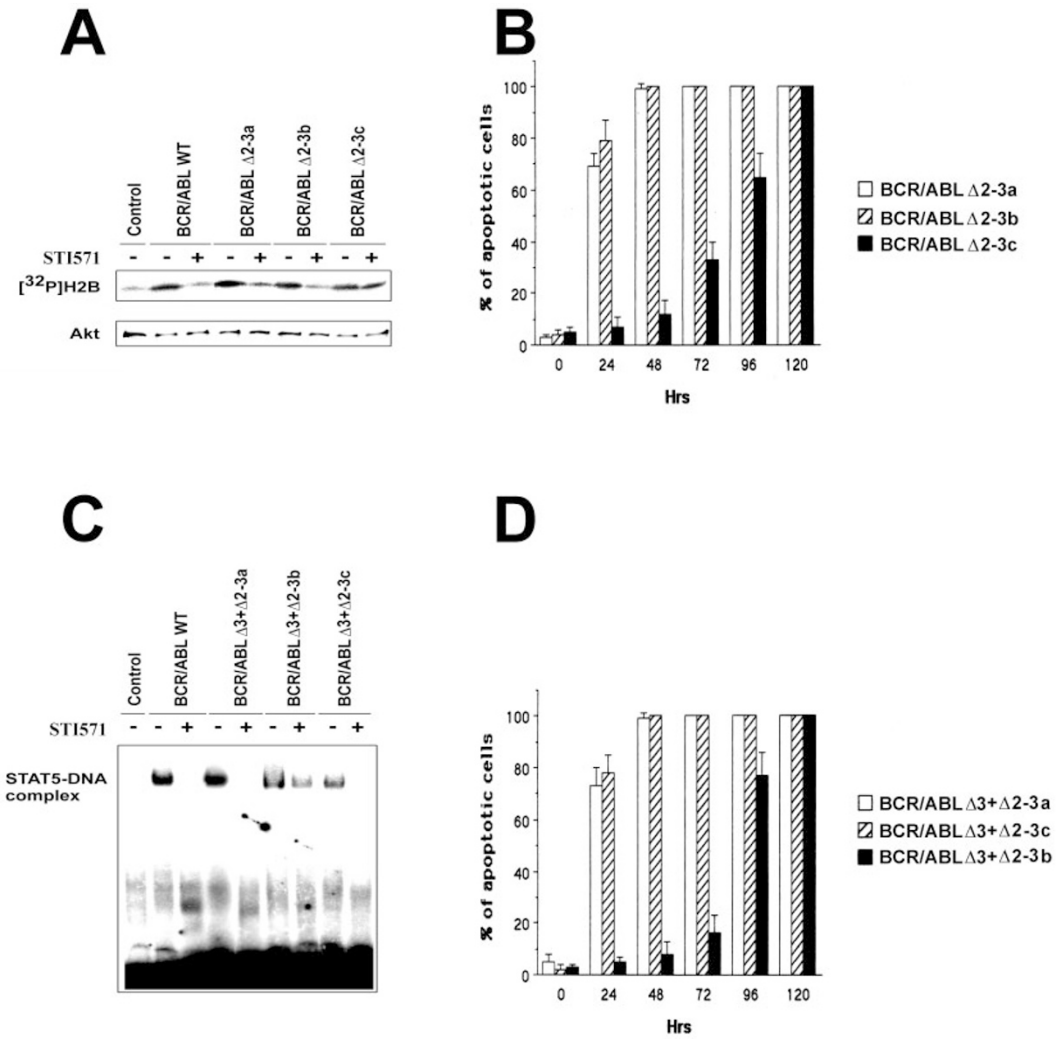

Figure 4 'Re-activation' of Akt and STAT5 may, or may not be dependent on BCR/ABL mutant tyrosine kinase activity. The ABL kinase activity in clones expressing BCR/ABL ΔSH2 mutant (**a,b**) or BCR/ABL ΔSH3 + ΔSH2 mutant (**c,d**) with 're-activated' Akt or STAT5, respectively, was (+) or was not (−) inhibited by STI571. Akt kinase activity (**a**) and STAT5 DNA binding activity (**c**) was assayed as previously described (Figures 1 and 2). Apoptosis (**b,d**) was examined in growth factor free conditions after inhibition of ABL kinase activity with STI571

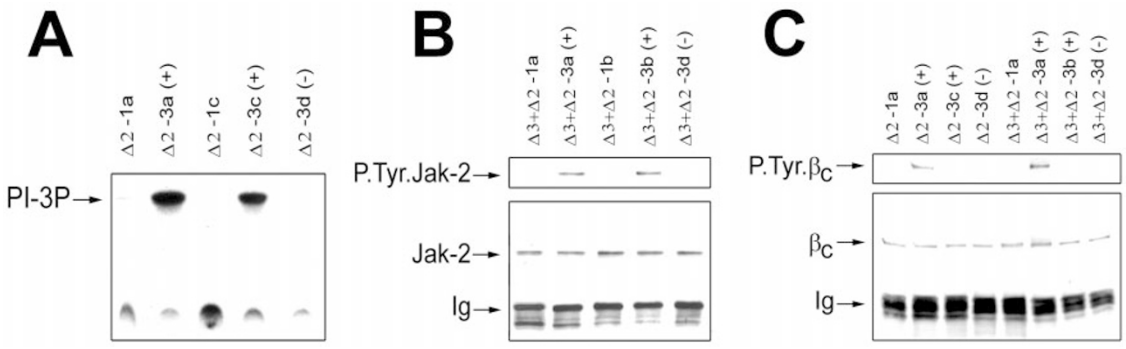

Figure 5 'Re-activation' of Akt and STAT5 is associated with stimulation of Pl-3k and Jak-2, respectively. Cells were starved from IL-3 for 5 h and Pl-3k activity (**a**), Jak-2 tyrosine phosphorylation (**b**) and IL-3/IL-5/GM-CSFRβ (β_c) tyrosine phosphorylation (**c**) was assayed by the *in vitro* kinase assay (**a**) or by immunoprecipitation with the specific antibody followed by Western blot assay with anti-P. Tyr antibodies (**b** and **c**, upper panels). Jak-2 and IL-3/IL-5/GM-CSFRβ was detected in the immunoprecipitates by Western blot assay with the specific antibodies (**b** and **c**, lower panels)

Discussion

BCR/ABL activates multiple signaling pathways leading to the transformation of hematopoietic cells (Raitano *et al*., 1997, Sattler and Salgia, 1997). These pathways are activated by several domains or amino acid residues of BCR/ABL such as SH1 (kinase domain), SH2, SH3, Y177, Y1294 or R1053 (Pendergast *et al*., 1993a,b; Afar *et al*., 1994; Goga *et al*., 1995; Cortez *et al*., 1995; Anderson *et al*., 1996; Skorski *et al*., 1997a; Salomoni *et al*., 1998; Nieborowska-Skorska *et al*., 1999), which are recognized as potential targets for the novel antitumor compounds. In this study we have shown that lack of activation of the BCR/ABL-dependent signaling pathway(s) due to the mutation/deletion of the particular BCR/ABL domain could be rescued by the alternative mechanisms. Activation of Akt serine/threonine kinase and STAT5 transcription factor is essential for leukemogenesis induced by BCR/ABL (Skorski *et al*., 1997a; Nieborowska-Skorska *et al*., 1999; DeGroot *et al*., 1999; Sillaber *et al*., 2000; Horita *et al*., 2000; Neshat *et al*., 2000). Stimulation of Akt may depend on the intact SH2 domain of BCR/ABL (Skorski *et al*., 1997a), but Akt was found activated in 32DcI3 cells expressing BCR/ABL ΔSH2 mutant after several weeks of *in vitro* culture. STAT5, which is activated by the signaling from the complex of SH3 + SH2 domains of BCR/ABL (Nieborowska-Skorska *et al*., 1999), was stimulated in several clones expressing BCR/ABL ΔSH3 + ΔSH2 mutant cultured *in vitro* for several weeks. Stimulation of Akt and STAT5 activity in cells expressing ΔSH2 or ΔSH3 + ΔSH2 BCR/ABL mutants, respectively, was observed after short (5 h) and prolonged (12 – 16 h) starvation of cells from IL-3 and serum (this study and data not shown, respectively). This 're-activation' of Akt and STAT5 was accompanied by increase of the resistance to apoptosis, induction of growth factor-independent proliferation and leukemogenesis in SCID mice. Moreover, inhibition of Akt and/or STAT5 activity by Akt or STAT5 dominant negative mutants, respectively, inhibited the 'transformed' phenotype of these cells, which again became sensitive to apoptosis induced by growth factor withdrawal and less leukemogenic in mice. Akt and STAT5 DNMs did not induce a complete reverse of the 're-activated' phenotype possibly due to the inefficient infection/expression of the mutants. Altogether, these studies show that 're-activation' of Akt and/or STAT5 was actually responsible for the increased leukemogenicity of the evolved cell lines.

'Re-activation' of the signaling pathways from the BCR/ABL SH3 + SH2 mutants leading to the stimulation of Akt and STAT5 was not enforced by the lack of IL-3, because cells were constantly cultured in the presence of cytokine. However, since BCR/ABL and IL-3 activate both unique and overlapping pathways of signal transduction (Matulonis *et al*., 1993), therefore some pathways induced by BCR/ABL wild-type may not be activated by BCR/ABL SH3 + SH2 mutants even in the presence of IL-3. Accordingly, the selection mechanisms could be activated in cells expressing BCR/ABL mutants and growing in the presence of IL-3, which will favor the changes in signaling to acquire the phenotype characteristic for BCR/ABL-transformed cells.

Experiments with the use of BCR/ABL inhibitor STI571 (Druker *et al*., 1996) clearly demonstrated that 're-activated' Akt or STAT5 in long-term cultured hematopoietic cells might, or might not be dependent on the kinase activity of BCR/ABL mutant. Therefore, at least two mechanisms, where the first is dependent on BCR/ABL kinase activity and the second one independent on the kinase, are responsible for these changes in signaling pathways. The observation that activation of MAPK and development of growth factor independence may require several passages of cells expressing BCR/ABL (Kabarowski *et al*., 1994) is in agreement with the first mechanism. The study of Klucher and colleagues (Klucher *et al*., 1998), who demonstrated that BCR/ABL-independent activation of STAT5 was due to unidentified activating mutations occurred upstream of STAT5 supports the second mechanisms. Interestingly, mutations 're-activating' Akt and STAT5 occurred in cells expressing BCR/ABL mutants but not in parental 32Dcl3 cells. This phenomenon could be explained by the finding that BCR/ABL confers a mutator phenotype (Canitrot *et al*., 1999).

Although precise mechanisms of 're-activation' of Akt and STAT5 in cells expressing BCR/ABL SH3 + SH2 mutants are not known, they were associated with the activation of PI-3k and Jak-2, the two direct upstream activators of Akt and STAT5, respectively (Franke *et al*., 1995; Nakamura *et al*., 1996). In addition, the ABL kinase-dependent 're-activation' of Akt and STAT5 in clones expressing BCR/ABL SH3 + SH2 mutant was accompanied by phosphorylation of the common β subunit of IL-3/IL-5/GM-CSF receptor, suggesting the involvement of IL-3-dependent signaling pathway. The role (if any) of this phenomenon has not been identified yet, but at least three mechanisms could be suggested: (i) BCR/ABL SH3 + SH2 mutants may acquire the ability to phosphorylate/activate IL-3/IL-5/GM-CSF receptor(s) pathways by direct interaction with the receptor (Wilson-Rawls *et al*., 1996); (ii) BCR/ABL SH3 + SH2 mutants may acquire the ability to stimulate cytoplasmic tyrosine kinases of the Src family such as Lyn and/or Hck, which can phosphorylate/activate IL-3/IL-5/GM-CSF receptor(s) pathways (Burton *et al*., 1997); (iii) cells expressing BCR/ABL SH3 + SH2 mutants, which initially are not able to produce IL-3 (Anderson and Mladenovic, 1996) may start to produce this cytokine and acquire the autocrine stimulation of IL-3 receptor pathways. The involvement of these mechanisms in 're-activation' of Akt and STAT5 is under investigation.

In addition, cells were constantly cultured in the presence of ciprofloxacin and had been screened periodically for Mycoplasma contamination to exclude the possibility that observed 're-activation' of Akt and/or STAT5 was due to the growth of Mycoplasma (Feng *et al*., 1999).

Accordingly, we believe that signaling pathways activated by BCR/ABL oncogenic tyrosine kinase may change during the course of the disease, resulting in plugging off or plugging in the BCR/ABL domain(s) from/to the downstream effectors. Therefore, some domains of BCR/ABL initially required for leukemogenesis may become expendable during the course of the disease (this study). It also seems possible that

BCR/ABL-dependent cells may evolve into BCR/ABL-independence due to the accumulation of additional genetic changes (this study and Klucher *et al*., 1998).

Growth of cells with 're-activated' Akt or STAT5 activity was still dependent on the tyrosine kinase activity of BCR/ABL mutants, because these cells died in apoptosis in the presence of STI571. Therefore, constitutive activation of Akt or STAT5 did not lead to growth factor independence, in agreement with our previous observations (Nieborowska-Skorska *et al*., 1999; Majewski *et al*., 1999). However, accumulation of genetic alterations inducing constitutive activation of multiple signaling proteins may trigger cell proliferation, which could be independent on the oncogene causing the disease (Klucher *et al*., 1998). For example, simultaneous activation of c-Myc transcriptional factor and Raf-1 serine/threonine kinase induced transformation of $p53^{-/-}$, hematopoietic cells (Metz *et al*., 1995). BCR/ABL activates c-Myc and Raf-1, which are essential for the leukemogenesis (Sawyers *et al*., 1992; Skorski *et al*., 1995a). In addition, loss of p53 function in BCR/ABL-positive cells is responsible for the malignant progression of chronic myelogenous leukemia from the relatively benign chronic phase to the fatal blast crisis (Skorski *et al*., 1996; Honda *et al*., 2000). Therefore, accumulation of genetic alterations leading to the constitutive activation of c-Myc and Raf-1 in the p53-negative BCR/ABL-transformed cells may induce BCR/ABL-independent 'secondary loop' of transformation and rendering leukemia cells resistant to the drugs targeting BCR/ABL. Recent finding demonstrating that simultaneous activation of STAT5 and Ras in human hematopoietic growth factor-dependent cell line enabled growth factor-independent proliferation (Matsumura *et al*., 1999) indicates that genetic alterations leading to the simultaneous activation of different signaling molecules (i.e. c-Myc + Raf-1 on $p53^{-/-}$ background or STAT5 + Ras) may stimulate leukemia cell proliferation independently on the oncogene causing the disease.

Our observations may be important for the planning of novel anti-tumor strategies. Theoretically, leukemia cells may develop the mechanism(s) of drug-resistance to the therapeutics targeting of a domain/motif of the oncogene by turning on alternative mechanisms of activation of the essential signaling pathways (Goga *et al*., 1995). It seems that combination of the compounds targeting BCR/ABL and its downstream effectors (Skorski *et al*., 1995b) or combination of a compound targeting BCR/ABL and a low dose of cytostatic (Skorski *et al*., 1993, 1997b) may benefit in more efficient elimination of leukemia cells carrying various genetic and metabolic alterations and may prevent early selection of drug-resistant cells.

Materials and methods

Cells

32Dcl3 growth factor-dependent murine myeloid precursor cells and clones expressing full-length p210BCR/ABL or p210BCR/ABL SH3 and/or SH2 domain mutants have been described before (Skorski *et al*., 1997a, 1998a; Nieborowska-Skorska *et al*., 1999). Cells were maintained in IMDM-CM (IMDM supplemented with 10% FBS, 2 mM L-glutamine, penicillin/streptomycin (100 μg/ml each), G418 (1 mg/ml), and 15% WEHI-conditioned medium (WEHI-CM) as a source of IL-3).

Apoptosis assay

Susceptibility to apoptosis induced by growth factor and serum withdrawal was measured as described (Skorski *et al*., 1997a, 1998a; Nieborowska-Skorska *et al*., 1999). In brief, cells (1×10^5/ml) were incubated in IMDM supplemented with 2 mM L-glutamine, penicillin/streptomycin and 0.1% BSA. The percentage of apoptotic cells was determined after 24 and 48 h of incubation using the TACS1 Klenow *in situ* apoptosis detection kit (Trevigen) according to the manufacturer's protocol.

Akt assay

Enzymatic activity of Akt was examined as described before (Skorski *et al*., 1997a; Majewski *et al*., 1999). Briefly, Akt was immunoprecipitated from growth factor and serum-starved cells and incubated with [γ-^{32}P]ATP and histone H2B as a substrate. Reaction mixtures were electrophoresed in SDS–PAGE, transferred onto nitrocellulose membranes (Protran) and exposed to X-ray film. The amount of immunoprecipitated Akt was determined by Western blot.

STAT5 assay

The DNA binding activity of STAT5 was examined by EMSA as previously described (Skorski *et al*., 1998a; Nieborowska-Skorska *et al*., 1999), using the FcγRI GAS motif as a probe.

PI-3k assay

PI-3k was assayed in anti-phosphotyrosine immunoprecipitates using [γ-^{32}P]ATP and phosphatidylinositol as a substrate as described (Skorski *et al*., 1995c). ^{32}P-labelled phosphatidylinositol-phosphate (PIP) was resolved by thin layer chromotography and visualized by autoradiography.

Immunoprecipitations

Cell lysates were prepared as described (Skorski *et al*., 1995c). Jak-2 and IL-3/IL-5/GM-CSFRβ were immunoprecipitated with anti-Jak-2 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with anti-IL-3/IL-5/GM-CSFRβ (K-17, Santa Cruz Biotechnology) antibodies, respectively. Immunoprecipitates were analysed by SDS–PAGE followed by Western blot assays using anti-P. Tyr antibodies (4G10 from Upstate Biotechnology, Lake Placid, NY, USA and PY20 from Oncogene Research Products, Cambridge, MA, USA) followed by anti-Jak-2 or anti-IL-3/IL-5/GM-CSFRβ antibodies.

Inhibition of BCR/ABL kinase activity

Cells (2×10^5/ml of IMDM supplemented with 0.1% BSA, L-glutamine and penicillin/ streptomycin) were incubated with 10 μM of STI571 (formerly known as CGP 57148B, generous gift of Novartis Pharma AG, Basel, Switzerland) for 12 h in 37°C. Inhibition of the BCR/ABL kinase activity was documented by the lack of detection of tyrosine phosphorylated proteins in total cell lysates analysed by SDS–PAGE followed by Western blotting with anti-P. Tyr antibodies (PY20 and 4G10).

Retroviral infections

Akt K179M and ΔSTAT5B mutants (Skorski *et al*., 1997a; Nieborowska-Skorska *et al*., 1999) were cloned into the pMX-puro retroviral construct. Infections were performed as previously described (Nieborowska-Skorska *et al*., 1999) with some modifications. To increase the concentration of the virus cells were suspended in medium collected from the culture of Bosc23 packaging cells transfected with an appropriate construct and have been co-cultivated on the monolayer of freshly transfected Bosc23 cells. Bulk cultures obtained 72 h after infection were incubated with puromycin for 48 h. Living (transfected) cells were selected by centrifugation on Lympholyte-M gradient medium (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and used for experiments.

Leukemogenesis in SCID mice

ICR SCID male mice (Taconic Farms, Inc., Germantown, NJ, USA) were injected intravenously with 10^6 32Dcl3 cells expressing the indicated BCR/ABL proteins. Terminally ill mice were sacrificed and examined for the development of leukemia as described before (Skorski *et al*., 1996; 1997a).

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