

Signal Transducer and Activator of Transcription (STAT)5 Activation by BCR/ABL Is Dependent on Intact Src Homology (SH)3 and SH2 Domains of BCR/ABL and Is Required for Leukemogenesis

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Summary

Signal transducer and activator of transcription (STAT)5 is constitutively activated in BCR/ABL-expressing cells, but the mechanisms and functional consequences of such activation are unknown. We show here that BCR/ABL induces phosphorylation and activation of STAT5 by a mechanism that requires the BCR/ABL Src homology (SH)2 domain and the proline-rich binding site of the SH3 domain. Upon expression in 32Dcl3 growth factor-dependent myeloid precursor cells, STAT5 activation-deficient BCR/ABL SH3+SH2 domain mutants functioned as tyrosine kinase and activated Ras, but failed to protect from apoptosis induced by withdrawal of interleukin 3 and/or serum and did not induce leukemia in severe combined immunodeficiency mice. In complementation assays, expression of a dominant-active STAT5B mutant (STAT5B-DAM), but not wild-type STAT5B (STAT5B-WT), in 32Dcl3 cells transfected with STAT5 activation-deficient BCR/ABL SH3+SH2 mutants restored protection from apoptosis, stimulated growth factor-independent cell cycle progression, and rescued the leukemogenic potential in mice. Moreover, expression of a dominant-negative STAT5B mutant (STAT5B-DNM) in 32Dcl3 cells transfected with wild-type BCR/ABL inhibited apoptosis resistance, growth factor-independent proliferation, and the leukemogenic potential of these cells. In retrovirally infected mouse bone marrow cells, expression of STAT5B-DNM inhibited BCR/ABL-dependent transformation. Moreover, STAT5B-DAM, but not STAT5B-WT, markedly enhanced the ability of STAT5 activation-defective BCR/ABL SH3+SH2 mutants to induce growth factor-independent colony formation of primary mouse bone marrow progenitor cells. However, STAT5B-DAM did not rescue the growth factor-independent colony formation of kinase-deficient K1172R BCR/ABL or the triple mutant Y177F+R522L+Y793F BCR/ABL, both of which also fail to activate STAT5. Together, these data demonstrate that STAT5 activation by BCR/ABL is dependent on signaling from more than one domain and document the important role of STAT5-regulated pathways in BCR/ABL leukemogenesis.

Key words: oncoprotein • domains • cooperation • transformation • leukemia

The *bcr/abl* chimeric oncogenes are generated by a reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome) that fuses a truncated *bcr* gene to sequences upstream of the second exon of *c-abl* (1). The *bcr/abl* genes encode the constitutively active p210 and p185 BCR/ABL tyrosine kinases (2, 3), which play essential roles in the pathogenesis of chronic myelogenous leukemia (CML)¹ and Philadelphia¹ (Ph¹) acute lymphoblastic leukemia (4).

Expression of BCR/ABL oncoproteins in hematopoietic cells induces resistance to apoptosis (5, 6), growth factor independence (7), alterations in cell-cell and cell-matrix interactions (8, 9), and leukemogenesis (10–12). This phenotype is associated with enhanced expression/activation of several effectors (13, 14), such as Ras (15, 16), Rac (17), Raf-1 (18), phosphatidylinositol-3 kinase (PI-3k; 19–21),

¹Abbreviations used in this paper: aa, amino acid(s); BrdU, 5-bromo-2'-deoxyuridine; CML, chronic myelogenous leukemia; CNS, central nervous sys-

tem; DAM, dominant-active mutant; DNM, dominant-negative mutant; EMSA, electrophoretic mobility shift assay; PI-3k, phosphatidylinositol-3 kinase; P.Tyr, phosphotyrosine; RT, reverse transcription; SH, src homology; STAT, signal transducer and activator of transcription; WT, wild-type.

Akt (21), Bcl-2 (22), nuclear factor (NF)- κ B (23), and signal transducers and activators of transcription (STATs; 24–27). Although the activity of most of these effectors is required for the BCR/ABL-mediated leukemogenesis, the role of STATs is unknown. Thus far, seven mammalian STATs (1, 2, 3, 4, 5A, 5B, and 6) have been identified. These seven STATs constitute a family of signal transduction proteins that, upon tyrosine phosphorylation, dimerize, translocate to the nucleus, and bind to specific response elements in the promoters of target genes to induce their transcription (28, 29). Among the STAT proteins, STAT5A and STAT5B are ~95% identical in amino acid (aa) sequence and have both distinct and redundant roles in cytokine responses (30–32). STATs are activated in cells stimulated with growth factors such as IL-2 (33, 34), IL-3 (35, 36), IL-5 (36), GM-CSF (36), erythropoietin (37), insulin (38), epidermal growth factor (38), growth hormone (40), and hepatocyte growth factor (41), and in cells expressing activated nonreceptor tyrosine kinases such as v-ABL (42), v-SRC (43), LCK (44), and BMX (45). STAT activity is regulated by tyrosine and also by serine phosphorylation (46, 47). In addition, COOH-terminal truncated forms of STAT described in different cell types (48–50) may act in a dominant-negative manner to regulate STAT activity.

The biological consequences of STAT activation in hematopoietic cells are largely unknown; however, a role for STATs in regulation of proliferation (48, 51, 52) and resistance to apoptosis (34) has been suggested. Constitutive activation of STATs has been found in malignant lymphoma and leukemia cells (53, 54), implicating these proteins in leukemogenesis.

STATs (mainly STAT5A and STAT5B, but also STAT1 and STAT3) are also activated in hematopoietic cell lines expressing BCR/ABL (24–27) and in CML cells (55). However, thus far no direct evidence of a role for STATs in BCR/ABL-dependent leukemogenesis has been obtained. We show here that most of the BCR/ABL-induced STAT activity is due to STAT5. Activation of STAT5 by BCR/ABL involves the SH3 and SH2 domains of BCR/ABL, and is distinct from mechanism(s) responsible for Ras stimulation. Moreover, STAT5 activation is involved in antiapoptotic activity and cell cycle progression induced by BCR/ABL, and is important for BCR/ABL-mediated leukemogenesis *in vitro* and *in vivo*.

Materials and Methods

Retroviral Constructs. The pSR α p210BCR/ABL wild-type (WT) and the kinase-deficient K1172R, Δ SH3, Δ SH2, P1013L, and R1053L BCR/ABL mutants have been described previously (9, 21). The Δ SH3+ Δ SH2 (deletion of both the SH3 and the SH2 domains), P1013L+ Δ SH2 (single aa substitution in the SH3 domain and deletion of the SH2 domain), Δ SH3+R1053L (deletion of the SH3 domain and single aa substitution in the SH2 domain), P1013L+R1053L (single aa substitutions in the SH3 and SH2 domains, respectively), p210BCR/ABL mutants were generated by replacing the EcoRI-KpnI (containing the SH3 domain) and the KpnI-BsrGI (containing the SH2 domain) fragments of BCR/ABL with the corresponding fragments containing the indicated mutation or lacking the region corresponding to the SH3 or the SH2 domain. All constructs were sequenced to

confirm the presence of specific mutations. The pSR α p185 BCR/ABL triple mutant (TM) (Y177F+R522L+Y793F) and Δ 176–426 mutant (56, 57) were obtained from Dr. A.M. Pendergast (Duke University Medical Center, Durham, NC). WT STAT5B and STAT5B dominant-active mutant (DAM) (H295R+S715F) were cloned into the pMX-puro retroviral vector carrying the puromycin-resistance gene (58). COOH-terminal truncated STAT5B (Δ STAT5) dominant-negative mutant (DNM) was a gift from Dr. A. Mui (DNAX, Palo Alto, CA) (48). The mutant was cloned into the pMX-puro vector.

Electroporation of 32Dcl3 Cells. Constructs were electroporated into 32Dcl3 growth factor-dependent murine myeloid precursor cells (59) growing in IMDM-CM (IMDM supplemented with 10% FBS, 2 mM l-glutamine, penicillin/streptomycin [100 μ g/ml each], and 15% WEHI-conditioned medium [WEHI-CM] as a source of IL-3). BCR/ABL and/or STAT5B-expressing clones were obtained after selection in G418 (1 mg/ml)- or puromycin (2 μ g/ml)-containing medium, respectively, and were maintained in IMDM-CM. Expression of BCR/ABL in G418-resistant mixed cell transfectants and in individual clones was confirmed by Western blot analysis with an anti-ABL antibody (see below). Ectopic expression of STAT5B-WT and STAT5B-DAM in the clones was confirmed by reverse transcription (RT)-PCR using primers spanning the EcoRI cloning site in the construct, since STAT5 expression was similar in the clones and in the nontransfected cells. In addition, STAT5B-DAM expression in transfected cells was confirmed by electrophoretic mobility shift assay (EMSA) detecting STAT DNA binding activity in growth factor- and serum-starved cells.

Retroviral Infections of 32Dcl3 Cells and Bone Marrow Cells. Infections were performed as previously described (21, 60) with some modifications. In brief, helper-free retroviruses were generated by transiently transfecting retroviral vectors into BOSC23 cells as described (61). 24 h after transfection, 5×10^6 bone marrow cells from C57BL/6TacBR mice (The Jackson Laboratory) treated 6 d before cell harvest with 5-fluorouracil (150 mg/kg body wt) were added to the monolayer of BOSC23 cells transfected with BCR/ABL- and/or STAT5B-containing vectors or with the insert-less vector and cocultivated for 72 h in 4 ml of IMDM supplemented with 10% FBS, l-glutamine, penicillin/streptomycin in the presence of recombinant IL-3, Kit ligand, and IL-6 (Genetics Institute), and polybrene (2 μ g/ml) as previously described (21, 60). Freshly established BCR/ABL-positive 32Dcl3 clones were infected in the presence of IL-3 by cocultivation with BOSC23 cells transfected with STAT5B-DNM containing vector or with insert-less vector. Bulk cultures of hematopoietic cells obtained 72 h after infection were used for the experiments. Expression of BCR/ABL was confirmed by Western blot analysis with anti-ABL antibody. STAT5B-WT expression was documented by RT-PCR using primers spanning the EcoRI cloning site. Expression of STAT5B-DAM was monitored by EMSA assay and by RT-PCR using primers spanning the EcoRI cloning site. Expression of STAT5B-DNM (Δ STAT) was detected by RT-PCR using primers spanning the NotI cloning site in the construct thus allowing amplification of a fragment corresponding to the ectopically expressed C-terminal truncated STAT5B, and by Western blot analysis with anti-STAT5 antibody raised against aa 451–649 (CAT. No. S21520; Transduction Labs.). Moreover, STAT5B-DNM was shown to inhibit BCR/ABL-induced STAT5 DNA binding activity in the transfected cells, and the samples in which the inhibition was >75% were used in the studies.

Western Blot Analysis. Expression of BCR/ABL and STAT5 was detected in total cell lysates by Western blot analysis with anti-ABL (Oncogene Science) and anti-STAT5 (Oncogene Sci-

ence; Santa Cruz Biotechnology, Inc.; and Transduction Labs.) antibodies, respectively. Tyrosine phosphorylated proteins and actin were detected using anti-phosphotyrosine (P.Tyr) antibodies (Upstate Biotechnology, Inc.; and Oncogene Science) and anti-actin (Santa Cruz Biotechnology, Inc.), respectively.

BCR/ABL Kinase Assay. Cells were starved of growth factors and serum for 3 h. BCR/ABL kinase activity was determined in anti-ABL immunoprecipitates from parental 32Dcl3 cells and wild-type or mutant BCR/ABL-expressing cells, using 5 μ g enolase as substrate (62).

Ras Assay. Ras activation was determined by measuring GTP-bound Ras as previously described (15).

STAT Functional Assays. The DNA binding activity of STAT was examined by EMSA as previously described (9) using the Fc γ RI GAS motif as a probe. The sequence of the probe and the nonspecific competitor were as described (9). Supershift assays were performed using anti-STAT1, -STAT3, and -STAT5 mAbs (Santa Cruz Biotechnology, Inc.). STAT5-dependent transactivation was examined by luciferase assay (36, 48). In brief, Tk-ts13 hamster fibroblasts were cotransfected with the expression vector (wild-type or mutant) BCR/ABL or with the insertless vector along with the STAT5-responsive luciferase reporter construct (β -casein-Luc) and the expression plasmids for STAT5B and β -galactosidase (β -Gal). 36 h after transfection, cells were starved from serum (0.1% BSA) for 48 h and harvested for the luciferase assay using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. For each transfection, luciferase activity was normalized using β -Gal activity as an internal control.

Apoptosis Assay. Susceptibility to apoptosis induced by growth factor and/or serum withdrawal was measured as described (17) with some modifications. In brief, cells (2×10^5 /ml) were incubated in IMDM supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 μ g/ml each), and 0.1% BSA or 10% FBS for 24 and 48 h. The percentage of apoptotic cells was determined by using the TACS1 Klenow in situ apoptosis detection kit (Trevigen) according to the manufacturer's protocol.

Cell Cycle Analysis. Cells (10^6) were starved of IL-3 (10% FBS) or IL-3 and serum (0.1% BSA) for 12 h and cell cycle analysis was performed as previously described (63) using propidium iodide to stain DNA.

DNA Synthesis. This parameter was determined using a 5-bromo-2'-deoxyuridine (BrdU) detection and labeling kit (Boehringer Mannheim). In brief, cells were starved of IL-3 (10% FBS) or IL-3 and serum (0.1% BSA) for 8 h and then incubated with 10 μ M BrdU for the next 4 h. Cytospin slides were prepared and fixed in 94.5% ethanol containing 5% acetic acid and 0.5% Triton X-100. BrdU was detected by staining with mouse anti-BrdU mAb followed by sheep anti-mouse IgG-FITC. Nuclei were counterstained with 500 μ g/ml bisbenzimidazole H33258 (Sigma Chemical Co.). Slides were mounted in Vectashield mounting medium (Vector Labs.) and the percentage of BrdU-positive cells was determined using a Zeiss microscope equipped for epifluorescence mode. 500 cells per cytospin were counted from randomly selected fields.

Leukemogenesis in SCID Mice. ICR SCID male mice (Taconic Farms, Inc.) were injected intravenously with 5×10^6 32Dcl3 cells expressing the indicated BCR/ABL and/or STAT proteins. 4 wk later, four to six mice per group were killed and organs were analyzed macroscopically and microscopically for the presence of leukemia (21). Terminally ill mice were also killed and examined for the development of leukemia. Tissue sections from bone marrow, spleen, liver, lungs, kidneys, and brain were fixed in phosphate-

buffered formalin and embedded in paraffin blocks. Two levels from each block were cut and stained with hematoxylin and eosin. In addition, selected slides were stained for chloroacetate esterase (Leder stain) to assess myeloid differentiation of blast cells.

Results

STAT5 Activation Requires Functional SH3 and SH2 Domains of BCR/ABL. To investigate mechanisms and functional consequences of STAT activation by BCR/ABL, we first identified BCR/ABL mutants defective in STAT activation. Based on the findings that v-Src requires an intact kinase domain and both SH3 and SH2 domains to stimulate STATs (43), the following BCR/ABL mutants were used: the kinase-deficient K1172R mutant; the Δ SH2 mutant lacking the entire SH2 domain (aa 1030–1120); the SH2 FLVRES motif mutant R1053L; the Δ SH3 mutant lacking the entire SH3 domain (aa 959–1020); the SH3 domain mutant P1013L; the Δ SH3+ Δ SH2 mutant lacking both the SH3 and SH2 domains (aa 959–1020 and aa 1030–1120); the P1013L+R1053L mutant carrying single aa substitutions in both the SH3 and the SH2 domains; and the Δ SH3+R1053L and P1013L+ Δ SH2 mutants lacking either the SH3 or the SH2 domain and containing a single aa substitution in the other domain.

After transfection of growth factor-dependent 32Dcl3 murine myeloid precursor cells, G418-resistant clones were selected and examined for the expression of BCR/ABL proteins (Fig. 1). G418-resistant BCR/ABL-expressing cell clones (three to six different clones for each BCR/ABL mutant) were then starved of growth factors and serum and nuclear extracts were examined for activation of STAT DNA binding activity using the STAT binding site from the Fc γ RI promoter as a probe. Cells expressing full-length BCR/ABL (WT) but not the control parental 32Dcl3 cells, or cells expressing the K1172R kinase-deficient BCR/ABL mutant showed STAT DNA binding activity (Fig. 2, top). The specificity of the DNA-protein complex was investigated by EMSA in which an excess of unlabeled specific or nonspecific oligonucleotide was used as competitor. The DNA-protein complex was not detectable, or was markedly inhibited in the presence of 100- or 10-fold excess of specific competitor, respectively (data not shown). STAT1, STAT3, STAT4, STAT5, and STAT6 have previously been shown to bind efficiently to the probe, and STAT1, STAT3, and STAT5 have been reported to be activated by p210 BCR/ABL (24–27, 55). In supershift assays performed to determine which STATs were involved in DNA-protein interaction, most of the complex was supershifted by the anti-STAT5 antibody, whereas no supershift was detected in the presence of the anti-STAT3 or the anti-STAT1 antibody (data not shown). In addition, when anti-STAT immunoprecipitates were blotted with the anti-P.Tyr antibody, STAT5 phosphorylation was prominent, STAT1 was weakly phosphorylated, and STAT3 was not detected in its phosphorylated form (data not shown). Thus, STAT5 represents the majority of the STAT activity induced by BCR/ABL, in accordance with other reports (24–27).

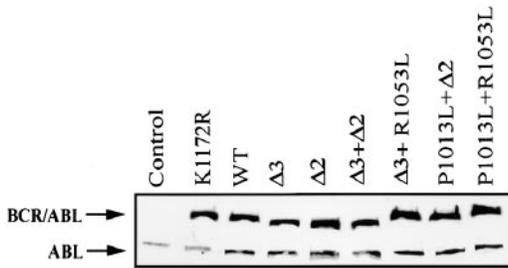


Figure 1. BCR/ABL expression in representative 32Dcl3 clones expressing WT or mutant BCR/ABL. BCR/ABL and c-Abl proteins were detected by Western blotting with anti-ABL mAb. Parental 32Dcl3 cells served as control.

BCR/ABL mutants deleted in either the SH3 (Δ SH3) or the SH2 (Δ SH2) domain, or those carrying a single aa substitution in the SH3 or the SH2 domain (P1013L or R1053L, respectively), were able to activate STAT5 DNA binding activity (Fig. 2, top, and data not shown). However, deletion of both the SH3 and the SH2 domains (Δ SH3+ Δ SH2 BCR/ABL mutant) completely abolished the ability to activate STAT5 (Fig. 2, top). Lack of STAT5 activation was also observed in cells expressing the P1013L+ Δ SH2 BCR/ABL mutant, whereas Δ SH3+R1053L and P1013L+R1053L BCR/ABL mutants activated STAT5 (Fig. 2, top).

Phosphorylation of STAT5 in IL-3 and serum-starved 32Dcl3 cells expressing wild-type and mutant BCR/ABL proteins was monitored by Western blot analysis of anti-STAT5 immunoprecipitates with anti-P.Tyr antibody and correlated with STAT5 DNA binding activity (Fig. 2, middle). Lack of STAT5 activation in clones expressing Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutants was not due to a general metabolic defect of the transfected cells, because it was fully restored by addition of IL-3 (data not shown).

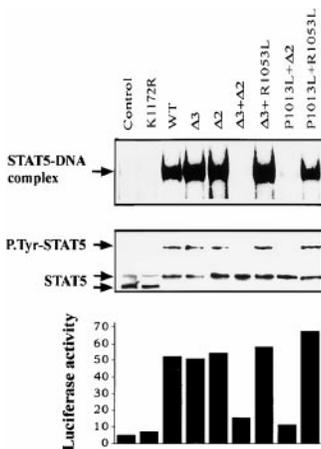


Figure 2. Activation of STAT5 by BCR/ABL mutants. Parental 32Dcl3 cells (Control) and cells expressing full-length (WT) or mutant (Δ 3 = Δ SH3, Δ 2 = Δ SH2) BCR/ABL were starved of IL-3 and serum for 5 h before assaying STAT DNA binding activity by EMSA using the GAS sequence as a probe (top). Results are representative of five experiments using individual clones and mixed cell transfectants before clonal selection. Tyrosine phosphorylation of STAT5 was detected by Western blot analysis with an anti-P.Tyr antibody of anti-STAT5 immunoprecipitates obtained using an antibody raised against the STAT5 NH₂ terminus

(middle, P.Tyr-STAT5). The presence of STAT5 in immunoprecipitates was confirmed by Western blotting with an anti-STAT5 antibody raised against aa 451–649 (middle, STAT5). (Bottom) STAT5 transactivation activity was measured by luciferase assay in Tk-ts13 cells transiently transfected with the indicated BCR/ABL expression plasmids along with the plasmids carrying STAT5 and the luciferase reporter gene driven by the STAT5-regulated β -casein promoter. Luciferase activity is expressed in arbitrary units. Results represent three independent experiments.

Parental 32Dcl3 cells and cells transfected with kinase-deficient K1172R BCR/ABL mutant expressed full-length and COOH-terminal truncated STAT5 proteins, as indicated by detecting two distinct bands in Western blotting (using the anti-STAT5 antibody raised against aa 451–649) of anti-STAT5 immunoprecipitates obtained with an antibody raised against the NH₂ terminus (aa 5–24) of STAT5 (Fig. 2, middle). The upper band was also detected by an antibody raised against the COOH terminus (aa 763–779) of STAT5B (data not shown). Interestingly, the COOH-terminal truncated STAT5 was usually detected in parental 32Dcl3 cells and in cells expressing the kinase-deficient BCR/ABL mutant but not in cells expressing wild-type BCR/ABL or the various SH3+SH2 BCR/ABL mutants retaining kinase activity (Fig. 2, middle).

To examine the ability of BCR/ABL mutants to modulate the transactivation activity of STAT5, a reporter plasmid carrying the luciferase gene under the control of the STAT5-regulated β -casein promoter (36, 48) was coexpressed in Tk-ts13 cells with wild-type STAT5 and the various BCR/ABL plasmids, and the transactivation ability of STAT5 was measured by luciferase assay. Stimulation of STAT5 transactivation activity by BCR/ABL (wild-type and mutants) correlated with their ability to induce STAT5 phosphorylation and DNA binding (Fig. 2, bottom).

Lack of STAT5 activation in cells expressing Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutants was not due to the inability of these mutants to function as tyrosine kinase, since similar (albeit not identical) patterns of tyrosine phosphorylated proteins were detected in lysates of cells expressing wild-type BCR/ABL or SH3+SH2 BCR/ABL mutants unable to activate STAT5 (Fig. 3, top), and immunoprecipitated BCR/ABL mutants were able to phosphorylate enolase in *in vitro* kinase assays (Fig. 3, middle). Likewise, lack of STAT5 activation was not the consequence of detectable defects in Ras activation since the SH3+SH2 BCR/ABL mutants, like WT BCR/ABL, were all able to activate Ras (Fig. 3, bottom).

Abrogation of STAT5 Activity Impairs BCR/ABL-dependent Protection from Apoptosis, Cell Cycle Progression, and Leukemogenesis. STAT5 activation-deficient BCR/ABL mutants (Δ SH3+ Δ SH2 and P1013L+ Δ SH2) failed to protect 32Dcl3 cells from apoptosis induced by withdrawal of IL-3 (10% FBS) or of IL-3 and serum (0.1% BSA) (Fig. 4, top). The BCR/ABL SH3+SH2 mutants (Δ SH3+R1053L and P1013L+R1053L), which retain the ability to activate STAT5, did not protect from apoptosis in IL-3- and serum-deprived medium (0.1% BSA), as only a minority of cells (15–25%) were alive after 48 h of culture (Fig. 4, top left). However, these mutants provided significant protection from apoptosis (50–60% of viable cells) in the presence of serum (10% FBS; Fig. 4, top right) and the surviving cells readily adapted to growth factor-free culture conditions and proliferated without IL-3 (data not shown).

To determine if inhibition of STAT5 activity interferes with the antiapoptotic effects of BCR/ABL, freshly established 32Dcl3 clones expressing BCR/ABL were infected with a retrovirus carrying STAT5B-DNM and were assessed

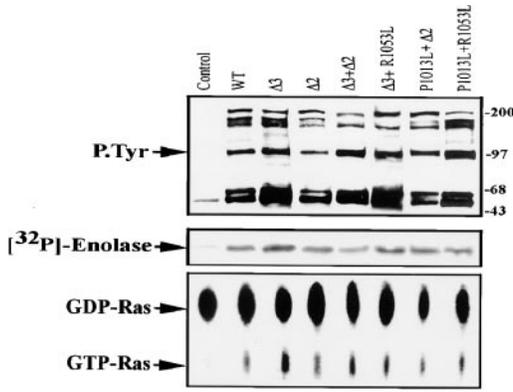


Figure 3. Tyrosine kinase activity and Ras activation by BCR/ABL proteins. Cells were treated as in Fig. 1 and the pattern of tyrosine phosphorylated proteins was analyzed in total cell lysates by Western blot analysis with anti-P.Tyr antibodies (top). In vitro kinase activity was assayed in anti-ABL immunoprecipitates using γ - ^{32}P ATP and enolase as substrate (middle). Ras activation was measured in a standard GTP-loading assay (bottom).

72 h later for apoptosis in cultures deprived of IL-3 (10% FBS) or IL-3 and serum (0.1% BSA). Approximately 60% of the cells infected with the STAT5B-DNM retrovirus underwent apoptosis 48 h after growth factor and/or serum withdrawal (Fig. 4, middle).

Cell cycle analysis after 12 h of starvation from IL-3 (10% FBS) or IL-3 and serum (0.1% BSA) revealed a reduced proportion of cells in S phase and an increase in the fraction of G0/G1 phase cells in STAT5 activation-deficient BCR/ABL mutant ($\Delta\text{SH3}+\Delta\text{SH2}$ and P1013L+ ΔSH2) expressing cells (Fig. 5, f and h) compared with those expressing BCR/ABL WT (Fig. 5 b) or BCR/ABL SH3+SH2 mutants capable of activating STAT5 (Fig. 5, d and e). Moreover, BrdU incorporation assay showed a reduced rate of DNA synthesis in cells expressing STAT5 activation-deficient BCR/ABL SH3+SH2 mutants (Fig. 5, f and h), as compared with those expressing BCR/ABL WT (Fig. 5 b) or BCR/ABL SH3+SH2 mutants activating STAT5 (Fig. 5, d and e). The changes in cell cycle distribution and DNA synthesis were dependent on the expression of mutant BCR/ABL and not on differences in growth

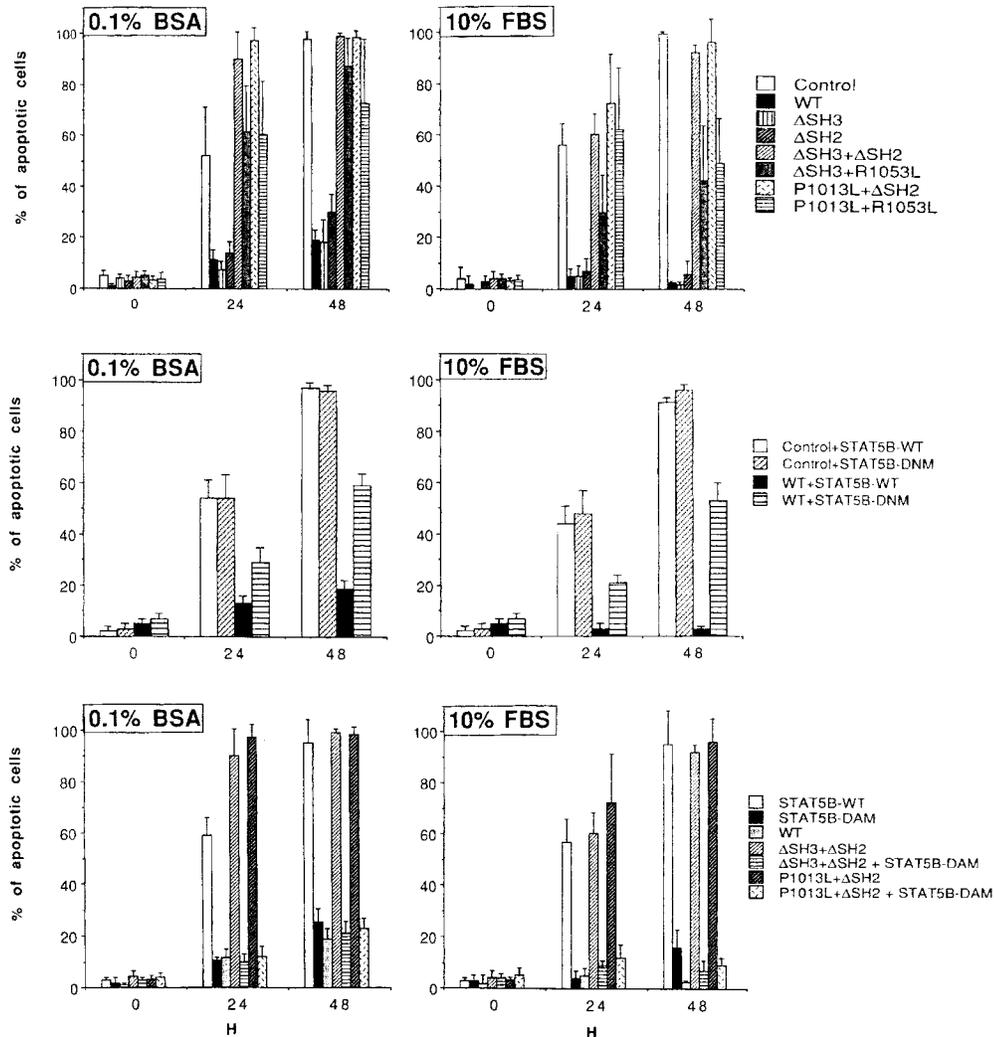
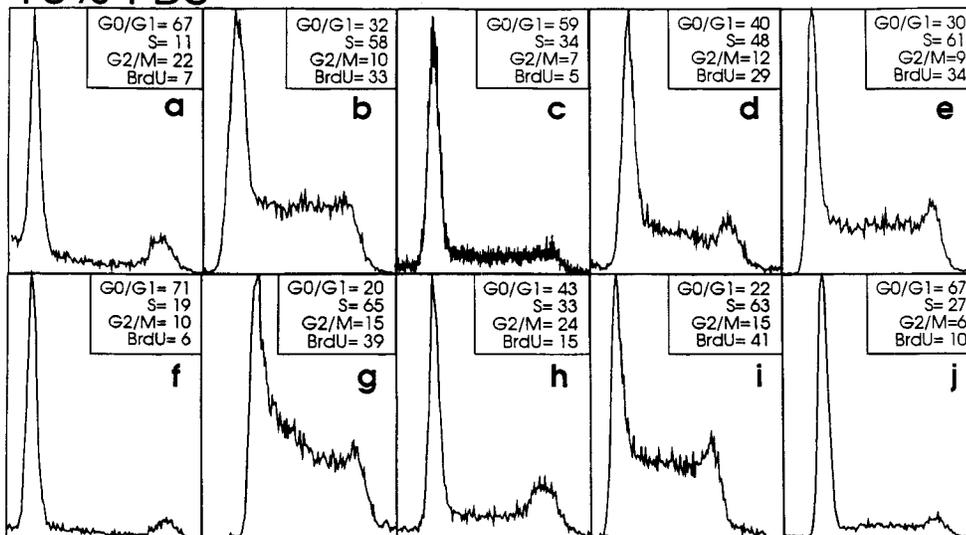


Figure 4. Antiapoptotic activity of BCR/ABL and/or STAT5B proteins. 32Dcl3 cells (Control) or cells expressing the indicated BCR/ABL and STAT5 proteins were starved of IL-3 (10% FBS) or IL-3 and serum (0.1% BSA), and assayed for apoptosis using in situ apoptosis detection kit. Results represent mean \pm SD from four different clones per group. WT, full-length BCR/ABL.

10% FBS



0.1% BSA

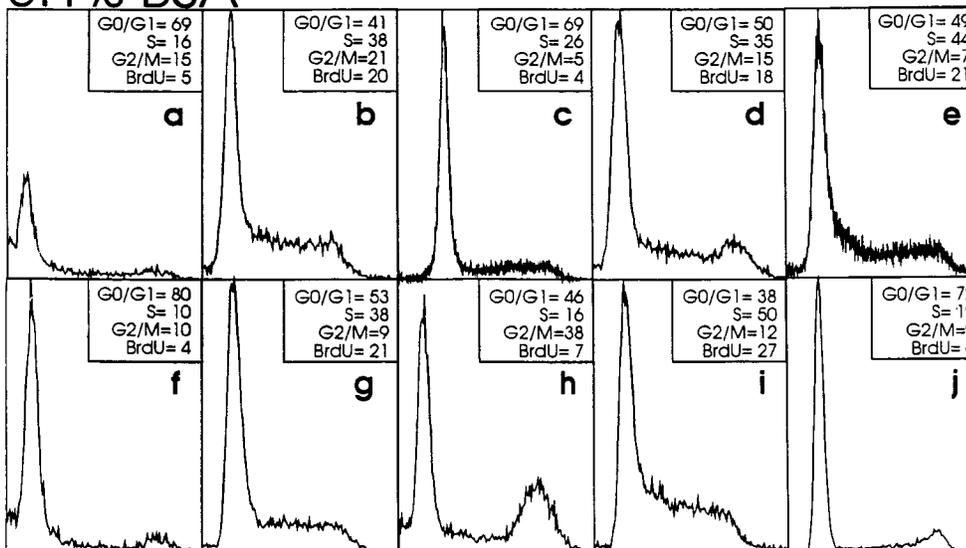


Figure 5. Effect of BCR/ABL and/or STAT5B proteins on cell cycle and DNA synthesis of 32Dcl3 cells. Cells were starved of IL-3 (10% FBS) or IL-3 and serum (0.1% BSA), and DNA content and DNA synthesis were determined by flow cytometry after staining with propidium iodide, and by immunocytochemistry to detect incorporated BrdU, respectively. 32Dcl3 parental cells (a) and clones expressing WT BCR/ABL (b), WT BCR/ABL and STAT5B-DNM (c), Δ SH3+R1053L BCR/ABL (d), P1013L+R1053L (e) Δ SH3+ Δ SH2 BCR/ABL (f), Δ SH3+ Δ SH2 BCR/ABL and STAT5B-DAM (g), P1013L+ Δ SH2 BCR/ABL (h), P1013L+ Δ SH2 and STAT5B-DAM (i), and STAT5B-DAM (j) were examined. Results represent three experiments using different clones. Percentage of cells in G0/G1, S, and G2/M phase, and of cells incorporating BrdU, are shown in upper right corners.

abilities of the clones, because all clones showed similar proliferation rate in the presence of IL-3 (data not shown).

Inhibition of STAT5 function in BCR/ABL-positive 32Dcl3 cells by transient expression of STAT5B-DNM caused a reduction of the percentage of cells in S and G2/M phase and an increase of the percentage of cells in G0/G1 phase (Fig. 5 c) in comparison to cells expressing BCR/ABL (Fig. 5 b) or BCR/ABL and WT STAT5B (data not shown).

Lack of STAT5 activation by BCR/ABL mutants correlated with their reduced leukemogenic potential. SCID mice injected with 32Dcl3 cells expressing WT BCR/ABL died after 4–7 wk (Fig. 6, top) due to leukemia as confirmed at necropsy. Mice inoculated with cells expressing BCR/ABL SH3 and/or SH2 mutants activating STAT5 (Δ SH3, Δ SH2, Δ SH3+R1053L, or P1013L+R1053L) succumbed to leukemia after 10–20 wk (Fig. 6, top). By con-

trast, mice injected with cells expressing STAT5 activation-deficient BCR/ABL mutants (Δ SH3+ Δ SH2 and P1013L+ Δ SH2) did not develop leukemia after 20 wk, as confirmed by histopathological examination of four mice per group, and remained leukemia-free during a 6-mo observation period.

Inhibition of STAT5 activity by transient transfection of the dominant-negative mutant impaired the leukemogenic potential of BCR/ABL-expressing 32Dcl3 cells (Fig. 6, middle). SCID mice injected with BCR/ABL-positive STAT5B-DNM transient transfectants survived longer (10–17 wk) than those inoculated with cells coexpressing BCR/ABL and WT STAT5B (5–7 wk).

Constitutively Active STAT5B Mutant Rescues the Antiapoptotic, Proliferative, and Leukemogenic Properties of STAT5 Activation-deficient BCR/ABL SH3+SH2 Mutants. To determine whether STAT5 is essential for the antiapoptotic

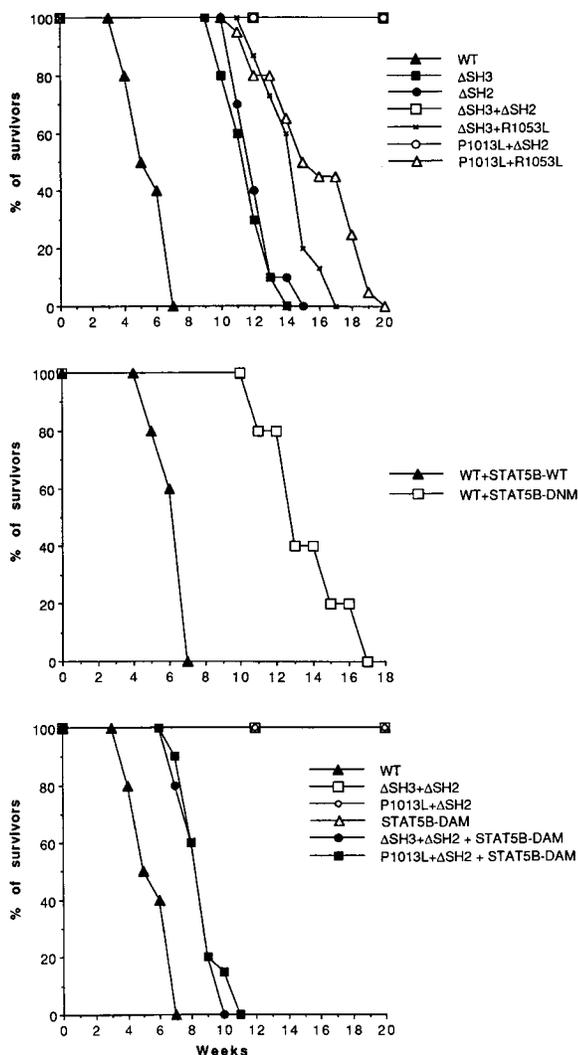


Figure 6. Survival of SCID mice injected with 32Dcl3 cells expressing various BCR/ABL and/or STAT5B proteins. Mice were injected intravenously with 5×10^6 cells expressing the indicated BCR/ABL and/or STAT5B proteins (3–5 clones, 5 mice/clone). Terminally ill mice were killed and analyzed for the presence of leukemia.

activity of BCR/ABL, STAT5B-DAM or STAT5B-WT was introduced into 32Dcl3 parental cells or cells expressing BCR/ABL SH3+SH2 mutants defective in STAT5 activation. 32Dcl3 cells expressing STAT5B-DAM, but not cells expressing STAT5B-WT, were transiently resistant to apoptosis induced by IL-3 and/or serum withdrawal (Fig. 4, bottom), but all died after 72–96 h. Cell cycle analysis after 12 h of starvation revealed that 32Dcl3 cells expressing STAT5B-DAM did not proliferate in the absence of IL-3 and/or serum (Fig. 5 j). In contrast, STAT5B-DAM permanently rescued the apoptotic phenotype of IL-3 and/or serum-starved 32Dcl3 cells expressing STAT5 activation-deficient BCR/ABL SH3+SH2 mutants (Fig. 4, bottom), stimulated DNA synthesis and cell cycle progression in serum- and/or IL-3-free medium (Fig. 5, g and i), and allowed IL-3-independent proliferation (data not shown).

To determine whether restoration of growth factor inde-

pendence by STAT5B-DAM in 32Dcl3 cells expressing the Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutant would also rescue their leukemogenic potential, SCID mice were injected intravenously with double-transfected cells and monitored for the development of leukemia. 4 wk after injection, organs taken from the mice were evaluated by visual inspection and light microscopy for the presence of leukemia. Consistent with previous studies (9), injection with WT BCR/ABL-expressing cells resulted in extensive leukemia involving both hematopoietic and nonhematopoietic organs in all four mice examined. Mice injected with 32Dcl3 cells expressing the Δ SH3+ Δ SH2 or the P1013L+ Δ SH2 BCR/ABL mutant showed no evidence of leukemia at 4 wk after cell inoculation (six and four mice per group examined, respectively) and remained alive after a 6-mo observation period. Similarly, mice injected with cells expressing STAT5B-DAM also showed no signs of leukemia at 4 wk after inoculation (five mice tested) and remained alive after 6 mo of observation. In contrast, all nine mice injected with 32Dcl3 cells coexpressing STAT5B-DAM and the Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutant developed leukemia within 4 wk. In bone marrow, spleen, liver, lungs, and kidneys, the leukemia resembled that caused by WT BCR/ABL, but was less extensive, e.g., liver typically showed scant periportal and sinusoidal infiltrates, and lungs showed patchy interstitial infiltrates without formation of frank, large tumors. Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL- and STAT5B-DAM coexpressing cells more frequently involved meninges (six out of nine) and even brain parenchyma (two out of nine), as compared with BCR/ABL WT-expressing cells, which only occasionally and focally involved meninges (one out of four mice) at 4 wk after leukemia cell injection. Leukemic cells in the terminally ill mice injected with cells coexpressing BCR/ABL mutant and STAT5B-DAM formed invasive parenchymal foci within the central nervous system (CNS) of 13 out of 15 animals analyzed, whereas only small meningeal foci were observed in 12 out of 20 terminally ill mice injected with BCR/ABL WT-expressing cells. Mice inoculated with cells expressing Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutants and STAT5B-DAM died of leukemia after 7–11 wk, whereas cells transformed with BCR/ABL WT killed the mice in 4–7 wk (Fig. 6, bottom).

STAT5 Activity Is Required for Growth Factor-independent Colony Formation of BCR/ABL-expressing Bone Marrow Cells. To determine whether STAT5 plays an essential role in BCR/ABL-mediated transformation of bone marrow cells, we tested whether STAT5B-DNM inhibits transformation of hematopoietic progenitor cells by WT BCR/ABL, and whether STAT5B-DAM rescues the impaired transformation potential of Δ SH3+ Δ SH2 and P1013L+ Δ SH2 BCR/ABL mutants. Bone marrow cells from 5-fluorouracil-treated mice were infected with retroviral vectors carrying the various BCR/ABL and/or STAT5B cDNAs, and transformation was assessed by growth factor-independent colony formation (21, 60). In the presence of threshold concentrations of recombinant murine IL-3 (0.1 U/ml),

a high number of colonies formed from marrow cells infected with a retrovirus encoding WT BCR/ABL; fewer colonies formed in the absence of IL-3 (Fig. 7, bottom). Coinfection of marrow cells with a STAT5B-WT or STAT5B-DAM induced a moderate increase in BCR/ABL-dependent colony formation (Fig. 7, bottom), whereas coinfection with STAT5B-DNM inhibited colony formation induced by WT BCR/ABL by ~60% and ~75% in the presence or absence of IL-3, respectively. Upon infection of mouse bone marrow cells with retroviruses carrying STAT5 activation-deficient BCR/ABL mutants (Δ SH3+ Δ SH2 and P1013L+ Δ SH2), no hematopoietic colonies formed in methylcellulose in the absence of IL-3 and only few (as in control groups) developed in cultures supplemented with IL-3 (Fig. 7, bottom). However, coinfection of marrow cells with the retroviruses carrying the Δ SH3+ Δ SH2 or P1013L+ Δ SH2 BCR/ABL mutant and STAT5B-DAM, but not STAT5B-WT, induced large (data not shown), and numerous (Fig. 7, bottom) colonies. Infection of marrow cells with the retrovirus carrying the STAT5B-DAM alone did not stimulate the formation of growth factor-independent colonies (Fig. 7, bottom). In addition, expression of STAT5B-DAM did not rescue the transformation-deficient phenotype of the K1172R kinase-deficient BCR/ABL mutant or of the BCR/ABL TM Y177F+R522L+Y793F (Fig. 7, bottom), both of which failed to

activate STAT5 in 32Dcl3 cells (Fig. 2 and data not shown) and in bone marrow cells (Fig. 7, middle).

Discussion

Activation of STAT5 is detected in Philadelphia¹ (Ph¹) cell lines (23–26), in hematopoietic cell lines ectopically expressing BCR/ABL (27), and in CML primary cells (55). However, the mechanism(s) of BCR/ABL-dependent activation of STAT5 and its role in BCR/ABL leukemogenesis are essentially unknown. In this study, we investigated the mechanism(s) of BCR/ABL-regulated STAT5 activation and assessed its role in BCR/ABL leukemogenesis.

Mechanisms of STAT5 Activation by BCR/ABL. In 32Dcl3 cells transfected with various BCR/ABL mutants, STAT5 activation was dependent on intact SH3 and SH2 domains that might be required for a direct interaction with STAT5 or with intermediate molecules linking structurally and/or functionally BCR/ABL and STAT5. Since only a small amount (if any) of BCR/ABL was usually detectable in complex with STAT5 (data not shown), it is possible that the BCR/ABL-STAT5 complex is too unstable for ready detection or that the interaction of BCR/ABL with intermediate molecules is the primary mechanism of STAT5 activation. In their COOH-terminal portions, STAT5A and STAT5B have two proline-rich regions that may be recognized by the SH3 domain of BCR/ABL or by an adaptor protein(s) serving as a bridge between BCR/ABL and STAT5. The interaction between the STAT5 P.Tyr(s) and the BCR/ABL SH2 domain does not seem to be involved in STAT5 activation, because the R1053L substitution in the FLVRES motif of the BCR/ABL SH2 domain, which reduces the ability of the SH2 domain to bind P.Tyr (64), did not interfere with STAT5 activation even when the BCR/ABL SH3 domain was deleted or carried the P1013L mutation, which impairs the interaction with proline-rich motifs (65). However, deletion of the entire SH2 domain from BCR/ABL in the context of a mutant lacking the SH3 domain or carrying the P1013L substitution prevented BCR/ABL-dependent STAT5 activation, suggesting that a portion of the BCR/ABL SH2 domain, distinct from the P.Tyr binding motif (FLVRES), is essential for this effect. One possibility is that the STAT5 SH2 domain recognizes one of the tyrosines in the BCR/ABL SH2 domain, or that the BCR/ABL SH2 domain interacts with STAT5 or with another intermediary protein(s) in a non-P.Tyr-dependent manner (57).

The BCR/ABL SH3+SH2 domains may also create a “pocket” required for the direct or indirect activation of STAT5. There is evidence to suggest intramolecular contact between the BCR/ABL SH3 and SH2 domains, collaboration between these domains, and mutual functional influence of one domain on the other (66).

The signaling pathways stimulated by BCR/ABL SH3 and SH2 domains that lead to STAT activation are unknown. RIN1, the Ras binding protein (67) that interacts simultaneously with both the SH3 and SH2 domains of BCR/ABL (68), might not be involved in this process be-

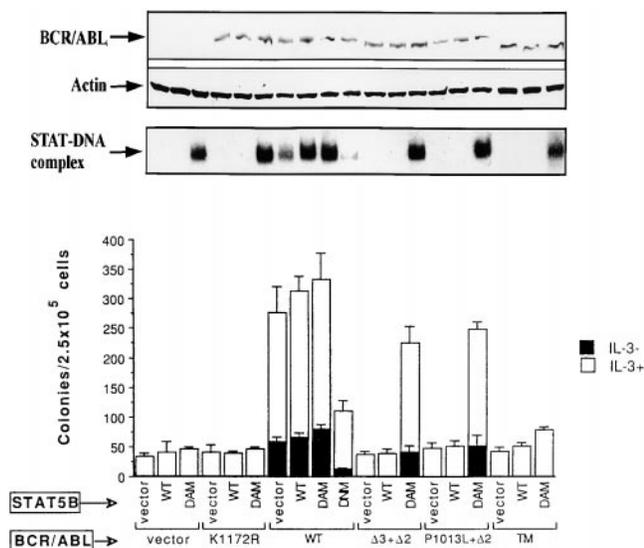


Figure 7. STAT5-DAM rescues the growth factor-independent colony formation of murine bone marrow cells expressing STAT5 activation-deficient BCR/ABL SH3+SH2 mutants. Murine bone marrow cells were coinfecting with BCR/ABL and/or STAT5 retroviruses and 72 h later were assayed for BCR/ABL expression, STAT5 DNA binding activity, and methylcellulose colony formation. (Top) Expression of BCR/ABL and actin proteins detected by Western blot analysis in total cell lysates from infected marrow cells. (Middle) STAT DNA binding activity measured by EMSA in nuclear extracts from cells starved of growth factors and serum for 12 h. (Bottom) Methylcellulose colony formation of infected cells cultured in the absence or presence of threshold concentrations (0.1 U/ml) of murine recombinant IL-3. Results (mean \pm SD) are from three independent experiments.

cause it does not coimmunoprecipitate with STAT5 (data not shown). The PI-3k/Akt pathway, which is affected by mutation/deletion of the BCR/ABL SH2 domain (21), is also probably not essential for STAT activation because no STAT activation was detected in 32Dcl3 cells expressing Akt dominant-active mutants (data not shown).

A recent report showed that the COOH-terminal portion of the v-ABL oncogene is required for the interaction with JAK1 and the activation of STAT5 (69), suggesting that BCR/ABL and v-ABL use different mechanisms to activate STAT5. Unlike v-Abl, BCR/ABL neither associates with nor phosphorylates JAK1 or JAK2 in hematopoietic cells (24, 26, 55, and our unpublished results). The mechanism(s) of BCR/ABL-dependent activation of STAT5 seems similar to that induced by v-SRC. Like BCR/ABL, v-SRC does not phosphorylate JAKs in 32Dcl3 cells (43). In addition, BCR/ABL-STAT5 and v-SRC-STAT5 complexes are not readily detectable (26, 43, and this paper), and both oncoproteins require the SH3 and the SH2 domains to activate STATs (43 and this paper). Perhaps the characterization of proteins interacting with the SH3-SH2 segment of BCR/ABL may shed light on the mechanism(s) of STAT5 activation, even if other BCR/ABL domains might be important in this process.

BCR/ABL-dependent STAT5 Activation Is Important for Protection from Apoptosis and Growth Factor Independence. The role of STAT5B in BCR/ABL-dependent protection from apoptosis and induction of growth factor-independent proliferation was assessed after perturbation of STAT5 activity using two different strategies: (a) coexpression of WT BCR/ABL and STAT5B-DNM; or (b) coexpression of STAT5 activation-deficient BCR/ABL mutants and STAT5B-DAM.

Transient expression of STAT5B-DNM induced apoptosis and arrested cell cycle progression in the majority of BCR/ABL-expressing cells cultured in the absence of serum and/or IL-3. This partial inhibitory effect could be due to expression levels of STAT5B-DNM insufficient to block STAT5 activity in some cells. However, it is more likely that more than one independent pathway is required for the reduced apoptosis susceptibility and the growth factor-independent proliferation of BCR/ABL-expressing cells. Indeed, the dominant-active STAT5B mutant induced transient protection from apoptosis in 32Dcl3 cells, but upon expression in cells cotransfected with STAT5 activation-deficient BCR/ABL SH3+SH2 mutants (Δ SH3+ Δ SH2 or P1013L+ Δ SH2), the double-transfectants were permanently protected from apoptosis and proliferated in a growth factor-independent manner.

Several proteins such as Bcl-2 (22), Bcl-X_L (70), Ras (71), and mitochondrial Raf (72) have been implicated in the transduction of antiapoptotic signals generated by BCR/ABL. Bcl-2 downmodulation was observed in cells expressing various BCR/ABL SH3+SH2 domain mutants, regardless of their ability to activate STAT5 (data not shown). The expression of functional GTP-bound Ras was upregulated in cells expressing STAT5 activation-deficient BCR/ABL mutants (this paper). Also, STAT5 was activated in cells expressing the Δ 176-426 BCR/ABL mutant,

which neither activates mitochondrial Raf-1 nor protects transfected cells from apoptosis induced by IL-3 withdrawal (72). There might be a functional link between STAT5 and Bcl-X_L, as the Bcl-X_L promoter contains a STAT5 binding site (73); however, the expression of Bcl-X_L was not regulated by BCR/ABL in growth factor-starved 32Dcl3 cells (data not shown). Thus, BCR/ABL-dependent activation of STAT5 may involve a novel antiapoptotic mechanism(s). Our data on the antiapoptotic effect of STAT5-DAM are consistent with previous reports indicating that STAT5 plays an important role in antiapoptotic pathways regulated by IL-2 (34) and that STAT5 regulates the expression of the A1 protein (52), an antiapoptosis member of the Bcl-2 family (74).

The Δ SH3+R1053L or P1013L+R1053L BCR/ABL mutant was able to activate STAT5 but protected only 50–60% of the cells from apoptosis induced by IL-3 withdrawal. Reduced susceptibility to apoptosis of 32Dcl3 cells expressing STAT5 activation-deficient BCR/ABL SH3+SH2 mutants (Δ SH3+R1053L and P1013L+R1053L) and STAT5B-DAM, in comparison to cells expressing BCR/ABL SH3+SH2 mutants activating endogenous STAT5 (Δ SH3+R1053L and P1013L+R1053L), may be due to several reasons. First, ectopically expressed STAT5B-DAM may stimulate its downstream effectors more effectively than endogenous STAT5 activated by BCR/ABL mutants. In accordance with this possibility, A1 mRNA levels appear to be higher in clones expressing the BCR/ABL Δ SH3+ Δ SH2 mutant and STAT5B-DAM than in clones transfected with the BCR/ABL Δ SH3+R1053L mutant (data not shown). Second, proapoptotic signaling molecules such as SHIP (75) could be differentially regulated by the various BCR/ABL SH3+SH2 mutants (76) and might interfere with antiapoptotic activity of STAT5. Third, proteins modulating STAT5 activity such as CRKL (77) or CIS (78) could be differentially activated in hematopoietic cells by the BCR/ABL SH3+SH2 mutants.

Cells expressing STAT5-activating BCR/ABL SH3+SH2 mutants (Δ SH3+R1053L or P1013L+R1053L) and surviving in growth factor-free medium became readily growth factor independent. Thus, although not sufficient, STAT5 activation might be necessary for BCR/ABL-dependent cell cycle progression (79). Indeed, expression of STAT5B-DAM in cells transfected with STAT5 activation-deficient BCR/ABL SH3+SH2 mutants not only restored protection from growth factor deprivation-induced apoptosis, but also significantly increased the percentage of cells synthesizing DNA, suggesting that STAT5 synergizes with STAT5 activation-deficient BCR/ABL SH3+SH2 mutants to stimulate growth factor-independent proliferation. However, it cannot be excluded that survival signals induced by constitutively active STAT5 are necessary to complement the mitogenic signals stimulated by the STAT5 activation-deficient BCR/ABL SH3+SH2 mutants. Some of the 32Dcl3 clones expressing STAT5 activation-defective BCR/ABL SH3+SH2 mutants became competent for STAT5 activation after long-term culture. This phenomenon was associated with the emergence of growth factor

independence, and could reflect the use of an alternative signaling pathway(s) by BCR/ABL mutant (80, 81), the reactivation of an autocrine loop for growth factor production which has been shown to require an intact BCR/ABL SH2 domain (82, 83), or the occurrence of secondary mutations (84) that may induce constitutive activation of STAT5. The requirement for STAT5 activation in BCR/ABL-induced abrogation of IL-3 dependence in 32Dcl3 cells is consistent with similar data in v-SRC-transfected cells (43). The importance of STAT5 in cell cycle progression was previously suggested, based on the suppression of DNA synthesis and proliferation in an IL-3-dependent BaF3 cell line expressing a STAT5 dominant-negative mutant (48). Moreover, STAT5B-DAM induced IL-3-independent proliferation of BaF3 cells (58). Also, bone marrow progenitors from STAT5A and STAT5A+STAT5B knockout mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation (32, 52).

A Functional STAT5 Activated by SH3 and SH2 Domains of BCR/ABL Is Required for Leukemogenesis. The involvement of STAT5 activation in BCR/ABL leukemogenesis was demonstrated by showing that: (a) expression of a dominant-negative STAT5B mutant suppressed the leukemogenic potential and the transformation (growth factor-independent colony formation) of BCR/ABL-expressing 32Dcl3 cells or bone marrow cells, respectively; and (b) a dominant-active STAT5B mutant rescued leukemogenic potential and growth factor-independent colony formation of 32Dcl3 cells or primary bone marrow cells, respectively, expressing STAT5 activation-defective BCR/ABL SH3+SH2 mutants.

BCR/ABL-dependent leukemogenesis of 32Dcl3 cells and transformation of primary mouse marrow cells was markedly, but not completely, inhibited by coexpression of dominant-negative STAT5B mutant. The simplest explanation for this partial inhibitory effect is that expression levels of STAT5B-DNM were insufficient to completely block STAT5 activity. Indeed, BCR/ABL-induced STAT5 DNA binding activity was not completely inhibited by coexpression of the dominant-negative STAT5 mutant. However, in light of the results of the complementation assays (see below) it seems likely that the full potential of BCR/ABL to transform bone marrow cells rests in the activation of several independent pathways.

The rescue of the leukemogenic potential of the STAT5 activation-deficient SH3+SH2 BCR/ABL mutant by dominant active STAT5 was not complete, as indicated by the slower development of disease in mice injected with mutant-expressing cells than in mice injected with cells expressing WT BCR/ABL. Most likely, other signaling pathways, such as the PI-3k/Akt pathway, are affected by deletion/mutation in the SH3+SH2 domains of BCR/ABL (21), and are not rescued by STAT activity.

The higher frequency of CNS involvement, compared with other organs, in mice injected with mutant BCR/ABL and STAT5B-DAM coexpressing cells might be due to STAT5-dependent transactivation of a gene(s) required for homing to CNS. On the other hand, the earlier death from leukemia in mice injected with WT BCR/ABL-

expressing cells might explain the apparent lower frequency of CNS involvement since large leukemic infiltrates in the CNS would not have formed at the time of necropsy.

The role of STAT5 in BCR/ABL-mediated transformation was also confirmed in transformation (growth factor-independent colony formation) assays with primary mouse bone marrow cells. STAT5 activation-deficient BCR/ABL SH3+SH2 mutants were completely defective in transformation; expression of dominant-active STAT5B mutant rescued the transformation potential of these BCR/ABL mutants, but not of the kinase-deficient K1172R BCR/ABL. However, the impaired leukemogenic potential of SH3+SH2 domain BCR/ABL mutants is not only due to lack of STAT5 activation. Indeed, the ability of STAT5-activating BCR/ABL SH3+SH2 mutants (Δ SH3+R1053L and P1013L+R1053L) to transform murine bone marrow cells was significantly diminished (data not shown), in accordance with previous findings indicating that intact SH3 and SH2 domains are required for BCR/ABL-mediated transformation (9, 21), and that STAT5B-DAM restored most but not all transformation potential of STAT5 activation-deficient BCR/ABL SH3+SH2 mutants (Δ SH3+ Δ SH2 and P1013L+ Δ SH2). Therefore, other signaling pathways regulated by BCR/ABL SH3+SH2 domains, in addition to STAT5 activation, are required for the full transformation potential of BCR/ABL. Interestingly, the transformation potential of the Y177F+R522L+Y793L BCR/ABL TM, which also does not activate STAT5, was not rescued by coexpression of STAT5B-DAM. This probably reflects the inability of activated STAT5 to rescue a BCR/ABL mutant defective in certain signaling pathways. For example, the STAT5 activation-deficient BCR/ABL SH3+SH2 mutants were competent for Ras activation, whereas the BCR/ABL TM was unable to induce an increase of functional GTP-bound Ras (56), suggesting the requirement for both Ras and STAT5 activation in the leukemogenic potential of BCR/ABL.

The mechanism(s) whereby STAT5-regulated pathways are involved in BCR/ABL-dependent transformation of hematopoietic cells is unknown, but the involvement of STAT5 effectors with a potential role in proliferation, survival, and transformation can be postulated based on the identification of several STAT5-regulated genes. For example, STAT5 induces expression of the A1 gene, a member of the Bcl-2 family that protects 32Dcl3 cells from apoptosis (74, 85). Bfl-1, the human homologue of A1, cooperates with the E1A oncogene in transformation (86), raising the possibility that A1 is activated by STAT5B-DAM in cells expressing STAT5 activation-deficient BCR/ABL SH3+SH2 mutants, and functions as a mediator of the leukemogenic potential of these double-transfectants. The proto-oncogene pim-1 is also regulated by STAT5 (48) and is involved in leukemic transformation (87). Also, Bfl-1 (A1) and pim-1 reportedly collaborate with c-myc (87) and c-myb (88), which are required for BCR/ABL transformation (89, 90).

In conclusion, this study demonstrates that intact BCR/ABL SH3 and SH2 domains are required for the induction of STAT5 activity and that a STAT5-dependent pathway(s) plays a crucial role in BCR/ABL leukemogenesis.

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