Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukemogenesis

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BCR/ABL oncogenic tyrosine kinase activates STAT5, which plays an important role in leukemogenesis. The downstream effectors of the BCR/ABL–STAT5 pathway remain poorly defined. We show here that expression of the antiapoptotic protein A1, a member of the Bcl-2 family, and the serine/threonine kinase pim-1 are enhanced by BCR/ABL. This up-regulation requires activation of STAT5 by the signaling from SH3+SH2 domains of BCR/ABL. Enhanced expression of A1 and pim-1 played a key role in the BCR/ABL-mediated cell protection from apoptosis. In addition, pim-1 promoted proliferation of the BCR/ABL-transformed cells. Both A1 and pim-1 were required to induce interleukin 3–independent cell growth, inhibit activation of caspase 3, and stimulate cell cycle progression. Moreover, simultaneous up-regulation of both A1 and pim-1 was essential for in vitro transformation and in vivo leukemogenesis mediated by BCR/ABL. These data indicate that induction of A1 and pim-1 expression may play a critical role in the BCR/ABL-dependent transformation. (Blood. 2002;99:4531-4539)

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Introduction

Emergence of the Philadelphia chromosome (Ph1) results from the t(9;22) reciprocal chromosomal translocation present in most, if not all, patients with chronic myelogenous leukemia (CML) and subsets of patients with an acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL). This translocation results in the formation of bcr/abl hybrid genes derived from relocation of the c-abl gene from chromosome 9 to the bcr gene locus on chromosome 22. The bcr/abl hybrid genes produce BCR/ABL fusion proteins (p230, p210, and p185) that transform immature hematopoietic cells in vitro and cause a CML or acute leukemia. The BCR/ABL oncoproteins display a constitutive tyrosine kinase activity, a feature necessary for their leukemogenic capacity. The BCR/ABL tyrosine kinase modulates signaling pathways activating several proteins including signal transducer and activator of transcription 5 (STAT5). Studies using STAT5 dominant- and dominant-negative mutants showed that STAT5 is essential for the transformation of hematopoietic cells by BCR/ABL. On the other hand, experiments performed on murine bone marrow cells from STAT5 knockout mice (STAT5 KO cells) indicated that although STAT5 was not essential for BCR/ABL-dependent in vitro transformation, it was important for development of BCR/ABL-induced myeloid leukemia in vivo. However, the results from STAT5 KO cells should be interpreted with caution because of the high level of redundancy in the signaling pathways in hematopoietic cells. Thus, it is possible that bone marrow cells (BMCs) in surviving STAT5 KO mice are able to develop overlapping pathways, which overcome the absence of STAT5. In accordance with this speculation, we observed that BCR/ABL-transformed cells were able to recover partially from expression of the STAT5B dominant-negative mutant, suggesting activation of redundant pathways. In conclusion, STAT5 seems to play an important role in the oncogenesis induced by BCR/ABL as well as other oncogenic tyrosine kinases such as TEL/JAK2 and NPM/ALK. The exact mechanisms regulated by STAT5 during cell transformation remain unclear.

STAT5 belongs to the family of STATs, which are latent transcription factors that become activated by phosphorylation on tyrosine and also on serine. The activated STATs dimerize, translocate to the nucleus, bind specific DNA elements, and induce transactivation of numerous genes. Activated STAT5 enhances the expression of various proteins including cyclin D1, bcl-xL, C1S, A1, pim-1, Id-1, OSM, c-fos, and c-jun. The role of cyclin D1, bcl-xL, C1S, and c-Jun in BCR/ABL-mediated transformation has already been established. This work is focused on A1 and pim-1, the 2 proteins regulating cell apoptosis and proliferation.

A1, a member of the Bcl-2 gene family has been shown to transiently protect from apoptosis 32D cells in vitro. Bcl-xL cells cooperate with the E1A oncogene in cell transformation. In addition, Bfl-1/A1 is able to suppress apoptosis induced by tumor necrosis factor α (TNF-α) and by ligation of an antigen to receptor. Mice lacking A1-a subtype of the A1 gene, display accelerated apoptosis of neutrophils. A1 is localized in the mitochondria and is able to inhibit mitochondrial...
depolarization and release of cytochrome c.\textsuperscript{42} The exact mechanism of A1 function is not known, but probably involves binding and inactivation of the proapoptotic proteins such as Bax.\textsuperscript{42} The above findings raise the possibility that A1 could be a mediator of the antiapoptotic activity and, perhaps, other functions of BCR/ABL.

Expression of pim-1 serine/threonine kinase correlated with cell mitogenesis and survival independent of growth factors.\textsuperscript{43} Pim-1 synergized with c-Myc in leukemogenesis\textsuperscript{44} and enhanced transcriptional activity of c-Myb.\textsuperscript{45} Both c-Myc and c-Mab are essential for BCR/ABL leukemogenesis.\textsuperscript{46,49} In addition, Cdc25A cell cycle phosphatase, a direct transcriptional target for c-Myc, is a substrate for pim-1 kinase\textsuperscript{50} and may tie pim-1 to the cell cycle machinery. Pim-1 may also be associated with protection of hematopoietic cells from apoptosis induced by genotoxic stress or growth factor withdrawal.\textsuperscript{51} Thus, pim-1 could potentially be involved in promotion of the cell cycle progression and inhibition of apoptosis in BCR/ABL-expressing cells.

We report here that expression of A1 and pim-1 is induced by BCR/ABL and that both are required in the BCR/ABL-mediated leukemogenesis.

Materials and methods

Plasmids

A1 and pim-1 complementary DNAs (cDNAs) in pME18S vector were obtained from Dr A. Mui (DNAX Research Institute, Palo Alto, CA). In addition, A1 cDNA in pBluescript was kindly sent by Dr M. Prystowsky (Albert Einstein College of Medicine, New York, NY). A1 sense and antisense (AS) cDNAs were cloned into pMSCV-neo retroviral construct. In addition, A1 AS was cloned into MigR1-IRES-GFP retroviral construct (gift of Dr W. Pear, University of Pennsylvania Medical School, Philadelphia). Flag-tagged pim-1 wild-type (WT) and the K67M kinase-defective mutant were from Dr S. Ness (University of New Mexico School of Medicine, Albuquerque). Both were cloned into pMX-puro and Mig-IRES-GFP retroviral constructs. BCR/ABL WT and BCR/ABL\textsuperscript{A1} mutant (lacking both SH3 and SH2 domains) were described before\textsuperscript{14} and subcloned into the MigR1-IRES-GFP plasmid. BCR/ABL was also subcloned into the pMX-puro plasmid.

Cells

The 32Dcl3 parental cells and cells expressing various BCR/ABL proteins were described before.\textsuperscript{14} 32DHN cells (expressing only hygromycin- and neomycin-resistance genes) and 32DHN-A1 cells overexpressing the A1 cDNA (sense, AS, or point-mutant) and puromycin- or neomycin-resistance sequences. Control cells were infected with the retroviruses containing the antibiotic resistance only. In general, GFP\textsuperscript{+} cells were co-cultivated with the Bosc23 cells transfected with the retroviral constructs for 72 hours in the presence of growth factors. To increase the infection efficiency an equal volume of fresh retroviral supernatant was added to the cocultivation medium every 24 hours (the retroviral titers were usually between 1 and 3 \times 10\textsuperscript{8} U/mL as determined by measuring the infection efficiency on Rat-2 cells\textsuperscript{52}). After infection cells were incubated for 4 days in the presence of growth factors, G418 and/or puromycin, and then spun down on Lympholyte-M (Cedarlane Laboratories, Hornby, ONT, Canada) to eliminate dead cells (about 20–30% of cells were recovered). GFP\textsuperscript{+}, G418, and/or puromycin-resistant cells were used for the experiments. In general, cell lines were infected as described.\textsuperscript{56} 32DHN, 32DHN-A1, FD/neom, and FD/mpim44 cells were infected with BCR/ABL-IRES-GFP, BCR/ABL\textsuperscript{A1}IRES-GFP, or IRES-GFP (control) retroviral constructs. Nonadherent green fluorescent protein\textsuperscript{-}resistant mixed populations were infected with pim-1(K67M)-IRES-GFP, A1(AS)-IRES-GFP, or IRES-GFP (control) viruses. 32Dcl3 cells were infected with pMX-puro or pMX-BCR/ABL-puro retrovirus. Freshly established puromycin-resistant mixed populations were infected with pim-1(K67M)-IRES-GFP, A1(AS)-IRES-GFP, or IRES-GFP (control) viruses. 32Dcl3 cells were infected with pMX-puro and pMX-BCR/ABL-puro retrovirus. Freshly established puromycin-resistant 32DHN-A1 clones overexpressing pim-1 (data not shown) were infected with the BCR/ABL\textsuperscript{A1}IRES-GFP or IRES-GFP retrovirus. GFP\textsuperscript{+} cells were obtained by sorting and used for experiments. 32DHN-A1 cells were infected with pMX-pim-1-puro. Freshly established puromycin-resistant 32DHN-A1 clones overexpressing pim-1 (data not shown) were infected with the BCR/ABL\textsuperscript{A1}IRES-GFP or IRES-GFP retrovirus. GFP\textsuperscript{+} cells were obtained by sorting and used for experiments.

Northern analysis

Cells were starved from IL-3 and serum (incubation in IMDM supplemented with 0.1% bovine serum albumin [BSA]) for 8 hours. Total RNA was isolated and probed for the presence of A1, pim-1, and GADPH using specific full-length cDNA probes end-labeled with (\textsuperscript{32}P)dCTP (NEN Life Science Products, Boston, MA).

Western analysis

Cells were solubilized in lysis buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 1 mM b-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM NaVO\textsubscript{4} and 10 \mu M lactate, each aprotinin and leupeptin). The lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and examined by Western analysis using primary antibodies recognizing the following proteins: A1 (T18 + C19, Santa Cruz Biotechnology, Santa Cruz, CA), pim-1 (C20 + N16, Santa Cruz), Flag-2 (Sigma Chemical, St Louis, MO), actin (C11, Santa Cruz), tyrosine phosphorylated proteins (PYt); PY20 from Oncogene Research Products, Cambridge, MA, and 4G10 from Upstate Biotechnology, Lake Placid, NY), c-ABL (Ab-3, Oncogene), active caspase 3 (CM1, generous gift from IDUN Pharmaceuticals, La Jolla, CA), kit (Trevigen, Gaithersburg, MD). Proliferation was examined by counting the cells excluding trypan blue.
Bad (K-17, Santa Cruz), Bax (Ab-5, Oncogene), Bcl-2 (N-17, Santa Cruz), and Bcl-xL (Transduction Laboratories, Lexington, KY). Species-specific secondary antibodies linked to the horseradish peroxidase (HRP) were from Amersham Life Sciences (Arlington Heights, IL). Bands were detected with an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences).

**pim-1 kinase reaction**

Kinase reaction was done essentially as described by Mochizuki and colleagues. Briefly, pim-1 was immunoprecipitated from the total cell lysates and reactions were carried out in a 30-μL volume containing 25 mM Hepes (pH 7.5), 10 mM MgCl₂, 0.5 mM dithiothreitol, 10 μCi (0.37 MBq) [γ-32P] adenosine triphosphate (ATP), 10 μM ATP, and 10 μg histone H1 (Roche Molecular Biochemicals, Indianapolis, IN). The samples were incubated at 22°C for 30 minutes and then reactions were terminated by the addition of 30 μL 2 times sample buffer (100 μM Tris-HCl [pH6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue). Samples were subjected to SDS-PAGE, followed by autoradiography.

**Leukemogenesis in mice**

C57Bl/6 mice (Taconic Farms, Germantown, NJ) received total body irradiation (450 rads). The next day they were injected intravenously with 10⁷ GFP⁺ BMCs infected with retroviruses carrying BCR/ABL (WT or ΔΔ mutant)-IRES-GFP followed by A1 and/or pim-1 cDNAs. Termally ill mice were killed and examined for development of leukemia as described. In brief, various organs (spleen, liver, lymph nodes, brain, lungs, kidneys, gastrointestinal tract, and skin) were harvested, fixed, embedded, sectioned, stained, and examined under the microscope. A chloroacetate esterase (Leder) staining confirmed myeloid differentiation of the leukemic cells in the selected tissue sections. Animal studies were approved by the Institutional Animal Care and Use Committee at Temple University.

**Results**

**The expression of A1 and pim-1 is regulated by BCR/ABL SH3+SH2-STAT5 pathway**

Our previous study revealed that signaling by BCR/ABL and in particular by its SH3+SH2 region activated STAT5, a signaling step essential for the BCR/ABL-mediated leukemogenesis. To define in more detail this novel cell transforming pathway, we examined expression of the 2 downstream effectors of STAT5: A1 and pim-1, respectively. For this purpose, messenger RNA (mRNA) and protein levels of A1 and pim-1 were determined in 32Dc13 parental cells and clones expressing either BCR/ABL WT, BCR/ABL ΔΔ mutant (SH3+SH2 deletion), which is unable to activate STAT5, STAT5 dominant-active mutant (DAM), STAT5 WT, or BCR/ABLΔΔ and STAT5-DAM. As shown in Figure 1A, expression of A1 and pim-1 on both mRNA and protein levels was markedly up-regulated in cells in which STAT5 was active (BCR/ABL WT, STAT5-DAM, BCR/ABLΔΔ, and STAT5-DAM) in comparison to cells with an inactive STAT5 (parental, STAT5-WT, BCR/ABLΔΔ). To confirm that A1 and pim-1 genes represent the BCR/ABL targets also in the patient-derived leukemic cells, their expression was analyzed in CML patient cells, before and after treatment with the ABL kinase inhibitor STI571. Western analysis clearly demonstrated that the primary leukemic cells expressed both A1 and pim-1 (Figure 1B). Furthermore, this expression was dependent on the BCR/ABL kinase activity, because it was profoundly diminished after cell treatment with STI571.

**Role of A1 and pim-1 in BCR/ABL-mediated growth factor independence**

Prevention of cell apoptosis and induction of growth factor independence are among the major functions of BCR/ABL. To examine the role of A1 and pim-1 in these processes, 2 types of experiments were performed. First, A1 or pim-1 proteins were up-regulated in cells expressing the BCR/ABLΔΔ mutant, and second, A1 protein and pim-1 kinase were down-modulated in the BCR/ABL⁺ cells by introduction of the A1 AS cDNA or kinase-deficient mutant of pim-1 (K67M).

Elevation of the expression of A1 protein in 32DHN-A1 cells to the level observed in BCR/ABL⁺ counterparts (Figure 2A, left panel) caused only a modest protection from apoptosis in growth factor-free medium (Figure 2A, right panel). Eventually all cells died in apoptosis after 5 days of incubation without growth factor. Similar up-regulation of A1 in the BCR/ABLΔΔ cells caused more pronounced early protection from apoptosis, but eventually all cells also died on day 5. In turn, down-regulation of A1 protein expression by transfection with the A1 AS cDNA, sensitized the BCR/ABL⁺ cells to apoptosis in the growth factor- and serum-free conditions (Figure 2B, triangles), but not in the absence of IL-3 and presence of serum (Figure 2B, squares).

Ectopic expression of pim-1 protein in FD/mpim44 cells at the level comparable to one seen in the BCR/ABL WT-transfected FD cells (Figure 3A) protected these cells from apoptosis in the growth factor-free medium during the first 3 days of culture, but eventually almost all cells died by day 5 (Figure 3B). Increased expression of pim-1 in the BCR/ABLΔΔ cells exerted a more significant antiapoptotic effect because only less than 40% of the cells died after 5 days. Although the FD/mpim44 cells were unable to proliferate in the absence of IL-3, their growth was partially re-established after transfection with the BCR/ABLΔΔ mutant (FD/mpim44 + BCR/ABLΔΔ cells; Figure 3C). The latter cells grew more slowly than the BCR/ABLWT⁺ cells, probably due to the fact that some of them were dying in apoptosis. To confirm the observation that pim-1 is important for growth factor independence of BCR/ABL⁺ cells, pim-1 (K67M) kinase-deficient mutant was expressed in BCR/ABLWT⁺ 32Dc13 cells. Expression of the FLAG-tagged pim-1 (K67M) protein and inhibition of the pim-1 kinase activity was confirmed by Western analysis and in vitro kinase assay, respectively (Figure 3D). The mutant was able to interfere with the BCR/ABL-mediated protection from apoptosis and caused the death of about 50%
of the transfected cells (BCR/ABL+pim-1[K67M] cells) in the absence of IL-3 (Figure 3E). This effect was associated with an about 2.5-fold decrease in the number of cells in the liquid culture as compared to the cells expressing BCR/ABL WT alone (Figure 3F). This observation was confirmed in the clonogenic assay performed in the methylcellulose semisolid medium: expression of the pim-1(K67M) markedly decreased the number and size of colonies formed by the BCR/ABL WT cells (data not shown).

Because A1 seems to be preferentially involved in protection from apoptosis and pim-1 appears to be engaged in both cell proliferation and apoptosis, we decided next to determine if the functions of A1 and pim-1 are complementary or overlapping.

Coexpression of A1 and pim-1 in 32DHN cells (Figure 4A) delayed apoptosis in the absence of IL-3, but almost all cells eventually died after 5 days of culture (Figure 4B). However, the coexpression of A1 and pim-1 rescued the factor-independent growth of the cell transformation–defective BCR/ABLΔΔ mutant. BCR/ABLΔΔ + A1 + pim-1 cells survived in the absence of IL-3 (Figure 4B) and displayed growth factor–independent proliferation comparable to that observed in the BCR/ABL WT cells (Figure 4C). Clonogenic assay performed in the semisolid medium confirmed this observation (data not shown). In conclusion, A1 and pim-1 seem to complement each other as downstream effectors of BCR/ABL in protection from apoptosis and promotion of growth factor independence.

Both A1 and pim-1 are required for BCR/ABL-mediated leukemogenesis

To determine if A1 or pim-1 or both play an essential role in BCR/ABL-mediated transformation of normal BMCs, we tested whether overexpression of A1 or pim-1 rescues the impaired growth of the cell transformation–defective BCR/ABLΔΔ mutant. BCR/ABLΔΔ + A1 + pim-1 cells survived in the absence of IL-3 and displayed growth factor–independent proliferation comparable to that observed in the BCR/ABL WT cells (Figure 4C). Clonogenic assay performed in the semisolid medium confirmed this observation (data not shown). In conclusion, A1 and pim-1 seem to complement each other as downstream effectors of BCR/ABL in protection from apoptosis and promotion of growth factor independence.

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transformation capacity of BCR/ABLΔΔ mutant, and whether down-modulation of A1 or pim-1 inhibits cell transformation mediated by BCR/ABL WT. BMCs from 5-FU-treated mice were infected with retroviral vectors carrying several different forms of BCR/ABL, A1, and pim-1, and analyzed in the in vitro colony formation assay in the growth factor–free medium. BMCs infected with BCR/ABL WT formed numerously colonies in the absence of IL-3 and many more arose in the presence of the threshold concentration of IL-3 (Figure 5A, group 5). The control, empty retrovirus-infected cells, did not form any colonies in the absence of IL-3 and only a few grew in the 0.1 U/mL IL-3 (Figure 5A, group 1). BCR/ABLΔΔ mutant alone did not induce any IL-3–independent colonies and did not increase the number of colonies in IL-3 in comparison to the control cells (Figure 5A, group 6). Furthermore, no colonies were found in the absence of IL-3 and no significant colony formation enhancement was noticed in the cells carrying either A1 or pim-1 (Figure 5, group 2 and group 3, respectively) in comparison to control (Figure 5A, group 1). When BCR/ABLΔΔ+ cells were infected with either A1 or pim-1 retrovirus, their clonogenic activity was not affected by A1 or was only moderately increased by pim-1 (Figure 5A, compare group 7 with group 6, and group 8 with group 6, respectively). However, coinfection with both A1 and pim-1 fully restored the impaired transforming capacity of the BCR/ABLΔΔ mutant as compared to BCR/ABL WT (Figure 5A, compare group 9 with group 5). Conversely, infection of BCR/ABL WT-expressing BMCs with either A1 AS cDNA or pim-1(K67M) mutant had no effect, or exerted only a mild effect on their clonogenicity, respectively (Figure 5B, compare group 2 with group 1, and group 3 with group 1, respectively). However, coinfection with both A1 antisense + pim-1(K67M) mutant exerted a profound negative effect on the BCR/ABL-induced colony formation (Figure 5B, compare group 4 with group 1).

To determine whether A1 and pim-1 also play an essential role in the BCR/ABL-mediated leukemogenesis in vivo, preirradiated syngeneic mice were injected with BMCs infected with BCR/ABL, A1, or pim-1 cDNAs as described above. All mice inoculated with the cells infected with BCR/ABL WT succumbed to leukemia (median survival time [MST] of the mice ± SD = 4.2 ± 0.3 weeks; Figure 6A). Histologic examination of the multiple organs (spleen, liver, lymph nodes, lung, skin, central nervous system) revealed the presence of AML in most organs. There was considerable variability in the degree of blast maturation among the mice as well as various involved sites. In one case, the histology resembled more transforming CML rather than an overt acute leukemia. All of the examined organs showed diffuse leukemic infiltrates and tissue destruction. The least differentiated cases displayed the highest mitotic and single-cell necrosis rates and areas of zonal necrosis. Myeloid origin of the blasts was confirmed in such cases by chloroacetateesterase stain. BMCs infected with the BCR/ABLΔΔ retrovirus were unable to induce leukemia in accordance with the
previous report. Infection of the BCR/ABLΔΔ cells with either A1 or pim-1 retrovirus or coinfection of normal BMCs with A1 and pim-1 retroviruses did not rescue leukemogenic activity of the BCR/ABL mutant (data not shown). However, coinfection of BCR/ABLΔΔ cells with both A1 and pim-1 led to the development of AML in all injected mice (MST = 5.4 ± 1.7 weeks; Figure 6A). There was similar histologic diversity in the degree of blast differentiation and similar pattern of organ involvement as in the mice injected with BCR/ABL WT-carrying cells. Poorly differentiated AML with high mitotic rate and single-cell and zonal necrosis was also seen. To confirm that A1 and pim-1 are essential for BCR/ABL leukemogenesis, the BMCs expressing BCR/ABL WT were coinfected with retroviruses carrying A1 AS cDNA and pim-1(K67M) kinase-deficient mutant. The simultaneous down-modulation of A1 and pim-1 impaired the leukemogenic capacity of the BCR/ABL WT BMCs (MST = 11.4 ± 2.2 weeks, P < .001) as compared to the BMCs expressing only BCR/ABL WT (MST = 4.2 ± 0.3 weeks) without changing the phenotype of disease. The above observations indicate that A1 and pim-1 are essential downstream molecules for BCR/ABL.

A1 and pim-1 work in concert to regulate apoptosis and cell cycle

A1 and pim-1 are potent regulators of apoptosis and cell cycle progression. Because they collaborate in BCR/ABL leukemogenesis, we decided to examine their effect on expression of proapoptotic and antiapoptotic genes and on cell cycle progression in the BCR/ABL cells. As parenteral and BCR/ABL-, A1-, and/or pim-1–carrying cells (cell clones are described in the legend to Figure 4) were starved from IL-3, time-dependent activation of caspase 3 was detected in the parental, A1, pim-1, BCR/ABLΔΔ, and BCR/ABLΔΔ + A1 cells, whereas in the BCR/ABL WT, A1 + pim-1, BCR/ABLΔΔ + pim-1, and BCR/ABLΔΔ + A1 + pim-1 cells caspase 3 was not activated during 12 hours of starvation from IL-3 (Figure 7A).

The high levels of unphosphorylated Bad protein correlated with caspase-3 activation, whereas Bax protein was generally not affected. Importantly, the antiapoptotic proteins Bcl-2 and Bcl-xL remained significantly up-regulated despite the IL-3 starvation in the BCR/ABL WT cells, but only the high expression of Bcl-2 was preserved in pim-1, A1 + pim-1, and BCR/ABLΔΔ + A1 + pim-1 cells, as compared to the parental and BCR/ABLΔΔ cells. However, after 72 hours starvation Bcl-2 was down-regulated and caspase 3 was activated in A1 + pim-1–transfected cells (data not shown).

Cell cycle analysis revealed that in the absence of IL-3 parental cells were composed almost totally of cells containing subdiploid amount of DNA (a signature of apoptosis), whereas the DNA content and cell cycle progression profile of the BCR/ABL WT cells were not significantly affected by the absence of IL-3 (Figure 7B). In turn, the IL-3–starved BCR/ABLΔΔ cells accumulated in the G1 and G2/M phases or displayed a subdiploid amount of DNA.

Figure 7. Both A1 and pim-1 are required to inhibit apoptosis and promote proliferation of the BCR/ABL-carrying cells. (A) Apoptotic proteins. Parental cells and cell clones expressing BCR/ABL proteins, A1 protein, and/or pim-1 protein were starved from IL-3 for 6 and 12 hours and the expression of proteins regulating apoptosis was detected by Western analysis. Bad phosphorylated (upper band) and unphosphorylated (lower band) proteins are visualized. Results represent 2 experiments. (B) Cell cycle. The same cells were incubated without IL-3 for 0 or 24 hours. DNA content was determined by flow cytometry after staining with propidium iodide. Results are representative of 3 independent experiments.
Under such conditions A1 and BCR/ABLΔΔ + A1 cells were arrested in G1 and G2/M phases with a small population already accumulated in subdiploid peak. Pim-1 and BCR/ABLΔΔ + pim-1 cells were mostly in G1 and G2/M phases with minimal accumulation in subdiploid fraction. A1 + pim-1 cells were arrested in the G1 and G2/M phases. Coexpression of both A1 + pim-1 caused a dramatic change in the cell cycle distribution of the BCR/ABLΔΔ cells by fully compensating for the IL-3 withdrawal as seen in the BCR/ABL WT cells.

Discussion

Activation of STAT5 seems important for the BCR/ABL-mediated leukemogenesis, especially for the development of myeloproliferative disease.14-17,20 The mechanisms operating downstream of the BCR/ABL→STAT5 pathway remain poorly characterized. STAT5 is a powerful transcriptional factor enhancing expression of genes, whose products are involved in regulation of apoptosis and proliferation.58 Because growth factor independence, defined as protection from apoptosis and stimulation of cell cycle progression in the absence of growth factors, is one of the major effects of the BCR/ABL-mediated cell transformation, we decided to examine the role of STAT5 downstream effectors in this process. Here we report that 2 such effectors, antiapoptotic protein A1 (homologue of human Bfl-1) and proto-oncogene pim-1, seem critical for the BCR/ABL leukemogenesis by acting in a complementary fashion. Coexpression of A1 and pim-1 restored the impaired leukemogenic capacity of the BCR/ABLΔΔ mutant by rendering the target myeloid cells growth factor independent in regard to resistance to apoptosis and ability to proliferate. Conversely, down-modulation of A1 and pim-1 retarded the BCR/ABL-dependent leukemogenesis. However, the exact mechanisms of function of A1 and pim-1 in the BCR/ABL-mediated signaling and cell transformation remain to be defined. It seems that A1 and pim-1 cowork downstream of BCR/ABL SH3 + SH2→STAT5 pathway.34 However, it is likely that lack of SH3 and SH2 domains from BCR/ABL may abrogate activation of the additional signaling pathways. Thus, A1 and/or pim-1 may complement not only STAT5, but also other signaling molecules. This hypothesis is supported by the findings that phosphatidylinositol-3 kinase (PI-3k) and nuclear factor-kB (NF-kB) can contribute to the activation of pim-1 and overexpression of A1, respectively.38,59

We speculate that enhanced A1 expression and pim-1-induced up-regulation of Bcl-2 expression could exert synergistic/additive antiapoptotic effects, as described before.29 A1 and Bcl-2 exert their function by heterodimerizing with the proapoptotic proteins to stabilize mitochondrial membranes and prevent the release of cytochrome c.41,42 Therefore, their functions may be simply additive. However, although both A1 and Bcl-2 can heterodimerize with Bax, only Bcl-2 appears to interact with Bad.42,60 Thus, Bcl-2 seems to regulate antiapoptotic pathways, which may not be accessible to A1. Nevertheless, A1 + pim-1 cells started to die after 72 hours of starvation, which was preceded by activation of caspase 3 and down-regulation of Bcl-2 (our unpublished data, May 2000). The latter phenomenon was at least partially due to the caspase 3–dependent cleavage of Bcl-2 because of the appearance of characteristic 22-kd Bcl-2 fragment,61 which can further accelerate apoptosis.62,63

In addition to its antiapoptotic effect, BCR/ABL also regulates the cell cycle.57 Pim-1 may contribute to this process through phosphorylation and activation of Cdc25A.50 Cdc25A, a phosphatase essential for G1→S transition, associates with, dephosphorylates, and activates the cell cycle kinase cyclin E-cdk2.64 Thus, pim-1–dependent activation of Cdc25A may contribute to G1→S transition,65 also in the BCR/ABL-carrying cells. Moreover, BCR/ABL→PI-3k→Akt pathway down-regulates p27kip1 and prevents inhibition of cyclin E-cdk2.66 Therefore, the BCR/ABL→PI-3k→Akt pathway down-regulates p27kip1, may exert a synergistic effect on the G1→S cell cycle progression in the leukemic cells. Furthermore, an additional proliferative signal may be delivered by the pim-1–dependent activation of c-Myc transcriptional factor,45 which is essential for the growth of Philadelphia chromosome–positive cells.47

Thus, it is probable that the combination of the strong antiapoptotic signaling (A1 and Bcl-2) with the mitogenic signal (Cdc25A and c-Myc) rescued transforming ability of the BCR/ABLΔΔ mutant. However, several mice given transplants of BCR/ABLΔΔ + A1 + pim-1 cells lived longer than these inoculated with BCR/ABL cells. Therefore, it is likely that in addition to A1 and pim-1, signaling by other STAT5 downstream effectors or even activation of different signaling pathways may be required for full reconstitution of the leukemogenic activity of BCR/ABLΔΔ mutant. Although our studies showed that A1 + pim-1 could rescue BCR/ABLΔΔ-mediated transformation of hematopoietic cells in vitro and leukemogenic activity in vivo without up-regulation of Bcl-xL, they should not underscore the potential role of Bcl-xL in BCR/ABL leukemogenesis.16,67 Even if the enforced overexpression of A1 and pim-1 in BCR/ABLΔΔ cells was similar to that observed in BCR/ABL WT cells, overexpression in the former cells is driven by strong retroviral promoters; conversely, overexpression in the latter cells is boosted by BCR/ABL-dependent stimulation of endogenous natural promoters. Thus, equal expression detected by Western analysis may not reflect discrete but significant differences between the expression profiles driven by retroviral promoters and BCR/ABL-mediated stimulation of endogenous promoters (eg, cell cycle–dependent changes). Another possibility is that A1, pim-1, and Bcl-xL may play cooperative and redundant functions downstream of BCR/ABL signaling. It is also worth noting that some signaling from BCR/ABLΔΔ mutant is still necessary for leukemogenesis, because A1 + pim-1 induced leukemias only when coexpressed with the mutant. p21Ras is a good candidate for this function because it is activated by the BCR/ABLΔΔ mutant14 and can cooperate with STAT5 in BCR/ABL-mediated transformation.58

In conclusion, our data demonstrate that A1 and pim-1 complement each other in BCR/ABL-mediated malignant cell transformation. They may, therefore, play an important role in the pathogenesis of CML and other, BCR/ABL-related hematopoietic disorders.

References

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Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukemogenesis

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