Small GTPase RAB45-mediated p38 activation in apoptosis of chronic myeloid leukemia progenitor cells

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Chronic myelogenous leukemia (CML) is characterized by a reciprocal chromosomal translocation (9;22) that generates the Bcr-Abl fusion gene. BCR-ABL transforming activity is mediated by critical downstream signaling pathways that are aberrantly activated by tyrosine kinases. However, the mechanisms of BCR-ABL anti-apoptotic effects and the signaling pathways by which BCR-ABL influences apoptosis in BCR-ABL-expressing cells are poorly defined. In this study, we found that treatment with ABL kinase inhibitors or depletion of BCR-ABL induced the expression of RAB45 messenger RNA and protein and induced apoptosis via reduction of mitochondrial membrane potential and p38 activation in CML cell lines and BCR-ABL+ progenitor cells from CML patients. Overexpressed RAB45 induced the activation of caspases-3 and -9 and reduced the expression of Survivin, XIAP, c-IAP1 and c-IAP2 in CML cells. Moreover, in colony-forming cells derived from CML-lymphoblast dehydrogenase44/CD34+ cells, treatment with ABL kinase inhibitors induced RAB45 expression and reduced mitochondrial membrane potential, resulting in inhibited colony formation of Bcr-Abl+ progenitor cells. The overexpression of RAB45 significantly decreased colony numbers and induced apoptosis through the activation of caspases-3 and -9. Furthermore, the overexpression of RAB45 increased the phosphorylation levels of p38, resulting in the induction of apoptosis and inhibition of proliferation of CML progenitor cells.

Our results identify a new signaling molecule involved in BCR-ABL modulation of apoptosis and suggest that RAB45 induction strategies may have therapeutic utility in patients with CML.

Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell malignancy that is characterized by the Philadelphia chromosome, which results from a reciprocal chromosomal translocation between the long arms of chromosomes 9 and 22 (t(9;22) (q34;q11) (1,2). The chimeric Bcr-Abl oncogene encodes the BCR-ABL oncoprotein that has highly deregulated, constitutive tyrosine kinase activity (3,4). The most common form of Bcr-Abl encodes a 210 kDa protein that is believed to play a critical role in the pathogenesis of CML (5). BCR-ABL dysregulates a variety of signaling pathways, including cell proliferation, differentiation and apoptosis. These signaling pathways play a role in BCR-ABL-mediated leukemogenesis. Of these multiple signal pathways, reversal of apoptosis is one of the most consistently observed effects of BCR-ABL. However, the mechanisms involved in this reversal of apoptosis are not yet fully understood.

BCR-ABL confers an apoptosis-resistant phenotype, which is critically dependent on its kinase activity, to several cell lines (6,7). Therefore, inhibition of tyrosine kinases by inhibitors such as STI571, AMN107 and BMS354825 induces apoptosis in BCR-ABL-expressing cells (8,9). In addition, it has been shown that BCR-ABL blocks the mitochondrial release of cytochrome c (10) and influences the expression of several pro- and anti-apoptotic proteins of the BCL-2 family that modulate mitochondrial apoptotic signals (11). The anti-apoptotic BCL-xL is induced by BCR-ABL through phosphorylation of Stat5, and inhibition of BCR-ABL kinase activity induces apoptosis by suppressing the capacity of Stat5 to interact with the bcl-x promoter (12). The anti-apoptotic BCL-2 has been also reported to be essential to the transforming potential of BCR-ABL (13,14) and its expression is upregulated via activation of Lyn (15). Moreover, the anti-apoptotic factor myeloid cell leukemia sequence 1, which belongs to the BCL-2 family, is constitutively expressed in a Ras/Raf/MEK-dependent manner via Ras activation in Bcr-Abl-positive cells (16). It has also been proposed that BCR-ABL can inhibit apoptosis by maintaining the pro-apoptotic Bad in its phosphorylated state (17) and that expression of the pro-apoptotic Bim probably has a role in imatinib-induced apoptosis through both FoxO3a-driven activation of Bim transcription (18) and the resistance of Bim to proteasome degradation (19–21). Thus, members of the BCL-2 family of proteins play pivotal roles in anti-apoptotic signaling pathways downstream of BCR-ABL. However, BCR-ABL has a much stronger anti-apoptotic effect than BCL-2 family proteins, indicating that additional or alternative survival pathways are involved in BCR-ABL anti-apoptotic effects (22). The inhibitor of apoptosis, survivin, blocks apoptosis by inhibiting caspases (23) and is aberrantly expressed in almost all cancers and hematopoietic malignancies, which correlates with poor prognosis (24). Depletion of survivin in several transformed cell models enhances apoptosis (25,26).

The Ras superfamily of small guanosine triphosphatases (GTPases) is broadly subdivided into five groups (Ras, Rho, Ran and Arf). Ras-associated binding (Rab)-GTPase proteins (Rabs) constitute the largest family of small Ras-like GTPases with >60 members in humans (27,28). Rabs generally possess the GTPase fold, composed of a six-stranded β-sheet flanked by five α-helices, that is common to all members of the Ras superfamily, which contributes to their interactions with effector proteins and to their mechanism of targeting to specific membranes (28). However, several Rabs have been implicated in the progression of various cancers and Ras, Raf and Rho-GTPases have also been implicated in oncogenesis (29). It has been reported that Rab1 is upregulated in tongue squamous cell carcinomas, Rab3 in cancers of the nervous system and Rab5 in malignant and metastatic lung cell adenocarcinomas (30). Moreover, it has been demonstrated that Rab25, which is closely related to Rab11 that regulates apical endocytosis and transcytosis in epithelial cells (31), is upregulated in certain ovarian and breast cancers and is associated with more aggressive forms of cancer and a lower patient survival rate (32). Rab25 has also been reported to be a tumor suppressor for colon carcinogenesis (33). A recent study identified Ras and EF hand domain containing (RASEF), which is also known as RAB45, as a potential tumor-suppressor gene in uveal and cutaneous melanoma and showed that RASEF is localized in the perinuclear area of these cells (34). However, little is known regarding RASEF function.

In the present study, we found that RAB45 is upregulated by treatment of cells with ABL kinase inhibitors, and we investigated the role of RAB45 in CML cell lines and in Bcr-Abl+ hematopoietic
RAB45-induced apoptosis in CML cells

progenitor cells derived from CML patients. We found that Bcr-Abl suppressed the expression of RAB45 in CML cells and that the ABL kinase inhibitor induced apoptosis via upregulation of RAB45 expression in CML cells and CML-derived progenitor cells. Interestingly, we also found that RAB45-mediated apoptosis is dependent on caspases-3 and -9 that are activated following a loss of mitochondrial membrane potential.

Materials and methods

Reagents

The ABL kinase inhibitors, imatinib mesylate (STI571) and AMN107, were kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). BMS54825 was kindly provided by Bristol-Myers Squibb (New York, NY). SB203580 and the pan-caspase inhibitor (Z-VAD-FMK) were purchased from Calbiochem (San Diego, CA). Each compound was prepared as a 10 mM stock solution in dimethyl sulfoxide and was stored at -20°C. Experiments were performed using 1000-fold dilutions of the stock solutions in reaction mixtures.

Cells and cell cultures

The human CML cell lines, K562 and Meg01, were purchased from the American Type Culture Collection (Manassas, VA). We established SHG3 cells from the bone marrow of a patient with CML (chronic phase) and YRK2 cells from the bone marrow of a patient with acute myeloid leukemia (AML) M5b (French–American–British classification). These cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 µg/ml streptomycin and 200 U/ml penicillin (Gibco BRL, Gaithersburg, MD) and were maintained in a humidified 5% CO2 atmosphere at 37°C.

Bone marrow samples

Prior to participation in the study, patients gave informed consent according to the Declaration of Helsinki. Samples of normal bone marrow were obtained from six healthy volunteers. Bone marrow was also obtained from 12 patients with CML in the first chronic phase. Mononuclear cells were isolated from bone marrow samples using Ficoll-Hypaque density gradient centrifugation. CML cells were obtained from patients before treatment with ABL kinase inhibitors was begun.

Leukemic blast cell purification from bone marrow-derived mononuclear cells based on aldehyde dehydrogenase activity and CD34 expression

For two-color staining, mononuclear cells were stained with an anti-CD34-phycocerythrin-conjugated antibody (Becton Dickinson, San Jose, CA) and the Aldefluor reagent (Stemcell Technologies, British Columbia, Canada) according to the manufacturer’s specifications. Cells were then separated using fluorescence-activated cell sorting (FACS) (Becton Dickinson). The Aldefluor substrate (0.625 µg/ml) was added to 2–7 × 106 cells per milliliter suspended in Aldefluor assay buffer and was incubated for 20–30 min at 37°C to allow conversion of the Aldefluor substrate to a fluorescent product that is retained within the cell because of its negative charge. Aldehyde dehydrogenase (ALDH)α cells were gated, and the CD34+ and CD34- cells in the gated ALDHα population were sorted using FACS. Sorted ALDHα/CD34+ and ALDHα/CD34- cell populations were collected in methylcellulose media (Methocult H4435; Stemcell Technologies).

Reverse transcription–polymerase chain reaction and quantitative real-time polymerase chain reaction

Total RNA was extracted from cells by using the RNeasy system (Qiagen, Tokyo, Japan), and 1 µg RNA was reverse transcribed by using a first strand complementary DNA (cDNA) synthesis kit (Roche, Indianapolis, IN). Polymerase chain reaction (PCR) was performed using a DNA thermal cycler (model PTC 200; MJ Research, Watertown, MA). The oligonucleotide sequences of the primers used were as follows: RAB45, sense 5’-TTGGCCTAGCTGGTGACGAGACGACG-3’, antisense 5’-GCCGCTGTGACGCGGCTCAGCT-3’; Bcr-Abl, sense 5’-GAGCGGAGCCTGCACAGAAGCCCAGGG-3’, antisense 5’-TTGAGTCCACACCTCAGTGTGC-3’. PCR conditions for RAB45, Bcr-Abl and GAPDH were 28 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. PCR products were electrophoresed in a 1.5% agarose gel containing 500 µg/l ethidium bromide and were visualized with UV light. Reverse transcription (RT)–PCR was performed in duplicate for each experiment. Quantitative real-time PCR was performed by using SYBER-Green dye and an ABI PRISM 7700 Sequence detector (Perkin-Elmer/Applied Biosystems, Foster City, CA).

Plasmid construction and RNA interference vectors

The full-length cDNA encoding human RAB45 was obtained using RT–PCR and human bone marrow cDNA as a template (BD Biosciences Clontech, Palo Alto, CA) and was cloned into the eukaryotic expression vector pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA). Sequences of recombinant cDNA were verified using automated sequencing.

The vectors for RNA interference specific for human RAB45 and Bcr-Abl were constructed based on the pGENE PUR I-L6 vector (pGENE Therapeutics, Takasu, Japan), according to the manufacturer’s instructions. We used the sequences of small interfering RNA (siRNA) directed against the Bcr-Abl (b3a2) transcript as reported previously (35) and the following targeting sequences: RAB45 small hairpin RNA (shRNA) #1: sense 5’-GACCGGAAAGAC- GAAAAADTGTT-3’ and antisense 5’-UUCUUCGCCUUCGUGUTTT-3’; RAB45 shRNA #2: sense 5’-GCUCGAGAACAACUUAATGTT-3’ and antisense 5’-UUUUGAUUGUUCUUGCCGTT-3’; Bcr-Abl shRNA #1: sense 5’-GAGGAGUGUUAGAACCCGUTTT-3’ and antisense 5’-AAGGGCUUUUGACUUCGACGU-3’. A scrambled shRNA sequence was sense 5’-UUUGAACGGAACGACGCUUATGTT-3’ and antisense 5’-UACUGUGAAGCCGCUAAGATGTT-3’ and was used as a control. Vectors were transfected into orthotopic liver cell lines; 2000 kit (Life Technologies, Gaithersburg, MD), according to the manufacturer’s instructions. The transfection procedure was repeated 12 h after the first transfection, and cells were harvested at 48 and 72 h after the initial transfection. Knockdown efficiency was consistently 50–60%, as determined by RT–PCR measurement of RAB45 and Bcr-Abl messenger RNA (mRNA).

Lentivirus construction and production

The full-length RAB45 and b3a2 Bcr-Abl cDNAs (kind gifts from Dr J. Y. Wang, University of California, San Diego, CA) were cloned upstream from the internal ribosomal entry site of the replication-deficient, self-inactivating lentiviral vector, pRRLsin-IRE5-EGFP. The RAB45 and Bcr-Abl-containing vectors were termed LV-RAB45 and LV-Bcr-Abl, respectively, and the control vector was termed LV-Con. All vector particles, pseudotyped with the vesicular stomatitis virus G glycoprotein, were produced using a three-plasmid expression system as described previously (36). The cell-free supernatants containing virus particles, LV-RAB45, LV-Bcr-Abl and LV-Con, were separately concentrated by ultracentrifugation at 28 000 r.p.m. for 2 h at 4°C in an SW28 rotor. Virus vector stocks were resuspended in complete Dulbecco’s modified Eagle’s medium and stored at -80°C. The titer of the virus vector pRRLsin-IRE5-EGFP was determined using a fluorescence-activated cell sorter (Becton Dickinson) and transfection procedure was repeated 12 h after the first transfection, and cells were harvested at 48 and 72 h after the initial transfection. Knockdown efficiency was consistently 50–60%, as determined by RT–PCR measurement of RAB45 and Bcr-Abl messenger RNA (mRNA).

Immunoprecipitation and western blot analysis

For immunoprecipitation and immunoblotting, total cell lysates were prepared from ABL kinase inhibitor-treated, DNA-transfected, shRNA-transfected cells or non-treated cells. Cells were lysed in a lysis buffer containing 1% Triton X-100, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated with the Abl-specific monoclonal antibody (Becton Dickinson) and trans- fected HeLa cells. To calculate titers, the number of target cells was multiplied by the percentage of enhanced green fluorescent protein-positive cells divided by the volume of the input virus and the titer of each concentrated lentivirus vector (LV-RAB45, LV-Bcr-Abl and LV-Con), which were 1.1 × 10^11, 1.7 × 10^11 and 2.2 × 10^11 gene-transducing units per milliliter, respectively.

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Cell proliferation and viability assays

To assess cell proliferation, K562 cells (1 × 10^6) were seeded in 24 well plates and were left untreated or were incubated with STI571 (1 μM), AMN107 (10 nM) or BMS354825 (1 nM) at 37°C. Cells were then transfected with RAB45 cDNA, shRNA #1 and #2 or scrambled shRNA. After 24, 48 and 72 h of incubation, cell proliferation was measured by counting viable cells using a hemocytometer. Cell viability was assessed by assay of trypan blue uptake of non-viable cells using a hemocytometer. The numbers of viable cells are reported as percentages of the numbers of viable cells in untreated control cells. Each data point was performed in triplicate, and the results are reported as mean counts ± SDs.

Detection of changes in mitochondrial membrane potential (ΔΨm)

To detect ΔΨm, cells (1 × 10^6 cells per well) that were transfected with RAB45 cDNA were incubated in 24 well plates. After 3 days, cells were labeled with DiOC6 (40 nM in culture medium) at 37°C for 20 min. After washing in phosphate-buffered saline, cellular uptake of DiOC6 was analyzed using flow cytometry.

Cell cycle analysis

DNA content analysis was performed using propidium iodide (Sigma-Aldrich, St Louis, MO) staining. Briefly, cells were transfected with RAB45 cDNA. Cells were cultured in 2 ml complete medium containing 1 × 10^6 cells at 37°C. After 7 days of incubation, the cells were washed twice with cold phosphate-buffered saline, fixed with 70% ethanol overnight before treatment with 100 μg/ml RNase A and then stained with 50 μg/ml propidium iodide. The relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France). The percentage of cells in sub-G1, G1, S and G2/M phases was calculated using the ModFit program (Becton Dickinson).

Colony-forming assay

ALDH^{+/−}CD34^{+} cells from CML bone marrow were treated with or without ABL kinase inhibitors, Z-VAD-FMK and SB203580, or were transfected with control siRNA, RAB45 shRNA or RAB45 cDNA. Human clonogenic progenitor assays were performed by plating purified populations of cells at cell densities ranging from 2 × 10^2 to 7 × 10^7 (ALDH^{+/−}CD34^{+}) into methylcellulose media (Methoct H4435). Colonies were enumerated under light microscopy (Zeiss, Muenchen, Germany) following incubation at 37°C, in 5% CO2, for 7–10 days.

Apoptosis assay

Apoptosis of RAB45 cDNA-transfected or untransfected cells that were treated with or without ABL kinase inhibitors and/or Z-VAD-FMK was assessed using an Annexin V staining kit (Clontech), according to the manufacturer’s specifications. Briefly, cells were rinsed once with binding buffer and were resuspended in 200 μl of binding buffer following which 5 μl of Annexin V and 10 μl of propidium iodide were added. Cells were incubated at room temperature in the dark for 15 min and were then analyzed using flow cytometry. The numbers of apoptotic cells are reported as percentages of the numbers of untreated control cells.

Caspase-3 activation assay

RAB45 cDNA-transfected or untransfected cells (3 × 10^6 cells per well) were left untreated or were treated without or with Z-VAD-FMK (100 μM) and/or STI571 (1 μM) in six well plates containing complete medium at 37°C for 24 h. The level of caspase-3 activity in the cells was measured using a CaspACE Assay System (Promega, Madison, WI) according to the manufacturer’s instructions, using a microplate reader.

Isolation of progenitor cells and quantitative real-time PCR and western blot analysis of the progenitor cells

Following colony formation in the colony-forming assays, each colony was harvested using a glass syringe and the cells of each colony were pooled and washed. Immunoprecipitation and western blotting of the cells were then performed using the appropriate antibody. The RNAeasy system was used to extract total RNA from ~2 × 10^6 cells from each colony. This RNA was used for RT-PCR and quantitative real-time PCR assays using RAB45-, Bcr-Abl- and GAPDH-specific primers.

Statistical analysis

Data are representative of at least three experiments that showed essentially similar results. These results are expressed as the means ± SDs from three independent experiments. The means were compared by using Student’s t-test. P values <0.01 were considered statistically significant.

Results

ABL kinase inhibitors induce RAB45 expression in CML cell lines

As shown in Figure 1A, the levels of RAB45 mRNA, assessed using RT–PCR, were low in CML cell lines (K562, Meg01 and SHG3 cells). Interestingly, we found that the expression of RAB45 mRNA increased in these three CML cell lines following treatment with ABL kinase inhibitors (STI571, AMN107 or BMS354825) for 24 h as compared with untreated cells. Moreover, RAB45 mRNA expression was significantly increased in these CML cell lines following transfection with Bcr-Abl siRNA #1 or #2, compared with control cells.

The RAB45 protein was constitutively expressed in all three CML cell lines (K562, Meg01 and SHG3 cells) (Figure 1B) although its expression in CML cell lines was at a lower level compared with that in AML cell lines (data not shown). Treatment with ABL kinase inhibitors or transfection with Bcr-Abl siRNA #1 or #2 also increased the protein levels of RAB45 in CML cells. Thus, ABL kinase inhibitors or knockdown of the BCR-ABL protein induced both RAB45 mRNA and protein expression. The ABL kinase inhibitors and knockdown of Bcr-Abl also decreased the levels of tyrosine phosphorylated Bcr-Abl (p-BCR-ABL) in CML cells. These results indicate that BCR-ABL expression negatively regulates RAB45 mRNA and protein expression.

Effects of RAB45 expression on CML cell proliferation

Since RAB45 expression was induced by ABL kinase inhibitors as well as by the knockdown of BCR-ABL in CML cells, we next examined the functional importance of RAB45 expression. We therefore transfected K562 cells with RAB45 cDNA and assessed the effects of RAB45 overexpression on CML cell proliferation (Figure 2A). When K562 cells were transfected with RAB45 cDNA, cell proliferation was inhibited compared with non-transfected cells. The ABL kinase inhibitor STI571 also significantly inhibited the proliferation of non-transfected K562 cells. In addition, the viability of K562 cells transfected with RAB45 cDNA or treated with STI571 was significantly decreased compared with control cells (Figure 2B). The expression of RAB45 mRNA was significantly increased by STI571 treatment compared with controls (Figure 2C). These results show that the ABL kinase inhibitor induces RAB45 mRNA expression and that RAB45 plays an important role in the inhibition of CML cell proliferation and decreased cell viability that is induced by the ABL kinase inhibitor.

We next investigated how RAB45 inhibits CML cell proliferation. FACS analysis of K562 cells on day 3 post-transfection with RAB45 cDNA indicated an increase in the sub-G1 population compared with non-transfected cells (Figure 2D and E). These results demonstrate that overexpression of RAB45 induces a significant increase in apoptosis in CML cells.

RAB45 induces apoptosis through the loss of mitochondrial membrane potential

We next determined the effect of RAB45 on markers of apoptosis. We first analyzed its effect on loss of mitochondrial membrane potential, which was determined by flow cytometric analysis of DiOC6 uptake. Loss of mitochondrial membrane potential is known to occur in apoptotic cells and precedes the activation of caspases. DiOC6 fluorescence of K562 and Meg01 cells was significantly reduced 2 days after transfection with RAB45 cDNA, compared with non-transfected cells (Figure 3A). In addition, a marked reduction in DiOC6 fluorescence of RAB45 cDNA-transfected cells compared with control cells was observed in a time-dependent manner (data not shown). We next examined the expression of various apoptotic regulatory proteins in RAB45-overexpressing CML cells by western blot analysis. As shown in Figure 3B, overexpression of RAB45 induced significant cleavage of Procaspases-3 and -9 and of PARP. Moreover, overexpression of RAB45 reduced the expression of survivin, XIAP, c-IAP1 and c-IAP2. Interestingly, no change in the expression of BCL-2 and BCL-xL proteins was observed in RAB45-overexpressing CML cells.
Fig. 1. RAB45 expression in leukemia cells. (A) CML cell lines (K562, Meg01 and SHG3) were left untreated or were treated with STI571 (1 μM), ANM107 (10 nM) or BMS354825 (1 nM) for 24 h. CML cells were harvested 3 days after transfection with scrambled shRNA or with Bcr-Abl shRNA #1 or #2. RT–PCR was then performed to detect RAB45 and Bcr-Abl mRNA expression. GAPDH mRNA expression is shown as an internal control. RT–PCR results representative of three independent experiments are shown (left panels). Relative levels of RAB45 mRNA expression in CMLs were measured (right panels). The expression levels of the target mRNAs were normalized to the expression of GAPDH mRNA. The results are expressed relative to the untreated control, which was set at 1. Each RT–PCR assay was performed at least three times, and the results are expressed as means ± SDs. *P < 0.01 compared with untreated or scrambled shRNA (control shRNA)-transfected control as indicated. (B) The effects of the indicated ABL kinase inhibitors and Bcr-Abl shRNA on RAB45 protein expression were assessed.
Thus, RAB45 overexpression induced the loss of mitochondrial membrane potential, activation of caspase cascades and a decrease in the expression of inhibitor of apoptosis proteins (IAPs).

Effects of deleted RAB45 expression on CML cell proliferation

To investigate whether decreased expression of RAB45 alters cell proliferation, we next assayed cell proliferation in cells in which RAB45 expression was decreased by RNA interference knockdown. Whereas STI571 significantly reduced the rate of proliferation in non-transfected and the scrambled shRNA-transfected K562 cells, knockdown of RAB45 reduced this STI571-mediated inhibition of proliferation in RAB45 shRNA #1- or #2-transfected K562 cells (Figure 4A). Moreover, knockdown of RAB45 also inhibited the STI571-mediated reduction of cell viability in RAB45 shRNA #1- or #2-transfected K562 cells (Figure 4B). Both RAB45 mRNA and protein expression were successfully reduced by RAB45 shRNA #1 and #2 in these experiments (Figure 4C and D). RAB45 shRNA #1 and #2 similarly reduced the effects of other ABL kinase inhibitors (AMN107 and BMS354825) (data not shown). Thus, these results show that RAB45 has an important role in the inhibition of CML cell proliferation that is caused by ABL kinase inhibitors.

RAB45 expression in primary CML progenitor cells

To confirm that the results obtained above regarding RAB45 in CML cell lines represented the situation in CML cells from patients, we first examined RAB45 mRNA expression in clinical specimens from CML patients. Hematopoietic progenitor cells were obtained from bone marrow using flow cytometry and were identified based on aldehyde dehydrogenase activity using the substrate Aldefluor. ALDHhi hematopoietic progenitor cells, which include CD34+ or Lin- cells, were selected based on side scatter and fluorescein isothiocyanate properties. CD34+ and CD34- progenitor cells in the ALDHhi population (ALDH hi/CD34+ or ALDH hi/Lin- cells) were then sorted using FACS. We isolated ALDHhi/CD34+ cells from bone marrow cells derived from six healthy volunteers and 12 CML patients, and representative data from a normal (#2) and a CML sample (#1) are shown in Figure 5A. In bone marrow cells derived from healthy volunteer #2, 95.1% of the population were ALDH low cells using western blotting. After 24 h treatment with the ABL kinase inhibitors or 5 days after transfection with scrambled shRNA or Bcr-Abl shRNA #1 or #2, total cell lysates from K562 (upper left), Meg01 (middle left) and SHG3 (bottom left) cells were prepared and analyzed by immunoblotting. Actin was immunoblotted as a loading control. Total cell lysates prepared from treated or non-treated CML cells were immunoprecipitated with anti-c-Abl antibodies and then immunoblotted with anti-c-Abl and anti-p-Tyr antibodies (right panels).
and 0.12% were ALDH\textsuperscript{hi} cells. Within the ALDH\textsuperscript{hi} population, 78.4% of the cells were CD34\textsuperscript{+} cells and 20.5% were CD34\textsuperscript{-} cells. There were no significant differences in the percentages of these populations among the six normal healthy volunteers. In contrast to the cell populations found in normal volunteers, 94.7% of the bone marrow cells derived from CML patient #3 were ALDH\textsuperscript{low} cells and 5.1% were ALDH\textsuperscript{hi} cells. Within the ALDH\textsuperscript{hi} cell population, 68.2% of the cells were CD34\textsuperscript{+} and 26.2% were CD34\textsuperscript{-} cells. There were no significant differences in the percentages of these cell populations among 12 CML specimens. In ALDH\textsuperscript{low}, ALDH\textsuperscript{hi}, ALDH\textsuperscript{hi}/CD34\textsuperscript{-} and ALDH\textsuperscript{hi}/CD34\textsuperscript{+} populations derived from CML patient #1, expression of RAB45 mRNA and protein was lower than that of normal ALDH\textsuperscript{hi} cells derived from healthy volunteer #2 (Figure 5B). A comparison of the RAB45 mRNA expression levels of all CML-derived ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells assayed with that in all normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells assayed is shown in Figure 5C. RAB45 mRNA was more strongly expressed in all normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} populations compared with its expression in all CML-derived ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells. The mean ratio of RAB45 mRNA expression to that of the internal control GAPDH in CML specimens was 0.22 ± 0.03. In contrast, the mean ratio of RAB45/GAPDH mRNA expression in normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells was 0.51 ± 0.08. Moreover, in all CML-derived ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells, STI571 treatment induced the expression of RAB45 protein and mRNA in all normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells. These results demonstrated that there was no significant difference in the expression levels of RAB45 mRNA and protein among CML specimens and that RAB45 mRNA and protein levels were decreased in all CML specimens analyzed compared with the levels in normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells. As shown in Figure 5E, treatment of purified ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells from CML patients (#1 and #5) with ABL kinase inhibitors for 24 h strongly induced RAB45 mRNA expression compared with untreated cells. On the other hand, in normal ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells (#2 and #4), treatment with STI571 did not affect the RAB45 mRNA expression. The other ABL kinase inhibitors (AMN107 and BMS354825) also did not affect the RAB45 mRNA expression (data not shown). The effects of the ABL kinase inhibitors on RAB45 and Bcr-Abl mRNA levels in ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells from two representative CML patients (#1 and #5) were assessed using quantitative real-time PCR. There were no significant differences in the levels of RAB45 mRNA induced by the different ABL kinase inhibitors in ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells derived from CML patients. These results demonstrated that the ABL kinase inhibitors induced RAB45 mRNA expression in BCR-ABL\textsuperscript{+} cells.

**RAB45 overexpression inhibited colony formation in CML-derived ALDH\textsuperscript{hi}/CD34\textsuperscript{+} progenitor cells**

We next examined the effect of overexpression of RAB45 on colony formation of ALDH\textsuperscript{hi}/CD34\textsuperscript{+} hemato poetic progenitor cells from all healthy volunteers (n = 6) and from all CML patients (n = 12) pre-treatment. We further examined the effect of STI571 on colony formation of RAB45 cDNA-transfected ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells of all healthy volunteers and all CML patients. ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells, which were not transfected or were transfected with RAB45 cDNA,
Fig. 4. The effects of RAB45 knockdown on K562 cell proliferation. K562 cells were transfected with scrambled shRNA, RAB45 shRNA #1, or RAB45 shRNA #2. Non-transfected cells were used as controls. After 48 h, transfected and control cells were treated with STI571 (1 μM) for 24, 48 and 72 h or were left untreated. The number of viable cells was then counted using a hemocytometer (A), and cell viability was assessed by counting the number of non-viable cells that showed trypan blue uptake. Cell viability is expressed as a percentage of the viability of untreated control cells, which was set at 100% (B). The RAB45 shRNA #1- or #2-transfected, scrambled shRNA or untransfected K562 cells that were treated with STI571 (1 μM) were collected and RAB45 mRNA levels were analyzed using quantitative real-time PCR. The mRNA levels were calculated relative to the mRNA expression of GAPDH (C). Results are means ± SDs from three independent experiments. * P < 0.01 compared with untreated control cells. (D) Representative western blot data of RAB45 protein expression are shown at 72 h after STI571 treatment.
Fig. 5. RAB45 expression in hematopoietic progenitor cells derived from CML patients. (A) Selection of ALDH\(^{hi}\)/CD34\(^{+}\) hematopoietic progenitor cells from the bone marrow (BM) of healthy volunteer (#2) and CML patient (#1). Regions P and Q denote populations of ALDH\(^{hi}\) and ALDH\(^{low}\) cells, respectively (left panels). Regions R and S denote populations of CD34\(^{+}\) and CD34\(^{-}\)/C0 cells, respectively, in the ALDH\(^{hi}\) population (region P). Negative control (light gray region) and CD34–phycoerythrin staining (dark gray region) (right panels). (B) RT–PCR (upper panels) and western blot (bottom panels) analyses were performed to determine RAB45 mRNA and protein levels, respectively, in ALDH\(^{low}\), ALDH\(^{hi}\), ALDH\(^{hi}/CD34^{+}\) and ALDH\(^{hi}/CD34^{-}\) populations derived from healthy volunteer...
#2 and CML patient #1, respectively. GAPDH and actin are shown as an internal control. The RT–PCR and western blotting data shown are representative of data of six healthy volunteers and 12 CML patients. (C and D) Summary of RAB45 mRNA (C) and protein (D) expression levels of normal ALDHhi/CD34+ cells (n = 6) and of ALDHhi/CD34+ cells derived from patients with CML (n = 12) with or without STI571 treatment. The cells were treated with STI571 (1 µM) for 24 h. Each RT–PCR assay was performed at least three times. RAB45 protein expression was analyzed by western blotting. The representative blot was displayed (D, upper panels). The levels of RAB45 protein were quantified by imaging densitometry and normalized to that of actin (bottom panel). *P < 0.01 compared with untreated control cells. (E) The mRNA expression of RAB45 and Bcr-Abl in sorted ALDHhi/CD34+ cells from two healthy volunteers (#2 and #4) and from two CML patients (#1 and #5), and the effects of treatment with ABL kinase inhibitors on the expression of these mRNAs in CML from patients were analyzed using quantitative real-time PCR. The data shown are representative of data of six healthy volunteers and 12 CML patients. The levels of target mRNAs were normalized to relative to GAPDH mRNA expression and are expressed relative to the level of the untreated control cells. Each RT–PCR assay was performed at least three times and the results are expressed as means ± SDs. *P < 0.01 compared with untreated control cells.
were treated with or without STI571 for 7–10 days, following which the number of colonies formed was counted. As shown in Figure 6A, the RAB45 overexpression moderately reduced the number of colonies in all normal ALDHhi/CD34+ cells. In contrast, overexpression of RAB45 in all CML-derived ALDHhi/CD34+ cells significantly decreased the number of colonies. Moreover, we examined the effect of ABL kinase inhibitors on colony formation of RAB45 cDNA- or shRNA-transfected ALDHhi/CD34+ cells of CML patient #1. ALDHhi/CD34+ cells, which were not transfected or were transfected with RAB45 cDNA or shRNA, were treated with or without ABL kinase inhibitor for 7–10 days, following which the number of colonies formed was counted. As shown in Figure 6B, the mean colony number obtained for normal #2 ALDHhi/CD34+ cells that were not transfected with RAB45 cDNA was 121 (range, 93–135). However, when RAB45 was overexpressed, the mean number of colonies was 89 (range, 71–97). Thus, RAB45 overexpression moderately reduced the number of colonies formed in normal ALDHhi/CD34+ cells. On the other hand, the mean colony number obtained for normal #2 ALDHhi/CD34+ cells that were treated with STI571 was 119 (range, 92–128). STI571 treatment did not reduce the number of colonies and induce the RAB45 mRNA expression compared with untreated normal ALDHhi/CD34+ cells. In contrast, overexpression of RAB45 in CML #1 ALDHhi/CD34+ cells significantly decreased the number of colonies formed from 182 (range, 167–195) to 47 (range, 31–56). Moreover, in these cells, the combination of STI571 treatment and RAB45 overexpression induced a greater decrease in the number of colonies than RAB45 overexpression alone. In addition, the decreased RAB45 expression in the RAB45 shRNA-transfected CML #1 ALDHhi/CD34+ cells did not affect the number of colonies formed, even though it weakened the STI571-mediated inhibitory effects for CML progenitor cell colony formation. There were no significant differences in the levels of RAB45 mRNA that were induced by STI571 or in the inhibitory effects of STI571, RAB45 cDNA and/or shRNA on colony formation among CML specimens. These findings suggest that RAB45 is constitutively expressed in both normal and CML ALDHhi/CD34+ cells, whereas RAB45 overexpression was more strongly suppressed in CML progenitor cells compared with normal progenitor cells. These results further show that RAB45 expression is induced by inhibition of BCR-ABL in CML progenitor cells and inhibits colony formation of progenitor cells from CML specimens. Moreover, DiOC6 fluorescence of ALDHhi/CD34+ cells from CML patients #1 and #5 was significantly reduced 5 days after transfection with RAB45 cDNA. In addition, a marked reduction in DiOC6 fluorescence of these cells was observed in a time-dependent manner (data not shown). In contrast, DiOC6 fluorescence was slightly reduced in normal #2 ALDHhi/CD34+ cells transfected with RAB45 cDNA (Figure 6C).

We next determined whether overexpression of RAB45 activated caspases, resulting in apoptosis of CML progenitor cells. In all CML-derived ALDHhi/CD34+ cells, RAB45 overexpression induced the activation of caspase-3. In contrast, the caspase-3 activation was slightly induced in all normal ALDHhi/CD34+ cells transfected with RAB45 cDNA (Figure 6D). As shown in Figure 6E, activation of caspase-3 was observed in RAB45-overexpressing CML ALDHhi/CD34+ cells. This caspase-3 activation, as well as the caspase-3 activation that was induced by STI571, was inhibited by 100 μM of the pan-caspase inhibitor Z-VAD-FMK. We further analyzed these cells for expression of the apoptotic marker Annexin V using FACS. At 2 days post-transfection, 50.8% of the RAB45-overexpressing CML ALDHhi/CD34+ cells were Annexin V positive. The levels of Annexin V in these cells were reduced (26.3%) by Z-VAD-FMK treatment for 24 h. Moreover, overexpression of RAB45 significantly induced the cleavage of caspase-3 and PARP. In contrast, caspase-3 cleavage was inhibited by Z-VAD-FMK in RAB45-overexpressing CML cells (Figure 6F). Thus, RAB45 overexpression in CML cells induced apoptosis via activation of caspases. In addition, RAB45 overexpression did not affect the levels of BCL-2 or BCL-XL proteins in CML progenitor cells (data not shown).

RAB45 induced caspase activation through p38 phosphorylation in CML ALDHhi/CD34+ progenitor cells

We therefore considered the potential mechanism by which RAB45 might induce apoptosis. It has been reported that phosphorylated p38 directly activates caspases or induces caspase-mediated cleavage of Raf, MEK and extracellular signal-regulated kinase proteins, resulting in apoptosis (38,39). We therefore investigated whether overexpression of RAB45 increased the phosphorylation levels of p38 in CML (#1)-derived ALDHhi/CD34+ cells (Figure 7A). When the CML-derived ALDHhi/CD34+ cells were treated with STI571 or were transfected with RAB45 cDNA, RAB45 protein expression was increased, and the phosphorylation levels of p38 were increased. On the other hand, in RAB45 knockdown CML ALDHhi/CD34+ cells, STI571-induced RAB45 expression was decreased and the phosphorylation of p38 was inhibited. Moreover, the p38 inhibitor, SB203580, inhibited the activation of caspase-3 and the cleavage of PARP in CML ALDHhi/CD34+ cells treated with STI571 and transfected with RAB45 cDNA (Figure 7B). Finally, we analyzed the phosphorylation status of p38 in all CML-derived ALDHhi/CD34+ cells transfected with RAB45 cDNA. In all CML-derived ALDHhi/CD34+ cells, the phosphorylation levels of p38 were increased (Figure 7C). These results show that RAB45 induced apoptosis through activation of p38 in CML ALDHhi/CD34+ cells.

Discussion

We investigated the role of RAB45 in cell growth inhibition of CML cells and CML-derived progenitor cells. In this study, we demonstrate that induction of RAB45 expression results in the loss of mitochondrial membrane potential, reduction in the expression of IAPs and activation of p38 and plays an important role in apoptosis of CML cells. BCR-ABL induces hematopoietic cell transformation (40) and protects cells from apoptosis induced by various stimuli (41). BCR-ABL transforming activity is mediated by critical downstream signaling pathways that are aberrantly activated by tyrosine kinases. However, the mechanisms by which BCR-ABL mediates its anti-apoptotic effects and the signaling pathways in BCR-ABL-expressing cells that lead to apoptosis are poorly defined. In this study, we found that treatment with BCR-ABL kinase inhibitors or depletion of BCR-ABL-induced the expression of RAB45 mRNA and protein and induced CML cell apoptosis via a reduction of mitochondrial membrane potential. We further showed that overexpression of RAB45 in CML cells inhibited their proliferation.

RABs have important roles in regulating signal transduction and cellular process such as differentiation, proliferation, vesicle transport, nuclear assembly and cytoskeleton formation (42). However, some RABs have been reported to be essential for the adhesion and migration of cancer cells (29–33), and understanding of the mechanism of action of RABs in cancer progression is limited. This is the first report that RAB45 induces apoptosis in CML cells.

The RAB45 gene is located on chromosome 9, area q21 (9q21), and is also known as RASEF or FLJ31614 (43). RAB45, which contains a coiled-coil motif at the midregion, a distinct N-terminal EF-hand domain and a C-terminal Rab-homology domain, is an atypical RAB GTPase. RAB45 binds guanine nucleotide tri/diphosphates via the GTPase. RAB45 is involved in the development of melanoma. Low RAB45 promoter region, and homozygosity and methylation of the RAB45 gene in primary tumors were associated with decreased survival (P = 0.019) (34). Moreover, the RAB45 gene product is one of the critical gene products that are affected by del(9q) in AML with t(8;21), and expression of RAB45 is significantly downregulated in AML cells compared with normal.
**Fig. 6.** RAB45 induced apoptosis through the loss of mitochondrial membrane potential in ALDH<sup>hi</sup>/CD34<sup>+</sup> hematopoietic progenitor cells derived from CML patients. (A) ALDH<sup>hi</sup>/CD34<sup>+</sup> cells (3 x 10<sup>3</sup> to 5 x 10<sup>3</sup> cells per plate) purified from normal healthy volunteers (n = 6) and CML patients (n = 12) were isolated and cultured in semisolid methylcellulose media (Methocult; H4435). The number of colonies formed by normal ALDH<sup>hi</sup>/CD34<sup>+</sup> cells, which were untransfected or transfected with RAB45 cDNA, were enumerated after 7–10 days of in vitro culture. The percentage of progenitor cells was evaluated as a percentage of the corresponding control that was expressed as 100% colony-forming units (CFU). Results are means ± SDs of three independent experiments. *P < 0.01 compared with untreated control cells. (B) ALDH<sup>hi</sup>/CD34<sup>+</sup> cells (3 x 10<sup>3</sup> to 5 x 10<sup>3</sup> cells per plate) purified from normal healthy volunteer #2 and CML patient #1 were isolated and cultured in semisolid methylcellulose media (Methocult; H4435). The number of colonies formed by normal ALDH<sup>hi</sup>/CD34<sup>+</sup> cells, which were treated with STI571 (1 μM), untransfected or transfected with RAB45 cDNA, and the number formed by CML-derived ALDH<sup>hi</sup>/CD34<sup>+</sup> cells, which were untransfected or transfected with RAB45 cDNA or RAB45 shRNA and were treated with or without STI571, were enumerated after 7–10 days of in vitro culture. The percentage of progenitor cells was evaluated as a percentage of the corresponding control that was expressed as 100% colony-forming units (CFU) (upper panel). Results are means ± SDs of three independent experiments. *P < 0.01 compared with untreated control cells. RAB45 and Bcr-Abl mRNA were detected using RT–PCR. GAPDH mRNA is shown as an internal control. Representative data are shown (middle panels). RAB45 mRNA expression was quantified using quantitative real-time PCR and is expressed relative to GAPDH gene expression (bottom panel). Data are shown as means ± SDs of triplicate cultures and are representative of three independent experiments. *P < 0.01 compared with untreated control cells. (C) Normal #2 (upper panel), CML #1 (middle panel) and CML #5 (bottom panel) ALDH<sup>hi</sup>/CD34<sup>+</sup> cells were transfected with RAB45 cDNA. After 5 days of transfection, mitochondrial membrane potential was determined by staining of the cells with DiOC6 followed by flow cytometric analysis. Negative control (mid-gray region), positive control (light gray region) and RAB45-overexpressing cells (dark gray region) are shown. Normal (n = 6) and CML (n = 12) ALDH<sup>hi</sup>/CD34<sup>+</sup> cells were untransfected or transfected with RAB45 cDNA. Cell lysates were then analyzed for caspase-3 activation. The fold changes in caspase-3 activity are shown relative to the value for the control cell. Results are means ± SDs of three independent experiments. *P < 0.01 compared with untreated control cells. (E) CML #1 ALDH<sup>hi</sup>/CD34<sup>+</sup> cells, which were untransfected or transfected with RAB45 cDNA, were left untreated or were treated for 24 h with STI571 (1 μM) and/or Z-VAD-FMK (100 μM) as indicated. Cell lysates were then analyzed for caspase-3 activation. The fold changes in caspase-3 activity are shown relative to the value for the control cell. Results are means ± SDs of three independent experiments. *P < 0.01 compared with untreated control cells. The percentage of Annexin V-positive cells was measured using FACs and was used as an indicator of apoptosis (middle panel). RAB45 mRNA expression was analyzed using quantitative real-time PCR and is expressed...
CD34-purified progenitor cells. These results are consistent with a model of tumor suppression that is mediated by haploinsufficiency of critical genes in del(9q) AML (43). We also found that \textit{RAB45} mRNA and protein levels are increased in CML cells (K562, Meg01 and SHG3 cells) treated with ABL kinase inhibitors. Moreover, knockdown of BCR-ABL also upregulated \textit{RAB45} mRNA and protein expression. Therefore, the observation that \textit{RAB45} is downregulated in leukemia cells prompted us to investigate its role in leukemia cell proliferation.

Cell proliferation of K562 cells was strongly inhibited by \textit{RAB45} overexpression and by treatment with STI571, whereas cell proliferation was moderately inhibited when these cells were transfected with \textit{RAB45} shRNA and then treated with STI571. Moreover, \textit{RAB45} overexpression also increased the population of apoptotic K562 cells.

relative to \textit{GAPDH} mRNA expression (bottom panels). Data are shown as means ± SDs of triplicate cultures and are representative of three independent experiments. *P < 0.01 compared with untreated control cells. (F) Expression of caspase-3, PARP and \textit{RAB45} protein in purified ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells from one CML patient (#1). ALDH\textsuperscript{hi}/CD34\textsuperscript{+} cells (3 × 10\textsuperscript{2} to 5 × 10\textsuperscript{2} cells per plate) were untransfected or were transfected with \textit{RAB45} cDNA, plated in semisolid methylcellulose media and were then treated for 24 h without or with STI571 (1 μM) or Z-VAD-FMK (100 μM). The data are representative of four number of CML patients. Western blot analysis was performed by using anti-caspase-3, anti-PARP and anti-\textit{RAB45} antibodies. The levels of actin served as a loading control.
These results indicate that RAB45 has an important role in ABL kinase inhibitor-mediated apoptosis of CML cells.

We found that RAB45 overexpression induced an increase in cell death that was due to an apoptotic process as demonstrated by a decrease in DIOC6 incorporation and activation of caspases-3 and -9. Furthermore, our results show that RAB45 overexpression reduced survivin, XIAP, and IAP1 expression, but it did not affect BCL-2 and BCL-xl expression in CML and CML-derived progenitor cells. The BCL-2 and IAP families are known to regulate apoptosis (43). BCR-ABL exerts anti-apoptotic effects by blocking mitochondrial release of cytochrome c through the BCL-2 family. Thus, aberrant BCR-ABL expression prevents the early translocation of members of this family to the mitochondria, maintains mitochondrial membrane potential and suppresses caspase activation (45). BCR-ABL also modulates apoptosis through modulation of Survivin levels. Survivin is highly expressed during blast crisis in CML patients (46) and elevated survivin levels are found in adriamycin-resistant K562 cells (47). BCR-ABL elevates Survivin and IAP like protein-2 (ILP-2) mRNA and protein expression and its effect on survivin is mediated via an effect on Survivin promoter activity. In addition, survivin disruption sensitizes CML cells to apoptosis induced by STI571 (48). These findings demonstrate that survivin plays an important role in the anti-apoptotic effects of BCR-ABL and that depletion of survivin expression promotes apoptosis in CML cells. Our results showed that RAB45 overexpression induced ABL kinase inhibitor-mediated apoptosis through activation of caspases-3 and -9 and reduction of the expression of survivin. In addition to survivin, expression of XIAP, c-IAP1 and c-IAP2 proteins was downregulated in RAB45-transfected K562 cells. Similar to survivin, these IAPs also play important roles in cell cycle progression and apoptosis and their downregulation might enhance apoptosis of CML cells in the RAB45-mediated apoptotic pathway.

We performed colony formation assays using ALDH+/CD34+ progenitor cells derived from normal healthy volunteers and CML patients. The number of colonies formed was not reduced when normal ALDH+/CD34+ progenitor cells were treated with STI571 (data not shown). In contrast, colony numbers were significantly reduced when ALDH+/CD34+ CML progenitor cells were transfected with RAB45 cDNA and treated with STI571. Moreover, when ALDH+/CD34+ CML progenitor cells were transfected with RAB45 siRNA, the inhibitory effects of the Abl kinase inhibitor on colony formation were decreased in colony-forming cells. In addition, RAB45 DNA transfection induced the loss of mitochondrial membrane potential in ALDH+/CD34+ CML progenitor cells. These results indicate that RAB45 overexpression reduced the number of colony-forming cells derived from CML progenitor cells through the intrinsic apoptotic pathway.

It has been reported that the p38 pathway is activated during treatment of BCR-ABL-expressing cells with STI571 (49). Moreover, dasatinib activates the p38 mitogen-activated protein kinase (MAPK) in CML cells and such activation is essential for the antileukemic effects of this dual kinase inhibitor (50). The p38 MAPK associates with various signaling cascades that regulate functional cellular responses generated in response to cytokines or stress signals (51). In BCR-ABL-expressing cells, p38 inhibition contributes to BCR-ABL transformation (52), suggesting a critical role for BCR/ABL-mediated p38 suppression in mediating its transforming ability. Our data demonstrate that the level of p38 MAPK phosphorylation was increased in RAB45-overexpressing CML progenitor cells. We also show that RAB45-mediated caspase-3 activation was inhibited by SB203580 treatment, suggesting that the mechanism by which RAB45 overexpression leads to apoptosis may involve induction of an increase in p38 MAPK activity.
ABL kinase inhibitors produce clinical responses in most CML patients (53). However, the development of drug resistance, due to increased expression of BCR-ABL through gene amplification or to mutations in the BCR-ABL catalytic domain, limits the efficacy of these drugs (54) and points to the need for additional treatment strategies. One approach to overcoming drug resistance would be to decrease BCR-ABL oncoprotein activity. Our results show that RAB45 enhances activation of caspases-3 and -9 through p38 phosphorylation, suggesting that modulation of RAB45 may be useful for decreasing BCR-ABL anti-apoptotic effects.

Our results identify a new signaling molecule in BCR-ABL modulation of apoptosis and suggest that strategies to induce RAB45 may have therapeutic utility in patients with CML. These findings raise the possibility that development of novel approaches to activate p38 in CML- or BCR-ABL-expressing cells may provide a novel therapeutic approach to enhance the effects of ABL kinase inhibitors on leukemic cells.

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References


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