Bcr-Abl signaling through the PI-3/S6 kinase pathway inhibits nuclear translocation of the transcription factor Bach2, which represses the antiapoptotic factor heme oxygenase-1

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The malignant phenotype of chronic myeloid leukemia (CML) is due to the abnormal tyrosine kinase activity of the Bcr-Abl oncoprotein. We have previously reported that expression of the Bach2 transcription factor, which induces apoptosis in response to oxidative stress, is greatly reduced in CML cells. Because these cells are resistant to apoptosis, we tested whether Bach2 could also be regulated through posttranslational mechanisms that promote inhibition of the apoptotic response to mutagenic stimuli in CML. We found that Bach2 is phosphorylated on S521 via the phosphatidylinositol-3/S6 kinase pathway, and substitution of this site to alanine leads to nuclear accumulation of the protein, indicating that this phosphorylation is important for its subcellular localization. Ectopic expression of the S521 mutant imparts greater impairment to CML cell growth than the wild-type factor. Furthermore, we showed that Bach2 transcriptionally represses heme oxygenase-1, an antiapoptotic factor up-regulated in CML. Because CML cells are known to produce high levels of intracellular reactive oxygen species, overexpression of heme oxygenase-1 resulting from inhibition of Bach2 activity may contribute to their genomic instability and leukemic phenotype. (Blood. 2007;109:1211-1219)

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of the hematopoietic stem cell caused by a t(9;22)(q34;q11) translocation that generates the Philadelphia (Ph) chromosome. This genetic defect, also found in 20% to 30% of adult acute lymphoblastic leukemias, results in the expression of Bcr-Abl, a fusion oncoprotein with uncontrolled tyrosine kinase activity. Imatinib mesylate inhibits the Bcr-Abl tyrosine kinase, suppresses the proliferation of CML progenitor cells, and induces apoptosis of Ph-positive cell lines. It is a highly effective drug and has become the first-choice treatment of CML. However, resistance to the inhibitor emerges in some patients, especially in advanced phases of CML (reviewed by Yoshida and Melo1 and Deininger et al2).

Bcr-Abl phosphorylates several substrates that activate multiple signaling pathways, including Ras, signal transducer and activator of transcription-5 (STAT-5), extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), Janus kinase 2 (Jak-2), phosphatidylinositol-3 kinase (PI-3K), and others.3 This abnormal signaling leads to the malignant cellular phenotype of CML, including increased proliferation, reduction of adhesion to the bone marrow stroma and extracellular matrix, and inhibition of the apoptotic response to mutagenic stimuli.4 DNA damage can be caused by oxidative stress arising when reactive oxygen species (ROSs) are not adequately removed from cells.5 Interestingly, it has been suggested that ROSs are increased in CML cells.6,7 Because a persistent increase of ROSs can lead to accumulation of DNA mutations, it may induce genomic instability as a long-term consequence.9,10 Thus, BCR-ABL–positive cells can survive under levels of oxidative stress that normally induce an apoptotic response in nonleukemic cells. However, the factors and mechanisms responsible for resistance to ROS-induced cell death in CML are largely unknown. Understanding these mechanisms may lead to insights on CML transformation and its prevention or treatment.

Bach2 is a transcription factor containing a basic leucine zipper, a BTB (broad complex Tramtrack bric-a-brac) domain, and a C/EBP homology domain.11,12 In the hematopoietic lineage, it is expressed mainly in B cells.13 Bach2 binds to the MARE, which contains the AP-1 binding site. Furthermore, Bach2 is important for the antibody response of B cells treated with chemotherapeutic drugs.
that induce intracellular ROSs and nuclear accumulation of Bach2.\textsuperscript{19} Accordingly, it is possible that inhibition of Bach2 may contribute to reduction of the apoptotic response to oxidative stress in Ph-positive cells.

Despite its down-regulation, Bach2 is still detectable at the protein level in Ph-positive cells.\textsuperscript{10} Therefore, it is possible that Bcr-Abl regulates it not only at the expression level but also through posttranslational mechanisms that promote inhibition of the apoptotic response to mutagenic stimuli. In this study, we show that Bcr-Abl exerts this function by preventing Bach2 dephosphorylation and consequent translocation to the cell nucleus in response to oxidative stress. We further identified a possible target gene of this transcription factor, heme oxygenase-1 (\textit{HO}-1), which may be related to the antia apoptotic phenotype of CML cells and to imatinib resistance.

**Materials and methods**

**Plasmids and in vitro mutagenesis**

A 475 bp DNA fragment encoding human Bach2 was isolated by polymerase chain reaction (PCR) and subcloned into \textit{BamH}I and EcoRI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA), resulting in pcDNA3.1-Bach2. pcDNA3.1-HABach2 was constructed by insertion of annealed oligonucleotides 5'-AGCTTGCGGCCGCGCTAGCGCCGCCACCATGTACCCATACAGTGTCCAGATTCGCCGCTTCG-3' encoding the influenza virus hemagglutinin (HA) into the HindIII and \textit{BamH}I sites of pcDNA3.1-Bach2. pcDNA3.1-HABach2T519A, pcDNA3.1-HABach2S521A, and pcDNA3.1-HABach2T817A were constructed by site-directed mutagenesis and express Bach2 mutant proteins. pEGFP-Bach2 and pEGFP-Bach2S521A express GFP-Bach2 and GFP-Bach2S521A fusion protein, respectively. pBSBach2 vectors were constructed by insertion of a \textit{BamH}I-EcoRI fragment of pcDNA3.1-HABach2 into pBlueScriptKS+ vector (Stratagene, La Jolla, CA) followed by site-directed mutagenesis and cloning of the mutated \textit{BamH}I-EcoRI fragments into pcDNA3.1-HABach2. Mutagenesis primers were as follows: 5'-GGAGGACCAGGAGGGGCTTCCAGCTCGGTCGCTAC-3' for T519A (pcDNA3.1-HABach2T519A), 5'-GGAGGACCAGGAGGGGCTTCCAGCTCGGTCGTCAC-3' for S521A (pcDNA3.1-HABach2S521A), and 5'-GGAGGACCAGGAGGGGCTTCCAGCTCGGTCGTCAC-3' for T817A (pcDNA3.1-HABach2T817A). These mutations were confirmed by sequencing. pEGFP-Bach2 was constructed by insertion of a \textit{BamH}I and Xhol fragment of pcDNA3.1-Bach2 into the same sites of pEGFP-C1 (Promega, Madison, WI). MIGRBach2 and MIGRBach2S521A were constructed by insertion of a \textit{BamH}I (blunt ended)-EcoRI fragment of pcDNA3.1-HABach2 or pcDNA3.1-HABach2S521A into the Xhol (blunt ended)-EcoRI cloning sites of MIGR1. The retrovirus vector MIGR1 encoding IRES-GFP was kindly provided by Dr W. Pear (University of Pennsylvania, Philadelphia). MIGRBach2 and MIGRBach2S521A encode \textit{BACH2}-IRES-GFP and \textit{BACH2S521A}-IRES-GFP, respectively. pCLAmpho was described previously.\textsuperscript{20} pcDNA3.1S6K2T410D and pcDNA3.1S6K1T412D were kindly donated by Dr O. Pardo (Cancer Research UK, London) and pcK7/HA6S6K1E389D3E\textsuperscript{11} and pcDNA3HAS6K2ΔCCT\textsuperscript{22} by Dr J. Blenis (Harvard Medical School, Boston, MA). pcDNA3.1HABach2T817A was constructed by insertion of an Xhol (blunt ended)-EcoRI fragment of pcK7/HA6S6K1E389D3E into the \textit{BamH}I (blunt ended)-EcoRI sites of pcDNA3.1. pG7basic was from Promega. hHO4.9Luc, hHO4.9_M1Luc, hHO4.9_M2Luc, and hHO4.9_M1+M2Luc\textsuperscript{23} were gifts from Drs G. Kronke and N. Leitinger (Medical University of Vienna, Austria, and University of Virginia, Charlottesville). pEGFP encodes a \textit{Renilla} luciferase.\textsuperscript{13}

**Cell culture and transfection**

BV173, Ramos, Namalwa, and Jurkat human lymphoid cell lines were cultured in RPMI 1640 medium (Gibco/Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS), 200 mM L-glutamine, 5000 IU/mL penicillin, and 5000 \mu g/mL streptomycin. 3T3 and 293T cells were cultured in Dulbecco modified Eagle medium (DMEM) (Gibco/Life Technologies) supplemented as for human lymphoid cell lines. 3T3 cells expressing Bcr-Abl, HAS6K1E389D3E, S6K2T410D, or vehicle (pcDNA3.1) were established by transfection of these expression plasmids by calcium phosphate or FuGENE6 (Roche Diagnostics, Lewes, United Kingdom) according to the manufacturer’s protocol, followed by selection and maintenance on 600 ng/mL G-418 sulfate (Gibco/Life Technologies).

**Reagents**

Imatinib mesylate was kindly provided by Dr E. Buchdunger (Novartis Pharma, Basel, Switzerland). LY294002 was purchased from Promega, wortmannin and diethyl maleate (DEM) from Sigma (Poole, United Kingdom), rapamycin from Cell Signaling Technology (Beverly, MA), and alkaline phosphatase from Roche Diagnostics. Monoclonal (2G11) and polyclonal (F6-2) anti-Bach2 antibodies were described previously.\textsuperscript{13} The antiphosphotyrosine antibody (4G10) was a kind gift from Dr B. Druker (Oregon Health & Science University, Portland, OR). Anti-AKT (9272), phospho-AKT (Ser473) (9271), p70 S6 kinase (9202), phospho-p70 S6 kinase (Thr389) (9205), S6 ribosomal protein (2212), and phospho-S6 ribosomal protein (Ser235/236) (2211) antibodies were obtained from Cell Signaling Technology; anticent antibody (A-2066) from Sigma; and the anti–HO-1 antibody from Stressgen Bioreagents (SAP-896; Victoria, BC, Canada). Horseradish peroxidase (HRP) swine anti–rabbit Ig (P0217) and anti–rat Ig (NA935) antibodies were purchased from DakoCytomation (Ely, United Kingdom) and Amersham Biosciences (Little Chalfont, United Kingdom, respectively).

**Immunoblotting**

Cells were lysed in 1% Triton X-100, 20 mM Tris-HCl [pH 8.2], 150 mM NaCl, 1 mM sodium metavanadate, 1 mM phenylmethylsulfonylfluoride, and 100 mM sodium fluoride, and the protein lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to membranes, the proteins were immunoblotted with primary and HRP-conjugated secondary antibodies and detected by enhanced chemiluminescence (Amersham Biosciences). Densitometry analysis was performed using Scion image software (Scion, Frederick, MD).

**Transduction of cells and fluorescence-activated cell sorter (FACS) analysis**

The 293T cells were transfected with the retrovirus vectors and pCLAmpho by calcium phosphate; the viral supernatants were harvested after 2 days and concentrated \times 10 by centrifugation. BV173 cells were infected with the concentrated virus particles and 4 \mu g/mL Polybrene. The percentage of GFP-positive cells was measured by flow cytometry (FACS) analysis (Becton Dickinson, Franklin Lakes, NJ). The collected data were analyzed by CellQuest software (Becton Dickinson).

**Reporter assay**

Jurkat cells were transfected with the indicated plasmids and pEF-CP with FuGENE6 (Roche Diagnostics), and after 24 hours the reporter gene activity was determined using the dual luciferase reporter assay system (Promega) and normalized for transfection efficiency using the activity of \textit{Renilla} luciferase as the standard internal control.

**Fluorescence microscopy**

The 3T3 cells were transiently transfected with the indicated plasmid by calcium phosphate and trypanized from the plates 2 days later for seeding onto coverslips coated with 0.1% gelatin (Sigma). After 1 or 2 additional days, they were treated with various drugs when indicated and fixed with 4% paraformaldehyde (Sigma). The coverslips were mounted in DAPI (4% paraformaldehyde (Sigma). The coverslips were mounted in DAPI (4% paraformaldehyde (Sigma). The coverslips were mounted in DAPI (4% paraformaldehyde (Sigma). The coverslips were mounted in DAPI.
antibody as previously reported\cite{14} and examined under a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Tokyo, Japan).

## Results

### Bach2 is phosphorylated in BCR-ABL–positive cells in vivo

Immunoblotting of total cell lysate from Ramos, a human Burkitt lymphoma cell line, with anti-Bach2 antibody after treatment with alkaline phosphatase showed the appearance of a faster-migrating Bach2 band (Figure 1A). This demonstrates that Bach2 is phosphorylated in B cells in vivo and that the phosphorylated and unphosphorylated protein can be resolved via SDS-PAGE. To determine whether Bach2 was phosphorylated by Bcr-Abl, BV173, a CML lymphoid blast crisis cell line, was treated with imatinib. In this case, Bach2 was dephosphorylated in BV173 but not in Ramos cells (Figure 1B), indicating that Bach2 is phosphorylated in the presence of an active Bcr-Abl kinase in Ph-positive cells.

### Bach2 is phosphorylated via the PI-3K/S6K pathway

Because Bcr-Abl is a tyrosine kinase, we assessed whether Bach2 is phosphorylated on tyrosine residues. Immunoblotting of immunoprecipitated Bach2 protein from BV173 cells with antiphosphotyrosine antibody did not reveal any bands (data not shown), suggesting that Bach2 is phosphorylated on serine (S) or threonine (T) residues by kinases downstream of Bcr-Abl. To determine the signaling pathways involved in Bach2 phosphorylation, Ramos and BV173 cells were treated with inhibitors of MEK (PD98059, U0126), Jak2 (AG490), Ras (L-744832), Src (PD166326), p38MAPK (SB203580), and PI-3K (LY294002). Immunoblotting with anti-Bach2 antibody showed dephosphorylation of Bach2 in both BCR-ABL–positive and –negative cells only upon treatment with LY294002 but not with the other kinase inhibitors (Figure 1C and Figure S2, which is available on the Blood website; see the Supplemental Figures link at the top of the online article). These results indicated that Bach2 is phosphorylated via PI-3K signaling pathways. Mammalian target of rapamycin (mTOR) is a downstream effector of PI-3/Akt and is inhibited by rapamycin. However, treatment with this kinase inhibitor showed only partial Bach2 dephosphorylation in 3T3 cells (Figure 1D, lane 5) compared with nearly complete dephosphorylation caused by LY294002 (Figure 1E, lane 3). Similar data were obtained in Ramos. These results suggested that Bach2 can be phosphorylated by a common kinase downstream of PI-3K and mTOR. However, no dephosphorylation of Bach2 was observed in BV173 cells treated with rapamycin (data not shown). This suggests that in Ph-positive cells, sole inhibition of mTOR may not be enough to repress phosphorylation of Bach2 because the Bcr-Abl–driven signaling of PI-3K may bypass mTOR and constitutively activate kinases further downstream, such as PDK1.\cite{24,25}

The S6 kinases, S6K1 and S6K2, are known downstream substrates of Akt, PDK1, and mTOR. They each have 2 isoforms, p70\textsubscript{S6K1} and p85\textsubscript{S6K2} for S6K1 and p70\textsubscript{S6K1} and p54\textsubscript{S6K2} for S6K2, which are generated by alternative splicing.\cite{26,27,28,29} We therefore examined whether \textit{S6K}s could phosphorylate Bach2 by using cells expressing constitutively active forms of S6K1 and S6K2 that have S and/or T residues substituted by acidic amino acids (aa’s) to mimic S6K phosphorylation.\cite{30,31} 3T3 cells were transfected with Bach2 and constitutively active \textit{S6K1\textsubscript{aa2}} (S6K1E389D3E) expression plasmids and serum starved. This resulted in phosphorylation of Bach2

![Figure 1. Bach2 is phosphorylated via the PI-3K/S6K pathway in vivo.](image-url)

(A) Lysates of Ramos cells were exposed (+) or not (-) to 20 units of alkaline phosphatase (AP) for 30 minutes. (B) BV173 and Ramos cells were exposed (+) or not (-) to 1 \( \mu \text{M} \) imatinib (IM) for 24 hours. (C) Ramos cells were either treated (+) or not (-) with 25 \( \mu \text{M} \) LY294002 (LY) or DMSO for the indicated time. BV173 cells were either treated (+) or not (-) with 50 \( \mu \text{M} \) LY or DMSO for 24 hours. (D) 3T3 cells were transiently transfected with pcDNA3.1Bach2. After serum starvation for 6 hours, the cells were either treated (+) or not (-) with 50 \( \mu \text{M} \) LY or 80 nM rapamycin (Rap) for 30 minutes and then stimulated (+) or not (-) with serum for 30 minutes. (E-F) 3T3 cells were transiently transfected with pcDNA3.1haBach2 together with pRK7, pRK7/HAS6K1E389D3E, pcDNA3.1, or pcDNA3.1S6K2T401D. After serum starvation for 6 hours, the cells were either treated (+) or not (-) with 50 \( \mu \text{M} \) LY for 30 minutes and then stimulated (+) or not (-) with serum for 30 minutes. (A) Total cell lysates were separated on SDS-PAGE and immunoblotted with a Bach2 monoclonal antibody, phospho-Akt (S473) (P-Akt), Akt, phospho-p70S6K (T389), or p70S6K antibodies. Phosphorylated (P-Bach2) and unphosphorylated Bach2 are indicated by the arrows. In panel E, the Bach2 strip for lanes 1 to 4 was taken from a 20-minute autoradiograph exposure and, for lanes 5 to 8, from a 5-minute exposure to compensate for different strengths of the Bach2 signal resulting from different efficiencies of cotransfection with the empty vector (pRK7) or the S6-K1 construct (pRK7/HAS6K1E389D3E). All images are representative of at least 3 independent experiments.
Identification of Bach2 phosphorylation sites

Candidate S6K phosphorylation sites on Bach2 were identified by Scansite screening for the Akt (R-X-R-X-X-S/T-X) phosphorylation consensus sequence because this is similar to that of S6K(R-R-X-R-X-S-X), which was not available in the software. Three candidate aa’s, T519, S521, and T817, were thus identified, and mutagenesis of these sites was then carried out by their individual substitution to alanine (A).

Immunoblotting of cell lysates from 3T3 cells expressing each of the Bach2 mutants demonstrated that the Bach2S521A protein, but not the others, resolved on SDS-PAGE with no apparent phosphorylated band (Figure 2A). The aa sequence around S521 is consistent with the S6K consensus phosphorylation sequence, which is conserved between humans, mice, and rats (Figure 2B and data not shown). To confirm whether S521 is phosphorylated downstream of the PI-3K pathway, 3T3 cells expressing wild-type Bach2 or the S521A mutant were serum starved, treated with LY294002, and activated by serum addition. This elicited phosphorylation of wild-type but not mutant Bach2 under conditions of Akt activation (Figure 2C, lanes 4 and 8, respectively). Similar results were obtained when the cells were exposed to rapamycin (data not shown). To confirm whether S521 is phosphorylated under conditions of oxidative stress, the cells were treated with diethyl maleate (DEM), a glutathione-depleting agent that increases ROS concentration in cells. In BCR-ABL–negative cells, GFP-Bach2 was localized predominantly in the cytoplasm under conditions of oxidative stress, the cells were treated with imatinib (Figure 3A), suggesting that the Bcr-Abl kinase activity does not regulate the subcellular localization of Bach2.

The subcellular localization of Bach2 is regulated at the phosphorylation level

Because the subcellular localization of Bach2 is crucial for its activity, we examined whether this phenomenon is also regulated by Bcr-Abl. First, the intracellular localization of endogenous Bach2 in BV173 cells was assessed by indirect immunofluorescence with an anti-Bach2 polyclonal antibody. This showed that the little Bach2 expressed in these cells was localized predominantly in the cytoplasm and nucleus but translocated to the nucleus when the cells were treated with imatinib (Figure 3A), suggesting that the Bcr-Abl kinase activity does regulate the subcellular localization of Bach2.

However, because expression of Bach2 is induced by treatment with imatinib, it was difficult to discriminate between the effect of this treatment on nuclear translocation due to overexpression or inhibition of phosphorylation of the endogenous protein. Therefore, we established 3T3 cells stably expressing BCR-ABL or control and transiently transfected with a vector expressing Bach2 fused to GFP. The subcellular localization of the GFP-Bach2 protein was visualized and categorized as shown in Figure S_, and the results are presented as the percentage of cells in which GFP-Bach2 localizes predominantly in the cytoplasm (C>N). To evaluate whether Bcr-Abl could retain GFP-Bach2 in the cytoplasm under conditions of oxidative stress, the cells were treated with DEM, in agreement with previous reports. In contrast, in Bcr-Abl–expressing cells, GFP-Bach2 was partially retained in the cytoplasm even in the presence of DEM. This retention was reversed when the cells were treated with imatinib for inhibition of Bcr-Abl kinase activity. These data suggest that the subcellular localization of Bach2 is regulated by its phosphorylation downstream of Bcr-Abl.

The cells were next treated with LY294002 to test whether PI-3K mediates localization of Bach2: as shown in Figure 4B, inhibition of this kinase allowed translocation of GFP-Bach2 from the cytoplasm to the nucleus both in BCR-ABL–positive and BCR-ABL–negative cells.
negative cells. This phenomenon was confirmed in B cells (which express endogenous Bach2) treated with LY94002 or with wortmannin, another PI-3K inhibitor (Figure 3B). Moreover, ectopic expression of S6K1 (S6K1E389D3E) and S6K2 (S6K2T401D) in 3T3 cells ensured that GFP-Bach2 was retained in the cytoplasm even in the presence of LY294002 (Figure 4C). These experiments demonstrate that the subcellular localization of Bach2 is regulated by both PI-3K and S6Ks.

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Figure 3. The subcellular localization of Bach2 is regulated by the PI-3K/S6K pathway. Representative images of Bach2 (left panels), DNA (middle panels), and merged images (right panels) are shown for each experiment. (A) BV173 cells were either exposed or not (control) to 10 μM imatinib (IM) for 24 hours. (B) Namalwa cells were untreated (control) or treated with 100 μM LY294002 (LY) or 1 μM wortmannin for 2 hours. (A-B) Cells were fixed and immunostained with anti-Bach2 polyclonal antibody and Hoechst 33342, the coverslips were mounted in DAPI-containing Vectashield (Vector Labs, Burlingame, CA), and the slides were observed under a 40×/1.0 NA oil objective through a Zeiss LSM510 confocal laser scanning microscope. (C) 3T3 cells were transiently transfected with pEGFPBach2 or pEGFPBach2S521A. The coverslips were mounted in DAPI-containing Vectashield, and the slides were examined under a 40×/1.0 NA oil objective through an Olympus BX-41 fluorescence microscope (Olympus, Tokyo, Japan). Images were captured using an Orca AG digital camera (Hamamatsu Photonics UK, Hertfordshire, United Kingdom) and Smartcapture X software (Digital Scientific, Cambridge, United Kingdom) and were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Figure 4. The fraction of cells with cytoplasmic retention of Bach2 varies in response to PI-3K/S6K signaling and oxidative stress. 3T3 cells were transiently transfected with pEGFPBach2 plasmids, and the subcellular localization of GFP-Bach2 was observed through a fluorescence microscope. (A) Percentage of BCR-ABL–positive (+) or –negative (−) 3T3 cells with cytoplasmic GFP-Bach2 when exposed to 0.5 μM imatinib (IM) for 24 hours, as indicated, and to 300 μM DEM (−DEM) or not (−DEM) for the last 3 hours before fixation. (B) Percentage of BCR-ABL–positive (+) or –negative (−) 3T3 cells with cytoplasmic GFP-Bach2 exposed to 25 μM LY294002 (−LY) or DMSO (LY) for 2 hours. (C-D) Percentage of 3T3 cells expressing HAS6K1E389D3E, S6K2T401D, or vehicle (pRK7 or pcDNA3.1) with cytoplasmic GFP-Bach2 when treated with 5 μM LY294002 (−LY) or DMSO (LY) for 2 hours (D) or treated (−DEM) or not (+DEM) with 300 μM DEM for 3 hours (D). (E) Percentage of cells stably expressing Bcr-Abl (+) or vehicle (−) with cytoplasmic GFP-Bach2 (WT) or GFP-Bach2S521A (S521A) treated or not with 300 μM DEM (−DEM) for 3 hours before fixation. At least 100 cells expressing GFP-Bach2 were counted in each experiment. Results are average ± standard deviation of 3 independent experiments. Comparison between the indicated groups shows a significant difference (*) or no significant difference (NS) by the Mann-Whitney U test (P < .05).
We next examined whether the phosphorylation status of Bach2 could also influence its subcellular localization in response to oxidative stress. When cells expressing the active forms of S6Ks were treated with DEM, there was less nuclear translocation of Bach2 than in the control cells (Figure 4D). These data suggest that the mechanism by which Bcr-Abl forces the retention of Bach2 in the cytoplasm in the presence of oxidative stress (Figure 4A) is its constitutive activation of the PI-3/S6K pathway.  

**SS21 is important for the regulation of Bach2 subcellular localization**

To confirm that the S6K target phosphorylation site is important for regulating Bach2 traffic in the cell, we examined the subcellular localization of the GFP-Bach2SS21A mutant. The phosphorylation-defective protein was localized mainly in the nucleus in the absence of any treatment, in contrast to the wild-type Bach2, which was confined predominantly to the cytoplasm in both BCR-ABL–positive and –negative cells (Figures 3C and 4E). Furthermore, Bcr-Abl failed to retain the mutant in the cytoplasm when cells were treated with DEM, in contrast to the retention of the wild-type protein (Figure 4A). These data strongly indicate that the subcellular localization of Bach2 is regulated by the phosphorylation of SS21 and that this mechanism is activated by Bcr-Abl.

**The Bach2SS21A mutant induces poorer survival of CML cells than the wild-type protein**

Bach2 has been previously shown to negatively regulate proliferation and induce apoptosis of BCR-ABL–negative cells. To examine whether the same phenomena could be observed in CML cells, BV173 cells were transduced with retroviruses expressing Bach2/IRES-GFP, Bach2SS21A/IRES-GFP, or IRES-GFP (control) only, and the percentage of cells expressing GFP was measured every 2 days, starting 3 days after transduction (Figure 5). In control cells, this percentage remained roughly constant throughout the culture period. In contrast, the fraction of cells expressing Bach2 progressively decreased, and this was even more marked for cells carrying the Bach2SS21A mutant. This demonstrates that Bach2 can impart a growth disadvantage to CML cells and that this is at least partly mediated by its phosphorylation status. Altogether, the data indicate that the mutant Bach2 causes greater impairment to cell survival than the wild-type protein, because the Bcr-Abl–induced phosphorylation cascade cannot ensure its retention in the cytoplasm. The constitutive migration to the nucleus in the presence of a Bcr-Abl–induced high level of ROSs may lead to increased cell death.

**Bach2 regulates the HO-1 promoter**

Although Bach2 has been shown to function as a proapoptotic factor, the target genes that effect this function have not been previously identified. Interestingly, it was recently reported that Bcr-Abl transcriptionally activates HO-1 expression, which has an antiapoptotic function and contributes to survival of BCR-ABL–positive cells. The expression of HO-1 is up-regulated by Bcr-Abl at the transcriptional level and is inhibited by treatment with imatinib. It is known that the promoter region of human HO-1, which is also recognized as an enhancer region in the murine gene, contains MARE sites regulated by Bach1, a close homolog of Bach2. Taken together, it is possible that Bach2 could inhibit HO-1 expression by binding to MAREs in its promoter region. Thus, in Ph-positive cells treated with imatinib, induction and dephosphorylation of Bach2 allowing it to translocate to the nucleus would result in repression of HO-1 and promotion of apoptosis. To test whether Bach2 regulates HO-1 expression, we ectopically expressed it in 293T cells, which normally do not express Bach2. Immunoblotting of total cell lysates with anti–HO-1 antibody showed that expression of HO-1 was clearly inhibited by Bach2, compared with the pattern in control vehicle-vector transected cells (Figure 6A). Next, we determined whether Bach2 could transcriptionally inhibit HO-1 promoter activity by carrying out a reporter assay with a luciferase gene construct containing a 4.9 kb human HO-1 promoter region. This region contains 2 MARE sites, one of which is also referred to as a CRE site, homologous to 2 MARE sites previously identified in the murine HO-1 enhancer region. As shown in Figure 6B, Bach2 expression significantly repressed the HO-1 promoter-dependent reporter gene activity (lanes 3 and 4). Cotransfection of a promoterless reporter plasmid did not elicit any effect, indicating that the Bach2-induced luciferase repression was mediated via the HO-1 promoter (lanes 1 and 2). Furthermore, either of 2 luciferase constructs each containing a single mutant MARE (M1 or M2) showed less inhibition than the 4.9 kb wild-type promoter fragment (lanes 5 to 7), and there was no repression by Bach2 if the promoter possessing double mutants (M1+M2) or of a deleted construct containing only a 3.8 kb promoter region devoid of any MAREs (lanes 8 to 12). Transfection with Bcr-Abl alone was found to affect the basal activity of the HO-1 promoter (data not shown), as had been previously shown by Mayerhofer et al. For this reason, it was not possible to identify the role of Bach2, under the influence of Bcr-Abl, on the HO-1 promoter. These data demonstrate that Bach2 may inhibit HO-1 expression via the 2 MAREs in the HO-1 promoter, suggesting that HO-1 is a transcriptional target of Bach2.

**Discussion**

Oncoproteins use several molecular mechanisms to render a cell phenotypically abnormal and endowed with a growth advantage. In the case of Bcr-Abl, constitutive activation of the PI-3K pathway is fundamental for its antiapoptotic effect. This is exerted through up-regulation and/or activation of prosurvival molecules such as Bcl-2, Bcl-XL, and Bcl-2 and down-regulation/suppression of proapoptotic factors such as Bad and Bim. In the present study, we show that
the transcription factor Bach2 is another important target of the PI-3K pathway in BCR-ABL–positive cells and that its defective function may contribute to their failure in undergoing apoptosis in the presence of oxidative stress.

We have observed that Bach2 is phosphorylated via PI-3K in B-lymphoid cells and that this can be reversed by treatment with LY294002. In Ph-positive lymphoid cells, excessive signaling via PI-3K leads to Bach2 existing predominantly in its phosphorylated form, with dephosphorylation being accomplished by inhibition of PI-3K. Because S6K1 has been shown to be activated in constitutively activated in phosphorylation of Bach2. Because S6K1 has been shown to be activated in constitutively activated S6Ks led to marked ectopic expression of constitutively activated S6Ks led to marked exacerbation of cell growth and are also regulated by MAPK/ERK.45-47 In CML cells, inhibition of Bach2 is crucial for regulating its phosphorylation status. Taken together, the data suggest that the activity of Bach2 may be tightly regulated by the PI-3K pathway during B-cell differentiation. In CML cells, inhibition of Bach2 could lead to a differentiation arrest in transformed B cells, but further studies are needed to clarify the issue.

Under oxidative stress, cells respond by activating signaling pathways that will determine their fate (ie, survival or apoptosis). In B cells, it is known that the Syk phosphotyrosine kinase activates the PI-3K survival pathway and enhances cellular resistance to oxidative stress-induced apoptosis. On the other hand, Syk also activates apoptosis through its downstream effector PLC-γ2.54 This observation suggests that cells try to survive in response to relatively low levels of oxidative stress but undergo apoptosis if the stimulus and induced damage are too strong to sustain survival. This is supported by recent observations that low levels of oxidative stress lead to activation of Akt, but higher doses may induce obstruction of the pathway.55 Although it has been shown that ROS-induced phosphorylation of S6Ks activate of these kinases may also follow the pattern described for Akt in response to higher levels of oxidative stress, because they are downstream of...
Akt. Therefore, in conditions of mild oxidative stress, Bach2 is phosphorylated by activated PI-3K/S6K and retained in the cytoplasm, allowing the cells to repair the damage and survive. Conversely, when cells find themselves under intense oxidative stress, Bach2 may be dephosphorylated through inhibition of the Akt/S6K pathway and translocates to the nucleus by disruption of Crm1-dependent nuclear export.12 The ROS-induced relocation of Bach2 to the nucleus may be only partially dependent on the S6K pathway and is likely to be also under concurrent control of the CLS and other mechanisms. In CML cells, constitutive phosphorylation of Bach2 induced by Bcr-Abl via Akt/S6Ks, as well as overall reduced expression of this transcription factor,16 may be major mechanisms by which they are resistant to oxidative stress. However, we cannot exclude at this time the role of mechanisms such as elevated expression of Bcl-XL, enhanced DNA repair, and overall reduced expression of this transcription factor,16,58 may be one of the important regulators that serve to bridge the gap between ROSs and HO-1.

The effector transcriptional targets of Bach2 involved in triggering the apoptotic process under conditions of oxidative stress are unknown. Here we show that HO-1 is one of the genes transcriptionally repressed by Bach2 and is likely to be involved in this process. Expression of HO-1 is regulated by several transcription factors, such as NF-E2–related factor 2 (Nrf2) and Bach1, both binding to the MAREs on its promoter.57,60 Nrf2 activates but Bach1 inhibits its expression, suggesting that MARE is an important binding element that is tightly regulated for transcription of HO-1. This is supported in our study and that of others by the fact that disruption of the 2 MAREs led to lower HO-1 promoter activity than that of the wild-type promoter, as shown in Figure 6B. HO-1 was originally identified as an enzyme that catalyzes oxidative degradation of heme to form biliverdin, carbon monoxide, and free ion. Its expression is induced by stress-inducing stimuli such as hypoxia, heavy metals, UV radiation, reactive nitric oxide, and ROSs.61 There is recent evidence that HO-1 also has cytoprotective and antiapoptotic functions against oxidative injury.61 Expression of HO-1 is high in various malignancies, and it has an antiapoptotic role in tumor growth.62-64 In CML, it is up-regulated by Bcr-Abl, and hemin-induced overexpression leads to in vitro resistance to imatinib.65 Because Bach2 is induced by treatment with imatinib in CML cells, this is likely to suppress the expression of HO-1, disabling its antiapoptotic function. Interestingly, in a recent microarray-based gene expression profiling of myeloma cells resistant to arsenic trioxide, a producer of ROSs, HO-1 was found as one of the most up-regulated and Bach2 the most down-regulated gene.65 These observations suggest that Bach2 may be one of the important regulators that serve to bridge the gap between ROSs and HO-1.

In summary, Bcr-Abl inhibits the nuclear translocation of Bach2 through phosphorylation of SS21 via the PI-3K/S6K pathway and therefore suppresses Bach2 proapoptotic function. Altogether, the data presented here combined with work by others are consistent with a model in which Bach2 is a crucial mediator of the response to mutagenic stimuli (Figure 7). Cells such as CML lymphoid blasts harboring too little and constitutively phosphorylated Bach2 are unable to trigger the necessary apoptotic response under conditions of oxidative stress, and this may allow the accumulation of harmful mutations that contribute to transformation of the leukemia.

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**Authorship**

Contribution: C.Y. performed experiments and wrote the draft manuscript; F.Y., D.E.S., S.M.H., D.I., and A.M. performed experiments; S.B. and K.L. provided advice on the design of the study, provided vital new reagents, and commented on the manuscript; and J.V.M. conceived and designed the study, supervised its execution, helped write the paper, and had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Bcr-Abl signaling through the PI-3/S6 kinase pathway inhibits nuclear translocation of the transcription factor Bach2, which represses the antiapoptotic factor heme oxygenase-1

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