Hematopoietic Malignancies Demonstrate Loss-of-Function Mutations of BAX


The BCL-2 gene family regulates the susceptibility to apoptotic cell death in many cell types during embryonic development and normal tissue homeostasis. Deregulated expression of anti-apoptotic BCL-2 can be a primary aberration that promotes malignancy and also confers resistance to chemotherapeutic agents. Recently, studies of Bax-deficient mice have indicated that the pro-apoptotic BAX molecule can function as a tumor suppressor. Consequently, we examined human hematopoietic malignancies and found that approximately 21% of lines possessed mutations in BAX, perhaps most commonly in the acute lymphoblastic leukemia subset.

Approximately half were nucleotide insertions or deletions within a deoxyguanosine (G8) tract, resulting in a proximal frame shift and loss of immunodetectable BAX protein. Other BAX mutants bore single amino acid substitutions within BH1 or BH3 domains, demonstrated altered patterns of protein dimerization, and had lost death-promoting activity. Thus, mutations in the pro-apoptotic molecule BAX that confer resistance to apoptosis are also found in malignancies.

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product. PCR amplification using primer pair C, D results in a 246-bp domain. Positions of PCR primers (A through D) are indicated. PCR amplification using primer pair A, B results in a 377-bp frame. Exons are numbered. BH1, BH2, and BH3 and transmembrane domains are shaded. Positions of PCR primers (A through D) are indicated. PCR amplification using primer pair A, B results in a 377-bp product. PCR amplification using primer pair C, D results in a 246-bp product.

**Fig 1.** BCL-2 homology (BH) domains within BCL-2 family members. (A) Schematic representation of BCL-2, BCL-XL, BAK, and BAX and the relative positions of the BH1, BH2, BH3, and BH4 domains and transmembrane region. (B) Schematic overview of BAX open reading frame. Exons are numbered. BH1, BH2, and BH3 and transmembrane domains are shaded. Positions of PCR primers (A through D) are indicated. PCR amplification using primer pair A, B results in a 377-bp product. PCR amplification using primer pair C, D results in a 246-bp product.

**RESULTS**

Substitution mutations. Twenty-nine cell lines derived from hematopoietic malignancies of various cell types were analyzed for substitutions in *BAX* by SSCP and sequence analysis. We found evidence for substitutions in 7 cell lines (Table 1). The Burkitt lymphoma (BL) cell line Daudi expressed a mutant BAX allele that contained a G108V mutation and a wild-type allele. This mutation substitutes the central glycine within the BH1 domain (Fig 1). The plasmacytoma (PC) cell line OPM1 expressed a mutant allele with a G11E mutation and a wild-type allele. This mutation substitutes an aspartic acid residue with glycine and disrupts a salt bridge in the BH1 domain. The acute lymphoblastic leukemia (ALL) cell line BCR-ALL expressed a G67R mutant allele as well as a wild-type allele. This mutation substitutes the central glycine within the BH1 domain, whereas the BH3 domain (Fig 1). The plasmacytoma (PC) cell line OPM1 expressed a mutant allele with a G11E mutation and a wild-type allele. This mutation substitutes an aspartic acid residue with glycine and disrupts a salt bridge in the BH1 domain. The acute lymphoblastic leukemia (ALL) cell line BCR-ALL expressed a G67R mutant allele as well as a wild-type allele. This mutation substitutes the central glycine within the BH1 domain, whereas the BH3 domain (Fig 1)...

**Transfection and Western blots.** Twenty micrograms of DNA of Xba-I linearized pSFFV-LTR neo vector expressing HA-BAX, HA-BAX<sup>G67R</sup>, or HA-BAX<sup>G108V</sup> was transfected into FL5.12 cells or cotransfected with 1 µg of linearized PGK-hygro vector DNA into FL5.12-BCL-2 cells. Stably transfected clones were selected for neomycin resistance (2 mg/mL G418) or hygromycin resistance (1 mg/mL). Transfected clones were analyzed for HA-BAX, HA-BAX<sup>G67R</sup>, or HA-BAX<sup>G108V</sup> expression by Western blot using polyclonal antiserum N20 (Santa Cruz, Santa Cruz, CA; 1:500) as primary antibody and goat-antirabbit HRPO (Caltag Labs; 1:2,000) as the secondary antibody. BCL-2 expression was detected by the 6C8 MoAb (1:250) and a secondary goat-antihamster HRPO antibody (Caltag Labs; 1:2,000). Immunoblots were developed by enhanced chemiluminescence (ECL; Amersham).

** Yeast two-hybrid analysis.** cDNAs cloned in pBTM116 or pACTII were cotransformed into yeast strain L40 (MATa trp1-901 leu2-3, 112 ade2 his3-D200 lys2::(lexA0p)-His3 ura3::(lexA)6-LacZ) using the lithium-acetate method. After transformation, yeast cells were plated on selective media (His<sup>b</sup>, Ura<sup>a</sup>, Leu<sup>b</sup>, and Trp<sup>b</sup>) and incubated at 30°C. After 2 to 4 days, yeast colonies were transferred to nitrocellulose filters and incubated for 1 minute in liquid N<sub>2</sub>. Filters were dried on Whatmann paper (Whatmann, Maidstone, UK) and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) substrate solution (60 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 40 mmol/L KCl, 1 mmol/L MgSO<sub>4</sub>, 50 mmol/L β-mercaptoethanol, 1 mg/mL X-Gal, pH 7.0) for 1 to 12 hours at 37°C.

**Alternatives.**

**Frameshift mutations.** SSCP analysis also noted a distinct, altered pattern common to four cell lines (KM3, CEM, Jurkat, and JM<sup>+</sup>; Table 1). DNA sequence analysis showed the same single nucleotide deletion (G<sub>7</sub>) in a simple tract of 8 deoxyguanosine residues (G<sub>7</sub>J14-121) encompassing codons 38 to 41 of human BAX (Table 1). This deletion was consistently detected in 3 or more independently amplified RT-PCR products from each cell. DNA sequence of four or five independent clones from these RT-PCR products demonstrate the same (G<sub>7</sub>) deletion. SSCP and DNA sequence analysis of the pre-B-cell
The presence of mutations in cell lines of hematopoietic malignancies as determined by SSCP and sequence analysis.

Abbreviations: BL, Burkitt’s lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; HD, Hodgkin’s disease; FL, follicular lymphoma; MM, multiple myeloma; PC, plasma cell leukemia; T-ALL, T-cell acute lymphoblastic leukemia; CML, chronic myeloid leukemia; M2 subtype.

The T-cell ALL line Jurkat and its derivative JM both demonstrated the G(9) insertion, whereas the others displayed an additional alteration on SSCP that, upon DNA sequencing, proved to be a single nucleotide insertion (G) 9 in guanine stretch (nt 114-121) on second allele. In Jurkat and JM cells (Table 1) and the presence of two mutated BAX alleles.

**Protein dimerization.** The BAX(67R) and BAX(108V) mutants found in HPB-ALL and Daudi reside in the conserved BH3 and BH1 domains, respectively, and consequently tested for dimerization capacity with BCL-2 family members by means of yeast two-hybrid analysis (Table 2). Although the binding of BAX(67R) to wild-type BAX appeared unaffected, it demonstrated enhanced dimerization with itself, BAX(67R). In contrast, the capacity to heterodimerize with BCL-2 or BCL-XL was lost. BAX(108V) did not form homodimers with itself, BAX(108V), but bound to wild-type BAX. BAX(108V) demonstrated enhanced binding to BCL-2 and BCL-XL. Thus, both mutations demonstrated altered, yet very distinct dimerization characteristics.

We next examined whether these BAX mutants demonstrated altered dimerization within mammalian cells. Stably transfected FL5.12-BCL-2 clones expressing comparable amounts of hemagglutinin epitope-tagged (HA) molecules HA-BAX, HA-BAX(67R), or HA-BAX(108V) were generated (Fig 2). When BCL-2 was immunoprecipitated from lysates, it coprecipitated HA-BAX and the endogenous BAX (Fig 2A, lane 1). BAX(108V) but not HA-BAX(67R) was also coprecipitated with BCL-2, confirming the interactions suggested in yeast two-hybrid (Fig 2A, lanes 2 and 3). A consistently higher ratio of HA-BAX(108V)/endogenous BAX compared with HA-BAX/ endogenous BAX in BCL-2 immunoprecipitates is consistent with the enhanced binding of BAX(108V) noted by yeast two-hybrid analysis (Table 2). In the reciprocal experiment, HA-BAX, HA-BAX(67R), and HA-BAX(108V) were immunoprecipitated with anti-HA MoAb 12CA5 and all heterodimerized to endogenous BAX. As before, HA-BAX and HA-BAX(108V) but not HA-BAX(67R) coprecipitated with BCL-2, confirming the pattern of interactions within mammalian cells (Fig 2).

**Functional analysis of BAX mutants.** To address the functional consequence of the BH1 and BH3 mutations, they were assessed in an interleukin-3 (IL-3) deprivation assay using the FL5.12 line. Clones of FL5.12 (Neo) or FL5.12-BCL-2 expressing comparable amounts of HA-BAX, HA-BAX(67R), or HA-BAX(108V) were identified by immunoblots (Fig 3A, B, and D). Addition of wild-type HA-BAX but not HA-BAX(67R) or HA-BAX(108V) was capable of promoting cell death in FL5.12-

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**Table 1. Summary of Mutation Analysis**

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<th>Cell Line</th>
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<th>Sequence</th>
<th>Protein</th>
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The presence of mutations in cell lines of hematopoietic malignancies as determined by SSCP and sequence analysis. The T-cell ALL line Jurkat and its derivative JM both displayed an additional alteration on SSCP that, upon DNA sequencing, proved to be a single nucleotide insertion (G)9 in guanine stretch (nt 114-121) on second allele. Results are based on at least two independent SSCP or DNA sequence analyses.

**Table 2. Yeast Two-Hybrid Interactions**

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<th>Interaction</th>
<th>pACTII</th>
<th>BAX</th>
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<th>G108V</th>
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<td>G67R</td>
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<tr>
<td>G108V</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>++</td>
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<td>BCL-XL</td>
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Yeast two-hybrid analysis of dimerization capacity of wild-type BAX versus BAX(67R) and BAX(108V). AD-fusion constructs are indicated across the top lane: control vector pACTII, BAX/C19, BAX(67R)/C18, BAX(108V)/C18, BCL-2/C22, BCL-XL/C19. DB-fusion constructs are indicated in the left hand column: BAX/C18, BAX(67R)/C18, BAX(108V)/C18, BCL-XL/C19. Protein interactions as measured by X-gal substrate conversion are indicated as ‘’–’’ for no interaction or ‘’+’’ for clear positive. Interactions that were enhanced are indicated as ‘’++’’ or ‘’+++’’. Interactions were tested in two to four independent experiments.
BCL-2 cells that were protected by BCL-2 (Fig 3C). Similarly, HA-BAX G67A and HA-BAX G108V did not substantially alter the survival of native FL5.12 cells, whereas wild-type HA-BAX clearly enhanced apoptosis (Fig 3E).

DISCUSSION

Prolonged cell survival with resistance to apoptosis can be a primary oncogenic event. Transgenic mice bearing a Bcl-2-Ig minigene that recapitulated the t(14;18) found in human follicular lymphoma display B-cell hyperplasia that progresses to high-grade lymphoma.33 Evidence is emerging that a principal contribution from the loss of p53 function is the elimination of a death pathway.34,35 Recent evidence suggests that BAX, a pro-apoptotic member of the BCL-2 family, can also qualify as a tumor suppressor. Bax-deficient mice display cellular expansions of neurons, lymphocytes, ovarian granulosa cells, and spermatogonia, reflecting the survival of cells that avoided developmental death.20 TGT121 transgenic mice that express a truncated T antigen that inhibits Rb but leaves p53 intact displayed an accelerated progression to malignancy upon a Bax-deficient background.25 It is of note that heterozygous Bax (+/-) mice also displayed an earlier onset of malignancy, suggesting that alteration of a single BAX allele could be of functional significance. An increase in focus formation was also documented in Bax-deficient versus wild-type fibroblasts when transfected with RAS and E1A.26 These experimental models argue that Bax can also be considered a tumor suppressor and that loss of this pro-apoptotic molecule promotes tumorigenesis.

We found mutations in BAX in approximately 21% (6 of 28 independent lines with confirmation of the mutation in Jurkat in its derivative JM) of human hematopoietic malignancy lines. No BAX alterations were noted in 35 normal individuals or in 8 Epstein-Barr virus–transformed lymphoblastoid lines, indicating that the observed alterations of BAX are not common polymorphisms or associated with immortalization. Approximately half of the mutations were frame-shifts confined to a single mononucleotide (G) tract (nt114-121). The existence of both insertions (G) and deletions (G) within the same leukemia (Jurkat and JM) favors a biallelic aberration and argues that the elimination of BAX is a selective advantage. Lack of detectable BAX protein in other leukemias with (G) deletions is compatible with a homozygous abnormality or perhaps loss of the second allele. Recently, Rampino et al28 described the presence of frameshift mutations in the identical (G) tract of BAX in about 50% of human colon adenocarcinomas with the microsatellite mutator phenotype (MMP). Some sporadic cancers and almost all cancers associated with the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) accumulate mutations in microsatellites of nucleotide repeats due to defects in human DNA mismatch repair genes, including hMSH2, hMLH1, hPMS1, and hPMS2.36 Msh-2–deficient mice progress to a precursor T-cell lymphoblastic lymphoma.37 Moreover, mutations have been noted in hMSH2 within human lymphoblastic lymphoma37 and in hMLH1 in a panel of lymphoid leukemia cell lines.38 This includes the CEM cell line,38 which demonstrated a frameshift mutation of BAX in this study (Table 1). In total, these studies indicate that a subset of lymphoblastic leukemia/lymphoma have a mutator phenotype and that BAX may represent one target.

In addition to the frameshift mutations, we found missense mutations, including BAXG67R in the BH3 and BAXG108V in the BH1 domain. Both mutations demonstrated abnormal dimerization characteristics but were nearly opposite in their patterns. This finding argues that the death agonist activity of BAX may not strictly correlate with the capacity to form any single set of homodimer or heterodimer pairs. Molecular modeling of the BAX BH3 a2-helix showed a classic amphipathic a helix. The G67R mutation would introduce a charged residue onto the hydrophobic face of this helix (Fig 4). NMR analysis of wild-type and mutant peptides of the BH3 a2-helix
of BAK indicated critical interactions with BCL-X\(_L\) through both hydrophobic and electrostatic interactions.\(^{39}\) The substitution of the central glycine in this \(\alpha\)-2-helix to arginine noted here has a much greater affect than an alanine substitution analyzed for BAK.\(^{39}\) The impact of the G67R mutation in BAX provides additional evidence that BH3 domains are critical for pro-apoptotic molecules.

The G108V substitution in the BH1 \(\alpha\)-5-helix of BAX also resulted in the loss of pro-apoptotic activity. While eliminating mutant/mutant dimerization, it, if anything, enhanced het-

![Fig 3. BAX\(^{G67R}\) and BAX\(^{G108V}\) have lost cell death-promoting activity. (A) Western blot analysis using anti-BAX polyclonal antiserum N20 on lysates of FL5.12-BCL-2 cells (Hygro) or HA-BAX\(_{\alpha}\), HA-BAX\(^{G67R}\), or HA-BAX\(^{G108V}\) stably transfected clones. (B) Western blot analysis of the same lysates as in (A) using anti-human BCL-2 MoAb 6C8. (C) Viability assay. Transfected clones described in (A) were deprived of IL-3 and viability was determined by trypan blue exclusion at 0, 0.5, 1, 2, 3, 4, and 5 days after IL-3 withdrawal and plotted as the mean percentage of survival \(\pm\) SEM. (D) Western blot analysis using anti-Bax polyclonal antiserum N20 on lysates of FL5.12 cells (Neo) or HA-BAX, HA-BAX\(^{G67R}\), or HA-BAX\(^{G108V}\) stably transfected clones. (E) Viability assay. Transfected clones described in (D) were deprived of IL-3 and viability was determined by trypan blue exclusion at 0, 12, 16, 20, 24, 36, and 48 hours after IL-3 deprivation and plotted as the mean percentage of survival \(\pm\) SEM.

![Fig 4. Three-dimensional structure of BH3 region of BAX. Views of a modeled surface of the BH3 amphipathic \(\alpha\) helix of BAX, calculated and displayed using GRASP. The G67R substitution as occurs in HPB-ALL is displayed at right. The surface is colored deep blue (23KBT) in the most negative, with linear interpolation for values inbetween. The model was generated using the protein building module (BUILDER) of INSIGHT II (Biosym, San Diego, CA) and minimized using DISCOVER, the forefield simulation mode.]
erodimerization with BCL-2 or BCL-X<sub>L</sub>. Previous substitution of this central glycine to alanine in BCL-2 (G145A) and in BCL-X<sub>L</sub> (G159A) eliminated both their heterodimerization with BAX and their anti-apoptotic activity. However, the comparable substitution in BAX (G108A) had no effect (unpublished observations). Thus, the strong effect of the G108V substitution was somewhat unexpected. The G108 residue resides in the long hydrophobic α5-helix felt to be part of the transmembrane helical cores responsible for the ion channel activity of BCL-X<sub>L</sub> and in similar approaches also for BAX. This provides an alternative role for this residue beyond protein interaction.

Oncogenes that promote proliferation contribute to cancer through gain-of-function alterations, whereas growth-inhibitory tumor-suppressor genes contribute principally through loss-of-function mutations. The gain-of-function alteration of the BCL-2-Ig translocation overexpressed the anti-apoptotic molecule BCL-2 in follicular lymphoma. This suggested that pro-apoptotic molecules could contribute to oncogenesis by loss-of-function mutations. The discovery of BAX mutations in a subset of colon carcinomas and in hematopoietic malignancies hereby provide such evidence. This adds evidence in human tumors to the prospective experiments using Bax-deficient mice. The loss of BAX function would confer resistance to programmed cell death within hematopoietic cells and could contribute to malignancy in several ways. Extended cell survival and resistance to apoptosis would enable cells to withstand additional genetic alterations. In this context, loss of BAX function could be a primary oncogenic aberration for which the Bax-deficient mice provide evidence. BAX mutations could also contribute to tumor progression or the establishment of cell lines. Finally, chemotherapy could have selected for the loss of BAX as BAX deficiency would confer chemoresistance.

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