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Functional Cooperation of the Proapoptotic Bcl2 Family Proteins Bmf and Bim In Vivo

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Bmf is a proapoptotic BH3-only member of the Bcl2-related protein family that is implicated in cell death caused by anoikis (23, 26, 27), arsenic trioxide (19), histone deacetylase inhibitors (33, 34), transforming growth factor β (24), and tumor necrosis factor alpha (8). Mice with a loss of Bmf expression exhibit B-cell hyperplasia and increased sensitivity to γ-radiation-induced B-cell lymphoma (14). These observations indicate that Bmf represents an important mediator of cell death signaling pathways.

The structure of Bmf includes a BH3 domain that is essential for apoptosis induction. In addition, Bmf contains a sequence motif that is required for interactions with dynein light chain 2 (DLC2), a component of the myosin V motor complex (23). The interaction of Bmf with DLC2 is required for the recruitment of Bmf to the cytoskeleton. The release of Bmf from complexes sequestered on the cytoskeleton may contribute to anoikis (23). Interestingly, this regulatory mechanism is shared by the related proapoptotic BH3-only protein Bim, which interacts via a similar sequence motif with dynein light chain 1 (DLC1), a component of the dynein motor complex (22).

The similarities between Bmf and Bim include the presence of a conserved phosphorylation site (Bmf Ser74 and Bim Thr112) that is a substrate for the c-Jun NH2-terminal kinase (JNK) (15). Data from biochemical studies indicate that the JNK-mediated phosphorylation of Bmf and Bim may increase apoptotic activity (15). Indeed, mice with a germ line point mutation in the Bmf gene (Thr112 replaced with Ala) exhibit decreased apoptosis (10). These studies indicate that Bmf and Bim may mediate, in part, proapoptotic signaling by JNK (3, 30).

Bmf represents an important mediator of cell death signaling. The purpose of this study was to examine the role of Bmf using mouse models with germ line defects in the Bmf gene, including mice with Bmf alleles that disrupt Bmf expression, prevent Bmf phosphorylation, or mimic Bmf phosphorylation.

MATERIALS AND METHODS

Mice. Bmfl−/− mice (1) and wild-type C57BL/6J mice were obtained from the Jackson Laboratories. We described Bmfl+/−/+, Bmfl−/−/−, Bmf−/−/−, and BmfnA5N/A5 mice on the C57BL/6J strain background previously (10). Mice with Bmf gene mutations were constructed by using standard methods. Mouse strain 129/SvEv (C57BL/6J) was used for all experiments. For all experiments, sex was matched and 2-μM ganciclovir (Syntex) and 2-μM ganciclovir (Syntex) were used as negative controls. When used, the genotypes of the mice were determined by Southern blot analysis using a random-primed Bmf cDNA probe.

Genotype analysis. The genotype at the Bmf locus was examined by Southern blot analysis using EcoRI-restricted genomic DNA by probing with a random-primed probe labeled probe (405 bp) that was isolated by PCR using a genomic bacterial artificial chromosomal clone as the template and the amplifiers 5′-TAGCTGTTTATCCTCCCAAC-3′ and 5′-GGCTGAAGAACCCGTGATG3′. The genotype was determined by using a PCR-based assay. The wild-type (306-bp) and knockout (402-bp) alleles were detected by PCR analysis of genomic DNA using the amplifiers 5′-AAGTAGAAAAACCCCTGACACCTTACC-3′, 5′-CCAACCTTTTATCATCGCCTG3′, and 5′-TGAGATGTTGGATTGCTGCA-3′. The wild-type (306-bp) and Ser74 point mutant...
Bmf alleles were detected by PCR amplification of genomic DNA with primers 5'-H11032-AAGTAGAAACCCCTGACACCTTACC-3' and 5'-H11032-CCAACCTTTATCATTTGCCCAGTC-3'. The genotype at Bmf codon 74 was also examined (see Fig. 3E) by PCR using amplimers that span exon 3 (5'-ATGGAGCCACCTCAGTGTGTGGAGG and 5'-AGTCTCTGGGGTTCCTCTGTCAC) and restriction digestion (NaeI and BamHI) to obtain a 284-bp fragment (wild type) or a 222-bp fragment (NaeI for BmfS74A and BamHI for BmfS74D).

Tissue culture. CD4 and CD8 T cells were purified from spleen and lymph nodes by negative selection by depleting cells expressing major histocompatibility complex class II, NK1.1, CD11b, and CD8 (for CD4 T-cell purification) or CD4 (for CD8 T-cell purification) (4, 5). The cells were cultured for 4 days in the presence of culture medium containing 5% fetal bovine serum. The number of viable cells was measured by trypan blue staining.

Flow cytometry. Cells (1 x 10^6) were incubated (30 min at 4°C) with R-phycocerythrin-conjugated anti-CD4 (L3T4), allophycocyanin-conjugated anti-CD8α (Ly-2), and fluorescein isothiocyanate-conjugated anti-B220 antibodies (Pharmingen); washed with phosphate-buffered saline plus 2% bovine serum albumin; fixed in phosphate-buffered saline plus 2% formaldehyde; and examined by flow cytometry.

RESULTS

Generation of mice with germ line mutations in the Bmf gene. We designed a targeting vector to disrupt the Bmf gene by the replacement of exon 3 and exon 4 (which encodes the BH3 domain) with a Neo' cassette (Fig. 1A). This vector was electroporated into ES cells, and clones with the Bmf gene correctly targeted by homologous recombination were identified by Southern blot analysis (Fig. 1B) and confirmed by PCR analysis (Fig. 1C). These ES cells were employed to create chimeric mice that were bred to obtain germ line transmission of the disrupted Bmf allele. The Bmf−/− mice were backcrossed to the C57BL/6J strain background (10 generations). Intercrosses of Bmf−/− mice generated wild-type, heterozygous, and homozygous knockout littermates in the expected Mendelian ratios. No obvious developmental defects in the male Bmf−/− mice were detected, although the spleen was moderately enlarged. This finding is consistent with data from a previous report that established a requirement of Bmf for B-cell homeostasis (14).

Failure of vaginal introitus development in Bmf−/− mice. Examination of female Bmf−/− mice demonstrated defects in uterovaginal development, including an imperforate vagina and hydrometrocolpos (Fig. 2). This phenotype was observed for 22% of female Bmf−/− mice and was not detected in female Bmf+/− mice. Previous studies demonstrated that the development of the vaginal introitus requires apoptosis of the vaginal mucosa (25) and that defects in apoptosis can result in an imperforate vagina (16, 25).
Role of JNK-mediated phosphorylation of Bmf. The apoptotic activity of Bmf may be regulated by phosphorylation. Indeed, the phosphorylation of Bmf on Ser74 was previously proposed to increase apoptotic activity (15). The phosphorylation of Bmf on this site is mediated by the JNK protein kinase (15). To test the role of Bmf phosphorylation, we constructed mice with germ line point mutations in the Bmf gene.

We designed a targeting vector to introduce a point mutation at the Bmf phosphorylation site at Ser74 (replacement with Ala or Asp) together with a floxed Neo' cassette in intron 3 (Fig. 3). ES cells with the correctly targeted Bmf alleles were identified by Southern blot analysis and confirmed by PCR analysis (Fig. 3). The floxed Neo' cassette was excised with Cre recombinase. Three ES cell clones with a LoxP site inserted into intron 3 of the Bmf gene were selected for the creation of chimeric mice (Bmf+/S74A, Bmf+/S74D, and Bmf+/WT). Germ line transmission of the mutated Bmf alleles was obtained, and the mice were backcrossed to the C57BL/6J strain background (10 generations). Intercrosses of the heterozygous mice generated wild-type, heterozygous, and homozygous mutant litters in the expected Mendelian ratios. No defects in uterovaginal development were detected in the homozygous female mutant mice (Bmf S74A/S74A, Bmf S74D/S74A, and Bmf S74D/WT). This observation indicates that phosphorylation on Ser74 may not be essential for normal Bmf activity.

Compound mutation of the Bmf and Bim genes. The Bmf protein is structurally similar to Bim. Both proteins contain a BH3 domain, a binding site that mediates interactions with cytoskeletal motor proteins, and sites of phosphorylation by JNK (15, 22, 23). These similarities indicate that compensation by Bim may contribute to the phenotype of Bmf mutant mice. We therefore made compound mutant mice with defects in both Bmf and Bim.

The Bmf and Bim genes are linked on mouse chromosome 2 (Fig. 4A). Progeny derived from crosses of Bim−/+ Bmf−/+ mice with Bmf−/− mice were screened for the genotype Bim−/+ Bmf−/−. Mice with the correct genotype were obtained with an efficiency of approximately 0.4%. These mice were crossed with a wild-type mouse to obtain Bim−/+ Bmf−/+ mice that have one wild-type chromosome 2 and one mutant chromosome 2. Intercrosses of these mice generated progeny with the genotypes Bim−/− Bmf−/−, Bim−/+ Bmf−/−, and Bim−/+ Bmf−/+ (Fig. 4B). Genotype analysis confirmed the presence of compound mutant Bim−− Bmf−/− mice (Fig. 4C). The number of double-knockout mice was reduced compared with the expected Mendelian inheritance (Fig. 4B), and these mice were smaller than wild-type and Bim−− Bmf−/+ littermates (Fig. 4E).

Examination of viable compound mutant Bim−− Bmf−/− mice demonstrated the persistence of interdigital tissue (Fig. 4D). Interdigital webs were observed on both the front and rear paws of all Bim−− Bmf−/− mice. No interdigital webbing was observed for Bmf−/− mice, Bim−/+ mice, or Bim−/+ Bmf−/+ mice. This finding suggests that Bim and Bmf serve partially redundant functions during paw development. Interdigital webs, like those found in Bim−− Bmf−/− mice (Fig. 4D), were also observed for Bax−− Bim−/+ mice (11) and Bax−− Bak−− mice (16).

We examined interdigital webbing in mice with other combinations of Bim and Bmf gene mutations, including mice lacking the sites of Jnk phosphorylation on both Bmf and Bim (BimT112A/T112A BmfS74A/S74A) and mice with an acidic mutation at the site of Bmf phosphorylation by JNK (Bim−− BmfS74D/S74D). No interdigital webbing was detected for these mice. This observation suggests that the persistence of interdigital webs requires the complete loss of function of both Bmf and Bim. This contrasts with the observed defects in uterovaginal development caused by a Bmf deficiency that were similar for Bmf−/− mice and compound mutant Bim−− Bmf−/− mice.

Bmf and Bim are required for normal lymphocyte homeostasis. The proapoptotic genes Bmf and Bim are implicated in the homeostatic maintenance of lymphocytes (14, 28). We therefore examined splenocytes from mice with Bmf and Bim gene defects (Fig. 5 and 6). We found a large increase in the number of B cells, CD4 T cells, and CD8 T cells in the spleen of...
Bim−/− mice, but Bmf−/− mice exhibited only a moderate increase in the number of B cells and CD8 T cells. However, no significant change in the number of splenocytes was detected in studies of mice with phosphorylation-defective Bmf proteins (BmfS74A/S74A and BmfS74D/S74D). Similarly, no increase in the number of splenocytes was detected for mice with defects in the sites of Bim phosphorylation by the extracellular signal-regulated kinase group of mitogen-activated protein kinases (BimEL/EL and BimS4A/S4A) (10). In contrast, a moderate increase in the number of B cells, CD4 T cells, and CD8 T cells was detected for mice with a defect in Bim phosphorylation by JNK (BimT112A/T112A) (10).

To test whether a mutation of the JNK phosphorylation site on Bmf (Ser74) might alter lymphocyte homeostasis in the...
In the context of altered Bim function, we examined splenocytes of mice with compound mutations in Bim and Bmf (Fig. 5 and 6). The combined loss of JNK phosphorylation of both Bim and Bmf (BimT112A/T112A BmfS74A/S74A mice) caused no further increase in the number of B cells or T cells beyond that detected for mice with defects in JNK phosphorylation of Bim alone (BimT112A/T112A mice). This observation suggested that Bmf phosphorylation on Ser 74 is not essential for Bmf function.

The phosphorylation of Bmf on Ser 74 may increase Bmf activity (15). However, a phosphomimetic mutation (replacement of Ser 74 with Asp) did not significantly change (P > 0.05) the number of splenocytes in BmfS74D/S74D mice (Fig. 5). If the phosphomimetic mutation caused only a moderate increase in Bmf function, we reasoned that this might be detected in mice with a loss of Bim function. Indeed, the number of B cells and T cells in the spleen of Bim+/− Bmf−/− mice was significantly decreased (P < 0.05) compared with that in the spleen of Bim−/− mice (Fig. 5 and 6). These data indicate that a
phosphomimetic mutation at Ser74 on Bmf can partially suppress the effects of a Bim deficiency, consistent with the conclusion that the phosphorylation of Bmf on Ser74 may cause a moderate increase in levels of Bmf apoptotic activity.

The mechanism that accounts for the phosphorylation-dependent increase in Bmf activity is unclear. However, it was previously established that Bmf interacts with the myosin V motor complex that may recruit Bmf to the cytoskeleton (23). The phosphorylation of Bmf on Ser74 disrupts the interaction of Bmf with DLC2, a component of the myosin V complex (15). The release of Bmf from the cytoskeleton may therefore contribute to an increased level of apoptotic activity caused by Bmf phosphorylation on Ser74.

**Functional cooperation of Bmf and Bim.** Comparison of the splenocytes from wild-type mice, Bmf+/−/− mice, Bim+/−/− mice, and Bim+/−/ Bmf+/−/− mice demonstrated that the combined loss of both Bmf and Bim caused a larger increase in cell number than did the loss of Bim or Bmf alone (Fig. 5 and 6). Flow cytometry demonstrated that Bim+/−/ Bmf+/−/− mice have increased numbers of B cells, CD4 T cells, and CD8 T cells in the spleen compared with wild-type mice (Fig. 6A). Similarly, Bim+/−/ Bmf+/−/− mice have increased numbers of CD4-positive, CD8-positive, and CD4/CD8 double-positive thymocytes compared with wild-type mice (Fig. 7). These observations suggest that Bmf and Bim may function cooperatively to maintain lymphocyte homeostasis.

The effect of Bim and Bmf gene ablation to change the relative number of B cells, CD4 T cells, and CD8 T cells in the spleen (Fig. 6) differs from the effect of the transgenic expression of BcI2 in hematopoietic cells that increases the total number of splenocytes (21). Furthermore, the increased percentage of CD4/CD8 double-positive thymocytes caused by Bim and Bmf gene mutation (Fig. 7) differs from the effects of transgenic BcI2 expression to increase the percentage of mature CD4 and CD8 T cells and decrease the percentage of immature CD4/CD8 double-positive T cells in the thymus (21). These observations indicate that Bim and Bmf exert stage-specific and cell type-dependent effects on lymphocyte homeostasis.

**FIG. 6.** Bim and Bmf deficiency causes an increase in the number of B cells and T cells in the spleen. (A and B) Spleen cells were examined by flow cytometry to identify B cells and T cells using antibodies to cell surface B220, CD4, and CD8 (means ± standard deviations). Statistically significant differences between wild-type and mutant mice are indicated (*, P > 0.05).
**DISCUSSION**

The mechanism of proapoptotic signaling by BH3-only proteins is not fully understood. However, it is established that Bim is able to interact with antiapoptotic members of the Bcl2 family (including Bcl2 and Bcl-xl) and displace the proapoptotic effector proteins Bax and Bak (31). Bim can also interact directly with Bax to induce apoptosis (6). Both mechanisms (interaction with antiapoptotic Bcl2/Bcl-xl and proapoptotic Bax) may contribute to Bim-induced cell death (18). It (interaction with antiapoptotic Bcl2/Bcl-xl and proapoptotic Bax) may contribute to Bim-induced cell death in vivo (18). These observations suggest that Bim and Bmf may cooperate to regulate the death of CD8 T cells.

**FIG. 7.** Effect of compound mutation of Bim and Bmf on thymocytes. Thymocytes isolated from wild-type and Bim<sup>-/-</sup> Bmf<sup>-/-</sup> mice were examined by flow cytometry using antibodies to cell surface CD4 and CD8 (means ± standard deviations; n = 6). Statistically significant differences between wild-type and mutant cells are indicated (*, P < 0.05).

**FIG. 8.** Bmf and Bim deficiency causes reduced T-cell apoptosis. Purified CD4 and CD8 T cells (1 × 10<sup>6</sup> cells) were incubated in medium (4 days), and the number of viable cells was counted (means ± standard deviations; n = 5). Statistically significant differences between wild-type and mutant cells are indicated (*, P < 0.05).
treatment with cytotoxic drugs (19, 29). Here we demonstrate that Bmf and Bim cooperate during apoptosis induction in vivo. This is illustrated by the finding of persistent interdigital webbing in Bim−/−Bmf−/− mice but not in Bim−/−Bmf−/−, Bmf−/− mice, or wild-type mice. Moreover, the sphenomegaly present in Bim−/−Bmf−/− mice was significantly greater than that in Bim−/− mice, Bmf−/− mice, or wild-type mice. Together, these data demonstrate functional cooperation between the proapoptotic proteins Bim and Bmf in vivo.

In conclusion, the results of our analysis demonstrate that Bmf and Bim exhibit partially redundant functions, that phosphorylation on Ser76 is not essential for Bmf activity, and that phosphorylation on Ser78 can contribute to increased Bmf activity in vivo.

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