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Et al.
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). 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 on the c-Jun NH2-terminal kinase (JNK) on Ser74, or mimic Bmf phosphorylation on Ser74. We report that the loss of Bmf causes defects in uterovaginal development, including an imperforate vagina and hydrometrocolpos. We also show that the phosphorylation of Bmf on Ser74 can contribute to a moderate increase in levels of Bmf activity. Studies of compound mutants with the related gene Bim demonstrated that Bim and Bmf exhibit partially redundant functions in vivo. Thus, developmental ablation of interdigital webbing on mouse paws and normal lymphocyte homeostasis require the cooperative activity of Bim and Bmf.

Materials and Methods

Mice. Bmf−/− mice (1) and wild-type C57BL/6j mice were obtained from the Jackson Laboratories. We described BmfΔELΔE3, BmfT112A/T112A, and Bmf3SA/3SA mice on the C57BL/6j strain background previously (10). Mice with Bmf gene mutations were constructed by using standard methods. Mouse strain 129/SvEv genomic bacterial artificial chromosome clones of the Bmf gene were isolated by hybridization analysis using a random-primed Bmf cDNA probe. Targeting vectors designed to disrupt the Bmf gene (Fig. 1A) or to introduce point mutations at Ser74 (replacement with Ala or Asp) (see Fig. 3A) were constructed with a floxed Neo cassette for positive selection and a thymidine kinase cassette for negative selection by using standard techniques. Embryonic stem (ES) cells were electroporated with these vectors and selected with 200 μg/ml G418 (Invitrogen) and 2 μM ganciclovir (Syntex). ES cell clones identified by Southern blot analysis were injected into C57BL/6j blastocysts to create chimeric mice that transmitted the mutated Bmf alleles through the germ line. The floxed Neo cassette was excised by using Cre recombinase. The mice were backcrossed to the C57BL/6j strain (Jackson Laboratories) for 10 generations. Homozygous Bmf mutant mice were obtained by crossing heterozygous Bmf mutant animals. The mice were housed in a facility accredited by the American Association for Laboratory Animal Care. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Genotype analysis. The genotype at the Bmf locus was examined by Southern blot analysis of EcoRI-restricted genomic DNA by probing with a randomly primed 32P-labeled probe (405 bp) that was isolated by PCR using a genomic bacterial artificial chromosome clone as the template and the amplifiers 5′-TACAGAGCCTGTAACCCAC-3′ and 5′-CGCAAGGAAATGTCCTACCTATGG-3′. The genotype was also determined by using a PCR-based assay. The wild-type (306-bp) and knockout (402-bp) Bmf alleles were detected by PCR analysis of genomic DNA using the amplifiers 5′-AAGTAGAAGAAACTGAGACACCTTACC-3′, 5′-CAGACCTTTATCATGCGCCAGTCT-3′, and 5′-TGAGATTGGAATGAGGCGGAG-3′. The wild-type (306-bp) and Ser74 point mutant

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Bmf alleles were detected by PCR amplification of genomic DNA with primers 5'-H11032-AAGTAGAAACCCCTGACACCTTACC-3' and 5'-H11032-CCAACCTTTATCATTGCCCAGTC-3'. The Bmf genotype at 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).
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, BmfS74D/S74D, and Bmf+/-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+/-. Bim-/- mice, or Bim+/-. Bmf+/-. 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<sup>−/−</sup> mice, but Bmf<sup>−/−</sup> 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 (Bmf<sup>S74A/S74A</sup> and Bmf<sup>S74D/S74D</sup>). 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 (Bim<sup>3SA/3SA</sup> and Bim<sup>EL/EL</sup>) (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 (Bim<sup>T112A/T112A</sup>) (10).

To test whether a mutation of the JNK phosphorylation site on Bmf (Ser<sup>74</sup>) might alter lymphocyte homeostasis 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 Ser74 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−/− BmfS74D/S74D 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 Ser$^{74}$ on Bmf can partially suppress the effects of a Bim deficiency, consistent with the conclusion that the phosphorylation of Bmf on Ser$^{74}$ 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 Ser$^{74}$ 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 Ser$^{74}$.

**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 Bcl2 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 Bcl2 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.
Bim and Bmf regulate survival of CD4 and CD8 T cells. To test the relative effects of Bim and Bmf on cell survival, we examined the effect of Bim and Bmf gene disruption during in vitro culture of T cells (Fig. 8). CD4 T cells were dependent primarily upon the Bim deficiency for survival during culture in vitro (Fig. 8). In contrast, CD8 T cells were partially protected by a deficiency of either Bim or Bmf (Fig. 8). Studies of compound mutant Bim−/− Bmf−/− CD8 T cells demonstrated increased viability in vitro compared with that of Bmf−/− CD8 T cells or Bim−/− CD8 T cells (P < 0.05). These observations suggest that Bim and Bmf may cooperate to regulate the death of CD8 T cells.

**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 effectors 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 in vivo (18). It is likely that Bmf may cause cell death by similar mechanisms.

The major phenotype that we detected in Bmf−/− mice was a defect in uterovaginal development, including an imperforate vagina and hydrometrocolpos. Vaginal introitus formation requires the apoptosis of the vaginal mucosa (25). Defects in apoptosis caused by the ectopic expression of the antiapoptotic protein Bcl2 (25) or the combined loss of expression of the proapoptotic proteins Bax and Bak (16) also caused an imperforate vagina. Similarly, the persistence of interdigital webbing observed for Bim−/− Bmf−/− mice (Fig. 3) was found in Bax−/− Bak−/− mice (16). These data suggest that the effect of Bmf on apoptosis may be mediated by Bax/Bak and negatively regulated by Bcl2 in vivo. It is interesting that the imperforate vagina and interdigital webbing phenotypes have not been described for Apaf-1−/− mice (2, 9, 32) or Casp9−/− mice (7, 13). This finding suggests that the cytochrome c pathway may not fully account for the proapoptotic actions of Bmf. One possibility is that this form of Bmf-induced apoptosis requires functional contributions from other mitochondrial proapoptotic molecules (e.g., AIF, Smac, and Endo G).

The JNK signaling pathway is implicated in cell death (3, 30). Targets of the JNK pathway include members of the Bcl2 protein family, including the antiapoptotic protein Mcl-1 (20) and the proapoptotic proteins Bmf and Bim (10, 15). JNK can trigger the rapid ubiquitin-mediated degradation of Mcl-1, leading to an increased sensitivity to stress-induced apoptosis (20). In contrast, JNK phosphorylation of Bmf and Bim was previously proposed to increase apoptotic activity (15). Indeed, germ line mutation of the Bim gene (replacement of the phosphorylation site at Thr112 with Ala) causes reduced apoptosis in vivo (10). Here we demonstrate that a mutation of the site of JNK phosphorylation on Bmf (Ser74 replaced with Ala) causes reduced apoptosis in vivo (10). We conclude that a mutation of the site of JNK phosphorylation on Bmf (Ser74 replaced with Ala) does not cause apoptotic phenotypes that resemble the effect of a Bmf deficiency, including an imperforate vagina and in a Bmf-deficient genetic background the persistence of interdigital webbing, or splenomegaly. These data demonstrate that the Bmf phosphorylation site at Ser74 is not essential for normal Bmf activity. Nevertheless, a phosphomimetic mutation at Ser74 was able to partially suppress splenomegaly caused by a Bim deficiency. We conclude that the phosphorylation of Bmf on Ser74 causes a moderate increase in levels of Bmf apoptotic activity in vivo.

A major conclusion of this study is that the related genes Bim and Bmf have partially redundant functions. This conclusion is consistent with previous studies that suggested cooperative actions of Bim and Bmf during cell death. Thus, it was suggested that lumen development in the breast epithelium may depend on the proapoptotic activity of both Bim (17) and Bmf (26). Similarly, both Bmf and Bim are implicated in cell death caused by infection with Neisseria gonorrhoeae (12) or...
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−/− Bmf−/− 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 Ser74 is not essential for Bmf activity, and that phosphorylation on Ser74 can contribute to increased Bmf activity in vivo.

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REFERENCES


