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Regulation of Bim by TCR Signals in CD4/CD8 Double-Positive Thymocytes

Anna Bunin,* Fatima W. Khwaja,† and Gilbert J. Kersh²*

Bim, a BH3-only Bcl-2 family member, is required for apoptosis of thymocytes in response to negative selection signals. Regulation of the apoptotic activity of Bim during negative selection is not understood. In this study we demonstrate that in murine thymocytes undergoing apoptosis in response to anti-CD3ε injection, levels of Bim protein expression do not change. In immature thymocytes, Bim is associated with mitochondria before stimulation and is not regulated by a change in subcellular localization during apoptosis. We also show that BimL is rapidly phosphorylated in thymocytes in response to CD3ε cross-linking both in vivo and in vitro, and that phosphorylation is sustained for at least 24 h. Analysis of MHC-deficient mice shows that phosphorylation of Bim occurs in CD4/CD8 double-positive thymocytes and does not depend on activation of mature T cells. We also find that TCR cross-linking on thymocytes induces an increase in the proportion of Bcl-xL bound to Bim at late time points. Our results favor a model in which strong TCR signals regulate the apoptotic activity of Bim by phosphorylation and subsequent changes in binding to Bcl-xL in immature thymocytes. The Journal of Immunology, 2005, 175: 1532–1539.

Developing T cells that are CD4/CD8 double positive (DP) or in the transition between DP and CD4 or CD8 single positive (SP) will undergo apoptosis if they receive a strong signal through the αβ TCR (1–3). Strong signals are delivered to thymocytes when the αβ TCR recognizes self peptide/MHC complexes with relatively high affinity. Thus, negative selection is one mechanism by which autoreactive T cells are eliminated (4). Failure to delete autoreactive cells in the thymus results in widespread autoimmunity (5, 6). In mature T cells, strong signals through the TCR can lead to cytokine production and proliferation, whereas the same stimulus in a DP thymocyte results in apoptosis. The mechanism by which TCR signals cause death in DP cells is poorly understood (7). Proximal TCR signals are required, but downstream of the initial phosphorylation events at the receptor much less is known.

A molecule that clearly plays a role in both thymocyte negative selection and death of peripheral T cells is Bim. Bim is a proapoptotic molecule that is related to the antiapoptotic protein Bcl-2 by the presence of a single BH3 (Bcl-2 homology) domain (8). Bim is part of a family of BH3-only proteins that promote apoptosis. Bim is thought to function by binding to Bcl-2 and/or Bcl-xL on the mitochondrial membrane and inhibiting their antiapoptotic function (8, 9). Three isoforms of Bim exist that result from alternative splicing (8). These are BimL, BimS, and BimL, BimS is the most abundant isoform in thymocytes and T cells, whereas BimS is almost undetectable (10, 11). DP thymocytes in Bim-deficient mice do not undergo apoptosis in response to TCR signals, and Bim-deficient animals develop multiorgan autoimmunity (9, 12). Bim is also essential for the deletion of CD4+CD24− thymocytes in response to TCR ligation (13). Thus, Bim is required for normal negative selection.

The mechanism by which TCR signals regulate Bim function is not known. However, Bim is expressed in numerous cell types, and several modes of regulation have been described for Bim. These include induction of its expression (14–18), translocation to mitochondria after release from the dynein motor complex (19), and phosphorylation (20–23). Although some studies have observed increases in Bim expression in thymocytes and T cells in response to TCR signals, a separate study using stimulation of murine peripheral T cells by superantigen in vivo found that Bim expression did not change after stimulation even though the superantigen stimulation in vivo led to Bim-dependent apoptosis (24). Whether other modes of Bim regulation come into play in thymocytes has not been reported, but in the superantigen model using peripheral T cells, Bim did not translocate to mitochondria (10). Thus, it is not clear how Bim is regulated in peripheral T cells or thymocytes.

In the present study we evaluated these potential mechanisms of Bim regulation in DP thymocytes by generating a strong signal through the TCR using anti-CD3ε mAb stimulation. We found that Bim protein levels do not change in response to TCR cross-linking, and Bim also does not translocate from the cytoskeleton to the mitochondria in response to TCR signaling. We did find that most of the Bim in thymocytes is associated with mitochondria in the absence of TCR signals, and that it is rapidly phosphorylated in response to TCR cross-linking. Phospho-Bim is maintained for at least 24 h after TCR cross-linking. Also at later time points (24 h), we found that TCR cross-linking increases the proportion of Bim bound to Bcl-xL. To control for glucocorticoid-mediated effects that may result from anti-CD3ε treatment in vivo, we also show that neither Bim phosphorylation nor Bim association with Bcl-xL is enhanced by dexamethasone treatment. Thus, our data are consistent with a model in which Bim apoptotic activity is regulated by phosphorylation, followed by increased Bim association with Bcl-xL at the time of apoptosis.
Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute. Mice with a genetic deletion of the β2-microglobulin gene (25) and mice that have all conventional MHC class II coding sequences deleted (26) were purchased from The Jackson Laboratory. The β2-microglobulin-deficient and MHC class II-deficient mice were bred together to generate MHC−/− mice. All procedures were approved by the Emory University institutional animal care and use committee.

Antibodies

Western blots were probed with mouse anti-Bcl-xL, hamster anti-TCRα (BD Pharmingen), rabbit anti-Bim (Sigma-Aldrich and Stressgen), mouse 60-kDa heat shock protein Ab (Stressgen), rabbit anti-Bcl-2 (N-19), mouse anti-HDAC-1 (H-11), rabbit anti-Bax (N-20; Santa Cruz Biotechnology), mouse anti-α-tubulin (Sigma-Aldrich), mouse anti-voltage-dependent anion channel-1 (Calbiochem), and rabbit anti-ERK1/2 (Promega). Immune complexes were revealed with HRP-conjugated goat anti-rabbit Fc-specific and sheep anti-mouse Fc-specific Abs at 1/10,000 (Jackson Immunoresearch Laboratories) and ECL reagents (Amersham Biosciences).

In vitro and in vivo TCR stimulation of thymocytes

Six-well plates were coated with 10 μg/ml goat anti-Armenian hamster IgG (Jackson Immunoresearch Laboratories), followed by PBS or 10 μg/ml hamster anti-CD3ε (2C11-145). Thymocytes were harvested from C57BL/6 mice or MHC-deficient mice (6–8 wk of age), transferred onto Ab-coated plates in serum-free medium, and centrifuged at 394 × g for 1 min. Plates were then placed at 37°C for 30 min. Cells remaining in suspension were removed, whereas adherent cells were lysed in 1% Triton X-100 lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM phenylmethylsulfate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate. Lysates were incubated on ice for 15 min and centrifuged at 16,000 × g, and supernatants were used for Western blot analysis.

Subcellular fractionation of thymocytes

Thymocytes (2.8 × 107) from C57BL/6 mice were harvested and subjected to fractionation essentially as described previously (10), except that extraction buffer III and benzonaze from the Subcellular Proteome Extraction kit (Calbiochem) were used to separate nuclear and cytoskeletal components.

Purification of mitochondria

Thymocytes (~7 × 106 cells/condition), were washed in TD buffer (133 mM NaCl, 5 mM KCl, 25 mM Tris-HCl (pH 7.5), and 0.7 mM NaH2PO4). Pellets were resuspended in RSB buffer (10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate) to give 10 times the original volume of the pellet. Cells were allowed to swell for 10 min before being disrupted with a Dounce homogenizer ( Kontes). After disruption, 2.5 × MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl (pH 7.5), and 5 mM EDTA) was added to make 1 × MS buffer and mixed gently. Homogenates were centrifuged three times at 1200 × g at 4°C, and the pellets were discarded each time. Resulting supernatants were centrifuged at 20,000 × g for 15 min at 4°C. Postnuclear, postmitochondrial supernatants were stored on ice until analysis. Pellets (heavy membrane fraction) were resuspended in sucrose-TE buffer (20% sucrose, 50 mM Tris-HCl (pH 7.5), and 10 mM EDTA) and layered on top of a sucrose step gradient containing 1.5 M sucrose, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA (bottom layer) and 1.0 M sucrose, 10 mM Tris-HCl, and 5 mM EDTA (pH 7.5; top layer). Step gradients were centrifuged at 100,000 × g for 1 h at 4°C. The interface between the top and bottom sucrose layers was collected using a Pasteur pipette. Volumes were made up to 1 ml with sucrose TE buffer, and samples were centrifuged at 20,000 × g for 15 min at 4°C. Mitochondrial pellets were resuspended in lysis buffer or directly in SDS sample buffer.

Alkaline treatment of membranes

The effect of alkaline extraction on heavy membranes isolated from postnuclear supernatants from C57BL/6 thymocytes (~3 × 106 cells/condition) was determined essentially as described previously (27).

Immunoprecipitation

Thymocytes were lysed at a concentration of 3 × 106 cells/ml in 0.2% Nonidet P-40 lysis buffer. Lysates were incubated on ice for 15 min, centri

trifuged at 16,000 × g for 5 min at 4°C, and precleared with protein A beads (Sigma-Aldrich). Bim was immunoprecipitated from lysates with 3 μg of rabbit anti-Bim Ab (Stressgen). Control lysates were incubated with 3 μg of normal rabbit IgG (Santa Cruz Biotechnology). Abhysates mixtures were gently rocked at 4°C for 1 h. Protein A beads (50 μl of a 50% (v/v) slurry) were added to each sample and rocked at 4°C for an additional hour. Protein A beads were washed with lysis buffer three times, then washed with PBS and transferred to fresh tubes; bound proteins were eluted with nonreducing 4 × SDS sample buffer by boiling for 5 min and submitted to Western blot analysis.

Acid phosphatase treatment

Protein A-rabbit anti-Bim immune complexes were prepared and washed three times in 1% Nonidet P-40 lysis buffer without phosphatase inhibitors. Lysates were made from stimulated or unstimulated thymocytes, incubated on ice for 15 min, and cleared of insoluble material by centrifuging for 5 min at 16,000 × g; Rabbit anti-Bim-protein A complexes were added to the lysates, and the mixtures were rocked at 4°C for 30 min. Immune complexes were then washed three times with 50 mM Pipes and 1 mM DTT (pH 6.0), resuspended in 100 μl of this buffer, and incubated for 10 min at 30°C. Acid potato phosphatase (Sigma-Aldrich) was then added to appropriate samples (10 μl/sample). Samples were incubated for 10 min at 30°C and for 1 min at 37°C, washed three times with 50 mM Pipes and 1 mM DTT (pH 6.0), resuspended in nonreducing SDS sample buffer, boiled, and analyzed by Western blot.

Two-dimensional (2D) gel electrophoresis

Bim was immunoprecipitated from ~7 × 106 thymocytes as described above. Protein A beads were washed with water three times, and pellets were dried in a Speed-Vac (Savant) with heat for 15 min. Proteins were eluted from protein A beads using 1 ml of isoelectric focusing lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 100 mM DTT, and 1/100 dilution of protease and phosphatase inhibitor mixtures; Sigma-Aldrich). The immunoprecipitated samples were concentrated to a final volume of 100 μl using Centricon-YM3 columns (Millipore). The first dimension was performed on an IPGphar system from Amersham Pharmacia using Immobiline dry strips (pH 3–10; 13 cm; Amersham Biosciences). For analytical gels, immobilized pH gradient dry strips were rehydrated overnight with 100 μl of total protein mixed with destreak rehydration buffer (8 M urea, 4% CHAPS, 100 mM DTT, 100 mM protease inhibitor, and 0.25% amphydles; Amersham Biosciences) to a final volume of 250 μl. Next, pre-focusing to remove ionic components of the samples was performed over 6 h by increasing the voltage linearly from 150 to 5,000 V. The prefocusing step was followed by focusing of proteins to a total of 100,000 Vh. Immobilized pH gradient strips were then equilibrated using equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris base (pH 8.8), and 20% glycerol), first containing 10 mg/ml DTT and then 25 mg/ml iodoacetamide for 30 min each, to reduce and alkylate the focused proteins. Once equilibrated, the gels were separated on the second dimension on 10% polyacrylamide gels with 2% SDS using the Protean II XL system from Bio-Rad. The gels were transferred to nitrocellulose membrane using the Criterion system from Bio-Rad and were analyzed by Western blot for Bim.

Results

The model system

To evaluate biochemical events in thymocytes responding to strong TCR signals, it is necessary to simultaneously activate the majority of thymocytes. In most of our experiments, we have done this by treating thymocytes with anti-CD3ε Ab both in vivo and in vitro. Two potential problems that can occur when using this approach are the activation of peripheral T cells and SP thymocytes. Activation of peripheral T cells results in the secretion of large amounts of cytokines. These cytokines do not directly kill DP thy

mocytes, but induce an increase in levels of endogenous glucocorticoids that is lethal to the DP cells (28) (A. Bunin and G. J. Kersh, manuscript in preparation). In light of this problem, in many of the experiments we compared the results obtained using anti-CD3ε treatment in vivo to those obtained using treatment with anti-CD3ε in vitro and treatment with dexamethasone in vivo. Activation of SP thymocytes can also complicate the results, because biochemical events observed in whole thymocytes responding to anti-CD3ε may be taking place primarily in the SP population. Therefore,
many of our experiments were performed using MHC-deficient mice that do not have any mature SP thymocytes, thus limiting the responding population to immature DP cells.

**Levels of Bim do not change in response to TCR cross-linking on thymocytes**

The analysis of Bim protein expression in several cell types has demonstrated that some cells express Bim at low levels, but in response to an apoptotic stimulus, Bim expression is markedly induced (14–16). However, in other cases Bim levels are unchanged after apoptotic stimulus (10, 24). To evaluate the possibility that Bim is regulated by a change in expression level in thymocytes after TCR cross-linking, we injected PBS or anti-CD3ε Ab into wild-type mice i.v.; harvested thymocytes at 6, 12, and 24 h after the injection; and analyzed thymocyte lysates by Western blot. As shown in Fig. 1A, Bim expression in wild-type thymocytes did not change in response to a strong signal through the TCR. By injecting anti-CD3ε Ab i.v., we consistently observed evidence of TCR signaling in thymocytes as early as 20 min after injection (data not shown) and evidence of apoptosis as early as 12 h after injection (Fig. 1B). In this experiment there are probably signals derived from anti-CD3ε acting on the DP cells directly as well as glucocorticoid-derived signals, but this combination of signals does not alter the total levels of Bim in the 24 h after stimulation.

*In thymocytes, Bim is not released from the microtubules after stimulation*

In various cell lines, Bim has been shown to be sequestered at the cytoskeleton (19). The release of Bim from the cytoskeleton has been shown to enable its proapoptotic activity. To determine whether translocation from the dynein motor complex to the mitochondria played a role in thymocyte apoptosis, we isolated cytosolic, membrane, nuclear, and cytoskeletal fractions from thymocytes 24 h after i.v. injection of anti-CD3ε or PBS and determined the relative amount of Bim in each fraction (Fig. 2). We found that nearly all Bim protein can be detected in fractions containing intracellular membranes (membrane and nuclear fractions) both before and after anti-CD3ε treatment. Only a small subset of total Bim is associated with the cytoskeleton in thymocytes, and Bim bound to the cytoskeleton does not get released after anti-CD3ε injection. In fact, we consistently observed a slight increase in Bim associated with the cytoskeleton after anti-CD3ε injection (Fig. 2). It is clear, however, that Bim translocation from cytoskeleton to mitochondria does not play a role in its regulation in thymocytes.

The proapoptotic activity of Bim is thought to be dependent on its interaction with antiapoptotic Bcl-2 and Bcl-xL at the mitochondria (8). Although we did not find evidence that Bim translocated from the cytoskeleton to the mitochondria in thymocytes, the possibility still remained that Bim might translocate to the mitochondria from other intracellular membranes after anti-CD3ε injection. To address this, we purified mitochondria from thymocytes isolated from mice injected with PBS or anti-CD3ε. Lysates of purified mitochondria, and fractions containing the remaining non-nuclear intracellular membranes were examined by Western blot for Bim expression. As expected, purified mitochondria contained abundant 60-kDa heat shock protein, a protein localized in the mitochondrial matrix (Fig. 3) (29). Similarly, a major portion of Bim was associated with the mitochondria in PBS-injected thymocytes, and this did not change in response to anti-CD3ε treatment.

**FIGURE 1.** TCR cross-linking on thymocytes does not induce expression of Bim<sub>wt</sub>. A, C57BL/6 mice were i.v. injected with 50 μg of anti-CD3ε. At the indicated times after injection, thymocytes were lysed, and proteins were analyzed by Western blot. B, C57BL/6 mice were injected with anti-CD3 or with PBS. At the indicated time points, the number of live thymocytes was determined using trypan blue exclusion.

**FIGURE 2.** Bim does not change its subcellular localization in response to a strong signal through the TCR. C57BL/6 mice were i.v. injected with PBS or 50 μg of anti-CD3ε. Twenty-four hours later, thymocytes were isolated and subjected to subcellular fractionation. Proteins from each resulting fraction were resolved by SDS-PAGE and analyzed by Western blot. The fractions were probed with four different Abs to verify the fractionation. As expected, Bax was detected in the cytoplasmic and membrane fractions, the TCR ε-chain was detected in the membrane fraction, histone deacetylase-1 (HDAC-1) was detected primarily in the nuclear fraction, and α-tubulin was detected in all fractions, including the cytoskeleton. The fractions are labeled as follows: CYT, cytoplasm; M, membrane; N, nucleus; CSK, cytoskeleton.
Bim is rapidly phosphorylated in vivo and in vitro in response to CD3ε cross-linking

Previous studies have shown that Bim can also be regulated by phosphorylation; however, Bim phosphorylation in response to a signal through the TCR has not been reported. Phosphorylation of Bim can result in either up- or down-regulation of the Bim pro-apoptotic function. For example, several studies have shown that Bim can be phosphorylated by the MAPK ERK, which results in a reduction of pro-apoptotic function (17). In some cases this has been attributed to the targeting of phospho-Bim for proteosomal degradation (22, 23). Thus, phosphorylation of Bim by ERK can promote cell survival. In contrast, in neurons, phosphorylation of Bim by the MAPK JNK on serine 65 has been shown to result in a reduction of proapoptotic function (17). In some cases this has been attributed to the targeting of phospho-Bim for proteosomal degradation (22, 23). Thus, phosphorylation of Bim by ERK can promote cell survival. In contrast, in neurons, phosphorylation of Bim by the MAPK JNK on serine 65 has been shown to result in a reduction of pro-apoptotic function (17). In some cases this has been attributed to the targeting of phospho-Bim for proteosomal degradation (22, 23).

To confirm that the slowly migrating form of Bim is indeed phosphorylated, we immunoprecipitated Bim from thymocytes harvested 30 min after the injection of PBS or anti-CD3ε and then treated with potato acid phosphatase. In PBS-injected animals, treatment with phosphatase resulted in faster migration of Bim on SDS-polyacrylamide gels (17, 20, 21). Thus, phosphorylation of Bim may play a role in its regulation during activation of MAPK pathways, we considered the possibility that phosphorylation of Bim may play a role in its regulation during activation of MAPK pathways, we considered the possibility that phosphorylation of Bim may play a role in its regulation during negative selection. To address this, we examined the electrophoretic mobility of Bim in response to anti-CD3ε stimulation. Although we did not observe a shift in Bim mobility in the data shown in Fig. 1, these gels were run for a relatively short amount of time to prevent Bim from migrating slower on SDS-polyacrylamide gels (17, 20, 21). Because TCR signaling during negative selection of thymocytes results in activation of MAPK pathways, we considered the possibility that phosphorylation of Bim may play a role in its regulation during negative selection. To address this, we examined the electrophoretic mobility of Bim in response to anti-CD3ε stimulation. Although we did not observe a shift in Bim mobility in the data shown in Fig. 1, these gels were run for a relatively short amount of time to observe BimEL and BimNL isoforms. As shown in Fig. 4A, when gels were run long enough to observe small mobility changes, thymocytes harvested 30 min after injection of PBS or anti-CD3ε had a considerable portion of Bim that migrated with reduced mobility in the anti-CD3ε-treated animals, and previous studies have attributed this shift to phosphorylation (15, 18, 19).

To confirm that the slowly migrating form of Bim is indeed phosphorylated, we immunoprecipitated Bim from thymocytes harvested 30 min after the injection of PBS or anti-CD3ε and then treated with potato acid phosphatase. In PBS-injected animals, treatment with phosphatase resulted in faster migration of Bim on SDS-PAGE (Fig. 4B). This suggests that Bim is phosphorylated to some extent in thymocytes before stimulation. The additional decrease in electrophoretic migration that is induced by TCR cross-linking in thymocytes disappears in Bim immunoprecipitates treated with phosphatase. This result confirms that retardation of migration displayed by BimEL in stimulated thymocytes is due to phosphorylation, and anti-CD3ε injection results in increased phosphorylation of BimEL in thymocytes.

The induction of phosphorylation in response to anti-CD3ε injection could be due to direct TCR cross-linking on thymocytes or to glucocorticoid production in response to peripheral T cell activation. To rule out a role for peripheral T cells in the change in Bim phosphorylation, we stimulated thymocytes in vitro with plate-bound anti-CD3ε. In vitro stimulation of thymocytes also resulted in a shift in electrophoretic mobility of BimEL (Fig. 4C). The stimulation of thymocytes in culture includes activation of both DP and SP thymocytes. It is possible that the increased Bim phosphorylation that we have observed is predominantly in the SP population or is dependent on products derived from the SP population. We therefore wanted to determine whether increased Bim phosphorylation could be observed in the DP population in the absence of SP stimulation. To this end we stimulated MHC-deficient thymocytes in vitro with plate-bound anti-CD3ε and analyzed Bim expression by Western blot. MHC-deficient thymocytes are >90% DP and do not contain any SP cells due to the absence of TCR ligands. Similar to normal thymocytes, we observed that...
slower migrating forms of Bim are induced in MHC-deficient thymocytes stimulated in vitro (Fig. 4D). To examine the time course of Bim phosphorylation, we stimulated wild-type thymocytes with plate-bound anti-CD3ε and analyzed thymocyte lysates at different time points after stimulation. Fig. 4E shows that Bim phosphorylation is sustained for at least 24 h after in vivo anti-CD3ε stimulation.

To further analyze Bim phosphorylation in the absence of peripheral T cell activation, we stimulated wild-type thymocytes in vitro with plate-bound anti-CD3ε for 30 min. Bim immunoprecipitates were subjected to 2D gel electrophoresis, followed by blotting with Bim-specific Ab. Fig. 4F shows that, consistent with our phosphatase treatment experiments (Fig. 4B), BimEL as well as Bimε1 exist as a set of species with different isoelectric points, consistent with varying amounts of phosphorylation. Upon stimulation with anti-CD3ε in vitro, BimEL, but not Bimε1, becomes hyperphosphorylated. This is reflected in the emergence of more acidic BimEL isoforms (indicated by arrows on the acidic side) and the disappearance of the most alkalic unphosphorylated BimEL species (indicated by the arrow on the alkaline side). These data indicate that Bim is rapidly phosphorylated in thymocytes in response to TCR signals. To rule out a role for peripheral T cell–dependent glucocorticoids in the induction of Bim phosphorylation, we treated mice with dexamethasone for 2 h in vivo and analyzed Bim on a 2D gel. Treatment of thymocytes with dexamethasone resulted in minimal induction of phosphorylation on BimEL.

Bim binds increased amounts of Bcl-xL in response to anti-CD3ε injection

It has been observed numerous times that BH3-only proteins bind prosurvival Bcl-2 family members (30). In mice with targeted deletion of the bcl-2 gene, the impairment of cell survival caused by the absence of bcl-2 was rescued when Bcl-2-deficient mice were crossed to Bim-deficient mice (31). This indicates that Bim is a critical promoter of cell death, and that Bcl-2 and Bim act in opposition to each other. Therefore, we analyzed the ability of Bim to bind Bcl-xL and Bcl-2 in thymocytes after anti-CD3ε treatment. Total levels of Bcl-2 in wild-type thymocytes were slightly increased with anti-CD3ε stimulation, whereas levels of Bcl-xL by Western blot using Bcl-2- and Bcl-xL-specific Abs. Immune complexes resolved on a gel and transferred onto a membrane were probed with Abs to Bcl-2 and Bcl-xL. C. Total levels of Bim, Bcl-2, and Bcl-xL in MHC−/− thymocytes were determined as described in A. D. In the absence of mature SP thymocytes, Bim binds increased amounts of Bcl-xL and Bcl-2. Bim binding to Bcl-xL and Bcl-2 in MHC−/− mice was analyzed as described in B.

Bim binds increased amounts of Bcl-2 in response to glucocorticoid treatment

We attempted to distinguish events that occur during glucocorticoid–induced apoptosis of thymocytes and apoptosis triggered by TCR cross-linking with regard to Bim binding to antiapoptotic Bcl-2 and Bcl-xL. Fig. 6A shows that expression levels of Bim and Bcl-2 in thymocytes treated with dexamethasone in vivo remain constant, whereas levels of Bcl-xL decrease. To estimate the amounts of Bcl-2 and Bcl-xL associated with Bim, we immunoprecipitated Bim and Bcl-2 and Bcl-xL, associated with Bim, we immunoprecipitated Bim and analyzed immunoprecipitates by Western blot using Bcl-2- and Bcl-xL–specific Abs. Fig. 6B shows that in thymocytes that have been treated with dexamethasone in vivo, Bim associates with a greater percentage of Bcl-2, whereas its interaction with Bcl-xL is completely abolished. Overall, these results show that increased binding of Bcl-2 by Bim can be induced by glucocorticoid. Thus, after anti-CD3ε treatment of wild-type mice, the increased Bim/Bcl-2 association may not be a direct result of TCR cross-linking on thymocytes, but may be secondary to peripheral T cell activation. This conclusion is supported by the
membrane fractions were extracted with neutral buffer or 0.2 M Na2CO3, and membrane fractions were obtained using differential centrifugation. Heavy hours later, thymocytes were isolated and homogenized, and heavy membranes were resolved by gel electrophoresis and analyzed by Western blot. Cytosol and microsome fractions were obtained from additional centrifugation experiments (Fig. 7). Thus, in this experiment, Bim is an integral membrane protein and its association with membranes (35) that was completely stripped by alkali treatment in our experiments (Fig. 7). This treatment strips away the proteins that are peripherally associated with membranes while leaving the integral membrane proteins intact (34). Fig. 7 illustrates that Bim is a transmembrane protein both before and after anti-CD3e injection. Bim is found exclusively in the heavy membrane fraction, and alkali treatment does not change its association with the membranes. This is in contrast to tubulin, a protein peripherally associated with membranes (35) that was completely stripped by alkali treatment in our experiments (Fig. 7). Thus, in this experiment, Bim behaves like a voltage-dependent anion channel, a transmembrane mitochondrial anion channel that resides in the outer mitochondrial membrane and is not stripped by alkali treatment (Fig. 7) (36).

**Discussion**

Bim has been shown to be critical for thymocyte negative selection (9), but its regulation by TCR signaling has not been described. Bim is unusual in that its proapoptotic activity can be regulated in multiple ways depending on the type of cell and the method of apoptosis induction. In several cell types, including thymocytes, it has been shown that Bim expression is induced by apoptotic stimuli, but it remains unclear whether this induction is critical for Bim-dependent apoptosis (14, 15, 37–39). In our system we have not found an increase in steady-state levels of Bim protein, up to 24 h after stimulation with anti-CD3ε. It is not clear why our results are in contrast with others, although differences in mouse strains and doses of anti-CD3ε may play a role. Interestingly, similar discrepancies have been found in studies that have examined the induction of Bim in mature T cells (24, 38).

In cells that express Bimα and Bimε constitutively, Bim can be found either at the mitochondria or associated with the microtubules. In the latter case, apoptotic stimuli cause the release of Bim from its sequestration at the microtubules (19). Once released, Bim relocates to the mitochondria. Studies of the translocation of Bim from microtubules to mitochondria have focused on the Bimε isoform, which is not the major isoform expressed in thymocytes. It has recently been reported that in nonapoptotic T cells, Bim localizes to the mitochondria and does not relocate in response to apoptotic stimuli (10). Similarly, we have found that the subcellular distribution of Bim in healthy and apoptotic thymocytes is the same. Moreover, we found that Bim is primarily associated with the mitochondria both before and after stimulation of thymocytes, and thus, its apoptotic activity cannot be regulated by redistribution to this organelle in the thymus.

Another potential mode of Bim regulation is phosphorylation. Four serines in Bim have been shown to be the targets of phosphorylation by both ERK and JNK (15, 18). ERK-mediated phosphorylation has been consistently correlated with a decrease in the apoptotic activity of Bim, either by targeting of Bim for degradation or by other mechanism (15, 22, 23). JNK activity has been found to both increase the expression of Bim and increase its cytotoxicity (20, 37). Surprisingly, phosphorylation of serine 65 (serine 69 in human Bim) by ERK or JNK has been found to both increase and decrease the proapoptotic activity of Bim (20, 23). This suggests that the phosphorylation can have different effects depending on the context.

We have found that Bimε, a phosphoprotein in resting thymocytes. This is in agreement with a previous study demonstrating that in resting thymocytes Bimε exists as four distinct phosphorylated species (11). Our results show that Bimε is rapidly hyperphosphorylated in response to TCR cross-linking on thymocytes both in vivo and in vitro. The hyperphosphorylation is sustained for at least 24 h after stimulation, a time at which a significant amount of apoptosis is occurring. We have considered the possibility that Bim phosphorylation results in an increase in Bim apoptotic activity. Activity of the MAPK JNK is thought to be critical for normal negative selection of thymocytes (40) and has also been shown to phosphorylate Bimε (20). It has been reported that Bimα and Bimε can be phosphorylated by JNK within the dynein L chain binding domain, resulting in Bim translocation to mitochondria and an increase in apoptotic activity (19). This domain is shared by both Bimα and Bimε isoforms. Our 2D electrophoresis experiments indicate that phosphorylation of dynein L chain binding domain, followed by the release of Bim from the microtubules, are not operational in thymocytes stimulated with anti-CD3ε.
dynein L chain binding domain is present in both BimEL and BimL, but only BimEL becomes phosphorylated in response to a strong signal through the TCR. Furthermore, we do not observe any translocation of Bim from the cytoskeleton to the mitochondria in response to anti-CD3e stimulation. However, there has been one report showing that BimEL phosphorylation by JNK can increase the apoptotic activity of BimEL independently of the dynein L chain binding domain. (20). Thus, it is possible that TCR signaling in thymocytes enhances Bim apoptotic activity via JNK-dependent phosphorylation.

We have also demonstrated that in thymocytes undergoing apoptosis, BimEL binds increasing amounts of Bcl-2 and Bcl-xL. The increased binding of Bcl-2 occurs in both dexamethasone-treated and anti-CD3e-stimulated thymocytes. The increased association with Bcl-xL takes place only in thymocytes that received a TCR signal. In addition, in MHC-deficient mice, Bim binds less Bcl-2 than in wild-type thymocytes. Taken together, these data suggest that Bcl-2 binding by Bim occurs during glucocorticoid-induced apoptosis, whereas increased binding of Bcl-xL is characteristic of apoptosis induced by TCR signaling. It is possible that BimEL phosphorylation results in an ability to bind these prosurvival molecules with greater affinity; however, the mechanism by which TCR signaling enhances Bim association with Bcl-xL remains unknown.

By binding an increasing proportion of Bcl-xL, Bim may shift the cell fate decision toward apoptosis by limiting the amount of free Bcl-xL. This is similar to a model proposed by Zhu et al. (10), who suggested that in mature T cells, Bim increases its association with Bcl-xL after an apoptotic stimulus. In their system, the levels of Bcl-2 were declining rather than slightly increasing as we observed in thymocytes. Something common to both thymocytes and mature T cells is that Bim increases its association with Bcl-xL after an apoptotic stimulus. Thus, in the T lineage, the amount of free Bcl-xL may be a critical survival factor.

A study by Bouillet et al. (9) also found that the association between Bcl-xL and Bim was increased after anti-CD3 stimulation of thymocytes, but differed from our results, in that they found that Bim expression was increased in response to TCR signals. The authors also found that Bcl-xL expression did not change. The reason for this discrepancy is not clear, but a common theme that can be derived from our study and those by Zhu and Bouillet is that in T-lineage cells undergoing apoptosis, the amount of available Bcl-xL bound by Bim is increased. Binding of Bcl-xL by Bim could impair the ability of Bcl-xL to counteract the apoptotic activity of Bax and Bak, thus promoting cell death.

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Disclosures

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References


