Bcl-2 Counters Apoptosis by Bax Heterodimerization-dependent and -independent Mechanisms in the T-cell Lineage*

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The effect of the cell death inhibitor Bcl-2 in relation to its capacity to dimerize with apoptosis promoter Bax or its homologs at their physiological expression levels was explored in the T-cell lineage. Transgenic mice expressing a BH1 mutant Bcl-2 (Bcl-2 mI-3), which fails to heterodimerize with proapoptotic members of the Bcl-2 family, such as Bax, were generated. Bcl-2 mI-3-protected immature CD4+8+ thymocytes from spontaneous, glucocorticoid and anti-CD3-induced apoptosis and altered T cell maturation, resulting in increased percentages of CD8hi and CD48+ thymocytes. In contrast, apoptosis of peripheral T-cells was unaffected by transgene expression. This correlated with their high Bax expression level and insensitivity to the caspase inhibitor, zVAD-fmk, a functional hallmark of Bax-like activity. Thus, within the T-cell lineage Bcl-2 can inhibit apoptosis independent of its association with Bax or its homologs; yet, above a threshold level of their physiologic proapoptotic activity, the capacity of Bcl-2 to heterodimerize with Bax or its homologs appears essential for it to counter cell death.

Apoptosis, or programmed cell death, is an evolutionarily conserved physiological process that ensures the elimination of unwanted or damaged cells from multicellular organisms (1, 2). Although apoptosis can be initiated by diverse physiological and experimental stimuli, ultrastructurally apoptotic cells are characterized by plasma membrane reorganization and blebbing, cell shrinkage and nuclear fragmentation, suggesting the convergence of these signals on a common final effector pathway (1, 3). Insight into the molecular nature of this effector mechanism has been initially derived from genetic studies of the nematode Caenorhabditis elegans. In the nematode, two autosomal recessive death effector genes, cad-3 and cad-4, are required for the death of all 131 cells destined to die during worm development (2, 4, 5), whereas an autosomal dominant death repressor gene, cad-9, is essential for cell survival (6). Cad-3 is related to a family of mammalian cysteine proteases (caspases) (5, 7), which are activated uniformly in mammalian apoptosis and required for certain aspects of cell death through the cleavage of a number of substrate proteins (for review, see Refs. 3 and 8). Cad-9 is a functional and structural homolog of Bcl-2 (9), the prototype member of the Bcl-2 protein family in vertebrates, which is able to inhibit the effect of many, but not all, apoptotic stimuli (for review, see Ref. 10). When expressed in mammalian cells, Ced-9 and the Bcl-2 functional homolog, Bcl-xL (11), can interact with (12–14) and inhibit the death-inducing function of Ced-4 (12), while recruiting it from the cytosol to intracellular membranes (13). In addition, Ced-4 can simultaneously interact with, and presumably activate, Ced-3 or its mammalian counterparts, interleukin 1β-converting enzyme (ICE; caspase 1) and Flice (caspase 8), biochemically linking Ced-9 and the Bcl-2 family to Ced-3 and the mammalian caspases (12). These findings suggest that apoptosis is precipitated by the proteolytic cleavage of one or several critical substrates, and Bcl-2 may function by blocking the activation of caspases by inactivating Apaf-1, the recently identified mammalian homolog of Ced-4 (15).

In mammalian cells, however, the control of apoptosis appears more complex than seen in C. elegans. For instance, mammals contain several genes encoding caspases and Bcl-2 homologs, whereas only a single essential copy of each type of gene has been identified to date in the nematode (2, 6). Also, Bcl-2 can prevent the mitochondrial release of an apoptogenic protease (AIF) (16), as well as of cytochrome c (17, 18), which, together with Apaf-1 can lead to caspase 3 activation (15, 19). More importantly, in vertebrates two functional classes of Bcl-2-related proteins exist which share highly conserved Bcl-2 homology 1 (BH1), 2 (BH2), and 3 (BH3) domains: antiapoptotic members (Bcl-2, Bcl-xL, McI-1, A1, Bcl-w, Bfl-1, Brag-1), which inhibit cell death, and proapoptotic members (Bax, Bak, Bad, Bik,Bid, and Hrk), which accelerate apoptosis and counter the death-repressive function of Bcl-2 or Bcl-xL upon receiving a death signal (for review, see Ref. 20). Several in vivo studies confirm (21–24) that in vertebrates the balance between death-promoting and death-repressing members of the Bcl-2 family contributes a critical checkpoint that determines the susceptibility of a cell to an apoptotic stimulus (for review, see Ref. 10).

The molecular mechanism(s) by which Bax and its homologs exert their death-promoting function at physiological expression levels is not clear. Inducible overexpression of Bax in yeast or in mammalian cells can trigger cell death in the absence of additional apoptotic stimuli (25–29). Of note, this Bax-induced apoptosis proceeds even when caspase activation is inhibited (28). Similarly, mutations within the BH1, BH2, and BH3 domains of Bcl-2 and Bcl-xL, which abrogate their function also cause loss of heterodimerization with Bax in mammalian cells (30–32). These data suggest that at high expression levels proapoptotic Bcl-2-related proteins possess the capacity to be directly cytotoxic and that Bcl-2 and Bcl-xL may have to form a protein complex with them to counter cell death. However, the abbreviations used are: BH1, BH2, BH3, Bcl-2 homology 1, 2, and 3 domains, respectively; wt, wild type; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal antibody; pAb, polyclonal antibody; FCS, fetal calf serum; IL, interleukin; TCR, T-cell receptor.
selected BH1 and BH2 mutants of Bcl-x<sub>L</sub> can exert their death-repressing activity even in the absence of heterodimerization with Bax (33). In mammalian cells, Bax can also directly compete with Ced-4 for association with Bcl-x<sub>L</sub>, together implying that at physiological expression levels Bax-like proteins may merely act as inert competitive inhibitors of Bcl-2 and its functional homologs (12).

To assess the antiapoptotic mechanism of Bcl-2 in relation to its capacity to dimerize with Bax and its homologs in a physiological context, transgenic mice expressing a BH1 mutant of Bcl-2 (Bcl-2<sup>ΔDH</sup>-mI-3) (30) in the T-cell lineage were generated. The varied sensitivity of thymocytes and peripheral T-cells to caspase inhibition (see below) suggested the suitability of this lineage to test this question. Bcl-2<sup>ΔDH</sup>-mI-3 (G145A) does not form heterodimers with proapoptotic members of the Bcl-2 family (30, 34), yet it protected immature CD4<sup>+</sup>8<sup>+</sup> thymocytes from spontaneous, glucocorticoid and anti-CD3-induced apoptosis. Despite this, transgene expression provided no protection against apoptosis of peripheral T-cells that displayed a high level of Bax expression and insensitivity to caspase inhibition. Thus, within the T-cell lineage Bcl-2 can inhibit cell death independent of association with Bax or its homologs; yet Bcl-2 must heterodimerize with Bax or its homologs above a threshold level of their physiologic cytotoxic activity to counter apoptosis.

**EXPERIMENTAL PROCEDURES**

**Construction of lck<sup>+</sup>-bcl-2ΔDH-mI-3 and Production of Transgenic Mice**—The construct was generated by insertion of a 0.75-kilobase fragment, containing the coding region of human bcl-2<sup>ΔDH</sup>-mI-3 cDNA (30) into the lck-hGH vector (35). The bcl-2<sup>ΔDH</sup>-mI-3 cDNA was inserted by blunt end ligation into the BamH I cloning site 3′ to the lck<sup>+</sup> promoter. The correct orientation was selected, and SfiI was used to prepare the 6.3-kilobase lck<sup>+</sup>-bcl-2ΔDH-mI-3 used for microinjection (see Fig. 2A). Transgenic mice were produced by DNA Transgenics on a C57BL/6J/SJL background. All animals were bred and maintained in a pathogen-free environment at the Northwestern University Experimental Animal Facility 2–12 weeks before analysis. The lck<sup>+</sup>-bcl-2ΔDH-mI-3 transgenic mice were provided by Dr. Stanley J. Korsmeyer (Washington University, St. Louis).

**Flow Cytometry, Reagents and Antibodies**—Flow cytometry reagents were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-mu-rine CD8 and anti-murine CD3, phycocyanin (PE)-conjugated anti-murine CD4, and anti-murine B220 (Pharmingen, San Diego); 6C8, human Bcl-2-specific hamster mAb (36), and the anti-murine CD3ε mAb (145-2C11, Pharmingen) were used. 651, a murine Bax-specific rabbit pAb (54), was a gift from Dr. Stanley J. Korsmeyer (Washington University). The 6C8 mAb was biotinylated as described previously (37). For Western immunostaining, the primary antibody dilutions were: 6C8 (1:100), biotinylated 6C8 (1:500), 651 (1:500). The primary antibodies were detected with species-specific biotinylated secondary antibodies (Pierce).

**Cell Surface Analysis**—For flow cytometry, cells were washed twice in staining buffer, 1% fraction 5 bovine serum albumin (Sigma) in phosphate-buffered saline. Each sample of 10<sup>6</sup> cells was stained in 100 μl of buffer. Primary incubation was with staining buffer alone (negative control) or with 1 μg of PE- or FITC-conjugated specific antibody for 30 min at 4 °C followed by washing with staining buffer. After additional washes in staining buffer, the samples were resuspended in 100 μl of staining buffer and analyzed on a FACScan analyzer (Becton-Dickinson, Mountain View, CA).

**Cell Culture and Viability Assays**—Murine lymphoid organs were placed in sterile ice-cold RPMI 1640 medium, and cells were prepared as described previously (38). Splenic T-cells were purified by negative selection with affinity chromatography, according to the manufacturer’s instructions (Cellec Mouse T-cell kit, Biotec Laboratories). T-cells were always 90–95% pure as judged by CD3, B220 double staining and were >95% viable. Jurkat cells (clone E6-1, ATCC) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Life Technologies, Inc.) and penicillin/streptomycin (100 units/ml). The interleukin-3 (IL-3)-dependent murine cell line FL5.12, a lymphoid progenitor clone, was maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% FCS and 25 IU/ml recombinant murine IL-3 (Genzyme). For induction of cell death FL5.12 cells were IL-3 deprived, as described (39), and Jurkat cells were treated with 100 ng/ml anti-human Fas mAb (Upstate Biotechnology) in the absence or presence of 100 μM z-Va-Ala-Asp-CH<sub>2</sub>F (zVAD-fmk) (Enzyme Systems Products). For induction of spontaneous apoptosis, thymocytes, splenic T-cells, and lymph-node cells were plated at 1 × 10<sup>6</sup> cells/ml in RPMI 1640 supplemented with 5% FCS in the absence or presence of 100 μM zVAD-fmk. At each time point cells were collected and stained with FITC-conjugated annexin-V/propidium iodide, according to the manufacturer’s instructions (CLONTECH). Flow cytometry was performed (FACScan), and cell populations negative for both annexin-V and propidium iodide were scored as viable.

**In Vivo Dexamethasone or Anti-CD3 Treatment**—0.5 ml of water-soluble dexamethasone (Sigma) or 50 μg of affinity-purified anti-CD3 mAb (145-2C11, Pharmingen) or vehicle (RPMI 1640) control was injected intraperitoneally into 6–8-week-old transgenic and control littermates. Thymi were removed 48 h after treatment, and cell suspensions were made by teasing in ice-cold RPMI 1640 and were stained for surface CD4, CD8, and CD3 within 2 h.

**Metabolic Labeling and Immunoprecipitations**—Before metabolic labeling thymocytes were washed once in prewarmed, serum-free, methionine-free Dulbecco’s medium (Life Technologies, Inc.). Cells were resuspended at 3–5 × 10<sup>6</sup> cells/ml in methionine-free Dulbecco’s medium supplemented with 10% dialyzed FCS. Metabolic labeling was performed with 40 μCi/ml [35S]methionine, [35S]cysteine (Trans-35S-label, ICN) for 9–12 h before. All steps of the immunoprecipitation of metabolically labeled or unlabeled cells with the 6C8 mAb were performed, as described previously (39). Gels containing immunoprecipitated [35S]methionine-labeled proteins were fixed with 10% glacial acetic acid and 30% methanol overnight and enhanced by impregnating with a commercial fluorography enhancing solution (Enhance, NEN Life Science Products) before autoradiography. Gels containing immunoprecipitated unlabeled proteins were electrotransferred overnight at 4 °C on polyvinylidene difluoride membranes.

**Western Blot Analysis**—Single cell suspensions were lysed in 150 mM NaCl, 10 mM Tris (pH 7.4), 1% Triton X-100 with 2 mg of aprotinin ml<sup>−1</sup> for 30 min at 4 °C. After centrifugation at 27,000 × g, the amount of protein in the supernatants was quantitated (D<sub>280</sub>, Protein Assay kit, Bio-Rad) or determined by immunoblotting. Bcl-2 and Bax expression levels, equal amounts of protein were electrotransferred overnight at 4 °C on polyvinylidene difluoride membranes. Immunostaining for Bcl-2 was performed, as described previously (39), and was developed with diazobenzidine (Bio-Rad) enhanced with nickel chloride (0.03%). Immunostaining for Bax was performed, as described previously (34) by utilizing the 651 pAb and was developed with enhanced chemiluminescence (ECL) (Amersham). Immunostaining for coimmunoprecipitated Bax was performed by utilizing the 651 pAb, and for Bcl-2 by utilizing biotinylated 6C8 mAb, as described previously (34, 37), and was developed with ECL.

**Results**

**Bax-like Activity in Splenic T-cells but Not in Thymocytes**—Recently, Bcl-2 was shown to be able to block apoptosis at least in part by inhibiting the mitochondrial release of cytochrome c (17, 18) or a mitochondrially derived apoptotic protease, AIF (16). Moreover, the Bcl-2 homolog, Bcl-x<sub>L</sub>, can interact with Ced-4 and block Ced-3 or Ced-4-induced apoptosis (12). Induction of cell death by all of these mechanisms can be inhibited by zVAD-fmk, an irreversible pseudosubstrate caspase inhibitor (12, 16, 19). zVAD-fmk was also shown to block Fas-induced caspase activation and cell death (40). In contrast, Bax-induced cell death was only partially effected by zVAD-fmk, as blocking IL-1β-converting enzyme-like protease activity prevented the cleavage of selected nuclear and cytosolic substrates, but cytoplasmic vacuolation and plasma membrane changes still occurred (28).

To examine the role of Bax, or Bax-like activity, on thymocytes and splenic T-cell apoptosis, the effect of zVAD-fmk on their spontaneous cell death was examined. As initial controls, Fas-mediated apoptosis of Jurkat cells and IL-3 deprivation-induced apoptosis of FL5.12 cells in the presence or absence of 100 μM zVAD-fmk were tested. As described previously (28), Jurkat cells treated with anti-Fas antibody died rapidly, a process that was completely prevented by simultaneous treatment with zVAD-fmk (Fig. 1A). The cleavage of PARP, a well
characterized marker of caspase cascade activation (41), was also prevented in the presence of zVAD-fmk in anti-Fas antibody-treated Jurkat cells (data not shown). In contrast, zVAD-fmk proved ineffective against cell death induced by IL-3 deprivation of FL5.12 cells (Fig. 1C), a cell line that possesses high amount of endogenous Bax (39). These data are in agreement with previous experiments in which IL-2 deprivation-induced apoptosis of thymocytes and splenic T-cells, suspensions of normal T-cells (Fig. 2B, top panel) and spleen (Fig. 2B, bottom panel) by Western immunoblot analysis utilizing the human Bcl-2-specific mAb, 6C8 (36). The tissue specificity of the lckm11-bcl-2 mI-3 transgene was examined by Western blot analysis, which failed to show transgene expression in non-lymphoid tissues, including the brain, heart, kidney, liver, and lung (data not shown). The thymus of transgenic animals contained a distinct cortex and medulla and was normal in size. The distribution of splenic red and white pulp was similar in transgenic and control littermate mice (data not shown). The two lines, 67 and 72, with the highest levels of human Bcl-2 mI-3 expression in thymocytes (Fig. 2B, top panel) and splenocytes (Fig. 2B, bottom panel) were characterized further and compared with the previously established lckm11-bcl-2 wt transgenic model (38).

Bcl-2 wt, but Not Bcl-2 mI-3, Heterodimerizes with Bax in Both Thymocytes and Peripheral T-cells—To confirm the inability of overexpressed Bcl-2 mI-3 to heterodimerize with endogenous proapoptotic Bcl-2 family members, such as Bax, coimmunoprecipitation experiments in bcl-2 wt and bcl-2 mI-3 thymocytes and splenic T-cells were performed. When [35S]methionine-labeled bcl-2 wt thymocytes were immunoprecipitated with the human Bcl-2-specific 6C8 mAb, a low amount of endogenous p21 protein was coprecipitated with human Bcl-2 (Fig. 3A, top panel, lane 2). Immunostaining of a Western blot of the same immunoprecipitate with the murine Bax-specific pAb, 651 (34), confirmed the identity of p21 as murine Bax (Fig. 3A, bottom panel, lane 2). Identical immunoprecipitations on [35S]methionine-labeled bcl-2 wt thymocytes revealed a lack of heterodimerization between Bcl-2 mI-3 and endogenous Bax (Fig. 3A, top panel, lane 3).

As metabolic labeling of splenic T-cells with [35S]methionine was not very effective, coimmunoprecipitation experiments on lysates of unlabeled thymocytes and splenic T-cells were performed (Fig. 3B). Equal amounts of protein lysates of bcl-2 wt and bcl-2 mI-3 thymocytes and splenic T-cells were immunoprecipitated with the human Bcl-2-specific 6C8 mAb, and Western blots of the immunoprecipitates were immunostained
with either biotinylated 6C8 mAb, for human Bcl-2 (Fig. 3B, top panel), or with the 651 pAb, for murine Bax (Fig. 3B, bottom panel). Thymocytes and splenic T-cells of both bcl-2 wt and bcl-2 mI-3 transgenics expressed comparable amounts of human Bcl-2, although the expression levels in thymocytes were somewhat higher (Fig. 3B, top panel). Bcl-2 immunoprecipitated either from bcl-2 wt thymocytes (Fig. 3B, lane 3) or splenic T-cells (Fig. 3B, lane 6) demonstrated heterodimerization with endogenous murine Bax, but the amounts of Bcl-2/Bax heterodimers were about 5-fold higher in splenic T-cells compared with those seen in thymocytes (Fig. 3B, bottom panel). Immunostaining of 6C8 immunoprecipitates from bcl-2 mI-3 thymocytes (Fig. 3B, lane 1) or splenic T-cell lysates (Fig. 3B, lane 4) demonstrated no association between Bcl-2 and Bax in either cell type.

**Higher Expression Level of Bax in Peripheral T-cells**—Despite a comparable level of Bcl-2 wt expression in thymocytes and splenic T-cells, the amount of endogenous Bax that coprecipitated with Bcl-2 wt in splenic T-cells was substantially higher than in thymocytes (Fig. 3B, bottom panel). Thus, we were interested to determine if this difference was due to varying expression levels of endogenous Bax or if it represented differential dimerization capacity between Bcl-2 and Bax within the two cell types.

Consequently, equal amounts of protein lysates of thymocytes and splenic T-cells from bcl-2 wt and bcl-2 mI-3 transgenic mice and control littermates were assessed for the expression level of endogenous Bax with the murine Bax-specific 651 pAb. No difference in endogenous Bax expression was seen between bcl-2 wt and bcl-2 mI-3 or nontransgenic thymocytes or splenic T-cells (Fig. 3C). However, endogenous Bax expression was about 5-fold higher in splenic T-cells compared with that seen in thymocytes of the bcl-2 wt and bcl-2 mI-3 transgenics and control littermates (Fig. 3C).

**Increased CD3^hi and CD4^-8^1 Thymocytes**—To assess the functional effect of enforced Bcl-2 mI-3 expression in the T-cell lineage thymic maturation was first studied. Expression of Bcl-2 mI-3 in the thymus did not substantially modify the number of thymocytes, yet it altered the distribution of thymocyte subsets. bcl-2 mI-3 transgenics uniformly demonstrated a ~4-fold increase in CD4^-8^1 single positive thymocytes (9.1 ± 1.4% versus 2.4 ± 0.3% of control thymocytes) (Fig. 4, top panel). Transgenic animals also displayed a ~1.4-fold in-
increased percentage of CD4^+ single positive cells (15.7 ± 2.0% versus 10.4 ± 1.8% of control thymocytes). The increase in CD4^+ thymocytes changed the average ratio of CD4^+ / CD4^- cells from 5:1 in control mice to 2:1 in transgenic mice. Immunoblot analysis revealed no difference in the amount of Bcl-2 mI-3 protein in CD4^+ versus CD4^- cells (data not shown). This phenotypic effect of expressing bcl-2 mI-3 in the thymus is similar to that seen with the bcl-2 wt transgene (38, 47), namely an increase in mature thymocytes predominantly skewed toward the CD8^+ subset.

Similarly, all transgenic animals had an increased percentage of CD3^hi/TCR^hi thymocytes (29.1 ± 3.5%) compared with control littermates (15.2 ± 1.8%) (Fig. 4, bottom panel). Transgenics displayed a reciprocal decrease in CD3^int-lo cells (Fig. 4, bottom panel). Thymocytes that have successfully completed thymic selection demonstrate increased CD3 expression, whereas CD3^int-lo cells represent immature thymocytes, most of which are believed to die while undergoing thymic selection. Of note, transgenic thymocytes also contained increased numbers of cells with an intermediate level of TCR and CD3. This intermediate stage following positive selection (48, 49).

**Fig. 4.** CD3, CD4, and CD8 expression in bcl-2 mI-3 transgenic and nontransgenic thymocytes. Top panels representative histograms of thymocytes stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs. The percentage of cells of each phenotype is indicated. Bottom panel, single-cell suspension of thymocytes from transgenic (solid line) and nontransgenic (dotted line) lines were stained with FITC-conjugated anti-CD3 mAb. The percentage of cells that are CD3^hi/lo and CD3^hi/hi is indicated. Similar results were obtained in both lines 67 and 72.

**Modestly Increased CD3^hi and CD4^+ T-cells**—Flow cytometric analysis of splenocytes from 6-week-old transgenic mice revealed an increase in both the number of T-cells (Thyl^+CD3^+ cells) and the percentage of CD8^+ cells (Fig. 5). The percentage of CD8^+ T-cells increased ~1.5-fold in young 6-week-old transgenic animals (18.6 ± 1.7% versus 12.1 ± 1.8%) (Fig. 5, top panel) but decreased to ~1.1-fold increment by 12 weeks of age (data not shown). The ratio of CD4^+ to CD8^+ T-cells in the splenocytes of 6-week-old transgenic mice averaged 1.2 compared with 1.5 in control littermate mice. Similar ratios of CD4^+ to CD8^+ T-cells in the lymph node cells were seen (data not shown).

Similarly, all 6-week-old transgenic animals had an increased percentage of CD3^hi/TCR^hi splenocytes compared with control littermates (45.8 ± 2.0% versus 31.1 ± 1.8% of control splenocytes) (Fig. 5, bottom panel). By 12 weeks of age the differences in CD3 expression level of transgenic and control animals diminished to the same extent as seen in CD4^+,CD8^+ expression (data not shown). Although the percentage of B cells in 6-week-old transgenic spleens was decreased by 15%, the absolute number of B cells was comparable in transgenic and control spleens. This phenotypic effect of expressing bcl-2 mI-3 in splenocytes is significantly weaker than that seen with the bcl-2 wt transgene where an increase in the CD8^+ subset is more pronounced (38, 47).

Increased Survival of bcl-2 mI-3 Transgenic Thymocytes but Not Transgenic Peripheral T-cells—Immature CD4^+ cortical thymocytes as well as peripheral T-cells die rapidly in culture (50), an effect that is countered by overexpression of Bcl-2 (38, 47) or Bcl-xL (51, 52). To assess the effects of bcl-2 mI-3 on the viability of these cells, suspensions of thymocytes, splenic T-cells, and lymph node cells from bcl-2 mI-3, bcl-2 wt, and control littermates were placed in vitro in RPMI 1640 medium supplemented with 5% fetal calf serum (Fig. 6). Western immunoblot on equal amounts of protein lysates of each sample demonstrated comparable Bcl-2 expression in thymocytes, lymph node cells, and splenic T-cells from bcl-2 mI-3 and bcl-2 wt mice (Fig. 6A), although Bcl-2 expression was slightly higher in thymocytes compared with peripheral T-cells.

Thymocytes from lck^-/bcl-2 wt mice demonstrated improved survival, whereas the majority of control thymocytes died within the first 3 days, as described previously (38, 47). Remarkably, the bcl-2 mI-3 thymocytes demonstrated survival rates similar to that seen with Bcl-2 wt expression (Fig. 6B). After 7 days, ~25% of bcl-2 mI-3 and bcl-2 wt thymocytes were still viable, whereas <1% of the normal thymocytes survived. Flow cytometric analysis at day 7 revealed the persistence of double positive and single positive thymocytes from both bcl-2 mI-3 and bcl-2 wt transgenic mice (data not shown). Similarly, in the presence of 1 × 10^-6 M dexamethasone both bcl-2 wt and bcl-2 mI-3 thymocytes showed improved survival (58.1 ± 3.8% and 53.2 ± 6.2% viability, respectively), whereas essentially all control thymocytes died within the first 24 h (data not shown).

Contrary to that seen with thymocytes, peripheral T-cells of bcl-2 mI-3 mice exhibited no increased survival in vitro (Fig. 6, C and D). Despite Bcl-2 mI-3 expression comparable to that
seen in thymocytes (Fig. 6A), splenic T-cells and lymph node cells from bcl-2 mI-3 mice died at a rate similar to that of control littersmates when placed in culture (Fig. 6, C and D). At the same time, peripheral T-cells from bcl-2 wt mice were remarkably resistant, as described previously (38, 47). After 7 days, ~36% of splenic T-cells and ~59% of lymph node cells from bcl-2 wt mice were still viable (Fig. 6, C and D).

bcl-2 mI-3 Thymocytes Are Resistant to Glucocorticoid and Anti-CD3-induced Apoptosis—As Bcl-2 mI-3 was able to block the spontaneous apoptosis of thymocytes in vitro, it was of interest to determine whether Bcl-2 mI-3 could also extend thymocyte survival in vivo. Triggering thymocytes with dexamethasone or anti-CD3 both in vivo and in vitro has been shown to induce apoptosis of primarily the immature CD4<sup>+</sup>CD8<sup>+</sup> cell population (53–56). Consequently, mice were treated with dexamethasone or anti-CD3, and thymocytes were evaluated 48 h later (Fig. 7).

Intraperitoneal injection of 0.5 mg of dexamethasone depleted a mean of 98% of thymocytes in control mice compared with vehicle treatment alone. However, bcl-2 mI-3 mice were markedly resistant with an average decrease of only ~25% with this dose. Flow cytometric analysis of the surviving thymocytes indicated that dexamethasone only slightly reduced the CD4<sup>+</sup>8<sup>+</sup> thymocytes in transgenic mice but almost com-
pletely eliminated the CD4\(^+\) population from control mice (Fig. 7, middle panel). Similarly, 48 h after the intraperitoneal injection of 50 \(\mu g\) of affinity-purified anti-CD3 monoclonal antibody, the number of CD4\(^+\)8\(^+\) thymocytes was decreased substantially (~85%) in control mice. The bcl-2 ml-3 mice were again resistant to in vivo anti-CD3 treatment, although a slight decrease in the number of CD4\(^+\)8\(^+\) thymocytes could be observed with an average decrease of ~40% with this dose (Fig. 7, bottom panel). Thus, Bcl-2 ml-3 proved capable of countering apoptosis of thymocytes both in vitro and in vivo.

**DISCUSSION**

In mammalian cells, death-promoting and death-repressing members of the Bcl-2 family readily form heterodimers with each other (for review, see Refs. 20 and 34), but the significance of this physical interaction to their respective function is controversial. Inducible overexpression of Bax in mammalian cells can in itself induce apoptosis that is countered by Bcl-2 (27–29). Also, selected mutations within the BH1, BH2, and BH3 domains of Bcl-2 and Bcl-x\(_{-}\) which disrupt their heterodimerization with proapoptotic Bcl-2 family members such as Bax or Bid (30, 31, 34) can also result in the abrogation of their function (30–32). These data argue that a critical level of Bax homodimers activates downstream effector molecules and that antagonists such as Bcl-2 prevent apoptosis by inactivating Bax through heterodimerization (30). However, recent experimental evidence has challenged and partially invalidated this model. Selected BH1 mutants of Bcl-x\(_{-}\), which are unable to dimerize with Bax, can still counter cell death (33). Also, Bcl-x\(_{-}\) can interact with mammalian caspases through Ced-4, a physical association that is influenced negatively by Bax (12). Moreover, bcl-2 can functionally substitute for ced-9 in *C. elegans* (57), an organism in which no bax homolog has been identified. These data offer an alternative hypothesis in which Bcl-2 and its functional homologs exert their action by preventing the formation of inactive Bcl-2:Ced-4 homolog (Apaf-1):caspase complexes. In turn, the death repressor function of such complexes may be neutralized by competition with an inert Bax molecule (12).

The data presented in this paper provide evidence for the validity of both models within the T-cell lineage. Our results demonstrate that depending on the physiological context Bcl-2 exerts its antiapoptotic function by one of two separate mecha-
Dual Antiapoptotic Function of Bcl-2 in the T-cell Lineage

Fig. 8. Model for the dual function of Bcl-2 and Bax.

External Apoptotic Signal

Bcl-2 Bcl-2

Bcl-2 Bax

Bax Bax

Internal Survival Signal

Execution of Apoptosis

Mechanisms: one that requires heterodimerization with proapoptotic Bax-like molecules and one in which such physical interaction is not required. To determine the antiapoptotic function of Bcl-2 in relation to its capacity to dimerize with Bax or its homologs at their physiological expression levels, we generated transgenic mice expressing a nondimerizing BH1 mutant of Bcl-2 (Bcl-2 mI-3) (G145A) in the T-cell lineage. Previous studies demonstrated that this mutant Bcl-2 is unable to counter apoptosis induced by growth factor deprivation or glucocorticoid treatment in cell lines possessing high levels of endogenous Bax (30). Yet, a similar BH1 mutation of ced-9 enhanced its survival promoting function in C. elegans (58), suggesting a dichotomy between the mammalian and nematode cell death machinery. Similarly, whereas in thymocytes spontaneous cell death was countered by zVAD-fmk, the spontaneous apoptosis of splenic T-cells remained unaffected by this caspase inhibitor (Fig. 1). Inasmuch as resistance to zVAD-fmk represents a hallmark of Bax or Bax-like activity (28), these results implicate an active apoptosis-inducing function of Bax or its homologs in peripheral T-cells but a lack of such activity in thymocytes. Thus, thymocytes appeared a reasonable candidate for a cell type in which Bcl-2 may function in a heterodimerization-independent fashion.

Bcl-2 mI-3 did not heterodimerize with Bax either in thymocytes or in peripheral T-cells, in agreement with previous findings (30) (Fig. 3). Yet, enforced thymic expression of Bcl-2 mI-3 protected immature CD4+8+ thymocytes from spontaneous, glucocorticoid and anti-CD3-induced apoptosis (Figs. 6 and 7). Bcl-2 mI-3 also altered thymocyte maturation and increased the percentages of CD3+ and CD4+8+ thymocytes (Fig. 4), both patterns being similar to that seen with wild type Bcl-2 (38, 47). Thus, within immature thymocytes, the antiapoptotic function of Bcl-2 is apparently independent of its capacity to heterodimerize with Bax or its homologs. In contrast, Bcl-2 mI-3 could not counter the apoptosis of peripheral T-cells whereas wild type Bcl-2 remained effective (Fig. 6). The loss of antiapoptotic activity of Bcl-2 mI-3 correlated with a higher Bax expression level in peripheral T-cells, and the amount of Bax that coprecipitated with wild type Bcl-2 proved significantly increased compared with that seen in thymocytes (Fig. 3). Thus, within peripheral T-cells the antiapoptotic function of Bcl-2 apparently requires a capacity to heterodimerize with Bax or its homologs.

These data together suggest a dual effector function for both death-promoting and death-repressing members of the Bcl-2 family (Fig. 8). As shown here within the T-cell lineage, Bcl-2 can counter apoptosis induced by external apoptotic stimuli, such as TCR engagement or glucocorticoid treatment, independent of its association with Bax or its homologs. Although thymocytes do express a low amount of endogenous Bax (Fig. 3), their rate of apoptosis induced by γ-irradiation or dexamethasone treatment is not altered in bax−/− mice (23). Yet, the same apoptotic stimuli in conjunction with enforced expression of Bax results in increased thymocyte cell death (59). Thus, a threshold level of Bax expression is apparently required for its death-accelerating function in thymocytes. Of note, external death signals themselves can alter the inherent Bcl-2 to Bax ratio (60). As spontaneous in vivo thymocyte apoptosis can be delayed by zVAD-fmk (Fig. 1), Bax in thymocytes seems to act as an inert competitive inhibitor, perhaps by competing for the Bcl-xL, expressed in immature CD4+8+ thymocytes (52). In contrast, spontaneous cell death of peripheral T-cells is apparently initiated by the active cytotoxicity of either Bax or one of its functional homologs, as it is not affected by zVAD-fmk (Fig. 1). Inactivation of Bax or its homologs in these cells can be achieved either by external survival signals or by heterodimerizing Bcl-2. Several mechanistic possibilities may account for the antiapoptotic effect of Bcl-2 in this case. In one scenario, Bcl-2 might simply act as an inert competitive inhibitor through disrupting the formation of Bax-like homodimers. Alternatively, Bcl-2-Bax heterodimers could possess a biochemical function that is diametrically opposed to Bax homodimers.

The identification of loss-of-function Bcl-2 mutants with intact Bax dimerization capacity (61) favors this latter possibility. In either case, whether Bax and its homologs are directly cytotoxic or only act as inert competitive inhibitors appears to depend on the presence of additional mediator molecules, such as proposed for the role of Bid (34).

Cell suicide is present in a variety of unicellular organisms, suggesting that apoptosis in multicellular organisms may have very primitive evolutionary origins. For instance, some strains of Escherichia coli will activate the expression of bacterial toxins that trigger cell death to ensure plasmid maintenance or as a response to external events by suicidal pore or channel formation through their plasma membrane (62–64). The demonstrated ion channel function of Bax and its inhibition by Bcl-2 at physiological pH (65) strongly implicate the evolutionary conservation of this mechanism in multicellular organisms. Yet, in the nematode C. elegans CED-9 appears to function by an alternative mechanism that involves blocking the activation of CED-3 through the formation of inactive CED-9-CED-4-CED-3 complexes (12).

Our data suggest the preservation of both pathways of cell death initiation in mammalian cells (Fig. 8): one that is initiated by Bax and its homologs and which may operate on the principles of unicellular pore-forming cell suicide systems, and one in which the activation of caspases plays a central role as seen in C. elegans. Bcl-2 can counter apoptosis when either pathway is involved but must heterodimerize with Bax and its homologs when cell death is initiated by their active cytotoxic function.

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REFERENCES

1. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) *Int. Rev. Cytol.* 68, 251–306.
2. Ellis, R. P., Yuan, J. Y., and Horvitz, H. R. (1991) *Annu. Rev. Cell Biol.* 7, 669–702.
3. Haney, P. K. (1996) *Immunity* 4, 195–201.
4. Yuan, J., and Horvitz, H. R. (1992) *Development* 116, 309–320.
5. Yang, J., Lai, S., Sedlak, T. W., Oliva, Z. N., Yang, E., Wang, K., Khan, M. S., and Korsmeyer, S. J. (1994) *Cell* 75, 641–652.
6. Cheng, E. H., Levine, B., Boise, L. H., Thompson, C. B., and Hardwick, J. M. (1998) *Nature* 398, 544–549.
7. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1982) *In Vitro* 18, 212–216.
8. Cherniavsky, A. M., and Dixit, V. M. (1996) *Curr. Biol.* 6, 755–765.
9. Yang, J., and Horvitz, H. R. (1992) *Nature* 359, 544–549.
10. Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997) *Cell* 88, 386–401.
11. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 597–608.
12. Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997) *Cell* 88, 1122–1129.
13. Wu, D., Wallen, H. D., and Nunez, G. (1997) *Science* 275, 641–652.
14. Yuan, J., and Horvitz, H. R. (1992) *Science* 259, 76, 653–656.
15. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Science* 275, 405–413.
16. Hunter, J. J., Bond, B. L., and Parslow, T. G. (1996) *Science* 270, 229–240.
17. Hunter, J. J., Bond, B. L., and Parslow, T. G. (1996) *Cell* 84, 195–202.
18. Shortman, K., Vremec, D., and Egerton, M. (1991) *J. Exp. Med.* 172, 635–645.
19. Shi, Y. F., Bissognette, R. P., Parfrey, N., Stalay, M., Kubo, T. R., and Green, D. R. (1991) *J. Immunol.* 146, 3340–3346.
20. Smith, C. A., Williams, G. T., Kingston, R. E., Jenkinson, E. J., and Owen, J. W. (1989) *Nature* 337, 181–184.
21. Vaux, D. L., Weisman, I. L., and Kim, S. K. (1992) *Science* 258, 1955–1957.
22. Hengartner, M. O., and Horvitz, H. R. (1994) *Nature* 368, 518–520.
23. Bhan, A. K., Dixit, V. M., and Reiners, E. L. (1997) *EMBO J.* 16, 2262–2263.
24. Li, T., Ramirez, K., and Palacios, B. (1996) *Cell Growth Differ.* 7, 107–114.
25. Tuckel-Szabo, C. L., Andjelic, S., Lacy, E., Elkon, B. K., and Nikolie-Zagje, J. (1996) *J. Immunol.* 156, 192–200.
26. Clark, T. N., and Korsmeyer, S. J. (1995) *J. Exp. Med.* 182, 821–828.
27. Elliott, J. L., Knudson, C. M., Johnson, E. M., Jr., Snider, D. R., and Korsmeyer, S. J. (1993) *Cell* 75, 229–240.
28. Grohmann, H., and Anderssen, B. (1979) *Cell. Immunol.* 39, 221–230.
29. Bhan, A. K., Dixit, V. M., and Reiners, E. L. (1997) *EMBO J.* 16, 2262–2263.
30. Shortman, K., Vremec, D., and Egerton, M. (1991) *J. Exp. Med.* 172, 635–645.
31. Bittner, M., Vida, I., and Schreiber, R. D. (1994) *Science* 266, 201–205.
32. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996) *Nature* 380, 529–532.
33. Visser, D. J., and Pickart, C. M. (1993) *EMBO J.* 12, 3340–3346.
34. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996) Genes Dev. 10, 2859–2869.
35. Vaux, D. L., Weisman, I. L., and Kim, S. K. (1992) *Science* 258, 1955–1957.
36. Hengartner, M. O., and Horvitz, H. R. (1994) *Nature* 368, 597–608.
37. Yang, J., Korsmeyer, S. J. (1996) *Cell* 88, 386–401.
38. Yang, J., Lai, S., Sedlak, T. W., Oliva, Z. N., Yang, E., Wang, K., Khan, M. S., and Korsmeyer, S. J. (1994) *Nature* 369, 621–625.
39. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Science* 275, 405–413.
40. Hunter, J. J., Bond, B. L., and Parslow, T. G. (1996) *Cell* 84, 195–202.
41. Shortman, K., Vremec, D., and Egerton, M. (1991) *J. Exp. Med.* 172, 635–645.
42. Yang, J., Korsmeyer, S. J. (1996) *Cell* 88, 386–401.
43. Yang, J., Korsmeyer, S. J. (1996) *Cell* 88, 386–401.