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Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis

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Expression of the pro-apoptotic molecule BAX has been shown to induce cell death. While BAX forms both homo- and heterodimers, questions remain concerning its native conformation *in vivo* and which moiety is functionally active. Here we demonstrate that a physiologic death stimulus, the withdrawal of interleukin-3 (IL-3), resulted in the translocation of monomeric BAX from the cytosol to the mitochondria where it could be cross-linked as a BAX homodimer. In contrast, cells protected by BCL-2 demonstrated a block in this process in that BAX did not redistribute or homodimerize in response to a death signal. To test the functional consequence of BAX dimerization, we expressed a chimeric FKBP–BAX molecule. Enforced dimerization of FKBP–BAX by the bivalent ligand FK1012 resulted in its translocation to mitochondria and induced apoptosis. Caspases were activated yet caspase inhibitors did not block death; cytochrome *c* was not released detectably despite the induction of mitochondrial dysfunction. Moreover, enforced dimerization of BAX overrode the protection by BCL-X$_L$ and IL-3 to kill cells. These data support a model in which a death signal results in the activation of BAX. This conformational change in BAX manifests in its translocation, mitochondrial membrane insertion and homodimerization, and a program of mitochondrial dysfunction that results in cell death.

Keywords: apoptosis/BAX/caspases/dimerization/mitochondria

Introduction

Programmed cell death or apoptosis is critical for both the development and maintenance of tissues (Thompson, 1995). A distinct genetic pathway apparently shared by all multicellular organisms governs apoptosis. The BCL-2 family of proteins constitutes a central checkpoint within this pathway. This family possesses both pro-apoptotic (BAX, BAD, BID, BAK, BCL-X$_S$, BIK, BIM, HRK) and anti-apoptotic (BCL-2, BCL-X$_L$, BCL-W, MCL-1, A1) molecules (Farrow and Brown, 1996; Kroemer, 1997). The ratio of anti- to pro-apoptotic molecules such as BCL-2/BAX determines the response to a death signal (Oltvai *et al*., 1993). Moreover, induction of BAX or BAK expression will initiate death in the absence of any additional signal (Xiang *et al*., 1996; McCarthy *et al*., 1997). Induction of BAX results in a downstream program of mitochondrial dysfunction as well as activation of caspases.

Most BCL-2 family members bear a hydrophobic C-terminal sequence and are intracellular membrane proteins most convincingly localized to mitochondria, endoplasmic reticulum and nuclear membrane (Hockenbery *et al*., 1990; Krajewski *et al*., 1993; de Jong *et al*., 1994). *In vitro* targeting experiments indicated that this C-terminal sequence serves as a signal anchor segment responsible for targeting BCL-2 to mitochondria (Nguyen *et al*., 1993). Despite the presence of a similarly hydrophobic C-terminus in BAX, it does not display *in vitro* targeting to mitochondria (I.S.Goping, A.Gross, J.N.Lavoie, M.Nguyen, R.Jemmerson, K.Roth, S.J.Korsmeyer and G.C.Shore, submitted). Perhaps related to this finding is recent evidence that a portion of BAX can be found in the soluble fraction of cells (Hsu *et al*., 1997; Wolter *et al*., 1997). A green fluorescent protein (GFP)-tagged BAX molecule was noted to redistribute from the cytosol to mitochondria following induction of apoptosis by staurosporine (Wolter *et al*., 1997).

A striking characteristic of many BCL-2 family members is their propensity to form homo- and heterodimers. Such interactions can be demonstrated in yeast two-hybrid and *in vitro* binding assays as well as by co-immunoprecipitation from membrane-solubilized mammalian cells (Oltvai *et al*., 1993; Sedlak *et al*., 1995; Zha *et al*., 1996). The family shares homology in four conserved domains designated BH1, BH2, BH3 and BH4 (Farrow and Brown, 1996). The multi-dimensional NMR and X-ray crystallographic structure of a BCL-X$_L$ monomer indicated that the BH1, 2 and 3 domains are in close proximity and create a hydrophobic pocket presumably involved in interactions with other BCL-2 family members (Muchmore *et al*., 1996). The NMR analysis of a BCL-X$_L$–BAK BH3 peptide complex revealed both hydrophobic and electrostatic interactions between the BCL-X$_L$ pocket and a BH3 amphipathic α-helical peptide from BAK (Sattler *et al*., 1997). However, several studies have provided evidence suggesting that pro- and anti-apoptotic molecules can function independently of one another. Genetic evidence indicates that BAX and BCL-2 are each capable of regulating death in the absence of the other partner (Knudson and Korsmeyer, 1997). Moreover, mutants of BCL-X$_L$ have been defined that fail to bind BAX or BAK but still repress cell death (Cheng *et al*., 1996). Another study found that a BAX mutant missing its BH3 domain failed to dimerize with BCL-X$_L$ or BAX but could still counter the death repressor activity of BCL-X$_L$ (Simonian *et al*., 1996b). Recently, admixture of soluble fractions from murine and human thymocytes

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only displayed mixed-species homodimers following the addition of detergent, suggesting that BAX in the soluble fraction of cells may be monomeric (Hsu and Youle, 1997). Thus, uncertainty exists as to the native conformation of these molecules *in vivo* and whether monomers, dimers or higher order multimers represent the active moiety that regulates apoptosis.

Here we examine the native conformation of BAX before and after a death signal, and the role of BAX homodimers in apoptosis. We note that in susceptible cells the cytosolic, monomeric BAX translocates to mitochondria in response to a death signal where it is a homodimerized, integral membrane protein. In cells protected from death by BCL-2 or BCL-X_L, the cytosolic BAX fails to redistribute or homodimerize. Finally, we utilize a chimeric FKBP–BAX molecule to demonstrate that enforced BAX dimerization is sufficient to induce its membrane translocation, mitochondrial dysfunction and apoptosis.

Results

BAX homodimerizes and translocates to the mitochondria following a death signal

Multiple approaches were utilized to assess the location of the intracellular protein BAX. Disruption of interleukin-3 (IL-3)-dependent FL5.12 hematopoietic cells using isotonic lysis conditions kept mitochondria intact with a retained outer membrane. A substantial portion of BAX was found consistently in the soluble S100 fraction (S) representing the cytosol as well as the mitochondria-enriched heavy membrane (HM) fraction as documented by the mitochondrial markers (porin/VDAC, outer mitochondrial membrane; cytochrome *c* oxidase, inner mitochondrial membrane) (Figure 1A, lanes 1 and 3). The low speed pellet (P1), comprised of residual whole cells, nuclei and some mitochondria, also displays BAX. In contrast, the anti-apoptotic BCL-2 molecule resides predominantly in the mitochondrial-rich HM and P1 fraction and is not found in the soluble (S) fraction (Figure 1A). Immunofluorescent confocal microscopy of viable FL5.12 cells confirmed that BAX resides in both mitochondria and cytosol (I.S.Goping, A.Gross, J.N.Lavoie, M.Nguyen, R.Jemmerson, K.Roth, S.J.Korsmeyer and G.C.Shore, submitted).

Following a death stimulus, the withdrawal of IL-3, most (~70%) of BAX moves from the cytosol to the mitochondrial HM fraction (Figure 1A). In contrast, the distribution of BCL-2 does not change following a death signal. Moreover, following a death signal, most of the mitochondrial BAX converts from being alkali sensitive to alkali resistant, indicative of an integral membrane position (I.S.Goping, A.Gross, J.N.Lavoie, M.Nguyen, R.Jemmerson, K.Roth, S.J.Korsmeyer and G.C.Shore, submitted).

To assess the conformation of BAX in IL-3-dependent FL5.12 cells, we stably expressed a Myc-tagged FKBP–BAX chimeric molecule. Immunoprecipitation from the S100 fraction of such cells using an anti-Myc antibody (Ab) failed to co-precipitate any endogenous BAX (Figure 1B). However, when NP-40 non-ionic detergent was added to the S100 fraction, endogenous BAX was co-precipitable. This confirms the monomeric conformation suggested for S100, cytosolic BAX (Hsu and Youle, 1997). Given the capacity of non-ionic detergent to enable the homodimerization of BAX, assessing the conformation of membrane-bound BAX required an approach without detergent. Consequently, we utilized homo-bifunctional protein cross-linkers to assess the conformation of mitochondrial associated BAX. Intact mitochondria were treated with either the membrane-permeable disuccinimidyl suberate (DSS) or membrane-impermeable bis(sulfosuccinimidyl) suberate (BS^3) non-cleavable primary amine cross-linker (Figure 1C). In the presence of IL-3, only a minor portion of BAX could be cross-linked as a 42 kDa presumptive homodimer with BS^3, which recognizes the extramembranous component of proteins (Figure 1C, lane 2). However, a death signal resulted in an ~2-fold increase in BS^3-cross-linkable BAX homodimers after 4 h and an ~10-fold increase after 12 h of IL-3 deprivation, as demonstrated by immunoprecipitation and Western analysis (Figure 1C, lane 4). Moreover, the membrane-permeable DSS cross-linker also detected BAX homodimers after IL-3 withdrawal (Figure 1C, lane 5). Experiments with a reversible cross-linker, dithiobis (succinimidylpropionate) (DSP), provided further evidence that the 42 kDa species represents a BAX homodimer (not shown). Cross-linking revealed an additional BAX immunoreactive band of ~26 kDa that may represent an associated small protein or intrachain cross-link. A series of immunoreactive bands between 70 and 200 kDa may represent additional protein partners and/or higher order multimers of BAX (Figure 1C). Several prominent higher molecular weight bands also possessed BCL-2, suggesting the presence of comparable amounts of BAX–BCL-2 hetero-multimers before and after a death signal (Figure 1D).

BAX does not homodimerize or translocate to mitochondria following a death signal in cells protected by BCL-2

To assess the relevance of BAX translocation and dimerization to cell death, we asked whether this process occurred in cells protected from death stimuli by adequate levels of BCL-2 or BCL-X_L. In FL5.12-BCL-2 cells, BAX is present in the S100, cytosolic fraction but fails to redistribute from cytosol to mitochondria at multiple time points following a death signal (Figure 2A). BCL-2 in these cells, as in parental cells, principally localizes to the HM and P1 fractions. Moreover, the mitochondria show no increase in cross-linkable BAX homodimers following a death signal (Figure 2B). Finally, FL5.12-BCL-2 cells demonstrate only a minimal amount of cross-linked ~46 kDa, apparent BAX–BCL-2 heterodimers, but more abundant BAX–BCL-2 hetero-multimers (Figure 2C).

Enforced dimerization of chimeric FKBP–BAX by FK1012 induces apoptosis in 293 cells

The redistribution and homodimerization of BAX in susceptible but not protected cells represents an attractive correlate with death. We next wished to determine if enforced dimerization of BAX would, in and of itself, induce apoptosis. Chimeric FKBP–BAX molecules tagged with either a Myc or a Glu–Glu (EE) epitope were co-expressed in 293 cells. Treatment with 0.3 μM of the cell-permeant bivalent ligand FK1012 (Spencer *et al*., 1993; Graef *et al*., 1997) for 15 min markedly increased

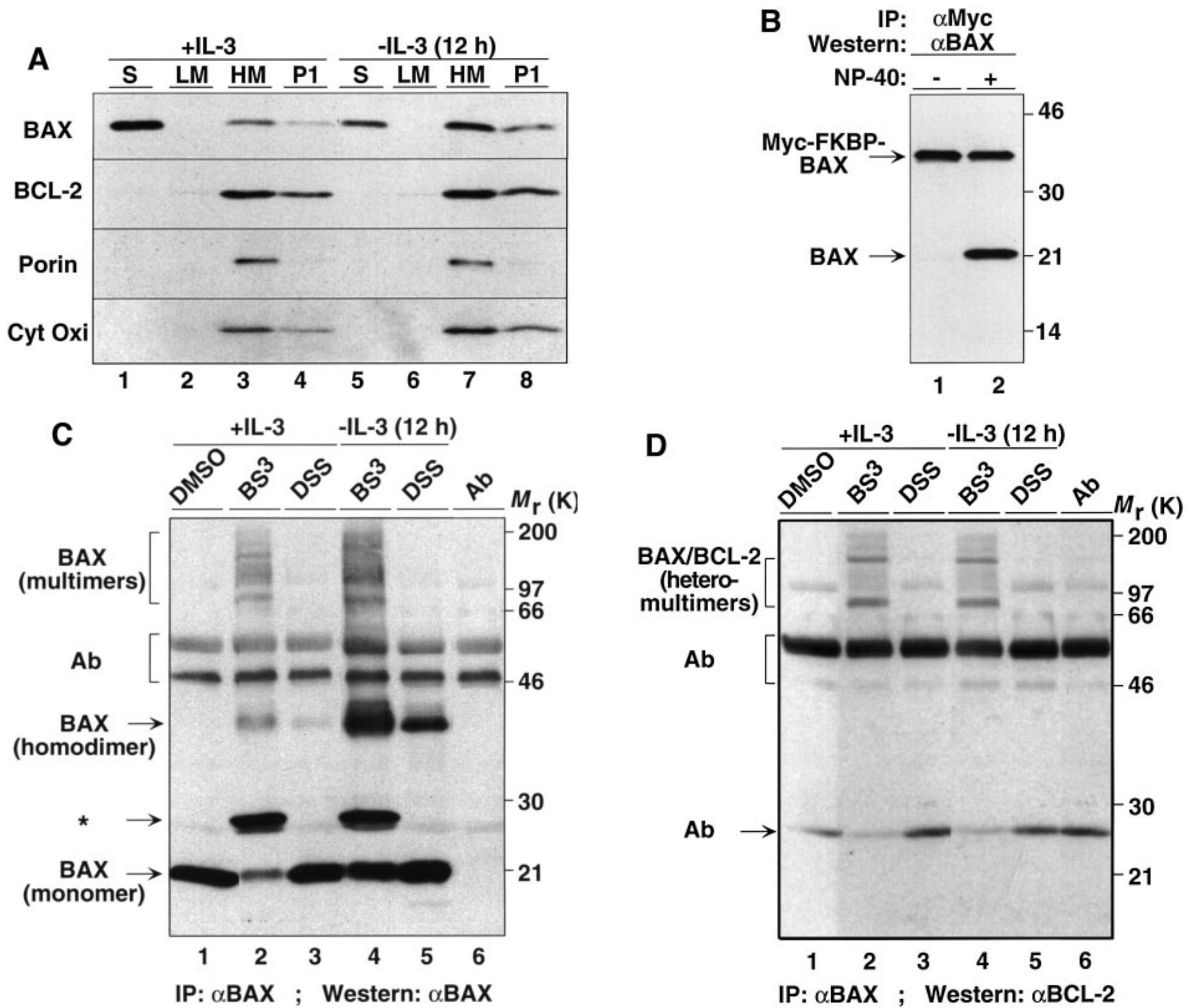

Fig. 1. Subcellular distribution and dimerization of BAX in FL5.12 cells. (**A**) Cytosol-to-heavy membrane redistribution of BAX following a death signal. FL5.12 cells in IL-3 (lanes 1–4) or deprived of IL-3 for 12 h (approximately the point of no return) (lanes 5–8) were suspended in isotonic buffer, homogenized and separated into soluble fraction (S), light membrane fraction (LM), heavy membrane fraction (HM) and low speed pellet (P1) by differential centrifugation. The fractions were analyzed by Western blot with anti-mBAX 651 Ab, anti-mBCL-2 3F11 mAb, anti-hPorin 31HL mAb (Calbiochem) and anti-cytochrome *c* oxidase subunit IV (Cyt Oxi) Ab. The P1 pellet contains residual whole cells, nuclei and mitochondria. The HM fraction is enriched for intact mitochondria. The LM fraction contains the endoplasmic reticulum and plasma membrane, and the soluble (S) fraction represents the cytosol. (**B**) Cytosolic BAX appears to be monomeric in the absence of detergent. The high speed soluble extract of FL5.12-BCL-X_L/Myc-FKBP–BAX cells without (lane 1) or with (lane 2) the addition of NP-40 was immunoprecipitated with anti-Myc 9E10 mAb (Santa Cruz) and analyzed by Western blot with anti-mBAX 651 Ab. (**C**) A marked increase in BAX homodimers following a death signal. Heavy membranes prepared from FL5.12 cells in IL-3 (lanes 1–3) or deprived of IL-3 for 12 h (lanes 4 and 5) were incubated in isotonic buffer and treated with the membrane-impermeable BS^3 (lanes 2 and 4) or membrane-permeable DSS (lanes 3 and 5) cross-linker, or with DMSO as a control (lane 1). After treatment, membranes were lysed, BAX was immunoprecipitated with anti-mBAX 4D2 mAb, size-fractionated by PAGE and analyzed by Western blot with anti-mBAX 651 Ab. The 4D2 mAb was run as a control (lane 6). The asterisk denotes an additional BAX immunoreactive 26 kDa band; Ab denotes IgG heavy chain. Immunoreactive cross-linked BAX species were confirmed by direct Western analysis of mitochondria and whole-cell lysates (not shown). (**D**) BCL-2 associates with BAX in mitochondria before and after a death signal. The blot shown in (C) was stripped and developed with anti-mBCL-2 3F11 mAb. Ab denotes IgG heavy or light chains.

the abundance of Myc/EE-FKBP–BAX homodimers (Figure 3A). To assess the effects of FKBP–BAX dimerization on the viability of 293 cells, we used a standard assay with a co-transfected luciferase reporter whose activity parallels the viability of cells (Wang *et al.*, 1996). Treatment of transfected 293 cells with 0.3 μM FK1012 for 4, 8 or 24 h dramatically increased cell death, reducing viability to 62, 52 or 51% of control cells, respectively (Figure 3B). Co-addition of 1 μM FK506, the univalent ligand, eliminated the enhanced death, indicating that induced apoptosis was due to homodimerization of FKBP–BAX. Another cell type, Rat-1a, demonstrated a similar response (not shown).

Enforced dimerization of FKBP–BAX results in its translocation to mitochondria and apoptosis, overriding the protection by BCL-X_L and IL-3

A more stringent test of the effectiveness of dimerization would be to assess cells protected by BCL-2 or BCL-X_L that stably express tolerated levels of FKBP–BAX. The soluble fraction from a FL5.12-BCL-X_L/Myc-FKBP–BAX stable clone treated with FK1012 for 15 min was separated by native polyacrylamide gel electrophoresis revealing a prominent gel shift of Myc-FKBP–BAX consistent with dimerization (Figure 4A). A molar excess of FK506 eliminated the shift, providing further evidence that the bivalent ligand promoted dimerization in the absence of

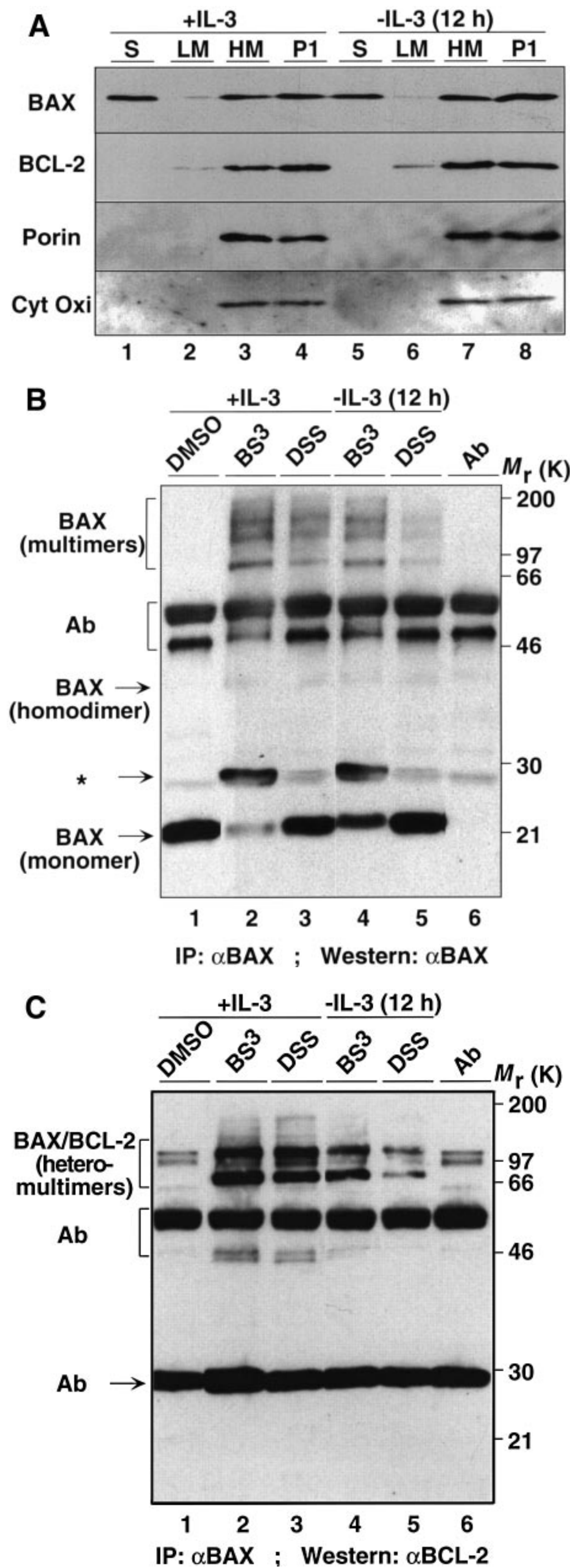

Fig. 2. Subcellular distribution and dimerization of BAX in FL5.12 cells protected by BCL-2. (**A**) BAX does not redistribute in FL5.12-BCL-2 cells following a death signal. The experimental procedure was identical to that in Figure 1A. (**B**) No increase in BAX homodimers in FL5.12-BCL-2 cells following a death signal. The experimental procedure was identical to that in Figure 1C. (**C**) FL5.12-BCL-2 cells demonstrate abundant BAX–BCL-2 hetero-multimers. The blot shown in (B) was stripped and redeveloped with anti-hBCL-2 6C8 mAb.

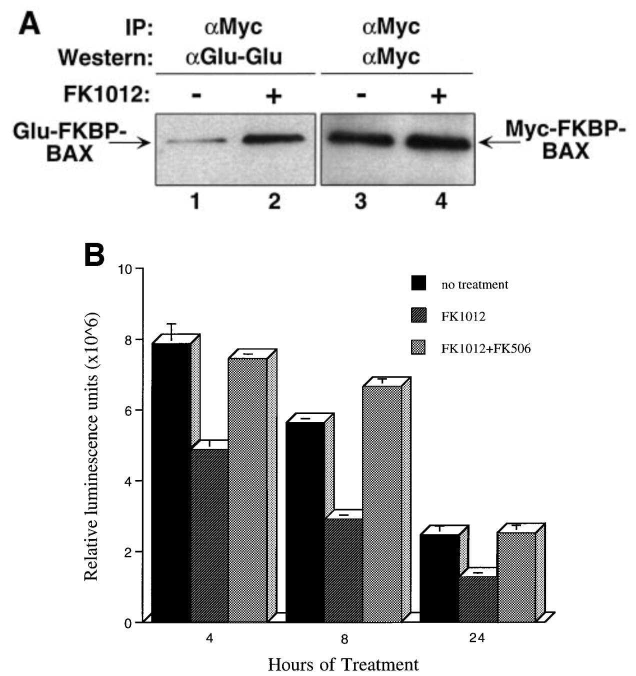

Fig. 3. Enforced dimerization of chimeric FKBP–BAX by FK1012 induces apoptosis. (**A**) FK1012 induces dimerization of FKBP–BAX in 293 cells. Myc-FKBP–BAX and Glu-FKBP–BAX were co-expressed transiently in 293 cells. Cells were treated with 0.3 μM FK1012H2 for 15 min (lanes 2 and 4) or not treated (lanes 1 and 3) before extraction. Anti-Myc 9E10 mAb (Santa Cruz) immunoprecipitates were immunoblotted with anti-Glu–Glu mAb (left panel) or with anti-Myc mAb (right panel). (**B**) Time course of FK1012-induced apoptosis in 293 cells expressing FKBP–BAX. 293 cells transiently expressing Myc-FKBP–BAX and a luciferase reporter (Wang *et al*., 1996) were treated with 0.3 μM FK1012H2 alone or in combination with 1 μM FK506 for the indicated times. After treatment, cells were extracted and assayed for luciferase activity. Cell viability is reflected by the percentage of luciferase activity (relative luminescence units) in treated cells compared with non-treated cells (Wang *et al*., 1996).

detergent. To determine if dimerization of BAX alters its localization, cells were subfractionated using isotonic lysis conditions. The distribution of FKBP–BAX parallels that of endogenous BAX, including its presence in the soluble cytosolic fraction (Figure 4B, lanes 1–4). Strikingly, 1 h post-treatment with FK1012, a complete redistribution of FKBP–BAX from cytosol to heavy membranes was noted (Figure 4B, lanes 5–8). Despite the modest amount of additional FKBP–BAX, compared with endogenous BAX, the FK1012-enforced dimerization and translocation proved capable of overriding the protection by BCL-X_L and IL-3 (Figure 4C). FK1012-induced death displays the hallmark morphologic features of apoptosis. A molar excess of FK506 eliminated this enhanced death (Figure 4C). The death of Jurkat T-cell clones stably expressing FKBP–BAX was also enhanced by FK1012 which, combined with the enhanced death of 293 and Rat-1a cells (Figure 3B), indicates that this effect is not lineage restricted.

Enforced dimerization of FKBP–BAX induces mitochondrial dysfunction but not detectable release of cytochrome c

Since enforced dimerization of BAX results in its translocation to mitochondria, we asked whether BAX dimers might induce alterations in mitochondrial function

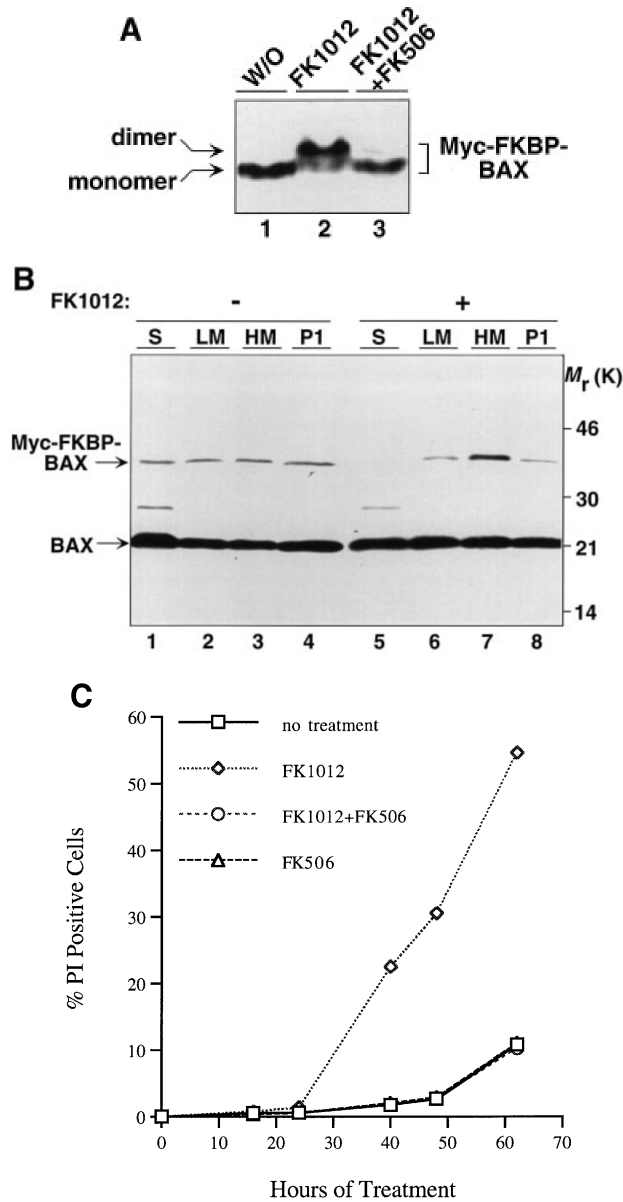

Fig. 4. FK1012 induces apoptosis in FL5.12 cells stably expressing FKBP–BAX and protected by BCL-X_L. (**A**) FK1012 induces dimerization of FKBP–BAX in FL5.12-BCL-X_L cells. FL5.12-BCL-X_L/Myc-FKBP–BAX cells were treated with 0.3 μM FK1012H2 alone (lane 2) or in combination with 1 μM FK506 (lane 3) for 15 min. The soluble fraction of cells was fractionated by native polyacrylamide gel electrophoresis and analyzed by Western blot with anti-Myc 9E10 mAb. (**B**) FK1012 induces cytosol-to-heavy membrane redistribution of FKBP–BAX in FL5.12-BCL-X_L cells. Cells were treated with 0.3 μM FK1012H2 for 1 h (lanes 5–8) or not treated (lanes 1–4), homogenized and subfractionated as described in Figure 1A. (**C**) FK1012 induces apoptosis in FL5.12-BCL-X_L cells expressing FKBP–BAX. Cells were cultured with 0.3 μM FK1012H2, 1 μM FK506 or a combination of both in the presence of IL-3. Viability was determined by propidium iodide at designated time points.

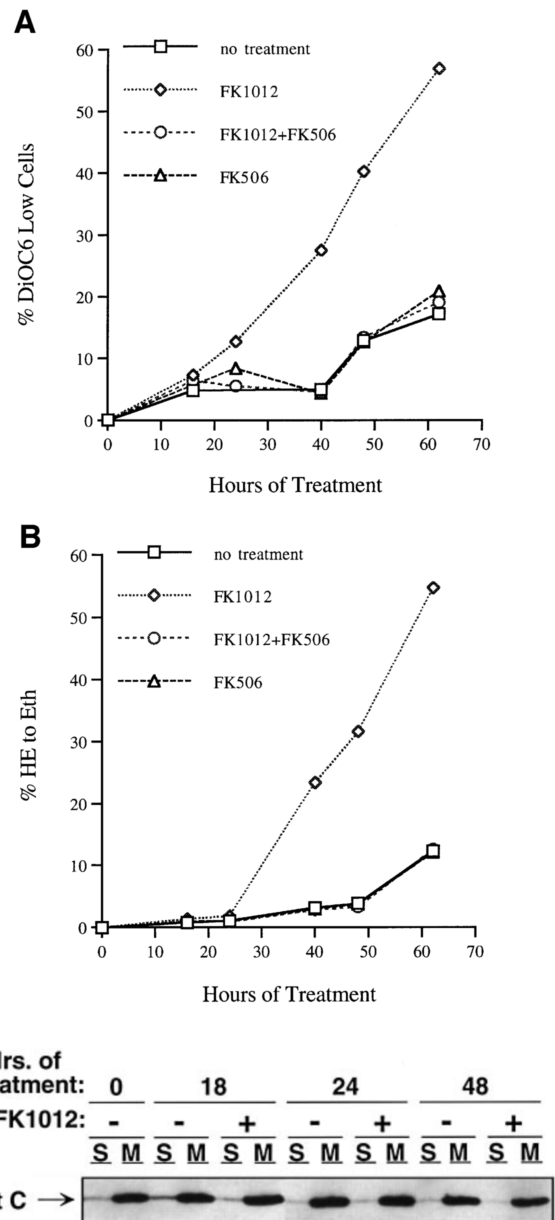

Fig. 5. Enforced dimerization of BAX results in mitochondrial dysfunction. (**A**) FK1012 induces reduction of the mitochondrial membrane potential ($\Delta\Psi_m$) in FL5.12-BCL-X_L/Myc-FKBP–BAX cells. Cells were treated with FK1012 and FK506 as described in Figure 4C. Aliquots of 1×10^6 cells were incubated with 40 nM 3,3′-dihexyloxacarbocynine iodide [$DiOC_6(3)$] and analyzed by cytofluorometry. The precentages reflect the reduction of $\Delta\Psi_m$ [$DiOC_6(3)$]. (**B**) FK1012 induces production of ROS in FL5.12-BCL-X_L/Myc-FKBP–BAX cells. The experimental procedure was identical to that in (A), except that cells were incubated with 2 μM hydroethidine. The precentages reflect ROS production (hydroethidine converted to ethidium). Data shown are representative of three experiments. (**C**) FK1012 treatment does not result in cytochrome *c* release from the mitochondria. FL5.12-BCL-X_L/Myc-FKBP–BAX cells were cultured in the absence or presence of 0.3 μM FK1012, homogenized at designated time points in isotonic buffer and subfractionated using differential centrifugation. The soluble (S) and heavy membrane (M) fractions were analyzed by Western blot with anti-cytochrome *c* (Cyt C) mAb (Pharmingen).

(Kroemer, 1997). Similarly to a system that induced expression of wild-type BAX (Xiang *et al.*, 1996), we noted a reduction in $\Delta\Psi_m$ within 24 h of enforced BAX dimerization (Figure 5A). The production of reactive oxygen species (ROS) as measured by hydroethidine, which principally detects superoxide anion, followed closely (Figure 5B). However, the reduction in $\Delta\Psi_m$ and production of ROS were not accompanied by a detectable release of cytochrome *c* from the mitochondria (Figure 5C).

Enforced dimerization of FKBP–BAX results in caspase activation but may not require this activity to induce death

To determine whether caspases were activated during BAX dimerization-induced death, the cleavage of specific fluorogenic peptide substrates DEVD-AFC for the caspase-3-like subset and LEHD-AMC for the caspase-9-like subset was measured (Thornberry *et al.*, 1997). Enforced dimerization of FKBP–BAX resulted in cleavage of both DEVD and LEHD (Figure 6A and B). Both enzymatic activities were blocked by pre-treatment with the pan-caspase inhibitor, zVAD-fmk. To assess whether activated caspases were required for FKBP–BAX-induced death, viability was measured following FK1012 treatment in the presence of zVAD-fmk. Strikingly, while caspase inhibition retarded the initiation, it did not prevent cell death (Figure 6C). It is of interest that zVAD-fmk had no effect on the parameters of mitochondrial dysfunction, including the reduction in $\Delta\Psi_m$ and the production of ROS (data not shown).

Discussion

These data demonstrate that BAX undergoes a conformational change in response to a death signal which includes its homodimerization and mitochondrial integral membrane insertion. In addition, a physiological death signal, the withdrawal of a survival factor, resulted in the translocation of BAX from cytosol to mitochondria in FL5.12 cells, supporting a recent observation of BAX redistributing to mitochondria in Cos-7 and L929 cells following treatment with staurosporine (Wolter *et al.*, 1997). Beyond this, the FKBP–BAX/FK1012 strategy argues that homodimerization of BAX enables its mitochondrial translocation and is sufficient to induce apoptosis. An examination of the cytosolic fraction utilizing tagged BAX molecules and cross-linkers did not detect any BAX homodimers in viable or IL-3-deprived cells (data not shown). These studies suggest a model in which cytosolic BAX represents an inactive monomer, perhaps reflecting a spontaneous inert configuration or conceivably held in that conformation by a tethering chaperone protein (Figure 7). A death stimulus would induce a conformational change in BAX and/or release BAX from a cytosolic chaperone, enabling its active configuration as a dimerized integral mitochondrial membrane protein.

Conversely, cross-linker analysis of cells protected by BCL-2 or BCL-X_L indicates that much of the BAX inherent to mitochondria is in BAX–BCL-2 hetero-multimers and much of overexpressed BCL-2 in homo-multimers (data not shown). Cytosolic BAX fails to translocate or homodimerize in the presence of protective levels of BCL-2, indicating a block at that step or at the death signal (Figure 7). However, the FKBP–BAX/FK1012 paradigm indicates that if BAX dimers can be induced they will eventually kill cells despite the presence of excess BCL-X_L and IL-3 survival factor. It is also possible that homodimerization of BAX functions as an amplification step.

Enforced dimerization of BAX results in a program of mitochondrial dysfunction which includes altered transmembrane potential and ROS production (Figure 5). However, cytochrome *c* was not released substantially,

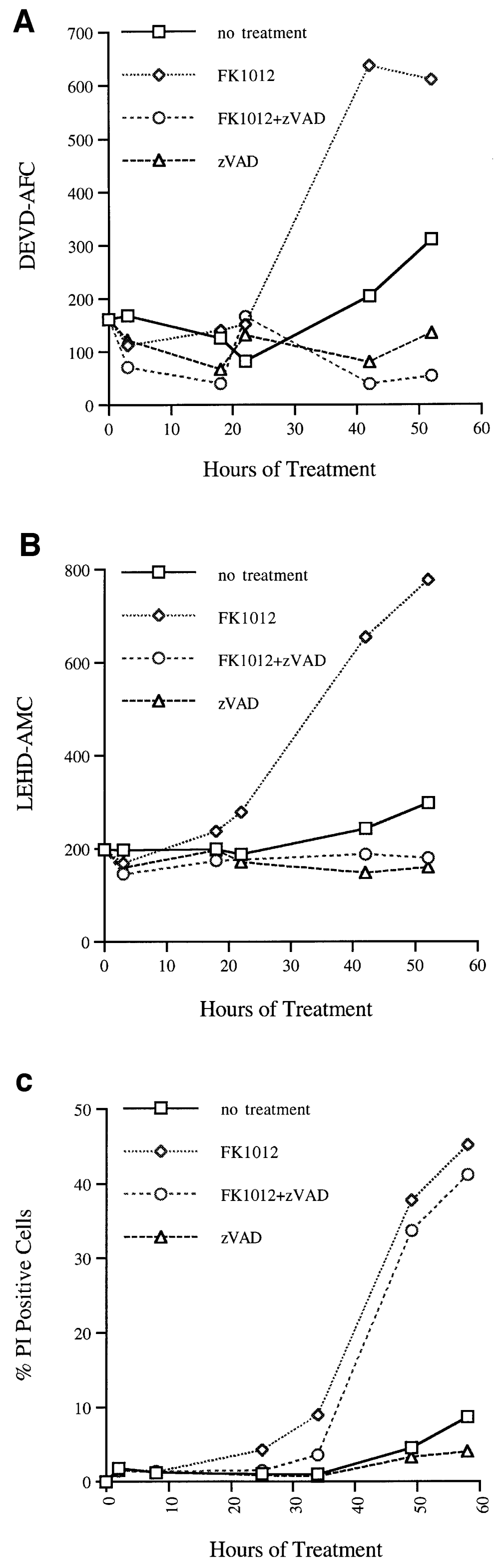

Fig. 6. FK1012 induces activation of caspases. (**A**) FL5.12-BCL-X_L/Myc-FKBP–BAX cells were cultured with 0.3 μM FK1012 or 50 μM zVAD-fmk or a combination. Caspase-3-like activity was measured using the fluorogenic substrate DEVD-AFC. (**B**) As in (A), but caspase-9-like activity was measured using the fluorogenic substrate LEHD-AMC. (**C**) The caspase inhibitor zVAD-fmk retards the onset, but does not prevent cell death induced by FK1012. FL5.12-BCL-X_L/Myc-FKBP–BAX cells were cultured as above and viability was determined by propidium iodide exclusion. Data shown are representative of three experiments.

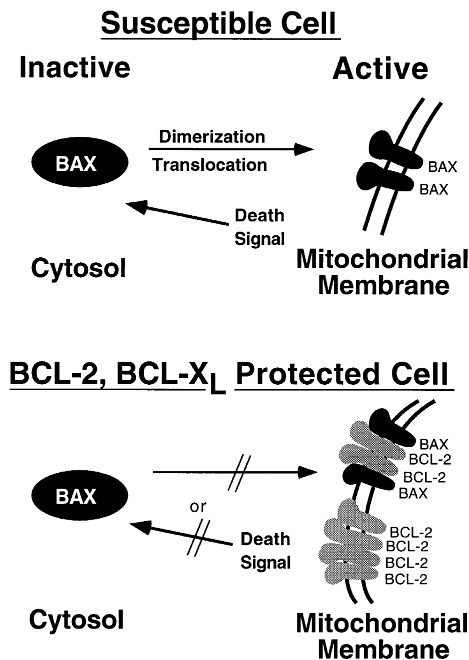

Fig. 7. Model of BAX translocation and dimerization in susceptible cells and those protected from death.

unlike in some other apoptotic programs (Li *et al.*, 1997). However, both caspase-3 and caspase-9 activity was induced by BAX dimerization, perhaps reflecting a cytochrome *c*-independent mechanism. While a pan-caspase inhibitor eliminated measurable caspase activity, it could not save the cells, nor did it alter the program of mitochondrial dysfunction that may be responsible for this death. We have shown previously that induction of wild-type BAX results in a similar program of mitochondrial dysfunction (Xiang *et al.*, 1996), although it may also eventually release cytochrome *c* (Pastorino *et al.*, 1998). In addition, the removal of IL-3 from FL5.12 cells, which results in the translocation of BAX to mitochondria and an increase of BAX dimers in mitochondria (Figure 1), also eventually results in mitochondrial dysfunction (Vander Heiden *et al.*, 1997). Taken together, these results suggest that enforced dimerization appears to activate BAX in a fashion that predominantly mimics the effect of a death signal.

The ability of BAX dimers to kill cells is consistent with genetic evidence that BAX can function independently of BCL-2 (Knudson and Korsmeyer, 1997). It is also compatible with rare BCL-X_L mutants (Cheng *et al.*, 1996) and BAX mutants (Simonian *et al.*, 1996a; Zha *et al.*, 1997) which fail to heterodimerize but still function. Moreover, we generated a point mutant of BAX that fails to form homodimers or heterodimers in classic binding assays, yet still inserts into mitochondrial membranes as a cross-linkable dimer and induces apoptosis (K.Wang and S.J.Korsmeyer, unpublished observations). The dimerization and mitochondrial membrane insertion of BAX may relate to the ability of BAX to form distinct ion conductive channels (Antonsson *et al.*, 1997; Reed, 1997; Schlesinger *et al.*, 1997), and ultimately result in the mitochondrial dysfunction that accompanies apoptosis. Alterations of BAX in response to a death signal appear to represent the activation of this pro-apoptotic molecule (Figure 7).

Materials and methods

Subcellular fractionation

FL5.12 cells were washed once in phosphate-buffered saline (PBS), resuspended in isotonic buffer A (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5) supplemented with protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotonin], and homogenized using a polytron homogenizer (Brinkmann Instruments) at setting 6.5 for 10 s. Nuclei and unbroken cells were separated at 120 *g* for 5 min as the low speed pellet (P1). This supernatant was centrifuged at 10 000 *g* for 10 min to collect the heavy membrane pellet (HM). This supernatant was centrifuged at 100 000 *g* for 30 min to yield the light membrane pellet (LM) and final soluble fraction (S). Hypotonic lysis was performed by resuspending the cells in hypotonic buffer [10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT)] and homogenized with a Dounce homogenizer.

Cross-linking

The heavy membrane fraction (0.5 mg of protein) was resuspended in buffer A, and BS^3 (in 5 mM sodium citrate buffer, pH 5.0; Pierce) or DSS (in dimethyl sulfoxide; Pierce) was added from a 10-fold stock solution to a final concentration of 10 mM (Partis *et al.*, 1983). After incubation for 30 min at room temperature, the cross-linker was quenched by addition of 1 M Tris–HCl pH 7.5 to a final concentration of 20 mM. After quenching, membranes were lysed in RIPA buffer and cleared by centrifugation at 12 000 *g*. Part of this lysate was analyzed directly by SDS–PAGE and Western blot and the remainder was immunoprecipitated with anti-mBAX 4D2 monoclonal antibody (mAb) and then size-fractionated by SDS–PAGE and analyzed by Western blot developed with anti-mBAX Ab (651), anti-mBCL-2 mAb (3F11) or anti-hBCL-2 mAb (6C8).

Expression plasmids

Human FKBP12 (Liu *et al.*, 1991) with a Myc (Evan *et al.*, 1985) or Glu–Glu (Martin *et al.*, 1995) epitope tag was ligated in-frame to the N-terminus of full-length murine BAX cDNA in pcDNA3 (Invitrogen) under the cytomegalovirus immediate early promoter to create the pFKBP–BAX plasmids.

Transient transfection system

Cell lines used included 293, an embryonic kidney line, and Rat-1a, a subline of the rat fibroblast cell line Rat-1 (Evan *et al.*, 1992). Transient transfections of cells and luciferase assays were performed as described (Wang *et al.*, 1996). Briefly, cells were allowed to grow to ~80% confluence in 12-well plates before transfection. The luciferase reporter plasmid (0.1 μg) was mixed with 0.1 μg of pFKBP–BAX plasmid and 3 μl of lipofectamine (Gibco-BRL) in a volume of 0.5 ml per transfection for 5 h. Twenty hours following transfection, cells were treated with 0.3 μM FK1012H2 (Graef *et al.*, 1997) alone or in combination with 1 μM FK506 for 4, 8 or 24 h, lysed and assayed for luciferase activity (luciferase assay system, Promega).

Stable transfection system

Cell lines used included FL5.12-BCL-X_L, an IL-3-dependent murine early hematopoietic cell line, and Jurkat, a human T-cell leukemia line. FL5.12-BCL-X_L cells were maintained in 10% fetal calf serum supplemented with 10% WEHI-3B conditioned media as a source of IL-3 (Lee *et al.*, 1982). Selection of stable transfectants was performed as described (Oltvai *et al.*, 1993).

A total of 1×10^5 FL5.12-BCL-X_L cells in the presence of IL-3 or Jurkat T cells were cultured with 0.3 μM FK1012H2 (1 mM stock solution in ethanol) alone or in combination with 1 μM FK506 (5 mM stock solution in methanol). Viability was determined at designated time points by propidium iodide exclusion. For mitochondrial potential and intracellular ROS production, 5×10^5 cells were incubated for 15 min at 37°C with 3,3′-dihexyloxacarbocynine iodide [$DiOC_6(3)$, 40 nM] or hydroethidine (2 μM; Molecular Probes) followed by FACScan (Becton Dickinson) analysis.

Caspase-3 and -9 activity assay

Cells were lysed in buffer B containing 5 mM EGTA, 5 mM EDTA, 10 μM digitonin, 2 mM DTT and 25 mM HEPES, pH 7.4. The lysates were clarified by centrifugation and the supernatants were used for enzyme assays. Enzymatic reactions were carried out in buffer B containing 20 μg of protein and 50 μM acetyl-Asp-Glu-Val-Asp-aminotrifluoromethylcoumarin (DEVD-AFC) or 50 μM acetyl-Leu-Glu-His-Asp aminomethylcoumarin (LEHD-AMC). The reaction mixtures were incubated at 37°C for 30 min, and fluorescent AFC or AMC formation was measured at excitation 400 nm and emission 505 nm using a FL500 microplate fluorescence reader (Bio-Tek).

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