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The Bcl-2 family proapoptotic protein, Bax, redistributes to the mitochondrion in response to varied stimuli, triggering loss of mitochondrial integrity and apoptosis. Suppression of MAPK kinase (MEK1) by the reagent UO126 in activated T cells maintained in the cytokine IL-2 disrupts cytoplasmic localization of Bax and cell survival. UO126 triggers mitochondrial translocation of ectopically expressed Bax-GFP, and both UO126 and dominant negative MEK1 (DN-MEK1) trigger increased apoptosis in Bax-GFP-expressing T cell lines. Because inhibition of PI3K or its target Akt also triggers mitochondrial translocation of Bax in T cells and apoptosis in Bax-transfected cell lines, we generated Bax deletion mutants to identify the region(s) that confers sensitivity to regulation by MEK1 and Akt. A deletion mutant (BaxΔ1–171) without the C terminus mitochondrial targeting sequence or an Akt target site (Ser184) localizes to the cytoplasm and triggers low level apoptosis that is enhanced by DN-Akt or DN-MEK1. A construct that lacks the first 29 aa (Bax629) largely localizes to mitochondria, is highly apoptogenic, and is not inhibited by Akt or MEK1. Furthermore, Bax629 overcomes IL-2-dependent survival in a T cell line, whereas Bax triggers comparatively low levels of apoptosis in these cells. Cytoplasmic localization and regulation by MEK1 and Akt are restored in a mutant deleted of the first 13 aa (BaxΔ613). Taken together, our results identify a region in the Bax N terminus that determines cellular localization regulated by MEK- and Akt-dependent signaling in T cells. The Journal of Immunology, 2004, 173: 6220–6227.

In mammalian cells apoptosis can be initiated by members of the TNF receptor family (1, 2) or by pathways integrated via the mitochondrion (3, 4). Members of the Bcl-2 family, which comprises both pro- and antiapoptotic proteins, are the principal regulators of mitochondrial integrity (5). Many proapoptotic members of this family are localized in the cytoplasm and translocate to the mitochondrion to initiate apoptotic cascades. The redistribution of proapoptotic proteins usually culminates in the loss of integrity of the mitochondrial outer membrane, culminating in the release of apoptogenic intermediates that propagate apoptotic pathways.

The Bcl-2 family proapoptotic protein Bax is critical for peripheral T cell homeostasis (6), and its overexpression results in decreased T cell number and survival (7). A combined deficiency of Bax and Bak compromises mitochondrionally regulated death pathways in T cells (6). Lymphocyte survival is regulated by cytokines, which activate multiple signal transduction pathways via interactions through the cytoplasmic domains of their receptor subunits (8–10). Thus, IL-2 receptor engagement results in the activation of MAPK kinase (MEK1) (11, 12) and PI3K signaling (12, 13) in T cells. MEK1 has been previously implicated in protection from various apoptotic signals. The mechanisms range from transcriptional or post-translational regulation of proteins (14–17) to the inhibition of apoptotic events after cytochrome c release (18, 19). The antiapoptotic function of PI3K has been described in multiple systems, and the serine-threonine kinase PKB/Akt is a key downstream target of PI3K in many models of suppression of apoptosis (20).

MEK1 or Akt regulation of Bax has been reported in diverse cellular systems (21–23), and the direct Akt-mediated phosphorylation of Bax as a mechanism of regulating its function has been recently demonstrated (23). However, most reports favor the model that Bax localization is regulated via its interactions with molecules such as 14-3-3 and Ku70 (24, 25) and other unidentified factors. A stretch of hydrophobic amino acids that may function as a mitochondrial localization sequence or transmembrane domain is present in the C-terminal region of Bax (26, 27). Additionally, various regions in the N terminus (25, 28) are implicated in the cytoplasmic sequestration of Bax. Thus, the multiple regulatory domains present in the molecule most likely facilitate tissue-specific regulation of Bax.

In one approach to understanding the regulation of Bax function, we have attempted to identify domains in Bax that confer sensitivity to regulatory signals that promote T cell survival. In this context we identify a region (spanned by aa 13–29) in the N terminus that is required for MEK1- and Akt-dependent regulation of Bax. Based on the analysis of various N and C terminus deletion mutants, we propose that MEK1 and/or Akt signaling requires the Bax N terminus to over-ride mitochondrial localization cues to sequester Bax in the cytosol of T cells.
Materials and Methods

Cells and reagents

The murine T cell lines 2B4 and d11S and the human lymphoblastoid cell line Jurkat were used in these experiments. CTEV-2, a murine T cell line dependent on exogenous IL-2 for survival, was also used in some experiments. The generation of activated T cells has been described previously (17). Abs to phosphorylated Akt (pAkt), pERK, Bax, and Bcl-xL were obtained from Cell Signaling Technology (Beverly, MA). Abs to Bax, inhibitor of apoptosis protein-1 (IAP-1), IAP-2, p38 MAPK, ERK, GFP, p65 NF-kB, and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Abs to GFP, cytochrome oxidase (Cox-4), cytochrome c, and Bcl-2 interacting mediator of cell death were obtained from BD Biosciences (Franklin Lakes, NJ). Digitonin was obtained from Sigma-Aldrich (St. Louis, MO), and all other chemicals were purchased from Calbiochem (San Diego, CA).

Plasmids

The mammalian expression vector encoding murine Bax was obtained from P. Vandenabeele (University of Ghent, Zvignaarde, Belgium). The Bax-GFP construct and the deletion mutants were made by PCR amplification and subcloning into the enhanced pGFP-N1 vector (BD Clontech, CA). The sequences of all constructs were verified by automated sequencing (International Center for Genetic Engineering and Biotechnology, New Delhi, India). CA-Akt, DN-Akt1 (K179M), and CA-MEK1 (Upstate, Lake Placid, NY) were also used. The DN caspase-9 construct was a gift from C. Vandenabeele (University of Ghent, Zvignaarde, Belgium).

The mammalian expression vector encoding murine Bax was obtained from P. Vandenabeele. For transient transfections, the expression vectors were mixed with Lipofectamine 2000 (Invitrogen) and lipofected into the cell lines at 25%. The cells were harvested, separated into cytosolic (Cyt) or mitochondrial (Mito) fractions, and analyzed by Western blot analysis for the distribution of Cox-4 and p38MAPK.

Whole cell lysates (0.5 × 10⁶ cells) or samples obtained from fractionation procedures were resolved by SDS-PAGE and analyzed by Western blot analysis using standard protocols (17). Densitometric analysis was performed using Image gauge software version 3.0 (National Institutes of Health). The fold change in expression was calculated relative to the loading control, which was usually p38MAPK in most experiments. In densitometric analysis of the subcellular distribution of the Bax deletion mutants, values represent the band intensity relative to the total intensities of the mitochondrial and cytosolic fractions. Where calculated, these values are entered below the blots in the figures.

Transfections and induction of apoptosis

Electroporation and analysis of apoptotic nuclear damage in GFP-transfected cells using Hoechst 33342 have been described previously (29). Routinely, transfection efficiencies ranged between 35 and 40% in the 2B4 and d11S cell lines, as estimated by the number of GFP-positive cells. In all transfection-based experiments, the analysis was designed to score only GFP-positive cells, thereby minimizing variations that may arise from differences in transfection efficiencies across multiple experiments. For transfections, 1 µg of GFP, 3 µg of Bax-GFP, or p65 NF-kB; 6 µg of CA-Akt, CA-MEK1, DN-caspase-9, or Bcl-2; and 8 µg of DN-Akt or DN-MEK1 were used. U0126 (10 µM) or LY294002 (10 µM) was added 12 h post-transfection. For UV treatment, cells were exposed to UV for 10 s, washed once with centrifugation, and continued in culture for 18 h. For analysis of T cell blasts, day 5 activated T cells were washed twice with PBS to remove bound IL-2 and pretreated with 40 µM U0126 for 30–45 min, followed by the addition of IL-2. Apoptotic nuclear damage was assessed 18 h after initiation of culture unless specified otherwise. All experiments were performed a minimum of three times unless specified otherwise.

Subcellular fractionation

A digitonin-based buffer was used to separate the membrane (mitochondrial) fraction from the soluble (cytoplasmic) fraction of cell lysates. Briefly, 10 × 10⁶ cells were pelleted by centrifugation, the pellet was resuspended in a digitonin-based buffer (40 µg/ml digitonin in 120 µM KCl, 10 mM NaCl, 1 mM KH₂PO₄, and 20 mM HEPES-Tris, pH 7.2, supplemented with protease inhibitors), and samples were kept on ice for 15 min. Samples were clarified by centrifugation at 7,500 × g to remove cell debris, followed by a centrifugation at 10,000 × g for 15 min. All manipulations were performed at 4°C. The resulting pellet was enriched in mitochondria, as confirmed by the mitochondrial marker protein Cox-4, and the supernatant was representative of the cytoplasmic fraction, confirmed by staining for Akt or NF-κB.

Cytochrome c release from isolated mitochondria

2B4 cells (20 × 10⁶) were washed once with chilled PBS and resuspended in 200 µl of intracellular medium (ICM; 1 mM KH₂PO₄, 120 mM KCl, 10 mM NaCl, and 20 mM HEPES, pH 7.2) containing 40 µg/ml digitonin (5% stock of digitonin in DMSO was made fresh for each assay). Samples were incubated on ice for 30 min, with gentle tapping of the tube at 5-min intervals to aid lysis. The samples were centrifuged at 700 × g for 5 min at 4°C to remove cell debris. The pellet was discarded, and the supernatant was subjected to another centrifugation at 10,000 × g for 15 min at 4°C. The pellet, which represents the mitochondrial fraction, was washed once with ICM and finally resuspended in 70 µl of ICM and used for subsequent analysis. Thirty microliters of each fraction was incubated with 200 ng of either mouse-specific binding protein (MBP) or MBP-Bax protein at 30°C for 20 min in a water bath. At the end of the incubation, the samples were centrifuged at 10,000 × g for 15 min at 4°C. Equivalent volumes of pellet (P) and the supernatant (Supn) fractions were boiled in 5× SDS lysis buffer, resolved on 12% SDS gels, and probed for cytochrome c and Cox-4 by Western blot analysis.

FIGURE 1. MEK1 regulates Bax translocation in activated T cells. A, Day 5 T cell blasts were washed and continued in culture in the presence (+IL-2) or the absence (No IL-2) of IL-2. After 6 h, cells from both conditions were harvested, separated into cytosolic (Cyt) or mitochondrial (Mito) fractions, and analyzed by Western blot analysis for the distribution of Bax and Cox-4, as described in Materials and Methods. Values below the blots represent the densitometric estimation of protein relative to the total intensity of the mitochondrial and cytosolic fractions. B, T cell blasts cultured in IL-2 were treated with 40 µM U0126 for 4 h. Total cell lysates were analyzed by Western blot analysis for the levels of phosphorylated ERK-1/2, and the expression of total ERK was used to establish parity of loading. C, T cell blasts were cultured as described in B. After 4 h, subcellular fractions were assessed for Bax expression by Western blot analysis. The purity of the fractions was confirmed by the distribution of Cox-4, and p38MAPK was used as the loading control (LC). D, Activated T cells were cultured with IL-2 in the presence or the absence of 40 µM U0126 for 24 h and scored for apoptotic nuclear damage.
Results

MEK1 regulates the cytoplasmic localization of Bax in activated murine T cells

In activated T cells that are sustained in culture by cytokines such as IL-2, Bax is normally localized in the cytoplasm (Fig. 1A). Cytokine deprivation triggers the mitochondrial translocation of Bax (Fig. 1A) and culminates in apoptosis (data not shown). Disruption of IL-2-dependent activation of MEK1 signaling (assessed by the loss of phosphorylation of its downstream target, ERK-1/2) using the reagent UO126 (Fig. 1B) triggered a redistribution of Bax to mitochondria (Fig. 1C). The distribution of the inner mitochondrial membrane protein Cox-4 was used to establish the purity of the mitochondrial fraction. As can be seen from the estimation of band intensities by densitometry, the amount of Bax in the mitochondrial fraction was 3-fold higher in cells treated with UO126 than in cells cultured with IL-2 alone. Additionally, UO126 attenuated, in part, IL-2-mediated survival of T cells (Fig. 1D). To further explore the molecular mechanism of MEK1 regulation of the subcellular distribution of Bax, we screened for T cell lines in which ectopically expressed Bax was regulated by cytoplasmic sequestration.

Ectopically expressed Bax is poorly apoptogenic in 2B4 and d11S cell lines

Ectopic expression of Bax induced low levels of apoptosis in the 2B4 T cell hybridoma (Fig. 2A, ■) compared with the human lymphoblastoid cell line Jurkat (Fig. 2A, □). The fusion protein, GFP-tagged Bax, was detected at the appropriate Mr (48 kDa) indicative of equivalent expression (Fig. 2A, inset, lanes 2 and 3, respectively). High endogenous levels of antiapoptotic proteins of the Bcl-2 family would render cells resistant to Bax-induced apoptosis. However, the levels of the antiapoptotic proteins, Bcl-xL, Bcl-2 (Fig. 2B), and the caspase antagonists, the IAPs, were comparable in the two cell lines (Fig. 2B). Bax-induced apoptosis in another T cell line, d11S, was marginally higher than that observed in 2B4 cells, but was substantially lower than that triggered in Jurkat cells (Fig. 2C). From a comparison of the expression of proapoptotic proteins in the Jurkat, d11S, and 2B4 cell lines, it was apparent that the expression of Bax or molecules such as Bcl 2 interacting mediator of cell death, which associate with Bax at the mitochondrial membrane, was higher in 2B4 and d11S than in the Jurkat cell line (Fig. 2D).

Resistance to Bax-induced apoptosis could also arise from defects in apoptotic signaling pathways that are regulated by the mitochrondion. 2B4 cells, however, were susceptible to UV- or etoposide-triggered apoptosis (Fig. 3A), and dexamethasone-induced apoptosis was inhibited by Bcl-2 overexpression in these cells (Fig. 3B), indicating that the intermediates required for mitochondria-mediated apoptotic signaling were functional in the cell line. As another more direct readout of mitochondrial function, we determined that purified Bax protein induced the release of cytochrome c from mitochondria isolated from the 2B4 cell line (Fig. 3C). Cytochrome c released into the supernatant by MBP-tagged Bax (Fig. 3C, lane 4) was clearly higher than that released by MBP alone (Fig. 3C, lane 2). Thus, it appeared that reduced levels of Bax-induced apoptosis in 2B4 and d11S cells could not be attributed to the increased expression of proapoptotic proteins in these cells or defects in mitochondrial signaling. Therefore, in subsequent experiments we tested the effect of modulating MEK-dependent signaling on Bax localization and Bax-induced apoptosis in 2B4 and d11S cells.

FIGURE 2. Comparison of sensitivity to Bax-induced apoptosis and the expression of pro- and antiapoptotic proteins in the 2B4, Jurkat, and d11S cell lines. A, 2B4 (■) or Jurkat (□) cells were transfected with GFP or Bax-GFP, and after 15 h, the GFP-positive cells were scored for apoptotic nuclear damage, as described in Materials and Methods. The data are representative of four independent experiments. Inset, Expression of GFP or Bax-GFP in 2B4 (lanes 1 and 2, respectively) and Bax-GFP in Jurkat (lane 3) using an Ab to GFP. B, 2B4 and Jurkat cell lysates were assessed for the expression of proapoptotic proteins by Western blot analysis. p38MAPK was used to establish parity of loading. C, d11S cells were transfected with GFP or Bax-GFP, and after 18 h, the GFP-positive cells were scored for apoptotic nuclear damage, as described in Materials and Methods. The data are representative of five independent experiments. D, Lysates of 2B4, Jurkat, and d11S cells were assessed for the expression of the indicated proapoptotic proteins as indicated by Western blot analysis. p38MAPK was used to establish parity of loading.

MEK1 regulates the cytoplasmic sequestration of Bax in 2B4 cells

Bax-GFP was largely present in the cytoplasm of 2B4 cells, as determined by the detergent-based subcellular fractionation of Bax-transduced 2B4 cells (Fig. 4A, lane 1). After the addition of UO126, there was a shift in the distribution of Bax-GFP to the mitochondrial fraction (Fig. 4A, lane 4). Thus, cell fractions enriched for Cox-4 expressed a higher proportion of total Bax-GFP after UO126 treatment (Fig. 4A).

Disruption of MEK1 signaling by UO126 or the expression of DN-MEK1 also culminated in Bax-mediated apoptosis in 2B4 cells (Fig. 4, B and C). The induction of apoptosis in cells cotransfected with Bax and treated with UO126 was inhibited by coexpression of Bcl-2 (Fig. 4D), consistent with the activation of mitochondria-dependent apoptotic signaling triggered by Bax. Coexpression of DN-MEK1 also enhanced sensitivity to Bax-mediated apoptosis in the d11S cell line (Fig. 4E).

Inhibition of PI3K triggers Bax translocation and apoptosis

Recent studies have shown that the kinase Akt/PKB sequesters Bax in the cytosol, thereby preventing Bax-induced apoptosis (22, 23). Because kinase activation and functions are often cell type and context specific, we examined the role of PI3K in the regulation of
Bax-induced apoptosis in 2B4 and d11S cells. Treating activated T cells with the PI3K inhibitor LY294002 triggered a redistribution of Bax to the mitochondrial fraction (Fig. 5A). Both LY294002 and the expression of dominant-negative Akt (DN-Akt) also revealed sensitivity to Bax-induced apoptosis in Bax-transfected 2B4 cells (Fig. 5, B and C). Apoptosis triggered by Bax and LY294002 was blocked by the coexpression of Bcl-2 (Fig. 5D). Similarly, cotransfection of DN-Akt enhanced Bax-induced apoptosis in the d11S cell line (Fig. 5E). Ectopic expression of both DN-Akt and DN-MEK1 did not result in a substantial increase in Bax-mediated apoptosis in 2B4 cells (Fig. 5F) compared with DN-MEK or DN-Akt alone, suggesting that both kinases may target one molecule or molecular complex to regulate Bax function.

MEK1 and Akt regulation of Bax deletion mutants

The findings to date indicate that Bax function can be regulated via Akt- and MEK1-dependent signaling. Post-transcriptionally this regulation could be effected by the phosphorylation of Bax, the phosphorylation of molecular interactors of Bax, or a combination of the two events. A recent study has described an Akt phosphorylation site (Ser184) in the Bax C terminus that sequesters Bax in the cytosol in neutrophils (23). However, aa 20–37 of the Bax N terminus function as a mitochondrial targeting sequence in a glioblastoma cell line (30). Therefore, we attempted to identify the regions in Bax that are required for regulation by MEK1 and Akt in T cells.

A Bax deletion mutant that is truncated at amino acid 171 (Bax1–171) and does not contain a stretch of hydrophobic amino acids at the C terminus was observed to largely localize to the cytoplasm (Fig. 6A) and triggered low levels of apoptosis (Fig. 6, D and E). This deletion also removes the recently described Akt phosphorylation site at Ser184. However, the addition of UO126 or LY294002 to Bax1–171-transfected cells triggered translocation of the deletion mutant to the mitochondrion (Fig. 6, B and C) and apoptosis in 2B4 cells (Fig. 6D). Additionally, both DN-MEK1 and DN-Akt increased the sensitivity of cells to Bax1–171-induced apoptosis (Fig. 6E). Apoptotic damage triggered by the coexpression of DN-Akt was lower than that triggered by the addition of...
LY294002 or by coexpressing DN-MEK1. However, it should be noted that despite deletion of the Akt phosphorylation site (Ser184), Akt-dependent regulation of Bax1–171 was not abrogated.

Deletion of the N-terminal aa 1–29 (Bax/H925429) resulted in a form of Bax that was primarily localized to the mitochondrion (Fig. 7, A and B) and triggered high levels of apoptosis in the 2B4 cell line (Fig. 7C). Apoptosis induced by Bax-829 was not inhibited by CA-Akt or CA-MEK (Fig. 7D), but was substantially reduced by DN-caspase-9 (Fig. 7E). Because the deletion of 29 aa may not completely disrupt the mitochondrial targeting sequence described in the N terminus (30), we generated a construct that was deleted for the first 37 aa. Bax/H925437 was not detected in the cytoplasmic fraction in 2B4 cells (Fig. 7F) and triggered high levels of apoptosis in the 2B4 and d11S cell lines (data not shown).

**FIGURE 5.** Suppressing PI3K-dependent Akt signaling triggers mitochondrial translocation of Bax and apoptosis. A, T cell blasts were cultured with IL-2 in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of LY294002 (40 μM). After 4 h, subcellular fractions of cytosol (Cyt)- or mitochondria (Mito)-enriched fractions were assessed for Bax and Cox-4 expression by Western blot analysis. B, 2B4 cells transfected with GFP or Bax-GFP were cultured for 18 h, continued in culture with or without LY294002 (10 μM) for an additional 8 h, and assessed for apoptotic nuclear damage. The inset shows the levels of pAkt in 2B4 cells cultured with or without LY294002 for 8 h. Akt served as the loading control (LC). C, 2B4 cells were transfected with GFP or Bax-GFP with and without DN-Akt. Apoptotic nuclear damage was assessed in GFP-positive cells after 18–24 h. Apoptotic damage in cells transfected with DN-Akt alone was not higher than that triggered by GFP; hence, the values are not presented.

**FIGURE 6.** Bax1–171-GFP distribution and apoptotic activity in the 2B4 cell line. A, 2B4 cells transfected with Bax1–171-GFP were subjected to subcellular fractionation after overnight culture. The cytosolic (Cyt) and mitochondria (Mit)-rich fractions were analyzed for GFP and Cox-4 expression by Western blot analysis. B and C, 2B4 cells transfected with Bax1–171-GFP were cultured for 18 h and then continued in culture with 20 μM LY294002 (B) or 25 μM U0126 (C) for an additional 6–8 h. Cells were subjected to subcellular fractionation and analyzed as described in A. The fractions were also probed for NF-κB (not shown), which was present only in the cytosol and whole cell lysate (WCL). D, 2B4 cells transfected with GFP or Bax1–171-GFP were cultured for 18 h and then continued in culture with or without U0126 (10 μM) or LY294002 (10 μM) for an additional 8 h before being assessed for apoptotic nuclear damage. Nuclear damage was normalized to GFP-transfected cells. E, 2B4 cells were transfected with GFP or Bax1–171-GFP with and without DN-MEK1 or DN-Akt. Apoptotic nuclear damage was assessed in the GFP-positive cells after 18–24 h.
However, a mutant that lacked the first 13 aa (Bax-13) was localized to the cytoplasm (Fig. 8A) and triggered low levels of apoptosis comparable to the full-length construct (Fig. 8D). Treatment with LY294002 or UO126 triggered mitochondrial translocation of the ectopically expressed protein (Fig. 8, B and C). Furthermore, cotransfection of Bax-13 with DN-MEK1 or DN-Akt triggered apoptosis in 2B4 cells (Fig. 8D). If the N terminus of Bax confers sensitivity to inhibition by MEK1 and Akt, we reasoned

**FIGURE 7.** Characterization of N-terminal Bax deletion mutants in the 2B4 cell line. A, Cells transfected with Bax-629-GFP were subjected to subcellular fractionation after overnight culture. The cytosolic (Cyt) and mitochondria (Mito)-rich fractions were analyzed for GFP, p38MAPK, and Cox-4 expression by Western blot analysis. B, Amounts of Bax-629-GFP and Bax-GFP present in the mitochondrial fraction relative to the total protein in the cytosolic and mitochondrial fractions. C, Cells were transfected with GFP, Bax, or Bax-629-GFP, and GFP-positive cells were assessed for apoptotic nuclear damage 15 h post-transfection. Nuclear damage plotted in the figure is normalized to GFP-transfected cells. The inset shows a Western blot analysis of 2B4 cells transfected with Bax-629-GFP or GFP. D, 2B4 cells were transfected with GFP, Bax-629-GFP, Bax-629-GFP plus CA-Akt, or Bax-629-GFP plus CA-MEK1. After 15 h, GFP-positive cells were scored for apoptotic nuclear damage. E, 2B4 cells transfected with GFP, Bax-629-GFP, or Bax-629-GFP and DN caspase-9 were assessed for the induction of apoptotic nuclear damage in GFP-positive cells after 15 h. F, Cells transfected with Bax-GFP or Bax-629-GFP were subjected to subcellular fractionation as described in A. The fractions were assessed for GFP, Cox-4, and p38MAPK. Lanes 5 and 6 are whole cell lysates.

**FIGURE 8.** Regulation of Bax-613-GFP in the 2B4 cell line. A, 2B4 cells transfected with Bax-613-GFP were subjected to subcellular fractionation as described in Fig. 7A. The fractions were probed for GFP, Cox-4, NF-κB, or Akt. B and C, 2B4 cells transfected with Bax-613-GFP were cultured for 18 h and then continued in culture with 20 μM LY294002 (B) or 25 μM UO126 (C) for an additional 6–8 h. Cells were subjected to subcellular fractionation and analyzed as described in A. D, Cells transfected with GFP and Bax-613-GFP in the presence or the absence of DN-MEK1 or DN-Akt were analyzed for induction of apoptotic nuclear damage 18 h after transfection. E, Apoptotic nuclear damage triggered by Bax-GFP and Bax-629-GFP in the CTEV-2 cell line was assessed 6 h after transfection.
that the cytokine IL-2-dependent survival should be abrogated by ectopic expression of Bax-Δ29. To this end we show that in the IL-2-dependent CTEV-2 cell line, cytokine-dependent survival is disrupted by Bax-Δ29 compared with the modest increase in apoptosis triggered by the enforced expression of Bax (Fig. 8E).

Discussion

Bax is a proapoptotic member of the Bcl-2 family that is sequenced in the cytoplasm in healthy cells (5, 31) and translocates to the mitochondrion to induce apoptosis. Thus, Bax falls into a class of apoptotic proteins that are not only regulated at their sites of action, i.e., the mitochondrion and endoplasmic reticulum, but are also the target of cellular survival strategies that sequester pro-apoptotic proteins away from the mitochondrion. In this study we report that the kinases MEK and Akt regulate the subcellular distribution of Bax via a region in the N terminus in T cells.

What is the physiological relevance of the regulation of Bax in T cells? Gene knockout approaches have demonstrated a requirement for Bax or Bak in activated T cell death (6). Activated T cells generated in the course of the immune response receive extracellular survival cues from costimulatory molecules and cytokines (32), and memory T cells that survive the deletion of Ag-reactive T cells reportedly express high levels of ERK-1/2 (33), which is a target of MEK1. Furthermore, sustained expression of Akt has been shown to promote the long term survival of activated T cells (34). Thus, the regulation of Bax by Akt or MEK1 is significant in the context of understanding activated T cell homeostasis.

The analysis reported in this study has been facilitated by the availability of T cell lines that sequester ectopically expressed Bax in the cytoplasm and are susceptible to Bax cytotoxicity when manipulated by signals that are not themselves apoptogenic to these cells. Thus, inhibition of MEK1 or Akt signaling by either pharmacological approaches or DN constructs triggered apoptosis only in Bax-transfected 2B4 and d11S cells. Although regulation of Bax function via Akt-dependent phosphorylation has been recently reported in neutrophils (23), we believe that the kinases may not regulate Bax by direct phosphorylation alone. Firstly, the C-terminal deleted construct Bax1–171 does not contain the phosphorylation site targeted by Akt (23); nonetheless, LY294002 and the coexpression of DN-Akt regulated Bax1–171 function and localization (Fig. 6). Secondly, the N-terminal deletion mutant Bax-Δ29 was not inhibited by CA-Akt (Fig. 7D), arguing against direct phosphorylation in the C-terminal region as the dominant mode of regulation of Bax in T cells. Finally, CA-MEK1 or CA-Akt did not inhibit Bax-induced apoptosis in the Jurkat cell line (data not shown). Given the potency of Bax, it is likely that multiple cellular mechanisms are in place to regulate Bax function. In this context it will undoubtedly be interesting to determine whether Ser184-mutated Bax is regulated by Akt and MEK1 in T cells.

The observation that the N-terminal truncation of 29 aa residues results in mitochondrial localization of Bax (Fig. 7, A and B) is consistent with the study by Wood and Newcombe (35). This is not in complete agreement with another study (36), which reported that deletion of aa 1–20 inhibited constitutive mitochondrial localization of a Bax isoform (Bax-Δ) and its apoptotic activity in a Bax-deficient gliblastoma cell line. Both the N- and C-terminal regions in Bax have been reported to interact with 14-3-3, a ligand that sequesters other proapoptotic proteins of the Bcl-2 family in the cytosol (24), and a stretch of 53 N terminus aa is implicated in the interaction with Ku70 (25). These differences suggest that the mechanism of Bax regulation may be cell and stimulus specific. It should be noted that despite the demonstrated association of Ku70 with Bax, the deletion of Ku70 did not result in spontaneous translocation of Bax to the mitochondrion (25), indicating that multiple cytosolic factors (cell type specific) probably regulate Bax sequestration.

Our experiments with the deletion mutants implicate a region spanned by aa 13–29 at the Bax N terminus in the regulation of cytoplasmic localization by MEK and Akt. A recent study has shown that N-terminally deleted Bax functions like a BH3-only protein, requiring endogenous Bax for induction of apoptosis (36). However, we detect little/no endogenous Bax in the Jurkat cell line and report higher and comparable levels of expression of the endogenous protein in 2B4 and d11S cells. Thus, it is unlikely that Bax-Δ29-induced apoptosis is mediated via the recruitment of endogenous Bax, because Jurkat cells are very susceptible to apoptosis induced by the deletion mutant (data not shown). Our experiments suggest a redundancy in the requirement for Akt- and MEK1-dependent signaling, and we hypothesize that Akt and MEK1 probably phosphorylate a cellular complex associated with Bax in T cells. The former may explain why the accumulation of activated T cells is not reported in mice genetically deficient for MEK1 or Akt.

In conclusion, our experiments in activated T cells show that one outcome of prosurvival signaling by cytokines is related to the regulation of Bax localization and thereby its apoptotic function. MEK-dependent regulation of Bax expression and translocation in primary activated T cells identifies a mechanism by which cytokines may block apoptosis by targeting premitochondrial events in T cells. Using an IL-2-dependent T cell line, we show that IL-2 could protect cells to some extent from Bax-induced apoptosis, but was unable to inhibit apoptosis triggered by the Bax-Δ29 deletion mutant (Fig. 8D). Ongoing experiments are directed toward the identification of molecules associated with the N terminal of Bax under the influence of Akt and MEK1 signaling.

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