Bcl-X$_L$ specifically activates Bak to induce swelling and restructuring of the endoplasmic reticulum

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Bcl-2 family members Bak and Bax constitute a mitochondrial gateway for multiple death pathways. Both proteins are also present in the endoplasmic reticulum where they control apoptosis through the regulation of calcium levels. We show here that reticular Bak has the additional capacity of modulating the structure of this organelle. Coexpression of Bak and Bcl-X$_L$ provokes extensive swelling and vacuolization of reticular cisternae. A Bak version lacking the BH3 domain suffices to induce this phenotype, and reticular targeting of this mutant retains the activity. Expression of upstream BH3-only activators in similar conditions recapitulates ER swelling and vacuolization if ryanodine receptor calcium channel activity is inhibited. Experiments with Bak and Bax-deficient mouse embryonic fibroblasts show that endogenous Bak mediates the effect, whereas Bax is mainly irrelevant. These results reveal a previously unidentified role of Bak in regulating reticular conformation. Because this activity is absent in Bax, it constitutes one of the first examples of functional divergence between the two multidomain homologues.

Introduction

Apoptotic cell death ensures the elimination of cells that become irrelevant or potentially damaging for the organism (Danial and Korsmeyer, 2004), and the Bcl-2 family of proteins plays a central role in the regulation of this process (Adams and Cory, 1998). This family includes both pro- and anti-apoptotic molecules sharing homology in any of the four Bcl-2 homology (BH) domains identified so far (BH1 to 4; Adams and Cory, 1998). The role of Bcl-2 proteins in the regulation of mitochondrial apoptosis has been clearly established (Green and Reed, 1998). However, functions in other subcellular compartments, like the ER, are beginning to be discovered (Breckenridge et al., 2003).

Based on both structural and functional criteria, three subgroups of Bcl-2 homologues have been identified. One of them includes pro-apoptotic effectors like Bak and Bax, characterized by containing three of the prototypical BH domains (BH1, BH2, and BH3; Adams and Cory, 1998; Reed, 1998). A functional BH3 domain has been shown to be critical for the apoptotic activity of these proteins (Chittenden et al., 1995a; Simonen et al., 1997). A second subgroup contains pro-apoptotic molecules structurally related by the presence of only one family domain corresponding to BH3. Members of this subfamily are thus known as BH3-only proteins (Bouillet and Strasser, 2002). The third subgroup includes anti-apoptotic homologues like Bcl-2 and Bcl-X$_L$, and a distinct structural feature is the presence of a BH4 domain in addition to BH domains 1, 2, and 3 (Adams and Cory, 1998; Reed, 1998). Bcl-2 family proteins have a propensity to dimerize, and the fine balance between pro- and anti-apoptotic members often defines whether a cell will survive or will commit to death in response to a particular insult (Reed, 1998; Danial and Korsmeyer, 2004).

Current knowledge about apoptotic signaling cascades supports the view that BH3-only molecules initiate the pathway, activating downstream effectors Bak and Bax to trigger mitochondrial apoptosis (Bouillet and Strasser, 2002). According to this model, apoptotic insults unleash BH3-only homologues and induce their translocation to the outer mitochondrial membrane (Bouillet and Strasser, 2002). Once in the mitochondria, these proteins are thought to bind preferentially anti-apoptotic members of the family (Cheng et al., 2001; Zong et al., 2001; Letai et al., 2002), saturating protective binding sites before they bind and activate Bak and Bax. This direct engagement of Bak and Bax has been established for BH3-only members BimEL and Bid (Desagher et al., 1999; Wei et al., 2000; Letai et al., 2002). Other homologues, like Bik or Bad, bind anti-apoptotic molecules only, thus exerting a sensitization effect (Letai et al., 2002). Activation of Bak and Bax involves modifications in their conformation (Hsu and Youle, 1997; Desagher et al., 1999; Griffiths et al., 1999) and assembly into oligomers
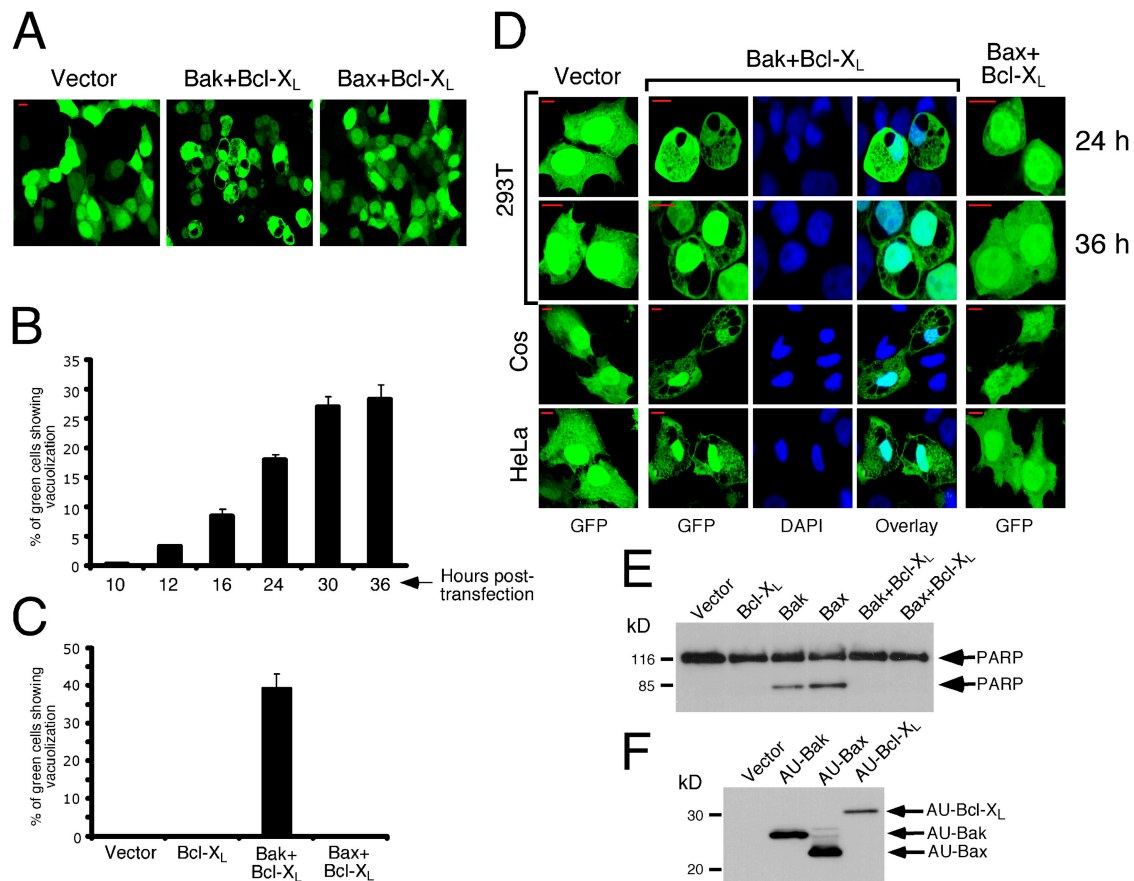
Figure 1. Coexpression of Bak and Bcl-X$_L$ induces cytoplasmic vacuolization. (A) Confocal analysis of cytoplasmic vacuolization induced by coexpression of Bak and Bcl-X$_L$. 293T cells were transfected with a mix of the indicated expression plasmids in combination with a plasmid expressing cytosolic GFP. 36 h after transfection, cells were fixed, mounted, and analyzed by confocal microscopy. (B) Time course of vacuolization induced by coexpression of Bak and Bcl-X$_L$. 293T cells were cotransfected with Bak, Bcl-X$_L$, and GFP as in A. At the indicated time points the proportion of GFP-expressing cells showing a detectable level of cytoplasmic vacuolization was determined in vivo by blindly counting green cells under an inverted fluorescence microscope. At least 400 cells were scored for each experimental point. Columns represent the percentage of GFP-expressing cells showing a vacuolated cytoplasm. Error bars show SDs of percentages obtained by counting at least eight different fields. (C) Quantitation of cells showing cytoplasmic vacuolization after transfection with Bak or Bax in combination with Bcl-X$_L$. 293T cells were transfected as in A, and analyzed as in B, 36 h after transfection. (D) Confocal analysis of cytoplasmic vacuolization induced by Bak and Bcl-X$_L$ in different cell types. The indicated cells were transfected with the same mix of plasmids as in A. 24 h (293T, as indicated) or 36 h (293T, HeLa, and Cos) later, cells were fixed, stained with DAPI, and mounted for confocal analysis. (E) Bcl-X$_L$ inhibits Bak and Bax-induced apoptosis to the same extent. 293T cells were transfected with the indicated expression plasmids. 24 h after transfection, cells were lysed and equal amounts of proteins were subjected to Western blotting using an anti-PARP antibody. (F) AU-Bak and AU-Bax are expressed to comparable levels. 293T cells were transfected as in E with the exception that a vector expressing the viral caspase inhibitor p35 was introduced in all experimental points to block death. 24 h after transfection, cells were lysed and equal amounts of proteins were resolved by PAGE and blotted with an anti-AU mAb.

(Eskes et al., 2000; Wei et al., 2000). These changes ultimately result in the release of mitochondrial apoptogenic factors into the cytoplasm, thus irreversibly launching the death process (Green and Reed, 1998; Danial and Korsmeyer, 2004).

Recently, Bak and Bax were shown to be essential for multiple apoptotic pathways (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001). In addition, both effectors show a substantial functional redundancy because cells deficient in only one of them remain sensitive to a variety of apoptotic stimuli. Consistent with this, mice deficient in either protein show mild phenotypes (Knudson et al., 1995; Lindsten et al., 2000), whereas animals deficient in both of them suffer major abnormalities (Lindsten et al., 2000). However, specific roles for Bak and Bax can be found in the literature. Some publications refer to a more prominent involvement of Bax in multiple cell death pathways in human cells (Zhang et al., 2000; The-

odorakis et al., 2002; Gillissen et al., 2003), whereas other reports describe a fundamental role for Bak in cell death induced by anticancer drugs (Wang et al., 2001a) or granzyme B (Wang et al., 2001b). Additional differences relate to their subcellular localization. In healthy cells, Bax is mainly a soluble protein (Suzuki et al., 2000) that stably associates with intracellular membranes upon an apoptotic insult (Hsu et al., 1997). On the contrary, Bak is permanently inserted into subcellular membranes and does not change its localization during apoptosis (Griffiths et al., 1999). Although these differences could reflect a differential regulation of pathways common to Bak and Bax, they may also suggest the existence of biological activities exclusively performed by each one of these molecules, which, for the most part, have not been described.

Both Bak and Bax are also localized in the ER (Nutt et al., 2002; Zong et al., 2003), an organelle that constitutes one

of the main calcium storages in the cell (Rizzuto et al., 2003). In fact, these effectors partially control apoptosis by regulating ER calcium levels (Breckenridge et al., 2003; Rizzuto et al., 2003). For example, Bak and Bax-deficient mouse embryonic fibroblasts (MEFs) show a reduced ER calcium concentration and a concomitant decreased response to apoptotic stimuli that use ER calcium for intracellular signaling (Scorrano et al., 2003). In turn, Bak and Bax expression promotes calcium release from ER stores thus leading to cell death (Nutt et al., 2002; Zong et al., 2003). Therefore, like in mitochondria, activities ascribed so far to Bak and Bax in the ER seem quite overlapping. In any event, the role of Bcl-2-family members in reticular cisternae has only recently been addressed, and the full scope of their functions in this compartment is probably far from complete.

Here, we describe a novel activity of Bak in the ER. When coexpressed with Bcl-X_L, Bak is able to induce dramatic conformational alterations and swelling of reticular cisternae. Due to an inhibitory function performed by ryanodine receptor (RyR) calcium channels, this phenomenon remains undetected if the signaling pathway is stimulated by upstream BH3-only activators. However, blockade of RyR activity by pharmacological agents reveals this underlying activity as a profuse ER swelling and vacuolization whose induction requires the endogenous expression of Bak. Interestingly, we find that Bax completely lacks this potential, indicating that the two multidomain homologues may not always carry out a completely overlapping range of cellular activities.

Results

Previous reports indicate that the enforced expression of Bak and Bax triggers spontaneous cell death (Chittenden et al., 1995b; Xiang et al., 1996), an activity opposed by anti-apoptotic homologues like Bcl-X_L (Chittenden et al., 1995a; Simonen et al., 1997). To look for functional differences between Bak and Bax we took advantage of this overexpression approach, and transfected both molecules under various conditions into 293T cells. A striking difference arose when the induced apoptotic process was inhibited by Bcl-X_L. Simultaneous expression of Bak and Bcl-X_L provoked a prominent cytoplasmic vacuolization revealed by the exclusion of cytosolic GFP (Fig. 1 A), whereas this phenotype was absent in cells coexpressing Bax and Bcl-X_L (Fig. 1 A). Time course experiments showed that the effect was first detectable between 12 and 16 h after transfection (Fig. 1 B), peaking at around 36 h after transfection (Fig. 1 B). At this time point there was no detectable vacuolization in cells transfected with Bax in the presence of Bcl-X_L, Bcl-X_L alone, or empty vector (Fig. 1 C). At later times cells lost viability as evidenced by an increased permeability to propidium iodide (unpublished data), a death process that progressed without detectable caspase-dependent proteolytic processing of poly(ADP-ribose) polymerase (PARP; unpublished data). This vacuolating capacity of Bak and Bcl-X_L was independent of the cell type, because different cell lines were similarly susceptible (Fig. 1 D). DNA staining with DAPI showed that at least one prominent cytoplasmic vacuole was
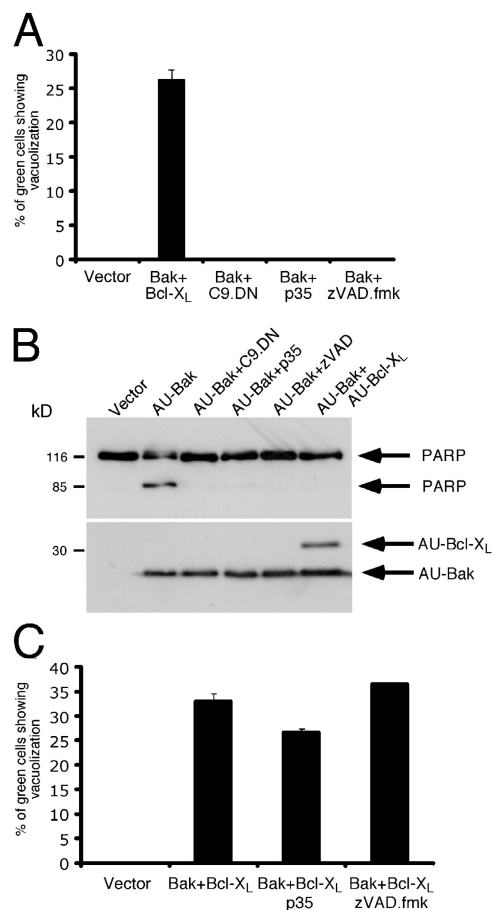

Figure 2. **Inhibition of Bak-induced apoptosis is not sufficient to reveal cytoplasmic vacuolization.** (A) Quantitation of vacuolated cells after transfection with Bak in the presence of different apoptotic inhibitors. 293T cells were transfected with a mix of the indicated plasmids and a GFP-expressing plasmid. C9.DN and p35 denote a dominant-negative version of caspase-9 and the viral caspase inhibitor p35, respectively. When indicated, the pan-caspase inhibitor z-VAD.fmk (100 μM) was added 1 h after transfection. Cytoplasmic vacuolization was scored as in Fig. 1 B. (B) Death inhibitors unable to reveal vacuolization block Bak apoptosis to the same extent as Bcl-X_L. 293T cells were transfected with a mix of the indicated expression plasmids. Z-VAD.fmk (100 μM) was added 1 h after transfection. Cells were lysed 24 h after transfection. Shown are blots probed with anti-PARP (top) and anti-AU (bottom) antibodies. The latter confirms equal transfection efficiency. (C) Quantitation of cells showing cytoplasmic vacuolization after transfection with Bak and Bcl-X_L in the presence of different inhibitors of apoptosis. 293T cells were transfected and treated as in A, and scored as in Fig. 1 B.

usually localized in close contact with the periphery of the nucleus (Fig. 1 D). The different potential of transfected Bak and Bax in this assay was not due to a dissimilar inhibitory capability of Bcl-X_L, because the proteolytic processing of PARP was equally blocked by Bcl-X_L in both cases (Fig. 1 E). In addition, no significant differences in Bak and Bax expression levels could account for the observed phenomena (Fig. 1 F).

Because Bak overexpression triggers efficient death (Fig. 1 E; Chittenden et al., 1995b), it is possible that the generation of cytoplasmic vacuolae is a simultaneous phenomenon normally obscured by the dominant apoptotic activity. In this scenario, cotransfected Bcl-X_L would reveal the phenotype by simply inhibiting cell death. A prediction of this model is that
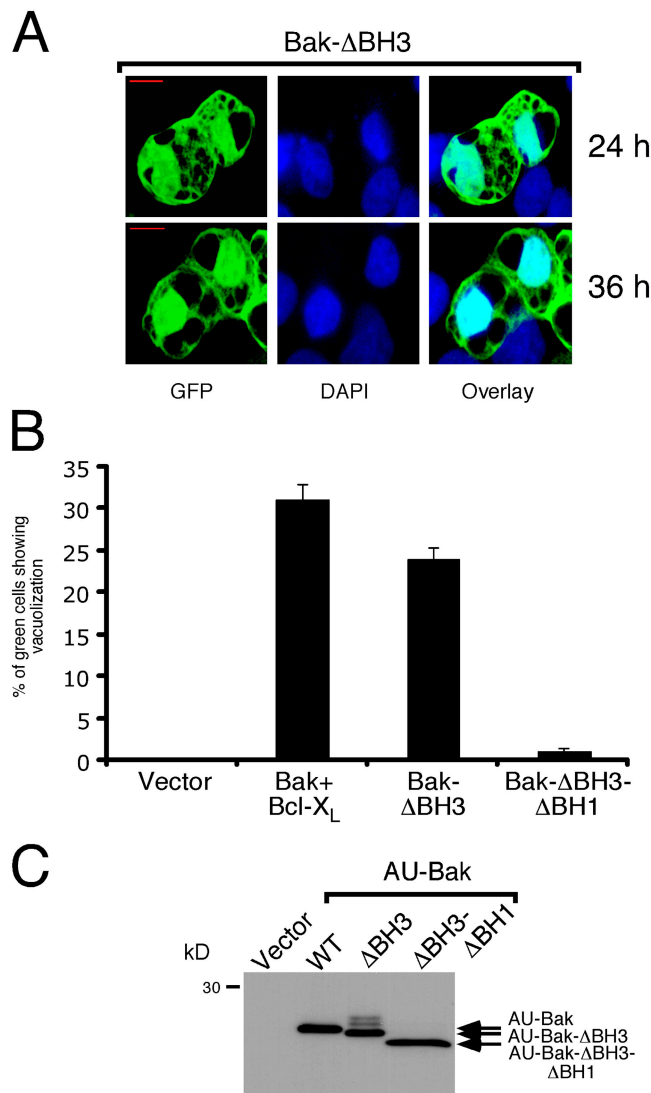
A

Bak-ΔBH3

GFP   DAPI   Overlay

24 h

36 h

B

C

Figure 3. **A version of Bak lacking the BH3 domain induces cytoplasmic vacuolization in the absence of cotransfected Bcl-X_L.** (A) Confocal analysis of cytoplasmic vacuolization induced by expression of Bak-ΔBH3. 293T cells were transfected with a mix of plasmids expressing Bak-ΔBH3 and GFP. Cells were fixed, mounted, and analyzed by confocal microscopy 24 or 36 h (as indicated) after transfection. (B) Quantitation of vacuolization in cells transfected with Bak-ΔBH3 or Bak-ΔBH3-ΔBH1. 293T cells were transfected with the indicated plasmids, along with a GFP-expressing plasmid. 36 h later the proportion of GFP-expressing cells showing vacuolization was determined as in Fig. 1 B. (C) AU-Bak, AU-Bak-ΔBH3, and AU-Bak-ΔBH3-ΔBH1 are expressed to comparable levels. 293T cells were transfected, lysed, and processed as in Fig. 1 F. Shown is a Western blot probed with an anti-AU antibody.

Bak expression should induce vacuolization in the presence of alternative blockers of apoptosis. However, treatment with different caspase inhibitors was not sufficient to reveal the phenotype in Bak-transfected cells (Fig. 2 A), although all tested reagents suppressed PARP processing to the same extent as Bcl-X_L did (Fig. 2 B). While this result could also indicate that caspases are necessary for vacuolization, caspase inhibition during coexpression of Bak and Bcl-X_L did not reduce the proportion of vacuolated cells (Fig. 2 C), ruling out a role for these proteases in the observed phenomenon. Together, these data

show that Bak normally lacks the capability of inducing cytoplasmic vacuolization, and suggest the need for an activation step provided by Bcl-X_L. Alternatively, Bcl-X_L, and not Bak, could be the effector molecule in this context.

Bcl-X_L has been shown to bind and inhibit Bak and Bax BH3 domains, thus antagonizing their apoptotic activities (Chittenden et al., 1995a; Simonen et al., 1997). Therefore, the capacity of Bcl-X_L to reveal the vacuolating potential of Bak might as well involve BH3 domain inhibition. To test this possibility, we created a deleted version of Bak lacking the BH3 domain (Bak-ΔBH3) and evaluated its ability to induce the observed phenomenon in the absence of Bcl-X_L. Transfection experiments indicated that this Bak mutant has an autonomous ability to induce cytoplasmic vacuolization (Fig. 3 A). Quantitation studies showed only a slight decrease in the percentage of vacuolated cells compared with cultures transfected with Bak and Bcl-X_L (Fig. 3 B). Both Bak versions were expressed to similar levels (Fig. 3 C). These data confirm that Bak, and not Bcl-X_L, is the active molecule in this context, and suggest that Bcl-X_L reveals this function by binding and inactivating the BH3 domain of Bak.

Interestingly, additional deletion of the BH1 domain blocked the vacuolating activity of Bak-ΔBH3 (Fig. 3 B) without affecting its expression level (Fig. 3 C) or intracellular distribution (unpublished data). This result suggests a role for Bak BH1 domain in inducing the phenotype. In addition, because both Bak versions (Bak-ΔBH3 and Bak-ΔBH3-ΔBH1) provoked a comparable loss of cell viability at late time points (measured by propidium iodide permeability; unpublished data), cytoplasmic vacuolization is probably not an epiphenomenon linked to this delayed death.

ER swelling is a morphological change associated with multiple cell death modalities (Van Cruchten and Van Den Broeck, 2002). To determine if vacuolae were topologically related to reticular cisternae, we created a version of RFP targeted to the ER lumen (erRFP). Control experiments established that erRFP completely colocalized with the ER marker calreticulin in normal cells (Fig. 4 A). Transfection of Bak in the presence of Bcl-X_L, or Bak-ΔBH3 alone, in combination with this construct showed that the induced vacuolae were completely filled with erRFP, clearly establishing their reticular origin (Fig. 4 B). A substantial fraction of the ER remained unchanged at early stages of the swelling process (Fig. 4 B, 293T cells, 24 h), but reticular cisternae seemed to be progressively incorporated into the altered structures as transfection evolved (Fig. 4 B). These results reveal the potential of Bak to regulate ER structure. Because vacuolae can often occupy a substantial area of the cell, their generation likely involves a swelling process, although some contribution of ER tubulae fusion cannot be ruled out.

To determine the subcellular localization of transfected Bak with respect to dilated ER cisternae we used AU-tagged versions of the different molecules. Anti-AU immunofluorescence stainings of cells transfected with AU-Bak in combination with untagged Bcl-X_L, or AU-Bak-ΔBH3 alone, revealed that vacuolae were often coated with a punctated pattern of AU reactivity (Fig. 5 A). This was particularly obvious in areas of
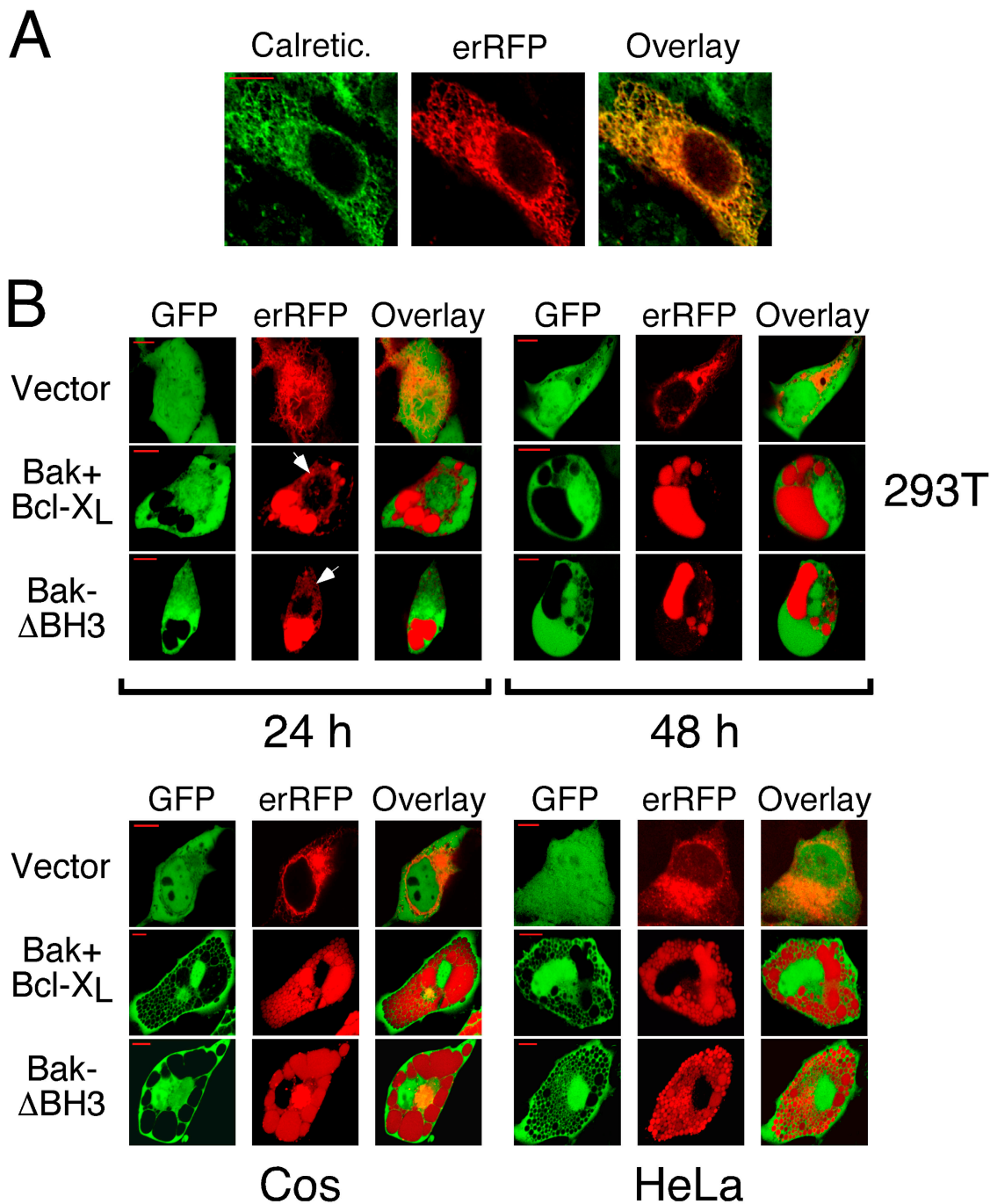
Figure 4. **Cytoplasmic vacuolae are swollen ER cisternae.** (A) Colocalization of erRFP with endogenous calreticulin. 293T cells were transfected with a plasmid encoding ER-targeted RFP (erRFP). 48 h later cells were fixed and stained with an anti-calreticulin antibody followed by an Alexa 488 (green)–coupled antibody, and analyzed by confocal microscopy. Shown is one representative example. (B) Vacuolae induced by transfection of Bak and Bcl-X$_L$, or Bak-ΔBH3, are filled with coexpressed erRFP. 293T, Cos, and HeLa cells were cotransfected with the indicated constructs and plasmids expressing GFP and erRFP. 24 h (293T cells, as indicated), or 48 h after transfection, cells were analyzed in vivo by confocal microscopy. Live cells were used since fixation resulted in the disappearance of erRFP fluorescence from vacuolae in mounted preparations. White arrows indicate reticular cisternae that remain unaffected at early time points in 293T cells.

direct apposition with the nucleus (Fig. 5 A). A variation of the same experiment showed a similar distribution of Bcl-X$_L$ (Fig. 5 A). These results indicate the existence of a topological link between effector molecules and swollen cisternae.

This idea was also supported by experiments where we artificially targeted Bak-ΔBH3 to different subcellular com-

partments. A chimeric version of Bak-ΔBH3 (Bak-ΔBH3-cb5) containing the transmembrane region of cytochrome b5, previously shown to confer exclusive ER targeting (Zong et al., 2003), fully retained the capacity to induce vacuolization (Fig. 5 B). In contrast, a form of Bak-ΔBH3 fused to the ActA peptide, which induces mitochondrial localization (Bak-ΔBH3-

Figure 5. **Involvement of ER-localized Bak in the generation of reticular swelling.** (A) Perivacuolar localization of AU-Bak, AU-Bcl-X$_L$, and AU-Bak-ΔBH3. 293T cells were transfected with the indicated constructs along with GFP. 36 h after transfection cells were fixed, stained with an anti-AU mAb followed by an anti–mouse-Cy3 (red) antibody, and mounted for confocal microscopy. N and V denote nuclei and vacuolae, respectively. (B) A version of Bak-ΔBH3 specifically targeted to the ER (Bak-ΔBH3-cb5) retains the capacity to induce ER swelling, whereas mitochondrial targeting (Bak-ΔBH3-ActA) results in a decreased activity. 293T or HeLa cells (as shown) were transfected with the indicated expression plasmids along with GFP. Z-VAD.fmk (100 μM) was added 1 h after transfection to reduce death. 36 h after transfection, cytoplasmic vacuolization was scored as in Fig. 1 B.
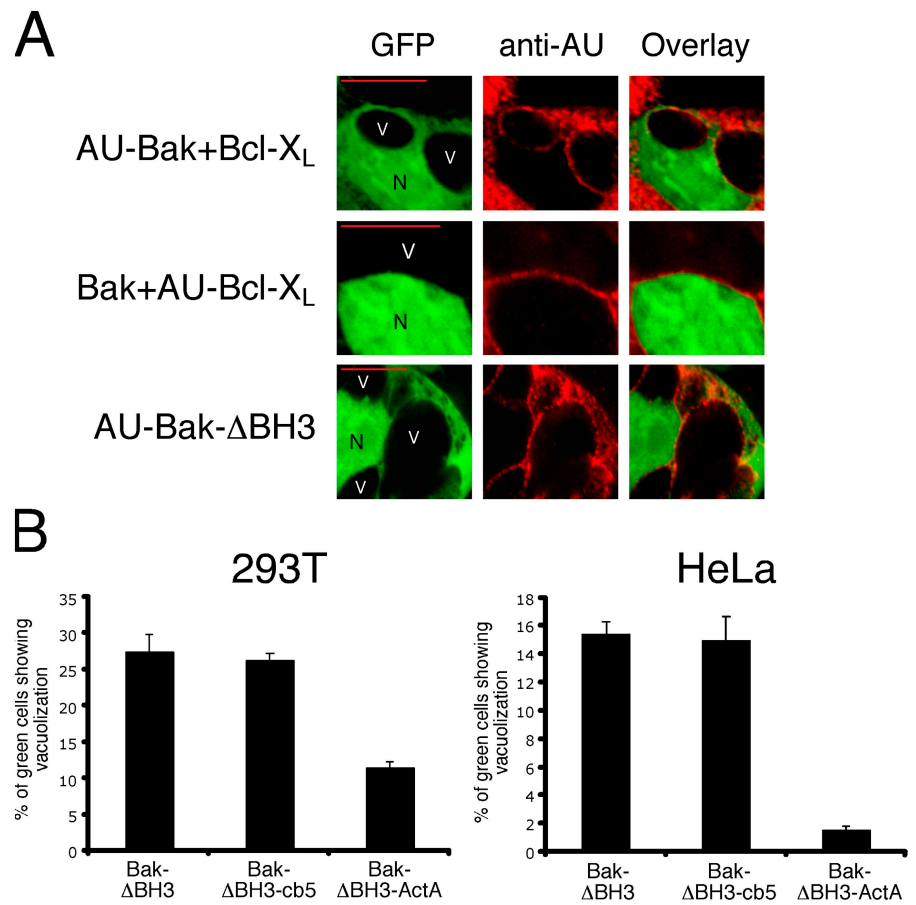
ActA; Zhu et al., 1996), showed a decreased activity (Fig. 5 B). Control immunostaining assays indicated that a portion of Bak-ΔBH3-ActA was surprisingly present in reticular membranes, as established by colocalization with calreticulin (unpublished data). This result suggests that human Bak may have additional ER targeting signals able to override ActA. Given this partially reticular localization, Bak-ΔBH3-ActA should not be expected to be completely incapable of inducing ER vacuolization, even if only reticular Bak is active in this context. The localization of the cb5 chimera was tightly reticular (unpublished data). Together, these results further underscore the notion that the ability of Bak to induce vacuolization is linked to its physical presence in the ER.

High calcium levels in the cytoplasm have been shown to induce ER compartmentalization and vacuolization (Subramanian and Meyer, 1997), pointing out to a role of calcium in the regulation of ER structure. Because Bcl-2 family members have the ability to alter ER calcium homeostasis (Nutt et al., 2002; Scorrano et al., 2003; Zong et al., 2003), the observed reticular swelling induced by Bak might be related to this activity. To test this hypothesis we used chemicals that influence calcium homeostasis in different ways. Chelation of extracellular calcium by EGTA, or treatment with the SERCA pump inhibitor thapsigargin, which allows a passive calcium leakage from ER stores (Breckenridge et al., 2003), had no effect in the degree of vacuolization (unpublished data). Dantrolene is a commercial drug that inhibits ER calcium release through the

RyR calcium channels (Zhao et al., 2001; Fill and Copello, 2002). This drug has been shown to reduce the cytosolic concentration of calcium in some systems (Jacobs et al., 1991). The presence of dantrolene during expression of transfected Bak and Bcl-X$_L$, or Bak-ΔBH3 alone, had the unexpected effect of exacerbating the vacuolated phenotype (Fig. 6 A). Vacuolae tended to occupy the vast majority of cytoplasmic space in the presence of dantrolene, whereas in its absence they were typically smaller (Fig. 6 A). In contrast, no change was observed in the cytoplasm of cells cotransfected with Bax and Bcl-X$_L$ or either Bak or Bax in the presence of apoptotic blockers like the caspase inhibitor p35 or a dominant-negative version of caspase-9 (unpublished data). This suggests that dantrolene only shows an effect in the presence of an underlying vacuolating stimulus.

Results obtained with dantrolene argue that the observed reticular changes are partially countered by the activity of RyR channels. If this model is true, RyR activation should provoke a reduction of vacuolae size. Among other cellular effects, caffeine activates these channels thus allowing the release of calcium from ER stores (Ozawa, 2001). Treatment of vacuolated cells with caffeine induced a prompt reduction of vacuolae size (unpublished data), and resulted in a lower number of cells presenting a detectable phenotype (Fig. 6 B). Together, these data support a role for RyR calcium channels in regulating Bak-induced ER structural changes and, as a consequence, suggest an involvement of calcium.
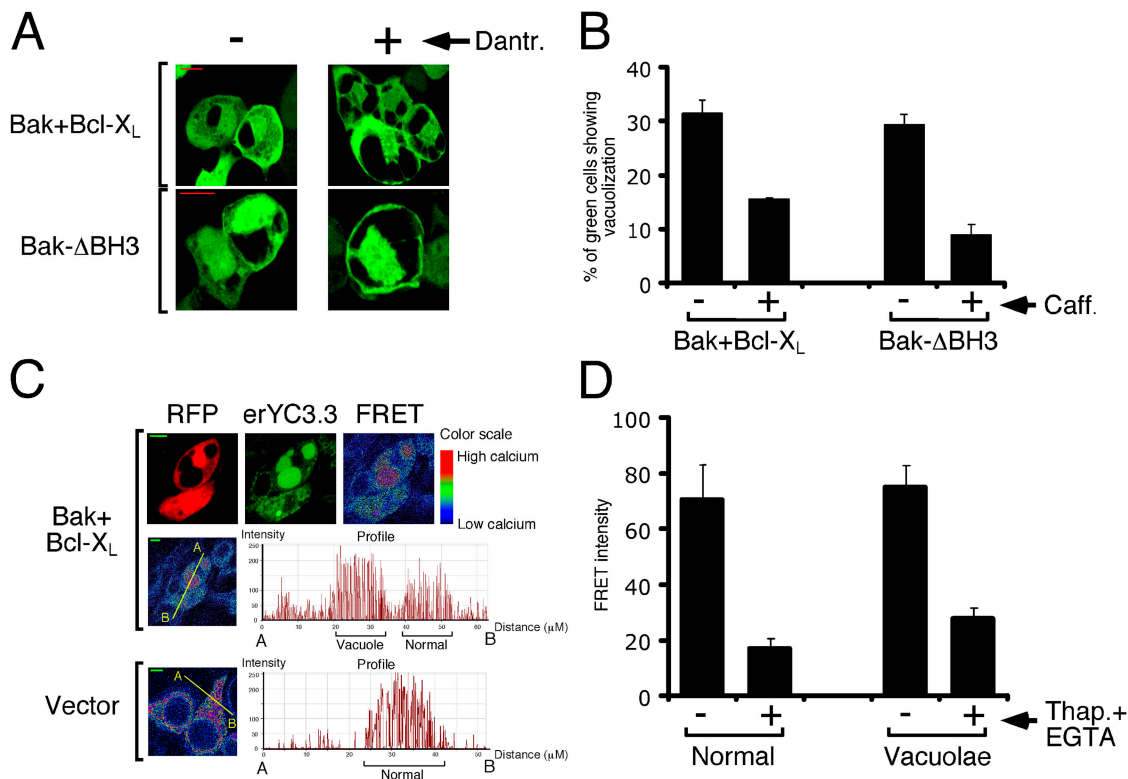
Figure 6. **Involvement of RyR calcium channel activity in the regulation of cytoplasmic vacuolization induced by Bak.** (A) Treatment with dantrolene potentiates vacuolization. 293T cells were transfected with a mix of the indicated plasmids and a plasmid expressing GFP. Where indicated, dantrolene (50 μM) was added 1 h after transfection. 36 h after transfection, cells were fixed, mounted, and analyzed by confocal microscopy. Shown are representative fields. The same magnification was used for both control and treated cells. (B) Caffeine treatment reduces vacuolization. 293T cells were transfected as in A. 36 h later, caffeine (20 mM) was added as indicated for 3.5 h, and cells were subsequently fixed and mounted. Quantitation of vacuolated cells was done as in Fig. 1 B. (C) Swollen ER cisternae and normal ER show similar calcium concentrations. 293T cells were transfected with the indicated plasmids in the presence of plasmids expressing cytosolic RFP and a reticular ratiometric calcium indicator (erYC3.3). 36 h after transfection, cells were analyzed in vivo by confocal microscopy to obtain FRET intensities of erYC3.3. In the top panel, RFP was used to locate a vacuolated cell in proximity to an unvacuolated neighbor with a similar level of transfection, and emission FRET ratios (535 nm/480 nm) were obtained for both cells. Shown are emissions in red (RFP), green (erYC3.3, 535 nm), and FRET ratios. The middle panel shows a profile of FRET intensities across the indicated yellow line, including both vacuolated (Vacuole) and unvacuolated (Normal) ER. The bottom panel shows a profile of ER FRET intensities of a cell transfected with an irrelevant vector and presenting similar erYC3.3 expression levels as cells in the top panel (measured by emission at 535 nm). (D) Treatment with thapsigargin and EGTA reduces erYC3.3 FRET intensities both in swollen and unaltered ER. 293T cells were cotransfected with Bak, Bcl-X$_L$, and erYC3.3. 36 h after transfection, both unvacuolated (Normal) and vacuolated (Vacuolae) cells were analyzed in vivo to obtain confocal FRET levels in the ER. After capturing FRET images for each untreated cell, 2 mM EGTA and 200 μM thapsigargin were simultaneously added to the culture, and reticular FRET images acquired after 15 min. This time point showed the maximum effect. To represent the data, mean values of FRET intensities across a chosen section of the ER were obtained for each cell in control and treated conditions. Shown are data averaged for at least five cells analyzed in each experimental condition, along with the corresponding SDs.

To explore this idea, we compared calcium concentrations inside vacuolae with those in normal ER. For this purpose, we cotransfected Bak and Bcl-X$_L$ along with an ER-targeted version of the ratiometric, low affinity calcium indicator yellow cameleon 3.3 (erYC3.3; Miyawaki et al., 1997) and RFP for identification of vacuolated cells. YC3.3 responds to changes in calcium concentrations by altering its spectral properties due to an intramolecular fluorescence resonance energy transfer (FRET) effect (Miyawaki et al., 1997). Confocal FRET measurements revealed that intensities inside swollen ER were similar to those present in unswollen cisternae of transfected neighboring cells (Fig. 6 C, top and middle). Comparable FRET levels were obtained when the erYC3.3 reporter was cotransfected with an irrelevant vector (Fig. 6 C, bottom). Treatment with EGTA and thapsigargin reduced FRET intensities in both dilated and normal ER (Fig. 6 D), thus confirming the capacity of erYC3.3 to detect calcium variations. These results indicate

that calcium concentrations present in swollen ER are similar to those in unaltered cisternae. Additional experiments showed no changes in cytoplasmic calcium induced by coexpression of Bak and Bcl-X$_L$, or Bak-ΔBH3 (unpublished data), measured at different times after transfection with the cytoplasmic, low affinity ratiometric indicator YC2.3 (Miyawaki et al., 1997).

This lack of correlation between reticular swelling and calcium variations, along with the inability of thapsigargin or EGTA to influence vacuolization (unpublished data), argue against a direct involvement of calcium in this phenomenon. Data showing an effect by dantrolene and caffeine suggest a model in which Bak-induced ER remodelling is countered by cytosolic calcium specifically mobilized by RyR channels.

Bak and Bax are physiologically activated by BH3-only homologues like BimEL or Bid (Bouillet and Strasser, 2002). To explore if these upstream effectors are capable of generating a vacuolated phenotype, we transfected cells with plasmids

Figure 7. **Coexpression of BimEL or tBid with Bcl-X$_L$ induces ER swelling in the presence of dantrolene.** (A) Quantitation of dantrolene-treated cells showing cytoplasmic vacuolization after transfection with BimEL or tBid, as a function of cotransfection with Bcl-X$_L$. 293T cells were transfected with a mix of the indicated constructs in the presence of plasmids expressing GFP and the apoptotic inhibitor p35. Dantrolene (50 μM) was added 1 h after transfection to all experimental points. 36 h later the proportion of GFP-expressing cells showing cytoplasmic vacuolization was determined as in Fig. 1 B. (B) ER swelling is the cause of cytoplasmic vacuolization induced by cotransfection of BimEL or tBid with Bcl-X$_L$, in the presence of dantrolene. 293T cells were transfected with plasmids expressing either Bim or tBid (as indicated), in combination with Bcl-X$_L$, GFP, and erRFP expression plasmids. Dantrolene (50 μM) was added 1 h after transfection. 36 h after transfection, cells were analyzed by in vivo confocal microscopy. (C) 293T cells express endogenous Bak and Bax. Cells were either transfected with the indicated DNAs or left untransfected, and lysed 24 h after transfection. Shown are Western blots probed with antibodies against Bak or Bax (top), and subsequently reprobed with an anti-AU mAb (bottom) as indicated. Lanes corresponding to transfected cells provide a size reference to identify the endogenous protein. Re-probing with an anti-AU mAb excludes sample contaminations across wells.
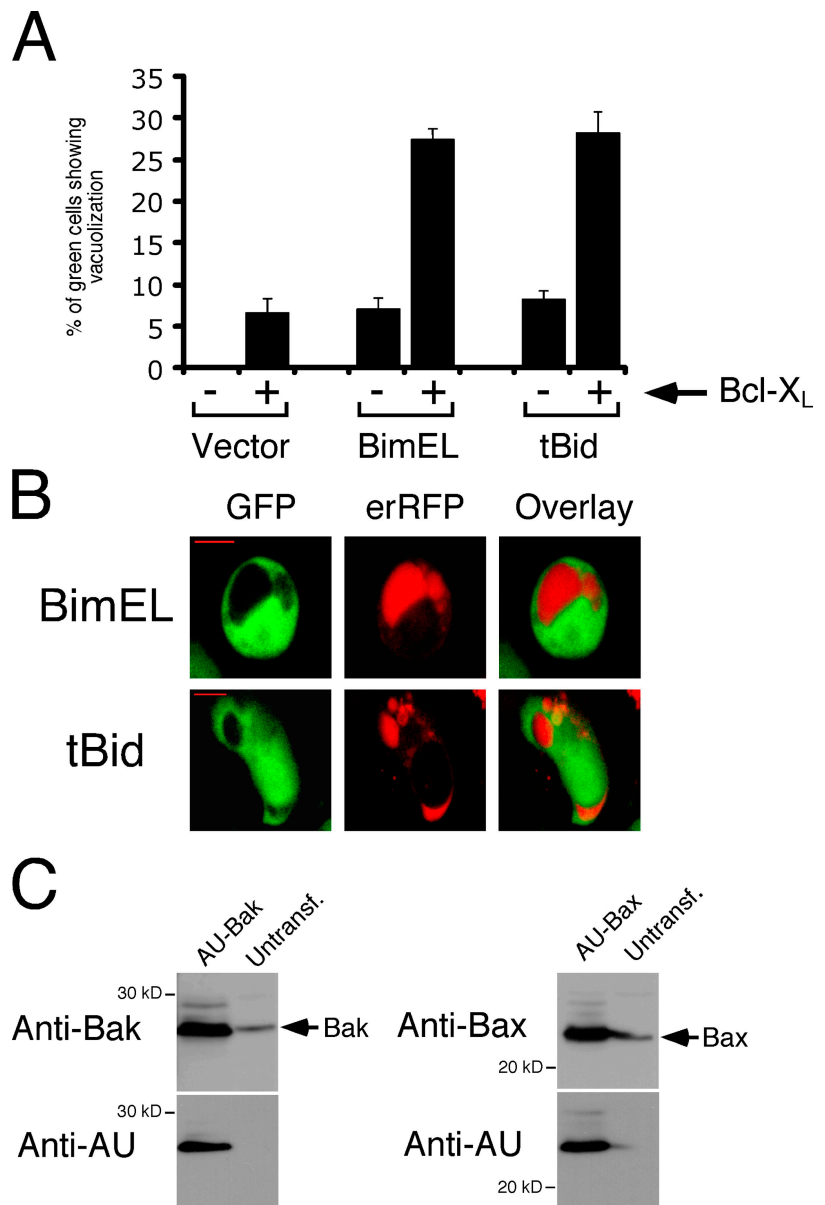
expressing either full-length BimEL or a truncated version of Bid (tBid) known to be constitutively active (Li et al., 1998). Coexpression of BimEL or tBid with Bcl-X$_L$ induced a low but detectable number of cells (~1%) to show cytoplasmic vacuolae (unpublished data), suggesting an incipient ER swelling. Given the potentiating effect of dantrolene (Fig. 6 A), we wondered if the drug could turn this weak effect into a vacuolated phenotype easier to identify. The presence of dantrolene from early on after transfection with BimEL or tBid in combination with Bcl-X$_L$ resulted in a substantial number of vacuolated cells (Fig. 7 A), whereas in the absence of Bcl-X$_L$ both molecules induced a more modest phenotype (Fig. 7 A). Cotransfection with erRFP confirmed the reticular origin of the induced vacuolae (Fig. 7 B). Therefore, upstream apoptotic activators can cause ER changes that remain undetectable with the techniques used unless amplified by inhibition of RyR channels. In light of our previous results this effect could be mediated by endogenous Bak, which, consistent with this model, is present in 293T cells (Fig. 7 C).

To test this possibility, we turned to *bak*$^{-/-}$ and *bax*$^{-/-}$ MEFs. Reminiscent of previous results with 293T cells (Fig. 7 A), transfection of BimEL or tBid in combination with Bcl-X$_L$ induced wild-type MEFs to show cytoplasmic vacuolization in the presence of dantrolene (Fig. 8 A). Vacuolae had a reticular origin as shown by experiments using cotransfected erRFP (unpublished data). Although this phenotype was basically unchanged in Bax-deficient MEFs (Fig. 8 A), the absence of Bak almost completely abolished it (Fig. 8 A). In addition, expression of Bak-ΔBH3 induced a comparable extent of cytoplasmic vacuolization in all tested MEFs (Fig. 8 B), indicating that endogenous full-length Bak is unnecessary for the activity of Bak-ΔBH3. All these results point to a model where upstream ER-remodelling signals are entirely transmitted through endogenous Bak, whereas Bax is for the most part inactive.
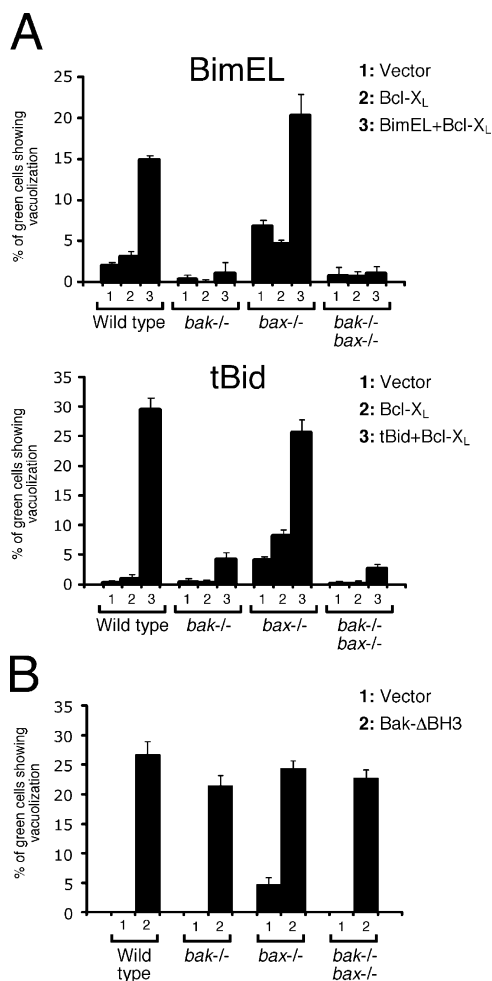
A

B

Figure 8. **Effect of endogenous Bak and Bax in cytoplasmic vacuolization.** (A) Bak-deficient MEFs are unable to generate ER swelling in response to coexpression of BimEL or tBid with Bcl-X_L in the presence of dantrolene. The different MEFs were transfected with a mix of the indicated plasmids and a plasmid expressing GFP. Transfection efficiencies ranged between 20 and 30%. Dantrolene (50 μM) was added 1 h after transfection to all wells. Z-VAD.fmk (100 μM) was also added 1 h after transfection to reduce background death. 48 h later the proportion of GFP-expressing cells showing cytoplasmic vacuolization was determined as in Fig. 1 B. Shown is one representative example of three independent experiments. (B) Expression of Bak-ΔBH3 induces cytoplasmic vacuolization irrespective of the endogenous expression of Bak or Bax. MEFs were cotransfected with the indicated plasmids and a plasmid expressing GFP. Transfection, treatments and scoring were done as in A.

Discussion

Results presented here describe a previously unrecognized activity of Bak in the ER. If activated by Bcl-X_L, overexpressed Bak induces swelling and vacuolization of ER cisternae. Endogenous Bak is also able to transmit similar signals when the pathway is stimulated by upstream BH3-only activators. However, in this case, ER changes are only detected as swollen cisternae after blockade of RyRs, indicating an inhibitory function performed by these calcium channels. Overexpression of signaling effectors is a widely used strategy that often provides hints about function, although excessive expression can lead to artifactual phenotypes. The fact that in our system endogenous Bak

can transmit ER changes initiated by BH3-only activators argues against an artifactual origin of the phenomenon induced by coexpression of Bak and Bcl-X_L. Together, these results point to a relevant function of Bak in the regulation of ER structure.

Data shown here also argue that Bcl-X_L can activate Bak to acquire a new function. In this novel functional status, Bak would have the capability to modulate reticular conformation, while its natural pro-apoptotic potential remains inhibited. However, previous reports suggest that these reticular changes might also have death sensitization consequences. For example, a Bak mutant lacking the BH3 domain, whose overexpression causes ER swelling in our hands, increases susceptibility to cell death induced by chemotherapy agents (Simonian et al., 1997), suggesting a link between both phenomena. Coincidentally, a naturally existing BH3-minus Bak version is able to kill cells if overexpressed (Kim et al., 2004). Furthermore, Bcl-X_L does not always block cell death, because in some systems it redirects apoptosis to alternative forms of cell demise such as necrosis (Shinoura et al., 1999). This is compatible with our own unpublished results indicating that, after suffering ER swelling, cells die in a nonapoptotic manner.

A substantial functional redundancy has been previously reported for Bak and Bax. Thus, cells deficient for only one of these mediators remain sensitive to multiple apoptotic inducers, whereas double-deficient cells are widely resistant (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001). In addition, only double knockout mice present acute phenotypes (Knudson et al., 1995; Lindsten et al., 2000). Consistent with this redundancy, an overlapping range of cellular activities has been ascribed to these molecules. Both mediators induce mitochondrial apoptosis by inserting into the outer mitochondrial membrane, by promoting their oligomerization, and ultimately by allowing the release of cytochrome *c* from the intermembrane space (Cory et al., 2003). Similarly, both proteins mediate ER-dependent apoptosis by inducing calcium release from reticular cisternae (Nutt et al., 2002). Our results showing that Bak has functions in the ER that are not shared by Bax suggest that this functional similarity may not always apply.

Activities uniquely ascribed to Bak or Bax could help explain some of the discrepancies found in the literature. Thus, despite a high degree of functional redundancy, only Bak is critical for apoptosis induced by chemotherapy drugs (Wang et al., 2001a) or granzyme B (Wang et al., 2001b), whereas Bax has a predominant role in other models (Zhang et al., 2000; Theodorakis et al., 2002; Gillissen et al., 2003). If cell-type or context-dependent cues can increase the relative importance of specific Bak and Bax activities with respect to common BH3-dependent pathways, this could explain why in some systems one molecule can be present but inactive whereas its counterpart is fully functional in transmitting death signals.

The accepted model for mitochondrial apoptosis places BH3-only molecules upstream of Bak and Bax in the signaling cascade (Bouillet and Strasser, 2002). We find that this epistatic sequence is maintained in the function of Bak that we describe here. However, how BH3-only molecules activate Bak to carry out a function that needs an additional activation step provided by Bcl-X_L poses some challenges. This

Bak-associated activity likely requires the formation of Bak–Bcl-X$_L$ complexes. Support for this notion comes from our data showing that the vacuolating potential of Bak is unmasked by deletion of the BH3 domain, precisely the region that Bcl-X$_L$ is known to bind and inhibit (Chittenden et al., 1995a). But in order to expose its BH3 domain and bind Bcl-X$_L$, Bak has to undergo a structural shift (Sattler et al., 1997), a change thought to be directly induced by BH3-only members Bim or tBid (Desagher et al., 1999; Wei et al., 2000; Letai et al., 2002). Therefore, the probable signaling logic in our system is that BH3-only proteins activate Bak to expose its BH3 domain for binding by Bcl-X$_L$. However, Bim and tBid are known to preferentially bind anti-apoptotic molecules, saturating protective sites before they activate Bak and Bax (Zong et al., 2001; Cheng et al., 2001; Letai et al., 2002). Obviously, this makes it difficult for activated Bak to find Bcl-X$_L$ molecules not previously occupied by BH3-only homologues. A plausible solution comes from reports showing that Bcl-X$_L$ is mainly a soluble protein that translocates to membrane fractions on apoptosis (Hsu et al., 1997). These data open the possibility that empty Bcl-X$_L$ molecules become available in the ER after Bak activation has occurred.

In addition, the activity described here may involve the formation of a molecular complex simultaneously including BH3-only homologues, Bak and Bcl-X$_L$. Although Bak is thought to mainly function as a BH3-domain donor, it contains a pocket that can be recognized by the BH3 domain of Bim and Bid (Letai et al., 2002). Whether Bak can bind at the same time BH3-only molecules and Bcl-X$_L$ is an interesting possibility that remains to be investigated.

Although the underlying biochemical nature of ER swelling in our system is unclear, a reasonable possibility is that it is due to an increased osmotic pressure caused by an inward ionic current. Because Bak has been shown to regulate ER calcium levels (Nutt et al., 2002; Scorrano et al., 2003; Zong et al., 2003), this ion could be involved. However, several lines of evidence argue against a direct role of calcium. First, Bax has been shown to regulate reticular calcium in the same manner as Bak (Nutt et al., 2002; Scorrano et al., 2003), and it is difficult to imagine how a Bak-specific function could be mediated by a common regulatory activity. Second, our own unpublished data indicate that treatment with thapsigargin (an agent that reduces ER calcium levels) does not influence vacuolae formation. Third, we have seen that calcium concentrations both inside vacuolae and in the cytosol remain unchanged in vacuolated cells.

Although calcium is unlikely to be directly involved, results showing that ER swelling is potentiated by dantrolene and inhibited by caffeine point to a regulatory role by RyR channels. Because dantrolene and caffeine respectively reduce (Jacobs et al., 1991) and increase (Ozawa, 2001) cytosolic calcium, a plausible model is that calcium specifically released by RyRs has the ability to counter the reticular alterations. In addition, it is interesting to note that, although reasonably selective, RyR channels are known to allow passage of other cations (Fill and Copello, 2002), thus raising the possibility that an ion other than calcium is involved in the described phenomena.

Other more trivial mechanisms are less likely to occur. For example, it has been described that insertion of the COOH-terminal tail-anchor of cytochrome b5 into reticular membranes causes ER conformational changes in yeast (Vergeres et al., 1993). The fact that deletion of the BH1 domain blocks this process excludes that Bak is unspecifically causing ER structural changes as a consequence of a similar phenomenon.

In summary, we describe here a novel cellular activity that can be unequivocally performed by Bak but not Bax. This exclusive function could explain why in some systems the functional redundancy that has been ascribed to both molecules does not apply. More generally, it provides some insight into the role of multidomain pro-apoptotic effectors in the ER, an organelle whose involvement in the regulation of cell death is just beginning to be dissected.

Materials and methods

Cell lines and reagents
293T, HeLa, and Cos cells were obtained from the American Type Culture Collection. S. Korsmeyer (Harvard Medical School, Boston, MA) provided the wild-type and Bak or Bax-deficient transformed MEFs (Wei et al., 2001). Cells were cultured at 37°C and a humidified 5% CO$_2$ atmosphere, in DME (Invitrogen) containing 10% heat-inactivated FBS (Invitrogen) and 100 U/ml of penicillin/streptomycin (Invitrogen). Caffeine, dantrolene, EGTA, and thapsigargin were obtained from Sigma-Aldrich. Z-VAD.fmk was obtained from Becton Dickinson.

DNA constructs and transfections
Bak, Bax, BimEL, and tBid cDNAs were amplified by PCR from a human primary T cell cDNA library. All cDNAs were verified by sequencing, contained the same gccaccatg Kozak consensus for initiation of translation, and were cloned into the pEAK series of mammalian expression plasmids (Edge Biosystems). The Bcl-X$_L$ construct was described elsewhere (Pimentel-Muiños and Seed, 1999). The caspase-9-dominant negative (C9.DN) and p35 expression plasmids (Rabizadeh et al., 2004) were provided by S. Rabizadeh (The Buck Institute for Age Research, Novato, CA). Bak-ΔBH3 was constructed by PCR, substituting the BH3 domain (amino acids 72–88, both deleted) for an EcoR1 site, which introduces amino acids EF instead. To create Bak-ΔBH3-ΔBH1, PCR was used to substitute the BH1 domain (amino acids 117–136) for a BamH1 site (amino acids GS). PCR was also used to generate both Bak-ΔBH3-cb5 and Bak-ΔBH3-ActA constructs. In brief, the previously described cb5 reticular (Zong et al., 2003) and ActA mitochondrial (Zhu et al., 1996) localization signals were inserted downstream amino acid 186 of human Bak-ΔBH3, using an engineered BbsI site. Template constructs containing mouse Bak-cb5 and human Bcl2-ActA were provided by C. Thompson (University of Pennsylvania, Philadelphia, PA) and D. Andrews (McMaster University, Hamilton, Canada), respectively. cDNAs were tagged at the NH$_2$ terminus by inserting the relevant PCR product downstream of a sequence encoding the AU1 peptide (DTYRYI). Constructs used in figures where AU tagging is essential (Figs. 1 F, 2 B, 3 C, 5 A, and 7 C) are labeled as such. All other results were reproduced with both tagged and untagged versions. ER-targeted RFP was created by PCR to introduce the COOH-terminal amino acid sequence SEKDEL, able to confer ER retention to heterologous proteins (Munro and Pelham, 1987). This fragment was ligated downstream the leader sequence of the surface molecule CD5, and cloned into the pEAK vector. A similar approach was used to build the ER-targeted version of the low affinity calcium indicator YC3.3 (Miyawaki et al., 1997), which was provided by R. Tsien (University of California, San Diego, La Jolla, CA).

Transfections of 293T cells were performed using the calcium phosphate precipitation method (Ausubel et al., 1987). DNA amounts were 1 μg for 24-well plates and 5 μg for 6-well plates. HeLa, Cos, and MEFs were always transfected in 24-well plates using Fugene (Roche) liposomes combined with 0.4 μg of DNA.

Immunofluorescence stainings, microscopy, and FRET analysis
In vivo quantitation of vacuolated cells and immunofluorescence studies were always performed in 24-well plates. For immunofluorescence, cells

were seeded onto poly-L-lysine (293T; Sigma-Aldrich) or tissue culture–treated (for Cos and HeLa cells; Fisher Scientific) coverslips, and transfected the next day. At the end of the experiment, coverslips were fixed in 4% PFA (Sigma-Aldrich). For DAPI and antibody stainings, cells were permeabilized in PBS containing 0.5% Igepal CA-630 detergent (Sigma-Aldrich) and 1% glycine to block free aldehyde groups. To stain with DAPI, an additional incubation in PBS with 1 μg/ml DAPI (Roche) was performed. For antibody stainings, preparations were blocked in 3% BSA diluted in PBS and stained for 1 h with an anti-AU1 mAb (2 μg/ml; Babco), or a polyclonal rabbit anti-calreticulin serum (1/100; Calbiochem). The respective secondary stainings were performed for 1 h using a 1/400 dilution of a Cy3-coupled goat anti–mouse serum (Jackson ImmunoResearch Laboratories) or an Alexa Fluor 488 goat anti–rabbit serum (Molecular Probes). Cells were mounted in medium containing Mowiol 4-88 (Calbiochem) prepared as described previously (Harlow and Lane, 1988).

Cytoplasmic vacuolization was usually scored in vivo under an inverted fluorescence microscope (Axiovert 135; Carl Zeiss MicroImaging, Inc.) using the 20× objective. For confocal studies we used the 488- and/or 543-nm bands of the Argon and Helium-Neon lasers, respectively, of a confocal microscope (model LSM-510; Carl Zeiss MicroImaging, Inc.). Images were imported using the LSM-510 software (Carl Zeiss MicroImaging, Inc.). For in vivo confocal microscopy, coverslips with transfected cells were placed in a microscopy quality glass-bottom plate (WillCo) and analyzed with the same confocal system. Confocal FRET measurements were done in vivo using the same technique, stimulating cells with the 458-nm band of the Argon laser. To calculate FRETs, the ratio of emission intensities at 535–480 nm was calculated for each pixel using the LSM-510 Zeiss software. Bars represent 10 μm in all micrographs.

Western blotting

Cells were seeded in 6-well plates and transfected the next day. At the end of the experiment, cells were lysed in a buffer containing 1% Igepal CA-630 detergent (Sigma-Aldrich), 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, and protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), and PMSF (1 mM; Roche). After a 5-min centrifugation, protein concentrations were measured using the Bradford method (Bio-Rad Laboratories). Equal amounts of protein were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with specific antibodies against PARP (clone C2-10, mAb; Becton Dickinson), AU1 (mAb; Babco), Bak (rabbit polyclonal; Becton Dickinson), or Bax (N-20, rabbit polyclonal; Santa Cruz Biotechnology, Inc.). After an additional incubation with appropriate secondary HRP-coupled antibodies (DakoCytomation), blots were developed by chemiluminescence using the ECL system (Amersham Biosciences). When reprobed, membranes were previously stripped for 15 min in a 7 M guanidinium hydrochloride (Sigma-Aldrich) solution.

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