Bax and Bak Promote Apoptosis by Modulating Endoplasmic Reticular and Mitochondrial Ca^{2+} Stores*

Alterations in intracellular Ca^{2+} homeostasis and cytochrome c release from mitochondria have been implicated in the regulation of apoptosis, but the relationship between these events remains unclear. Here we report that enforced expression of either Bax or Bak via adenoviral gene delivery results in the accumulation of the proteins in the endoplasmic reticulum (ER) and mitochondria, resulting in early caspase-independent BCL-2-sensitive release of the ER Ca^{2+} pool and subsequent Ca^{2+} accumulation in mitochondria. The inhibition of ER-to-mitochondrial Ca^{2+} transport with a specific inhibitor of mitochondrial Ca^{2+} uptake attenuates cytochrome c release and downstream biochemical events associated with apoptosis. Bax and Bak also directly sensitize mitochondria to cytochrome c release induced by immediate emptying of ER Ca^{2+} pool. Our results demonstrate that the effects of the “multidomain” proapoptotic BCL-2 family members Bak and Bax involve direct effects on the endoplasmic reticular Ca^{2+} pool with subsequent sensitization of mitochondria to calcium-mediated fluxes and cytochrome c release. These effects modulate the kinetics of cytochrome c release and apoptosis.

Caspase activation during apoptosis is regulated by the release of mitochondrial polypeptide activators including cytochrome c (1), SMAC/Diablo (2, 3), and AIF (4, 5). Antia apoptotic members of the BCL-2 family prevent caspase activation by blocking factor release (4, 6, 7), whereas proapoptotic members of the family promote release (8–11). Indeed, very recent work (12) has demonstrated that the expression of either Bax or Bak, members of the “multidomain” subfamily of proapoptotic BCL-2 homologs, is absolutely required for cytochrome c release and caspase activation in mouse embryonic fibroblasts that are exposed to a wide array of stimuli. However, the biochemical mechanisms underlying the effects of BCL-2 family polypeptides are still unclear.

Another large body of evidence implicates alterations in intracellular Ca^{2+} homeostasis in the control of apoptosis. Early work (13) demonstrated that endogenous nucleolus activation proceeds via a Ca^{2+}-dependent mechanism in thymocytes and certain other cell types exposed to a wide range of stimuli, and more recent studies (14–17) suggest that BCL-2-sensitive depletion of the ER Ca^{2+} pool is an early event in apoptosis. Other studies (18, 19) have shown that BCL-2 regulates mitochondrial Ca^{2+} homeostasis and prevents Ca^{2+}-induced cytochrome c release (20). BCL-2 constitutively associates with both the mitochondrial (21–23) and ER (24–26) membranes via a conserved “transmembrane” domain localized within its C terminus (27, 28). On the other hand, Bax appears to reside within the cytosol in resting cells but translocates to membrane fraction(s) particularly mitochondria during apoptosis (23, 29, 30) via a mechanism that is also dependent upon its C-terminal transmembrane domain (30). Whether or not Bax and/or other members of the BCL-2 family accumulate within the ER has not been addressed directly.

Other recent work (31) has shown that ER Ca^{2+} release has immediate effects on mitochondrial function. Close contacts exist between mitochondria and the sites of ER Ca^{2+} release, such that ER Ca^{2+} release leads to rapid Ca^{2+} accumulation in mitochondria (32, 33). Under normal conditions, mitochondrial Ca^{2+} uptake appears to serve as an activating signal to increase metabolism (32). However, one group has shown that mitochondrial Ca^{2+} uptake promotes cytochrome c release in cells exposed to the proapoptotic agent, staurosporine (34, 35). Given previous work implicating BCL-2 in the control of ER Ca^{2+} homeostasis, we wondered whether or not proapoptotic members of the BCL-2 family might exert their effects through modulation of intracellular Ca^{2+} stores.

EXPERIMENTAL PROCEDURES

Materials—The esterified peptide caspase inhibitor ZVAD (O-Me) was purchased from Enzyme System Products, Inc. (Dublin, CA). MG-132 and RU-360 were from Calbiochem. Anti-Fas antibody, CH-11, was from Kamiya Biomedical (Seattle, WA). Staurosporine, thapsigargin, and all other chemicals were obtained from Sigma.

Cell Lines and Tissue Culture—The PC-3 human prostatic adenocarcinoma line was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum antibiotics, sodium pyruvate, and 10 mM Hepes, pH 7.4. The PC-3 cells were stably transfected with human BCL-2 using a plasmid, pCI-BCL-2, generously provided by Dr. John C. Reed, The Burnham Institute. Cells were incubated for 3 h with 10 μg of plasmid in 2 ml of serum-free medium containing 60 μl of liposomes (Transfast, Promega, Inc., Madison, WI). The transfection medium was

* This work was supported in part by NCI, National Institutes of Health Grants CA69876 (to D. J. M.), PO1-CA78778-01A1 (to J. A. R., and S. G. S.), 2P50-CA09790-04 (to J. A. R.), and Cancer Immunobiology Training Grant CA09598 (to L. K. N.). Additional funding was provided by a grant from the Tobacco Settlement Funds as appropriated by the Texas State Legislature (Project B) and The W. M. Keck Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Received for publication, July 19, 2001, and in revised form, December 5, 2001. Published, JBC Papers in Press, December 6, 2001, DOI 10.1074/jbc.M106817200

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then diluted in normal tissue culture medium, and cells were incubated for an additional 24 h without selection. The medium was then replaced with 1 mg/ml Geneticin (G418, Invitrogen), and cells were maintained in this medium until obvious colonies appeared on the tissue culture dishes. Individual colonies were harvested and expanded, and BCL-2 expression was confirmed by immunoblotting with a monoclonal anti-BCL-2 antibody (6C8, generously provided by Dr. Timothy McDonnell, M. D. Anderson Cancer Center). Resistance to apoptosis was also verified in cells incubated for 24–48 h with staurosporine or thapsigargin.

Adenovirus-mediated Transduction—The construction of the Ad/GT-Bak, Ad/GT-Bax, Ad/GT-LacZ, and Ad/GV16 vectors was reported previously (36). The Ad/GT-Bak or Ad/GT-Bax vector was constructed by placing Bak or Bax cDNA downstream of the GAL4/TATA promoter (GT) to generate the shuttle plasmid pAd/GT-Bak or Bax. This plasmid was cotransfected into 293 cells along with a 35-kb ClaI fragment purified from human adenoviral type 5 to generate the Ad/GT-Bak or Ad/GT-Bax vector. Bak or Bax gene expression can then be induced in target tissues by a coadministration of the Ad/GT-Bak or Ad/GT-Bax vector with the second adenoviral vector in our system, Ad/GV16, which produces the GAL4/GV16 fusion protein. Purified Ad/GT-Bak or Ad/GT-Bax was obtained by expanding the virus in 293 cells, harvesting the supernatant of those cells, and then subjecting the supernatant to ultracentrifugation on a cesium chloride gradient. Virus titers were determined by optical absorbancy at 260 nm. Lysates were centrifuged for 5 min in an ice-cold buffer containing 25 mM Tris and 5 mM MgCl2, pH 7.4. Lysates were centrifuged for 5 min at 16,000 × g, supernatants were mixed with 1× Laemmli’s reducing SDS-PAGE sample buffer, and polypeptides were transferred to nitrocellulose membranes. The transduction efficiencies of adenoviral vectors in various cancer cell lines were determined by infecting cells with Ad/GT-LacZ and then determining the titers needed to transduce at least 80% cells. These levels were achieved in PC-3 cells following treatment with Ad/GT-Bak (2000 viral particles) Ad/GV16 (1000 viral particles), Ad/GT-Bax (2000 viral particles) Ad/GV16 (1000 viral particles), and Ad/GT-LacZ (2000 viral particles) Ad/GV16 (1000 viral particles).

DNA Fragmentation Analysis—We measured DNA fragmentation by propidium iodide staining and fluorescence-activated cell sorter analysis as described previously (37). Cells were harvested, pelleted by centrifugation, and resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4 °C for 16 h and vortexed prior to fluorescence-activated cell sorter analysis (FL-3 channel, Becton-Dickinson FACScan, Mountain View, CA).

Cytochrome c Release Measurements—The release of cytochrome c from mitochondria was measured by immunoblotting as described previously (38). Cells were harvested by centrifugation and gently lysed for 5 min in an ice-cold buffer containing 25 mM Tris and 5 mM MgCl2. The transduction efficiencies of adenoviral vectors in various cancer cell lines were determined by infecting cells with Ad/GT-LacZ and then determining the titers needed to transduce at least 80% cells. These levels were achieved in PC-3 cells following treatment with Ad/GT-Bak (2000 viral particles) Ad/GV16 (1000 viral particles), Ad/GT-Bax (2000 viral particles) Ad/GV16 (1000 viral particles), and Ad/GT-LacZ (2000 viral particles) Ad/GV16 (1000 viral particles).

Quantification of Intracellular Ca2+ in Prostate Cancer Cells—Cells plated on 22 × 30-mm glass coverslips were loaded with 10 μM fura-2 acetoxyethyl ester (Molecular Probe, Inc.) for 1 h at 37 °C with humidified air (5% CO2). The coverslips were washed thoroughly with phosphate-buffered saline and mounted on a 1.5-mL volume chamber (cells facing upward). The chamber was placed on an epifluorescence/phase-contrast microscope for Ca2+ imaging and quantitation. Cells were bathed in 1 mL of Hank’s Balanced Salt Solution without Ca2+ at room temperature. After a baseline [Ca2+]i was established, cells were then treated with thapsigargin (5 μM) to empty [Ca2+]i stores.

An INCA work station (Intracellular Imaging, Inc.) was used to quantify [Ca2+]i levels based on fura-2 fluorescence. The INCA software allowed the subtraction of background fluorescence. Fluorescence was monitored using a ×20 fluorescence objective. Cells were illuminated alternately at excitation wavelengths of 340 and 380 nm using a xenon arc lamp. The emitted fluorescence was monitored at 511 nm with a video camera, and the calculated free [Ca2+]i was determined using the cell-free calibration curve. The data were collected with INCA software (Win 3.1 version).

Spectrofluorometric Analysis of Mitochondrial Ca2+—Cells were pelleted and resuspended in 5 mL of complete RPMI 1640 medium. 50 μg of Rhod-2 acetoxyethyl ester (Molecular Probes) was diluted to 0.5 μg/ml in Me2SO. Cells were loaded with Rhod-2 acetoxyethyl ester for 45 min, and the washed cells were analyzed in a spectrofluorimeter (Model LS 50 B, PerkinElmer Life Sciences) at 540 nm excitation and 585 nm emission. The analysis of washed MitoTracker-counterstained cells by confocal microscopy confirmed that the vast majority of Rhod-2 fluorescence was associated with mitochondria. Furthermore, preincubation with the mitochondrial uncoupler CCCP reduced fluorescence levels to base line and completely blocked the increases in fluorescence normally observed following stimulation with thapsigargin. To obtain fluorescence maxima and minima, cells were sequentially incubated with

![Fig. 1](http://www.jbc.org/Downloadedfrom)

**Fig. 1.** Kinetics of cytochrome c release induced by adenoviral Bak or Bax. A, constructs were added to PC-3 as described under “Experimental Procedures,” and time-dependent expression of each polypeptide was measured by immunoblotting. Loading was controlled by immunoblotting with anti-actin antibody. Cytosolic extracts were also prepared for analysis of cytochrome c release, and DNA fragmentation was assessed in gently permeabilized (0.1% Triton X-100) cells by propidium staining and flow cytometry. Experiments were performed in triplicate. B, effects of Ad-Bax. Experiments were conducted as outlined in A.

![Fig. 2](http://www.jbc.org/Downloadedfrom)

**Fig. 2.** Bak and Bax localize to the endoplasmic reticulum and mitochondria. PC-3 cells were infected with Ad-Bax or Ad-Bak as described under “Experimental Procedures.” After 12 h, cells were stained for each polypeptide using phycoerythrin-conjugated secondary antibodies (red) and counterstained with either MitoTracker Green or an antibody to the ER-specific protein GRP-78/BIP with an Alexa 594-conjugated (green) secondary antibody, and fluorochromes were detected by confocal microscopy (×60 objective).
Bax- and Bak-induced Ca\(^{2+}\) Fluxes in Apoptosis

Bax- and Bak-induced Ca\(^{2+}\) uptake and subsequent cytochrome c release. To investigate this possibility, we overexpressed Bax or Bak in human PC-3 prostate adenocarcinoma cells with adenoviral vectors Ad-Bak and Ad-Bax. We decided on this approach, because it allowed us to study the effects of Bax and Bak in isolation. In addition, we designed our experiments to reproduce the kinds of increases in protein expression (2–4-fold) observed in cells exposed to more conventional proapoptotic stimuli (i.e., DNA-damaging agents) (39–41). Transduction of the PC-3 cells resulted in detectable increases in Bak or Bax by 10–12 h (Fig. 1). Protein expression was followed closely by the release of cytochrome c from the mitochondria and DNA fragmentation both by 12 h (Fig. 1), consistent with previous results (36, 42). To determine whether Bax and Bak accumulated within mitochondria and the endoplasmic reticulum, we characterized their subcellular localizations by immunofluorescence confocal microscopy using antibodies specific to Bax, Bak in conjunction with mitochondrial (MitoTracker), or endoplasmic reticular (antibody to GRP-78/BIP) probes. The results confirmed that both proteins traffic to both organelles, although some diffuse cytosolic staining was also detected (Fig. 2).

We next determined the effects of Bak or Bax expression on ER Ca\(^{2+}\) pool content. Substantial reductions (>50%) in ER Ca\(^{2+}\) levels were detected as early as 10 h after transduction with Ad-Bax or Ad-Bak, whereas infection with a control vector Ad-β-galactosidase had no effect (Fig. 3, A and B). Bak and Bax were not expressed until 10 h, and [Ca\(^{2+}\)]\(_{ER}\) calcium was not detectable after 12 h. Therefore, even though [Ca\(^{2+}\)]\(_{ER}\) was significantly depleted in cells treated with Ad-Bax or Ad-Bak compared with controls at 10 h, there were still measurable amounts of Ca\(^{2+}\) within the ER at this time point that were equal to or greater than the concentration of Ca\(^{2+}\) within the mitochondria. The loss of ER Ca\(^{2+}\) induced by Bax (data not shown) or Bak was unaffected by pretreatment with a pan-caspase inhibitor ZVADfmk (Fig. 3C) but was prevented by the overexpression of BCL-2 in PC-3 transfectants (Bcl-2.9) (Fig. 3D). The overexpression of BCL-2 also blocked the release of cytochrome c from the mitochondria and delayed DNA fragmentation (Fig. 4).

As noted above, an efflux of Ca\(^{2+}\) from the ER can lead to

Examples of several typical traces showing thapsigargin-induced Ca\(^{2+}\) increases ([Ca\(^{2+}\)]\(_E\)) at different times after Ad-Bak treatment. [Ca\(^{2+}\)]\(_E\) was quantified by stimulating cells with thapsigargin (7.5 μM) in the absence of extracellular Ca\(^{2+}\). The difference between basal and peak fluorescence after TG stimulation is equivalent to [Ca\(^{2+}\)]\(_E\). B, PC-3 cells were treated with adenoviral Bak, Bax, or β-galactosidase control for 10–12 h, and [Ca\(^{2+}\)]\(_E\) was measured as described above. C, ER calcium depletion is caspase-independent. PC-3 cells were pretreated with the caspase inhibitor ZVAD (20 μM) for 30 min. Cells were then exposed to Ad-Bak for 12 h, and [Ca\(^{2+}\)]\(_E\) was quantified as described above. D, ER calcium depletion is Bcl-2-sensitive. PC-3 and PC-3-Bcl-2.9 cells were treated with Ad-Bak and Ad-Bax for 10 h, and [Ca\(^{2+}\)]\(_E\) was measured by the thapsigargin release method as described above.
coupled increases in Ca\(^{2+}\) levels within mitochondria. Therefore, we assessed the effects of Bak and Bax on mitochondrial Ca\(^{2+}\) concentrations in cells loaded with the Ca\(^{2+}\)-sensitive dye, Rhod-2. The expression of either protein induced a significant increase in mitochondrial Ca\(^{2+}\) accumulation, although Bax produced significantly more Ca\(^{2+}\) uptake than Bak (\(p < 0.01\)) (Fig. 5A). The increases in mitochondrial calcium levels were almost completely suppressed in cells pretreated with a specific inhibitor of mitochondrial Ca\(^{2+}\) uptake (RU-360) (43) (Fig. 5A). In contrast, mitochondrial Ca\(^{2+}\) levels were unchanged following infection with the control vector Ad-\(\beta\)-galactosidase or in the BCL-2 transfectants infected with Ad-Bak or Ad-Bak (Fig. 5B). Importantly, RU-360-mediated inhibition of mitochondrial Ca\(^{2+}\) uptake attenuated the cytochrome c release and DNA fragmentation induced by Ad-Bax or Ad-Bak (Fig. 5, C and D), demonstrating that cytochrome c release occurred via a Ca\(^{2+}\)-sensitive mechanism.

The ER Ca\(^{2+}\) ATPase inhibitor, thapsigargin, is often employed to induce rapid pharmacologic emptying of the ER Ca\(^{2+}\) pool (reviewed in Ref. 44). Thapsigargin does induce apoptosis in a variety of different cell types, but a significant lag period is invariably observed between thapsigargin-induced emptying of ER Ca\(^{2+}\) pool and the first biochemical end points of apoptosis (12, 45–47). To test whether direct effects of Bax and/or Bak on mitochondria might sensitize them to cytochrome c release induced by emptying of ER pool, we measured cytochrome c release in cells that had been pretreated with Ad-Bax or Ad-Bak for 10 h. At this time point, Bax and Bax protein accumulation was readily detected, but ER Ca\(^{2+}\) release was submaximal and cytochrome c release had not yet occurred. Cytochrome c release was not observed in untreated cells or in cells infected with Ad-\(\beta\)-galactosidase (Fig. 5E). Strikingly, however, exposure to Ad-Bax- or Ad-Bak-sensitized mitochondria to Ca\(^{2+}\) induced cytochrome c release, such that the release was induced by thapsigargin within 5 min (Fig. 5E). Together with the results presented above, the data demonstrate that Bak and Bak regulate Ca\(^{2+}\)-induced cytochrome c release by promoting the release of Ca\(^{2+}\) from the ER pool and by sensitizing mitochondria to the effects of this release. Although not absolutely required for cytochrome c release, ER Ca\(^{2+}\) release appears to augment the kinetics of cytochrome c release. The regulation of SMAC/Diablo and other proteins may further refine this process, which was set up initially by ER Ca\(^{2+}\) release from the multidomain proapoptotic Bcl-2 family members, Bak and Bax.

The release of protein factors from mitochondria plays a central role in cellular commitment to apoptosis, but the biochemical mechanisms controlling the release remain obscure. A growing consensus suggests that proapoptotic and antiapoptotic members of the BCL-2 family are involved. The results of this study support this conclusion and identify one candidate mechanism for their effects. Specifically, our data demonstrate that two proapoptotic members of the BCL-2 family, Bak and Bax, promote early emptying of the endoplasmic reticular Ca\(^{2+}\) pool and subsequent accumulation of Ca\(^{2+}\) within the mitochondria. These effects are blocked by the overexpression of BCL-2 but are not dependent upon caspase activation. Importantly, a selective inhibitor of mitochondrial Ca\(^{2+}\) uptake attenuated the cytochrome c release and DNA fragmentation induced by either protein, providing direct evidence for a causal role for Ca\(^{2+}\) uptake in apoptosis. Furthermore, cells overexpressing Bak or Bax were sensitized to cytochrome c release induced by thapsigargin, a compound that directly stimulates direct and complete emptying of ER Ca\(^{2+}\) pool through the inhibition of the ER Ca\(^{2+}\) ATPase. Together, our data indicate that Bak and Bak simultaneously promote alterations in intracellular Ca\(^{2+}\) compartmentalization and sensitize mitochondria to cytochrome c release induced by these alterations. A role for mitochondrial Ca\(^{2+}\) uptake in promoting cytochrome c release is consistent with other recent studies. For example, Hajnoczky and coworkers (34, 35) showed that staurosporine sensitized mitochondria to cytochrome c release induced by inositol 1,4,5-trisphosphate-induced emptying of the ER Ca\(^{2+}\) pool, and independent work by Gogvadze and colleagues (48) demonstrates that Bak promotes Ca\(^{2+}\)-mediated cytochrome c release in isolated liver mitochondria.

Although BCL-2 did not affect steady-state levels of Ca\(^{2+}\) in the ER or mitochondria, it did inhibit the effects of Bax and Bak on ER Ca\(^{2+}\) release and subsequent mitochondrial Ca\(^{2+}\) uptake. These observations suggest that BCL-2 acts primarily to antagonize the effects of Bax and Bak in our cells and does not exert direct effects of its own. However, it should be stressed that other laboratories have reported different effects.
of BCL-2 on intracellular Ca\(^{2+}\) pools (16, 19, 49, 50). For example, at least three groups have shown that BCL-2 lowers the steady-state level of Ca\(^{2+}\) within the ER, which they argued inhibits apoptosis by reducing Ca\(^{2+}\) efflux across the ER membrane (16, 19, 49). In contrast, another group (50) showed that the overexpression of Bcl-2 increased steady-state levels of ER Ca\(^{2+}\). On the other hand, Murphy et al. (18) concurred that BCL-2 had no significant effect on steady-state Ca\(^{2+}\) levels in their cells, but they found that BCL-2 potentiated Ca\(^{2+}\) uptake by mitochondria, an observation that stands in opposition to our results. Although we cannot explain these contrasting findings at present, they are probably related to the use of different cellular model systems. Hematopoietic cells and excitable cells (neurons) are likely to regulate intracellular Ca\(^{2+}\) compartmentalization very differently from epithelial cells. Other groups (51) have argued that BCL-2 is capable of interacting with a variety of different proteins, some of which are involved in Ca\(^{2+}\)-associated signal transduction (i.e. the Ca\(^{2+}\)-dependent protein phosphatase, calcineurin). It is conceivable that cellular context dictates precisely how BCL-2 will influence intracellular Ca\(^{2+}\) pools. Importantly, the connection between ER Ca\(^{2+}\) pool emptying and apoptosis is not disputed.

Although our studies establish a role for Bax and Bak in regulating ER and mitochondrial Ca\(^{2+}\) fluxes, they do not directly identify the proximal mechanism(s) involved. Most of the available information on the biochemical mechanisms underlying the actions of BCL-2 family members has come from studies describing their effects on mitochondria and planar lipid bilayers. Specifically, proapoptotic and antiapoptotic members of the family appear to bind to and regulate components of the permeability transition pore (9, 10), and permeability transition pore opening has been implicated in cytochrome c release in many model systems (reviewed in Ref. 52). In addition, a caspase-processed form of the BH3-only family member, Bid (tBID), can promote oligomerization of Bax or Bak to form transmembrane channels capable of directly allowing for passage of cytochrome c into the cytoplasm (53, 54). Finally, in vitro studies (55–58) (reviewed in Ref. 59) have shown that BCL-2, BCL-X\(_L\), Bax, and Bak can form smaller ion-selective channels in planar lipid membranes. It is conceivable that large pore formation induced by oligomerization of Bax or Bak within the ER membrane directly stimulates Ca\(^{2+}\) release and/or that Ca\(^{2+}\) release is regulated by the ion channel properties of the two proteins. Our data also do not formally rule out the possi-
bility that ER Ca$^{2+}$ release occurs secondarily to earlier alterations within mitochondria. Importantly, the characterization of BCL-2 family conductance properties suggested that BCL-2 and BCL-X$r$ form pores that are selective for monovalent cations (Na$^+$ and K$^+$), whereas Bak and Bax form pores selective for monovalent anions (Cl$^-$) (58). Unless these properties are dramatically modified in vivo, these observations argue against the idea that Bax and Bak directly form Ca$^{2+}$-selective channels in the ER membrane.

Our results also demonstrated that Bak produced significantly higher increases in mitochondrial Ca$^{2+}$ than did Bak. To date, the multidomain members of the BCL-2 family have been thought of as largely redundant, but hints of biochemical selectivity have emerged recently. For example, studies in mouse embryo fibroblasts lacking Bax, Bak or both proteins demonstrated that one of them must be expressed for the physiological properties of the ER. Previous work (60) has shown that increases in Ca$^{2+}$ cause a dramatic decrease in protein mobility within the ER, and this may in part explain the rigidity of the membrane Bax compartment observed within apoptotic cells.
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*J. Biol. Chem.* 2002, 277:9219-9225.
doi: 10.1074/jbc.M106817200 originally published online December 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106817200

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