Bax-mediated Ca$^{2+}$ Mobilization Promotes Cytochrome $c$ Release during Apoptosis

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Previous studies have demonstrated that Ca$^{2+}$ is released from the endoplasmic reticulum (ER) in some models of apoptosis, but the mechanisms involved and the functional significance remain obscure. We confirmed that apoptosis induced by some (but not all) proapoptotic stimuli was associated with caspase-independent, BCL-2-sensitive emptying of the ER Ca$^{2+}$ pool in human PC-3 prostate cancer cells. This mobilization of ER Ca$^{2+}$ was associated with a concomitant increase in mitochondrial Ca$^{2+}$ levels, and neither ER Ca$^{2+}$ mobilization nor mitochondrial Ca$^{2+}$ uptake occurred in Bax-null DU-145 cells. Importantly, restoration of DU-145 Bax expression via adenoviral gene transfer restored ER Ca$^{2+}$ release and mitochondrial Ca$^{2+}$ uptake and dramatically accelerated the kinetics of staurosporine-induced cytochrome $c$ release, demonstrating a requirement for Bax expression in this model system. In addition, an inhibitor of the mitochondrial Ca$^{2+}$ uniporter (RU-360) attenuated mitochondrial Ca$^{2+}$ uptake, cytochrome $c$ release, and DNA fragmentation, directly implicating the mitochondrial Ca$^{2+}$ changes in cell death. Together, our data demonstrate that Bax-mediated alterations in ER and mitochondrial Ca$^{2+}$ levels serve as important upstream signals for cytochrome $c$ release in some examples of apoptosis.

Commitment to cell death via apoptosis appears to require the activation of members of a family of aspartate-directed cysteine proteases known as caspases. Caspase activation is controlled in part by changes within mitochondria that lead to the release of polypeptide factors, including cytochrome $c$ (1) and Smac/Diablo (2, 3), which promote caspase activation by independent and complementary mechanisms. Antiapoptotic members of the BCL-2 family can prevent the mitochondrial alterations leading to factor release and caspase activation (4–6), whereas proapoptotic members of the family promote factor release (7–10). A subfamily of proapoptotic BCL-2 family polypeptides may in fact be required for cytochrome $c$ release because mouse embryonic fibroblasts derived from Bax$^{-/-}$–Bak$^{-/-}$ mice fail to display cytochrome $c$ release or downstream biochemical features of apoptosis after exposure to a variety of death stimuli in vitro (11). However, the biochemical mechanisms underlying the effects of BCL-2 family polypeptides on mitochondria are still unclear.

Another large body of evidence implicates alterations in intracellular Ca$^{2+}$ homeostasis in the control of apoptosis. We demonstrated in early studies that endogenous endonuclease activation proceeds via a Ca$^{2+}$-dependent mechanism in thymocytes and certain other cell types exposed to a wide range of stimuli (12). However, more recent studies indicate that alterations in subcellular Ca$^{2+}$ compartmentalization can be detected in apoptotic cells that do not display global increases in cytosolic Ca$^{2+}$ concentration. Specifically, work by Lam et al. (13) demonstrated that glucocorticoid-induced apoptosis was associated with early emptying of the endoplasmic reticulum Ca$^{2+}$ pool in W7MG1 mouse lymphoma cells (13). Parallel, independent efforts by Baffy et al. (14) showed that the same was true in interleukin-3-dependent 32D hematopoietic progenitor cells after withdrawal of IL-3 (14). Importantly, ER Ca$^{2+}$ pool depletion was not observed in cells transfected with BCL-2 (14–18), suggesting that part of BCL-2’s antiapoptotic activity involves maintenance of the ER Ca$^{2+}$ store. However, precisely how ER Ca$^{2+}$ pool depletion might contribute to the regulation of apoptosis remains unclear.

Given the central role of mitochondria in the commitment to apoptosis, it is possible that ER Ca$^{2+}$ pool depletion triggers secondary changes in mitochondrial Ca$^{2+}$ levels that contribute to cytochrome $c$ release and cell death. Close contacts exist between mitochondria and sites of ER Ca$^{2+}$ release (19), such that ER Ca$^{2+}$ release leads to rapid Ca$^{2+}$ accumulation within mitochondria (20, 21). Furthermore, Hajnoczky and co-workers (22, 23) have shown that 1,4,5-inositol trisphosphate-mediated ER Ca$^{2+}$ release results in mitochondrial Ca$^{2+}$ increases that play an important role in promoting mitochondrial permeability transition and cytochrome $c$ release in cells undergoing apoptosis in response to staurosporine treatment. We recently used adenoviral gene transfer to demonstrate that apoptosis induced by overexpression of Bax (and to a lesser extent, Bak) was associated with alterations in the ER and mitochondrial...
Ca$_2^+$ stores in human PC-3 prostate adenocarcinoma cells (24). Inhibition of mitochondrial Ca$_2^+$ uptake attenuated Bax-induced cytochrome c release and DNA fragmentation (24), strongly suggesting that the Ca$_2^+$ fluxes participated directly in caspase activation and cell death. Whereas our study provided some of the first evidence that Bax and Bak can affect intracellular Ca$_2^+$ stores, it did not provide direct evidence that the effects were important for "endogenous" signals for apoptosis, such as staurosporine, Fas engagement, or exposure to DNA-damaging agents.

Here we characterized the effects of several well-known pro-apoptotic stimuli on ER and mitochondrial Ca$_2^+$ fluxes in human PC-3 prostate adenocarcinoma cells and investigated whether or not these fluxes played a role in promoting cytochrome c release. In addition, we used another, Bax-deficient human prostate cancer cell line (DU-145) to determine whether or not these Ca$_2^+$ fluxes were dependent on cellular Bax expression. The results confirm and extend our previous observations and demonstrate that at least two mechanistically distinct pathways for cytochrome c release can exist within a given cell type, one that is dependent on ER-to-mitochondrial Ca$_2^+$ fluxes and another that is not.

**EXPERIMENTAL PROCEDURES**

**Materials**—The esterified peptide caspase inhibitor Z-VAD (O-Me) was purchased from Enzyme System Products, Inc. (Dublin, CA). MG-132 and RU-360 were from Calbiochem. Anti-Fas antibody (CH-11; IgM) 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) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and 10 mM Hepes (pH 7.4). Stable transfectants overexpressing human BCL-2 were generated as described previously (24) and maintained in RPMI 1640 medium supplemented with 1 mg/ml Geneticin (G418; Invitrogen). Expression of BCL-2 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**—Construction of the Ad/GT-Bax, Ad/GT-LacZ, and Ad/GV16 vectors was reported previously (25). The Ad/GT-Bax vector was constructed by placing Bax cDNA downstream of the GAL4/TATA promoter (GT) to generate the shuttle plasmid pAd/GT-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-Bax vector. Bak or Bax gene expression can then be

**Fig. 1.** Depletion of the ER Ca$_2^+$ store is associated with apoptosis induced by some stimuli. A, quantification of ER Ca$_2^+$ in PC-3 cells exposed to staurosporine or Adriamycin. Cells were incubated with 1 μM staurosporine or 1 μg/ml Adriamycin for the times indicated. One h before analysis, cells were loaded with 5 μM fura-2 AM and transferred to Ca$_2^+$-free medium, and thapsigargin-induced Ca$_2^+$ increases were used as an indirect measure of ER Ca$_2^+$ content as described previously (13, 52). Representative traces (10–20 cells/trace) are shown. The arrow indicates the time point of thapsigargin (TG) addition. B, quantification of ER Ca$_2^+$ content in cells exposed to various stimuli. Cells were incubated for the times indicated with 1 μM staurosporine, 1 μg/ml doxorubicin, 10 μG-MG-132, or 1 μg/ml anti-Fas antibody (CH-11), and ER Ca$_2^+$ content was measured as described above. Mean ± S.E.; n = 3–13. Significant ER Ca$_2^+$ depletion (*, p < 0.001) was observed in staurosporine-treated cells after 2 h and in Adriamycin-treated cells after 8 h.
induced in target tissues by coinfection of the Ad/GT-Bax vector with the second adenoviral vector in our system, Ad/GV16 (which produces the GAL4/GV16 fusion protein). Purified 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 absorbance at $A_{594}$. One unit $= 10^{12}$ viral particles/ml. 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% of the cells. Those levels were achieved in PC-3 cells after treatment with Ad/GT-Bax (2000 viral particles) and Ad/GV16 (1000 viral particles) and treatment with Ad/GT-LacZ (2000 viral particles) and Ad/GV16 (1000 viral particles).

**DNA Fragmentation Analysis**—We measured DNA fragmentation by propidium iodide staining and FACS analysis as described previously (26). Cells were harvested, pelleted by centrifugation, and resuspended in phosphate-buffered saline containing 50 $\mu$g/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 h and vortexed before FACS analysis (BD PharMingen FACSScan; FL-3 channel).

**Spectrofluorometric Caspase-3 Quantification**—Cells were lysed in caspase-3 buffer (100 mM Hepes (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mM EDTA) with freshly added 10 mM diithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and a complete mini-tablet (Roche Molecular Biochemicals) for 1 h at 4°C. Lysates were incubated at 37°C for 1 h in a total of 2 ml of caspase-3 buffer containing 25 $\mu$g aspartate-glutamate-valine-aspartate aminomethyl coumarin (Bachem, King of Prussia, PA). Samples were excited at 380 nm and read at 460 nm in an RF-1501 spectrofluorometer (Shimadzu Scientific Instruments, Columbia MD). Relative fluorescence was calculated by subtracting the blank fluorescence (buffer plus substrate only) from the sample fluorescence and dividing by the protein content of the sample (sample – blank/ protein; fluorescence unit/ $\mu$g protein).

**Cytochrome c Release Measurements**—Release of cytochrome c from mitochondria was measured by immunoblotting as described previously (27). Cells were harvested by centrifugation and gently lysed for 5 min in an ice-cold buffer containing 25 mM Tris and 5 mM MgCl$_2$ (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 extracts from equal numbers of cells ($10^8$ – $10^9$) were resolved by 15% SDS-PAGE. Polyacrylamide gels were transferred to nitrocellulose membranes (0.2 $\mu$m; Schleicher & Schuell), and cytochrome c was detected by immunoblotting with the monoclonal antibody clone 7H8.2C12 (BD PharMingen).

**Quantification of Intracellular Ca$^{2+}$**—Cells plated on 22 × 30-mm glass coverslips were loaded with fura-2-acetoxyethyl ester (AM) (10 $\mu$M; Molecular Probes) for 1 h at 37°C with humidified air (5% CO$_2$). The coverslips were washed thoroughly with phosphate-buffered saline and mounted on a 1.5-ml-volume chamber (with the cells facing upward). The chamber was placed on a epifluorescence/phase-contrast microscope for Ca$^{2+}$ imaging and quantification. Cells were bathed in 1 ml of phosphate-buffered saline without Ca$^{2+}$ at room temperature. Identical results were obtained when cells were bathed in Hanks’ balanced salt solution containing 0.5 mM EGTA. After a baseline cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}]_{c}$) was established, cells were then treated with thapsigargin (5 $\mu$m) to empty [Ca$^{2+}]_{cytosol}$ stores. An INCA work station (Intracellular Imaging, Inc.) was used to quantify [Ca$^{2+}]_{c}$, levels based on fura-2 fluorescence. The INCA software allowed for subtraction of background fluorescence. Fluorescence was monitored using a 2×20 fluorescence objective. Cells were illuminated at alternating 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 [Ca$^{2+}]_{c}$ was determined using the cell-free calibration curve. The data were collected with INCA software (Win 3.1 version).

**Spectrofluorometric Analysis of Mitochondrial Ca$^{2+}$**—Cells were pelleted and resuspended in 5 ml of RPMI 1640 medium containing 0.2% fetal bovine serum. Rhod-2 AM (50 $\mu$g; Molecular Probes) was diluted to 0.5 $\mu$g/ml in Me$_2$SO. Cells were loaded with Rhod-2 AM for 45 min, and the washed cells were analyzed in a spectrofluorometer (PerkinElmer Life Sciences model LS 50 B) at 550 nm excitation and 578 nm emission. 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 FCCP reduced fluorescence levels to baseline and completely blocked the increases in fluorescence normally observed after stimulation with thapsigargin. To obtain fluorescence maxima and minima, cells were sequentially incubated with detergent and EGTA (10 mM, final concentration) in the presence of saturating concentrations of extracellular Ca$^{2+}$. No differences in minimum Ca$^{2+}$-dependent fluorescence were observed when FCCP was substituted for EGTA in these studies. Intramitochondrial Ca$^{2+}$ concentrations were calculated by the following formula: [Ca$^{2+}]_{i} = (F - F_{\text{min}})/F_{\text{max}} - F_{\text{d}}$ (Rhod-2) where $F = $ fluorescence.

**Statistical Analyses**—Values are the means ± S.E. The number of experiments is shown in the legend of each figure. Statistical analysis was performed by analysis of variance with Neuman-Keuls post hoc comparison.

**RESULTS AND DISCUSSION**

Previous work has shown that certain proapoptotic stimuli cause depletion of the endoplasmic reticular Ca$^{2+}$ store (13–16). To determine the prevalence and kinetics of this phenomenon, we treated human PC-3 prostate adenocarcinoma cells with several common death-inducing agents and measured their effects on ER Ca$^{2+}$ levels. Cells were preincubated with various death-promoting stimuli and loaded with the Ca$^{2+}$-sensitive fluorescent dye fura-2 AM, and ER Ca$^{2+}$ content was measured indirectly by releasing the pool with the ER Ca$^{2+}$ ATPase inhibitor thapsigargin. Representative fluorescence traces obtained using this technique in cells treated with staurosporine or the cancer chemotherapeutic agent Adriamycin (doxorubicin) are presented in Fig. 1A, and quantitative data obtained in several experiments are presented in Fig. 1B. Both staurosporine and Adriamycin promoted ER Ca$^{2+}$ release several hours before cytochrome c release was first detected, but the proteasome inhibitor MG-132 and the anti-Fas antibody CH-11 did not (Fig. 1B). Thus, ER Ca$^{2+}$ release is associated with apoptosis induced by some (but not all) proapoptotic stimuli.
The commitment phase of apoptosis involves activation of the caspase family of cysteine proteases. To determine whether or not ER Ca\(^{2+}\) pool depletion was a consequence of caspase activation, we measured staurosporine-induced pool depletion in cells preincubated with the pan-caspase inhibitor zVADfmk (20 \(\mu\)M). The inhibitor had no effect on ER Ca\(^{2+}\) release (Fig. 2B), although control experiments confirmed that it completely blocked caspase activation (measured using a fluorogenic dye; Fig. 2B). These results confirm that ER Ca\(^{2+}\) release occurs upstream of caspase activation.

As described above, previous studies have shown that BCL-2 localizes to the ER, where it may regulate ER Ca\(^{2+}\) homeostasis. To test the effects of BCL-2 in our model system, we stably transfected the PC-3 cells with BCL-2 and characterized their responses to staurosporine. Immunoblotting confirmed that the transfectants expressed high levels of BCL-2 (Fig. 3A), and functional studies demonstrated that staurosporine-induced DNA fragmentation was reduced in the transfectants (Fig. 3B). Consistent with some of the earlier studies, staurosporine failed to induce ER Ca\(^{2+}\) pool depletion in the BCL-2 transfectants (Fig. 3C), an effect that was associated with reduced staurosporine-stimulated cytochrome c release from mitochondria (Fig. 3D).

Recent studies have shown that pseudosynaptic contacts exist between the ER and mitochondrial Ca\(^{2+}\) pools (19), such that release of Ca\(^{2+}\) from ER stores can result in rapid Ca\(^{2+}\) uptake by mitochondria (20, 21). We therefore investigated the effects of staurosporine on mitochondrial Ca\(^{2+}\) levels in PC-3 cells loaded with the mitochondrial Ca\(^{2+}\) probe Rhod-2 AM; representative fluorescence traces obtained using this technique are presented in Fig. 4A. Ca\(^{2+}\)-independent (background) fluorescence was measured by treating cells with the protonophore CCCP, which completely releases the mitochondrial Ca\(^{2+}\) pool, and levels of this background fluorescence were nearly indistinguishable in the cells under all conditions examined (Fig. 4A). Control experiments also confirmed that all of the Rhod-2 was confined to mitochondria. Exposure of PC-3 cells to staurosporine resulted in a significant increase in mitochondrial Ca\(^{2+}\) concentration that was completely abolished by overexpression of BCL-2 (Fig. 4B). To investigate the relationship between mitochondrial Ca\(^{2+}\) uptake and cytochrome c release, we compared the levels of cytochrome c release observed in staurosporine-treated cells in the absence or presence of a chemical inhibitor of the mitochondrial Ca\(^{2+}\) uniporter (RU-360). A representative immunoblot obtained in these experiments is shown in Fig. 4C, and quantitative results obtained in multiple experiments are presented in Fig. 4D. RU-360 significantly reduced staurosporine-induced cyto-
chrome c release (Fig. 4, C and D) and DNA fragmentation (Fig. 4F), but it had no effect on Fas-mediated cytochrome c release (Fig. 4E). Together, these results demonstrate that mitochondrial Ca2+ uptake is important for staurosporine-induced apoptosis and confirm that Fas-mediated apoptosis occurs via Ca2+-independent mechanisms, at least in the PC-3 model system.

We recently showed that overexpression of Bax via adenoviral gene transfer resulted in early, caspase-independent depletion of the ER Ca2+ pool and apoptosis (24). These observations suggested to us that staurosporine’s effects on the ER and mitochondrial Ca2+ pools might be mediated by Bax. To directly test this hypothesis, we characterized the effects of staurosporine on ER (Fig. 5A) and mitochondrial (Fig. 5B) Ca2+ pools in the Bax-deficient human prostate cancer cell line DU-145. Consistent with the hypothesis, staurosporine failed to induce significant ER Ca2+ pool depletion or mitochondrial Ca2+ uptake in the parental DU-145 cells. Importantly, reconstitution of Bax expression via adenoviral gene transfer restored staurosporine’s effects on the ER (Fig. 5A, right bars) and mitochondrial (Fig. 5B, right bars) Ca2+ pools and dramatically accelerated the kinetics of staurosporine-induced cytochrome c release (Fig. 5C). Because mitochondrial Ca2+ uptake appears to be involved in promoting cytochrome c release and

Fig. 4. Effects of staurosporine on mitochondrial Ca2+ levels. A, representative fluorescence traces. Cells were exposed to 1 μM staurosporine (ST) for 4 h in the absence or presence of 10 μM RU-360. Cells were then loaded with Rhod-2 AM, and mitochondrial Ca2+ was estimated based on Rhod-2 fluorescence as described under “Experimental Procedures.” After baseline fluorescence levels were obtained, cells were exposed to CCCP to empty mitochondrial Ca2+ stores. RFU, relative fluorescence intensity (arbitrary units). B, effects of BCL-2 on staurosporine-induced increases in mitochondrial Ca2+. Parental PC-3 cells or BCL-2 transfectants were incubated with 1 μM staurosporine for 4 h. Cells were loaded with Rhod-2 AM, and mitochondrial Ca2+ was measured in a spectrofluorometer. Once baseline fluorescence values were obtained, cells were exposed to CCCP to obtain a fluorescence minimum and then lysed in the presence of extracellular Ca2+ to obtain a fluorescence maximum. Raw fluorescence values were then used to derive Ca2+ concentrations using the Rhod-2 Ca2+ dissociation constant essentially as described previously (24). Mean ± S.E.; n = 4. C and D, RU-360 attenuates staurosporine-induced but not anti-Fas-induced cytochrome c release. Cells were preincubated with the indicated concentrations of RU-360 for 30 min. Cells were then incubated with or without 1 μM staurosporine for 8 h, and cytosolic cytochrome c was analyzed by immunoblotting. Results of densitometric quantification of cytochrome c release in cells treated with 1 μM staurosporine (D) or 1 μg/ml anti-Fas antibody (CH-11) (E) with or without RU-360 are shown. Note that 10 μM RU-360 inhibited thapsigargin-induced mitochondrial Ca2+ uptake by 50% in separate experiments (Fig. 6). Mean ± S.D.; n = 3. F, inhibition of DNA fragmentation by RU-360. PC-3 cells were preincubated with 10 μM RU-360 for 30 min before exposure to 1 μM staurosporine, and DNA fragmentation was quantified by propidium iodide staining and FACS analysis at 12 h. Mean ± S.E.; n = 3. *, p < 0.01 versus control.

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downstream features of apoptosis, these Bax-mediated Ca\(^{2+}\) changes appeared to directly promote apoptotic cell death.

Finally, we turned our attention to the paradoxical observation that staurosporine stimulated apoptosis more rapidly than the Ca\(^{2+}\)-mobilizing agent thapsigargin, even though the effects of thapsigargin on intracellular Ca\(^{2+}\) fluxes were more rapid than those of staurosporine. Specifically, thapsigargin caused a faster ER Ca\(^{2+}\) release and subsequent RU-360-sensitive mitochondrial Ca\(^{2+}\) uptake in PC-3 cells (Fig. 6A), but it did not promote cytochrome c release (Fig. 6C) or DNA fragmentation (Fig. 6B) until much later (18 h after exposure).

These kinetics of cytochrome c release and DNA fragmentation are consistent with previous findings (11, 28–30). These data suggested to us that staurosporine’s effects on mitochondrial Ca\(^{2+}\) might cooperate with other effects (i.e., pore formation and interaction with the permeability transition pore complex) to promote cytochrome c release. To directly test this possibility, we measured thapsigargin-induced cytochrome c release in PC-3 cells that had been pretreated with stauro-

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**Fig. 5.** A, staurosporine-induced ER Ca\(^{2+}\) depletion is Bax-dependent. Left bars, control DU-145 cells were incubated in medium alone or with 1 μM staurosporine for 8 h, and ER Ca\(^{2+}\) levels were measured by thapsigargin release as described in Fig. 1. Mean ± S.E.; n = 4–13. Right bars, DU-145 cells were transduced with adenoviral β-galactosidase (Ad-β-Gal) or adenoviral Bax (Ad-Bax) for 10 h as outlined under “Experimental Procedures.” Cells were then exposed to 1 μM staurosporine for 1 h, and ER Ca\(^{2+}\) were measured by thapsigargin release as described in Fig. 1. Mean ± S.E.; n = 4–13. *p < 0.001 versus control. Although ER Ca\(^{2+}\) levels were similar to those of controls at 10 h, note that controls (18 h) accumulated Bax results in ER Ca\(^{2+}\) pool depletion, consistent with our earlier observations (24). An adenoviral control (β-galactosidase) had no effect on staurosporine-induced Ca\(^{2+}\) release. B, Bax is required for staurosporine-induced mitochondrial Ca\(^{2+}\) increases. Left bars, Bax −/− DU-145 cells were exposed to staurosporine for 4 h, and mitochondrial Ca\(^{2+}\) concentrations were quantified using Rhod-2 AM as described in Fig. 2. Mean ± S.E.; n = 4–13. Right bars, transduction with Bax restores staurosporine-induced mitochondrial Ca\(^{2+}\) fluxes. DU-145 cells were transduced with a control vector (Ad-β-Gal) or Ad-Bax for 10 h. Cells were then exposed to staurosporine for 1 h, and mitochondrial Ca\(^{2+}\) levels were quantified using Rhod-2 as described above. A statistically significant increase in mitochondrial Ca\(^{2+}\) (*, p < 0.001 versus controls) was observed only in the staurosporine-treated DU-145 cells transduced with Bax. Note that Bax itself induced increases in mitochondrial Ca\(^{2+}\) at 12 h (*, p < 0.01), consistent with our previous findings (24). C, Bax accelerates staurosporine-induced cytochrome c release in DU-145 cells. Cells transduced with Bax (10 h after infection) were exposed to staurosporine for 45 min, and cytochrome c release, Bax expression, and an internal loading standard (actin) were measured by immunoblotting. The results shown are from one experiment that was representative of three replicates.
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sporine for 4 h. At this time point, staurosporine-induced ER Ca2+ release was submaximal (Fig. 1, A and B), and no cytochrome c release had occurred (Fig. 6D). Thapsigargin induced a nearly immediate release of cytochrome c (within 5–15 min) in the staurosporine-pretreated cells (Fig. 6D), confirming that staurosporine sensitized mitochondria to efflux of Ca2+ from the ER. The mechanisms probably involved direct effects of Bax and/or other proapoptotic members of the BCL-2 family because previous work has established that Bax translocates to mitochondria in response to staurosporine exposure (31–33). In addition, exogenous Bax sensitizes mitochondria to Ca2+-mediated cytochrome c release, but Ca2+-independent effects of Bax are also involved (24, 34).

Whereas our results establish a novel role for Bax-mediated Ca2+ changes in apoptosis, they do not identify the biochemical mechanisms involved in the effects of Bax on Ca2+. Previous studies have shown that Bax can form transmembrane ion channels in planar lipid bilayers (Refs. 35–38; reviewed in Ref. 39) and tetramerize in response to proapoptotic signals to form larger pores capable of directly accommodating cytochrome c release (40, 41). Thus, one simple explanation for our data would be that Bax forms transmembrane cation (i.e., Ca2+) channels that allow for Ca2+ to flow down its concentration gradient from the ER into mitochondria. However, previous studies have shown that the channels initially formed by Bax in lipid bilayers are selective for monovalent anions (Cl–) rather than divalent cations such as Ca2+ (39). Thus, it is possible that Bax must assemble into higher order structures to exert its effects on ER and mitochondrial Ca2+ and that its Ca2+-independent effects on mitochondria involve some other activity of the protein. The latter may be explained by previous studies that demonstrated that proapoptotic BCL-2 family members interact with components of the permeability transition pore (8, 9), which is a Ca2+-sensitive, mitochondrial polypeptide complex that has been implicated in some pathways of cytochrome c release by other investigators (42). Recent work by others has demonstrated that Bak and Bax play redundant roles in promoting cytochrome c release in mouse embryonic fibroblasts (11), and our own previous findings demonstrated that both Bak and Bax can promote ER Ca2+ pool emptying and mitochondrial Ca2+ uptake (24). We were therefore surprised that staurosporine failed to cause alterations in the ER or mitochondrial Ca2+ pools in DU-145 cells because these cells retain wild-type Bak. We did confirm that staurosporine-induced Ca2+ fluxes were attenuated in mouse embryonic fibroblasts derived from Bax−/− mice (data not shown). However, it was difficult to obtain satisfactory Ca2+ dye loading in the cells, and our failure to detect residual Bak-mediated Ca2+ changes may have been limited by the lower sensitivity of our methods in the cells. We have no reason to expect that other “multidomain” proapoptotic BCL-2 family members are incapable of mediating effects on Ca2+ similar to those described here, although our experience with Bak and Bax in PC-3 cells suggests that Bax is significantly more potent (24). It is possible that the involvement of Ca2+ fluxes in cytochrome c release varies not only with the proapoptotic stimulus in question but also with cellular background because human prostate cancer cells are notoriously sensitive to Ca2+-mediated apoptosis.

Although BCL-2 did not affect steady-state levels of Ca2+ in the ER or mitochondria in the PC-3 cells, it did inhibit the effects of staurosporine on ER Ca2+ release and subsequent mitochondrial Ca2+ uptake. These observations suggest that BCL-2 acts primarily to antagonize the effects of proapoptotic stimuli in our cells and does not exert direct effects of its own on either Ca2+ pool. It should be stressed, however, that other laboratories have reported different effects of BCL-2 on intracellular Ca2+ pools. For example, at least three groups have shown that BCL-2 lowers the steady-state level of Ca2+ within the ER, which they argued inhibits apoptosis by reducing Ca2+ efflux across the ER membrane (15, 18, 43). In contrast, another group showed that overexpression of BCL-2 increased steady-state levels of ER Ca2+ (44). On the other hand, Murphy et al. (17) concurred that BCL-2 had no significant effect on steady-state Ca2+ levels in their cells, but they found that it potentiated Ca2+ 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 Ca2+ compartmentalization very differently from epithelial cells. Furthermore, recent studies have demonstrated that high and low levels of BCL-2 can exert different effects on cells (45, 46). It is therefore possible that we would have observed direct effects of BCL-2 on ER and/or mitochondrial Ca2+ pools in PC-3 cells expressing different levels of BCL-2 protein. BCL-2 is capable of interacting with a variety of different proteins, some of which are involved in Ca2+-associated signal transduction (i.e., the Ca2+-dependent protein phosphatase calcineurin) (47), and BCL-2’s activity is regulated by phosphorylation (48–51). It therefore appears likely that cellular context dictates precisely how BCL-2 will influence intracellular Ca2+ pools. Additional efforts are required to more precisely define the biochemical mechanisms involved in the regulation of intracellular Ca2+ compartmentalization by the different members of the BCL-2 family.

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